Effects of replacing maize silage with lucerne silage and lucerne silage chop length on rumen function and milk fatty acid composition

Interpretive summary

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Thomson

Including a longer chop length lucerne silage in dairy cow diets had positive effects on rumination time per unit feed intake and daily rumination time was highest when longer chop lucerne silage was fed at higher inclusion rates. Longer chopped lucerne silage may be beneficial for diets where low rumen pH is a concern. In addition, higher lucerne levels in cow diets improved milk fatty acid profile in terms of human health, potentially increasing its value for human consumption.

Effects of replacing maize silage with lucerne silage and lucerne silage chop length on rumen function and milk fatty acid composition

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ABSTRACT

The objective of this study was to investigate whether higher lucerne (*medicago sativa;* alfalfa) silage inclusion rate and longer lucerne chop length improves rumen function through increased provision of physically effective fiber, when included in a maize and lucerne silage-based total mixed ration. Diets were formulated to contain a 50:50 forage:concentrate ratio (dry matter [DM] basis) and be isonitrogenous and contain equal levels of neutral detergent fiber (320 g/kg). The forage portion of the offered diets was comprised of maize and lucerne silage DM in proportions (w/w) of either 25:75 (high lucerne; HL) or 75:25 (low lucerne; LL). Second cut lucerne was harvested and conserved as silage at either a long (L) or a short (S) chop length (geometric mean particle lengths of 9.0 and 14.3 mm, respectively). These variables were combined in a 2 x 2 factorial arrangement to give four treatments (HLL, HLS, LLL, LLS) which were fed in a 4 x 4 Latin square design study to four rumen-cannulated, multiparous, Holstein dairy cows in mid-lactation. Effects on dry matter intake (DMI), chewing behaviour, rumen volatile fatty acid (VFA) concentration, rumen pH, rumen and fecal particle size, milk production and milk fatty acid (FA) profile were measured. Longer chop length increased rumination times/kg DMI (+2.8 min/kg) relative to the S chop length, with HLL diets resulting in the most rumination chews. Rumen concentrations of total VFA, acetate, and n-valerate were higher for the HLS diet than the other three diets, while rumen propionate concentration was lowest for the HLL diet. Physically effective fiber (particles >4 mm) percentage in the rumen mat was increased when L chop length was fed regardless of lucerne inclusion rate. No effect of treatment was observed for milk yield although milk protein concentration was increased by L for the LL diet (+1.6 g/kg) and decreased by L for the HLL diet (-1.4 g/kg). Milk fat concentrations of total *cis*-18:1 (+3.7 g/100g FA) and 18:3 n-3 (+0.2 g/100g FA) were greater with HL. In conclusion, longer lucerne silage chop length increased time spent ruminating per kg DMI, but had no effect on rumen pH in the present study. Increasing dietary lucerne

inclusion rate had no effects on rumination activity or rumen pH, but decreased the ratio of n-6:n-3 polyunsaturated fatty acid concentrations in milk fat.

Keywords: lucerne, silage, rumination, rumen health, milk fatty acids, effective fiber,

INTRODUCTION

The physical form of a total mixed ration (**TMR**) can affect rumen function and the efficiency of digestion in lactating dairy cows (Allen, 1997). Lucerne silage is thought to promote rumen health as it contains high NDF and ADF concentrations as well as having a higher natural buffering capacity (based on cation exchange capacity) than silages such as maize or ryegrass (McBurney *et al.*, 1983). Factors that are considered markers of rumen health include pH, volatile fatty acid (**VFA**) profile, time spent ruminating (increasing saliva production), and consistency of the rumen mat (Weidner and Grant, 1994; Plaizier *et al.*, 2008; Zebeli *et al.*, 2012). For optimal rumen health, highly fermentable concentrate feedstuffs must be adequately balanced by forage physically effective fiber (**peNDF**) in TMR.

Physically effective fiber is defined as the NDF present within the long forage particles (Mertens, 1997) and can be increased by lengthening forage particle size. However, relationships between particle size and the rumen environment are complex and different particle sizes can play different roles, such as rumen mat formation and stimulation of rumination, although there are conflicting views within the literature on the relative effectiveness of different particle sizes. For example, Zebeli *et al.* (2012) suggested that all particles greater than 1.18 mm are effective at stimulating rumination whereas only particles greater than 8 mm form the structure of the rumen mat; whereas Heinrichs (2013) suggested that only particles greater than 4 mm should be considered physically effective. Furthermore, an oversupply of long particles has been shown, in some instances, to reduce DMI and milk

yield, possibly through excessive rumen fill (Kononoff and Heinrichs, 2003) and reduced surface area for bacterial attachment and thus digestibility (Zebeli *et al.*, 2008). Therefore, the optimum dietary inclusion rate (**IR**) of individual forages may vary depending on their chop lengths (**CL**). To this end, the main objective of this study was to evaluate the effect of two IRs of lucerne silage within a maize and lucerne silage-based TMR with two different lucerne CLs on parameters associated with rumen health and function. A secondary objective was to examine whether any changes in diet composition and rumen fermentation were associated with changes in milk yield and composition.

MATERIALS AND METHODS

Forage Harvesting and Clamp Sampling

The present study formed part of a larger trial reported previously (Thomson et al., 2017) utilizing the same dietary treatments and a larger cohort of cows. In brief, the lucerne silage used was a second cut crop, harvested in the calendar year before the present study at an estimated 10 % bloom, windrowed, and wilted for 48 h to produce a high DM concentration (576 g/kg) silage. Alternate swaths originating from the same field area were used to create two silages with differing chop length (CL), long (**L**) and short (**S**) by altering the knife arrangement of a precision chop forage harvester (Claas Jaguar, Claas Group, Harsewinkel, Germany) from a theoretical CL of 14 mm (shortest setting) to 19 mm (longest setting) which produced silages of 9.0 and 14.3 mm geometric mean particle length respectively; assessed usin a Penn State Particle Separator (**PSPS**) (Heinrichs, 2013). The L and S chopped material was ensiled separately in identical adjacent clamps. Maize silage for the study was taken from a crop of mixed varieties harvested in the year before the present study and ensiled in a concrete-walled clamp with no additive. The geometric mean particle length for the maize silage was determined to be 10 mm.

