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Effect of replacing grass silage with red clover silage on ruminal lipid metabolism in lactating cows fed diets containing a 60:40 forage-to-concentrate ratio

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ABSTRACT

Diets based on red clover silage (RCS) typically increase the concentration of polyunsaturated fatty acids (PUFA) in ruminant milk and meat compared with grass silages (GS), an effect that has been attributed to higher activity of polyphenol oxidase in red clover, promoting ruminal escape of dietary lipid. Four multiparous Finnish Ayrshire cows in mid lactation fitted with rumen cannulas were used in a 4 × 4 Latin Square design with 21-d experimental periods to evaluate the effects of incremental replacement of GS with RCS on ruminal lipid metabolism, using the omasal sampling technique in combination with Cr-EDTA, Yb acetate, and indigestible neutral detergent fiber as markers. Treatments comprised total mixed rations offered ad libitum containing 600 g of forage/kg of diet dry matter, with RCS replacing GS in a ratio of 0:100, 33:67, 67:33, and 100:0 on a dry matter basis. Silages contained a high proportion of lipid as nonesterified fatty acids (NEFA), with no difference between forage species (75 and 73% for GS and RCS, respectively). Substitution of GS with RCS had no influence on the intakes of NEFA, polar lipid, triacylglycerol, diacylglycerol, monoacylglycerol, or total fatty acids (FA), but altered the ingestion of specific FA. Replacing GS with RCS decreased linearly 18:3n-3 and increased linearly 18:2n-6 intakes. Changes in the proportion of RCS in the diet had no effect on the amounts or on the relative proportions of different lipid fractions at the omasum. On average, NEFA, polar lipid, triacylglycerol, diacylglycerol, and monoacylglycerol accounted for 80, 12, 4.4, 2.4, and 0.8% of total FA in omasal digesta, respectively. Replacement of GS with RCS increased linearly the amount of esterified and nonesterified 18:3n-3 at the omasum. Flows of *cis*-9 18:1 and 18:2n-6 were also increased linearly in response to RCS in the diet, whereas 3,7,11,15-tetramethyl-16:0 at the omasum was decreased. Replacing GS with RCS in the diet decreased linearly the lipolysis of dietary esterified lipids in the rumen from 85 to 70%. Effects on lipolysis due to forage species were also associated with linear decreases in apparent ruminal 18:3n-3 biohydrogenation from 93 to 85% and a trend toward lowered biohydrogenation of *cis*-9 18:1 and 18:2n-6 in the rumen. However, forage species had no effect on the flow of bound phenols formed as a consequence of polyphenol oxidase activity at the omasum. In conclusion, despite minimal differences in the extent of lipolysis in silo, lipid and constituent FA in RCS were less susceptible to ruminal lipolysis and biohydrogenation compared with GS.
Key words: red clover, grass, lipolysis, biohydrogenation

INTRODUCTION

Meat and milk from ruminants are characterized by relatively high proportions of SFA and low amounts of PUFA, due at least in part to extensive lipolysis and biohydrogenation of dietary unsaturated FA in the rumen (Dewhurst et al., 2006; Shingfield et al., 2010). Ruminant-derived foods are an important source of lipid in the human diet (Shingfield et al., 2008b) and, therefore, increasing interest exists in developing nutritional strategies for altering the composition of ruminant milk and meat to improve long-term human health. Evidence from clinical and biomedical studies suggest that increasing milk fat 18:3n-3 and decreasing 12:0, 14:0, and 16:0 FA concentrations may lower cardiovascular disease risk and insulin resistance in humans without requiring a change in consumer eating habits (WHO, 2003; Shingfield et al., 2008b).

It is well established that replacing grass silage (**GS**) with red clover (*Trifolium pratense* L.) silage increases 18:2n-6 and 18:3n-3 concentration in milk (Dewhurst et al., 2006; Vanhatalo et al., 2007; Moorby et al., 2009)

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because of higher escape of these PUFA from the rumen (Dewhurst et al., 2003; Lee et al., 2003; 2006). However, the mechanisms explaining the influence of forage species on ruminal metabolism and higher transfer of PUFA from the diet into milk are not well defined, although several mechanisms have been postulated (Lee et al., 2007, 2010).

Red clover has a higher polyphenol oxidase (**PPO**) activity compared with grasses (Van Ranst et al., 2011). Inherent differences in the activity of this enzyme have been suggested to contribute to the effects of forage species on ruminal biohydrogenation of unsaturated FA due to an inhibition of plant-mediated lipolysis arising from the formation of electrophilic quinones liberated during the action of PPO on endogenous phenols (Lee et al., 2004; Van Ranst et al., 2009b). Quinones readily react with cellular nucleophiles, resulting in the binding and complexing of phenols with specific functional groups of lipid in forages, which may result in the physical entrapment of forage lipid within protein-phenol matrices (Lee et al., 2010). The aim of the present study was to provide further insight into the influence of forage species on the transformations of dietary lipid in the rumen of lactating cows by examining the effects of incremental replacement of GS with RCS on the flow of bound phenols, esterified lipid fractions, and NEFA at the omasum. Four cows were used in a 4 × 4 Latin square design to test the hypothesis that the effects of RCS on milk fat composition are related to lower lipolysis and biohydrogenation of dietary unsaturated FA in the rumen as a consequence of inherent differences in PPO activity between forage species.

MATERIALS AND METHODS

Forage Management

Grass silage was prepared on June 12 to 13, 2007, from primary growths of 3- and 4-yr leys of mixed timothy (*Phleum pratense* L. 'Tuukka') and meadow fescue (*Festuca pratensis* Huds. 'Antti' and 'Ilmari'). Swards were established using a seed mixture (Boreal Plant Breeding Ltd., Jokioinen, Finland) comprising 54% timothy and 46% meadow fescue. On April 17, 2007, grass leys were fertilized per hectare with 96 kg of N, 4 kg of K, 11 kg of Ca, 4 kg of Mg, 11 kg of S, 74 g of B, and 4 g of Se. Red clover silage (**RCS**) was prepared on September 5, 2007, from secondary growths of single-year leys (cultivar Jokioinen; Boreal Plant Breeding Ltd.). Growth of red clover was not fertilized before cropping. Forages were grown in Jokioinen (60°49′N, 23°28′E), cut using a mower conditioner, and harvested at a DM content of 221 and 194 g/kg for grass and red clover, respectively, with a precision chop harvester, and ensiled in bunker silos with a formic acid-based additive (760 g of formic acid and 55 g of ammonium formate, AIV 2 Plus; Kemira Ltd., Helsinki, Finland) applied at a rate of 5 L/t of fresh herbage. Mean herbage yields were 3,600 and 2,300 kg of DM/ha for grass and red clover leys, respectively. Bunker silos were opened on January 28, 2008.

Animals, Experimental Design, and Experimental Diets

All experimental procedures were approved by the National Animal Ethics Committee (Hämeenlinna, Finland) in accordance with guidelines established by the European Community Council Directives 86/609/EEC.. Four multiparous Finnish Ayrshire cows (*Bos p. taurus*; 599 ± 26.0 kg of BW, 108 ± 5.8 DIM, and producing 30.0 ± 1.84 milk/d) fitted with rumen cannulas (100-mm i.d.; Bar Diamond, Inc., Parma, ID) were allocated at random to experimental diets according to a 4 × 4 Latin square design with 21-d periods. Experimental treatments consisted of TMR containing 600 g of forage/kg of diet DM with RCS replacing GS in the diet in the DM ratio (RCS:GS) of 0:100, 33:67, 67:33, and 100:0. Forages were supplemented with a standard concentrate formulated to meet or exceed ME and protein requirements of lactating cows producing 35 kg of ECM/d (MTT Agrifood Research Finland, 2006). Formulation of experimental diets is presented in Table 1. Diets were offered ad libitum as 4 equal meals at 0600, 0800, 1800, and 2000 h. Cows were housed in a dedicated metabolism unit equipped with individual tie-stalls with continuous access to water and milked twice daily at 0700 and 1645 h.

Measurements and Sampling

Individual cow intakes were recorded daily throughout the experiment, but only measurements for the last 7 d were used for statistical analysis. During this period, representative samples of silage and concentrates for chemical analysis were collected, composited, and stored at −20°C. Additional samples for lipid analysis were also collected and stored at −80°C. The chemical composition of feeds, including the correction of silage DM content for volatile losses was determined using standard procedures (Halmemies-Beauchet-Filleau et al., 2013). The concentration of indigestible NDF (**iNDF**) was determined in duplicate by incubating 0.5- to 1-g samples within polyester bags (60 × 120 mm, 17-μm pore size) in the rumen for 12 d (Ahvenjärvi et al., 2000). The OM content of the indigestible residue was determined by ashing at 600°C for 18 h.

Table 1. Formulation of experimental diets

Ingredient, % of DM	Treatment[1]			
	GS	GRC	RCG	RCS
Grass silage	60	40	20	
Red clover silage		20	40	60
Rolled barley	18	18	18	18
Molassed sugar beet pulp	9	9	9	9
Solvent-extracted rapeseed meal	11.5	11.5	11.5	11.5
Vitamins and minerals[2]	1.5	1.5	1.5	1.5

[1]GS = grass silage; RCS = red clover silage; GRC = 2:1 mixture of GS and RCS, respectively; RCG = 1:2 mixture of GS and RCS, respectively.

[2]Declared as containing (g/kg) calcium (205), magnesium (72), sodium (85), phosphorus (27), zinc (1.46), manganese (0.35), copper (0.27); (mg/kg) iodine (39), cobalt (27), selenium (20); (IU/g) retinyl acetate (120), cholecalciferol (25), and DL-tocopheryl acetate (0.34; Onni, Melica Finland Ltd., Vaasa, Finland).

Samples of ruminal fluid (n = 8) were collected via the rumen cannula at 1.5-h intervals starting at 0600 h on d 13 of each period, filtered through 2 layers of cheesecloth, and analyzed for pH, VFA, and ammonia-N concentrations (Halmemies-Beauchet-Filleau et al., 2013). To assess rumen protozoal numbers, a 10-mL subsample of filtered rumen fluid was taken and preserved with 30 mL of aqueous NaCl (0.9% wt/vol) containing 10% (vol/vol) formaldehyde. Samples for each sampling time were composited and replicate (n = 6) measurements of protozoal numbers were made using a Fuchs-Rosenthal counting chamber (Fortuna W. G. Co., Wertheim, Germany).

