THE EFFECT OF CONDENSED TANNINS UPON PROTEIN DEGRADATION IN THE RUMEN AND ON ANIMAL PRODUCTION IN SHEEP FED FRESH *LOTUS CORNICULATUS*

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BYENG-RYEL MIN

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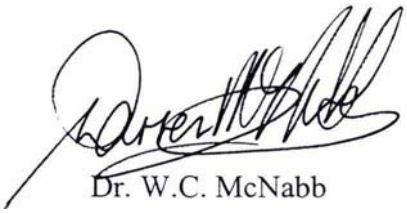
DECLARATION

The studies presented in this thesis were completed by the author while a post-graduate student in the Institute of Food, Nutrition and Human Health, College of Science, Massey University, Palmerston North, New Zealand. This is all my own work and the views presented are mine alone. Any assistance received is acknowledged in the thesis.

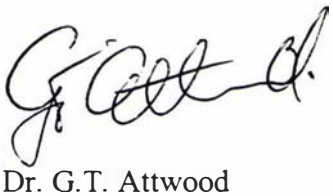
I certify that the substance of this thesis has not been already submitted for any degree and is not being currently submitted for any other degree. I certify that to the best of my knowledge any help received in preparing this thesis, and all sources used, have been acknowledge in this thesis.

Byeng-Ryel Min
PhD Candidate

Professor T.N. Barry
Chief Supervisor



Dr. W.C. McNabb
Co-supervisor



Dr. G.T. Attwood
Co-supervisor

ABSTRACT

A series of *in vitro*, *in sacco* and *in vivo* indoor and grazing experiments were conducted at Massey University and AgResearch Grasslands, Palmerston North, New Zealand to study the effect of condensed tannins (CT) in *Lotus corniculatus* (Birdfoot trefoil; CV. Grasslands Goldie) upon protein digestion in the rumen and on animal production. Aspects studied included effects of CT upon proteolytic bacterial activity, protein solubilization and degradation in the rumen and wool production and reproduction in grazing sheep. The studies also investigated the potential of *L. corniculatus* compared with perennial ryegrass/white clover pasture (hence referred as to pasture).

The nutritional effects of CT in *L. corniculatus* were assessed by administrating polyethylene glycol (PEG; MW 3500) into the rumen of one group of sheep (PEG sheep; CT-inactivated), whilst a separate group of sheep received water (control sheep; CT-acting). PEG selectively binds with CT, preventing the CT from binding plant proteins in the rumen, so that effects of CT can be determined by comparing CT-acting sheep with PEG sheep. The productivity of mixed age ewes in grazing trials was measured in two experiments in the summer of 1995/1996 (Chapter 2) and the summer/autumn of 1997 (Chapter 3), to evaluate the effects of CT in *L. corniculatus* upon efficiency of animal production. A rotational grazing system with restricted feed allowance was used in both experiments.

1. During 1995/1996 (Chapter 2), a grazing trial was conducted to evaluate the effect of CT in *L. corniculatus* on wool growth and on wool processing characteristics in sheep fed close to maintenance for 125 days during summer and autumn (20 December 1995 until 25 April 1996). Half the ewes received twice daily supplements of PEG. The *Lotus corniculatus* contained 32 g total nitrogen (N) and 28 g total CT/kg dry matter (DM) and had an *in vitro* organic matter digestibility of 0.70. Action of CT reduced rumen ammonia concentration ($P < 0.05$) and reduced blood plasma urea concentration ($P < 0.01$) but increased blood plasma cysteine concentration ($P < 0.05$) compared to their counterparts receiving PEG supplementation. The concentration of blood plasma methionine was unaffected by CT. The CT had no effect on voluntary feed intake (VFI) and average liveweight gain ($P > 0.05$) but increased both clean fleece weight ($P < 0.05$) and staple length ($P < 0.001$). The CT also reduced dag percentage ($P < 0.05$) and tended to reduce wool yellowness ($P = 0.07$) relative to sheep receiving PEG. There were no significant effects of CT on fiber diameter (μm),

staple breaking force (Newtons), bulk density (cm^3/g) or wool resilience (cm^3/g). It was concluded that the action of CT in sheep fed *L. corniculatus* increased the efficiency of wool production, with more wool being produced at the same feed intake.

