Effect of forage conservation method on ruminal lipid metabolism and microbial ecology in lactating cows fed diets containing a 60:40 forage-to-concentrate ratio

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ABSTRACT

The effect of forage conservation method on ruminal lipid metabolism and microbial ecology was examined in 2 complementary experiments in cows. Treatments comprised fresh chopped grass, barn-dried hay, or untreated (UTS) or formic acid-treated silage (FAS) prepared from the same grass sward. Preparation of conserved forages coincided with the collection of samples from cows offered fresh grass. In the first experiment, 5 multiparous Finnish Ayrshire cows (229 d in milk) were used to compare the effects of feeding diets based on grass followed by hay during 2 consecutive 14-d periods separated by a 5-d transition during which extensively wilted grass was fed. In the second experiment, 5 multiparous Finnish Ayrshire cows (53 d in milk) were assigned to 1 of 2 blocks and allocated treatments according to a replicated 3 × 3 Latin square design with 14-d periods to compare the effects of hay, UTS, and FAS. Cows received 7 or 9 kg/d of the same concentrate in experiments 1 and 2, respectively. Conservation of grass by drying, but not ensiling, decreased forage fatty acid content primarily due to losses of 18:2n-6 and 18:3n-3. Compared with grass, feeding hay had no effect on dry matter intake (DMI), rumen pH, or fermentation characteristics, other than increasing ammonia content, but lowered whole-tract organic matter and fiber digestibility (experiment 1). Relative to hay, silage increased DMI, rumen volatile fatty acid (VFA) concentrations, and molar proportions of butyrate, and decreased molar acetate proportions (experiment 2). Compared with UTS, FAS increased DMI, had no effect on rumen ammonia or VFA concentrations,

but tended to lower rumen pH and the molar ratio of lipogenic to glucogenic VFA. Conservation method had no substantial effect on ruminal or whole-tract digestibility coefficients. Compared with fresh grass and silages, hay decreased lipolysis and biohydrogenation (BH) of dietary unsaturates in the rumen, resulting in similar flows of 18:2n-6 and 18:3n-3, but lower amounts of *trans*-11 18:1 and Δ 11,13 18:2 at the omasum. The extent of silage fermentation had minimal influence on ruminal lipid metabolism. Treatments were not associated with changes in the relative abundance of specific bacteria known to be capable of BH or rumen protozoal numbers. In conclusion, conservation method altered forage lipids, the extent of lipolysis and BH in the rumen, and the flow of fatty acids at the omasum, in the absence of substantial changes in ruminal *Butyrivibrio* populations.

Key words: *Butyrivibrio*, cow, forage conservation, ruminal biohydrogenation

INTRODUCTION

Ruminant meat and milk are characterized by high relative proportions of SFA and low proportions of PUFA due, at least in part, to extensive biohydrogenation of dietary unsaturated FA in the rumen (Dewhurst et al., 2006; Kim et al., 2009; Shingfield et al., 2012a). In humans, consumption of excessive amounts of SFA, 12:0, 14:0, and 16:0, in particular, is associated with an increase in cardiovascular disease risk and development of insulin resistance and dyslipidemia (WHO, 2003; Shingfield et al., 2008b). Milk and dairy products are typically a major source of medium-chain and total SFA in the Western diet (Hulshof et al., 1999; Kris-Etherton et al., 2000), generating considerable interest in lowering the amounts of medium-chain SFA, and increasing *cis*-9 18:1, 18:2n-6, conjugated linoleic acid (CLA), and 18:3n-3 in ruminant milk to improve long-term human health (refer to Shingfield et al., 2008b).

Concentrations of medium-chain SFA are known to be lower, and those of *cis*-9 18:1, *trans*-11 18:1, and

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cis-9,trans-11 CLA are higher in milk from pasture compared with hay or silage (Dewhurst et al., 2006; Elgersma et al., 2006; Chilliard et al., 2007), but the underlying mechanisms are not well defined. Even though wilting of grass is associated with extensive losses of unsaturated FA (Dewhurst and King, 1998; Boufaïed et al., 2003a; Shingfield et al., 2005), the concentrations of 18:2n-6 and 18:3n-3 in milk on grass hay-based diets are often similar to that from pasture (Dewhurst et al., 2006; Ferlay et al., 2006; Coppa et al., 2011). Differences in forage ensiling method have relatively minor effects on forage FA content, unless secondary fermentation in silo is extensive (Dewhurst and King, 1998; Boufaïed et al., 2003a; Shingfield et al., 2005), but milk from silage-based diets typically contain lower concentrations of PUFA compared with fresh grass or hay (Dewhurst et al., 2006; Chilliard et al., 2007). The efficiency of transfer of 18:3n-3 from the diet into milk is higher on hay- than grass silage-based diets (Shingfield et al., 2005), with the implication that forage conservation method influences the extent of ruminal escape of forage PUFA. Furthermore, recent studies have provided evidence that shifts in rumen microbial communities may, at least in part, contribute to dietary induced differences in ruminal lipid metabolism and outflow of FA (Huws et al., 2010; Shingfield et al., 2012b).

The aim of the present study was to examine the influence of conservation method on forage lipids, ruminal lipid metabolism, rumen microbial ecology, and flows of FA at the omasum in lactating cows fed diets based on fresh or conserved grasses prepared simultaneously from the same swards of timothy and meadow fescue. Measurements of plasma lipid concentrations, mammary lipogenesis, and milk fat composition will be reported in a companion paper [A. Halmemies-Beauchet-Filleau, P. Kairenius, S. Ahvenjärvi, V. Toivonen, P. Huhtanen, A. Vanhatalo, D. I. Givens (University of Reading, Reading, UK), and K. J. Shingfield, unpublished data].

MATERIALS AND METHODS

Forage Management

All experimental forages were prepared from the same primary growths of 3-yr leys of timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) grown in Jokioinen, Finland (60°49'N, 23°28'E). On April 25, 2005, grass leys were fertilized with N and K at a rate of 96 and 4 kg/ha, respectively. Experiment 1 started on June 6, 2005. On each morning over a 7-d period (June 13 to 19, 2005) corresponding to d 8 to 14 of experiment 1, approximately 600 kg of grass was collected using a precision chop forage harvester and treated with a for-

mic acid-based additive (0.5 L/t; AIV II Plus; Kemira Ltd., Helsinki, Finland) in an attempt to ensure that the composition of chopped grass resembled, as far as possible, parent herbage. Once collected, fresh chopped grass was transported immediately to a dedicated metabolism unit and stored at +4°C before feeding out to minimize oxidative deterioration. On June 15, 2005, corresponding to d 10 of the first experiment (Figure 1), an area of the same pasture used for zero grazing was cut using a mower-conditioner and conserved as hay or silage. Grass used for the production of hay was wilted extensively in the field over a 4-d period to a final DM content of 750 g/kg, baled, transported to a barn and left to dry further at ambient temperature for 5 d before being fed on d 20 of experiment 1. Grass assigned for the production of silage was cut using a mower-conditioner, wilted in the field for 3 to 4 h to a DM content of 235 g/kg, before being collected with a precision-chop harvester and ensiled in bunker silos. Differences in silage fermentation characteristics were achieved by ensiling grass directly with no additive (untreated, **UTS**) or using a formic acid-based additive (**FAS**; AIV II Plus) applied at a rate of 6 L/t of grass to restrict fermentation in silo. The chop length of fresh grass and grass silages averaged 3 to 4 cm. Dried hay was chopped (average chop length of 5 cm) immediately before feeding out.

Animals, Experimental Design, and Experimental Diets

All procedures involving animals were approved by the Animal Experiment Committee of MTT Agrifood Research Finland in accordance with the Use of Vertebrates for Scientific Purposes Act of 1985. Five multiparous lactating Finnish Ayrshire cows (605 ± 25.7 kg of BW and 229 ± 31.7 DIM) fitted with rumen cannulas (100-mm i.d.; Bar Diamond Inc., Parma, ID) were used in the first experiment. Another group of 5 rumen-fistulated Finnish Ayrshire cows (617 ± 25.8 kg of BW and 53 ± 5.3 DIM) were recruited for the second experiment. For both experiments, cows were housed in a dedicated metabolism unit fitted with individual stalls with continuous access to fresh water.

In experiment 1, the effects of feeding diets based on fresh chopped grass and barn dried hay were compared over 2 consecutive 14-d periods (June 6 to July 8, 2005). During the first period, cows were allowed to strip graze the same pasture used for conservation for 7 d before being transported to the metabolism unit and then zero grazed for 7 d. During the second 14-d period, cows received diets based on barn-dried hay. During the transition from fresh chopped formic acid-treated grass to grass hay, cows were offered extensively wilted grass

Figure 1. Scheduling of ensilage or drying of mixed timothy and meadow fescue swards relative to dietary treatments offered to cows in experiment 1.

for 5 d obtained from the same grass swaths baled to produce hay. This transition between treatments was introduced to provide enough time for the drying and chemical stabilization of hay and also allow cows to adjust to the switch from fresh grass to dried hay (Figure 1). Experiment 2 (October 29 to December 9, 2005) evaluated the effects of feeding diets based on hay, UTS, or FAS. Cows were assigned to 1 of 2 blocks according to stage of lactation and milk yield and allocated to treatments according to a replicated 3×3 Latin square design with 5 cows, 3 treatments, and 3 periods, each 14 d in duration. For both experiments, forages were offered *ad libitum* in amounts to ensure 10% refusals. To minimize potential oxidative deterioration of fresh grass in particular during feeding, all forages were offered as frequent meals (approximately 4 to 6 times per day) of 2 to 3 or 10 to 15 kg for hay or fresh grass and silages, respectively. Forages were supplemented with a common standard concentrate (Table 1) at 7 or 9 kg/d for experiments 1 and 2, respectively, offered in equal amounts at 0600, 0800, 1800, and 2000 h. Concentrates were fed to meet or exceed nutrient requirements (MTT Agrifood Research Finland, 2004), in an amount to result in similar dietary forage:concentrate ratios between experiments. Cows were milked at 0700 and 1645 h.

Measurements and Sampling

Samples of intact fresh grass were collected by hand directly from grass leys at the same time swards were cut for the preparation of conserved forages on June 15, 2005 (Figure 1). Once collected, samples were

separated into 2 equal subsamples. One subsample for the determination of FA content was immediately immersed in liquid nitrogen, freeze dried, milled, and stored at -80°C before analysis. The other subsample was stored at -20°C and submitted for the measurement of DM, ash, N, indigestible NDF (**iNDF**), NDF, and water-soluble carbohydrate content.

