Effects of willow (Salix spp.) browse upon ewe reproduction and rumen microbiology under drought feeding conditions

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CERTIFICATE OF REGULATORY COMPLIANCE

This is to certify that the research carried out in the Doctoral Thesis entitled "Effects of willow (*Salix spp.*) browse upon ewe reproduction and rumen microbiology under drought feeding conditions", in the Institute of Veterinary, Animal and Biomedical Sciences at Massey University, New Zealand and AgResearch (Grasslands), New Zealand:

a. is the original work of the candidate, except as indicated by appropriate attribution in the text and/or in the acknowledgements.

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This is to certify that the research carried out for my Doctoral thesis entitled "Effects of willow (*Salix spp.*) browse upon ewe reproduction and rumen microbiology under drought feeding conditions", in the Institute of Veterinary, Animal and Biomedical Sciences at Massey University, New Zealand is my own work and that the thesis material has not been used in part of whole for any other qualification.

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ABSTRACT

A series of grazing experiments were conducted in the summer/autumn of 2003 and 2004 at Massey University's Riverside dryland farm near Masterton in Wairarapa on the East Coast of NZ, to study the effects of grazing willow fodder blocks (6,000 stems/ha) upon the production and reproductive performance of ewes relative to ewes grazing drought pastures. Drought pastures were simulated in this study and included short drought pasture and long drought pasture. Pasture with a low pre-grazing mass of approximately 1500 kg DM/ha, a dead matter content of >50 % and a sward height of 5-7 cm was defined as short drought pasture typical of drought conditions. Long drought pasture was similar to pasture growing in the willow fodder blocks, with a pre-grazing pasture mass of >4000 kg DM/ha, a sward height of > 30cm and a dead matter content of 30–60 %. Willow fodder blocks were established on low-lying wet, marshy areas of the farm that had very low or zero productivity in the undeveloped state. Pasture development in the fodder blocks was noticed with the growth of unsown grasses and legumes, as the areas dried up following the planting of willow stakes, due to evapotranspiration from the trees. Forage in the willow fodder blocks included both trees and pasture that was grown under the trees. The nutritive value of short drought pasture was low with an ME of 8 MJ/kg DM; long drought pasture ranged between 8-10 MJ ME/kg DM; willow pasture contained 8 MJ ME/kg DM in 2003 and 10 MJ ME/kg DM in 2004. The nutritive value of edible willow tree (<5 mm diameter) was superior to drought pasture with an ME of >10 MJ/kg DM. The concentrations of the secondary compounds such as condensed tannins (CT; 30-40 g/kg DM) and phenolic glycosides (PG; 15-35 g/kg DM) were higher in willow

trees compared to their concentrations (CT; 2-3 g/kg DM) and (PG; 2-9 g/kg DM) in control drought pastures.

Experiments involving short drought pasture, long drought pasture and willow fodder blocks as treatment groups were grazed by ewes for 10 weeks in regular breaks from mid February to early May. Ewes were mated during this period and were joined together after mating and grazed on normal pasture until weaning. Live weight (LW) change and body condition score (BCS) were recorded throughout the experiments, whilst reproductive performance of ewes was measured as the number of lambs recorded at ultrasound pregnancy scanning, lambing, docking and weaning. Measurements on wool production were also recorded at weaning.

In 2003, experimental ewes grazed control drought pastures (short and long) and willow fodder blocks (restricted and full access) as treatment groups (n=100 ewes/group; Chapter 2). Ewes grazing short drought pasture had an allowance of 0.8 kg DM/ewe/d whilst ewes with restricted access had an allowance of 0.8 kg DM/ewe/d from drought pasture and 0.4 kg DM/ewe/d from willow fodder blocks. Ewes in full access treatment group had no access to pasture but were confined to willow fodder blocks at an allowance of 2.0 kg DM/ewe/d, which was the same allowance given to long drought pasture ewes. Ewes grazing short drought pasture lost weight at approximately 100g/d and recorded a low reproductive rate (90 lambs weaned/100 ewes mated) with a high proportion of single lamb births. Live weight loss was significantly reduced to 40 g/d in ewes grazing willow fodder blocks (full access) with a 20% units increase in reproductive rate due to more multiple births ($P<0.05$). Ewes grazing long drought pasture performed intermediate to ewes with full access to fodder blocks and ewes grazing short drought pasture, whilst ewes with

restricted access performed similar to ewes grazing short drought pasture. In 2004 (Chapter 3), the restricted access to willow fodder blocks treatment was eliminated from the study and the number of ewes was increased to 165 ewes per treatment group. Performance of ewes grazing short drought pasture was similar to that of ewes grazing short drought pasture in 2003, with ewes loosing live weight (40g/d) and a low reproductive rate (90 lambs weaned/100 ewes mated) whilst ewes grazing long drought pasture gained LW (54 g/d) and had a higher reproductive rate ($P<0.05$). Ewes grazing willow fodder blocks performed better than ewes grazing short drought pasture by maintaining LW and their reproductive rate was intermediate to ewes grazing short and long drought pasture.

In 2005, a short grazing trial with rumen fistulated sheep was conducted to study the effect of supplementing willow to ewes grazing drought pastures upon plasma amino acid concentrations (Chapter 4) and upon the microbiology of the rumen (Chapter 5 and 6). Grazing occurred during summer/autumn for 10 weeks with two treatment groups; control (short drought pasture; n=7) at an allowance of 0.8 kg DM/ewe/d and ewes grazing short drought pasture at 0.8 kg DM/ewe/d plus a supplement of fresh willow at 1.4 kg fresh willow/ewe/d (n=7). Blood samples for the quantification of plasma amino acids were collected at week 5 and 10, with LW and BCS measured at fortnightly intervals. Short drought pasture in this experiment had a low pasture mass (2000 kg DM/ha) and a low nutritive value (8 MJ/kg DM), whilst willow had a higher ME of 10 MJ/kg DM. Both groups of ewes lost live weight at the rate of 50 g/d. Plasma concentration of 3 methyl histidine (3-MTH; 88 vs 127 μ mole/L) at week 5 and non essential amino acids (NEAA; 1082 vs 1417 μ mole/L) at week 5 and (1155 vs 1324 μ mole/L) at week 10, were substantially lower ($P<0.05$) in

willow supplemented ewes than control ewes. It was concluded that the increased reproductive rate from willow supplementation in ewes grazing drought pasture might be partly explained by reduced body protein catabolism, besides also increasing plasma branched chain amino acids (BCAA) and essential amino acids (EAA) concentrations.

To investigate the effects of willow supplementation on rumen microbes, rumen samples were collected during the 2005 experiment with fistulated ewes over a 10 week period. The study involved the use of a molecular technique (Chapter 5), denaturing gradient gel electrophoresis (DGGE), to compare the rumen microbial populations between the control and supplemented ewes and a cultivation technique (Chapter 6) to study the effect on rumen bacteria of ewes grazing drought pastures with and with out willow supplementation. DGGE analysis of the V3 region of 16*S* ribosomal RNA genes in DNA extracted from samples of rumen contents taken fortnightly over a 10 week feeding period showed a distinct difference in banding patterns between treatment groups which progressively developed over time, showing rumen microbial adaptation to willow supplementation. However, phylogenetic analysis of the DNA sequences retrieved from the DGGE bands from willow-supplemented and control ewes did not cluster by treatment group. It was deduced that willow supplementation induced a change in rumen bacterial populations through selecting sub-populations of organisms already present in the rumen. The changes in the rumen bacterial populations is attributed to the ability of these bacteria to metabolise secondary compounds in willow such as phenolic glycosides and flavanoid monomers and their ability to resist the inhibitory effects of condensed tannins.

The cultivation study involved enumeration, isolation and purification of bacterial colonies on Complete Carbohydrate, Salicin, Xylan, Cellulose and Willow media followed by full characterisation of a representative set of pure bacterial cultures. Total bacterial counts on the above media at week 5 and week 10 were generally lower in willow-supplemented ewes compared to control ewes and the 16*S* rRNA gene sequences of the majority of isolates characterised from both Salicin and Xylan media, were most closely related to species from the *Pseudobutyrivibrio* genus. Isolates from Willow medium clustered as two distinct groups. One group (mostly isolated from control ewes) was made up of mainly of organisms not usually associated with the rumen and probably represent non-resident organisms that are passing through the rumen. The other group of bacteria were mainly retrieved from willow-supplemented ewes and were most closely related to species of the *Olsenella* genus. Compared to bacteria isolated on Salicin and Xylan media, isolates on Willow medium showed little ability to ferment various carbohydrates or trypticase (hydrolysed protein) but were able to utilise secondary compounds from willow.

It was concluded that willow fodder blocks are useful sources of supplementary fodder for mating ewes during drought situations. Both the field and microbiological studies showed adaptation to the willow supplementary diet, including the detection of *Olsenella*-like bacteria for the first time in the rumen. It is suggested that the principal purpose of the rumen investigation is the degradation of secondary compounds present in willow.

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LIST OF ABBREVIATIONS

BCAA	Branched chain amino acid
BCS	Body condition score
CA	Chlorogenic acid
CC	Complete carbohydrate
CP	Crude protein
Cr2O3	Chromium sesquioxide
CT	Condensed tannins
d	day
DGGE	Denaturing gradient gel electrophoresis
DM	Dry matter
DMI	Dry matter intake
DOMD	Digestible organic matter
DON	Deoxy Nivalenol
DSMD	Days of soil moisture deficit
EAA	Essential amino acid
ELISA	Enzyme linked immunosorbent assay
ENSO	El Nino - Southern oscillation phenomenon
EU	European union
FM	Flavanoid monomer
FV	Feeding value
GDP	Gross domestic product
GLM	Generalised linear model
ha	hectare
HCL	Hydrochloric acid
hd	Head
HPLC	High performance liquid chromatography
HT	Hydrolysable tannins
IPO	Interdecadal pacific oscillation index
IVDMD	Invitro dry matter digestibility
LHV	Lower heating value

LIG	Lignin
LW	Live weight
LWC	Live weight change
MAF	Ministry of agriculture and forestry
ME	Metabolisable energy
MTH	Methyl histidine
MW	Molecular weight
NAN	Non ammonia nitrogen
ND	Not determined
NDF	Neutral detergent fibre
NE	Net energy
NEAA	Non-essential amino acid
NH3	Ammonia
NIV	Nivalenol
NIWA	National institute for water and atmospheric research
NV	Nutritive value
NZ	New Zealand
OM	Organic matter
OMD	Organic matter digestibility
OR	Ovulation rate
P	Probability
PED	Potential evapotranspiration deficit
PEG	Poly ethylene glycol
PG	Phenolic glycoside
SAS	Statistical analysis system
SE	Standard error
SMD	Soil moisture deficit
SOI	Southern oscillation index
TLC	Thin layer chromatography
UDP	Undegradable dietary protein
VFI	Voluntary feed intake

CHAPTER 1.
A LITERATURE REVIEW

1.0 INTRODUCTION

Agriculture is a major industry sector in the NZ Economy. The total land area is 27.1 million ha, with pastoral farming covering over 13.5 million ha (grazing, arable, fodder and fallow land), being more than 80 per cent of the occupied land area and nearly half of the total land area (MAF, 2003). Sheep and beef farming extend over 10 million ha and dairy farming a further 2 million ha. The livestock population from 1980 to 2005 is shown in Table 1.1.

Table 1.1 Changes in the domestic livestock population in NZ from 1980 to 2005 (millions)

	1980	1985	1990	1995	2000	2005
Sheep	68,772	67,854	57,852	48,816	42,260	40,145
Dairy Cattle	2,969	3,308	3,441	4,090	4,794	5,156
Beef Cattle	5,162	4,613	4,593	5,183	4,276	4,393
Deer	104	320	976	1,179	1,542	1,592
Goats	53	427	1063	256	147	99[a]

{Source: Annual Review of the NZ Sheep and Beef Industry/NZ Meat and Wool Boards' Economic Service. (1980 – 2005)}

[a] Goat population not available for 2005 and number represents 2004

The data reveal a decreasing number of sheep over this period, while farmed deer and dairy cattle numbers increased. NZ's temperate climate and topography are ideal for the extensive grassland farming of the country's 40 million sheep, providing favourable pastoral conditions and freedom to range and graze good quality pasture. One of the reasons for the decline in breeding ewe numbers was the removal of livestock subsidies in the mid 1980's.

Table 1.2 Changes in productivity of the NZ sheep industry from 1980 to 2005.

Year	1980	1985	1990	1995	2000	2005
Lambing (%)	99.2	98.5	100.4	104.3	116.1	123.2
Wool production/ewe (kg)	5.54	5.27	5.28	5.51	5.72	5.65
Lambs slaughtered/annum (000-head)	28692	39961	25149	26684	26050	25079
Lamb carcass weight (kg)	13.61	12.52	13.71	14.83	16.61	17.62

{Source: Annual Review of the NZ Sheep and Beef Industry/NZ Meat and Wool Boards' Economic Service. (1980 – 2005)}

The number of lambs slaughtered for export has remained relatively static since 1990 at 25 million. At the same time, however, farm productivity has improved enormously. The national average lambing percentage has increased from 100 percent to 123 percent (that is, 123 lambs from every 100 ewes), and the average lamb carcass weight has gone up about 3 kg or 24 percent (Table 1.2). A 30 percent decrease in sheep numbers has been accompanied by a 52 percent increase in lambs produced per breeding ewe. However, under some circumstances, grassland farming may put the welfare of sheep at risk, as they may be exposed to inclement weather. Unless care is taken the health of individual animals may be compromised in the interest of general flock welfare. World-wide, welfare considerations are becoming increasingly important for the animal husbandry. In addition, 60% of NZ's foreign exchange earnings come from farming, based on a low-cost agricultural system and feeding of pasture. This enables NZ farmers to produce milk, beef, lamb, venison, wool and goat fibre, at about 40% of the costs of European or American agricultural systems.

Table 1.3 Contribution of pastoral agriculture to NZ's export earnings (FOB (free on board)Value of NZ's exports ($ Million))

Year	1980	1985	1990	1995	2000	2005
Lamb	746.1	1019.2	957.7	1043.9	1530.2	2110.3
Mutton	108.1	131.3	135.8	152.8	168.6	263.3
Total wool	892.6	1475.4	1315.9	1252.1	801.9	971.8
Total dairy products	991.2	1697.2	2511.1	3256.7	4700.3	5689.9
Total pastoral based exports	3664.3	6049.8	7090.6	8102.7	9797.2	12,603.1
Total agricultural based exports	3851.4	6585.4	8170.1	9432.6	12419.9	15229.6

{Source: Annual Review of the NZ Sheep and Beef Industry/NZ Meat and Wool Boards' Economic Service. (1980 – 2005)}

All sheep and beef farms are run on low input pasture grazing systems. This is with the principal sown pasture used being an 80:20 mix of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). This low cost system enables NZ farmers to supply high quality pasture-fed meat and wool to world markets at competitive prices. Since the sheep industry is a major contributor to the national economy (Table 1.3), research is required aimed at improving the efficiency of sheep production.

The present study is aimed at looking for nutritional solutions to drought, which is a problem for sheep farmers in the dry East coast areas of NZ, especially during the Feb/March/April flushing and mating time in late summer/autumn. The study investigates tree forage as a supplement in drought conditions when the ewes are grazing low quality pasture during mating.

1. 1. SHEEP PRODUCTION

The sheep population in NZ is 39.7 million animals (June 2003), comprising 27.1 m breeding ewes, 7.7 m ewe hoggets, and 4.4 m wethers and rams (MAF, 2003). The major breeds are Romney 46%, Coopworth 13%, Perendale 8%, Corriedale 5%, Merino 4% and cross breeds and other breeds 24%. The average lambing percentage is 124%; however there is considerable variation among flocks and some of the very best producers having a lamp crop of over 200% lambs (Smith and Knight, 1998).

1.1.1 Annual Cycle

The annual cycle of production and management in sheep is built round a breeding season in March/April, lambing in August/September, and weaning in November/December. The live weight targets for breeding ewes are depicted in Fig 1.1. The animal body weight acts as the main buffer between pasture production and

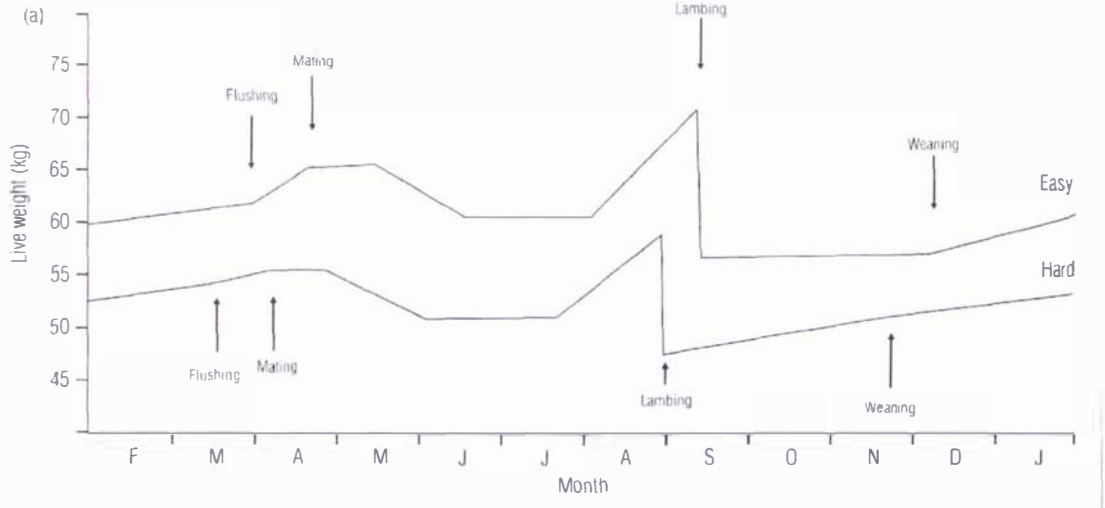


Fig 1.1 Live weight targets through the annual cycle of breeding ewes on easy summer wet country (Easy) and harder hill country (Hard) (Matthews et al., 1999)

feed requirements and management of the sheep farm to increase animal production is focussed on mating and lambing live weight targets, weaning date, flushing before mating and culling ewes. In the drier East coast areas of both the North and South

Islands, including Wairarapa, the aim is to achieve maximum production through the spring and early summer and then to reduce feed demand over the summer when drought reduces pasture production. However, feeding levels immediately prior to mating (flushing) are important as ewe live weight changes at this time are highly correlated with lambing rate in the following season.

1.1.2 Seasonal priorities

Nutrition is one of the most significant environmental influences on reproductive performance of sheep. Management priorities and nutrition in any season must ensure high production in the short term, without penalizing production in the longer term. Recommendations have been developed for each season to try to optimise and improve pasture allowance at critical times of the productive cycle of the ewe (Smeaton et al., 1985). The recommended nutritional allowances and the sward measurements (Rattray et al., 1987; Matthews et al., 1999) are given in Table 1.4.

1.1.2.1 Summer/ Early Autumn (Weaning to mating)

The period between weaning and the next mating is important for good reproductive efficiency in the following season and for good wool production. Live weight of the ewe flock at mating will determine ovulation rate and thus lambing percentage. Therefore ewes need to eat 1.0 to 1.3 kg of dry matter per day (average quality) to maintain a conditions score 2.5 to 3.5 during summer (Geenty, 1997). It is desirable to have the flock as heavy as possible and 'flushed' by gaining 100 to 150 g/d live weight for at least 3 weeks prior to mating. Most effective flushing is achieved with pastures of around 2500 kg DM/ha, with the ewes offered 3-4 kg DM/ewe/d and grazing to a post-grazing pasture mass of 1500 kg DM/ha. Ewes need to be in good body weight and condition (CS3) for high ovulation rates at mating (Rattray et al., 1980).

Table 1.4 Seasonal recommended allowances of pasture cover, pasture length and DM quantities for a breeding ewe

	Weaning to mating	**Mating to early pregnancy**	**Mid pregnancy**	**Late pregnancy and lambing**	**Lactation**
Season	Summer/ Early Autumn	Late Autumn	Winter	Early Spring	Late Spring
Months	December/ Early March	Mid March/May	June/August	August/September	October/November
Condition score	2.5 – 3.5	2.5 – 3.5	2.5 – 3.5	2.5	2.0
Recommended pasture cover (kg DM/ha)					
Pre grazing mass	1200-1400	1400-1800	1400-1800	1500-2000	2000-2500
Post grazing mass	900-1000	1200-1400	400-500	600-800	1400-1600
Pasture length (cm)					
Pre Grazing	5-7	7-9	7-9	8-10	>10
Post Grazing	1-2	2-3	1-2	2-3	4-5
Recommended daily intake (kg DM/day)	1.0	1.4	1.0	1.3	1.8
Recommended ME intake (MJ/day)	8	15.2	11	14.56	21.6
ME concentration (MJ/kg DM)	8	10.8	11	11.2	12

Adapted from Rattray et al.(1987); Matthews et al.(1999)

1.1.2.2 Late Autumn (Mating and early Pregnancy)

Continuing high ewe live weight and condition score during mating means good ovulation rate and higher lambing percentage. Thompson et al. (1990) showed that ovulation rate was more dependent on ewe live weight at oestrus than on previous changes. Placental development is strongly linked to lamb birth weight. Underfeeding can reduce cotyledon numbers and development, thus reducing the transfer of nutrients from the ewe to the lamb (Dingwall et al., 1987), leading to lower lamb birth weights. The major factors affecting foetal growth are ewe nutrition and the size of the placenta. Ewes need 1.0 to 1.3 kg of dry matter per day (average to good quality) to hold body condition during mating and early pregnancy (Geenty, 1997). Environmental stresses and cold weather can reduce embryo survival. Repeated stress such as yarding and handling increases embryonic loss (Doney et al., 1976). Stressed ewes may lose up to 30% of potential embryos. Shearing can coincide with mating when two shearings are done per year. If ewes are shorn just prior to putting rams out they stop oestrus cycling for about three weeks (Geenty, 1997).

Therefore, management practises to minimise stress (yarding, shearing, sudden feed changes) during mating – early pregnancy, to avoid upsetting oestrus, to maximise embryonal survival, and shearing, should not be undertaken from two weeks before until two weeks after mating (Geenty, 1997).

1.1.2.3 Winter and Early spring

This period covers management in the second and third 50 days of pregnancy (from day 51 of pregnancy to lambing), to minimise health problems in pregnancy and prepare for good lamb survival and ewe milk production. Ultra sound scanning is usually done in this period (between 60 and 90 days of pregnancy) and allows the

farmer to identify dry ewes for culling and to separate ewes with multiple pregnancies for preferential feeding and lambing management (Geenty, 1997).

The effect of ewe live weight gain during the first 50 days of pregnancy has been shown to increase lamb birth weight by 46g for every one kg of ewe weight gained (Orleans- Probee and Beatson, 1989) in both single and multiple pregnancies. This effect was not apparent during the second 50 days of pregnancy, whilst during the final 50 days lamb birth weight increased to 111g/kg of ewe live weight gain. About 70% of foetal growth occurs in the last third of pregnancy, greatly increasing ewe energy requirements. Therefore, extra energy is required (above maintenance) by pregnant ewes. Increased ewe feed requirements above maintenance during the final 60 days of pregnancy are 0.1 to 0.5 kg DM per day for singles and 0.2 to 0.9 kg DM for multiples (Geenty and Rattray, 1987).

Management priorities for the ewe flock over winter will depend on pasture reserves, ewe live weight and condition and expected lambing percentage. Recent experiments show shearing earlier in pregnancy may result in increased lamb birth weight (McCutcheon et al., 1983). However recent NZ work shows that shearing by day 70 of pregnancy increases birth weight of single and multiple lambs. The ewes should be vaccinated (anthelmintic) at least two weeks prior to lambing and trace element deficiencies should be treated during this period (Geenty, 1986).

1.1.2.4 Early Spring (Lambing)

Lambing time is the 'crunch' period when benefits from all the work done before and during mating and throughout pregnancy can be realised with a good lambing percentage.

Managemental practices like setting lambing paddocks, avoiding cold wet sides, steep hills and prevailing storms are crucial in this period to achieve a good

lambing percentage. Lamb birth weight is the dominant factor in survival of both singles and multiples (Hinch et al., 1985). Optimum birth weights for lamb survival range to 3.9 to 5.0 kg for single lambs and 3.2 to 4.5 kg for twin lambs (Hight and Jury, 1970). Lambs weighing less than 3.0 kg or more than 6.5 kg at birth have a very low survival rate (Hight and Jury, 1970, Dalton et al., 1980). Birth takes about an hour from fluid release, varying from ten minutes to over three hours (Kilgour, 1982). A delay of over ten minutes has been associated with a longer time from birth to the lamb's first drink (Arnold and Morgan, 1975). Most ewes leave the lambing site within 24 hours but some remain up to 72 hours after birth (Kilgour, 1982). Tagging and lamb handling may frighten first-lambers from the lambing site before bonding is established. Low pasture cover (less than 1000 kg DM/ha) often results in ewe mis-mothering, as ewes are inclined to wander to graze immediately after lambing. Target pasture cover at lambing should be around 1200 kg DM/ha (typically about 2-3 cm high for spring pasture) and ideally pasture should begin growing rapidly early in lactation. Lactating ewes and their lambs usually take top priority, and live weight gains of over 200 g/d for twin lambs and closer to 300 g/d for single lambs can be achieved with pasture allowances of about 6 kg DM/ewe/d and post grazing pasture mass of 1400-1600 kg DM/ha (Rattray and Jagusch, 1978).

1.1.2.5 Late Spring (Lactation)

Ewe nutrition during lactation is important in maintaining or increasing ewe live weight as well as milk production and lamb growth (Rattray and Jagusch, 1978). NZ trials comparing July and September lambing dates show late lambing ewes produce more milk at peak lactation around week four (2.9 kg/day) than those that lamb earlier (2.3 kg/day) and have higher lamb weaning weights (Geenty, 1986). This study suggests better matching of feed supply and demand for later lambing ewes

results in ewes having higher live weights at lambing and better levels of nutrition during early lactation. Ewes cannot be expected to milk well without sufficient pasture and good body condition. During early lactation post-grazing pasture cover should be around 1400-1600kg DM/ha of 4-5 cm length (Geenty and Rattray, 1987).

1.1.3 Pasture production

Pasture is the source of most of the nutrients consumed by NZ grazing livestock, with the two most frequently sown species being perennial ryegrass and white clover in a 80:20 ratio. Grass species provide the majority of the herbage produced by the pasture but the legume species are the keystone of the pastoral system because they fix atmospheric nitrogen and often have greater nutritive value than grasses. Species used in a pasture need to complement each other so the balance between grasses and legumes is maintained. The seasonal supply of herbage must meet the requirements of the grazing animals so it is imperative that the total annual pasture DM production and the pattern of pasture production throughout the year be defined. These patterns of pasture production are basic information for feed planning, as the type of flock management and the feeding required will depend on seasonal growth of pasture.

