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Published in:
Journal of Animal Science

DOI:
10.2527/jas.2014-8813

Publication date:
2015

Citation for published version (APA):
koivonen, E., Jaakkola, S., Terttu, H., Lampi, A-M., Halmemies-Beauchet-Filleau, A., Lee, M. R., Winters, A., Shingfield, K., & Vanhatalo, A. (2015). Effects of Plant Species, Stage of Maturity, and Level of Formic Acid Addition on Lipolysis, Lipid Content, and Fatty Acid Composition during Ensiling. *Journal of Animal Science*, *93*, 4408-4423. https://doi.org/10.2527/jas.2014-8813

Published September 8, 2015

Effects of plant species, stage of maturity, and level of formic acid addition on lipolysis, lipid content, and fatty acid composition during ensiling[1]

E. Koivunen,*† S. Jaakkola,* T. Heikkilä,† A.-M. Lampi,‡ A. Halmemies-Beauchet-Filleau,*† M. R. F. Lee,§[2] A. L. Winters,§ K. J. Shingfield,†§ and A. Vanhatalo*[3]

*Department of Agricultural Sciences, P.O. Box 28, FI-00014 University of Helsinki, Finland; †Natural Resources Institute Finland (Luke), Green Technology, FI-31600 Jokioinen, Finland; ‡Department of Food and Environmental Sciences, P.O. Box 28, FI-00014 University of Helsinki; and §Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth, Ceredigion, SY23 3EB, UK

ABSTRACT: Forage type and management influences the nutritional quality and fatty acid composition of ruminant milk. Replacing grass silage with red clover (RC; *Trifolium pratense* L.) silage increases milk fat 18:3*n*-3 concentration. Red clover has a higher polyphenol oxidase (PPO) activity compared with grasses, which has been suggested to decrease lipolysis *in silo* and *in vivo*. The present study characterized the abundance and fatty acid composition of esterified lipid and NEFA before and after ensiling of grass and RC to investigate the influence of forage species, growth stage, and extent of fermentation *in silo* on lipolysis. A randomized block design with a 2 × 3 × 4 factorial arrangement of treatments was used. Treatments comprised RC or a mixture of timothy (*Phleum pratense* L.) and meadow fescue (*Festuca pratensis* Huds.) harvested at 3 growth stages and treated with 4 levels of formic acid (0, 2, 4, and 6 L/t). Lipid in silages treated with 0 or 6 L/t formic acid were extracted and separated into 4 fractions by TLC. Total PPO activity in fresh herbage and the content of soluble bound phenols in all silages were determined. Concentrations of 18:3*n*-3 and total fatty acids (TFA) were higher ($P < 0.001$) for RC than for grass. For both forage species, 18:3*n*-3 and TFA content decreased linearly ($P < 0.001$) with advancing growth stage, with the highest abundance at the vegetative stage. Most of lipid in fresh RC and grass herbage (97%) was esterified, whereas NEFA accounted for 71% of TFA in both silages. Ensiling resulted in marginal increases in TFA content and the amounts of individual fatty acids compared with fresh herbages. Herbage total PPO activity was higher ($P < 0.001$) for RC than grass (11 vs. 0.11 μkatal/g leaf fresh weight). Net lipolysis during ensiling was extensive for both forage species (660 to 759 g/kg fatty acid for grass and 563 to 737 g/kg fatty acid for RC). Formic acid application (0 vs. 6 L/t) resulted in a marked decrease ($P = 0.026$) in net lipolysis during the ensiling of RC, whereas the opposite was true ($P = 0.026$) for grass. In conclusion, results suggest that formic acid addition during the ensilage of RC decreases lipolysis *in silo*. For both plant species, total PPO activity was not associated with the extent of lipolysis *in silo*. However, bound phenols formed via PPO activity appear to have a role in protecting lipid and protein against degradation in grass and lowering proteolysis of RC during ensiling.

Key words: grass, formic acid, lipolysis, polyphenol oxidase, red clover, stage of maturity

J. Anim. Sci. 2015.93:4408–4423
doi:10.2527/jas2014-8813

[1]The authors gratefully acknowledge laboratory staff in the Department of Agricultural Science, University of Helsinki, Helsinki, Finland, and technical staff at Green Technology, Natural Resources Institute Finland (Luke), Jokioinen, Finland, for assistance in chemical analysis and the ensiling of experimental forages. Authors appreciate the contributions of Leena Luukkainen and Ilkka Simpura at the University of Helsinki and Vesa Toivonen at Natural Resources Institute Finland (Luke) for lipid analysis. This research was supported by the Finnish Funding Agency for Technology and Innovation, Helsinki, Finland, Valio Ltd.., Helsinki, Finland, Raisioagro Ltd., Raisio, Finland, and Kemira Ltd., Helsinki, Finland.

[2]Present address: School of Veterinary Science, University of Bristol, Langford, Somerset, BS40 5DU, UK.

[3]Corresponding author: aila.vanhatalo@helsinki.fi

Received December 17, 2014.
Accepted July 13, 2015.

INTRODUCTION

Ruminant milk and meat are important sources of lipids for humans. Many studies have examined the potential of plant oil, oilseed, and marine lipid supplements to alter the fatty acid composition of ruminant products (Shingfield et al., 2013) but less is known about the effect of forage management (Dewhurst et al., 2006).

Replacing grass silage with red clover (**RC**; *Trifolium pratense* L.) silage increases milk 18:3*n*-3 content (Dewhurst et al., 2006; Moorby et al., 2009, Halmemies-Beauchet-Filleau et al., 2014), especially when RC is harvested at an early growth stage (Vanhatalo et al., 2007), due to a higher ruminal escape of both esterified and nonesterified 18:3*n*-3 (Lee et al., 2006; Halmemies-Beauchet-Filleau et al., 2013b). Red clover has a higher polyphenol oxidase (**PPO**) activity than grasses, which has been suggested to lower lipolysis *in silo* and *in vivo* (Lee et al., 2004; Van Ranst et al., 2011). Most of the lipid in herbages is present as phospholipids and galactolipids in the thylakoid membranes of chloroplasts. Due to the action of plant and microbial lipases, the majority of fatty acids in silage is nonesterified and, therefore, more susceptible to ruminal biohydrogenation (Van Ranst et al., 2011). The amount of 18:3*n*-3 and total fatty acids (**TFA**) in herbage is highest when the leaf blade proportion is high, which declines with advancing maturity (Boufaïed et al., 2003; Van Ranst et al., 2009b). Lee et al. (2009b) reported a decrease in PPO activity as RC matured; however, little is known about the role of plant maturity on PPO activity in grass and on lipolysis *in silo* under different fermentation conditions (Van Ranst et al., 2009a).

This study examined lipid and fatty acid composition before and after ensiling of RC and grass harvested at 3 stages of maturity that were treated with no additive or formic acid. The experiment tested the hypothesis that decreases in lipolysis during ensiling of RC compared with grass mediated by a higher activity of PPO could be further lowered by harvesting at an early growth stage and through the restriction of silage fermentation by formic acid.

MATERIAL AND METHODS

Experimental Design, Forage Management, and Sample Collection

A randomized block design with a 2 × 3 × 4 factorial arrangement of treatments was used to evaluate the effects of 2 plant species, 3 growth stages, and 4 additive treatments on silage chemical composition and fermentation characteristics. Changes in silage lipid composition were investigated according to a 2 × 3 x 2 factorial arrangement of treatments that included 2 plant species, 3 growth stages, and 2 additive treatments.

Grass and RC swards were harvested at 3 stages of maturity. The first harvest was made at the vegetative growth stage and the last two harvests at weekly intervals thereafter (Table 1). Grass was harvested on June 5, 13, and 25, 2008, from the primary growth of mixed timothy (*Phleum pratense* L. cv. Tuukka) and meadow fescue (*Festuca pratensis* Huds. cv. Antti). The grass sward was established using a seed mixture (Boreal Plant Breeding, Ltd., Jokioinen, Finland) comprising (by weight) 54% timothy and 46% meadow fescue. On April 24, 2008, the grass sward was fertilized with 370 kg/ha of inorganic fertilizer (Yara Suomi Ltd., Espoo, Finland) containing 26 kg N, 0 kg P, 1 kg K, 3 kg Ca, 1 kg Mg, 3 kg S, 20 g B, and 1 g Se per 100 kg. Primary growth of RC (*Trifolium pratense* L. cv. Jokioinen; Boreal Plant Breeding, Ltd.) was harvested on June 25, July 4, and July 14, 2008. Soil testing indicated adequate P and K (20.8 and 292.4 mg/L soil, respectively), and therefore, no fertilizer was applied. Forages were grown in Jokioinen (60°49′ N, 23°28′ E) and cut at a height of 8 cm and DM content of 235, 212, and 216 g/kg fresh weight (**FW**) for grass and 153, 153, and 205 g/kg FW for RC at the 3 stages of maturity, respectively. Fresh forage was collected from 3 separate areas within the sward at the time of ensiling and composited before silage production. Samples of fresh-cut grass and RC were wilted for an equal amount of time to achieve a target DM content of between 250 and 300 g/kg FW before ensiling. The wilting time in swaths varied between 3 h 30 min and 4 h 15 min for grass. Where necessary, wilting of RC was accelerated indoors by passing ambient air through the cut herbage to ensure that total wilting time was the same for both plant species. The total amount of time for on-field wilting of swaths and that indoors varied between 3 h 45 min to 4 h 45 min for RC.

