Modeling degradation ratios and nutrient availability of anthocyanidin-accumulating *Lc*-alfalfa populations in dairy cows

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ABSTRACT

Dairy cattle eating fresh or ensiled alfalfa as the main portion of their diet often have low protein efficiency because of the rapid initial rate of ruminal protein degradation of fresh and ensiled alfalfa. Ruminal protein degradation of alfalfa might be reduced by introducing a gene that stimulates the accumulation of mono- or polymeric anthocyanidins in alfalfa. The objectives of this study were to fractionate protein and carbohydrates by in situ and chemical approaches, to evaluate in situ ruminal degradation characteristics and synchronization ratios, to determine protein availability to dairy cattle using the 2007 digestible intestinal protein/rumen-degraded protein balance (DVE/OEB) protein system, and to determine net energy for lactation using the Dutch net energy for lactation (VEM) system for 3 newly developed transgenic winter hardy anthocyanidin-accumulating *T₁ Lc*-alfalfa populations. These *T₁ Lc*-alfalfa populations, called *T₁BeavLc1*, *T₁RambLc3*, and *T₁RangLc4*, had an average anthocyanidin accumulation of 163.4 µg/g of DM, whereas AC Grazeland (selected for a low initial rate of degradation) did not accumulate anthocyanidin. The basic chemical composition of the original samples, soluble and potentially degradable fractions, and degradation characteristics of crude protein and carbohydrates were similar in *T₁ Lc*-alfalfa and AC Grazeland. The undegradable in situ crude protein and neutral detergent fiber fraction had 1.3% lower CP and 4.8% lower CHO, respectively, in *T₁ Lc*-alfalfa compared with the amounts in AC Grazeland. The *T₁ Lc*-alfalfa had a 0.34 MJ/kg of DM higher calculated net energy for lactation and 1.9% of CP higher buffer soluble protein compared with that in AC Grazeland. By the protein evaluation model, it was predicted that *T₁ Lc*-alfalfa tended to have 11.9, 6.9, and 8.4 g/kg of DM higher rumen degradable protein,

OEB, and intestinal available protein, respectively, compared with the amounts in AC Grazeland. The hourly OEB included an initial and substantial peak (oversupply) of protein relative to energy, which was highest in *T₁RangLc4* and lowest in *T₁RambLc3*. The hourly OEB between 4 and 24 h was similar and more balanced for all 4 alfalfa populations. In conclusion, *T₁ Lc*-alfalfa accumulated anthocyanidin, tended to have higher predicted intestinal protein availability, and had higher predicted net energy of lactation availability for dairy cattle than did AC Grazeland.

Key words: anthocyanidin-accumulating alfalfa, DVE/OEB 2007 protein system, ruminal protein-to-energy ratio and synchronization, dairy cattle

INTRODUCTION

Alfalfa (*Medicago sativa* L.), also known as lucerne, is one of the main forages in the world, which makes it an important protein source in ruminant rations. When alfalfa is grazed, the costs for milk and meat production are decreased (Rotz, 1996). However, the rapid initial rate of ruminal protein degradation, which is prevalent in freshly ingested alfalfa pasture, makes protein the most limiting nutritional factor for the ruminant (Barry, 1981; Dhiman and Satter, 1993; Broderick, 1995) and is the main cause of pasture bloat, an often fatal disorder (Howarth, 1975; McMahon et al., 2000). Rapid protein degradation also causes an imbalance between the supply of N and energy in the rumen for microbial protein synthesis. Excess ruminal protein is mainly absorbed as ammonia (NH₃) by the rumen and is mainly lost to the animal (Broderick, 1995; Yu et al., 2004). Excessive ruminal protein degradation also results in a smaller portion of dietary protein escaping to the lower digestive tract, a process which is required for optimum animal performance of high-producing cattle (Dhiman and Satter, 1993; Klopfenstein, 1996).

Secondary plant metabolites such as monomeric anthocyanidins and polymeric anthocyanidins (condensed tannins) may decrease ruminal protein degradation by

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precipitating dietary protein (Stafford, 1990; Wang et al., 2006a) and increasing the portion of protein escaping ruminal degradation (Broderick, 1995; Aerts et al., 1999). At the low pH present in the abomasum, protein is released from the polymeric-anthocyanidin/protein complex, which might increase the availability of amino acids for absorption in the small intestine (Waghorn et al., 1987; Bermingham et al., 2001). However, polymeric anthocyanidins do not accumulate in alfalfa forage (Goplen et al., 1980). Using a whole-farm model, it was predicted that protein supplementation could be decreased by 60% if alfalfa accumulated polymeric anthocyanidins in the forage. This, in turn, would increase net return by 12% for dairy farms (Bouton, 2008).

Ray et al. (2003) transformed alfalfa with a maize anthocyanidin-regulating *βHLLH LEAF COLOR (Lc)* gene to develop new genotypes that accumulate monomeric or polymeric anthocyanidins in the forage. Wang et al. (2006b) found that the initial *in vitro* rate of nitrogen and DM degradation for T_0 *Lc*-alfalfa genotypes was decreased compared with that of their nontransgenic parent genotype. However, their survival was poor under western Canadian conditions, and 3 of these genotypes had to be crossed with western Canadian varieties to develop winter-hardy T_1 *Lc*-alfalfa populations. Coulman et al. (2000) reviewed the development of AC Grazeland, an alfalfa variety with a low initial rate of degradation, reduced bloat incidence, reduced leaf-to-stem ratio, and thicker leaf cell walls (Coulman et al., 1998, 2000), but this variety does not feature enhanced forage anthocyanidins. The lower initial degradation rate of AC Grazeland has the potential to change the site of nutrient supply in the digestive tract of the ruminant, but this has not been tested.

Feed material can be evaluated by *in vivo* experiments. However, when sample amount is limited or a large number of treatments is tested, one can use modeling as an alternative approach. Several mathematical models are available for diet formulation of dairy cattle. Input values for these models can be generated by chemical analysis or *in vitro* and *in situ* techniques (Sniffen et al., 1992; Tamminga et al., 2007). With these input values, one can calculate nutrient availability in the rumen for microbial protein synthesis, nutrient availability in the small intestine for absorption by the animal, and amount of nutrients completely lost to the animal. The recently revised (2007) digestible intestinal protein/rumen-degraded protein balance (**DVE/OEB**) protein system uses a more advanced *in situ* fractionation scheme to fractionate CP and carbohydrates (**CHO**) compared with that of previous protein systems. This revised protein system uses individual CP and CHO fractions to calculate rumen-degradable fractions and rumen microbial protein synthesis (**MCP_r**); previous

protein systems used total rumen available energy to calculate MCP_r (Tamminga et al., 2007). Thus, the new DVE/OEB system provides a more detailed evaluation of feed digestion in the ruminant.

The objectives of this study were to evaluate transgenic winter-hardy anthocyanidin-accumulating T_1 *Lc*-alfalfa populations and the nontransgenic alfalfa variety AC Grazeland for nutrient availability to dairy cattle using modeling. We fractionated protein and energy of these 2 types of germplasm by *in situ* and chemical approaches and evaluated *in situ* ruminal degradation characteristics and synchronization ratios between protein and energy in the rumen. We also calculated protein availability in the rumen and small intestine based on *in situ* input values using the updated 2007 DVE/OEB protein system. Finally, we calculated energy values for lactation based on the Dutch net energy for lactation (**VEM**) system.