1.1.3.1 Seasonal Pattern

The seasonal pattern of pasture production of ryegrass/white clover pastures has been measured extensively through out NZ (Radcliffe, 1979). Patterns of pasture production fall into four major environmental categories:

1. Moist summer and mild winter, typical of much of the West Coast of South Island and North West, North Island,

2. Dry summer and mild winter, typical of the drier areas of East Coast, North Island,

3.Moist summer, cold winter, typical of Southland, South Island foot hill country and North Island high hill country and

4. Dry summer, cold winter, typical of East coast, South Island and Wairarapa.

The four growth patterns reflect the environment, mainly the regulating effects of temperature and moisture stress on radiation (light energy) driving plant growth.

The Figure 1.2 depicts the seasonal growth of pasture of moist summer and mild winter, a typical pattern observed in Northland and South Auckland. The pasture is available throughout the year are required for planning feed requirements of the flock throughout the productive period.

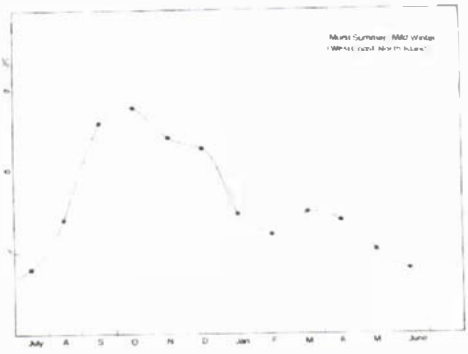


Figure 1.2 Pasture growth curve (moist summer / mild winter areas)[a]

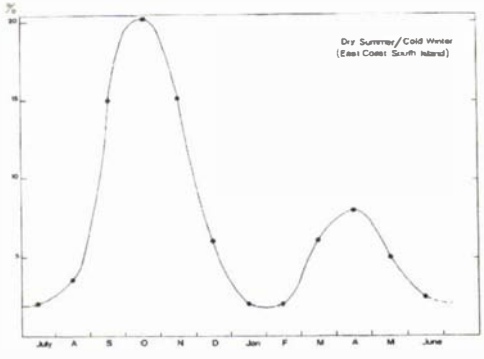


Figure 1.3 Pasture growth curve (dry summer / cold winter areas)[b]

[a, b] : Radcliffe et al. (1974-1978)

Figure 1.3 indicates a dry summer and cold winter which is mostly commonly found in the region of Wairarapa, where the pasture production drops to minimum levels with low pasture growth during summer and there is a probability of drought over the late summer/autumn period.

1.1.3.2 Sward Characteristics

The sward characteristics most likely to affect herbage and animal production, and therefore of most importance to management, are surface height and herbage mass.

1.1.3.2.1 Sward surface height

It is conventionally defined as the average height of the uppermost leaves in an undisturbed sward canopy measured with the simple sward stick. It is the best predictor of bite size and intake (Hodgson, 1990).

1.1.3.2.2 Herbage mass

It is defined as the weight of herbage dry matter (DM) per unit ground area (kg DM/ha) measured to ground level.

The recommended allowances of surface heights and herbage mass differentiated to pre and post grazing masses (kg DM/ha) are given in the Table 1.4.

1.1.4 Grazing systems

Farm subdivision provides the basis for pasture control and energy utilisation in spite of several constraints involved in subdivision. Conventionally there are two grazing systems, viz., Continuous stocking systems, in which animals remain on the same pasture for substantial periods of time or for a whole season and the other is the Rotational grazing system, in which paddocks are grazed in sequence, each for a short period (varying from hours to days). Set stocking is a special case of continuous stocking in which a fixed number of animals remain on a specified area for a prolonged period of time (Hodgson, 1990). Dairying systems generally use rotational grazing methods, whereas lamb producers favour a system of continuous stocking from lambing to weaning in order to maximise lamb performance. (Matthews et al., 1999).

Rotational grazings particularly over the winter months, where feeding levels are relatively low and therefore pasture intake restricted, gave rise to a number of different rotational grazing systems. Strip grazing where a temporary electric fence is used to offer a strip of pasture in a paddock each day to the herd/flock, normally with out a back fence to keep animals off previously grazed pasture.

Block grazing differs from strip grazing to the extent that the herd/flock is offered pasture on a daily basis with both front and a back temporary electric fence, and daily blocks are rectangular in shape. This allows control of extremely high grazing intensities of animals with severely restricted intakes and pastures grazed to very low residuals, while minimising the area and extent of pasture damage.

On/off grazing is where animals are offered their daily pasture allowance (three to four hours grazing) and then removed from a pasture to a hard stand-off area, wintering pad or sacrifice area. This reduces treading damage during grazing and leaves higher grazing residuals to enhance pasture regrowth.

1.1.4.1 Continuous stocking system

Herbage intake and animal performance increase at a progressively declining rate towards a maximum value as sward height increases. This maximum value is called the critical height (point C in Figure 1.4). Herbage intake and animal performance may be expected to start to decline when the surface height of the sward falls below 6-7 cm for sheep (Matthews et al., 1999).

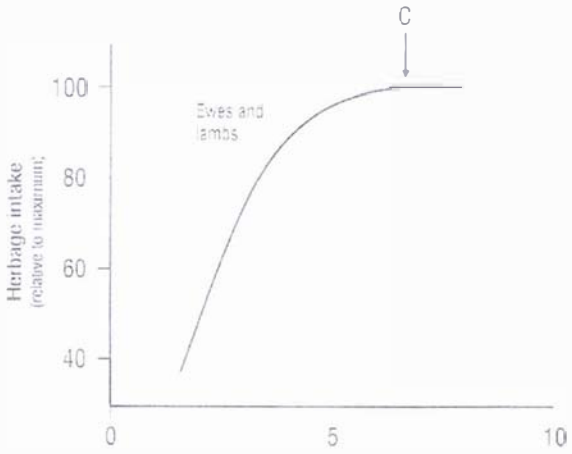


Figure 1.4 Sward surface height (cm) is expressed against herbage intake. Point C depicts critical height of the sward surface when animal performance starts to decline (Matthews et al., 1999)

1.1.4.2 Rotational grazing system

Herbage intake and animal performance is related to the daily allowance of herbage DM. The daily allowance is arrived at by dividing the number of animals per unit area into the herbage mass. Animal performance increases at a declining rate with increasing allowance, usually reaching a plateau at a daily DM allowance equal to 10-12% of the animal's body weight. Sward conditions required to maintain specified levels of animal performance are given in the Table 1.5.

Table 1.5 Sward conditions required to maintain the performance of ewes as per their metabolic condition.

Animal class and performance	**Sward surface height (cm)**	**Herbage mass (kg DM/ha)**	**Energy value of herbage consumed (MJ ME/kg DM)**
Ewe plus twins, Early Lactation	7-8	1400-1600	11-12
Dry ewe	3-4	800-1000	10.5-11.5

Adapted from (Matthews et al., 1999).

1.2. DROUGHT

1.2.1 Definition

In pastoral farming, when pasture production declines as a result of lack of rainfall, to the point where daily rate of pasture growth is substantially less than the daily requirements of livestock and if this situation prevails for a long time, then this culminates in a drought (Crump, 1984). Plant growth depends on the soil water reservoir (amount of moisture available to plants). When this reservoir loses more water from evaporation and gets close to wilting point (where plant growth ceases), a deficit for water is created in the soil, called soil moisture deficit (SMD) (Barringer and Lilburne, 1999). Thus, days of soil moisture deficit (DSMD) is a measure of drought (Tony, 2001).

1.2.2 Occurrence

Climate variability has a significant impact on primary industries in NZ, including livestock farming. Droughts, floods, and other extreme weather events all have an impact on primary production, and ultimately, profitability (Daw, 1999)

1.2.2.1 Influence of Climatic factors

Increasing climate variability and trends over NZ are influenced by three major systems, all operating on different time scales. Firstly, year-to year climate variability in the NZ region is significantly affected by the El Nino/ Southern Oscillation (ENSO) phenomenon. Secondly, climate systems that operate on time scales of approximately two decades have been identified and termed the Interdecadal Pacific Oscillation (IPO). Finally, global warming operating for approximately the last 100 years has caused a mean surface temperature rise of 0.4-0.8 ^{o} C since the second half of the 19th century (Salinger, 2000).

1.2.2.1.1 Effects of ENSO

NZ's climate is dominated by a Pacific–wide fluctuation in the patterns of atmospheric pressure, ocean temperature, trade winds and rainfall – The ENSO phenomenon. The main indicators of this are a band of warmer than average ocean water along the Pacific equator between the International Dateline and South America, and a negative value of the Southern Oscillation Index (SOI). This index is based on the atmospheric pressure gradient between Darwin and Tahiti (Sonzaf, 2003).

During EI Nino conditions, NZ experiences stronger or more frequent winds from the west in summer, often leading to drought in the East Coast areas and more rain in the west. In winter, the winds tend to be more from the south, bringing colder conditions to both the land and the surrounding ocean. In spring and autumn, south-westerlies tend to be stronger or more frequent, providing a mix of the summer and winter effects (Salinger, 2000).

A La Nina phase occurs when the index is positive and the band of ocean water is cooler than average (Sonzaf, 2003). NZ tends to experience more north-easterly winds, which bring moister, rainy conditions to the northeast parts of North Island, and drier conditions in the south east of the South Island (Salinger, 2000).
Figure 1.5 shows the EI Nino and La Nina events that have occurred between 1989 and 2003, and the extent of droughts as expressed in terms of DSMD deviations from the long-term average value. The deviations from the average value indicate occurrence of a drought as happened in 1997/98 (an El Nino event), 1998/99 (La Nina) and 2002/03 (El Nino). However, the deviations need not necessarily result in drought as there was deviation in 2000/01(La Nina) but no incidence of drought.

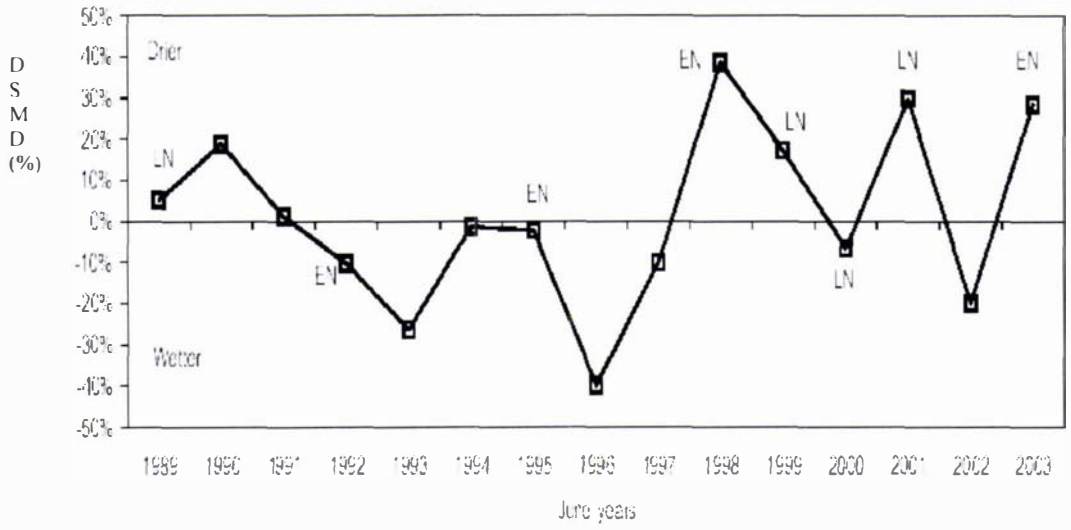


Figure 1.5 Percentage DSMD deviations from 1989 till 2003 due to El Nino and La Nina events. DSMD: Days of soil moisture deficit; EN: El Nino; LN: La Nina events. (Sonzaf, 2003)

In the year 2003 a moderate El Nino climate pattern was observed, which resulted in an atypical drought (one in 30 years) in the east coast as well as western districts of Taranaki to Wellington, shown by the high DSMD in February and March (summer/autumn) of 2003 in Figure 1.6.

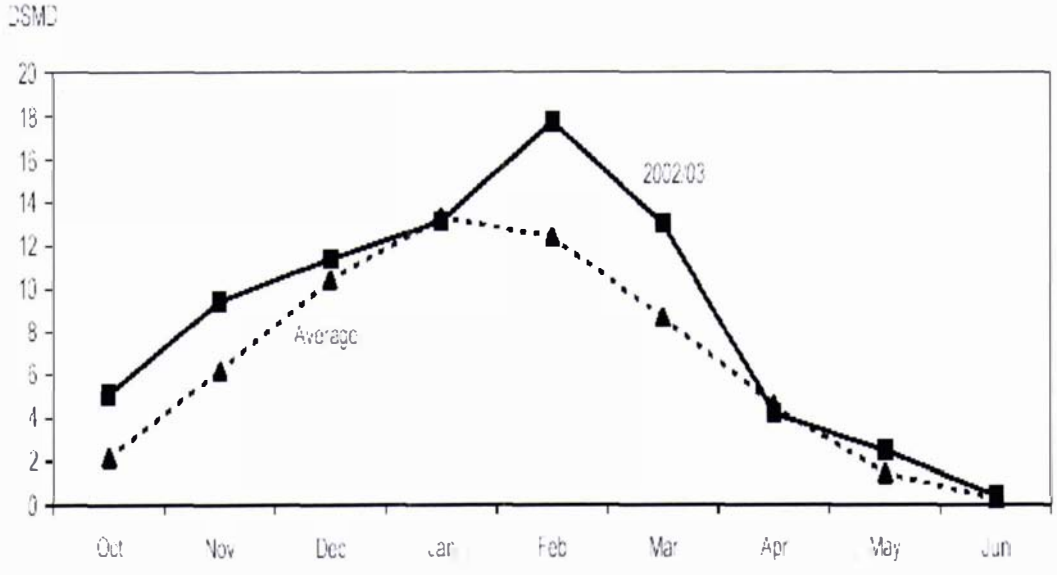


Figure 1.6 The monthly DSMD deviation (%) for NZ for the year 2002/03. DSMD: Days of soil moisture deficit; (Sonzaf, 2003)

East Coast droughts are common during El Nino, but can also happen in non – El Nino years and the severity of East Coast droughts vary from one El Nino to another. ENSO accounts for less than 25% of the year to year variance in seasonal rainfall and temperature in NZ (Salinger, 2000). In Wairarapa, the presence of an El Nino event increases the chance of summer drought and a La Nina event increases the chance of an autumn drought (Harkness, 2000).

1.2.2.1.2 Interdecadal Pacific Oscillation

A newly determined climatic feature that shifts climate every one to three decades is the Inter-decadal Pacific Oscillation (Power et al., 1999). There is a tight coupling between the ocean and atmosphere with the main centre of action in sea surface temperatures in the North Pacific, with an opposing weaker centre just south of the Eastern Pacific (Salinger, 2000). IPO generally has two phases; the positive phase where south-westerlies dominate the NZ area and the negative phase which brings north-easterly winds to NZ (Salinger, 2000).

Figure 1.7 depicts the positive and negative phases of IPO against decades, a pattern that was identified in NZ. Based on this, three phases have been identified during the 20th century, with two well-documented climate shifts in NZ during this time period that can be linked to the IPO: a positive phase (1922-1946), a negative phase (1947-1976) and the most recent positive phase (1977-onwards). The second climate phase (a negative IPO index) that occurred in 1947 brought more anticyclones to the east of the South Island and more easterly winds over the north. Temperatures rose slightly, rainfall increased in the north and east, and decreased in the west and south. The more recent climate phase (a positive IPO index) that occurred in 1977 resulted in more anticyclones over northern areas of the country, with westerly winds

strengthening over southern regions. This shift only had a small effect on temperatures, but rainfall increased in the north, west, south and southeast of the South Island, and decreased in the north of the North Island (Salinger, 2000).

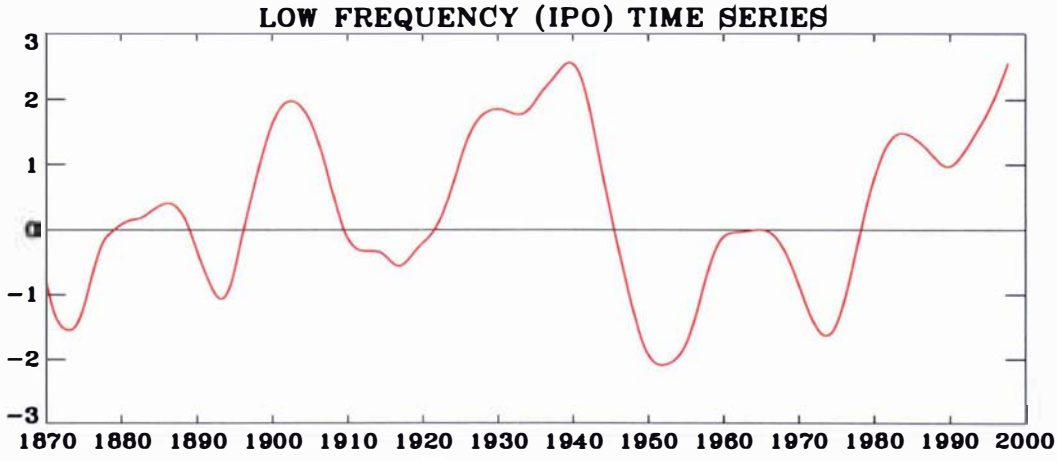


Figure 1.7 Index denoting the phases of the Interdecadal Pacific Oscillation Index (IPO; Salinger 2000)

1.2.2.1.3 Global warming

This is the phenomenon by which, the Earth becomes warmer as a result of an increase in concentration of greenhouse gases in the atmosphere, resulting in climate change. The main greenhouse gases caused by human activity are carbon dioxide, methane, nitrous oxide and some synthetic industrial gases.

Global warming has resulted in increases in global mean surface temperature between 0.4 and 0.8°C in the last 100 years, compared with the second half of the 19th century (Reid, 1992; Salinger, 1992) as shown in Figure 1.8. The largest rises on average over the last 100 years have been in the summer (0.9 ^{o} C) with the other seasons showing an increase of 0.7 ^{o} C. The ten globally averaged warmest years all occurred since 1983 (Salinger, 2000). The temperatures are expected to increase by 1 ^{o} C to 3.5 ^{o} C by 2100 (NIWA, 2003).

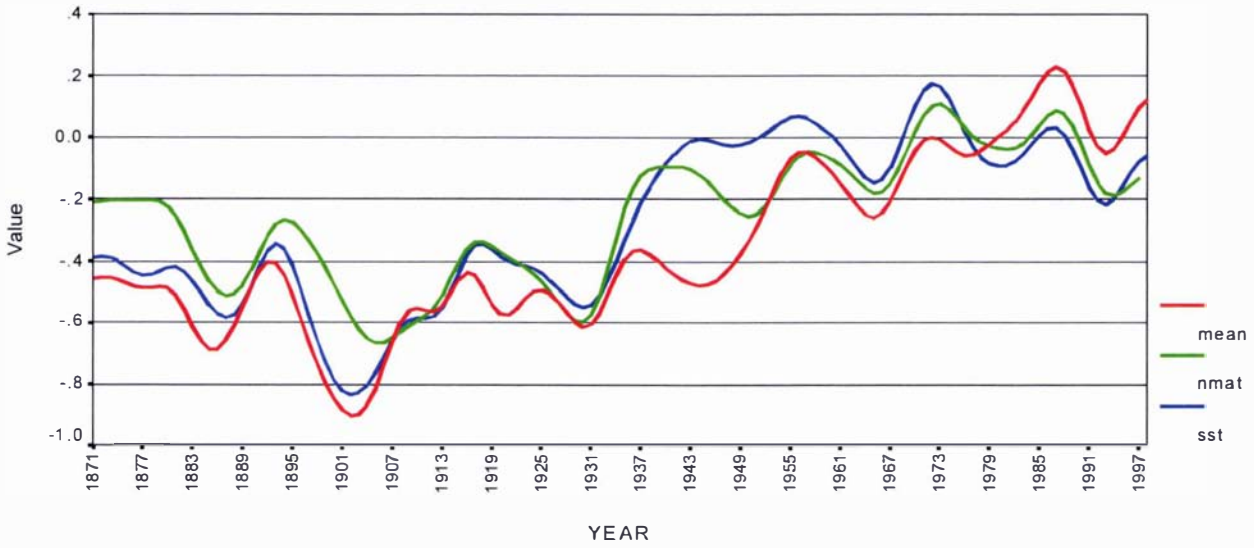


Figure 1.8 Smoothed NZ surface air and surrounding marine temperatures 1871-1998 (°C) compared with the 1961-1990 reference period. Mean = surface air temperature, nmat = night marine air temperature, sst = sea surface temperature (Salinger 2000)

The combined effects of this increase in temperature, together with ENSO and IPO is predicted to result in climate change and lead to more natural disasters like droughts and floods. These trends also indicate a greater incidence of severe droughts in East Coast areas (Salinger, 2000).

1.2.3 Effect on farming

The most significant effect of drought on pastoral farms is the loss in livestock production due to shortages in available fodder and reduction in feed quality. The possibility of summer and autumn drought is a regular feature of East Coast farming in NZ and has been intensified due to the severe climatic shifts; thus the cost of the drought to the farmer and the nation has increased significantly (Moore et al., 2003).

1.2.3.1 Loss at National level

The economic loss caused by severe drought is felt over many years. In the first year, additional stock is sent for slaughter as farmers reduce capital stock. Stock tend to be retained on farm to build up replacements following drought culling, leading to reduced throughput of meat processing chains (Crump, 1984). The cost of purchasing replacement stock also increases, due to an increase in demand for stock following the end of the drought (Moore et al., 2003).

The Ministry of Agriculture and Forestry (MAF) estimated the likely farm gate cost of the 1997/98 El Nino drought to be $256 million during that season (Daw, 1999). This agrees with the figures quoted by Ward (1999), $ 260 million, who also estimated the loss of income for the following 1998/99 farming year as $ 233 million, giving a total production loss of approximately $ 490 million. These figures are estimated for farm gate costs only and it is important to consider the effect of drought on upstream and down stream products. The down stream value–added agricultural products, whose value tends to be three times that of on-farm returns, would also have been significantly reduced (Daw, 1999). The drought in 1997/98 caused a decrease in total farm gate revenue of 2.6% and a figure of 2.2% has been estimated for the 1998/99 season (Ward, 1999). These figures apply to the whole of NZ. This had a significant effect on NZ's Gross Domestic Product (GDP), as farm gate returns are estimated to contribute up to 5% of GDP, whereas valued-added agricultural products contribute 15%. Hence, the impact of El Nino drought conditions is larger than just on-farm effects.

1.2.3.2 Loss at Individual Farm level

In a typical summer/autumn East Coast drought, as happened on Riverside Farm in Wairarapa, a gross margin analysis was calculated in 2003 using Riverside Farm as a model.

Table 1.6 Comparison of reproductive rates at ultrasound scanning over the three field experiments for experimental control ewes grazing simulated drought pasture during mating versus commercially farmed ewes grazing non-drought pastures during mating at Massey University Riverside Farm in Wairarapa

	Reproductive Rate at Scanning			
	Drought Pasture 'Control'	**Riverside Farm Commercial Management**	**Reduction Due to Drought**	**Percentage Reduction Due to Drought**
2001	122	176	54	30.7
2002	133	153	20	13.1
2003	128	167	39	23.4
Mean	**127.7%**	**165.3%**	**37.6**	**22.4%**

Adapted from (McWilliam, 2004)

The data on reproductive rate at scanning was worked on drought pasture with comparison to non drought pastures on the farm. The analysis (Table 1.6) showed a 22.4% reduction in scanning rate, over three years, in ewes suffering drought conditions during mating (McWilliam, 2004). A pre grazing pasture mass of around 1100 kg DM/ha with a low digestibility of 50% is defined as drought pasture and feeding the livestock with this low quality drought pasture would result in live weight loss and reduced body condition score. Reduced weight loss at the time of mating ewes severely affects the reproductive rate by reducing ovulation rate (Smith and Knight, 1998; Mc William et al., 2004). This contributes to the loss of reproductive rate in ewes fed on drought pasture as shown in the Table 1.6.

Besides the reproductive rate, the number of lambs weaned and sold, total wool production also is reduced when the stock is fed on drought pasture during mating (Table 1.7) as indicated in the model which used actual data for the drought pasture only, or control ewes from each of the three grazing trials and compares this with Riverside Farm commercial data. The economic analysis showed a 20% reduction in wool production caused by drought and on the whole would reduce sheep production income by $14.12/ewe or $35,300 per annum for Riverside Farm (Table 1.7).

Table 1.7 Estimated cost of a drought using Massey University Riverside Farm, Wairarapa, as a model (J. Stantiall, Agricultural Consultant, Wilson & Keeling Ltd.)

	Gross Margin Analysis	
	Drought	**Normal**
Number of Ewes	2,500	2,500
Scanning Rate	127.7 %	165.3 %
Pre- and Post-natal Lamb Mortality	20 %	23 %
Weaning Rate	102.3 %	127.3 %
Number Lambs Sold	1,912	2,534
Lamb Live Weight at Sale	32.3 kg	30.0 kg
Total Wool Weight	10,000 kg	12,500 kg
Sheep Gross Margin	$137,776	$173,082
Sheep Gross Margin / Ewe	$55.11	$69.23
Cost Of Drought / Ewe	**$14.12**	
Farm Gate Loss	**$35, 306**	

Adapted from (McWilliam, 2004).

In summary, the nation as a whole and the individual farm is severely affected by drought, due to the climatic variability caused by ENSO, IPO and Global warming climatic factors. As droughts are predicted to be more severe in the future, it is inevitable that farmers must develop more robust drought management plans to ensure the viability of the farm and maintenance of livestock productivity.

1.3. WILLOWS AS A SUPPLEMENTARY FEED IN NZ FARMS

Willows (*Salix spp)* have been introduced and extensively planted in New Zealand to control soil erosion on hill pastoral farms (Wilkinson, 1999) and to a lesser extent for shelter, shade and supplementary forage for livestock. Many of these attributes make willows potentially useful for silvipastoral systems in New Zealand hill country, where soil erosion is widespread and low rainfall in summer results in low pasture production (Oppong et al., 2001).

The willows include some 300 species, most of which are indigenous to temperate regions of the Northern Hemisphere, though some are native to South Africa and South America.

