Characterization of Anthocyanidin-Accumulating *Lc*-Alfalfa for Ruminants: Nutritional Profiles, Digestibility, Availability and Molecular Structure, and Bloat Characteristics

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by

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Summary

Grazing cattle on alfalfa (*Medicago sativa* L.) would be economically beneficial, but its rapid initial rate of protein degradation results in pasture bloat, low efficiency of protein utilization and excessive N pollution into the environment. Introducing a gene that stimulates the accumulation of mono/polymeric anthocyanidins might reduce the ruminal protein degradation rate and reduce bloat related foam stability. The overall objective of this thesis was to evaluate newly developed anthocyanidin-accumulating *Lc*-alfalfa progeny for nutritional properties (composition, site of degradation and molecular structure), environmental emissions and bloat characteristics.

The objective of the first study was to determine survival and phytochemical and chemical profiles of *Lc*-alfalfa progeny (Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4) and their non-transgenic (NT) parental cultivars (Beaver, Rambler and Rangelander). *Lc*-alfalfa forage accumulated enhanced amounts of anthocyanidin, with an average concentration of 197.4 µg/g DM, while proanthocyanidin (i.e. condensed tannins) were not detected. Both of these metabolites were absent in the NT-parental varieties. *Lc*-alfalfa progeny had ~3 % less crude protein (CP) and ~3 % more carbohydrates (CHO), which resulted in their 11 g/kg lower N:CHO ratio compared with NT-alfalfa. Total rumen-degradable N:CHO ratio based on chemical analysis was 12.9 g/kg lower in *Lc*-alfalfa compared with NT-alfalfa.

The objective of the second study was to evaluate *in vitro* degradation, fermentation and microbial-N partitioning of three forage color phenotypes [green, light purple-green (LPG) and purple-green (PG)] within *Lc*-progeny and their parental green NT-alfalfa varieties. Purple-green-*Lc* alfalfa accumulated more anthocyanidin than Green-*Lc* with LPG-*Lc* intermediate. Gas, methane and ammonia accumulation rates were slower for the two purple-*Lc* phenotypes compared with NT-alfalfa with Green-*Lc* intermediate. Effective degradable DM and N were lower in the three *Lc*-phenotypes compared with NT-alfalfa. Anthocyanidin concentration correlated negatively with gas and methane production rates and effective degradability of DM and N.

The objectives of the third study were to evaluate *in situ* ruminal degradation characteristics and synchronization ratios, and to model protein availability to dairy cattle and net energy for lactation of three *Lc*-alfalfa progenies, Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4 and the cultivar AC Grazeland (selected for a low initial rate of ruminal degradation). Anthocyanidin accumulation was on average 163.4 µg/g DM in the three *Lc*-progeny while AC Grazeland 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 *Lc*-alfalfa and AC Grazeland. The undegradable *in situ* crude protein and neutral detergent fiber fraction were, respectively, 1.3 %CP and 4.8 %CHO lower in the three *Lc*-progeny compared with AC Grazeland. *Lc*-alfalfa had a 0.34 MJ/kg DM higher net energy for lactation and tended to have a 11.9, 6.9 and 8.4 g/kg DM higher rumen degradable protein, rumen degraded protein balance and intestinal available protein, respectively, compared with AC Grazeland,. The hourly rumen degraded protein balance included an initial and substantial peak (over-supply) of protein relative to energy which was highest in Rang*Lc*4 and lowest in Ramb*Lc*3. The hourly rumen degraded protein balance between 4 and 24 h was similar and more balanced for all four alfalfa populations.

The objective of the fourth study was to determine foam formation and stability *in vitro* from aqueous leaf extracts of three *Lc*-alfalfa progeny (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4), parental NT-alfalfa and AC Grazeland (bloat reduced cultivar) harvested in the field at 07:00 or 18:00 h. Anthocyanidin accumulation averaged 247.5 μg/g DM in the leaves of the three *Lc*-progeny. There was an interaction between population and harvest time for the foam parameters. Initial foam volume (0 min) and final foam volume (150 min) at 07:00 h were lower for AC Grazeland compared with all other treatments and lower for Rang*Lc*4 compared with the other two *Lc*-progeny at 0 min and NT-alfalfa at 150 min; while from the 18:00 h harvest, initial foam volume was larger for NT-alfalfa and final foam volume was larger for Ramb*Lc*3 compared with AC Grazeland, Beav*Lc*1 and Rang*Lc*4. Foam formation correlated positively (R = 0.30 to 0.44) with leaf DM content, leaf extract protein and ethanol-film content, spectroscopic vibration intensity due to all carbohydrates (CHO_{VI}) and amide I:amide II ratio and negatively (R = -0.33 and -0.34; $P<0.05$) with α-helix:β-sheet ratio and amide I:CHO_{VI}. Final foam volume correlated negatively (R = -0.53 to -0.25; $P<0.05$) with leaf extract pH, spectroscopic vibration intensity due to all protein structures, structural carbohydrates (SC_{VI}) and lipids (CH_2 and CH_3 asymmetric stretching) and amide I:CHO_{VI} ratio and corelated positively (R = 0.39 to 0.44; $P<0.05$) with CHO_{VI}, amideI:SC_{VI} ratio and CHO_{VI}:SC_{VI} ratio.

In conclusion, all *Lc*-alfalfa progeny and phenotypes accumulated anthocyanidin in their forage. *Lc*-alfalfa progeny had lower protein and higher carbohydrate content which improved the nitrogen to carbohydrate balance compared to their parental NT-alfalfa cultivars. Rate of fermentation and effective degradability *in vitro* reduced for both purple anthocyanidin-

accumulating *Lc*-alfalfa phenotypes compared with NT-alfalfa. Intestinal protein availability tended to be higher and net energy for lactation was higher from *Lc*-alfalfa progeny for dairy cattle compared with AC Grazeland. Foaming properties were reduced in *Lc*-alfalfa progeny compared with parental non-transgenic alfalfa but not compared with AC Grazeland. However, differences between the *Lc*-alfalfa progeny and other cultivars were small. Therefore, further increases in mono/polymeric anthocyanidin accumulation in alfalfa are required in order to develop an alfalfa cultivar with superior nutritional and bloat preventing characteristics compared to currently available alfalfa cultivars.

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This thesis is

Dedicated

to

all the people that gave me positive inspiration to

continue my studies

with the biggest supporters being

my parents

Wim and Annie Jonker

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Abbreviations

Alfalfa treatment

AG	Alfalfa cultivar AC Grazeland
Lc	Maize leaf color anthocyanidin regulatory gene
LPG	Light purple-green
NT	Non-transgenic
PG	Purple-green
AAFC	Agriculture and Agri-Food Canada
AOAC	**Association of Official Analytical Chemists (Basic chemicals)**
ADF	Acid detergent fiber
ADIP	Acid detergent insoluble protein
ADL	Acid detergent lignin
BSP	Buffer soluble protein
CHO	Carbohydrates
CP	Crude protein
EE	Ether extract
EIR	Ethanol insoluble residue
ESC	Ethanol soluble carbohydrates
NDF	Neutral detergent fiber
NDIP	Neutral detergent insoluble protein
NDSF	Neutral detergent soluble fiber
NFC	Non-fiber carbohydrates
NPN	Non protein N
OM	Organic matter
RNSP	Residual non starch polysaccarides
APE	^{15}N atom % excess
ATP	Adenosine triphospate
CNCPS	**Cornell Net Carbohydrate and Protein System (fractions)**
CA	Soluble carbohydrate fraction

CA3	Organic acids
CA4	Sugars (i.e. ESC)
CB	Potentially degradable carbohydrates
CB2	Soluble fiber
CB3	Potentially degradable fiber
CC	Undegradable fiber
DRUC	Intestinally digestible rumen undegradable carbohydrates
PA	Soluble protein fraction (i.e. NPN)
PB	Potentially degradable true protein
PB1	Rapidly degradable true protein
PB2	Intermediately degradable true protein
PB3	Slowly degradable true protein
PC	Undegradable protein
CFIA	Canadian Food Inspection Agency
DVE/OEB	**Dutch protein modeling system**
DVE	Intestinally digestible protein (i.e. metabolizable protein)
DMCP	Intestinally digestible microbial protein
DMFP	Endogenous protein losses in the faeces
DRUP	Intestinally digestible rumen undegradable protein
FOMr	Fermentable organic matter in the rumen
MCPr	Microbial protein synthesis in the rumen
OEB	Rumen degraded protein balance
FA	Fatty acids
FPCM	Fat and protein corrected milk
FTIR	**Fourier Transform Infrared Vibration Spectroscopy**
CHO_{VI}	Vibration intensity due to all carbohydrates structures
SC_{VI}	Vibration intensity due to structural carbohydrates

In situ/exponential model

a	Asymptotic accumulation
AP	Average production
c	Fractional production rate
D	Potentially degradable fraction
ED	Ruminal effectively degradable (i.e. extent of ruminal degradation and RD)
Kd	Fractional degradation rate
Kp	Fractional passage rate
Lag	Lag time before ruminal degradation or fermentation
RD	Rumen degradable
RU	Rumen undegradable
S	Soluble fraction
SW	Soluble washable fraction
U	Undegradable fraction
W	Washable fraction
WI	Washable insoluble fraction
MN	Microbial nitrogen
PEG	Polythylene glycol (proanthocyanidin inactivator)
PPC	Protein precipitating capacity of proanthocyanidin
VEM	**Feed energy unit for lactation (Dutch energy system)**
tDOM	Total tract digestible organic matter
GE	Gross energy
ME	Metabolizable energy
NE_l	Net energy for lactation
VFA	**Volatile fatty acids**
BCFA	Branch chain fatty acids
NGR	Ratio between non-glucogenic and glucogenic VFA

Chapter 1

Introduction

Fresh pasture is the cheapest source of dietary nutrients and consequently results in low cost for milk and meat produced by cattle that are grazed on fresh pasture (Lacefield, 1996; Rotz, 1996; Taweel et al., 2006). Alfalfa (*Medicago sativa* L.; also known as lucerne), is one of the main forages grown in the world (Hanson et al., 1988). Alfalfa is a winter hardy drought tolerant N-fixing legume with a good longevity, high yield (McMahon et al., 2000; Popp et al., 2000), high nutrient levels, high digestibility, unique structural to non-structural components ratio (Yu et al., 2003b), high dry matter intake (Thornton and Minson, 1973; Allen, 2000) and high animal productivity per hectare (Popp et al., 2000). However, the main limitation of fresh alfalfa is its excessively rapid initial rate of protein degradation in the rumen (Broderick, 1995; Yu et al., 2004) which is the main cause of digestive disorders like pasture bloat (Popp et al., 2000) and creates an imbalance between the supply of ruminal N and energy required for microbial protein synthesis (Kingston-Smith and Theodorou, 2000; Yu et al., 2004). The imbalance decreases animal protein efficiency (Broderick, 1995; Kingston-Smith et al., 2003) and results in excessive release of N into the environment (Wattiaux and Karg, 2004; Lapierre et al., 2005). 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). Therefore protein can be the most limiting nutritional factor for high producing animals when the diet is composed mainly of fresh alfalfa pasture (Barry, 1981; Dhiman and Satter, 1993; Broderick, 1995).

Characteristic bloat-free legume forages like sainfoin (*Onobrychis viciifolia*.L) and birdsfoot trefoil (*Lotus corniculatus*.L) contain proanthocyanidin which are also known as condensed tannins (Majak et al., 1995; Aerts et al., 1999a; McMahon et al., 2000). Proanthocyanidins complex with protein in the rumen and reduce ruminal protein degradation, which can increase the efficiency of protein utilization (Broderick, 1995; Aerts et al., 1999a) and eliminate pasture bloat related foam stabilization (Fay et al., 1980; Tanner et al., 1995). However, these alternative proanthocyanidin accumulating legumes have a lower seed germination, poorer pasture longevity and lower DM yield per hectare compared with alfalfa (Howarth, 1975). Using a whole farm model, it was predicted that protein supplementation could be decreased by 60% if alfalfa accumulated proanthocyanidin in its forage (Bouton, 2008). This, in turn, would increase net return by 12% for dairy farms (Bouton, 2008) and eliminate the economic losses related to cattle pasturing on alfalfa. Therefore it would be very beneficial to develop alfalfa that produces

moderate amounts of proanthocyanidin in its forage. Alfalfa contains proanthocyanidin in its seed coat (Kaupai-Abyazani et al., 1993b) proving the presence of a complete flavonoid pathway in alfalfa. Ray et al. (2003) transformed alfalfa with a maize anthocyanidin regulating *βHLH LEAF COLOR* (*Lc*) gene to develop new genotypes which accumulated anthocyanidin and proanthocyanidin in its forage. Wang et al. (2006b) found that the initial *in vitro* rate of nitrogen and dry matter degradation for these T_0 *Lc*-alfalfa genotypes was reduced compared to their non-transgenic parent genotype. However, their survival was poor under western Canadian conditions, and three of these genotypes had to be crossed with western Canadian cultivars in order to develop hardy T_1 *Lc*-alfalfa populations and to facilitate the movement of the *Lc*-gene into a broader spectrum of alfalfa breeding germplasm.

Two hypotheses were tested in this thesis: a) newly developed T_1 *Lc*-alfalfa progeny will accumulate anthocyanidin and proanthocyanidin in its forage, b) these secondary metabolites will decrease ruminal protein degradation and fermentation and consequently reduce bloating characteristics, improve ruminal N to energy balance, increase ruminal escape protein and reduce environmental emissions.

The hypotheses were tested by determining detailed protein and energy profiles (Chapter 3), *in vitro* fermentation-degradation characteristics, *in vitro* microbial synthesis and partitioning, *in vitro* environmental emissions (Chapter 4), *in situ* protein and energy degradation, balance and synchronization, modeling site of nutrient availability and animal performance (Chapter 5) and bloat related foam formation and stability and its relation to alfalfa leaf vibration spectroscopic features (Chapter 6) of three T_1 *Lc*-alfalfa progeny.

Chapter 2

Development of Alfalfa that Accumulates Moderate Amounts of Proanthocyanidin (Condensed Tannins) in its Forage to Improve Ruminant Protein Metabolism, Reduce Environmental Pollution and Guarantee Bloat Safety: A Comprehensive Literature Review

2.1. Introduction

Alfalfa (*Medicago sativa* L.) is one of the main forages grown in the world (Hanson et al., 1988). Alfalfa is a winter hardy drought tolerant N-fixing legume with a good longevity, high yield (McMahon et al., 2000; Popp et al., 2000), high nutrient levels, high digestibility, unique structural to non-structural components ratio (Yu et al., 2003b), high dry matter intake (Thornton and Minson, 1973; Allen, 2000) and high animal productivity per hectare (Popp et al., 2000). However, its main limitation is its excessively rapid initial rate of protein degradation in the rumen which results in pasture bloat and inefficient use of protein by the animal with consequent excessive excretions of nitrogen into the environment (Broderick, 1995; Popp et al., 2000; Yu et al., 2004). Proanthocyanidins (also known as condensed tannins) are secondary plant metabolites that can bind with protein and reduce the rate and extent of ruminal protein degradation (Barry and McNabb, 1999). However, these secondary metabolites do not accumulate in alfalfa forage (Goplen et al., 1980). The objectives of this review are firstly to describe the events involved in the rapid release of protein from alfalfa forage and its effect on ruminant nutrition, environmental pollution and pasture bloat. Secondly, to describe occurrence, structure, functions and benefits of moderate amounts of proanthocyanidin in pasture forages. Finally, to describe the development of alfalfa which accumulates moderate amounts of proanthocyanidins.

2.2. Rapid Alfalfa Protein Degradation and Consequences

2.2.1. Mechanisms for the Release of Protein from Alfalfa Forage

Alfalfa has an excessively rapid initial rate of protein degradation compared to grasses and many legumes like cicer milkvetch (*Astragalus cicer* L.), sainfoin (*Onobrychis viciifolia* L.), birdsfoot trefoil (*Lotus corniculatus*.L), big trefoil (*Lotus pedunculatus* L. also known as *Lotus major* L. and *Lotus uliginosus* L.) and sulla (*Hedysarium coronarium* L.) but similar to white clover (*Trifolium repens* L.; also known as ladino clover), red clover (*Trifolium pratense*.L) and wheat pasture forage (*Triticum aestivum*) (Hoffman et al., 1993; Djouvinov et al., 1998; Julier et al., 2003). The main portion of forage proteins is located in leaf mesophyll cells (Fig. 2.1). Soluble proteins can be divided into fraction I proteins [Ribulose-1,5-*bis*phosphate Carboxylase (Rubisco)] which makes up 30-50 % of soluble protein, fraction II protein (several proteins present in small amounts, e.g plant enzymes and hormones) and chloroplast membrane proteins

(Howarth et al., 1977). On a plant cell level, rapidly degradable N fractions in plants are non-protein N (NPN; e.g. NH_4^+ and NO_3^- which are mainly found in the plant cell vacuole) and cytoplasmic and soluble protein (mainly Rubisco in alfalfa which is mainly found in the plant cell chloroplast; Fig. 2.1) (Boudon and Peyraud, 2001). The first mechanism by which these soluble protein fractions are released from the plant cell is ingestive chewing during livestock grazing. Chewing cracks the cuticle and plasma membrane, destroys mesophyll cells and reduces the forage particle size. The N components from the vacuoles were found to be released at a faster rate and larger extent than N from the chloroplasts (Boudon and Peyraud, 2001; Boudon, 2002). White clover was found to have a faster release of the total cell content and intracellular N from the plant cell after ingestive chewing (before entering the rumen) than three grass species (Acosta et al., 2007) and alfalfa and red clover had a faster release of soluble proteins after ingestive chewing than sainfoin (Table 2.1) (Mangan et al., 1976). Epidermal and mesophyll cells of alfalfa and white and red clover were found to be thinner and less resistant to mechanical rupture than those of birdsfoot trefoil, sainfoin and cicer milkvetch (Table 2.1) (Howarth et al., 1978; Lees et al., 1981; Lees, 1984). Waghorn et al. (1989) found that ~ 40 % of perennial ryegrass and ~20 % of alfalfa plant cells reached the rumen intact after ingestion. It is believed that over 50 % of plant cells reach the rumen intact (Kingston-Smith and Theodorou, 2000).

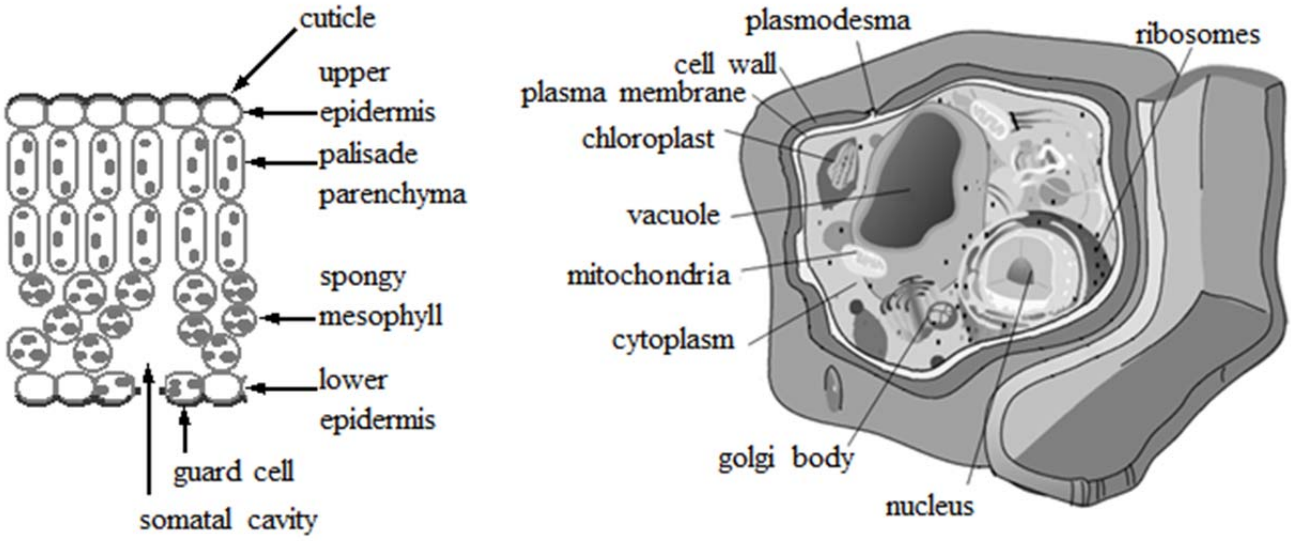


Fig. 2.1. Plant tissue (left) and plant cell (right) structrue. Figures adapted from: hcs.ohio-state.edu/hcs300/anat3.htm and commons.wikimedia.org/wiki/File:Eukaryota_cell_strucutre.PNG

Some plant cells are still intact and metabolically active when entering the rumen. The conditions in the rumen (temperature, anaerobic nature and microbial colonization) cause a stress reaction in the viable plant cells and result in a release of protein, due to in plant metabolic (e.g. proteinase) activity (Kingston-Smith and Theodorou, 2000; Attwood, 2005). Proteolytic activity indexes of fresh plant tissue incubated over a gelatin substrate gel was high for *Medicago* species (mean 0.56, for alfalfa 0.77) and *Avena* (oat 0.71) but low for the *Trifolium* (with clover 0.24, red clover 0.17), *Triticum* (wheat 0.08), *Lolium* (perennial ryegrass 0.22) and *Lotus* (birdsfoot trefoil 0.14, big trefoil 0.16) species (Table 2.1) (Pichard et al., 2006). Thus it seems that forages with high ruminal protein degradation do not necessarily have higher proteolytic activity. Kingston-Smith et al. (2003) found that ions which are stored in the vacuole (e.g. Ca^{2+}, Mg^{2+}, NH_4^+ and NO_3^-) are released when fresh plant material was incubated in H_2O at 39 ºC (rumen temperature) without microbes (Table 2.1). This indicates that membranes become weaker and more permeable over time. Probably absorption of H_2O into the plant tissue is involved (cells swell and burst) and allows small molecules including ions, soluble carbohydrates and small proteins to escape from the plant cell (Kingston-Smith et al., 2003). The rate at which ions were released was fast for white and red clover and sainfoin, slow for birdsfoot trefoil and intermediate for alfalfa (Kingston-Smith et al., 2003).

Intact plant cells have to be ruptured before microbes can enter (Cheng et al., 1980). Many of these plant cells will be ruptured during re-mastication (rumination) of the cud by the ruminant (Boudon, 2002). When new plant material enters the rumen, microbes will attach to it within five minutes (McAllister et al., 1994). Microbes will try to invade the plant cell through the stomatal cavity, lenticels and disrupted cells, after which intracellular micro-colonies are formed that can easily disrupt and digest mesophyll cells from the inside out (Cheng et al., 1980; McAllister et al., 1994). The time needed to disrupt leaf tissue and to invade and digest mesophyll cells was shorter for alfalfa, and white and red clover than for birdsfoot trefoil, sainfoin and cicer milkvetch (Table 2.1) (Howarth et al., 1979; Cheng et al., 1980; Howarth et al., 1982). The breakdown of plant particles in the rumen was found to be faster for alfalfa than for perennial ryegrass (*Lolium perrene* L.) (Waghorn et al., 1989).

Overall, alfalfa protein was found to have a higher solubility, faster degradation rate and larger extent of ruminal degradation than protein of sainfoin and grasses (Yu et al., 2004; Aufrère et al., 2008). Besides having plant cells that are more resistant to rupture, legumes like

sainfoin, birdsfoot trefoil, big trefoil and sulla contain proanthocyanidins which reduce protein degradation and availability in the rumen (detailed discussion in section 2.3.3) (Barry and McNabb, 1999). However, proanthocyanidins are not synthezised in the aerial parts of alfalfa and are therefore not present in their forage (Goplen et al., 1980).

Table 2.1. Composition, tissue disruption and release of nutrients in leaves of several temperate forage legumes

	Legume species					
	alfalfa	White clover	Red clover	Birdsfoot trefoil	Sainfoin	Cicer milkvetch
Leaf epidermis + cuticle thickness						
Upper (µm)[1]	1.05	1.30	1.04	1.21	1.54	ND
Lower (µm)[1]	0.93	1.31	0.89	1.22	2.04	ND
Upper (µm)[2]	21	26	23	30	25	32
Leaf tissue						
Cavities air space (%)[1]	21.2	15.8	10.8	23.8	22.6	ND
Leaf cell wall strength[3]	weak	weak	mid	strong	mid	Mid
Leaf tissue strength[3]	low	low	low	low	high	High
Intact leaf mesophyll cells (no.)[4]	48	7	1	1167	570	811
Microbial tissue disruption (%)[5]	88	94	89	ND	ND	65
Release of plant cell constituents						
Potassium (%)[6]	57	ND	50	ND	64	ND
Kd conductivity (%/h)[7]	59	71	75	34	98	ND
Rubisco (%)[6]	46	ND	16	ND	17	ND
Soluble CP (%)[6]	24	ND	20	ND	0	ND
Proteolytic activity index[8]	0.77	0.24	0.17	0.14	ND	ND

ND is not determined

[1]Epidermis + cuticle thickness and air space were measured (Fig. 2.1) in leaf cross sections by electron microscopy (Kingston-Smith et al., 2003).

[2]Epidermis + cuticle thickness measured in leaf sections under a coverslip in 0.55 *Mm* mannitol solution using photographic transparency (shadow graph with stage micrometer scale) (Lees et al., 1982).

[3]Tissue and cell wall strength determined as chlorophyll released after mechanical disruption by shaking leaves in a tube with glass beads, by a ground glass tissue grinder or by sonication (Lees et al., 1982).

[4]Leaves crushed between two layers of nylon cloth in a mortar with buffer using a pestle. Intact filtrate mesophyll cells were counted with a hemocytometer and light microscope (Howarth et al., 1978)

[5]Disappearence of green leaf tissue DM after 8 h of ruminal *in situ* incubations (Howarth et al., 1982).

[6]Release of plant cell constituent after ingestive mastication (Mangan et al., 1976).

[7]Change in conductivity (rate) due to release of minerals from leaves incubated in H_2O at 39 ºC (Kingston-Smith et al., 2003).

[8] Proteolytic activity of fresh plant tissue incubated over a gelatin substrate gel (Pichard et al., 2006).

However, alfalfa has higher seed germination, higher pasture longevity and higher grazing tolerance compared with legumes like sainfoin, birdsfoot trefoil and cicer milkvetch (Howarth,

1975). Compared with grasses, alfalfa has a higher animal DM intake and requires lower fertilizer inputs (Thornton and Minson, 1973). Compared with other legumes and grasses, alfalfa has a higher DM yield and animal production per hectare (Gesshe and Walton, 1981; Popp et al., 2000).

2.2.2. Protein Degradation, N to Energy Balance and N Excretion

The amino acids that are absorbed in the small intestine of ruminants originate from intestinal digested microbial bodies (protein), intestinal digested dietary protein that escaped rumen microbial degradation and endogenous protein (Tamminga et al., 1994). Microbial-N that enters the small intestine ranges from 60 to up to 90 % of total N entering the intestine (Broderick et al., 2010). For their growth, rumen microbes require N, energy and essential minerals. Carbohydrates are the main source of energy used by microbes (Bergman, 1990; Hoover and Stokes, 1991). Optimal ratio between ruminal available N and energy required for microbial growth was found to be approximately 25 g N/kg DM degraded in the rumen (Beever et al., 1986), 25 g N/kg OM degraded in the rumen (Czerkawski, 1986) or 32 g N/kg CHO degraded in the rumen (Tamminga et al., 1990; Sinclair et al., 1991).

In freshly harvested alfalfa at different stages of growth, rumen degradable N:DM ratio (RD_N:RD_{DM}) ranged from 31-to-49 g N/kg DM with extent of ruminal DM degradation ranging from 55 to 80 % which is lower than the range of 76 to 90 % for extent of ruminal CP degradation (Table 2.2). NRC (2001) recommends that a total diet consists for 14-18 % of CP with 62-65 % of CP consisting of rumen degradable CP, while Broderick et al. (2010) found (analysis of 32 studies with 122 diets) dietary CP of 15 % from which 70 %CP in the rumen degradable form to be optimum. The forage CP content, rumen degradable CP and imbalance between ruminal degradable N and DM tends to decrease with advancing plant maturity stages (Table 2.2).

The rate and extent of alfalfa protein degradation are not only excessive, but the supplied amount of rumen available energy from alfalfa is also not sufficient to support optimal conditions for microbial growth. Degradation of fibrous carbohydrates is slower in alfalfa than for non-fiber carbohydrates (NFC). For two varieties of alfalfa at three stages of growth (pre-bud, late-bud and early flower stages) fractional degradation rate of neutral detergent fiber (NDF) ranged from 4.7-to-7.6 %/h which is slower than the range of 7.8-to-9.5 %/h found for

NFC (Yu et al., 2004). The CP properties of alfalfa improve with advancing maturity, but NDF content will increase as well, which counterbalances the improved CP properties in a negative fashion. Therefore, the imbalance between the release of N and energy in the rumen can be decreased by decreasing protein content, solubility or degradation rate and/or increasing content, solubility or degradation rate of carbohydrates.

Table 2.2. Protein quality characteristics for freeze-dried alfalfa, grass and sainfoin.

			Ruminal CP degradation				
	RD_{DM}[1]	CP	Rate	Solubility	Extent	$RD_N:RD_{DM}$[1]	Ref.[2]
	(%DM)		(%/h)	(%CP)		(g/kg)	
Alfalfa							
Vegetative	65-80	20-27	15-34	40-60	80-90	40-49	1, 2, 3, 4, 5
Bud	55-77	17-21	15-29	41-47	80-83	34-41	2, 3, 5
Early flower	57-59	17-19	17-31	40-46	75-81	37-41	1, 2, 3
Full flower	58-60	15-16	12-14	41-47	73-79	31-32	3, 5
Early pod	55-58	16	15-17	52	78-82	36	4, 5
Grass							
Tillering	66-70	20-26	17	32-45	79-82	39-50	3
Elongation	52-62	15-21	10-15	27-47	68-82	31-47	3
Heading	50-60	12-14	11-12	36-56	70-76	26-30	3
Flowering	38-40	7-10	7-11	43-53	61-70	18-27	3
Sainfoin							
Vegetative	55	16	11.3	16	59	28	2
Early flower	48	12	14.1	20	63	26	2

[1]RD_{DM} is rumen degradable DM; $RD_N:RD_{DM}$ is rumen degradable N:DM ratio.
[2]Data in the table was obtained from in situ studies reported by: 1. Aufrère et al. (2000), 2. Aufrère et al. (2008), 3. Elizalde et al. (1999), 4. Faría-Mármol et al. (2002), and 5. González et al. (2001).

The excess protein released into the rumen above microbial requirements is mainly deaminated into ammonia (NH_3), energy and volatile fatty acids (mainly propionate and branch chain fatty acids) by rumen microbes (Tamminga, 1979; Kaneda, 1991). The energy is used for microbial growth and excess ruminal NH_3 is absorbed across the rumen wall and converted in the liver mainly into urea at the cost of energy (Van Duinkerken et al., 2005; Tamminga, 2006). Urea-N can re-enter the rumen via secreted saliva or by direct diffusion/active transport from blood into the rumen where it is converted back into NH_3 by ureolytic bacteria (Rémond et al., 1996; Lapierre and Lobley, 2001). When sufficient energy is available, NH_3 is used for microbial

protein synthesis; otherwise when energy is insufficient, NH_3 will be mainly lost to the animal and excreted via urea in the urine (Lapierre et al., 2005; Van Duinkerken et al., 2005). At different levels of protein intake from total mixed rations with different levels of N and ruminal N degradability and different forage:concentrate ratios, urine-N excretion ranges from 133-to-592 g/kg N intake (Hoekstra et al., 2007). Excreted urinary urea-N is easily volatilized to NH_4 or converted to NO_3^- which contribute to environmental acidification and eutrophication, respectively (Van Duinkerken et al., 2005; Hoekstra et al., 2007). In addition to these environmental drawbacks, high blood NH_3 and urea levels also have a negative effect on reproduction and fertility of cattle (Tamminga, 2006).

Valkeners et al. (2004) found that an unsychronized 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. Some recent research suggests that diets with oscillating dietary protein (unsynchronized but balanced) are utilized more efficiently, probably because of increased utilization of recycled urea-N into microbial cells (Cole, 1999; Archibeque et al., 2007; Kiran et al., 2011). However, as described before, the release of excess N relative to energy in the rumen is both unsynchronized and unbalanced and will lead to an inefficient use of dietary N by the animal and excessive excretion of N into the environment. Conversion of protein into energy results not only in protein losses for the animal but it also yields less energy (13.6 mole ATP/kg CP) than derived from fermentation of carbohydrates (23.9-27.3 mole ATP/kg CHO) for microbial growth (Tamminga, 1979; Tamminga et al., 2007). Moreover, excessive ruminal protein degradation results in a reduced 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).

2.2.3. Alfalfa and Pasture Bloat

Pasture bloat arises from rumen fermentation gases which become trapped in a viscous stable protein foam that prevents normal eructation of microbial fermentation gasses by the animal (Cockrem et al., 1987; Tanner et al., 1995). The accumulation of gas in the rumen causes dissention of the rumen and exerts pressure on organs like the lungs, heart and vagus nerve. This can lead to the death of the animal under severe conditions (Clarke and Reid, 1974; Cheng et al., 1998). Even considering the relatively low number of animals grazed on alfalfa in North

America, cattle mortality due to pasture bloat was estimated to be as high as 1.5 % with economic losses estimated at $ 125 million a year (Howarth et al., 1986; Majak et al., 2003b).

Rumen conditions that favor the formation of stable protein foams include the nature of the surface active materials at the gas-liquid interface provided by the rumen environment (pH, viscosity, ionic composition, soluble protein concentration, and microbial species composition) and adequate gas production (Howarth, 1975; Clark et al., 1992). Table 2.3 shows some rumen and animal characteristics of cattle prone to pasture bloat compared with animals that do not bloat on the same pasture.

Table 2.3. Characteristics of cattle prone to pasture bloat compared with non-bloating animals

Bloating cattle	
Decreased dry matter intake	(Majak et al., 1986, 1995)
Decreased saliva production	(Mangan, 1958; Mendel and Boda, 1961)
Increased saliva viscosity	(Mendel and Boda, 1961)
Decreased clearance rate liquid and particles	(Majak et al., 1983, 1986)
Rumen liquid of bloating cattle	
Decreased Na^+	(Hall et al., 1988)
Increased Ca^{2+}, Mg^{2+} and K^+	(Hall et al., 1988)
Increased viscosity	(Gutierrez et al., 1961; Meyer and Bartley, 1971)
Increased ethanol precipitated film with higher CP content	(Gutierrez et al., 1963; Min et al., 2006)
Increased buoyancy of particle matter	(Majak et al., 1983, 1986)
Increased small particle retention	(Majak et al., 1983, 1985, 1986)
Increased foam volume and stability	(Cockrem et al., 1987; Tanner et al., 1995)
Similar protein concentration	(Majak et al., 1983, 1985)

Increased viscosity of the rumen liquid may be due to increased protein, carbohydrate, and nucleic acid concentrations (Gutierrez et al., 1963), viscous saliva (Mangan, 1958) and slime produced by certain microbes (Gutierrez and Davis, 1962; Min et al., 2006). The increased viscosity of rumen liquid makes it more difficult for feed particles to move within the liquid and increases particle matter buoyancy with consequent increased particle retention (chlorophyll) in the rumen (Majak et al., 1983; Majak et al., 1986; Clauss et al., 2006). The viscous film at the gas-liquid interpface can be harvested by methanol precipitation. This film of bloating cattle on legume pastures (white clover) had a CP and CHO composition (63 % and 15 %, respectively) similar to foam from a bloating animal (Bartley and Bassette, 1961; Gutierrez et al., 1963).

Because positive correlations between pasture bloat and alfalfa soluble protein content have been reported frequently, it is generally accepted that rapid initial release of protein from alfalfa is involved in its propensity to cause pasture bloat (Howarth et al., 1977; Lees, 1992; Majak et al., 1995). Soluble proteins which are rapidly released into the rumen liquid are thought to form a viscous film at the gas-liquid interface and this could act as an active agent for the formation of foam that might result in frothy bloat (Majak et al., 1995; Min et al., 2006). The proteolytic bacterial strain *Streptococcus bovis* cultured on a protein extract (from wheat forage) was found to have a high growth rate and to produce large amounts of slime (Min et al., 2006). Proteolytic microbial populations in the rumen of cattle grazing perennial ryegrass/white clover pasture were found to consist of 61 % *S. bovis* like strains (Attwood and Reilly, 1995). So, part of the viscous film formed at the gas liquid interface might originate from protein degrading microbes that produce a viscous slime. However, *in vitro* foam stability of aqueous leaf extracts was previously found to be related to *in vitro* foam stability from rumen liquid of bloating animals and to *in vivo* bloat incidence in cattle (Mangan, 1959; Pressey et al., 1963). This suggests that slime produced by microbes is not required for the formation of a viscous film at the gas liquid interface and thus not required for the formation of stable foam in the rumen.

Incidence of bloat in grazing cattle is higher when offering alfalfa at a vegetative stage than at advanced stages of maturity (Thompson et al., 2000). Vegetative alfalfa has a higher leaf to stem ratio, higher protein content and higher digestibility compared with more mature alfalfa (Luckett and Klopfenstein, 1970; Gutek et al., 1974). In general, pasture bloat occurs within two to three hours after moving cattle to a bloat inducing pasture (Howarth, 1975; Hall and Majak, 1989). Cattle graze the pasture canopy from the top to the bottom of the plants removing around 35 % of the sward height per bite (Rook, 2000). The top of the canopy has a higher leaf to stem ratio than the bottom of the canopy (Gutek et al., 1974). So, at the initial stage of grazing cattle consume mainly leaves. Leaves have a higher soluble protein content, higher ruminal digestibility, lower fiber content and higher foaming properties compared with stems (Cooper et al., 1966; Luckett and Klopfenstein, 1970; Tremblay et al., 2002). Thus the higher soluble protein content and digestibility in leaves and vegetative growth might explain the higher bloat incidence shortly after allocating cattle to a new pasture or to a vegetative pasture. However, the protein content in rumen liquid of bloating cattle was not different than that of non-bloating

cattle on the same pasture. This suggests that other components are involved in the stabilization of bloat related foam (Majak et al., 1983, 1985; Howarth et al., 1986).

Alfalfa leaves and younger vegetative growth were found to have a higher saponin content compared with stems and more mature growth (Pedersen et al., 1967). Saponins form a stable froth when shaken in water. When saponins are released into the rumen liquid, they reduce smooth muscle activity and thereby reduce rumen motility. This reduces the rumen digesta passage rate and may directly reduce or stop the eructation reflex (Klita et al., 1996; Mathison et al., 1999). Saponins released in the rumen liquid are extensively degraded by saponin degrading microbes (e.g. *Butyrivibrio*) which were found to produce a viscous slime (Gutierrez and Davis, 1962). However, cattle grazing a high saponin (1.9 %) alfalfa cultivar were no more prone to pasture bloat than cattle grazing a low saponin (0.8 %) cultivar, and administration of alfalfa juice with saponins to cattle and sheep did not produce bloat (Ferguson and Terry, 1955; Majak et al., 1980). Besides, bloat occurs on wheat pastures as well and wheat forage does not contain saponins (Min et al., 2007; Akins et al., 2009). However, some reported studies detected bloat signals like distention of the rumen and animal distress when administering alfalfa saponin extracts to ruminants (Lindahl et al., 1954; Mathison et al., 1999). These findings suggest that saponins might be involved in some cases of frothy bloat, but they are not required for the formation of stable foam in the rumen. Saponin content, ratio between sapogenins and sapogenin structure differ among alfalfa cultivars and might determine the propensity to develop pasture bloat (Pedersen et al., 1967, 1973; Sen et al., 1998). Little is known about the effect of environment, season, climatic conditions and growth conditions on saponin content, the ratio between individual sapogenins and on sapogenin structure in alfalfa (Sen et al., 1998).

A number of factors appear to influence foam strength. Previously, foam strength was found to be maximum around pH 5 to 6 (Buckingham, 1970; Wang and Kinsella, 1976). Positively-charged ions (Ca^{2+}, Mg^{2+}) may increase foam stability by attracting negatively charged soluble proteins in rumen liquid (Stifel et al., 1968a; Clarke and Reid, 1974; Hall et al., 1988). Pectins and simple saccharides are also thought to increase the viscosity of rumen liquid (Conrad et al., 1958; Pressey et al., 1963) while higher fiber content is associated with a lower forage digestibility, a condition which reduces the amount of foaming compounds released (Jung et al., 1997). Soluble proteins, small particles and positively charged ions may form a complex at the gas liquid interface and stabilize ruminal foam (Majak et al., 1983; Howarth et al., 1986).

2.2.4. Management Strategies to Reduce Bloat Incidence on Alfalfa Pastures

Grazing management strategies found to reduce the incidence of pasture bloat include allocating cattle to a new pasture in the afternoon instead of the early morning, continuous grazing instead of interrupted grazing, strip grazing to force the animals to graze the whole plants in a short period (lower part of the plant contains less protein and is less digestible) (Majak et al., 1995, 2001) and offering alfalfa in an advanced stage of maturity (Thompson et al., 2000). Even though these grazing management strategies are associated with decreased severity and occurrence of bloat, pasture bloat can occur in the afternoon, with continuous grazing and at full bloom (Howarth, 1975).

Anti-bloat agents which contain oils and alfalfa forage lipids destabilize foam related to bloat (Stifel et al., 1968b; Min et al., 2007). Non-bloating alfalfa forage has a higher lipid (mainly located in the chloroplast) content relative to protein (Stifel et al., 1968b). Anti-foaming agents, like oils and detergents, have been used with success to prevent pasture bloat, but their effectiveness depends on intake which cannot be guaranteed when supplied as free choice (Cheng et al., 1998; Majak et al., 2001).

The risk of pasture bloat is negligible when cattle graze pastures with grass. Feeding corn silage or grass hay to cattle before turning them onto alfalfa pasture reduces the incidence of bloat substantially (Bretschneider et al., 2007; Majak et al., 2008). However, beef cattle and sheep are usually not (or not regularly) supplemented during the grazing season. Moreover, in general, grass pastures have a lower nutritional value, lower DM yield and higher fertilization costs compared to alfalfa under North American conditions (Howarth, 1975). Therefore, alfalfa can be grown in mixtures with grass so that livestock can benefit from the positive attributes of both forage families, but even mixtures have limitations. Orchard grass-alfalfa mixtures (25-to-50% grass) reduced the incidence of bloat, but did not fully eliminate it (Majak et al., 2003a; Veira et al., 2010), likely because of plant selection by cattle. Rutter et al. (2006) found that cattle and sheep grazed on mixed grass-legume pastures (perennial ryegrass/white clover) prefer eating legumes (*ca.* 70%), especially in the morning.