Forages were chopped to average length of 1 to 2 cm using a laboratory chopper (Walter u. Wintersteiger KG, Ried im Innkreis, Austria) and ensiled in laboratory silos (from 95 to 115 g DM per 120 mL) with a formic acid based additive (containing 850 g formic acid/kg and 150 g water/kg) applied at a rate of 0, 2.4, 4.7 or 7.1 L/t FW (equivalent to 0, 2, 4 or 6 L of 100% formic acid/t FW). Each silage treatment was prepared in triplicate. Following the application of ensiling additive, silos were sealed with a rubber stopper and a plastic screw cap, stored at room temperature (20 ± 2.0°C), and protected from light. Gas production for each silo was monitored throughout the ensiling period to assess fermentation losses. The volume of gases produced was determined using a needle and syringe twice daily during the first 3 d of ensiling but at less frequent intervals as gas production diminished. Data are reported as cumulative gas production in milliliters per gram of ensiled DM. All silos were

Table 1. Dry matter yield and chemical composition of herbage at the time of ensiling

	Herbage					
	Plant species					
	Timothy–meadow fescue			Red clover		
	Growth stage			Growth stage		
	I	II	III	I	II	III
	Sampling date					
	June 5	June 13	June 25	June 25	July 4	July 14
	Stage of maturity					
Item	Vegetative	Early boot	Late boot early head	Vegetative	Early boot	Bloom
DM yield, kg DM/ha	3,995	4,876	7,128	–	5,814	6,560
DM, g/kg	255	264	303	216	209	238
DOMD,[1] g/kg DM	738	713	645	688	655	633
Ash, g/kg DM	70	64	55	102	99	88
NDF, g/kg DM	505	566	636	309	343	395
CP, g/kg DM	182	153	118	232	214	204
Water-soluble carbohydrates, g/kg	162	136	117	95	101	131
Soluble N, g/kg N	311	341	388	217	272	231
Buffering capacity, mEq/kg DM	531	450	370	686	656	661

[1]DOMD = the digestible OM content in the silage DM.

opened simultaneously on January 19, 2009, after 6 to 7 mo of storage. For the determination of DM yield (kg/ha), herbage was harvested from 4 areas of 50 by 50 cm at a height of 8 cm and weighed. Yields of DM (kg/ha) were recorded for each sampling time, except for the first harvest of RC, due to reasons unrelated to this study.

Samples of fresh herbage for lipid and PPO determinations were obtained in the field from untreated herbage. Three replicate samples were collected for each treatment. For lipid analysis, samples of intact plants (approximately 300 g FW) were cut at a height of 8 cm using scissors. For PPO analysis, leaves of RC and grass were collected (150 g FW) at the same time. Samples collected for both analyses were placed in a plastic bag, immediately flash frozen in liquid N, and stored at –80°C. Additional samples were also collected for the determination of gross chemical composition and stored at –20°C. Samples of fresh herbage and silages were freeze-dried at –80°C (Christ γ 2-20; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 48 h, passed through a hammer mill (Sakomylly KT-3100; Koneteollisuus Ltd., Helsinki, Finland) fitted with a 1-mm mesh, and submitted for lipid and bound phenol analysis.

Chemical Analysis of Fresh Herbage and Silage

The DM concentration of samples was determined by drying samples at 105°C for 24 h. Oven DM concentrations of silages were corrected for the loss of volatiles (Huida et al., 1986). Digestible OM content in silage DM was determined using a method based on pepsin-cellulase solubility (Nousiainen et al., 2003). Results were calculated using a correction to convert pepsin-cellulase solubility to *in vivo* OM digestibility using a prediction equation derived from an extensive data set of *in vivo* digestibility of silages determined in sheep fed at maintenance (Huhtanen et al., 2006).

Ash concentration was determined in a muffle furnace at 600°C for 16 h (Heraeus K1253; Heraeus GmbH, Hanau, Germany). Determination of NDF was performed in the presence of sodium sulfite according to Van Soest et al. (1991) using a Tecator Fibertec system (1020 hot extractor and 1021 cold extractor; FOSS, Hillerød, Denmark). Concentrations of NDF included residual ash. Nitrogen content and the content of soluble N were determined by the Kjeldahl method (AOAC, 1995) using a Tecator Auto Digestion unit and copper as a catalyst and a Kjeltec Auto 2300 Analyzer (FOSS). Buffering capacity was determined according to Playne and McDonald (1966). Lactic acid (Barker and Summerson, 1941), water-soluble carbohydrate (**WSC**; Somogyi, 1945; Salo, 1965), ammonia N (NH_3; McCullough, 1967), and ethanol contents were measured using colorimetric methods (Shimadzu UV-VIS mini 1240, Shimadzu Europa GmbH, Duisburg, Germany). Ethanol was analyzed using a commercial kit (catalog number 176,290; Boehringer Mannheim, Darmstadt, Germany). Silage VFA was measured from a water extract containing 5% (wt/wt) formic acid. Before analyses, extracts were filtered through an acrodisc liquid chromatography 13-mm syringe filter with a 0.45-μm polyvinylidene difluoride membrane (Pall, East Hills, NY). The content of VFA was measured with a gas chromatograph (model 7890A; Agilent Technologies, Beijing, China) equipped with a flame-ionization detector, automatic injector (7683; Agilent Technologies), and 25-m capillary column (320-μm i.d., 0.5-μm film, and operating temperature range of 60 to 250°C; Agilent J&W HP-FFAP, 19091F-112LTM; Agilent Technologies, Santa Clara, CA) using a

temperature gradient program and helium as a carrier gas at a flow rate of 12.5 mL/min. Following splitless sample injection (1 μL), column temperature was maintained at 90°C for 0.5 min, increased at a rate of 4°C/min to 150°C, held at 150°C for 0.5 min, and increased at 120°C/min to a final temperature of 170°C, which was maintained for 1 min. Injector and detector temperatures were maintained at 180 and 240°C, respectively. Concentrations of VFA were calculated without internal standard using an external standard method.

Lipid Analysis

Due to the large number of samples generated, only silages prepared with 0 or 6 L formic acid/t FW were submitted for lipid analysis. Fatty acid content of herbage and silage was determined using nonesterified 19:0 (N-5252; Sigma-Aldrich, Helsinki, Finland) as an internal standard and esterified 16:0 (T-5888; Sigma-Aldrich) as an external standard. Following the addition of 100 μL of internal standard (15 mg of 19:0/100 mL chloroform), lipid in 1 g of freeze-dried forages was extracted 3 times using 17 mL of a mixture (2:1, vol/vol) of chloroform and methanol. Pigments in organic extracts were removed by activated carbon before fractionation or determination of TFA composition.

Lipids were separated into polar lipid (**PL**), triacylglycerol (**TAG**), diacylglycerol (**DAG**), monoacylglycerol (**MAG**), and NEFA fractions by TLC (Lee et al., 2004). It was not possible to separate PL from MAG due to elution as a single band during TLC analysis and, therefore, the amounts of both fractions (polar lipid + monoacylglycerol [**PL+MAG**]) are reported. Analysis by TLC was performed using preparative silica plates (200 by 200 mm and 1 mm thickness; number 1.13895.0001; Merck KGaA, Darmstadt, Germany) that were developed for 60 min using a mixture of hexane, diethyl ether, and acetic acid (70:30:2 by volume). Bands were visualized under UV light after spraying with a 0.2% (wt/vol) solution of 2',7'-dichlorofluorescein (D-9053; Sigma-Aldrich) in methanol and identified using 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., Elysian, MN), and soybean L-α-phosphatidylcholine (37-0102; Larodan Fine Chemicals AB) standards. Each band was individually removed from the plate and transferred to clean test tubes. The fatty acids of each recovered fraction were converted to fatty acid methyl esters (**FAME**) by a 1-step extraction–transesterification procedure using toluene as solvent (Sukhija and Palmquist, 1988) and incubations with 2% (vol/vol) methanolic sulfuric acid at 70°C for 2 h.

The FAME profile in a 1-μL sample at a split ratio of 1:40 was determined using a gas chromatograph (model 5890; Hewlett-Packard, Wilmington, DE) equipped with a flame-ionization detector, automatic injector (7672A; Hewlett-Packard), and 100-m CP-Sil 88 fused silica capillary column (Chrompack 7489; Varian BV, Middelburg, The Netherlands) using a temperature gradient program and helium as a carrier gas operated at constant pressure (0.5 MPa) at a flow rate of 0.5 mL/min. Following sample injection, column temperature was maintained at 110°C for 2 min, increased at a rate of 30°C/min to 170°C, held at 170°C for 54 min, and increased at 30°C/min to a final temperature of 220°C, which was maintained for 50 min. Injector and detector temperatures were maintained at 240 and 260°C, respectively. Peaks were identified by comparison of retention times with an authentic FAME standard (GLC number 463; Nu-Chek Prep, Inc.). Peak area percentages of FAME were converted to fatty acid weight percentages based on theoretical response factors (Wolff et al., 1995).