Willows may be conveniently divided into 3 groups: tree willows, osier or basket willows and sallows or shrub willows. Of the three groups, tree willows are considered to have significant nutritive value and therefore considered as a feed supplement to grazing livestock (Kemp et al., 2001; McWilliam, 2004). Tree willows have single stems and lanceolate leaves with some species reaching 25 metre in height. Some of the more common varieties found in New Zealand include crack willow (*S. fragilis)*, golden willow (*S. alba var.vitellina),* weeping willow (*S. babylonica*) and navajo willow/globe willow (*S. matsudana).* The latter was a successful introduction from North China and is widely planted for soil conservation,

shelter and as an ornamental plant. When hybridised with *S. alba* from Europe, considerable hybrid vigour is produced, and a number of clones developed are widely being planted in New Zealand.

1.3.1 Varieties

The need for improved varieties of willow species and clones to prevent soil conservation in was recognised in NZ. Crosses between *S. matsudana* (N.China) and *S. alba* (Europe) enhances hybrid vigour, with some clones growing 50% faster than parents. The first 3 clones: NZ 1001"Cannock", NZ 1002 "Aokautere" and NZ 1003 "Te Awa" were released in 1975. In 1980 a further series of *S. mastusdana* × *alba* hybrids were released for soil conservation planting for wind breaks. These were NZ 1040 "Tongoio", NZ 1130 "Hiwinui", NZ 1143 "Adair", NZ 1149 "Wairakei", NZ 1179 "Makara" and NZ 1194 "Moutere" (Streamland, 1987).

1.3.2 Utilisation

1.3.2.1 Soil Conservation

Willows and poplars check soil erosion on hills, which is a widespread problem in New Zealand. Willows reduce this by binding the soil with their fibrous root system and by transpiring water from deep in the soil profile (transpiration) and improving the health of soil. They increase soil pH by 0.5 to 1.0 units and increase the concentration of calcium at the soil surface. The leaf fall adds organic matter to soil and compensates for lower pasture production (Kemp, 2001).

1.3.2.2 Shade and shelter

Animals must maintain body temperature within a tight range otherwise they expend energy to keep warm or cool (Palmer et al., 2003). The provision of shelter, particularly against wind, is known to alleviate many of the adverse effects of inclement winter conditions on livestock (Flanagan, 1995). Under cold conditions,

providing shelter can improve growth rate and ovulation rate in cattle and sheep (Lynch & Donelly, 1980) and reduce lamb mortality and abortions induced by hypothermia (Alexander et al., 1980). Wilkinson (1999) concluded that a row of poplars and willow trees can be used to shelter livestock.

1.3.2.3 Supplementary Feed

Willows can be fed as a supplement during dry summers and willows fed to livestock have a similar nutritive value to lucerne hay (McCabe and Barry, 1988). The willows *S. kinuyanagi* and *S. matusdana* × *alba* were selected for soil conservation in New Zealand, but have potential as supplementary livestock forage in summer and autumn (Hathaway, 1986; McCabe and Barry, 1988; Douglas et al., 1996). After 30 years of planting, it is estimated that 1.44 million trees of willow/poplar are available as a source of supplementary fodder on the North Island East Coast (D.J. Cameron, Personal communication). The yield of edible forage (leaves plus stem ≤ 5 mm diameter) per tree from widely spaced trees ranged from 1-25 kg dry matter (DM) per tree depending on tree age (Kemp et al., 2001), and up to 5.9 tonnes DM/ha is produced from densely planted fodder blocks (Hathaway, 1986).

1.3.3 Methods of feeding

There are several methods available for feeding willows to livestock. These include grazing livestock on fallen leaves, feeding them on pruned lower branches and foliage and the grazing of fodder blocks. The widespread methods adopted by farmers in NZ are as follows.

1.3.3.1 Cut and supplementation with willows

Willow fodder can be used as a supplementary forage to livestock on farms by cutting some or all of the foliage of variously aged, widely spaced trees, planted mainly for soil conservation (Douglas et al., 2003). Cut forage of *S. matsudana* × *alba* was suggested by McCabe and Barry (1988) as a nutritionally acceptable supplement to pasture during summer.

Moore et al., (2003) supplemented willow stem cuttings to beef cattle grazing dry summer sparse pasture and recommended that willow can reduce live weight loss when fed for at least 55 days. The diameter of the willow eaten (Figure 1.9) as well as the DM content of the willow offered, continuously increased over the experimental period ($P<0.05$), resulting in the amount of willow DM eaten/cow per day continuously increasing throughout the experiment. The diameter of willow eaten by beef cattle ranged from 4 to 8 mm.

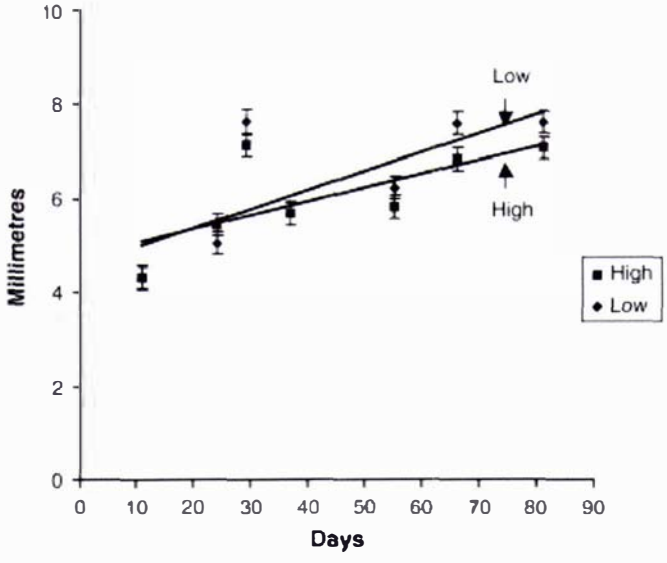


Figure 1.9 The diameter of supplementary willow consumed by beef cattle when grazing low quality drought pasture. (■) High supplementation received 8 kg fresh/cow/day; (♦) Low supplementation received 4 kg fresh willow/cow/day; (I)-S.E. Adapted from (Moore et al., 2003)

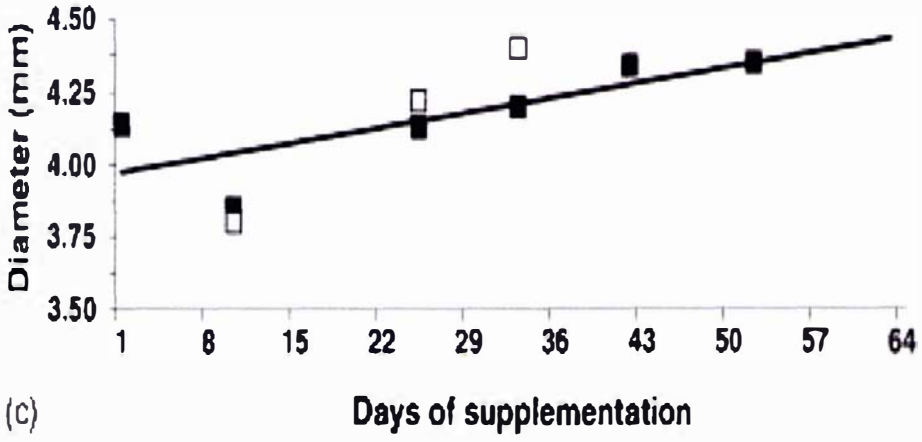


Figure 1.10 Change in stem diameter of supplementary Tangoio willow consumed by ewes grazing low quality drought pasture. (■) Long supplementation (63 days); (□) short supplementation (31 days). Adapted from (McWilliam et al., 2005b)

McWilliam et al. (2005b) fed cut willows as a supplementary forage to ewes that were mated on drought pasture and concluded that feeding willows to ewes led to increased reproductive rate besides reducing live weight loss. The diameter of the willow eaten increased (3.8 mm to 4.5mm) with time (Figure 1.10) and the dry matter of the willow offered also increased with time.

1.3.3.2 Fodder blocks

Growing of shrub or tree species in rows in association with pasture, is another practical option for farms (Douglas et al., 1996). Large scale planting of willows originally relied on using rooted stem cuttings, but these are expensive. An alternative is to use unrooted stem cuttings which were as productive as rooted cuttings, whilst being cheaper to establish and easier to handle (Zsuffa, 1992). Establishment of fodder blocks can be achieved by vertically planting cuttings referred to as 'wands' or 'stakes', which are often 1.0 to 1.2 m long, with diameters of 15-25mm and 20-40mm, respectively (Van Kraayenoord, 1984). Douglas et al. (2003) suggested that 2m poles, with more potential growing points and greater potential energy reserves for shoot growth, could enhance yield per tree, and per hectare. Further, a quarter to

one-third burial of the pole in the ground provides satisfactory plant survival and growth in a range of environments. These stem cuttings (stakes) have been used as potential browse plants in dry summer conditions (Oppong et al., 1996). Douglas et al. (1996) concluded that willows could be cut, carried and used in coppicing and recommended that farmers could establish special purpose forage banks of the willows, which could be cut or grazed when required. The authors reported that, under a range of cutting heights and cutting frequencies at dry and moist sites, total biomass of Tangoio (2,700 stems/ha) above cutting height averaged 1.2 to 4.3 t DM/ha/year, of which about 25% was edible.

1.3.4 Nutritive value

The nutritive value of feed and its voluntary intake in livestock reflects its feeding value for grazing ruminants. The nutritive value of willows has become the focus of studies investigating the use of willows as a supplementary feed in dry summer conditions, as it is abundantly available and cheaper when compared to other supplements used in hill country. It was observed that CP concentration of edible browse of willow species was 165 to 210 (g/kg DM). Moreover, in summer, the edible fodder (leaves and fine stems) had considerable nutritive value and was adequate for the maintenance of sheep, goats and red deer and better than low quality pasture (typical of summers) (McCabe and Barry, 1988, Kemp et al., 2001). Although the green leaves of willows have a higher nutritive value than the stems, livestock also tend to eat the stems of approximately 5mm or less diameter (Kemp et al., 2001). Senesced or dead leaves had a lower nutritive value, whilst freshly fallen leaves had OMD of 0.5 and ME of 7-8 MJ/kg DM. Bark also carries a low nutritive value with CP of 30-50g/kg DM, OMD of 0.55-0.65 and ME of 9 MJ/kg DM (Kemp et al., 2001).

1.3.4.1 Supplementary feed

The willows *S. kinuyanagi* and *S. matsudana* × *alba* are mostly used for soil conservation in NZ; they have considerable nutritive value and form a potential supplementary feed in summer and autumn (Hathaway 1986; McCabe and Barry 1988; Douglas et al., 1996). Edible forage production (leaf and edible stem) ranged from 1-6 t DM/ha, values to 0.3-2.5 kg DM/tree (Hathaway 1986; Douglas et al., 1996). The OMD, CP and ME of willows were found to be higher in spring than summer. A 10% decrease in OMD of edible tree forage was observed in summer which was attributed to the maturation of thin stems (Kemp et al., 2003).

1.3.4.2 Primary compounds

The N content in willows (17.8 g/kg DM) was low but not lower than the threshold concentration of (< 13 g/kg DM), which can limit voluntary intake of adult ruminants (McCabe and Barry, 1988) and the ratio of readily fermentable to structural carbohydrate was substantially lower in willows than forage kale (2.60) and vegetative white clover (1.26) (McCabe and Barry, 1988), but was higher (0.65 vs 0.2) than summer pasture (McWilliam et al., 2005a and b). Willow has a high concentration of lignin (182 g/kg DM) compared to other temperate forages, which is a limiting factor for organic matter digestibility. The reason for the high content of readily fermentable carbohydrate was deduced to be the presence of secondary chemical compounds present in willows and these were mainly phenolic glycosides (e.g. Salicin containing glucose) and condensed tannins. Table 1.8 summarises the nutritive value of willow.

Table 1.8 Nutritive value of edible DM (leaf plus stem≤ 5mm diameter) of willows. OMD is organic matter digestibility, ME is metabolisable energy

Cultivar/Season	Crude Protein (g/kg DM)	OMD	Ash (g/kg DM)	ME (MJ/kg DM)
Tangoio	171	0.69		9.9
Tangoio	132	0.72	64	10.3
Kinuyanagi	71	0.65	16	9.7
Willow/Dec	119	0.71	47	10.4
Willow/March	84	0.65	33	9.6
Willow/Spring	170	0.79	76	11.6
Willow/summer	142	0.64	64	9.8

Adapted from Kemp et al.(2003)

Willows were fed as a supplement to low quality summer pasture to beef cattle (Moore et al., 2003) and to sheep (McWilliam et al., 2005a&b) and the nutritive value of willows was found to be consistently superior to low quality summer pasture in both the studies, particularly for ME concentration (Table 1.9). Low OMD values in the willow consumed by cattle can be explained by cattle eating willow with thicker diameter (8mm) than sheep (4mm).

Table 1.9 Nutritive value of willow supplements and drought pasture, to beef cattle (Moore et al., 2003) and ewes (McWilliam et al., 2005a&b)

Author	Total Nitrogen (g/kg DM)	OMD	ME (MJ/kg DM)	NDF (g/kg DM)	Lignin (g/kg DM)
Beef cows (Moore et al., 2003)					
Willow	18.1	0.582	9.3	473.6	116.6
Drought pasture	22.2	0.592	8.4	571.7	35.1
Ewes (McWilliam et al., 2005a&b)					
Willow[1]	26.3	0.68	10.1	381.3	134.3
Drought pasture[1]	24.5	0.53	7.5	570.6	36.5
Willow[2]	24.7	0.70	10.4	355	107
Drought pasture[2]	15.9	0.49	7.2	603	39

[1] Study conducted in 2002
[2] Study conducted in 2003

1.3.4.3 Secondary Compounds

Secondary compounds can be classified into 3 groups: carbon based phenolics, terpenes and nitrogen-containing compounds such as alkaloids (Taiz and Zeiger, 1991). The term 'phenolic' is used to define substances that posses one or more hydroxyl (OH) substituents bonded onto an aromatic ring; compounds that have several phenolic substituents are referred to as polyphenols (Waterman and Mole, 1994). Simple phenolics contain an aromatic ring substituted by one or more hydroxyl groups. Complicated phenolics have additional functional groups such as an ester, methyl, acetyl or sugar moieties. Phenolic glycosides are less toxic and are usually stored in vacuoles as water soluble compounds (Hosel, 1981). Willows and other Salicaceous plant species produce two main secondary chemicals: phenolic glycosides and condensed tannins (Orians et al., 2000). Condensed tannins are phenolic polymers known to reduce protein solubility, while phenolic glycosides are phenolic monomers (Lindroth, 1991; Per Hallgren et al., 2003). Some willow species produce condensed

tannins, others produce mostly phenolic glycosides and others produce both classes of compounds (Julkunen-Tiitto, 1986, 1989; Orians and Fritz, 1995; Orians et al., 2000).

1.3.4.3.1 Phenolic glycosides

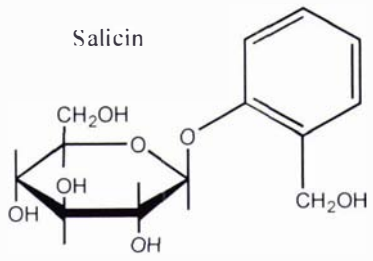


Figure 1.11 The chemical structure of Salicin molecules (MW=286.27)

Salicylates are phenolic glycosides found in willows and poplars, members of Salicaceae family (Lindroth and Pajutee, 1987, Julkunen-Tiitto, 1989). These substances contain a backbone structure of salicin, 2-*O*-β-D-glucoside of salicyl alcohol, attached to a variety of substituents, such as benzyl, acetyl and/or hydroxycyclohexenone groups (Figure 1.11).

Salicylates are often found in high levels in the bark and leaves of plants, but not in roots (Julkunen Tiitto, 1989). Salicin and salicortin are the most widespread salicylates but higher substituted salicylates such as salicortin, acetylsalicortin and tremulacin are characteristic compounds of the Salicaceae family (Julkunen-Tiitto, 1989, Pierpoint, 1994). Biosynthesis of salicylates seems to be complex and it was concluded that Salicin is derived from either of two precursors namely, *t*- cinnamic acid or benzoic acid. Complex salicylates are degraded to salicin either through preservation of samples, extraction procedures or rupture of the leaf by chewing. Therefore, salicin is the key intermediate in the metabolism of salicylate, acting as a degradation product and a precursor of higher substituted salicylates (Ruuhola, 2001).

Salicin concentrations vary between cultivars of the same species with a recorded range of 0 to 84 g/kg DM in willow cultivars analysed using TLC methods (Markham, 1971). McWilliam (2004) reported a value of Salicin (0.9 and 2.0) and other phenolic glycosides (32.7 and 17.2) g/kg DM in two experiments respectively, using HPLC methods.

Edwards (1978) concluded that willow/poplar species with higher concentrations of Salicin, had reduced palatability in marsupial brush tail possums (*Trichosurus vulpecula*) (Figure 1.12). However, intake of willow is not entirely dependent on Salicin concentration but also depends on other factors such as smell and appearance.

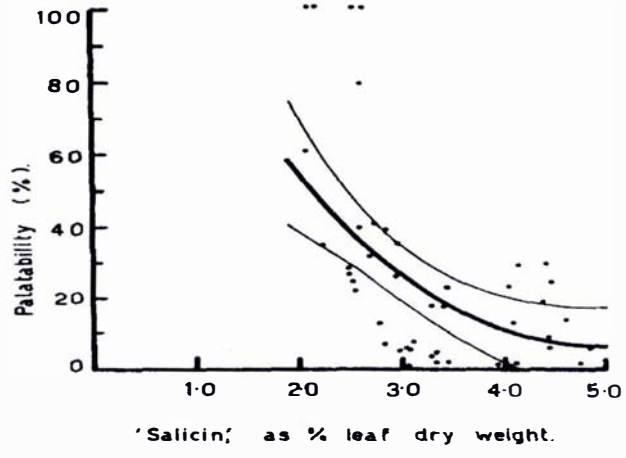


Figure 1.12 Salicin as a percentage of dry weight versus palatability as a percentage of leaves partly or wholly consumed by possums. The upper and lower lines are 5% confidence limits. Adapted from Edwards (1978)

Pass and Foley (2000) conducted several experiments to investigate the regulation of salicin intake, by studying pre-ingestive factors (taste) to post-ingestive (nutritional or toxic) effects in brushtail possums (*T. vulpecula*) when fed with salicin-containing diets. Their experiments showed that possums regulate their salicin intake so as not to exceed a threshold level of 1.9±0.1 g/kg DM/day (Figure 1.13). DMI was found to decrease in the initial phase (6 days of feeding salicin rich diets) (Figure

1.13), however, this reduction in DMI was reduced on prolonged feeding (20 days). They concluded that the negative correlation between the food preference and phenolic glycoside concentrations of a range of plant material was due to compounds other than salicin.

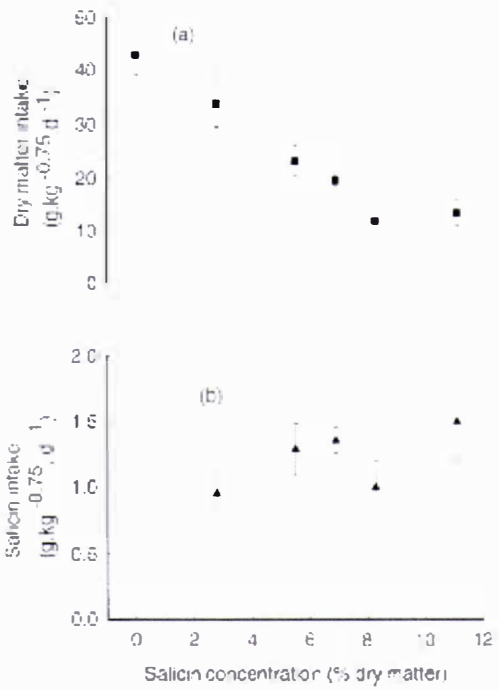


Figure 1.13 Intake of (a) dry matter (DM) and (b) salicin in common brushtail possums fed diets with variable concentrations of salicin. (Mean ± SE; n =6) Adapted from (Pass and Foley, 2000)

1.3.4.3.2 Condensed Tannins

Tannins are phenolic secondary compounds that are widespread in the plant kingdom. Tannins exist mainly as condensed (CT) and hydrolysable (HT) forms; the HT molecule contains a carbohydrate as a central core, which is often D-glucose and the carboxyl groups of these carbohydrates are esterified with phenolic groups such as gallic acid (gallotannins) or ellagic acid (ellagitannins), and unlike CT, their degradation products can be absorbed from the small intestine of animals, and can be potentially toxic to ruminants (Kumar and D'Mello, 1995, Min et al., 2001).

The CT or proanthocyanidins (PA) are the most common type of tannin found in forages such as legumes, trees and shrubs. CT are complexes of oligomers and polymers of flavanoid units (flavan-3-ols, flavan-3, 4-diols and biflavans) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis (Hagerman and Butler, 1991; Min et al., 2001). CT are found in most plant tissues (Min et al., 2001) but most often in the leaves and stems of plants.

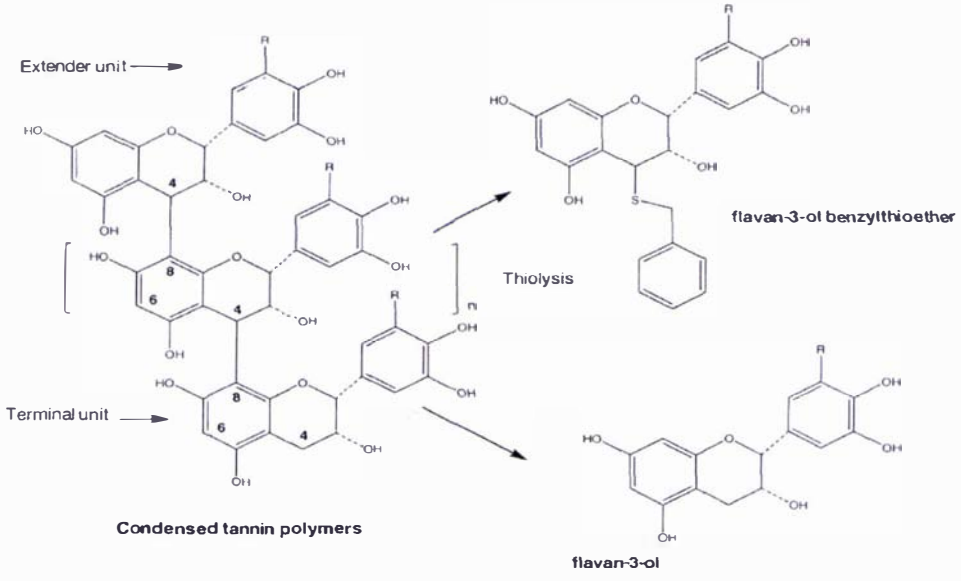


Figure 1.14 Thiolysis reaction of condensed tannin polymers with procyanidin (R=H) and/or prodelphinidin (R=OH) units. The trans-stereochemistry is associated with catechin and gallocatechin (not shown), while cis-stereochemistry is associated with epicatechin and epigallocatechin. All terminal units in the polymer were released as flavan-3-ols, and the extender unit as flavan-3-ol benzylthioethers (Meagher et al., 2004)

1.3.4.3.2.1 Concentration of CT

Condensed tannins are commonly found in legumes such as *Lotus pedunculatus* (Big trefoil), *Lotus corniculatus* (Birdsfoot trefoil) and *Hedysarum coronarium* (Sulla). They are present in only trace amounts in *Trifolium repens* (white clover) and *Trifolium pratense* (red clover) mainly due to the CT content of the flowers. They are also found in trees like *Salix* sp and *Populus* sp (Table 1.10).

1.3.4.3.2.2 Effects of CT

Condensed tannins play a significant role in the nutrition of animals, and may have both adverse and beneficial effects on nutrient utilization, health and production. CT in forage bind to plant protein and protect it from digestion in the rumen, thus making it available for digestion and utilization in the abomasum and small intestine (Waghorn et al., 1990; Norton, 1999). Another possible effect of CT is related to the stimulation of salivary flow in animals (Van Soest, 1994). The ideal concentration of CT in forage legumes generally ranges from 20–40 g/kg of DM, at which level they bind dietary proteins during mastication and protect the protein from microbial attack in the rumen (Barry, 1985; Barry and McNabb, 1999). CT concentrations above 50 g/kg DM can act as anti-nutritional factors in plant material fed to ruminants (McLeod, 1974; Wang et al., 1996) and at high concentrations (60–90 g/kg), they may have an adverse effect on intake or rumen function (Barry, 1985; Norton and Ahn, 1997).

1.3.4.3.2.3 Voluntary feed intake

The effects of CT on feeding value can be regarded as the sum of the effects on voluntary feed intake, on the digestive process and on the metabolism of absorbed nutrients. CT do not affect voluntary feed intake (VFI) of ruminants grazing forages with moderate concentrations of CT (34-45 g/kg DM). However, decrease in VFI of

27% in sheep grazing *L. pedunculatus* with high CT concentrations (63 and 106 g/kg DM) was reported by Barry & Duncan (1984). Smaller depressions in VFI of 12% were also reported in sheep grazing *L. pedunculatus* with only 55g CT/kg DM (Waghorn et al., 1994). This is consistent with plant CT production being a defensive mechanism against pathogenic microorganisms, insects and grazing herbivores (Waghorn et al., 1994).

1.3.4.3.2.4 Digestibility and metabolism of nutrients

1.3.4.3.2.4.1 Protein

When ruminants are fed with common fresh temperate forages such as perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*), about 75% of the feed protein is degraded by rumen microorganisms mainly to ammonia (NH_3). Most of this nitrogenous product is utlilised by rumen bacteria in microbial protein synthesis (Barry et al., 2001). When the animals are fed with fresh forages, not all the rapidly produced NH_3 is utilized by ruminal bacteria, but approximately 30% of the nitrogen eaten is absorbed as ammonia through the rumen wall to be eliminated as urea in the urine. Therefore, the duodenal flow of NAN (non ammonia nitrogen) is about 65% of the total N eaten for ruminants fed high quality fresh forages (Barry and McNabb, 1999; Min et al., 2003).