Individual cow intakes were recorded daily throughout each experiment, but only measurements for the last 5 d were used for statistical analysis. Representative samples of feeds were collected daily over the last 5 d of each period and stored at -20°C . The chemical composition of feeds, including the correction of silage DM content for volatile losses, omasal digesta, and feces were determined using standard procedures (Shingfield et al., 2001). The concentration of **iNDF** of feeds, digesta, and feces was determined in duplicate by incubating 0.5- to 1-g samples within polyester bags (60×120 mm, $17\text{-}\mu\text{m}$ 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.

Samples of ruminal fluid ($n = 8$) were collected via 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 (Shingfield et al., 2002). To assess rumen protozoal numbers, a 10-mL subsample of filtrate was taken and preserved with 30 mL of 0.9% (wt/vol) aqueous NaCl containing 10% (vol/vol) CH_2O . Samples for each sampling time were composited and replicate ($n = 6$) measurements of protozoal numbers were made

Table 1. Chemical composition of dietary ingredients

Composition	Experiment 1			Experiment 2				
	Fresh herbage ¹	Chopped grass	Grass hay	Concentrate ²	Grass hay	Untreated silage ³	Formic acid-treated silage ⁴	Concentrate ²
DM, %	18.4	18.4	87.0	88.9	83.8	23.8	24.3	88.1
% of DM								
OM	92.0	92.5	95.8	92.0	92.9	91.7	92.3	92.0
CP	13.5	13.7	13.5	16.9	13.8	15.6	15.0	16.5
NDF	58.5	59.3	62.3	23.8	62.8	54.3	53.8	26.8
iNDF	6.04	7.14	7.63	5.23	7.35	6.05	5.59	5.31
Water-soluble carbohydrates	1.12	1.22	1.04		1.08	1.75	4.28	

¹Refers to intact fresh herbage collected at the time of harvest.

²Concentrate in pelleted form was formulated (% on DM basis) from rolled barley (52), molassed sugar beet pulp (25), solvent-extracted rapeseed meal (20; Raisio Feed Ltd., Raisio, Finland), and a proprietary vitamin and mineral supplement (3; Onni; Melica Finland Ltd., Vaasa, Finland), 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); and (IU/g) retinyl acetate (120), cholecalciferol (25), and DL- α -tocopheryl acetate (0.34).

³Mean fermentation characteristics of untreated silage: pH 4.21; % of DM: lactic acid, 9.08; acetic acid, 3.60; propionic acid, 0.12; and butyric acid, 0.35; % of total N: ammonium-N, 9.52.

⁴Mean fermentation characteristics of restrictively fermented silage: pH 3.99; % of DM: lactic acid, 5.71; acetic acid, 2.35; propionic acid, 0.02; and butyric acid, 0.16; % of total N: ammonium-N, 4.79.

using a counting chamber (Fuchs-Rosenthal, Fortuna, Germany).

Samples of ruminal digesta used for microbial population analysis were collected from 4 sites within the rumen-reticulum at 1500 and 0900 h on d 11 and 13, respectively. Immediately after removal from the rumen, a 50-g subsample was taken and placed in liquid nitrogen before storage at -80°C . Subsamples were composited for each animal within period before quantitative PCR (qPCR) analysis. Extraction of total genomic DNA followed the manufacturer's instructions (Promega Corp., Madison, WI), except that cell lysis was carried for 2×45 s at level 6 of the FastPrep instrument (Qbiogene, Cambridge, UK). Final DNA was recovered in 100 μL of water, quantified by its absorbance at 260 nm, and stored at -20°C . The integrity of the DNA was checked on 1% (wt/vol) agarose gels and the purity was assessed from the 260/280 nm ratio. Purity of DNA was considered acceptable with ratios >1.8 . For qPCR reactions, the DNA was diluted 10 fold, except for the determination of total bacteria when 100-fold dilutions were used. Quantitative PCR was performed using Sigma JumpStart PCR mixes with or without SYBR Green dye. Conditions of the PCR were as follows: denaturation (95°C) for 3 min, followed by 40 cycles at 95°C for 15 s and 60 s at the respective annealing temperature, except for the *Butyrivibrio proteoclasticus* and the *Butyrivibrio hungatei* primer sets, where the following protocol was used: initial denaturation (95°C) for 3 min, followed by 40 cycles at 95°C for 15 s and 30 s at the respective annealing temperature and 30 s at 72°C (Supplemental Table 1, available online at <http://www.journalofdairyscience.org>). The specificity of the primers and probes was validated on the same set of strains used in earlier reports, as well as 30 additional members of the *Butyrivibrio* group and a further 70 strains of ruminal bacteria held in the culture collection at the Rowett Institute of Nutrition and Health (Aberdeen, UK) as outlined elsewhere (Shingfield et al., 2012b). Calibration was carried out using DNA from a mixture of known target organisms. Responses were also tested using the same DNA added to DNA extracted from omasal digesta. Results were analyzed using the iCycler iQ detection system software (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK).

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) and Yb-acetate (7.5 g) were administered directly into the rumen of each cow at 1800 h on d 8 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 kg) and infused via separate infusion lines into the rumen at a constant rate for 120 h until 1800 h on d 13.

Spot samples (500 mL) of digesta entering the omasal canal were collected 4 times daily at 3-h intervals on d 11 through 13 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 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 analysis were stored at -80°C . All digesta phases were analyzed for DM, OM, Cr, Yb, and iNDF content. Concentrations of Cr and Yb were determined by inductively coupled plasma atomic emission spectrophotometry. 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 lipid analysis.

Whole-tract apparent digestibility coefficients were determined by total fecal collection over 96 h, starting at 1800 h on d 9 of each experimental period. Subsamples (5%, wt/wt) were stored at -20°C until analyzed for DM, OM, NDF, iNDF, N, Cr, and Yb using same methods as for feeds and omasal digesta.

Lipid Analysis

Fatty acid content of feeds and omasal digesta were determined using internal standards based on 13:0 in esterified or nonesterified form due to low abundance in analyzed samples and the elution of the 13:0 methyl ester in the absence of interference with other FA methyl esters (**FAME**) during gas chromatography analysis. Following the addition of 1 mL of internal standard (0.1 wt/vol of tridecanoic acid in absolute ethanol; N-13A; Nu-Chek Prep Inc., Elysian, MN) and 1 mL of deionized water, the pH of 100 mg of freeze-dried samples of

forages and reconstituted omasal digesta was adjusted to 2.0 with 2 M hydrochloric acid. Lipid was extracted using a mixture (4 mL; 3:2, vol/vol) of hexane and isopropanol. Extractions were repeated and the organic phases recovered combined. Nonesterified FA were separated from other lipid classes by solid-phase extraction using aminopropyl-bonded silica cartridges (500 mg Bond Elut NH_2 ; Varian Inc., Lake Forest, CA) and transesterified to FAME using 1% sulfuric acid in methanol as a catalyst (Shingfield et al., 2003). 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 incubations with chloroform and 2% (vol/vol) sulfuric acid in methanol at 70°C for 2 h for forages or 2% (vol/vol) sulfuric acid in methanol at 50°C for 1 h for omasal lipids (Shingfield et al., 2003). Total lipid in freeze-dried samples of concentrates were converted to FAME using a one-step extraction-transesterification procedure using 2% (vol/vol) sulfuric acid in methanol as a catalyst with chloroform at 70°C for 3 h and tritridecanoin (T-135; Nu-Chek Prep Inc.) as an internal standard (Shingfield et al., 2003).

The FAME prepared from lipid in feeds and omasal digesta were quantified using a gas chromatograph (model 6890; Hewlett-Packard Co., 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 88, Chromopack 7489; Chromopack International BV, Middelburg, the Netherlands). 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 a carrier gas operated at constant pressure (137.9 kPa) at a flow rate of 0.5 mL/min (Shingfield et al., 2003). Isomers of 18:1 were further resolved in a separate analysis under isothermal conditions at 170°C (Shingfield et al., 2003). Peaks were identified by comparison of retention times with authentic FAME standards (Shingfield et al., 2003; Halmemies-Beauchet-Filleau et al., 2011). Methyl esters of FA not available as commercial standards were identified based on GC-MS of 4,4-dimethylxoline derivatives prepared from FAME. Preparation of 4,4-dimethylxoline derivatives, parameters used for GC-MS analysis, and interpretation of 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 (Schröder and Vetter, 2011). The distribution of CLA isomers in omasal digesta was determined by HPLC (model 1090; Hewlett-Packard Co.) using 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 and 0.1% (vol/vol) acetonitrile in heptane as the mobile phase (Halmemies-Beauchet-Filleau et al., 2011).

Calculations

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

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

Apparent biohydrogenation of unsaturated FA in the rumen was calculated as

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

Statistical Analysis

Treatment means in experiment 1 were evaluated by a paired *t*-test (SAS version 9.2.; SAS Institute Inc., Cary, NC). In all cases, a 95% confidence interval was used as the default for the hypothesis test, whereas assumptions of normality were validated using the univariate procedure. Differences between treatment means were considered to reflect the effect of forage conservation by drying.

For experiment 2, data were analyzed by ANOVA for a replicated 3 × 3 Latin square with 5 cows to evaluate 3 treatments over 3 experimental periods, with a model that included the fixed effects of treatment and period and random effects of block and cow within block using PROC MIXED of SAS. Sums of squares were further separated into single degree-of-freedom contrasts to test for the significance of forage conservation by drying versus ensiling (method = hay vs. UTS and FAS) and extent of fermentation in silo (extent = UTS vs. FAS). 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

Chemical Composition of Feeds

The chemical composition of experimental feeds is shown in Table 1. All conserved forages were of high

nutritional quality, whereas, by design, the fermentation characteristics of UTS were typical of extensively fermented grass silage. The FA content and composition of chopped fresh grass treated with formic acid fed during the first period of experiment 1 was similar to that of intact parent herbage (Table 2). Most FA in parent herbage and chopped grass were esterified (ca. 89%), whereas the ratio of NEFA to total FA in hay and silages was much higher and similar (over 55%) after storage over an equal period of time. Drying of grass was associated with a substantial decrease in forage FA content due, in the main, to extensive losses of 18:2n-6 and 18:3n-3 (58 and 70%, respectively; experiment 1), whereas ensiling resulted in minimal FA losses (Table 2). For all forages, 18:3n-3 was the major (40–55%) FA, with 18:2n-6 (16–19%) and 16:0 (14–21%) being relatively abundant (Table 2). Concentrate supplements were relatively rich in 18:2n-6 (on average, 42% of FA), *cis*-9 18:1 (24%), and 16:0 (15%), with few differences in FA composition and content between experiments (Table 2).

