

The effect of alfalfa (Medicago sativa) silage chop length and inclusion rate within a total mixed ration on the ability of lactating dairy cows to cope with a feed withholding and refeeding challenge

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1 Interpretive summary

2 **The effect of alfalfa (*Medicago sativa*) silage chop length and inclusion rate within a total**
3 **mixed ration on the ability of lactating dairy cows to cope with a feed withholding and**
4 **refeeding challenge**

5

6 Thomson

7 Cows fed diets containing a lower concentration of alfalfa silage (replacing corn silage)
8 experienced greater reductions in rumen pH following a six hour feed withholding/refeeding
9 challenge than those fed higher alfalfa concentration diets and also suffered greater short-term
10 milk loss on the day of the challenge. Lower rumen pH in animals fed a long chop length
11 compared to a shorter chop length raised questions over the effect of long forage particles in
12 the diet during and following short-term feed deprivation. This research highlights the
13 importance of maintaining feeding routines and ensuring adequate feed access throughout the
14 day in dairy systems.

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ACIDOSIS MITIGATION POTENTIAL OF ALFALFA SILAGE

The effect of alfalfa (*Medicago sativa*) silage chop length and inclusion rate within a total mixed ration on the ability of lactating dairy cows to cope with a short-term feed withholding and refeeding challenge

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Keywords: alfalfa, chop length, acidosis, feed withholding, rumen challenge,

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ABSTRACT

35 The objectives of the study were (i) to test whether 6 h feed deprivation followed by refeeding
36 induces an acidosis challenge in dairy cattle and (ii) to quantify the acidosis challenge
37 mitigation potential of increased alfalfa silage concentration in the diet. Alfalfa silage
38 constituted either 25 or 75% of forage dry matter (DM) replacing corn silage (low alfalfa or
39 high alfalfa; LA or HA), and was chopped to either 14 or 19 mm theoretical length (short or
40 long; S or L). Dietary treatments LAS, LAL, HAS or HAL were offered to four rumen-
41 cannulated Holstein dairy cattle (161 d in milk; 5th - 6th parity) in a 4 x 4 Latin square design
42 study with 21 d periods. Starch concentration was 69 g/kg DM higher for LA diets than HA
43 diets. Feed was withheld for 6 h followed by ad libitum refeeding on d 18 of each period.
44 Measurements of DM intake, milk yield and composition, rumen pH, and eating and rumination
45 behaviour were taken on one baseline day, the challenge day and two further recovery days.
46 After refeeding, rumen pH was reduced in cows fed LA diets but not HA diets. Feeding LAL
47 resulted in the greatest subclinical acidosis risk (pH < 5.8 for 355 minutes on the 1st recovery
48 day). Animals fed LA produced 4.4 L less milk on the challenge day in comparison to baseline.
49 It was concluded that short-term feed deprivation detrimentally affected rumen health and milk
50 yield in dairy cattle normally fed ad libitum but had no effect on DM intake or milk
51 composition. Feeding alfalfa silage in place of corn silage mitigated acidosis risk due to
52 interrupted feed supply, likely due to a combination of lower starch concentration in HA diets,
53 greater effective fiber concentration, and higher buffering capacity of alfalfa relative to corn
54 silage.

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56

INTRODUCTION

57 Lactating dairy cow diets are often formulated to include a high concentration of rapidly
58 fermented non-fiber carbohydrate (**NFC**) as a source of energy to support milk production
59 (Lechartier and Peyraud, 2011). However, such diets can also decrease rumen pH through
60 greater rate of production of VFA (Allen, 1997). In circumstances where pH remains below 5.8
61 for 3 consecutive hours, a dairy cow is purported to suffer from Sub-Acute Rumen Acidosis
62 (**SARA**), a condition that can reduce milk yield and milk fat concentration (Plazier et al., 2008).
63 Dietary strategies to increase the resilience of dairy cattle to SARA include feeding forages
64 with high buffering capacities (e.g. Alfalfa, *Medicago sativa*) or increasing the concentration
65 of physically effective fiber (**peNDF**) in the diet by lengthening forage chop length (McBurney
66 et al., 1983; Zebeli et al., 2006). Physically effective fiber is defined as the NDF contained
67 within particles that are longer than the critical particle size for rumen escape (which recent
68 research suggests is 4 mm although was historically defined as 1.18 mm [Oshita et al., 2004;
69 Maulfair and Heinrichs, 2012]) and therefore can contribute to the rumen mat (Mertens, 2000).
70 A lower rumen pH has also been linked with changes in cow feeding behaviour and the adoption
71 of coping mechanisms, including showing preferences for long particles in the diet (Maulfair
72 et al., 2013; DeVries et al., 2008) or for supplementary hay (Kmicikewycz and Heinrichs, 2015).

73 Experimentally, the stability of rumen pH can be tested by induction of a rumen
74 fermentation challenge. This is typically achieved through the addition of a large quantity of a
75 rapidly degradable carbohydrate to the diet such as cereal grains or alfalfa pellets (Krause and
76 Oetzel, 2005; Colman et al., 2013). However, it is unclear whether such a method accurately
77 replicates conditions that cause SARA, and furthermore, may not provide an appropriate model
78 for evaluating dietary mitigation strategies. An alternative approach to instigate a rumen
79 challenge is deprivation of feed for a period of several hours (Oetzel, 2007). A period of fasting
80 is then followed by a period of overeating when access to feed is returned, termed ‘refeeding’
81 (Chilibroste et al., 2007). Periods of feed deprivation lasting up to 6 h may be relatively

82 common in a commercial setting, for instance, where there is insufficient feed or pasture
83 allocation, feeding equipment failure, or removal of the animal's access to feed for routine
84 processes such as milking or health checks. However, relatively little is known about the
85 severity of the effect of such events on rumen function and milk production. Studies in the
86 literature have examined the effect of longer periods of fasting such as 12 to 48 h (Chelikani et
87 al., 2004; Oetzel, 2007; Toerien and Cant, 2007) that generally result in high levels of temporary
88 milk yield loss, however, we are not aware of any studies that have examined the effects of
89 shorter fasting periods in dairy cattle that would be more representative of commercial practice.
90 Therefore, the aims of the present study were (i) to test whether 6 h feed deprivation followed
91 by refeeding induces an acidosis challenge and (ii) to examine the effect of varying inclusion
92 rate (**IR**) and chop length (**CL**) of alfalfa silage, replacing corn silage in a TMR on resilience
93 to a feed withholding and refeeding challenge.

94

95

MATERIALS AND METHODS

Forage Harvesting and Clamp Sampling

97 The present study formed part of a larger research trial that utilised the same dietary treatments
98 and observed their effects on milk yield, dry matter intake, diet digestibility, and rumen function
99 under non-challenging conditions, in a larger cohort of cows and over a longer time period, as
100 reported previously (Thomson et al., 2017a,b). In brief, alfalfa silage was harvested as a second
101 cut crop at an estimated 10 % bloom in July 2014 and conserved in concrete-walled clamp. The
102 crop was wilted for 48 h and ensiled, producing a high DM (576 g/kg fresh weight) silage. Two
103 CLs (long; **L** and short; **S**) were created from material collected in alternate swaths by altering
104 the knife arrangement of the precision chop forage harvester (Claas Jaguar, Claas Group,
105 Harsewinkel, Germany) from a theoretical chop length of 14 mm (shortest setting) to 19 mm
106 (longest setting) that were ensiled in two adjacent clamps. An additive was applied (Axcool

107 Gold containing *L. Buchneri*; 2 L/Tonne; Biotal, Cardiff, UK) to prevent heating in the clamp.
108 Samples for chemical composition analysis (Sciantec Analytical Services, Cawood, UK) were
109 obtained using a clamp corer. A detailed analysis of the particle length profile of the silages
110 produced (mean 14.3 and 9.0 mm for L and S, respectively) has been published previously
111 (Experiment 2; Thomson et al., 2017b). Corn (*Zea mays*) silage for the study was taken from a
112 commercial crop of mixed varieties harvested in autumn 2014 which was chopped by the forage
113 harvester (Model FR700, New Holland Ltd, Turin, Italy; theoretical chop length of 18 mm) and
114 ensiled as described for the alfalfa clamps (geometric mean particle length of 10 mm determined
115 using a Penn State Particle Separator; **PSPS** [Heinrichs, 2013]).