Diets

A TMR with 50:50 ratio of forage:concentrate (DM basis) was fed. The forage was comprised of maize and lucerne silage at IRs (DM basis) of either 25:75 (high lucerne; **HL**) or 75:25 (low lucerne; **LL**), respectively. The two IRs (LL or HL) and the two CL (**L** or **S**) were combined in a 2 x 2 factorial arrangement to give four treatments (**HLL, HLS, LLL, LLS**). Diets were formulated (Thomas, 2004) to be isonitrogenous (170 g CP/kg DM) and contain similar levels of NDF (320 g/kg DM) through variation in the inclusion rates of soy hulls and rapeseed meal, based on preliminary analysis of core silage samples and reference values for other components. Maize meal was included at higher rates in the HL diet to offset the reduction in maize silage starch inclusion (Table 1), however, starch concentration was still greater in LL diets than HL diets (Table 2) and predicted metabolisable energy concentration was lower in HL than LL diets (11.5 and 12.0 MJ/kg DM, respectively).

Animals

Four multiparous Holstein-Friesian dairy cows in mid lactation (161 d in milk, s.e.m. ± 23.1), producing 39.7 L/d milk yield (s.e.m ± 6.2 L), in 6^{th} parity (s.e.m ± 0.3) on average at the start of the study were used. Cows weighed 741 kg (s.e.m. ± 13.9) at the start of the study and gained 25 kg (s.e.m. ± 34.6) on average over the study duration. Cows were fitted in a previous lactation with rumen cannula (Bar Diamond, Parma, Idaho, USA). Animals were randomly assigned to one of four initial treatments according to a 4x4 Latin square design balanced for carryover effects with 21 day periods. All regulated animal procedures used were licensed and monitored by the UK Government Home Office under the Animal (Scientific Procedures) Act 1986. Animals were housed in a cubicle yard and individually fed once daily for *ad libitum* intake through Insentec RIC feeders (Insentec B.V., Marknesse, The Netherlands) during

weeks 1 and 2 of each period. Cubicles were bedded with wood shavings and continuous access to water was provided. In the final week of each period animals were housed and milked in individual tie stalls situated adjacent to the cubicle yard to facilitate sampling. Animals were given 3 days to acclimatise to the stalls before sampling began. While in tie stalls, animals were fed twice daily at 1000 and 1600 h for *ad libitum* intake (10 % refusals). Refusals were taken daily at 0930 h.

Experimental Routine

Intake and Diet analysis. Weights of feed offered and refused were taken during days 14 – 17 of each period and the DM of both determined by oven drying at 100°C for 24 h. Bulked daily grab samples of the TMR and diet components fed were frozen at -20 °C until analysed. Diet components were analysed for DM, nitrogen (**N**), ash, NDF and ADF, starch, and water soluble carbohydrates (**WSC**) as described previously (Kliem *et al.*, 2008) and concentrations for each TMR were calculated based on constituent inclusion rates. Crude protein concentration was calculated by multiplying N (g/kg DM) by 6.25. The fatty acid (**FA**) profile of the TMR was determined using dried and ground (1 mm screen; Cyclotec Mill; Foss Systems, Hillerød, Denmark). TMR samples from each cow in each period. A one step extraction-transesterification procedure was performed as described previously (Kliem *et al.*, 2008) using methyl heneicosanoate in toluene as the internal standard. A sample of each TMR was analysed in triplicate for particle size distribution using a PSPS (fresh weight basis). The PSPS used had sieves with holes measuring 4 mm, 8 mm and 19 mm in diameter and a bottom pan. Material from each sieve was collected, bulked over each of the triplicate sub-samples, and oven-dried at 60ºC for 72 h to give a DM correction. Average particle size of the sample was calculated as described previously (Heinrichs, 2013). During the sample week of each period, each cow was fitted with a chew-monitoring headcollar and supporting analytical software (Rumiwatch,

ITIN+HOCH GmbH, Fütterungstechnik, Liestal, Switzerland) capable of detecting jaw movements through pressure on the noseband and categorising them as either eating, ruminating or drinking (Ruuska *et al.*, 2016).

Milk Yield and Composition. Cows were milked twice daily at 0630h and 1630h. Representative 30 ml milk samples, preserved using potassium dichromate, were taken at six consecutive milkings between days 15 and 18 of each period and 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). On day 18 a further sample was taken at each milking and stored at -20ºC before being thawed, pooled according to milk yield, and analysed for FA profile. Lipid was extracted from 1 ml of milk using ethanol, hexane and diethylether and transesterified to FA methyl esters (**FAME**) using methanolic sodium methoxide with subsequent FAME separation using a gas chromatograph (**GC**; 3400, Varian Inc., Palo Alto, CA, USA) equipped with a flame-ionisation detector as described previously (Kliem *et al.*, 2008). Concentrations of FA are presented as g/100g total FA. Apparent recovery rates for 18:3 n-3 and 18:2 n-6 were calculated as the daily yield of these FA in milk as a percentage of the daily amount ingested in feed based on mean DMI, milk yield and milk composition data for each cow in each period.

Rumen and Fecal Sampling. On day 15 of the treatment period 100ml samples of rumen liquor were taken at just prior to feeding and then at 0.5, 1.0, 1.5, 2.0 h post feeding and at each subsequent hour until 2200 h making a total of 15 samples. Rumen samples were collected by aspiration using a 50 ml catheter tip syringe through a coarse filtered sample tube as described previously (Dittmann *et al.*, 2016). The fluid was mixed immediately and the pH was measured (HANNA instruments, Woonsocket) after which a subsample was acidified to pH <2 using

concentrated H_2SO_4 and stored at -20 ºC prior to analysis for ammonia (NH_3) using a segmented flow analyzer as described previously (Sutton *et al.*, 2000). A further non-acidified subsample was immediately placed in a freezer (-20 ºC) until analysed for VFA concentration using GC (3400, Varian Inc., Palo Alto, CA, USA) procedures as described previously (Erwin *et al.*, 1961).