2.2.5. Selection of Alfalfa with Reduced Protein Degradation

From 1979 onwards, selection at Agriculture and Agri-Food Canada (AAFC) for alfalfa with a low initial rate of degradation was conducted by incubating the top 15 cm of alfalfa plants *in situ* in nylon bags for 4 h in the rumen of fistulated cows. The population of the fourth selection cycle was released as a new cultivar in 1997 under the name AC Grazeland (Coulman et al., 2000). AC Grazeland harvested at a vegetative stage had a reduced initial rate of degradation, increased NDF and acid detergent fiber content, lower leaf-to-stem ratio and increased leaf epidermis and mesophyll wall thickness compared with the alfalfa cultivar Beaver (Goplen et al., 1993; Coulman et al., 1998, 2000; Tremblay et al., 2000). In grazing experiments, AC Grazeland at the vegetative stage reduced the incidence and severity of pasture bloat on average by 56% compared to Beaver but it did not fully prevent it (Berg et al., 2000; Coulman et al., 2000; Majak et al., 2001). A similar selection program in Argentina (Basigalup et al., 2003) produced a third selection cycle population called Carmina which reduced the incidence and severity of pasture bloat by 25% (Bernaldez et al., 2009). Thus further improvements in protein degradation characteristics are required in order to develop a higher quality alfalfa cultivar.

A range of methods has been be used for rapid selection of alfalfa samples for different protein properties. The *in vitro* inhibitor method showed a difference of 41, 38 and 31 g/kg N and 4.2 %/h for soluble N, potentially degradable N, ruminal escape protein and degradation rate, respectively, among 19 alfalfa cultivars from different parts of the world while each had a similar total N content (Broderick and Buxton, 1991). Using the same method, rumen undegradable N differed 21 g/kg N and N degradation rate differed by 1.9 %/h among 27 Canadian cultivars grown in two years and *in vitro* DM digestibility (Tilley and Terry method) of these same cultivars differed by 38 g/kg DM (Tremblay et al., 2000). Protein fractions generated by wet chemical analysis as used in the Cornell Net Carbohydrate and Protein System (CNCPS) correlated with *in vitro* inhibitor rumen undegradable CP (Tremblay et al., 2003).

Individual amino acids and individual proteins differ in ruminal degradation rate. So protein degradation rates could be decreased by increasing the expression of genes that produce amino acids with a low degradation rate (Tabe et al., 1993, 1995). Protein secondary and tertiary structures affect the extent and rate of rumen degradability as well. The number of disulphide (S-S) bonds in the tertiary protein structure were found to affect the extent and rate of protein degradation (Ørskov, 1986; McNabb et al., 1994). Sulfur containing amino acids have disulfide

bonds which are less degradable in the rumen. Mid-IR vibration spectroscopy determined that protein with more α-helices relative to β-sheets and protein with less amide I relative to amide II vibration had reduced ruminal protein degradability (Doiron et al., 2009; Yu and Nuez-Ortín, 2010). Twenty-six alfalfa proteins from several genotypes of three alfalfa populations showed a range of degradability using fluorescence 2D gel electrophoresis combined with mass spectrometry (Chen et al., 2009). For nine of these proteins, more than 75% remained after 45 min of *in situ* ruminal incubation, while twelve other proteins where more than 50% digested after 45 min, and others were intermediate. After 120 min incubation, 80% remained from four proteins and less than 50% remained from 14 proteins. The main protein (41% of the original sample) with a low degradability after 120 min was the Rubisco large subunit (Chen et al., 2009). Previously the Rubisco large subunit was found to have a faster ruminal degradation rate than the Rubisco small subunit (Tanner et al., 1994; Min et al., 2000). Selecting alfalfa plants based on protein structure or protein composition might offer new ways to develop alfalfa with a reduced ruminal degradation rate. Proanthocyanidins are also known to reduce the rate and extent of ruminal protein degradation (Barry and McNabb, 1999.

2.3. Proanthocyanidin Structure, Function and Benefits

2.3.1. Occurrence of Proanthocyanidins

Proanthocyanidins are oligomeric and polymeric linked flavonoid units synthesized in the flavonoid pathway (Fig. 2.2). The name proanthocyanidin comes from the red anthocyanidin formed after polymer cleavage and acid oxidation upon heating in acid (Marles et al., 2003). Monomeric flavonoids are synthesized in the cytosol and transported into the vacuole to form end products like proanthocyanidin and anthocyanin (Schwinn and Davies, 2004). Proanthocyanidins are found in leaves, stems, flowers and seeds of some forage legumes (Lees et al., 1993; McMahon et al., 2000). Some forage legumes like sainfoin and birdsfoot trefoil contain proanthocyanidin in all of these plant parts (Bate-Smith, 1973b; Lees et al., 1993; Theodoridou et al., 2010), while in alfalfa it accumulates only in the seed coat (Koupai-Abyazani et al., 1993b) and in white and red clover only in the flower (Sivakumaran et al., 2004). In sainfoin leaves, proanthocyanidin concentration was higher, with a higher degree of polymerization and higher prodelphidin content (Fig. 2.3), than in stems (Lees et al., 1993;

Theodoridou et al., 2010). During sainfoin leaf development, proanthocyanidin concentration, molecular weight and degree of polymerization increase until the leaves start to unfold, after which these compounds decrease until senescence (Koupai-Abyazani et al., 1993a; Lees et al., 1995). Berard et al. (2011) also found a similar pattern for forage of sainfoin, but in forage of birdsfoot trefoil, purple prairie clover (*Dalea pupurea* L.) and several *Trifolium* species, proanthocyanidin content was higher at a more advanced stage of maturity.

In addition to the growth stage of the plant, proanthocyanidin concentration seems to be influenced by the environmental conditions in which it grows. Big trefoil accumulated more proanthocyanidin when grown at 30 ºC than at 20 ºC in a growth cabinet (Lees, 1994). Proanthocyanidin content and protein binding capacity was higher in temperate and tropical legumes growing in low fertility soils compared with high fertility soils (Barry and Forss, 1983; Barry and Duncan, 1984a; Tiemann et al., 2010b) and higher in the dry season than the wet season for a tropical legume forage in Columbia (Tiemann et al., 2010a). On the western Canadian prairies, growth season did not affect proanthocyanidin content of temperate legumes (Berard et al., 2011). Herbivore and insect foraging on proanthocyanidin-containing plants caused wound-induced up regulation of flavonoid pathway regulatory genes with concomitant increase in proanthocyanidin accumulation in aspen trees (*Populus termuloides* Michx.) (Peters and Constabel, 2002) and turtle grass (*Thalassia testudinum* L.) (Arnold et al., 2008).

2.3.2. Proanthocyanidin Synthesis and Structure

Proanthocyanidin shares the early and middle steps of the flavonoid pathway with the plant pigment anthocyanin (Fig 2.2). The flavonoid pathway starts by the condensation and subsequent cyclization of one molecule of 4-coumaroyl CoA (synthezised in the phenylpropanoid pathway from phenylalanine via cinnamic acid and coumaric acid) and three molecules of malonyl CoA (which is formed by carboxylation of acetyl CoA) to form chalcone. Flavonoids, starting with chalcone, contain a 15 carbon backbone (C15) in a C6-C3-C6 skeleton which contains two phenyl rings (A ring, originating from 3 x malonyl CoA cyclization and B ring, originating from phenylalanine). These two rings are connected by a three carbon bridge which forms a third ring (C ring) by isomerization in the next step of the pathway towards naringenin (Fig 2.3).

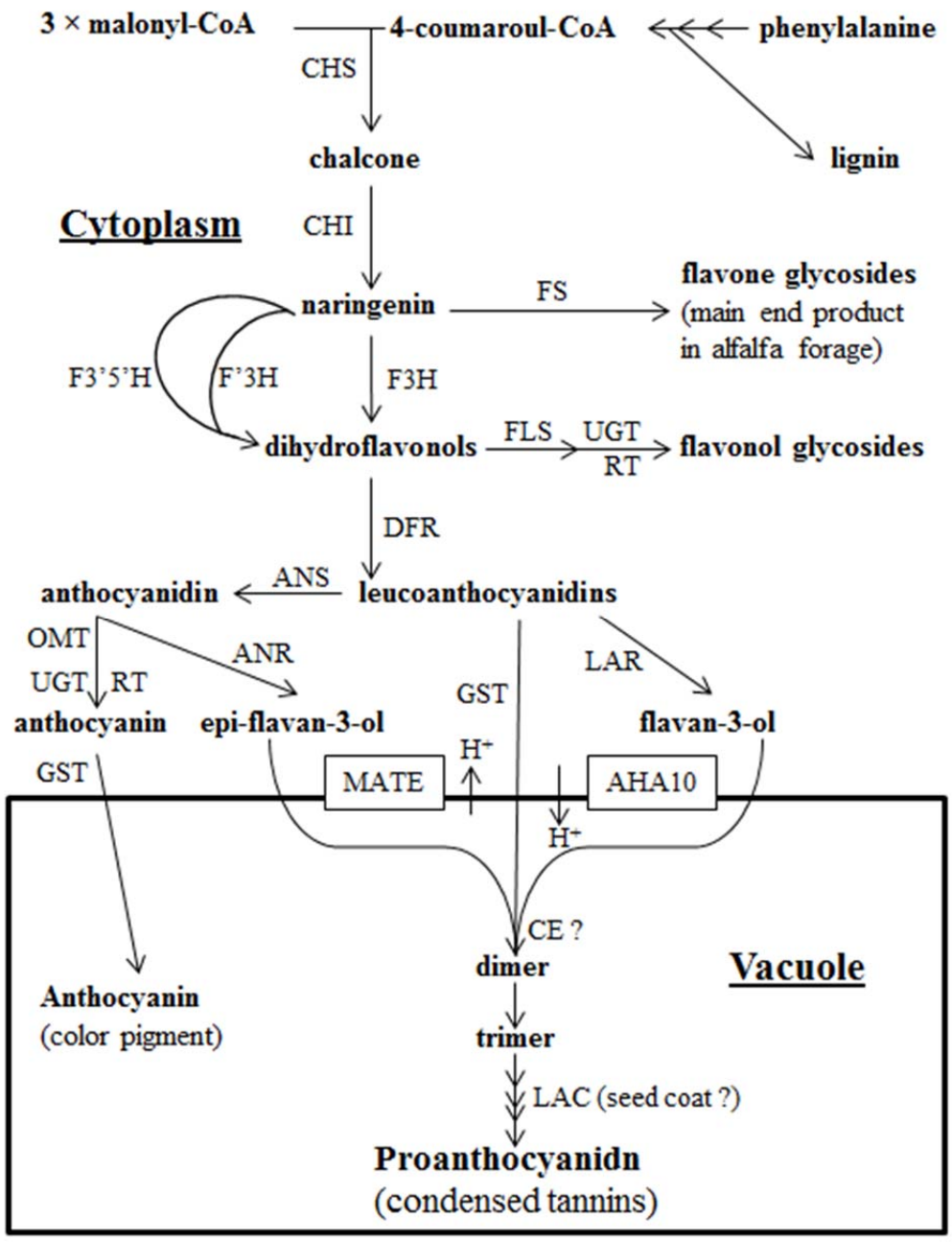
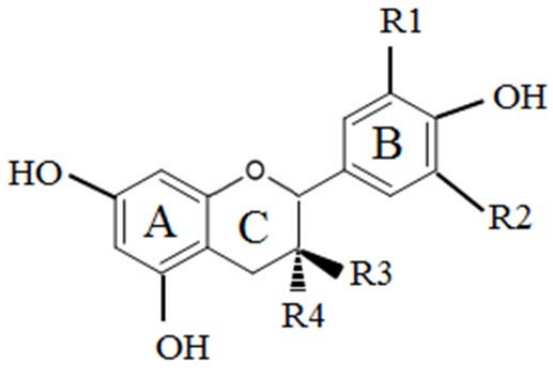
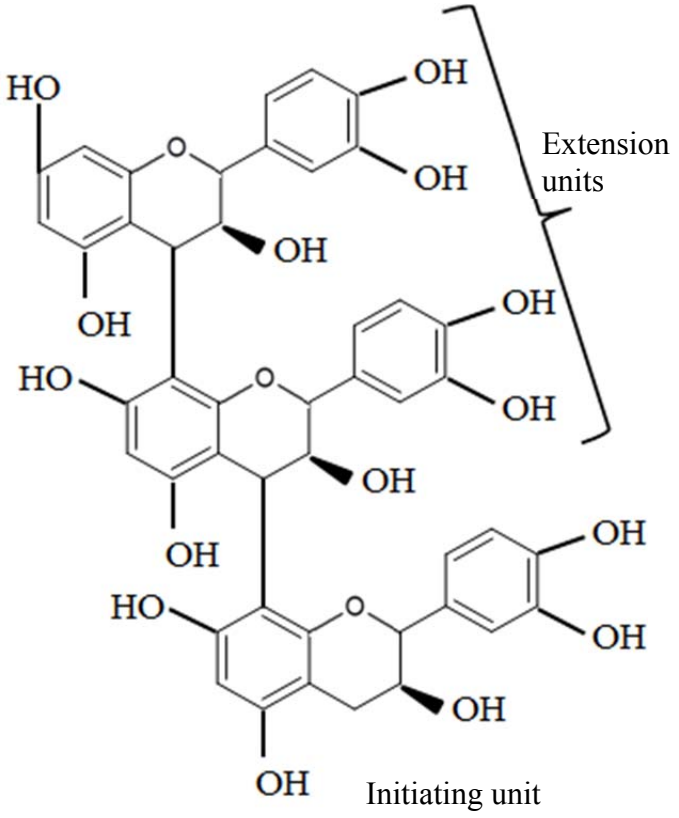


Fig. 2.2. Biosynthetic pathway of anthocyanin and proanthocyanidin. Abbreviations are for enzymes involved in the flavonoid pathway towards the synthesis of proanthocyanidin; CHS is chalcone synthase, CHI is chalcone isomerase, FS is flavone synthase, F3H is flavanone-3-hydroxylase, F'3H is flavonoid 3' hydroxylase, F3'5'H is flavonoid 3'5' hydroxylase, FLS is flavonoid synthase, UGT is UDP dependent glucosyltransferase, RT is rhamnosyl transferase, DFR is dihydroflavonol 4-reductase, ANS is anthocyanidin synthase, ANR is anthocyanidin reductase, LAR is leucoanthocyanidin reductase, OMT is *O*-methyltransferase, GST is glutathione S transferase, MATE is multidrug and toxic compound extrusion-type transporter, AHA10 is plasma membrane H^+-ATPase, CE, condensing enzyme, LAC is laccase-like flavonoid oxidase. This figure was prepared with information from Kleindt et al. (2010) and Zhao et al. (2010).

Dihydroflavonols and leucoanthocyanidin are formed in the next two steps of the pathway by hydroxylation of C3 (C ring) and reduction of C4 (C ring), respectively (Winkel-Shirley, 2001; Marles et al., 2003; He et al., 2008). Alfalfa forage is restricted to the accumulation of upper pathway flavonol glycosides which are formed from dihydroflavonols via flavonols (Stochmal et al., 2001), where as the alfalfa seed coat has a full flavonoid pathway through to proanthocyanidins (Koupai-Abyazani et al., 1993b) The building blocks of proanthocyanidin are flavan-3,4-diols (leucoanthocyanidins) which form a dimer with either flavan-3-ols (e.g. (+)-catechin and (+)-gallocatechin) (Winkel-Shirley, 2001; Tanner et al., 2003) or epi-flavan-3-ols (e.g. (-)-epi-catechin and (-)-epi-gallocatechin) (Fig. 2.3). Anthocyanidins (e.g. delphinidin and cyanidin) are the precursors for both epi-flavan-3-ols and anthocyanin (Marles et al., 2003; Xie et al., 2004).

(epi)flavan-3-ol **Proanthocyanidin**





	R1	R2	R3	R4
Procyanin:				
Catechin	H	OH	H	OH
Epicatechin	H	OH	OH	H
Prodelphinidin:				
Gallocatechin	OH	OH	H	OH
Epigallocatechin	OH	OH	OH	H

Fig. 2.3. Structure of (epi)flavan-3-ol and substitution patterns of proanthocyanidins found in legumes. This figure was prepared with information from Marles et al. (2003).

2.3.3. Effects of Proanthocyanidin in Ruminant Protein Metabolism

Proanthocyanidins are stored in the vacuole to prevent interaction with enzymes involved in plant metabolic processes. Vacuoles in forage legumes which contain proanthocyanidin are more abundant under the adaxial epidermis extending into the mesophyll and more frequently around the stomata (Fig. 2.1) (Lees et al., 1982; Lees et al., 1993). Microbes normally attach very rapidly to new feed that enters the rumen. When proanthocyanidins are present in the plant cell, attachment of microbes to the plant/feed tissue is much slower which decreases the invasion of plant tissue (cells) by microbes (Fig. 2.4) (Cheng et al., 1980). Therefore plant tissue (cells) which contains proanthocyanidins are ruptured more slowly and less extensively than plant tissue (cells) that do not contain proanthocyanidin. This reduces the accessibility of the cell content and fiber components for microbial utilization (Cheng et al., 1980; McAllister et al., 1994).

When the vacuole is ruptured through chewing or microbial digestion, proanthocyanidin can bind with surrounding proteins; mainly proteins from within the plant tissue, but also dietary, salivary and microbial protein (Fig. 2.4). The proanthocyanidin-protein complex is very resitant to digestion and utilization by ruminal microbes (Tanner et al., 1994; Min et al., 2000).

During ingestive chewing, large amounts of soluble protein are released (ruptured) from proanthocyanidin-free forages like alfalfa, perennial ryegrass and red clover, but not from proanthocyanidin-accumulating sainfoin forage. However, when PEG (polyethylene glycol; proanthocyanidin inactivator) is added, approximately 60% of the soluble protein in sainfoin forage is released, indicating that proanthocyanidins in sainfoin forage are responsible for the lower release of soluble proteins compared with the other forages tested (Mangan et al., 1976). During ingestive mastication of birdsfood trefoil and sulla by sheep, 14 and 21 g/kg DM extractable proanthocyanidin were converted into 11 and 12 g/kg DM protein bound and 3 and 6 g/kg DM free proanthocyanidin, respectively (Terrill et al., 1992a; Min et al., 1998). The proanthocyanidins that bind to protein are considered beneficial for the animal because they increase the protein flow to the lower digestive tract (Barry and Manley, 1986; Waghorn et al., 1987a) while free proanthocyanidins are considered negative because they negatively affect fiber digestion (especially hemicellulose) (Barry and Manley, 1986; Mangan, 1988). The proanthocyanidins which were still extractable after ingestive mastication were probably located in plant cells that were not ruptured yet. For birdsfoot trefoil, 17 % of the original extractable proanthocyanidin was still present after ingestive mastication in contrast to sulla (31 %) (Terrill

et al., 1992a; Min et al., 1998), which might indicate that plant tissue from birdsfoot trefoil is more easily ruptured than that of sulla.

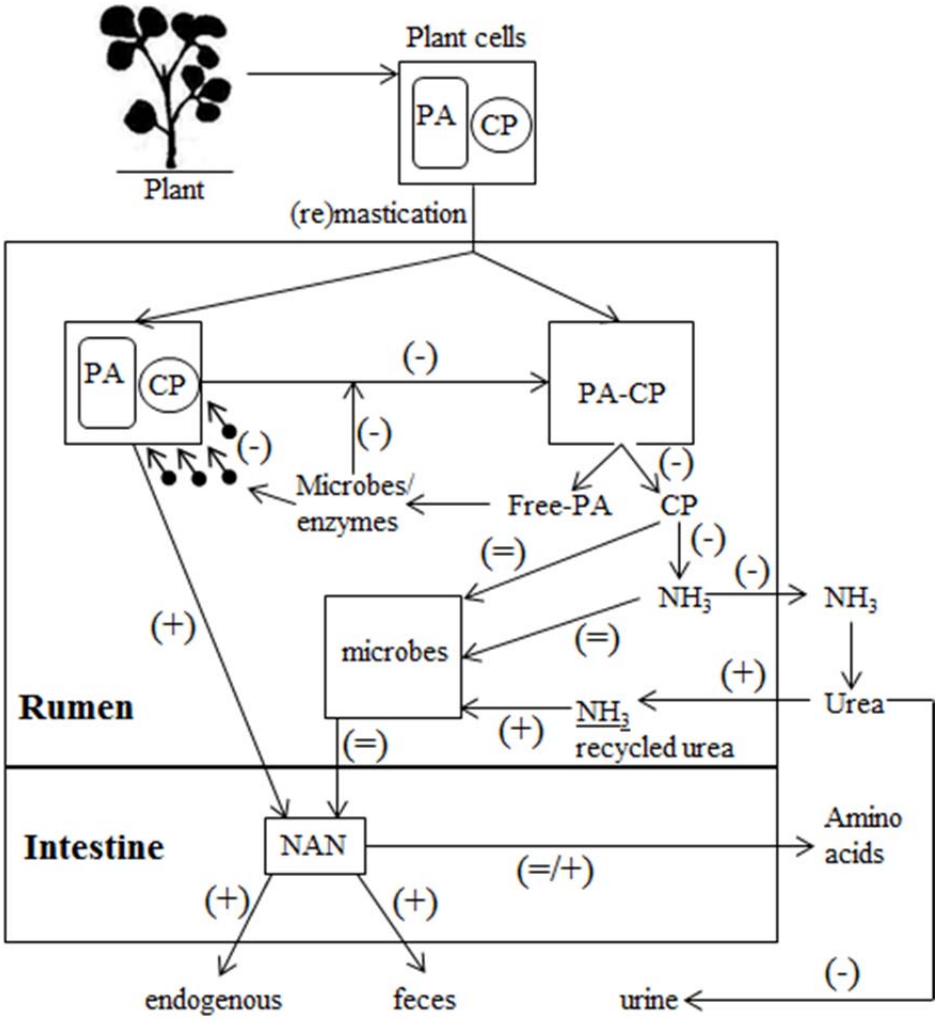


Fig. 2.4. Schematic flow chart of protein (CP) digestion from proanthocyanidin (PA)-containing forage. Symbols between brackets represent the effect of PA-containing vs. PA-free forage on protein flow: + is increased flow, - is decreased flow and = is similar flow. NAN is Non-ammonia N. This figure was prepared with information from the following references (Cheng et al., 1980; Waghorn et al., 1987b; McAllister et al., 1994; Waghorn et al., 1994; Bermingham et al., 2001).

Protein precipitation by proanthocyanidin is mostly based on hydrogen bonding between the hydroxyl groups (OH) of proanthocyanidin and amino group (-NH) of peptides or other substrates (Siebert et al., 1996) or hydrophobic interactions between the phenol ring of proanthocyanidin and the carboxyl group (COOH) of protein. Ionic interaction and covalent bonding occurs less frequently (McMahon et al., 2000). Proanthocyanidin can also bind to

metals, essential amino acids, carbohydrates, digestive enzymes and microbes, but with a lower affinity than for protein (Chung et al., 1998; Aron and Kennedy, 2008).

Normally, the ruminal degradation of the large sub-unit of Rubisco is faster than ruminal degradation of the small sub-unit of Rubisco (Tanner et al., 1994; Min et al., 2000). The presence of proanthocyanidin from sainfoin decreases the degradation of the large sub-unit of Rubisco more than it affects degradation of the small sub-unit of Rubisco (Tanner et al., 1994; Min et al., 2000). However, proanthocyanidins from birdsfoot trefoil and big trefoil do not differ in their ability to reduce microbial degradation of the small and large sub-unit of Rubisco (Aerts et al., 1999b). These suggest that proanthocyanidins from different forage legumes differ in their biological activity on ruminal Rubisco degradation.

Proanthocyanidins that dos not bind with protein is referred to as free proanthocyanidin. This free proanthocyanidin can directly interact with minerals, microbial populations and microbial enzymes and reduce the overall proteolytic activity (protein degradation) in the rumen (Scalbert, 1991; Jones et al., 1994; Tanner et al., 1994; McNabb et al., 1998; Min et al., 2002). Proanthocyanidins inhibit microbial fibrolytic, ureolytic and proteolytic enzyme activity and inhibit the growth of fungi, protozoa and some bacteria species (Scalbert, 1991; Jones et al., 1994; Min et al., 2002). Some proteolytic bacteria species are affected by proanthocyanidin, while other species seem unaffected (Jones et al., 1994; Min et al., 2002, 2005a). For example, proanthocyanidin promoted the growth of proanthocyanidin resistant gram-negative bacteria in the rat gastrointestinal tract, which was composed of 50 % resistant microbes after three weeks of offering the proanthocyanidin-containing test feed (Smith and Mackie, 2004). Microbial growth in the presence of proanthocyanidin might be decreased because of reduced availability of essential nutrients (e.g. amino acids and minerals), reduced total nutrient availability (e.g. CHO and CP), complexes formed with microbial membrane lipoproteins, and direct interactions with microbial body metabolism (Scalbert, 1991). Protozoa numbers are decreased in the presence of proanthocyanidin in the diet (Yanez Ruiz et al., 2004; Vaithiyanathan et al., 2007). Protozoa increase overall organic matter digestibility, are highly proteolytic, degrade insoluble proteins and predate on bacteria (increasing ruminal-N turnover) and reside in the rumen for a longer period than bacteria (Veira, 1986; Jouany, 1996). However, the total flow of microbial-CP to the lower digestive tract is not decreased when proanthocyanidins are present in forage (Fig 2.4) (Waghorn et al., 1994; Min et al., 2002). In the latter study, proathocyanidin-resistant

microbial growth and/or reduced protozoa number improves microbial efficiency. Defaunation of protozoa from the rumen on its own was previously found to increase microbial protein flow to the lower digestive tract (Veira, 1986; Jouany, 1996).

Sheep fed with proanthocyanidin-accumulating forage have a lower ruminal NH_3 and soluble protein concentration than sheep fed the same diet plus PEG (Waghorn et al., 1987b; Waghorn et al., 1994; Min et al., 2002). This can be the result of reduced dietary protein availability and overall proteolytic activity in the rumen (Min et al., 2002). Several studies also found that when proanthocyanidin accumulation increases, the protein content of the forage decreases (Barry and Manley, 1984b; Miller and Ehlke, 1995; Grabber, 2009), which reduces the direct oversupply of protein. Sheep with a lower ruminal NH_3 concentration have higher urea-N recycling and higher incorporation of recycled urea-N into microbial mass (Fig. 2.4) (Waghorn et al., 1994; Kiran and Mutsvangwa, 2011). This might be another explanation of why presence of proanthocyanidin in the diet does not decrease overall flow of microbial-CP to the lower digestive tract. Decreased ruminal NH_3 concentration in cattle fed proanthocyanidin-containing forage decreases urinary-N output and increases fecal-N output (Fig. 2.4) (Waghorn, 2008; Woodward et al., 2009; Theodoridou et al., 2010). Fecal-N is less prone to volatilization and leaching into ground water than urine-N, therby reducing environmental impact of animal N excretion (Hoekstra et al., 2007; Woodward et al., 2009).

2.3.4. Protein Precipitating Capacity of Proanthocyanidin and its Effect on Intestinal Protein Adsorption

The composition of proanthocyanidin varies with linkage between the flavonoid monomers (C4 to C6 or C4 to C8) and with stereochemical variation at carbons 2, 3 and 4 and the number of hydroxyl groups on the A and B ring (Fig. 2.3). These differences in proanthocyanidin composition affect its molecular structure and influence the capability to interact with other molecules, such as protein. Protein precipitating capacity (PPC) by proanthocyanidin increases when proanthocyanidins are at a higher concentration or have a higher molecular weight, a larger degree of polymerization or a higher prodelphinidin:procyanidin ratio (Table 2.4) (Bate-Smith, 1973a; Jones et al., 1976; Horigome et al., 1988; McAllister et al., 2005). Jones et al. (1976) found that PPC increased with increased prodelphidin content when testing several *Trifolium* species and big trefoil.

Table 2.4. Proanthocyanidin concentration, structure and protein precipitating capacity of several temperate forage legumes

Trait	Legume species					
	Sainfoin	Birdsfoot trefoil	Big trefoil	Alfalfa	White clover	Red clover
	Forage					
Proanthocyanidin (g/kg)[1]						
Extractable	44	7-36	61	0	ND	0.4
Protein-bound	38	9-13	14	0.5	ND	0.6
Fiber bound	5	2-3	1	0	ND	0.7
Total	87	21-47	77	0.5	6-12	1.7
	Forage			Seed	Flower	
Molecular weight (kDA)[2]	5.1	3.3	4.4	3.6	ND	ND
No. of monomers[3]	7	7	8	5	10	9
Main polymer[3]	Pdelph	Pcyanid	Pdelph	Pcyanid	Pdelph	Pcyanid
Extender unit (%)[3]						
Catechin	0	0	0	0	0	6
Epicatechin	12	67	19	92	0	81
Gallocatechin	13	0	13	0	39	6
Epigallocatechin	60	30	64	0	56	7
Terminal unit (%)[3]						
Catechin	21	82	50	92	0	95
Epicatechin	28	16	20	0	0	5
Gallocatechin	34	0	20	0	48	0
Epigallocatechin	17	0	10	0	52	0
Proanthocyanidn (μg) required to precipitate one mg protein[2]						
Alfalfa Rubisco	50	80	72	108	ND	ND
Bovine serum albumin	269	436	323	348	ND	ND

ND is not determined

[1]Values for sainfoin and birdsfoot trefoil from Scharenberg et al. (2007), for birdsfoot trefoil and big trefoil from Terrill et al. (1992b), for birdsfoot trefoil, alfalfa and red clover from Jackson et al. (1996) and for white clover from Burggraaf et al. (2008).

[2]Molecular weight and protein precipitating capacity (μg proanthocyanidin needed to precipitate 1 mg of alfalfa Rubisco protein or bovine serum albumin) adapted from McAllister et al. (2005)

[3]Values for sainfoin from Koupai-Abyazani et al. (1993c), for birdsfoot trefoil from Foo et al. (1996), for big trefoil from Foo et al. (1997), for alfalfa seed coat from Koupai-Abyazani et al. (1993b), and for white and red clover from Sivakumaran et al. (2004); no. of monomers is the average number of monomers in proanthocyanidin; Pdelph is prodelphidinin and Pcyanid is procyanidin.

McAllister et al. (2005) found that PPC with alfalfa Rubisco protein and bovine serum albumin (BSA) was highest for proanthocyanidin from sainfoin, and lowest for alfalfa seed coat

proanthocyanidin with Rubisco and lowest for birdsfoot trefoil proanthocyanidin with BSA (Table 2.4).

Aerts et al. (1999b) suggested based on their results that proanthocyanidin molecular weight was relatively more important for PPC, while monomer composition of proanthocyanidin was relatively more important in the interaction with microbes.

Proanthocyanidins form stable complexes with proteins from different sources at a pH between 3.5 and 7.0 (Jones and Mangan, 1977) which occurs in the rumen (Bergman, 1990) and ileum (Boerner et al., 1987). The total amount of dietary protein escaping ruminal degradation into the lower digestive tract was found to be higher for proanthocyanidin-containing forage without PEG than in the presence of PEG (Waghorn et al., 1987b; Waghorn et al., 1994; Bermingham et al., 2001). Protein is released from the proanthocyanidin complex at a pH of < 3 (Jones and Mangan, 1977) which occurs in the abomasum (Wheeler and Noller, 1977; Van Winden et al., 2002) and proximal duodenum (Boerner et al., 1987) and at a pH of > 8 which occurs with pancreatic secretion (Mangan, 1988).

The change in site of protein digestion due to dietary proanthocyanidin results in a overall increased digestion and absorption of amino acids in the small intestine of sheep eating birdsfoot trefoil (Waghorn et al., 1987a,b) and sulla (Bermingham et al., 2001) but not when sheep eat big trefoil (Waghorn et al., 1994) and sainfoin (Bermingham et al., 2001). Kariuki and Norton (2008) found that proanthocyanidin from *Leucaena leucocephala* L. had a lower PPC with bovine serum albumin but this complex had a higher true digestibility between abomasum and distal ileum than when proanthocyanidin originated from *Leucaena pallida* L. The data in Table 2.4 indicates a higher PPC with bovine serum albumin and proanthocyanidin from sainfoin and big trefoil than from birdsfoot trefoil. Based on the results of Kariuki and Norton. (2008), the lower PPC of proanthocyanidin from birdsfoot trefoil might result in higher digestibility of protein which was bound to proanthocyanidin between abomasum and distal ileum than from sainfoin and big trefoil. These might be an explanation why amino acid absorption in the small intestine increased when feeding birdsfoot trefoil and not when feeding sainfoin and big trefoil.

2.3.5. Other Effects of Proanthocyanidin in Ruminant Diets

Besides a negative relationship with protein, other relationships exist between proanthocyanidin and nutritional comonents of forage. A positive correlation exists in some

forages between proanthocyanidin accumulation and forage lignin content (Barry and Manley, 1986); forage lignin content is negatively related to its digestibility (Jung et al., 1997). In general, volatile fatty acid production is either similar or reduced by proanthocyanidin-containing forage but with a reduced acetate:propionate ratio (Wang et al., 2006a; Waghorn, 2008) indicating increased availability of glucogenic precursors for production. Feeding forage that contains proanthocyanidins decreases methane emissions in sheep grazing sulla and big trefoil (Waghhorn et al., 2002; Waghorn and Clark, 2006) and in dairy cows grazing sulla and birdsfoot trefoil (Woodward et al., 2000, 2002, 2004). The decreased methane emission with proanthocyanidin-containing forage is probably due to a reduced amount of forage substrate fermented in the rumen, a shift in fermentation end products (reduced H^+-producing acetate and more H^+-utilizing propionate), and/or direct inhibition of the growth of methanogenic bacteria or a decrease in symbiotic-associated protozoa numbers (Scalbert, 1991; Johnson and Johnson, 1995; Bhatta et al., 2009). Reducing methane emissions reduces the contribution to global warming and improves the energy efficiency of the animal (Johnson and Johnson, 1995; Moss et al., 2000). However, methane emissions from steers grazing sainfoin was only reduced in one of three years compared with steers grazing alfalfa/grass mixtures (Iwaasa, 2007, 2008). The addition of up to 2% quebracho proanthocyanidin to beef cattle forage-based diets also did not lower methane emission (Beauchemin et al., 2007). Additional studies are required to understand the differences in methane emission from animals grazing forages with different proanthocyanidin structure-concentrations and nutrient composition and cultivated in different climates and different geographical locations.

Flavonoids from the lower part of the flavonoid pathway, including anthocyanidin and proanthocyanidin, have antimicrobial activity on pathogenic gram-negative bacteria (Puupponen-Pimiä et al., 2001), as well as strong antioxidant activity (Aron and Kennedy, 2008; Butelli et al., 2008), anti-inflammatory activity (Rice-Evans et al., 1996) and the ability to change cell signaling pathways (Williams et al., 2004). Livestock consuming these flavonoids might experience beneficial effects important for the overall health of the animal.

Sheep and cattle offered birdsfoot trefoil or big trefoil with proanthocyanidin concentrations between 2 and 5 % showed improved animal performance (Table 2.5). This could be the result of improved energy efficiency due to reduced methane (energy) emission and/or reduced energy cost for urea synthesis, increased amino acid absorption in the small intestine, or improved

overall animal health status. However, when the proanthocyanidin concentration in birdsfoot trefoil and big trefoil rises above 5%, animal performance decreases due to decreased dry matter intake and/or excessively decreased digestion and availability of nutrient over the whole digestive tract (Aerts et al., 1999a; Waghorn, 2008). Sainfoin (hay) seems to be a palatable forage which is preferred by ruminants even if it has high proanthocyanidin levels (Hartnell and Satter, 1979; Parker and Moss, 1980; Khalilvandi-Behroozyar et al., 2010). Sainfoin might not have a reduced dry matter intake and animal performance at proanthocyanidin levels above 5%.

Table 2.5. Effect of feeding birdsfoot trefoil and big trefoil with proanthocyanidin concentrations between 2 and 5 % on performance of sheep and cattle

Trait	Reference
Sheep	
Increased wool growth	(Wang et al., 1996a,b; Min et al., 1998, 1999)
Increased milk yield	(Wang et al., 1996a)
Increased ovulation rate/no. lambs born	(Min et al., 1999; Ramírez-Restrepo et al., 2005a)
Increased lamb weight gain	(Wang et al., 1996b; Ramírez-Restrepo et al., 2005b)
Reduced intestinal parasite load	(Min et al., 1999; Min and Hart, 2003)
Cattle	
Increased milk production	(Harris et al., 1998; Woodward et al., 1999, 2004, 2009)
Increased milk protein production	(Harris et al., 1998; Woodward et al., 1999)
Decreased milk fat production	(Woodward et al., 1999)
Reduced milk saturated fatty acids	(Turner et al., 2005)
Increased milk omega-3 fatty acids	(Turner et al., 2005)
Increased weight gain	(Wen et al., 2002)

Many characteristic bloat-free legumes contain proanthocyanidin (Fay et al., 1980; Lees, 1992; Coulman et al., 2000). The proanthocyanidin-protein complex decreases the release of protein in the rumen. This reduces the amount of protein available at the gas-liquid interface (Tanner et al., 1994; Aerts et al., 1999b) and decreases foam formation and stability (Fay et al., 1980; Tanner et al., 1995; Min et al., 2005b) and substrate availability for ruminal microbes, with a consequent reduction in gas production (McMahon et al., 2000; Min et al., 2005b). Lysis of protozoa and gram-negative bacteria in the rumen releases foam provoking material and exotoxins which may play a role in the formation of pasture bloat (Clarke and Reid, 1974; Nagaraja et al., 1976). Numbers of protozoa and gram-negative bacteria are decreased by proanthocyanidin as described above. Also, the growth of the viscous slime-producing bacteria *S. bovis* is impaired by the

presence of proanthocyanidin (Min et al., 2006). According to Li et al. (1996), bloat-provoking legumes should contain a proanthocyanidin concentration of approximately 0.5%, or higher, in order to be bloat-safe.

2.4. Proanthocyanidin in Alfalfa Forage

2.4.1. Alfalfa-Proanthocyanidin Containing Forage Mixtures

Proanthocyanidins do not usually accumulate in alfalfa forage (Goplen et al., 1980), but they can be introduced into livestock diets by mixing proanthocyanidin containing forage species into alfalfa pastures (Wang et al., 2006a). In grazing experiments, mixed alfalfa pastures with 9.1 to 35.5% sainfoin (1.8 to 8.6 g/kg proanthocyanidin) reduced the incidence of bloat as the percentage of sainfoin in the pasture increases. Cattle grazing this mixed pasture had decreased ruminal ammonia concentration, decreased acetate:propionate ratio and decreased ruminal proteolytic activity (Wang et al., 2006a). However, mixing sainfoin into alfalfa pastures does not fully eliminate pasture bloat (McMahon et al., 1999; Wang et al., 2006a), likely because of competition between plant species (changing the proanthocyanidin content ingested by the animal) and/or plant selection by grazing cattle (Wang et al., 2006a). Performance of grazing sheep was improved in birdsfoot trefoil/alfalfa mixed pastures (0.8-1.0% proanthocyanidin) compared with pure alfalfa pastures, but was superior in pure birdsfoot trefoil pastures (3-6% proanthocyanidin) (Douglas et al., 1995). When forage contains up to 9% proanthocyanidins, approximately 90% of it complexes with host plant constituents (mainly protein) during digestion and only 10% is released as free-proanthocyanidins (Barry and Manley, 1986). Free-proanthocyanidins are required in the mixture to improve the degradation characteristics of non-proanthocyanidin accumulating forage species; its limitation may explain the limited benefit of mixing proanthocyanidin accumulating forages with alfalfa. Proanthocyanidin content of the co-forage should probably be very high (>9 %) in order to be successful at affecting ruminal degradability from mixtures with alfalfa (Barry and McNabb, 1999). Therefore it would be beneficial to develop alfalfa that produces moderate amounts of proanthocyanidin in its forage to improve ruminant livestock production.

2.4.2. Relation of the Lower Flavonoid Pathway to Stimulate Anthocyanin and Proanthocyanidin

The synthesis of proanthocyanidin in the flavonoid pathway is regulated by many regulatory genes at the transcriptional level. The regulatory genes involved in the flavonoid pathway code for helix-loop-helix proteins (*βHLH*), *Myb*-like proteins and WD40-like proteins. The complex relation between regulatory genes and structure proteins involved in the regulation of the flavonoid pathway of *Arabidopsis* is displayed in Fig 2.5.

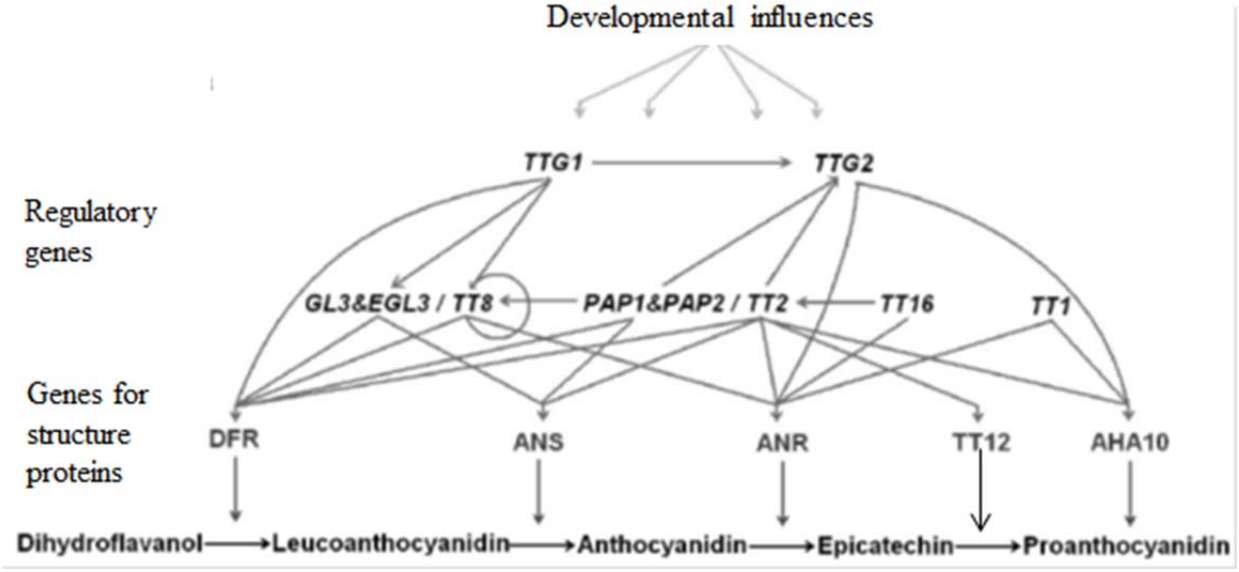


Fig. 2.5. Interrelation among the regulatory genes and the genes for structure proteins of *Arabidopsis thaliana* adapted from He et al. (2008).