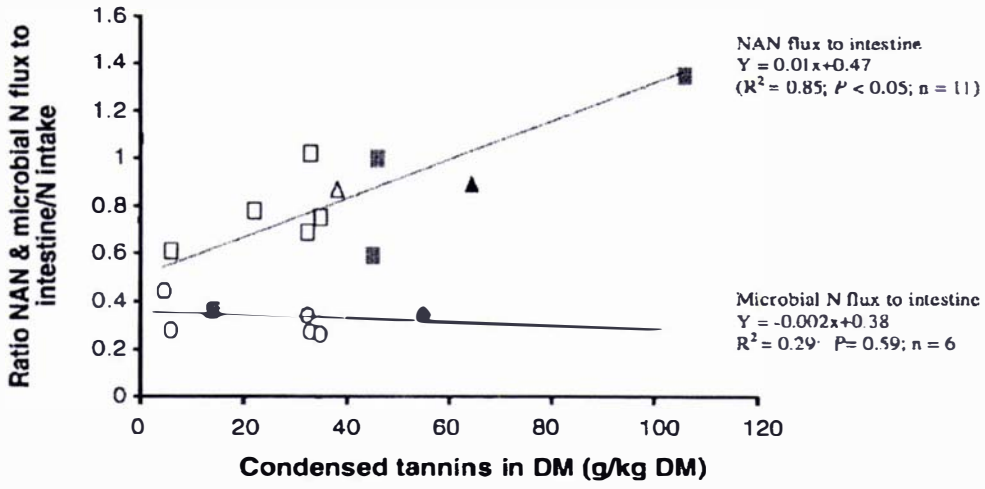
Table 1.10 Concentration of secondary compounds (condensed tannins and others) in temperate forage species with pastoral value for New Zealand farming systems

Forage	Total condensed tannin content (g/kg DM)	Other known plant secondary compounds
Grasses[1]		
Lolium perenne (perennial ryegrass)	1.8	Endophyte alkaloids 12–30 mg/kg DM
Legumes[1]		
Lotus corniculatus (birdsfoot trefoil)	47	0
Lotus pedunculatus (big trefoil)	77	0
Hedysarum coronarium (sulla)	84	0
Trifolium repens (white clover)	3.1	Cyanogenic glycosides
Trifolium pratense (red clover)	1.7	Iso-flavones 7–14 g/kg DM
Medicago sativa (lucerne)	0.5	Coumestrol 0–100 mg/kg DM
Herbs[1]		
Chicorium intybus (chicory)	4.2	Sesquiterpene lactones 3.6 g/kg DM
Sanguisorba minor (sheeps burnet)	3.4	0
Plantago lanceolata (plantain)	14	Iridoid glycosides
		Catalpol 8 g/kg DM
		Acubin 22 g/kg DM
Trees[2]		
Salix spp (willow)	27-52	Phenolic glycosides (17-33 g/kg DM)
Populus spp (poplar)	19.3	Phenolic glycosides 14.4 g/kg DM

[1] Ramirez-Restrepo and Barry (2005), [2] McWilliam (2004)

With CT-containing *Lotus* sp, duodenal NAN flow (an index of the amount of feed protein leaving the rumen), increases linearly with increasing CT concentration (Figure 1.15) and equals N intake at a CT concentration of approximately 50 g/kg DM, without affecting microbial protein production. This is because CT reduce both solubilization and degradation of forage protein by rumen microorganisms, specially the principal leaf protein, ribulose-bisphosphate carboxylase/oxygenase (Rubisco; fraction 1 leaf protein) (Barry and McNabb, 1999).

Besides decreasing the protein degradation in the rumen, the CT in both *Lotus corniculatus* and *Lotus pedunculatus* increased the flow of essential amino acids (EAA) out of the abomasum by 50% and 30% respectively, as deduced from PEG supplementation studies. However, apparent digestibility of EAA in the small intestine was reduced 10% by CT in *L. pedunculatus*, whilst CT in *L. corniculatus* increased net absorption of EAA by 60% without affecting apparent digestibility in the small intestine. These differences were due to differences in the species, concentration, structure, molecular weight (MW) and hence reactivity of the CT. CT from *L. pedunculatus* exihibit a greater affinity for feed protein, decreasing the percentage of protein being released in the small intestine for digestion and absorption.



Adapted from (Min et al., 2003)

Figure 1.15 The relationship between condensed tannin concentration in the dry matter of forage species with the ratio of non-amonia-nitrogen (NAN) flowing at the abomasum or duodenum and microbial N flow at the abomasum or duodenum per unit of N eaten by sheep. (□), *L.corniculatus*; (■), *L. pedunculatus*; (Δ), sainfoin; (▲), sulla, (○), *L. corniculatus*; (●), *L. pedunculatus*.

1.3.4.3.2.4.2 Fibre digestion

After mastication, a large proportion of the CT becomes bound to plant protein, and a small amount remains as free CT. In plants like *L. pedunculatus* with 90g CT/Kg DM, normally 90% of the CT binds to plant protein and 10% remains as free CT; this concentration is the limit where the protein-binding system becomes saturated, and increments in total CT concentration above 90g/Kg DM remain as "free tannins"(Barry and McNabb, 1999). High concentrations of the free CT enhance the reaction of these compounds with bacterial enzymes and bacterial cell walls, which can interfere with the transport of nutrients into the cell and affect fermentation of other nutrients like carbohydrates. The magnitude of these effects may vary with each type of CT (McSweeney et al., 2001b; Min et al., 2003). Moreover, high concentrations of free CT may reduce fiber digestion either by complexing with lignocellulose and preventing microbial digestion or by directly inhibiting the cellulolytic microorganisms, although this effect does not always result in impairment

of ruminal microbial protein synthesis because the total population of fungi, protozoa and proteolytic bacteria might not be affected (McSweeney et al., 2001b). This explains why very high concentrations of CT from *L. pedunculatus* are associated with reduced rumen degradation of structural carbohydrates, especially hemicellulose (Barry and Manley, 1984).

1.3.4.3.2.4.3 Animal Production

The major benefit from CT is through protection of proteins from degradation in the rumen and making it available in the abomasum. The basic effect of CT affects all other production responses directly or indirectly and influences livestock productivity through increases in protein reaching the small intestine. Forages containing CT such as *Lotus corniculatus*, when fed to grazing ruminants, have been shown to have the capacity to protect feed protein from ruminal digestion and thereby increased production performance in ruminants (Table 1.11). Most of the experiments involved PEG supplementation that binds to CT, have shown that the major reason for the effect of lotus on productive performance of ewes is due to its CT content, which accounts for 50% of the reproductive improvements (Table 1.11; Min et al., 1999; Min et al., 2001).

Table1.11 Livestock production responses to feeding CT-containing forages such as *Lotus corniculatus* and *Salix* and *Populus sp.*, relative to a control non CT-containing forages.

	Dietary source	Production response	Mechanism of action
Wool production	*L. corniculatus* and *L. pedunculatus* Vs. Temperate grass and white clover	10% increase in wool production and quality of wool is greatly improved	Reduced the degradation of sulphur-containing amino acids in rumen (McNabb et al., 1993; Wang et al., 1994).
Milk Production	Cattle *L. corniculatus* Vs. Rye grass	30% increase in milk production and 10% increase in milk protein during late lactation	(Woodward et al., 1999)

	Ewes *L. corniculatus*	21% increase in milk production and 14% increase in milk protein during mid and late lactation	(Wang et al., 1996)
Meat Production	*L. corniculatus* Vs. ryegrass/white clover	Experiment I: 37% increase in live weight gain and 20% increase in weaning weight Experiment II: 52% increase in live weight gain and 32% increase in weaning weight	Higher OMD and ME and increased protein absorption from the action of CT. (Ramirez-Restrepo et al., 2004)
Reproduction	*L. corniculatus* Vs. ryegrass/white clover	25% increase in lambing percentage, with 50% of it is explained by CT in the diet.	Due to increases in the plasma concentration of BCAA and EAA by 57% and 52% respectively (Min et al., 1999; Min et al., 2001).
Post natal lamb mortality	Willow and Poplar supplementation of ewes grazing short drought pasture	Reduced from 18% to 12%	(McWilliam et al., 2005b)
Anthelminthic	*L. pedunculatus*, *L. corniculatus*, *H. coronarium* fed as supplements	reduced the rate of larval development (eggs to L3 larvae) by 91%, reduced the number of eggs hatching by 34%, and decreased the motility of L3 larvae by 30%.	CT nematode interactions, or directly by interfering with parasite egg hatching and development to infective stage larvae. Molan et al. (2000)

1.4 THE RUMEN MICROBIAL ECOSYSTEM

The main ruminants of agricultural importance in New Zealand are cattle, sheep, deer and goats. Ruminants are defined by their possession of a sac known as the rumen (Van Soest, 1994), a capacious pre-gastric fermentation chamber, that sustains a rich community of microorganisms which rapidly colonize and digest feed particles (Hobson, 1997). Plants contain complex carbohydrate polymers (cellulose and hemi-celluloses) that are indigestible to most animals but are hydrolyzed and fermented by a range of microorganisms to volatile fatty acids (VFAs) as the end products of fermentation in the rumen. These VFAs include acetic, propionic and butyric acids and form the major metabolic fuel for the ruminant. Microbial cells that flow out of the rumen are the main source of protein and amino acids when absorbed in the lower digestive tract of the animal (Hungate, 1984).

Rumen microbes that are involved in the breakdown of plant material include a range of species of bacterial, archaeal, protozoal, fungal and phage which are diverse and vary with time, animal and environmental factors (Edwards et al., 2004). The microbial species so far reported from the rumen have been cultured on various media (Krause and Russell, 1996) using rumen inocula and represent only about 11% of the total bacterial populations present in the rumen as estimated from analysis of small subunit ribosomal RNA gene libraries (Edwards et al., 2004). Therefore rumen microbes currently in culture do not represent the entire microbial diversity present in the rumen. The failure of conventional culture techniques to retrieve all or even the majority of micro-organisms is commonly encountered when attempting to cultivate microbes from an environment and is known as the "Great Plate Count Anomaly" (Staley and Konopka, 1985).

A complete review of the microbes associated with the degradation of plant material in the rumen is beyond the scope of this literature review. However, the focus of the thesis is the supplemental feeding of willow to sheep under drought conditions. Therefore those microbes that are most likely to be associated with degradation of prominent components of willow (i.e., cellulose, hemicellulose) and the metabolism of its secondary compounds (PGs, FMs and CTs) are summarized here.

1.4.1 Fibre degrading bacteria

The main function of the rumen is to digest structural carbohydrates within plant material, a process which is carried out by a complex community of fibrolytic rumen microorganisms. The predominant cellulose-digesting bacteria are the Gram-negative bacterium *Fibrobacter succinogenes*, and two species of Gram-positive bacteria, *Ruminococcus albus* and *R. flavefaciens*. Species of the *Butyrivibrio* and *Pseudobutyrivibrio* genera form a group of highly xylanolytic Gram positive bacteria inhabiting the rumen, which have a central role in hemicellulose digestion (Stewart et al., 1997). Other cellulolytic and hemicellulolytic bacteria include *Prevotella* species, *Eubacterium cellulosolvens* and the anerobic rumen fungi such as *Neocallimastix* species (Orpin and Joblin, 1997) and the rumen protozoa (Williams and Coleman, 1997). These organisms produce a range of hydrolytic enzymes that attack the various chemical linkages found within plant structural carbohydrates. Cellulases and xylanases are the most common fibrolytic enzymes secreted by fibrolytic bacteria (Henrissat and Bairoch, 1993) and are classified based on similarities in primary amino acid sequence and secondary structure (Gilkes et al., 1991, Henrissat and Bairoch, 1993).

1.4.2 Degradation of phenolic compounds in the rumen

Phenolic compounds like PGs, FMs and CTs in plants are mostly esterified to cell wall polysaccharides but also occur as soluble phenolics esterified to sugars which are readily liberated during digestion in the rumen (Lowry et al., 1993). These phenolic compounds undergo transformations in the rumen which are mainly hydrolytic and reductive. The effect of phenolic compounds on rumen microbes depends on their structure and molecular weight (Lowry et al., 1996). PGs or esters are cleaved releasing aglycones and methoxy groups are lost by methyl ether cleavage and by demethoxylation (McSweeney et al., 1994). However, higher aryl ethers are resistant to breakdown by microbes. Reductive dehydroxylation results in loss of phenol properties of the compound which then ceases to be a phenolic (Lowry et al., 1996). Most of the information reported on the effects of plant phenolics in the rumen relate to CTs and these are summarized below.

1.4.2.1 Effects of CT on microbes

1.4.2.1.1 Anti-microbial effects

CTs are generally considered inhibitory to microorganisms at varying concentrations (Lowry et al., 1996) and are thought to cause inhibition in several ways. CTs form complexes with the surface of bacterial cells and with bacterial enzymes, which can alter bacterial growth and reduce proteolytic enzyme activities (Jones et al., 1994) and also reduce the number of cellulolytic bacteria of *F. succinogenes*, *Ruminococcus species* (McSweeney et al., 2001a). The relative proteolytic activities of rumen microorganisms and their responses to CTs are also influenced by diet (Waghorn et al., 1987, 1994). Molan et al. (2001) compared the effect of CTs from *Lotus pedunculatus* and *Lotus corniculatus* on the growth of proteolytic rumen bacteria and observed that CT's from *L. pedunculatus* were more

inhibitory to proteolytic bacteria at increasing concentrations (200-600 μg/ml) than CTs from *L. corniculatus*. Increased inhibitory effects were explained by the higher molecular weight (MW; 2200) and the predominance of prodelphinidin type subunits with epigallocatechin in *L. pedunculatus* compared to the predominance of procyanidin-type subunits with epicatechin in *L. corniculatus*. Min et al. (2002) concluded that the inhibitory effects of CTs in *L. corniculatus* were due to reduced Rubisco (fraction 1 of leaf protein) degradation and rumen bacterial growth which depended on the level of CTs, the bacterial species and on the protein substrate. The astringent (enzyme inhibition and substrate deprivation) property of CT can induce the formation of CT-enzyme complexes. When purified enzymes (cellulases, pectinases, xylanases, peroxidase, laccase and glycosyltransferase) were mixed with CTs, microbial enzymes were inhibited (Scalbert, 1991). CTs may also form complexes with metal ions, thus restricting their availability to rumen bacteria and causing inhibition. This is significant for the activity of metalloenzymes such as peroxidase and laccase which are vital for the growth of microorganisms (Scalbert, 1991).

1.4.2.1.2 Resistance to CTs

Some rumen bacterial species are reported to be resistant to the action of CTs. *Streptococcus caprinus/gallolyticus* produced an extracellular polysaccharide (EPS) as a means of protection against tannins in the growth medium while *Selenomonas ruminantium* K2 secreted tannin-inducible tannin acylhydrolase when grown on CTs as the sole source of carbon (Brooker et al., 1999). The mechanism of protection in tannin-resistant species of *Lactobacillus, Butyrivibrio* and *Enterobacteriacae* is not known (Brooker et al., 1999). Bacteria within the *Cytophaga-Flexibacter-Bacteroides* (CFB) group formed approximately 90% of rumen microbial population in goats fed

mulga (*Acacia aneura)* which contained 50-240 g/kg DM of tannins (Plumb et al., 1999). This study concluded that the CFB bacterial group had more tannin-tolerant or tannin-degrading properties. In other studies, bacterial strains appeared to vary in their sensitivity to CTs. *P. ruminicola* B_14 and *Ruminobacter amylophilus* WP225 were resistant to CTs from *O. viciifolia* (Jones et al., 1994) while strains of *Bacteroides*, *Porphyromonas* and *Prevotella* species were less affected by CTs (McSweeney et al., 2001a). The growth rates of *Prevotella* species C21a, *Butyrivibrio* species (C211a) and *C. proteoclasticum* $B316^T$ were not significantly decreased even at high CT concentrations (>200 μg/ml; Min et al., 2005).

1.4.2.2 Effects of other phenolic compounds on rumen microbes

1.4.2.2.1 Inhibitory effects

Simple phenolic compounds such as free phenol have been reported to suppress fibrolytic activity in the rumen while simple compounds like catechol and gallic acid were more toxic to *Cellvibrio fulvus* and *Bacillus subtilis* when compared with higher molecular weight condensed tannins (Sivaswamy and Mahadevan, 1986). The number of hydroxyls attached to the B ring of flavanols affects their activity. A trihydroxy B ring (gallocatechins) being more inhibitory to *Streptococcus*, *Clostridium*, *Proteus*, and *Staphylococcus* species than catechin with a dihydroxy B ring (Hara and Watanabe, 1989, Sakanaka et al., 1989).

1.4.2.2.2 Resistance to phenolic compounds

Plant phenolic compounds are either degraded by rumen microbes to simple substances for their utilisation or are converted by the microbes to less toxic substances that could be excreted (Lowry et al., 1996). Some plant phenols like flavonols and flavones can be degraded by gut microbes via the opening of the phenolic ring structure. Krumholz and Bryant (1986) reported the formation of acetate

from quercitin (flavonol) and acetate-releasing fermentation appears to be a detoxification mechanism for some phenolic compounds which produces a phenylacetic acid residue (Lowry et al., 1996). Tagasaste (*Chamaecytisus palmensis*, also known as tree Lucerne), is a fodder shrub grazed by livestock as a supplement to pasture in southern Australia (Lefroy et al., 1997). Tagasaste contains phenolic compounds as flavones that occur in glycoside form as *c*-glycosides vitexin (apigenin 8-*c*-glucoside) and iso-vitexin (luteolin 8-*c*-glucoside) (Edwards, 1999). These phenolic glycosidic forms had a positive influence on rumen fermentation and increased the total microbial population while the flavone aglycones (apigenin and luteolin) without attached glucose failed to show the increase in microbial populations This effect on rumen fermentation was attributed to the utilisation of the glucoside component of the PGs by rumen micro-organisms (Edwards, 1999).

1.4.3 Molecular techniques used for microbial community analysis

The above mentioned bacterial species involved with fibre degradation or metabolism of plant secondary compounds have all been isolated from the rumen using cultivation techniques. As mentioned previously, these organisms probably represent a fraction of the actual microbial diversity present and therefore only reveal a small part of plant-microbial interactions in the rumen. In recent times, a range of molecular techniques predominantly based on the analysis of 16*S* rRNA genes have been developed to characterise microbial communities in natural environments. These include Denaturing Gradient Gel Electrophoresis (DGGE) ((Muzyer et al., 1993, Donskey et al., 2003), Terminal Restriction Fragment Length Polymorphism (Kuske et al.,2002), Length Heterogeneity PCR (Ritchie et al., 2000, Suzuki et al., 1998), automated rRNA Intergenic Spacer Analysis (Fisher and Tripplet, 1999), Ribosomal Intergenic Spacer Length Polymorphism (Erikkson et al., 2003, Yu and Mohn, 2001),

Amplified rDNA Restriction Analysis (Vaneechoutte et al., 1993), and Metagenome Sequence Analysis (Handelsman, 2004) are now widely used. These techniques have also been applied to the rumen ecosystem, (Edwards et al., 2004; Deng et al., 2007) to evaluate the microbial diversity and identify uncultivated rumen microbes (Deng et al., 2007).

The earliest example of the use of a molecular technique for bacterial detection in the rumen is that of Stahl et al. (1988) who used a membrane-based hybridisation technique that involved species-specific and group-specific 16*S* ribosomal RNA targeted oligonucleotide hybridisation probes to enumerate various strains of *Bacteroides succinogenes* and *Lachnospira multiparus*-like organisms in the bovine rumen after the addition of an antibiotic monensin in the diet. The study was able to compare the predominance of type strains in the rumen before, during and after monensin addition to the diet and used this technique to successfully monitor the complex microbial communities in the rumen. The same hybridisation principle was used with a genomic DNA-targeted probe to follow the fate of *Bacteroides ruminicola* strain B_14 inoculated into the rumen (Attwood et al., 1988). Subsequently oligonucleotide probes were used to enumerate *Butyrivibrio* (Forster et al., 1997) groups from the rumen of cattle, sheep and deer (Forster et al., 1997) while *Ruminococci* were detected (Krause et al., 1999) from adult sheep and immature lamb rumen (Krause et al., 2000). Oligonucleotide probes that targeted *Ruminococcus, Fibrobacter*, the *Bacteroides-Porphyromoas-Prevotella* group, and anerobic fungi were used to identify rumen microbial populations in sheep fed with CT containing *Calliandra calothyrsus* (McSweeney et al., 2001a). Fluorescently-labelled oligonucleotide probes targeting ribosomal RNA that can permeabilise intact cells have also been used for fluorescent *in situ* hybridisation (FISH) for bacterial

identification and enumeration. Species-specific *Fibrobacter* probes were used to identify 14 strains in rumen samples (Amann et al., 1990). McSweeney et al. (1993) used a radiolabelled or fluorescent dye-conjugated oligonucleotide probe to detect and enumerate *Synergistes jonesii* 78-1 in pure cultures and mixed culture chemostats that simulated the rumen ecosystem. These hybridisation techniques are useful to detect and enumerate bacteria from rumen samples but they are laborious and the bacterial detection limit is 10^4 to 10^6 bacteria per ml of rumen fluid (Deng et al., 2007).

Fluorescent probes have also been used to detect specific sequences in DNA extracted from rumen samples. Schofield et al. (1997) used fluorescently-labelled probes known as "Molecular Beacons" to detect *R. albus* and *F. succinogenes*. Molecular Beacons have a short complementary sequence of nucleotides attached to the 5' and 3' ends of the probe sequence which allows a stem-loop structure to form in solution. A fluor and a suitable quencher molecule are attached to each end of the probe and are held in close proximity to each other. In the absence of target sequence, the fluorescence is quenched but when a DNA target is present, the complementary part of the single-stranded loop hybridizes to it and the stem structure is disrupted. Fluorescence appears when the quencher molecule is physically separated from the fluor and indicates binding of the probe to its target DNA.

The advent of PCR technology allowed exponential amplification of specific stretches of DNA and resulted in rapid techniques for microbial detection. Because PCR amplifies DNA in an exponential manner it is difficult to use quantitatively. A modified version of PCR called competitive PCR (cPCR) allows quantification of PCR products (Leser 1995, Leser et al., 1995) relative to an internal control. This technique was used to detect *C. proteoclasticum* (Reilly and Attwood, 1998) and proteolytic bacteria (Reilly et al., 2002) in cattle rumen samples on low and high

nitrogen with and without supplementation of carbohydrates in the diet. Reilly and Attwood (1998) concluded that cPCR could be used to reliably quantify 2.5 x 10^3 cells of a specific bacterial population within rumen samples. The same cPCR technique was also used to quantitate the rumen bacterium *Butyrivibrio fibrisolvens* OB156 (Kobayashi, et al., 2000) and *F. succinogenes*, *R. albus* and *R. flavefaciens* (Koike and Kobayashi, 2001). Although cPCR is sensitive, the whole process is tedious and involves discrete but complex steps requiring separate image analysis and cannot handle the processing of large number of samples. Both hybridisation and cPCR techniques involved the use of probes that were based on sequences determined from organisms previously cultured from the rumen. As mentioned earlier, cultured rumen bacteria are not a true representation of the bacterial diversity present in the rumen. This limitation has been addressed through the construction and comparative sequence analysis of 16*S* rRNA gene libraries (Whitford et al., 1998; Tajima et al., 1999, 2000). Typically, rumen samples are collected, DNA is extracted and 16*S* rRNA genes are PCR-amplified, cloned and sequenced. Comparison of 16*S* rRNA gene sequences retrieved in this manner indicate that mostly novel rumen bacteria were found and that the majority of the sequences were similar to those of the low G+C, Gram positive bacterial group and the *Prevotella-Bacteroides* group (Whitford et al., 1998). A similar comparative phylogenetic analysis was conducted on methanogens in the rumen (Whitford et al., 2001). The sequences fell into 3 clusters, and only part of one cluster was closely related to the known rumen methanogen, *Methanobrevibacter ruminantium*. All of the other sequences represented either new species of *Methanobrevibacter* or *Methanosphaera* (previously not detected in the rumen) or distantly related to species of *Methanosarcina*. These phylogenetic studies

concluded that new probes and quantitative PCR methods are required to identify the true diversity of methanogens present in the rumen.

Further improvements in PCR technology resulted in the development of a technique known as Real-time PCR (RT-PCR). RT-PCR is a quanititative DNA amplification technique that is rapid and relatively simple (Holland et al. 1991; Higuchi et al. 1993). One form of the technique uses a fluorescent dye (SYBR Green I) that intercalates with, and fluoresces only with double-stranded DNA. When incorporated into a PCR, its fluorescence when binding to double stranded DNA prior to the denaturation step can be used to quantify the amount of DNA present. This technique was used by Tajima et al. (2001) to monitor shifts in 12 bacterial species in the rumen during diet transition. The same technique was used to determine fungal population in cattle rumen (Denman and McSweeney, 2006) while the proportion of cultured rumen species in the dominant group of *Prevotella* has also been quantified (Stevenson and Weimer, 2007). A different type of RT-PCR, known as the TaqMan assay, was adopted using fluorescent reporter dyes in the enumeration of *Megasphaera elsdenii* (Ouwerkerk et al., 2002; Klieve et al., 2003). This method involves the use of PCR primers and an internal probe that is 5'-labelled with a fluorescent reporter dye and 3'-labelled with a quencher. The 5'-3' exonuclease activity of the Taq polymerase is used to cleave the reporter dye during each amplification phase of the PCR. Collection of the fluorescent signal throughout the PCR allows an accurate quantification of the specific amplification product. This method was also adopted by Alexander et al., (2004, 2006) in determining the fate of transgenic DNA in sheep rumen. Modified PCR-based techniques like cPCR and RT-PCR are highly sensitive for bacterial enumeration but also involve the use of expensive equipment and reagents. Their use in enumeration of rumen microbes is

also restricted by the limited number of specific primers and internal control DNAs available for rumen bacterial species (Deng et al., 2007). The use of DNA microarrays in identification and quantification of target sequences that bind to a probe on a microarray has been used to analyse community composition in microbial ecosystems (Cho and Tiedje, 2002). This technique was used to study the effect of *Acacia angustissima* on rumen bacterial populations in Merino wethers (Krause et al., 2004).

To enumerate different microbial species present at any given time and measure the dynamics involved in population shifts within the rumen over time, fingerprinting-type techniques are required (Muyzer, 1999). Restriction Fragment Length Polymorphism (RFLP) is one such example of a general molecular fingerprinting technique, which can be adapted for use on amplified of 16*S* rRNA gene sequences. Wood et al. (1998) used RFLP of amplified16*S* rRNA genes to determine the genetic composition of *Bacteroides* and *Prevotella* populations in sheep and cow rumen samples and estimated they represented 12 and 62 % respectively. They also observed that no cultured isolate was available for the predominant ribotypes of *Bacteroides* and *Prevotella* obtained using RFLP. Similarly, McSweeney et al. (1999) identified 15 genotypes that were proteolytic and ammonia-producing bacteria from the rumen of sheep and goats fed on CT containing *Calliandra calothyrsus.*

The DGGE technique has been one of the most widely used molecular community fingerprinting techniques as it gives a good overview of the changes in microbial community composition and helps in identification of individual members through recovery and sequencing of the amplified products (Curtis, 1998; Diez et al., 2001). Several reports have been published on the use of DGGE in the rumen ecosystem. Rumen fibrolytic bacteria have been analysed (Cann et al., 1996), as has

the bacterial diversity associated with corn or hay-fed animals (Kocherginskaya et al., 2001). The technique was also used to detect *Oscillospira* species in different ruminants (Mackie et al., 2003), to screen ciliate populations in the sheep rumen (Regensbogenova et al., 2004), to assess the effect of day length on sheep rumen microbes (McEwan et al., 2005) or the effects of disodium fumarate on rumen microbes (Mao et al., 2007). In DGGE, a single hypervariable (V) region of the 16*S* ribosomal RNA gene is typically used. An analysis of the relative usefulness of the V1, V3, V1 to V3, and V6 to V8 regions of the 16*S* ribosomal RNA gene indicated that V3/V1 for short amplicons and V3 to V5/V6 to V8 for longer amplicons provided more reliable information (Yu and Morrison, 2004). DGGE data can be interpreted in a quantitative manner using the Shannon-Wiener diversity index, and via analysis of richness (number of bands in a lane), and evenness (calculated from Shannon-Wiener index) (Yu and Morrison, 2004). There are some limitations associated with DGGE such as a single isolate can produce multiple bands by DGGE (Satokari et al., 2001), and conversely, a single band within a DGGE gel may represent multiple populations (Yang and Crowley, 2000). Furthermore, the typical length of amplified sequence (200-500 bp) represents only a fraction of the whole 16*S* rRNA gene, thus constraining identification of bacteria to higher taxonomic levels (Nübel et al., 1996).