116

117 *Diets*

118 Diets comprised a TMR with 50:50 ratio of forage:concentrate on a DM basis (Thomson et al.,
119 2017a,b), in which the forage portion consisted of corn and alfalfa silage at IRs (DM basis) of
120 either 25:75 (high alfalfa; **HA**) or 75:25 (low alfalfa; **LA**), respectively. These treatments were
121 combined with the two alfalfa silage CLs in a 2 x 2 factorial arrangement to give four treatments
122 (**HAL, HAS, LAL, LAS**) that were formulated to be isonitrogenous (170 g CP/kg DM) and
123 contain similar levels of NDF (320 g/kg DM). The reduction in corn starch associated with the
124 lower corn silage inclusion in HA diets was partially offset by increasing the concentration of
125 corn meal (Table 1), however for the experimental diets fed, starch concentration was still lower
126 in the HA diets (Table 2).

127

128 *Animals*

129 Four multiparous Holstein dairy cows, previously prepared with rumen fistulae (Bar Diamond
130 rumen cannula; Parma, Idaho, USA), in mid-lactation (161 d in milk, SE \pm 23.1) weighing 739
131 kg (SE \pm 13.9), and 7 - 9 years of age (5th - 6th parity), were randomly assigned to one of four

132 initial treatments according to a 4 x 4 Latin square design balanced for carryover effects with
133 21 d periods. All procedures were licensed and monitored by the UK Government's Home
134 Office under the Animal (Scientific Procedures) Act 1986. The experimental design and
135 replication employed was based on variance and expected treatment effects for key variables
136 observed in previous studies (Reynolds et al., 2014). During adaptation weeks (weeks 1 and 2
137 of each period) animals were housed in a cubicle yard and individually fed once daily for ad
138 libitum intake (10% refusals) through Insentec RIC feeders (Insentec B.V., Marknesse, The
139 Netherlands). Continuous access to water was provided. From d 12 of each period animals were
140 housed and milked in individual tie stalls to facilitate sampling. Animals were allowed to
141 acclimatise to the stalls for 3 d prior to sampling beginning on d 15. While in tie stalls, animals
142 were offered their daily feed allocation in two halves at 1000 h and 1600 h. Refusals were taken
143 daily at 0930 h. Between d 15 – 18 measurements of rumen function under non-challenging
144 conditions were performed including rumen VFA and ammonia concentrations, rumen pH,
145 rumen mat particle distribution and faecal particle distribution that have been reported
146 previously (Thomson et al., 2017b). The feeding routine differed on d 18 of each period when
147 a refeeding challenge was simulated (described below). While in tie stalls, each cow was also
148 fitted with a rumination headcollar (ITIN+HOCH GmbH, Fütterungstechnik, Liestal,
149 Switzerland) to measure eating and rumination behaviour as described previously (Ruuska et
150 al., 2016).

151

152 *Experimental Routine*

153 *SARA induction protocol.* Baseline measurements of all variables were taken on d 16 of each
154 period (other than rumen pH, which was measured on d 15 because other measurements being
155 performed on d 16 that have been reported separately). On d 18 of each period, refusals from
156 the previous day were removed from the cows one hour early (0830 h) to begin a period of

157 fasting. Feed was withheld for 6 h until 1430 h when half the daily diet allocation was offered
158 followed by the second half two hours later at 1630 h. On d 19 refusal and feeding routine was
159 returned to that of d 17. To summarise, the timetable for the week 3 of each period was as
160 follows:

161 D15: Basal rumen pH recorded (coinciding with sampling of rumen liquor, reported separately)

162 D16: Basal DMI, milk yield, and eating and rumination behaviour measurements

163 D17: Rest day with refusals removed one hour early the following morning

164 D18: Feed withheld until 1430h followed by refeeding

165 D19: Recovery day 1, original feeding routine resumed

166 D20: Recovery day 2

167 D21: No measurements, rest allowed before diet change.

168

169 *Intake and diet analysis.* The weight and dry matter concentration of feed offered and refused
170 were measured during d 14 – 21 of each period for each cow. A daily grab sample of each TMR
171 and the TMR constituents was bulked across the sampling week for each diet in each period
172 (16 samples in total). Dry matter concentration of feed was determined by oven drying at 100
173 °C for 24 h. Samples of the TMR constituents for each diet in each period were stored frozen at
174 -20 °C until analysed for DM, nitrogen (**N**; using the macro kjeldahl method; AOAC 954.01
175 [AOAC, 2000]), ash (by combustion at 500 °C for 16 hours), NDF and ADF (expressed
176 inclusive of residual ash; Mertens et al., 2002; Robertson and Van Soest, 1981), starch (Fuller,
177 1967; Macrae and Armstrong, 1968), and water soluble carbohydrates (**WSC**) as described
178 previously (Reynolds et al., 2014; Kliem et al., 2016). Concentrations (g/kg DM) of CP, NDF,
179 ADF, ash, starch and WSC in each TMR were calculated based on constituent inclusion rates.
180 A sample of each TMR from each period was analysed for particle size distribution using a
181 Penn State Particle Separator (**PSPS**, sieve apertures measuring 19 mm, 8 mm and 4 mm in

182 diameter and a bottom pan). A dry matter correction for material retained on each sieve was
183 obtained (Thomson et al. 2017a). Average particle size of the sample was calculated as
184 described previously (Heinrichs, 2013) and peNDF was calculated as the proportion of particles
185 (DM corrected) greater than the threshold length (4, 8, or 19 mm) multiplied by the NDF
186 concentration of the diet (Mertens, 1997; Farmer et al., 2014). The chemical and physical
187 composition of the diets is shown in Table 2 for reference but has been discussed in detail
188 previously (Thomson et al., 2017b).

189

190 *Milk Yield and Composition.* Cows were milked twice daily at 0630 h and 1630 h and milk
191 samples, preserved using potassium dichromate, were analysed for fat, protein, casein, lactose,
192 urea, and somatic cell count (SCC) by mid infra-red spectroscopy on a CombiFoss machine
193 (National Milk Laboratories, Chippenham, Wiltshire, UK). The CombiFoss machine combines
194 both the Fossomatic 5000 and Milkoscan 6000 (both Foss, Hilleroed, Denmark) and utilises the
195 entire mid-infra red wavelength spectrum. Morning and afternoon milk samples were scanned
196 separately. Only data from d 16 (baseline), 18 (challenge), 19 (recovery day 1) and 20 (recovery
197 day 2) were statistically analysed.