Omasal digesta flow was assessed using the omasal sampling technique (Ahvenjärvi et al., 2000) and Cr-EDTA, Yb acetate, and iNDF as markers for liquid, small particles, and large particles, respectively (Shingfield et al., 2008a). Chromium-EDTA was prepared using standard procedures (Shingfield et al., 2008a), whereas Yb acetate was obtained from a commercial source (Dasico A/S, Birkerød, Denmark). To facilitate rapid marker equilibration in the rumen, priming doses of Cr-EDTA (1,500 mL; 590 mg of Cr) and Yb acetate (7.5 g) were administered directly into the rumen of each cow at 1800 h on d 14 of each experimental period. Following the priming dose, Cr-EDTA (1,000 mL/d) and Yb acetate (5.8 g) were dissolved in distilled water (6 L) and infused via separate infusion lines into the rumen at a constant rate for 120 h until 1800 h on d 21.

Spot samples (500 mL) of digesta entering the omasal canal were collected 3 times daily at 4-h intervals on d 17 through 20 using the omasal sampling device (Ahvenjärvi et al., 2000). Sampling started at 0600 h and was advanced by 1 h each day to cover a 12-h period considered representative for the entire feeding cycle. Samples were stored immediately after collection at −20°C. At the end of the experiment, digesta samples were thawed in cold water, composited, and separated into large particle, small particle, and liquid phases by filtration and centrifugation as outlined elsewhere (Ahvenjärvi et al., 2000). Each phase was freeze dried and stored at −20°C, whereas subsamples collected for FA and bound phenol analysis were stored at −80°C. All digesta phases were analyzed for DM, OM, Cr, Yb, and iNDF. The chemical composition of omasal digesta was determined using same methods as for feeds. Concentrations of Cr and Yb were determined by an inductively coupled plasma atomic emission spectrophotometer (Thermo Jarrell Ash-Baird 14033700 IRIS Advantage ICOPS, Thermo Jarrell Ash Corp., Franklin, MA). Concentrations of NDF and ADF were measured in small and large particle phases, whereas the liquid phase was submitted for VFA and ammonia N determinations. Based on marker concentrations, the relative proportions of the liquid, small particle, and large particle fractions in true digesta were calculated using the digesta reconstitution technique (Ahvenjärvi et al., 2000). Thereafter, appropriate amounts of freeze-dried digesta previously stored at −80°C were weighed to yield a 20-g composite sample and submitted for bound phenol and lipid analysis.

Bound Phenol Analysis

Total protein and protein-bound phenol was determined in reconstituted freeze-dried omasal digesta (ca. 500 mg of DM) using a modified Lowry/Potty assay following extraction with 2 mL of phosphate/citrate (McIlvaine buffer), pH 7.0, containing 0.5% (wt/vol) lithium dodecyl sulfate and 75 m*M* ascorbic acid. Extracts were sonicated for 5 min, boiled for 3 min, and centrifuged at 15,000 × *g* for 10 min at 4°C. Soluble protein was precipitated from 1 mL of supernatant with 1 mL of 20% trichloroacetic acid and 0.4% phosphotungstic acid, incubated for 30 min at 4°C, and centrifuged at 10,000 × *g* for 10 min at 4°C. The protein pellet, which included bound phenol, was dissolved in 6 mL of 0.1 *M* NaOH and analyzed for protein and

bound phenol content by running a duplicate assay in the presence and absence of copper ions using BSA as an internal standard. First, 50 μL of Lowry reagent A (0.45 *M* Na_2CO_3 and 0.17 m*M* sodium potassium tartrate in 0.14 *M* NaOH) was mixed with 50 μL of sample in a microplate well, and 50 μL of reagent A (including 0.15 m*M* $CuSO_4$) was mixed with 50 μL of sample in a corresponding well of a second microplate. Both plates were incubated for 10 min at room temperature, after which 50 μL of 1 N Folin-Ciocalteu reagent was added, and absorbance at 650 nm was measured (Bio-Rad Benchmark microplate reader; Bio-Rad Laboratories, Hemel Hempstead, UK) after a 30-min incubation. The assay response without copper is mainly due to the amount of bound phenol, whereas the response with copper is due to protein and bound phenol content. By estimating the protein component of the response, it was possible to calculate the concentration of bound phenol (Winters and Minchin, 2005).

Lipid Analysis

Fatty acid content of feeds and omasal digesta were determined using 13:0 in nonesterified and 23:0 in esterified form as internal standards, due to the low abundances in analyzed samples and the elution of the 13:0 and 23:0 methyl esters in the absence of interference with other FA methyl esters (**FAME**) during GC analysis. Following the addition of internal standards [1 mL of tridecanoic acid in absolute ethanol, N-13A, 0.1 wt/vol (Nu-Chek Prep Inc., Elysian, MN) and 0.2 mL of tritricosanoin in hexane, T-185, 0.1 wt/vol (Nu-Chek Prep Inc.)] and 3 mL of deionized water, the pH of 600 mg of freeze-dried samples of feeds and reconstituted omasal digesta was adjusted to 2.0 with 2 *M* hydrochloric acid. Lipid was extracted using a mixture (12 mL; 3:2; vol/vol) of hexane and isopropanol. Extractions were repeated and the organic phases recovered combined. After washing with 6 mL of deionized water and drying over anhydrous sodium sulfate, lipid in organic extracts were separated into polar lipid (**PL**), triacylglycerol (**TAG**), diacylglycerol (**DAG**), monoacylglycerol (**MAG**), and NEFA fractions by thin-layer chromatography (Lee et al., 2004) using preparative silica plates (200 × 200 mm and 1-mm thickness; no. 1.13895.0001; Merck KGaA, Darmstadt, Germany). Plates were developed for 75 min using a mixture of hexane, diethyl ether, and acetic acid (70:30:2 vol/vol) and the separated bands were visualized under UV light after spraying with a 0.2% (wt/vol) solution of 2′,7′-dichlorofluorescein (D-9053; Sigma-Aldrich, Helsinki, Finland) in methanol. Bands corresponding to TAG, DAG, MAG, NEFA, and PL were located using authentic tripalmitin (T-5888; Sigma-Aldrich), 1,3-dipalmitin (D-1639; Sigma-Aldrich), monopalmitin (31-1600; Larodan Fine Chemicals AB, Malmö, Sweden), palmitic acid (N-16-A; Nu-Chek Prep Inc.), and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (T-6517; Sigma-Aldrich) standards. Each band was removed manually from the plate and transferred to a clean test tube. Lipid was recovered from silica by rinsing twice with a mixture (1:2:1 vol/vol) of methanol, hexane, and 5% (wt/vol) aqueous sodium chloride. Extracts were dried over sodium sulfate and evaporated to dryness under oxygen-free nitrogen. Methyl esters of PL, TAG, DAG, and MAG fractions were prepared by incubation with 2 mL of hexane and 40 μL of fresh methylation reagent (1.75 mL methanol and 0.4 mL of Na-methoxide solution; Fluka reagent 71748, Sigma-Aldrich) at 20°C for 5 min. Nonesterified FA were transesterified to FAME using 1% (vol/vol) sulfuric acid in methanol as a catalyst (Halmemies-Beauchet-Filleau et al., 2013).

Total lipid in feeds and omasal digesta were determined in a separate analysis. Following the addition of 1 mL of internal standard (0.1 wt/vol of tridecanoic acid, N-13A; Nu-Check Prep Inc.; in absolute ethanol) and 1 mL of deionized water, and adjusting sample pH to 2.0 using 2 *M* hydrochloric acid, lipid in 100 mg of feed or omasal digesta was extracted using a mixture (4 mL; 3:2, vol/vol) of hexane and isopropanol. Extractions were repeated and both organic extracts were combined, rinsed with 2 mL of deionized water, dried using anhydrous sodium sulfate, and evaporated to dryness under a stream of oxygen-free nitrogen. The FAME of total lipid were prepared using a 2-step base-acid catalyzed procedure based on incubations with sodium methoxide in methanol, followed by 1% (vol/vol) sulfuric acid in methanol (Halmemies-Beauchet-Filleau et al., 2013).

The FAME prepared from lipid in feeds and omasal digesta were quantified using a gas chromatograph (model 6890; Hewlett-Packard, Wilmington, DE) equipped with a flame-ionization detector, automatic injector, split injection port and a 100-m fused silica capillary column (0.25-mm i.d.) coated with a 0.2-μm film of cyanopropyl polysiloxane (CP-SIL; 7489; Chromopack, Middelburg, the Netherlands). The total FAME profile in a 2-μL sample at a split ratio of 1:50 was determined using a temperature gradient program and hydrogen as the carrier gas operated at constant pressure (137.9 kPa) at a flow rate of 0.5 mL/min. Isomers of 18:1 were further resolved in a separate analysis under isothermal conditions at 170°C. Peaks were identified by comparison of retention times with authentic FAME standards. Methyl esters of FA not available as commercial standards were identified based on GC-MS of 4,4-dimethyloxoline (DMOX) derivatives prepared from FAME. Preparation of DMOX derivatives, pa-

rameters used for GC-MS analysis, and interpretation mass spectra were in accordance with earlier reports (Halmemies-Beauchet-Filleau et al., 2011). Relative retention time and order of elution was used to differentiate between diastereomers of 3,7,11,15-tetramethyl-16:0 (Halmemies-Beauchet-Filleau et al., 2013). The distribution of conjugated linoleic acid (**CLA**) isomers in omasal samples was determined using a HPLC system (model 1090; Hewlett-Packard) equipped with 4 silver-impregnated silica columns (ChromSpher 5 Lipids, 250 × 4.6 mm, 5-μm particle size; Varian Ltd., Walton-on-Thames, UK) coupled in series. Methyl esters of CLA were separated under isothermal conditions at 22°C using 0.1% (vol/vol) acetonitrile in heptane at a flow rate of 1 mL/min and monitoring column effluent at 233 and 210 nm (Halmemies-Beauchet-Filleau et al., 2011).

Fatty acid composition was expressed as a weight percentage of total FA using theoretical relative response factors (Halmemies-Beauchet-Filleau et al., 2011). Concentrations of CLA isomers were calculated based on proportionate peak area responses determined by HPLC and the sum of *trans*-7,*cis*-9 CLA, *trans*-8,*cis*-10 CLA, and *cis*-9,*trans*-11 CLA weight percentage determined by GC analysis.

Calculations

Lipolysis of esterified lipid in the rumen was calculated assuming negligible incorporation of 18:3n-3 in membrane lipid of rumen bacteria, protozoa, and fungi (Vlaeminck et al., 2006) as follows:

$$[(\text{intake of esterified 18:3n-3, g/d} - \text{flow of esterified 18:3n-3 at the omasum, g/d})/\text{intake of esterified 18:3n-3, g/d}] \times 100.$$

Apparent biohydrogenation of unsaturated FA in the rumen was calculated as follows:

$$[(\text{intake, g/d} - \text{flow at the omasum, g/d})/\text{intake, g/d}] \times 100.$$

Statistical Analysis

Data were analyzed by ANOVA with a model that included the fixed effects of treatment and period and the random effect of cow using PROC MIXED of SAS (version 9.2; SAS Institute Inc., Cary, NC). Sums of squares for treatment effects were further separated using orthogonal contrasts into single-degree-of-freedom comparisons to test for the significance of linear and quadratic components of the response to incremental replacement of GS with RCS. Least squares means are reported, with treatment effects declared significant at $P \leq 0.05$, and P-values between 0.05 and 0.10 considered a trend toward significance.