In maize (*Zea mays*) several genes regulate its flavonoid pathway. These code for *βHLH* proteins (*Sn*, *B-Peru, Lc*), *Myb*-like proteins (*C1*) and WD40-like proteins (*PAC1*) (Quattrocchio et al., 2006). Over-expression of the maize *βHLH* gene in birdsfoot trefoil increased the number of proanthocyanidin-containing cells by 50 fold and its leaf concentration was increased by 1% (Robbins et al., 2003).

Alfalfa contains proanthocyanidin in the seed coat (Koupai-Abyazani et al., 1993b) proving the existence of the flavonoid pathway in alfalfa. Resently, alfalfa was transformed with the *Myb*-like legume anthocyanin-producting gene (*LAP1*) from *Medicago truncatula* (Peel et al., 2009). Small but stable amounts of proanthocyanidin-like structures without (-)-epicatechin extension units and multiple glycosylated conjugates of cyanidin were detectable in this deep-

purple *LAP1*-transformed alfalfa. In addition, large number of regulatory genes involved in the flavonoid pathway were induced (Peel et al., 2009).

Ray et al. (2003) transformed alfalfa with three flavonoid pathway regulatory genes of maize (*C1, Lc,* and *B-Peru*). Only the *Lc* (*βHLH*) regulatory gene stimulated the accumulation of anthocyanidin under field conditions (97-136 µg/g DM) (Wang et al., 2006b) and anthocyanidin (152 µg/g fresh) and proanthocyanidin (307 µg/g fresh; ~1.5 g/kg DM) after indoor shift to high light intensity (Ray et al., 2003). A detailed phytochemical analysis of these genotypes indicated variation in flavone glycosides between the *Lc* (A01-88)-genotypes and in one genotype a two-fold increase in total saponin content (Oleszek and Gruber; unpublished data). Three of these *Lc*-alfalfa genotypes grown in the field had a reduced initial rate of nitrogen and DM degradation *in vitro* in rumen liquid compared to their non-transgenic parent genotype, but the extent of N and DM degradation was unaffected (Wang et al., 2006b). However, proanthocyanidin concentration in the *LAP1*-alfalfa and *Lc*-alfalfa genotypes was far below levels needed to guarantee bloat safety and to beneficially affect protein metabolism. Hence, further improvements are required to develop a alfalfa cultivar that accumulates a higher amount of proanthocyanidin in its forage.

Chapter 3

Nutrient Composition and Degradation Profiles of Anthocyanidin-Accumulating *Lc*-Alfalfa Progeny*

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3.1. Abstract

Alfalfa (*Medicago sativa* L.) is one of the most used forages in the world but suffers from the disadvantage of having poor protein utilization by the animal. The poor protein utilization is the result of excessive ruminal protein degradation which might be reduced by the protein precipitating capacity of mono and polymeric (pro)anthocyanidin. The objective of this study was to determine the effects of the *Lc*-transgene on plany survival, mono/polymeric anthocyanidin and chemical profiles in three *Lc*-alfalfa progeny. These were compared with their non-transgenic (NT) parental cultivars, Rangelander, Rambler, and Beaver. *Lc*-alfalfa forage accumulated enhanced amounts of anthocyanidin, with an average concentration of 197.4 µg/g DM, while proanthocyanidin was not detected. Both of these metabolites were absent in the NT-parental cultivars. *Lc*-alfalfa had a lower (24.8 vs. 27.3 %DM) crude protein (CP) and higher (58.3 vs. 55.5 %DM) carbohydrate (CHO) concentration, which resulted in the decreased N:CHO ratio (68.1 vs. 79.2 g/kg) compared with NT-alfalfa. Slowly degradable N:CHO ratio was decreased by 5.9 g/kg and total rumen-degradable N:CHO ratio was decreased by 12.9 g/kg in *Lc*-alfalfa compared with NT-alfalfa. In conclusion, *Lc*-gene transformation resulted in the accumulation of anthocyanidin, decreased total protein content, increased total carbohydrate content and improved the balance between nitrogen and carbohydrates in *Lc*-alfalfa progeny compared to their parental NT-alfalfa cultivars.

Key words: Alfalfa, Anthocyanidin, *Lc*-transgene, Protein and carbohydrate sub-fractions, Nitrogen-to-carbohydrate degradation ratios

3.2. Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important forages in the world (Smith et al., 2000; Wang et al., 2006b) because of high yield and high nutrient levels (McMahon et al., 2000; Popp et al., 2000; Yu et al., 2003a,b). A major disadvantage of grazing cattle on alfalfa is its poor protein utilization (Broderick, 1995; Yu et al., 2004) and its tendency to cause pasture bloat (Popp et al., 2000; Wang et al., 2006b). Pasture bloat arises from rumen fermentation gases which become trapped in a stable protein foam that prevents normal eructation of gas via the oesophagus by the animal (Cockrem et al., 1987; Tanner et al., 1995).

Characteristic bloat-free legume forages like sainfoin (*Onobrychis viciifolia*), sulla (*Hedysarium coronarium*), birdsfoot trefoil (*Lotus corniculatus*), and big trefoil (*Lotus pedunculatus*) contain proanthocyanidin (Majak et al., 1995; Aerts et al., 1999; McMahon et al., 2000). Proanthocyanidin complexes with protein in the rumen and reduces ruminal protein degradation, which can increase the efficiency of protein utilization (Broderick, 1995; Aerts et al., 1999) and reduce *in vitro* foam stability (Fay et al., 1980; Tanner et al., 1995). Forage species with proanthocyanidin reduce the incidence of bloat, but do not fully eliminate it in mixed alfalfa pastures because of species competition in the mixture and/or specific plant selection by cattle (Wang et al., 2006a). Therefore, development of an alfalfa cultivar with moderate amounts of proanthocyanidin would be highly beneficial for ruminant livestock producers who wish to graze cattle on alfalfa pastures.

In most alfalfa germplasm, proanthocyanidin accumulates in the seed coat (Koupai-Abyazani et al., 1993b) and the forage is restricted to accumulating upper flavonoid pathway flavone glycosides (Stochmal et al., 2001). Ray et al. (2003) transformed alfalfa with the 2.4 kb maize anthocyanidin regulating *LEAF COLOR* (*Lc*) gene. T_0 *Lc*-alfalfa genotypes usually have a purple-green phenotype and stimulate the accumulation of monomeric anthocyanidin and small amounts of polymeric anthocyanidin in the forage under outdoor light intensities or cold temperatures. Wang et al. (2006a) showed that field grown T_0 *Lc*-alfalfa genotypes had a reduced initial rate of nitrogen (N) and dry matter (DM) degradation *in vitro* compared to their NT-parent genotype, but the extent of N and DM degradation was unaffected. However, the eastern Canadian adapted background of T_0 *Lc*-alfalfa compromised survival under cold climate conditions prevalent in western Canada.

In 2006, three T_0 *Lc*-alfalfa genotypes with similar saponin concentration and profile and unique flavonoid profiles (Gruber et al. unpublished data) were cross-pollinated with three western Canadian cultivars to develop new winter-hardy T_1 populations and to facilitate the movement of the *Lc*-gene into a broader spectrum of alfalfa breeding germplasm. The objective of the study was to determine survivability, mono/polymeric anthocyanidin and basic chemical and nutritional profiles of these newly developed *Lc*-populations and compared to their non-transgenetic (NT) western Canadian parent cultivars.

3.3. Material & Methods

3.3.1. Plant Material

T_1 transgenic *Lc*-alfalfa populations were developed at Forage Genetics International, Wisconsin, by manual crossing. Crosses were made between three T_0 transgenic *Lc*-alfalfa genotypes, A01-88-01, A01-88-09 and A01-88-19 and several pre-selected genotypes, each from three non-transgenic western Canadian-adapted alfalfa cultivars (Beaver, Rambler and Rangelander) to generate Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4 (Table 3.1). The male T_0 parents, A01-88-01 (*Lc*1), A01-88-09 (*Lc*3) and A01-88-19 (*Lc*4) described in Wang et al. (2006b) were three genotypes out of a range of independently generated transgenic plants developed previously by introducing the 2.4 kb maize (*Zea mays*) *β-Helix-Loop-Helix* (*bHLH*) *LEAF COLOR* (*Lc*) gene into an eastern Canadian alfalfa genotype N4-2-2 (also known as A01). The inserted *Lc*-gene version contained a 200 bp 5'-untranslated region which enhanced anthocyanidin accumulation in alfalfa (Ray et al., 2003). The female parents were elite winter-hardy representative genotypes selected in 2005 from three western Canadian adapted alfalfa cultivars Beaver, Rambler and Rangelander. Six MB5 Beaver genotypes, two MB5 Rambler genotypes, and two MB5 Rangelander genotypes were selected from plants established in 2004 at a Manitoba nursery. Seeds from these female parent genotypes were bulked per cultivar and used as *Lc*-alfalfa population. Parental western Canadian adapted cultivar control populations Beaver, Rambler and Rangelander used in our study were randomly selected from nurseries in Saskatoon. The experiment was set up in a completely randomized design with three harvest replications used for the three NT-alfalfa populations and four (harvest one was split up in two pools) replications for the three *Lc*-alfalfa populations.

The *Lc* and NT alfalfa populations were grown from seed for approximately six months in a greenhouse. Sheared plants were transplanted on August 24, 2006 and June 5, 2007 into an experimental field (75 cm inter-row and 15 cm inter-plant spacing) approved by the Canadian Food Inspection Agency (CFIA). The field site was located in the dark brown soil zone at the Saskatoon Research Centre farm of Agriculture and Agri-Food Canada (AAFC). Survival rates were calculated for the *Lc*-populations as the number of surviving plants at the first mid-summer harvest in 2007, 2008 and 2009. Survival rate was not calculated for NT-populations because of the small number of plants planted.

Table 3.1. Alfalfa population identity, *Lc*-transgene, phenotype and no. of plants used

		Lc transgene[1] (no. plants)			Phenotype (no. plants)	
Alfalfa population origin	Name	+	-	*nt*	Purple-green	Green
A01-88-01 x Beaver	Beav*Lc*1	52	11	16	25	38
Beaver	NT1	0	9	0	0	9
A01-88-09 x Rambler	Ramb*Lc*3	70	5	11	35	40
Rambler	NT3	0	9	0	0	9
A01-88-19 x Rangelander	Rang*Lc*4	53	14	7	33	34
Rangelander	NT4	0	8	0	0	8

[1]Number of plants with or without an *Lc*-transgene (2007): +, present; -, not present; *nt*, not tested (plants died before first harvest).

Alfalfa forage was harvested manually with shears at *ca.* 5 cm above ground level in 2007 on July 4 and 25 and August 15 and 25 and in 2008 on July 24 and August 13 at a vegetative pre-bud stage of growth (according to CFIA-imposed confined field trail conditions). Alfalfa plants in 2007 were harvested individually into plastic bags, placed immediately on dry ice, stored at -80 °C and analyzed for the *Lc*-transgene by a polymerase chain reaction (PCR) using *Lc*-specific primers 5`- GGC CAA GAG CGC GTC CAT TCA G -3` (forward) and 5`- CCG CCG GCC ACC ACT TTC TTC -3` (reverse). Subsequently, *Lc*-positive plants were phenotyped in the field based on color (green and purple-green; Table 3.1) and the 2007 harvests were pooled per phenotype within each population to obtain sufficient material for crude chemical and nutritional analysis. Phenotypes per population for each 2008 harvest were analyzed separately. Purple-green *Lc* samples and green NT samples were freeze-dried and ground to pass through a 1 mm screen (Retsch SM-3000, Brinkmann Instruments, ON, Canada). Green *Lc*-positive transgenic plants were not analyzed in this study.

3.3.2. Phytochemical and Chemical Analysis

Extractable proanthocyanidin was determined as anthocyanidin released from the supernatant of 1 g ground forage extracted with 80 % aqueous MeOH, bound to polyvinylpolypyrrolidone (Sigma Aldrich, Mississauga, Canada), and hydrolyzed with butanol:HCl (70:30 v/v) after a wash to remove interfering anthocyanidin (Watterson and Butler, 1983). Unextractable proanthocyanidin was determined as anthocyanidin released after butanol:HCl hydrolysis on the 80% aqueous MeOH centrifugation pellet. Anthocyanidin was measured as the differential between the absorbance measured at 550 nm in a SPECTRAmax PLUS^{384} spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) after hydrolysis of duplicate samples at 70 °C and room temperature for 1 h. The anthocyanidin found in the room temperature sample is regarded to originate from easy hydrolysable anthocyanins while proanthocyanidin are hydrolyzed to anthocyanidin at 70 °C. Therefore, concentrations were calculated relative to a standard curve of extracted alfalfa Rambler seed coat proanthocyanidin (Ray et al., 2003), and the differential absorbance (70 °C sample – room temperature sample) was used to remove interference from any residual anthocyanidin.

Anthocyanidin concentration was determined as described by Wang et al. (2006b) on 0.5 g of ground forage extracted in 30 mL of methanol-HCl (99:1 v/v) at 4 °C for 24 h. The anthocyanidin extract was filtered through a Whatman #1 filter paper (Whatman, Middlesex, UK) and the filtrate absorbance measured at 530 nm against methanol:HCl (99:1 v/v) in an Aquamate spectrophotometer (Thermo Scientific, Rochester, NY, USA) relative to a standard curve of cyanidin chloride (Alexis Biochemicals, San Diego, CA, USA). Mean absorbance for the NT-alfalfa extracts was subtracted from the *Lc*-alfalfa samples to adjust for background absorbance not due to anthocyanidin.

Alfalfa forage was analyzed for crude nutritional profiles according to standard procedures described by the Association of Official Analytical Chemists (AOAC, 1990). Dry matter and ash residue weights were determined after oven drying of forage samples at 135 ºC for 2 h (AOAC 930.15) and ashing of forage samples at 550 ºC overnight (AOAC 942.05), respectively. Ether extract weight (EE) was determined after refluxing forage in ethoxyethane for 6 h, followed by oven drying of the extract at 100 ºC for 30 min (AOAC 954.02). Crude protein (CP = N x 6.25) was measured by Kjeldahl-N analysis on a Kjeltic 1030 N autotitrator (FOSS, Höganäs, Sweden) using $CuSO_4$ as a catalyst (AOAC 984.13).

Neutral detergent fiber (NDF; AOAC 2002.04) and acid detergent fiber (ADF; AOAC 973.18) were determined as filter residue weights after boiling alfalfa forage samples for 1 h in beakers either in neutral detergent solution supplemented with heat-stable α-amylase (Ancom Technology, Macedon, NY, USA) or in acid detergent solution, followed by vacuum filtration through a Whatman #54 filter paper and oven drying of the filter residue at 105 ºC for 2 h according to Van Soest et al. (1991). Acid detergent lignin (ADL) was determined as the residue weight after soaking the ADF filter bag residue in sulphuric acid for 3 h according to the ANKOM A200 Filter Bag technique (Ankom Technology, Fairport, NY, USA), followed by nine washes with water and oven drying of the filter bag residue at 105 ºC for 2 h (AOAC 973.18). Neutral detergent-insoluble protein (NDIP) and acid detergent-insoluble protein (ADIP) were determined by Kjeldahl-N analysis of the NDF and ADF filter residue, respectively, as described by Licitra et al. (1996). Neutral detergent fiber and ADF were adjusted to more accurately reflect true neutral and acid fiber components (NDF, composed mainly of ADF + hemicellulose; ADF, composed mainly of ADL + cellulose) by subtracting unextractable protein NDIP and ADIP, respectively (but not ash).

Ethanol-insoluble residue (EIR) was determined as the filter residue weight after extracting 0.5 g of forage sample in 100 ml of 80 % ethanol at toom temperature for 4 h, followed by filtration through a Whatman #54 filter paper and oven drying of the filter residue at 105 ºC for 2 h (Hall et al. 1997; 1999). The 80 % ethanol filtrate was analyzed for ethanol-soluble carbohydrates (ESC) by measuring the absorbance at 490 nm in a SPECTRAmax PLUS^{384} spectrophotometer using a phenol-sulfuric acid assay (DuBois et al., 1956; Buysse and Merckx, 1993) and a sucrose standard (Sigma Aldrich, Mississauga, Canada). Ethanol-insoluble residue CP was determined by Kjeldahl-N analysis of the EIR filter residue as described by Hall et al. (1997; 1999). Ethanol insoluble residue was adjusted to reflect its true fiber component (EIR, composed mainly of NDF + soluble fiber) by subtracting ethanol unextractable protein (but not ash). Neutral detergent-soluble fiber (NDSF) was calculated by subtracting NDF from EIR (Hall et al. 1997; 1999).

Non-protein N (NPN) was calculated as the difference between total CP and trichloroacetic acid (TCA) precipitable-CP after precipitation of true protein from ground forage incubated overnight with 10 % TCA, followed by filtration through Whatman #54 filter paper and Kjeldahl-N analysis of the filter residue (Licitra et al., 1996). Soluble crude protein (BSP) was

calculated as the difference in CP between total CP and buffer residue-CP after extraction of ground forage with sodium bicarbonate/phosphate buffer (12.2 g/L $NaH_2PO_4.H_2O$ and 8.91 g/L $Na_2B_4O_7.10H_2O$, pH 6.7) at 39 ºC for 1 h, followed by filtration through Whatman #54 filter paper and Kjeldahl-N analysis of the filter residue (Roe et al., 1990).

Non-fiber carbohydrates (NFC, composed mainly of ESC, organic acids, NDSF and starch, [NFC =100– (NDF + CP + EE + Ash)] and total carbohydrates [CHO = 100 - (CP + EE + Ash)] were calculated according to NRC-Dairy (2001).

3.3.3. Protein and Carbohydrate Profiling

The Cornell Net Carbohydrate and Protein System (CNCPS) was used to model the division of alfalfa CP and CHO each into five sub-fractions based on degradation characteristics (Sniffen et al., 1992; Fox et al., 2004; Lanzas et al., 2007a; Tylutki et al., 2008). Crude protein was divided into five sub-fractions (Table 3.2): instantaneously solubilized protein A (PA), completely undegradable CP (PC) and true CP fraction (PB). True protein fraction PB was further sub-divided based on degradation characteristics into rapidly degradable (PB1), intermediately degradable (PB2), and slowly degradable (PB3) true protein.

While CNCPS_v6 normally divides CHO into eight sub-fractions (Lanzas et al. 2007a; Tylutki et al. 2008), in the case of fresh alfalfa, volatile fatty acids (CA1) and lactic acid (CA2) are both not present and starch (CB1) is present only in small amounts (Hall et al., 1999; Lanzas et al., 2007a). Hence, CHO in this study was divided into five sub-fractions (Table 3.2): intermediately degradable CA3, composed mainly of organic acids like citric, malic, and aconitic acids; rapidly degradable CA4, composed mainly of simple sugars; intermediately degradable CB2, composed mainly of soluble fibers such as pectic polysaccharides, β-glucans and fructans (and small amounts of starch) (Hall et al., 1997; 1999); slowly degradable CB3, composed of available NDF; and undegradable CC, composed of completely undegradable NDF (Lanzas et al. 2007a; Tylutki et al. 2008).

Rumen-degradable CP (RD_{CP}) and rumen-degradable CHO (RD_{CHO}) were calculated based on the CNCPS sub-fractions using fractional rate of degradation (Kd) values given for legume pasture (Table 3.2) (Sniffen et al., 1992; Grabber, 2009b). One should address that these Kd legume pasture values are of normal non-transgenic pastures that do not have altered anthocyanidin accumulation in its forage. Rumen-undegradable CP (RU_{CP}) and rumen-

undegradable CHO (RU_{CHO}) were calculated by subtracting RD_{CP} and RD_{CHO} from total CP and total CHO content, respectively. Intestine-digestible rumen-undegradable CP (DRUP) and intestine-digestible rumen-undegradable CHO (DRUC) were calculated by subtracting the completely undegradable CP (PC) and completely undegradable CHO (CC) fraction from RU_{CP} and RU_{CHO}, respectively (Aufrère and Guérin, 1996).

Nitrogen:carbohydrate (N:CHO) ratios were calculated for the original unfractionated sample (N:CHO), the soluble rapidly degradable fraction (PA:CA), the insoluble slowly degradable fraction (PB:CB) and the total rumen-degradable fraction (RD_N:RD_{CHO}).

Table 3.2. Calculation and fractional rate of degradation (Kd) of protein and carbohydrate sub-fractions

Fraction	Calculation	Kd (%/h)[1]
Carbohydrate sub-fraction (%DM)		
CA3[2]	NFC – (ESC + NDSF)	5
CA4	ESC	40
CB2[2]	EIR – NDF	35
CB3	NDF – CC	8
CC	(NDF × (ADL/NDF) × 2.4) / CHO × 100	-
Rumen degradable carbohydrates (%DM)		
RD_{CHO}	Σ CHO sub-fractions × Kd / (Kd + Kp), where Kp is fractional rate of passage which is assumed to be 4.5 %/h	
Protein sub-fraction (%DM)		
PA	NPN	200
PB1	BSP – PA	20
PB2	100 – (PA + PB1 + PB3 + PC)	15
PB3	NDIP – PC	8
PC	ADIP	-
Rumen degradable protein (%DM)		
RD_{CP}	Σ CP sub-fractions × Kd / (Kd+Kp), where Kp is fractional rate of passage which is assumed to be 4.5 %/h	

[1]Fractional rate of degradation of CHO sub-fractions adapted from legume pasture values reported in Lanzas et al. (2007a) and fractional degradation rates of CP sub-fractions adapted from legume pasture values reported in the CNCPS v_6.1 feed library (www.cncps.cornell.edu).
[2]If the calculation of CA3 (organic acids) resulted in a negative value then CA3 was assumed to be zero and CB2 (soluble fiber) was calculated as CHO – (NDF + ESC).

3.3.4. Statistical Analysis

The effect of the *Lc*-transgene on nutritional factors was analyzed using PROC MIXED of SAS (2003). The statistical models used were:

$$Y_{ijk} = \mu + T_i + H_j + \varepsilon_{ijk} \quad (3.1)$$

$$Y_{ijk} = \mu + P_i + M_j + \varepsilon_{ijk} \quad (3.2)$$

where, Y_{ijk} is the dependent variable, μ is the general mean, T_i is the fixed effect of treatment (i = 6; Beaver, Beav*Lc*1, Rambler, Ramb*Lc*3, Rangelander and Rang*Lc*4), H_j is the random effect of harvest year (j = 2; 2007 and 2008), P_i is the fixed effect of *Lc*-progeny (i = 3; Beav*Lc*1, Ramb*Lc*3, and Rang*Lc*4), M_j is the random effect of first mid-summer harvest survival rate of each year (j = 3; 2007, 2008 and 2009) and ε_{ijk} is the residual error.

Model 3.1 was used for the analysis of chemical composition and CNCPS sub-fractions and degradation characteristics and model 3.2 was used for the analysis of survival rate. Three harvest replications were used for the three NT-alfalfa populations and four replications for the three *Lc*-alfalfa populations (harvest one was split up in two pools).

Pearson's correlations coefficient between anthocyanidin and other parameters were analyzed using the PROC CORR of SAS (2003). Reported values are least squares (LS) means. The Fisher's protected least significant difference (LSD) test was used for multiple treatment comparisons with letter groupings obtained using the SAS pdmix800 macro (Saxton, 1998). Contrasts in SAS (Steel et al., 1996) were used to compare the mean of $NT^{1,3,4}$-alfalfa (Beaver + Rambler + Rangelander) with the mean of $Lc^{1,3,4}$-alflafa (Beav*Lc*1 + Ramb*Lc*3 + Range*Lc*4); $NT^{1,3,4}$-alfalfa with Beav*Lc*1; $NT^{1,3,4}$-alfalfa with Ramb*Lc*3; and $NT^{1,3,4}$-alfalfa with Rang*Lc*4. For the different statistical tests, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$, unless otherwise mentioned.

3.4. Results

3.4.1. Survival and Anthocyanidin Content of Lc-Alfalfa

Field survival after three successive winters indicated that plants with the Rambler background (Ramb*Lc*3) and Rangelander background (Rang*Lc*4) survived the climate in western Canada (Saskatoon) more successfully than those with the Beaver background (Beav*Lc*1) (Table

3.3). All forage samples derived from individual purple-green populations accumulated anthocyanidin (Table 3.3), with a mean of 197.4 µg/g DM and a range of 42.5-to-349.0 µg/g DM. This anthocyanidin accumulation was higher (P<0.001) than anthocyanidin concentrations in NT-alfalfa populations, which did not contain detectable amounts of anthocyanidin within our sample populations (Table 3.3). However, purple-green *Lc*-alfalfa samples in the Ramb*Lc*3 background accumulated on average only 60% of the anthocyanidin accumulated in the Beav*Lc*1 and Rang*Lc*4 populations. Concentrations of extractable and unextractable polymeric anthocyanidins were below the detection limit when tested in 1 g of sample from individual purple-green alfalfa populations.

Table 3.3. Anthocyanidin content and survival rate of *Lc*-alfalfa and parental cultivars

Alfalfa population	Anthocyanidin		Cumulative survival[2]			
	Monomeric	Polymeric[1]	2007	2008	2009	Mean
	(µg/g DM)		(% of initial plants planted)			
Beav*Lc*1	223.5[a]	nd	81.0	50.6	44.3	77.0[b]
Beaver	0.0[b]	nd	ND	ND	ND	ND
Ramb*Lc*3	136.2[a]	nd	88.4	72.1	67.4	87.8[a]
Rambler	0.0[b]	nd	ND	ND	ND	ND
Rang*Lc*4	232.6[a]	nd	89.2	87.5	67.6	88.2[a]
Rangelander	0.0[b]	nd	ND	ND	ND	ND
SEM	38.32	ND	ND	ND	ND	5.89
P value	<0.001	ND	ND	ND	ND	0.034

[a,b] Means with different letters within the same column differ significantly (P<0.05).
[1] nd is not detectable; ND is not determined.
[2]Survival rate: Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4 plants alive at first mid-summer harvest in 2007, 2008 and 2009 and mean survival per year, survival of control plants was not calculated because of limited number of control plants (Beaver, Rambler and Rangelander) planted.

3.4.2. Basic Chemical Composition of Lc and NT Alfalfa

All six alfalfa populations had similar DM, ash, EE and NFC with mean %DM values of 18.7, 13.3, 3.8 and 29.9, respectively (Table 3.4). Crude protein content of $Lc^{1,3,4}$-alfalfa was lower (P<0.03) when compared to $NT^{1,3,4}$-alfalfa (24.8 vs. 27.3 %DM), while individual *Lc*-populations had a numerically lower CP content compared with individual NT-populations. A trend (P<0.07) towards higher NDF was observed for $Lc^{1,3,4}$-alfalfa compared to the $NT^{1,3,4}$-alfalfa (29.1 vs. 24.5 %DM), while individual *Lc*-populations each had a numerically higher NDF content compared with individual NT-populations. Anthocyanidin content tended to

correlate negatively with CP (R=-0.34; $P<0.10$) and correlated positively with CHO (R=0.44; $P<0.05$).

Table 3.4. Basic chemical composition of *Lc*-alfalfa and parent cultivars

Alfalfa population	DM	Ash	EE	CP	NDF[2]	NFC[2]
	%DM					
Beav*Lc*1	18.9	12.8	3.8	24.8	28.4	30.3
Beaver	19.9	13.5	4.0	28.6	23.8	30.1
Ramb*Lc*3	18.6	13.2	3.7	24.7	29.6	28.8
Rambler	18.0	13.3	3.6	26.1	25.9	31.2
Rang*Lc*4	18.1	13.4	4.0	24.9	29.3	28.5
Rangelander	18.5	13.6	3.8	27.2	23.8	30.7
SEM	0.79	0.53	0.27	1.44	2.59	2.30
P value	0.366	0.930	0.633	0.185	0.508	0.939
	---------- Contrast *P* values----------					
[1]$NT^{1,3,4}$ vs. $Lc^{1,3,4}$	0.576	0.462	0.967	0.021	0.062	0.443

[1]$NT^{1,3,4}$ vs. $Lc^{1,3,4}$ = contrast between mean of three non-transgenic alfalfa populations (Beaver, Rambler, Rangelander) and mean of three *Lc*-alfalfa progeny (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4).
[2]NDF = nitrogen adjusted NDF, calculated as: NDF – NDIP; NFC = Non fiber carbohydrates, calculated as: 100 - (ash + CP + EE + NDF).

3.4.3. Protein and Carbohydrate Degradation Characteristics of Lc and NT Alfalfa

All six alfalfa populations had a similar rapidly degradable and slowly degradable protein sub-fractions (Table 3.5), with mean %CP values of 56.4 (PA), 10.8 (PB1), 67.0 (BSP), 29.4 (PB2), 1.9 (PB3), and 3.7 (NDIP). All six alfalfa populations also had similar RD_{CP}, RU_{CP} and intestinal digestible DRUP with mean %DM values of 22.8, 3.2 and 2.8, respectively. A trend towards a higher PC (2.0 vs.1.5 %CP; $P=0.10$) was observed for $Lc^{1,3,4}$-alfalfa compared to $NT^{1,3,4}$-alfalfa, while all individual *Lc*-populations had a numerically higher PC content than individual NT-populations. Anthocyanidin correlated negatively with PB3 (R=-0.46; $P<0.04$).

Total CHO content of $Lc^{1,3,4}$-alfalfa was higher ($P<0.01$) compared to $NT^{1,3,4}$-alfalfa (58.3 vs. 55.5 %DM). Most carbohydrate sub-fractions were similar for the six alfalfa populations (Table 3.6), with mean values of 2.4 (CA3), 7.2 (CA4), 40.2 (ADF) and 26.4 (CC) %CHO; 33.9 (RD_{CHO}) 23.3 (RU_{CHO}) and 8.1 (DRUC) %DM; and 23.4 (ADL) %NDF.

Table 3.5. Protein sub-fractions and degradation profiles of *Lc*-alfalfa and parental cultivars

Alfalfa population	CP (%DM)	Rapidly degradable CP			Slowly degradable CP			RD_{CP}[3] (%DM)	Rumen undegradable CP		
		PA[2]	PB1[2]	BSP	PB2[2]	PB3[2]	NDIP		RU_{CP}[3]	DRUP[3]	PC[2]
		(%CP)			(%CP)				(%DM)		(%CP)
Beav*Lc*1	24.8	55.6	10.0	65.6	30.4	2.1	4.1	23.0	3.3	2.9	2.0
Beaver	28.6	53.9	13.6	67.3	29.6	1.8	3.2	23.3	3.3	2.9	1.3
Ramb*Lc*3	24.7	57.6	9.2	66.8	29.6	1.6	3.6	21.7	3.0	2.5	2.0
Rambler	26.1	58.2	9.8	67.8	28.9	1.7	3.4	22.5	3.1	2.7	1.6
Rang*Lc*4	24.9	55.9	11.2	67.0	29.1	2.0	3.9	22.7	3.1	2.7	2.0
Rangelander	27.2	57.1	10.8	67.6	28.8	2.0	3.7	23.4	3.2	2.8	1.6
SEM	1.44	14.78	1.61	15.36	15.80	0.66	0.80	2.21	0.68	0.65	0.35
P value	0.185	0.513	0.511	0.633	0.878	0.915	0.958	0.937	0.39	0.258	0.651
	---------- Contrast *P* value ----------										
$^{1}NT^{1,3,4}$ vs. $Lc^{1,3,4}$	0.021	0.995	0.346	0.201	0.510	0.961	0.500	0.577	0.672	0.375	0.100

[1]$NT^{1,3.4}$ vs. $Lc^{1,3,4}$ = contrast between mean of three non-transgenic alfalfa populations (Beaver, Rambler, Rangelander) and mean of three *Lc*-alfalfa progeny (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4).

[2]Protein sub-fractions calculated according the CNCPS model: PA = CP that is instantaneously solubilized (NPN); PB1 = CP soluble in borate-phosphate buffer (BSP) and precipitated with trichloroacetic acid (BSP – NPN); PC = undegradable CP recovered with ADF (ADIP); PB3 = calculated as the difference between the portions of CP recovered with NDF and ADF (NDIP – ADIP); PB2 = calculated as total CP minus the sum of fractions PA, PB1, PB3 and PC (Fox et al. 2004; Sniffen et al., 1992; Tylutki et al. 2008).

[3]Protein degradation characteristics, calculated according the CNCPS model: RD_{CP} = Rumen degradable protein [Σ CP sub-fraction × Kd/(Kd+Kp)]; RU_{CP} = Rumen undegradable protein (CP – RD_{CP}); DRUP = Digestible RU_{CP} (RU_{CP} – PC).

Table 3.6. Carbohydrate sub-fractions and degradation profiles of *Lc*-alfalfa and parental cultivars

Alfalfa population	CHO (%DM)	Rapidly degradable CHO (%CHO)			Slowly degradable CHO (%CHO)			RD_{CHO}[3] (%DM)	Rumen undegradable CHO (%DM)			
		CA3[2]	CA4[2]	CB2[2]	NDF	ADF	CB3[2]		RU_{CHO}[3]	DRUC[3]	CC[2] (%CHO)	ADL (%NDF)
Beav*Lc*1	58.6[a]	2.2	7.6	42.0	48.3	41.1	22.4	33.7	22.9	7.9	25.9	22.4
Beaver	53.9[c]	1.8	8.5	46.1	44.3	39.2	16.7	33.7	22.8	7.9	27.0	25.4
Ramb*Lc*3	58.4[a]	2.8	6.2	40.4	50.6	42.0	23.5	34.3	24.3	8.9	27.1	22.3
Rambler	57.1[ab]	2.8	6.9	45.7	45.3	37.9	21.8	34.9	22.4	8.4	22.9	21.3
Rang*Lc*4	57.8[ab]	2.1	7.5	39.9	50.5	42.3	21.8	33.3	23.8	8.1	28.7	23.8
Rangelander	55.5[bc]	2.8	7.0	46.1	44.8	38.9	17.7	33.2	23.5	7.3	26.5	25.2
SEM	1.22	2.22	1.22	3.61	4.13	4.67	4.01	1.59	2.38	0.49	3.75	3.13
P value	0.025	0.744	0.673	0.629	0.776	0.963	0.230	0.974	0.982	0.339	0.880	0.523
	---------- Contrast *P* value ----------											
[1]$NT^{1,3,4}$ vs. $Lc^{1,3,4}$	0.003	0.837	0.636	0.091	0.158	0.378	0.064	0.916	0.651	0.296	0.530	0.424

[a-c]Means with different letters within the same column differ significantly ($P<0.05$).

[1]$NT^{1,3,4}$ vs. $Lc^{1,3,4}$ = contrast between mean of three non-transgenic alfalfa populations (Beaver, Rambler, Rangelander) and mean of three *Lc*-alfalfa progeny (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4).

[2]Carbohydrate sub-fractions calculated according the CNCPS_v6 model: CA3 = organic acids (OA = NFC - ESC); CA4 = simple sugars (80% ethanol soluble CHO; ESC); CB2, soluble fiber (NDSF = EIR - NDF); CB3 = available neutral detergent fiber (NDF – CC); CC, unavailable NDF [NDF × ADL (%NDF) × 2.4)/CHO] (Lanzas et al., 2007a; Tylutki et al. 2008).

[3]Carbohydrate degradation characteristics, calculated according to the CNCPS_v6 model: RD_{CHO} = Rumen degradable carbohydrate [Σ CHO sub-fracti Kd / (Kd + Kp)]; RU_{CHO} = Rumen undegradable carbohydrate (total CHO – RD_{CHO}); DRUC = Intestinal digestible rumen undegradable carbohydrate (RU_{CHO} – CC).

Lc[1,3,4]-alfalfa tended to have a lower (P<0.10) soluble fiber content (CB2, 40.8 vs. 46.0 %CHO) and a trended to have a higher (P<0.07) slowly degradable fiber content (CB3, 22.6 vs. 18.7 %CHO) compared with NT[1,3,4]-alfalfa. Each individual *Lc*-population had numerically higher slowly-degradable fiber (NDF, ADF and CB3) and numerically lower soluble fiber (CB2) than individual NT-populations.

3.4.4. Nitrogen to Carbohydrate Ratios of Lc and NT Alfalfa

All six alfalfa populations had a similar, rapidly degradable N:CHO ratio (PA:CA), with a mean value of 559 g/kg (data not shown). However, the *Lc*[1,3,4]-alfalfa had lower (P<0.02) total N:CHO ratio (68.1 vs. 79.2 g/kg), a lower (P<0.03) insoluble potentially-degradable PB:CB ratio (45.7 vs. 51.6 g kg^{-1}) and lower (P<0.02) total rumen-degradable RD_N:RD_{CHO} ratio (102.5 vs. 115.4 g/kg) compared to NT[1,3,4]-alfalfa (Table 3.7). These three N:CHO ratios were numerically highest in Beaver compared to the other five populations. Anthocyanidin correlated negatively with the ratios N:CHO, PA:CA and RD_N:RD_{CHO} (R=-0.43 to -0.38; P<0.10).

Table 3.7. Nitrogen to carbohydrate ratios of *Lc*-alfalfa and parental cultivars

Alfalfa population	Nitrogen to CHO ratio (g N/kg CHO)[2]		
	N:CHO	PB:CB	RD_N:RD_{CHO}
Beav*Lc*1	67.7	45.2^{bc}	99.3^{b}
Beaver	85.7	57.7^{a}	126.5^{a}
Ramb*Lc*3	67.7	43.8^{c}	102.7^{b}
Rambler	73.3	44.9^{bc}	104.0^{b}
Rang*Lc*4	69.0	48.2^{bc}	105.5^{b}
Rangelander	78.6	52.2^{ab}	115.6^{ab}
SEM	5.73	17.94	8.10
P value	0.088	0.027	0.046
	---------- Contrast P value ----------		
[1]NT[1,3,4] vs. *Lc*[1,3,4]	0.011	0.022	0.016

[a,b]Means with different letters within the same column differ significantly (P<0.05).
[1]NT[1,3,4] vs. *Lc*[1,3,4] = contrast between mean of three non-transgenic alfalfa populations (Beaver, Rambler, Rangelander) and mean of three *Lc*-alfalfa progeny (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4).
[2]N:CHO = nitrogen to carbohydrate ratio; PA:CA = soluble rapidly degradable N:CHO ratio; PB:CB = Insoluble slowly degradable N:CHO ratio; RD_N:RD_{CHO} = total rumen degradable N:CHO ratio.

3.5. Discussion

3.5.1. Survival and Anthocyanidin Content of Lc-Alfalfa

One of the main limitations of fresh grazed alfalfa is its excessively rapid initial rate of protein degradation in the rumen, resulting in an imbalance between the supply of N and CHO in the rumen, required for microbial protein synthesis (Broderick 1995; Yu et al. 2004). Mono/polymeric anthocyanidins can form complexes with proteins and affect the degradation behavior of CP in the rumen (Stafford, 1990; Wang et al., 2006b). Earlier, several T_0 *Lc*-alfalfa genotypes containing the *Lc*-maize anthocyanin regulatory gene were developed to stimulate momo/polymeric anthocyanidin-accumulation in alfalfa forage (Ray et al. 2003), Normally, polymeric-anthocyanidins accumulate only in alfalfa seed and monomeric anthocyanidin accumulation is mainly limited to flowers or to small amounts accumulated in stems when the plant is under stress (Koupai-Abyazani et al. 1993b). However, these new T_0 *Lc*-alfalfa genotypes had poor survival rates in the climate of the western Canadian prairies as these were adapted to a milder climate found in eastern Canada. Hence, three of these new genotypes with similar saponin concentration and profile, were selected for their unique flavonoid profiles (Gruber et al., unpublished data) and crossed with hardy western Canadian cultivars.

Rambler (Ramb*Lc*3) and Rangelander (Rang*Lc*4) populations survived the Saskatoon climate more successfully than plants with Beaver (Beav*Lc*1) background. *Lc*-populations also accumulated anthocyanidin over a broader and higher range (42.5-to-349.0 µg/g DM) than that found previously in the T_0 *Lc*-alfalfa parent genotypes (96.9-to-136.4 µg/g DM) (Wang et al., 2006b). This was true even when the cyanidin standard, field site, plant harvested growth stage and harvest cycle (mid-summer and late summer) were harmonized for the T_0 and T_1 plants. These data suggest that individual genetic background and potentially the transgene location within the alfalfa genome, and/or the harvest year, have impacted *Lc*-transgene expression. Combining each T_0 *Lc*-genotype with the same set of alfalfa elite NT genotypes and additional harvests over a range of growth conditions are necessary to determine the full scope and limitations of the mono/polymeric anthocyanidin trait in *Lc*-alfalfa.

3.5.2. Protein and Carbohydrate Sub-fractions and Degradation Profiles of Lc and NT Alfalfa

Development of the three crossed *Lc*-alfalfa populations enabled the evaluation of forage nutritional parameters over two harvest years from the same plants. Ruminal protein degradation characteristics were determined for the *Lc*-populations using the CNCPS, a system which models the division of CP into five sub-fractions, PA, PB1, PB2, PB3 and PC, based on different rates and extent of degradation in ruminants. These rates for the individual sub-fractions are derived from *in vitro* and *in vivo* studies (Fox et al. 2004; Sniffen et al. 1992). Soluble protein fractions PA (NPN) and PB1 (BSP-NPN) each are rapidly degraded in the rumen and mainly available in the RD_{CP} pool (Sniffen et al., 1992). Insoluble protein fraction PB2 is presumed to have an intermediate ruminal degradation rate and PB3 (NDIP-ADIP) a slow ruminal degradation rate. Varying amounts of these two rumen-insoluble fractions (PB2, PB3) escape ruminal degradation and move to the lower digestive tract (Sniffen et al., 1992; Lanzas et al., 2007b).