198

199 *Rumen pH.* An indwelling pH meter (Sentix 41-3 probe, WTW Trifthof, Weilheim, Upper
200 Bavaria) attached to a weight (200 g) and connected to the rumen cannula using nylon cord (50
201 cm) was placed within the rumen of each animal for 24 h beginning just prior to feeding (0930
202 h) on d 15 of each period until refusals were removed at 0930 h on d 16 to establish baseline
203 patterns of rumen pH, and inserted again at 0830 h on d 18 (challenge day), remaining within
204 the rumen until 0930 h on d 21. The probe was calibrated before every insertion by immersion
205 in solutions of pH 4 and 7. After use, the probe was re-immersed in the calibration solutions
206 and any drift was calculated as the given value subtracted from the true pH of the solution. Drift

207 greater than 0.3 pH units was considered the upper threshold for inclusion however no readings
208 greater than this value were found in the present study and therefore all data were included. The
209 pH probe was attached to a datalogger (ph340i, WTW, Trifthof, Weilheim, Upper Bavaria)
210 with readings recorded every 10 minutes. Time spent at < pH 6.2 and < pH 5.8 were calculated
211 for each day for each cow in each period. Readings were then averaged over each hour for
212 further analysis, beginning on the hour for Baseline, and Recovery days 1 and 2, and at the half
213 hour mark for challenge day to coincide with feeding times. Any measurements within the first
214 hour of insertion (0830 to 0930 h) were not included in statistical analysis due to differences in
215 the start time of each cow.

216

217 *Statistical analysis*

218 Average daily data starting at morning feeding, 1000 h, were calculated for 4 phases (days) of
219 week 3: Baseline (d 15/16), Challenge (d 18), Recovery 1 (d 19), and Recovery 2 (d 20).
220 Averages for each cow, treatment, and day (D) combination were analysed to determine fixed
221 effects of period, alfalfa IR, alfalfa CL, D (as a repeated measure) and their interactions (IR×CL,
222 IR x D, CL x D and IR×CL x D), and random effects of cow using mixed models procedures
223 of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). The ‘SLICE’ option was used to show
224 treatment effects for each day. Least squares means (LSM) for each treatment, and effects of
225 IR, CL and IR×CL interactions within each day, are presented separately. Within each
226 treatment, means for challenge or recovery days were compared to the baseline value for that
227 treatment using the PDIFF option within the LSMEANS statement of the Mixed procedure. For
228 measurements of eating time and relative rumen pH within each D, the same model was used
229 except day was replaced with hour (H, a repeated measure) and each day was analysed
230 separately. The covariance structure giving the best fit (out of compound symmetry, compound
231 symmetry heterogenous, unstructured or spatial power) was chosen for each variable using the

232 bayesian information criterion. Compound symmetry and spatial power were the most common
233 structures of best fit.

234 For rumen pH a baseline value for each hour of a 24 h period (starting at morning
235 feeding, d15, 1000 h) was taken for each cow on each treatment that was then subtracted from
236 the hourly mean at the same time point for each subsequent phase to analyse and present each
237 hourly value relative to baseline. The data was transformed in this way to ensure the magnitude
238 of any effects could be compared between animals with differing baseline rumen pH levels.
239 For example, the nadir pH observed during baseline varied between cows from 5.76 – 6.22
240 (mean of all treatments for each animal) and similarly basal daily mean rumen pH ranged from
241 6.48 – 6.76 between animals. Therefore, presenting data as time below a certain threshold was
242 judged to be of lesser importance than pH change relative to baseline. A mean of relative pH
243 for each day was also analysed (with the challenge day subdivided into ‘fast’ and ‘refeeding’)
244 to determine fixed effects of period, alfalfa IR, alfalfa CL, and IR×CL interaction, and random
245 effects of cow using mixed models procedures with each day and sub-phase tested separately.
246 For rumen pH parameters there were no effects of period and therefore it was judged that
247 recovery time was sufficient in between challenges to prevent carryover effects.

248 Effects of treatment on diet chemical and physical composition were analysed
249 separately using values for each bulked diet sample in each period ($n = 16$ bulked samples
250 originating from d 15-21). Fixed effects of period, alfalfa IR, alfalfa CL, IR×CL interaction and
251 random effect of cow was utilised also using mixed models procedure of SAS with period as a
252 repeated measure.

253

254

RESULTS

255 *Baseline treatment effects*

256 The effect of treatment on diet chemical composition in the present study (Table 2) and particle
257 size have been reported previously (Thomson et al., 2017a and 2017b). Briefly, the
258 concentration of starch was 69 g/kg DM greater in LA diets than in HA diets by design ($P <$
259 0.04), whereas ADF concentration was 36 g/kg DM greater in HA diets ($P < 0.01$). Increasing
260 CL from S to L increased the proportion of particles retained on both the 8 and 19 mm sieves
261 of the PSPS by 36 and 43 g/kg DM respectively whilst reducing the proportion that was retained
262 on the 4 mm sieve and in the bottom pan (all $P < 0.02$). Both greater IR and greater CL of alfalfa
263 increased or tended to increase peNDF concentrations using 4, 8 and 19 mm threshold lengths
264 ($P < 0.06$) relative to a low IR and a short CL.

265 We found no effect of diet on daily mean rumen pH for which the average across all
266 treatments was 6.36 (Table 3), or on daily time spent at less than pH 6.2 or pH 5.8, nor were
267 there any time points during the baseline day in which there was an effect of treatment on rumen
268 pH. Following feeding at baseline, rumen pH showed a downwards trend reaching a nadir
269 between 9 and 13 h post morning feeding followed by a return to pre-feeding levels between
270 15 and 22 h post feeding (Figure 1a). Baseline eating patterns, showed an increase in time spent
271 eating (20 - 40 min/h) in the first hour after fresh feed was offered (at both 1000 and 1600 h),
272 followed by a reduction in time spent eating in the second hour post feeding to roughly 10
273 min/h, a rate that was sustained throughout the daytime hours (Figure 1b). Between 13 and 19
274 h post feeding < 5 min/h eating occurred that corresponded to the rise in rumen pH shown in
275 Figure 1a. Dry matter intake, milk yield, milk composition and the yield of milk solids showed
276 no effect of treatment during the baseline phase (Table 4). Both daily mean time spent eating
277 (Table 5) and transient eating patterns were similar for all dietary treatments at baseline. Cows
278 fed HAL diets had more daily mean rumination chews and spent more time ruminating per day
279 than cows fed either LAL or HAS, while cows fed LAS had an intermediate number of
280 ruminating chews (IR \times CL; $P < 0.04$). Cows fed HAL diets also showed a tendency to spend

281 the greatest time ruminating per day compared to other dietary treatments (IR×CL; $P < 0.07$).
282 Hourly patterns of rumination indicate a level profile of rumination for all treatments
283 throughout the day with 10 - 30 minutes spent ruminating each hour (Figure 1c).

284

285 *Challenge effect on rumen pH and eating patterns*

286 Relative rumen pH increased steadily during the feed withholding period for all diets
287 (figure 2a). There was no effect of treatment on the mean relative pH (Table 3) nor at any
288 individual time-points over the fasting phase. At the peak of the fasting phase, mean rumen pH
289 across the treatments ranged from 6.8 to 7.2. A steep fall in rumen pH on all treatments occurred
290 with the refeeding event. Over the first hour post re-feeding, relative rumen pH in cows fed the
291 LAL diet decreased to the baseline level in comparison to the other three diets ($P < 0.03$) where
292 relative pH remained elevated above baseline levels until 2 h post refeeding, which coincided
293 with the second offering of feed. At 8 - 12 h post refeeding, rumen pH of cows fed LA diets fell
294 to lower levels than HA relative to their baseline values (IR effects $P < 0.04$), whilst HAS
295 remained closer to baseline than HAL (IR×CL interaction; $P < 0.04$). Cows fed HAS diets
296 maintained a rumen pH that was close to baseline pH throughout the refeeding period: 0.04 pH
297 units higher than baseline over the entire refeeding phase. Cows fed LA diets had a rumen pH
298 0.16 pH units lower on average over the refeeding phase than HA diets relative to their own
299 baseline values ($P < 0.008$; Table 3) and spent on average 97 minutes at pH <5.8 compared
300 with 30 minutes for cows fed HA diets.