MATERIALS AND METHODS

Alfalfa Material

The T_1 transgenic *Lc*-alfalfa populations (Jonker et al., 2010) used in this study were developed at Forage Genetics International (West Salem, WI) by manually crossing 3 T_0 transgenic *Lc*-alfalfa genotypes (*Lc1*, *Lc3*, and *Lc4*; Wang et al., 2006b) with several genotypes selected for winter hardiness from 3 nontransgenic western Canada-adapted alfalfa varieties (Beaver, Rambler, and Rangelander) to generate T_1 Beav*Lc1*, T_1 Ramb*Lc3*, and T_1 Rang*Lc4*, respectively. The T_1 *Lc*-alfalfa populations were compared with alfalfa variety AC Grazeland, which was selected earlier for a low initial rate of degradation and has been shown to reduce bloat in grazing cattle (Coulman et al., 2000). The T_1 *Lc*-alfalfa populations were grown for approximately 6 mo from seed in a greenhouse. Sheared alfalfa plants were then transplanted into rows (75 cm between rows; 15 cm between plants) in the dark brown soil in a Canadian Food Inspection Agency (**CFIA**)-approved experimental field at the Saskatoon Research Centre farm of Agriculture and Agri-Food Canada (AAFC) on August 24, 2006, and June 5, 2007. The AC Grazeland plants were dug up in the first week of June 2008 from a breeder's field at AAFC Indian Head (SK, Canada) and transplanted into the experimental field with T_1 *Lc*-alfalfa populations.

Field-grown alfalfa plants were harvested manually with shears at *ca.* 5 cm above ground level on July 24 and August 13 of 2008 at a vegetative pre-bud stage (according to CFIA-imposed confined field trail regulations). Transgenic T_1 *Lc* plants were sorted visually in the field based on forage color (green and purple-green).

Purple-green ^{11}C -alfalfa and AC Grazeland forage were stored at -20°C pending analysis. Transgenic plants had been analyzed previously for the presence of the *Lc* gene (Jonker et al., 2010).

In Situ Rumen Incubation

For in situ rumen incubations, 3 rumen fistulated, nonpregnant, dry Holstein-Friesian cows were used, which had been reviewed and approved by the CFIA and by the Animal Care Committee of the University of Saskatchewan (animal use protocol # 19910012). Cows were individually housed in pens at the experimental farm of the University of Saskatchewan (Saskatoon, SK, Canada) and were cared for according to the Canadian Council on Animal Care guidelines (CCAC, 1993). The cows had free access to water and were fed 15 kg of DM/d of a TMR twice daily in equal portions at 0800 and 1600 h. The TMR consisted of (% of DM) 55% barley silage, 12.5% alfalfa hay, 5% dehydrated alfalfa, and 27.5% concentrates, as described in Yu et al. (2009).

Prior to in situ incubations, frozen alfalfa samples were freeze-dried and ground to pass through a 2-mm screen using a cyclonic mill (Retsch SM-3000, Brinkmann Instruments, ON, Canada). In situ ruminal degradation kinetics were determined as described by Yu et al. (2004), using number-coded nylon bags (10×20 cm, pore size 40 μm , Nitex 03-41/31 monofilament open mesh fabric, ScreenTech, Mississauga, ON, Canada). Approximately 5 g of freeze-dried alfalfa sample was placed into each bag, resulting in a sample-to-bag surface ratio of approximately 28 mg/cm^2 . Filled bags were incubated in the rumen for 72, 36, 12, 6, and 2 h by the "all-out method." Immediately after retrieval, all bags were placed in a bucket with cold tap water to stop microbial fermentation and then washed 5 times manually in cold tap water, followed by oven drying at 55°C for 48 h.

The 0-h incubation samples were washed by the procedure described by Azarfar et al. (2007a) to fractionate the washable (**W**) fraction into a truly soluble (**S**) washable fraction and a washable insoluble (**WI**) fraction. Briefly, the 0-h bags containing ca. 5 g of sample were placed into 250-mL polypropylene tubes with 20 mL of distilled water per 1 g of alfalfa present in the bag and shaken at 150 rpm. After 1 h, the nylon bags were removed from the tubes and dried at 55°C for 48 h. The remaining washout fraction in the tube was filtered through filter paper (Whatman #54; pore size 20 μm) and the filter paper with residue (**WI**) was dried in an oven for 24 h at 55°C . Because the **WI** residue on the filter paper was less than 0.001 g (data not shown), for the purpose of modeling, it was assumed that the

W fraction equaled the S fraction. Incubation residues from the treatment bags were combined within time per incubation run.

Chemical Analysis

Prior to chemical analysis, original freeze-dried alfalfa samples and dried rumen-incubated residues were ground to pass through a 1-mm screen using a centrifugal mill (Retsch ZM-100, Brinkmann Instruments). Original alfalfa samples and rumen-incubated residues were analyzed for DM, ash, and CP according to AOAC (1990). Crude protein ($\text{N} \times 6.25$) was analyzed by flash combustion using a Leco FP 528 analyzer (Leco Corporation, St. Joseph, MI). The NDF (determined with heat-stable α -amylase for original samples and rumen-incubated residues, and with sodium sulfite for rumen-incubated residues), ADF, and acid detergent lignin (**ADL**) concentrations were determined by the method of Van Soest et al. (1991). The NDF and ADF residues for original samples were corrected for protein-determined neutral detergent-insoluble protein and acid detergent-insoluble protein (**ADIP**) content using the Kjeldahl-N method (Kjeltec 1030 N autotitrator, Foss, Höganäs, Sweden) as described by Licitra et al. (1996). Nonprotein N remaining soluble in trichloroacetic acid and CP soluble in borate/phosphate buffer (**BSP**) were analyzed in original samples by the Kjeldahl-N method (Licitra et al., 1996). Additionally, original samples were analyzed for fat content by ether extraction (**EE**; AOAC, 1990) and for ethanol-soluble carbohydrates according to Hall et al. (1999). Total CHO content was calculated in % of DM as $100 - (\text{ash} + \text{EE} + \text{CP})$ according to the NRC (2001).

Protein and Carbohydrate Profiling

Protein and carbohydrates each were fractionated into soluble fractions, insoluble potentially degradable fractions, and undegradable fractions based on chemical analysis using the Cornell Net Carbohydrate and Protein System (**CNCPS**; Sniffen et al., 1992; Tylutki et al., 2008) and based on in situ incubations described in the 2007 version of the DVE/OEB system (Tamminga et al., 2007; Table 1). Degradation rates (K_d) for the potential degradable (**D**) fraction of CP, NDF, residual nonstarch polysaccharides (**RNSP**), and OM were calculated using an exponential mathematical model as described by Robinson et al. (1986):

$$R(t) = U + D \times \exp^{-K_d(t-lag)},$$

where R is the residue (%) of the incubated sample, U is the undegradable fraction, t is time of rumen incuba-

tion (h), and *lag* is the lag time before degradation initiates. The parameters of the exponential model were calculated using PROC NLIN (nonlinear) from SAS (SAS Institute, 2003) with iterative least squares regression (Gauss-Newton method). Rumen-degradable fractions were calculated as described in Table 2. Rumen degradability was calculated for each in situ fraction (S, WI, and D) as described in Table 2, with values for fractional rate of passage (K_p) and K_d described in Table 1. Hourly rumen degradability was calculated as

$$\text{In situ fraction} \times K_d / (K_d + K_p) \times \left[1 - \exp^{-t(K_d + K_p)} \right]$$

Ratios of N:CHO (g/kg) were calculated for total as well as hourly rumen-degradable N-to-CHO fractions.

Modeling Nutrient Availability

The 2007 DVE/OEB protein system (Tamminga et al. (2007) was used to predict feed protein quality using equations which calculate OEB and DVE (also called metabolizable protein in the CNCPS protein evaluation system; Sniffen et al., 1992; Table 2).