Recognising the weaknesses of the DGGE technique, it remains a powerful technique for following overall changes in microbial populations in complex communities. Therefore we believe the DGGE technique is the most appropriate technique to allow observation of the changes in bacterial populations that are likely to occur in animals that are adapted to eating a willow-supplemented diet.

1. 5 FEEDING VALUE OF FORAGES

1.5.1 Definition

Feeding value is defined as the animal production obtained from grazing a particular forage under unrestricted conditions (Ulyatt, 1973) with its components being the voluntary feed intake (VFI) and nutritive value (NV). Nutritive value is the level of production obtained per unit of food consumed and is a function of the digestive processes and the efficiency of utilisation of digested nutrients. Feeding value is generally expressed as the relative growth rate in young animals and as relative milk yield in lactating animals.

Feeding values of a range of forage grasses and legumes were studied at Palmerston North, NZ (Table 1.12). Feeding values of legumes are generally higher than grasses in NZ pastures with white clover, being higher than other legumes (Ulyatt 1981). The feeding value of grasses generally declines from annuals to perennials.

Table 1.12 The comparative feeding value in terms of sheep live-weight gain of some pasture species grown in NZ. All forage feeding values expressed relative to white clover as 100%. Adapted from Ulyatt (1981)

	Number of studies	Comparative feeding value
Legumes		
White clover (Grasslands Huia)	14	100
Lotus pedunculatus (Grasslands Maku)	6	84
Sainfoin (Melrose)	2	84
Lucerne (Wairau)	10	82
Red Clover (Grasslands Hamua)	5	71
Red Clover (Red West)	2	69
Red Clover (Grasslands Pawera)	4	65
Grasses		
Italian ryegrass(Grasslands Paroa)	1	83
Short-rotation ryegrass (Grasslands Mana)	11	77
Timothy, common	5	67
Perennial ryegrass (Grasslands Ariki)	2	58
Perennial ryegrass (Grasslands Ruanui)	16	52
Browntop (Common spring)	1	52
Browntop (summer)	1	43

Grazing systems in NZ are based on mixed swards with a major proportion of grasses dominated by perennial ryegrass (*Lolium perenne)* and a legume, which is typically white clover (*Trifolium repens),* forming a minor proportion (approximately 20%) of the pasture DM (Ramirez and Barry, 2005). White clover has a higher feeding value when compared to perennial ryegrass; increases in milk production were recorded when dairy cattle were fed with white clover compared to perennial ryegrass (Table 1.13)

Table 1.13 Effect of feeding white clover or perennial ryegrass diets on intake and milk yield of dairy cows

	White clover	**Perennial ryegrass**
DM Intake (kg/d)	15.9	12.0
DM Digestibility	0.72	0.66
Digestible DMI (kg/d)	11.4	7.9
Milkfat yield (kg/d)	0.67	0.51

Adapted from Rogers et al.(1982)

Pasture composition varies seasonally as influenced by temperature and rainfall, producing a cyclical growth rate, herbage mass and herbage quality (Stevenson et al., 2003). This often results in a leafy sward in spring, followed by stem and seedhead formation in late spring and a reduction in quality during summer when growth rate is often restricted by low soil moisture availability. Sward growth and quality improves in autumn, but pasture growth rates decrease markedly in winter (Moller et al., 1996). Levels of milk yield and DM intake were positively correlated with the quality of pasture when dairy cows were fed on a good medium quality pasture (Table 1.14).

Table 1.14 Effect of feeding lactating cows maintaining weight (LW; 550 kg) on good and medium quality pasture on milk production and DM intake. Adapted from Waghorn and Clark (2004)

	Good pasture	Medium pasture
Milk yield (kg/d)	23	12
DM Intake (kg/cow/d)	17.5	14
DM digestibility	0.76	0.69
CP (g/kg DM)	240-280	150-180
ME (MJ/kg DM)	12.5	11.1

1.5.2 Feeding value of drought pasture in NZ

Pasture production is low with a high dead matter content and low nutritive value in dry summers, typical of drought situations (McCabe and Barry, 1988). Mc William et al. (2005) reported a year-to-year variation in composition of drought pastures during mating of ewes in late summer/early autumn (Table 1.15). Pastures differed between years in pasture masses as well as in the chemical composition. The authors reported that pasture masses were low with high dead matter content and low nutritive value in all three years. The presence of zearalenone, produced by *Fusarium* fungi in the low quality drought pastures was recorded, with highest values in 2003 and correspondingly reduced the nutritive value compared to 2001 and 2002. Performance of ewes was reduced substantially both in live weight gain and also in lambing percentage, when the ewes were mated on these low quality pastures (Table 1.15). The normal scanning values for ewes mated on normal non- drought pastures on Riverside farm is 165% (McWilliam, 2004).

Table 1.15 Variation in the control drought pasture between years and its effect on calculated dry matter intake (DMI) and on animal performance. Data refers to pastures on Massey University's Riverside Farm in the Wairarapa, NZ, between mid Feb and late April (Late summer/autumn).

	2001	2002	2003
Herbage mass (kg DM/ha)			
Pre grazing	1040	941	1261
Post grazing	531	456	821
Pre-grazing dead matter content (%)	84	62	78
Composition of diet selected			
CP[1] (g/kg DM)	111	156	99
OMD[2]	0.52	0.54	0.49
ME[3] (MJ/kg DM)	7.6	7.7	7.2
Zearalenone (mg/kg DM)	0.58	0.16	1.5
DMI (kg/d)	0.67	0.59	0.47
Performance of ewes			
LWC[4] during mating(g/d)	-82	-103	-147
Reproductive rate as lambs born/100 ewes mated	121	131	124

[1] CP, Crude protein, [2] OMD, organic matter digestibility
[3] ME, metabolisable energy, [4] LWC, live weight change
Data adapted from McWilliam et al. (2005b)

1.5.3 Feeding value of tree forages

Willow and poplar stem cuttings have recently been used to supplement livestock on NZ farms in times of drought. McWilliam (2004) studied the effect of feeding willow and poplar cuttings to ewes mated on drought pasture in late summer/autumn in 3 different years. The authors reported a consistent superior performance of ewes in production as well as in reproduction through reduction in live weight loss and increases in lambing %, when ewes were supplemented willow and poplar stem cuttings in addition to drought pasture (Table 1.16). Supplementing willow and poplar also reduced the post-natal lamb mortality in all three consecutive years when compared to the control treatment.

Table 1.16 The effect of supplementing ewes (100/group) grazing low quality drought pasture with willow/poplar (1.4 kg fresh/ewe/d for approximately 70 d) during mating upon reproductive performance (lambs/100 ewes mated) and lamb mortality between birth and weaning adjusted to equal birth rank. Data collected on Massey University's Riverside Farm in the Wairarapa, NZ.

	Control	Supplemented willow or poplar
Experiment I –2001 poplar supplementation		
Live weight change (g/d)	-82	-67
Reproductive rate/100 ewes mated		
Scanning	122	163
Lambing	121	155
Weaning	96	125
Post-natal mortality (%)	20.3	16.3
Range	(13.8, 28.7)	(11.2, 23.2)
Experiment II – 2002 willow supplementation		
Live weight change (g/d)	-103	-86
Reproductive rate/100 ewes mated		
Scanning	132	148
Lambing	131	148
Weaning	106	126
Post-natal mortality (%)	17.3	12.1
Range	(11.5, 25.2)	(7.7, 18.5)
Experiment III – willow supplementation		
Live weight change (g/d)	-147	-96
Reproductive rate/100 ewes mated		
Scanning	128	128
Lambing	124	127
Weaning	103	116
Post-natal mortality (%)	16.0	8.0
Range	(10.4, 23.8)	(4.3, 14.4)
Overall post-natal lamb mortality	17.8	11.7
Range	(14.1, 22.3)	(8.7, 15.5)

Data adapted from (McWilliam et al., 2005b)

Although, cutting and supplementing willow and poplar stem cuttings proved to be beneficial to livestock in drought situations; the process of cutting and supplementing was time consuming and labor intensive. Willow poles (stakes) were being planted as fodder blocks on unproductive land and these areas used as fodder blocks. There is no information available on the nutritive value that is obtained from the fodder blocks or the effects on animal performance (i.e. relative feeding value).

Thus, measuring the animal production from the willow fodder blocks relative to that obtained from drought pasture can be an index of their relative FV particularly during dry summers.

1.5.4 Problems associated calculated intakes in grazing Experiments

Nutrient intake is a major indicator of nutritional status in grazing animals. Intakes are commonly measured from animal-based techniques using indigestible markers such as alkanes (internal markers), and chromium sesquioxide (Cr_2O_3, external marker) to estimate faecal output, which is then divided by 1- diet digestibility (Mayes and Dove, 2000). Animal based techniques are based on quantification of fragments of plant material in extrusa from oesophageal-fistulated animals, stomach contents, digesta and faeces. These methods offer advantages over plant-based techniques in that they give a measure of intake by individual animals within a group. The most widely used animal-based method, in a pasture based system is the separate estimation of faecal output from a dosed marker and diet digestibility from *in vitro* incubation of oesophageal extrusa or plucked samples (Mayes and Dove, 2000). This method is associated with limitations, ranging from the inadequacy of the *in vitro* technique to problems associated with the estimation of faecal output (Dove and Mayes, 1996); a small error in digestibility can result in a large error in 1-digestibility (i.e., indigestibility). The measurement of intake by the plant-wax alkane method is confined to two dietary components (Doves and Mayes, 1991, 1996) ie., grasses or grasses/legume associations. Another potential source of error is that markers are assumed to be indigestible; this is not always the case with alkanes, where a small amount may be digested and this varies with the chain length of the individual alkane (Dove et al., 2000). Dillon and Stakelum (1989) reported a diurnal variation in faecal concentration of alkanes which was greater in once –daily

dosing to twice daily dosing in dairy cows. However, these animal-based intake estimates when applied to grazing Experiments might show a degree of inaccuracy owing to the limitations of the available measuring techniques (Mayes and Dove, 2000) and the range of assumptions that have to be made. McWilliam et al. (2005a and 2005b) measured DM intake by pasture sampling technique (indirect method), as the difference between the pre-grazing and post-grazing pasture masses per week and expressed as DM/ewe/d. This calculated DM intake is an apparent value. The intakes of CP, CT and PG were also calculated by multiplying their concentrations in the diet by the DMI and hence also represent apparent values. Ulyatt (1973) calculated that differences in voluntary intake accounted for approximately 50% of the variation between individual forages in feeding value.This method seems to be effective when the stocking density is high, effective in short grazing intervals (weekly) and when pasture growth in between the two measurements is assumed to be minimal as in drought conditions. However, the method gives an apparent value of intake for a group of animals and not for individual animals, could be associated with errors including sample losses and assumes there is no pasture growth between measurements (McWilliam, 2004). In large field experiments that involve supplements such as willow stem cuttings, calculation of intakes by pasture sampling technique seem to be more appropriate measure when compared to marker-based methods. As the latter involves a wide range of assumptions that need to be made, unknown alkane content of plant species and the differences in faecal recovery rates of odd and even-chain alkanes between pasture and forage feeding becomes cumbersome (Swainson et al., 2005).

1.6. CONCLUSIONS

- NZ's economy is mainly based on farming (sheep, dairy, beef and deer) run on low-input pasture grazing systems. Pasture grown in NZ comprise 80% perennial ryegrass and 20% white clover. Research is focussed on increasing sheep production to combat the increasing demand for sheep meats.

- Sheep production follows an annual cycle with mating in March/April, lambing in Aug/Sep and weaning in December. Good nutrition is vital during mating and in lactation to achieve a better performance and to maintain the live weight of sheep throughout the annual cycle.

- Pasture production in NZ is seasonal. In East Coast regions including Wairarapa, the weather pattern falls into the category of dry summer and cold winter with minimal pasture production during summer and winter. Autumn droughts are predicted to be common in these regions.

- Climatic variability results in extreme weather events such as droughts and floods which affect primary production. The climate in NZ is governed by three climatic systems, which operate in different time scales viz., ENSO (year to year), IPO (over decades) and global warming over centuries. All these patterns predict climatic variability in the near future and culminate in droughts or floods.

- Climatic variability thus affects farming at both the national level as well as at the individual farm level. Management plans to counteract drought remain a task for the farmers; either to reduce the feed demand by selling the stock at an early stage or provide supplementary feed during the deficit periods.

- Willows (*Salix spp*) have been bred in NZ for over 20 year ago, mainly for soil erosion control and to provide shelter for livestock. New clones have been evolved and the most widespread clone is *S. matsudana* × *alba* used for soil conservation.

- Willows are also being used by farmers in NZ as supplementary feed during feed scarcity and drought conditions. They are being cut and used as supplement for livestock. Planting willows as fodder blocks is starting on NZ farms, to use as fodder blocks, in addition to soil conservation.

- Willows have a considerable nutritive value and form a potential supplementary feed in dry summers. The edible forage production ranged from 0.3 to 25 kg DM/tree per year. The total nitrogen content is approximately 18g/kg DM and metabolisable energy is approximately 10 MJ/kg DM. This nutritive value can sustain livestock at maintenance.

- A feature of willows is the presence of secondary compounds such as condensed tannins and phenolic glycosides, including Salicin. Higher concentrations of these compounds might have deleterious effects but a value of 27-52 g/kg DM of CT and 17-33g/kg DM of Phenolic glycosides in *Salix spp* was found to have beneficial effects on livestock.

- Rumen microbes strip the glycosyl part from the phenolic glycoside (as a source of energy) and this produces an enhanced effect on rumen fermentation. CT in *Lotus sp*, reduces the protein degradation in the rumen and increases the amino acid availability in the small intestine; similar effects may be produced by the CT in willows. The presence of secondary compounds in willows are thought to contribute significantly to the performance of animals, although at higher concentrations can cause detrimental effects on the animal performance.

- Drought pasture is low in nutritive value (7.5 MJ ME/kg DM) and reduces reproductive efficiency, which is the salient feature in profitable sheep farming. Providing feed supplements is necessary to maintain reproductive efficiency of ewes when the ewes are mated on drought pasture.

- Cutting and supplementing willows as supplementary feed to livestock is an option during dry summers; but is time consuming and labor intensive.

Planting willows as fodder blocks in unproductive areas (i.e., wet, low lying and swampy areas or eroding hillsides) of the farm seems to be a better option and provides feed for grazing sheep not only from the trees but also pasture growing as an under cover to the trees.

- Willow fodder blocks provide good supplementary feed to livestock but the efficiency of utilisation of both herbage and trees in the fodder block is dependent on the grazing management used. Therefore, research is necessary to study the nutritive value from willow fodder blocks along with a suitable managemental plan to efficiently utilise fodder blocks and maximise ewe production and reproduction under drought conditions.

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Plate 1. Preparation of short drought pasture

a. Before grazing

b. Grazing with cattle

c. Simulated short drought pasture

Plate 2. Establishment of willow fodder blocks

a. Site selection

b. Site preparation

c. Planting stakes

d. Established willow fodder blocks

Plate 3. Experiment 1 in 2003

a. Established willow fodder blocks (3 year old) being grazed by ewes

b. Willow fodder block showing start and end of a break

Plate 4. Experiment 2 in 2004

a. Willow fodder blocks – Primary growth (Feb/march)

b. Willow fodder blocks – regrowth (April/may)

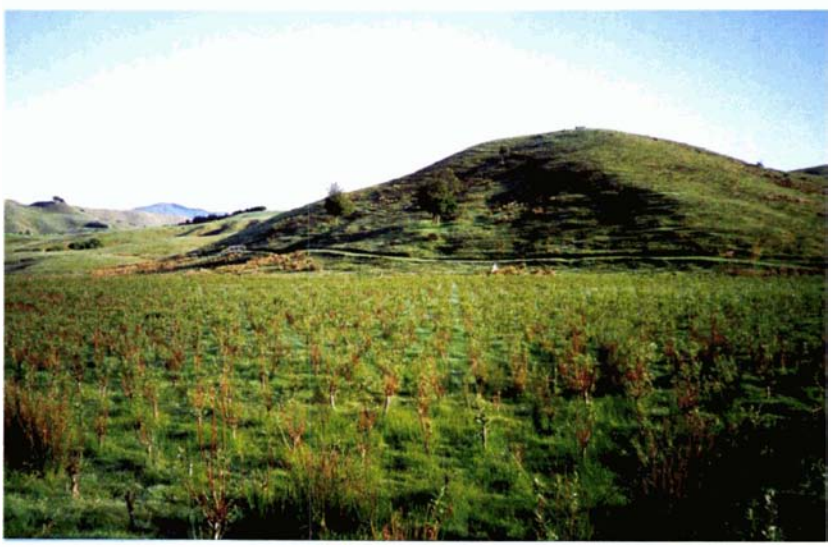

Plate 5. Ewes grazing willow fodder blocks in Experiment 2

a. Ewes grazing primary growth

b. Ewes grazing regrowth

Plate 6. Short grazing Experiment with fistulated ewes in 2005

a. Fistulated ewes grazing short drought pasture supplemented with willow stem cuttings

b. Control fistulated ewes grazing short drought pasture

CHAPTER 2.

EFFECTS ON EWE REPRODUCTION OF GRAZING WILLOW FODDER BLOCKS DURING DROUGHT

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2.1 ABSTRACT

A grazing experiment was conducted in the summer/autumn of 2003 to determine the effect of grazing on willow fodder blocks at 6,000 stems/ha during mating, relative to control ewes grazed on drought pasture, upon ewe production and reproduction. The fodder blocks contained a mixture of herbage and small trees. Grazing occurred over 10 weeks, from 19 February including 3 cycles of mating, with four groups of 100 ewes, comprising short drought pasture typical of drought pasture, long drought pasture typical of the pasture growing in the willow fodder blocks, short drought pasture with restricted access to willow fodder blocks (restricted access) and full access to willow fodder blocks (fenced on the willow fodder blocks all the time; full access). After mating, the four groups were joined and managed as one group until weaning in late November 2003. Ewe live weight (LW) and body condition score (BCS) change and reproductive rate at foetal ultra-sound scanning, lambing, docking and weaning were measured. Ewe wool production and staple length were measured at weaning. Short drought pasture had a pre-grazing mass of 1639 kg dry matter (DM)/ha with a dead matter content of 60%; typical of drought conditions. Herbage in the willow fodder blocks was similar to both control drought pastures (short and long) in nutritive value, with an organic matter digestibility (OMD) of 0.50. Tree yields were low in the fodder blocks, but they had higher concentrations of all secondary compounds, including 30 g condensed tannin (CT)/kg DM and OMD was higher, at 0.72. CT concentration was higher in the fodder block herbage than in short and long control drought pasture (5.0 vs. 2.5 g/kg DM)

Substantial LW loss occurred in the short control group (101 g/day), and reproductive rate was low, as would occur in severe drought conditions. Full access to fodder blocks lowered LW loss to 40g/day and increased reproductive rate by approx 20% units, with more ewes giving birth to twin lambs. Restricted access ewes had a low

reproductive rate, similar to the short control group. Reproductive rate in full access treatment was slightly higher than in the long control group, despite similar calculated DM intakes in both groups. Calculated crude protein and CT intakes were higher for full access ewes than for any other groups, due to contributions from both the herbage and the trees; this may have increased the flow of undegradable dietary protein (UDP) to the small intestine and so have contributed to the increased fecundity of this group. Full access to willow fodder blocks proved beneficial in increasing ewe reproductive rate. However, both pasture and trees need to be managed as a tree/pasture system in order to produce herbage of higher nutritive value and more efficiently utilise willow fodder blocks as a supplementary feed.

2.2 INTRODUCTION

Droughts, floods and weather events can all have adverse impacts on primary production, and ultimately profitability (Daw, 1999). Climatic predictions indicate that droughts will be more frequent, and more severe, in the East Coast regions of New Zealand in the future (Salinger, 2000). Willows (*Salix spp)* have been introduced, and extensively planted, in New Zealand to control soil erosion on hill pastoral farms (Wilkinson, 1999) and, to a lesser extent, to provide shelter, shade and supplementary forage for livestock. Their multi-purpose attributes make willows potentially useful for silvopastoral systems on New Zealand hill country where soil erosion is widespread, and low rainfall in summer results in low pasture production (Oppong et al., 2001). The edible fodder of willow trees i.e., (leaves and fine stems) in summer is adequate for maintenance of sheep, goats and red deer, and is generally higher in nutritive value than low quality summer pasture (McCabe and Barry, 1988, Kemp et al., 2001). Moore et al. (2003) found that willow supplementation of beef cattle grazing dry summer pastures reduced live weight (LW) loss under prolonged summer drought conditions. McWilliam et al. (2003, 2004) established that supplementing ewes grazing drought pasture with poplar and willow cuttings during mating reduced LW loss and increased reproductive performance, although cutting and supplementing willows to sheep and cattle grazing drought pastures can be intensive.

Growing of shrub species in rows, in association with pasture, is a practical option (Douglas et al., 1996). Large scale planting of willows originally relied on using rooted stem cuttings, but these are expensive. An alternative is to use unrooted stem cuttings, which were as productive as rooted cuttings, whilst being cheaper to establish and easier to handle (Zsuffa, 1992). Establishment of fodder blocks can be achieved by vertically planting cuttings referred to as 'wands' or 'stakes', which are

often 1.0 to 1.2 m long, with diameters of 15 to 25 mm and 20 to 40 mm, respectively (Van Kraayenoord et al., 1986). The yield of edible forage (i.e., leaves plus stem ≤ 5 mm diameter)/tree from widely spaced trees ranged from 1 to 25 kg dry matter (DM)/tree depending on tree age (Kemp et al., 2001), and up to 5.9 tonnes DM/ha is produced from densely planted fodder blocks (Douglas et al., 2003). These stem cuttings (stakes) have been used as potential browse plants in dry summer conditions (Oppong et al., 1996), who recommended that farmers establish special purpose forage blocks of the willows, which could be cut or grazed when required.

The present study aimed to reduce labour costs in using supplementary tree fodder during droughts by establishing densely planted willows as fodder blocks to be harvested by grazing livestock. The objective was to investigate effects of grazing ewes on these willow fodder blocks during mating, as an alternative to drought pasture, to increase reproductive rate.

2.3 MATERIALS AND METHODS

2.3.1. Experimental Design

A grazing study using 400 mixed age Romney ewes was conducted at Massey University's Riverside Farm, near Masterton (New Zealand) on the North Island East Coast. Ewes grazed simulated drought pastures with access to established willow fodder blocks with 6,000 stems/ha. The experimental areas were grazed for 10 weeks, from 19 February 2003 to 30 April 2003 (i.e., late summer/autumn), including 3 cycles of mating, with ewes randomly assigned to one of four treatment groups of 100 ewes. The treatments were short drought pasture with a pre-grazing pasture mass of 1200-1400 kg DM/ha and a sward height of 5-7 cm (short control; typical of drought conditions), long drought pasture with a pasture mass of approximately 4000-5000 kg DM/ha and a sward height of 30-35 cm (long control; typical of the pasture growing

in the willow fodder blocks), restricted access to willow fodder blocks i.e., ewes grazed short drought pasture but with limited access to willow fodder blocks (restricted access) and full access to willow fodder blocks i.e., ewes were completely grazed on willow fodder block and had no access to pasture outside the fodder blocks (fenced on the willow fodder blocks all the time; full access). The feed in the willow fodder blocks was comprised of trees and herbage grown underneath the trees. Each group of ewes grazed on separate plots. After mating, the four groups were joined and managed as one group until weaning in late spring. The LW and body condition score (BCS) of ewes were measured regularly throughout the experiment up to weaning, whilst reproductive rate was measured as a percentage of the total number of ewes exposed to the ram at ultra-sound pregnancy scanning (mid gestation period), lambing (birth of lambs spread over 2 months), docking (lamb tail removal) and weaning. Conception rate was recorded as ewes pregnant/100 ewes mated and fecundity was expressed as lambs born/100 ewes lambing.

2.3.2 Ewes

Romney ewes of similar age, size and weight were randomly assigned to the four treatment groups and individually tagged, scored for BCS (Jefferies, 1961) and weighed to ensure that the initial average LW of each group was similar. All ewes were vaccinated with Salvexin ™ + B (Schering-Plough Animal Health Ltd., Upper Hutt, Wellington, New Zealand) before the experiment to prevent salmonella infection and were given Eweguard ™ (Fort Dodge New Zealand Ltd., Auckland, New Zealand) prior to lambing, a combination 6-in-1 vaccine and anthelmintic drench.

The short and the long control groups were fed on pasture alone and had no access to willow fodder blocks. The DM allowance provided to ewes in both willow fodder block groups was a combination of DM from trees and herbage present in the

willow fodder blocks. The short drought pasture (herbage) and restricted access groups were offered 0.8 kg DM/ewe/d of low quality drought pasture. The restricted access group was offered an additional 0.4 kg DM/ewe/d from the willow fodder blocks (trees and herbage), while the long control (herbage only) and full access to willow fodder blocks (trees and herbage) groups were offered 2.0 kg DM/ewe/d each, respectively. During the mating period (i.e., March 15 to 30 April), two Suffolk rams were run with each group of 100 ewes and rams were randomly reassigned to the groups every two weeks, to ensure that a ram breakdown did not affect reproductive results.

Ewes were scanned for pregnancy using ultrasound on 9 June 2003 (winter) and non pregnant ewes were sent to the abattoir. Ewes lambed between 12 August 2003 and 30 September 2003 (late winter/early spring) and reproductive data was recorded at lambing. Lambs were docked (tails removed) on 15 October 2003 and weaned on 25 November 2003. Ewes were shorn and wool production data was collected in December 2003 (early summer).