301 Cows spent a greater proportion of time eating in the 3 h following refeeding than during
302 the same period after the initial feed was offered at baseline (57 % vs 29 % of each hour was
303 spent eating in 0-3 h post feeding respectively; Figure 2b). At 4 h post refeeding eating intensity
304 reduced for cows fed all diets, although at 6 h post refeeding cows fed the LAS diet again spent
305 a high proportion of time eating in comparison to cows fed other diets ($P < 0.01$). Following

306 this, cows on all diets continued to eat at a fluctuating rate between 0-20 min/h (Figure 2b).
307 Rumination pattern indicated a slightly larger reduction in rumination between 0 - 4 h post
308 refeeding than at mealtimes on other days during the observation period for cows on all
309 treatments. In the hour prior to refeeding cows fed LA diets ruminated very little (< 5 minutes)
310 in comparison to cows fed HA that continued to ruminate for between 15 and 25 minutes during
311 the hour ($P < 0.04$).

312

313 *Recovery from the rumen challenge*

314 On recovery day 1 the rumen pH of all cows recovered close to baseline levels prior to morning
315 feeding. However, post feeding, the rumen pH of cows fed LA diets again decreased relative to
316 their baseline values leading to multiple hours in which there were effects of IR. At 31 h post
317 refeeding the rumen pH of cows fed LAS diets returned to basal values whereas cows fed LAL
318 diets continued to show reduced relative rumen pH until 36 h post refeeding (IR×CL
319 interactions $P < 0.04$). Cows fed HAS diets continued to show a rumen pH pattern close to
320 baseline while cows fed HAL diets were marginally lower than baseline values (Figure 2a).
321 Mean relative rumen pH for the recovery day 1 phase demonstrated that cows fed LA and L
322 diets had reduced relative pH in comparison to HA and S diets (effect of IR $P < 0.001$; effect
323 of CL $P < 0.03$) which was also reflected in cows fed LA spending longer at pH < 5.8 than
324 cows fed HA.

325 On recovery day 2 there were no significant differences in relative rumen pH between
326 treatments or any hours in which treatment differences occurred although the relative rumen
327 pH of cows fed LAL diets continued to be the lowest of the four treatments and on average 0.17
328 pH units below baseline values for that diet (Table 3). Over both recovery days, eating and
329 rumination patterns appeared similar to those observed at baseline. Some fluctuation led to

330 significant effects on time spent eating and ruminating during these days but overall, differences
331 were slight and not sustained.

332

333 *Induction of SARA*

334 Taking the definition of SARA to be a period of 3 consecutive hours where rumen pH is less
335 than 5.8, then we observed 6 bouts of SARA within the data set of which 2 bouts were in the
336 same cow when fed the LAS diet and the remaining 4 were in 3 cows when fed the LAL diet
337 (with 1 cow experiencing 2 separate bouts on this diet). Of these 6 bouts of SARA, 2 occurred
338 on the day of the challenge (1 LAS and 1 LAL) and 4 occurred on recovery day 1 (1 LAS and
339 3 LAL). No episodes of SARA were observed in cows fed HA diets.

340

341 *Challenge effect on intake and milk production*

342 On the day of the challenge, DMI was similar to that consumed on baseline day (Table 4) as
343 was daily mean time spent eating and ruminating (Table 5) despite the pattern of eating during
344 the day being altered as described earlier. A numerical decline in intake was observed between
345 the Challenge Day and Recovery Day 2 for cows fed LAL and HAL diets, resulting in animals
346 fed L eating 2.7kg/d less than animals fed S on Recovery Day 2 ($P < 0.05$).

347 Milk yield was reduced in cows fed LAS and LAL diets on challenge day relative to
348 milk yield at baseline ($P < 0.05$), by 4.5 kg and 4.3 kg respectively, although yield was not
349 significantly lower than that of cows fed the HA diets on the challenge day. The reduction in
350 milk yield on LA diets on this day, also led to significant reductions in milk protein yield
351 compared to baseline for these treatments. On recovery day 1 and 2 milk yield for all treatments
352 was not statistically different ($P > 0.05$) from baseline levels. Concentrations of milk protein
353 were unaffected by treatment and day. The milk fat yield of cows fed LAS and HAL diets on

354 recovery day 2 was higher than baseline ($P < 0.05$), and furthermore the milk fat yield for HAL
355 cows on that day was greater than that of any other dietary treatment (IR×CL; $P < 0.04$).

356

357

DISCUSSION

358 *The effect of a refeeding challenge on eating patterns and rumen pH*

359 During the fasting phase, prior to re-feeding, we observed increased rumen pH for all animals,
360 likely because of rumen VFA being absorbed and not replaced due to a lack of substrate for
361 fermentation, and perhaps as an effect of salivation while the animals were waiting for feed to
362 be offered. In support of this, cows were shown to continue ruminating during the fasting
363 period. Following refeeding, animals exhibited a three-hour period in which a high proportion
364 of time was spent eating across all treatments in comparison to the baseline day (57 % vs 29 %
365 of each hour was spent eating in 0-3 h post feeding respectively; Figure 2b). An increase in
366 eating intensity following feed deprivation is consistent with the findings of other studies
367 (Oetzel, 2007; Patterson et al., 2008) and has been linked with low rumen fill prior to refeeding
368 (Gregorini et al., 2007). This over-eating episode resulted in a rapid decrease in rumen pH such
369 that 3 h after refeeding animals had reached the same rumen pH as was observed 7 h after
370 feeding on the baseline day. We attribute this accelerated decline in rumen pH to acid load from
371 the ingested feed and from VFAs produced from fermentation of the same. Furthermore, high
372 feed intake in a short time-period would have increased the supply of rapidly degraded starch
373 and sugars to the microbial population, especially within the LA diet that contained a greater
374 concentration of starch from corn silage. Total VFA concentration in the rumen is dependent
375 on the rate at which VFA are produced in comparison to the rate at which VFA can be absorbed
376 through the ruminal epithelium, be neutralised by saliva, or are removed from the rumen by
377 passage. There are various absorption mechanisms that facilitate VFA removal from the rumen
378 however the most predominant are bicarbonate-dependant transport (Aschenbach et al., 2011)

379 and passive diffusion (Chibisa et al., 2016). For the latter, a low VFA concentration in the
380 rumen, such as that created by short-term feed deprivation, would reduce VFA removal rate
381 initially until a sufficient diffusion gradient was established. Simultaneously, recent research
382 suggests that such conditions are likely to also favour increased production rate of VFA by
383 microbes that benefit from a diffusion gradient that swiftly removes VFA from their boundary
384 layer (Russell et al., 2009; Mason and Stuckey, 2016). Therefore the swift decline in rumen pH
385 observed is likely to be a combined effect of increased microbial productivity combined with
386 reduced ability to remove VFA from the rumen through absorption. Another longer term study
387 also noted a reduction in epithelial absorption rate during and after feed restriction that was
388 attributed to reduced blood flow due to feed deprivation (e.g. 5 d feed restriction followed by
389 refeeding; Zhang et al., 2013); however this is unlikely to be the case in our study where feed
390 was only withheld for 6 h. There are few previous studies in which withholding and refeeding
391 TMR have been examined. Studies have examined effects in grazing animals (Chilibroste et
392 al., 2007), but there is still a lack of data on rumen kinetics to explain the mechanisms
393 underpinning responses to such a challenge and further work is required to fully understand
394 responses in TMR-fed animals.