The NE_L of alfalfa was calculated using equations from the VEM energy system described in Table 2 (Van Es, 1978). However, ME was calculated as described on the Dutch Product Board of Animal Feed (PDV) website (PDV, 2005) because the PDV recommended replacing the original ME equation of the VEM energy system described by Van Es (1978) for roughage like alfalfa with this new ME equation (Table 2).

Equations from the CVB (2005; Dutch Central Feed Office, part of PDV) were used to calculate potential fat/protein-corrected milk production (**FPCM**) from NE_L (**FPCM_** NE_L) and DVE (**FPCM_** DVE). Fat/protein-corrected milk is defined as milk with 4% of fat and 3.3% of protein (CVB, 2005). Intake NE_L /DVE was calculated for a 650-kg Holstein-Friesian cow in second or later lactation with a DMI of 15 kg/d grazing on alfalfa pasture (Castillo et al., 2006). This type of cow requires 864 KJ/d (772 KJ/d + 12% of extra energy required for grazing) and 119 g of DVE/d for maintenance (CVB, 2005). Intake NE_L /DVE minus maintenance NE_L /DVE leaves any remaining NE_L /DVE available for milk production, and this available NE_L /DVE can be used to calculate the potential FPCM using the following 2 equations (CVB, 2005):

$$FPCM_NE_L = 440 \times FPCM + 0.73 \times FPCM^2 \text{ and}$$

$$FPCM_DVE = 1.396 \times P + 0.000195 \times P^2,$$

where P is milk protein production per day (g/d).

Statistical Analysis

The effect of alfalfa populations on measured parameters was analyzed using PROC MIXED of SAS 9.2 (SAS Institute, 2003). The statistical model used to analyze the chemical composition of the original sample was

$$Y_{ij} = \mu + T_i + H_j + \varepsilon_{ij},$$

where Y_{ij} is the dependent variable, μ is the general mean, T_i is the fixed effect of treatment ($i = 4$; T1 BeavLc1, T1 RambLc3, T1 RangLc4, and AC Grazeland), H_j is the random effect of harvest date ($j = 2$; July 24 or August 13, 2008), and ε_{ij} is the residual error.

The statistical model used to analyze in situ fractions and degradation and calculated nutrient availability was

$$Y_{ij} = \mu + T_i + R_j + \varepsilon_{ij},$$

where Y_{ij} is the dependent variable, μ is the general mean, T_i is the fixed effect of treatment ($i = 4$; T1 BeavLc1, T1 RambLc3, T1 RangLc4, and AC Grazeland), R_j is the random effect of incubation run ($j = 2$; 1 and 2), and ε_{ij} is the residual error. Differences between in situ and chemical fractions were analyzed by a paired *t*-test using PROC TTEST of SAS 9.2 (SAS Institute, 2003). The Fisher protected LSD test was used for multiple treatment comparisons using the LSMEAN statement of SAS 9.2 (SAS Institute, 2003) with letter groupings obtained using the SAS pdmix800 macro (Saxton, 1998). Contrasts in SAS were used to compare the mean of $^{T1}Lc^{1,3,4}$ -alfalfa (T1 BeavLc1 + T1 RambLc3 + T1 RangLc4) with AC Grazeland. For the different statistical tests, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$, unless otherwise stated.

RESULTS AND DISCUSSION

Anthocyanidin Concentration and Fiber Levels in ^{T1}Lc -Alfalfa and AC Grazeland

The 3 ^{T1}Lc -alfalfa populations harvested in 2008 had an average forage anthocyanidin level of 163.4 μ g/g of DM, whereas AC Grazeland forage harvested from the same field at the same time did not accumulate anthocyanidins at all (Table 3). The anthocyanidin level of these ^{T1}Lc -populations was higher than that previously found for their T_0 Lc -alfalfa parent genotypes harvested in 2001 and 2002 at the same field site (96.9 to 136.4 μ g/g of DM; Wang et al., 2006b), but lower than the anthocyanidin content (232 μ g/g of DM) of the same ^{T1}Lc -populations harvested in 2007 (Jonker et al., 2010).

Table 1. Chemical and in situ fractionation of proteins and carbohydrates, degradation rates, passage rates, and ATP yield

	Chemical analysis ¹		In situ ²		DVE/OEB		
Trait	Chemical fractions	Calculation	In situ fraction	Description	K _d ³ (%/h)	K _p ⁴ (%/h)	ATP yield ⁵ (mol/kg)
Carbohydrate fraction (% of CHO)							
Soluble	CA	CHO – (CB2 +CB3 + CC)	S _{ESC}	80% of ethanol-soluble CHO	2.0	0.11	23.9
Potentially degradable	CB2	RNSP ⁶	WI _{RNSP} ⁶	100 – (0h_ASH + 65% × EE + 0h_CP + NDF + ESC)	2.5 × K _{dRNSP}	0.08	23.9
Undegradable	CB3	NDF – CC	D _{RNSP} ⁶	100 – (WI _{RNSP} + U _{RNSP})	K _{dRNSP}	0.0139 + 0.1775 × K _{dRNSP}	27.3
			D _{NDF} ⁶	100 – (WI _{NDF} + U _{NDF})	K _{dNDF}	0.0139 + 0.1775 × K _{dNDF}	27.3
	CC	(NDF × (ADL/NDF) × 2.4)/CHO × 100	U _{RNSP} ⁶	100 – (72h_ASH + U _{CP} + U _{NDF})	—	—	—
			U _{NDF}	72-h residue	—	—	—
Protein fraction (% of CP)							
Soluble	PA	NPN	S _{CP}	Soluble washable fraction	2.0	0.11	13.6
Potentially degradable	PB	CP – (NPN + ADIP)	WI _{CP}	Washable CP – S _{CP}	K _{dCP}	0.08	13.6
Undegradable	PC	ADIP	D _{CP}	100 – (WI _{CP} + U _{CP})	K _{dCP}	0.06	13.6
			U _{CP}	72-h residue	—	—	—

¹Carbohydrate (CHO) and CP fractions calculated by the Cornell Net Carbohydrate and Protein System (CNCPS) system based on chemical analysis in % of CHO and CP, respectively. CA = soluble CHO; CB2 = potentially degradable soluble fiber; CB3 = potentially degradable NDF; CC = undegradable CHO; PA = soluble CP; PB = potentially degradable CP; PC = undegradable CP (Sniffen et al., 1992). ADL = acid detergent lignin; ADIP = acid detergent-insoluble protein.

²In situ CHO and CP fractions used in the intestinal digestible protein/rumen-degraded protein balance (DVE/OEB) system in % of CHO and CP, respectively. S = truly soluble washable fraction; WI = washable insoluble fraction; D = potentially degradable fraction; U = completely undegraded fraction; EE = ether extraction. Subscript letters: ESC = CHO soluble in 80% of ethanol; RNSP = residual nonstarch polysaccharides (Tamminga et al., 2007).

³K_d, the fractional degradation rate of the D fractions, was calculated by the exponential model of Robinson et al. (1986), whereas K_d values for the S and WI fractions were adapted from Tamminga et al. (2007).

⁴K_p is the fractional passage rate; values and equations were adapted from Tamminga et al. (2007).

⁵Energy content (ATP) of each in situ fraction was adapted from Tamminga et al. (2007).

⁶RNSP content was calculated as 100 – (ash + EE + CP + NDF + ESC). Correction factors 65, 44, 17, and 3% of the original EE, as described in Tamminga et al. (2007), were applied to calculate RNSP for the 0-, 2-, 6-, and 12-h incubation residues, respectively.