RESULTS

Distribution and Composition of Lipid in Forages and Concentrates

The chemical composition of experimental feeds is shown in Table 2. Both silages were well preserved and of high nutritive and fermentation quality. Total FA content or the relative distribution of lipid fractions did not differ between RCS and GS. On average, NEFA, TAG, PL, DAG, and MAG fractions accounted for 74, 11, 10, 4.8, and 0.7% of total FA in ensiled forages. In contrast, TAG was the major lipid fraction in concentrates, contributing to ca. 79% of total FA. Compared with GS, RCS contained lower 18:3n-3 and higher 18:2n-6 concentrations (43 vs. 52 and 22 vs. 17% of total FA, respectively; Table 3). Concentrates predominated in 16:0, *cis*-9 18:1, and 18:2n-6 (12, 30, and 36% of total FA, respectively; Table 3).

The profile and amount of FA differed between specific lipid fractions in ensiled forages (Table 3). For PL of GS and RCS, 18:3n-3 was the principal FA (on average, 76%), but it was present in lower proportions for NEFA and neutral lipid fractions. Despite differences in enrichment among lipid fractions, the majority (70%) of 18:3n-3 in RCS and GS was present in nonesterified form, with smaller amounts in PL (16%). In contrast, the contribution of PL to total *cis*-9 18:1 and 18:2n-6 content in RCS and GS was low (3–5%). However, enrichment of 18:2n-6 in NEFA and neutral lipid fractions of silage was relatively high (13–31% of FA), whereas the abundance of *cis*-9 18:1 was low in all lipid fractions. For NEFA, DAG, and MAG fractions, 16:0 accounted for between 14 and 21% of total FA, whereas the relative enrichment in PL and TAG fractions was much lower. The abundance of S3,R7,R11,15-tetramethyl-16:0 was higher in GS than RCS, the majority being in nonesterified form. Concentrates contained also trace amounts of S3,R7,R11,15-tetramethyl-16:0, principally as TAG.

Intake of Dietary Lipid Fractions and FA

Intake of DM tended ($P = 0.06$) to be higher when GS and RCS were fed together rather than when offered separately, but forage species had no significant effect ($P > 0.05$) on the intake of dietary lipid fractions or total FA (Table 4). For all diets, silage represented

Table 2. Chemical composition of dietary ingredients

Item	Grass silage	Red clover silage	Concentrate
pH	3.97	4.00	
DM, %	23.6	23.3	89.8
In DM, %			
Ash	8.72	10.3	5.27
CP	15.6	20.0	20.1
NDF	52.9	33.9	22.1
Indigestible NDF	9.82	9.16	4.30
Water-soluble carbohydrates	3.29	2.08	6.84
Lactic acid	6.32	10.1	
Acetic acid	1.97	2.49	
Propionic acid	0.02	0.02	
Butyric acid	0.04	0.01	
FA	2.32	2.43	1.96
NEFA	1.74	1.77	0.14
Polar lipid	0.23	0.23	0.20
Triacylglycerol	0.24	0.29	1.55
Diacylglycerol	0.10	0.13	0.07
Monoacylglycerol	0.02	0.02	0.01
Distribution of FA among lipid fractions, %			
NEFA	74.9	72.8	7.22
Polar lipid	10.0	9.39	10.0
Triacylglycerol	10.3	11.7	78.6
Diacylglycerol	4.21	5.30	3.51
Monoacylglycerol	0.64	0.82	0.65
In total N, %			
Ammonium-N	4.46	5.28	
Soluble N	61.5	38.4	

the major source of NEFA in the diet, whereas the majority of dietary TAG originated from concentrate. Owing to differences in the FA composition of silages, incremental replacement of GS with RCS in the diet decreased linearly ($P < 0.05$) the intake of 18:3n-3 (NEFA and TAG fractions) and increased linearly ($P < 0.05$) 18:2n-6 ingestion (NEFA, DAG, and MAG fractions; Table 4 and Figure 1). Forage species had no influence ($P > 0.10$) on total *cis*-9 18:1 intake (Table 4). Overall, the magnitude of changes in FA intake in response to the substitution of GS with RCS were relatively minor (Table 4; Supplemental Tables S1–S5; http://dx.doi.org/10.3168/jds.2013-6872).

The majority of 18:3n-3 supplied by the diet was principally in the form of NEFA, whereas the majority of 18:2n-6 was supplied as TAG and NEFA fractions (Figure 1). Furthermore, the contribution of PL to total dietary intakes of 18:3n-3 was higher compared with 18:2n-6 (on average, 14–16% and 8–9%, respectively; Figure 1). Most of the *cis*-9 18:1 supplied by the diet was in the form of TAG (Figure 1).

Rumen Fermentation

Forage species had no effect ($P > 0.10$) on rumen pH, VFA concentration, or ruminal protozoal numbers. Replacing GS with RCS in the diet decreased linearly ($P < 0.02$) molar acetate, valerate, isovalerate, and caproate proportions, and increased linearly ($P < 0.01$) the molar proportion of propionate in rumen VFA (Table 5). Offering silages as a mixture tended to increase ($P = 0.07$) rumen ammonia N concentrations compared with RCS or GS as sole forages (Table 5).

Ruminal Lipolysis and Biohydrogenation

Replacing GS with RCS decreased linearly ($P < 0.01$) lipolysis of dietary esterified lipids in the rumen from 85 to 70% (Table 6). Furthermore, these changes were associated with lowered ($P < 0.01$) 18:3n-3 biohydrogenation in the rumen and tended ($P = 0.08$) to decrease linearly the extent of ruminal *cis*-9 18:1 and 18:2n-6 biohydrogenation (Table 6).

Ruminal FA Balance

On average, the amount of total FA at the omasum was 164 g/d higher (Tables 4 and 7) compared with intake, due in the most part, to a net synthesis of 16:0 (24 g/d), 18-carbon FA (94 g/d), and 13- to 18-carbon odd- and branched-chain FA (**OBCFA**; collectively 15:0, 17:0, *anteiso* 13:0, 15:0, and 17:0, and *iso* 13:0, 14:0, 15:0, 16:0, 17:0, and 18:0) in the rumen, with no differences among treatments ($P > 0.10$; data not

Table 3. Relative proportions and abundance of selected FA in nonesterified, polar, and neutral lipid fractions of dietary ingredients

	Grass silage		Red clover silage		Concentrate	
Item	% of FA	g/kg of DM	% of FA	g/kg of DM	% of FA	g/kg of DM
NEFA						
16:0	20.1	3.49	20.9	3.70	22.6	0.32
18:0	1.27	0.22	2.62	0.46	4.58	0.06
cis-9 18:1	3.49	0.61	3.12	0.55	21.8	0.31
18:2n-6	17.2	2.99	22.6	4.00	35.3	0.50
18:3n-3	49.9	8.68	40.1	7.09	5.11	0.07
S3,R7,R11,15-tetramethyl-16:0	0.030	0.005	0.019	0.003	0.042	0.001
Polar lipids						
16:0	8.14	0.19	7.41	0.17	17.1	0.33
18:0	0.85	0.02	1.47	0.03	0.96	0.02
cis-9 18:1	1.42	0.03	1.19	0.03	23.3	0.45
18:2n-6	8.75	0.20	8.98	0.20	47.1	0.92
18:3n-3	75.5	1.76	76.2	1.74	5.98	0.12
S3,R7,R11,15-tetramethyl-16:0	0.040	0.001	0.033	0.001	0.039	0.001
Neutral lipids						
Triacylglycerol						
16:0	7.60	0.18	10.3	0.29	10.4	1.60
18:0	1.32	0.03	2.13	0.06	1.47	0.23
cis-9 18:1	6.78	0.16	8.36	0.24	32.5	5.03
18:2n-6	24.6	0.59	27.5	0.79	34.4	5.32
18:3n-3	53.7	1.28	35.1	1.00	8.82	1.36
S3,R7,R11,15-tetramethyl-16:0	0.055	0.001	0.031	0.001	0.075	0.012
Diacylglycerol						
16:0	17.3	0.17	17.0	0.22	20.2	0.14
18:0	3.32	0.03	2.90	0.04	3.56	0.03
cis-9 18:1	5.53	0.05	5.18	0.07	19.1	0.13
18:2n-6	23.9	0.23	30.7	0.40	43.7	0.30
18:3n-3	36.7	0.36	33.2	0.43	5.67	0.04
S3,R7,R11,15-tetramethyl-16:0	0.193	0.002	0.069	0.001	0.131	0.001
Monoacylglycerol						
16:0	13.9	0.02	16.8	0.03	24.7	0.03
18:0	5.01	0.01	4.83	0.01	5.50	0.01
cis-9 18:1	9.45	0.01	3.92	0.01	15.9	0.02
18:2n-6	12.9	0.02	21.3	0.04	40.2	0.05
18:3n-3	34.0	0.05	34.2	0.07	3.85	0.01
S3,R7,R11,15-tetramethyl-16:0	0.430	0.001	0.204	<0.001	0.205	<0.001
Total						
16:0	17.4	4.05	18.2	4.42	12.4	2.43
18:0	1.35	0.31	2.49	0.60	1.74	0.34
cis-9 18:1	3.75	0.87	3.68	0.89	30.2	5.94
18:2n-6	17.4	4.03	22.3	5.43	36.1	7.09
18:3n-3	52.2	12.1	42.5	10.3	8.13	1.60
S3,R7,R11,15-tetramethyl-16:0	0.043	0.010	0.026	0.006	0.071	0.014

presented) other than a linear decrease ($P < 0.04$) in OBCFA synthesis [from 32 to 26 g/d (SE = 3.6)] when RCS replaced GS in the diet. Irrespective of forage species, ruminal disappearance of *cis*-9 18:1, 18:2n-6, and 18:3n-3 accounted for up to 76% of the net appearance of 18:0 and *trans*-18:1 at the omasum.