2.3.3 Forages

2.3.3.1 Pasture Management

Perennial ryegrass/white clover pasture was prepared to simulate drought conditions by temporary desiccation of normal pasture using glyphosate applied at a low rate (i.e., 2 litres/ha Roundup; Monsanto New Zealand Ltd., Wellington, New Zealand) so that the dead matter content of the herbage was approx 60% with low initial mass of 1,500 kgDM/ha.

Pasture was rotationally grazed in 10 breaks (i.e., areas) each lasting 7 d, using front and back electric fences. All treatment groups were moved to a new break on the same day. Water was provided ad libitum to all groups from moveable water troughs.

The plot area (ha) for each break was calculated as:

n x DM allowance x TGD / initial pasture mass– 500

Where: n is the number of ewes, DM allowance in kg DM/ewe/d, and TGD is the total grazing days for each break.

In the equation, the figure of 500 is the minimum level to which sheep can graze, and it was subtracted from the initial pasture mass to determine the amount of edible forage available. In the case of ewes grazing willow fodder blocks, DM allowance used refers to the combined total of trees and herbage growing in the willow fodder blocks.

2.3.3.2 Management of willow fodder blocks

2.3.3.2.1 Site selection

Rush infested swamps and low lying wet areas that were unfit for production were identified on the Riverside Farm as sites to establish willow fodder blocks. These areas were mechanically mowed to remove the rushes and the regrowth sprayed with glyphosate approximately 1 month later, followed by ripping using a tractor to break up the soil, open the ground in lines and make root development easier. Prior to development, herbage in these areas was very minimal (no edible forage mass).

2.3.3.2.2 Willow fodder blocks

Four such areas of one hectare were planted with willow trees spaced at 1.2 m (i.e., 6,000 trees/ha) giving rise to four established willow fodder blocks. The species used were *Salix matsudana* Koidz. × *alba* L. (hybrid willow) clone 'Tangoio' (NZ 1040), a drought – tolerant hybrid tree willow developed in New Zealand, and *Salix matsudana* Koidz. × *alba* L. clone 'Moutere' (NZ 1184). Willow stakes (0.7 m long) were planted 0.35 m below the surface. These small trees were 1.5 to 2.5 years old at the time of the experiment.

Prior to the experiment, these willow fodder blocks were grazed with sheep first, and then with cattle, in May 2002 (i.e., end Autumn), and the trees were cut back to stump height during June 2002. As more grazing occurred during the winter of 2002, a good cover of herbage developed in the willow fodder blocks from volunteer species, mainly grasses with some legumes and herbs.

2.3.4 Forage Measurements

2.3.4.1 Pasture

Pre and post-grazing herbage mass in both control (short and long) pastures and willow fodder blocks were determined immediately before and after grazing each break, respectively, by cutting 8 random quadrats per treatment group/break to ground level, washing, and then drying the herbage at 80°C for 18 to 24 hours. 6 exclusion cages, approximately 1.0 × 0.5 × 0.5 m were placed over the herbage, including willow fodder blocks, in each break before grazing. Hand-plucked diet selected samples were then collected from the exclusion cages, after grazing, by simulating the diet actually consumed by ewes. These samples were stored at -20° C for subsequent nutritive analysis of the diet selected. Samples typical of pasture diet selected were collected before and after grazing each break for dissection into green and dead matter content.

2.3.4.2 Willow measurements

The mass of willow per ha was estimated before grazing each break by cutting 4 trees/break, selected at random, to stump level, cutting the material into approximately 2 cm lengths and drying. Willow material remaining after grazing was similarly estimated. Four round exclusion cages (2m height × 0.7 m diameter) per break were placed around individual trees, for both restricted and full access to willow fodder blocks treatments. At the conclusion of grazing each break, samples were collected that corresponded to the willow diet selected by the grazing ewes and pooled

by break. Representative samples were cut into 2 cm lengths and stored at -20°C for nutrient analysis.

2.3.5 Animal measurements

Mean initial LW and BCS was similar between the four groups with the short control, long control, restricted access and the full access groups weighing (mean ± SE) 55.7±0.45 kg, 55.6±0.45 kg, 55.8±0.44 kg, and 55.7±0.45 kg respectively, with body condition scores of 2.8±0.06, 2.9±0.06, 2.8±0.05 and 2.7±0.06 for the four treatment groups. Ewes were weighed fortnightly using electronic scales (Tru-test, Auckland, New Zealand) during the period of supplementation and body condition score from 1 to 5, was assessed monthly (Jefferies, 1961). Following the supplementation period, ewes were weighed and body condition scored monthly except during lambing period, until shearing in December. Reproductive data collected during the lambing period included lamb birth date, birth weight, birth rank and sex. Lamb weaning weights were recorded and scanning, lambing, docking (lamb tail removal), and weaning proportions calculated.

Ewe fleeces were weighed at shearing to determine greasy fleece weight, with samples of 200 to 300 g collected from both the left and right mid-side areas for staple length (mm) measurements.

2.3.6 Laboratory Analyses

Willow and pasture samples of diet selected were stored at -20°C, freeze-dried and ground to pass a 1 mm diameter sieve. Total N concentration was determined using the Dumas method (Leco Corporation, USA 1994) and organic matter (OM) by ashing samples for 16 h at 550°C. Neutral detergent fibre (NDF) was determined by the detergent procedures of Robertson and Van Soest (1981) and Van Soest et al. (1991) with alpha amylase (BDH, Poole, UK) added during extraction and values are

expressed with residual ash. Sodium sulphite was not added. In vitro OM digestibility (OMD) was determined by the enzymatic method of Roughan and Holland (1977), using separate standard curves prepared from in vivo values for forages and from willow fed to sheep. Metabolisable energy (ME) in the diet select samples was calculated as 16.3 x digestible OM / 100g DM (DOMD; Drew and Fennessy, 1980).

Samples were analysed for acetone/water-extractable, protein-bound and fibre-bound condensed tannin (CT) fractions, using the butanol-HCL colorimetric procedure (Terrill et al., 1992) and total CT concentration was calculated by summing the three fractions. All CT concentrations were determined using CT extracted from *Lotus pedunculatus* as a reference standard (Jackson et al., 1996). Pasture and willow diet selected samples were analysed for zearalenone by enzyme linked immunosorbent assay (ELISA), which detects total zearalenone (zearalenone plus α- and β-zearalenol; Towers 1997). Willow and pasture diet selected samples were analysed for salicin and concentrations of other phenolic glycosides, using the high-performance liquid chromatographic procedure of Meier et al. (1988) a method that allowed measurement of catechin and epicatechin, other flavenoid monomers, and chlorogenic acid.

2.3.7 Statistical analyses

Mean and standard errors for each of the variables that described the chemical composition of the diet selected by the ewes in each of the treatments were obtained using the GLM procedure in SAS (2001) fitting a linear model that considered the fixed effect of treatment. Repeated data of individual LW and BCS of the ewes were analysed using the MIXED procedure of SAS (2001) fitting a mixed model including the fixed effects of treatment and day of measurement and the random effect of ewe with a compose symmetric structure of covariances within ewes (Littell et al., 1996). Least-squares means for reproductive rate at scanning, lambing, docking and weaning

were obtained for each treatment using PROC MIXED of SAS (2001). Reproductive performance was expressed as the number of lambs born as a proportion of the number of ewes mated. PROC GENMOD was used to run a categorical analysis to compare the proportion of ewes bearing singles and multiples between treatments, assuming a binomial distribution with a logit transformation of the data. Lamb birth and weaning weights were analysed using PROC MIXED of SAS (2001) fitting a linear model including the fixed effect of treatment, sex and birth rank. Greasy fleece weight and staple length of wool data were analysed using PROC MIXED of SAS (2001) fitting a linear model that considered the fixed effects of treatment, lambing week, weaning rank and weaning weight as a covariable within each treatment.

Regression equations for change in NDF and OMD in the willow diet selected with time were estimated for restricted and full access using the GLM procedure in SAS (2001). Lamb mortality data were analysed with a generalised linear model (PROC GENMOD of SAS, 2001) assuming a binomial distribution (0 = dead, 1 = alive) considering the fixed effect of treatment, sex and birth rank (i.e., single, twin or triplet). Data were transformed using the logit transformation and least square means and 95% confidence interval were back transformed into the nominal scale.

2.4 RESULTS

Pre and post grazing herbage mass for short drought pasture were 1639 kg DM/ha and 745 kg DM/ha respectively (Table 2.1), with a pre-grazing dead matter content of 66%, typical of drought pastures. Long drought pasture had a similar dead matter content. Pre-grazing herbage masses in the willow fodder blocks were high, approximately 5,500 kg DM/ha, with a dead matter content of approximately 50%, whilst tree yields were low at 550 kg DM/ha.

Short drought pasture and long drought pasture from the diet selected samples were similar in chemical composition (Table 2.2) and of low nutritive value, with a high NDF content of 600 g/kg DM, a low OMD of approximately 0.50 and a ME value of approximately 7.5 MJ/kg DM, typical of drought conditions. The herbage present in the willow fodder blocks was comparable to both the short and long drought pastures in chemical composition, indicating a low nutritive value. However, the selected browse in the willow fodder blocks were higher in digestibility (0.72) and in ME (10.7 MJ/kg DM) and therefore superior in nutritive value to control drought pastures.

Changes with time in NDF and OMD content of willow diet selected by ewes grazing restricted and full access willow fodder blocks are given in Figure 2.1. NDF increased with time, whilst OMD decreased, with homogenous ($P>0.05$) slopes and intercepts, between restricted and full access groups. OMD decreased with increasing NDF concentration (Figure 2.2), the relationship being similar for the restricted and full access groups ($P>0.05$).

Secondary compounds (Table 2.3) were in both short and long control drought pastures, but at low concentrations. Herbage from willow fodder blocks had a higher concentration of secondary compounds than drought pastures, with nearly double the concentration of CT (5 g/kg DM vs. 2.5 g/kg DM) and higher concentrations of flavenoid monomers (3.3 vs. 2.0 g/kg DM). Trees from the fodder blocks were unique

Table 2.1 Pre-grazing and post-grazing mass (kg DM/ha) and dead matter content of control (short and long) drought pasture and willow fodder blocks grazed during the experiment (mean values with standard errors)

	Control		Willow fodder blocks				
			Restricted access[4]			Full access[5]	
	Short drought pasture (5-7 cm)[6]	Long drought pasture (30-35 cm)[6]	Short drought pasture (5-7 cm)[6]	Herbage (30-35cm)[6]	Trees	Herbage (30-35cm)[6]	Trees
Forage mass (kg DM/ha)							
Pre-grazing[1]	1639 ± 169.5	3776 ± 284.9	1587 ± 196.5	5672 ± 205.1	555 ± 119.1	5206 ± 258.1	549 ± 108.3
Post-grazing[1]	745 ± 158.2	2065 ± 202.0	857 ± 120.9	2843 ± 326.9	339 ± 62.7	2958 ± 117.4	370 ± 68.7
Dead matter content (%)[3]							
Pre-grazing[1]	66.2 ± 9.40	63.5 ± 3.36	62.5 ± 7.49	52.1 ± 3.85		50.6 ± 4.98	
Post-grazing[2]	79.2 ± 8.23	85.5 ± 3.14	78.2 ± 7.15	87.2 ± 4.12		80.1 ± 3.49	

[1] n = 10 measurements per treatment
[2] n = 6 samples per treatment
[3] Percentage of total forage mass
[4] ewes were grazed on short drought pasture but had limited access to willow fodder block (herbage + trees)
[5] ewes were grazed on willow fodder block all the time (herbage + trees) [6] Approximate pre-grazing sward height

Table 2.2 Chemical composition and nutritive value of the pasture and willow diet selected (g/kg DM) by ewes grazing low quality control drought pastures (short and long) and willow fodder blocks (mean values with standard errors)[1]

	Control		Willow fodder blocks				
			Restricted Access			Full Access	
	Short drought pasture	Long drought pasture	Short drought pasture	Herbage	Trees	Herbage	Trees
Total N	22.7 ± 2.16	16.0 ± 1.10	22.4 ± 1.92	17.6 ± 0.80	17.4 ± 0.67	18.0 ± 1.01	15.5 ± 1.05
NDF[2]	588.1 ± 24.12	648.7 ± 21.11	606.0 ± 20.70	594.5 ± 20.58	357.9 ± 14.81	587 ± 17.67	370.4 ± 18.20
OM[3]	899.4 ± 13.83	931.9 ± 7.08	920.8 ± 4.41	920.6 ± 4.41	928.8 ± 2.59	920.6 ± 2.92	926.6 ± 1.79
OMD[4]	0.53 ± 0.01	0.50 ± 0.01	0.52 ± 0.01	0.52 ± 0.01	0.72 ± 0.01	0.54 ± 0.01	0.72 ± 0.02
DOMD[5]	0.47 ± 0.01	0.45 ± 0.01	0.47 ± 0.01	0.47 ± 0.01	0.66 ± 0.01	0.5 ± 0.01	0.66 ± 0.02
ME[6] (MJ/kg DM)	7.7 ± 0.23	7.4 ± 0.19	7.8 ± 0.15	7.7 ± 0.21	10.8 ± 0.23	8.0 ± 0.18	10.7 ± 0.32

P

Pasture measurements made on hand plucked samples of diet selected.

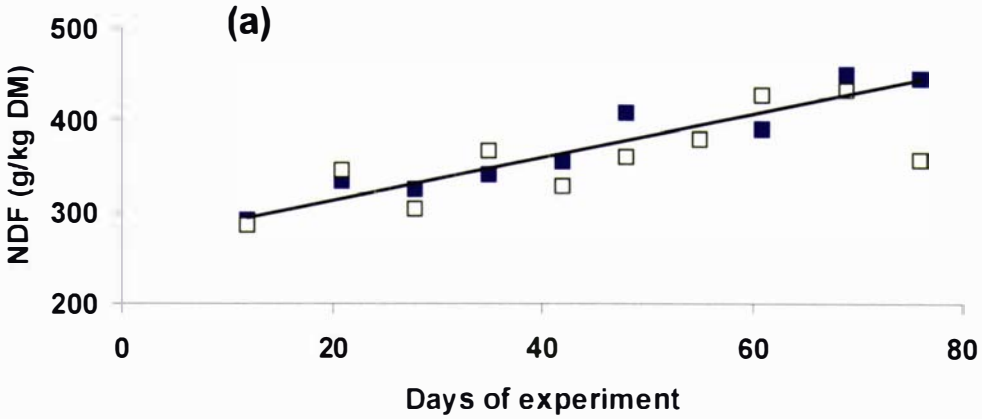
Willow tree measurements made on hand cut samples from trees (stem diameter < 7mm) of diet selected.

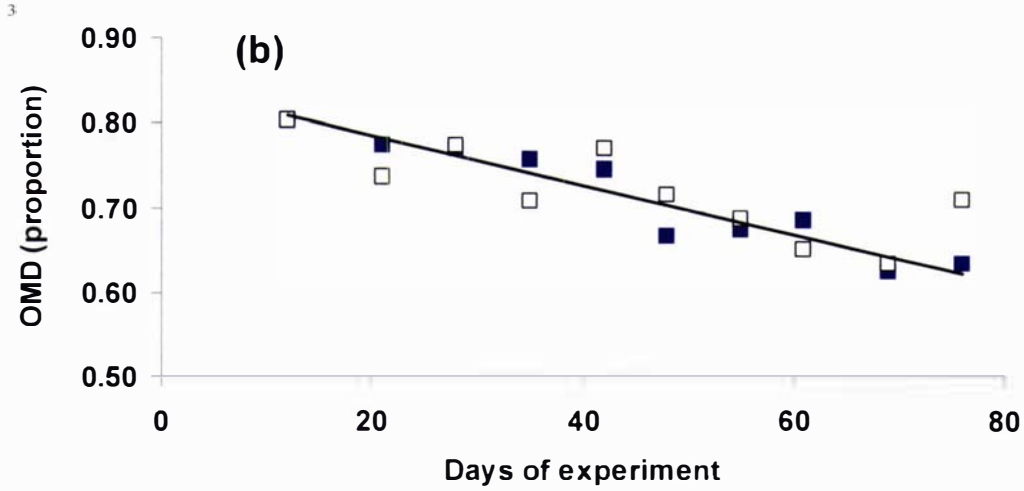
[1] n: 10 samples per treatment; [2] NDF: Neutral Detergent Fibre; [3] OM: Organic matter; [4] OMD: Organic matter digestibility in vitro; [5] DOMD: Digestible Organic matter in the dry matter in vitro; [6] ME: Metabolisable energy.

Table 2.3 Secondary compound content of the pasture and willow diet selected (g/kg DM) by ewes grazing low quality control drought pastures (short and long) and willow fodder blocks (mean values with standard errors)[1]

	Control		Willow fodder blocks		
			Restricted Access [2,3]	Full Access	
	Short drought pasture	Long drought pasture	Trees	Herbage	Trees
Condensed Tannins	2.6 ± 0.01	2.4 ± 0.01	34.1 ± 0.29	5.0 ± 0.11	30.1 ± 0.25
Catechin + Epicatechin	0.06 ± 0.019	0.05 ± 0.014	0.67 ± 0.144	0.07 ± 0.01	0.78 ± 0.221
Other Flavenoid monomers	2.23 ± 1.11	1.99 ± 0.9	11.47 ± 1.84	3.32 ± 0.37	13.53 ± 1.54
Salicin	ND	ND	1.7 ± 0.31	ND	1.7 ± 0.26
Other phenolic glycosides	1.69 ± 0.74	1.85 ± 0.96	13.17 ± 0.76	1.94 ± 0.24	14.35 ± 0.52
Chlorogenic acid	0.13 ± 0.087	0.06 ± 0.027	0.56 ± 0.131	0.13 ± 0.01	0.93 ± 0.210
Zearalenone (mg/kg)	0.39 ± 0.194	0.18 ± 0.040	0.22 ± 0.018	0.11 ± 0.007	0.27 ± 0.012

[1] n: 5 samples per treatment
[2] values for short drought pasture consumed by restricted access sheep is assumed to be similar to that consumed by the control short drought pasture group
[3] values for restricted access browse block herbage consumed is assumed to be similar to herbage consumed by full access group
ND : not detectable

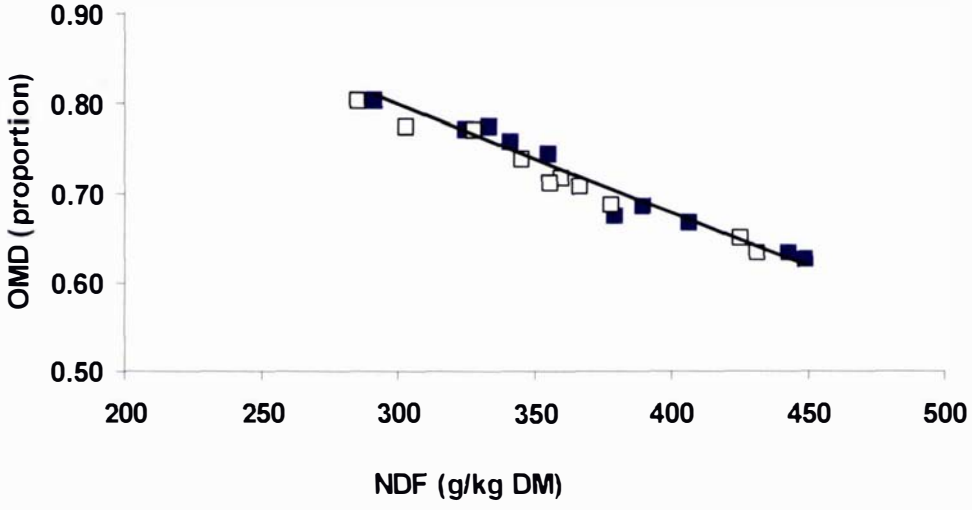




NDF = 275.3 + 1.99 days (SE: 14.72, 0.300; P: ***, ***) (1)

OMD = 0.82 – 0.002 days (SE: 0.015, 0.0003; P: ***, ***) (2)

Figure 2.1 Change in (a) neutral detergent fibre concentration (NDF) and (b) organic matter digestibility (OMD) in samples of willow selected by sheep grazing willow fodder blocks. (□) restricted access; (■) full access.



OMD = 1.14 – 0.001 NDF (SE: 0.207, 0.00005; P: ***, ***) (3)

Figure 2.2 Decrease in organic matter digestibility (OMD) with increase in neutral detergent fibre (NDF) concentration in samples of willow selected by sheep grazing willow fodder blocks. (□) restricted access; (■) full access.

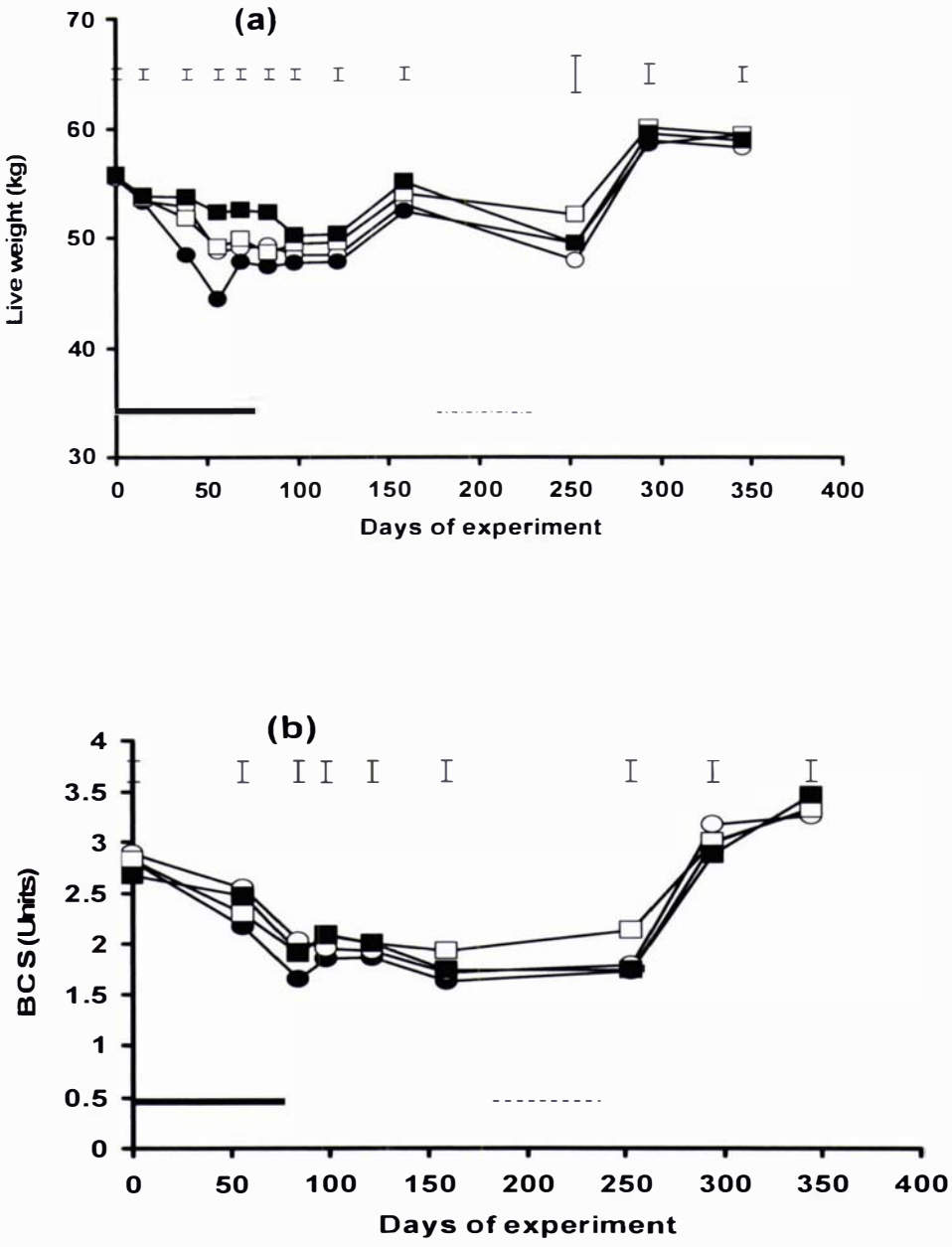


Figure 2.3 Changes in (a) mean ewe live weight and (b) body condition score in ewes grazed on control drought pastures (short and long) and willow fodder blocks (restricted and full access). The solid line indicates the experimental grazing period (75 days). The broken line indicates lambing. (●) short control; (○) long control; (□) restricted access; (■) full access; (I) indicates pooled standard error.

amongst all the treatments in their very high concentrations of secondary compounds, including CT (30 g/kg DM), flavenoid monomers (12 g/kg DM), salicin (1.7 g/kg DM) and other phenolic glycosides (13 g/kg DM).

Ewes grazing short drought pasture during mating had a large LW loss of approx 100 g/d and a loss of BCS of 1.17 units, typical of drought conditions (Table 2.4). Full access to willow fodder blocks markedly reduced loss of both LW and BCS during mating to 41 g/day and 0.75 unit, respectively ($P<0.05$). LW loss in the restricted access and long control groups was intermediate between the full access and short control groups. LW and BCS (Figure 2.3) increased when the treatment groups were joined at the conclusion of mating at 75 days and no differences were evident between the four groups at lambing and weaning.

The reproductive rate of ewes grazing short drought pasture was low (Table 2.4) throughout the experiment from scanning until weaning, typical of drought conditions. Reproductive rate increased ($P<0.05$) in ewes with full access to willow fodder blocks during mating, with the restricted access and long control groups being intermediate but different from short drought pasture and the full access groups (Table 2.4).

Reproductive rate was not affected (Table 2.5) by conception rate (i.e., ewes pregnant/100 ewes mated) but was affected ($P<0.05$) by fecundity (i.e., lambs born/100 ewes lambing), with the proportion of ewes bearing multiple lambs being higher ($P<0.05$) in the full access group vs. the short drought pasture group. Mean lambing date was similar in all treatment groups. Total lamb mortality (Table 2.5) was only numerically lower in the full access, restricted access and long drought pasture groups compared with the short drought pasture group.

There were no consistent differences between treatments in birth weight and weaning weight of lambs (Table 2.6). Greasy fleece weight and staple length were generally

similar in all groups and there were no affects due to grazing willow fodder blocks (Table 2.7).

2.5 DISCUSSION

Establishment of willow fodder blocks in areas of the farm that had negligible pasture production has had beneficial effects on sheep performance. The present study showed that grazing sheep on willow fodder blocks for all of the mating period (i.e., full access group) under drought conditions resulted in reduced LW loss and increased reproductive rate by 20% units, due to increasing the proportion of ewes bearing multiple lambs.