Polyphenol Oxidase Activity

For the PPO assay, the enzyme was extracted from plant tissue according to Winters and Minchin (2001) and assayed using the method of Robert et al. (1995). In brief, leaf material (approximately 0.5 g) was ground in liquid N and 2 mL McIlvaine buffer at pH 7.0 containing 75 mmol/L of ascorbic acid to inhibit PPO activity. Extracts were centrifuged at 15,000 × *g* for 10 min at 4°C. The recovered supernatant (1.0 mL) was desalted by passing through a Bio-Gel P6DG column (1.5 by 6 cm; Bio-Rad, Hemel Hempstead, UK) equilibrated with McIlvaine buffer (pH 7.0) containing no ascorbic acid and centrifuged at 2,500 × *g* for 6 min at 4°C. Enzyme activity was determined using methylcatechol as a substrate prepared in 2.5 mol/L HCl at pH 6.0 to prevent oxidation before incubation with the PPO enzyme. Active PPO content in RC was determined by incubating 10 μL of the eluted fraction with 15 μL of 0.001 mmol/L of copper sulfate, 10 mmol/L of methylcatechol, and 1.5 mL of McIlvaine buffer and measuring the change in absorbance at 420 nm over 60 s, using a Pharmacia Biotech Ultraspec 4000 UV spectrophotometer (Pharmacia Biotech, Cambridge, UK) coupled to Swift II software (Pharmacia Biotech; Biochrom Ltd, Cambridge, UK) using methylcatechol as a blank control. Total PPO activity (sum of active and latent) in RC was calculated by repeating the assay by including 0.25% (wt/vol) of sodium dodecyl sulfate in the McIlvaine buffer. For grass species, the analysis was performed without sodium dodecyl sulfate given that PPO in grass is present only in the active form (Winters et al., 2003; Lee et al., 2009a). Enzyme reaction rate was defined as the amount of enzyme that produced 1 μmol

of quinone/s (μkatal) based on the absorption at 420 nm of a known concentration of quinones formed through the reaction of methylcatechol and sodium periodate with a conversion factor of μkatal = 0.0453 × change in optical density (Lee et al., 2010). Mean active and total PPO activity is expressed on a FW basis.

Soluble Bound Phenol Assay

Total protein and protein-bound phenol contents in freeze-dried silages (0.5 g) were determined using a modified assay (Winters and Minchin, 2005) after extraction with 2 mL of McIlvaine buffer, pH 7.0, containing 0.5% (wt/vol) lithium dodecylsulfate and 75 mmol/L ascorbic acid (Lee et al., 2004). Soluble protein was precipitated from 1 mL of supernatant with 1 mL of 20% trichloroacetic acid and 0.4% (wt/vol) phosphotungstic acid, incubated for 30 min at 4°C, and centrifuged at 10,000 × *g* for 10 min at 4°C. The protein pellet that included bound phenol was dissolved in 6 mL of 0.1 mol/L NaOH and analyzed for protein and bound phenol content by running a duplicate assay in separate microplate wells in the presence and absence of copper ions using BSA as an internal standard. 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, Hercules, CA) following a 30-min incubation. The assay response without copper is mainly due to bound phenol content, whereas the response with copper is due to a combination of protein and bound phenol content. By estimating the protein component of this response, it was possible to calculate the concentration of bound phenol (Winters and Minchin, 2005).

Calculations and Statistical Analysis

Owing to the inability to separate PL and MAG, the potential recycling of NEFA released during lipolysis of PL and possible incorporation into TAG via the activity of diacylglycerol acyltransferase (**DGAT**) could not be accounted for. Therefore, net lipolysis (g/kg fatty acid) *in silo* was calculated as the difference between the proportion of fatty acids as NEFA in fresh chopped herbage and that in the resultant silage (fatty acids as NEFA in silage [g/kg FA] – fatty acids as NEFA in fresh herbage [g/kg fatty acid]). Esterified fatty acids were present in the PL+MAG, TAG, and DAG fractions.

Data were analyzed by ANOVA using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC) with the following model: $Y_{ij} = \mu + S_i + \varepsilon_{ij}$, in which Y_{ij} = observation, μ = the general mean, S_i = the effect of the treatment ($i = 1, \ldots, 24$), and ε_{ijk} = the experimental error term. Treatment effects were separated, when appropriate, using orthogonal contrasts into single degree of freedom comparisons to test for 1) effects of plant species (timothy–meadow fescue vs. RC; type [**T**], 2) linear and quadratic components due to growth stage (**G**), and 3) linear, quadratic, and cubic components due to formic acid addition (**A**). For measurements of silage fatty acid concentration, only the contrast between the 0 and 6 L/t of formic acid addition could be tested. Furthermore, the interactions between the main treatment effects (T, G, and A) were also evaluated.

Relationships between soluble bound phenols, net lipolysis of esterified lipids *in silo*, PPO activity, and ammonia N for fresh herbage or silage were evaluated by linear regression analysis using PROC REG in SAS. Associations between parameters at $P > 0.05$ are not reported. Treatment effects were considered significant at $P < 0.05$.

RESULTS

Chemical Composition of Plant Material and Silage Quality

In general, the DM content of the grass was higher than that of RC (Table 1). Grass contained more NDF and less ash and CP at all growth stages compared with RC. Furthermore, the content of NDF in both forage species increased and those of ash and CP decreased with advancing maturity. The content of WSC was higher in grass compared with RC at the first and second growth stage, whereas the opposite was true for the third growth stage. The content of WSC decreased in grass and increased in RC with advancing maturity. The proportion of soluble N in total N was higher in grass than in RC for all growth stages. For grass, the solubility of N increased with advancing maturity, whereas this parameter for RC was not directly related to stage of maturity. Buffering capacity was higher for RC than for grass at all growth stages and was found to decrease with advancing maturity in grass with little change in RC.

Plant species had no effect ($P > 0.05$) on the content of WSC or pH of resulting silages (Table 2). The concentration of lactic acid was higher in RC silage than in grass silage ($P = 0.002$), which decreased linearly ($P \leq 0.001$) with advancing maturity for both plant species and in a quadratic manner to formic acid addition. For RC silages, the proportion of ammonia N in total N was lower ($P < 0.001$) compared with grass silages. For both plant species, incremental formic acid addition decreased in a quadratic manner ($P \leq 0.019$) the proportion of ammonia N in total N and the concentration of VFA and increased that of WSC. However, the effects of formic acid application on silage pH were complex and dependent on interactions ($P = 0.022$) between plant species and growth stage.

Fatty Acid Concentration and Distribution of Lipid in Fresh Herbage

Quantitatively, 16:0, 18:2*n*-6, and 18:3*n*-3 were the most abundant fatty acids (940 g/kg of TFA) in fresh RC and grass, with 18:3*n*-3 being the most abundant, accounting for between 650 and 740 g/kg of TFA (Table 3). The majority of fatty acid (930 g/kg) in RC and grass was recovered in the unresolved PL and MAG fraction. For both plant species, the PL+MAG fraction contained a high proportion of 18:3*n*-3 (from 699 to 809 g/kg of fatty acid). In general, 16:0, 18:2*n*-6, 18:3*n*-3, and TFA concentrations (g/kg DM) were higher ($P \leq 0.017$) in RC than in grass. Furthermore, the proportion of total lipid as PL+MAG was higher ($P = 0.002$) in RC compared with grass, whereas the proportion of NEFA was lower ($P = 0.002$). The proportions of DAG and TAG were dependent on plant species, growth stage, and their interactions ($P \leq 0.037$). Concentrations of 16:0 and TFA in DM for grass and RC decreased in a quadratic manner ($P \leq 0.004$), respectively, with advancing growth stage. Concentration of 18:3*n*-3 in DM for grass and RC decreased linearly ($P < 0.001$) with advancing growth stage, whereas the concentration of 18:2*n*-6 in grass decreased in a quadratic manner ($P < 0.05$) with advancing maturity. For RC, 18:2*n*-6 concentration was higher ($P \leq 0.001$) in the second growth stage compared with the first or third growth stages.

Fatty Acid Concentration and Distribution of Lipid in Silage

The relative abundance and composition of fatty acids in silage (Table 4) reflected that of the corresponding fresh herbage. For both RC and grass, 18:3*n*-3 was the major fatty acid (from 540 to 620 g/kg of TFA) that, together with 16:0 and 18:2*n*-6, made up, on average, 920 g/kg of TFA in silages. However, the contribution of 18:3*n*-3 to TFA was, on average, 14 to 18 and 13 to 23% lower in silages than in the corresponding fresh grass and RC herbage, respectively. Silages contained higher amounts of most individual fatty acids and TFA compared with fresh herbages (on average, 13.3 vs. 20.9 and 15.4 vs. 19.3 g TFA/kg DM for grass and RC, respectively). Concentrations of 18:3*n*-3 and TFA were lower ($P < 0.001$) and the concentration of 18:2*n*-6 was higher ($P < 0.001$) in RC silages compared with grass silages. The amount of 16:0 in silage was dependent on plant species, growth stage, and their interaction ($P < 0.001$).