Bound Phenols, Lipid Fractions, and FA at the Omasum

Forage species had no significant effect ($P > 0.05$) on the flow of DM or bound phenols at the omasum (Table 7). Replacing GS with RCS in the diet had no substantial influence ($P > 0.05$) on the amount or on the relative proportions of major lipid fractions at the omasum, other than increasing ($P < 0.02$) NEFA flow when a mixture of GS and RCS were fed (Table 7). On average, NEFA, PL, TAG, DAG, and MAG fractions contributed to 80, 12, 4.4, 2.4, and 0.8% of total FA in omasal digesta, respectively (Table 7). Replacing GS with RCS increased linearly ($P < 0.05$) the flow of 18:3n-3 in all lipid fractions at the omasum (Figure 2). The majority of 18:3n-3 at the omasum was present as PL (42–46%), with smaller contributions from NEFA (22–26%), TAG (17–20%), and DAG (8–14%). Ruminal escape of MAG accounted only for 2 to 3% of total 18:3n-3 at the omasum (Figure 2). Substituting GS for RCS increased ($P < 0.05$) linearly 18:2n-6 at the omasum (in NEFA and DAG fractions), with relatively equal contributions from NEFA, TAG, and PL fractions (Figure 2).

Table 4. Effect of replacing grass silage with red clover silage in the diet on total FA intake in lactating cows

	Treatment[1]					*P*-value[2]	
Item	GS	GRC	RCG	RCS	SEM	L	Q
DMI, kg/d	19.9	20.1	20.1	18.4	1.06	0.06	0.06
Intake, g/d	446	452	464	441	22.6	0.98	0.17
NEFA	222	224	231	219	11.6	0.85	0.11
Polar lipids	44.6	44.3	45.2	42.2	2.17	0.30	0.33
Neutral lipid							
Triacylglycerol	159	161	164	157	8.2	0.83	0.25
Diacylglycerol	17.6	18.9	20.5	20.6	1.23	0.08	0.59
Monoacylglycerol	2.85	3.11	3.38	3.36	0.274	0.20	0.59
12:0	0.58	0.55	0.54	0.48	0.030	<0.001	0.30
14:0	1.44	1.39	1.34	1.21	0.069	<0.001	0.19
16:0	69.2	71.4	73.9	71.1	3.62	0.25	0.16
cis-9 16:1[3]	1.81	1.97	2.16	2.18	0.113	0.005	0.33
trans-3 16:1	4.07	4.54	5.14	5.26	0.268	<0.001	0.22
18:0	6.63	7.87	9.22	9.79	0.454	<0.001	0.20
cis-9 18:1	60.4	60.8	61.2	57.8	2.95	0.27	0.22
cis-11 18:1	14.4	14.2	14.3	13.2	0.68	0.06	0.19
18:2n-6	108	115	121	120	6.0	0.017	0.26
18:3n-3	160	153	149	133	7.46	<0.001	0.18
20:0	1.69	2.24	2.82	3.14	0.160	<0.001	0.40
3S,7R,11R,15-tetramethyl-16:0	0.24	0.22	0.21	0.18	0.010	<0.001	0.19
cis-11 20:1	1.43	1.37	1.31	1.17	0.059	<0.001	0.08
22:0	2.32	2.40	2.52	2.43	0.127	0.22	0.30
24:0	1.50	2.07	2.69	3.06	0.140	<0.001	0.29
26:0	1.29	1.11	0.95	0.72	0.051	<0.001	0.46
28:0	2.06	1.90	1.78	1.49	0.090	<0.001	0.30
30:0	1.52	1.39	1.27	1.04	0.070	<0.001	0.38
Other[4]	6.81	9.17	11.7	13.2	0.625	<0.001	0.30
Σ SFA	90.6	95.3	101	99.3	4.96	0.017	0.18
Σ MUFA	84.1	85.1	86.4	81.9	4.15	0.55	0.20
Σ PUFA	271	272	276	260	13.6	0.31	0.19
Total	446	452	464	441	22.6	0.98	0.18

[1]Refers to diets containing a 60:40 forage:concentrate ratio based on grass silage (GS), red clover silage (RCS), or a 2:1 (GRC) or 1:2 (RCG) mixture (DM basis) of GS and RCS, respectively.

[2]Significance of linear (L) and quadratic (Q) components of response to incremental replacement of GS with RCS in the diet.

[3]Co-elutes with *anteiso* 17:0.

[4]Contains *anteiso* 13:0, *iso* 14:0, *cis*-9 14:1, 15:0, *anteiso* 15:0, *iso* 15:0, *iso* 16:0, *cis*-6+7 16:1, *trans*-9 16:1, 17:0, *iso* 17:0, *iso* 18:0, *cis*-14 18:1, *cis*-9,*trans*-12 18:2, *trans*-9,*cis*-12 18:2, *trans*-9,*trans*-12 18:2, 18:2 double bond position and configuration indeterminate, 18:3n-6, *cis*-9,*trans*-12,*cis*-15 18:3, *cis*-9,*trans*-12,*trans*-15 18:3, unresolved *cis*-9,*cis*-12,*trans*-15 18:3 and *trans*-9,*trans*-12,*cis*-15 18:3, 18:4n-3, 19:0, *cis*-10 19:1, Δ10,13 19:2 double bond geometry indeterminate, Δ10,13,16 19:3 double bond geometry indeterminate, *cis*-8 20:1, *cis*-9 20:1, 21:0, 20:2n-6, 20:3n-3, 20:3n-6, 20:4n-3, 20:4n-6, 20:5n-3, 22:2n-6, 22:4n-6, *cis*-15 24:1, 25:0, 27:0, and 29:0.

Replacement of GS with RCS in the diet increased ($P < 0.05$) linearly *cis*-9,*trans*-11,*cis*-15 18:3 at the omasum (Table 8). For all diets, the vast majority of *cis*-9,*trans*-11,*cis*-15 18:3 and isomers of CLA in omasal digesta were present in nonesterified form (Table 8). Forage species had no effect ($P > 0.05$) on total CLA flow (Table 8), but altered the distribution of CLA isomers at the omasum, with *cis*-9,*trans*-11 being the most abundant CLA isomer (Table 8 and Supplemental Table S11; http://dx.doi.org/10.3168/jds.2013-6872). Substituting RCS for GS in the diet increased linearly ($P < 0.03$) *cis*-9,*trans*-11 CLA and decreased ($P < 0.02$) *trans*-10,*cis*-12, *trans*-11,*cis*-13, and *trans*-9,*trans*-11 CLA at the omasum (Table 8 and Supplemental Table S11). Incremental replacement of GS with RCS in the diet also increased linearly ($P < 0.05$) the flow of several nonesterified *cis* 18:1 (Δ9,11,12,16) isomers at the omasum (Supplemental Table S6), but had no significant effect ($P > 0.05$) on the amount of esterified *cis*-9 18:1 at the omasum (Figure 1). Most *trans* 18:1 isomers were detected in omasal digesta as NEFA (94–96%), with *trans*-11 18:1 being the most abundant in the NEFA and esterified fractions, other than for TAG, in which *trans*-15 18:1 predominated (Table 8 and Supplemental Tables S6–S10). However, offering forages as a mixture increased ($P \leq 0.04$) the flow of certain *trans*-18:1 (Δ5–8, 11) at the omasum (as NEFA and total; Table 8 and Supplemental Tables S6 and S11) compared with GS and RCS alone.

On average, 18:0 accounted for 47% of the total amount of FA at the omasum, being recovered almost exclusively (97–98%) as NEFA (Table 8). Mixtures of GS and RCS increased ($P < 0.05$) the flow of 18:0 (as NEFA and total; Table 8 and Figure 2) compared

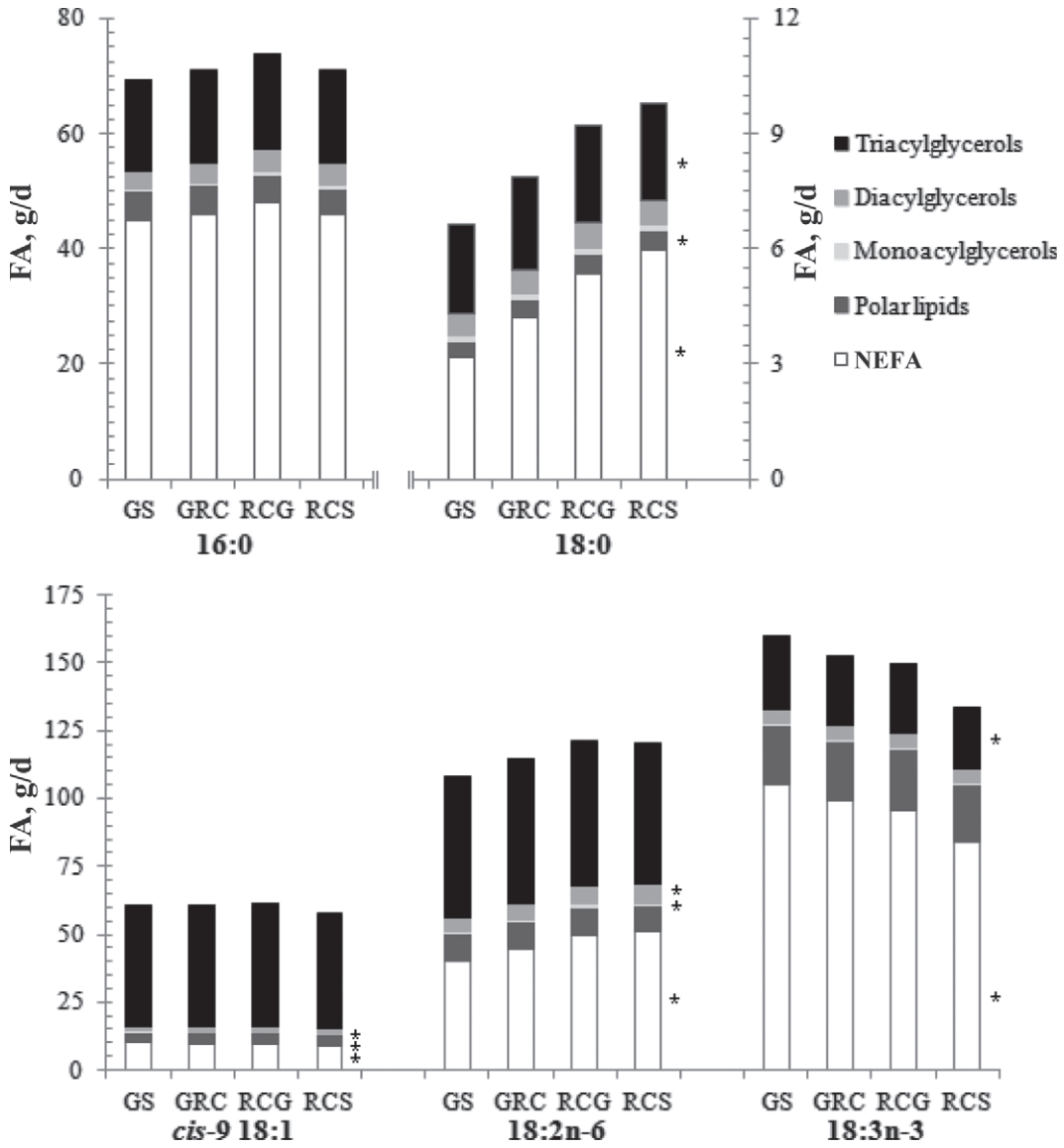

Figure 1. Effect of replacing grass silage with red clover silage in the diet on the contribution of different lipid fractions to 16:0, 18:0, *cis*-9 18:1, 18:2n-6, and 18:3n-3 intakes in lactating cows. * Indicates linear changes ($P < 0.05$) within lipid classes across treatments. GS = grass silage; RCS = red clover silage; GRC = 2:1 mixture of GS and RCS, respectively; RCG = 1:2 mixture of GS and RCS, respectively.

with either forage alone. Forage species had no effect ($P > 0.10$) on total 16:0 at the omasum (Table 8), whereas mixtures of GS and RCS increased the flow of nonesterified 16:0 and decreased ($P < 0.05$) the amount of 16:0 as TAG at the omasum (Figure 2). On average, 60 to 68% of 16:0 in omasal digesta was recovered as NEFA, with 22 to 28% in the form of PL (Figure 2).

