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

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

Thomson

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 challenge than those fed higher alfalfa concentration diets and also suffered greater short-term milk loss on the day of the challenge. Lower rumen pH in animals fed a long chop length compared to a shorter chop length raised questions over the effect of long forage particles in the diet during and following short-term feed deprivation. This research highlights the importance of maintaining feeding routines and ensuring adequate feed access throughout the day in dairy systems.

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

A. L. Thomson, D. J. Humphries, L. A. Crompton, and C. K. Reynolds

Sustainable Agriculture and Food Systems Research Division, Centre for Dairy Research, School of Agriculture, Policy and Development, University of Reading, Whiteknights Road, Reading, RG6 6AR

Corresponding author: C. K. Reynolds. Email: c.k.reynolds@reading.ac.uk

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

ABSTRACT

The objectives of the study were (i) to test whether 6 h feed deprivation followed by refeeding induces an acidosis challenge in dairy cattle and (ii) to quantify the acidosis challenge mitigation potential of increased alfalfa silage concentration in the diet. Alfalfa silage constituted either 25 or 75% of forage dry matter (DM) replacing corn silage (low alfalfa or 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-cannulated Holstein dairy cattle (161 d in milk; 5^{th} - 6^{th} parity) in a 4 x 4 Latin square design study with 21 d periods. Starch concentration was 69 g/kg DM higher for LA diets than HA diets. Feed was withheld for 6 h followed by ad libitum refeeding on d 18 of each period. Measurements of DM intake, milk yield and composition, rumen pH, and eating and rumination behaviour were taken on one baseline day, the challenge day and two further recovery days. 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 1^{st} recovery day). Animals fed LA produced 4.4 L less milk on the challenge day in comparison to baseline. It was concluded that short-term feed deprivation detrimentally affected rumen health and milk yield in dairy cattle normally fed ad libitum but had no effect on DM intake or milk composition. Feeding alfalfa silage in place of corn silage mitigated acidosis risk due to interrupted feed supply, likely due to a combination of lower starch concentration in HA diets, greater effective fiber concentration, and higher buffering capacity of alfalfa relative to corn silage.

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Forage Harvesting and Clamp Sampling

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

Gold containing *L. Buchneri*; 2 L/Tonne; Biotal, Cardiff, UK) to prevent heating in the clamp. Samples for chemical composition analysis (Sciantec Analytical Services, Cawood, UK) were obtained using a clamp corer. A detailed analysis of the particle length profile of the silages produced (mean 14.3 and 9.0 mm for L and S, respectively) has been published previously (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 harvester (Model FR700, New Holland Ltd, Turin, Italy; theoretical chop length of 18 mm) and ensiled as described for the alfalfa clamps (geometric mean particle length of 10 mm determined using a Penn State Particle Separator; **PSPS** [Heinrichs, 2013]).

Diets

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

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 kg (SE ± 13.9), and 7 - 9 years of age (5^{th} - 6^{th} parity), were randomly assigned to one of four

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

Experimental Routine

SARA induction protocol. Baseline measurements of all variables were taken on d 16 of each period (other than rumen pH, which was measured on d 15 because other measurements being performed on d 16 that have been reported separately). On d 18 of each period, refusals from 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 ºC for 24 h. Samples of the TMR constituents for each diet in each period were stored frozen at
174 -20 ºC until analysed for DM, nitrogen (**N**; using the macro kjeldahl method; AOAC 954.01
175 [AOAC, 2000]), ash (by combustion at 500 ºC 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).

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

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

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

Statistical analysis

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

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

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

RESULTS

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

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 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 ($P < 0.04$).

Recovery from the rumen challenge

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

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

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.

Challenge effect on intake and milk production

On the day of the challenge, DMI was similar to that consumed on baseline day (Table 4) as was daily mean time spent eating and ruminating (Table 5) despite the pattern of eating during the day being altered as described earlier. A numerical decline in intake was observed between the Challenge Day and Recovery Day 2 for cows fed LAL and HAL diets, resulting in animals 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; $P < 0.04$).