The majority of fatty acids (710 g/kg of TFA) in silage were present as NEFA. For both plant species, the concentration of 18:3*n*-3 in the PL+MAG fraction (from 810 to 860 g/kg of TFA) of silage was particularly high compared with that in fresh herbage. The proportion of NEFA was lower ($P < 0.001$) in RC silages for all growth stages when formic acid was used, whereas the opposite was true ($P < 0.001$) for grass silages for all growth stages. In addition, the proportion of NEFA in grass silage increased linearly ($P \leq 0.018$) with advancing maturity, but for RC, the proportion of NEFA was dependent on both growth stage and application of formic acid ($P \leq 0.018$). Addition of formic acid decreased ($P < 0.05$) the concentrations of 16:0, 18:3*n*-3, and TFA in RC silage DM prepared from all growth stages. However, the influence of formic acid on the 18:2*n*-6 concentration in RC silage and on the concentrations of 16:0, 18:2*n*-6, 18:3*n*-3, and TFA in grass silage were dependent on growth stage ($P \leq 0.029$). Ensiling grass with formic acid increased ($P = 0.005$) the proportion of fatty acids in NEFA and PL+MAG and decreased ($P = 0.005$) the proportion of fatty acids in DAG and TAG fractions ($P = 0.005$). Ensiling RC with formic acid was associated with an increase ($P = 0.005$) in the proportion of fatty acids in DAG and PL+MAG and a decrease ($P = 0.005$) in the proportion of fatty acids as NEFA and TAG.

Lipolysis during Ensiling

Lipolysis was extensive during the ensilage of RC and grass (Fig. 1). Use of formic acid lowered net lipolysis of esterified lipid during ensilage of RC but increased lipolysis during the ensilage of grass ($P < 0.001$ for interaction of plant species and formic acid addition). Increases in forage maturity were associated with a linear increase in net lipolysis for grass ($P = 0.026$), whereas the reverse was true for RC ($P = 0.026$). For grass silages, the extent of lipolysis was positively associated with the proportion of ammonia N in total N ($P \leq 0.018$; Table 5).

Polyphenol Oxidase Activity and Soluble Bound Phenol Content

Total (active + latent) PPO activity was markedly higher for RC than for grass (Fig. 2), being increased in a quadratic manner ($P \leq 0.016$) by advancing growth stage in RC. The content of soluble bound phenol was marginally higher ($P < 0.001$) in RC than in grass silages, except for ensiled grass harvested at the third growth stage and treated with 0 or 2 L/t of formic acid (Fig. 3A and 3B). Furthermore, the content of soluble bound phenols was dependent on 3 factors ($P < 0.001$ for interaction of plant species, growth stage, and formic acid). For RC silages prepared without formic acid, the content of soluble bound phenols in silages was negatively associated ($P = 0.004$; Table 5) with total PPO activity of parent herbage, whereas for grass silages prepared with formic acid, a negative relationship ($P < 0.001$) existed between net lipolysis and soluble bound phenol content during ensiling.

Table 2. Effect of plant species, growth stage, and formic acid addition on silage chemical composition and fermentation characteristics

	Silage												SEM	Significant effects[1]
	Growth stage													
	I				II				III					
	Formic acid, L/t													
Item	0	2	4	6	0	2	4	6	0	2	4	6		
Timothy–meadow fescue														
DM, g/kg	261	265	263	255	276	274	283	269	310	311	305	301		
pH	4.22	4.19	4.02	4.14	4.26	4.10	4.09	4.17	4.22	4.58	4.33	4.85		
CP, g/kg DM	171	175	174	177	151	149	142	146	120	125	117	116		
WSC,[2] g/kg DM	14.1	6.1	26.1	141.7	17.5	26.7	18.1	151.5	32.0	89.1	113.7	92.8		
Lactic acid, g/kg DM	94.1	69.0	65.3	17.6	97.4	66.9	55.8	3.7	95.6	64.4	18.0	8.8		
VFA total,[3] g/kg DM	18.4	14.2	13.8	6.4	28.1	20.4	14.1	5.1	11.7	7.4	6.9	3.3		
Acetic acid, g/kg DM	16.0	14.2	13.8	6.4	23.9	20.4	14.1	5.1	11.7	7.4	6.9	3.3		
Butyric acid, g/kg DM	<0.1	<0.1	<0.1	<0.1	0.3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1		
Ethanol, g/kg DM	5.31	5.13	4.82	1.79	3.90	3.49	4.15	0.61	1.93	1.53	1.32	2.06		
Lactic acid,[4] g/kg	839	830	825	698	780	766	797	399	891	883	717	715		
Ammonia N, g/kg N	64.6	31.5	23.7	20.4	78.8	33.7	26.1	20.7	96.1	79.8	35.8	72.8		
Gas, mL/g DM	13.8	14.7	10.2	1.0	13.2	6.5	8.4	0.0	10.1	5.5	2.1	13.6		
Red clover														
DM, g/kg	245	241	248	233	233	222	226	219	254	253	250	247	3.4	T, G_{lq}, A_{lqc}, T × G_l, and T × G_q
pH	4.34	4.25	4.24	4.34	4.28	4.18	4.21	4.29	4.24	4.23	4.35	4.31	0.076	G_{lq}, A_q, T × G_l, T × A_c, and G_l × A_l[5]
CP, g/kg DM	218	217	230	216	208	186	197	205	172	169	169	170	4.5	T, G_l, T × A_c, and G_q × A_q
WSC, g/kg DM	27.3	26.9	28.2	122.1	18.7	6.1	29.7	118.9	42.1	55.1	90.4	134.7	13.83	G_{lq}, A_{lqc}, and G_{lq} × A_c[6]
Lactic acid, g/kg DM	98.6	92.9	64.3	10.6	108	88.9	61.2	11.4	99.7	88.6	34.5	10.9	6.81	T, G_l, A_{lq}, T × A_q, and G_l × A_{qc}
VFA total,[3] g/kg DM	20.5	21.2	19.8	6.7	22.9	20.3	17.5	6.1	18.5	23.2	10.9	5.1	3.10	T, G_{lq}, A_{lq}, T × G_q, and G_q × A_l
Acetic acid, g/kg DM	20.5	21.2	19.8	6.7	22.9	20.3	17.5	6.1	18.5	23.2	10.9	5.1	2.42	T, G_{lq}, A_{lq}, T × G_q, and G_q × A_l
Butyric acid, g/kg DM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.05	
Ethanol, g/kg DM	1.79	3.44	4.70	0.72	1.75	4.59	4.75	1.02	2.56	2.26	2.77	0.31	0.615	T, G_l,, A_{lqc}, T × G_l, T × A_q, and G_{lq} × A_q[5]
Lactic acid,[4] g/kg	828	812	765	589	825	814	778	648	847	793	720	672	36.9	G_q, A_{lq}, T × G_q, and G_l × A_{qc}[7,8]
Ammonia N, g/kg N	54.9	41.0	15.7	25.3	59.4	47.1	31.9	21.4	60.6	49.3	30.7	23.3	5.19	T, G_{lq}, A_{lqc}, T × G_{lq}, T × A_q, and G_{lq} × A_q[6,9]
Gas, mL/g DM	9.8	9.0	11.4	0.3	7.7	10.8	8.5	0.6	8.5	7.3	3.4	0.0	1.96	T, G_l, A_l, T × G_q, G_l × A_l, and G_l × A_q[5,6,10]

[1]Refers to the significance ($P < 0.05$) of effects due to plant species (timothy–meadow fescue vs. red clover; type [T]), growth stage (linear and quadratic effects [G_l and G_q, respectively]), formic acid addition (linear, quadratic and cubic effects [A_l, A_q, and A_c, respectively]), and their interactions.

[2]WSC = water-soluble carbohydrates.

[3]Contains acetic, propionic, butyric, isovaleric, valeric, and caproic acids.

[4]Lactic acid as a proportion of fermentation end products (g/kg).

[5]Refers to the significance ($P < 0.05$) of the effects due to T × G_l × A_l interactions.

[6]Refers to the significance ($P < 0.05$) of the effects due to T × G_l × A_q interactions.

[7]Refers to the significance ($P < 0.05$) of the effects due to T × G_q × A_l interactions.

[8]Refers to the significance ($P < 0.05$) of the effects due to T × G_l × A_q interactions.

[9]Refers to the significance ($P < 0.05$) of the effects due to T × G_l × A_c, interactions.

[10]Refers to the significance ($P < 0.05$) of the effects due to T × G_q × A_c interactions

Table 3. Distribution of lipid fractions (g/kg of total fatty acids), fatty acid composition of lipid fractions (g/kg of fatty acids within the fraction or as a proportion of total fatty acids), and content of major fatty acids (g/kg DM) in fresh grass and red clover herbages at 3 stages of growth