On day 15 and 16 of each period spot samples (approx. 500 g) of feces voided were collected and bulked, until sufficient material (approx. 3 kg daily) was obtained. Up to six samples were obtained per day providing that the feces were uncontaminated by bedding. Furthermore, on day 17, a grab sample of the rumen mat (approx. 3 kg) was obtained at 4 h post feeding. The sample was taken by vertically removing handfuls of material until the liquid phase in the ventral rumen was reached, with each handful immediately placed into a collection bucket. Bulked samples of rumen and fecal material were mixed and a subsample of each was oven-dried at 60 ºC for 72 h. Subsamples were then sieved using an adaptation of the wet sieving procedure described by Kononoff and Heinrichs (2003) using three sieves of 1, 2 and 4 mm diameter. Sieves were manually shaken while held under a cold water tap at a fixed flow rate for 30 seconds. Any material passing through the 1 mm sieve could not be retained and was assumed to be very small or soluble – a value for this was obtained as the difference between the starting dry weight and the combined dry weight of the other three fractions. A minimum of four replicates per sample were sieved with resulting material on each sieve being collected and bulked across replicates. Material from each sieve was analyzed for DM and NDF concentration by oven drying each fraction at 60 ºC for 72 h followed by subsequent determination of NDF concentration as described for feed samples.

Rumen pH. A weighted (300 g) indwelling pH meter (Sentix 41-3 probe, WTW Trifthof, Weilheim, Upper Bavaria) inserted through the bung of the rumen cannula and anchored to 50

cm of nylon cord was placed within the rumen of each animal for 24 h beginning just prior to feeding on day 15 of each period until refusals were removed at 0930 h on day 16. The probe was calibrated before every insertion and checked for drift after use through immersion in standard solutions of pH 4 and 7. The pH probe was attached to a datalogger (ph340i, WTW, Trifthof, Weilheim, Upper Bavaria) with readings recorded every 10 minutes. Readings were further averaged over each hour for analysis.

Data Analysis

Feed conversion efficiency (**FCE**) was calculated as estimated milk energy yield (Tyrrell and Reid, 1965) divided by DMI. Dietary peNDF was calculated as the percentage of particles greater than either 4, 8 or 19 mm (measured using a PSPS) multiplied by total dietary NDF for each TMR in each period on a DM basis. All measured daily mean variables were averaged for each cow and treatment combination and analysed using mixed models procedures of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) to determine fixed effects of period, lucerne IR, lucerne CL, and IR and CL interaction, and random effects of cow, with period included as a repeated effect. For rumen VFA, NH_3 and pH measurements the effect of time (T) was also included in the model as a repeated effect, with cow (period) as the subject, and tested for interactions with IR and CL (TxIR, TxCL, TxIRxCL) with the 'SLICE' option used to test for treatment effects at each time point. For each variable the covariance structure giving the best fit for repeated effects of period or of time were selected according to the structure giving the smallest Bayesian Information Criterion (from compound symmetry, heterogeneous compound symmetry, auto-regressive, heterogeneous autoregressive, unstructured, or spatial power covariance; spatial power was always used for time-based data where there were unequal spacing between spot measurements). Data from one cow in period 4 was removed as her DMI

and milk yield did not fully recover following mastitis that occurred during the adaptation period.

RESULTS

Diet Composition

Concentrations of DM, OM and ADF (Table 2) were higher in HL diets than LL diets (all $P < 0.03$) while starch and water soluble carbohydrate concentrations were lower (both $P < 0.04$) despite inclusion of maize meal in the HL diets. The HL diets also had lower concentrations of *cis*-9 18:1 ($P < 0.03$) and 18:2 n-6 FA ($P < 0.01$), while the concentration of 18:3 n-3 was higher ($P < 0.02$). Furthermore, for HL diets there was a significant effect of CL on 18:3 n-3 concentration, with a greater concentration measured in HLS diets than HLL diets, whereas CL had no effect in LL diets (IR x CL interaction, $P < 0.03$). Overall the HL diets contained less total FA than LL diets ($P < 0.04$). The HLL diet contained more than double the concentration of very long particles (>19 mm) relative to the other diets, an effect mirrored by concentrations of $peNDF_{>19mm}$ (IR x CL interaction, $P < 0.009$). The concentration of $peNDF_{>8mm}$ was mainly influenced by CL, with L diets containing 3.5 % more $peNDF_{>8mm}$ than S diets ($P < 0.03$), while HL and L both increased $peNDF_{>4mm}$ ($P < 0.004$). A longer lucerne CL also increased concentration of ADF ($P < 0.007$) and decreased WSC concentration ($P < 0.02$) relative to a shorter CL.

Rumination Patterns and Rumen Parameters

Cows fed the L diets spent more time ruminating per unit DMI ($P < 0.04$) and also tended to chew a greater number of times during rumination ($P < 0.09$) than when fed S diets. The greatest number of rumination chews per day was observed for the HLL diet (CL x IR

interaction, $P < 0.05$), whereas the greatest eating chews per day was observed for the LLS diet (IR x CL interaction, $P < 0.05$), which contained the least physically effectively fiber.

Over 12 h post feeding, HL diets increased the concentration of rumen NH_3 relative to LL diets ($P < 0.001$; Table 4). Additionally, both CL and IR affected the rumen VFA profile in this time period, with LL diets increasing concentration of propionate ($P < 0.01$) and i-butyrate ($P < 0.007$) and reducing acetate:propionate ratio ($P < 0.001$), while total VFA and n-butyrate concentration were greater in S diets than L diets ($P < 0.03$). The HLS diet resulted in higher concentrations of total VFA, acetate and n-valerate concentrations than the other three diets as indicated by CL x IR interactions (all $P < 0.009$; Table 4). In the case of propionate, LLS, LLL and HLS diets showed similar concentrations with an average of 25 mM whereas feeding the HLL diet resulted in a lower propionate concentration of 20 mM (CL x IR interaction, $P < 0.004$).