Forage species also influenced the flow of OBCFA at the omasum that were present principally in nonesterified form or as components of the PL fraction (Figure 3 and Supplemental Table S11; http://dx.doi.org/10.3168/jds.2013-6872). Substitution of GS for RCS in the diet increased in a quadratic manner ($P < 0.05$) 17:0 (in NEFA fraction), but decreased linearly ($P < 0.05$) *anteiso* 17:0 (in PL and DAG fractions), *iso* 15:0 (in NEFA, PL, TAG, and DAG fractions), and *iso* 17:0 (in NEFA, PL, and DAG fractions) at the omasum (Figure 3 and Supplemental Table S11). Furthermore, replacing GS with RCS decreased linearly ($P < 0.05$) the flow of nonesterified and esterified (in PL and DAG fractions) S3,R7,R11,15-tetramethyl 16:0 at the omasum (Figure 3).

DISCUSSION

It is well established that RCS increases the flow of PUFA escaping the rumen compared with GS in lactating and growing cattle (Dewhurst et al., 2003;

Table 5. Effect of replacing grass silage with red clover silage in the diet on rumen fermentation characteristics in lactating cows

	Treatment[1]					*P*-value[2]	
Item	GS	GRC	RCG	RCS	SEM	L	Q
pH	6.73	6.81	6.73	6.90	0.101	0.19	0.50
Ammonia-N, mmol/L	6.33	7.17	6.95	6.43	0.440	0.95	0.07
VFA total, mmol/L	104	105	111	110	4.9	0.25	0.73
Molar VFA proportion, mmol/mol							
Acetate	657	653	646	631	5.1	0.010	0.32
Propionate	186	193	206	223	4.8	<0.001	0.30
Butyrate	111	108	105	106	2.9	0.18	0.58
Isobutyrate	9.31	8.70	8.48	8.23	0.481	0.11	0.68
Valerate	15.8	15.5	15.1	14.1	0.48	0.019	0.45
Isovalerate	14.3	14.6	12.8	13.0	0.96	0.011	0.83
Caproate	6.85	6.47	5.99	4.94	0.309	0.003	0.29
Molar ratio							
Acetate:propionate	3.55	3.38	3.14	2.83	0.103	0.002	0.50
(Acetate + butyrate):propionate	4.15	3.94	3.65	3.31	0.116	<0.001	0.53
Protozoa, $\times 10^5$ U/mL	3.78	3.93	3.78	3.18	0.569	0.31	0.38

[1]Refers to diets containing a 60:40 forage:concentrate ratio based on grass silage (GS), red clover silage (RCS), or a 2:1 (GRC) or 1:2 (RCG) mixture (DM basis) of GS and RCS, respectively.

[2]Significance of linear (L) and quadratic (Q) components of response to incremental replacement of GS with RCS in the diet.

Lee et al., 2003, 2006), but the mechanisms explaining this phenomenon are not known. Novel features of this experiment included a detailed assessment of all lipid fractions in omasal digesta and quantitative estimates of the contribution of nonesterified and esterified lipids to FA flows at the omasum. Furthermore, the amount of bound phenols in omasal digesta as a measure of oxidation was determined allowing inferences to be drawn on the possible influence of inherent differences in PPO activity between forage species on ruminal lipid metabolism in lactating cows.

Forage Lipid and FA Composition

Most of the lipid in grasses and legume forages is present as phospholipids and glycolipids located within thylakoid membranes of chloroplasts (Lee et al., 2009; Van Ranst et al., 2010; Buccioni et al., 2012). Typically, ensiling has minimal influence on the total FA content of forages, but substantially alters the distribution of FA in specific lipid fractions (Dewhurst et al., 2006; Vanhatalo et al., 2007; Van Ranst et al., 2010). Changes arising during fermentation in silo are characterized by a substantial decrease in the relative abundance of polar membrane lipid and an increase in NEFA, TAG, DAG, and MAG fractions attributable to the activity of plant and microbial lipases (Lee et al., 2004; Van Ranst et al., 2009a, 2010). For both RCS and GS, NEFA, TAG, PL, DAG, and MAG fractions accounted for, on average, 74, 11, 10, 4.8, and 0.7% of total FA, with no evidence to suggest that forage species had a major influence on the extent of lipolysis in silo. Several reports examining the ensilage of forages in laboratory-scale silos (Van Ranst et al. 2009a), bunker silos (Vanhatalo et al. 2007), or as round bales (Lee et al., 2006) have shown that RCS typically contains higher proportions of esterified lipid and lower proportions of NEFA than GS. Lower plant-mediated lipolysis during the ensilage of red clover than grasses has been attributed to a higher activity of PPO (Van Ranst et

Table 6. Effect of replacing grass silage with red clover silage in the diet on ruminal lipolysis and apparent biohydrogenation of 18-carbon unsaturated FA in lactating cows

	Treatment[1]					*P*-value[2]	
Item	GS	GRC	RCG	RCS	SEM	L	Q
Lipolysis, %	84.7	80.6	77.7	69.8	2.11	0.003	0.40
Apparent ruminal biohydrogenation, %							
cis-9 18:1	59.3	59.0	58.3	53.9	2.12	0.08	0.29
18:2n-6	77.6	77.6	77.2	73.8	1.28	0.08	0.21
18:3n-3	93.3	91.0	89.0	84.8	0.94	<0.001	0.32

[1]Refers to diets containing a 60:40 forage:concentrate ratio based on grass silage (GS), red clover silage (RCS), or a 2:1 (GRC) or 1:2 (RCG) mixture (DM basis) of GS and RCS, respectively.

[2]Significance of linear (L) and quadratic (Q) components of response to incremental replacement of GS with RCS in the diet.

Table 7. Effect of replacing grass silage with red clover silage in the diet on the amount of bound phenols in omasal digesta, distribution of FA in omasal lipid, and on the flow of FA at the omasum as NEFA, polar, and neutral lipids in lactating cows

	Treatment[1]					*P*-value[2]	
Item	GS	GRC	RCG	RCS	SEM	L	Q
DM, kg/d	14.2	13.9	14.2	12.8	1.04	0.08	0.25
Bound phenols, mg/d	39.3	44.6	40.5	40.9	10.87	0.98	0.66
Lipid fraction, %							
NEFA	78.3	80.6	81.5	79.0	1.15	0.56	0.08
Polar lipids	13.8	12.1	11.4	12.4	0.85	0.26	0.15
Neutral lipid							
Triacylglycerol	4.54	3.95	4.04	5.15	0.318	0.21	0.033
Diacylglycerol	2.54	2.59	2.24	2.25	0.135	0.49	0.45
Monoacylglycerol	0.90	0.75	0.84	0.90	0.119	0.97	0.40
FA, g/d							
NEFA	471	512	519	464	36.4	0.84	0.014
Polar lipids	81.6	76.2	72.7	73.8	6.51	0.35	0.60
Neutral lipid							
Triacylglycerol	27.4	24.8	25.9	30.1	3.00	0.45	0.23
Diacylglycerol	15.3	16.4	14.4	14.9	1.51	0.54	0.80
Monoacylglycerol	5.46	4.63	5.43	5.02	0.712	0.99	0.78
Total FA	600	634	638	589	43.8	0.67	0.06

[1]Refers to diets containing a 60:40 forage:concentrate ratio based on grass silage (GS), red clover silage (RCS), or a 2:1 (GRC) or 1:2 (RCG) mixture (DM basis) of GS and RCS, respectively.

[2]Significance of linear (L) and quadratic (Q) components of response to incremental replacement of GS with RCS in the diet.

al., 2011). The PPO enzyme is a stress-activated copper metalloprotein that catalyzes the oxidation of endogenous phenols to quinones in the presence of oxygen. Quinones formed during the action of PPO are highly reactive, electrophilic molecules that may covalently modify and crosslink a variety of nucleophilic cellular constituents, such as proteins, amines, and amides (Igarashi and Yasui, 1985). Binding with specific sites on forage proteins results in the formation of protein-bound phenols (Van Ranst et al., 2011).

A lack of difference in the proportion of total FA in PL or NEFA fractions between ensiled forages in this experiment may, at least to some extent, be related to the rather long storage of RCS and GS of 22 and 34 wk, respectively, before feeding out. Over shorter storage intervals (of 16 to 18 and 19 to 21 wk), the concentration and relative abundance of NEFA in GS and RCS prepared in bunker silos, respectively, was lower compared with current measurements, whereas the proportion of esterified lipid was marginally higher for ensiled red clover than a mixture of timothy and meadow fescue (Vanhatalo et al., 2007). Activation of latent PPO through the mechanical damage of red clover has been shown to lower lipolysis during the initial few days postensiling (Van Ranst et al., 2010). However, the protection of plant lipids to lipolysis was found to diminish after 60 d in silo, possibly due to increased activity of microbial lipases (Van Ranst et al., 2010). Protection of esterified lipid via PPO-mediated denaturation of plant lipases can be expected to be limited to the first 24 h after cutting and ensiling. The PPO enzyme has been shown to be rapidly denatured following quinone binding, such that additional lipolysis thereafter arises from the activities of microbial rather than plant lipases (Lee et al., 2013). In addition to forage species and duration of silage storage, other factors including cultivar, growth stage, DM content at ensiling, and the extent of fermentation in silo may also contribute to lipolysis during ensiling (Vanhatalo et al., 2007; Van Ranst et al., 2009a,b).

Irrespective of forage species, 18:3n-3 was the predominant FA in PL, whereas the abundance of 16:0, *cis*-9 18:1, and 18:2n-6 in PL was much lower, confirming previous comparisons of parent herbage and corresponding silages (Van Ranst et al., 2009a; Buccioni et al., 2012).