395 Despite the reduced window of time when animals were allowed access to feed on the
396 challenge day (18.5 h), there was no difference in the quantity of feed consumed or total minutes
397 spent eating in comparison to baseline days, again highlighting that eating rate post-refeeding
398 was increased in comparison to basal eating rate. Milk yield was reduced on the day of the
399 challenge for all diets, and significantly so for LA diets, which might indicate there was a
400 carryover effect of the fasting period for these diets, or that the increased rate of feed
401 consumption after refeeding reduced the efficiency of energy capture from the diet.
402 Concentrations of fat and protein within the milk were largely unaffected, other than an
403 unexpected rise in milk fat concentration seen on recovery day 2 in both LAS and HAL diets,

404 however this is likely to be due to slightly reduced milk yield on these treatments since total fat
405 yield was unaffected. It should also be borne in mind that that using a single day as a baseline
406 value may not have fully accounted for day to day variation in our study.

407

408 *The acidosis mitigation potential of the dietary treatments*

409 In the present study, cows fed diets comprising a high IR of alfalfa silage were less affected by
410 the rumen challenge than those with a low IR, despite there being no difference in rumen pH
411 profile between the diets at baseline. Alfalfa silage provided more effective fiber (Table 2) to
412 the diet than the corn silage and has also been reported previously to have a higher cation
413 exchange capacity than corn (McBurney et al., 1983) and therefore a combination of these two
414 factors could explain the increased ability of the cows to buffer against low rumen pH.
415 Furthermore, alfalfa often contains a higher proportion of indigestible, lignified, stem in
416 comparison to other forages that may reduce rumen passage rate and maintain rumen fill for
417 longer providing a better environment for continued microbial activity and facilitating a slow
418 rate of VFA production in the rumen during the period of feed deprivation (Dewhurst et al.,
419 2003). In support of this, the present study showed that cows fed HA diets spent more time
420 ruminating during the fast period than those fed LA. This may have enhanced the rate of
421 microbial adaptation to refeeding, reduced any disruption of epithelial function, and therefore
422 reduced negative effects on milk yield. The LA diets also contained a higher concentration of
423 starch that would have contributed to reduced rumen pH at refeeding. The difference in starch
424 concentration between the two diets may also have altered utilisation of dietary nutrients,
425 particularly nitrogen. We observed no incidence of SARA in cows fed HA diets confirming
426 that feeding alfalfa at the higher IR of 375 g/kg diet DM, and consequently feeding less corn
427 silage and starch, was successful at mitigating acidosis risk in comparison to the lower inclusion
428 rate. Milk loss in cows fed LA diets on the day of the challenge (4.4 kg/d) was a decrease of

429 14.3 % compared to baseline yield, which represents a cost to the farmer if animals were
430 regularly fasted for similar periods (6 h continuous). Furthermore the work of Dohme et al.
431 (2008) suggests the severity of acidosis can increase where challenges are repeated in quick
432 succession, although this was not evident in our study as there was no significant or numerical
433 ($P > 0.2$) effect of period on time spent at $\text{pH} < 6.2$. This is likely due methodological
434 differences as Dohme et al. (2008) induced challenges 14 d apart, as opposed to 21 d in the
435 present study, and the effect of the challenges imposed by Dohme et al. (2008) were greater
436 (using 4 kg of barley grain consumed within 1 h to induce acidosis) with nadir pH in the range
437 of 5.13 – 5.53 versus 5.41 – 6.22 observed on recovery day 1 in our study. Furthermore, Dohme
438 et al. (2008) also noted increased severity of subsequent acidosis challenges when cows were
439 in early lactation as opposed to mid-lactation.

440 Evidence from jaw movement monitors in the present study confirmed that the long
441 chop length increased rumination activity as would be expected, however, animals fed diets
442 containing L chop alfalfa silage had lower ruminal pH on average on recovery day 1 than
443 animals fed S, with those fed LAL diets having the greatest and most prolonged reduction in
444 ruminal pH in comparison to the other diets. In this regard, our findings contrast with previously
445 published work suggesting a positive correlation between rumen pH and peNDF concentration
446 (Zebeli et al., 2006) that has been attributed to increased rumination supplying more saliva to
447 the rumen, although these relationships were generated from studies where no feed withholding
448 and refeeding challenge was applied. Lengthening chop length can negatively affect diet
449 uniformity and allow increased sorting against longer particles, which would contain the most
450 peNDF (Leonardi and Armentano, 2003), however, this is unlikely to explain the lower rumen
451 pH of cows on L diets on recovery day 1, as animals have previously been shown to increase
452 selection of longer particles in response to a rumen challenge (DeVries et al., 2008). The
453 beneficial effect of peNDF is thought to be the result of increased stimulation of rumination

454 producing saliva to buffer the rumen, and, in line with this, HAL diets did increase rumination
455 however we did not observe the same effect in the other diets, including LAL, where the
456 concentration of peNDF was lower. Longer particles would have required rumination to aid
457 digestion after ingestion, however in our study rumination was reduced during the refeeding
458 event while eating was prioritised, an effect which has also been observed in previous refeeding
459 work (Chilibroste et al., 2007), meanwhile smaller forage particles and concentrates can be
460 broken down without the need for further rumination chewing. This delay in rumination due to
461 overeating may have reduced the ability of animals fed LAL to digest the forage portion of the
462 diet. It is also possible that fiber digestion was impaired as a result of the low pH conditions
463 affecting microbial populations (Grant and Mertens, 1992). Reductions in DMI in animals fed
464 the long CL diets on both recovery day 1 and 2 relative to those fed short CL diets (a difference
465 that was not observed at baseline) also supports this explanation as reducing fiber digestibility
466 of dietary alfalfa has previously been linked to reduced appetite (Getachew et al., 2011; Fustini
467 et al., 2017) likely due to increased feeling of satiety. However, if fiber digestion was reduced,
468 the lack of an effect on milk composition suggests the effect was short-lived. Based on the
469 negative effect of increasing peNDF provision through increased chop length, it is likely the
470 mitigation effect of high alfalfa IR was attributable to the buffering capacity of alfalfa, increased
471 rumen fill during the feed withholding phase and reduced diet starch concentration, rather than
472 any effect of peNDF *per se*.

473 In the LAL diet, effects of the challenge continued throughout recovery day 1 despite a
474 return to baseline feeding patterns, with DMI also reduced for this diet on recovery day 2. The
475 timeline is similar to that observed previously in the literature (Oetzel, 2007) where a cow faced
476 with a 12 hour fast followed by a refeeding challenge took 60 h for rumen pH to return to pre-
477 fast levels. The extended number of days over which significant effects were seen despite no

478 further challenges being applied highlights the need for rumen pH to be observed over several
479 days when investigating induced SARA experimentally.

480

481

CONCLUSIONS

482 We conclude that a relatively short fast (6 h) followed by a refeeding event, in which a day's
483 allocation of feed equal to the pre-fast level was offered ad libitum, was sufficient to induce
484 SARA in 4 out of 8 observations where low alfalfa diets were fed. However, a high rate of
485 alfalfa inclusion within the diet combined with a lower dietary starch concentration mitigated
486 the acidosis risk, and was particularly effective when the alfalfa silage was chopped to a shorter
487 length. We attribute this mitigation effect to (i) buffering capacity provided by the alfalfa, (ii)
488 less degradable alfalfa fractions providing rumen substrate during the fast, and (iii) reduced
489 dietary starch concentration, rather than increased effective fiber provision, as a longer particle
490 length led to greater reductions in rumen pH after refeeding. Milk lost from cows fed diets with
491 lower inclusion rates of alfalfa would represent a significant financial loss if such a refeeding
492 challenge were to occur regularly, highlighting the need to ensure uniformity of feeding routines
493 in ad libitum TMR feeding systems for dairy cows on a day to day basis.