Rumen propionate concentration was consistently lower throughout the 12 h time period with the HLL diet compared with the other diets with significant differences recorded at 1300, 2000, 2100, and 2200 h ($P < 0.05$, figure 1a). The HLS diet resulted in a higher rumen concentration of both acetate and total VFA at certain time points, but the effect was inconsistent (Figure 1b, 1c). Despite these effects on VFA profile, there were no significant effect of treatments on average, minimum, or maximum rumen pH measured over the same 24 h period, although, the mean pH during 12 h post-feeding did show a tendency for HLL diets to have an elevated pH in comparison to the means of the other three diets (IR x CL interaction, $P < 0.06$; Figure 1d).

For samples of rumen mat, feeding L increased the DM percentage of large particles (>4mm) by 14 % units ($P < 0.002$; Table 5) and decreased the DM percentage of medium particles (2-4 mm) by 3.8 % units ($P < 0.002$). The percentage of medium length particles in the mat was greatest when the HLS diet was fed (IR x CL interaction, $P < 0.008$ on a DM basis

and < 0.001 on an NDF basis). On an NDF basis, feeding HL diets led to more small particles (1-2 mm) retained within the rumen mat than LL diets ($P < 0.05$). Fecal particle structure was largely unaffected by treatment diets, except for an increase in NDF retained on the 1 mm sieve when HL versus LL diets were fed ($P < 0.03$). There was also a tendency for cows fed HL diets to void feces with a higher DM concentration ($P < 0.06$).

Intake, Milk Yield and Composition

There was no consistent effect of CL or IR on DMI although LLL resulted in a lower DMI relative to LLS (IR x CL interaction, $P < 0.03$; Table 6). Milk yield and the yield of milk solids was not affected by diet, although milk protein yield tended to be greater for LL versus HL diets ($P < 0.063$). Milk protein concentration was increased by longer CL with the LL diets and decreased by longer CL with the HL diets (CL x IR interaction, $P < 0.001$), whilst overall milk protein concentration was higher for LL than HL diets ($P < 0.033$). Inclusion rate affected concentrations of some milk FA (Table 7). Milk concentrations of total *cis*-18:1 isomers (mainly comprised of *cis*-9 18:1) and 18:3 n-3 were both higher for diets containing a higher IR (both $P < 0.006$). Cows fed HL diets also produced milk with higher 4:0 and lower 10:0 concentration relative to cows fed LL (both $P < 0.04$). The apparent recovery of 18:2 n-6 was increased by 3.8 % where HL diets were fed ($P < 0.04$). Total MUFA concentration was higher in the HLL diet than in the LLL or HLS diet (IR x CL interaction $P < 0.04$). A longer CL of lucerne tended to increase 18:1 and decrease 18:3 n-3 concentrations in comparison to the shorter CL (both $P < 0.07$). In addition, longer CL increased n-6:n-3 PUFA concentration ratio in milk fat ($P < 0.018$).

DISCUSSION

Diet Physical Properties and Rumen Function

Particle size distribution in the diet was affected by both lucerne IR and CL (Table 2). Heinrichs (2013) suggested that all dietary particles greater than 4 mm contribute to formation of the rumen mat however the model of Zebeli *et al.* (2012) proposes that only particles greater than 8 mm in length promote increased rumination. In this study, both HL and L increased diet peNDF measured in all particles sizes. The longer lucerne CL, but not lucerne IR, tended to increase both rumination chews per unit DMI and the concentration of particles >4 mm within the rumen mat which suggests agreement with the proposed model of Zebeli *et al.* (2012) and is consistent with findings of a number of studies that have examined the effect of CL of the diet (Beauchemin *et al.*, 1994; Clark and Armentano, 2002; Teimouri Yansari *et al.*, 2004). From these data it could be concluded that although HL inclusion did increase effective fiber concentration in the diet relative to LL, the effect of CL on rumination was greater than the effect of IR. This may be due in part to the lack of effect of IR on particles >4 mm in the rumen mat. In the case of rumination, numbers of chews and time spent ruminating were highest for the HLL diet (although the effect did not always reach statistical significance). However, rumination activity did not always correlate with peNDF concentration as might be expected. For example, cows fed the LLS diet chewed more when eating than cows fed the other diets and also chewed more when ruminating relative to both LLL and HLS diets. Differences might be partly attributed to increased uniformity of the diet altering particle prehension or preventing cows sorting against the longer particles as has been observed previously in CL studies (Kmicikewycz and Heinrichs, 2015). Regardless of the cause, the higher chewing activity in the LLS diet could have led to a higher saliva production, which could explain why the daily mean pH of cows fed this diet were comparatively high.

Rumen propionate concentrations in cows fed the HLL diet were consistently lower than in cows fed the other diets over a 12 h period after morning feeding. The reduction in propionate concentration might be attributed to reduced starch intake combined with longer

CL reducing the rate of production or that the increased rumination seen in cows fed HLL diets led to higher levels of saliva production thus increasing the provision of bicarbonate, which is involved in the removal of VFAs from the rumen by epithelial absorption (Dijkstra *et al.*, 2012). In contrast cows fed the HLS diet had the highest total VFA concentration and also the highest acetate concentration. The HLS diet, with a higher concentration of short particles, may have facilitated a greater rate of fermentation in the rumen leading to a more rapid supply of volatile fatty acids (Allen, 1997). The observation that the rumen mat of cows fed HLS diets had a greater proportion of 2-4 mm particles and a lower concentration of particles >4 mm than other diets at 4 h after feeding may support this explanation. Particles >4 mm within the rumen mat are thought to play a role in trapping smaller particles within the rumen for longer, allowing increased digestion of nutrients (Zebeli *et al.*, 2012).