	Herbage							
	Plant species							
	Timothy–meadow fescue			Red clover				
	Growth stage							
Item[1]	I	II	III	I	II	III	SEM	Significant effects[2]
g/kg of total fatty acids								
NEFA	46.4	37.1	44.0	31.0	26.5	21.0	4.89	T
PL+MAG	891	916	920	934	933	931	7.1	T and T × G_l
DAG	41.5	31.4	18.9	21.2	20.4	23.8	3.12	T, G_l, and T × G_l
TAG	20.8	15.9	17.1	14.0	20.0	22.7	2.64	T × G_l
16:0	89.9	95.1	88.5	95.0	100.3	106.3	1.78	T, G_l, and T × G_l
18:0	6.60	6.50	7.10	11.3	11.8	12.0	0.390	T
cis-9 18:1	23.5	24.7	26.0	16.1	15.7	15.2	1.13	T
18:2*n*-6	116	107	105	129	171	176	3.5	T, G_{lq}, and T × G_{lq}
18:3*n*-3	725	728	733	723	678	656	6.3	T, G_l, and T × G_l
g/kg of fatty acids in NEFA								
16:0	156	336	255	142	252	342	61.9	G_l
18:0	20.4	0.0	33.4	50.9	54.2	54.6	18.88	T
cis-9 18:1	29.5	0.0	54.6	47.7	12.0	0.0	13.84	G_q and T × G_l
18:2*n*-6	170	94.3	169	284	300	187	27.10	T
18:3*n*-3	296	324	214	364	377	268	31.5	T and G_{lq}
16:0	84.5	90.1	79.5	91.1	101.3	105.8	2.86	T, G_q, and T × G_l
18:0	5.10	5.70	4.70	9.80	12.4	11.3	0.650	T and G_q
cis-9 18:1	14.5	18.7	15.2	10.8	10.7	9.5	1.21	T
18:2*n*-6	100	93.5	90.1	113	157	161	3.63	T, G_{lq}, and T × G_{lq}
18:3*n*-3	789	784	809	767	707	699	7.3	T, G_{lq}, and T × G_l
g/kg of fatty acids in DAG								
16:0	199	215	331	263	252	239	61.3	
18:0	9.90	0.00	1.50	10.7	13.7	0.00	8.200	
cis-9 18:1	94.0	96.0	30.6	48.1	13.7	0.0	15.98	T and G_l
18:2*n*-6	252	301	197	343	357	317	26.4	T and G_q
18:3*n*-3	445	387	440	336	364	445	80.2	
g/kg of fatty acids in TAG								
16:0	119	205	177	167	139	104	28.1	
18:0	0.00	0.00	0.00	10.8	0.00	0.00	4.400	
cis-9 18:1	79.9	54.4	32.2	24.1	31.7	0.0	30.58	
18:2*n*-6	300	345	288	337	379	386	33.9	
18:3*n*-3	399	374	354	419	411	485	65.9	
g/kg DM								
14:0	0.05	0.06	0.04	0.02	0.02	0.02	0.006	T
15:0	0.01	0.01	0.02	0.02	0.04	0.03	0.009	T
16:0	1.43	1.30	0.92	1.63	1.65	1.35	0.046	T, G_{lq}, and T × G_l
17:0	0.01	0.01	0.01	0.02	0.03	0.03	0.006	T
18:0	0.10	0.09	0.07	0.19	0.19	0.15	0.006	T and G_l
cis-9 18:1	0.37	0.34	0.27	0.28	0.26	0.19	0.011	T and G_l
cis-11 18:1	0.07	0.04	0.05	0.02	0.02	0.02	0.001	T
18:2*n*-6	1.85	1.46	1.09	2.21	2.82	2.24	0.081	T, G_{lq}, and T × G_{lq}
18:3*n*-3	11.6	9.95	7.58	12.4	11.2	8.33	0.410	T and G_l
20:0	0.03	0.03	0.03	0.02	0.03	0.03	0.002	T and G_l
22:0	0.05	0.04	0.04	0.03	0.03	0.03	0.004	T
24:0	0.04	0.03	0.03	0.04	0.04	0.03	0.003	T and G_l
26:0	0.08	0.05	0.04	0.02	0.02	0.02	0.004	T, G_l, and T × G_l
28:0	0.15	0.10	0.06	0.04	0.02	0.03	0.007	T, G_l, and T × G_l
Other[3]	0.16	0.15	0.10	0.21	0.12	0.18	0.026	
TFA	16.0	13.7	10.3	17.2	16.5	12.7	0.51	T and G_{lq}

[1]DAG = diacylglycerol; PL+MAG = polar lipid + monoacylglycerol; TAG = triacylglycerol; TFA = total fatty acids.

[2]Refers to the significance ($P < 0.05$) of effects due to plant species (timothy–meadow fescue vs. red clover; type [T]), growth stage (linear effect [G_l] and quadratic effect [G_q]), and their interactions.

[3]Contains 12:0, *cis*-9 16:1, 20:2 *n*-6, 20:3 *n*-3, 20:4 *n*-6, 21:0, 22:2 *n*-6, 22:4*n*-6, and *cis*-15 24:1.

Table 4. Distribution of lipid fraction (g/kg of total fatty acids), fatty acid composition of lipid fractions (g/kg of fatty acids within the fraction or as a proportion of total fatty acids), and content of major acids (g/kg DM) in grass and red clover silages harvested at 3 stages of growth

	Silage													
	Plant species													
	Timothy meadow fescue						Red clover							
	Growth stage													
	I		II		III		I		II		III			
	Formic acid, L/t addition													
Item[1]	0	6	0	6	0	6	0	6	0	6	0	6	SEM	Significant effects[2]
g/kg of total fatty acids														
NEFA	706	737	717	749	756	806	743	615	716	637	760	584	18.5	T, A, T × G_l, and T × A
PL+MAG	109	183	134	178	91.7	113	110	183	134	161	125	224	19.67	A and T × G_l
DAG	78.6	53.8	70.9	48.8	47.8	40.9	50.2	132	60.9	160	48.5	153	4.81	T, G_q, A, T × G_l, T × A, and G_l × A
TAG	107	26.4	78.6	24.0	105	40.4	96.8	69.7	89.3	42.4	66.9	39.1	9.05	A, T × G_l, and T × A
16:0	156	142	160	141	154	145	152	159	154	157	163	164	2.8	T, G_l, A, and T × A
18:0	10.3	9.00	10.7	8.66	10.2	9.15	15.8	16.1	16.9	15.2	17.9	17.2	3.811	T, G_l, A, T × G_l, and G_q × A
cis-9 18:1	24.7	26.8	12.6	34.4	30.1	30.6	17.8	20.5	15.0	17.1	17.0	19.2	1.96	T, G_q, A, T × A, and G_q × A^3
18:2*n*-6	155	153	151	160	157	163	183	202	173	202	193	220	5.1	T, A, and T × A
18:3*n*-3	606	623	610	610	597	603	586	555	590	552	561	540	7.7	T, G_l, A, and T × A
g/kg of fatty acids in NEFA														
16:0	199	166	204	164	196	168	181	200	185	200	200	209	5.5	T, A, and T × A
18:0	11.9	9.25	13.3	9.59	8.65	10.1	17.3	17.9	19.2	16.7	20.7	19.5	1.417	T
cis-9 18:1	21.7	26.1	9.82	31.7	20.1	22.5	15.5	20.4	14.9	15.9	14.9	19.0	4.181	T and A
18:2*n*-6	141	162	137	167	102	171	186	211	179	223	201	234	15.6	T and A
18:3*n*-3	575	600	574	587	616	587	564	515	562	515	527	486	15.9	T, A, T × G_l, and T × A
g/kg of fatty acids in PL+MAG														
16:0	94.3	74.9	79.5	82.0	86.9	79.9	68.7	77.9	67.8	68.0	72.7	73.2	2.59	T, G_q, and T × $A^{3,4}$
18:0	11.9	9.25	13.3	9.59	8.65	10.1	17.3	17.9	19.2	16.7	20.7	19.5	1.417	T
cis-9 18:1	21.7	26.1	9.82	31.7	20.1	22.5	15.5	20.4	14.9	15.9	14.9	19.0	4.181	T and A^3
18:2*n*-6	74.4	77.3	65.2	72.5	77.2	65.8	67.2	57.4	59.3	51.0	67.1	59.2	3.80	T, G_q, and A
18:3*n*-3	808	823	855	814	836	854	855	845	861	863	844	855	8.4	T, G_l, T × G_l, and G_q × A^3
g/kg of fatty acids in DAG														
16:0	147	223	137	229	157	192	192	210	207	210	194	220	6.7	T, A, and T × $A^{3,4}$
18:0	24.7	15.4	0.00	4.3	0.00	0.00	24.6	24.5	22.7	17.4	21.3	27.8	6.430	T, G_l, and T × G_l
cis-9 18:1	30.9	38.8	11.6	32.4	14.9	102	24.9	36.0	23.9	26.9	86.5	34.6	21.17	T × A^4
18:2*n*-6	192	283	176	311	212	243	285	343	265	312	274	351	8.7	T, A, T × G_q, T × A, and G_q × $A^{3,4}$
18:3*n*-3	603	439	607	423	614	464	440	378	459	357	425	357	16.7	T, A, and T × A
g/kg of fatty acids in TAG														
16:0	95.5	107	92.3	132	103	111	108	107	103	91.7	108	125	5.34	A, T × G_q, and T × A^3
18:0	2.88	18.7	0.00	0.00	0.00	0.00	10.3	0.00	14.3	0.00	0.00	0.00	3.101	T × G_q, T × A, and G_q × A^4
cis-9 18:1	45.7	44.1	11.3	49.1	38.1	37.5	20.7	7.7	18.8	0.00	0.00	0.00	12.236	T
18:2*n*-6	286	285	291	315	279	245	261	331	244	317	307	335	18.5	A, T × G_q, and T × A

(continued)

Table 4. (cont.)