The actual mechanism of the increased reproductive rate in ewes grazing willow fodder blocks (full access) during mating is probably multifactorial. A likely reason is the increase in DM intake in the full access group by (0.46 kg DM/ewe/day), compared with the short drought pasture group (Table 2.8). An additional reason, is the higher calculated CP and CT intakes of full access ewes compared to short control ewes, which is likely to have increased the supply of undegradable dietary protein (UDP); McWilliam et al. (2004) similarly deduced that additional amino acids absorbed from the small intestine was likely to be a contributing factor to the increased reproductive rate from supplementing ewes with poplar species when grazing short drought pasture during mating. Reduced LW loss and BCS in ewes of the full access group during the experimental period is due to their higher DM and ME intakes, consistent with the reduced LW loss in ewes supplemented with poplar stem cuttings when grazing drought pastures (McWilliam et al., 2004).

Table 2.4 Live weight change (g/day) and body condition score change (units) during the 75 day experimental grazing period, together with reproductive rate (expressed as a percentage of the total number of ewes exposed to the ram) in ewes grazing control (short and long) drought pastures and willow fodder blocks (mean values with standard errors)

	Control		Willow fodder blocks		
	Short Drought pasture	**Long Drought pasture**	**Short + restricted Access**	**Full Access**	**SEM**
Change in live weight (g/day)	- 101 [b]	- 75[a]	- 86 [ab]	- 41 [c]	3.7
Change in BCS (units)	- 1.17 [a]	- 0.86 [b]	- 0.92 [b]	- 0.75 [b]	0.046
Reproductive rate:					
Scanning	124[a]	134[ab]	129[a]	148[b]	5.92
Lambing	122[a]	131[ab]	125[ab]	137[b]	6.20
Docking	92[a]	111[b]	108[ab]	114[b]	6.82
Weaning	90[a]	105[ab]	103[ab]	109[b]	6.68

[a, b, c] Means within rows with different superscripts differ significantly ($p < 0.01$)

Table 2.5 The effect of grazing ewes for 75 days, including mating, on control (short and long) drought pasture and willow fodder blocks on conception rate, fecundity, mean lambing date and total lamb mortality from birth to weaning(mean values with standard errors)

	Control		Willow fodder blocks		SEM
	Short Drought pasture	Long Drought pasture	Restricted Access	Full Access	
Conception rate[1]	92	90	98	95	2.30
Fecundity[2]					
Singles	66.0[ab]	55.6[ac]	69.8[b]	50.0[c]	5.02
Twins	34.0[ab]	44.4[ac]	30.2[b]	50.0[c]	5.06
Average lambing date	22 Aug	23 Aug	21 Aug	21 Aug	0.7
Total lamb mortality[3]	23.5	16.8	15.4	18.1	
Range	(16.7 – 32.0)	(11.2 – 24.4)	(10.0 -23.0)	(12.5 – 25.6)	

[1] Expressed as ewes pregnant per 100 ewes mated
[2] Expressed as ewes per 100 ewes lambing
[3] Adjusted by sex and birth rank
[4] ns = non significant ($P>0.05$)
[a, b, c] Means within rows differ significantly ($P<0.05$)

Table 2.6 The effect of grazing ewes on control (short and long) drought pasture and willow fodder blocks for 75 days during the late summer/autumn, including mating, on lamb birth and weaning weights (kilograms; mean values and standard errors)

	Control		Willow fodder blocks		SEM
	Short Drought pasture	Long Drought pasture	Restricted Access	Full Access	
Birth weight					
Single male	5.7	5.9	5.6	5.7	0.23
Single female	5.2	5.2	5.3	4.8	0.16
Twin male	4.3	4.6	4.5	4.8	0.33
Twin female	4.0	4.3	4.3	4.4	0.14
Weaning weight					
Single male	27.3	28.9	22.0	29.5	2.18
Single female	21.6	24.1	29.5	20.7	2.21
Twin male	18.2	21.0	23.0	20.2	1.92
Twin female	16.7	18.2	20.8	20.8	1.89

Table 2.7 The effect of grazing ewes on control (short and long) drought pasture and willow fodder blocks for 75 days during the late summer/autumn, including mating, on whole –year wool production and staple length (mean values and standard errors)

	Control		Willow fodder blocks		SEM
	Short Drought pasture	Long Drought pasture	Restricted Access	Full Access	
Greasy Fleece Weight (kg)					
Single bearing ewes	3.1	3.2	3.2	3.1	0.07
Twin bearing ewes	3.1	2.9	3.1	3.2	0.10
Average for all ewes	3.1	3.0	3.1	3.2	0.07
Staple length (mm)					
Single bearing ewes	133	135	135	138	2.28
Twin bearing ewes	133	135	135	139	3.49
Average for all ewes	133	135	135	138	2.10

In contrast to the full access group, restricted access to willow fodder blocks during mating reduced LW loss in ewes, but did not increase the reproductive rate, when compared with the short control group. The effect on LW loss can be explained by the small increase in DM and ME intake relative to the short control group. Increases in CP and CT intake on the restricted access treatment were minimal and, when combined with the small increases in DM and ME intake, were not enough to increase reproductive rate. Managing ewes on the restricted access treatment was labour intensive, suggesting it is not a practical option to utilise willow fodder blocks.

Although ewes in the full access group performed exceptionally well, compared to ewes grazing short drought pasture, reproductive rate in the full access group was higher than for long drought pasture fed at the same allowance. DM intakes were also similar in the two treatments, but intakes of ME and CP were slightly greater for the full access group, due to the contribution from the trees and CT intakes were substantially greater for full access compared with long control ewes, due to the contributions from both trees and herbage growing in the willow fodder blocks. The herbage in the fodder blocks is a particularly good source of CT and the plants responsible need to be identified in future studies. Increases in reproductive rate are usually accompanied by increases in lamb mortality, with losses of approximately 15% for single born lambs, 25% for twins and 35% for triplets under New Zealand pastoral farming conditions (Barry et al., 2004).

Table 2.8 The effect of ewes grazing for 75 days during the late summer/autumn, including mating, on low quality control drought pastures (short and long) and willow fodder blocks on calculated dry matter intake (kg DM/ewe/day), calculated metabolisable energy (ME) intake (MJ ME/ewe/day), calculated crude protein (CP) intake (g/ewe/day) and calculated condensed tannin (CT) intake (g/ewe/day) and phenolic glycosides (g/ewe/day) (mean values with standard errors)

	Control		Willow fodder blocks				
			Restricted Access			Full Access	
	Short drought pasture	Long drought pasture	Short drought pasture	Herbage	Trees	Herbage	Trees
DM intake[a]	0.42 ± 0.074	0.88 ± 0.058	0.31 ± 0.119	0.21 ± 0.028	0.01 ± 0.004	0.82 ± 0.103	0.06 ± 0.017
Total DM intake	0.42	0.88		0.53		0.88	
ME intake[b]	3.2 ± 0.58	6.5 ± 0.48	2.5 ± 0.96	1.5 ± 0.27	0.2 ± 0.05	6.7 ± 0.88	0.7 ± 0.20
Total ME intake	3.2	6.5		4.2		7.4	
CP intake[c]	60.1 ± 12.02	88.71 ± 9.09	47.9 ± 19.33	20.8 ± 3.51	1.68 ± 0.49	90.4 ± 10.06	7.0 ± 2.01
Total CP intake	60.1	88.7		70.4		97.4	
CT intake[d]	1.07 ± 0.147	1.90 ± 0.376	0.78 ± 0.381[1]	1.08 ± 0.291[2]	0.58 ± 0.256	4.38 ± 1.130	1.87 ± 0.635
Total CT intake	1.1	1.9		2.4		6.3	
Phenolic glycosides[e]	0.67 ± 0.330	1.70 ± 0.948	0.80 ± 0.571	0.03 ± 0.013	0.23 ± 0.09	1.67 ± 0.441	1.05 ± 0.292
Total phenolic glycosides	0.67	1.7		1.04		2.62	

[a] Estimated from pasture mass measurements before and after grazing; [b] DM intake × ME concentration in MJ/kg DM
[c] DM intake × CP concentration in g/kg DM; [d] DM intake × CT concentration in g/kg DM; [e] DM intake × PG concentration in g/kg DM
[1] CT values for short drought pasture consumed by restricted access sheep is assumed to be similar to control short drought pasture
[2] CT values for restricted access browse block herbage consumed is assumed to be similar to herbage consumed by full access group

Supplementation of ewes with poplar and willow when grazing drought pastures during mating not only increased reproductive rate through scanning and lambing percentages, but also reduced post-natal lamb mortality from 18% to 12% (McWilliam, 2004). Ramírez-Restrepo et al. (2005) reported a reduction of post-natal lamb mortality from 24.1 to 19.5%, when ovulation rate in ewes linearly increased from 173 to 200% with increased time (0 to 6 weeks) grazing on *L. corniculatus* before mating, and there was an increase in reproductive rate. In the present study, the restricted and full access groups tended to have reduced the lamb mortality (15% and 18% post-natal lamb mortality), compared with 23% in the short control group, despite a numerical increase in ewes bearing multiple lambs in the full access group, but this trend failed to reach statistical significance ($P>0.05$). The mechanism for the reduction in post-natal lamb mortality is unknown, although it has been suggested that it may be due to increased intestinal absorption of essential amino acids, which is a characteristic of feeding CT-containing forages, at a critical point in early embryonic development (Barry et al., 2004).

The low pre-grazing and post-grazing masses with high dead matter content (60%) and low digestibility values of approximately 0.50, in the short control is indicative of typical drought pasture common in dry summers in East Coast regions in New Zealand. It results in substantial LW loss, of approximately 100 g/day (Mc William et al., 2004) which reduces the reproductive rate substantially (McWilliam, 2004).

A major weakness of the willow fodder blocks was the low nutritive value of the herbage that accumulated in them, which was similar to that of control drought

pasture (approx 0.50 digestible). A contributing reason was the early closure of the fodder blocks in mid August 2002, giving a 6 month growing season for the pasture to accumulate before the start of the experiment. With an aim to improve the quality of herbage in the willow fodder blocks, closure in the following study was delayed until October, 2003, giving them a four month growing season, before being used as feed at the time of mating(Autumn) in 2004.

The key feature in planning the present study was to utilise areas that were low lying, wet, and had the lowest productivity, as sites to establish willow fodder blocks. In the unimproved state, prior to development as fodder blocks, these areas were rush-infested swamps with poor drainage and very little pasture cover. A surprising feature of their planting with trees was that these areas markedly dried and a cover of non-sown grasses, legumes and herbs developed under the grazing management that was applied. Willow fodder blocks were advantageous over cutting and supplementing willow prunings as the later process is labour intensive (Moore et al., 2003).

2.6 CONCLUSIONS AND RECOMMENDATIONS

More effective utilisation of these fodder blocks as supplementary feed for grazing ewes during mating could be achieved by adopting a better grazing management regime that aims at improving the quality of the herbage, while maintaining the above-ground height of trees. A system of 3 grazings/year is proposed (i.e., a first grazing preferably with lambs during late spring/early summer (December/January) is an option to control the height of both the grasses and the trees). Grazing in late summer/early autumn (March) with ewes during mating

(second grazing) followed by clean up with cattle in late autumn (May) is proposed, to further reduce stem height, followed by manual topping to the desired height if necessary. The fodder blocks would then be set stocked with sheep (third grazing) during winter/early spring to control the herbage mass and minimise dead matter. The biomass from the trees in the fodder blocks is expected to increase with age by a factor of 2.6/year (Douglas et al., 2003). Therefore, maximising the yield from trees and improving the quality of pasture growing under the trees, should be investigated in future experiments.

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CHAPTER 3.

WILLOW FODDER BLOCKS - AN ALTERNATE FORAGE TO LOW QUALITY PASTURE FOR MATING EWES DURING DROUGHT

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3.1 ABSTRACT

Two grazing experiments were conducted in the East Coast region of the North Island of NZ with ewes grazing on willow fodder blocks or control grass-based pastures during mating. Experiment I was conducted for 86 days with three treatments; short drought pasture (5-7 cm), long drought pasture (25-30 cm) and full access to willow fodder blocks which also contained long pasture (25-30 cm). Experiment II was conducted for 35 days with two treatments; control pasture and full access to willow fodder blocks. All ewe groups were combined at the end of mating and grazed on pastures until lambing and weaning. Live weight (LW) was recorded fortnightly during experimental grazing, body condition score (BCS) was scored monthly, and reproductive data was recorded at ultra-sound pregnancy scanning, lambing, docking and weaning. In Experiment I, short drought pasture and long drought pasture contained, respectively, a metabolisable energy (ME) of 8.2 and 9.6 MJ /kg dry matter (DM) and only traces of condensed tannins (CT). Herbage in the willow fodder blocks was intermediate in ME to short drought pasture and long drought pasture, whilst trees in willow fodder blocks contained 38 g of CT /kg DM and had a ME of approximately 10 MJ/kg DM. Willow fodder block herbage was consistently of higher legume content than drought pasture of similar mass. Ewes grazing short drought pasture lost 40g/d of LW and had a low reproductive rate, as would occur in a drought situation, whilst ewes grazing long drought pasture gained LW and had a higher reproductive rate. Ewes grazing willow fodder blocks maintained LW and their reproductive performance was intermediate to ewes mated on long drought pasture and short drought pasture. Calculated intakes of DM, ME and crude protein (CP) were low for ewes grazing short drought pasture and higher and similar for the other two groups, while intake of secondary compounds was much

higher for ewes grazing willow fodder blocks. In Experiment II, ewes mated on willow fodder blocks had lower LW gain than ewes mated on control pasture, but reproductive performance was similar for both groups. Willow fodder blocks are a useful source of supplementary feed during droughts, but the grazing management that optimizes animal performance needs further research.

3.2 INTRODUCTION

Summer and autumn drought is a regular feature of East Coast farming in New Zealand (NZ) and this has intensified due to recent climatic shifts (Salinger, 2000), thereby increasing the cost of drought to the farmer and NZ (Moore et al., 2003). McWilliam (2004) reported a loss of $14 NZ per ewe per annum at the farm level when ewes were mated on drought type pastures compared to normal pastures.

Willows (*Salix spp)* have been introduced, and extensively planted, in NZ to control soil erosion on hill pastoral farms (Wilkinson, 1999) and, to a lesser extent, to provide shelter, shade and supplementary forage for livestock. In response to severe droughts, feeding willows to grazing livestock as a silvipastoral system by farmers has increased. McWilliam et al. (2004; 2005) studied effects of supplementing poplar and willow tree trimmings to ewes grazing drought pasture during mating and reported a consistent reduction in LW loss, and increased reproductive efficiency, through increases in conception rate and fecundity as well as decreases in lamb mortality. However, cutting and supplementing the tree prunings was labor intensive.

Willow stakes can be planted as fodder blocks in wet areas of farms that have low economic value in an undeveloped state (Douglas et al., 2003), and could be used as a supplementary feed for the livestock. Pitta et al. (2005) showed that grazing these fodder blocks with ewes during mating could reduce labour costs, whilst increasing the reproductive rate. The study also showed that ewe numbers per treatment needed to be increased from 100 to 165, to more effectively detect statistically significant effects of fodder blocks upon reproductive efficiency. However, planting willows as fodder blocks posed management problems caused by high biomass and poor nutritive value of herbage growing in them due to using a long interval between grazings to protect the willows from excessive browsing.

It is possible that these management problems could be minimised if the willow fodder block is considered as a tree-pasture system, with grazing management to suit both species. The present study aimed at managing the willow fodder block as a tree-pasture system, with the objective being to improve the quality of pasture through better management, especially late closure (i.e., removal of all livestock), reduced intervals between grazings, and to better define effects upon ewe fecundity and lamb mortality by increasing ewe numbers per treatment.

3.3 MATERIALS AND METHODS

3.3.1 General

Two experiments were conducted in 2004 with mixed age Romney ewes in the Wairarapa region of New Zealand, on the East Coast of the North Island. Both involved effects on mating ewes grazing willow fodder blocks relative to control ewes grazing grass-based drought pastures. Experiment 1 was conducted on Massey University's Riverside Farm, near Masterton, whilst Experiment 2 was conducted at the Fernglen farm, near Riversdale. Masterton is approximately 40 km inland, whilst Riversdale is on the East Coast and is hotter and drier during summer than Masterton. Both farms are classified as summer dry in NZ.

3.3.2 Experiment 1

3.3.2.1 Experimental design

Mixed age Romney ewes grazed simulated drought pasture or established willow fodder blocks (6,000 stems/ha) in 12 weekly breaks (i.e., experimental areas), from 25 February 2004 to 14 May 2004 (late summer/autumn), including 3 cycles of mating (n=167 ewes per group). Drought pastures (long and short) were simulated on shallow soils on the Riverside Farm whilst willow fodder blocks were established in rush infested swamps that had little productive potential in the undeveloped state. The

treatments were short drought pasture (short control; typical of drought conditions; approx 5-7 cm tall), long drought pasture (long control; typical of pasture growing in the willow fodder blocks; approx 25-30 cm tall), and full access to willow fodder blocks (grazed on trees and 25-30 cm tall herbage in willow fodder blocks with no access to outside pasture). Each group of ewes grazed separate plots. After mating, the three groups were combined and managed as one group until weaning in late spring. The LW and body condition score (BCS) of ewes were measured regularly throughout the experiment up to weaning in late November (2004), whilst reproductive rate was measured at ultra-sound pregnancy scanning, lambing, docking (i.e., lamb tail removal) and weaning.

3.3.2.2 Animals

Romney ewes of similar age, size and LW were randomly assigned to the three treatment groups and individually tagged, scored for BCS and weighed to ensure that the initial average LW of each group was similar. All ewes were vaccinated with Salvexin ™ + B (Schering-Plough Animal Health Ltd., Upper Hutt, Wellington, NZ) before the experiment to prevent salmonella poisoning and were given Eweguard ™ (Fort Dodge New Zealand Ltd., Auckland, NZ) prior to lambing, a combination 6-in-1 vaccine and anthelmintic drench.

The short and the long control groups were fed on pasture alone and had no access to willow fodder blocks. The DM allowance provided to the ewes in the willow fodder block was a combination of DM from trees and herbage present in the willow fodder blocks. The short drought pasture (herbage) group were offered 0.8 kg DM/ewe/day, while the long control (herbage alone) and full access to willow fodder blocks (trees and herbage) groups were offered 2.0 kg DM/ewe/day each, respectively. Grazing on willow fodder blocks (approximately 6 ha) occurred in two rotations for 12 weeks. First rotation of grazing on willow fodder blocks was 10

breaks on primary growth (approximately 6 ha) and then two breaks were grazed on regrowth (approximately 1.5 ha), until leaf fall in mid May when the experiment concluded. During the mating period (i.e., March 12 to 30 April), two Suffolk rams were run with each group of 167 ewes, and rams were randomly re-assigned to the groups every two weeks to ensure that an ineffective ram did not affect reproductive results.

Ewes were scanned for pregnancy using ultrasound on the 2 June 2004 (winter) and dry ewes were sent to the abattoir. Ewes lambed between 2 August 2004 and 30 of September 2004 (i.e., late winter/early spring) and reproductive data were recorded at lambing. Lambs were docked (tail removal) on 6 October 2004 and weaned on 16 November 2004. Ewes were shorn and wool production data were collected on 4 November 2004. Ewe fleeces were weighed at shearing to determine the greasy fleece weight, with samples of 200-300 g collected from both the left and right mid-side areas for staple length (mm) measurements.

3.3.2.3 Forages

3.3.2.3.1 Preparation of drought pasture

Long drought pasture comprised of perennial ryegrass/white clover pasture (*Lolium perenne and Trifolium repens)*, grown on very shallow, stony soil. Drought conditions were simulated by allowing the grasses to grow long (25-30 cm) and mature until development of seed heads, thus decreasing quality of the sward. Short drought pasture was prepared by grazing the above mentioned areas with cows and non-experimental ewes to reduce pasture mass to approximately 1300 kg DM/ha, thereby giving low sward height (5-7 cm) with a high (> 50%; i.e., 500 g/kg) dead matter content and low nutritive value, typical of drought conditions.

3.3.2.3.2 Preparation of willow fodder blocks

Selection of sites for the fodder blocks, site preparation and planting willow stakes have been described by Pitta et al. (2005). A two step grazing procedure was followed in managing willow fodder blocks, which were grazed by sheep, and then with cattle, to consume residual pasture and reduce height of the willow trees, followed by manual topping of the trees to the stump level in May 2003. They were then set stocked with sheep during winter and during lambing (Aug/Sept), to graze pasture growing in the fodder blocks. The animals were removed from the fodder blocks in early spring (7th October) 2003 (i.e., closure), when the buds on the trees started to sprout. The willow fodder blocks were then shut from grazing until mid Feb'2004.

3.3.2.3.3 Grazing management

All treatment groups were moved in breaks (i.e., experimental areas) and were shifted to a new break on the same day. Pasture and fodder blocks were rotationally grazed in 12 weekly breaks, using front and back electric fences. Short and long drought pastures were simulated as mentioned above and pre grazing pasture mass were measured. Plot area (ha) for each break was calculated using equation 1 as:

$$\frac{(167 \text{ ewes}) \times (\text{daily DM allowance}) \times (\text{no. of days/break})}{\text{Initial pasture mass (kg DM/ha} - 500)} \quad (1)$$

In this equation, the figure of 500 kgDM/ha was deducted, because it is as low as sheep are able to graze. In the case of ewes grazing willow fodder blocks, the DM allowances used refers to the combined total of trees and herbage growing in the willow fodder blocks. Water was provided ad libitum to all groups from moveable water troughs.

3.3.3 Experiment II

3.3.3.1 Experimental design

Mixed-age Romney ewes (n=100) grazed willow fodder blocks or control pasture (n=100) in rotational 5 day breaks for 4 weeks from 27 February to 29 March 2004 during mating. At the conclusion of the grazing period, all ewes were joined and grazed on pasture until ultra sound scanning on 27 May, 2004. The LW and BCS of ewes were measured regularly throughout the experiment, whilst reproductive rate was measured by ultra-sound pregnancy scanning.

3.3.3.2 Animals

Romney ewes of similar age, size and LW were randomly assigned to two treatment groups (100 in each) and individually tagged, scored for BCS and weighed to ensure that the initial average LW of each group was similar. The control ewes were rotationally grazed on the hill country pasture along with non experimental sheep at Fernglen Farm. The LW and BCS of the control ewes was recorded at the start and end of experimental grazing.

The willow fodder block (1.73 ha) was divided into 4 blocks of 0.39, 0.46, 0.46 and 0.42 ha each, and ewes grazed the entire block in two rotations of 6 breaks. The first rotation was comprised of 5 days grazing in each of 4 breaks whilst, in the second rotation, breaks 1 and 2 were combined as break 5, and breaks 3 and 4 were combined as break 6 and grazed for 3-4 days each. Ewes were exposed to a ram during the experimental grazing and were then merged with the whole farm stock at the conclusion of the experiment on 29th March, 2004.

3.3.3.3 Pasture and willow fodder blocks

Perennial grass and white clover comprised the major component of the pasture grazed by control ewes, which is typical of the pasture grown and grazed on flat and rolling hills in East Coast of NZ. Preparation of the willow fodder block at

Fernglen was similar to the preparation of willow fodder blocks at Riverside as in (Experiment 1).

3.3.4 Forage Measurements

Measurements and analysis on pasture and willow trees were similar in both experiments and are described together.

3.3.4.1 Pasture

Pre and post- grazing herbage mass in both control pastures and willow fodder blocks was determined immediately before and after grazing each break, by cutting 8 random quadrats per treatment group per break to ground level, washing and then drying the herbage at 80°C for 18-24 hours. Eight exclusion cages, approximately 1.0 × 0.5 × 0.5 m were placed over the herbage, including willow fodder blocks, in each break before grazing. Hand plucked diet selected samples were then collected from the exclusion cages, after grazing, by simulating the feeding behaviour of the ewes. These samples were stored at -20° C for nutritive analysis of the diet selected.

3.3.4.2 Willow measurements

Mass of willow per ha was estimated before grazing by cutting 8 trees per break selected at random to the level of the stumps, cutting the material into approximately 2 cm lengths and drying. Willow material remaining after grazing was similarly estimated. Diameter of the chewed willow was determined at the end of each grazing plot, using electronic callipers, with 75 measurements made per plot for both the leader shoots and basal branches. Eight exclusion cages (2m high and 0.7 m diameter) per break were put around individual trees in each break. At the conclusion of grazing each break, samples were collected that corresponded to the diet selected by the grazing animals and pooled per break. Representative samples were cut into 2

cm lengths and stored at -20°C for later analysis of nutritive value. The DM in the willows was also estimated from the diet select samples collected in every break.

3.3.5 Laboratory Analyses

Willow and pasture diet select samples were freeze-dried and ground to pass a 1 mm diameter sieve. Total N concentration was determined using the Dumas method (Leco Corporation, USA 1994) and organic matter (OM) by ashing samples for 16 h at 550°C. Neutral detergent fibre (aNDF) was determined by the detergent procedures of Robertson and Van Soest (1981) and Van Soest et al. (1991), with alpha amylase added (BDH, Poole, UK) during extraction and values expressed with residual ash. Sodium sulphite was not added. *In vitro* OM digestibility (OMD) was determined by the enzymatic method of Roughan and Holland (1977), using separate standard curves prepared from *in vivo* values for forages and from willow grazed by sheep. Metabolisable energy (ME) in the diet select samples was calculated as 16.3 x digestible organic matter / 100 g DM (DOMD; Drew & Fennessy 1980).

Willow and pasture samples were analysed for acetone/water-extractable, protein-bound and fibre-bound condensed tannin (CT) fractions, using the butanol-HCL colorimetric procedure (Terrill et al., 1992), and the total CT concentration was then calculated by summing the three fractions. All CT concentrations were determined using CT extracted from *Lotus pedunculatus* as a reference standard (Jackson et al., 1996). Pasture and willow diet selected samples were analysed for zearalenone by enzyme linked immunosorbent assay (ELISA), which detects total zearalenone (zearalenone plus α- and β-zearalenol; Towers 1997). Willow and pasture diet selected samples were analysed for salicin and the concentration of other phenolic glycosides, using the high-performance liquid chromatographic procedure of

Meier et al. (1988). This method also allowed measurement of catechin and epicatechin, other flavenoid monomers and chlorogenic acid.

3.3.6 Statistical Analyses

All animal data was analysed using individual animals as the statistical unit and, for logistical reasons it was not possible to use areas of land as the statistical unit. Whilst it is realised that this results in confounding between nutritional treatments and areas of land, it is believed not to have altered the conclusions that can be drawn from the present study due partly to the large number of animals per land area as well as the overwhelming impact of treatment on the land areas utilised.

Mean and standard errors for each of the variables describing chemical composition of the diet selected in each of the treatments were obtained from the GLM procedure of SAS (2003). Repeated data of individual LW and BCS of the ewes were analysed using the MIXED procedure of SAS (2003) fitting a mixed model including fixed effects of treatment and day of measurement and random effect of ewe with a compose symmetric structure of covariances within ewes (Littell et al., 1996).