DISCUSSION

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

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

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

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

The acidosis mitigation potential of the dietary treatments

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

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

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

producing saliva to buffer the rumen, and, in line with this, HAL diets did increase rumination however we did not observe the same effect in the other diets, including LAL, where the concentration of peNDF was lower. Longer particles would have required rumination to aid digestion after ingestion, however in our study rumination was reduced during the refeeding 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 broken down without the need for further rumination chewing. This delay in rumination due to overeating may have reduced the ability of animals fed LAL to digest the forage portion of the diet. It is also possible that fiber digestion was impaired as a result of the low pH conditions affecting microbial populations (Grant and Mertens, 1992). Reductions in DMI in animals fed the long CL diets on both recovery day 1 and 2 relative to those fed short CL diets (a difference that was not observed at baseline) also supports this explanation as reducing fiber digestibility of dietary alfalfa has previously been linked to reduced appetite (Getachew et al., 2011; Fustini et al., 2017) likely due to increased feeling of satiety. However, if fiber digestion was reduced, the lack of an effect on milk composition suggests the effect was short-lived. Based on the negative effect of increasing peNDF provision through increased chop length, it is likely the mitigation effect of high alfalfa IR was attributable to the buffering capacity of alfalfa, increased rumen fill during the feed withholding phase and reduced diet starch concentration, rather than 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 pre-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 ## ACKNOWLEDGEMENTS
496 Funding for the present study was provided by Agriculture and Horticulture Development
497 Board's Dairy Division (AHDB Dairy) as part of the Soils, Forage and Grassland Partnership.
498 Thanks are given to Dr. Marcus Clauss of the University of Zurich for kindly loaning
499 rumination monitoring and to Dr. Marie Dittmann (University of Reading) for assisting in its
500 usage. Further thanks go to the technical staff at the Centre for Dairy Research (Arborfield,
501 UK) for their many inputs, including Mr. C. Green, Mr. P. Kirton, and Ms. D. Cockman.

REFERENCES

Allen, M. S. 1997. Relationship between fermentation acid production in the rumen and the requirement for physically effective fiber. *J. Dairy Sci.* 80:1447-1462. http://dx.doi.org/10.3168/jds.S0022-0302(97)76074-0

AOAC (2000). Official Methods of Analysis. 17th ed.: Association of Analytical Communities, Washington, DC, USA.

Aschenbach, J. R., Penner, G. B., Stumpff, F. and G. Gabel, 2011. Ruminant nutrition symposium: Role of fermentation acid absorption in the regulation of ruminal pH. *J. Anim. Sci.* 89:1092-1107. http://dx.doi.org/10.2527/jas.2010-3301

Chelikani, P. K., Ambrose, J. D., Keisler, D. H. and J. J. Kennelly, 2004. Effect of short-term fasting on plasma concentrations of leptin and other hormones and metabolites in dairy cattle. *Domest. Anim. Endocrinol.* 26:33-48. http://dx.doi.org/10.1016/j.domaniend.2003.08.003

Chibisa, G. E., Beauchemin, K. A. and G. B. Penner, 2016. Relative contribution of ruminal buffering systems to pH regulation in feedlot cattle fed either low- or high-forage diets. *Animal* 10:1164-72. http://dx.doi.org/10.1017/s1751731115002888

Chilibroste, P., Soca, P., Mattiauda, D. A., Bentancur, O. and P. H. Robinson, 2007. Short term fasting as a tool to design effective grazing strategies for lactating dairy cattle: a review. *Australian Journal of Experimental Agriculture* 47:1075-1084. http://dx.doi.org/10.1071/ea06130

Colman, E., Khafipour, E., Vlaeminck, B., De Baets, B., Plaizier, J. C. and V. Fievez, 2013. Grain-based versus alfalfa-based subacute ruminal acidosis induction experiments: Similarities and differences between changes in milk fatty acids. *J. Dairy Sci.* 96:4100-4111. http://dx.doi.org/10.3168/jds.2012-6109