18:3*n*-3	570	480	577	504	580	606	585	537	597	583	586	540	20.6	G_l, A, T × G_{lc}, and T × A
g/kg DM														
14:0	0.10	0.09	0.10	0.09	0.08	0.08	0.06	0.06	0.05	0.04	0.05	0.04	0.006	T and G_l
15:0	0.11	0.08	0.50	0.16	0.14	0.18	0.35	0.46	0.38	0.40	0.26	0.47	0.075	T, G_q, T × G_q, T × A, and G_q × A
16:0	3.73	3.27	3.40	3.06	2.62	2.67	3.30	2.86	3.27	3.11	3.10	2.62	0.070	G_{lq}, A, T × G_{lc}, and G_l × $A^{3,4}$
17:0	0.14	0.14	0.18	0.15	0.12	0.14	0.31	0.35	0.30	0.28	0.33	0.30	0.023	T, G_l, A, T × G_l, and G_q × A
18:0	0.24	0.21	0.23	0.19	0.17	0.17	0.34	0.29	0.36	0.30	0.34	0.27	0.010	T, G_l, A, T × A, and G_q × A
cis-9 18:1	0.59	0.62	0.28	0.74	0.51	0.56	0.39	0.37	0.32	0.34	0.32	0.31	0.044	T, G_l, A, and G_{lq} × A^3
cis-11 18:1	0.15	0.11	0.04	0.12	0.11	0.12	0.05	0.03	0.07	0.01	0.06	0.05	0.014	T, G_q, T × G_q, and T × A^3
18:2*n*-6	3.71	3.53	3.21	3.46	2.66	2.98	3.97	3.64	3.68	4.00	3.68	3.35	0.105	T, G_{lq}, T × G_l, and G_q × A
18:3*n*-3	14.5	14.3	13.0	13.2	10.1	11.1	12.8	10.0	12.6	11.0	10.7	8.6	0.51	T, G_{lq}, A, T × G_l, and T × A
20:0	0.05	0.04	0.04	0.04	0.06	0.06	0.06	0.05	0.07	0.05	0.07	0.05	0.010	T, G_q, and G_q × A^3
22:0	0.12	0.10	0.11	0.10	0.09	0.11	0.10	0.08	0.10	0.08	0.10	0.07	0.005	T, G_l, A, T × A, and G_l × $A^{3,4}$
24:0	0.07	0.06	0.08	0.06	0.08	0.07	0.13	0.09	0.14	0.10	0.12	0.09	0.004	T, G_q, A, T × G_{lq}, and T × A
26:0	0.11	0.10	0.10	0.09	0.08	0.08	0.03	0.03	0.04	0.03	0.03	0.03	0.005	T, G_l, A, and T × G_l
28:0	0.23	0.22	0.20	0.18	0.16	0.14	0.05	0.06	0.06	0.04	0.05	0.04	0.007	T, G_l, A, and T × G_l
Other[5]	0.25	0.25	0.41	0.25	0.16	0.19	0.36	0.31	0.42	0.65	0.33	0.30	0.048	T and G_q^3
TFA	23.9	23.0	21.4	21.6	17.0	18.4	21.8	18.0	21.3	19.9	19.1	16.0	0.66	T, G_{lq}, T × G_l, A, and T × A

[1]DAG = diacylglycerol; PL+MAG = polar lipid + monoacylglycerol; TAG = triacylglycerol; TFA = total fatty acids.

[2]Refers to the significance ($P < 0.05$) of effects due to plant species (timothy–meadow fescue vs. red clover; type [T]), growth stage (linear effect [G_l] and quadratic effect [G_q]), formic acid addition (0 vs. 6 L/t; A), and their interactions.

[3]Refers to the significance ($P < 0.05$) of effects due to T × G_q × A interactions.

[4]Refers to the significance ($P < 0.05$) of effects due to T × G_l × G_q interactions.

[5]Contains 12:0, *cis*-9 14:1, *cis*-9 16:1, 20:2 *n*-6, 20:3 *n*-3, 21:0, 22:2 *n*-6, and *cis*-15 24:1.

For both plant species, total PPO activity was not associated ($P > 0.10$) with net lipolysis during ensiling. However, herbage PPO activity was positively related ($P = 0.012$) to the proportion of ammonia N in total N for RC silages prepared without formic acid. In contrast, there was a tendency ($P = 0.088$) for a negative relationship between RC herbage PPO activity and the proportion of ammonia N in total N of RC silages prepared with formic acid. The content of soluble bound phenols was negatively related ($P \leq 0.023$) to the proportion of ammonia N in total N for grass prepared with formic acid and for RC silages prepared without formic acid.

DISCUSSION

Silage Fermentation Characteristics

Ensiling was performed to achieve a target silage DM concentration of between 250 and 300 g/kg. Irrespective of growth stage, it was possible to meet these targets for grass silages. However, the higher moisture content of RC and the need to standardize wilting time for both plant species resulted in lower DM concentrations of RC silages than of grass silages. Both the lower DM content and the higher buffering capacity resulted in the ensiling of RC being more challenging compared with grass. Nevertheless, all experimental silages were well preserved, as indicated by the relatively low concentrations of VFA and ammonia N. Silage pH values varied around 4.0 to 4.9 as could be expected due to high amount of formic acid used and relatively high DM content of grass silages, especially those prepared from the third growth stage. There was no evidence of secondary fermentation. In the absence of formic acid, more extensive fermentation of grass harvested at the second and third growth stage was associated with silage containing higher VFA concentrations and greater proportions of ammonia N in total N compared with other silages. The use of formic acid restricted fermentation *in silo* as indicated by the increased concentration of residual sugars, decreased concentrations of lactic

Table 5. Relationships[1] between net lipolysis of esterified lipid *in silo* (g/kg of total fatty acids) and total polyphenol oxidase (PPO) activity (μkatal/g fresh weight of leaf) in fresh grass and red clover herbages and corresponding soluble bound phenol (mg/g DM) and ammonia nitrogen (g/kg of total nitrogen) in silages

Independent variable (*x*)	Dependent variable (*y*)	No.[2]	Intercept	SE	*P*-value	Slope	SE	r^2_{adj}[3]	*P*-value
Timothy–meadow fescue									
Bound phenols in silages prepared with formic acid	Net lipolysis during ensiling with formic acid	9	79.8	1.04	<0.001	–9.059	1.1396	0.886	<0.001
Ammonia N in silages prepared without formic acid	Net lipolysis during ensiling without formic acid	9	53.6	4.96	<0.001	1.884	0.0613	0.514	0.018
Ammonia N in silages prepared with formic acid	Net lipolysis during ensiling with formic acid	9	68.9	1.18	<0.001	0.083	0.0247	0.563	0.012
Bound phenols in silages prepared with formic acid	Ammonia N in silages prepared with formic acid	9	94.9	20.99	0.003	–66.706	23.0506	0.480	0.023
Red clover									
Bound phenols in silages prepared without formic acid	Ammonia N in silages prepared without formic acid	9	66.9	2.99	<0.001	–4.030	1.3635	0.492	0.021
Total PPO activity in herbages	Ammonia N in silages prepared without formic acid	9	53.4	1.67	<0.001	0.463	0.1383	0.560	0.012
Total PPO activity in herbages	Bound phenols in silages prepared without formic acid	9	3.1	0.27	<0.001	–0.092	0.2222	0.670	0.004

[1]Only linear regressions between parameters at $P < 0.05$ are reported.

[2]Number of observations.

[3]r^2_{adj} = adjusted R^2.

acid and total VFA, and lowered proportion of ammonia N in total N, findings that are consistent with earlier reports in the literature (Kung et al., 2003; Jaakkola et al., 2006). A rapid decrease in pH lowers proteolysis *in silo* by inhibiting plant proteases and microbial growth. Decreased proteolysis minimizes AA degradation and, as a consequence, lowers volatile N losses (McDonald and Whittenbury, 1973; Charmley, 2001; Alves et al., 2011). Concentrations of lactic acid were higher in RC silages compared with grass silages that are presumably due to the higher buffering capacity of RC.

Effect of Plant Species and Stage of Maturity on Fatty Acid Composition of Herbage

In the present study, most of the fatty acids (from 890 to 930 g/kg) in RC or mixed timothy and meadow fescue were present as components of polar and neutral lipid recovered in the unresolved PL+MAG fraction during TLC analysis. It is well established that the majority of lipid in fresh herbage is contained in phospholipids and galactolipids that are located within thylakoid membranes of chloroplasts (Lee et al., 2004; Van Ranst et al., 2009b; Buccioni et al., 2012).

Grass had lower concentrations of 16:0, 18:2*n*-6, 18:3*n*-3, and TFA than RC in agreement with previous comparisons of RC with perennial ryegrass (*Lolium perenne*; Van Ranst et al., 2009a,b). In contrast, earlier investigations reported no difference in fatty acid composition between RC, timothy, or cocksfoot (*Dactylis glomerata*; Boufaïed et al., 2003). Few studies have characterized variation in herbage fatty acid content across growing seasons. It is therefore possible that in addition to plant genetics, differences in environmental conditions, management practices such as fertilizer application, and herbage maturity may contribute to the extent of differences in fatty acid composition reported for grasses compared with RC (Boufaïed et al., 2003; Dewhurst et al., 2006; Mir et al., 2006).