2. Another grazing trial (Chapter 3) was conducted to study the effects of CT in *L. corniculatus* upon VFI, concentration of plasma metabolites, reproductive efficiency and wool production in ewes during two synchronised oestrous cycles in autumn 1997. The ewes were restricted to maintenance feeding for the first 12 days of each oestrous cycle and then increased to *ad-libitum* for the last five days before ovulation. The experiment was of 2 x 2 factorial design, using two types of forage (*L. corniculatus vs.* pasture), with half the ewes grazing each forage being given twice daily oral PEG supplementation. A rotational grazing system with 200 mixed aged dry ewes (52±0.88 kg/ewe) was used. The *Lotus corniculatus* contained 17 g total CT/kg DM in the diet selected, with only trace amounts of total CT present in pasture. Ewes grazing *L. corniculatus* had higher plasma concentrations of branched chain amino acids (BCAA; 57 %) and essential amino acids (EAA; 52 %) than sheep grazing pasture. Again CT in *L. corniculatus* had no effect on mean VFI. The PEG supplementation had no effect upon ovulation rate (OR; 1.33 *vs.* 1.35) and lambing percentage (1.36 *vs.* 1.36 %) of the ewes grazing pasture. The CT increased both OR (1.78 *vs.* 1.56) and lambing percentage (1.70 *vs.* 1.42 %) in the ewes grazing *L. corniculatus* relative to sheep supplemented with PEG. Increases in OR and lambing % of ewes grazing *L. corniculatus* were due to increases in fecundity (more multiple ovulations and less single ovulations), with no effect on ewes cycling/ewes mated. Compared to ewes grazing pasture, ewes grazing *L. corniculatus* had increased clean fleece weight (19 %). It was concluded that action of CT in the lotus diet was partly responsible for the increased efficiency of reproduction, with more lambs being produced at the same VFI.

3. *In situ* and *in vitro* rumen incubations (Chapter 4) were used to determine the effect of CT on both the solubilization and degradation of Rubisco (ribulose-1,5-*bis*phosphate carboxylase/oxygenase; *EC* 4.1.1.39; fraction 1 leaf protein) from white clover (*Trifolium repens*; 0.3 g CT/kg DM) and *Lotus corniculatus* (22.1 g CT/kg DM). The sheep used for the experiments were fed either white clover or *L. corniculatus*. The loss of DM and neutral detergent fibre (NDF), total N and Rubisco from polyester bags suspended in the rumen of sheep was used as a measurement of solubilisation. The effect of CT extracted from *L. corniculatus* on the degradation of Rubisco from white clover was measured by *in vitro*

incubations with rumen fluid obtained from the same fistulated sheep fed either white clover or *L. corniculatus*.

In the absence of PEG, the solubilisation of Rubisco from *L. corniculatus* was less rapid than the solubilisation of this protein from white clover when each forage was incubated in the rumen of sheep fed the same diet. Addition of PEG tended to increase the solubilisation of Rubisco from *L. corniculatus*, suggesting that CT slowed the rates of solubilization of Rubisco from this forage. The action of CT did not inhibit the *in situ* loss of NDF from either white clover or *L. corniculatus*. In the absence of PEG, the *in vitro* degradation of Rubisco from *L. corniculatus* was slower when compared to the degradation of this protein from white clover; PEG addition increased the degradation of Rubisco from *L. corniculatus*, but not from white clover, showing that CT was the causal agent. The addition of CT extracted from *L. corniculatus* markedly depressed the degradation of Rubisco from white clover, with the effect being completely reversible by PEG. The large subunit (LSU) of Rubisco was consistently degraded at a faster rate than the small subunit (SSU) and added CT had a greater effect in slowing the degradation of the LSU compared to the SSU. It was concluded that the action of CT from *L. corniculatus* reduces the digestion of protein in the rumen of sheep through a minor effect on solubilization and a major effect on degradation. The main effects of CT on protein solubilization and degradation seemed to be produced locally by CT present in plant tissue.