Positive changes in rumination, physically effective fiber concentration, and rumen mat structure are often associated with a rise in rumen pH (Zebeli *et al.*, 2006). However, in the present study, no effects of lucerne silage IR or CL on daily mean rumen pH were observed although there was a tendency for increased pH over the first 12 h post-feeding in the HLL diet. This contrasts with numerous studies investigating CL that have reported decreased rumen pH when average particle size is decreased (Kononoff and Heinrichs, 2003; Bhandari *et al.*, 2008) although the effect is not always seen (Beauchemin and Yang, 2005; Suarez-Mena *et al.*, 2013), perhaps indicating that a plateau can be reached above which greater inclusion of peNDF in the diet ceases to affect rumen pH. This is in agreement with Zebeli *et al.* (2006) who also observed a plateau in the relationship between peNDF and rumen pH at 30 % peNDF in diet DM, however the threshold length for peNDF measured using PSPS has since been increased from 1.18 mm to 4 mm preventing a fair comparison with the present study. Similar to our study, altered ratios of maize silage to lucerne hay in the diet had no effect on rumen pH in lactating dairy cows in a study by Akbari-Afjani *et al.* (2014). Differences in the response

of rumen pH to lucerne silage IR and CL may be influenced by the extent to which the basal diet represents a rumen pH challenge. For instance, in this study daily mean rumen pH was above 6.3 and minimum values were above 5.9, indicating that the threshold for SARA was never reached, even though the LLS, LLL and HLS diets contained less than 18 % $peNDF_{>8mm}$ which is proposed to be the threshold below which SARA risk increases (Zebeli *et al.*, 2010). Zebeli *et al.* (2010) proposes that SARA risk can be avoided even in high starch diets by balancing rumen degradable starch (RDS) concentration with an equal or greater concentration of peNDF. In this study the ratio of $peNDF_{>4mm}$ to estimated total RDS supply was 0.96, 1.11, 1.54 and 1.60 for the LLS, LLL, HLS and HLL diets, respectively, which may explain why there was little effect of treatment diets on rumen pH. However, the range from minimum to maximum pH was greatest in the LLS diet (0.87 pH units) with a smaller range observed in HL diets (0.67 pH units on average), which could be a consequence of the higher buffering capacity of the lucerne silage.

Effect on Milk Yield and Composition

Diets used in this study had little effect on milk or milk constituent yield. The decrease in milk protein concentration for the HLL versus HLS diets was observed when these diets were fed to a larger group of cows (Thomson et al, 2017) and reflected the lower starch concentration provided by diets with high concentration of lucerne silage. Milk yield and milk composition can also be negatively affected by lower digestibility of lucerne-based diets leading to a reduction of fermentable energy to drive microbial protein synthesis (Sinclair *et al.*, 2015).

Substituting legumes for other forages such as grass has been shown to alter the FA profile of milk in ways which can be advantageous to milk quality (Dewhurst *et al.*, 2006). Lucerne silage contains a higher concentration of 18:3 n-3 than maize silage (Onetti *et al.*, 2002; Benchaar *et al.*, 2007), which was consistent with the present study. The higher

concentration of 18:3 n-3 FA in HL diets was probably the main reason for the higher concentration of 18:3 n-3 in milk fat from cows fed HL diets (Khiaosa-ard *et al.*, 2015), which led to a reduced n-6:n-3 FA ratio. The reduction in the n-6:n-3 FA ratio in milk fat observed in the present study is consistent with previous reports of effects of increased dietary lucerne IR (Dhiman *et al.*, 1999; Sinclair *et al.*, 2015). For instance, in the study of Sinclair *et al.* (2015), the concentration of 18:3 n-3 increased by 0.6 g/kg FA when lucerne silage IR was increased from 40 to 60 % of offered forage DM; which is in line with the present study where a larger increase in lucerne concentration (50 % units of forage DM) resulted in a 1.6 g/kg FA increase in 18:3 n-3 concentration in milk fat. In the case of 18:2 n-6, HL diets supplied a lower concentration than LL diets, however due to a greater recovery rate in these diets, 18:2 n-6 concentration was numerically greater in the milk from cows fed HL diets. Khiaosa-ard *et al.* (2015) proposed that recovery rate of 18:2 n-6 is exponentially increased where a low rate of this FA (<10 g/kg of diet DM) is supplied by the diet. As HL diets were below the 10g/kg 18:2 n-6 threshold this might explain increased efficiency of dietary 18:2 n-6 transfer to milk fat observed in the present study. High lucerne silage diets also increased milk fat *cis*-9 18:1 concentrations despite containing less *cis*-9 18:1 per kg DM. Around 50 % of *cis*-9 18:1 in milk fat is derived from the action of mammary Δ^9 desaturase on 18:0 from the circulation (Enjalbert *et al.*, 1998), which arises following complete biohydrogenation of dietary unsaturated 18-carbon FA. This is reflected in the numerically higher concentration of 18:0 in milk fat from cows fed HL diets. As HL diets contained less total unsaturated 18-carbon FA than LL diets, the difference in *cis*-9 18:1 is probably due to the rumen environment promoted by the HL diets, which likely favoured complete biohydrogenation. There was relatively little effect of CL on the milk fatty acid profile. The ratio of n-6:n-3 PUFA concentrations in milk fat was decreased for S compared to L diets. In addition, there were tendencies for lower concentration of 18:3 n-3 and higher concentration of 18:1 *c*9 when L was fed relative to S. This may suggest

399 that L diets create a rumen environment that leads to more complete biohydrogenation of
400 dietary PUFA, perhaps through a reduction of rumen passage rate. Similarly, Dhiman *et al.*
401 (1999) observed a small increase in total 18:1 isomer concentration (+2.1 g/kg FA) when coarse
402 alfalfa hay was fed in place of finely ground alfalfa hay, which is similar to the present study
403 where total 18:1 concentration increased by 2.8 g/kg FA when L was fed in place of S.
404

405 **CONCLUSION**

406 In the present study, feeding a higher lucerne silage IR and longer lucerne silage CL increased
407 the dietary concentration of peNDF. Longer lucerne silage CL, but not greater IR, increased
408 $peNDF_{>4mm}$ in the rumen mat and rumination activity. However, there were no effects of
409 dietary treatments on rumen pH, despite LL diets being higher in starch and lower in physically
410 effective fiber. Whilst lucerne silage IR had no effects on rumination activity or rumen pH in
411 the present study, greater IR and shorter CL both decreased the ratio of n-6:n-3 PUFA
412 concentrations in milk fat.
413

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421

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Table 1 Ingredients used to create experimental total mixed rations.