Total CP concentration found for samples of both $Lc^{1,3,4}$-alfalfa and $NT^{1,3,4}$-alfalfa was within the same range reported earlier (22.6-to-27.4 %DM) for alfalfa forage harvested at a vegetative stage of growth (Elizalde et al. 1999; González et al. 2001; Wang et al. 2006b). Soluble CP fraction BSP (PA+PB1) and the intermediate-degradable PB2 did not differ between $Lc^{1,3,4}$-alfalfa and $NT^{1,3,4}$-alfalfa populations, with average CP values of 67.0 and 29.4 %, respectively. However, BSP for both $Lc^{1,3,4}$-alfalfa and $NT^{1,3,4}$-alfalfa was much higher and the less soluble PB2 fraction was much lower than values reported in other studies for alfalfa at several stages of growth, with BSP values ranging from 38.4-to-41.1 %CP and PB2 values ranging from 52.0-to-57.1 %CP (Elizalde et al., 1999; Grabber, 2009b). The total CP content was lower in $Lc^{1,3,4}$-alfalfa compared with that in $NT^{1,3,4}$-alfalfa but CP sub-fractions PA, PB1, PB2 and PB3 were similar between $Lc^{1,3,4}$-alfalfa and $NT^{1,3,4}$-alfalfa. The lack of difference in CP sub-fractions between $Lc^{1,3,4}$-alfalfa and $NT^{1,3,4}$-alfalfa populations is most likely an indication that anthocyanidin concentration in $Lc^{1,3,4}$-alfalfa was not high enough to affect CP fractionation or that the chemical procedures used to fractionate CP failed to evaluate the effect of anthocyanidin accumulation in alfalfa forage. Previously, a negative relation between polymeric-anthocyanidin and CP content was found in birdsfoot trefoil (Barry and Manley, 1984b; Miller and Ehlke, 1995; Grabber, 2009a). The T_0 *Lc*-alfalfa parent genotypes that accumulated anthocyanidin had also a numeric lower CP content of 8.5 g/kg DM than their NT-parent A01 (Wang et al., 2006b).

The accumulation of anthocyanidin in *Lc*^{1,3,4}-alfalfa might be an explanation for the lower CP content in *Lc*^{1,3,4}-alfalfa compared to NT^{1,3,4}-alfalfa.

Undegradable protein fraction PC (ADIP) is regarded to be completely unavailable for the ruminant (Sniffen et al., 1992; Aufrère and Guérin, 1996). Fiber associated protein fractions, NDIP and PC (ADIP) of all populations in our study (3.7 and 1.8 %CP, respectively) was substantially lower than earlier reported values for alfalfa harvested at a vegetative stages of growth with values ranging from 4.1-to-14.1 %CP for NDIP and 2.1-to-4.6 %CP for ADIP (Elizalde et al., 1999; González et al., 2001; Faría-Mármol et al., 2002; Wang et al., 2006b).

In addition to affecting ruminal CP degradation, mono/polymeric anthocyanidin may also influence ruminal CHO degradation either directly or by impairing the growth of polysaccharide degrading microbes (McMahon et al., 2000; McAllister et al., 2005). Ruminal CHO degradation characteristics were determined using CNCPS_v6, a system which models the division of CHO into five sub-fractions, CA3, CA4, CB2, CB3 and CC, based on different rates and extent of degradation in ruminants. Carbohydrate fraction CB2 (NDSF) has an intermediate degradation rate and fraction CB3 has a slow degradation rate, both from which varying amounts escape ruminal degradation to move to the lower digestive tract (Lanzas et al. 2007a; Sniffen et al. 1992). Fraction CB2 tended to be lower and fraction CB3 tended to be higher in *Lc*^{1,3,4}-alfalfa compared to NT^{1,3,4}-alfalfa, while the overall CHO content in *Lc*^{1,3,4}-alfalfa was higher. These differences did not result in a change in site of carbohydrate degradation between the six alfalfa populations. Overall, mean fiber contents of NDF and ADF in our study (27.2 and 23.2 %DM, respectively) were similar to mean NDF and ADF contents (26.5 and 21.1, respectively) of T_0 *Lc*-alfalfa parental clones grown at the same field site in earlier years (Wang et al., 2006b). However NDF and ADF values in both of these studies were lower than those reported in other studies, in which NDF ranged from 29.4-to-48.8 %DM and ADF ranged from 24.2-to-37.9 %DM for alfalfa at different stages of growth (Elizalde et al., 1999; González et al., 2001).

Fiber fractions increase and cellular protein content decreases with increasing plant maturity (Elizalde et al., 1999; Beever et al., 2000; González et al., 2001). The lower content of all fiber and fiber associated fractions (PB2, NDIP, PB3, ADIP, CB3, NDF and ADF) and the higher soluble (cellular) protein content for all populations in our study may be the result of our use of a less mature forage canopy compared to earlier studies (Elizalde et al., 1999; González et al., 2001; Grabber, 2009b). Harvests were mandated at a very young stage in our plots due to

confined field trail restrictions imposed by CFIA. In addition, wider spacing of plants in our plots compared with spacing used in a standard seeded alfalfa forage plot would result in shorter plants, which have a relatively lower stem:leaf ratio compared with taller alfalfa plants (Julier et al., 2000). Stems have a higher fiber content and lower protein and digestibility than leaves (Luckett and Klopfenstein, 1970; Tremblay et al., 2002).

3.5.3. Ruminal Nitrogen to Carbohydrate Synchronization of Lc and NT Alfalfa

Ruminal available dietary CHO and CP are needed for microbial protein synthesis (Hoover and Stokes, 1991; Stern et al., 1994). Optimum N:CHO balance for microbial growth is around 32 g N/kg CHO (Tamminga et al., 1990; Sinclair et al., 1991). A lower N:CHO ratio will result in a decreased microbial growth (protein synthesis) and a higher N:CHO ratio will result in deamination of excess CP into energy and NH_3 (Van Duinkerken et al., 2005). The decreased N content and increased CHO content found in $Lc^{1,3,4}$-alfalfa resulted in an reduction in the N:CHO ratio (68.1 vs. 79.2 g/kg) compared with $NT^{1,3,4}$-alfalfa. In addition, $Lc^{1,3,4}$-alfalfa had a reduced insoluble potentially degradable PB:CB ratio (45.7 vs. 51.6 g/kg) and rumen-degradable RD_N:RD_{CHO} ratio (102.5 vs. 115.4 g/kg) compared with NT-alfalfa. These reduced N:CHO ratios in $Lc^{1,3,4}$-alfalfa are still sufficient for an optimal microbial protein synthesis and will reduce the deamination of CP into NH_3 compared to $NT^{1,3,4}$-alfalfa. Excess ruminal NH_3 will be lost for the ruminant through absorption across the rumen wall, detoxification into urea at the cost of energy, and excretion with the urine into the environment (Van Duinkerken et al., 2005). Therefore, the reduced N:CHO ratios in $Lc^{1,3,4}$-alfalfa may result in an improved protein efficiency, reduced N excretion into the environment and reduced energy expenditure for NH_3 to urea conversion compared to $NT^{1,3,4}$-alfalfa. However, *in vivo* experiments should be conducted to confirm that $Lc^{1,3,4}$-alfalfa improves protein efficiency in cattle compared to NT-alfalfa.

3.6. Conclusions

The presence of the *Lc*-transgene in the T_1 crossed western Canadian-adapted alfalfa populations resulted in the accumulation of an average of 197.4 μg/g DM anthocyanidin in purple-green forage harvested over two consecutive years. Polymeric-anthocyanidin were not detected in the first year and therefore not analyzed in the second year. In contrast, neither monomeric anthocyanidin nor polymeric anthocyanidin were detected in any of the non-

transgenic western Canadian parental alfalfa populations. The transgenic populations developed from the cultivars Rangelander and Rambler appeared to be more suitable germplasm, since a higher percentage of plants survived after the three experimental years as compared to those developed from the cultivar Beaver. The *Lc*-transgene decreased total protein content, increased total carbohydrate content and tended to increase fiber fractions in the T_1 crossed *Lc*-alfalfa populations compared with the non-transgenic alfalfa populations. Although modest changes, they resulted in an improved ruminal synchronization between nitrogen and carbohydrates in the crossed T_1 *Lc*-alfalfa populations. The present data indicated that presence of the *Lc*-transgene in the T_1 crossed western Canadian-adapted alfalfa populations improved ruminal balance between nitrogen and carbohydrates which will result in an increased efficiency of protein utilization by the animal and our data suggest that additional modifications to increase mono/polymeric anthocyanindin-accumulation could further improve these nutritional parameters.

Chapter 4

Fermentation, Degradation and Microbial Nitrogen Partitioning for Three Progeny and Three Forage Color Phenotypes within Anthocyanidin-Accumulating *Lc*-Alfalfa*

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4.1. Abstract

Alfalfa has the disadvantage of having a rapid initial rate of protein degradation which may result in pasture bloat, low efficiency of protein utilization and excessive pollution of N into the environment from cattle. Introducing a gene that stimulates the accumulation mono/polymeric anthocyanidins might reduce the ruminal protein degradation rate and methane emission. The objectives of this study were to evaluate *in vitro* degradation, fermentation and microbial-N partitioning of three purple *Lc*-progeny (Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4) and three forage color phenotypes [green, light purple-green (LPG) and purple-green (PG)] within *Lc*-progeny and to compare them to parental green non-transgenic (NT) alfalfa.

Purple-green-*Lc* accumulated more anthocyanidin compared with Green-*Lc* with LPG-*Lc* intermediate. Volatile fatty acids (VFA) and potentially degradable DM and N did not differ between the three phenotypes and NT-alfalfa. Gas, methane and ammonia accumulation rates were slower for the two purple-*Lc* phenotypes compared with NT-alfalfa, while Green-*Lc* was intermediate. Effective degradable DM and N were lower in the three *Lc*-phenotypes compared with NT-alfalfa. Anthocyanidin concentration was negatively correlated with gas and methane production rates and effective degradability of DM and N.

Anthocyanidin accumulation was similar among the three purple *Lc*-progeny with an average concentration of 232 μg/g DM. Gas production rate was faster for Ram*Lc*3 than the other two *Lc*-progeny and faster for Rang*Lc*4 than Beav*Lc*1. Methane production rate tended to be faster for Ramb*Lc*3. Ammonia accumulation was faster but total ammonia accumulation and branch chain fatty concentration were lower for Beav*Lc*1. Total VFA accumulation was higher for Rang*Lc*4, propionate was higher and acetate lower for Ramb*Lc*3, and butyrate was lower and acetate higher for Beav*Lc*1. Microbial growth and effective degradable DM was higher for Beav*Lc*1 than Rang*Lc*4.

In conclusion, the *Lc*-alfalfa progeny and phenotypes accumulated anthocyanidin. Fermentation and degradation parameters indicated a reduced rate of fermentation and effective degradability for both purple anthocyanidin-accumulating *Lc*-alfalfa phenotypes compared with non-transgenic alfalfa. Fermentation profiles differed among the three *Lc*-progeny without a consistent best one.

Key words: Anthocyanidin-accumulating alfalfa, *in vitro* fermentation and degradation, protein metabolism, microbial nitrogen partitioning, methane.

4.2. Introduction

The primary nutrients absorbed and utilized by ruminants are microbial protein and volatile fatty acids (VFA, i.e. energy), both of which are end-products of rumen microbial fermentation (Bergman, 1990; Van Soest, 1994). Ruminal fermentation also produces end-products like methane (CH_4) and ammonia (NH_3) which decrease energy and protein efficiency of the animal (Weimer, 1998). Methane and NH_3 emission by ruminants are contributors to global warming and environmental acidification and eutrophication, respectively (Johnson and Johnson, 1995; Van Duinkerken et al., 2005). Legumes which contain proanthocyanidin reduce CH_4 and NH_3 formation during ruminal fermentation (Waghorn et al., 1987b, 2002; Woodward et al., 2004). These reduced emissions probably result from the interaction of proanthocyanidins with protein and other nutrients and the resulting reduction in nutrient availability for microbial fermentation in the rumen. Moreover, proanthocyanidins interact with specific microbial populations, such as protozoa and their associated methanogenic bacteria and with proteolytic bacteria (Min et al., 2002; Yanez-Ruiz et al., 2004). The reduced protein degradation rate due to proanthocyanidins improves the efficiency of protein utilization and reduces the risk of pasture bloat in grazing cattle (Wang et al., 2006a).

Proanthocyanidins do not accumulate in alfalfa forage (*Medicago sativa* L.), one of the main forage crops utilized for ruminant production (Goplen et al., 1980). Alfalfa transformed with a maize anthocyanidin-regulating *βHLH LEAF COLOR* (*Lc*) gene accumulated mono/polymeric anthocyanidin in the forage and had a reduced initial rate of nitrogen and dry matter degradation *in vitro* (Ray et al., 2003; Wang et al., 2006b). However, survival of these T_0 *Lc*-alfalfa genotypes was poor under western Canadian conditions, and three of these genotypes had to be crossed with western Canadian cultivars in order to develop hardy T_1 *Lc*-alfalfa progeny.

Ruminal fermentation characteristics are best evaluated by *in vivo* experiments. However, when sample amount is limiting and/or a large number of treatments are tested, one can use *in vitro* techniques to simulate ruminal fermentation. In this study we had access to limited quantities of plant material selected from a small-scale field trial of hardy transgenic anthocyanidin-accumulating *Lc*-alfalfa progeny and their parental non-transgenic alfalfa cultivars. The samples were suspended in rumen liquid and evaluated for their *in vitro* fermentation and degradation characteristics and for microbial-N partitioning using the gas production and filter bag techniques.

4.3. Material & Methods

4.3.1 Plant Material

Second generation transgenic (T_1) *Lc*-alfalfa crossed progeny Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4 used in this study were developed at Forage Genetics International (Wisconsin, USA) and compared with commercially available parental non-transgenic (NT) alfalfa cultivars Beaver, Rambler and Rangelander (Section 3.3.1). Seeds from *Lc*-alfalfa progeny populations and NT-alfalfa cultivars were sown initially in a greenhouse and then transplanted into a Canadian Food Inspection Agency (CFIA) approved experimental field at the Saskatoon Research Centre farm of Agriculture and Agri-Food Canada (AAFC). Plants, which had been genotyped earlier in the field for the presence of the *Lc*-gene were phenotyped by forage color [green, light purple-green (LPG) and purple-green (PG)] within each of the three *Lc*-progeny populations (Section 3.3.1). Segregating non-transgenic plants were discarded. Field grown alfalfa plants were harvested manually with shears at *ca.* 5 cm above ground level on July 4 and 25, and August 15 and 25 of 2007 at a vegetative pre-bud stage according to CFIA-imposed confined field trail regulations. The four harvests were pooled into one sample per phenotype within each *Lc*-progeny population (3 phenotypes × 3 *Lc*-progeny) and for each NT-alfalfa cultivar (3 cultivars) to obtain sufficient material for *in vitro* incubations and analysis of original samples for their chemical and phytochemical composition. Prior to conducting *in vitro* incubations, each pooled sample was freeze-dried and ground to pass through a 1 mm screen using a cyclonic mill (Retsch SM-3000, Brinkmann Instruments, ON, Canada). Ground material for each phenotype within a population was mixed thoroughly.

4.3.2. Experiment 1: In Vitro Gas Production Technique: Incubations, Sampling and Analysis

Cumulative gas production incubations as described by Wang et al. (2006b), were performed in three runs at the Lethbridge Research Centre of AAFC. Rumen liquid was collected two hours after morning feeding from two red Angus heifers at the Lethbridge Research Center farm of AAFC (diet: 33% barley silage, 67% alfalfa hay and minerals). Heifers were cared for according to the Canadian Council on Animal Care standards (CCAC, 1993). Rumen liquid was strained twice through four layers of cheesecloth and then combined with mineral buffer (1:2 v/v) (Menke et al., 1979) and ^{15}N labeled ammonium sulfate (Cambridge Isotope Laboratories Inc.

Andover, USA). Four replicates of each substrate (3 *Lc*-phenotypes and NT-alfalfa × 3 populations × 4 vials) were incubated in 125 mL vials containing substrate (0.5 g) and inoculum (40 mL) in a manually operated *in vitro* system for 48 h. Gas production was measured at 4, 8, 12, 24 and 48 h of incubation using a water displacement device (Fedorak and Hurdley, 1983). Before gas production measurements, headspace gas was collected with a 20 mL syringe (15 mL at 4, 8 h; 10 mL at 12, 24, 48 h) and injected into a 6.8 mL vacuume container (Labco Ltd. High Wycombe, Buckinghamshire, UK) for later CH_4 analysis on a CP-4900 micro gas chromatograph with a thermal conductivity detector (Varian Canada Inc., Mississauga, ON), a 10-m PoraPLOT PPQ no.1 BF column and with the column oven at 37 °C and argon as a carrier gas. Standards with CH_4 concentrations of 0.24, 1, 5, 10, 15 and 40% were used to fit the standard curve (BOC Gases, Calgary, AB) (Wang et al., 2008).

Quadruplicate vials for each treatment and triplicate control vials (containing inoculum only) were withdrawn from the incubator after 0, 4, 12, 24, and 48 h of incubation. The vial contents were processed to determine true DM digestibility and true N digestibility, accumulation of microbial-N (MN) accumulation, volatile fatty acids (VFA) accumulation and ammonia (NH_3) concentration as described by Wang et al. (2006b). The first three tubes of each treatment at each incubation time were centrifuged at 28,000 *g* for 30 min, after which the supernatant was combined with 65% trichloracetic acid (TCA) for NH_3 analysis (1.66:0.14 v/v) and with 25% metephosphoric acid for VFA analysis (1.5:0.3 v/v). The pellet left in each tube was washed twice using 20 mL of ddH_2O followed by centrifugation at 28,000 *g* for 30 min to remove excess ^{15}N not incorporated into microbial-N (MN). The fourth tube of each treatment at each incubation time was used to isolate microbial cells as follows: tube was centrifuged at 400 *g* for 10 min; supernatant was transferred to a new tube and centrifuged at 28,000 *g* for 30 min to pellet microbes from the liquid; microbial pellet left in the tube was washed twice using 20 mL of ddH_2O, followed by centrifugation again at 28,000 *g* for 30 min (Wang et al., 2006b). Ammonia was analyzed by the phenol-hypochlorite method (Weatherburn, 1967). Volatile fatty acids, including acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate were measured by gas chromatography as described by Wang et al. (1998). The ratio between non-glucogenic and glucogenic VFA (NGR) was calculated as: NGR = (acetate + 2 × butyrate + valerate)/(propionate + valerate) (Ørskov, 1975). Branched-chain VFAs (iso-butyrate + iso-valerate), are assumed to originate exclusively from protein fermentation (Azarfar et al., 2007). The amount of NH_3 and

VFAs present in the buffered rumen fluid before incubation was subtracted from the amount measured at sampled incubation times.

Sample residues after incubation were freeze dried, weighed for DM determination, and analyzed for total N content and ^{15}N atom % excess (APE). Total N content and ^{15}N APE portion in total N were determined by flash combustion (Model 1500; Carlo Erba Instruments, Milan, Italy) with isotope ratio mass spectrometry (VG Isotech, Middlewich, England).

4.3.3. Experiment 2: In Vitro Filter Bag Technique: Incubations, Sampling and Analysis

To prepare filter bags for incubation, ANKOM F57 filter bags (ANKOM Technology, Fairport, NY, USA) were soaked in acetone for 5 min to remove surfactant that may negatively affect microbial activity. To begin the incubations, filter bags with 0.5 g sample and empty bags (blank control) were transferred into 2.5 L jars (per treatment; 4 phenotypes × 3 populations) followed by addition of 1.6 L of pre-warmed (39 ºC) mineral buffer (in g/L: 8.33 KH_2PO_4, 0.42 $MgSO_4 \cdot 7H_2O$, 0.42 NaCl, 0.083 $CaCl_2 \cdot H_2O$, 0.42 urea, 2.5 Na_2CO_3 and 0.17 $Na_2S \cdot 9H_2O$ + 1.67 g/L labeled ^{15}N ammonium sulfate) and 0.4 L rumen liquid (prepared from the same animals described above). Jars were incubated in DAISY II incubators (ANKOM Technology) at 39 ºC under continuous rotation. Triplicate bags with sample residue and duplicate blank bags were withdrawn from the fermentation jars at 4, 8, 12 and 24 h of incubation and processed as described by Wang et al. (2006b) to determine DM, total N and ^{15}N APE in N. At the same time points, inoculum (12 mL) was sampled from each fermentation jar and processed to determine liquid associated MN (Wang et al., 2006b). The 0 h bags were incubated at 39 °C for 30 min in mineral buffer without rumen liquid to fractionate the washable fraction into a washable truly soluble fraction (SW) and a washable insoluble fraction which was added back to the residue left in the bag (Wang et al., 2006b).

Triplicate filter bags from each treatment and incubation time were placed into stomach bags and 15 mL ddH_2O was added and stomach bags with content processed for 60 sec in a Stomacher 400 laboratory paddle blender at 206 rpm (Seward Medical Limited, London, UK). Next, filter bags were manually squeezed and liquid released in the stomach bag transferred to a tube. The filter bags with residue were washed twice with 7.5 mL ddH_2O in the stomach bag and liquid combined into the same tube. The tube with liquid from the washes and liquid sampled from the digestion jars were centrifuged as described in section 4.3.2 to isolate microbes from

the liquid. Microbial pellets and filter bag residues were freeze dried, weighed for DM determination, ground in a ball grinder to a fine powder (Retsch MM-200, Brinkmann Instruments, ON, Canada), and analyzed for total N and ^{15}N APE in N as described in section 4.3.2.

True DM digestibility, true N digestiblity, MN firmly attached to feed particles and MN loosely attached onto feed particles and liquid associated MN were calculated as described by Wang et al. (2006b).

4.3.4. Chemical Analysis

Ground alfalfa sample was analyzed for dry matter (DM; AOAC 930.15), ash (AOAC 942.05), ether extract (EE; AOAC 954.02) and crude protein (CP; Kjeldahl-N × 6.25; AOAC 984.13) according to the procedures of AOAC (1990). Acid detergent fiber (ADF) and neutral detergent fiber with heat-stable α-amylase (NDF) were analyzed according to procedures of Van Soest et al. (1991). Neutral detergent insoluble CP (NDIP) and acid detergent insoluble CP were determined according to the procedures of Licitra et al. (1996) by the Kjeldahl-N method. Non-protein nitrogen (NPN) was analyzed by the Kjeldahl-N method after extraction of ground sample in water and precipitation with trichloroacetic acid (Licitra et al., 1996). The values of NDF and ADF presented in this paper were adjusted for CP, but not for ash. The non-fiber carbohydrates (NFC) were calculated as 1000 – (NDF + CP + EE + Ash) and total carbohydrates (CHO) as 1000 - (CP + EE + Ash). Anthocyanidin extractable in methanol:HCl (99:1 v/v) was analyzed by the method described in section 3.3.2 with cyanidin as a standard.

4.3.5. Calculations and Statistical Analysis

True DM digestibility and true N digestibility were calculated as:

$$\frac{S - (R - MW)}{S} \times 100 \qquad (4.1)$$

Where, S is DM or N incubated (g), R is residual DM or N after incubation (g), MW is the calculated microbial DM (g) in the residue. The MW was calculated as (APE in RN / APE in MN) × RN × 6.25/ MP, where APE is the atom % excess of ^{15}N, RN is N content of incubation residue (g), MN is microbial N of the fermentation residue (g), and MP is a conversion factor to convert microbial CP to microbial DM (N % microbial pellet × 6.25). Liquid associated MN and

loosely attached MN were calculated by multiplying the weight of the isolated microbial pellet by the N % in the microbial pellet (Wang et al., 2006b).

Kinetic parameters of gas, CH_4, VFA and NH_3 accumulation (Exp. 1) were calculated using the following exponential model described by McDonald (1981):

$$P = a(1 - exp^{-c(t-lag)}) \quad (4.2)$$

Where, P is gas, VFA, CH_4 and NH_3 accumulation at time (t), *a* is asymptotic production, *c* is fractional production rate (%/h), t is incubation time (h) and *lag* is initial delay in onset of production (h). Average production at half of its asymptotic value was calculated as:

$$\text{Average production} = \frac{a \times c}{2 \times (\ln 2 + c \times lag)} \quad (4.3)$$

Where, ln2 is 0.693 (Lopez et al., 1999). Dry matter and N degradation rates and undegradable fractions (exp. 2) were calculated using the exponential model described by Robinson et al. (1986):

$$R(t) = U + D(exp^{-Kd(t-lag)}) \quad (4.4)$$

where R is residue (%) of the incubated sample, U is undegradable fraction (%), D is potentially degradable fraction calculated as: [100% - (0 h washout fraction) – U], Kd is fractional degradation rate (%/h), t is incubation time (h) and *lag* is initial delay in onset of degradation (h). Effective degradability (ED) of DM and N were calculated as:

$$ED = W + D \times \frac{Kd}{Kd + Kp} \quad (4.5)$$

where fractional passage rate (Kp) is assumed to be 4.5 %/h (Ørskov and McDonald, 1979). The parameters of both exponential models were calculated using PROC NLIN (non-linear) from (SAS, 2003) with iterative least squares regression (Gauss–Newton method).

All data were statistically analyzed using PROC MIXED of SAS 9.2 (2003). The statistical models used were:

$$Y_{ij} = \mu + P_i + \varepsilon_{ij} \quad (4.6)$$

$$Y_{ijk} = \mu + P_i + H_j + P_i \times H_j + \varepsilon_{ijk} \quad (4.7)$$

where, Y_{ij} is the dependent variable, μ is the general mean, P_i is the fixed effect of phenotype (i = 4; NT-alfalfa, Green-*Lc*, LPG-*Lc* and PG-*Lc*) or progeny (i = 3; Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4), H_j is the fixed effect of incubation time (j = 5; 0 … 48 h), $P_i \times H_j$ is the interaction between phenotype or progeny and incubation time and ε_{ij} is the residual error.

Model 4.6 was used to analyze sample chemical composition and output data from the two exponential models and model 4.7 was used to analyze *in vitro* fermentation end-products and microbial-N accumulation. Populations within each phenotype (phenotype comparison; n=3) and the two purple phenotypes (PG and LPG) within progeny (progeny comparison; n=2) were regarded as experimental replicates for the chemical composition data. Populations within each phenotype (n=3) or purple phenotype within progeny (n=2) and vials (n=4) or bags (n=3) incubated per sample treatment were used as replicate for *in vitro* fermentation/degradation parameters and microbial-N accumulation.

Fisher's protected least significant difference (LSD) test was used for multiple treatment comparisons using the LSMEAN statement of SAS 9.2 (2003) and letter groupings were obtained using the pdmix800 macro (Saxton, 1998). A contrast statement from SAS 9.2 (2003) was used to compare the mean of both purple-*Lc* phenotypes (LPG-*Lc* + PG-*Lc*) with NT-alfalfa and the mean of all three *Lc*-phenotypes (Green-*Lc* + LPG-*Lc* + PG-*Lc*). Pearson's correlation coefficients between anthocyanidin and other parameters were determined using the PROC CORR of SAS (2003). For all statistical tests, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$, unless otherwise stated.

4.4. Results

4.4.1. Anthocyanidin Content and Chemical Composition

All three *Lc*-alfalfa phenotypes tested (but not NT-alfalfa) accumulated anthocyanidin pigments within their forage (Table 4.1), even though the level in the Green-*Lc* was not sufficient to show a purple phenotype.

Table 4.1. Anthocyanidin and nutritional composition for three forage color phenotypes and three progeny within *Lc*-alfalfa and for non-transgenic (NT) alfalfa.

Trait[1]	Phenotype[2]				SEM	*P* value	NT v P-*Lc*[3]	NT v *Lc*[4]	Progeny			SEM	*P* value
	NT-alfalfa	Green-*Lc*	LPG-*Lc*	PG-*Lc*			*P* value		Beav *Lc*1	Ramb *Lc*3	Rang *Lc*4		
Anthocyanidin (µg/g DM)	0.0^{c}	103.9^{bc}	180.4^{ab}	282.5^{a}	38.76	0.005	0.001	0.003	271.6	136.7	286.1	56.30	0.264
Ash (g/kg DM)	134.7^{a}	124.0^{b}	126.3^{b}	132.7^{a}	0.18	0.008	0.045	0.009	127.5	129.5	131.5	3.20	0.707
EE (g/kg DM)	40.3	37.3	40.0	39.3	0.29	0.882	0.855	0.676	36.0	39.5	43.5	2.27	0.212
DM (g/kg)	957.3^{b}	972.0^{a}	958.3^{b}	957.7^{b}	0.33	0.037	0.873	0.200	955.4	958.4	960.6	6.09	0.838
CHO (g/kg DM)	571.0^{c}	609.0^{a}	595.0^{ab}	581.3^{bc}	0.58	0.008	0.042	0.007	593.5	591.0	580.0	7.38	0.480
NFC (g/kg DM)	322.0	324.3	289.3	317.0	0.93	0.093	0.136	0.304	323.5	299.5	286.5	15.15	0.348
NDF (g/kg DM)	248.7^{c}	284.3^{b}	306.7^{a}	264.3^{bc}	0.63	0.001	0.002	0.001	270.0	292.0	293.0	21.07	0.712
ADF (g/kg DM)	206.7^{b}	244.3^{a}	246.7^{a}	207.3^{b}	0.8	0.01	0.072	0.023	211.5	233.5	236.0	21.84	0.712
CP (g/kg DM)	254.0^{a}	229.7^{c}	238^{bc}	246.3^{ab}	0.45	0.025	0.067	0.016	243.0	239.0	245.0	5.48	0.754
NPN (g/kg DM)	108.3^{a}	88.7^{b}	95.7^{ab}	103.7^{a}	0.41	0.039	0.121	0.031	94.5	99.5	105.0	6.42	0.575
NDIP (g/kg DM)	8.0	7.0	8.3	7.3	0.06	0.366	0.812	0.506	8.5	8.0	7.0	0.87	0.534
ADIP (g/kg DM)	4.3	5.0	5.3	4.7	0.03	0.163	0.096	0.081	5.0	5.0	5.0	0.58	1.00
GP TND (%)	80.3	74.3	73.9	75.5	1.88	0.075	0.020	0.012	72.8	74.0	76.8	1.72	0.293
GP TDMD (%)	83.8^{a}	84.2^{a}	78.8^{b}	80.3^{ab}	1.56	0.050	0.032	0.142	80.7	79.7	78.2	1.40	0.471

[a-c]Means with different letters within the same row differ ($P<0.05$).

[1]EE, ether extract; CHO, total carbohydrates calculated as: DM - (ash + EE + CP); NFC, non-fiber carbohydrates calculated as: DM - (ash + EE + CP + NDF); aNDF, neutral detergent fiber with heat stable α-amylase and adjusted for CP; ADF, acid detergent fiber adjusted for CP; NPN, nitrogenous compounds soluble in water and not precipitated by trichloroacetic acid; NDIP, neutral detergent insoluble CP; ADIP, acid detergent insoluble CP; GP TDMD and GP TND, true DM and N digestibility , respectively, after 48 h incubation by the *in vitro* gas production technique.

[2]Transgenic *Lc*-plants of progenies Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4 were visually sorted in the field based on forage color: green, light purple-green (LPG) and purple-green (PG) and compared with their green non-transgenic (NT) parental varieties Beaver, Ramble and Rangelander.

[3]NT vs. P-*Lc*, comparison of NT-alfalfa with the mean of the two purple-*Lc* phenotypes (LPG and PG).

[4]NT vs. *Lc*, comparison of NT-alfalfa with the mean of the three *Lc* phenotypes (Green, LPG and PG).

Extractable and unextractable polymeric anthocyanidins measured by the butanol:HCl method could not be measured in these *Lc*-alfalfa samples as described in section 3.3.2. Among the three *Lc*-phenotypes, PG-*Lc* anthocyanidin accumulation ranged from 157-to-394 µg/g DM which was higher than the range of 47-to-151 µg/g DM for Green-*Lc* (P<0.05) with the range of 117-to-230 µg/g DM for LPG-*Lc* intermediate. Anthocyanidin concentration in the three *Lc*-progeny was similar with an average of 231.5 µg/g DM (Table 4.1). However, anthocyanidin accumulation was numerically twice as high in Beav*Lc*1 and Rang*Lc*4 compared with Ramb*Lc*3.

All *Lc*-phenotypes and NT-alfalfa had similar EE, NFC, NDIP, and ADIP (Table 4.1). Total CHO content and fiber fractions (NDF and ADF) were higher in Green-*Lc* and LPG-*Lc* (P<0.05) compared with NT-alfalfa while PG-*Lc* was intermediate. Total CP content and NPN were lower for the mean of all three *Lc*-phenotypes (P<0.05) and individually lower for Green-*Lc* and LPG-*Lc* (P<0.05) compared with NT-alfalfa, while PG-*Lc* was intermediate (Table 4.1). True N digestibility (Exp. 1) was lower (P<0.05) for the mean of all three *Lc*-phenotypes and mean of both purple-*Lc* phenotypes compared with NT-alfalfa. Dry matter content of Green-*Lc* was higher (P<0.05) than in the other three phenotypes. True DM digestibility (Exp. 1) was lower for LPG-*Lc* and the mean of the two purple-*Lc* phenotypes (P<0.05) compared with NT-alfalfa and Green-*Lc*. All chemical constituents measured in the original sample were similar among the three *Lc*-progeny (Table 4.1).

4.4.2 Fermentation End-Products from the In Vitro Gas Production Technique (Exp. 1)

The three *Lc*-phenotypes were analyzed for differences in their gas and methane accumulation profiles using an *in vitro* gas production technique. Total gas production, determined by finding the asymptote after fitting the gas production to an exponential model, was similar between NT-alfalfa and PG-*Lc*, intermediate for LPG-*Lc* and slightly lower (P<0.05) for Green-*Lc* (Table 4.2). Gas production rate was slower for the mean of all three *Lc*-phenotypes (P=0.008) and individually for each purple-*Lc* phenotype (P<0.05) compared with NT-alfalfa. This was consistent with a lower average gas production (i.e. ½ max of the asymptote) for all three individual *Lc*-phenotypes (P=0.03) compared with NT-alfalfa. Anthocyanidin concentration correlated negatively with gas production rate (R=-0.409; P=0.013).

Table 4.2. Production characteristics of fermentation end-products for three forage color phenotypes and three progeny within *Lc*-transgenic alfalfa and for non-transgenic (NT) alfalfa (Exp. 1).

Trait[1]	Phenotype[2]					NT v P-*Lc*[3]	NT v *Lc*[4]		Progeny				
	NT-alfalfa	Green-*Lc*	LPG-*Lc*	PG-*Lc*	SEM	*P* value	*P* value		Beav *Lc*1	Ramb *Lc*3	Rang *Lc*4	SEM	*P* value
Gas production													
a (mL/g DM)	197.9^{a}	190.4^{b}	193.6^{ab}	196.5^{a}	2.04	0.045	0.329	0.109	206.3^{a}	181.3^{b}	177.7^{b}	2.46	<0.001
c (%/h)	14.4^{a}	13.3^{ab}	12.4^{b}	12.6^{b}	0.05	0.027	0.004	0.008	13.1^{c}	16.5^{a}	15.0^{b}	0.08	<0.001
AP (mL)	19.4^{a}	17.8^{b}	17.4^{b}	17.9^{b}	0.43	0.027	0.005	0.003	17.2^{b}	19.0^{a}	18.8^{a}	0.43	<0.001
Methane production													
a (mL/g TDMD)	31.7	29.7	30.2	31.1	0.79	0.323	0.319	0.178	30.8	30.9	30.2	1.15	0.900
c (%/h)	11.0	10.1	9.9	9.8	0.30	0.068	0.009	0.012	9.5	10.5	9.5	0.33	0.066
AP (mL)	2.05	1.85	1.82	1.94	0.087	0.278	0.137	0.102	1.83	2.01	1.80	0.118	0.422
Ammonia accumulation													
a (mg/g N)	495.5^{b}	540.8^{ab}	596.4^{a}	571.9^{a}	19.89	0.007	0.001	0.003	489.0^{b}	617.5^{a}	645.9^{a}	13.57	<0.001
c (%/h)	6.6^{a}	6.7^{a}	5.2^{b}	5.0^{b}	0.04	0.001	0.003	0.048	5.8^{a}	4.5^{b}	5.1^{b}	0.23	0.004
AP (mg)	42.2^{b}	48.2^{a}	41.3^{b}	39.4^{b}	0.25	0.003	0.311	0.064	41.0	39.2	40.8	1.08	0.428
Volatile fatty acids													
a (mmol/g DM)	8.2	9.4	9.1	8.8	0.64	0.638	0.366	0.255	7.9^{b}	8.3^{b}	10.3^{a}	0.39	0.001
c (%/h)	9.5	11.9	12.8	9.2	1.90	0.686	0.685	0.562	13.0	11.6	8.37	2.24	0.313
AP (mmol)	0.53	0.73	0.73	0.54	0.073	0.090	0.221	0.100	0.70	0.67	0.55	0.11	0.541

^{a,b}Means with different letters within the same row differ (P<0.05).

[1]*In vitro* gas production system parameters calculated by the following exponential model: $P = a(1 - \exp^{-c(t-lag)})$, where P is either gas, methane, ammonia or volatile fatty acids produced, *a* is asymptotic production, *c* is fractional production rate (%/h), t is incubation time (h) and *lag* is initial delay in onset of production (h). Average production (AP) at half of its asymptotic value was calculated as: $a \times c(2(\ln 2 + c \times lag)$, ln2 is 0.693.

[2]Transgenic *Lc*-plants of progenies Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4 were visually sorted in the field based on forage color: green, light purple-green (LPG) and purple-green (PG) and compared with their green non-transgenic (NT) parental varieties Beaver, Ramble and Rangelander.

[3]NT vs. P-*Lc*, comparison of NT-alfalfa with the mean of the two purple-*Lc* phenotypes (LPG and PG).

[4]NT vs. *Lc*, comparison of NT-alfalfa with the mean of the three *Lc* phenotypes (Green, LPG and PG).

Total and average methane production were similar among the three phenotypes and NT-alfalfa, but methane production rate was slower for the mean of the two purple-*Lc* phenotypes (*P*=0.009) and the mean of three *Lc*-phenotypes (*P*=0.01) compared with NT-alfalfa (Table 4.2). Anthocyanidin concentration correlated negatively with methane production rate (R=-0.507; *P*=0.002).

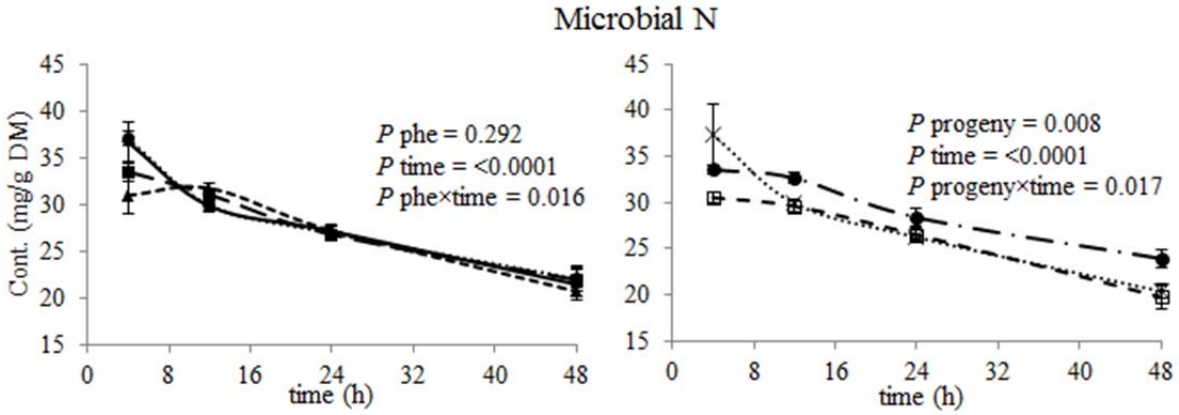

Fig. 4.1. Microbial-N accumulation (Exp. 1). Left graph, NT-alfalfa ( ) and forage color phenotypes Green-*Lc* ( ), light purple-green-*Lc* ( ) and purple-green-*Lc* ( ) within *Lc*-alfalfa progeny; Right graph, Beav*Lc*1 ( ), Ramb*Lc*3 ( ) and Rang*Lc*4 ( ). Vertical bars represent the standard error. *P* values represent the effect of phenotype or progeny, time and the interaction between phenotype or progeny × time.

Total gas production was higher for Beav*Lc*1 (P<0.001) compared with Ramb*Lc*3 and Rang*Lc*4 (Table 4.2). Gas production rate was faster for Ramb*Lc*3 (*P*<0.001) compared with the other two *Lc*-progeny and higher for Rang*Lc*4 (*P*<0.001) compared with Beav*Lc*1. Average gas production was higher for Ramb*Lc*3 and Rang*Lc*4 (*P*<0.001) compared with Beav*Lc*1. Total and average methane production was similar between the three *Lc*-progeny. Methane production rate tended to be faster for Ramb*Lc*3 (*P*=0.066) compared with Beav*Lc*1 and Rang*Lc*4 (Table 4.2).

Total NH_3 accumulation (the asymptote calculated after curve fitting) was higher for both purple-*Lc* phenotypes (*P*=0.007) and the mean of all three *Lc*-phenotypes (*P*=0.003) compared with NT-alfalfa (Table 4.2). Anthocyanidin concentration had a positive correlation with total NH_3 accumulation (R=0.380; *P*=0.02). The higher NH_3 accumulation occurred even though the NH_3 accumulation rate was lower for both purple-*Lc* phenotypes (*P*=0.004) compared with NT-alfalfa.