4. Eleven strains of proteolytic rumen bacteria (Chapter 5) were used to determine the effect of CT extracted from *Lotus corniculatus* on the *in vitro* proteolysis of Rubisco protein, bacterial specific growth rate and maximum optical density (ODmax). Effects of CT on the rate of Rubisco proteolysis (%/h) were determined through making measurements in the presence and absence of PEG. *Streptococcus bovis* strain NCFB 2476 and B315, *Butyrivibrio fibrisolvens* strain WV1 and C211a, *Prevotella ruminicola* strain 23 and C21a, *Clostridium proteoclasticum* B316^{T}, *Ruminococcus albus* 8, *Fibrobacter succinogenes* S-85, *Eubacterium* sp. strain C12b and C124b were tested against 1.5 mg CT/ml for Rubisco proteolysis and were examined with 0, 50, 100, 200, 400, and 600 μg CT/ml for bacterial growth measurements.

In general, the presence of CT markedly depressed the degradation of both the LSU and SSU of Rubisco, with the effect being completely reversible by PEG. However, the rates of proteolysis per hour for both sub-units of Rubisco varied considerably between individual bacterial species and subunits of Rubisco. In the absence of CT, *S. bovis* strain NCFB 2476 and B315 and *P. ruminicola* like-strain C21a appeared to be most active in both LSU and

SSU degradation, while *P. ruminicola* 23, *Eubacterium* sp. strain C12b and C124b, *C. proteoclasticum* B316^T, *B. fibrisolvens* strain WV1 and C211a had moderate to lower rates of LSU and SSU degradation. In the presence of CT, *S. bovis* strain NCFB 2476 and B315 and *P. ruminicola*-like strain C21a appeared to be most active in both LSU and SSU breakdown.

Most bacterial strains showed significantly ($P < 0.05$-0.01) decreased specific growth rate and OD_{max} with increasing CT concentrations. However, some of the strains, *C. proteoclasticum* B316^T and *R. albus* 8 showed transient increases in specific growth rate at low concentrations of CT (between 50 to 100 μg CT/ml), but not at high concentrations of CT. In terms of specific growth rate, addition of CT at low concentrations (50-200 μg CT/ml), *S. bovis* NCFB 2476, *Eubacterium* sp. C124b and *F. succinogenes* S-85 were most affected compared to the minus CT controls, while *P. ruminicola* sp. C21a and *C. proteoclasticum* B316^T were not greatly inhibited at the highest concentrations of CT. The degree of inhibition of both bacterial growth and Rubisco degradation in the presence of CT varied considerably between individual bacterial species and will be discussed in Chapter 5. It was concluded that action of CT from *L. corniculatus* reduces both the rate of Rubisco proteolysis and the growth rate of proteolytic rumen bacteria, but the magnitude of the CT effect differed between strains used.

5. Twelve six month old Romney sheep were fistulated in the rumen and abomasum and fed *Lotus corniculatus* (32 g CT/kg DM), to examine the effects of CT on proteolytic rumen bacterial populations and on quantitative N digestion in the rumen. Half the animals were given continuous intraruminal infusions of PEG. In the first part of the experiment, the populations of four proteolytic rumen bacteria were enumerated directly from rumen samples using a competitive polymerase chain reaction (cPCR) technique. During pre-feeding on a perennial ryegrass/white clover pasture diet, populations of *C. proteoclasticum* B316, *Eubacterium* sp. C12b, *S. bovis* B315 and *B. fibrisolvens* C211a were 1.6×10^8, 2.7×10^8, 7.1×10^6 and 1.2×10^6 per ml respectively. When the diet was changed from pasture to *L. corniculatus* (average of 8 h to 120 h), the average populations of *C. proteoclasticum* B316, *Eubacterium* sp. C12b, *S. bovis* B315 and *B. fibrisolvens* C211a from the same animals were decreased significantly ($P < 0.001$) to 5.1×10^7, 1.5×10^8, 2.6×10^6 and 1.0×10^6 per ml, respectively. When the PEG was infused into the rumen of sheep fed *L. corniculatus*, the populations of proteolytic bacteria were significantly increased ($P < 0.01$-0.001) compared to the CT-acting group. Rumen proteinase activity, concentrations of rumen ammonia and