494

495

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647

648 **Table 1** Ingredients used in diet formulation

Item	Diet	
	LA	HA
Ingredients, g/kg DM		
Alfalfa silage ¹	125	375
Corn silage ²	375	125
Concentrate blend ³		
Cracked Wheat	80	80
Corn Meal	54	97
Unmolassed Sugar Beet Feed	40	40
Soy Hulls	82	108
Soybean Meal	100	65
Rapeseed Meal	100	65
Molasses	10	10
Dicalcium phosphate	5	5
Salt	5	5
Dairy Mineral ⁴	10	10
Megalac ⁵	15	15

649 LA, low alfalfa diet; HA, high alfalfa diet;

650 ¹ long chop alfalfa silage composition: 593 g/kg DM; 164 g/kg DM CP; 397 g/kg DM NDF; 348g/kg DM ADF;
 651 108 g/kg DM Ash; and 10 g/kg DM water soluble carbohydrate. Short chop alfalfa silage composition: 566 g/kg
 652 DM; 167 g/kg DM CP; 385 g/kg DM NDF; 326 g/kg DM ADF; 108 g/kg DM Ash; and 17 g/kg DM water
 653 soluble carbohydrate.

654 ² Corn silage composition: 383 g/kg DM; 63 g/kg DM CP; 387 g/kg DM NDF; 223g/kg DM ADF; 37 g/kg DM
 655 Ash; 357 g/kg DM starch; and 25 g/kg DM water soluble carbohydrate.

656 ³ HA concentrate composition: 911 g/kg DM; 199 g/kg DM CP; 278 g/kg DM NDF; 172 g/kg DM ADF; 67 g/kg
 657 DM Ash; 247 g/kg DM Starch and 57 g/kg DM water soluble carbohydrate. LA concentrate composition: 884
 658 g/kg DM; 241 g/kg DM CP; 272 g/kg DM NDF; 171 g/kg DM ADF; 71 g/kg DM Ash; 195 g/kg DM Starch and
 659 67 g/kg DM water soluble carbohydrate.

660 ⁴ Contained vitamin A (400,00 IU/kg), vitamin D (80,000 IU/kg) and vitamin E (2,000 IU/kg), manganese (2.2
 661 g/kg), calcium (230 g/kg), zinc (5.2 g/kg), phosphorous (20 g/kg), magnesium (40 g/kg), sodium (95 g/kg),
 662 copper (1.2 g/kg), and selenium (30 mg/kg).

663 ⁵ Megalac rumen protected fat supplement (Volac International ltd., Royston, UK)

664

665 **Table 2** The chemical and physical composition of four total mixed rations containing a high
 666 (HA) or low (LA) concentration of alfalfa silage at a long (L) or short (S) chop length
 667 (Thomson et al. 2017b).

Item	Diet				SEM	<i>P</i> value ¹		
	LAS	LAL	HAS	HAL		IR	CL	IR×CL
Chemical composition, g/kg DM								
Oven DM, g/kg	555	571	610	632	5.0	0.022	0.065	0.364
Ash	62	63	78	77	0.6	0.001	0.471	0.070
CP	164	163	168	167	3.5	0.200	0.710	0.945
NDF	311	322	335	340	4.8	0.115	0.221	0.510
ADF	202	208	237	245	1.5	0.004	0.007	0.322
Starch	234	235	164	168	7.0	0.039	0.680	0.780
WSC ²	37	35	35	32	0.7	0.006	0.020	0.371
Particle size distribution ³								
Material retained, g/kg DM								
19mm	32 ^a	50 ^a	53 ^a	121 ^b	7.5	0.001	0.001	0.007
8mm	364 ^a	419 ^b	374 ^{ac}	391 ^c	5.0	0.129	0.012	0.026
4mm	165 ^a	135 ^b	187 ^c	126 ^b	2.4	0.033	0.001	0.004
Bottom pan	438	398	379	363	5.0	0.001	0.010	0.094
Mean particle size ⁴ , cm	0.50	0.56	0.54	0.65	0.014	0.001	0.001	0.099
peNDF ⁵ , g/kg DM								
peNDF _{>19mm}	10.3 ^a	16.4 ^a	17.4 ^a	40.4 ^b	2.68	0.001	0.001	0.009
peNDF _{>8mm}	123	148	138	182	2.7	0.056	0.030	0.137
peNDF _{>4mm}	172	199	205	213	3.8	0.003	0.004	0.051

668 ^{a,b} Where there is a significant interaction, values within a row with different superscripts differ significantly at
 669 *P*<0.05.

670 ¹IR, Inclusion rate; CL, chop length; IR×CL, interaction between IR and CL.

671 ²WSC, water soluble carbohydrate.

672 ³ Particle size distribution measured using a Penn State Particle Separator with three sieves: 19, 8 and 4 mm
 673 diameter.

674 ⁴ Mean particle size was determined using the recommended equation of Penn State University (Heinrichs,
 675 2013).

676 ⁵ Physically effective neutral detergent fiber (peNDF) determined as the proportion of particles in the total mixed
 677 ration (TMR) greater than the threshold length (specified in subscript) multiplied by the NDF concentration of
 678 the TMR (Mertens, 1997).

679

680 **Table 3** Mean relative rumen pH of lactating dairy cows fed a total mixed ration containing a
 681 high (HA) or low (LA) concentration of alfalfa silage at a long (L) or short (S) chop length
 682 prior to, during, and following a rumen challenge that involved a 6 hour fast followed by a
 683 refeeding challenge.

Phase ²	Diet				SEM	<i>P</i> value ¹		
	LAS	LAL	HAS	HAL		IR	CL	IR×CL
Baseline daily rumen pH	6.30	6.38	6.31	6.43	0.130	0.785	0.396	0.828
Relative rumen pH ³								
Challenge day								
Fast	+0.43	+0.42	+0.46	+0.38	0.098	0.905	0.517	0.592
Refeeding	-0.15	-0.21	+0.04	-0.08		0.007	0.115	0.643
Recovery day 1	-0.20	-0.41	-0.01	-0.11		0.001	0.023	0.443
Recovery day 2	-0.01	-0.17	0.02	0.01		0.241	0.365	0.428
Minutes below pH 6.2 ⁴								
Baseline	531	390	428	348	209.3	0.700	0.561	0.921
Challenge day								
Fast	-	-	-	-		-	-	-
Refeeding	761	463	353	328		0.168	0.398	0.426
Recovery day 1	1008*	880*	438	408		0.017	0.677	0.097
Recovery day 2	551	612	435	357		0.343	0.965	0.769
Minutes below pH 5.8 ⁵								
Baseline	25	33	23	3	11.9			
Challenge day								
Fast	0	0	0	0				
Refeeding	93	100	45	15				
Recovery day 1	135	355	15	18				
Recovery day 2	35	113	20	57				

684 * Where a value differs significantly ($P < 0.05$) from a baseline value for that treatment (not applicable to
 685 relative rumen pH).