526 DeVries, T. J., Dohme, F. and K. A. Beauchemin, 2008. Repeated ruminal acidosis challenges
527 in lactating dairy cows at high and low risk for developing acidosis: Feed sorting. *J.*
528 *Dairy Sci.* 91:3958-3967. http://dx.doi.org/10.3168/jds.2008-1347
529 Dewhurst, R. J., Fisher, W. J., Tweed, J. K. S. & Wilkins, R. J. 2003. Comparison of grass
530 and legume silage for milk production. 1. Production responses with different levels
531 of concentrate. *J. Dairy Sci.* 86:2598-2611.
532 Dohme, F., DeVries, T. J. and K. A. Beauchemin, 2008. Repeated ruminal acidosis challenges
533 in lactating dairy cows at high and low risk for developing acidosis: Ruminal pH. *J.*
534 *Dairy Sci.* 91:3554-3567. http://dx.doi.org/10.3168/jds.2008-1264
535 Farmer, E. R., H. A. Tucker, H. M. Dann, K. W. Cotanch, C. S. Mooney, A. L. Lock, K.
536 Yagi, and R. J. Grant. 2014. Effect of reducing dietary forage in lower starch diets on
537 performance, ruminal characteristics, and nutrient digestibility in lactating Holstein
538 cows. J. Dairy Sci. 97:5742-5753.
539 Fuller, K. W. 1967 Automated determination of sugars. *In:* Automation in analytical
540 chemistry. European Technicon Symposia, Paris, France, 2-4 November 1966, pp. 57-
541 61.
542 Fustini, M., Palmonari, A., Canestrari, G., Bonfante, E., Mammi, L., Pacchioli, M. T.,
543 Sniffen, G. C. J., Grant, R. J., Cotanch, K. W. and A. Formigoni, 2017. Effect of
544 undigested neutral detergent fiber content of alfalfa hay on lactating dairy cows:
545 Feeding behavior, fiber digestibility, and lactation performance. *J. Dairy Sci.*
546 100:4475-4483. http://dx.doi.org/10.3168/jds.2016-12266
547 Getachew, G., Ibanez, A. M., Pittroff, W., Dandekar, A. M., McCaslin, M., Goyal, S., Reisen,
548 P., DePeters, E. J. and D. H. Putnam, 2011. A comparative study between lignin down
549 regulated alfalfa lines and their respective unmodified controls on the nutritional

characteristics of hay. *Anim. Feed Sci. Technol.* 170:192-200. http://dx.doi.org/10.1016/j.anifeedsci.2011.09.009

Grant, R. J. and D. R. Mertens, 1992. Development of buffer systems for ph control and evaluation of ph effects on fiber digestion invitro. *J. Dairy Sci.* 75:1581-1587.

Gregorini, P., Gunter, S. A., Masino, C. A. and P. A. Beck, 2007. Effects of ruminal fill on short-term herbage intake rate and grazing dynamics of beef heifers. *Grass Forage Sci.* 62:346-354. http://dx.doi.org/10.1111/j.1365-2494.2007.00589.x

Heinrichs, A. J. 2013. The Penn State Particle Separator. Penn State Extension. Accessed Nov. 15, 2016. http://extension.psu.edu/animals/dairy/nutrition/forages/forage-quality-physical/separator

Kliem, K. E., Humphries, D. J., Reynolds, C. K., Morgan, R. and D. I. Givens, 2016. Effect of oilseed type on milk fatty acid composition of individual cows, and also bulk tank milk fatty acid composition from commercial farms. *Animal* 11:354-364. http://dx.doi.org/10.1017/S1751731116001403

Kmicikewycz, A. D. and A. J. Heinrichs, 2015. Effect of corn silage particle size and supplemental hay on rumen pH and feed preference by dairy cows fed high-starch diets. *J. Dairy Sci.* 98:373-385. http://dx.doi.org/10.3168/jds.2014-8103