Nutrient Intake and Digestibility

Fresh Grass Versus Grass Hay. Replacing fresh grass with hay had no effect ($P > 0.05$) on DMI, or on the ingestion of OM or CP, but tended to ($P = 0.09$) increase NDF and potentially digestible NDF (pdNDF) intake (Table 3). The intake of most FA, 16:0, *cis*-9 18:1, 18:2n-6, and 18:3n-3, in particular, was decreased ($P \leq 0.05$) when hay replaced fresh grass in the diet (on average, 24, 17, 22, and 61%, respectively; Table 3). Substituting fresh grass for hay in the diet had no effect ($P > 0.10$) on apparent digestion of OM, NDF, or N in the rumen, but lowered ($P < 0.04$) whole-tract OM and NDF digestibility coefficients (Table 4).

Grass Hay Versus Grass Silage. Intakes of DM and CP were higher ($P < 0.04$) in cows fed silage compared with hay (Table 3). Because of the differences in DMI and the fatty content between conserved forages, the intakes of *cis*-9 18:1, 18:2n-6, 18:3n-3, and total FA were substantially higher ($P < 0.01$) in cows fed silage compared with hay (Table 3). Feeding silage relative to hay had no effect ($P > 0.10$) on ruminal or whole-tract digestibility of OM and NDF, but improved ($P < 0.05$) whole-tract N digestion (Table 4).

Extent of Silage Fermentation. Application of an ensiling additive was associated with increased ($P < 0.05$) intake of DM, OM, pdNDF, and water-soluble carbohydrates relative to UTS (Table 3). However, the intake of FA was similar ($P > 0.10$) for FAS and UTS (Table 3). Restricting silage fermentation had no effect ($P > 0.05$) on nutrient digestibility compared with UTS (Table 4).

Table 2. Fatty acid content of dietary ingredients

FA, g/kg of DM	Experiment 1							Experiment 2						
	Fresh herbage ¹		Chopped grass		Grass hay		Concentrate	Grass hay		Untreated silage		Formic acid-treated silage		Concentrate
	NEFA	TFA ²	NEFA	TFA	NEFA	TFA	TFA	NEFA	TFA	NEFA	TFA	NEFA	TFA	TFA
12:0	0.01	0.19	0.01	0.19	0.01	0.05	0.01	0.01	0.04	0.02	0.16	0.02	0.13	0.02
14:0	0.02	0.10	0.02	0.10	0.03	0.04	0.05	0.03	0.04	0.05	0.09	0.05	0.08	0.06
<i>cis</i> -9 14:1	ND ³	0.04	ND	0.05	ND	0.01	0.01	ND	0.01	ND	0.03	ND	0.03	ND
16:0	0.39	3.58	0.52	3.58	0.85	1.94	3.93	0.85	1.60	2.61	3.80	2.77	3.29	4.68
<i>cis</i> -9 16:1	0.01	0.04	0.01	0.05	0.01	0.03	0.15	0.01	0.02	0.04	0.05	0.04	0.04	0.16
<i>trans</i> -3 16:1	0.01	0.49	0.02	0.40	0.02	0.15	0.02	0.04	0.10	0.25	0.37	0.26	0.30	0.02
18:0	0.08	0.25	0.12	0.29	0.14	0.19	0.35	0.14	0.14	0.26	0.30	0.23	0.25	0.47
<i>cis</i> -9 18:1	0.09	0.97	0.09	1.02	0.11	0.32	6.29	0.11	0.25	0.44	0.80	0.52	0.71	7.42
<i>cis</i> -11 18:1	0.01	0.09	0.01	0.10	0.02	0.05	1.57	0.02	0.01	0.02	0.03	0.02	0.02	1.54
Σ <i>trans</i> 18:1	ND	ND	ND	ND	ND	ND	0.14	ND	ND	ND	ND	ND	ND	0.18
18:2n-6	0.32	4.40	0.36	4.27	0.51	1.83	11.2	1.03	1.44	1.91	3.88	2.45	3.47	13.0
18:3n-3	1.20	12.8	1.03	12.5	1.09	3.80	2.04	2.08	3.06	6.84	13.2	7.89	11.7	2.24
20:0	0.01	0.12	0.01	0.15	0.02	0.12	0.12	0.03	0.08	0.03	0.12	0.03	0.10	0.14
<i>cis</i> -11 20:1	ND	ND	ND	ND	ND	ND	1.57	ND	ND	ND	ND	ND	ND	0.22
22:0	0.04	0.22	0.04	0.25	0.06	0.16	0.10	0.06	0.12	0.11	0.21	0.11	0.19	0.10
24:0	0.03	0.13	0.03	0.15	0.04	0.08	0.11	0.04	0.06	0.08	0.13	0.08	0.12	0.12
26:0	0.07	0.18	0.07	0.21	0.07	0.10	0.02	0.07	0.09	0.11	0.18	0.10	0.14	0.02
28:0	0.21	0.36	0.21	0.39	0.19	0.22	ND	0.18	0.17	0.28	0.32	0.27	0.20	ND
Σ Other ⁴	0.28	0.74	0.32	0.89	0.27	0.52	0.79	0.29	0.37	0.49	0.63	0.49	0.60	0.87
Σ SFA	1.10	5.63	1.32	5.90	1.61	3.25	4.84	2.09	2.55	3.86	5.68	3.93	4.90	5.76
Σ MUFA	0.15	1.24	0.18	1.33	0.19	0.50	8.69	0.29	0.38	0.61	1.04	0.71	0.94	9.90
Σ PUFA	1.53	17.4	1.40	16.9	1.61	5.71	13.4	3.13	4.56	8.79	17.1	10.4	15.2	15.5
Total	2.77	24.8	2.91	24.6	3.43	9.62	27.0	5.53	7.59	13.5	24.3	15.3	21.4	31.3

¹Refers to intact fresh herbage collected at the time of harvest.

²TFA = total FA.

³ND = not detected.

⁴Contains 15:0, 17:0 *iso*, *cis*-11 16:1, *trans*-13 16:1, *cis*-13 18:1, 18:2 undetermined double-bond position and configuration, 18:3 undetermined double-bond position and configuration, *cis*-9 20:1, 20:2n-6, *cis*-11 22:1, *cis*-13 22:1, *cis*-15 22:1, 23:0, *cis*-15 24:1, 25:0, 29:0, 30:0, 32:0, and 6 unidentified FA.

Table 3. Effect of fresh grass or conserved forages on nutrient intake in lactating cows

Intake	Experiment 1				Experiment 2				<i>P</i> -value ⁵	
	Grass	Hay	SEM ¹	<i>P</i> -value	Hay	UTS ²	FAS ³	SEM ⁴	Method	Extent
kg/d										
Forage DM	10.7	12.3	0.77	0.11	11.8	12.3	13.9	1.03	0.038	0.029
Total DM	16.7	18.4	0.792	0.10	19.8	20.2	21.8	1.03	0.038	0.029
OM	15.6	17.1	0.799	0.15	18.3	18.6	20.1	0.95	0.06	0.024
CP	2.56	2.67	0.121	0.43	2.95	3.22	3.40	0.153	0.003	0.10
NDF	7.94	9.12	0.506	0.09	9.59	8.80	9.54	0.573	0.18	0.06
pdNDF ⁶	6.84	7.87	0.444	0.09	8.29	7.63	8.35	0.505	0.26	0.042
Starch	1.94	2.22	0.021	0.002	2.71	2.68	2.71	0.011	0.06	0.023
Water-soluble carbohydrates	1.72	1.65	0.101	0.51	1.78	0.75	1.10	0.064	<0.001	<0.001
FA, g/d										
12:0	2.10	0.76	0.150	<0.001	0.67	2.11	2.11	0.164	<0.001	0.98
14:0	1.44	0.84	0.080	0.002	0.94	1.59	1.58	0.089	<0.001	0.93
<i>cis</i> -9 14:1	0.56	0.17	0.036	<0.001	0.09	0.42	0.41	0.036	<0.001	0.81
16:0	62.4	47.5	2.84	0.007	56.5	82.7	84.8	3.77	<0.001	0.64
<i>cis</i> -9 16:1	1.46	1.23	0.040	0.005	1.54	1.92	1.88	0.048	<0.001	0.43
<i>cis</i> -11 16:1	0.12	0.10	0.001	<0.001	0.16	0.15	0.15	0.001	1.00	1.00
18:0	5.34	4.43	0.233	0.017	5.38	7.26	7.37	0.306	<0.001	0.76
<i>cis</i> -9 18:1	49.7	41.4	0.986	0.002	62.0	68.4	69.1	0.78	<0.001	0.45
<i>cis</i> -11 18:1	11.1	9.55	0.144	<0.001	12.3	12.6	12.6	0.03	<0.001	0.82
Σ <i>trans</i> 18:1	0.92	0.73	0.010	<0.001	1.38	1.45	1.38	0.001	1.00	1.00
18:2n-6	114	89.3	3.59	0.003	121	149	153	3.9	<0.001	0.41
18:3n-3	148	58.4	9.79	<0.001	55.1	175	187	13.3	<0.001	0.48
20:0	2.39	2.14	0.263	0.11	2.05	2.55	2.61	0.124	0.006	0.69
<i>cis</i> -11 20:1	1.15	1.12	0.010	0.041	1.70	1.81	1.75	0.001	1.00	1.00
22:0	3.36	2.59	0.194	0.017	2.20	3.31	3.54	0.241	0.004	0.49
24:0	2.36	1.64	0.120	0.004	1.67	2.47	2.60	0.161	0.004	0.60
26:0	2.39	1.36	0.160	0.003	1.22	2.31	2.26	0.172	<0.001	0.83
28:0	4.22	2.70	0.302	0.008	2.02	3.87	4.06	0.315	<0.001	0.62
Σ Other	13.3	9.76	0.656	0.006	10.2	12.6	13.8	0.737	0.008	0.24
Σ Unidentified	0.96	0.71	0.012	<0.001	1.20	1.29	1.27	0.001	1.00	1.00
Σ SFA	93.2	68.7	4.64	0.007	76.4	114	117	5.77	<0.001	0.64
Σ MUFA	68.4	57.2	1.31	0.001	83.2	90.9	92.2	1.05	<0.001	0.36
Σ PUFA	265	150	13.5	0.001	178	327	344	17.3	<0.001	0.46
Total	427	276	19.4	0.002	339	533	554	24.1	<0.001	0.49

¹Standard error of the mean for n = 10 measurements; error degree of freedom = 4.²Untreated silage.³Silage ensiled using a formic acid-based additive.⁴Standard error of the mean for n = 15 measurements; error degree of freedom = 6.⁵Significance of effect due to forage conservation method (drying vs. ensiling; method) or extent of silage fermentation (extensively vs. restrictively fermented silage; extent).⁶Potentially digestible NDF.

Rumen Fermentation

Fresh Grass Versus Grass Hay. Replacing fresh grass with hay in the diet had no effect ($P > 0.10$) on rumen pH, resulted in higher ($P < 0.02$) ruminal ammonia-N concentrations, and tended to increase ($P = 0.08$) rumen VFA concentrations (Table 5). Molar proportions of individual VFA in rumen fluid were similar ($P > 0.10$) for diets based on fresh grass or hay diets, other than higher ($P < 0.01$) molar valerate proportions for hay diets (Table 5).