Table 2. Protein and energy system equations to calculate intestinal digestible protein (DVE), rumen-degraded protein balance (OEB), and NE_L

Trait	Calculation
Protein system ruminal phase ¹ (g/kg of DM)	
OEB	$MCP^{RDP} - MCP^{FOMr}$
Rumen-degradable CP (RD _{CP})	$[S_{CP} \times K_{ds}/(K_{ds} + K_{ps})] + [D_{CP} \times K_{pd}/(K_{pd} + K_{ps})]^2$
Rumen-undegraded CP (RU _{CP})	$CP - RD_{CP}$
Microbial CP from RD _{CP} (MCP _r ^{RDP})	RD_{CP}
Rumen-fermentable OM (FOMr)	$\Sigma K_d/(K_d + K_p) \times \text{in situ fractions}$
Microbial CP from FOMr (MCP _r ^{FOMr})	$1/Y = (M/GR + 1/Y_{max})^3$
Protein system intestinal phase ¹ (g/kg of DM)	
DVE	$DMCP + DRUP - DMFP$
Intestinal available MCP (DMCP)	$0.85 \times 0.75 \times MCP^{FOMr 4}$
Intestinal available RUP (DRUP)	$RU_{CP} - U_{CP}$
Endogenous fecal CP losses (DMFP)	$0.075 \times [DM - (tDOM + DASH)]^5$
Total digestible ash (DASH)	$ASH \times 0.65$
Total digestible OM (tDOM)	$OM - U_{OM}$
Energy system (KJ/kg of DM)	
Gross energy (GE)	Analyzed by Bomb calorimetry
ME ⁶	$14.94 \times tDOM + 18.98 \times EE - 1.478 \times NDF - 0.97 \times ESC$
Metabolizability ⁷ (q)	$100 \times ME/GE$
Net energy for lactation ⁷ (NE _L)	$0.6 \times [1 + 0.004 \times (q - 57)] \times 0.9752 \times ME$

¹Parameters were calculated according to Tamminga et al. (2007).

²S_{CP} = truly soluble washable CP (g/kg of DM); D_{CP} = potentially degradable CP (g/kg of DM); K_d = fractional degradation rate (%/h); K_p = fractional passage rate (%/h).

³Y = microbial DM yield; M = microbial maintenance requirements (1.365 mmol of ATP/g bacteria per h for the D fractions, 4.095 mmol of ATP/g of bacteria per h for the S and WI fractions); GR = fractional growth rate (affected by ATP yield of specific in situ fractions, K_p, and M for specific in situ fractions); Y_{max} = maximum microbial growth yield (assumed to be 32 g of microbes/mol of ATP; microbes contain 62.5% of CP); a correction of 0.20 is applied to adjust the final microbial protein mass for predation by protozoa (Pirt 1965).

⁴The factor 0.85 represents the true protein content of MCP_r and the factor 0.75 represents the digestibility of MCP in the small intestine (Tamminga et al., 1994).

⁵The factor 0.075 assumes that 75 g of protein is lost in the feces per 1 kg of undigested DM that passes through the digestive tract (Tamminga et al., 1994).

⁶Metabolizable energy equation adapted from the PDV (2005) website. EE = ether extraction; ESC = carbohydrate soluble in 80% of ethanol.

⁷Equations adapted from Van Es (1978).

Fiber fraction concentrations, NDF, ADF, and ADL tended ($P < 0.10$) to be higher in AC Grazeland compared with their concentrations in the 3 ^{T1}Lc^{1,3,4}-alfalfa populations (59.1 vs. 54.3, 53.0 vs. 47.7, and 14.6 vs. 11.0% of CHO, respectively; Table 3). Tremblay et al. (2000) screened 27 alfalfa varieties over 2 consecutive years and found that AC Grazeland always had a higher fiber (NDF, ADF) content compared with that of all other varieties tested. This higher fiber content of AC Grazeland likely resulted from its reduced leaf-to-stem ratio and thicker leaf cell walls (Coulman et al., 1998, 2000).

Comparison of In Situ and Chemical Protein Fractionations of ^{T1}Lc-Alfalfa and AC Grazeland

The S protein fractions in both CNCPS and DVE/OEB systems are presumed to have a fractional degradation rate of 200%/h. Nonprotein N (PA) was similar in all 3 ^{T1}Lc-alfalfa populations and AC Grazeland (average of 62.9% of CP; Table 3), as were the soluble CP (S_{CP}) fractions and the fractional degradation rates (K_{dCP}) of potentially degradable CP (D_{CP}; Table 4).

However, the S_{CP} fraction of ^{T1}RamLc3 was numerically lower (approximately 6.7%) and the D_{CP} and K_{dCP} of the same population were numerically higher (approximately 8.1% and approximately 3.1%/h, respectively) compared with those of the other 3 populations.

The S_{CP} mean of 34.9% of CP for all alfalfas used in this study was similar to values reported by Aufrère et al. (2000) and Elizalde et al. (1999b) for alfalfa harvested at a vegetative stage of growth. However, the NPN value for PA averaged approximately 28% of CP higher ($P < 0.0001$) than did the in situ S_{CP} value. The S_{CP} was determined after solubilizing the alfalfa sample in water for 4 h at room temperature. In contrast, PA was determined after solubilizing the sample in water with TCA for 30 min, after which the pH was adjusted to 2 and the solution incubated over night at room temperature. Because TCA precipitates peptides larger than 10 AA units long (Licitra et al., 1996), amino acids and small peptides would be present as contaminants in the PA fraction and could explain the high PA value.

Overall, BSP was higher ($P < 0.04$) in ^{T1}Lc^{1,3,4}-alfalfa forage compared with that in AC Grazeland forage (72.6 vs. 70.7% of CP; Table 3). When individual popu-

Table 3. Phytochemical and chemical composition for ^{T1}Lc-alfalfa and AC Grazeland (AG)

Trait	Population				Model		Lc vs. AG ¹
	^{T1} BeavLc1	^{T1} RambLc3	^{T1} RangLc4	AG	SEM	P-value	P-value
Anthocyanidin (μg/g of DM)	175.3	135.7	179.2	0.0	58.85	0.113	0.034
DM (%)	17.6	18.3	18.2	18.4	1.04	0.345	0.375
Ash (% of DM)	12.9	12.4	13.6	13.7	1.10	0.084	0.108
EE ² (% of DM)	4.0	3.4	3.6	3.6	0.17	0.186	0.723
CP (% of DM)	28.7	28.2	29.1	27.3	2.22	0.313	0.130
Protein subfractions ³							
NPN (% of CP; PA)	64.0	66.4	59.8	61.5	1.90	0.289	0.512
BSP (% of CP)	73.3	73.0	71.4	70.7	0.46	0.064	0.037
PB (% of CP)	34.7	31.9	38.5	36.7	2.11	0.290	0.544
NDIP (% of CP)	4.1	3.8	3.9	4.2	1.37	0.817	0.518
ADIP (% of CP; PC)	1.7	1.6	1.7	1.8	0.69	0.684	0.311
CHO ⁴ (% of DM)	54.5	55.0	54.5	55.45	1.05	0.506	0.308
Carbohydrate subfractions ⁵							
ESC (% of CHO; CA4)	7.4	5.8	7.0	6.1	2.00	0.662	0.582
RNSP (% of CHO; CB2)	38.3	39.8	39.0	34.9	5.54	0.302	0.105
NDF (% of CHO)	54.3	54.4	54.0	59.1	7.32	0.276	0.086
ADF (% of CHO)	49.4	46.7	46.9	53.0	7.20	0.174	0.064
ADL (% of CHO)	10.9	10.5	11.9	14.6	2.34	0.239	0.077
N:CHO (g/kg)	84.6	82.2	87.0	78.9	8.60	0.392	0.189

¹Lc vs. AC Grazeland (AG) = comparing the mean of 3 transgenic ^{T1}Lc-alfalfa populations (^{T1}BeavLc1, ^{T1}RambLc3, ^{T1}RangLc4) with variety AC Grazeland (selected for a low initial rate of degradation).

