

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

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

5

6 Thomson

7 Cows fed diets containing a lower concentration of alfalfa silage (replacing corn silage) experienced greater reductions in rumen pH following a six hour feed witholding/refeeding 8 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.

15	ACIDOSIS MITIGATION POTENTIAL OF ALFALFA SILAGE
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20	The effect of alfalfa (Medicago sativa) silage chop length and inclusion rate within a total
21	mixed ration on the ability of lactating dairy cows to cope with a short-term feed
22	withholding and refeeding challenge
23	
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33	Keywords: alfalfa, chop length, acidosis, feed witholding, 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 long; S or L). Dietary treatments LAS, LAL, HAS or HAL were offered to four rumen-40 cannulated Holstein dairy cattle (161 d in milk; 5th - 6th parity) in a 4 x 4 Latin square design 41 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 resulted in the greatest subclinical acidosis risk (pH < 5.8 for 355 minutes on the 1st recovery 47 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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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.

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MATERIALS AND METHODS

96 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 commercial crop of mixed varieties harvested in autumn 2014 which was chopped by the forage 112 113 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

Four multiparous Holstein dairy cows, previously prepared with rumen fistulae (Bar Diamond
rumen cannula; Parma, Idaho, USA), in mid-lactation (161 d in milk, SE ± 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 diameter and a bottom pan). A dry matter correction for material retained on each sieve was
obtained (Thomson et al. 2017a). Average particle size of the sample was calculated as
described previously (Heinrichs, 2013) and peNDF was calculated as the proportion of particles
(DM corrected) greater than the threshold length (4, 8, or 19 mm) multiplied by the NDF
concentration of the diet (Mertens, 1997; Farmer et al., 2014). The chemical and physical
composition of the diets is shown in Table 2 for reference but has been discussed in detail
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

bayesian information criterion. Compound symmetry and spatial power were the most commonstructures 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 - 6.22240 (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.

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

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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×CL; P < 0.04). Cows fed HAL diets also showed a tendency to spend the greatest time ruminating per day compared to other dietary treatments (IR×CL; P < 0.07). Hourly patterns of rumination indicate a level profile of rumination for all treatments 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 then 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 this, cows on all diets continued to eat at a fluctuating rate between 0-20 min/h (Figure 2b). Rumination pattern indicated a slightly larger reduction in rumination between 0 - 4 h post refeeding than at mealtimes on other days during the observation period for cows on all treatments. In the hour prior to refeeding cows fed LA diets ruminated very little (< 5 minutes) in comparison to cows fed HA that continued to ruminate for between 15 and 25 minutes during 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.

On recovery day 2 there were no significant differences in relative rumen pH between treatments or any hours in which treatment differences occurred although the relative rumen pH of cows fed LAL diets continued to be the lowest of the four treatments and on average 0.17 pH units below baseline values for that diet (Table 3). Over both recovery days, eating and 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, differences331 were slight and not sustained.

332

333 Induction of SARA

Taking the definition of SARA to be a period of 3 consecutive hours where rumen pH is less than 5.8, then we observed 6 bouts of SARA within the data set of which 2 bouts were in the same cow when fed the LAS diet and the remaining 4 were in 3 cows when fed the LAL diet (with 1 cow experiencing 2 separate bouts on this diet). Of these 6 bouts of SARA, 2 occurred on the day of the challenge (1 LAS and 1 LAL) and 4 occurred on recovery day 1 (1 LAS and 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).

Milk yield was reduced in cows fed LAS and LAL diets on challenge day relative to milk yield at baseline (P < 0.05), by 4.5 kg and 4.3 kg respectively, although yield was not significantly lower than that of cows fed the HA diets on the challenge day. The reduction in milk yield on LA diets on this day, also led to significant reductions in milk protein yield compared to baseline for these treatments. On recovery day 1 and 2 milk yield for all treatments was not statistically different (P > 0.05) from baseline levels. Concentrations of milk protein were unaffected by treatment and day. The milk fat yield of cows fed LAS and HAL diets on recovery day 2 was higher than baseline (P < 0.05), and furthermore the milk fat yield for HAL cows on that day was greater than that of any other dietary treatment (IR×CL; P < 0.04).

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DISCUSSION

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

During the fasting phase, prior to re-feeding, we observed increased rumen pH for all animals, 359 360 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 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 work (Chilibroste et al., 2007), meanwhile smaller forage particles and concentrates can be 459 460 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.

In the LAL diet, effects of the challenge continued throughout recovery day 1 despite a return to baseline feeding patterns, with DMI also reduced for this diet on recovery day 2. The timeline is similar to that observed previously in the literature (Oetzel, 2007) where a cow faced with a 12 hour fast followed by a refeeding challenge took 60 h for rumen pH to return to prefast 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 several479 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.

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648 Table 1 Ingredients used in diet formulation

	D	iet
Item	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;

¹long 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.

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

Ash; 357 g/kg DM starch; and 25 g/kg DM water soluble carbohydrate.

656 ³HA concentrate composition: 911 g/kg DM; 199 g/kg DM CP; 278 g/kg DM NDF; 172 g/kg DM ADF; 67 g/kg

bM 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).

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

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).

	Diet				P value ¹			
Item	LAS	LAL	HAS	HAL	SEM	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
СР	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°	5.0	0.129	0.012	0.026
4mm	165 ^a	135 ^b	187°	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 P < 0.05.

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

671 ²WSC, water soluble carbohydrate.

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

⁴ Mean particle size was determined using the recommended equation of Penn State University (Heinrichs, 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 u

Table 3 Mean relative rumen pH of lactating dairy cows fed a total mixed ration containing a

high (HA) or low (LA) concentration of alfalfa silage at a long (L) or short (S) chop length
prior to, during, and following a rumen challenge that involved a 6 hour fast followed by a

prior to, during, and following a rumen challenge that involved a 6 hour fast followed by arefeeding challenge.

	Diet				P value ¹			
Phase ²	LAS	LAL	HAS	HAL	SEM	IR	CL	IR×CL
Baseline daily rumen								
pН	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				

* Where a value differs significantly (P < 0.05) from a baseline value for that treatment (not applicable to

relative rumen pH).

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

² The fast period combines measurements from 0930 h until 1430 h on the day of the challenge during which time animals were not allowed to access feed (note, the start of the feed withdrawal was 0830 h however the time taken to insert rumen pH probes meant that data for this hour was incomplete so was not included in the

analysis). The refeeding period combines measurements from 1430 h on the day of the challenge until 0930 h the
following morning. After which the subsequent two 24 h periods are termed recovery day 1 and recovery day 2
that both begin at 1000 h.

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.

⁴ All cows spent either low or no time below pH 6.2 during the fast sub-phase and therefore this sub-phase was

removed from statutical analysis to prevent non-normaility of the remaining dataset.

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

700 Table 4 Daily mean intake, milk production and milk composition of lactating dairy cows fed

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

refeeding challenge.

	Diet					P value ¹		
Item ²	LAS	LAL	HAS	HAL	SEM	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	36.5ª	35.3ª	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 P < 0.05.

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

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

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).

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.

	Diet				<i>P</i> value			
Item ¹	LAS	LAL	HAS	HAL	SEM	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/a Basalina day	on cab	27.58	04.08	25 Ab	2.01	0.414	0.052	0.020
Challen and an	27.6	27.5"	24.2ª	35.4°	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 723 724 the subsequent 24 h periods (d 19 and d 20 respectively both beginning 1000 h).







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.