Advances in maturity decreased 18:2*n*-6, 18:3*n*-3, and TFA content of grass and RC that has also been demonstrated previously (Dewhurst et al., 2001; Boufaïed et al., 2003; Van Ranst et al., 2009b). Leaf tissue contains the majority of the lipid in plants (Harfoot, 1981), such that alterations in the fatty acid concentrations of herbage due to cutting date are probably related to decreasing the leaf:stem ratio as the plant matures (Elgersma et al., 2005; Dewhurst et al., 2006; Mir et al., 2006). Concentrations of all major fatty acids in DM decreased in both herbages with advancing maturity. However, the fatty acid composition of grass remained relatively constant, whereas advances in maturity of RC were associated with a marked increase in the proportion of 18:2*n*-6 (from 12 to 18 g/100 g fatty acid) and a decline in 18:3*n*-3 (from 72 to 66 g/100 g fatty acid). Earlier reports have indicated that advances in the maturity of ryegrass or timothy typically decrease the proportion of 18:3*n*-3 in plant lipids, whereas the effects on the proportion of 18:2*n*-6 were inconsistent (Dewhurst et al., 2001; Boufaïed et al., 2003; Elgersma et al., 2003). For RC, postponing harvest date was shown to increase the content of both 18:2*n*-6 and 18:3*n*-3 (Vanhatalo et al., 2007). The reasons for the discrepancies between our findings and earlier studies are not obvious but may be related

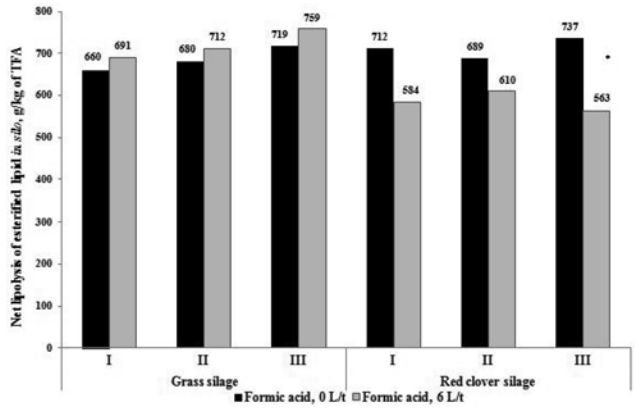

Figure 1. Net lipolysis of esterified lipid *in silo* (g/kg of total fatty acids [TFA]) of mixed timothy and meadow fescue and red clover silages in growth stages I, II, and III. *Significant effects on net lipolysis; T, G_l, A, T × G_l, and T × A refer to the significance ($P < 0.05$, SEM 18.56) of effects due to plant species (timothy–meadow fescue vs. red clover; type [T]), growth stage (linear effect [G_l]), formic acid addition (A), and their interactions.

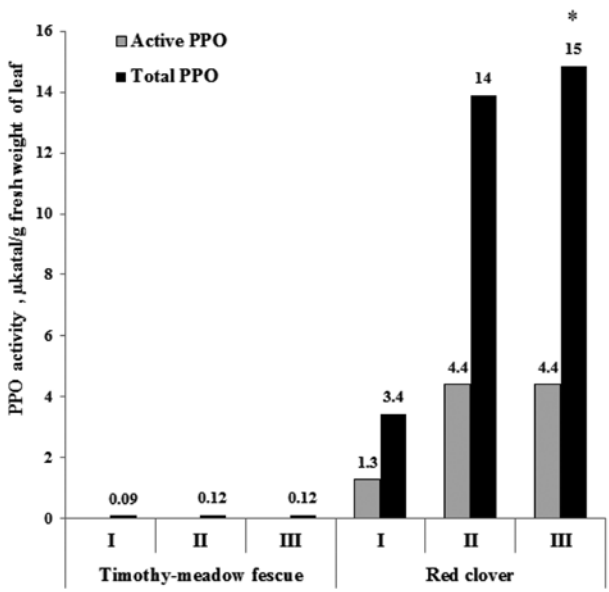

Figure 2. Polyphenol oxidase (PPO) activity (μkatal/g fresh weight of leaf) of mixed timothy and meadow fescue and red clover herbages in growth stages I, II, and III. *Significant effects on total PPO activity; T, G_{lq}, T × G_l, and T × G_q refer to the significance ($P < 0.05$, SEM 0.89) of effects due to plant species (timothy–meadow fescue vs. red clover; type [T]), growth stage (linear effect [G_l] and quadratic effect [G_q]), and their interactions.

to differences in the physiological senescence of plant species, cultivars, weather, and agronomical conditions.

Effect of Ensiling on Forage Fatty Acid Concentration and Composition

Ensiling caused major changes in the distribution of esterified lipids and NEFA in both RC and grass. Extensive hydrolysis of esterified plant lipid resulted in a substantial decrease in the amount of fatty acids present in polar membrane lipids (PL+MAG) that was accompanied by an increase in NEFA, DAG, and TAG fractions. Such changes are known to occur during the ensiling of grasses and RC (Vanhatalo et al., 2007; Van Ranst et al., 2010; Halmemies-Beauchet-Filleau et al., 2013a) due to the activity of plant and microbial lipases *in silo* (Lee et al., 2004; Van Ranst et al., 2009b, 2010). Furthermore, the marked increase in the proportion of 18:3*n*-3 in TAG during ensiling is an indication of the stress-induced DGAT enzyme being active that catalyses the conversion of DAG released from degraded membranes into TAG (Kaup et al., 2002; Lung and Weselake, 2006). Grass silage has been shown to contain higher levels of TAG compared with RC silage, an observation attributed to the denaturation of the DGAT enzyme in RC by binding with reactive quinones formed during the action of PPO on endogenous phenols (Lee et al., 2006). However, in the present study, there was no difference in the amount of TAG in silages (g/kg of TFA) between the forage species. Due to rapid decrease in forage pH *in silo*, formic acid application substantially lowered the proportion of silage TFA as TAG compared with the non-formic acid treatment for both forage species consistent with a low pH leading to an immediate inhibition of the DGAT enzyme (Van Ranst et al., 2009a). Of particular note, treating RC silage with formic acid caused a remarkable increase in the DAG fraction and decreased the amount of NEFA compared with untreated silage. It seems that the initial degradation of PL (removal of phosphoric acid or galactose units) proceeded as expected, but subsequent lipolysis and secondary TAG synthesis were substantially decreased. However, treating grass silage with formic acid lowered the amount of DAG and increased NEFA content. Differences in activities of species-specific plant or microbial enzymes *in silo* as influenced by the rate and extent of decreases in silage pH may explain the differential effect of formic acid on the abundance of DAG and NEFA in silages prepared from grass and RC. Such effects may be related to the higher buffering capacity of RC than grass that offers more resistance to a rapid decline in pH during the initial stages of ensiling.

Silages had higher concentrations of major fatty acids (16:0, 18:2*n*-6, and 18:3*n*-3) and TFA in DM than fresh herbage, consistent with earlier reports (Boufaïed et al., 2003; Van Ranst et al., 2009b). In contrast, some studies reported no difference (Halmemies-Beauchet-Filleau et al., 2013a), whereas others reported lower major fatty acid and TFA content for silages compared with parent herbage (Elgersma et al., 2003; Vanhatalo et al., 2007). Discrepancies between studies most probably originate from differences in herbage wilting time, wilting conditions, or the timing of herbage sampling before ensiling. Higher silage fatty acid content relative to parent herbage can be attributed to either a loss of DM (Mayne and

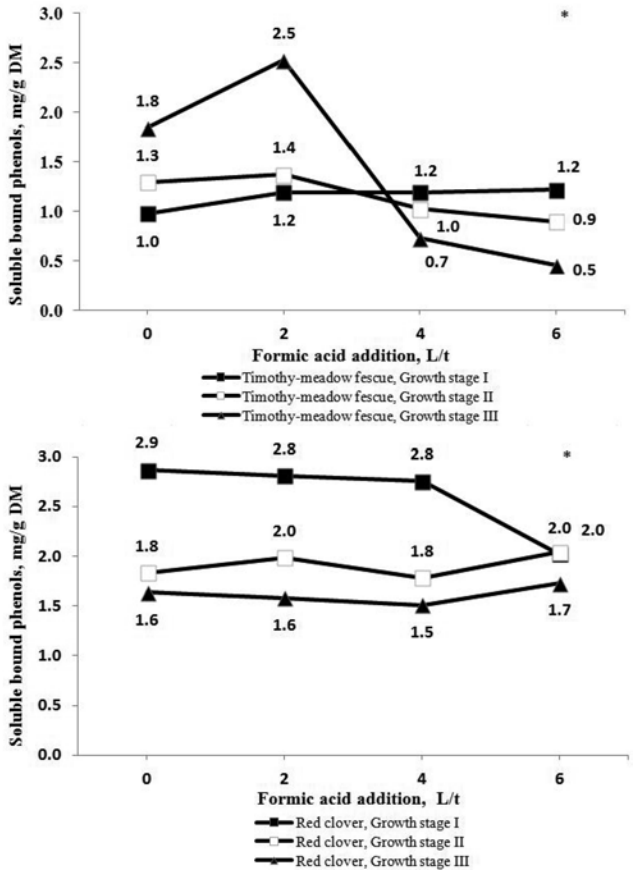

Figure 3. Concentration of soluble bound phenol (mg/g DM) in mixed timothy and meadow fescue and red clover silages in growth stages I, II, and III. *Significant effects on concentration of soluble bound phenol; T, G_{lq}, A_{lqc}, T × G_l, T × A_{lc}, G_{lq} × A_{lc}, T × G_l × A_l, and T × G_l × A_q refer to the significance ($P < 0.05$, SEM 0.16) of effects due to plant species (timothy–meadow fescue vs. red clover; type [T]), growth stage (linear effect [G_l] and quadratic effect [G_q]), formic acid addition (linear effect [A_l], quadratic effect [A_q], and cubic effect [A_c]), and their interactions.