	Diet[1]	
Item	LL	HL
Ingredients, g/kg DM		
Lucerne silage	125	375
Maize silage	375	125
Concentrate blend		
Cracked Wheat	80	80
Maize 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	10	10
Megalac[2]	15	15

[1] LL = low lucerne diet; HL = high lucerne diet;

[2] 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 (4:1 ratio with maize silage, DM basis; HL) or low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Chemical composition, g/kg DM								
DM, g/kg	555	571	610	632	5.0	0.022	0.065	0.364
OM	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
Fatty acid profile, g/kg DM								
16:0	8.82^{a}	7.13^{b}	7.21^{b}	7.51^{b}	0.391	0.089	0.061	0.014
18:0	1.03	1.03	0.98	0.86	0.091	0.247	0.453	0.494
18:1 *c*9	8.43	8.75	6.76	4.82	0.828	0.023	0.302	0.201
18:2 n-6	10.9	10.6	8.9	7.0	0.74	0.003	0.077	0.182
18:3 n-3	1.51^{c}	1.73^{c}	2.40^{a}	2.07^{b}	0.034	0.012	0.336	0.027
Total fatty acids	33.3	33.3	29.4	23.5	2.42	0.038	0.218	0.248
Particle size distribution[3]								
Material retained, %DM								
19mm	3.2^{b}	5.0^{b}	5.3^{b}	12.1^{a}	0.75	0.001	0.001	0.007
8mm	36.4^{c}	41.9^{a}	37.4^{bc}	39.1^{b}	0.50	0.129	0.012	0.026
4mm	16.5^{b}	13.5^{c}	18.7^{a}	12.6^{c}	0.24	0.033	0.001	0.004
Bottom pan	43.8	39.8	37.9	36.3	0.50	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], %DM								
$peNDF_{>19mm}$	1.03^{b}	1.64^{b}	1.74	4.04^{a}	0.268	0.001	0.001	0.009
$peNDF_{>8mm}$	12.3	14.8	13.8	18.2	0.27	0.056	0.030	0.137
$peNDF_{>4mm}$	17.2	19.9	20.5	21.3	0.38	0.003	0.004	0.051
n	3	4	4	4				

[1] IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL;
[2] WSC = water soluble carbohydrate.
[3] Particle size distribution measured using a Penn State Particle Separator with three sieves: 19, 8 and 4mm diameter.
[4] Mean particle size was determined as described by Heinrichs (2013).
[5] peNDF determined as the proportion of particles (DM basis) greater than the threshold length (specified in subscript) multiplied by NDF concentration.
[a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at $P<0.05$.

Table 3 Rumination activity and eating patterns of lactating dairy cows fed total mixed rations containing a high (4:1 ratio with maize silage, DM basis; HL) or low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Time spend								
Ruminating, min/d	447	453	445	566	41.9	0.106	0.077	0.097
Eating, min/d	290	223	239	221	34.8	0.362	0.205	0.441
Ruminating, min/kg DMI	19.4	20.7	19.0	23.4	1.18	0.341	0.035	0.140
Eating, min/kg DMI	12.9	10.2	10.0	9.5	1.89	0.132	0.208	0.375
Number of chews								
Ruminating, ‘000/d	27.9^{a}	26.9^{b}	25.6^{c}	34.9^{a}	2.69	0.192	0.081	0.043
Eating, ‘000/d	19.0^{a}	12.3^{b}	12.6^{b}	13.1^{ab}	2.37	0.093	0.081	0.050
Ruminating, ‘000/kgDMI	1.27	1.24	1.09	1.40	0.090	0.882	0.147	0.097
Eating, ‘000/kgDMI	0.84^{a}	0.55^{b}	0.54^{b}	0.58^{b}	0.123	0.052	0.087	0.042
n	3	4	4	4				

[1] IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL.

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

570 **Table 4** Rumen pH, volatile fatty acid profile and ammonia concentration of lactating dairy
571 cows fed total mixed rations containing a high (4:1 ratio with maize silage, DM basis; HL) or
572 low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19
573 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Manual samples[2]								
Ammonia, mg/L	90	96	156	141	14.1	0.001	0.664	0.331
Rumen pH	6.25	6.24	6.22	6.38	0.141	0.186	0.103	0.058
VFA Profile, mM								
Total VFA	118^{b}	121^{b}	130^{a}	114^{b}	7.11	0.296	0.030	0.002
Acetate	72.3^{b}	74.4^{b}	81.6^{a}	74.2^{b}	3.39	0.013	0.132	0.009
Propionate	24.1^{a}	25.9^{a}	24.5^{a}	20.2^{b}	2.58	0.009	0.181	0.004
n-Butyrate	16.4	15.1	17.8	15.0	1.52	0.253	0.002	0.185
i-Butyrate	0.70	0.77	1.03	0.87	0.064	0.006	0.526	0.097
n-Valerate	1.82^{b}	2.07^{b}	2.55^{a}	1.91^{b}	0.201	0.009	0.062	0.001
i-Valerate	1.35	1.41	1.65	1.19	0.143	0.737	0.136	0.054
Caproate	0.71	1.00	0.95	0.74	0.161	0.927	0.760	0.067
Acetate:Propionate	3.10	3.05	3.37	3.74	0.257	0.001	0.155	0.079
n	3	4	4	4				
24h pH measurements[3],								
Average pH	6.52	6.38	6.31	6.43	0.281	0.764	0.973	0.622
Maximum pH	6.84	6.70	6.68	6.81	0.148	0.702	0.916	0.142
Minimum pH	5.97	5.88	6.02	6.14	0.127	0.254	0.378	0.686
n	3	4	4	4				

574 [1] IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL; VFA = volatile fatty acids.
575 [2] The least squares mean of measurements taken at -0.5, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12h post
576 morning feeding in each cow in each period.
577 [3] pH measurements were taken every 10 minutes over a 24h period with an indwelling pH meter with data
578 averaged every hour for analysis.
579 [a,b] Values within a row with different superscripts differ significantly at $P<0.05$.
580