soluble N were decreased significantly ($P < 0.05$-0.001) in the CT-acting compared to the PEG treatment group.

In the quantitative N studies, the principal effects of CT were to reduce rumen N digestibility ($P < 0.05$) and ammonia pool size, and to increase the flow of non-ammonia nitrogen (NAN) to the abomasum. Dry matter intake and DM digestibility were unaffected. The N intake, rumen NAN and microbial NAN pool sizes were similar in both CT-acting and PEG sheep. Non-microbial NAN fluxes to the abomasum were significantly higher ($P < 0.01$) in the CT-acting sheep than in the PEG sheep, but microbial NAN flux to the abomasum was unaffected by treatment. It was concluded that *L. corniculatus* CT reduced forage protein degradation in the rumen, and increased the flow of undegraded feed NAN to the abomasum. Proteolytic bacterial populations seemed to be reduced by CT, but these changes did not effect the total rumen microbial NAN pool or abomasal microbial NAN flux. Therefore, more protein was potentially available for absorption from the small intestine.

6. This study is the first to report that action of CT increased reproductive efficiency in grazing ewes. It is also the first study to show that action of CT decreased proteolytic bacterial populations measured directly from rumen samples using cPCR techniques. Feeding forages containing CT such as *L. corniculatus* has been shown to reduce proteolysis in the rumen, with the mechanisms being to slightly reduce protein solubilization, to markedly reduce protein degradation and to reduce the populations of proteolytic bacteria. CT increased NAN flux into the abomasum (in indoor studies) and increased animal production in grazing ewes without affecting VFI, thus improving the efficiency of animal production. It is concluded that forage CT can be used to increase the efficiency and sustainability of livestock production from grazed forages.

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AA	Amino acids
BCAA	Branched amino acids
BCF	Bound condensed tannins
BSA	Bovine serum albumin
BW	Body weight
Cr-EDTA	Chromium ethylenediaminetetra acetic acid
CT	Condensed tannins
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
EAA	Essential amino acids
ECT	Extractable condensed tannins
FCT	Fibre bound condensed tannins
FSH	Follicle stimulating hormone
FV	Feeding value
g	Gram
GLM	General linear model
ha	Hectare
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HT	Hydrolysable tannins

IRL	Irreversible loss rate
K_f	Efficiency of utilization of ME for fattening
K_g	Efficiency of utilization of ME for growth
K_l	Efficiency of utilization of ME for lactation
Km	Efficiency of utilisation of ME for maintenance
LH	Luteinizing hormone
LWG	Liveweight gain
ME	Metabolisable energy
MW	Molecular weight
N.Z.	New Zealand
N	Nitrogen
NAN	Non ammonia nitrogen
NV	Nutritive value
OF	Oesophageal fistulae
OM	Organic matter
OMD	Organic matter digestibility
OR	Ovulation rate
cPCR	Competitive polymerase chain reaction
PRG	Perennial ryegrass/white clover pasture
PEG	Polyethylene glycol
RPM	Revolutions per minute
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39)
SE	Standard error
SEM	Standard error of mean
SAS	Statistical analysis system
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
UK	United Kingdom
VFA	Volatile fatty acid
VFI	Voluntary feed intake
V/V	Volume by volume
W/V	Weight by volume

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