Gordon, 1986) and volatile components such as VFA and CO_2 during silage preparation and fermentation *in silo* or a loss of soluble components in silage effluent (Boufaïed et al., 2003). In the present experiment, no effluent was produced. It is notable that the difference in the TFA content of fresh herbage and corresponding silage was much smaller than the difference in fermentation and gas losses between the untreated and formic acid–treated silages. Measurements of fatty acid content were not made for wilted herbage at the time of ensiling but from the fresh intact plant material samples intended for lipid fraction analyses. It is possible that the loss of nutrients during wilting may, at least in part, explain the observed differences in TFA content between fresh herbage and silage.

Changes in the concentration of individual fatty acids in DM during ensiling were species specific. In fresh herbage, the concentrations of 16:0, 18:2*n*-6, 18:3*n*-3, and TFA in DM were higher in RC compared with grass, whereas for silages, the amount of 16:0 did not differ between plant species and 18:2*n*-6 was higher and 18:3*n*-3 and TFA were lower for RC than for grass. Unless extensive secondary fermentation occurs, ensiling has little effect on herbage fatty acid concentration and composition, and therefore, the major losses of 18:3*n*-3 could be expected to occur during wilting (Dewhurst and King, 1998; Elgersma et al., 2005; Halmemies-Beauchet-Filleau et al., 2013a). Oxidative losses of PUFA during wilting are associated with the activity of the lipoxygenase system initiated by damage to plant tissues. Plant lipases hydrolyze esterified lipid in damaged membranes to release fatty acids that are converted to hydroperoxy PUFA, which can be further oxidized into a range of volatile compounds including aldehydes and alcohols (Dewhurst et al., 2006). More extensive 18:3*n*-3 losses in RC compared with grass may be attributed, at least in part, to the marginally longer wilting time before ensiling and more extensive fermentation *in silo*, as would be indicated by higher concentrations of fermentation acids in RC silage compared with grass silage. However, the content of butyric acid was low in all RC silages, indicating that the higher losses of PUFA were not due to secondary fermentation *in silo*. It is also possible that differences in plant enzyme activity could also explain the higher losses of 18:3*n*-3 during ensiling of RC than grasses.

Silages contained higher amounts of 15- to 18-carbon SFA than parent herbages that may be related to the synthesis of fatty acids by microbes *in silo*. Microbial lipid is known to be relatively rich in 15- to 18-carbon SFA compared with plant material (Vlaeminck et al., 2006). Furthermore, microbial fatty acid synthesis may also contribute, at least in part, to the higher 16:0, 18:0, and TFA concentrations of RC silages prepared without formic acid addition compared with silage prepared with formic acid, due to a higher abundance of fermentative microbes.

Grass and RC silage has been reported to contain *trans* 18:1 (Vanhatalo et al., 2007) and *trans* 18:2 isomers (Lough and Anderson, 1973; Alves et al., 2011), but concentrations in the present study were below the limit of detection. More extensive fermentation in farm-scale than laboratory silos along with higher fatty acid concentrations in fresh herbage used for ensiling in previous studies (Vanhatalo et al., 2007) may explain this discrepancy.

Ensiling with formic acid resulted in marginal changes in the TFA, 16:0, 18:2*n*-6, and 18:3*n*-3 contents of grass silage and 16:0 and 18:2*n*-6 contents of RC silage but substantially decreased the amounts of TFA and 18:3*n*-3 in RC silage. Preparing grass silage with formic acid or formalin has, in some cases, resulted in decreased 18:2*n*-6, 18:3*n*-3, and TFA concentrations compared with the untreated control (Dewhurst and King, 1998; Boufaïed et al., 2003), which may be related to smaller losses of DM in restrictively fermented silages, whereas others have reported similar fatty acid contents and composition, irrespective of the use

a formic acid–based additive (Halmemies-Beauchet-Filleau et al., 2013a; Van Ranst et al., 2009a). In this investigation, less extensive fermentation *in silo* and lower DM losses as indicated by decreased gas production *in silo* may explain the lower concentration of 18:3*n*-3 and TFA in RC silage treated with formic acid compared with untreated RC silage. However, there were no major differences between the RC and grass silages with respect to extent of fermentation *in silo* or changes in gas production in response to formic acid addition. Therefore, the rather minor differences in gas production and fermentation during the ensiling of both forage species do not explain the decrease in the relative abundance of 18:3*n*-3 for RC treated with formic acid.

Lipolysis and Proteolysis Influenced by Polyphenol Oxidase Activity

Formic acid–treated RC contained higher proportions of esterified lipid and lower proportions of NEFA than grass silage, which is in agreement with earlier investigations (Lee et al., 2007; Vanhatalo et al., 2007; Van Ranst et al., 2009b). Differences in the distribution of lipid are indicative of lower plant-mediated lipolysis in RC than in grass that has been attributed to a higher PPO activity (Dewhurst et al., 2006; Lee et al., 2008; Van Ranst et al., 2011). The PPO enzyme is a stress-activated copper metalloprotein that catalyses the oxidation of endogenous phenols to quinones in the presence of oxygen (Lee et al., 2010). 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). Three hypotheses have been proposed to explain the possible mode of action for PPO in lowering lipolysis of esterified lipid in RC, namely 1) denaturation of plant lipases through quinone binding, 2) entrapment of lipids within protective protein-phenol complexes, and 3) direct binding of quinones to nucleophilic sites in PL (Van Ranst et al., 2011) that would prevent lipid from entering the active site of the lipase.

Measurements of total PPO activity for RC were similar to or higher than values in previous reports (Lee et al., 2009a, 2013). Both active (not statistically tested) and total PPO activities increased substantially with advancing growth stage of RC, such that approximately 38, 32, and 30% of PPO in RC was present in the active form at growth stages I, II and III, respectively. This agrees with earlier findings (Lee et al., 2009b) reporting that for RC harvested at 4 and 8 wk of regrowth, active PPO represented 29 and 81% of total PPO, respectively. It was speculated from these measurements that the large variation in the activation of latent PPO was due to an increasing proportion of senescent material as the crop matures that activates the enzyme. Furthermore, a higher proportion of active PPO can also be expected when plant is exposed to pathogens and abiotic stress (Van Ranst et al., 2011).

No positive relationship existed between herbage total PPO activity and the content of soluble bound phenols in RC silage. This may, at least in part, be due to the underestimation of silage bound phenol content due to the loss of protein solubility as a result of extensive binding (Lee et al., 2013). In the present study, the level of bound phenols in RC silages was considerably lower than would be expected on the basis of bound phenol formation in RC following PPO activation (Lee et al., 2013). Activation of PPO through mechanical damage of RC has been shown to decrease lipolysis during the initial few days after ensiling, but the protection of plant lipids against to hydrolysis was found to diminish after extensive fermentation *in silo* (Van Ranst et al., 2010). In this investigation, the ensiling period was rather long, which may explain the lack of association between herbage total PPO activity and soluble bound phenol content for formic acid treated RC silages or between enzyme activity and net lipolysis for untreated and treated RC silages. It is also possible that formic acid may damage or degrade the phenolic complexes, given that total PPO activity in grass was only associated with increased soluble bound phenol content of untreated silage. Formic acid application may result in more extensive cell membrane damage and, as a consequence, more extensive lipolysis during the ensilage of grass than that for RC. Formic acid addition is known to disrupt cell structure in grasses (Winters et al., 1987), but there are no reports for forage legumes, including RC.

Proteolysis *in silo* as adjudged from the proportion of ammonia N in total N was found to be lower for RC compared with grass silages in this investigation. Earlier studies have shown that the activity of PPO inhibits proteolysis in RC (Jones et al., 1995; Sullivan and Hatfield, 2006), either by active inhibition of proteases or through decreases in protein solubility due to protein-phenol binding (Winters and Minchin, 2001; Lee et al., 2008). A negative association between the content of silage soluble bound phenol and ammonia N for untreated RC silages and formic acid treated grass silages may be attributed to partial bound phenol-mediated protection of forage proteins against endogenous and microbial proteases *in silo*.

On the basis of the positive relationship between proportion of ammonia N in total N and net lipolysis of esterified lipid *in silo* for grass, there appears to be some evidence of a common mechanism regulating the extent of lipolysis and proteolysis *in silo*. However, enzymes produced during extensive microbial growth *in silo* may also be capable of metabolizing these complexes predisposing forage lipid to lipolysis in extensively fermented silages.

In conclusion, concentrations of 18:3*n*-3 and TFA were higher in fresh RC than in a mixture of timothy and meadow fescue grasses. For both species, the content of 18:3*n*-3 and TFA decreased with advancing growth stage, being greatest at the vegetative stage, which corresponds to the highest leaf:stem ratio. Ensiling resulted in a higher content of TFA and individual fatty acids in silage compared with fresh herbage, possibly due to DM losses during wilting before ensiling and during fermentation *in silo*. The majority of fatty acids in fresh herbage were esterified, whereas NEFA predominated in silage. For both fresh and ensiled forages, the PL+MAG fraction contained the highest proportion of 18:3*n*-3. Active and latent PPO concentrations were higher for RC than for grass. Current findings suggest that formic acid addition during the ensiling of RC decreases lipolysis *in silo*. Total PPO activity of both plant species was not associated with the lipolysis *in silo*. However, bound phenols formed via PPO activity appear to have a role in protecting lipid and protein against degradation in grass and the protein in RC from proteolysis during ensiling.

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