686 ¹IR, Inclusion rate; CL, chop length; IR×CL, interaction between IR and CL;

687 ² The fast period combines measurements from 0930 h until 1430 h on the day of the challenge during which
 688 time animals were not allowed to access feed (note, the start of the feed withdrawal was 0830 h however the time
 689 taken to insert rumen pH probes meant that data for this hour was incomplete so was not included in the
 690 analysis). The refeeding period combines measurements from 1430 h on the day of the challenge until 0930 h the
 691 following morning. After which the subsequent two 24 h periods are termed recovery day 1 and recovery day 2
 692 that both begin at 1000 h.

693 ³ Relative rumen pH calculated hourly as rumen pH measurement minus the corresponding baseline
 694 measurement (Thomson et al. 2017b) at the same hour of the day for each cow on each treatment in each phase.

695 ⁴ All cows spent either low or no time below pH 6.2 during the fast sub-phase and therefore this sub-phase was
 696 removed from statistical analysis to prevent non-normality of the remaining dataset.

697 ⁵ For minutes below pH 5.8 a large number of values were 0 and therefore the data did not display a normal
 698 distribution, nor could a meaningful transformation be achieved, therefore data are presented as arithmetic
 699 means.

700 **Table 4** Daily mean intake, milk production and milk composition of lactating dairy cows fed
 701 a total mixed ration containing a high (HA) or low (LA) concentration of alfalfa silage at a
 702 long (L) or short (S) chop length prior to, during, and following a 6 hour fast followed by a
 703 refeeding challenge.

Item ²	Diet				SEM	<i>P</i> value ¹		
	LAS	LAL	HAS	HAL		IR	CL	IR×CL
Dry Matter Intake, kg/d								
Baseline day	25.5	21.3	22.5	24.4	1.44	0.998	0.359	0.133
Challenge day	25.2	23.7	24.5	23.6		0.764	0.352	0.810
Recovery day 1	25.3	21.5	23.1	22.2		0.548	0.085	0.251
Recovery day 2	23.1	19.6	23.7	21.8		0.289	0.049	0.130
Milk Yield, kg/d								
Baseline day	31.7	30.1	27.8	29.2	5.89	0.654	0.992	0.938
Challenge day	27.2*	25.8*	25.9	27.3		0.985	0.994	0.994
Recovery day 1	31.9	29.5	29.0	30.8		0.879	0.954	0.978
Recovery day 2	30.9	28.2	29.0	27.0		0.775	0.664	0.962
Milk fat, g/kg								
Baseline day	32.6	35.2	35.3	33.2	3.04	0.766	0.832	0.305
Challenge day	38.7	38.4	34.0	34.8		0.218	0.948	0.638
Recovery day 1	34.7	35.3	35.8	35.8		0.588	0.817	0.939
Recovery day 2	37.0* ^a	36.5 ^a	35.3 ^a	40.3* ^b		0.292	0.046	0.033
Milk fat yield, kg/d								
Baseline day	1.07	1.04	0.94	1.03	0.208	0.701	0.897	0.963
Challenge day	1.06	1.00	0.84	0.95		0.485	0.886	0.864
Recovery day 1	1.11	1.05	1.04	1.09		0.946	0.996	0.991
Recovery day 2	1.12	1.01	1.01	1.07		0.892	0.898	0.968
Milk protein, g/kg								
Baseline day	31.3	31.5	30.9	30.9	1.16	0.602	0.923	0.952
Challenge day	31.4	31.0	30.2	29.5		0.209	0.583	0.585
Recovery day 1	30.3	30.8	30.2	29.1		0.364	0.764	0.637
Recovery day 2	30.6	30.4	30.5	29.1		0.546	0.490	0.755
Milk protein yield, kg/d								
Baseline day	0.99	0.95	0.84	0.87	0.173	0.440	0.996	0.869
Challenge day	0.85*	0.80*	0.77	0.80		0.808	0.954	0.990
Recovery day 1	0.96	0.91	0.87	0.89		0.740	0.920	0.982
Recovery day 2	0.94	0.87	0.88	0.75		0.571	0.549	0.857

704 ^{a,b} Where there is a significant interaction, values within a row with different superscripts differ significantly at
 705 $P < 0.05$.

706 * Where a value differs significantly ($P < 0.05$) from a baseline value for that treatment.

707 ¹ IR, Inclusion rate; CL, chop length; IR×CL, interaction between IR and CL.

708 ² Baseline data was collected on d 16 and the challenge day was d 18 (starting at 1000 h) of each period, during
 709 which animals spent 4.5 h of the day fasting (post a 1.5 h period during which refusals were removed early to
 710 make a total fast of 6 h) and a 17.5 h period in which feed was offered ad libitum. Recovery days 1 and 2 were
 711 the subsequent 24 h periods (d 19 and d 20 respectively both beginning 1000 h).

712

713 **Table 5** Eating and rumination behaviour of lactating dairy cows fed a total mixed ration
 714 containing a high (HA) or low (LA) concentration of alfalfa silage at a long (L) or short (S)
 715 chop length prior to, during, and following a 6 hour fast followed by a refeeding challenge.

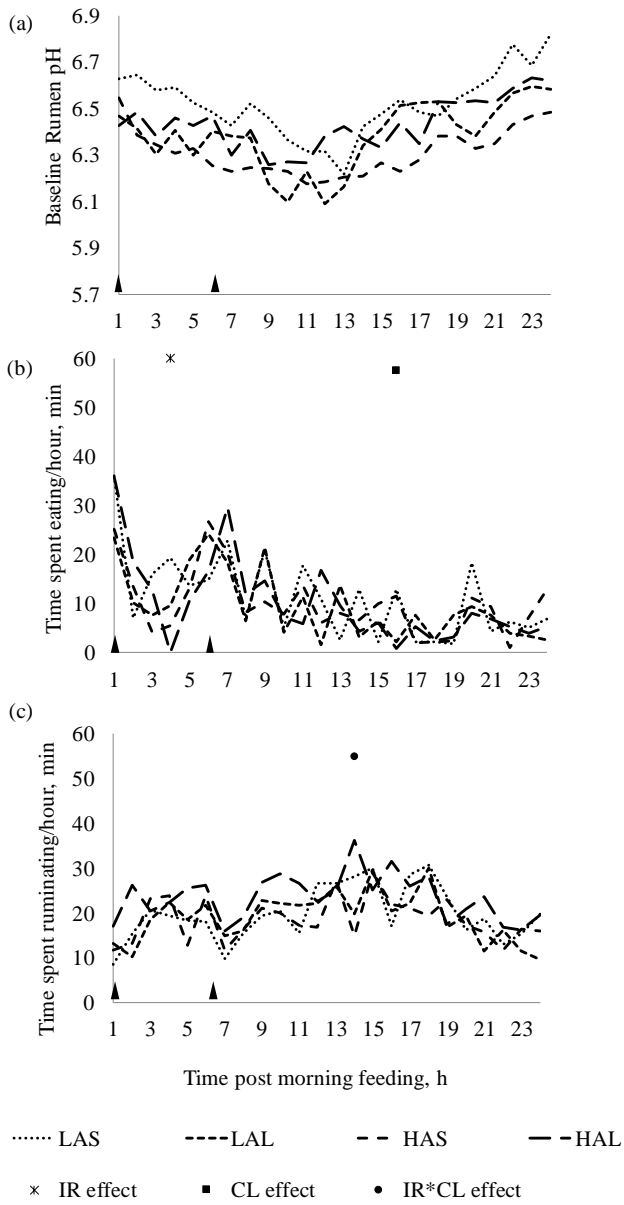
Item ¹	Diet				SEM	P value		
	LAS	LAL	HAS	HAL		IR	CL	IR×CL
Eating chews '000/d								
Baseline day	17.8	12.1	12.8	14.0	2.56	0.509	0.351	0.392
Challenge day	16.2	12.0	15.4	14.5		0.707	0.283	0.626
Recovery day 1	15.4	9.8	13.5	11.4		0.950	0.120	0.410
Recovery day 2	14.4	11.1	11.9	15.0		0.791	0.961	0.605
Eating time, min/d								
Baseline day	268	225	339	239	35.0	0.795	0.490	0.817
Challenge day	250	217	267	227		0.681	0.271	0.665
Recovery day 1	241	177	249	189*		0.768	0.073	0.305
Recovery day 2	229	184	217	222		0.703	0.551	0.808
Ruminating chews '000/d								
Baseline day	27.6 ^{ab}	27.5 ^a	24.2 ^a	35.4 ^b	3.01	0.414	0.052	0.038
Challenge day	28.7	26.2	26.1	34.2		0.336	0.319	0.124
Recovery day 1	31.0	29.0	27.8	32.0		0.958	0.691	0.673
Recovery day 2	30.1	26.1	28.1	32.7		0.459	0.934	0.479
Ruminating time, min/d								
Baseline day	442	460	421	574	47.1	0.281	0.056	0.065
Challenge day	464	432	439	548		0.303	0.389	0.198
Recovery day 1	499	484	469	520		0.944	0.686	0.835
Recovery day 2	494	438	478	533		0.414	0.991	0.591