Krause, K. M. and G. R. Oetzel, 2005. Inducing subacute ruminal acidosis in lactating dairy cows. *J. Dairy Sci.* 88:3633-3639. https://doi.org/10.3168/jds.S0022-0302(05)73048-4

Lechartier, C. & Peyraud, J. L. (2011). The effects of starch and rapidly degradable dry matter from concentrate on ruminal digestion in dairy cows fed corn silage-based diets with fixed forage proportion. *Journal of Dairy Science* **94**(5), 2440-2454.

573 Leonardi, C. and L. E. Armentano, 2003. Effect of quantity, quality, and length of alfalfa hay
574 on selective consumption by dairy cows. *J. Dairy Sci.* 86:557-564.
575 http://dx.doi.org/10.3168/jds.S0022-0302(03)73634-0
576 Macrae, J. C. and D. G. Armstrong, 1968. Enzyme method for determination of alpha-linked
577 glucose polymers in biological materials. *J. Sci. Food Agric.* 19:578-81.
578 http://dx.doi.org/10.1002/jsfa.2740191006
579 Mason, P. M. and D. C. Stuckey, 2016. Biofilms, bubbles and boundary layers – A new
580 approach to understanding cellulolysis in anaerobic and ruminant digestion. *Water*
581 *Res.* 104:93-100. http://dx.doi.org/10.1016/j.watres.2016.07.063
582 Maulfair, D. D. and A. J. Heinrichs, 2012. Review: Methods to measure forage and diet
583 particle size in the dairy cow. *The Professional Animal Scientist* 28:489-493.
584 Maulfair, D. D., McIntyre, K. K. and A. J. Heinrichs, 2013. Subacute ruminal acidosis and
585 total mixed ration preference in lactating dairy cows. *J. Dairy Sci.* 96:6610-6620.
586 http://dx.doi.org/10.3168/jds.2013-6771
587 McBurney, M. I., Vansoest, P. J. and L. E. Chase, 1983. Cation-exchange capacity and
588 buffering capacity of neutral-detergent fibers. *J. Sci. Food Agric.* 34:910-916.
589 http://dx.doi.org/10.1002/jsfa.2740340903
590 Mertens, D. R. 1997. Creating a system for meeting the fiber requirements of dairy cows. J.
591 Dairy Sci. 80:1463-1481.
592 Mertens, D. R. 2000. physically effective NDF and its use in dairy rations explored.
593 *Feedstuffs* 10:11-14.
594 Mertens, D. R., Allen, M., Carmany, J., Clegg, J., Davidowicz, A., Drouches, M., Frank, K.,
595 Gambin, D., Garkie, M., Gildemeister, B., Jeffress, D., Jeon, C. S., Jones, D., Kaplan,
596 D., Kim, G. N., Kobata, S., Main, D., Moua, X., Paul, B., Robertson, J., Taysom, D.,
597 Thiex, N., Williams, J. and M. Wolf, 2002. Gravimetric determination of amylase-