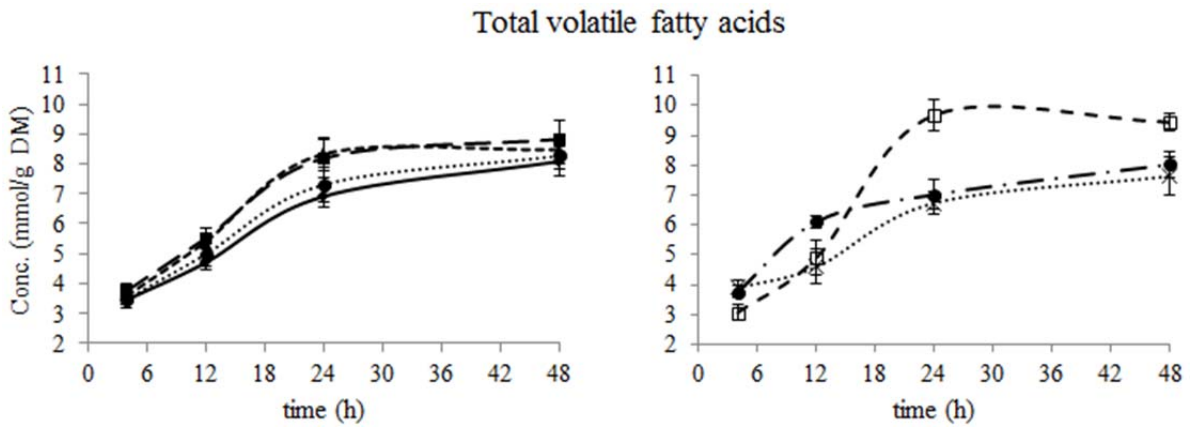

Fig. 4.2. Total volatile fatty acid accumulation (Exp. 1). Left graph, NT-alfalfa ( ) and forage color phenotypes Green-*Lc* ( ), light purple-green-*Lc* ( ) and purple-green-*Lc* ( ) within *Lc*-alfalfa progeny; Right graph, Beav*Lc*1 ( ), Ramb*Lc*3 ( ) and Rang*Lc*4 ( ). Vertical bars represent the standard error.

Microbial-N accumulation decreased over 48 h of incubation (P<0.001), and with a phenotype × time interaction (P=0.016; Fig. 4.1). Total VFA production (at the asymptote) and VFA production rate were similar for the three *Lc*-phenotypes and NT-alfalfa, although a trend towards increased average VFA production (P=0.09) was noted for Green-*Lc* and LPG-*Lc* (Table 4.2). Production appeared to reach a maximum level more quickly between 12 and 24 h for LGP-*Lc* and Green-*Lc* compared with PG-*Lc* and NT-alfalfa (Table 4.2).

Total NH_3 accumulation was higher for Ramb*Lc*3 and Rang*Lc*4 (P<0.001) compared with Beav*Lc*1 (Table 4.2), while the converse was true for ammonia accumulation rate (P=0.004). Total VFA production was higher for Rang*Lc*4 (P=0.001) compared with Beav*Lc*1 and Ramb*Lc*3 (Table 4.2). Average NH_3 accumulation, VFA production and VFA production rate were similar between the three *Lc*-progeny (Fig 4.2).

When considering phenotypes, among the individual VFA fractions, there was a phenotype × time interaction for acetate (P=0.035), propionate (P=0.035), non-glucogenic:glucogenic VFA ratio (NGR; P=0.0005) and branch chain FA (P=0.01) (Fig. 4.3). Branched chain FA initially increased similarly for all four phenotypes, but after 24 h kept increasing for the *Lc*-phenotypes and leveled off for NT-alfalfa. Profiles for other VFA during this initial 24 h of incubation changed similarly for all four phenotypes (acetate remained steady, butyric increased, and propionate decreased). Differences between the *Lc*-alfalfa and NT-alfalfa became apparent after 24 h, where the non-glucogenic:glucogenic VFA ratios for NT-alfalfa continued to rise while the

Lc-alfalfa phenotypes remained level. Butyrate concentration was higher (P<0.001) in the three *Lc*-phenotypes compared with NT-alfalfa (Fig. 4.3).

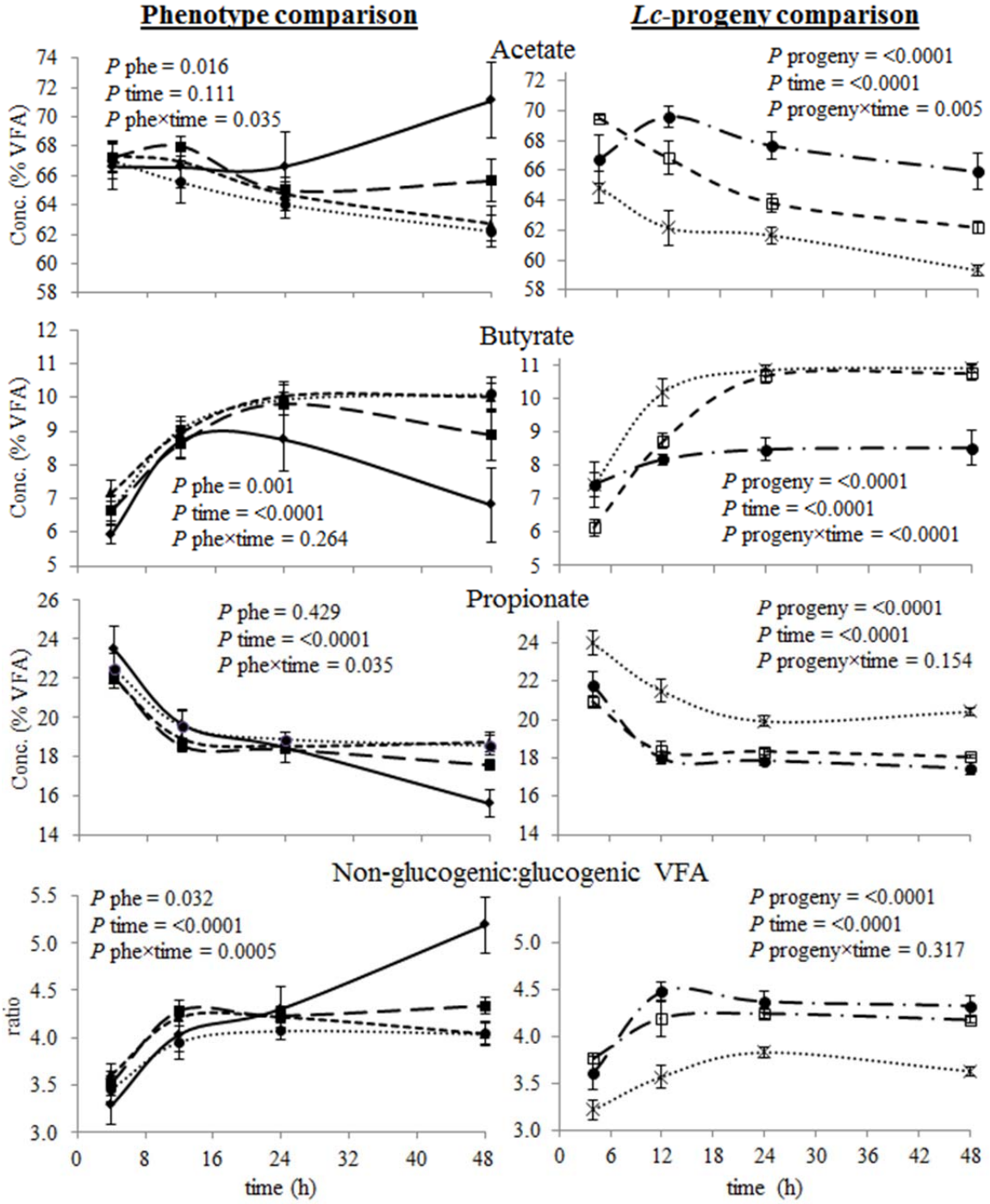

Fig. 4.3. Accumulation of volatile fatty acid (VFA) components (Exp. 1). NT-alfalfa (—◆—) and forage color phenotypes Green-*Lc* (—■—), light purple-green-*Lc* (- -▲- -) and purple-green-*Lc* (···●···) within *Lc*-alfalfa progeny (Left); Beav*Lc*1 (—●- -), Ramb*Lc*3 (···×···) and Rang*Lc*4 (- -⊟- -) (Right). Vertical bars represent the standard error. P values represent the effect of phenotype or progeny, time and the interaction between phenotype or progeny × time.

When considering *Lc*-progeny, there was a *Lc*-progeny × time interaction for acetate (P=0.005) and butyrate (P<0.001) (Fig 4.3). Acetate concentration for Ramb*Lc*3 and Range*Lc*4 decreased during incubation, while in Beav*Lc*1 it increased from 4 to 12 h of incubation and then decreased (Fig 4.3). Butyrate concentration of Beav*Lc*1 remained fairly steady during incubation, while for Ramb*Lc*3 and Range*Lc*4, it increased from 4 to 24 h of incubation after which it leveled (Fig. 4.3). Propionate concentration was higher and non-glucogenic:glucogenic VFA ratio was lower for Ramb*Lc*3 (P<0.001) compared with Beav*Lc*1 and Rang*Lc*4 (Fig. 4.3). Branch chain FA were lower for Beav*Lc*1 (P<0.001) compared with Ramb*Lc*3 and Rang*Lc*4. Branch chain FA concentration for all four alfalfa phenotypes followed an identical pattern, increasing between 4 and 24 h of incubation, after which branch chain FA of the three *Lc*-phenotypes continued to rise in a linear fashion, while branch chain FA for NT-alfalfa reached a plateau (Fig. 4.4).

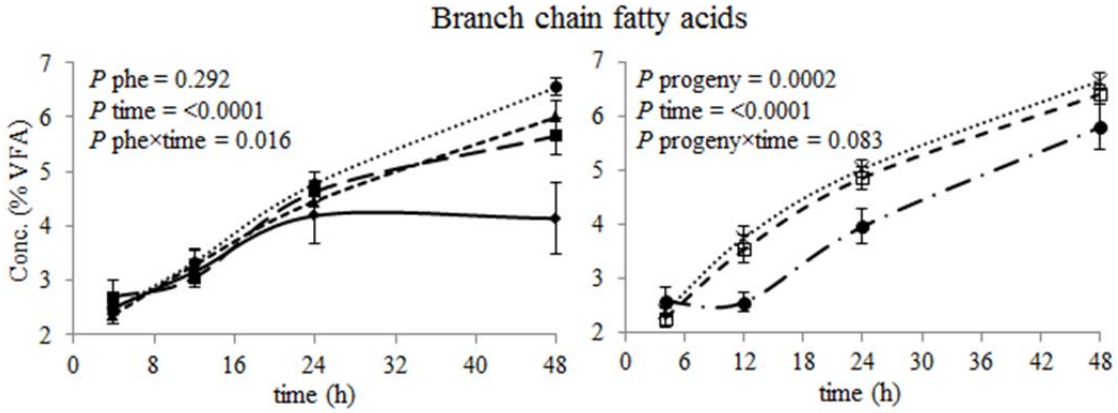

Fig. 4.4. Branch chain fatty acid accumulation (iso-Butyrate + iso-Valerate; Exp. 1). Left graph, NT-alfalfa ( ) and forage color phenotypes Green-*Lc* ( ), light purple-green-*Lc* ( ) and purple-green-*Lc* ( ) within *Lc*-alfalfa progeny; Right graph, Beav*Lc*1 ( ), Ramb*Lc*3 ( ) and Rang*Lc*4 ( ). Vertical bars represent the standard error. P values represent the effect of phenotype or progeny, time and the interaction between phenotype or progeny × time.

4.4.3. Degradation Characteristics from the Filter Bag Technique (Exp. 2)

Dry matter and N (protein) degradation characteristics were determined for samples placed in filter bags in a DAISY II incubator. The truly soluble washable DM and N were lower for the mean of all three *Lc*-phenotypes (P<0.001) and individually for LPG-*Lc* and Green-*Lc* (P<0.05) compared with NT-alfalfa (Table 4.3).

Table 4.3. Dry matter and nitrogen fractionation and degradation characteristics for three forage color phenotypes and three progeny within *Lc*-transgenic alfalfa and for non-transgenic (NT) alfalfa (Exp. 2).

Trait[1]	Phenotype[2]				SEM	*P* value	NT v P-*Lc*[3]	NT v *Lc*[4]	Progeny			SEM	*P* value
	NT-alfalfa	Green-*Lc*	LPG-*Lc*	PG-*Lc*			*P* value		Beav *Lc*1	Ramb *Lc*3	Rang *Lc*4		
Dry matter													
SW (%)	34.8^{a}	32.4^{b}	31.3^{c}	33.0^{ab}	0.31	<0.001	<0.001	<0.001	31.8	31.5	33.1	0.47	0.053
D (%)	36.2	36.7	34.8	34.6	0.83	0.221	0.147	0.280	37.2^{a}	34.8^{a}	32.3^{b}	0.85	0.003
U (%)	29.0^{c}	30.9^{bc}	33.9^{a}	32.4^{ab}	0.81	0.001	<0.001	0.001	31.1	33.7	34.6	0.96	0.054
Kd (%/h)	11.7^{ab}	10.2^{b}	13.6^{a}	11.0^{ab}	0.60	0.035	0.893	0.656	11.2	12.9	11.3	0.91	0.354
ED_{DM} (g/kg DM)	581.7^{a}	560.9^{b}	543.1^{c}	547.4^{c}	3.49	<0.001	<0.001	<0.001	551.6^{a}	547.6^{ab}	537.6^{b}	3.64	0.037
Nitrogen													
SW (%)	33.9^{a}	32.2^{b}	29.1^{c}	32.2^{b}	0.53	<0.001	<0.001	<0.001	31.1	30.0	30.6	0.78	0.630
D (%)	38.6	44.8	40.7	40.8	1.64	0.062	0.298	0.070	45.0^{a}	42.2^{a}	36.0^{b}	1.64	0.003
U (%)	27.5^{a}	23.0^{b}	30.3^{a}	27.1^{ab}	1.55	0.015	0.540	0.684	23.9^{b}	27.9^{b}	33.4^{a}	1.71	0.003
Kd (%/h)	11.7^{b}	9.6^{b}	14.6^{a}	10.9^{b}	0.90	0.001	0.329	0.981	9.8^{b}	13.2^{ab}	15.0^{a}	1.32	0.041
ED_{N} (g/kg DM)	25.0^{a}	22.9^{b}	22.8^{b}	22.6^{c}	0.58	0.014	0.002	0.001	23.7	23.2	22.8	0.38	0.276
ED_{N}:ED_{DM} (g/kg)	43.0	40.8	41.9	39.6	1.76	0.576	0.311	0.279	43.0	42.4	42.3	0.77	0.844

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

[1]*In vitro* filter bag DM and N degradation characteristics: SW truly soluble washable fraction (0 h incubation); D, potentially degradable fraction calculated as: 100-(SW+U); U, undegradable fraction; Kd, fractional degradation rate; ED_{DM} and ED_{N}, effective degradability of DM and N, respectively, calculated as: SW+D×Kd/(Kd+Kp), where passage rate (Kp) was assumed to be 4.5%/h. Kd and U were calculated by an exponetial model: $R(t) = U+D(\exp^{-Kd(t-lag)})$

[2]Transgenic *Lc*-plants of progenies Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4 were visually sorted in the field based on forage color: green, light purple-green (LPG) and purple-green (PG) and compared with their green non-transgenic (NT) parental varieties Beaver, Ramble and Rangelander.

[3]NT vs. P-*Lc*, comparison of NT-alfalfa with the mean of the two purple-*Lc* phenotypes (LPG and PG).

[4]NT vs. *Lc*, comparison of NT-alfalfa with the mean of the three *Lc* phenotypes (Green, LPG and PG).

These were both negatively correlated with a rise in undegradable DM (R=-0.45; P<0.001) but not with undegradable N, which was highly variable between the three *Lc*-phenotypes. Among the three *Lc*-phenotypes, undegradable DM and N were lower for Green-*Lc* (P<0.05) compared with LPG-*Lc* while PG-*Lc* was intermediate.

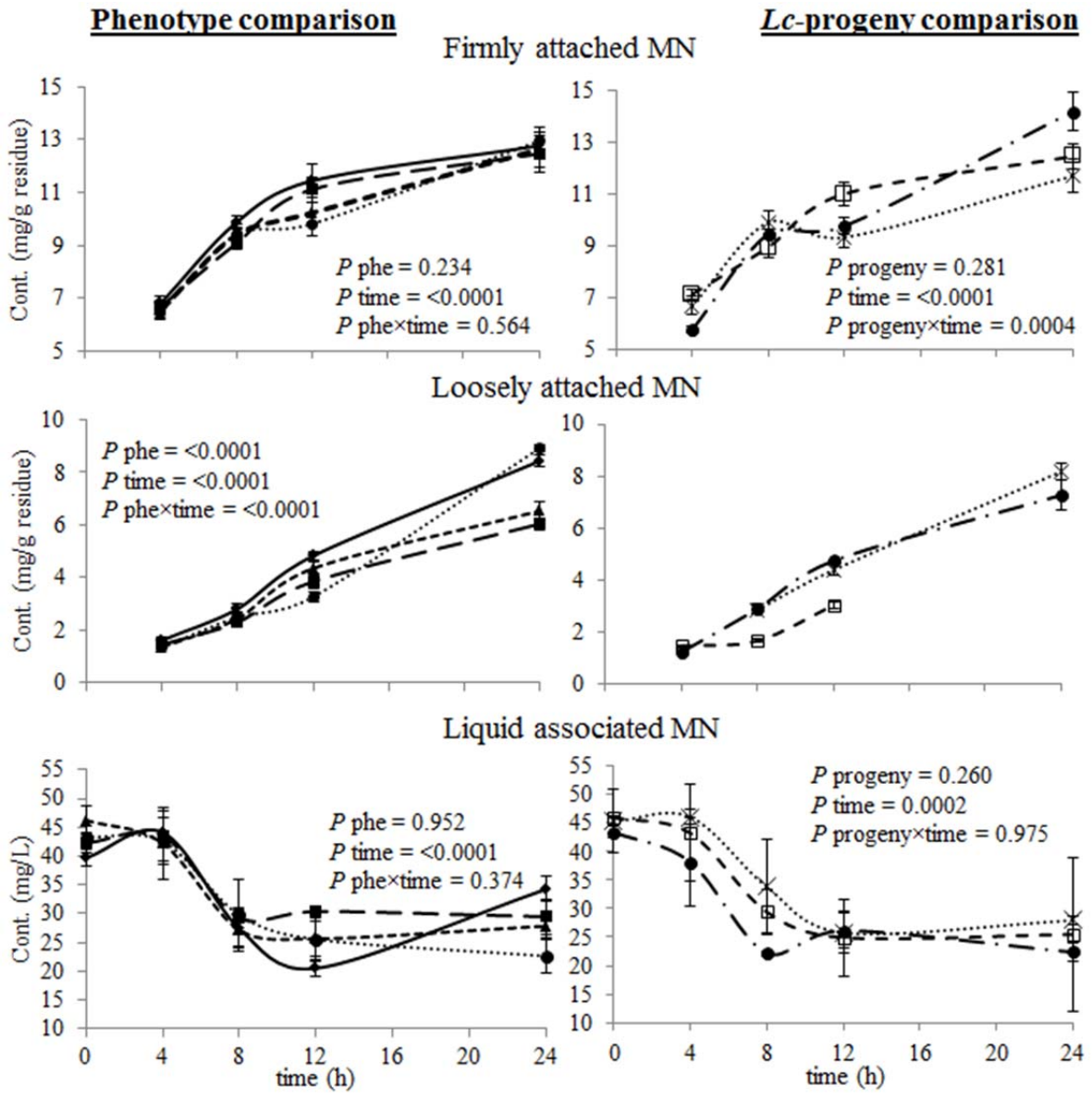

Fig. 4.5. Microbial partitioning (Exp. 2). NT-alfalfa ( ) and forage color phenotypes Green-*Lc* ( ), light purple-green-*Lc* ( ) and purple-green-*Lc* ( ) within *Lc*-alfalfa progeny (Left); Beav*Lc*1 ( ), Ramb*Lc*3 ( ) and Rang*Lc*4 ( ). Vertical bars (invisible behind some point symbols) represent the standard error. P values represent the effect of phenotype or progeny, time and the interaction between phenotype or progeny × time. Too many data points were missing for statistical *Lc*-progeny comparison of loosely attached MN.

None of these parameters appeared to correlate with potentially degradable N and DM, neither of which were different between the four phenotypes. The DM and N degradation rates were slower (P<0.05) for Green-*Lc* compared with LPG-*Lc*. Effective degradability of DM and N were lower for the three *Lc*-phenotypes (P<0.01) compared with NT-alfalfa. Among the *Lc*-phenotypes, effective degradability of DM was lower for the two purple *Lc*-phenotypes compared with Green-*Lc* and effective degradability of N was lower in PG-*Lc* compared with Green-*Lc* and LPG-*Lc*. Anthocyanidin content correlated negatively with effective degradability of DM (R=-0.637; P<0.001) and effective degradability of N (R=-0.476; P=0.005). The ratio between effective degradable N and DM was similar for the three *Lc*-phenotypes and NT-alfalfa (Table 4.3).

Truly soluble washable N and DM, undegradable DM, fractional degradation rate of DM, effectively degradable N and ratio between effectively degradable N:DM were similar between the three *Lc*-progeny (Table 4.3). Potentially degradable N and DM were higher in Beav*Lc*1 and Ramb*Lc*3 (P=0.003) compared with Rang*Lc*4. Effectively degradable DM was higher for Beav*Lc*1 (P=0.037) than Rang*Lc*4 with Ramb*Lc*3 intermediate. Undegradable N was higher (P=0.003) and N degradation rate was faster (P=0.041) for Rang*Lc*4 compared with Beav*Lc*1 and Ramb*Lc*3.

Microbial nitrogen (MN) is synthesised by microbes that utilize liquid-associated material and microbes that digest and utilize plant residues. Microbial-N in the different rumen fractions was determined using the DAISY II filter bag method. All MN parameters changed as a function of time, with firmly attached MN, loosely attached MN and total particle-associated MN rising immediately and fairly steadily over the incubation period, while liquid-associated MN displayed an initial four-hour lag period before declining quickly to a plateau by eight hours. During and by the end of the fermentation period, liquid-associated and firmly attached MN had not changed between the three *Lc*-phenotypes and NT-alfalfa (Fig. 4.5). However, loosely attached MN and consequently total particle associated MN differed between phenotypes, with LPG-*Lc* and Green-*Lc* being consistently lower than NT-alfalfa. There was a phenotype × time interaction (P<0.001) for loosely attached MN and particle associated MN (Fig. 4.5).

There was a *Lc*-progeny × time interaction (P<0.001) for firmly attached MN (Fig. 4.5). Liquid-associated MN displayed an initial four-hour lag period before declining quickly to a

plateau by eight hours. During the fermentation period, liquid-associated and firmly attached MN had not changed between the three *Lc*-progeny (Fig. 4.5).

4.5. Discussion

4.5.1. Anthocyanidin Within the Three Lc-Alfalfa Progeny and Phenotypes

Most of the plants from the the three newly developed *Lc*-alfalfa progeny survived the harsh western Canadian climate over three years (Section 3.4.1) and were found to express three different color phenotypes within each progeny, Green-*Lc*, light purple-green-*Lc* (LPG-*Lc*) and purple-green-*Lc* (PG-*Lc*). Therefore, the *Lc*-alfalfa progeny populations were split into these three phenotypes and tested for their range of anthocyanidin. Anthocyanidin accumulation ranged from 47-to-349 μg/g DM when all three phenotypes for all progeny were considered. This was a broader range and a higher level than the range of 97-to-136 μg/g DM previously found for the T_0 *Lc*-parent genotypes grown earlier in the same field in 2001 and 2002 as discussed in more detail in section 3.5.1 (Wang et al., 2006b).

4.5.2. Protein Fermentation and Degradation for Lc-Alfalfas and NT-Alfalfa

Ruminal microbial fermentation of proteins results in the formation of branch chain FA and NH_3 (Williams et al., 2005). Previously, polymeric-anthocyanidin were found to decrease the formation of branch chain FA and NH_3 in the rumen (Waghorn et al., 1987b; Vasta et al., 2009). In our study, fermentation of the two purple *Lc*-phenotypes resulted in a slower NH_3 accumulation rate and lower 0 h filter bag soluble washout fraction compared with NT-alfalfa. This suggests lower availability of N for the conversion into NH_3 by microbes at the initiation of incubation with the two purple *Lc*-phenotypes compared with NT-alfalfa. However, this was not consistent with a lower NPN (directly available N analyzed in original sample) content and lower N degradation rate (Exp. 2) for the two purple-*Lc* phenotypes compared with NT-alfalfa. Moreover, the ranking of individual *Lc*-alfalfa phenotypes was inconsistent for the different parameters. Previously it was found that NPN could give variable results when predicting the filter bag washout fraction (Section 5.4.2), which might explain the different ranking of phenotypes for soluble washable N compared with NPN in the current study. Rang*Lc*4 had a slower NH_3 accumulation rate but faster N degradation rate compared with Beav*Lc*1. While

deamination into NH_3 is partly affected by direct interaction of polymeric anthocyanidin with specific proteolytic microbial species (Min et al., 2002), the initial release of N from the feed is mainly affected by the strength and disruption of plant cell walls (Lees et al., 1982; Acosta et al., 2007). These may explain the difference in ranking of NH_3 accumulation rate compared with the filter bag degradation rates of N.

Fiber degrading (cellulolytic) microbes produce the non-glucogenic organic acid acetate as a by-product of cellulose degradation and utilize branched chain FA and NH_3 as their main N source (Hoover, 1986; Russell et al., 1992). When considering individual purple *Lc*-progeny, Beav*Lc*1 had a lower branch chain FA from 12 h of incubation and lower total NH_3 accumulation compared with Ramb*Lc*3 and Rang*Lc*4. Moreover, branch chain FA concentration by 48 h of incubation as well as the asymptote of NH_3 accumulation were higher for the two purple-*Lc* phenotypes compared with NT-alfalfa. The NH_3 in a closed *in vitro* system originates from microbial proteolysis and deamination of sample/microbes to form NH_3, balanced with its utilization for microbial protein synthesis (Wang et al., 2006b). Our results suggest that either more N was available for fermentation and/or less NH_3 was utilized for microbial growth for the two purple *Lc*-phenotypes. However, the lower CP content and lower rumen degradability of N for the two purple *Lc*-phenotypes causes a ~13 g/kg DM lower CP release into rumen compared with NT-alfalfa with no difference in batch culture accumulation of MN (Exp. 1). The turnover rate of microbial-N may have been lower for the two purple *Lc*-phenotypes between 24 and 48 h of incubation. Moreover, acetate concentration was lower at 48 h of incubation for the three *Lc*-phenotypes. This suggests that fewer fiber-degrading microbes were present (NH_3 and branch chain FA utilizing microbes) in fermentations with these *Lc*-phenotypes compared with NT-alfalfa. This was partly supported by a lower loosely attached microbial-N and total particle microbial-N within the residue remaining at 24 h, but not by firmly attached microbial-N. Extending the batch fermentation in the DAISY II fermenter (Exp. 2) until 48 h of incubation should give additional support to this hypothesis, since the main deviation in fermentation end-products occurred from 24-to-48 h during the *in vitro* gas production incubations. However, when interpreting the results from 24 to 48 of incubation one should be cautious because an increased portion of the VFAs produced might be from fermentation of lysed microbial debris and thus not from the fermentation of test feed (Blümmel and Ørskov, 1993).

4.5.3. Substrate Availability and Methane Emission from Lc-Alfalfas and NT-Alfalfa

Methane is one of the greenhouse gases contributing to global warming and causing energy loss for cattle. Therefore, it would be beneficial to reduce CH_4 emission. One strategy to reduce methane emission is by reducing the rumen degradability of the diet without reducing total tract degradability, which can be achieved by a slower feed degradation rate and/or faster passage of feed particles (Johnson and Johnson, 1995; Moss et al., 2000). Average gas production and fractional gas production rate (which are related to the digestibility of DM; Dijkstra et al., 2005) and effective degradability of DM were lower for the two purple *Lc*-phenotypes compared with NT-alfalfa (Green-*Lc* intermediate). This indicates slower and less available ruminal substrate for microbial use from the DM of the two purple *Lc*-phenotypes. Average gas production and fractional gas production rate were lower for Beav*Lc*1 compared with the other two *Lc*-progeny, but total gas production and effectively degradable DM were highest in Beav*Lc*1.

Reducing ruminal substrate availability might decrease CH_4 emission through the reduction in H_2 production as a result of reduced fermentation of nutrients into acetate and butyrate (Johnson and Johnson, 1995; Moss et al., 2000). The non-glucogenic:glucogenic VFA ratio (NGR) is an indication for the balance between H_2 producing (acetate and butyrate) and H_2 utilizing (propionate) processes in the rumen, as well as an indication of precursors available for gluconeogenesis (France and Dijksta, 2005). The NGR was similar between 4 and 24 h of incubation for the three *Lc*-phenotypes and NT-alfalfa. However, between 24 and 48 h of incubation, the ratio increased for NT-alfalfa while remaining constant for the three *Lc*-phenotypes (Fig. 4.2). A lower NGR at 48 h for the *Lc*-phenotypes supports the hypothesis of lower H_2 availability for CH_4 production.

The NGR was lower during incubation for Ramb*Lc*3 compared with Beav*Lc*1 and Rang*Lc*4. This suggests that Ramb*Lc*3 produce less H_2 and utilizes more of H_2. However, the difference in DM digestibility and NGR did not result in differences in CH_4 production among the three *Lc*-progeny.

4.5.4. Fermentation Profiles from Lc-Alfalfas and NT-Alfalfa

Lower nutrient availability (lower average production and rate of gas production, lower effectively degradable DM) within the *Lc*-phenotypes suggests that one could expect a lower VFA production. However, none of the total VFA production parameters differed between the

Lc-phenotypes and NT-alfalfa. An earlier report suggested that polymeric-anthocyanidins could alter microbial composition during batch culture incubations to utilize available nutrients more efficiently (Makkar, 2005). Only monomeric-anthocyanidin and not polymeric-anthocyanidin, as measured by standard methods, were detectable in our *Lc*-phenotypes, and accumulation of microbial-N in the *in vitro* gas production system (Exp. 1) did not differ between the four phenotypes. Within the individual purple *Lc*-progeny, Rang*Lc*4 had a higher total VFA production, but lower total gas production and lower effectively degradable DM compared with Beav*Lc*1 and Ramb*Lc*3. Microbial-N (Exp. 1) tended to be higher for Beav*Lc*1 from 8 to 48 h of incubation compared with the other two *Lc*-progeny. These data suggest that fermentation from Rang*Lc*4 might be more efficient than from the other two *Lc*-progeny.

Individual VFA components followed a similar pattern through the course of incubation for the two purple-*Lc*-phenotypes, which differed from the pattern of NT-alfalfa and to a lesser extent Green-*Lc*, especially between 24 to 48 h of incubation. Acetate production is promoted by cellulose fermentation, butyrate production by soluble fiber (hemicellulose and pectin) fermentation, and propionate production by starch and protein fermentation (Bannink et al., 2006). Acetate concentration for the *Lc*-phenotypes decreased gradually over the 48 h of incubation, while acetate concentration for NT-alfalfa increased during this time period, especially after 24 h. Acetate concentration of Beav*Lc*1 was higher from 12 h of incubation than of Ramb*Lc*3 with RangLc4 in between. This suggests that between 24 and 48 h of incubation for phenotypes comparison and between 12 and 48 h for *Lc*-progeny, microbes fermented relatively less cellulose from the three *Lc*-phenotypes and from Ramb*Lc*3, respectively. Butyrate concentration increased rapidly from 4 to 12 h of incubation for all phenotypes, after which the butyrate concentration of two purple *Lc*-phenotypes reached a plateau and that of NT-alfalfa decreased. Butyrate concentration of Ramb*Lc*3 and Rang*Lc*4 increased from 4 to 24 h after which it reached a plateau that was higher than the butyrate concentration of Beav*Lc*1. This suggests that microbes could ferment relatively more soluble fiber from the two purple *Lc*-phenotypes and Ramb*Lc*3 and Rang*Lc*4 later in incubation (i.e., after 12 h) and that the major portion of soluble fiber in NT-alfalfa and Beav*Lc*1 are fermented within the initial phase of incubation (before 12 h).

Propionate concentration decreased rapidly and equivalently between 4 and 12 h of incubation for all four phenotypes, after which the propionate concentration of NT-alfalfa decreased

gradually while remaining constant for the three *Lc*-phenotypes. Propionate concentration was higher during the whole course of incubation for Rambl*Lc*3 compared with Beav*Lc*1 and Rang*Lc*4. Since starch concentration in alfalfa is close to zero (Hall et al., 1999), the differences in propionate patterns suggest that microbes could ferment relatively more protein from the three *Lc*-phenotypes compared with NT-alfalfa. This was supported by the higher branch chain FA concentrations and NH_3 accumulation from *Lc*-phenotypes after 24 h of incubation, as described above. However, protein fermentation characteristics of the three *Lc*-progeny did not relate to propionate concentration. The patterns of change in acetate, butyrate and propionate throughout the course of incubation suggest that after 24 h of incubation most of the more easily fermentable substrate within NT-alfalfa had been fermented.

4.5. Conclusion

Degradation and fermentation characteristics were determined using *in vitro* gas production and filter bag techniques to compare green, light purple-green and purple-green transgenic *Lc*-alfalfa accumulating different levels of anthocyanidin with their green non-transgenic anthocyanidin free parents and to compare the three purple *Lc*-progeny with each other. The three *Lc*-progeny populations had different fermentation-degradation characteristics, but inconsistencies in improvements arose among the three progeny. A lower soluble washable nitrogen fraction, slower rate of gas and ammonia accumulation, and slower rate of dry matter disappearance are parameters that indicated a reduced initial rate of dry matter and nitrogen degradation for the two purple *Lc*-phenotypes compared with non-transgenic alfalfa. Decreased rate of methane production for the two purple *Lc*-phenotypes likely resulted from the lower dry matter degradability (effective degradability of dry matter and related gas production parameters) and lower hydrogen producing fermentation end-products (acetate and butyrate) compared with non-transgenic alfalfa.

Chapter 5

Modeling Degradation Ratios and Nutrient Availability of Anthocyanidin-Accumulating *Lc*-Alfalfa Progeny in Dairy Cows*

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5.1. Abstract

Dairy cattle eating fresh or ensiled alfalfa as the main portion of their diet often have a 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/polymeric anthocyanidin 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 DVE/OEB protein systems and to determine net energy for lactation using the VEM energy system for three newly developed transgenic winter hardy anthocyanidin-accumulating *Lc*-alfalfa progeny. These *Lc*-alfalfa progeny, called Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4, had an average anthocyanidin accumulation of 163.4 µg/g DM while 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 *Lc*-alfalfa and AC Grazeland. The undegradable *in situ* crude protein and neutral detergent fiber fraction were, respectively, 1.3 %CP and 4.8 %CHO lower in *Lc*-alfalfa compared with AC Grazeland. *Lc*-alfalfa had a 0.34 MJ/kg DM higher net energy for lactation and 1.9 %CP higher buffer soluble protein compared with AC Grazeland. By the protein evaluation model it was predicted that *Lc*-alfalfa tended to have a 11.9, 6.9 and 8.4 g/kg DM higher rumen degradable protein, rumen degraded protein balance and intestinal available protein compared with AC Grazeland, respectively. The hourly rumen degraded protein balance included an initial and substantial peak (over-supply) of protein relative to energy which was highest in Rang*Lc*4 and lowest in Ramb*Lc*3. The hourly rumen degraded protein balance between 4 and 24 h was similar and more balanced for all four alfalfa populations. In conclusion, *Lc*-alfalfa accumulated anthocyanidin, tended to have a higher intestinal protein availability and had a higher net energy of lactation availability for dairy cattle compared with AC Grazeland.

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

5.2. Introduction

Alfalfa (*Medicago sativa* L.), is one of the main forages in the world which makes it an important protein source in ruminant rations. When alfalfa is grazed, cost 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 (NH_3) 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 anthocyanidin and proanthocyanidin (condensed tannins) may decrease ruminal protein degradation by precipitating dietary protein (Stafford, 1990; Wang et al., 2006b) 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 proanthocyanidin/protein complex which might increase the availability of amino acids for absorption in the small intestine (Waghhorn et al., 1987; Bermingham et al., 2001). However, polymeric-anthocyanidin does not accumulate in alfalfa forage (Goplen et al., 1980).

Ray et al. (2003) transformed alfalfa with a maize anthocyanidin regulating *βHLH LEAF COLOR* (*Lc*) gene to develop new genotypes which accumulate monomeric/polymeric anthocyanidin in the forage. Wang et al. (2006a) found that the initial *in vitro* rate of nitrogen and dry matter degradation for T_0 *Lc*-alfalfa genotypes was reduced compared to their non-transgenic parent genotype. However, their survival was poor under western Canadian conditions, and three of these genotypes had to be crossed with western Canadian cultivars in order to develop winter-hardy T_1 *Lc*-alfalfa populations. Coulman et al. (2000) reviewed the development of AC Grazeland, an alfalfa cultivar 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 cultivar 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 limiting and/or a large number of treatments are 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 and/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 DVE/OEB (2007) protein system uses a more advanced *in situ* fractionation scheme to fractionate crude protein (CP) and carbohydrates (CHO) compared with previous protein systems. This revised protein system uses individual CP and CHO fractions to calculate ruminal degradable fractions and ruminal microbial protein synthesis (MCPr); previous protein systems used total rumen available energy to calculated MCPr (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 progeny and non-transgenic alfalfa cultivar AC Grazeland for nutrient availability to dairy cattle using modeling. We fractionated protein and energy of these two 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 VEM energy system.

5.3. Material & Methods

5.3.1. Alfalfa Material

T_1 transgenic *Lc*-alfalfa populations (Section 3.3.1) used in this study were developed at Forage Genetics International, Wisconsin by manually crossing three T_0 transgenic *Lc*-alfalfa genotypes (*Lc*1, *Lc*3 and *Lc*4) (Wang et al., 2006b) with several genotypes selected for winter

hardiness from three non-transgenic western Canadian-adapted alfalfa cultivars (Beaver, Rambler and Rangelander) to generate Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4, respectively. *Lc*-alfalfa populations were compared with alfalfa cultivar AC Grazeland which has been selected earlier for a low initial rate of degradation and shown to reduce bloat in grazing cattle (Coulman et al., 2000). The *Lc*-alfalfa populations were grown for approximately six months 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. 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 *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 *Lc*-plants were sorted visually in the field based on forage color (green and purple-green). Purple-green *Lc*-alfalfa (high anthocyanidin accumulation and reduced fermentation/degradation rates) and AC Grazeland forage was stored at -20 °C pending analysis. Transgenic plants had been analyzed previously for the presence of the *Lc*-gene (Section 3.3.1).

5.3.2. In Situ Rumen Incubation

For *in situ* rumen incubations, three rumen fistulated, non-pregnant, dry Holstein Frisian cows were used, which had been reviewed and approved by 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 the Canadian Council on Animal Care guidelines (1993). The cows had free access to water and were fed 15 kg DM/day total mixed ration twice daily in equal portions at 08:00 and 16:00 h The total mixed ration consisted in %DM of 55% barley silage, 12% alfalfa hay, 5% dehydrated alfalfa and 27.5% concentrates as described in Yu et al. (2009a).

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, Mississagua, ON, Canada). Approximately 5 g of freeze-dried alfalfa sample was placed into each bag, resulting in a sample-to-bag surface ratio of ~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 five times manually in cold tap water followed by oven drying at 55 ºC for 48 h.

The 0 h incubation samples were washed by the procedure described by Azafar et al. (2007a) to fractionate the washable fraction (W) into a truly soluble washable fraction (S) and a washable insoluble fraction (WI). Briefly, the 0 h bags containing *ca*. 5 g of sample were placed into 250 mL polypropylene tubes with 20 mL distilled water per 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 ºC for 48 h. The remaining washout fraction in the tube was filtered through filter paper (Wathman #54; pore size 20 μm) and the filter paper with residue (washable insoluble fraction; WI) was dried in an oven for 24 h at 55 ºC. Since 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 equalled the S fraction. Incubation residues from the treatment bags were combined within time per incubation run.

5.3.3. 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, ON, Canada). Original alfalfa samples and rumen incubated residues were analyzed for dry matter (DM), ash and CP according to (AOAC, 1990)). Crude protein (N × 6.25) was analyzed by flash combustion using a Leco FP 528 analyzer (Leco Corporation, St Joseph, MI, USA). Neutral detergent fiber (NDF; determined with heat stable α-amylase for original samples and rumen incubated residues and with sodium sulphite for rumen incubated residues), acid detergent fiber (ADF) and acid detergent lignin (ADL) 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 (NDIP) and acid detergent insoluble protein (ADIP) content using the Kjeldahl-N method (Kjeltic 1030 N autotitrator, Foss,

Höganäs, Sweden) as described by Licitra et al. (1996). Non-protein N (NPN) remaining soluble in trichloroacetic acid and CP soluble in borate/phosphate buffer (BSP) were analyzed in original sample 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 (ESC) according to Hall et al. (1999). Total CHO content was calculated in %DM as 100 – (ash + EE + CP) according to NRC-Dairy (2001).

5.3.4. Protein and Carbohydrate Profiling

Protein and carbohydrates each were fractionated into a 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) and based on *in situ* incubations described in the 2007 version of DVE/OEB (Tamminga et al., 2007) (Table 5.1). Degradation rates (Kd) for the potential degradable (D) fraction of CP, NDF, residual non-starch polysaccharides (RNSP) and organic matter (OM) were calculated using an exponential mathematical model as described by Robinson et al. (1986):

$$R(t) = U + D \times \exp^{-Kd(t-lag)} \quad (5.1)$$

Where, R is the residue (%) of the incubated sample, U is undegradable fraction (72 h incubation residue), t is time of rumen incubation (h), and *lag* is the lag time before degradation initiates. The parameters of the exponential model were calculated using PROC NLIN (non-linear) from SAS (2003) with iterative least squares regression (Gauss–Newton method). Rumen degradable (RD) fractions were calculated as described in Table 5.2. Rumen degradability was calculated for each *in situ* fraction (S, WI and D) as described in Table 5.2, with values for fractional rate of passage (Kp) and Kd described in Table 5.1. Hourly rumen degradability was calculated as:

$$\textit{In situ} \text{ fraction} \times Kd/(Kd + Kp) \times [1 - \exp^{-t(Kd+Kp)}] \quad (5.2)$$

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

Table 5.1. Chemical and *in situ* fractionation of proteins and carbohydrates, degradation rates, passage rates and ATP yield.