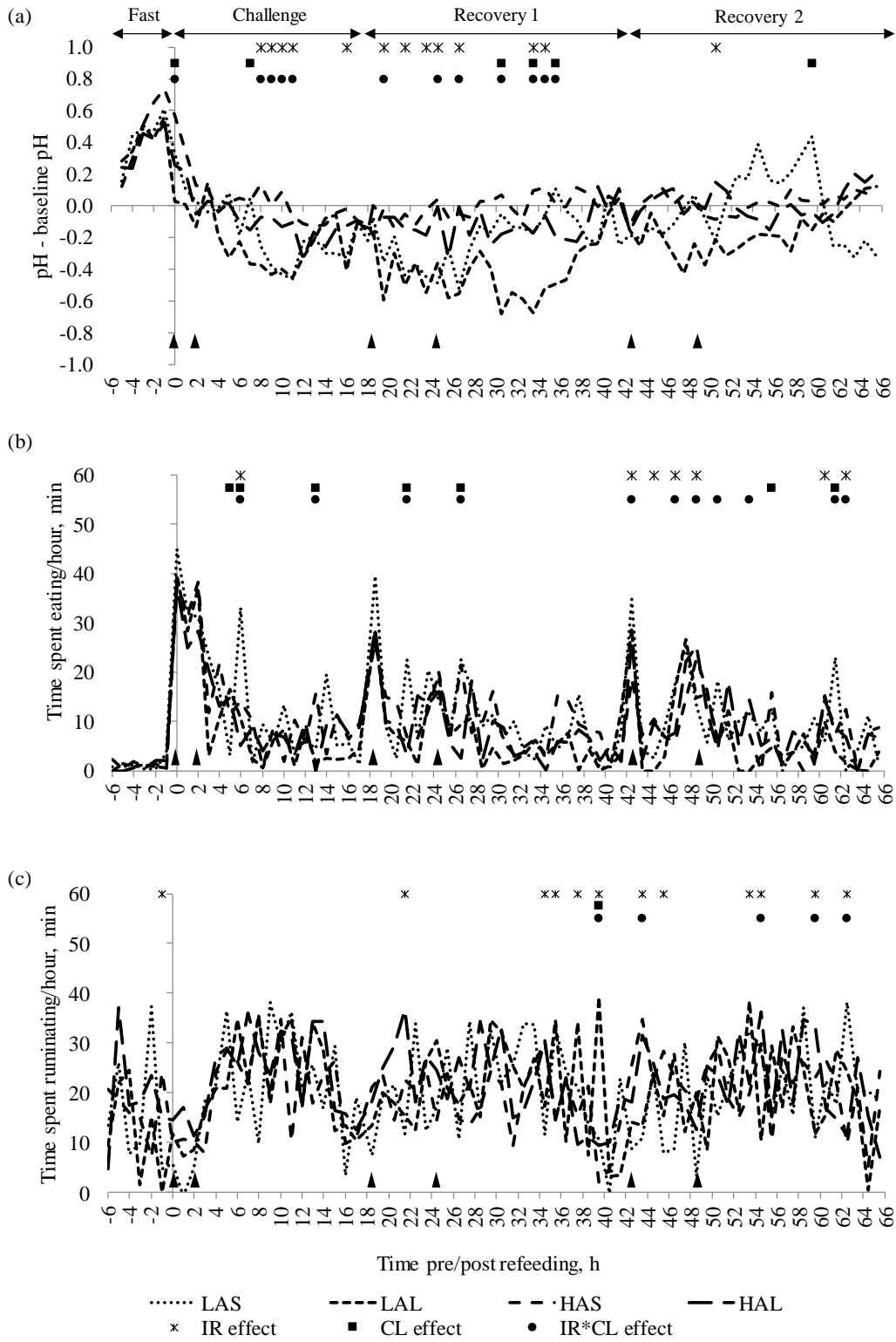
716 ^{a,b} Where there is a significant interaction, values within a row with different superscripts differ significantly at
 717 $P < 0.05$

718 * Where a value differs significantly ($P < 0.05$) from a baseline value for that treatment.

719 ¹ IR, Inclusion rate; CL, chop length; IR×CL, interaction between IR and CL.

720 ² Baseline data was collected on d 16 and the challenge day was d 18 (starting at 1000 h) of each period, during
 721 which animals spent 4.5 h of the day fasting (post a 1.5 h period during which refusals were removed early to
 722 make a total fast of 6 h) and a 17.5 h period in which feed was offered ad libitum. Recovery days 1 and 2 were
 723 the subsequent 24 h periods (d 19 and d 20 respectively both beginning 1000 h).
 724
 725





730 **Figure captions**

731

732 **Figure 1** Hourly mean (a) rumen pH (Thomson et al., 2017b), (b) time spent eating , and (c)
733 time spent ruminating of lactating dairy cows, fed a total mixed ration containing a high (HA)
734 or low (LA) concentration of alfalfa silage at a long (L) or short (S) chop length, over a 24 h
735 baseline period beginning at 1000 h (hour 1). Baseline values were measured over a single 24
736 h period two (for eating pattern) or three (for rumen pH) days prior to a feed
737 deprivation/refeeding challenge being administered. Black triangles indicate time points at
738 which half a daily allocation of feed was offered. Hours at which there was a significant effect
739 of alfalfa inclusion rate (IR), alfalfa chop length (CL) or their interaction, analysed using Mixed
740 Models procedure of SAS, are marked.

741

742 **Figure 2** Hourly mean (a) relative rumen pH, (b) time spent eating, and (c) time spent
743 ruminating of lactating dairy cows, fed a total mixed ration containing a high (HA) or low
744 (LA) concentration of alfalfa silage at a long (L) or short (S) chop length, over a 72 h period
745 beginning at 0830 h on day 18 of the period, when feed was withheld for 6 h followed by a
746 refeeding challenge at 1430 h. The hour beginning 1430 is represented by 0 on the *x* axis.
747 Black triangles indicate time points at which half a daily allowance of feed was offered.
748 Hours at which there was a significant effect of alfalfa inclusion rate (IR), alfalfa chop length
749 (CL) or their interaction, analysed using Mixed Models procedure of SAS, are marked.