598 treated neutral detergent fiber in feeds with refluxing in beakers or crucibles:
599 Collaborative study. *Journal of Aoac International* 85:1217-1240.
600 Oetzel, G. 2007. Sub-acute Ruminal Acidosis in dairy Herds: Physiology, Pathophysiology,
601 Milk Fat Response, and Nutritional Management. *In,* Proceedings of the 40th meeting
602 of the American Association of Bovine Practitioners, September 17th 2007,
603 Vancouver, CA.
604 Oshita, T., Nonaka, K., Kume, S. and T. Nakui, 2004. Effects of forage type on particle size
605 distribution of ruminal digesta and faeces of non-lactating cows fed high quality
606 forage. *Livest. Prod. Sci.* 91:107-115.
607 http://dx.doi.org/10.1016/j.livprodsci.2004.07.015
608 Patterson, D. M., McGilloway, D. A., Cushnahan, A., Mayne, C. S. and A. S. Laidlaw, 1998.
609 Effect of duration of fasting period on short-term intake rates of lactating dairy cows.
610 *Animal Science* 66:299-305.
611 Plaizier, J. C., Krause, D. O., Gozho, G. N. and B. W. McBride, 2008. Subacute ruminal
612 acidosis in dairy cows: The physiological causes, incidence and consequences. *Vet. J.*
613 176:21-31. http://dx.doi.org/10.1016/j.applanim.2015.11.005
614 Reynolds, C. K., Humphries, D. J., Kirton, P., Kindermann, M., Duval, S. and W. Steinberg,
615 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and
616 nitrogen balance of lactating dairy cows. *J. Dairy Sci.* 97:3777-3789.
617 http://dx.doi.org/10.3168/jds.2013-7397
618 Robertson, J. B. and P. J. Van Soest, 1981. The detergent system of analysis and its
619 application to human foods. *In: James, W., Theander, O. (Eds), The analysis of dietary*
620 *fribe in food.* Marcer Decker Inc, NY, USA.

621 Russell, J. B., Muck, R. E. and P. J. Weimer, 2009. Quantitative analysis of cellulose
622 degradation and growth of cellulolytic bacteria in the rumen. *Fems Microbiol. Ecol.*
623 67:183-197. http://dx.doi.org/10.1111/j.1574-6941.2008.00633.x
624 Ruuska, S., Kajava, S., Mughal, M., Zehner, N. and J. Mononen, 2016. Validation of a
625 pressure sensor-based system for measuring eating, rumination and drinking behaviour
626 of dairy cattle. *Appl. Anim. Behav. Sci.* 174:19-23.
627 http://dx.doi.org/10.1016/j.applanim.2015.11.005
628 Thomson, A. L., Humphries, D. J., Jones, A. K. and C. K. Reynolds, 2017a. The effect of
629 varying proportion and chop length of alfalfa silage in a corn silage-based total mixed
630 ration on diet digestibility and milk yield in dairy cattle. *Animal [article in press]*.
631 http://dx.doi.org/10.1017/S175173111700129X
632 Thomson, A. L., Humphries, D. J., Kliem, K. E., Dittmann, M. T. and C. K. Reynolds, 2017b.
633 Effects of replacing corn silage with alfalfa silage and alfalfa silage chop length on
634 rumen function and milk fatty acid composition. *J. Dairy Sci. [article in press]*.
635 http://dx.doi.org/10.3168/jds.2017-12914
636 Toerien, C. A. and J. P. Cant, 2007. Duration of a severe feed restriction required to
637 reversibly decrease milk production in the high-producing dairy cow. *Can. J. Anim.*
638 *Sci.* 87:455-458.
639 Zebeli, Q., Tafaj, M., Steingass, H., Metzler, B. and W. Drochner, 2006. Effects of physically
640 effective fiber on digestive processes and milk fat content in early lactating dairy cows
641 fed total mixed rations. *J. Dairy Sci.* 89:651-668. http://dx.doi.org/10.3168/jds.S0022-
642 0302(06)72129-4
643 Zhang, S., Albornoz, R. I., Aschenbach, J. R., Barreda, D. R. and G. B. Penner, 2013. Short-
644 term feed restriction impairs the absorptive function of the reticulo-rumen and total

645 tract barrier function in beef cattle. *J. Anim. Sci.* 91:1685-1695.

646 http://dx.doi.org/10.2527/jas.2012-5669

647

Table 1 Ingredients used in diet formulation

	Diet	
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

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

[1] long chop alfalfa silage composition: 593 g/kg DM; 164 g/kg DM CP; 397 g/kg DM NDF; 348g/kg DM ADF; 108 g/kg DM Ash; and 10 g/kg DM water soluble carbohydrate. Short chop alfalfa silage composition: 566 g/kg 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 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.