Grass Hay Versus Grass Silage. Replacing hay with silage in the diet had no effect ($P > 0.10$) on rumen pH, but increased ($P < 0.03$) rumen VFA con-

centrations (Table 5). Compared with hay, feeding diets based on silage resulted in higher ($P < 0.01$) molar proportions of butyrate, caproate, and branched-chain VFA in the rumen fluid, and lowered ($P < 0.01$) the molar proportion of acetate (Table 5).

Extent of Silage Fermentation. Using an ensiling additive tended to lower ($P = 0.08$) rumen pH and molar propionate proportions, increased ($P < 0.01$) molar butyrate proportions, but had no effect ($P > 0.10$) on rumen VFA concentrations (Table 5).

Nutrient Flow at the Omasum

Replacing fresh grass with hay in the diet had no major influence ($P > 0.05$) on the flow of nutrients at

Table 4. Effect of fresh grass or conserved forages on rumen and whole-tract apparent digestibility coefficients in lactating cows

Digestibility, %	Experiment 1				Experiment 2				<i>P</i> -value ⁵	
	Grass	Hay	SEM ¹	<i>P</i> -value	Hay	UTS ²	FAS ³	SEM ⁴	Method	Extent
Rumen										
OM	48.4	45.2	1.92	0.18	47.6	50.5	51.2	3.06	0.19	0.78
Nitrogen	−14.6	−17.5	5.42	0.62	−7.6	5.4	3.4	5.29	0.015	0.65
NDF	69.1	68.5	0.92	0.54	66.3	66.8	68.3	2.47	0.52	0.48
pdNDF ⁶	77.4	77.6	0.70	0.39	73.6	73.1	74.8	2.25	0.87	0.40
Whole tract										
OM	79.5	76.6	0.88	0.029	75.5	77.1	76.9	1.46	0.11	0.78
Nitrogen	72.0	70.2	1.02	0.15	67.8	70.6	68.7	1.72	0.043	0.07
NDF	74.7	71.2	1.10	0.035	71.2	71.4	71.0	2.18	0.99	0.82
pdNDF	82.1	80.6	1.12	0.07	80.2	80.0	79.5	1.98	0.71	0.73

¹Standard error of the mean for n = 10 measurements; error degree of freedom = 4.²Untreated silage.³Silage ensiled using a formic acid-based additive.⁴Standard error of the mean for n = 15 measurements; error degree of freedom = 6.⁵Significance of effect due to forage conservation method (drying vs. ensiling; method) or extent of silage fermentation (extensively vs. restrictively fermented silage; extent).⁶pdNDF = potentially digestible NDF.

the omasum, other than altering the flow of specific FA (Table 6). Compared with hay, diets based on UTS or FAS had no effect on OM, NDF, pdNDF, N, NAN, or VFA flow, but increased ($P < 0.01$) the amount of NEFA and total FA at the omasum (Table 6). Omasal flows of FA exceeded intake, on average, by 10, 86, and 26 g/d in cows fed diets based on fresh grass, hay, and silage, respectively (Tables 3 and 6). A higher flow of FA at the omasum relative to intake in cows fed hay was

due, in the most part, to net synthesis of 16:0 (8 g/d), 18-carbon FA (54 g/d), and odd- and branched-chain FA (**OBCFA**; 26 g/d) in the rumen, whereas synthesis of OBCFA (ca. 32 g/d) accounted for the net increase in cows fed fresh grass or grass silage (Tables 3 and 6; Supplemental Table 2, available online at <http://www.journalofdairyscience.org>).

On average, 72, 75, and 84% of FA at the omasum were present as NEFA in cows fed fresh grass, hay, or

Table 5. Effect of fresh grass or conserved forages on rumen fermentation characteristics in lactating cows

Item	Experiment 1				Experiment 2				<i>P</i> -value ⁵	
	Grass	Hay	SEM ¹	<i>P</i> -value	Hay	UTS ²	FAS ³	SEM ⁴	Method	Extent
pH	6.55	6.45	0.082	0.23	6.12	6.14	6.00	0.060	0.46	0.08
Ammonia-N, mmol/L	3.84	5.95	0.530	0.017	5.79	7.67	7.50	1.143	0.10	0.88
Total VFA, mmol/L	100	109	3.9	0.08	123	129	133	2.3	0.026	0.30
Molar proportion, mmol/mol										
Acetate	684	677	5.4	0.22	678	640	643	5.2	<0.001	0.59
Propionate	178	170	4.7	0.17	183	192	172	7.6	0.89	0.08
Butyrate	111	118	5.8	0.25	105	123	141	3.2	<0.001	0.003
Isobutyrate	6.88	6.88	0.438	1.00	7.48	9.59	8.80	0.298	0.002	0.07
Valerate	9.12	17.7	0.684	<0.001	15.1	15.0	15.3	0.51	0.84	0.55
Isovalerate	7.52	6.53	0.551	0.15	7.90	14.6	13.2	0.720	0.002	0.23
Caproate	3.44	3.87	0.596	0.52	3.01	6.89	6.52	0.464	<0.001	0.51
Molar ratio (Acetate + butyrate)/propionate	4.48	4.69	0.145	0.229	4.34	3.99	4.59	0.212	0.85	0.06

¹Standard error of the mean for n = 10 measurements; error degree of freedom = 4.²Untreated silage.³Silage ensiled using a formic acid-based additive.⁴Standard error of the mean for n = 15 measurements; error degree of freedom = 6.⁵Significance of effect due to forage conservation method (drying vs. ensiling; method) or extent of silage fermentation (extensively vs. restrictively fermented silage; extent).

grass silage, respectively (Table 6). Most of the 18:2n-6 and 18:3n-3 escaping the rumen was esterified (74–83 and 74–87%, respectively), whereas the majority of 3,7,11,15-tetramethyl-16:0, 18-carbon biohydrogenation intermediates, and 18:0 were nonesterified. Irrespective of diet, 18:0 was the major FA in omasal digesta, representing ca. 50% of total FA, with 16:0 as the next most abundant (13–16%).

Fresh Grass Versus Grass Hay. Replacing fresh grass with hay resulted in numerical, but nonsignificant ($P > 0.10$) decreases in the flow of 16:0, 18:0, *cis*-9 18:1, 18:2n-6, 18:3n-3, and total FA at the omasum (Table 6). Furthermore, fresh grass resulted in higher ($P < 0.05$) *trans*-11 18:1, *trans*-13+14 18:1, and *cis*-11 18:1 at the omasum and tended ($P \leq 0.09$) to increase the flow of *trans*-6 to -9 18:1 and *cis*-15 18:1 (Table 7). Substituting fresh grass for hay also increased ($P < 0.01$) 18-carbon oxygenated FA at the omasum (Table 6). Conservation of grass by drying was also associated with alterations in the amount of CLA and nonconjugated 18:2 isomers at the omasum. Fresh grass tended to increase ($P \leq 0.08$) *trans*-11,*cis*-15 18:2 and Δ 11,13 CLA flow, but decreased ($P \leq 0.05$) *cis*-9,*trans*-11 CLA, and *trans*-10,*cis*-12 CLA at the omasum (Table 7). Compared with hay, fresh grass tended ($P = 0.07$) to increase 3R,7R,11R,15-tetramethyl-16:0 at the omasum (Table 6). In contrast, diets based on hay rather than fresh grass tended ($P \leq 0.10$) to increase omasal flow of several other OBCFA, including *anteiso* 15:0, *anteiso* 17:0, *iso* 13:0, *iso* 15:0, and *iso* 16:0 (Supplemental Table 2, available online at <http://www.journalofdairyscience.org>).

Replacing fresh grass with hay decreased ($P < 0.01$) the lipolysis of esterified dietary lipid in the rumen by 7.1 percentage units (Table 8), lowered ($P < 0.01$) ruminal 18:2n-6 and 18:3n-3 biohydrogenation, and tended ($P = 0.07$) to decrease the biohydrogenation of *cis*-9 18:1 in the rumen (Table 8).

Grass Hay Versus Grass Silage. Flows of 16:0 and 18:0 at the omasum were increased ($P < 0.01$) in cows fed diets based on silage relative to hay (Table 6). Replacing hay with silage increased ($P \leq 0.05$) 18:3n-3 and *cis*-9,*trans*-11,*cis*-15 18:3, but had no effect ($P > 0.10$) on *cis*-9 18:1 and 18:2n-6 at the omasum (Table 6). However, diets based on grass silage resulted in higher ($P \leq 0.03$) flows of 10-*oxo*-18:0, *cis*-18:1 (Δ 12–16), and *trans*-18:1 (Δ 4–9, 11–16), and lower ($P < 0.01$) 15-*oxo*-18:0 at the omasum relative to hay (Tables 6 and 7). The type of conserved forage offered to cows also influenced the amount and distribution of CLA and nonconjugated 18:2 isomers entering the omasum (Table 7). Grass silage increased ($P < 0.01$) Δ 11,15 and Δ 12,15 18:2, *trans*-12,*trans*-14 CLA, Δ 11,13 CLA, and Δ 13,15 CLA, but decreased

($P \leq 0.02$) *cis*-12,*trans*-14 CLA and *trans*-10,*cis*-12 CLA at the omasum.

Grass conservation method had relatively minor effects on omasal OBCFA flow (Supplemental Table 2, available online at <http://www.journalofdairyscience.org>). Compared with hay, grass silage resulted in higher ($P < 0.01$) flows of *anteiso* 13:0 and 3,7,11,15-tetramethyl-16:0 and lower ($P < 0.01$) *iso* 14:0 at the omasum (Table 6 and Supplemental Table 2). Replacing hay with grass silage in the diet was associated with more extensive ($P < 0.04$) lipolysis of esterified lipids and biohydrogenation of 18:2n-6 and 18:3n-3 in the rumen (Table 8).

Extent of Silage Fermentation. Ensiling method had only minimal influence on the abundance of FA at the omasum (Tables 6 and 7; Supplemental Table 2, available online at <http://www.journalofdairyscience.org>). Restricting fermentation in silo had no effect ($P > 0.10$) on total 16:0, 18:0, *cis*-18:1, 18:2n-6, and 18:3n-3 (Table 6), but tended ($P \leq 0.08$) to increase *trans*-10 18:1, *trans*-10,*cis*-12 CLA, and Δ 11,13 CLA at the omasum (Table 7). Compared with UTS, diets based on FAS lowered ($P < 0.01$) 10-*oxo* 18:0 and increased ($P < 0.01$) 3R,7R,11R,15-tetramethyl-16:0 at the omasum (Table 6). Both the type and extent of silage fermentation had no effect ($P > 0.10$) on the extent of lipolysis of esterified dietary lipid or on the biohydrogenation of 18-carbon unsaturated FA in the rumen (Table 8).