Traits	Chemical analysis[1]		*In situ*[2]		DVE/OEB		
	Chemical fraction	Calculation	*In situ* fraction	Description	Kd[3] (/h)	Kp[4] (/h)	ATP yield[5] (mol/kg)
Carbohydrate fraction (%DM)							
Soluble	CA4	CHO – (CB2 + CB3 + CC)	S_{ESC}	80% ethanol soluble CHO	2.0	0.11	23.9
Potential degradable	CB2	RNSP[6]	WI_{RNSP}[6]	100 – (0h_ASH + 65% × EE + 0h_CP + NDF + ESC)	$2.5 \times Kd_{RNSP}$	0.08	23.9
			D_{RNSP}[6]	100 – (WI_{RNSP} + U_{RNSP})	Kd_{RNSP}	$0.0139+0.17 \times Kd_{RNSP}$	27.3
	CB3	NDF – CC	D_{NDF}	100 – (WI_{NDF} + U_{NDF})	Kd_{NDF}	$0.0139+0.17 \times Kd_{NDF}$	27.3
Undegradeble	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 (%DM)							
Soluble	PA	NPN	S_{CP}	soluble washable fraction	2.0	0.11	13.6
Potential degradable	PB	CP – (NPN + ADIP)	WI_{CP}	Washable CP – S_{CP}	Kd_{CP}	0.08	13.6
			D_{CP}	100 – (WI_{CP} + U_{CP})	Kd_{CP}	0.06	13.6
Undegradeble	PC	ADIP	U_{CP}	72 h residue	-	-	-

[1]Carbohydrate (CHO) and protein (CP) fractions calculated by the CNCPS system based on chemical analysis in %CHO an CP, respectively: CA, soluble CHO; CB2, potential degradable soluble fiber; CB3, potential degradable NDF; CC, undegradeble CHO; PA, soluble CP; PB, potential degradable CP; PC, undegradeble CP (Sniffen et al. 1992).

[2]*In situ* CHO and CP fractions used in the DVE/OEB protein system in %CHO and CP, respectively: capital letters: S, truly soluble washable fraction; WI, washable insoluble fraction; D, potentially degradable fraction; U, completely undegraded fraction. Lower case letters: $_{ESC}$, CHO soluble in 80% ethanol; $_{RNSP}$, residual non-starch polysaccharides; $_{NDF}$, neutral detergent fiber; $_{CP}$, crude protein (Tamminga et al. 2007).

[3]Kd, fractional degradation rate of the D fractions was calculated by the exponential model: $R(t) = U + D \times \exp^{-Kd(t-lag)}$ (Robinson et al. 1986) while Kd values for the S and WI fraction were adapted from Tamminga et al. (2007).

[4]Kp is fractional passage rate values and equations where adapted from Tamminga et al. (2007).

[5]Energy content (ATP, adenosine triphosphate) of each *in situ* fraction was adapted from Tamminga et al. (2007).

[6]RNSP, residual non-starch polysaccharides was calculated as 100 - (ash + EE + CP + NDF + ESC). A 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.

5.3.5. Modeling Nutrient Availability

The 2007 DVE/OEB protein system (Tamminga et al. 2007) was used to predict feed protein quality using equations which calculate rumen-degraded protein balance (OEB) and the amount of true protein digested in the small intestine (DVE) (also called metabolizable protein in the CNCPS protein evaluation system; Sniffen et al., 1992; NRC, 2001) (Table 5.2).

The net energy for lactation (NE_l) of alfalfa was calculated using equations from the VEM energy system described in Table 5.2 (Van Es, 1978). However, metabolizable energy (ME) was calculated as described on the Dutch Product Board of Animal Feed (PDV, 2005) website because the PDV recommended to replace the original ME equation of the VEM enegy system described by van Es (1978) for roughage like alfalfa with this new ME equation (Table 5.2).

Equations from the CVB (2005) 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% fat and 3.3% protein (CVB, 2005). Intake NE_l/DVE were calculated for a 650 kg Holstein Frisian cow in second or later lactation with a dry matter intake of 15 kg/day grazing on alfalfa pasture (Castillo et al., 2006). This type of cow requires 864 KJ/day (772 KJ/day + 12% extra energy required for grazing) and 119 g DVE/day 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 fat/protein-corrected milk production using the following two equations (CVB, 2005):

$$\text{FPCM-NE}_l = 440 \times \text{FPCM} + 0.73 \times \text{FPCM}^2 \quad (5.3)$$

$$\text{FPCM-DVE} = 1.396 \times \text{Protein} + 0.000195 \times \text{Protein}^2 \quad (5.4)$$

5.3.6. Statistical Analysis

The effect of alfalfa populations on measured parameters was analyzed using PROC MIXED of SAS 9.2 (2003). The statistical models used were:

$$Y_{ij} = \mu + T_i + H_j + \varepsilon_{ij} \quad (5.5)$$

$$Y_{ij} = \mu + T_i + R_j + \varepsilon_{ij} \quad (5.6)$$

where, Y_{ij} is the dependent variable, μ is the general mean, T_i is the fixed effect of treatment (i = 4; Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4 and AC Grazeland), H_j is the random effect of harvest date (j

= 2; July 24 or August 13, 2008), R_j is the random effect of incubation run (j = 2; 1 and 2) and ε_{ij} is the residual error.

Table 5.2. DVE/OEB protein and VEM energy system equations to calculate intestinal available protein (DVE), rumen degradable protein balance (OEB) and net energy for lactation (NE_l).

Traits		Calculation
Protein system ruminal phase (g/kg DM)[1]		
Rumen degraded CP balance	OEB	$MCP^{RDP} - MCP^{FOMr}$
Rumen degradable CP	RD_{CP}	$(S_{CP} \times Kd_S / (Kd_S + Kp_S)) + (D_{CP} \times Kp_D / (Kd_D + Kp_D))$[2]
Rumen undegraded CP	RU_{CP}	$CP - RD_{CP}$
Microbial CP from RD_{CP}	$MCPr^{RDP}$	RD_{CP}
Rumen fermentable OM	FOMr	$\sum Kd / (Kd + Kp) \times$ *in situ* fractions
Microbial CP from FOMr	$MCPr^{FOMr}$	$1 / Y = (M / GR + 1 / Y_{max})$[3]
Protein system intestinal phase (g/kg DM)[1]		
Intestinal available protein	DVE	DMCP + DRUP – DMFP
Intestinal available MCP	DMCP	$0.85 \times 0.75 \times MCP^{FOMr}$ [4]
Intestinal available RUP	DRUP	$RU_{C\,P} - U_{CP}$
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 DM)		
Gross energy	GE	Analyzed by Bomb calometrie
Metabolizable energy[6]	ME	$14.94 \times tDOM + 18.98 \times EE - 1.478 \times NDF - 0.97 \times ESC$
Metabolizability[7]	q	$100 \times ME / GE$
Net energy for lactation[7]	NE_l	$0.6 \times [1 + 0.004 \times (q - 57)] \times 0.9752 \times ME$

[1]Parameters were calculated according Tamminga et al. (2007).
[2]S_{CP}, truly soluble washable CP (g/kg DM); D_{CP}, potentially degradable CP (g/kg DM); Kd, fractional degradation rate (%/h); Kp, fractional passage rate (%/h).
[3]Y, microbial DM yield; M, microbial maintenance requirements (1.365 mmoles of ATP/g bacteria/h for the D fractions, 4.095 mmoles of ATP/g bacteria/h for the S and WI fractions); GR, fractional growth rate (affected by ATP yield of specific *in situ* fractions, passage rate and microbial maintenance requirements for specific *in situ* fractions); Y_{max}, maximum microbial growth yield (assumed to be 32 g microbes/mole ATP, microbes contain 62.5 %CP); a correction of 0.20 is applied to adjust the final microbial protein mass for predation by protozoa (Pirt 1965).
[4]The factor 0.85 represents the true protein content of MCPr and the factor 0.75 represents the digestibility of MCP in the small intestine (Tamming et al., 1994).
[5]The factor 0.075 assumes that 75 g of protein is lost in the faeces per kg of undigested DM that passes through the digestive tract (Tamminga et al., 1994).
[6]Metabolizable energy equation adapted from the PDV (2005) website.
[7]Equations adapted from Van Es (1978).

Model 5.5 was used to analyze the chemical composition of the original sample and model 5.6 was used to analyze *in situ* fractions and degradation and calculated nutrient availability. For model 5.5 harvest date was used as the replicate (n=2) and form model 5.6 incubation run (n=2).

Differences between *in situ* and chemical fractions were analyzed by a paired t-test using PROC TTEST of SAS 9.2 (2003). The Fisher's protected least significant difference (LSD) test was used for multiple treatment comparisons using the LSMEAN statement of SAS 9.2 (SAS, 2003) with letter groupings obtained using the pdmix800 macro (Saxton, 1998). Contrasts in SAS were used to compare the mean of three *Lc*-alfalfa progeny (Beav*Lc*1 + Ramb*Lc*3 + Range*Lc*4) 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.

5.4. Results & Discussion

5.4.1. Anthocyanidin Concentration and Fiber Levels in Lc-Alfalfa and AC Grazeland

The three *Lc*-alfalfa populations harvested in 2008 had an average forage anthocyanidin level of 163.4 μg/g DM, while AC Grazeland forage harvested from the same field at the same time did not accumulate anthocyanidin at all (Table 5.3). The anthocyanidin level of these *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 μg/g DM) (Wang et al., 2006b), but lower than the anthocyanidin content (232 μg/g DM) of the same *Lc*-populations harvested in 2007 (Section 4.4.1).

Fiber fractions, NDF, ADF and ADL tended ($P<0.10$) to be higher in AC Grazeland compared with the mean of three *Lc*-alfalfa populations (59.1 vs. 54.3, 53.0 vs. 47.7, and 14.6 vs. 11.0 %CHO, respectively) (Table 5.3). Tremblay et al. (2000) screened 27 alfalfa cultivars over two consecutive years and found that AC Grazeland always had a higher fiber (NDF, ADF) content compared to all other cultivars 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).

5.4.2. Comparison of In Situ and Chemical Protein Fractionations of Lc-Alfalfa and AC Grazeland

The soluble protein fractions in both CNCPS (PA) and DVE/OEB (S_{CP}) systems are presumed to have a fractional degradation rate of 200 %/h. Non-protein N (PA) was similar in all three *Lc*-alfalfa populations and AC Grazeland (average of 62.9 %CP; Table 5.3), as were the soluble CP (S_{CP}) fractions and the fractional degradation rates (Kd_{CP}) of potentially degradable CP (D_{CP}; Table 5.4). However, the S_{CP} fraction of ^{T1}Ram*Lc*3 was numerically lower (~6.7 %) and the D_{CP} and Kd_{CP} of the same population were numerically higher (~8.1% and ~3.1 %/h, respectively) compared to the other three populations.

The S_{CP} mean of 34.9 %CP for all alfalfas used in this study was similar to values reported by Aufrère et al. (2000) and Elizalde et al. (1999) for alfalfa harvested at a vegetative stage of growth. However, the NPN value for PA averaged ~28 %CP higher (P<0.001) than the *in situ* S_{CP} value. The S_{CP} was determined after solubilizing alfalfa sample in water for 4 h at room temperature (RT). In contrast, PA was determined after solubilizing sample in water with trichloracetic acid (TCA) for 30 min, after which the pH was adjusted to 2 and the solution incubated over night at RT. Since TCA precipitates peptides larger than 10 amino acid 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, buffer-soluble protein (BSP) was higher (P<0.04) in the mean of three *Lc*-alfalfa forages compared to AC Grazeland forage (72.6 vs. 70.7 %CP; Table 5.3). When individual populations were considered, a trend towards highest BSP (P=0.06) was observed for Beav*Lc*1 and lowest for AC Grazeland (Table 5.3). The mean BSP concentration for all four alfalfa populations was higher than values reported in other studies, in which values ranged from 38.4-to-41.1 %CP (Elizalde et al., 1999; Grabber, 2009). Previously in section 3.5.2, 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 CFIA. However, mean *in situ* S_{CP} values were similar to other values reported for alfalfa (Elizalde et al,. 1999; Aufrere et al., 2000). Elizalde et al. (1999) found only slightly higher soluble protein in BSP than in washable CP (W_{CP}) from *in situ* analysis (38.9 vs. 36.6 %CP) in alfalfa harvested at several stages of growth. Therefore, BSP gave a fairly reliable estimate of the *in situ* washable W_{CP} fraction in the study of Elizalde et al. (1999). Thus, BSP gives variable results when compared to *in situ* W_{CP}.

Table 5.3. Phytochemical and chemical composition for *Lc*-alfalfa and AC Grazeland (AG).

	Population				Model		*Lc* vs. AG[1]
Traits	Beav*Lc*1	Ramb*Lc*3	Rang*Lc*4	AG	SEM	*P* value	*P* value
Anthocyanidin (μg/g 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 (%DM)	12.9	12.4	13.6	13.7	1.10	0.084	0.108
EE (%DM)	4.0	3.4	3.6	3.6	0.17	0.186	0.723
CP (%DM)	28.7	28.2	29.1	27.3	2.22	0.313	0.130
Protein sub-fractions (%CP)[2]							
NPN i.e. PA	64.0	66.4	59.8	61.5	1.90	0.289	0.512
BSP	73.3	73.0	71.4	70.7	0.46	0.064	0.037
PB	34.7	31.9	38.5	36.7	2.11	0.290	0.544
NDIP	4.1	3.8	3.9	4.2	1.37	0.817	0.518
ADIP i.e. PC	1.7	1.6	1.7	1.8	0.69	0.684	0.311
CHO[3] (%DM)	54.5	55.0	54.5	55.5	1.05	0.506	0.308
Carbohydrate sub-fraction (%CHO)[4]							
ESC i.e. CA4	7.4	5.8	7.0	6.1	2.00	0.662	0.582
RNSP i.e. CB2	38.3	39.8	39.0	34.9	5.54	0.302	0.105
NDF	54.3	54.4	54.0	59.1	7.32	0.276	0.086
ADF	49.4	46.7	46.9	53.0	7.20	0.174	0.064
ADL	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

[1]*Lc* vs. AC Grazeland (AG), comparing the mean of three transgenic *Lc*-alfalfa populations (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4) with var. AC Grazeland (selected for a low initial rate of degradation).
[2]NPN, nitrogenous compounds soluble in water and not precipitated by trichloroacetic acid, called PA in CNCPS; BSP, crude protein soluble in phosphate buffer; PB, true potential degradable protein calculated in %DM as CP – (NPN + ADIP); NDIP, neutral detergent insoluble CP; ADIP, acid detergent insoluble CP, called PC in CNCPS.
[3]CHO, total carbohydrate content calculated as: DM – (ash + EE + CP).
[4]ESC, CHO soluble in 80% ethanol, called CA4 in CNCPS; RNSP, residual non-starch polysaccharides calculated as CHO – (NDF + ESC), called CB2 in CNCPS; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin.

All four alfalfas had similar completely undegradable ADIP fractions with an average of 1.7 %CP (Table 5.3). The ADIP values were on average 7.4 %CP lower ($P<0.001$) than the values for undegradable CP with the *in situ* method (U_{CP}). Moreover, U_{CP} values were lower ($P<0.05$) in Ramb*Lc*3 and Rang*Lc*4 compared with AC Grazeland while U_{CP} in Beav*Lc*1 did not differ from the other three alfalfa populations (Table 5.4). The higher U_{CP} compared with ADIP may be

caused by contamination of the 72 h *in situ* residue with microbial protein and/or more protein was solubilized during acid detergent fibre (ADF) determination of original alfalfa samples than during the 72 h *in situ* rumen incubations.

The ADIP values for all alfalfas in our study was lower than previously reported ADIP values for alfalfa harvested at a vegetative stage of growth (2.1-to-4.6 %CP). While our *in situ* undegradable U_{CP} fractions were in a similar range (5.6-to-15.6 %CP) as those previous reported (Elizalde et al., 1999; 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., 1999; 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 Haugen et al. (2006) and Pichard and Van Soest (1977).

All four alfalfa populations in this study had similar potentially degradable PB values calculated by the CNCPS system (average 35.5 %CP) (Table 5.3). The potentially degradable CP fraction D_{CP} measured by the *in situ* method averaged 56.1 %CP and was similar for all alfalfas in this study, but there was a trend towards higher D_{CP} ($P<0.10$) in Ramb*Lc*3 compared to Range*Lc*4 (Table 5.4). The D_{CP} in our study was similar to D_{CP} values reported by Aufrère et al. (2000) and Elizalde et al. (1999) for vegetative alfalfa. The Kd_{CP} 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, while the Kd_{CP} for vegetative alfalfa was higher (ranging from 17.9-to-34.1 %/h) compared with alfalfa forage at more mature stages of growth (Elizalde et al., 1999; Aufrère et al., 2000; González et al., 2001). The potentially degradable CP (PB) fraction calculated by the CNCPS method was 20.6% lower ($P<0.001$) than the *in situ* D_{CP} fraction by the DVE/OEB 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 to alfalfa tested in other studies where they found higher PB values (68.7 and 71.7 %CP, respectively) for alfalfa (Elizalde et al., 1999a; Grabber, 2009). Yu et al. (2003a,b) found PB values (40.7-to-55.5 %CP) in a similar range to *in situ* D_{CP} values (43.6-to-52.6 %CP) for alfalfa harvested at different stages of growth. So, in the study of Yu et al. (2003a,b), PB gave a good estimate for D_{CP}.

Table 5.4. *In situ* protein and energy fractions and ruminal degradation for *Lc*-alfalfa and AC Grazeland (AG).

Traits[1]	Population				Model		*Lc* vs. AG[2]
	Beav*Lc*1	Ramb*Lc*3	Range*Lc*4	AG	SEM	*P* value	*P* value
Total CP (%DM)	28.7	28.2	29.1	27.3	2.22	0.313	0.130
In situ protein fractions and degradation							
S_{CP} (%CP)	38.9	28.6	36.6	35.3	2.46	0.107	0.806
D_{CP} (%CP)	52.0	63.0	54.6	54.9	2.57	0.097	0.536
Kd_{CP} (%/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} (%CP)	9.1^{ab}	8.5^{b}	8.8^{b}	9.8^{a}	0.22	0.048	0.016
Total CHO (%DM)	54.5	55.0	54.5	55.5	1.05	0.506	0.308
In situ carbohydrate fractions and degradation							
S_{ESC} (%CHO)	7.4	5.8	7.0	6.1	-	-	-
WI_{RNSP} (%CHO)	13.2	12.8	15.1	12.1	3.00	0.870	0.643
D_{RNSP} (%CHO)	22.4	24.9	22.3	20.4	2.88	0.711	0.429
Kd_{RNSP} (%/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} (%CHO)	27.9	30.1	28.0	30.0	0.49	0.053	0.082
Kd_{NDF} (%/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} (%CHO)	2.6	2.0	1.4	2.4	0.30	0.146	0.294
U_{NDF} (%CHO)	26.7^{b}	24.4^{c}	26.3^{b}	29.1^{a}	0.49	0.011	0.004
Total OM (%DM)	87.1	87.6	86.4	86.3	1.10	0.084	0.108
In situ organic matter fractions and degradation							
W_{OM} (%OM)	23.8	21.5	24.5	23.2	2.93	0.763	0.997
D_{OM} (%OM)	54.6	58.9	55.2	53.2	2.99	0.396	0.289
Kd_{OM} (%/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} (%OM)	21.6^{b}	19.7^{c}	20.3^{c}	23.6^{a}	0.21	<0.001	<0.001

[a-c] Means with different letters within the same row differ ($P<0.05$).

[1]Capital letters: S, washable soluble fraction; W, washable fraction; WI, washable insoluble fraction; D, potential degradable fraction calculated as: 100 – (S + WI + U); U, undegradable fraction (72h incubation residue); Kd, fractional degradation rate; lag, lag time before fermentation initiated. Subscript letters: $_{CP}$, crude protein; $_{ESC}$, CHO soluble in 80% ethanol; $_{RNSP}$, residual non-starch polysaccharides; $_{NDF}$, neutral detergent fiber; $_{OM}$, organic matter. Kd and lag were calculated by an exponential model: $R(t) = D \times \exp^{-Kd\times(t-lag)}$.

[2]*Lc* vs. AC Grazeland (AG), comparing the mean of three transgenic *Lc*-alfalfa populations (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4) with var. AC Grazeland (selected for a low initial rate of degradation).

5.4.3. Ruminal Protein Availability of Lc-Alfalfa and AC Grazeland

The DM weight of the alfalfa *in situ* WI protein fractions was < 0.001 %DM for all four 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 by-product ingredients lay within a range from 1.9-to-29.3 %CP, but was minimal (below 1.0 %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 which could escape through the pores of the nylon bag during washing.

The rumen degradable CP (RD_{CP}) content was similar for each of the four alfalfa population, but there was a trend towards a higher RD_{CP} when the mean of three *Lc*-alfalfas (P<0.10) was compared with AC Grazeland (22.6 vs. 21.4 %DM) (Table 5.5). The mean RD_{CP} for all alfalfa populations was on average 79.3 %CP. This was within the range of 71.2-to-89.6 %CP reported previously for alfalfa harvested at a vegetative stage of growth (Elizalde et al., 1999; Aufrère et al., 2000; González et al., 2001; Faría-Mármol et al., 2002). Since ADL and other fiber fractions tended to be higher (P<0.10) in AC Grazeland compared with the mean of three *Lc*-alfalfas and increased ADL decreases the digestibility of forages (Jung et al., 1997), the enhanced fiber fractions in AC Grazeland may have had greater impact on RD_{CP} than the anthocyanidin levels in the mean of three *Lc*-alfalfa forages have on protein precipitation.

5.3.4. Comparison of In Situ and Chemically Carbohydrate Fractions of Lc-Alfalfa and AC Grazeland

All *in situ* derived CHO fractional degradation rates, lag times and fractions, except for undegradable fibre (U_{NDF}), were similar (P>0.05) between our four alfalfas tested (Table 5.4). The U_{NDF} was higher (P<0.04) for AC Grazeland compared with the mean of three *Lc*-alfalfas (29.1 vs. 25.8 %CHO). When individual *Lc*-progeny were assessed, U_{NDF} was lower (P<0.01) for Ramb*Lc*3 compared with Beav*Lc*1 and Rang*Lc*4. Opposite, Ramb*Lc*3 and AC Grazeland trended to have higher (P<0.10) *in situ* potential degradable fiber (D_{NDF}) compared to Beav*Lc*1 and Rang*Lc*4 (Table 5.4). When the CNCPS system was compared, undegradable fiber (CC) values for all four 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 systems (Table 5.4). While the trend in AC Grazeland towards higher ADL content (P<0.08) (Table 3) may have had a negative impact on NDF digestibility (Jung et al., 1997), differences between the U_{NDF} and

D_{NDF} for Ramb*Lc*3 and the other two *Lc*-alfalfa populations may result from the numerically lower anthocyanidin content in Ramb*Lc*3 (Table 5.3).

In the CNCPS system, a fractional degradation rate of 8 %/h is assumed for potentially degradable NDF (i.e. CB3) of alfalfa pasture (Tylutki et al., 2008). In our study, the *in situ* fractional degradation rate of D_{NDF} for all four alfalfa populations was similar with an average 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 5.4) and were consistent with values reported by Yu et al. (2004) for alfalfa. All four alfalfa populations had similar RNSP (i.e. CB2) fractions (P>0.05), but RNSP was numerically higher for individual *Lc*-alfalfa populations compared with AC Grazeland (Table 5.3). The fractional degradation rate of RNSP (Kd_{RNSP}) for alfalfa forage averaged 20.1 %/h and was highly variable between the populations. This Kd_{RNSP} in our study was two-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., 2007a). Moreover, the CNCPS system has only one RNSP fraction, CB2, while *in situ* methods fractionate RNSP into a washable insoluble fraction (WI_{RNSP}), potentially degradable and undegradablefractions. Since the WI_{RNSP} fraction of alfalfa forage is assumed to have a Kd of 250 %/h, this would substantially increase the Kd of the total RNSP fraction (Tamminga et al., 2007), if the WI_{RNSP} fraction formed major part of the RNSP.

5.4.5. Modelling ruminal Energy Availability of 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 (Kd_{OM}), and lag time before the initiation of D_{OM} fermentation were similar between our four alfalfa populations (Table 5.4) and resulted in a similar RD_{OM} (Table 5.5). However, undegradable OM (U_{OM}) was higher (P<0.001) in AC Grazeland compared to the mean of the mean of three *Lc*-alfalfas, and U_{OM} within the three *Lc*-populations was higher (P < 0.001) in Beav*Lc*4 compared to Ramb*Lc*3 and Range*Lc*4 (Table 5.4). The mean RD_{OM} content of all four populations was 58.4 %DM. This was slightly lower than the range (63.2-to-72.0 %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 μm), which would allow more insoluble feed particles to escape from the bag which would overestimate the W_{OM} fraction.

Table 5.5. Modelling DVE/OEB rumen microbial nutrient availability and intestinal protein availability for *Lc*-alfalfa and AC Grazeland (AG).

Traits[1]	Population				Model		*Lc* vs. AG[2]
	Beav*Lc*1	Ramb*Lc*3	Rang*Lc*4	AG	SEM	*P* value	*P* value
Ruminal phase							
RD_{CP} (g/kg DM)	226.0	223.9	227.0	213.8	4.73	0.313	0.095
RD_{CHO} (g/kg DM)	313.8	325.0	305.7	302.3	5.40	0.130	0.116
RD_{OM} (g/kg DM)	579.9	586.6	565.6	546.8	16.05	0.416	0.175
FOMr (g/kg DM)	539.8	548.9	532.7	516.1	9.98	0.267	0.102
$MCPr^{FOMr}$ (g/kg 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}	0.86	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 DM)	26.2	23.9	25.5	26.8	0.62	0.104	0.086
U_{DM} (g/kg DM)	233.5^b	217.4^c	223.1^c	251.5^a	1.78	<0.001	<0.001
DMFP (g/kg DM)	17.5^b	16.3^c	16.7^c	18.9^a	0.14	<0.001	<0.001
RU_{CP} (g/kg DM)	60.7	58.2	64.1	59.5	4.73	0.837	0.791
DRUP (g/kg DM)	34.5	34.3	38.6	32.7	4.72	0.836	0.594
DMCP (g/kg DM)	53.8	55.7	49.5	49.8	3.01	0.471	0.412
DVE (g/kg DM)	70.9	73.7	71.3	63.6	3.21	0.169	0.054

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

[1]RD_{CP}, rumen degradable protein; RD_{OM}, rumen degradable organic matter; RD_{CHO}, rumen degradable carbohydrates; RD_N, rumen degradable nitrogen; FOMr, rumen fermentable organic matter; $MCPr^{FOMr}$, ruminal microbial protein synthesis from FOMr; OEB, rumen degraded protein balance; U_{CP}, completely undegradeble protein; U_{DM}, completely undegradeb;e dry matter; DFMP, endogenous protein lost into the faeces; RU_{CP}, rumen undegradeble protein; DRUP, intestinal available RU_{CP}; DMCP, intestinal available microbial protein; DVE, total intestinal available protein, see Table 2 for calculations (Tamminga et al. 2007).

[2]*Lc* vs. AC Grazeland (AG), comparing the mean of three transgenic *Lc*-alfalfa populations (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4) with var. AC Grazeland (selected for a low initial rate of degradation).

In the 2007 DVE/OEB system, FOMr is calculated as the sum of all rumen degradable fractions which was similar for all four alfalfa populations with a numerically trend towards lower FOMr in AC Grazeland and a mean FOMr of 53.4 %DM (Table 5.5). The FOMr was 3.5%

lower (P<0.001) than RD_{OM} (Table 5.5). To calculate RD_{OM}, it is assumed that the washable (S and WI) *in situ* fractions are completely degraded in the rumen, while the calculation FOMr assumes that a portion of the washable *in situ* fraction escapes ruminal degradation. Since the W_{OM} fraction used in the calculation of RD_{OM} was ~3.0% higher than the sum of 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 5.5). Unlike FOMr, the calculation for RD_{OM} does not consider that microbes cannot utilize EE, which was on average 3.7 %DM (Table 5.3).

5.4.6. Ruminal Protein:Energy Ratios and Synchronization of Lc-Alfalfa and AC Grazeland

The total rumen degradable RD_N:RD_{CHO} ratio was lower for Ramb*Lc*3 compared to Beav*Lc*1 (P<0.01), which in turn, had a lower RD_N:RD_{CHO} ratio than Rang*Lc*4, which had the highest ratio (P<0.01; Table 5.5). There was a trend towards, Ramb*Lc*3 and AC Grazeland having a lower (P<0.10) OEB (balance between MCPr calculated based on protein and energy) compared with Range*Lc*4 and Beav*Lc*1 (Table 5.5). 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 three *Lc*-alfalfa populations and AC Grazeland were evaluated for hourly RD_N:RD_{CHO}, a bi-phasic profile was observed for all four types of germplasm and included an initial and substantial peak (over-supply) of N relative to energy within the first two hours after feeding, followed by a slow decline from 2 to 24 h after feeding (Fig. 5.1A). This latter time period shows a relatively synchronized and balanced ratio when the hourly OEB method is evaluated (Fig. 5.1B). The OEB after one hour was least synchronization (P<0.01) for Beav*Lc*1 and Rang*Lc*4, followed by AC Grazeland and Ramb*Lc*3, after which the OEB between 2 to 24 h was similar for all four alfalfa populations. The more synchronized OEB for Ramb*Lc*3 was likely due to a numerically lower highly degradable S_{CP} fraction compared to the other three populations. [The S_{CP} fraction is almost completely gone after one hour]. 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 pattern (Tas et al., (2006), but the magnitude of imbalance for alfalfa was much higher than for perennial ryegrass.

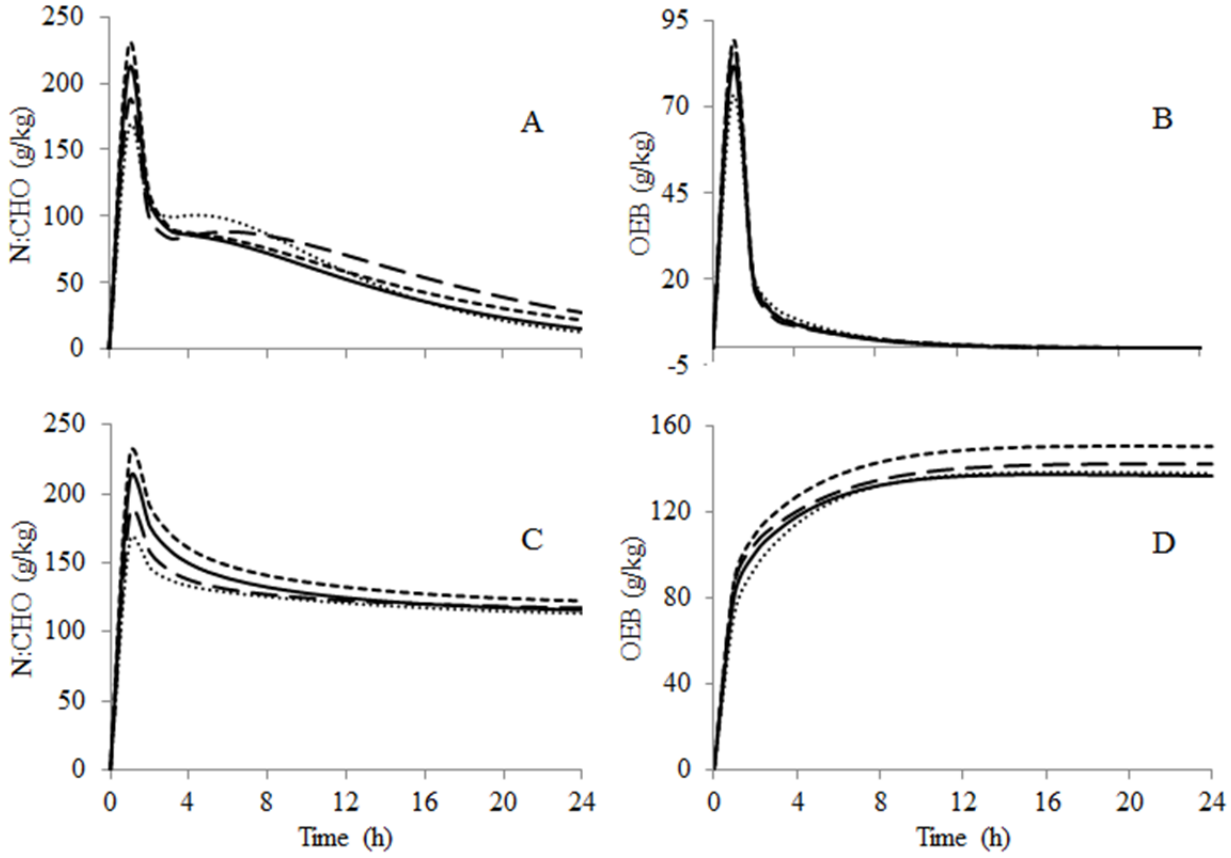

Fig. 5.1. Degradation ratios of Beav*Lc*1(— —), Ramb*Lc*3 (·····), Rang*Lc*4 (- - - - -) and AC Grazeland (———). **A**: hourly ratio of rumen degradable nitrogen to carbohydrate (N:CHO); **B**: hourly rumen degraded protein balance (OEB); **C**: cumulative rumen degradable N:CHO; **D**: cumulative OEB.

Excessive protein supplied, above microbial requirements, from alfalfa within the first two hours 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 unsychronized 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 Fig. 5.1C,D suggests that urea recycling will be ineffective for the four alfalfa populations, with AC Grazeland and Ramb*Lc*3 being more effective than Beav*Lc*1 and Rang*Lc*4. 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 three 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 two hours after ingestion will occur at least three times a day.

5.4.7. Modelling Intestinally Available Protein of Lc-Alfalfa and AC Grazeland

The predicted microbial protein synthesis in the rumen (MCPr) and therefore predicted intestinally available MCP (DMCP) was similar between the four alfalfa populations (Table 5.5). Since rumen undegradable CP (RU_{CP}) was similar between the four alfalfa populations, predicted intestinally available RU_{CP} (DRUP) was also similar between the four alfalfa populations (Table 5). Completely undegradable DM (U_{DM}) and predicted endogenous protein loss in the faeces (DMFP) each were higher (P<0.001) for AC Grazeland compared to the mean of the three *Lc*-alfalfa populations (Table 5.5). When individual populations were considered, both U_{DM} and predicted DMFP were higher (P < 0.001) for Beav*Lc*1 compared with Ramb*Lc*3 and Rang*Lc*4. There was a trend towards a higher predicted intestinally available protein DVE (P<0.10) when the mean of *Lc*-alfalfa was compared to AC Grazeland (7.2 vs. 6.4 %DM). The calculated parameters DMCP, DRUP, DMFP and DVE in our study were similar to values from alfalfa reported by Yu et al. (2003a).

5.4.8. Total Tract Energy Availability of Lc-Alfalfa and AC Grazeland

Gross energy was similar between the four alfalfa populations and total digestible OM (tDOM) was highest for Ramb*Lc*3 and Rang*Lc*4, followed by Beav*Lc*1 and lowest for AC Grazeland (P<0.001; Fig. 5.2). The higher tDOM resulted in a higher (P<0.001) predicted NE_l for the mean of the three *Lc*-alfalfa populations compared to AC Grazeland (6.2 vs. 5.8 MJ/kg DM). These predicted NE_l values were much higher than the NE_l value of 5.1 MJ/kg DM reported in CVB (2005) feed tables for fresh alfalfa. However, the feed tables do not describe the stage of growth at alfalfa harvest.

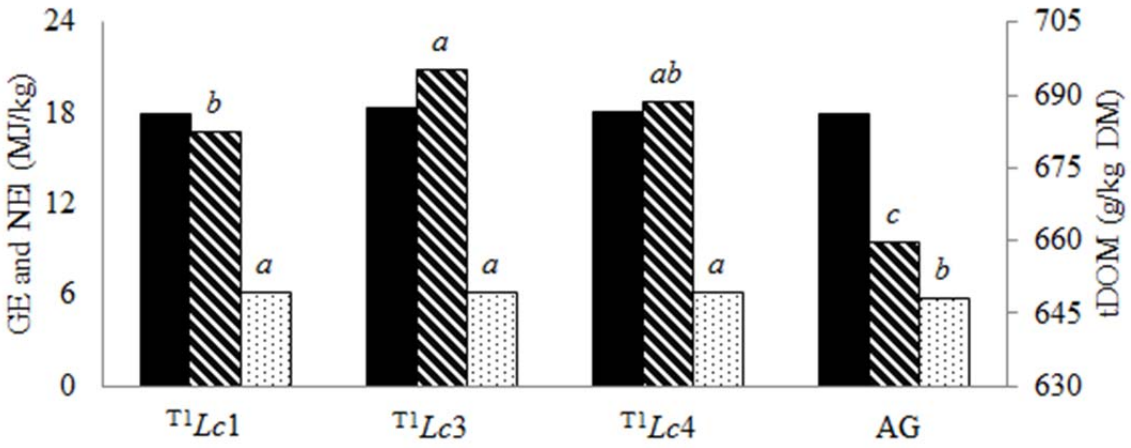

Fig. 5.2. Energy content of Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4 and AC Grazeland (AG) as calculated according to Van Es (1978). GE, gross energy ( ); tDOM, total tract digestible organic matter ( ); NE_l, net energy for lactation ( ). [a-c]Bars with different letters within the same component differ (P<0.05).

5.4.9. Calculated Milk Production of Lc-Alfalfa and AC Grazeland

The higher predicted NE_l for the mean of three *Lc*-alfalfa populations (Fig. 5.2) resulted in a higher calculated fat/protein corrected milk production based on NE_l (FPCM-NE_l) for these populations compared with AC Grazeland (16.5 vs. 14.9 kg; Fig. 5.3). Because of the trend towards higher predicted intestinal available protein (DVE) when the mean of the three *Lc*-alfalfa populations was compared to AC Grazeland, also a trend towards higher (P<0.10) calculated fat/protein-corrected milk production based on DVE (FPCM-DVE) was observed in the mean of three *Lc*-alfalfas (19.1 vs. 16.7 kg/day). The FPCM calculated based on NE_l was on average 2.4 kg/day lower than FPCM based on DVE. This means that for a 650 kg Holstein Frisian cow with a dry matter intake of 15 kg/day, 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 eight dairy farms that graze their dairy cattle on alfalfa pasture. They found an average milk production of 15.1 kg milk of dairy cows with an average dry matter intake of 15 kg/day (Castillo et al., 2006). Their *in vivo* results are similar to the calculated results we found using modelling methods.

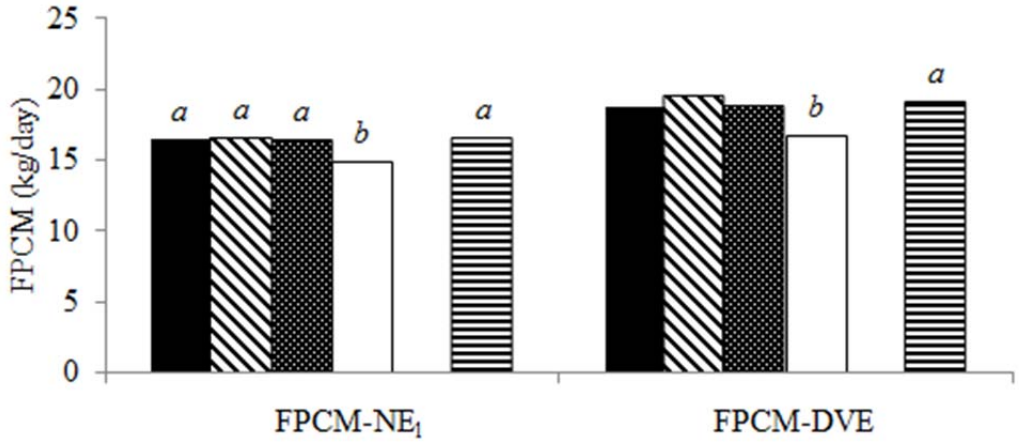

Fig. 5.3. Fat/protein corrected milk production (FPCM) from net energy for lactation (NE_l) and intestinal available protein (DVE) calculated according CVB (2005) for a Holstein Frisian cow of 650 kg with a dry matter intake of 15 kg/day from Beav*Lc*1 (■), Ramb*Lc*3 (▨), Rang*Lc*4 (▩), AC Grazeland (□) and the mean of the three $Lc^{1,3,4}$-alfalfas (▤). ^{a,b}Bars of FPCM-NE_l with different letters differ (P<0.05); Bars of the mean $Lc^{1,3,4}$-alfalfa and AC Grazeland for FPCM-DVE differ (P<0.10).

5.5. Conclusion

Forage nutritional characteristics were predicted using the CNCPS system, the 2007 DVE/OEB protein system and VEM energy system to compare anthocyanidin-accumulating *Lc*-alfalfa populations with cultivar AC Grazeland, which had been selected earlier for an lower initial rate of degradation. The *Lc*-transgenic populations had an average forage anthocyanidin concentration of 163.4 µg/g DM, while AC Grazeland did not accumulate anthocyanidin. Crude protein, neutral detergent fiber and residual non-starch polysaccharide content were similar in forage of the three *Lc*-alfalfa populations and AC Grazeland. AC Grazeland had higher undegradable *in situ* crude protein and neutral detergent fiber content compared with the *Lc*-alfalfa populations, and there was a trend towards higher predicted rumen degradable protein content, rumen degraded protein balance and intestinally available protein content for *Lc*-alfalfa. When comparing individual *Lc*-alfalfa populations, Ramb*Lc*3 had numerically the lowest soluble and undegradable and numerically highest potential degradable *in situ* protein and carbohydrate fractions of all *Lc*-alfalfa populations which resulted in a numerically lower predicted rumen degraded protein balance and numerically higher predicted intestinally available protein in Ramb*Lc*3. The hourly rumen degraded protein balance synchronization differed only after one hour for the four populations, and the net energy for lactation was higher in *Lc*-alfalfa compared with AC Grazeland. The trend towards a higher predicted intestinal available protein and a

higher predicted net energy for lactation in the three *Lc*-alfalfa populations resulted in a higher calculated milk production compared with AC Grazeland.