[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 DM Ash; 247 g/kg DM Starch and 57 g/kg DM water soluble carbohydrate. LA concentrate composition: 884 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 67 g/kg DM water soluble carbohydrate.

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

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

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).

	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
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 [1]IR, Inclusion rate; CL, chop length; IR×CL, interaction between IR and CL.
671 [2] WSC, 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.

	Diet					*P* value[1]		
Phase[2]	LAS	LAL	HAS	HAL	SEM	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 [1]IR, 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 statutical anlaysis to prevent non-normaility 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 transofrmation be acheived, therefore data are presented as arithmetic
699 means.

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 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]	36.5[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

[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P < 0.05$.

* 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.

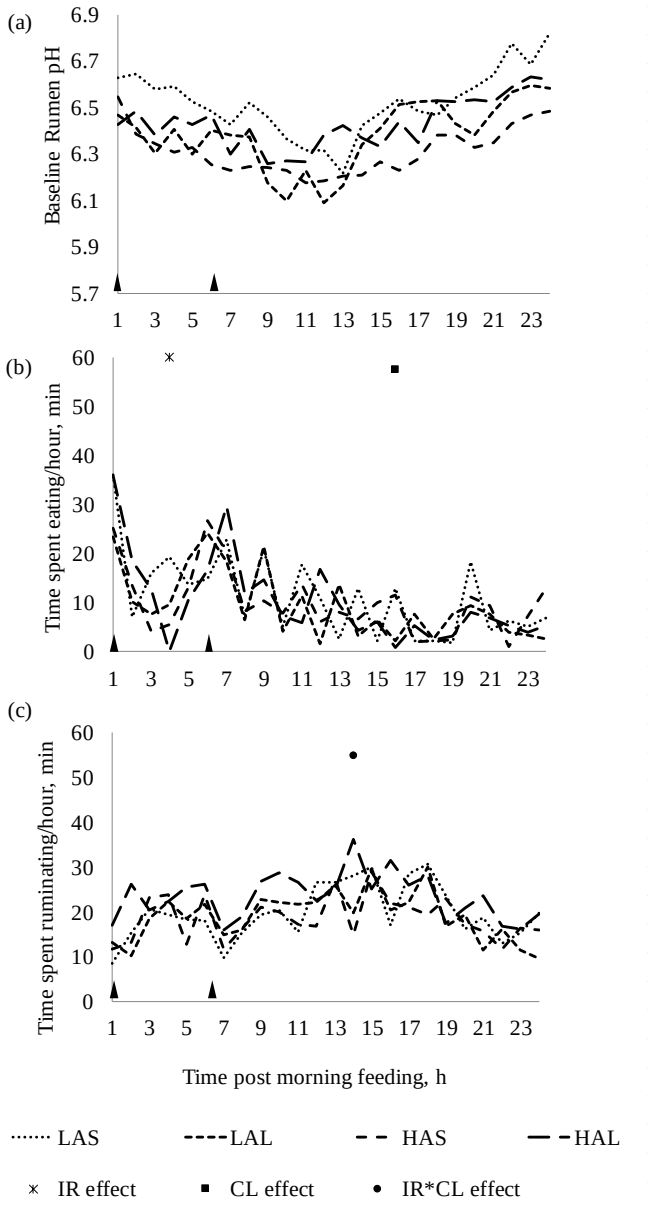
[2] Baseline data was collected on d 16 and the challenge day was d 18 (starting at 1000 h) of each period, during which animals spent 4.5 h of the day fasting (post a 1.5 h period during which refusals were removed early to make a total fast of 6 h) and a 17.5 h period in which feed was offered ad libitum. Recovery days 1 and 2 were 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					*P* 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/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