Rumen Microbial Ecology

Treatments had no effect ($P > 0.10$) on ruminal protozoal counts in experiments 1 and 2 (Table 9). The specificity of different primer sets, 2 in combination with probes, to members of the *Butyrivibrio*/*Pseudobutyrvibrio* group is shown in Supplemental Figure 1 (available online at <http://www.journalofdairyscience.org>). Primary qPCR measurements corresponded to the *Butyrivibrio*-related subgroups vaccenic acid 1 (VA1), stearic acid (SA)+VA2, atypical (ATYP), and VA2 (Paillard et al., 2007), together with *Streptococcus bovis* and *Megasphaera elsdenii*. Even though the method using *Cpr* primers and probe was designed to detect only *B. proteoclasticus* (Paillard et al., 2007), the group that includes *B. hungatei* (VA2) was also detected. The *Bhu* primers detected only the *B. hungatei* group. Accordingly, *B. proteoclasticus* numbers were calculated as *Cpr*-*Bhu*. Total *Butyrivibrio*/*Pseudobutyrvibrio* numbers were the sum of *Cpr*, *AtB*, *Bhu*, and *Bfi*. The *Sbo* and *Mel* primers/probe detected all the strains of *Strep. bovis* and *M. elsdenii*, respectively, in the Rowett collection, but did not amplify DNA from other ruminal bacteria.

Table 6. Effect of fresh grass or conserved forages on the flow of nutrients at the omasum in lactating cows

Flow	Experiment 1				Experiment 2				<i>P</i> -value ⁵	
	Grass	Hay	SEM ¹	<i>P</i> -value	Hay	UTS ²	FAS ³	SEM ⁴	Method	Extent
OM, ⁶ kg/d	8.10	9.36	0.511	0.07	9.51	9.19	9.78	0.833	0.95	0.29
NDF, kg/d	2.47	2.88	0.190	0.10	3.20	2.92	3.03	0.276	0.36	0.70
pdNDF, ⁷ kg/d	1.55	1.77	0.152	0.24	2.16	2.05	2.12	0.205	0.67	0.77
N, g/d	471	501	30.6	0.39	505	486	522	46.1	0.99	0.14
NAN, g/d	469	498	30.3	0.40	499	480	517	45.3	0.96	0.14
VFA, g/d	1,326	1,357	134.5	0.83	1,711	1,764	1,924	214.6	0.42	0.41
NEFA, g/d										
12:0	0.22	0.21	0.017	0.63	0.28	0.24	0.26	0.015	0.07	0.18
14:0	1.58	1.05	0.644	0.46	1.14	1.22	1.25	0.067	0.09	0.65
16:0	35.7	33.4	4.84	0.66	36.7	52.2	54.4	3.22	<0.001	0.21
<i>cis</i> -7 16:1	0.17	0.15	0.018	0.34	0.11	0.06	0.06	0.007	<0.001	0.46
<i>cis</i> -8 16:1	0.08	0.09	0.009	0.58	0.04	0.11	0.01	0.014	<0.001	0.17
18:0	195	161	12.6	0.06	202	279	309	20.2	<0.001	0.043
10- <i>oxo</i> 18:0	0.26	0.60	0.067	0.009	0.61	5.56	1.73	0.613	0.007	0.005
13- <i>oxo</i> 18:0	0.17	0.57	0.055	0.002	0.49	0.74	0.47	0.134	0.47	0.20
15- <i>oxo</i> 18:0	<0.00	0.14	0.030	<0.001	0.12	0.06	0.05	0.016	0.020	0.54
<i>cis</i> -9 18:1	8.19	7.51	1.394	0.65	6.39	6.56	6.74	0.747	0.64	0.78
Σ <i>cis</i> -18:1	11.7	11.0	1.591	0.69	9.72	12.2	12.8	1.14	0.007	0.44
Σ <i>trans</i> -18:1	31.6	20.5	3.04	0.022	26.7	45.1	52.2	3.79	<0.001	0.09
18:2n-6	3.02	4.44	0.175	0.002	3.89	4.28	4.47	0.506	0.26	0.68
Σ 18:2 ⁸	7.46	7.30	0.622	0.81	6.16	9.00	10.6	0.897	<0.001	0.07
Σ CLA ⁹	3.06	4.79	0.576	0.040	3.08	3.78	4.29	0.418	0.049	0.30
18:3n-3	1.38	1.51	0.151	0.43	1.21	1.87	2.04	0.204	0.003	0.37
<i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15 18:3	1.17	1.68	0.195	0.07	0.55	0.75	0.84	0.144	0.033	0.42
20:0	2.38	2.71	0.134	0.07	3.26	3.46	3.83	0.277	0.009	0.020
3S,7R,11R,15-tetramethyl-16:0	0.53	0.88	0.162	0.10	1.02	5.33	4.41	0.464	<0.001	0.07
3R,7R,11R,15-tetramethyl-16:0	1.01	0.80	0.073	0.06	0.70	0.71	0.97	0.165	<0.001	<0.001
20:2n-6	0.04	0.03	0.003	0.16	0.03	0.04	0.04	0.006	0.11	0.60
22:0	1.93	2.24	0.119	0.07	2.43	2.71	3.03	0.279	0.005	0.034
24:0	1.70	2.00	0.114	0.06	2.34	2.59	2.82	0.259	0.005	0.06
26:0	3.48	4.50	0.383	0.06	5.80	9.77	10.7	1.382	<0.001	0.18
28:0	2.30	2.70	0.252	0.19	3.58	5.52	6.12	0.742	<0.001	0.17
Σ SFA	258	225	16.9	0.14	271	377	408	27.8	<0.001	0.09
Σ MUFA	45.1	33.2	4.56	0.07	38.2	59.4	67.4	4.92	<0.001	0.10
Σ PUFA	10.1	10.5	0.95	0.66	11.1	15.4	17.8	1.58	0.003	0.13
Total	314	271	21.7	0.12	322	459	499	31.3	<0.001	0.09
Total FA, g/d										
12:0	1.77	0.62	0.202	0.005	0.90	0.87	0.87	0.071	0.64	0.94
14:0	7.17	2.88	0.590	0.002	2.90	2.88	3.23	0.240	0.27	0.07
<i>cis</i> -9 14:1	0.58	0.42	0.076	0.11	0.54	0.55	0.54	0.092	0.92	0.95
16:0	66.8	56.9	5.40	0.14	62.0	75.6	78.5	7.30	<0.001	0.30
<i>cis</i> -7 16:1	0.49	0.34	0.100	0.20	0.27	0.22	0.20	0.043	0.07	0.50
<i>cis</i> -8 16:1	0.47	0.48	0.107	0.90	0.37	0.43	0.40	0.065	0.29	0.74
<i>cis</i> -9 16:1	0.52	0.37	0.067	0.10	0.46	0.42	0.33	0.051	0.18	0.22
18:0	207	175	16.1	0.12	214	290	314	26.8	<0.001	0.12
10- <i>oxo</i> 18:0	0.20	0.60	0.046	<0.001	0.73	6.46	1.98	0.698	0.006	0.004
13- <i>oxo</i> 18:0	0.22	0.73	0.068	<0.001	0.84	0.83	0.51	0.166	0.42	0.20
15- <i>oxo</i> 18:0	<0.00	0.18	0.010	<0.001	0.16	0.08	0.04	0.021	0.003	0.14
<i>cis</i> -9 18:1	14.9	14.2	0.86	0.45	15.6	16.0	16.5	1.81	0.58	0.70
Σ <i>cis</i> -18:1	21.0	19.2	1.30	0.25	21.7	23.9	24.9	2.27	0.08	0.51
Σ <i>trans</i> -18:1	36.2	25.0	4.05	0.06	30.9	47.8	55.7	4.13	<0.001	0.08
18:2n-6	18.2	17.2	0.771	0.28	18.3	17.8	18.7	2.40	1.00	0.51
Σ 18:2 ⁸	24.7	21.0	1.73	0.11	21.4	23.4	26.0	2.73	0.046	0.15
Σ CLA ⁹	3.70	5.25	0.737	0.11	4.12	4.85	5.38	0.511	0.038	0.27
18:3n-3	10.4	7.81	1.226	0.11	6.83	7.69	7.89	1.043	0.05	0.84
<i>cis</i> -9, <i>trans</i> -11, <i>cis</i> -15 18:3	1.43	2.15	0.278	0.07	0.91	1.16	1.18	0.209	0.044	0.91
20:0	3.11	3.30	0.205	0.41	3.87	3.30	4.13	0.754	0.78	0.22
3S,7R,11R,15-tetramethyl-16:0	0.82	1.19	0.288	0.27	1.24	6.12	5.19	0.584	<0.001	0.11
3R,7R,11R,15-tetramethyl-16:0	1.23	0.91	0.124	0.07	0.72	0.84	1.13	0.187	0.005	0.006
20:2n-6	0.09	0.05	0.011	0.025	0.10	0.05	0.06	0.018	0.006	0.36
22:0	2.94	2.74	0.221	0.44	2.85	3.11	3.29	0.370	0.049	0.31

Continued

Table 6 (Continued). Effect of fresh grass or conserved forages on the flow of nutrients at the omasum in lactating cows

Flow	Experiment 1				Experiment 2				<i>P</i> -value ⁵	
	Grass	Hay	SEM ¹	<i>P</i> -value	Hay	UTS ²	FAS ³	SEM ⁴	Method	Extent
22:6n-3	0.17	0.12	0.011	0.012	0.15	0.18	0.18	0.018	0.028	0.94
24:0	2.56	2.52	0.206	0.87	2.58	2.82	2.87	0.321	0.09	0.76
<i>cis</i> -15 24:1	1.18	0.94	0.082	0.039	1.04	1.17	1.20	0.132	0.06	0.66
26:0	4.66	5.44	0.583	0.26	6.02	10.0	10.4	1.496	<0.001	0.55
28:0	3.46	3.28	0.484	0.72	3.61	5.36	5.73	0.721	<0.001	0.32
30:0	2.45	1.98	0.291	0.19	2.08	2.70	2.99	0.354	0.003	0.14
Σ SFA	336	283	25.9	0.12	331	446	460	42.0	<0.001	0.24
Σ MUFA	61.6	47.9	5.59	0.08	56.5	75.8	84.7	6.27	0.002	0.12
Σ PUFA	36.8	31.2	3.16	0.15	33.5	37.4	40.6	4.48	0.033	0.22
Total	437	365	34.3	0.11	422	551	587	51.5	<0.001	0.19

¹Standard error of the mean for n = 10 measurements; error degree of freedom = 4.

²Untreated silage.

³Silage ensiled using a formic acid-based additive.