²Ether extraction.

³NPN = nitrogenous compounds soluble in water and not precipitated by TCA, called PA in the Cornell Net Carbohydrate and Protein System (CNCPS); BSP = CP soluble in phosphate buffer; PB = true potentially degradable protein calculated in % of DM as CP – (NPN + ADIP); NDIP = neutral detergent-insoluble CP; ADIP = acid detergent-insoluble CP, called PC in CNCPS.

⁴CHO = total carbohydrate content calculated as DM – (ash + EE + CP).

⁵ESC = CHO soluble in 80% of ethanol, called CA4 in CNCPS; RNSP, residual nonstarch polysaccharides calculated as CHO – (NDF + ESC), called CB2 in CNCPS; ADL = acid detergent lignin.

lations were considered, a trend toward highest BSP ($P = 0.06$) was observed for ^{T1}BeavLc1 and lowest for AC Grazeland (Table 3). The mean BSP concentration for all 4 alfalfa populations was higher than values reported in other studies, in which values ranged from 38.4 to 41.1% of CP (Elizalde et al., 1999b; Grabber, 2009). Previously, in Jonker et al. (2010), we proposed that the high BSP in our alfalfa populations might result from harvesting young plant material rather than more mature material due to a ban on flowering imposed by the CFIA. However, mean in situ S_{CP} values were similar to other values reported for alfalfa (Elizalde et al., 1999b; Aufrère et al., 2000). Elizalde et al. (1999b) found only slightly higher soluble protein in BSP than in washable CP (W_{CP}) from in situ analysis (38.9 vs. 36.6% of CP) in alfalfa harvested at several stages of growth. Therefore, BSP gave a reliable estimate of the in situ washable W_{CP} fraction in the study of Elizalde et al. (1999b). Thus, BSP gives variable results when compared with in situ W_{CP}.

All 4 alfalfas had similar completely undegradable ADIP fractions with an average of 1.7% of CP (Table 3). The ADIP values were, on average, 7.4% of CP lower ($P < 0.0001$) than the values for undegradable CP (U_{CP}) with the in situ method. Moreover, U_{CP} values

were lower ($P < 0.05$) in ^{T1}RambLc3 and ^{T1}RangLc4 compared with those in AC Grazeland, whereas U_{CP} values in ^{T1}BeavLc1 did not differ from those in the other 3 alfalfa populations (Table 4). The higher U_{CP} compared with ADIP may be caused by contamination of the 72-h in situ residue with microbial protein or more protein was solubilized during ADF determination of the original alfalfa samples than during the 72-h in situ rumen incubations.

The ADIP values for all alfalfa in our study were lower than previously reported ADIP values for alfalfa harvested at a vegetative stage of growth (2.1 to 4.6% of CP), whereas our in situ undegradable U_{CP} fractions were in a similar range (5.6 to 15.6% of CP) as those previously reported (Elizalde et al., 1999b; González et al., 2001; Faría-Mármol et al., 2002; Wang et al., 2006b). Moreover, ADIP values were 1.6 to 10.5% lower in these studies compared with in situ U_{CP} values (Elizalde et al., 1999b; González et al., 2001; Faría-Mármol et al., 2002). Therefore, our results confirm those of other studies in which ADIP was lower than the in situ undegradable U_{CP} fraction. This implies that ADIP might not be a reliable predictor for undegradable U_{CP} in alfalfa as stated previously by Pichard and Van Soest (1977) and Haugen et al. (2006).

Table 4. In situ protein and energy fractions and ruminal degradation for ^{T1}Lc-alfalfa and AC Grazeland (AG)

Trait ¹	Population				Model		Lc vs. AG ²
	^{T1} BeavLc1	^{T1} RambLc3	^{T1} RangeLc4	AG	SEM	P-value	P-value
Total CP (% of DM)	28.7	28.22	29.10	27.33	2.215	0.313	0.130
In situ protein fractions and degradation							
S _{CP} (% of CP)	38.9	28.6	36.6	35.3	2.46	0.107	0.806
D _{CP} (% of CP)	52.0	63.0	54.6	54.9	2.57	0.097	0.536
K _{dCP} (%/h)	19.3	23.0	18.1	19.9	4.07	0.850	0.970
lag _{CP} (h)	0.52	0.03	0.00	0.00	0.26	0.500	0.582
U _{CP} (% of CP)	9.1 ^{ab}	8.5 ^b	8.8 ^b	9.8 ^a	0.22	0.048	0.016
Total CHO (% of DM)	54.5	55.0	54.5	55.5	1.05	0.506	0.308
In situ carbohydrate fractions and degradation							
S _{ESC} (% of CHO)	7.4	5.8	7.0	6.1	—	—	—
W _{IRNSP} (% of CHO)	13.2	12.8	15.1	12.1	3.00	0.870	0.643
D _{RNSP} (% of CHO)	22.4	24.9	22.3	20.4	2.88	0.711	0.429
K _{dRNSP} (%/h)	23.4	26.2	13.8	16.8	4.83	0.361	0.484
lag _{RNSP} (h)	1.07	1.16	0.00	0.62	0.66	0.626	0.876
D _{NDF} (% of CHO)	27.9	30.1	28.0	30.0	0.49	0.053	0.082
K _{dNDF} (%/h)	10.6	8.1	7.9	9.1	2.02	0.783	0.923
lag _{NDF} (h)	1.16	0.00	0.04	0.74	0.69	0.521	0.652
U _{RNSP} (% of CHO)	2.6	2.0	1.4	2.4	0.30	0.146	0.294
U _{NDF} (% of CHO)	26.7 ^b	24.4 ^c	26.3 ^b	29.1 ^a	0.49	0.011	0.004
Total OM (% of DM)	87.1	87.6	86.4	86.3	1.10	0.084	0.108
In situ organic matter fractions and degradation							
W _{OM} (% of OM)	23.8	21.5	24.5	23.2	2.93	0.763	0.997
D _{OM} (% of OM)	54.6	58.9	55.2	53.2	2.99	0.396	0.289
K _{dOM} (%/h)	17.0	17.0	13.4	13.8	2.77	0.702	0.572
lag _{OM} (h)	0.83	0.00	0.25	0.00	0.43	0.500	0.485
U _{OM} (% of OM)	21.6 ^b	19.7 ^c	20.3 ^c	23.6 ^a	0.21	<0.001	<0.001

^{a-c}Means with different superscripts within the same row differ ($P < 0.05$).

¹S = washable soluble fraction; W = washable fraction; WI = washable insoluble fraction; D = potentially degradable fraction calculated as $100 - (S + WI + U)$; U = undegradable fraction (72-h incubation residue); CHO = total carbohydrate content; K_d = fractional degradation rate; lag = lag time before fermentation initiated. Subscript letters: ESC = CHO soluble in 80% of ethanol; RNSP = residual nonstarch polysaccharides. K_d and lag were calculated by the exponential model of Robinson et al. (1986).

²Lc vs. AC Grazeland (AG) = comparing the mean of 3 transgenic ^{T1}Lc-alfalfa populations (^{T1}BeavLc1, ^{T1}RambLc3, ^{T1}RangeLc4) with variety AC Grazeland (selected for a low initial rate of degradation).