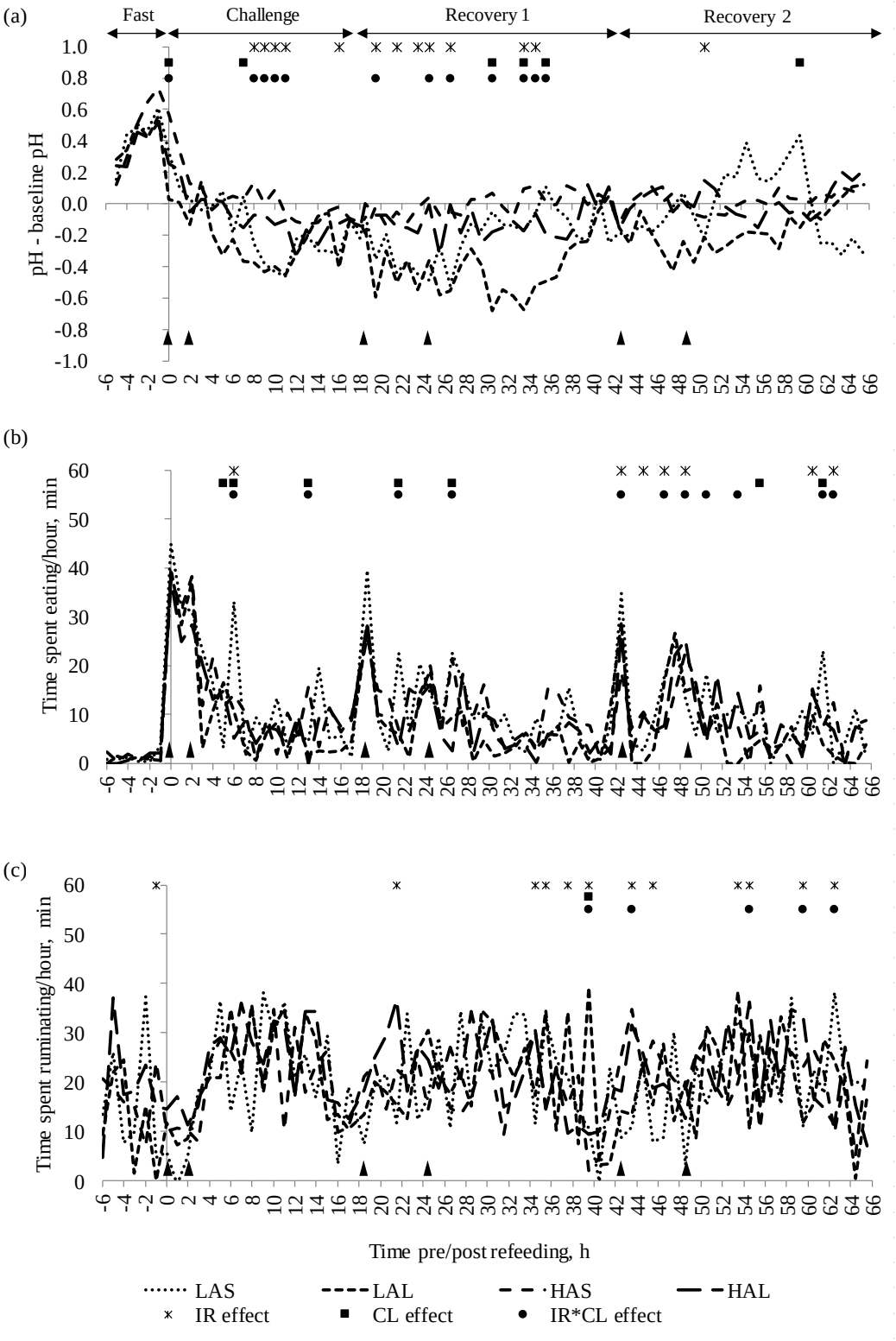
716 [a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at
717 $P < 0.05$
718 * Where a value differs significantly ($P < 0.05$) from a baseline value for that treatment.
719 [1] IR, Inclusion rate; CL, chop length; IR×CL, interaction between 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

726 Thomson **Figure 1**



727

728 Thomson **Figure 2**



729

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.