Intake of FA

Marginal differences in the consumption of FA among treatments reflected differences in the distribution and composition of lipid fractions in GS and RCS and the numerical increases in DMI when GS and RCS were fed together compared with GS and RCS alone. Replacing GS with RCS has, in some cases, resulted in similar 18-carbon PUFA intakes (Vanhatalo et al., 2007), whereas in others it lowered (Dewhurst et al., 2003; Moorby et al., 2009) or increased (Lee et al., 2003, 2006, 2008) the intake of 18:3n-3. Several factors may account for these differences, including species and cultivar of red clover and grasses, maturity at ensiling, duration and extent of wilting and fermentation in silo,

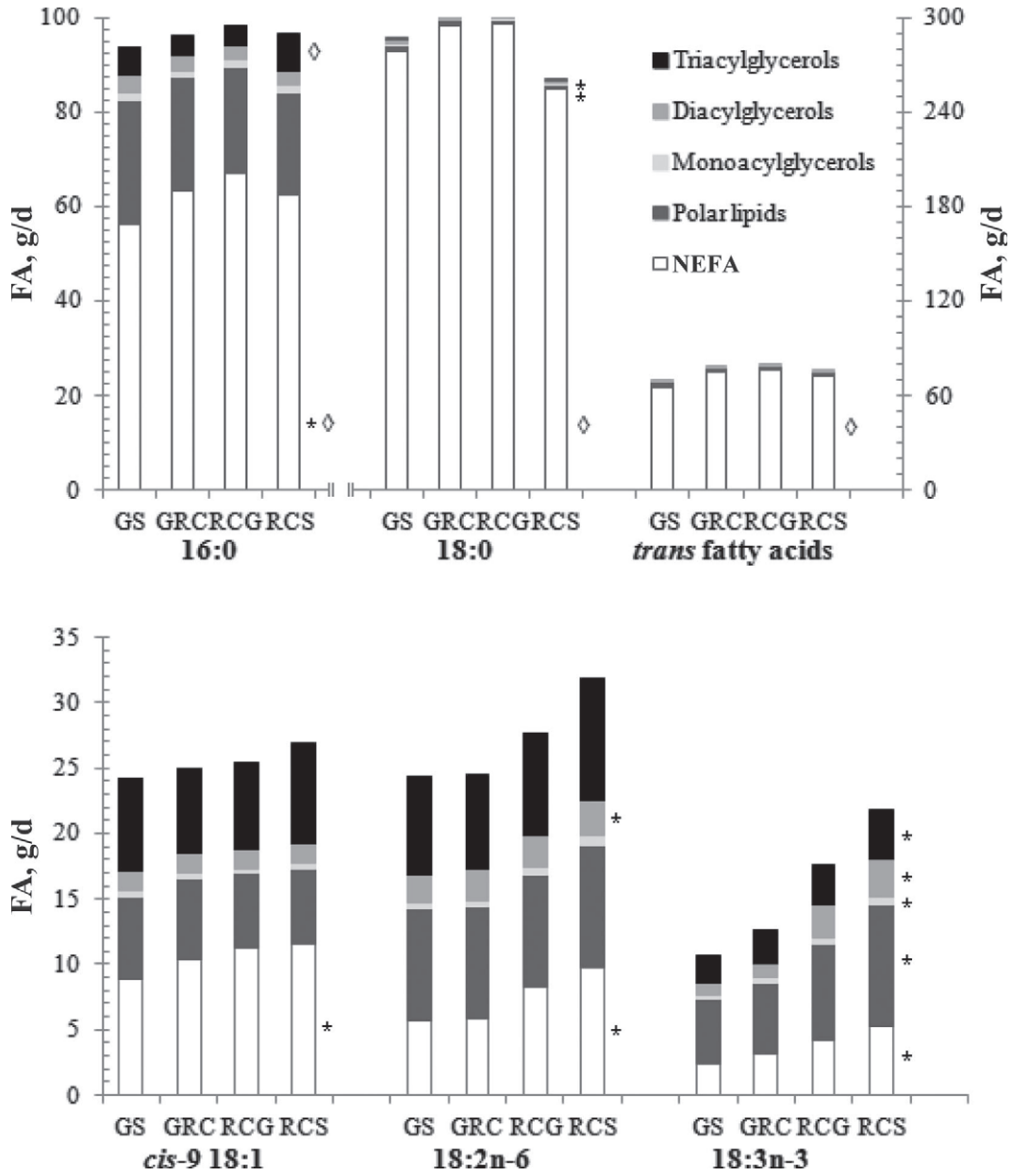

Figure 2. Effect of replacing grass silage with red clover silage in the diet on the contribution of different lipid fractions to the flow of 16:0, 18:0, total *trans* FA, *cis*-9 18:1, 18:2n-6, and 18:3n-3 at the omasum in lactating cows. * Indicates linear changes ($P < 0.05$) within lipid classes across treatments; ◊ indicates quadratic changes ($P < 0.05$) within lipid classes across treatments. GS = grass silage; RCS = red clover silage; GRC = 2:1 mixture of GS and RCS, respectively; RCG = 1:2 mixture of GS and RCS, respectively.

as well as the overall influence of forage species on DMI and the composition and amount of concentrates in the diet (Boufaïed et al., 2003; Dewhurst et al., 2006).

Rumen Fermentation

Forage species had no effect on rumen pH or total VFA concentrations, whereas replacing GS with RCS in the diet shifted rumen fermentation toward the production of propionate at the expense of acetate. Replacing GS with RCS has had no influence on rumen fermentation (Dewhurst et al., 2003; Vanhatalo et al., 2006), or promoted the formation of acetate at expense of propionate and butyrate (Vanhatalo et al., 2009) in lactating cows. Variation in the composition and fermentation characteristics of ensiled forages may, at least in part, contribute to these differences. In the present study, ensiled red clover had higher lactic

Table 8. Effect of replacing grass silage with red clover silage in the diet on the flow of nonesterified and total FA at the omasum of lactating cows

	Treatment[1]					*P*-value[2]	
Flow, g/d	GS	GRC	RCG	RCS	SEM	L	Q
NEFA, g/d							
12:0	0.25	0.25	0.28	0.25	0.034	0.93	0.56
14:0	1.04	1.18	1.20	1.10	0.115	0.46	0.10
16:0	56.0	63.3	67.2	62.6	4.76	0.048	0.032
cis-9 16:1	0.22	0.23	0.28	0.28	0.040	0.17	0.89
trans-9 16:1	0.01	0.00	0.01	0.01	0.004	0.93	0.19
trans-12 16:1	0.09	0.08	0.08	0.06	0.011	0.22	0.72
trans-13 16:1	0.25	0.33	0.44	0.51	0.041	<0.001	0.82
18:0	279	295	296	255	18.7	0.11	0.015
cis-9 18:1	8.84	10.4	11.3	11.6	1.163	0.013	0.34
Σ *cis* 18:1	15.4	18.2	19.5	19.6	2.10	0.013	0.19
trans-11 18:1	24.0	27.7	27.3	24.4	2.04	0.88	0.023
Σ *trans* 18:1	52.2	59.9	60.6	57.2	5.43	0.14	0.036
18:2n-6	5.67	6.89	8.30	9.78	1.002	0.005	0.86
Σ 18:2[3]	12.4	14.7	16.1	16.7	1.77	0.014	0.40
cis-9,*trans*-11 CLA	1.88	1.98	2.06	2.32	0.241	0.025	0.48
Σ CLA	3.88	4.02	4.12	4.09	0.422	0.47	0.68
18:3n-3	2.38	3.23	4.21	5.28	0.560	0.003	0.80
cis-9,*trans*-11,*cis*-15 18:3	1.00	1.12	1.20	1.45	0.175	0.040	0.60
18:4n-3	1.18	1.19	1.18	1.20	0.015	0.58	0.71
20:0	4.15	5.13	5.83	5.68	0.357	<0.001	0.026
S3,R7,R11,15-tetramethyl-16:0	4.17	4.06	3.29	2.35	0.302	<0.001	0.026
R3,R7,R11,15-tetramethyl-16:0	1.53	1.86	1.96	1.78	0.094	0.07	0.025
cis-9 20:1	0.07	0.07	0.07	0.07	0.010	0.65	0.94
cis-11 20:1	0.35	0.28	0.26	0.26	0.054	0.17	0.46
trans-11 20:1	0.11	0.17	0.08	0.07	0.045	0.38	0.44
20:2n-6	0.06	0.07	0.11	0.12	0.011	<0.001	0.96
20:3n-3	0.19	0.22	0.22	0.18	0.011	0.52	0.009
20:3n-6	0.00	0.01	0.01	0.01	0.002	0.53	0.54
20:4n-3	0.00	0.00	0.00	0.01	0.005	0.22	0.35
20:4n-6	0.00	0.02	0.02	0.00	0.009	0.89	0.16
22:0	3.25	3.51	3.49	3.06	0.246	0.29	0.022
22:2n-6	0.02	0.03	0.02	0.03	0.004	0.56	0.89
24:0	2.94	3.60	4.04	3.94	0.286	<0.001	0.025
cis-15 24:1	0.88	0.78	0.61	0.38	0.043	<0.001	0.06
26:0	8.31	7.10	4.82	2.14	0.571	<0.001	0.14
28:0	4.91	4.79	4.33	2.84	0.374	<0.001	0.026
30:0	2.86	2.27	3.36	2.46	0.223	0.10	0.002
Unidentified	1.85	2.13	1.88	1.65	0.153	0.09	0.035
Σ *trans*	65.6	74.8	75.8	72.0	6.95	0.13	0.049
Σ SFA	380	408	411	357	26.7	0.24	0.015
Σ MUFA	70.2	80.8	82.7	79.2	7.71	0.07	0.046
Σ PUFA	20.1	23.6	26.2	28.1	2.91	0.012	0.65
Total FA, g/d							
12:0	1.12	0.95	0.99	0.99	0.134	0.48	0.43
14:0	4.90	4.61	4.15	4.27	0.312	0.039	0.35
16:0	93.5	96.2	98.3	93.3	6.61	0.92	0.31
cis-9 16:1	0.60	0.59	0.66	0.65	0.071	0.35	0.95
trans-9 16:1	0.02	0.01	0.02	0.02	0.005	0.64	0.30
trans-12 16:1	0.32	0.26	0.29	0.27	0.027	0.37	0.49
trans-13 16:1	0.46	0.64	0.81	0.98	0.078	<0.001	0.98
18:0	287	302	302	261	19.1	0.09	0.017
cis-9 18:1	24.5	24.9	25.4	26.9	2.20	0.25	0.71
Σ *cis* 18:1	35.9	36.9	37.7	39.1	3.47	0.26	0.93
trans-11 18:1	25.8	29.2	28.8	25.8	2.05	0.92	0.025
Σ *trans* 18:1	55.5	62.9	63.3	59.7	5.52	0.23	0.042
18:2n-6	24.3	25.5	27.7	31.9	2.51	0.024	0.47
Σ 18:2[3]	31.6	33.9	36.0	39.3	3.28	0.035	0.83
cis-9,*trans*-11 CLA	1.90	2.01	2.08	2.34	0.243	0.026	0.50
Σ CLA	3.94	4.08	4.16	4.12	0.426	0.52	0.67
18:3n-3	10.7	13.7	16.4	20.6	1.811	0.002	0.68