All 4 alfalfa populations in this study had similar potentially degradable true protein (PB) values calculated by the CNCPS system (average 35.5% of CP; Table 3). The potentially degradable CP fraction D_{CP} measured by the in situ method averaged 56.1% of CP and was similar for all alfalfas in this study, but a trend was observed for higher D_{CP} ($P < 0.10$) in ^{T1}RambLc3 compared with ^{T1}RangeLc4 (Table 4). The D_{CP} values in our study were similar to D_{CP} values reported by Aufrère et al. (2000) and Elizalde et al. (1999b) for vegetative alfalfa. The K_{dCP} for the D_{CP} fractions reported in other studies had a fairly large range (12.2 to 34.1%/h) for alfalfa at several stages of growth, whereas the K_{dCP} for vegetative alfalfa was higher (ranging from 17.9 to 34.1%/h) compared with that for alfalfa forage at more mature stages of growth (Elizalde et al., 1999b; Aufrère et al., 2000; González et al., 2001). The potentially degradable CP (PB) fraction calculated by the CNCPS method was 20.6% lower ($P < 0.0001$) than the in situ D_{CP} fraction by the DVE/OEB⁰⁷ system. Potentially degradable PB in our alfalfa germplasm was low, mainly as a result of a high NPN

fraction and only a slightly lower ADIP compared with alfalfa tested in other studies where they found higher PB values (68.7 and 71.7% of CP, respectively) for alfalfa (Elizalde et al., 1999a; Grabber, 2009). Yu et al. (2003a,b) found PB values (40.7 to 55.5% of CP) in a similar range to in situ D_{CP} values (43.6 to 52.6% of CP) for alfalfa harvested at different stages of growth. So, in the study of Yu et al. (2003a,b), PB gave a good estimate of D_{CP}.

Ruminal Protein Availability of ^{T1}Lc-Alfalfa and AC Grazeland

The DM weight of the alfalfa in situ WI protein fractions was <0.001% of DM for all 4 of our alfalfas (data not shown). Azarfar et al. (2007b) and Gierus et al. (2005) found that the WI protein fractions for different concentrates and wet byproduct ingredients lay within a range from 1.9 to 29.3% of CP, but was minimal (below 1.0% of CP) for grass silage. The trace levels of WI protein in our trials suggests that freeze-dried alfalfa, similar to grass silage, contains very few small particles

Table 5. Modeling ruminal and intestinal nutrient availability for T1 Lc-alfalfa and AC Grazeland (AG)

Trait ¹	Population				Model		Lc vs. AG ²
	T1 BeavLc1	T1 RambLc3	T1 RangLc4	AG	SEM	P-value	P-value
Ruminal phase							
RD _{CP} (g/kg of DM)	226.0	223.9	227.0	213.8	4.73	0.313	0.095
RD _{CHO} (g/kg of DM)	313.8	325.0	305.7	302.3	5.40	0.130	0.116
RD _{OM} (g/kg of DM)	579.9	586.6	565.6	546.8	16.05	0.416	0.175
FOMr (g/kg of DM)	539.8	548.9	532.7	516.1	9.98	0.267	0.102
MCP ^{rFOMr} (g/kg of DM)	84.5	87.3	77.6	78.1	4.71	0.470	0.412
RD _N /RD _{CHO} (g/kg)	115.2 ^b	110.2 ^c	118.8 ^a	113.1 ^{bc}	0.858	0.009	0.175
OEB (g/kg)	141.5	136.6	149.4	135.6	2.72	0.069	0.093
Intestinal phase							
U _{CP} (g/kg of DM)	26.2	23.9	25.5	26.8	0.62	0.104	0.086
U _{DM} (g/kg of DM)	233.5 ^b	217.4 ^c	223.1 ^c	251.5 ^a	1.78	<0.001	<0.001
DMFP (g/kg of DM)	17.5 ^b	16.3 ^c	16.7 ^c	18.9 ^a	0.14	<0.001	<0.001
RU _{CP} (g/kg of DM)	60.7	58.2	64.1	59.5	4.73	0.837	0.791
DRUP (g/kg of DM)	34.5	34.3	38.6	32.7	4.72	0.836	0.594
DMCP (g/kg of DM)	53.8	55.7	49.5	49.8	3.01	0.471	0.412
DVE (g/kg of DM)	70.9	73.7	71.3	63.6	3.21	0.169	0.054

^{a-c}Means with different superscripts within the same row differ ($P < 0.05$).

¹RD_{CP} = rumen-degradable protein; RD_{OM} = rumen-degradable OM; RD_{CHO} = rumen-degradable carbohydrates; RD_N = rumen-degradable N; FOMr = rumen-fermentable OM; MCP^{rFOMr} = ruminal microbial protein synthesis from FOMr; OEB = rumen-degraded protein balance; U_{CP} = completely undegradable protein; U_{DM} = completely undegradable DM; DMFP = endogenous protein lost into the feces; RU_{CP} = rumen-undegradable protein; DRUP = intestinal available RU_{CP}; DMCP = intestinal available microbial protein; DVE = intestinal digestible protein.

²Lc vs. AC Grazeland (AG) = comparing the mean of 3 transgenic T1 Lc-alfalfa populations (T1 BeavLc1, T1 RambLc3, T1 RangLc4) with variety AC Grazeland (selected for a low initial rate of degradation).

that could escape through the pores of the nylon bag during washing.

The rumen-degradable CP (RD_{CP}) content was similar for each of the 4 alfalfa populations, but a trend toward a higher RD_{CP} was found when the mean of T1 Lc^{1,3,4}-alfalfa ($P < 0.10$) was compared with that of AC Grazeland (22.6 vs. 21.4% of DM; Table 5). The mean RD_{CP} for all alfalfa populations was, on average, 79.3% of CP. This was within the range of 71.2 to 89.6% of CP reported previously for alfalfa harvested at a vegetative stage of growth (Elizalde et al., 1999b; Aufrère et al., 2000; González et al., 2001; Faría-Mármol et al., 2002). Because ADL and other fiber fractions tended to be higher ($P < 0.10$) in AC Grazeland than in T1 Lc^{1,3,4}-alfalfa, and increased ADL decreases the digestibility of forages (Jung et al., 1997), the enhanced fiber fractions in AC Grazeland may have had a greater effect on RD_{CP} than the anthocyanidin levels in T1 Lc^{1,3,4}-alfalfa forage have on protein precipitation.

Comparison of In Situ and Chemical Carbohydrate Fractions of T1 Lc-Alfalfa and AC Grazeland

All in situ-derived CHO fractional degradation rates, lag times, and fractions, except for undegradable fiber (U_{NDF}), were similar ($P > 0.05$) between our 4 alfalfas tested (Table 4). The U_{NDF} was higher ($P < 0.04$) for AC Grazeland compared with that in T1 Lc^{1,3,4}-alfalfa (29.1 vs. 25.8% of CHO). When individual T1 Lc

populations were assessed, U_{NDF} was lower ($P < 0.01$) for T1 RambLc3 compared with that for T1 BeavLc1 and T1 RangLc4. In contrast, T1 RambLc3 and AC Grazeland trended to have higher ($P < 0.10$) in situ potentially degradable fiber (D_{NDF}) compared with that in T1 BeavLc1 and T1 RangLc4 (Table 4). When the CNCPS system was compared, undegradable fiber (CC) values for all 4 alfalfa populations were consistent with the combined values for undegradable in situ U_{NDF} + undegradable in situ RNSP (U_{RNSP}) fractions from the DVE/OEB system (Table 4). Whereas the trend in AC Grazeland toward higher ADL content ($P < 0.08$; Table 3) may have had a negative effect on NDF digestibility (Jung et al., 1997), differences between the U_{NDF} and D_{NDF} for T1 RambLc3 and the other 2 T1 Lc-alfalfa populations may have resulted from the numerically lower anthocyanidin content in T1 RambLc3 (Table 3).

In the CNCPS system, a fractional degradation rate of 8%/h is assumed for potentially degradable NDF (CB3) of alfalfa pasture (Tylutki et al., 2008). In our study, the in situ fractional degradation rate of D_{NDF} for all 4 alfalfa populations was similar with an average of 8.9%/h. Our in situ results were consistent with the value assumed by the CNCPS system, but higher than the range of 4.7 to 7.26%/h reported by Yu et al. (2004) for alfalfa harvested at several stages of growth.