Chapter 6

Foam Stability of Leaves from Anthocyanidin-Accumulating *Lc*-Alfalfa and Relation to Molecular Structures Detected by FTIR Vibration Spectroscopy*

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6.1. Abstract

Foam stability related to pasture bloat from alfalfa pastures might be reduced by introducing a gene that stimulates the accumulation of mono/polymeric anthocyaindin. The objective of this study was to determine foam formation (at 0 min) and stability (at 150 min) from aqueous leaf extracts of three transgenic *Lc*-alfalfa progeny (Beav*Lc*1, Rambl*Lc*3, Rang*Lc*4), parental non-transgenic (NT) alfalfa and AC Grazeland (bloat reduced cultivar) harvested at 07:00 or 18:00 h. Leaf anthocyanidin accumulation was similar among the three *Lc*-alfalfa progeny and between 07:00 h and 18:00 h with an average of 248 µg/g DM. Initial foam volume and stability were lower for Rang*Lc*4 and AC Grazeland at 07:00 h ($P<0.05$) and for Beav*Lc*1, Rang*Lc*4 and AC Grazeland at 18:00 h ($P<0.05$) compared with NT-alfalfa. Amide I:amide II ratio, carbohydrate vibration intensity (CHO_{VI}) and extract ethanol-film and protein content correlated positively ($R<0.30$; $P<0.05$) and α-helices:β-sheets ratio correlated negatively ($R=0.43$; $P<0.01$) with initial foam volume. Final foam volume correlated negatively ($R<0.35$; $P<0.05$) with structural carbohydrate (SC_{VI}) and lipid vibration intensity and amide I:CHO_{VI} ratio and positively ($R<0.35$; $P<0.05$) with CHO_{VI}, amide I:CHO_{VI} ratio and CHO_{VI}:SC_{VI} ratio. The mean of three *Lc*-progeny had a higher lipid vibration intensity, higher SC_{VI}, lower CHO_{VI} and lower CHO_{VI}:SC_{VI} ratio compared with NT-alfalfa ($P<0.05$). Rang*Lc*4 had more beneficial foaming properties compared with Rambl*Lc*3, probably due to lower vibration intensity associated with protein structures and lower amide I:amide II ratio ($P<0.05$). Amide I:amide II and CHO_{VI}:SC_{VI} ratios were lower and amide I:CHO_{VI} ratio higher for AC Grazeland compared with the mean of the *Lc*-progeny ($P<0.05$). In conclusion, *Lc*-alfalfa accumulated anthocyanidin and had reduced foam stability compared with NT-alfalfa, while Rang*Lc*4 and AC Grazeland had the lowest foam stability.

Key words: Anthocyanidin-accumulating alfalfa, foam formation and stability, pasture bloat, FTIR vibration spectroscopy molecular structure.

6.2. Introduction

Alfalfa (*Medicago sativa* L.) is the major forage crop grown in many parts of the world. If beef or dairy cattle could be safely grazed on alfalfa, the cost of milk and meat production would be lower compared with feedlot or indoor dairy operations and management costs currently associated with alfalfa grazing would be eliminated (Rotz, 1996). However, pasture bloat is a real and often fatal risk to cattle grazing pure alfalfa at present (Majak, et al., 1995, 2001). In bloat, rapid ruminal protein degradation of alfalfa causes the formation of viscous stable foam which entraps fermentation gases and prevents normal eructation (Cockrem, et al., 1987; Tanner, et al., 1995). The resulting accumulation of fermentation gases causes distention of the rumen which exerts pressure on organs like the heart and lungs until the animal suffocates in extreme cases (Clarke and Reid, 1974). Normally, pasture bloat occurs within two to three hours after the bloat inducing forage is offered to cattle, and it occurs more frequently when offering cattle a new pasture in the early morning compared with the late afternoon (Hall and Majak, 1989; Majak, et al., 1995). Components thought to be involved in the formation and stabilization of bloat-related foams are specific fractions of soluble proteins and carbohydrates and small particles while lipids and flavonoid polymers inhibit these foaming properties (Clarke and Reid, 1974; Howarth, 1975; Hall, et al., 1988). However, none of these individual fractions has been identified as the main cause of pasture bloat. Structural features associated with protein, carbohydrates and lipids are detectable by mid-IR vibration spectroscopy, but not by traditional chemical methods (Yu, 2004; Doiron, et al., 2009). Hence, IR spectroscopy may give useful information about bloat reducing properties in forages.

The alfalfa cultivar AC Grazeland was previously selected for a low initial rate of degradation, and this cultivar reduced the incidence of bloat *in vivo* by means of thicker cell walls (Goplen, et al., 1993; Coulman, et al., 2000). Many bloat-free legumes also contain polymeric-anthocyanidins (Aerts, et al., 1999; McMahon, et al., 2000). These metabolites destabilize bloat-related foams (Fay, et al., 1980; Tanner, et al., 1995). Therefore, alfalfa (which usually does not accumulate polymeric anthocyanidin in forage) was transformed with a maize anthocyanidin regulating *βHLH LEAF COLOR* (*Lc*) gene. This caused the accumulation of monomeric anthocyanidin in alfalfa forage under field conditions and mono/polymeric anthocyanidin in plants transferred from normal to high light intensities (Ray, et al., 2003; Wang, et al., 2006b). However, survival of these T_0 *Lc*-alfalfa genotypes was poor under western

Canadian conditions. Therefore, three of these genotypes were crossed with genotypes of hardy western Canadian cultivars to generate hardier T_1 *Lc*-alfalfa progeny.

The objective of this study was to determine basic nutritional composition, molecular structures (using vibration spectroscopy), and physicochemical properties affecting foam formation and stability of leaves from transgenic T_1 *Lc*-alfalfa progeny, parental non-transgenic (NT) alfalfa cultivars and the cultivar AC Grazeland harvested in the field in early morning or late afternoon. Foam formation and stability parameters were correlated with vibration depended molecular structures and leaf composition.

6.3. Material & methods

6.3.1. Plant Material and Harvest Conditions

Transgenic (T_1) *Lc*-alfalfa crossed progeny, Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4, were developed at Forage Genetics International (Wisconsin, USA) as described in section 3.3.1. and compared with their parental non-transgenic (NT) western Canadian adapted cultivars Beaver, Rambler and Rangelander, and with alfalfa the cultivar AC Grazeland (Coulman et al., 2000) .

Prior to planting in the field, *Lc* and NT alfalfa populations were grown for approximately six months from seed in a greenhouse. Sheared plants were clipped and then transplanted into rows (75 cm between rows; 15 cm between plants) in dark brown chernozemic soil in a Canadian Food Inspection Agency (CFIA) approved experimental field block at the Saskatoon Research Centre farm of Agriculture and Agri-Food Canada (AAFC) on August 24, 2006 and June 3, 2009. AC Grazeland plants were dug up in the first week of June 2008 from a seed field at AAFC Indian Head, SK, Canada. One half of the AC Grazeland plants was directly transplanted into the experimental field with *Lc*-alfalfa progeny and the other half was established in the greenhouse and transplanted into the second block of the experimental field on June 3, 2009. Alfalfa plants were harvested manually with shears at *ca.* 5 cm above ground level at 07:00 h and 18:00 h on August 20 of 2009 at a vegetative pre-bud stage (according to CFIA-imposed regulations restricting flower buds from forming). [Harvested transgenic *Lc*-plants expressed a purple-green phenotype, while all other alfalfas were green]. Immediately after harvest, leaves were separated from stems and stored at -20 °C pending analysis. In the foam stability

experiment, each of the three NT-alfalfa cultivars (Beaver, Rambler and Rangelander) were considered as one replicate due to insufficient material per cultivar.

6.3.2. Leaf Extract Preparation, Foam Stability Measurements and Extract Analysis

Foams were prepared *in vitro* from 20 g of frozen alfalfa leaves homogenized in a commercial blender for 2 × 1 min (Oster model: BCBG08-B20-033) with 100 mL ddH_2O, followed by filtration through four layers of cheese cloth and centrifugation at 400 *g* for 10 min. In triplicate, 8 mL aliquots of supernatant were transferred to 15 mL screw-cap volumetric tubes per treatment, after which tubes were shaken by hand for 30 sec according to Kapp and Bamforth (2002) and Wei and Liu (2000). [In a pilot trial with different dilution rates and filtration methods we found that these parameters gave the largest foam volume (data not shown)]. Foam volume was measured at 0 min (directly after shaking), followed by 5 min intervals for the first 30 min after form formation, 10 min intervals from 30 to 60 min, 15 min intervals from 60 to 120 min and at 150 min. [In the pilot trial foam disappearance was negligible after 150 min]. Foam volume was defined by the distance (mm) from the top of the foam to the top of the liquid interface. Foam disappearance rate was calculated using the following exponential model (Ørskov and McDonald, 1979):

$$R(t) = a \times \exp^{-c \times t} \qquad (6.1)$$

Where, R is remaining foam (%) at incubation time t, *a* is initial foam volume (mL), *c* is fractional foam disappearance rate (%/h) and t is incubation time (h). This allowed us to characterize foam formation (0 min) and foam stability (foam disappearance rate and final foam volume at 150 min).

The leaf extract used for the foaming trial was analyzed for pH, protein content, viscosity and ethanol-film formation. Protein content of the leaf extract was analyzed by the Kjeldahl-N method (N × 6.25; AOAC 984.13). Viscosity was analyzed (after centrifugation of the leaf extract at 12,700 *g* for 5 min) on a Brookfield digital viscometer set at speed setting 4 with 40 rpm and maintained at 39.9 ºC (Model DV-III, Brookfield Engineering Laboratories, Stoughton, MA, USA). [Viscosity of ddH2O was 0.72 cP]. Pure ethanol was added to supernatants (1:1 v/v), after centrifugation at 16,000 g for 15 min, and then the mixture was stored at 4 ºC for 24 h to develop a viscous film that floats to the surface. The ethanol precipitated film was harvested by centrifugation at 16,000 *g* for 15 min, after which the supernatant was discarded and the pellet

(film) was weighed after drying at 55 ºC for 24 h. [The amount of this ethanol-film (pellet) was previously related to pasture bloat (Gutierrez, et al., 1963; Min, et al., 2006)].

6.3.3. Leaf Nutritional Analysis

Alfalfa leaf samples were freeze-dried and ground to pass through a 1 mm screen using a cyclonic mill (Retsch SM-3000, Brinkmann Instruments, ON, Canada). Leaf samples were analyzed for dry matter (DM; AOAC 930.15), ash (AOAC 942.05) and CP (Kjeldahl-N × 6.25; AOAC 984.13) according to the procedures of AOAC (1990). Neutral detergent fiber with heat stable α-amylase (NDF) was analyzed by the ANKOM filter bag (A200) technique (ANKOM Technology, Fairport, NY, USA) and neutral detergent insoluble CP (NDIP) was analyzed according to Licitra et al. (1996) by the Kjeldahl-N method. The values of NDF presented in this paper were adjusted for NDIP, but not for ash. Anthocyanidin extractable in methanol:HCl (99:1 v/v) was analyzed by the spectrophotometric method described by in section 3.3.2. with cyanidin as a standard.

6.3.4. Molecular Structures Analyzed by Fourier Transformed Infrared Vibration Spectroscopy

Fourier transformed infrared vibration spectroscopy (FTIR) was performed using a JASCO FT/IR-4200 with a ceramic IR light source and a deuterated L-alanine doped triglycine sulfate detector (JASCO Corporation, Tokyo, Japan) equipped with a MIRacle^{TM} attenuated total reflectance accessory module and outfitted with a ZnSe crystal and pressure clamp (PIKE Technologies, Madison, WI, USA). Spectra were generated from the mid-IR region (4000-800 cm^{-1}) using JASCO Spectramanager II software with a spectral resolution of 4 cm^{-1}. Functional spectral bands associated with protein, carbohydrate and lipid molecular structures were assigned according to published studies (see below) and identified with OMNIC 7.2 software (Spectra Tech, Madison, WI, USA).

Unique primary protein features found in peptide bonds (C-O, C-N and N-H) include amide I (~80% C=O and ~20% C-N stretching vibration; centered at a wavelength of *ca.* 1655 cm^{-1}) and amide II (~60% N-H bending vibration, ~40% C-N stretching vibration; centered at *ca.* 1550 cm^{-1}) (Fig. 6.1), which are detectable as two absorption peaks within the wavelength region from *ca.* 1720-to-1485 cm^{-1} (Fig. 6.2) (Barth, 2007; Jackson and Mantsch, 2000; Wetzel, et al., 2003). Absorption peak heights for secondary protein structure α-helices (*ca.* 1660 cm^{-1}) and β-sheets

(*ca.* 1630 cm^{-1}) (Fig. 6.1) are detectable in the amide I area using the second derivative function of OMNIC 7.2.

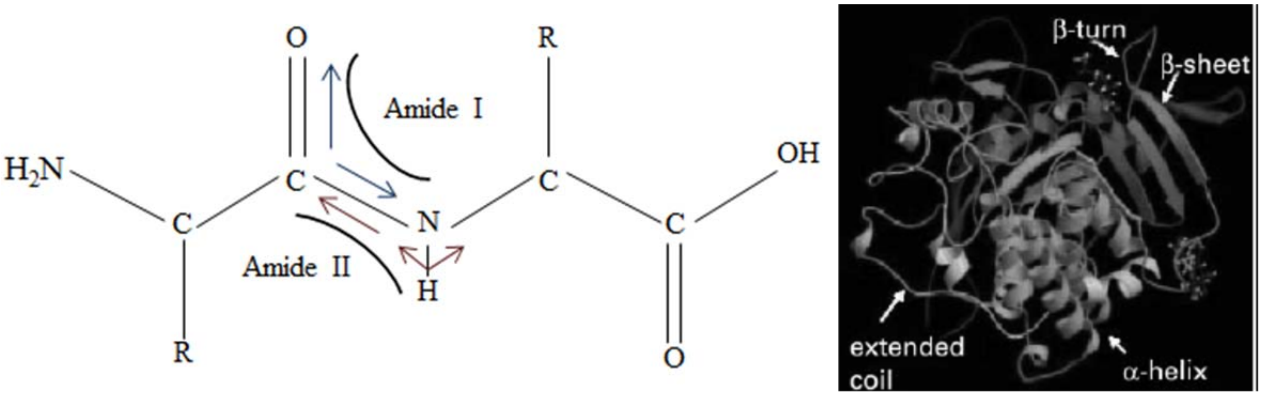

Fig. 6.1. Protein vibration spectroscopy features. Left, amide I and II bonds responsible for the vibration motions; Right, protein secundary structures. Figures adapted/prepared from Mantsch and Chapman (1996) and Dumas (2003).

The vibration absorption peak from all carbohydrates structures (CHO_{VI}) associated with C-O and C-C stretching vibration and C-O-H deformation is detectable at wavelengts from *ca.* 1180-to-900 cm^{-1} (Mathlouthi and Koenig, 1987; Wetzel, et al., 1998). The vibration absorption peak characteristic of structural carbohydrates (SC_{VI}; β-glucans, hemicellulose, cellulose) is detectable from *ca.* 1585-to-1188 cm^{-1} (Fig. 6.2) (Wetzel, et al., 1998; Wetzel and LeVine, 2001). Lipid features are detectable from *ca.* 3000-to-2770 cm^{-1} due to vibration absorption of CH (asymmetric stretching of CH_2 at *ca*. 2920 cm^{-1} and CH_3 at *ca*. 2955 cm^{-1}) (Wetzel, et al., 1998; Yu, et al., 2005).

6.3.5. Statistical Analysis

Data was statistically analyzed using PROC MIXED of SAS 9.2 (2003) using the statistical model:

$$Y_{ijk} = \mu + P_i + H_j + P_i \times Hj + \varepsilon_{ijk} \qquad (6.2)$$

where, Y_{ijk} is the dependent variable, μ is the general mean, P_i is the fixed effect of population (i = 5; NT-alfalfa, Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4 and AC Grazeland), H_j is the fixed effect of harvest time (j = 2; 07.00 and 18.00 h), $P_i \times H_j$ is the interaction between population and harvest time and ε_{ijk} is the residual error.

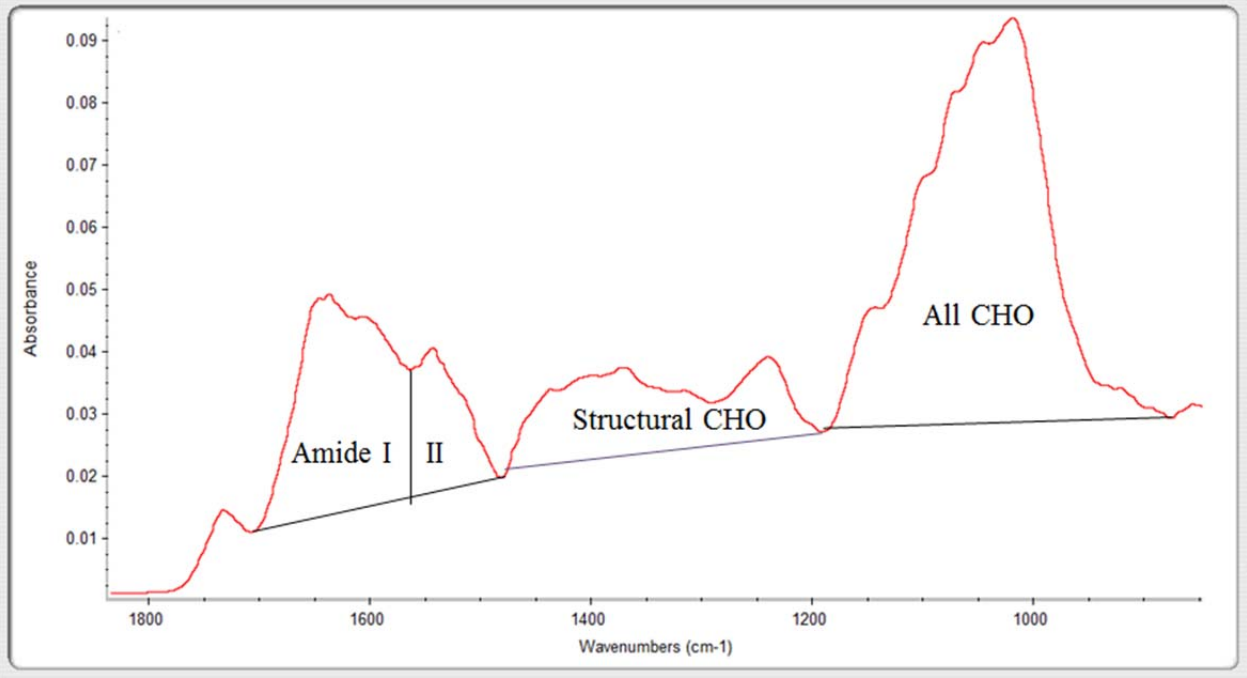

Fig. 6.2. Vibration spectrum in the finger print region from alfalfa with peak areas of amide I, amide II, structural carbohydrates (CHO) and all carbohydrates determined in OMNIC 7.2 software.

The blocks in the field (n=2) were regarded as replicates for the three *Lc*-alfalfa progeny and AC Grazeland and parental cultivars were each regarded as one replicate for NT-alfalfa (n=3) for the leaf composition and vibration spectroscopy data. For output data from the exponential model, leaf extract composition and foaming properties, blocks in the field (n=2); cultivar for each NT-alfalfa (n=3) plus foaming tubes (n=3) per treatment were each regarded as replicates.

The Fisher's protected least significant difference (LSD) test was used for multiple treatment comparisons using the LSMEAN statement of SAS 9.2 (2003) with letter groupings obtained using the SAS pdmix800 macro (Saxton, 1998). A contrast statement from SAS 9.2 (2003) was used to compare NT-alfalfa and AC Grazeland with the mean of the three *Lc*-progeny (Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4). Pearson's correlation coefficient between foaming properties and other parameters were analyzed using PROC CORR of SAS 9.2 (2003) by the Pearson correlation method. For the different statistical tests, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$, unless otherwise stated.

6.4. Results & Discussion

6.4.1. Foam Formation and Foam Stability of Lc-Alfalfa Related to Anthocyanidin Content

Pasture bloat is caused by the formation of stable foam in the rumen (Clarke and Reid, 1974; Howarth, 1975). In the current study, frozen leaves from three new populations of western Canadian adapted *Lc*-alfalfa were homogenized in a blender with water to perform a foam stability test.

Table 6.1. Basic chemical and phytochemical composition of leaves from *Lc*-transgenic, non-transgenic (NT) and AC Grazeland (AG) alfalfa harvested at 07:00 and 18:00 h.

	DM	anthocyanidin	ash	NDF[1]	CP	NDIP[1]	WSP[2]
	g/kg	µg/g DM	g/kg DM				
Population							
NT-alfalfa	246.0^{a}	0.0^{b}	197.6^{a}	211.7^{a}	304.2^{c}	27.4^{a}	163.5
Beav*Lc*1	216.4^{b}	256.9^{a}	130.3^{b}	170.3^{ab}	349.2^{a}	24.7^{ab}	172.3
Ramb*Lc*3	221.2^{b}	249.5^{a}	123.0^{b}	157.6^{b}	341.4^{a}	22.9^{bc}	190.4
Rang*Lc*4	225.2^{b}	236.0^{a}	127.5^{b}	154.3^{b}	338.4^{ab}	24.8^{ab}	190.4
AC Grazeland	213.4^{b}	0.0^{b}	124.2^{b}	156.8^{b}	318.1^{bc}	18.7^{c}	177.1
SEM	0.67	62.41	1.59	1.44	0.72	0.14	1.30
Harvest time							
07:00 h	203.7^{b}	141.5	163.4^{a}	189.8^{a}	342.6^{a}	25.7^{a}	179.6
18:00 h	245.2^{a}	155.5	117.6^{b}	150.4^{b}	317.9^{b}	21.6^{b}	177.9
SEM	0.42	39.47	1.00	0.91	0.45	0.09	0.82
Statistics	---------- *P* values----------						
Population	0.018	0.013	0.012	0.042	0.003	0.006	0.496
Time	<.0001	0.807	0.007	0.010	0.002	0.005	0.888
Population×time	0.276	0.705	0.378	0.508	0.171	0.047	0.657
	----------Contrast *P* values----------						
Lc vs. NT[3]	0.004	0.002	0.001	0.005	<0.001	0.038	0.149
Lc vs. AG[3]	0.364	0.006	0.887	0.825	0.013	0.005	0.649

[a-c]Means with different letters within the same column differ (P<0.05).
[1]NDF, neutral detergent fiber analyzed including α-amylase and corrected for neutral detergent insoluble protein (NDIP).
[2]WSP, protein solubilized during homogenization of 20 g leaves in a blender with 100 mL ddH_2O.
[3]*Lc* vs. NT or AG, comparing the mean of three transgenic *Lc*-alfalfa progeny (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4) with commercial available non-transgenic (NT) alfalfa or AC Grazeland (bloat reduced cultivar).

These *Lc*-progeny all contained the maize *Lc* regulatory gene which stimulated the accumulation of low levels of anthocyanidin compared with their parental genotypes (NT) (Table 6.1). The leaf NDF, NDIP, and ash were lower (P<0.05) and crude protein (Table 6.1) and WSP (blender-solubilized protein; Table 6.3) were higher (P<0.05) in these new *Lc*-alfalfa populations compared with parental NT-alfalfa (Table 6.1).

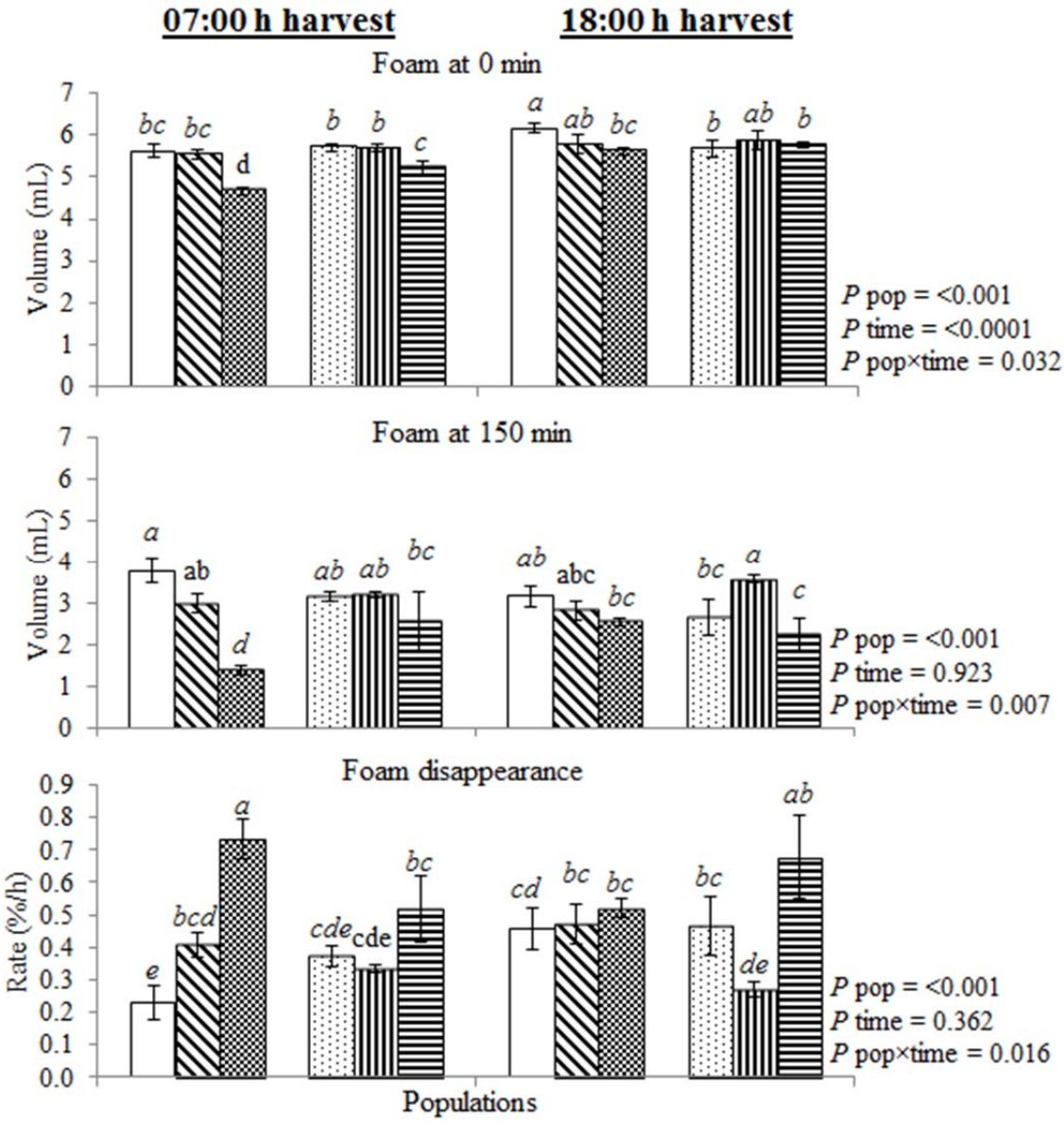

Fig. 6.3 Foam stability characteristics of leaf extracts from NT-alfalfa (□),the mean of three *Lc*-alfalfa progeny (▨), AC Grazeland (▩), Beav*Lc*1 (⊡), Ramb*Lc*3 (▥) and Rang*Lc*4 (▤) harvested at 07:00 and 18:00 h. Vertical bars represent standard error; *a-e* Bars with different letters differ (P<0.05).

Leaves have a higher foaming potential than stems (Cooper, et al., 1966). Hence, leaves of the new anthocyanidin-accumulating populations were tested for their foaming properties and compared with their anthocyanidin-free parental genotypes. Foams formed from leaves harvested at 07:00 h had lower initial (0 min) and final (150 min) foam volumes for Rang*Lc*4 compared with foams from the other two *Lc*-progeny at 0 min and from NT-alfalfa at 150 min (Fig. 6.3). This resulted in a faster foam disappearance rate at 07:00 h for the mean of the three *Lc*-progeny (similar between the three *Lc*-progeny) compared with NT-alfalfa (Fig. 6.3-5). From the 18.00 h harvest, initial foam volume was largest for NT-alfalfa ($P<0.05$) and final foam volume was more stable (larger) for Ramb*Lc*3 ($P<0.01$) compared with Rang*Lc*4 (Fig. 6.3). Foam disappearance rate at 18:00 h was similar between the mean of three *Lc*-progeny and NT-alfalfa (Fig. 6.5), but slower for Ramb*Lc*3 compared with the other two *Lc*-progeny (Fig. 6.4).

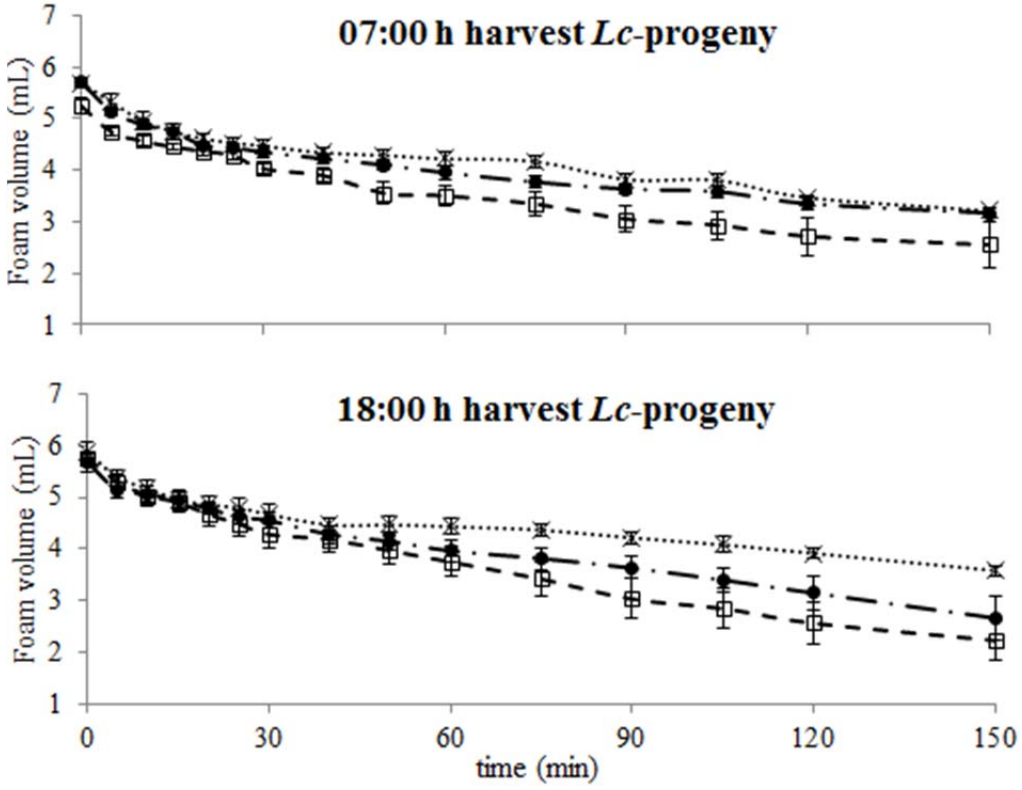

Fig. 6.4. Foam disappearance of leaf extracts from Beav*Lc*1 (—●- -), Ramb*Lc*3 (···×···) and Rang*Lc*4 (– □ –) harvested at 07:00 and 18:00 h. Vertical bars represent the standard error.

Foam stability of leaf extract was previously related to *in vitro* foam stability for rumen liquid of bloating animals and to bloat incidence in cattle (Mangan, 1959; Pressey, et al., 1963).

Foaming and bloating properties are reduced when alfalfa forage is supplemented or grown in mixtures with forage-containing polymeric-anthocyanidins (McMahon, et al., 1999; Wang, et al., 2006a). These polyphenols also break up foam *in vitro* (Tanner, et al., 1995; Min, et al., 2005).

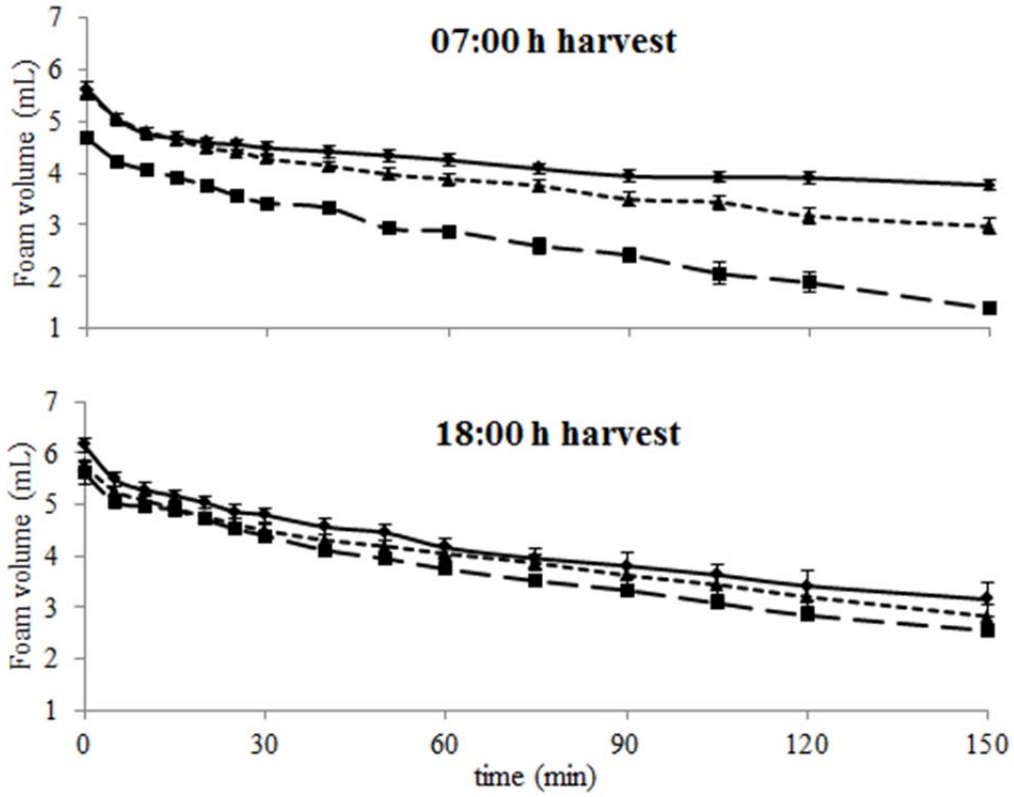

Fig. 6.5. Foam disappearance of leaf extracts from the mean of three *Lc*-alfalfa progeny ( ), NT-alfalfa ( ) and AC Grazeland ( ) harvested at 07:00 and 18:00 h. Vertical bars represent the standard error.

Anthocyanidin accumulation was similar among the three *Lc*-progeny (average of 248 µg/g DM) and between the morning and evening harvests (Table 6.1). Foam volumes and foam disappearance rates were poorly correlated with anthocyanidin levels found in the transgenic and parental populations (Table 6.2). Our correlation data suggests that anthocyanidin levels presently found in the *Lc*-alfalfa populations contribute little to their foaming properties. Hence, other factors in these plants needed to be uncovered and tested for their role in this trait.

6.4.2. Structural Properties Affecting Foam Formation and Stability of Lc-alfalfa

Overall, initial and final foam volumes were lower and foam disappearance rate tended to be faster for the mean of the three *Lc*-progeny compared with NT-alfalfa especially for the 07:00 h harvest. Since anthocyanidins did not correlate with these foam characteristics, we investigated structural components in leaf extracts (i.e., lipid, carbohydrate, and protein structures) to determine whether they impact foaming properties. Asymmetric vibration associated with CH_2 and CH_3 (mainly related to lipids) correlated negatively (R=-0.38 and -0.36, respectively, $P<0.01$) with final foam volume and positively with foam disappearance rate (R=0.44 and 0.37, respectively, $P<0.01$), but these correlations were modest (Table 6.2). Asymmetric vibration associated with CH_2 was higher for Beav*Lc*1 and Range*Lc*4 ($P=0.004$) compared with Ramb*Lc*3 and NT-alfalfa. The CH_2 and CH_3 asymmetric vibrations were higher for the mean of the three *Lc*-progeny ($P=0.006$ each) compared with NT-alfalfa and higher at 18:00 h ($P=0.01$) compared with 07:00 h. Anti-bloat agents containing oils, as well as lipid content in alfalfa forage, have each been found to destabilize foam related to bloat (Stifel, et al., 1968b; Min, et al., 2007).

The FTIR vibration intensities associated with CHO (CHO_{VI}) correlated positively with both initial and final foam volume (R = 0.30 and 0.39, respectively, $P<0.01$). The vibration intensity due to structural carbohydrates (SC_{VI}) correlated negatively (R=-0.41; $P<0.01$) and CHO_{VI}: SC_{VI} ratio correlated positively (R=0.44; $P<0.01$) with final foam volume (Table 6.2). These data imply that carbohydrates other than those involved in SC_{VI} (i.e. cellulose, hemicellulose, β-glucans) are involved in foam formation and stabilization. Vibration intensity peaks associated with all carbohydrates structures in general were higher for NT-alfalfa ($P=0.004$) compared with the three *Lc*-progeny and higher at 18:00 h ($P=0.006$) compared with 07:00 h. Structural carbohydrate-associated peaks were higher for the three *Lc*-progeny ($P<0.001$) compared with NT-alfalfa and higher at 18:00 h ($P<0.001$) compared with 07:00 h (Table 6.4). Previously, it was suggested that pectins and simple saccharides stabilize bloat-related foams (Conrad, et al., 1958; Pressey, et al., 1963). Fiber content is negatively associated with forage digestibility (Jung, et al., 1997) and reduces the amount of foaming compounds released. Thus, the lower initial and final foam volume for the mean of the three *Lc*-progeny compared with NT-alfalfa could be due in part to lower total CHO_{VI}, higher structural SC_{VI}, a lower CHO_{VI}:SC_{VI} ratio and higher asymmetric vibration from lipid CH_2 and CH_3 bands.

Table 6.2. Correlation between foam stability parameters and leaf chemical composition, leaf extract composition and leaf molecular structure of alfalfa populations used in this study.

	Foam at 0 min		Foam at 150 min		Disappearance rate	
Trait	CORR R	P value	CORR R	P value	CORR R	P value
Leaf composition						
DM	0.53	<0.01	0.20	0.10	-0.06	0.63
Anthocyanidin	0.11	0.38	-0.17	0.18	0.19	0.12
CP	-0.28	0.02	-0.13	0.28	0.03	0.80
NDIP	0.14	026	0.37	<0.01	-0.31	0.01
NDF	-0.09	0.48	0.42	<0.01	-0.44	<0.01
WSP	-0.10	0.41	-0.44	<0.01	0.42	<0.01
Leaf extract composition[1]						
pH	-0.22	0.07	-0.52	<0.01	0.47	<0.01
WSP	0.35	<0.01	-0.29	0.02	0.38	<0.01
Ethanol-film	0.44	<0.01	-0.28	0.02	0.37	<0.01
Viscosity	-0.34	<0.01	-0.29	0.02	0.30	0.02
Leaf vibration spectroscopy molecular structure[2]						
Amide I	-0.06	0.65	-0.32	0.01	0.23	0.06
Amide II	-0.18	0.15	-0.30	0.01	0.18	0.14
α-helices	-0.16	0.19	-0.25	0.04	0.14	0.26
β-sheets	-0.12	0.33	-0.26	0.03	0.16	0.19
CHO_{VI}	0.30	0.01	0.39	<0.01	-0.35	<0.01
SC_{VI}	0.04	0.74	-0.41	<0.01	0.39	<0.01
CH_2 asymmetric	0.18	0.14	-0.38	<0.01	0.44	<0.01
CH_3 asymmetric	0.16	0.20	-0.36	<0.01	0.37	<0.01
Vibration spectroscopy ratios[2]						
Amide I:II	0.43	<0.01	0.13	0.31	0.03	0.80
α-helix: β-sheet	-0.33	0.01	-0.13	0.28	0.02	0.86
AmideI:CHO_{VI}	-0.34	0.01	-0.53	<0.01	0.43	<0.01
AmideI:SC_{VI}	-0.12	0.32	0.39	<0.01	-0.45	<0.01
CHO_{VI}:SC_{VI}	0.06	0.64	0.44	<0.01	-0.43	<0.01
CH_2:CH_3	0.09	0.45	-0.18	0.16	0.25	0.05

[1]WSP is aqueous leaf extract protein content; Ethanol-film is film precipitated by ethanol
[2]CHO_{VI} and $SC_{VI}$is vibration intensity mainly absorbed due to carbohydrates and structural carbohydrate structures, respectively; CH_2 and CH_3 asymmetric stretching vibration originate mainly from lipids.

Range*Lc*4 had the lowest final foam volume and the fastest foam disappearance rate at both harvest times compared with the other two *Lc*-alfalfa progeny and NT-alfalfa, especially compared with Rambl*Lc*3. The major difference measurable between Rang*Lc*4 and Rambl*Lc*3 was

the lower vibration intensity associated with protein structures amide I and II (i.e. α-helices, β-sheets) and the lower amide I:amide II ratio in Rang*Lc*4 (Table 6.4). These data indicate that protein structures differed between the two *Lc*-progeny. Combined together, the lipid, carbohydrate, and protein structural differences contribute to the unique nature of each of the three *Lc*-alfalfa populations and likely help to specify the foaming differences that are measurable compared with the NT-parental population.

6.4.3. Other Physicochemical Properties Affecting Foam Formation and Stability

Pasture bloat occurs more frequently when cattle are offered a new pasture in the early morning compared with the late afternoon (Hall and Majak, 1989; Majak, et al., 1995). Field-grown *Lc*-alfalfa plants often appeared to have a darker purple-green coloration in the morning compared with the evening (personal observation by M.Y. Gruber) and foam properties differed between the two harvest times for these plants, even though anthocyanidin levels did not change measurably (Table 6.1). Therefore, we harvested alfalfa in the early morning and late afternoon and determined other differences in physicochemical properties of alfalfa leaf extracts to see if any correlated with *in vitro* foaming properties for each of the two harvest times.

Initial foam volume, blender-solubilized protein content (WSP) and ethanol-film weight were higher for leaf extracts from evening harvests ($P<0.01$) compared to early morning harvests (Fig. 6.3; Table 6.3) for all alfalfa populations. The WSP and ethanol-film weight were similar between the *Lc*-alfalfas and NT alfalfa populations (Table 6.2). However, these two parameters correlated modestly with initial foam volume and foam disappearance rate, but were only weakly correlated (negatively) with final foam volume (Table 6.2). These data suggest that higher extract protein content and higher ethanol-film formation have the potential to influence the formation of larger foams, but do not stabilize foam. The incidence of bloat was positively correlated with soluble protein content of alfalfa forage in other studies (Howarth, et al., 1977; Majak, et al., 1995). However, the soluble protein concentration in these studies was similar in rumen liquid of bloating cattle compared to non-bloating cattle (Majak, et al., 1985; Howarth, et al., 1986). Therefore, these authors concluded that soluble proteins were involved in the formation of foam, but not the onset. Foam and ethanol-precipitated film from the rumen of bloating cows in other studies consisted both for ~63 % of CP (Bartley and Bassette, 1961;

Gutierrez, et al., 1963). Ethanol precipitated film was also higher in bloating cattle than in non-bloating cattle (Gutierrez, et al., 1963; Min, et al., 2006).