Continued

Table 8 (Continued). Effect of replacing grass silage with red clover silage in the diet on the flow of nonesterified and total FA at the omasum of lactating cows

	Treatment[1]					*P*-value[2]	
Flow, g/d	GS	GRC	RCG	RCS	SEM	L	Q
cis-9,*trans*-11,*cis*-15 18:3	1.03	1.13	1.21	1.45	0.176	0.046	0.60
18:4n-3	0.19	0.20	0.19	0.20	0.015	0.48	0.85
20:0	4.47	5.47	6.21	6.11	0.387	<0.001	0.040
S3,R7,R11,15-tetramethyl-16:0	4.64	4.49	3.66	2.66	0.319	<0.001	0.026
R3,R7,R11,15-tetramethyl-16:0	1.61	1.94	2.02	1.85	0.097	0.09	0.025
cis-9 20:1	0.09	0.09	0.09	0.08	0.012	0.93	0.76
cis-11 20:1	0.55	0.43	0.41	0.46	0.091	0.45	0.32
trans-11 20:1	0.11	0.19	0.09	0.08	0.046	0.36	0.44
20:2n-6	0.11	0.13	0.18	0.19	0.015	<0.001	0.90
20:3n-3	0.30	0.34	0.33	0.29	0.018	0.47	0.036
20:3n-6	0.04	0.04	0.04	0.05	0.006	0.09	0.68
20:4n-3	0.00	0.00	0.00	0.01	0.005	0.20	0.36
20:4n-6	0.01	0.02	0.03	0.01	0.009	0.75	0.17
22:0	3.53	3.82	3.82	3.42	0.266	0.57	0.026
22:2n-6	0.03	0.03	0.03	0.03	0.004	0.70	0.88
24:0	3.23	3.98	4.41	4.41	0.329	<0.001	0.041
cis-15 24:1	1.03	0.92	0.73	0.48	0.050	<0.001	0.06
26:0	8.45	7.23	4.94	2.25	0.572	<0.001	0.14
28:0	5.05	4.93	4.44	2.91	0.378	<0.001	0.23
30:0	3.01	3.44	3.50	2.57	0.225	0.07	0.002
Unidentified	1.90	2.19	1.94	1.69	0.158	0.09	0.030
Σ *trans*	66.2	75.0	75.5	71.7	6.75	0.19	0.049
Σ SFA	457	477	474	419	30.3	0.09	0.027
Σ MUFA	95.4	104	105	103	9.27	0.24	0.23
Σ PUFA	46.1	51.7	56.7	64.6	5.41	0.009	0.75

[1]Refers to diets containing a 60:40 forage:concentrate ratio based on grass silage (GS), red clover silage (RCS), or a 2:1 (GRC) or 1:2 (RCG) mixture (DM basis) of GS and RCS, respectively.

[2]Significance of linear (L) and quadratic (Q) components of response to incremental replacement of GS with RCS in the diet.

[3]Excluding isomers of conjugated linoleic acid (CLA).

acid concentrations and lower amounts of NDF relative to ensiled grass, which may explain the observed changes in rumen fermentation characteristics when RCS replaced GS in the diet. Isobutyrate, valerate, and isovalerate formed from fermentation of valine, proline, and leucine, respectively, as well as acetate and caproate are essential growth factors for certain cellulolytic rumen bacteria (Cotta and Hespell, 1986). Decreases in molar proportions of acetate, valerate, isovalerate, and caproate in response to RCS in the diet observed in the present study were accompanied by lower flows of several 15- and 17-carbon branched-chain FA and microbial NAN (data not presented) at the omasum.

Ruminal Lipolysis and Biohydrogenation

Lipolysis through the action of plant or microbial lipases is a prerequisite for biohydrogenation, a phenomenon that serves to minimize the toxic effects of unsaturated FA on the growth of rumen bacteria (Lourenço et al., 2010). Replacing GS with RCS progressively lowered lipolysis of dietary esterified 18:3n-3 in the rumen, with the implication that the rate of NEFA release would also be decreased. Alterations in the extent of lipolysis may, at least in part, explain the decrease in ruminal 18:3n-3 biohydrogenation when RCS replaces GS in the diet (Dewhurst et al., 2003; Lee et al., 2003, 2006). Replacing GS with RCS also lowered *cis*-9 18:1 and 18:2n-6 biohydrogenation, but to a much lesser extent compared with 18:3n-3, which appears to be related to differences in the abundance of these FA in PL compared with other esterified lipid fractions between forage species. Concentrates in this experiment contained low amounts of 18:3n-3, whereas total intake of 18:3n-3 from PL and TAG fractions averaged 22 and 25 g/d, respectively. Flows of 18:3n-3 contained in these fractions at the omasum of 7.0 and 2.8 g/d, indicate that ruminal escape of 18:3n-3 in PL was more than double than that as TAG, with evidence that RCS promoted ruminal escape of 18:3n-3 in PL and TAG fractions compared with GS.

Studies in vitro have also demonstrated lowered lipolysis of esterified lipid in red clover than grasses during incubations with rumen fluid (Lee et al., 2004, 2007; Van Ranst et al., 2010). Based on these findings it has been suggested that formation of membrane lipid entrapped in protein-bound phenol matrices could offer a physical barrier to rumen bacterial lipases (Lee et al., 2010). A lower degradability of dietary protein in the rumen, together with a decrease in hindgut N digest-

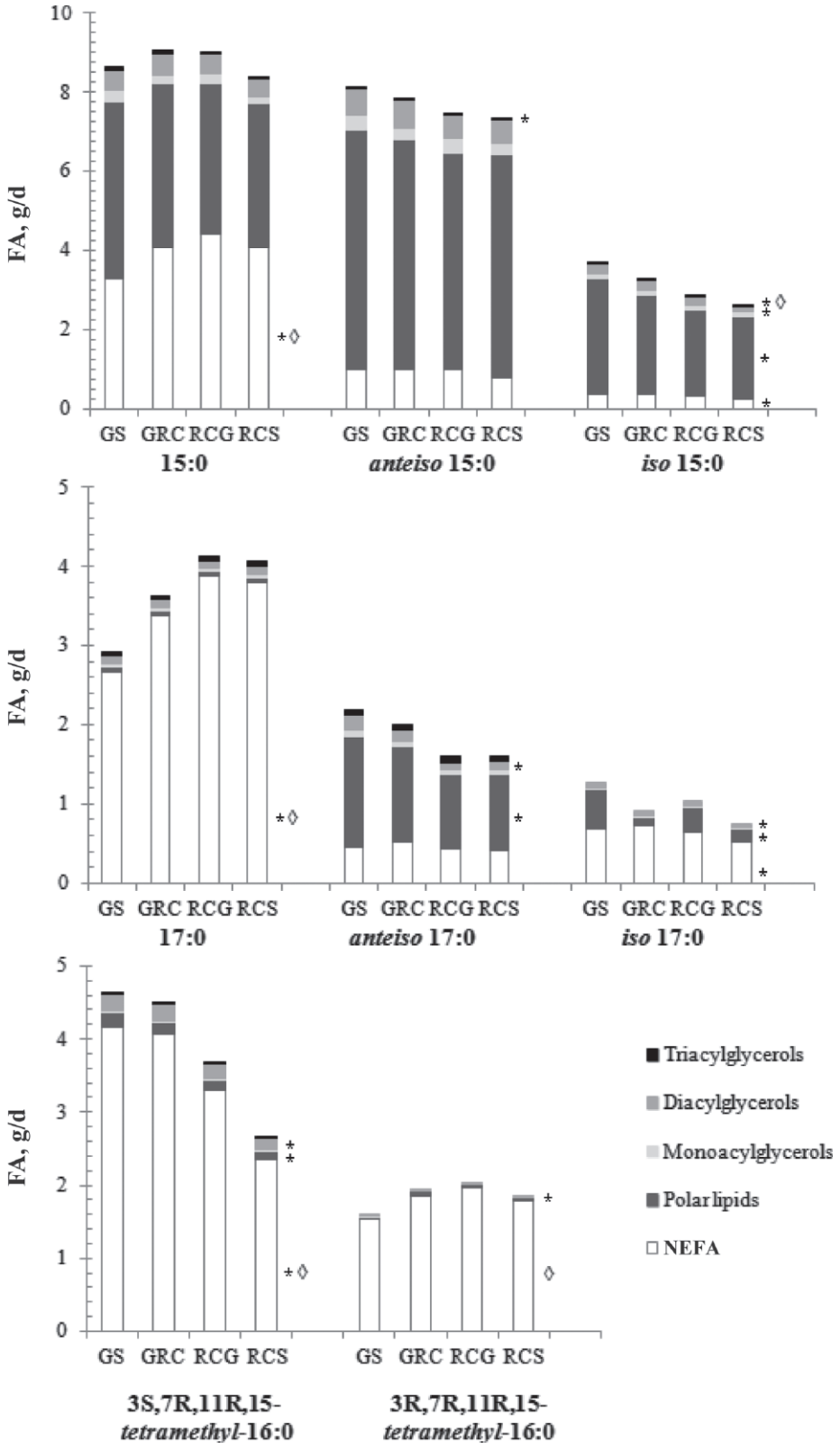

Figure 3. Effect of replacing grass silage with red clover silage in the diet on the contribution of different lipid fractions to the flow of selected odd- and branched-chain FA at the omasum in lactating cows. * Indicates linear changes ($P < 0.05$) within lipid classes across treatments; ◊ indicates quadratic changes ($P < 0.05$) within lipid classes across treatments. GS = grass silage; RCS = red clover silage; GRC = 2:1 mixture of GS and RCS, respectively; RCG = 1:2 mixture of GS and RCS, respectively.

ibility when RCS replaced GS (data not presented), would tend to support a mechanism involving protein complexing. Bound phenols are widely used as a proxy of PPO activity and oxidation both in silo and in the rumen (Lee et al., 2004, 2009), with higher concentrations reported in red clover compared with grasses (Van Ranst et al., 2009b). Flows of bound phenols at the omasum did not differ among treatments, which, combined with similar lipolysis in silo for RCS and GS, suggest that products of PPO were not a major factor contributing to the influence of forage species on ruminal lipolysis in the present experiment. However, measurements of bound phenol can potentially underestimate PPO activity due to the lowered solubility of protein bound phenol or result in overestimates in the presence of high concentrations of free phenols such as phenolic amino acids and plant phenols (e.g., phaselic acid and clovamide; Lee et al., 2013). It is, therefore, difficult to draw definitive conclusions on the possible role of PPO on the observed differences between forage species on ruminal lipid metabolism, solely on the basis of bound phenols at the omasum.