In situ-derived potentially degradable RNSP (D_{RNSP}) fractions were similar between the alfalfa populations (Table 4) and were consistent with values reported by

Yu et al. (2004) for alfalfa. All 4 alfalfa populations had similar RNSP (CB2) fractions ($P > 0.05$), but RNSP was numerically higher for individual ^{T1}Lc-alfalfa populations compared with that in AC Grazeland (Table 3). The fractional degradation rate of RNSP (K_{dRNSP}) for alfalfa forage averaged 20.1%/h and was highly variable between the populations. This K_{dRNSP} in our study was 2-fold higher than the range (7.83 to 9.48%/h) reported for alfalfa forage by Yu et al. (2004), but 1.7-fold lower than the 35%/h value assumed for alfalfa CB2 fractions in the CNCPS system (Lanzas et al., 2007). Moreover, the CNCPS system has only one RNSP fraction, CB2, whereas in situ methods fractionate RNSP into a washable insoluble fraction (WI_{RNSP}) and potentially degradable and undegradable fractions. Because the WI_{RNSP} fraction of alfalfa forage is assumed to have a K_d of 250%/h, this would substantially increase the K_d of the total RNSP fraction (Tamminga et al., 2007), if the WI_{RNSP} fraction formed a major part of the RNSP.

Modeling Ruminal Energy Availability of ^{T1}Lc-Alfalfa and AC Grazeland

Rumen degradable OM (RD_{OM}) can be used to predict fermentable OM (FOMr). Using this method, in situ washable OM (W_{OM}), potentially degradable OM (D_{OM}), fractional degradation rate of OM (K_{dOM}), and lag time before the initiation of D_{OM} fermentation were similar between our 4 alfalfa populations (Table 4) and resulted in a similar RD_{OM} (Table 5). However, the undegradable OM (U_{OM}) mean was higher ($P < 0.001$) in AC Grazeland compared with the mean in ^{T1}Lc^{1,3,4}-alfalfa, and U_{OM} within the 3 ^{T1}Lc populations was higher ($P < 0.001$) in ^{T1}BeavLc4 compared with that in ^{T1}RambLc3 and ^{T1}RangeLc4 (Table 4). The mean RD_{OM} content of all 4 populations was 58.4% of DM. This was slightly lower than the range (63.2 to 72.0% of DM) reported by Yu et al. (2003a) for alfalfa. The higher RD_{OM} values for alfalfa reported by Yu et al. (2003a) were likely the result of a higher W_{OM} fraction, a lower U_{OM} fraction, and a lower passage rate (4.0 vs. 4.5%/h in our study) chosen by Yu et al. (2003a) for the calculation of RD_{OM} . The larger W_{OM} fraction most likely is due to a larger nylon bag pore size used (53 μ m), which would allow more insoluble feed particles to escape from the bag and would overestimate the W_{OM} fraction.

In the 2007 DVE/OEB system, FOMr is calculated as the sum of all rumen-degradable fractions, which was similar for all 4 alfalfa populations with a numerical trend toward lower FOMr in AC Grazeland and a mean FOMr of 53.4% of DM (Table 5). The FOMr was 3.5% lower ($P < 0.001$) than the RD_{OM} (Table 5).

To calculate RD_{OM} , it is assumed that the washable (S and WI) in situ fractions are completely degraded in the rumen, whereas the calculation FOMr assumes that a portion of the washable in situ fraction escapes ruminal degradation. Because the W_{OM} fraction used in the calculation of RD_{OM} was approximately 3.0% higher than the sum of the washable in situ fractions ($S_{CP} + WI_{RNSP}$) used for the calculation of FOMr, ruminal escape of W cannot fully explain the difference between FOMr and RD_{OM} (Table 5). Unlike FOMr, the calculation for RD_{OM} does not consider that microbes cannot use EE, which was, on average, 3.7% of DM (Table 3).

Ruminal Protein:Energy Ratios and Synchronization of ^{T1}Lc-Alfalfa and AC Grazeland

The total rumen-degradable $RD_N:RD_{CHO}$ ratio was lower for ^{T1}RambLc3 compared with that of ^{T1}BeavLc1 ($P < 0.01$), which in turn, had a lower $RD_N:RD_{CHO}$ ratio than did ^{T1}RangeLc4, which had the highest ratio ($P < 0.01$; Table 5). A trend existed toward ^{T1}RambLc3 and AC Grazeland having a lower ($P < 0.10$) OEB (balance between MCPr calculated based on protein and energy) compared with that of ^{T1}RangeLc4 and ^{T1}BeavLc1 (Table 5). The $RD_N:RD_{CHO}$ ratios in this study ranged from 110 to 119 g/kg and were 3.5-fold higher than the 32 g/kg required for optimal microbial protein synthesis (Tamminga et al., 1990; Sinclair et al., 1991) and the OEB in this study ranged from 136 to 149 g/kg, which is much higher than the optimum of zero required for efficient utilization of ruminal N (Tamminga et al., 1994, 2007).

When our 3 ^{T1}Lc-alfalfa populations and AC Grazeland were evaluated for hourly $RD_N:RD_{CHO}$, a biphasic profile was observed for all 4 types of germplasm and included an initial and substantial peak (oversupply) of N relative to energy within the first 2 h after feeding, followed by a slow decline from 2 to 24 h after feeding (Figure 1A). This latter time period shows a relatively synchronized and balanced ratio when the hourly OEB method is evaluated (Figure 1B). The OEB after 1 h was least synchronized ($P < 0.01$) for ^{T1}BeavLc1 and ^{T1}RangeLc4, followed by AC Grazeland and ^{T1}RambLc3, after which the OEB between 2 to 24 h was similar for all 4 alfalfa populations. The more synchronized OEB for ^{T1}RambLc3 was likely due to a numerically lower highly degradable S_{CP} fraction compared with that in the other 3 populations (the S_{CP} fraction is almost completely gone after 1 h). The hourly rumen-degradable $RD_N:RD_{CHO}$ ratio of perennial ryegrass, which is characterized by a high soluble protein content and high initial rate of degradation as alfalfa, follows a similar

Figure 1. Degradation ratios of alfalfa populations T^1 BeavLc1 (---), T^1 RambLc3 (.....), T^1 RangLc4 (— · —), and AC Grazeland (—). A) Hourly ratio of rumen-degradable N-to-carbohydrate ratio (N:CHO); B) hourly rumen-degraded protein balance (OEB); C) cumulative rumen-degradable N:CHO; D) cumulative OEB.

pattern (Tas et al., 2006), but the magnitude of imbalance for alfalfa was much higher than for perennial ryegrass.

Excessive protein supplied, above microbial requirements, from alfalfa within the first 2 h of ruminant ingestion will mainly be deaminated into NH_3 by rumen microbes, then absorbed by the rumen wall and converted into urea in the liver at the cost of energy (van Duinkerken et al., 2005). Urea-N can re-enter the rumen via secreted saliva or direct diffusion into the rumen, where it is converted back into NH_3 . When sufficient energy is available, NH_3 can be used for microbial protein synthesis; otherwise, NH_3 will be mainly lost to the animal and excreted via urea in the urine (Lapierre and Lobley, 2001). Valkeners et al. (2004) found that an unsynchronized N-to-energy supply during parts of the day can be balanced by urea-N recycling when the overall N-to-energy supply is balanced on a daily basis.