Table 5 Rumen and fecal particle size distribution of lactating dairy cows fed total mixed rations containing a high (4:1 ratio with maize silage, DM basis; HL) or low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Rumen particle profile								
Total DM, g/kg	182	149	166	159	10.3	0.789	0.116	0.250
Total NDF, g/kg DM	583	579	584	550	14.2	0.385	0.183	0.259
Material retained, %DM								
<1mm or soluble[2]	36.1	28.6	30.0	28.5	3.45	0.297	0.148	0.311
1mm – 2mm	14.5	15.0	19.3	15.7	2.02	0.400	0.593	0.500
2mm – 4mm	14.8[b]	12.3[c]	17.1[a]	12.0[c]	0.46	0.021	0.002	0.008
>4mm	34.6	44.8	33.8	43.3	2.27	0.482	0.002	0.840
Material retained, %NDF								
<1mm or soluble[2]	19.9	9.2	10.5	7.7	3.63	0.129	0.072	0.259
1mm – 2mm	19.2	17.8	24.3	21.8	1.95	0.045	0.280	0.764
2mm – 4mm	17.7[b]	15.5[c]	22.0[a]	14.9[c]	0.61	0.003	0.001	0.001
>4mm	43.4	57.4	42.7	56.2	2.80	0.662	0.001	0.901
Fecal particle profile								
Total DM, g/kg	147	145	156	157	0.68	0.060	0.834	0.752
Total NDF, g/kg DM	474	488	442	433	1.57	0.168	0.855	0.486
Material retained, %DM								
<1mm or soluble[2]	51.3	51.7	54.1	53.1	2.51	0.323	0.878	0.760
1mm – 2mm	18.5	21.4	21.6	22.7	1.45	0.075	0.118	0.425
2mm – 4mm	11.5	13.3	14.1	11.4	1.72	0.845	0.733	0.216
>4mm	14.6	13.1	12.7	12.9	0.85	0.143	0.213	0.111
Material retained, %NDF								
<1mm or soluble[2]	31.7	33.6	22.6	25.8	5.09	0.152	0.637	0.893
1mm – 2mm	30.7	33.7	39.9	40.4	2.76	0.024	0.484	0.624
2mm – 4mm	18.8	17.6	22.4	20.1	1.37	0.058	0.187	0.587
>4mm	14.8	13.4	16.0	15.2	6.96	0.344	0.416	0.817
n	3	4	4	4				

[1] IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL.

[2] >1mm or soluble material calculated as the starting amount minus material retained on each of the three sieves.

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

589 **Table 6** Intake, milk yield, milk composition and feed conversion efficiency of lactating
590 dairy cows fed total mixed rations containing a high (4:1 ratio with maize silage, DM basis;
591 HL) or low (1:4 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a
592 long (19 mm; L) or short (14mm; S) chop length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
DMI, kg/d	25.1[a]	22.4[b]	23.1[ab]	24.4[ab]	1.08	0.991	0.152	0.027
Milk yield, kg/d	29.3	29.4	29.1	28.4	3.93	0.519	0.704	0.646
Milk composition, g/kg								
Fat	34.4	34.9	35.7	34.6	1.35	0.648	0.798	0.472
Protein	30.0[c]	31.6[a]	31.2[b]	29.8[c]	0.64	0.033	0.560	0.001
Lactose	45.0	44.6	45.5	44.6	0.60	0.572	0.190	0.642
Casein	22.8[b]	24.4[a]	23.9[a]	22.2[b]	0.88	0.051	0.763	0.019
Urea, mg/kg	276	257	270	281	40.6	0.591	0.799	0.393
Component yield, kg/d								
Fat	1.02	1.02	1.00	0.98	0.139	0.215	0.519	0.745
Protein	0.92	0.93	0.86	0.83	0.120	0.063	0.850	0.520
Lactose	1.32	1.37	1.29	1.22	0.184	0.172	0.859	0.401
Casein	0.70	0.71	0.66	0.64	0.095	0.096	0.773	0.570
n	3	4	4	4				

593 [1]IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL;
594 [2] FCE = feed conversion efficiency.
595 [a,b] Where there is a significant interaction, values within a row with different superscripts differ significantly at
596 $P<0.05$.
597

598

599 **Table 7** Milk fatty acid profile of lactating dairy cows fed total mixed rations containing a
600 high (4:1 ratio with maize silage, DM basis; HL) or low (1:4 ratio with maize silage, DM
601 basis; LL) concentration of lucerne silage at a long (19 mm; L) or short (14mm; S) chop
602 length.