Lowered lipolysis with RCS in the diet may also occur due to decreases in rumen retention time of forage particles. The rate of DM outflow from the rumen has been reported to be higher for RCS-containing diets compared with GS (Dewhurst et al., 2003). More recent investigations have also indicated increased ruminal potentially digestible NDF passage and digestion rates for diets based on RCS than GS (Kuoppala et al., 2009; Bayat et al., 2010). These observations highlight the possibility that changes in ruminal digestion kinetics may also contribute to higher ruminal escape of dietary PUFA in lactating cows fed diets based on RCS. Nevertheless, biohydrogenation of unsaturated FA has been demonstrated to be consistently lower for red clover than grasses during incubations with ruminal fluid (Lee et al., 2007; Van Ranst et al., 2010), which would infer some form of protection from metabolism in the rumen, other than simple differences in ruminal outflow rate.

Forage species may also alter the distribution of feed particles in the rumen, with higher proportions of small particles (<0.038 mm) being reported in cows fed RCS than GS (Bayat et al., 2010). Earlier studies reported no difference in rumen particle size distribution in cows offered GS and RCS (Dewhurst et al., 2003), but these observations were made on the basis of a wider range in the size of small particles (<0.106 mm) compared with subsequent investigations. In addition to ruminal escape, substantial decreases in the forage particle size may inhibit the adherence of ruminal bacteria (Buccioni et al., 2012), with feed particles being the active site for biohydrogenation in the rumen (Harfoot and Hazlewood, 1988) or promote the ingestion of chloroplasts by rumen protozoa. Protozoa contain relatively high amounts of unsaturated FA (Lourenço et al., 2010) and thereby represent a means of redirecting dietary lipids from extensive lipolysis and biohydrogenation in the rumen. However, the selective retention of protozoa in the rumen, particularly on high-forage diets (Huws et al., 2012), suggests that other mechanisms are much more likely to account for the effects of forage species on ruminal lipid metabolism.

Ruminal Balance of FA

On all diets, the flow of FA at the omasum exceeded intake consistent with a net gain due to the microbial FA synthesis de novo in cattle fed diets containing low amounts of lipid (Doreau and Ferlay, 1994; Vlaeminck et al., 2006; Schmidely et al., 2008). Flow of 16:0 in PL exceeded intake by ca. 17 to 21 g/d, indicating incorporation of 16:0 from the diet into microbial membrane lipids or synthesis of 16:0 de novo by ruminal bacteria, or both (Doreau and Ferlay, 1994; Vlaeminck et al., 2006). Comparison of the intake and flow at the omasum of FA in PL also indicate that several *trans* 18-carbon biohydrogenation intermediates may serve as substrates for incorporation into microbial lipid membranes. Forage species had limited influence on ruminal FA balance, other than lowered synthesis of certain OBCFA (15:0, 17:0, *iso* 13:0, 14:0, 15:0, 16:0, 17:0, and 18:0, and *anteiso* 13:0, 15:0, and 17:0) in response to RCS inclusion in the diet, and a numerically higher net 18-carbon FA balance when RCS and GS were fed as a mixture than when offered separately. In growing cattle, FA at the duodenum have been in some (Lee et al., 2003, 2006), but not in all, cases (Lee et al., 2008) lower than intake on diets containing RCS.

Flow of Lipid and FA at the Omasum

Lipid Fractions. The majority of FA reaching the rumen were nonesterified, confirming extensive lipolysis of dietary lipid in the rumen and limited incorporation and esterification of NEFA into microbial lipids (Harfoot and Hazlewood et al., 1988; Vlaeminck et al., 2006). In ruminants, NEFA contribute to ca. 80% of total FA leaving the rumen (Atkinson et al., 2006; Halmemies-Beauchet-Filleau et al., 2013). Polar lipids represented the second-most-abundant lipid fraction at the omasum that comprise plant-derived phospholipids and glycolipids escaping ruminal metabolism (Harfoot and Hazlewood, 1988) and microbial membrane lipids (Doreau and Ferlay, 1994). In the current study, relatively small amounts of FA escaped the rumen within TAG, DAG, and MAG fractions, consistent with much earlier re-

ports in the literature (Harfoot and Hazlewood, 1988). Extensive hydrolysis of TAG also explains marginal increases in postruminal flow of all *cis* unsaturated PUFA when plant oils and processed oilseeds are fed (Doreau and Ferlay, 1994; Glasser et al., 2008).

Most of the 18-carbon all-*cis* unsaturated FA at the omasum escaped the rumen as components of esterified lipid fractions, whereas their biohydrogenation intermediates *trans*-18:1, non-methylene-interrupted 18:2, and CLA and end product 18:0 were almost exclusively in nonesterified form. Earlier investigations have also reported that the majority of 18-carbon all-*cis* unsaturated FA at the omasum or duodenum are esterified (Atkinson et al., 2006; Halmemies-Beauchet-Filleau et al., 2013). These findings indicate that increases in PUFA supply arise principally from dietary lipid escaping lipolysis rather than biohydrogenation in the rumen.

FA. Replacing GS with RCS resulted in progressive increases in the amount of 18:3n-3 in all lipid fractions at the omasum. Irrespective of forage species, ca. 43% of 18:3n-3 at the omasum was in the form of PL, suggesting that a significant part of 18:3n-3 escaped the rumen as a component of chloroplast membranes. Substitution of GS for RCS in the diet also increased *cis*-9 18:1 and 18:2n-6 at the omasum, principally due to higher ruminal escape of these FA in nonesterified form. It seems that the reasons for higher escape of *cis*-9 18:1 and 18:2n-6 in NEFA compared with 18:3n-3 is related to the distribution of these FA in forage lipids, and the relatively high abundance of *cis*-9 18:1 and 18:2n-6 in TAG of concentrate ingredients. Furthermore, ruminal biohydrogenation of *cis*-9 18:1 and, to a lesser extent 18:2n-6, is often lower than that of 18:3n-3 (Glasser et al., 2008) possibly due to preferential storage of these FA in the vacuoles of rumen bacteria (Bauchart et al., 1990) or direct incorporation into membrane lipid of the rumen microbes (Doreau and Ferlay, 1994; Vlaeminck et al., 2006).

Overall, the influence of forage species on ruminal metabolism of 18-carbon unsaturated FA was due, in the most part, to lowered lipolysis of esterified lipid in ensiled red clover compared with a mixture of grasses. Rates at which NEFA were released in the rumen were not measured, but it seems plausible that differences in lipolysis would also influence the kinetics of ruminal biohydrogenation. Increases in *cis*-9,*trans*-11 CLA and *cis*-9,*trans*-11,*cis*-15 18:3 at the omasum suggest that the initial isomerization of 18:2n-6 and 18:3n-3, the reduction of primary biohydrogenation intermediates, or possibly both occur at a much lower rate when RCS replaces GS in the diet. Earlier investigations have reported that RCS increases *cis*-9,*trans*-11 CLA accumulation in vitro (Loor et al., 2003) or at the duodenum in lactating cows (Dewhurst et al., 2003) compared with GS. However, it remains unclear whether the differences in the profile of biohydrogenation intermediates is solely a function of altered lipolysis or a reflection of a much broader influence of forage species on ruminal digestion kinetics and passage rates. Nevertheless, current measurements offer no support that following initial isomerization, nonesterified 18-carbon PUFA are less susceptible to biohydrogenation when RCS replaces GS in the diet. In the current experiment, mixtures of GS and RCS resulted in higher flows of 18:0, several *trans* 18:1 (Δ5–8, 11), and *trans*-11,*cis*-15 18:2 at the omasum than when RCS or GS was the sole forage in the diet, which may, at least in part, be associated with the positive associative effects on intake.

Compared with GS, RCS also increased the amount of nonesterified *cis*-18:1 (Δ11,12) and *trans*-18:1 (Δ12–16) at the omasum, that were associated with concomitant decreases in *trans*-10,*cis*-12 CLA, *trans*-11,*cis*-13 CLA, and *trans*-9,*trans*-11 CLA, suggesting subtle changes in the relative importance of minor biohydrogenation pathways between forage species. Such differences, although small, may reflect the influence of forage species on ruminal lipolysis, passage rate, or microbial ecology. Recent studies have shown that replacing GS with RCS alters the diversity and abundance of ruminal bacteria capable of lipolysis and biohydrogenation (Huws et al., 2010). Replacing GS with RCS in the diet increased 17:0 and decreased *iso* 15:0 and *iso* 17:0 at the omasum, confirming earlier observations in growing cattle (Lee et al., 2008). Based on differences in the FA composition of rumen bacteria, it has been suggested that the amount of *iso* 15:0 and *iso* 17:0 in ruminal contents could serve as a marker of *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens* populations (Vlaeminck et al., 2006), the latter exhibiting phospholipatic activity and responsible for biohydrogenation in the rumen (Lourenço et al., 2010).

Ensiled red clover contained lower concentrations of 3S,7R,11R,15-tetramethyl-16:0 compared with GS and resulted in a lower net synthesis of 3S,7R,11R,15-tetramethyl-16:0 in the rumen consistent with earlier reports in growing cattle (Lee et al., 2006), whereas net ruminal synthesis of the minor 3R,7R,11R,15-tetramethyl-16:0 diastereomer was similar among diets. Appearance of 3,7,11,15-tetramethyl-16:0 in ruminal digesta arises from bacterial hydrogenation of the phytol moiety of chlorophyll a and b (Patton and Benson, 1966). Typically, RCS and GS contain similar amounts of chlorophyll (Lee et al., 2006), suggesting that one or more attributes of red clover may protect chlorophyll in chloroplasts from conversion to 3S,7R,11R,15-tetramethyl-16:0 in the rumen.

CONCLUSIONS

Replacing GS with RCS in the diet increased the flow of 18:2n-6 and 18:3n-3 and modified the relative abundance of specific biohydrogenation intermediates at the omasum, changes that were associated with decreases in the lipolysis of esterified lipids in the rumen. The majority of 18:2n-6 and 18:3n-3 at the omasum originated from ruminal escape of polar and neutral lipid, suggesting that the influence of forage species may be mediated via several interrelated mechanisms, including possible formation of protein complexes as a consequence of PPO activity, alterations in digestion kinetics and forage particle size distribution in the rumen, and ruminal microbial ecology.

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