However, the cumulative protein and energy synchronization shown in Figure 1C and D suggests that urea recycling will be ineffective for the 4 alfalfa populations, with AC Grazeland and T^1 RambLc3 being more effective than T^1 BeavLc1 and T^1 RangLc4. These data are consistent with other research showing that alfalfa requires decreased ruminal protein release or additional ruminal energy inputs (Yu et al., 2003a). One should also note that cows graze in 3 main grazing bouts per day (Gibb et al., 1998; Taweel et al., 2004). Therefore, in a 24-h period, the excessively high protein supply within the first 2 h after ingestion will occur at least 3 times per day.

Modeling Intestinally Available Protein of T^1 Lc-Alfalfa and AC Grazeland

The predicted microbial protein synthesis in the rumen (MCP_r) and, therefore, predicted intestinally avail-

Figure 2. Energy content of alfalfa populations ^{T1}BeavLc1, ^{T1}RambLc3, ^{T1}RangLc4, and AC Grazeland (AG) as calculated according to Van Es (1978). GE = gross energy (solid bars); tDOM = total-tract digestible OM (striped bars); NE_L = net energy for lactation (dotted bars). Bars with different letters (a–c) within the same component differ ($P < 0.05$).

able MCP (DMCP) was similar between the 4 alfalfa populations (Table 5). Because rumen undegradable CP (RU_{CP}) was similar between the 4 alfalfa populations, predicted intestinally available RU_{CP} (DRUP) was also similar between the 4 alfalfa populations (Table 5). Completely undegradable DM (U_{DM}) and predicted endogenous protein loss in the feces (DMFP) each were higher ($P < 0.001$) for AC Grazeland compared with the mean of the 3 transgenic ^{T1}Lc^{1,3,4}-alfalfa populations (Table 5). When individual populations were considered, both U_{DM} and predicted DMFP were higher ($P < 0.001$) for ^{T1}BeavLc1 than for ^{T1}RambLc3 and ^{T1}RangLc4. A trend existed toward a higher predicted intestinally available protein DVE ($P < 0.10$) when the mean of ^{T1}Lc^{1,3,4}-alfalfa was compared with that of AC Grazeland (7.2 vs. 6.4% of DM). The calculated parameters DMCP, DRUP, DMFP, and DVE in our study were similar to values from alfalfa reported by Yu et al. (2003a).

Total Tract Energy Availability of ^{T1}Lc-Alfalfa and AC Grazeland

Gross energy was similar between the 4 alfalfa populations and total digestible OM (tDOM) was highest for ^{T1}RambLc3 and ^{T1}RangLc4, followed by ^{T1}BeavLc1, and lowest for AC Grazeland ($P < 0.001$; Figure 2). The higher tDOM resulted in a higher ($P < 0.001$) predicted NE_L for the mean of the 3 ^{T1}Lc^{1,3,4}-alfalfa

populations compared with that of AC Grazeland (6.2 vs. 5.8 MJ/kg). These predicted NE_L values were much higher than the NE_L value of 5.1 MJ/kg reported in the CVB (2005) feed tables for fresh alfalfa. However, the feed tables do not describe the stage of growth at alfalfa harvest.

Calculated Milk Production of ^{T1}Lc-Alfalfa and AC Grazeland

The higher predicted NE_L for the 3 ^{T1}Lc^{1,3,4}-alfalfa populations (Figure 2) resulted in a higher calculated FPCM based on NE_L (FPCM_{NE_L}) for these populations compared with that for AC Grazeland (16.5 vs. 14.9 kg; Figure 3). Because of the trend toward higher predicted DVE, when the mean of the 3 ^{T1}Lc^{1,3,4}-alfalfa populations was compared with that of AC Grazeland, a trend toward higher ($P < 0.10$) calculated FPCM based on DVE (FPCM_{DVE}) also was observed in ^{T1}Lc^{1,3,4}-alfalfa (19.1 vs. 16.7 kg/d). The FPCM calculated based on NE_L was, on average, 2.4 kg/d lower than the FPCM based on DVE. This means that for a 650-kg Holstein-Friesian cow with a DMI of 15 kg/d, NE_L intake would be the first limiting factor for milk production using the alfalfas in this study.

Castillo et al. (2006) did a survey on 8 dairy farms that graze their dairy cattle on alfalfa pasture. They found an average milk production of 15.1 kg of milk for dairy cows with an average DMI of 15 kg/d (Castillo

Figure 3. Fat/protein-corrected milk production (FPCM) from NE_L and intestinal digestible protein (DVE) calculated according to CVB (2005) for a Holstein-Friesian cow of 650 kg with a DMI of 15 kg/d from alfalfa populations T¹BeavLc1 (solid bars), T¹RambLc3 (diagonally striped bars), T¹RambLc4 (dotted bars), AC Grazeland (open bars), and the mean of the 3 T¹Lc^{1,3,4}-alfalfas (horizontally striped bars). Bars of FPCM-NE_L with different letters (a, b) differ ($P < 0.05$); bars of the mean T¹Lc^{1,3,4}-alfalfa and AC Grazeland for FPCM-DVE differ ($P < 0.10$).

et al., 2006). Their in vivo results are similar to the calculated results we found using modeling methods.

Application

The trend toward a higher predicted DVE and a higher predicted NE_L in the 3 T¹Lc-alfalfa populations resulted in a higher calculated milk production compared with that of AC Grazeland. However, differences between the T¹Lc-alfalfa and AC Grazeland were modest and were not the result of a shift in the site of nutrient degradation. Therefore, more substantial modifications to increase mono/polymeric anthocyanidin accumulation over the levels present in T¹Lc-alfalfa are needed to develop an alfalfa variety with superior nutritional characteristics compared with currently available alfalfa varieties. Such modifications could include the development of Lc-alfalfa plants with multiple copies of the Lc-transgene or introduction of additional regulatory genes that promote mono/polymeric anthocyanidin accumulation. Such genes could include the maize *C1 MYB* regulatory gene (which is a partner to *Lc*; Paz-Arez et al., 1987), other WD40 partner proteins (Pang et al., 2009), and the *Medicago truncatula* *LAP1 MYB* regulatory gene (Peel et al., 2009). Additional basic research to clearly define the final condensation and vacuolar uptake steps of polymeric anthocyanidin biosynthesis and to find new regulatory genes would also shed light on additional genes that may be useful to support the development of polymeric anthocyanidin-accumulating

alfalfa. These developments depend on new functional genomics strategies in *Medicago truncatula* (Pang et al., 2007) and the discovery of new *transparent testa* mutants in *Arabidopsis* (Robinson et al., 2009).

CONCLUSIONS

Forage nutritional characteristics were predicted using the CNCPS system, the 2007 DVE/OEB protein system and VEM energy system to compare T¹Lc-alfalfa populations with raised forage anthocyanidins with the variety AC Grazeland, which had been selected earlier for a lower initial rate of degradation. The T¹Lc-transgenic populations had an average forage anthocyanidin concentration of 163.4 µg/g of DM, whereas AC Grazeland did not accumulate anthocyanidin. Crude protein, NDF, and residual nonstarch polysaccharide content were similar in the forage of the 3 T¹Lc-alfalfa populations and AC Grazeland. AC Grazeland had higher undegradable in situ CP and NDF content compared with that of the T¹Lc-alfalfa populations, and a trend toward higher predicted rumen degradable protein content, rumen OEB and DVE content for T¹Lc-alfalfa was noted. When comparing individual T¹Lc-alfalfa populations, T¹RambLc3 had numerically the lowest soluble and undegradable and highest potentially degradable in situ protein and CHO fractions of all of the T¹Lc-alfalfa populations, which resulted in a numerically lower predicted rumen OEB and numerically higher predicted

DVE in T_1 RambLc3. The hourly rumen OEB synchronization differed only after 1 h for the 4 populations, and the NE_L was higher in T_1 Lc-alfalfa compared with that for AC Grazeland.

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