Table 6.3. Leaf extract composition of *Lc*-transgenic, non-transgenic (NT) and AC Grazeland (AG) alfalfa harvested at 07:00 and 18:00 h.

	pH	WSP[1]	Ethanol-film[2]	Viscosity
		mg/mL		cP
Population				
NT-alfalfa	6.64	7.04	6.65	2.08
Beav*Lc*1	6.66	7.86	7.00	2.13
Ramb*Lc*3	6.60	7.95	7.15	2.19
Rang*Lc*4	6.63	8.15	7.49	2.23
AC Grazeland	6.74	7.18	6.80	2.30
SEM	0.079	0.364	0.307	0.108
Harvest time				
07:00 h	6.62	6.98^{b}	6.42^{b}	2.15
18:00 h	6.69	8.29^{a}	7.62^{a}	2.22
SEM	0.050	0.230	0.194	0.069
Statistics	----------*P* values----------			
Population	0.810	0.161	0.362	0.596
time	0.381	0.002	0.001	0.526
Population×time	0.479	0.283	0.316	0.506
	----------Contrast *P* values----------			
Lc vs. NT[3]	0.921	0.028	0.103	0.364
Lc vs. AG[3]	0.278	0.089	0.282	0.372

[a-d]Means with different letters within the same column differ ($P<0.05$).
[1]WSP, protein solubilized during homogenization of 20 g leaves in a blender with 100 mL ddH_2O.
[2]Ethanol-film, film precipitated by ethanol.
[3]*Lc* vs. NT or AG, comparing the mean of three transgenic *Lc*-alfalfa progeny (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4) with parental non-transgenic (NT) alfalfa or AC Grazeland (bloat reduced cultivar).

In the original sample, WSP was similar and total CP content higher from the morning harvest compared with the evening harvest (Table 6.1). However, leaf DM content was ~42 g/kg higher for the evening harvest and correlated with foam formation. These data mean that 20 g of freshly frozen leaves from the evening harvest consisted of 0.2 g more CP and WSP than 20 g leaves from the morning harvest. Hence, dry matter itself appears to influence the ability of alfalfa to foam by providing more constituents per portion of fresh material.

Table 6.4. FTIR spectroscopic vibration intensities associated with leaf molecular structures of *Lc*-transgenic, non-transgenic (NT) and AC Grazeland (AG) alfalfa harvested at 07:00 – 18:00 h.

	Amide I[1]	Amide II[1]	α-helix[1]	β-sheet[1]	CHO_{VI}[2]	SC_{VI}[2]	$^3CH_2$	$^3CH_3$
IR intensity	Area		Height		Area		Height	
Population								
NT-alfalfa	3.56^{c}	1.28^{c}	0.033^{c}	0.035^{c}	10.11^{a}	2.62^{c}	0.016^{c}	0.005
Beav*Lc*1	3.74^{bc}	1.40^{bc}	0.036^{bc}	0.038^{bc}	8.33^{b}	3.10^{b}	0.020^{a}	0.006
Ramb*Lc*3	4.11^{ab}	1.50^{b}	0.039^{ab}	0.041^{ab}	9.04^{b}	3.18^{b}	0.017^{bc}	0.006
Rang*Lc*4	3.73^{c}	1.32^{c}	0.035^{c}	0.037^{c}	9.00^{b}	3.06^{b}	0.019^{ab}	0.005
AC Grazeland	4.34^{a}	1.67^{a}	0.042^{a}	0.042^{a}	8.97^{b}	3.49^{a}	0.017^{bc}	0.006
SEM	0.140	0.051	0.001	0.001	0.338	0.115	0.0009	0.0002
Harvest time								
07:00 h	3.75^{b}	1.38^{b}	0.036^{b}	0.037^{b}	8.67^{b}	2.80^{b}	0.017^{b}	0.005^{b}
18:00 h	4.04^{a}	1.49^{a}	0.039^{a}	0.040^{a}	9.51^{a}	3.38^{a}	0.019^{a}	0.006^{a}
SEM	0.089	0.032	0.0008	0.0009	0.214	0.073	0.0006	0.0001
Statistics	---------- *P* values----------							
Population	0.001	<.0001	<.0001	0.001	0.004	<.0001	0.004	0.056
Time	0.026	0.023	0.007	0.034	0.006	<.0001	0.011	0.001
Population×time	0.889	0.444	0.430	0.790	0.009	0.428	0.064	0.565
	----------Contrast *P* values----------							
Lc vs. NT[4]	0.041	0.019	0.005	0.019	0.000	<.0001	0.006	0.006
Lc vs. AG[4]	0.004	<.0001	0.002	0.014	0.639	0.006	0.222	0.732

[a-c]Means with different letters within the same column differ ($P<0.05$).
[1]Vibration intensity mainly absorbed due to protein structures (baseline 1707-1481 cm^{-1}) with the peak region for Amide I at 1707-1563 cm^{-1} and for Amide II at 1563-1481 cm^{-1} and the peak height of α-helix at 1653 cm^{-1} and of β-sheet at 1634 cm^{-1}.
[2]Vibration intensity mainly absorbed due to carbohydrates structures (CHO_{VI}; baseline and peak region 1193-859 cm^{-1}) and due to structural carbohydrates structures β-glucans, hemicellulose and cellulose (SC_{VI}; baseline and peak region1483-1193 cm^{-1}).
[3]Intensity absorbed due to CH_2 (2920 cm^{-1}) and CH_3 (2955 cm^{-1}) asymmetric vibration (baseline 3000-2770 cm^{-1}), these structures are mainly present in lipids.
[4]*Lc* vs. NT or AG, comparing the mean of three transgenic *Lc*-alfalfa progeny (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4) with commercial available non-transgenic (NT) alfalfa or AC Grazeland (bloat reduced cultivar).

Previously, alfalfa foam stability was maximum from pH 5 to 6 (Buckingham, 1970; Wang and Kinsella, 1976). The pH of our new *Lc*-alfalfas and NT-populations was similar among all four populations and between morning and evening harvests (mean of 6.6) (Table 6.3). However, the pH of the aqueous leaf extract correlated more negatively with final foam volume (R=-0.52; $P<0.01$) and more positively with foam disappearance rate (R=0.47; $P<0.01$) than with initial

foam volume. Thus, pH might have affected foam stability, even though variation in pH was small and not significantly different amoung alfalfa populations tested.

6.4.4. Foam Formation and Stability of Lc-Alfalfa Compared with AC Grazeland Check Cultivar

The cultivar AC Grazeland has become a standard livestock forage check cultivar, since it is a cultivar with a reduced bloat incidence *in vivo* in grazing cattle (Berg et al., 2000; Coulman et al., 2000). The germplasm base for this cultivar was previously selected for a low initial rate of degradation by an *in situ* nylon bag method (Goplen et al., 1993), thus not directly for its foaming properties. This cultivar also has a reduced leaf-to-stem ratio, thicker leaf cell walls and higher fiber fractions (NDF and ADF) (Goplen, et al., 1993; Tremblay, et al., 2000). Initial and final foam volumes were lower and foam disappearance rate was faster for AC Grazeland harvested at 07:00 h compared with the three *Lc*-progeny and their mean (Fig. 6.3-5). [Only leaves were evaluated to eliminate the effect of different leaf to stem ratios between these germplasm types]. The NDF, which is the fiber fraction that includes the majority of hemicellulose, cellulose and lignin (Van Soest, et al., 1991) was similar between AC Grazeland and the three *Lc*-progeny (Table 6.1). However the FTIR vibration intensity peak from structural carbohydrates SC_{VI} was lower for the *Lc*-alfalfas compared with AC Grazeland (Table 6.4). The SC_{VI} peak includes vibration intensity due to hemicellulose, cellulose and β-glucans (Wetzel, et al., 1998; Yu, et al., 2005). These differences in structural carbohydrates suggest that leaves of *Lc*-alfalfa may contain less β-glucans compared with AC Grazeland. Other physicochemical features (e.g. pH, WSP, ethanol-film and viscosity) of the leaf extracts used for the foam stability tests were similar for *Lc*-alfalfas and AC Grazeland (Table 6.3) and would not contribute to the differences in foam stability noted for AC Grazeland. However, DM and NDIP were lower in AC Grazeland, and crude protein was higher to some extent (Table 6.1). Therefore, protein structural features were also evaluated between the *Lc*-alfalfas and AC Grazeland.

Initial foam volume for all the alfalfas tested correlated positively with amide I: amide II ratios and negatively with α-helix:β-sheet ratios (Table 6.2). These modest correlations suggest that proteins with less amide I (*ca*. 80% C-O stretching vibration + 20% C-H stretching vibration) relative to amide II (*ca*. 60% N-H bending vibration and 40% C-N stretching) and proteins with more α-helices relative to β-sheets might have a lower potential to form foams. Vibration intensity associated with all protein structural features (amide I and II, α-helices, β-

sheets) was higher for AC Grazeland compared with the three *Lc*-progeny, especially with Beav*Lc*1 and Range*Lc*4 (P<0.01) (Table 6.4). Plant material harvested at 18:00 h also had a higher vibration intensity associated with all these protein structures compared with material from the 07:00 h harvest (P<0.05).

Table 6.5. Ratios of vibration spectroscopy parameters detecting leaf molecular structures for *Lc*-transgenic, non-transgenic (NT) and AC Grazeland (AG) alfalfa harvested at 07:00 and 18:00 h.

		Amide I:II	α-helix: β-sheet	AmideI: CHO_{VI}	AmideI: SC_{VI}	CHO_{VI}: SC_{VI}	CH_2: CH_3
Population	Time (h)			Ratio[1]			
NT-alfalfa	07:00	2.78^{b}	0.95^{c}	0.32^{g}	1.56^{a}	5.08^{a}	3.76
Beav*Lc*1	07:00	2.69^{bc}	0.95^{c}	0.48^{b}	1.27^{cd}	2.64^{bcd}	3.72
Ramb*Lc*3	07:00	2.72^{bc}	0.96^{c}	0.46^{b}	1.37^{b}	3.00^{bc}	3.32
Rang*Lc*4	07:00	2.77^{b}	0.96^{c}	0.43^{cde}	1.28^{cd}	3.03^{bc}	3.77
AC Grazeland	07:00	2.65^{c}	0.96^{c}	0.52^{a}	1.30^{c}	2.51^{d}	3.61
NT-alfalfa	18:00	2.77^{b}	0.95^{c}	0.39^{f}	1.23^{de}	3.16^{b}	3.22
Beav*Lc*1	18:00	2.65^{c}	0.98^{b}	0.42^{de}	1.15^{g}	2.73^{bcd}	4.04
Ramb*Lc*3	18:00	2.79^{ab}	0.96^{c}	0.45^{bcd}	1.23^{def}	2.72^{bcd}	2.89
Rang*Lc*4	18:00	2.88^{a}	0.95^{c}	0.40^{ef}	1.17^{fg}	2.92^{bcd}	3.60
AC Grazeland	18:00	2.54^{d}	1.01^{a}	0.45^{bc}	1.19^{efg}	2.63^{cd}	2.45
SEM[1]		0.032	0.006	0.010	0.021	0.181	0.392
Statistics		----------*P* values----------					
Population		<.0001	<.0001	<.0001	<.0001	<.0001	0.147
Time		0.792	0.001	0.005	<.0001	0.001	0.112
Population×time		0.008	<.0001	<.0001	<.0001	<.0001	0.442

[a-g]Means with different letters within the same column differ (P<0.05).
[1]Abbreviations are explained in table 6.3.

The amide I to amide II ratio was higher for Ramb*Lc*3 and Rang*Lc*4 (and similar to NT-alfalfa) at 18:00 h (P=0.01) compared with AC Grazeland at 07.00 h and Beav*Lc*1 and AC Grazeland at 18:00 h (Table 6.5). While α-helix:β-sheet ratios were similar for all the alfalfa populations at the 07:00 h harvest, AC Grazeland harvested at 18:00 h (P<0.001) had a higher α-helix:β-sheet ratio compared with Beav*Lc*1 and these two populations were both higher than the other two *Lc*-progeny and NT-alfalfa. Thus, in this study, there was more variation in protein molecular features than in a previous study where we found similar leaf protein molecular structures between Beav*Lc*1 and Beaver (Yu et al., 2009b). Protein with less amide I relative to

amide II was found to have reduced ruminal protein degradability and therefore an increased amount of protein that escaped to the lower digestive tract (Yu and Nuez-Ortín, 2010). Proportionally higher α-helices to β-sheets was previously found to slow down and reduce protein degradability in the rumen and increase metabolizable protein available for dairy cows (Doiron, et al., 2009; Yu, 2004). In an *in situ*/nutritional modeling trial with forage of the three *Lc*-alfalfa progeny and AC Grazeland harvested in 2008, we found that AC Grazeland tended to have a lower ruminal degradability of protein compared with the three *Lc*-progeny (Section 5.4.3) which might have been the result of lower amide I relative to amide II and/or more α-helices relative to β-sheets that we found in this study.

Amide I to CHO_{VI} ratios correlated negatively with initial (R=-0.34; P=0.01) and final (R=-0.53; P<0.01) foam volume (Table 6.2). These modest correlations suggest that CHO_{VI} may be more important for the formation and stabilization of foam than amide I. The amide I to CHO_{VI} ratio was lower for the three *Lc*-alfalfas at 07:00 h and Beav*Lc*1 and Ramb*Lc*3 at 18:00 h (P<0.001) compared with AC Grazeland at both harvest times, respectively (Table 6.5). The ratio of CHO_{VI}:SC_{VI} was lowest for AC Grazeland compared with the other germplasm (Table 6.5), which indicates that the modification in the *Lc*-alfalfa progeny is not sufficient enough to meet this check cultivar level of protein and carbohydrate structures which are associated with lower foam stability as described before.

6.5. Conclusion

Bloat related foam formation and stability was tested by an *in vitro* shaking method with aqueous leaf extracts of three new transgenic *Lc*-alfalfa progeny that accumulated on average 248 μg/g DM forage anthocyanidin. These progeny plants were compared first with parental non-transgenic (NT) alfalfa and secondly with AC Grazeland, the bloat reduced cultivar check, and for harvests in the early morning and late afternoon. Foam formation in general was reduced with a low leaf extract protein and ethanol-film concentration and lower vibration intensity due to all carbohydrate structures and proteins structures with less amide I relative to amide II and more α-helices relative to β-sheets. Foam stability was in general lower by increased pH, increased vibration intensity due to structural carbohydrates and lipids, reduced amide I to structural carbohydrates ratio and increased amide I to all carbohydrates structures ratio. Foam formation was higher for forage harvested in the late afternoon, but foam stability was similar

between morning and afternoon harvests. Foam formation and stability were not correlated with anthocyanidin levels, but these foaming properties were lower overall for the three *Lc*-progeny compared with NT-alfalfa, but still not as low as for the AC Grazeland bloat reduced check cultivar. When ranking the individual *Lc*-progeny, Range*Lc*4 had lower foam than Ramb*Lc*3, with Beav*Lc*1 intermediate. Phytochemical analysis of their parental *Lc*-genotypes showed distinct differences in flavonoid glycosides and saponins which may specify these individual differences in foam stability (Gruber and Olezek, unpublished). Regardless, our results suggest that the accumulation of mono or polymeric anthocyanidins needs to be increased substantially in *Lc*-alfalfa, or other factors specifying protein and carbohydrate structures need to be modified or selected in order to develop a alfalfa cultivar with superior bloat-preventing characteristics. Our study also points to Fourier Transformed Infrared vibration spectroscopy as a useful rapid low-cost technique, which does not require the use of chemicals, to select alfalfa plants with structural properties that reduce foam formation and stability.

Chapter 7

General Discussion

7.1. Introduction

Grazing alfalfa is the most cost-effective ruminant production system (Rotz, 1996). However, the use of alfalfa for grazing is limited because of its propensity to cause the potentially fatal metabolic disorder, pasture bloat (Howarth, 1975). The main cause of bloat from alfalfa is its excessively rapid initial rate of ruminal protein degradation that also causes low animal protein efficiency with consequent excessive nitrogen losses into the environment (Broderick, 1995; Yu et al., 2004). Ruminal protein degradation is decreased by mono/polymeric anthocyanidin which can bind protein directly and/or interaction with specific microbes in the rumen (Aerts et al., 1999; Min et al., 2002; Wang et al., 2006a). However, alfalfa does not accumulate mono/polymeric anthocyanidin in its forage (Goplen et al., 1980). Therefore, alfalfa was transformed with a maize anthocyanidin regulating *βHLH LEAF COLOR* (*Lc*) gene to stimulate the lower part of the flavonoid pathway (Ray et al., 2003). By this transformation, four T_0 *Lc*-alfalfa genotypes were generated that accumulated monomeric anthocyanidin under field conditions and mono/polymeric anthocyanidin indoor in a shift to high light trial (Ray et al., 2003; Wang et al., 2006a). These T_0 *Lc*-genotypes had a reduced initial rate of nitrogen and dry matter degradation *in vitro* in rumen liquid (Wang et al., 2006a). However, the eastern Canadian background of T_0 *Lc*-alfalfa compromised survival under western Canada conditions. Therefore, three T_0 *Lc*-alfalfa genotypes were crossed with three western Canadian cultivars to develop new hardy T_1 *Lc*-progeny (Beav*Lc*1, Ramb*Lc*3 and Rang*Lc*4) and to facilitate the movement of the *Lc*-gene into a broader spectrum of alfalfa breeding germplasm.

7.2. Phytochemical Composition

The plants in the field were found to express three different color phenotypes within each progeny, Green-*Lc*, light purple-green-*Lc* (LPG-*Lc*) and purple-green-*Lc* (PG-*Lc*). During the 2007 harvest season, the *Lc*-alfalfa progeny were split into these three phenotypes and tested for their range of forage mono/polymeric anthocyanidin. All three *Lc*-alfalfa phenotypes tested (but not parental non-transgenic (NT) alfalfa) accumulated anthocyanidin pigments within their forage, even though the level in the green-*Lc* was not sufficient to show a purple phenotype (Chapter 4). Among the three *Lc*-phenotypes, PG-*Lc* anthocyanidin accumulation ranged from 157-to-394 μg/g DM which was higher than the range of 47-to-151 μg/g DM for Green-*Lc* with the range of 117-to-230 μg/g DM for LPG-*Lc* intermediate. Extractable and unextractable

polymeric-anthocyanidins were below the detection limit when tested in 1 g of sample from individual *Lc*-alfalfa progeny and phenotypes. Only purple-green forage (combined LPG-*Lc* + PG-*Lc*) was harvested for the 2008 harvest season and Green-*Lc* was discarded. The three *Lc*-alfalfa progeny (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4) harvested in 2008 had an average forage anthocyanidin level of 163.4 μg/g DM, while AC Grazeland forage harvested from the same field at the same time did not accumulate anthocyanidin at all (Chapter 5). The anthocyanidin level of these purple-green *Lc*-progeny was ~1.4 times higher in the 2007 compared with the 2008 harvest.

When combining the 2007 and 2008 harvests, all forage samples derived from individual purple-green populations accumulated anthocyanidin with a mean of 197.4 μg/g DM and a range of 42.5-to-349.0 μg/g DM (Chapter 3). Purple-green *Lc*-alfalfa samples in the Ramb*Lc*3 background accumulated on average 136 μg/g DM (range 43-to-229 μg/g DM) which was only 60 % of the anthocyanidin accumulated in the Beav*Lc*1 (mean 224 and range 124-to-349 μg/g DM) and Rang*Lc*4 (mean 233 and range 128-to-342 μg/g DM) progeny. The second generation (T_1) *Lc*-progeny used in this study accumulated anthocyanidin over a broader and higher range than previously found for the T_0 parental genotypes grown earlier at the same field in 2001 and 2002 (97-to-136 μg/g DM) (Wang et al., 2006b). This was true even when the cyanidin standard, field site, plant harvest stage, and harvest cycle (mid-summer and late summer) were the same for the T_0 and T_1 *Lc*-plants. This suggests that individual genetic backgrounds, potentially, the transgene location within the alfalfa genome, and/or the harvest year, have impacted *Lc*-transgene expression or expression of other flavonoid pathway regulatory genes.

In 2009, only alfalfa leaves from forage harvested at 07:00 and 18:00 h were analyzed for their anthocyanidin content. Anthocyanidin content averaged 248 μg/g DM and ranged from 76-to-483 μg/g DM and was similar between the 07:00 and 18:00 h harvests on the same day (Chapter 6). The anthocyanidin content in the *Lc*-leaves harvested in 2009 tended to be higher than in the whole *Lc*-forage harvested in 2007 and 2008. Leaves may contain more anthocyanidin than stems. All transgenic forage overall expressed a darker purple-green phenotype in 2009 (personal observation). This might be due to the wet and cool growth season in Saskatoon in 2009.

7.3. Nutritional Profile

The main nutritional properties of a feed for ruminats are its protein and energy content and the balance and site at which these are released (rumen or lower digestive tract). Chemical analysis can give information about the nutritional composition of feed in terms of protein carbohydrates and lipids. Chemical analysis of forage from the 2007 and 2008 harvests revealed that the three anthocyanidin-accumulating *Lc*-alfalfa progeny had a higher carbohydrate (CHO) and lower protein (CP) content compared with NT-alfalfa, which resulted in a decreased N:CHO ratio (Chapter 3). A negative correlation between polymeric-anthocyanidin and forage CP content was found in several studies (Barry and Manley, 1984b; Miller and Ehlke, 1995). Parental T_0 *Lc*-genotypes also had a numerically lower CP compared with their eastern Canadian parental NT-alfalfa genotype N4-4-2 (Wang et al., 2006a). This might explain the reduced CP content in our anthocyanidin accumulation *Lc*-progeny compared with their western Canadian parental NT-alfalfas.

Fiber fractions (neutral and acid detergent fiber) were numerically higher in all individual *Lc*-progeny compared with the three western Canadian NT-alfalfas. This might be due to interaction of the enhanced secondary metabolite fractions with the fiber analysis method used (Makkar et al., 1995) and lignin was previously found to increase with increasing polymeric-anthocyanidin accumulation (Baray and Manley, 1986). However, the higher fiber fraction could also be the result of the genetic background of the NT-parent (N4-4-2) of the first generation T_0 *Lc*-progeny (Ray et al., 2003) that was crossed into the T_1 *Lc*-progeny. This laboratory genotype was selected from a eastern Canadian alfalfa adapted cultivar for its tissue culture and transformability characteristics. The nutritional properties of N4-4-2 have not been tested under western Canadian conditions and by crossing it with cultivars that were selected for improved nutritional and plant characteristics in western Canada, it might have reduced the quality of the newly developed T_1 *Lc*-progeny compared. We did not include the genotype N4-4-2 in our studies because it did not survive under western Canadian conditions (Wang et al., 2006a).

7.4. Nutrient Fermentation-Degradation

Chemical analysis can determine exact nutritional composition, but gives no information about the site and extent of nutritient degradation. Alfalfa is known for its rapid initial rate of protein degradation in the rumen. This can make protein a limiting nutritional factor for ruminant

production, because the rapid protein degradation causes an oversupply of N relative to energy in the rumen for microbial protein synthesis and limits the amount of dietary protein escaping to the lower digestive tract which is required for high-producing ruminants (Dhiman and Satter, 1993; Broderick, 1995; Klopfenstein, 1996). Mono/polymeric anthocyanidin reduce the rate and extent of protein degradation in the rumen, increasing the amount of protein escaping to the lower digestive tract and increasing amino acid absorption in the small intestine (Waghorn et al., 1987b; Aerts et al., 1999; Wang et al., 2006a). The extent of degradation in the rumen is expressed by the rumen degradable or effective degradable fraction of a constituent, and speed of degradation is expressed by the fractional degradation rate, soluble fractions (filter bag) and fractional production rate (gas production system).

Protein and carbohydrate sub-fractions were similar between all three *Lc*-progeny and all three NT-populations when calculated by the CNCPS system. This resulted in similar rumen degradable CP and CHO for all six populations (Chapter 3). Fractional degradation rates of the different CP and CHO fractions as calculated from the *in situ* method were similar between the three *Lc*-progeny and AC Grazeland (Chapter 5), which suggests that it was appropriate to use literature values for the CNCPS degradability calculations. Effective degradable N and DM correlated negatively with anthocyanidin, and they were lower for the three *Lc*-phenotypes (Green, LPG, PG) compared with NT-alfalfa. Effective degradable N was lower for the purple-green *Lc*-phenotype compared with the other two *Lc*-phenotyes and effective degradable DM was lower for the two purple *Lc*-phenotypes compared with green-*Lc* (Chapter 4). The lower effective degradable N and DM resulted mainly from a reduction in directly available soluble washable N and DM fractions in the *Lc*-phenotypes compared with NT-alfalfa. The two purple *Lc*-phenotypes had a slower gas production rate (related to DM degradation), slower ammonia accumulation rate (gas production method) and lower soluble washout of N and DM (*in vitro* filter bag) compared with NT-alfalfa. This suggests a lower availability of N for the conversion into NH_3 by the microbes at the initiation of incubation with the two purple *Lc*-phenotypes compared with NT-alfalfa.

Protein content, solubility, potentially degradability and degradation rate were similar between the three *Lc*-progeny and AC Grazeland which resulted in a similar rumen degradable CP content for the three *Lc*-progeny and AC Grazeland (Chapter 5). However, there was a trend towards a higher rumen degradable CP when the mean of three *Lc*-alfalfas were compared with

AC Grazeland (22.6 vs. 21.4 %DM). Since lignin (ADL) and other fiber fractions tended to be higher in AC Grazeland compared with the mean of three *Lc*-alfalfas and an increased lignin content decreases the digestibility of forages (Jung et al., 1997), the higher fiber fractions in AC Grazeland may have had greater impact on it rumen degradable CP than the anthocyanidin levels in the three *Lc*-alfalfa forages have on protein precipitation.

7.4. Microbial Protein Efficiency and Animal Nitrogen Excretion

Microbes in the rumen require N and energy for their growth (Hoover and Stokes, 1991). The CNCPS rumen degradable N:CHO ratios ranged from 99-to-127 g/kg (Chapter 3), the *in situ* rumen degradable N:CHO ratio ranged from 110-to-119 g/kg (Chapter 5) and were 3.5-fold higher than the ~32 g/kg required for optimal microbial protein synthesis (Sinclair et al., 1991; Tamminga et al., 1994). *In vitro* filter bag effective degradable N:DM ratio ranged from 40-to-43 g/kg (Chapter 4) which was two times higher than the ~25 g/kg required for optimal microbial protein synthesis (Beever et al., 1986). The CNCPS rumen degradable N:CHO ratio was lower (improved) for the mean of three *Lc*-progeny compared with the mean of three NT-alfalfa populations, mainly because of the lower CP and higher CHO in *Lc*-progeny (Chapter 3). When the three *Lc*-alfalfa populations and AC Grazeland were evaluated for the hourly rumen degradable N:CHO ratio, a bi-phasic profile was revealed which included an initial and substantial over-supply of N relative to energy within the first two hours after feeding, followed by a more synchronized and balanced rumen degradable N:CHO ratio from 2 to 24 h after feeding (Chapter 5).

Excessive protein supplied from alfalfa above microbial requirements 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). Therefore, the reduced rumen degradable N:CHO ratios found in our *Lc*-alfalfa may result in an improved animal protein efficiency, reduced N excretion into the environment and reduced energy expenditure for NH_3 to urea conversion. This is supported by a lower NH_3 accumulation rate for the two purple *Lc*-phenotypes compared with NT-alfalafa (Chapter 4) without having compromised microbial protein syntheis in any of these puple-green *Lc*-alfafas compared with NT-alfalfa (Chapter 4) and AC Grazeland (Chapter 5).

7.5. Bloat Properties

Pasture bloat is caused by the formation of stable foam in the rumen (Clarke and Reid, 1974; Howarth, 1975). Rapid ruminal protein degradation of alfalfa causes viscous foamy rumen contents to entrap fermentation gases and prevent their normal eructation (Cockrem et al., 1987; Tanner et al., 1995). Previously it was found that polymeric anthocyanidin reduces the stability of bloat-related foam (Tanner et al., 1995). As discussed in section 7.3, purple-green *Lc*-alfalfa had a slower NH_3 accumulation rate and lower effective degradable protein content *in vitro* compared with NT-alfalfa (Chapter 4), but fractional protein degradation rate and rumen degradable protein content were similar between *Lc*-alfalfa and AC Grazeland (Chapter 5). These suggest that *Lc*-alfalfa progeny have a reduced amount of foam-promoting protein in the rumen compared with NT-alfalfa, but are similar to AC Grazeland.

We tested foam formation and stability with aqueous extracts *in vitro* from leaves harvested at 07:00 h and 18:00 h (Chapter 6). At 07:00 h Rang*Lc*4 and AC Grazeland had the lower initial and final foam volume compared with NT-alfalfa, Beav*Lc*1 and Ramb*Lc*3. While at 18:00 h, initial foam volume was lower for Beav*Lc*1, Rang*Lc*4 and AC Grazeland compared with NT-alfalfa and final foam volume was lower for Rang*Lc*4 compared with NT-alfalfa and Ramb*Lc*3. Results suggest that *Lc*-progeny (especially Rang*Lc*4) have improved bloat-preventing characteristics compared with NT-alfalfa, but not compared with AC Grazeland.

7.6. Comparing Individual Lc-Progeny

Field survival after three successive winters indicated that Ramb*Lc*3 and Rang*Lc*4 survived the climate in western Canada (Saskatoon) more successfully than Beav*Lc*1 (Chapter 3). Beaver is a tap-rooted cultivar, while Rambler and Ranglander are creeping-rooted cultivars. In general creeping-rooted cultivars are more winter hardy, and Rambler was previously found to be more winter hardy than Beaver (Gossen et al., 1992). However, we should take into account that the plants were not planted in a normal inter-row spacing of 15-60 cm, but in a inter-row spacing of 75 cm which affects the micro-climate. The Saskatchewan forage crop production guide advises not to cut alfalfa after the middle of August because the plants will not have time to restore energy reserves before the first frost starts. When energy reserves are not sufficient, alfalfa is more sensitive to winter kill (Gov.SK.Ca, 2010). Perhaps, Beaver is more sensitive to later cutting and larger inter-row spacing than Rambler and Rangelander.

Nutrient profile of forage harvested in 2007 and 2008 was similar among the three *Lc*-progeny tested (Chapter 3). Total gas production (related to DM digestibility) and effectively degradable DM were higher for Beav*Lc*1 compared with Rang*Lc*4, while gas production rate was fastest for Ramb*Lc*3 (Chapter 4). While ammonia accumulation rate was faster, N degradation rate was slower and branch chain FA concentration lower for Beav*Lc*1 compared with Ramb*Lc*3 and Rang*Lc*4 and effectively degradable N was similar between the three *Lc*-progeny. In the *in situ*/modeling trial (Chapter 5), Ramb*Lc*3 had a numerically lower soluble and undegradable, and numerically higher potentially degradable CP and CHO fractions while having similar total CP and CHO content compared with Beav*Lc*1 and Rang*Lc*4. These resulted in a numerically lower predicted rumen degraded protein balance (balance between microbial synthesis based on energy or protein) and numerically higher intestinally available protein for lactation production in Ramb*Lc*3.

As described in section 7.6, Range*Lc*4 had numerically the lowest final foam volume and fastest foam disappearance rate at both harvest times, especially compared with Ramb*Lc*3. The main difference in parameters measured between Rang*Lc*4 and Ramb*Lc*3 was the lower vibration intensity associated with protein structures (amide I and II, α-helix, β-sheet) in Rang*Lc*4 which indicates that protein structures differed between the two.

8.1. Overall Conclusions

- All three T_1 *Lc*-alfalfa progeny accumulated anthocyanidin over three harvest years.
- The three *Lc*-alfalfa progeny had a lower protein and higher carbohydrate content and tended to contain more fiber in their forage compared with parental non-transgenic alfalfa harvested in 2007 and 2008.
- The two purple-green *Lc*-alfalfa phenotypes showed a reduced rate of fermentation and effective degradability compared with parental non-transgenic alfalfa (2007 harvest).
- The three *Lc*-alfalfa progeny gave a higher nutrient availability for animal production (net energy for lactation and intestinally digestible protein) and lower undegradable protein and carbohydrate fractions compared with AC Grazeland (2008 harvest).
- The three *Lc*-alfalfa progeny (especially Rang*Lc*4) had reduced bloat related foaming properties compared with parental non-transgenic alfalfa but not compared with AC Grazeland (2009 harvest).

8.1. Future Research Directions

Several distinct differences and inconsistencies arose between the individual *Lc*-progeny during the four studies and between the *Lc*-phenotypes in chapter 4. Rang*Lc*4 had improved bloat preventing properties and Beav*Lc*1 and Ramb*Lc*3 had improved ruminal protein metabolism properties *in vitro* and *in situ*, respectively. These progeny and phenotypes were only distinguished by their monomeric-anthocyanidin concentrations in our studies since we could not detect proanthocyanidin by standard chemical assays. Small amounts of polymeric-anthocyanidin like structures without (-)-epicatechin extension units and multiple glycosylated conjugates of cyanidin were detectable in deep purple *LAP1*-transformed alfalfa by sophisticated analytical chemistry techniques (Peel et al., 2009). Use of such techniques, as well as Fourier-Transformed Infra-Red spectroscopy, could shed new light on what has transpired chemically within the *Lc*-alfalfa progeny and phenotypes. Such knowledge will point to which transgenic *Lc*-progeny and phenotype is the most desirable to advance in a plant breeding programme in which further stimulation of the flavonoid pathway is required to develop an alfalfa cultivar with superior fermentation and degradation characteristics. Such modifications could include:

- Development of *Lc*-alfalfa plants with multiple copies of the *Lc*-transgene.

- 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), *Medicago truncatula LAP1 MYB* regulatory gene (Peel et al., 2009) and other WD40 partner proteins (Quattrocchio et al., 2006).
- 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. Such research may 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).

9. References

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10. List of Publications

10.1. Peer Reviewed Articles

Jonker, A., Gruber, M.Y., McCaslin, M., Wang, Y., Coulman, B., McKinnon, J.J., Christensen, D.A., Yu, P., 2010. Nutrient composition and degradation profiles of anthocyanidin-accumulating *Lc*-alfalfa populations. Can. J. Anim. Sci. 90:401-412.

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Jonker, A., Gruber, M.Y., Wang, Y., Narvaez, N., Coulman, B., McKinnon, J.J., Christensen, D.A., Azarfar, A., Yu, P., 2011. Fermentation, degradation and microbial nitrogen partitioning for three forage color phenotypes within anthocyanidin-accumulating *Lc*-alfalfa progeny. Anim. Feed. Sci. Technol. (*Submitted*).

Jonker, A., Gruber, M.Y., Wang, Y., Coulman, B., McKinnon, J.J., Christensen, D.A., Yu, P., 2011. Foam stability of leaves from anthocyanidin-accumulating *Lc*-alfalfa and relation to molecular structures detected by FTIR vibration spectroscopy. Grass Forage Sci. (*Submitted*).

10.2. Conference Abstracts

Jonker, A., Yu, P., Wang, Y., Gruber, M.Y., 2009. Chemical profiles and protein and carbohydrate subfractions of new crossed and winter-hardy anthocyanidin-accumulating alfalfa populations transformed with the maize *βHLH* (*Lc*) regulatory gene in ruminants: comparison with non-transgenic alfalfa. ADSA-CSAS-ASAS Joint Annual Meeting, July 12-16, Montreal, QC, Canada. J. Anim. Sci. 87 (E-Suppl. 2)/J.Dairy Sci. 92 (E-Suppl. 1), 32-33.

Jonker, A., Yu, P., Wang, Y., Gruber, M.Y., 2009. Total digestible nutrient and energy values of new crossed and winter-hardy anthocyanidin-accumulating alfalfa populations transformed with the maize *βHLH* (*Lc*) regulatory gene in ruminants: comparison with non-transgenic alfalfa. ADSA-CSAS-ASAS Joint Annual Meeting, July 12-16, Montreal, QC, Canada. J. Anim. Sci. 87, (E-Suppl. 2)/J.Dairy Sci. 92, (E-Suppl. 1), 32.

Jonker, A., Gruber, M.Y., Wang, Y., Yu, P., 2010. Protein and carbohydrate degradation characteristics and ratios of anthocyanidin-accumulating *Lc*-alfalfa and alfalfa selected for a

low initial rate of degradation in grazing cattle, pp 195. WSASAS 4th Grazing Livestock Nutrition Conference, July 8-10, Estes Park, CO, USA.

Jonker, A., Gruber, M.Y., Wang, Y., Yu, P., 2010. Modeling degradation characteristics and nutrient availability of anthocyanidin-accumulating *Lc*-alfalfa and alfalfa selected for a low initial rate of degradation in dairy cows. ADSA-PSA-AMPA-CSAS-ASAS Joint Annual Meeting, July 11-15, Denver, CO, USA. J. Anim. Sci. 88, (E-Suppl. 2)/J. Dairy Sci. 93, (E-Suppl. 1)/Poult. Sci. 89, (E-Suppl. 1), 153.

Jonker, A., Gruber, M.Y., Wang, Y., Yu, P., 2010. *In vitro* ruminal fermentation characteristics of anthocyanidin-accumulating *Lc*-alfalfa. ADSA-PSA-AMPA-CSAS-ASAS Joint Annual Meeting, July 11-15, Denver, CO, USA. J. Anim. Sci. 88, (E-Suppl. 2)/J. Dairy Sci. 93, (E-Suppl. 1)/Poult. Sci. 89, (E-Suppl. 1), 478-479.

Jonker, A., Gruber, M.Y., Wang, Y., Azarfar, A., Damiran, D., Yu, P., 2010. Anthocyanidin-accumulating alfalfa transformed with the maize *Lc*-regulatory gene for future cattle nutrition, pp 317. 10th Agricultural Biotechnology International Conference (ABIC), September 12-15, Saskatoon, SK, Canada.

Jonker, A., Gruber, M.Y., Wang, Y., Yu, P., 2010. Pasture bloat related foam stability of anthocyanidin-accumulating *Lc*-alfalfa extract. 31th Western Nutrition Conference (WNC), September 21-23, Saskatoon, SK, Canada.

Jonker, A., Gruber, M.Y., Wang, Y., Yu, P., 2011. Bloat related foam stability of anthocyanidin-accumulating *Lc*-alfalfa and relation to FTIR vibration spectroscopic features. CSAS-SCSA Annual Meeting, May 4-5, Halifax, NS, Canada. Can. J. Anim. Sci. (*In press*).

Jonker, A., Gruber, M.Y., Wang, Y., Christensen, D.A., McKinnon, J.J., Yu, P., 2011. Fermentation and microbial protein synthesis from anthocyanidin-accumulating *Lc*-alfalfa in rumen liquid. ADSA-ASAS Joint Annual Meeting, July 10-14, New Orleans, LA, USA. J. Anim. Sci./J. Dairy Sci. (*in press*).

11. Appendix

Resently, the Dutch DVE/OEB protein model was updated. A comparison was made between the original (1994) and updated (2007) DVE/OEB model to determine the differences.

Table not published. Modelling DVE/OEB (1994) intestinal protein availability for [T1]*Lc*-alfalfa and AC Grazeland.

Traits[1]	Population				Model		*Lc* vs. AG[3]
	Beav*Lc*1	Ramb*Lc*3	Rang*Lc*4	AG	SEM[2]	*P* value	*P* value
Ruminal phase							
RD_{CP}^{94} (g/kg DM)	225.8	222.2	226.1	212.8	5.09	0.346	0.113
RD_{OM} (g/kg DM)	579.9	586.6	565.6	546.8	16.05	0.416	0.175
$FOMr^{94}$ (g/kg DM)	582.1^{ab}	601.7^{a}	588.0^{a}	563.6^{b}	6.10	0.049	0.019
$MCPr^{FOMr94}$ (g/kg DM)	87.3^{ab}	90.3^{a}	88.2^{a}	84.5^{b}	0.92	0.049	0.019
OEB^{94} (g/kg)	138.5	131.9	137.9	128.3	4.21	0.375	0.182
Intestinal phase							
RU_{CP}^{94} (g/kg DM)	60.9	60.0	64.9	60.4	5.09	0.807	0.980
$DRUP^{94}$ (g/kg DM)	34.8	36.1	39.5	33.6	5.10	0.861	0.622
$DMCP^{94}$ (g/kg DM)	55.7^{ab}	57.5^{a}	56.2^{a}	53.9^{b}	0.59	0.049	0.019
DVE^{94} (g/kg DM)	72.9	77.3	79.0	68.7	4.53	0.464	0.213

[1]RD_{CP}, rumen degradable protein; RD_{OM}, rumen degradable organic matter; RD_{CHO}, rumen degradable carbohydrates; RD_{N}, rumen degradable nitrogen; FOMr, rumen fermentable organic matter; $MCPr^{FOMr}$, ruminal microbial protein synthesis from FOMr; OEB, rumen degraded protein balance; U_{CP}, completely undegradeble protein; U_{DM}, completely undegradeble dry matter; DFMP, endogenous protein lost into the faeces; RU_{CP}, rumen undegradeble protein; DRUP, intestinal available RU_{CP}; DMCP, intestinal available microbial protein; DVE, total intestinal available protein, see Table 2 for calculations (Tamminga et al. 1994).

[2]SEM, standard error of the mean; $^{a\text{-}c}$ Means with different letters within the same row differ (P<0.05).

[3]*Lc* vs. AC Grazeland (AG), comparing the mean of three transgenic *Lc*-alfalfa populations (Beav*Lc*1, Ramb*Lc*3, Rang*Lc*4) with var. AC Grazeland (selected for a low initial rate of degradation).

Table not published: Difference between DVE/OEB 1994 and 2007 system

	DVE/OEB system				t-test
Trait[1]	1994	2007	difference	SEM	*P* value
RDP	210.4	211.2	-0.8	0.25	0.021
RUP	60.8	60.0	0.8	0.25	0.021
FOMr	585.6	558.6	27.0	3.18	<0.0001
MCPr	87.8	84.9	3.0	2.02	0.183
DMCP	56.0	54.1	1.9	1.30	0187
DRUP	29.6	31.9	-2.4	0.27	<0.0001
DVE	67.6	68.0	-0.4	1.37	0.760
OEB	122.6	126.3	-3.8	2.08	0.115

[1]See previous table for explanation of the abbreviations used