⁴Standard error of the mean for n = 15 measurements; error degree of freedom = 6.

⁵Significance of effect due to forage conservation method (drying vs. ensiling; method) or extent of silage fermentation (extensively vs. restrictively fermented silage; extent).

⁶Measurements do not include VFA.

⁷pdNDF = potentially digestible NDF.

⁸Excludes isomers of CLA.

⁹CLA, conjugated linoleic acid.

Experimental treatments had no major influence ($P > 0.10$) on specific populations of rumen bacteria known to be capable of biohydrogenation (Table 9). However, replacing fresh grass with hay in the diet was associated with an increase ($P = 0.02$) in ruminal *Strep. bovis* populations. Numbers of *Propionibacterium acnes* and *M. elsdenii* were below the limit of detection (10^{-7} of total bacteria) in all samples.

DISCUSSION

Novel aspects of the present study include measurements of rumen microbial ecology and FA flows at the omasum in cows during zero grazing or fed conserved forages prepared simultaneously from the same grass ley. Cows were offered hay in both experiments allowing for indirect inferences on the impact of ensiling relative to zero grazing in the absence of differences due to maturity or chop length. The experimental design used in the present investigation can be criticized due to the confounding effects of treatment with time in experiment 1. However, minimizing variation in herbage maturity and composition was considered more important to understanding the effect of forage conservation, than time-related effects associated with short experimental periods. Maturity is a major determinant of the lipid content and composition of grasses (Boufaïed et al., 2003a; Elgersma et al., 2006; Vanhatalo et al., 2007) and

known to influence ruminal biohydrogenation and the flow of FA at the duodenum in lactating cows (Doreau et al., 2007). However, for cows in established lactation fed the same diet, changes in milk fat composition over time are marginal (Roy et al., 2006; Shingfield et al., 2006; Mohammed et al., 2011), and by implication, time-related effects on ruminal lipid metabolism and microbial ecology are also relatively small compared with the differences expected between diets based on fresh and conserved forages.

DMI, Digestibility, and Rumen Fermentation

Replacing fresh grass with grass hay had no effect on nutrient digestion in the rumen, but lowered whole-tract OM and NDF digestibility, which may be related to numerical differences in DMI and the relatively short transition between grazing and housing of cows in experiment 1. In general, precision chopping and prewilting often stimulate higher intakes of grass silage, whereas the relatively high NDF content may explain the lower DMI of cows fed hay than silage in this experiment (Allen, 1996; Huhtanen, 1998). Conservation of grass by ensiling or drying had no influence on nutrient digestion, which is in broad agreement with previous findings (Jaakkola and Huhtanen, 1993; Holden et al., 1994). Higher DMI for FAS than UTS in the absence of altered ruminal or whole-tract nutrient digestibility

Table 7. Effect of fresh grass or conserved forages on the flow of 18:1 and 18:2 FA at the omasum in lactating cows

Flow, g/d	Experiment 1				Experiment 2				<i>P</i> -value ⁵	
	Grass	Hay	SEM ¹	<i>P</i> -value	Hay	UTS ²	FAS ³	SEM ⁴	Method	Extent
18:1										
<i>cis</i> -11 18:1	3.79	2.98	0.276	0.044	3.75	3.48	3.72	0.301	0.33	0.18
<i>cis</i> -12 18:1	1.02	1.02	0.158	0.96	1.23	1.90	1.91	0.172	0.003	0.92
<i>cis</i> -13 18:1	0.24	0.23	0.048	0.98	0.24	0.35	0.37	0.045	0.029	0.68
<i>cis</i> -15 18:1	0.56	0.39	0.083	0.09	0.40	1.20	1.30	0.140	<0.001	0.46
<i>cis</i> -16 18:1	0.44	0.40	0.055	0.52	0.45	0.92	1.05	0.082	<0.001	0.17
<i>trans</i> -4 18:1	0.24	0.12	0.067	0.15	0.20	0.24	0.29	0.024	0.011	0.036
<i>trans</i> -5 18:1	0.19	0.15	0.028	0.16	0.19	0.25	0.29	0.024	0.020	0.22
<i>trans</i> -6+7+8 18:1	1.37	1.14	0.089	0.07	1.44	1.97	2.16	0.183	0.004	0.29
<i>trans</i> -9 18:1	0.96	0.75	0.084	0.07	0.85	1.30	1.43	0.132	0.002	0.27
<i>trans</i> -10 18:1	2.26	1.89	0.251	0.22	2.72	2.84	3.53	0.368	0.15	0.08
<i>trans</i> -11 18:1	20.1	12.5	2.47	0.037	15.0	18.9	22.9	1.01	0.021	0.12
<i>trans</i> -12 18:1	1.95	1.83	0.203	0.61	2.15	3.53	4.12	0.342	<0.001	0.12
<i>trans</i> -13+14 18:1	4.47	2.93	0.538	0.046	4.16	8.74	10.3	0.915	<0.001	0.18
<i>trans</i> -15 18:1	2.02	1.66	0.233	0.21	1.84	4.54	4.81	0.434	<0.001	0.55
<i>trans</i> -16 18:1 ⁶	2.59	2.06	0.319	0.17	2.27	5.45	5.81	0.481	<0.001	0.47
Nonconjugated 18:2										
<i>cis</i> -9, <i>cis</i> -15 18:2 ⁶	0.34	0.13	0.044	0.010	0.16	0.19	0.26	0.031	0.07	0.05
<i>cis</i> -11, <i>cis</i> -14 18:2	0.96	0.15	0.075	<0.001	0.19	0.28	0.31	0.023	0.005	0.40
<i>cis</i> -12, <i>cis</i> -15 18:2	0.11	0.10	0.039	0.74	0.08	0.28	0.30	0.042	0.004	0.62
<i>cis</i> -9, <i>trans</i> -12 18:2	0.11	0.13	0.048	0.78	0.10	0.08	0.11	0.014	0.94	0.10
<i>trans</i> -9, <i>cis</i> -12 18:2	0.25	0.17	0.067	0.30	0.19	0.23	0.30	0.035	0.09	0.16
<i>trans</i> -11, <i>cis</i> -15 18:2	3.51	2.18	0.549	0.08	1.80	3.35	4.46	0.360	<0.001	0.029
<i>trans</i> -12, <i>cis</i> -15 18:2	0.14	0.12	0.048	0.75	0.14	0.23	0.31	0.024	<0.001	0.028
<i>trans</i> -9, <i>trans</i> -12 18:2	0.15	0.11	0.069	0.64	0.12	0.12	0.15	0.025	0.57	0.42
<i>trans</i> -11, <i>trans</i> -15 18:2	0.55	0.28	0.136	0.12	0.28	0.74	1.03	0.099	0.003	0.09
Conjugated 18:2										
<i>cis</i> -9, <i>cis</i> -11 CLA ⁷	0.04	0.06	0.016	0.28	0.04	0.04	0.04	0.014	0.86	0.96
<i>cis</i> -9, <i>trans</i> -11 CLA	2.43	4.35	0.640	0.041	2.71	2.71	2.71	0.413	1.00	1.00
<i>cis</i> -12, <i>trans</i> -14 CLA	0.02	0.02	0.007	0.55	0.06	0.03	0.04	0.005	0.002	0.07
<i>trans</i> -10, <i>cis</i> -12 CLA	0.09	0.14	0.019	0.05	0.28	0.17	0.24	0.016	0.012	0.037
<i>trans</i> -11, <i>cis</i> -13 CLA	0.37	0.11	0.066	0.016	0.16	0.36	0.58	0.052	0.002	0.018
<i>trans</i> -13, <i>cis</i> -15 CLA	0.04	0.03	0.004	0.23	0.04	0.11	0.13	0.013	0.002	0.46
<i>trans</i> -8, <i>trans</i> -10 CLA	0.003	<0.000	0.003	0.38	0.01	0.02	0.02	0.006	0.16	0.97
<i>trans</i> -9, <i>trans</i> -11 CLA	0.18	0.24	0.038	0.23	0.25	0.21	0.24	0.031	0.56	0.58
<i>trans</i> -10, <i>trans</i> -12 CLA	0.03	0.03	0.002	0.81	0.08	0.08	0.08	0.004	0.34	0.24
<i>trans</i> -11, <i>trans</i> -13 CLA	0.38	0.20	0.063	0.06	0.37	0.76	0.88	0.058	<0.001	0.08
<i>trans</i> -12, <i>trans</i> -14 CLA	0.12	0.08	0.031	0.27	0.14	0.33	0.40	0.032	<0.001	0.06
<i>trans</i> -13, <i>trans</i> -15 CLA	ND ⁸	ND			<0.00	0.02	0.03	0.003	<0.001	0.042

¹Standard of the mean for n = 10 measurements; error degree of freedom = 4.²Untreated silage.³Silage ensiled using a formic acid-based additive.⁴Standard error of the mean for n = 15 measurements; error degree of freedom = 6.⁵Significance of effect due to forage conservation method (drying vs. ensiling; method) or extent of silage fermentation (extensively vs. restrictively fermented silage; extent).⁶Coelutes with *cis*-10 19:1.⁷CLA = conjugated linoleic acid.⁸ND = not detected.

represents a typical response to restricting fermentation in silo with a formic acid-based additive during the ensilage of grass (Huhtanen, 1998).

Conservation method had no major influence on ruminal pH, but influenced ruminal VFA concentrations, which can be explained by differences in DMI both between and within experiments. Substituting fresh grass with hay had little effect on rumen fermentation pat-

tern, whereas rumen fermentation on grass silage diets was characterized by relatively high molar proportions of butyrate and low proportions of acetate in rumen VFA, as could be expected based on earlier reports (Jaakkola and Huhtanen, 1993; Holden et al., 1994; Mohammed et al., 2009). A higher molar proportion of butyrate, trend toward lowered molar proportions of propionate, and decrease in the ratio of glucogenic

Table 8. Effect of fresh grass or conserved forages on apparent lipolysis and biohydrogenation of unsaturated FA in the rumen of lactating cows

Item	Experiment 1				Experiment 2				<i>P</i> -value ⁵	
	Grass	Hay	SEM ¹	<i>P</i> -value	Hay	UTS ²	FAS ³	SEM ⁴	Method	Extent
Lipolysis, %	93.4	86.3	0.83	0.001	80.2	93.3	91.7	2.49	0.005	0.64
Biohydrogenation, %										
<i>cis</i> -9 18:1	70.1	65.8	1.67	0.07	74.8	76.7	75.9	2.68	0.43	0.69
18:2n-6	84.1	80.7	0.43	0.002	84.6	88.1	87.7	1.68	0.038	0.80
18:3n-3	93.0	86.6	0.84	0.002	87.3	95.6	95.9	0.97	<0.001	0.79

¹Standard error of the mean for n = 10 measurements; error degree of freedom = 4.²Untreated silage.³Silage ensiled using a formic acid-based additive.⁴Standard error of the mean for n = 15 measurements; error degree of freedom = 6.⁵Significance of effect due to forage conservation method (drying vs. ensiling; method) or extent of silage fermentation (extensively vs. restrictively fermented silage; extent).

to lipogenic VFA for FAS compared with UTS is consistent with reports in the literature (Huhtanen, 1998).