	Diet					*P* value[1]		
Item	LLS	LLL	HLS	HLL	SEM	IR	CL	IRxCL
Fatty acid profile, g/100g FA								
4:0	2.49	2.49	2.75	2.76	0.134	0.001	0.894	0.871
6:0	1.71	1.69	1.73	1.69	0.054	0.670	0.894	0.765
8:0	1.11	1.07	1.02	0.99	0.054	0.168	0.426	0.987
10:0	2.73	2.63	2.44	2.27	0.194	0.034	0.300	0.750
12:0	3.36	3.28	2.19	2.70	0.656	0.159	0.700	0.603
14:0	11.3	11.2	10.4	10.7	0.30	0.084	0.631	0.307
16:0	32.0	32.9	31.7	32.4	1.17	0.461	0.161	0.807
18:0	8.88	8.19	9.05	9.11	0.628	0.266	0.501	0.427
18:1 *c*9	17.1	17.6	20.3	21.6	0.51	0.008	0.099	0.253
18:1 total cis	18.5	19.0	21.8	23.0	0.47	0.006	0.065	0.172
18:1 total trans	3.49	3.36	3.43	3.61	0.258	0.621	0.884	0.405
18:2 n-6	2.46	2.40	2.67	2.69	0.176	0.141	0.869	0.780
18:2 total excluding CLA	2.94	2.87	3.25	3.17	0.203	0.127	0.681	0.967
18:2 total CLA	0.77	0.76	0.74	0.78	0.077	0.957	0.838	0.577
18:3 n-3	0.37	0.35	0.57	0.47	0.018	0.002	0.066	0.197
20:0	0.14	0.14	0.14	0.16	0.018	0.400	0.649	0.560
20:1 total cis	0.08	0.09	0.10	0.13	0.027	0.238	0.392	0.773
20:2 n-6	0.04	0.03	0.04	0.04	0.006	0.425	0.519	0.276
20:3 n-6	0.07	0.07	0.06	0.10	0.030	0.794	0.392	0.373
20:4 n-6	0.14	0.15	0.22	0.13	0.029	0.338	0.126	0.096
20:5 n-3	0.03	0.03	0.05	0.04	0.003	0.012	0.349	0.630
22:0	0.14	0.13	0.46	0.11	0.030	0.885	0.177	0.497
22:4 n-6	0.06	0.06	0.05	0.05	0.008	0.332	0.743	1.000
22:5 n-3	0.07	0.08	0.08	0.06	0.015	0.675	0.759	0.380
Summary, g/100g FA[2]								
Total SFA	66.5	67.5	67.5	65.2	0.83	0.385	0.377	0.170
Total MUFA	28.7[ab]	28.0[b]	27.7[b]	29.8[a]	0.62	0.250	0.103	0.036
Total cis MUFA	23.8	23.9	25.3	24.7	0.98	0.196	0.780	0.653
Total trans MUFA	4.07	3.93	4.06	4.18	0.277	0.568	0.967	0.509
Total PUFA	4.53	4.53	5.06	4.96	0.303	0.093	0.826	0.833
Total n-3 PUFA	0.65	0.65	0.86	0.76	0.090	0.106	0.464	0.560
Total n-6 PUFA	2.89	2.84	3.11	3.12	0.195	0.177	0.901	0.854
Ratio n-6:n-3 PUFA	4.72	5.00	3.31	3.77	0.413	0.002	0.018	0.438
Total unsaturates	33.6	32.6	32.4	34.8	0.82	0.409	0.326	0.145
Total trans-fats excluding CLA	4.54	4.41	4.64	4.67	0.297	0.434	0.801	0.717
Recovery rates, %								
Apparent recovery 18:2 n-6	10.3	10.4	12.3	16.0	1.63	0.034	0.212	0.250

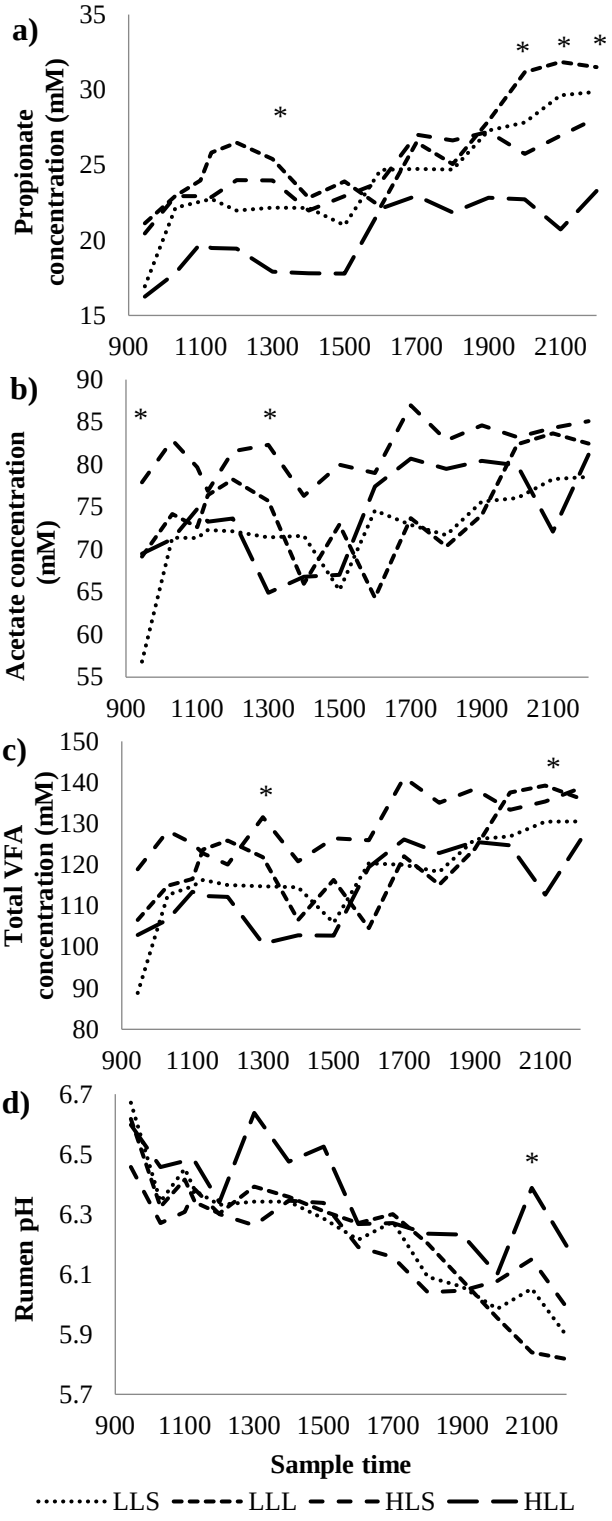
Apparent recovery 18:3 n-3	10.3	10.2	10.1	9.3	1.33	0.576	0.682	0.725
n	3	4	4	4				

[1]IR = Inclusion rate; CL = chop length; IRxCL = interaction between IR and CL;

[2]FA = fatty acid.

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

Thomson **Figure 1**



613 **Figure captions**
614
615 **Figure 1** The rumen concentrations of (a) acetate, (b) propionate (c) total volatile fatty acids
616 and (d) pH of lactating dairy cows just prior to, and until 12 h post morning feeding when fed
617 total mixed rations containing forage with a high (3:1 ratio with maize silage, DM basis; HL)
618 or low (1:3 ratio with maize silage, DM basis; LL) concentration of lucerne silage at a long
619 (19 mm; L) or short (14mm; S) chop length. Significant effects of time ($P < 0.05$) are marked
620 (*).