Forage Lipid and FA Compositions

Most of the lipids in grasses in the form of phospholipids and glycolipids are located within thylakoid membranes of chloroplasts (Harfoot and Hazlewood, 1988). During cutting or mastication, the damage to plant tissues activates endogenous lipases, causing the release of nonesterified 18:2n-6 and 18:3n-3 from disrupted membranes (Dewhurst et al., 2006). In the present experiment, mechanical cutting and the application of a formic acid-based additive resulted in rather marginal increases in the proportion of total FA as NEFA relative to intact grass.

Conservation of grass by drying resulted in substantial decreases in FA content, principally due to losses of 18:2n-6 and 18:3n-3, confirming earlier reports (Boufaïed et al., 2003a; Shingfield et al., 2005; Ferlay et al., 2006). Losses of FA during hay making can be explained by prolonged wilting stimulating extensive lipolysis and oxidation of membrane lipids, largely due to the activity of lipoxygenases (Dewhurst et al., 2006). Plant lipoxygenases catalyze the incorporation of molecular oxygen in nonesterified 18:2n-6 and 18:3n-3, forming 9- and 13-hydroperoxy PUFA that are highly reactive and rapidly metabolized into series of oxylipins, including volatile leaf aldehydes and alcohols, hydroxy- and epoxy-FA, and jasmonates (Feussner and Wasternack, 2002; Mosblech et al., 2009). Leaf shatter may also contribute to the loss of FA during hay mak-

Table 9. Effect of fresh grass or conserved forages on rumen microbial population in lactating cows

Population, % of total eubacteria ¹	Experiment 1				Experiment 2				<i>P</i> -value ⁶	
	Grass	Hay	SEM ²	<i>P</i> -value	Hay	UTS ³	FAS ⁴	SEM ⁵	Method	Extent
Total <i>Butyrivibrio</i> + <i>Pseudobutyrvibrio</i>	0.91	0.73	0.198	0.53	0.77	0.89	0.89	0.344	0.78	0.99
<i>Butyrivibrio proteoclasticus</i>	0.65	0.52	0.010	0.55	0.48	0.41	0.41	0.186	0.73	0.99
<i>Butyrivibrio fibrisolvens</i> + <i>Pseudobutyrvibrio</i>	0.10	0.12	0.050	0.73	0.16	0.38	0.32	0.118	0.20	0.74
Atypical <i>B. fibrisolvens</i>	0.032	0.020	0.0087	0.24	0.080	0.032	0.071	0.0416	0.58	0.52
<i>Butyrivibrio hungatei</i>	0.126	0.072	0.0311	0.16	0.057	0.075	0.090	0.0277	0.44	0.71
<i>Streptococcus bovis</i>	0.008	0.048	0.0097	0.016	0.022	0.006	0.012	0.0111	0.37	0.70
Protozoal counts, 10 ⁵ /mL of rumen fluid	2.07	2.87	0.203	0.13	2.26	2.04	1.97	0.286	0.50	0.86

¹Populations determined based on quantitative PCR using primers and probes designed to target different members of the *Butyrivibrio*/*Pseudobutyrvibrio* group and *Strep. bovis*.²Standard error of the mean for n = 10 measurements; error degree of freedom = 4.³Untreated silage.⁴Silage ensiled using a formic acid-based additive.⁵Standard error of the mean for n = 15 measurements; error degree of freedom = 6.⁶Significance of effect due to forage conservation method (drying vs. ensiling; method) or extent of silage fermentation (extensively vs. restrictively fermented silage; extent).

ing (Dewhurst et al., 2006). Although the FA content of hay was similar between experiments 1 and 2, the relative proportion of FA as NEFA increased from 36 to 73%, indicating that lipolysis continues during the storage of dried grasses.

Ensiling had relatively minor effects on the FA content and composition of mixed timothy and meadow fescue, reflecting the relatively short wilt, but increased forage NEFA content, consistent with previous findings (Dewhurst et al., 2006; Vanhatalo et al., 2007). Increases in NEFA can be explained by the hydrolysis of glycolipid and phospholipids catalyzed by plant (Dewhurst et al., 2006) and microbial lipases (Van Ranst et al., 2010). In earlier investigations, application of formic acid has been reported to result in higher silage 18:3n-3 and total FA concentrations (Dewhurst and King, 1998; Boufaïed et al., 2003a; Shingfield et al., 2005), but there was no evidence that the extent of fermentation in silo influenced the lipid composition of grass silage in this experiment.

Ruminal Lipolysis and Biohydrogenation

For all diets, apparent lipolysis of esterified lipid and biohydrogenation of *cis*-9 18:1, 18:2n-6, and 18:3n-3 in the rumen was extensive and in the range of earlier estimates (Doreau and Ferlay, 1994; Palmquist et al., 2005; Shingfield et al., 2010).

Fresh Grass Versus Grass Hay. Plant cell walls have to be disrupted by mastication and microbial digestion before lipolysis can take place, a process considered rate limiting for ruminal biohydrogenation of unsaturated FA (Palmquist et al., 2005). Despite a higher proportion of FA as NEFA, replacing fresh grass with hay was associated with lowered apparent lipolysis of dietary lipid in the rumen. Previous investigations have reported a slower rate of disappearance of 18:3n-3 from dried than fresh timothy during incubation with rumen fluid, an effect suggested to be related to lower rates of DM digestion associated with drying (Boufaïed et al., 2003b). Even though the kinetics of digestion in the rumen were not determined in this experiment, relatively small differences in the extent of ruminal OM or NDF digestion do not appear to explain the 7.1 percentage unit decrease in lipolysis in cows fed hay compared with zero grazing. It has been suggested that a higher activity of endogenous lipases during the ingestion of fresh grasses contributes to the higher ruminal lipolysis and biohydrogenation compared with conserved forages (Doreau et al., 2005). Alternatively, the removal of water during drying may result in the entrapment of chloroplasts by surrounding cell walls, introducing a physical barrier to microbial lipases and, thereby, lower lipolysis of esterified plant lipid in the rumen.

Consistent with more extensive lipolysis, apparent ruminal biohydrogenation of 18:2n-6 and 18:3n-3 were higher (3.4 and 6.4 percentage units, respectively) for the fresh grass relative to the hay diet. Earlier reports have demonstrated that biohydrogenation of PUFA is higher for fresh than dried grass in sheep (Doreau et al., 2005) and during incubations with rumen fluid (Boufaïed et al., 2003b). The decrease in the extent of ruminal *cis*-9 18:1 and 18:2n-6 biohydrogenation due to drying of grass was less significant compared with that of 18:3n-3 that may, at least in part, be attributed to the lower proportion of *cis*-9 18:1 and 18:2n-6 intake originating from polar lipids in forage relative to triacylglycerols in concentrate supplements, whereas forages were the principle source of 18:3n-3 on all diets. Recent investigations have shown that in cows fed diets similar to the FAS treatment, ca. 60, 26, 11, and 4% of total esterified 18:3n-3 at the omasum was in the form of polar lipid, triacylglycerols, diacylglycerols, and monoacylglycerols, respectively (Halmemies-Beauchet-Filleau et al., 2012). Corresponding values for esterified *cis*-9 18:1 and 18:2n-6 escaping the rumen were 41, 46, 10, and 3% and 46, 40, 11, and 3%, respectively (Halmemies-Beauchet-Filleau et al., 2012).

Grass Hay Versus Grass Silage. Replacing grass hay with grass silage was associated with more extensive lipolysis and biohydrogenation of 18-carbon PUFA in the rumen, findings that are in agreement with measurements in sheep (Doreau et al., 2005) and in vitro studies (Boufaïed et al., 2003b). Even though the extent of lipolysis in hay and silages during storage and FA intake from concentrates were similar across diets, the FA and NEFA content of hay is inherently lower due to extensive wilting, resulting in the amount of FA from forage being lower than concentrates in cows fed hay-based diets. On average, NEFA accounted for ca. 35% of FA supplied by FAS and UTS, whereas NEFA represented 19% of FA intakes in cows fed hay-based diets. Biohydrogenation does not provide rumen microbes with a source of energy, but instead represents a survival mechanism for sustaining the microbial community and rumen function through the removal of the toxic effects of dietary unsaturated FA on bacterial growth (Lourengo et al., 2010). More extensive biohydrogenation of 18:2n-6 and 18:3n-3 on grass silage-based diets could be expected due to the higher susceptibility of nonesterified than esterified PUFA to biohydrogenation, and the higher overall intake of unsaturated FA compared with diets based on hay. It appears that the influence of drying compared with ensiling on plant-mediated lipolysis and the relative abundance of esterified FA and NEFA can, at least in part, account for the observed differences between forage conservation methods on ruminal lipolysis and biohydrogenation.

Ruminal lipolysis was lower and the biohydrogenation of 18-carbon unsaturates marginally higher in cows fed hay-based diets in experiment 2 than in experiment 1, which may be related to the ingestion of a greater proportion of FA from forage as NEFA in the second compared with the first experiment. Alternatively these differences may arise as a consequence of higher DMI or simply reflect inherent variance in ruminal lipid metabolism between individual cows.

Extent of Silage Fermentation. Even though lipolysis in silo was more extensive for FAS than UTS, restricting silage fermentation had little influence on the extent of lipolysis or biohydrogenation in the rumen.

Flow of FA at the Omasum

For all diets, most of the FA (72–85%) entering the omasum were NEFA, consistent with earlier reports (Noble, 1981). However, the majority of 18:2n-6 and 18:3n-3 at the omasum were esterified, with the implication that PUFA in forages escape the rumen principally as components of the chloroplastic membrane (Kim et al., 2009). Virtually all the 18-carbon biohydrogenation intermediates, 18:0, and longer-chain SFA entered the omasal canal as NEFA, which could be expected, given that a free carboxyl group is considered a prerequisite for biohydrogenation (Harfoot and Hazlewood, 1988). Both 16:0 and *cis*-9 18:1 left the rumen as esterified FA and NEFA in almost equal amounts, which can be explained by both the contribution of microbial synthesis *de novo* and ruminal escape of dietary lipid to the flow of these FA at the omasum.

Consistent with the extensive ruminal biohydrogenation of *cis*-9 18:1, 18:2n-6, and 18:3n-3, 18:0 was the predominant FA in omasal digesta, whereas *trans*-11 and *cis*-9,*trans*-11 were the most abundant isomers of *trans* 18:1 and CLA, respectively, which is typical for cows fed grass silage-based diets (Shingfield et al., 2003, 2012b).

Fresh Grass Versus Grass Hay. Despite a higher intake, the flow of 18:2n-6 and 18:3n-3 at the omasum was not substantially higher during zero grazing. These observations point toward the drying of grass offering a degree of protection of PUFA from metabolism in the rumen, confirming earlier investigations *in vitro* (Boufaïed et al., 2003b). Flows of *cis*-9,*trans*-11 CLA and *cis*-9,*trans*-11,*cis*-15 18:3 were lower at the omasum in cows fed fresh grass compared with hay, whereas those of specific 18:1, 18:2, and CLA isomers were higher. Differences in the profile of initial and secondary intermediates of 18:2n-6 and 18:3n-3 biohydrogenation, suggest that the conservation of grass by drying influences both the rate of NEFA release and the kinetics

of isomerization and reduction reactions in the rumen. Such an interpretation is also supported by the lower proportions of biohydrogenated 18-carbon unsaturated FA recovered as 18:0 in omasal digesta of cows fed hay relative to fresh grass.

Flows of *trans*-11 18:1 at the omasum in cows fed fresh grass were double those for hay. Abundance of *trans*-11 18:1 in ruminal digesta was reported to be 4-fold higher in cows at pasture compared with those fed grass silage, with measurements in zero-grazed cows being intermediate (Mohammed et al., 2009). Differences in *trans*-11 18:1 concentrations at pasture relative to zero grazing were attributed to the selective grazing of leafy material enriched in chloroplasts, suggesting that larger differences in *trans*-11 18:1 at the omasum could be expected between cows at pasture compared with hay-based diets. However, several FA oxidation products formed following mechanical damage to plant cells, including 1,2-dimethylethyl hydroperoxide and *trans*-2-decenal have been reported to influence the biohydrogenation of unsaturated FA and microbial communities during incubations of grass silage with bovine rumen fluid *in vitro* (Lee et al., 2007). It is possible that oxylipins found in green odor contribute to potential differences in ruminal lipid metabolism in cows at pasture relative to zero grazing and may also, at least in part, account for the differences in lipolysis and biohydrogenation between cows fed diets based on fresh grass and grass hay.

The appearance of 3,7,11,15-tetramethyl-16:0 in omasal digesta arises from the sequential reduction and oxidation of the phytol moiety of chlorophyll a, b, and d released during microbial digestion of cell walls (Schröder and Vetter, 2011). Fresh grass resulted in a higher ratio of the SRR:RRR 3,7,11,15-tetramethyl-16:0 diastereomers at the omasum (0.67 vs. 1.31), which is consistent with the relative abundance of these FA in milk fat of cows at pasture or fed hay-based diets (Baars et al., 2012).

Grass Hay Versus Grass Silage. Even though the intakes of *cis*-9 18:1, 18:2n-6, and 18:3n-3 were higher from grass silage compared with hay, only the flow of 18:3n-3 at the omasum was marginally increased. In sheep, grass silage and grass hay supplying the same amount of PUFA were found to result in similar flows of 18:2n-6 and 18:3n-3 at the duodenum (Doreau et al., 2003). Ensiling, rather than drying, of grass was associated with an increase in 18:0 and several 18-carbon biohydrogenation intermediates at the omasum. It remains unclear if these differences are solely explained by differences in unsaturated FA intake from forage, or also related to the changes in lipolysis and the rates at which NEFA are released into the rumen. Ensiling, rather than drying, of grass was also associated with a

higher SRR:RRR ratio and total flow of 3,7,11,15-tetramethyl-16:0 diastereomers at the omasum, which is consistent with the changes of these diastereomers in milk in cows fed diets based on grass silage or grass hay (Baars et al., 2012).

Extent of Silage Fermentation. Restricting silage fermentation had no effect on 18:0, *cis*-9 18:1, 18:2n-6, and 18:3n-3 at the omasum, but increased or tended to elevate the amounts of specific biohydrogenation intermediates including *trans*-10 18:1, *trans*-10,*cis*-12 CLA, and Δ 11,13 CLA. Overall, there was no substantive evidence to suggest that the extent of fermentation in silo has a major influence on lipolysis or biohydrogenation of 18-carbon unsaturated FA in the rumen.

Ruminal Balance of FA

Flow of total FA at the omasum exceeded dietary intake to a variable extent for all experimental diets, confirming a net synthesis of FA in the rumen of cows fed diets containing low amounts of lipid (Doreau and Ferlay, 1994; Schmidely et al., 2008). Most of the increase was related to OBCFA originating from synthesis *de novo* by rumen microbes (Vlaeminck et al., 2006), with a significant contribution from 16:0 and 18:0 for hay-based diets. Ruminal lipolysis and biohydrogenation of *cis*-9 18:1, 18:2n-6, and 18:3n-3 could explain up to 77 and 82% of the net synthesis of 18:0 and *trans*-18:1 in the rumen of cows fed hay in experiments 1 and 2, respectively, whereas these accounted for the net appearance of 18:0 and *trans*-18:1 at the omasum in cows fed fresh grass and grass silage. Similarly, in cows fed perennial ryegrass, the flow of total FA at the duodenum exceeded intake, whereas the intakes of *cis*-9 18:1, 18:2n-6, and 18:3n-3 could quantitatively explain 18:0 and *trans* 18:1 biohydrogenation intermediate formation (Doreau et al., 2007).

Rumen Microbial Ecology

Protozoa. Conservation of grass by drying was not associated with changes in ruminal protozoal numbers, consistent with recent comparisons of the rumen microbial communities in cows at pasture or fed a TMR (de Menezes et al., 2011). However, no attempt was made in the present study to characterize the genetic diversity of rumen protozoal populations or the outflow of protozoa from the rumen. Protozoal numbers in the rumen can be expected to be higher in cows at pasture relative to those offered hay or grass silage-based diets (Holden et al., 1994). Indirect comparisons suggest that the number of protozoa in the rumen is lower during zero grazing or from hay-based diets compared with grazing (Dehority and Orpin, 1988). Similar protozoal

counts in the rumen of cows receiving diets based on grass silage or grass hay is in agreement with previous comparisons of drying and ensilage of grass (Jaakkola and Huhtanen, 1993; Holden et al., 1994) and investigations of the influence of fermentation in silo (Jaakkola et al., 2006).

Bacteria. Bacteria are generally recognized to be the principal organisms responsible for biohydrogenation, whether free living or associated with protozoa (Harfoot and Hazlewood, 1988; Lourenço et al., 2010). Ruminal bacteria capable of hydrogenating unsaturated FA have been classified into group A and B bacteria. Group A bacteria reduce 18:2n-6 and 18:3n-3 to *trans*-11 18:1 and group B bacteria hydrogenate 18-carbon PUFA to 18:0 (Harfoot and Hazlewood, 1988; Kim et al., 2009; Lourenço et al., 2010). Modern phylogenetic analysis has revealed that culturable group A bacteria comprise a range of related species that include *Butyrivibrio fibrisolvens*, *B. hungatei*, and several *Pseudobutyrvibrio* spp. (Paillard et al., 2007; Boeckaert et al., 2008). *Butyrivibrio fibrisolvens* is also known to exhibit galacto- and phospholipase activity (Harfoot and Hazlewood, 1988). Group B bacteria form a tight grouping in which strains cluster together close to *B. proteoclasticus* (van de Vossenberg and Joblin, 2003; Wallace et al., 2006). Other, as yet uncultivated bacteria may also be involved in the final reduction of 18:1 to 18:0 (Huws et al., 2011; Toral et al., 2012).

Rumen bacterial numbers are similar for animals at pasture or fed hay of similar chemical composition (Dehority and Orpin, 1988). In the present experiment, ruminal balances of OBCFA, as well as the flow of microbial OM and NAN at the omasum and the energetic efficiency of microbial protein synthesis (data not shown) were similar across treatments, which could be interpreted as evidence of no major differences in microbial counts between treatments.

Current measurements indicated that the *Butyrivibrio* + *Pseudobutyrvibrio* group comprised 0.73 to 0.91% of total bacterial DNA, consistent with recent estimates in cattle (Boeckaert et al., 2008; Huws et al., 2010; Shingfield et al., 2012b). Higher numbers of *B. proteoclasticus* over other species from the same group have also been reported, based on the analysis of omasal digesta from lactating cows (Shingfield et al., 2012b) and ruminal digesta of growing sheep (Toral et al., 2012). However, alterations in the flow of 18-carbon biohydrogenation intermediates and 18:0 at the omasum associated with forage conservation method were not accompanied by significant changes in populations of rumen *Butyrivibrio* capable of biohydrogenation, which could have been expected in view of the differences in forage chemical composition and rumen fermentation characteristics. Replacing fresh grass with

hay in the diet was associated with an increase in the ruminal *Strep. bovis* population, which may explain, at least in some part, the higher amount of 18-carbon oxygenated FA at the omasum. *Streptococcus bovis* is known to hydrate rather than hydrogenate *cis*-9 18:1 and 18:2n-6 (Hudson et al., 1995, 1998). However, differences in the flow of 18-carbon oxygenated FA at the omasum between cows fed hay or grass silages were not accompanied by alterations in *Strep. bovis* populations, suggesting that other species may also hydrate FA in the rumen. *Enterococcus faecalis* is one such species (Hudson et al., 1998). It remains uncertain whether the conservation of grass influences other as yet uncultured species involved in the transformation of dietary lipid in the rumen. Recent experiments based on deep sequencing of 16S rRNA gene amplicons have reported significant changes in bacterial and archaeal, but not protozoal, communities in the rumen of cattle following a switch from pasture to a diet based on maize silage, grass silage, and straw (de Menezes et al., 2011). Further studies based on modern molecular techniques may provide further insight into the influence of conserving grass by drying or ensiling on the genetic diversity and community structure of microbial populations in the rumen.

CONCLUSIONS

Drying of grass resulted in substantial decreases in FA content, 18:2n-6 and 18:3n-3 in particular, whereas ensiling resulted in minimal losses. Both drying and ensiling of grass were associated with extensive lipolysis. Compared with fresh grass or grass silage, feeding diets based on grass hay lowered the extent of lipolysis and biohydrogenation of unsaturated FA in the rumen, leading to similar flows of 18:2n-6 and 18:3n-3, but lower amounts of *trans*-11 18:1 and Δ 11,13 18:2 at the omasum, with evidence to suggest that drying protects chloroplastic PUFA against ruminal metabolism. The extent of silage fermentation had minor effects on ruminal lipid metabolism. Changes in the omasal flow of FA due to grass conservation method were not associated with substantial changes in rumen protozoal counts or specific populations of ruminal bacteria known to be capable of biohydrogenation.

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