The LW change was analysed using the MIXED procedure of SAS (2003) with a linear model that considered effect of treatment. Least-squares means for reproductive rate at scanning, lambing, docking and weaning were obtained for each treatment using the MIXED procedure of SAS (2003). Reproductive performance was expressed as the number of lambs born as a proportion of the number of ewes mated. The PROC GENMOD (SAS, 2003) was used to run a categorical analysis to compare the proportion of ewes bearing singles and multiples lambs between treatments, assuming a binomial distribution with a logit transformation of the data. Lamb mortality data were analysed with a generalised linear model (PROC GENMOD of SAS, 2003) assuming a binomial distribution (0 = dead, 1 = alive) considering the fixed effect of treatment, sex and birth rank (i.e., single, twin or triplet). Data were

transformed using the logit transformation and least square means and 95% confidence interval were back transformed into the nominal scale. Lamb birth and weaning weights were analysed using the PROC MIXED of SAS (2003) fitting a linear model including fixed effects of treatment, sex and birth rank. Calendar lambing week was considered as a covariable. Least squares means and their standard errors were obtained for multiple comparisons between treatments. Greasy fleece weight and staple length of wool data were analysed using PROC MIXED of SAS (2003) fitting a linear model that considered the fixed effects of treatment, lambing week, weaning rank and weaning weight as a covariable within each treatment. Regression equations for changes in DM, diameter of chewed willow and CT in the willow diet selected with time were estimated for full access using the GLM procedure in SAS (2003).

3.4 RESULTS

3.4.1 Experiment I

Pre and post-grazing pasture mass for short drought pasture was 1486 and 664 kg DM/ha with a pre grazing dead matter content of 570 g/kg (Table 3.1), typical of drought conditions. Pre and post-grazing pasture masses in herbage from willow fodder blocks and long drought pasture for the first 8 weeks (i.e., primary growth) were higher, with similar pre-grazing dead matter content (300 g/kg) in both. Tree yields in willow fodder blocks were low, contributing approximately 0.150 of their total DM yield (Table 3.1). Regrowth herbage in both long drought pasture and willow fodder blocks, which was grazed for 2 weeks only, had lower values for both pre and post-grazing herbage mass.

Grasses dominated green herbage in short and long drought pasture (Table 3.1), with little contribution from legumes in short drought pasture. The mean content

of legumes (Table 3.1) was 240 g/kg in the green herbage from willow fodder blocks (primary growth), which was higher than the legume content in the long drought pasture (122 g/kg). The same legume trend occurred in regrowth herbage, whilst dead matter content was greatly reduced in the regrowth herbage in willow fodder blocks.

Short drought pasture had a low nutritive value from the selected diet samples (Table 3.2) with a high NDF, a low OMD (0.57) and a low ME (8.0 MJ/ kg DM), which was typical of drought conditions. Long drought pasture had a higher nutritive value with a higher OMD (0.65) and higher ME (9.6 MJ/ kg DM) (Table 3.2). Primary growth herbage from the willow fodder block was intermediate between short drought pasture and long drought pasture in nutritive value, but the regrowth was of higher nutritive value. The nutritive value of primary growth trees in willow fodder blocks was higher than herbage in OMD (0.67) and ME (9.9) but yield was low (Table 3.2).

Table 3.1 Pregrazing and post grazing mass (kg DM/ha) and dead matter content from primary and secondary growth of control (short and long) drought pasture and willow fodder blocks grazed during the experiment (mean values with standard errors) (Experiment I)

	Control		Willow fodder block	
	Short drought pasture	**Long drought pasture**	**Herbage**	**Trees**
Primary growth				
n	10	8	9	9
Pre-grazing mass	1486± 93.1	4256 ● 306.3	5724± 330.4	814 ± 79.5
Post grazing mass	664± 57.6	2401 ± 262.2	3605 ± 304.3	470 ± 52.6
Botanical Composition (g/kg)				
Dead Matter content	569 ± 66.9	269 ± 31.0	306 ± 31.7	
Grasses [1]	914±16.9	756±44.5	578±39.3	
Legumes[1]	62±14.0	127±27.7	240±40.5	
Others[1]	24±12.8	117±28.0	182±29.7	
Regrowth				
n		2	2	2
Pre-grazing mass		3883 ± 800.2	3369 ± 69.8	226 ± 75.4
Post grazing mass		1429 ± 68.6	1333 ± 63.0	101± 19.5
Botanical Composition (g/kg)				
Dead Matter content		296 ± 69.9	150 ± 34.0	
Grasses [1]		851± 22.2	819 ± 20.5	
Legumes[1]		88 ± 40.1	122 ± 8.9	
Others[1]		61 ± 17.9	59 ± 29.4	

1. Proportion of green matter content.

Table 3.2 Chemical composition and nutritive value of the pasture and willow diet selected (g/kg DM) by ewes grazing low quality control drought pastures (short and long) and willow fodder blocks (primary and secondary growths) (mean values with standard errors) (Experiment 1)

	Control		Willow fodder block			
			Primary growth		Regrowth	
	Short drought pasture	Long drought pasture	Herbage	Trees	Herbage	Trees
n	10	10	9	9	2	2
N	24.2±1.21	25.5±1.91	20.0±0.95	13.6±0.89	38.2±0.80	16.3±3.19
aNDF[1]	550.7±19.89	460.5±18.81	512.0±14.40	417.4±16.23	383.7±8.06	528.4±60.57
OM[2]	885.0±4.86	917.4±2.31	915.9±2.30	942.7±2.42	889.6±3.35	947.0±6.72
OMD[3]	0.57±0.017	0.65±0.016	0.60±0.012	0.67±0.012	0.74±0.010	0.57±0.045
DOMD[4]	0.50±0.147	0.59±0.014	0.59±0.010	0.61±0.010	0.65±0.007	0.52±0.040
ME[5] **(MJ/ kg DM)**	8.16±0.240	9.56±0.229	8.77±0.167	9.87±0.169	10.61±.0.121	8.49±0.656

Pasture measurements made on hand plucked samples of diet selected.
Willow tree measurements made on hand cut samples from trees (stem diameter < 7mm) of diet selected.
[1] aNDF: Neutral Detergent Fibre; [2] OM: Organic matter; [3] OMD: OM digestibility *in vitro*; [4] DOMD: Digestible OM in the DM *in vitro*;
[5] ME: Metabolisable energy.

Table 3.3 Secondary compound content of the pasture and willow diet selected (g/kg DM) by ewes grazing low quality control drought pastures (short and long) and willow fodder blocks (mean values with standard errors)[1] (Experiment I)

	Control		Willow fodder blocks	
	Short drought pasture	**Long drought pasture**	**Herbage**	**Trees**
Condensed Tannins	1.9±0.10	2.0±0.16	3.6±0.87	38.3±4.12
Catechin + Epicatechin	0.11±0.033	0.20±0.018	0.14±0.043	2.85±0.479
Other Flavenoid monomers	5.23±1.488	10.16±1.938	4.21±0.389	14.21±2.826
Salicin	ND[2]	ND[2]	ND[2]	1.86±0.498
Other phenolic glycosides	4.44±0.976	9.21±1.195	5.13±1.247	36.53±6.085
Chlorogenic acid	0.24±0.047	0.65±0.113	0.26±0.055	1.09±0.301
Zearalenone (mg/kg)	0.13±0.011	0.12±0.010	0.16±0.006	0.31±0.053

[1] n: 5 samples per treatment
[2] ND: Not detected

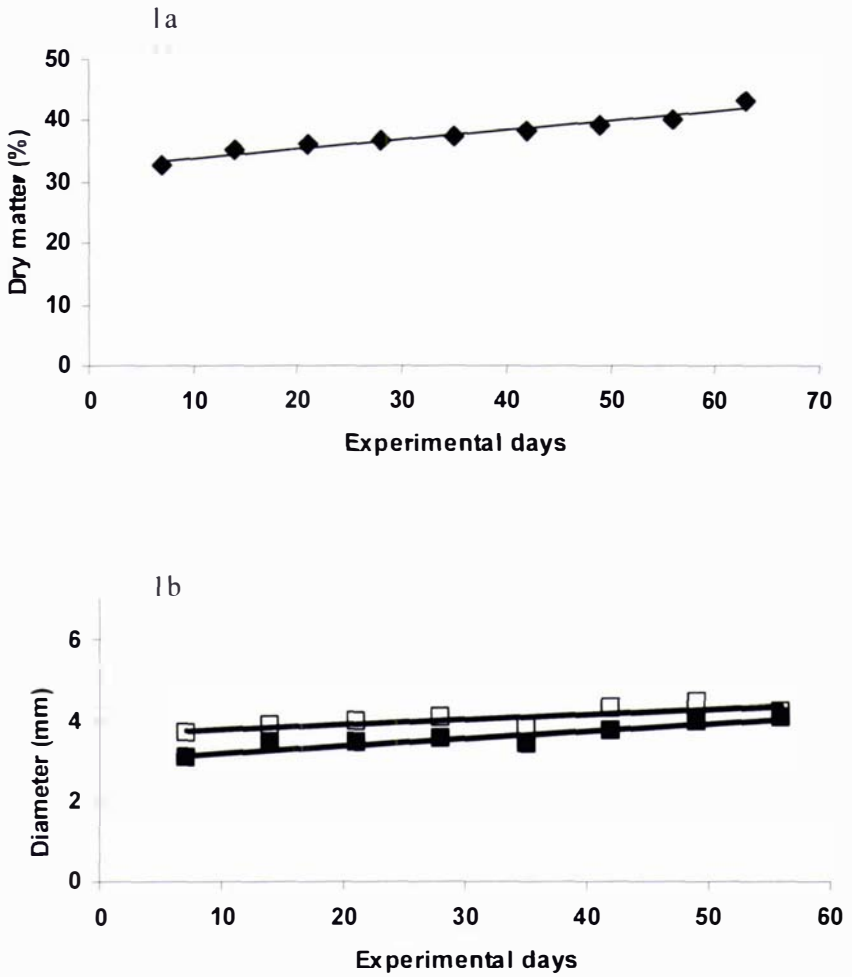

Figure 3.1. Experiment 1. Increase in (a) Dry matter (DM) content expressed as (%) (b) Diameter (D) of chewed willow (mm) with time during experimental grazing in samples of willow selected by ewes grazing willow fodder blocks. (♦) DM%; (□) leader shoots; (■) basal shoots.

DM in trees eaten (Fig 3.1a; Equation 2) and diameter (D; mm) of chewed willow (Fig 3.1b; Equations 3 and 4) increased with time (d), with the difference between regression intercepts being significant ($P < 0.05$) for central and basal shoots.

DM = 32.26 + 0.153 days (SE: 0.619, 0.0157; P: ***, ***) (2)

D (central shoot) = 3.70 + 0.011 days (SE: 0.159, 0.0034; P: ***, **) (3)

D (basal shoot) = 3.05 + 0.017 days (SE: 0.092, 0.0026; P: ***, ***) (4)

Secondary compound concentrations in short and long drought pastures were low. Herbage in willow fodder blocks had nearly double the concentration of CT (3.6 g/ kg DM) when compared to short (1.9 g/kg DM) and long drought pasture (2.0 g/kg DM). Trees from willow fodder blocks were unique in their secondary compounds, with a CT concentration of 38.3 g/kg DM and phenolic glycoside concentration of 36.5 g/kg DM. Salicin was present only in the trees in the willow fodder blocks at 1.9 g/kg DM (Table 3.3).

CT concentration in volunteer lotus, a legume growing in the fodder block herbage was high, and decreased with time, whilst the tree CT concentration (Figure 3.2) increased with time.

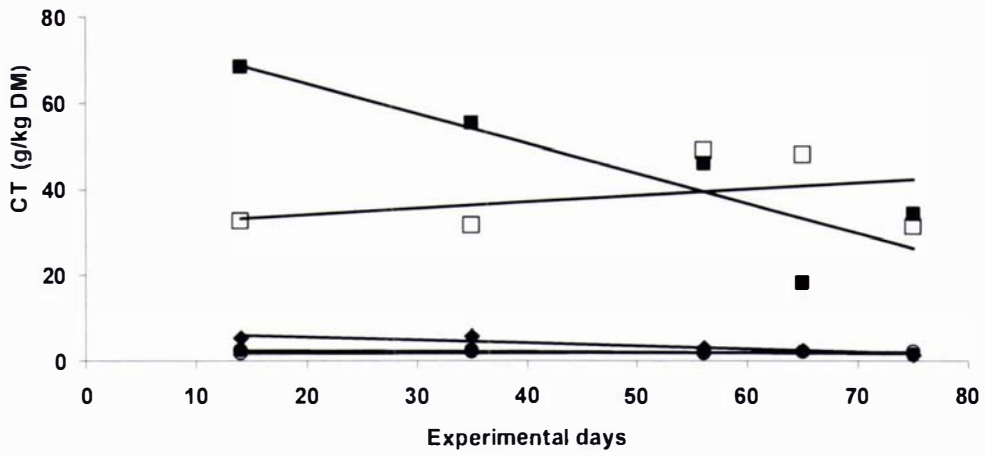

Figure 3.2 Change in CT concentration (g/kg DM) with time in short control pasture(○); long control pasture(●); willow trees (□); lotus legume present in willow pasture (■); and willow fodder block pasture (♦).(Experiment I)

Ewes grazing short drought pasture (Table3.4) during mating lost 40 g LW/ day and their reproductive rate was low (Table 3.5). Relative to ewes grazing short drought pasture, ewes grazing long drought pasture during mating gained LW ($P<0.05$) and had a higher reproductive rate, with a higher conception rate and better fecundity ($P<0.05$). Full access to fodder blocks was intermediate to short drought pasture and long drought pasture in ewe LW change ($P<0.05$) and overall reproductive rate ($P<0.05$), with the ewes maintaining LW during mating (Table 3.4 and 3.5); conception rate ($P>0.05$) was similar to ewes grazing long drought pasture (Table 3.5), whilst fecundity was similar to ewes grazing short drought pasture ($P>0.05$) (Table 3.5).

The LW and BCS (Figure 3.3a and b) increased when the treatment groups were combined at the conclusion of mating at 86 days and no differences were evident between the three groups at weaning.

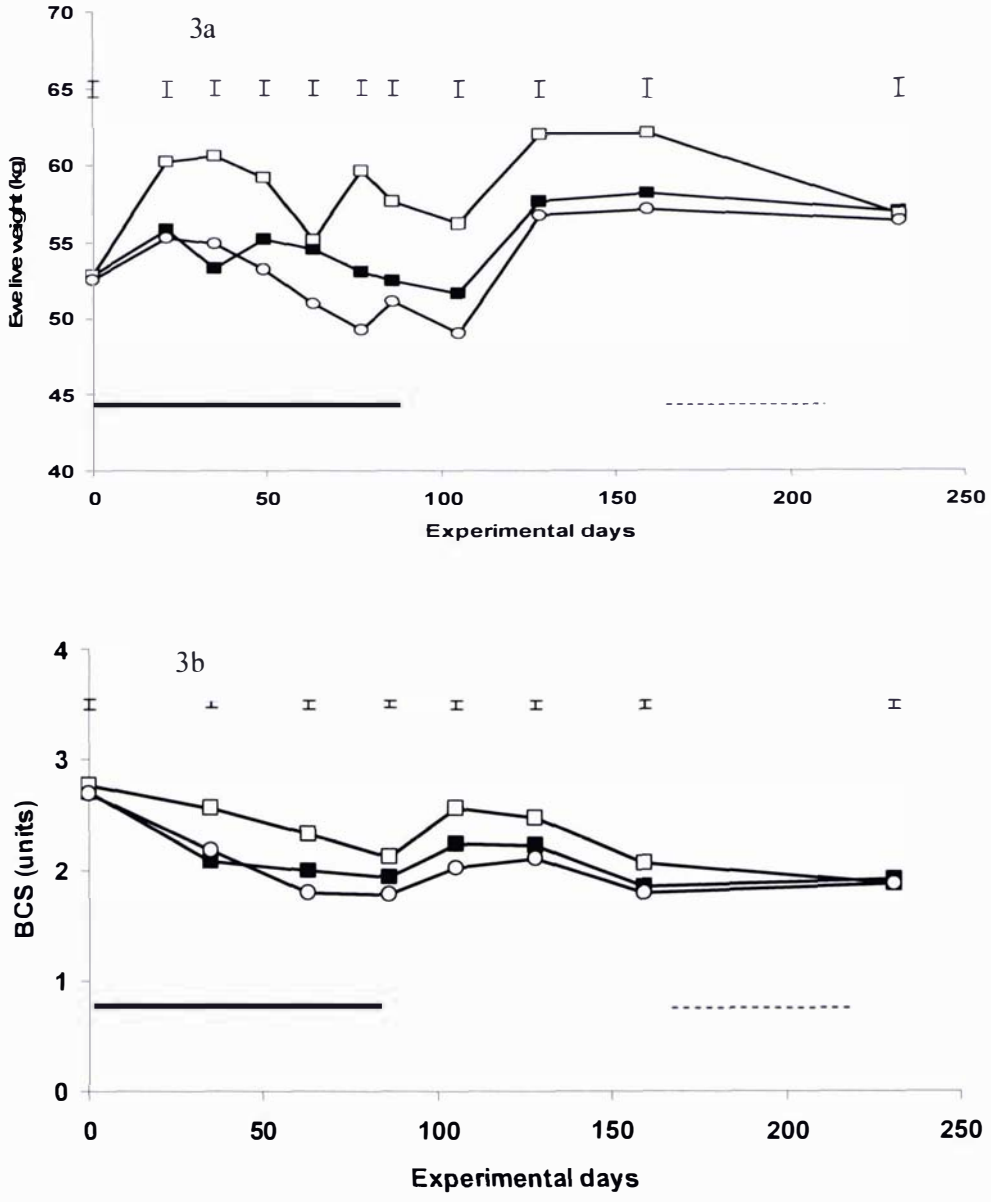

Figure 3.3 Changes in (a) mean ewe live weight and (b) body condition score in ewes grazed on control drought pastures (short and long) and willow fodder blocks (full access). The solid line indicates the experimental grazing period (86 days). The broken line indicates lambing. (○) short control; (□) long control; (■) full access to fodder blocks; (I) indicates pooled standard error. (Experiment 1)

Mean lambing date (Table 3.5) was similar in ewes mated on long drought pasture (19 August, 2004) or with full access to fodder blocks (20 August, 2004), but was delayed by four days when mated on short drought pasture (23 August, 2004) ($P<0.05$). Total lamb mortality (lambing to weaning) was 20 lambs per 100 lambs born on short drought pasture, as would happen in drought conditions, and was similar for ewes mated on long drought pasture. However, total lamb mortality was higher for ewes mated with full access to fodder blocks ($P<0.05$) compared to the two control groups. This difference in lamb mortality was associated with a sudden occurrence of inclement weather during lambing.

There were no consistent differences due to nutrition at mating amongst the treatments upon lamb birth and weaning weights (Table 3.6). Greasy fleece weight was similar in short drought pasture (2.49 kg) and full access groups (2.54 kg), but was higher for ewes mated on long drought pasture (2.76 kg; $P<0.05$). Staple length was not affected by nutritional treatments.

3.4.2 Experiment II

Pre and post-grazing masses of the herbage in willow fodder blocks (Table 3.7) was high and similar to that for willow fodder blocks in Experiment I. Both experiments had a similar pre-grazing dead matter content of approximately 300 g/kg. However, the control pasture was not a drought pasture, as it represented a typical pasture from the Wairarapa region of that year, with a pre-grazing pasture mass of 3,000 kg DM/ha, a low pre-grazing dead matter content of 180 g/kg and a high nutritive value (OMD 0.72; ME 10.3 MJ/kg DM). Fodder block herbage had a lower OMD (0.63) and ME (9.0 MJ/kg DM), whilst tree nutritive value was higher and similar to that in Experiment I. The CT content in trees was also similar to that in Experiment I.

Table 3.4 Changes in live weight (g/day) and body condition score (BCS) over the experimental feeding period, when ewes were grazed on control (short and long) pastures and willow fodder blocks (mean values with standard errors) (Experiment I)

		Control		Willow fodder block
	Days	**Short drought pasture**	**Long drought pasture**	**Full access**
Live weight change				
Primary growth + regrowth[1]	86	-17.0±3.34[c]	54.4±3.33[a]	-5.0±3.31[b]
Primary growth only[2]	77	-43.1±3.90[c]	86.8±3.92[a]	2.3±3.89[b]
Body condition score	86	-0.93±0.033[c]	-0.66±0.032[a]	-0.77±0.032[b]

[1]. Live weight change over the entire experimental period
[2]. Live weight change excluding the regrowth grazing period
[a, b & c]. Means within rows with different superscripts differ significantly (P <0.05)

Table 3.5 The effect of grazing ewes for 86 days, including mating, on control (short and long) drought pasture and willow fodder blocks on reproductive rate (expressed as a proportion of the total number of ewes exposed to the ram), conception rate, fecundity, mean lambing date and total lamb mortality at birth and weaning(mean values with standard errors) (Experiment I)

	Control		Willow fodder blocks	SEM
	Short Drought pasture	Long Drought pasture	Full Access	
Reproductive rate(no of lambs/100 ewes mated)				
Scanning	135[c]	173[a]	147[b]	
Lambing	116[c]	157[a]	134[b]	
Weaning	90[b]	113[a]	89[b]	
Conception rate[1]	91 [b]	98 [a]	96 [a]	1.61
Fecundity[2]				
Singles	53.3[b]	28.8[a]	49.7[b]	3.85
Twins	46.7 [b]	71.2 [a]	50.3 [b]	3.85
Average lambing date	23 Aug [b]	19 Aug [a]	20 Aug [a]	0.8
Lamb mortality[3] **(lambs dead/100 lambs born)**				
Birth	2.5 [b]	4.2 [b]	10.4 [a]	
(Range)	(1.0-2.6)	(2.3-7.5)	(6.9-15.4)	
Weaning	19.8 [b]	20.6 [b]	27.9 [a]	
(Range)	(14.6-26.3)	(15.9-26.2)	(22.1-34.4)	

[1] Expressed as ewes pregnant per 100 ewes mated, [2] Expressed as ewes per 100 ewes lambing
[3] Adjusted by sex, birth rank and lambing week, [a,b&c] Means within rows differ ($P<0.05$)

Table 3.6 The effect of grazing ewes on control (short and long) drought pasture and willow fodder blocks for 86 days during the late summer/autumn, including mating, on lamb birth and weaning weights (kilograms; mean values and standard errors) (Experiment 1)

	Control		Willow fodder blocks	SEM
	Short Drought pasture	Long Drought pasture	Full Access	
Birth weight				
Single male	6.2	6.0	6.0	0.13
Single female	5.6 [a]	5.1 [b]	5.8 [a]	0.16
Twin male	5.0 [ab]	4.8 [b]	5.2 [a]	0.09
Twin female	4.5	4.6	4.4	0.09
Weaning weight				
Single male	22.0	20.6	23.4	1.99
Single female	23.4	17.5	20.5	2.25
Twin male	17.5 [b]	16.6 [b]	22.0 [a]	1.61
Twin female	23.9 [a]	17.4 [b]	21.6 [a]	1.45

[a, & b] Means within rows with different superscripts differ ($P<0.05$)

Table 3.7 Pregrazing and post grazing mass (Kg DM/ha), dead matter content and Chemical composition of control pasture and willow fodder block (primary and regrowth) grazed during the experimental period of 30 days (mean values and tandard errors) (Experiment II).

	Control	Willow fodder block	
	Pasture	Pasture	Trees
Pasture mass and Dead matter content			
Pre-grazing mass	3081 ± 127.8	4978 ± 67.0	717 ± 177.0
Post-grazing mass	1789 ± 224.5	2999 ± 633.2	391 ± 89.4
Dead matter (g/kg)	182 ● 35.4	338 ± 30.6	
Chemical composition (Primary growth)			
N (g/kg DM)	32.9 ± 0.71	25.3 ± 1.40	18.0 ± 1.54
OM (g/kg DM)	882.8 ± 4.45	879.8 ± 3.97	926.2 ± 5.06
OMD	0.72 ± 0.008	0.63 ± 0.013	0.73 ± 0.014
DOMD	0.63 ± 0.009	0.56 ± 0.012	0.67 ± 0.010
ME(MJ/kgDM)	10.30 ± 0.147	9.06 ± 0.196	10.94 ± 0.171
CT(g/kgDM)	ND[1]	ND[1]	38.90 ± 2.051
Chemical composition (Regrowth)			
N (g/kg DM)		33.97	35.30
OM(g/kg DM)		863.2	911.0
OMD		0.666	0.805
DOMD		0.578	0.727
ME(MJ/kgDM)		9.42	11.85
CT(g/kgDM)		ND[1]	19.00

[1] ND: Not determined.

Table 3.8 Live weight change (g/day) and body condition score change (units), during the 30 day period of grazing control and willow fodder block and reproductive data at ultra sound scanning expressed as a percentage of the total number of ewes exposed to the ram (mean values and standard errors) (Experiment II)

	Control pasture	**Willow fodder block**
Animal body weight and condition score		
Initial liveweight (kg)	70.3 ± 0.57	70.3 ± 0.59
Liveweight change (g/d)	113.3 ± 6.53	32.2 ± 6.47
Initial BCS (units)	4.23 ± 0.032	4.15± 0.041
Change in BCS (units)	0.07 ± 0.047	-0.23± 0.047
Reproductive rate (no of lambs/100 ewes mated)		
Scanning	185	181
Conception rate[1]	93 ± 2.55	97 ± 1.71
Fecundity[2]		
Singles	20.4	21.7
Twins	79.6	78.3

[1] Expressed as ewes pregnant per 100 ewes mated
[2] Expressed as ewes per 100 ewes lambing

The LW gain of ewes grazing control pasture was 113 g/d, whilst grazing willow fodder blocks increased ewe LW gain by only 32 g/d ($P<0.05$). Reproductive rate at scanning was high, and similar in both the groups (Table 3.8), with no consistent differences in conception rate and fecundity at scanning.

3.5 DISCUSSION

Mating on willow fodder blocks was beneficial to sheep production and increased LW gain, conception rate and proportion of lambs born, compared to ewes mated on short drought pasture, as also found by Pitta et al. (2005), but was not as effective as mating on long drought pasture. Therefore, a mixed diet of herbage and trees was not as effective as a full diet of herbage fed at the same allowance under the conditions of this experiment.

The superior performance of ewes mated on long drought pasture relative to ewes mated on short drought pasture was due to its higher OMD, ME and legume content, relative to short drought pasture, and to its higher calculated ME intake (Table 3.9), producing increases in LW gain, lambing proportion and wool production. Ewes grazed on willow fodder blocks during mating had intermediate values in OMD and ME for primary growth fodder block herbage, but similar calculated total ME intake to long control ewes (Table 3.9), and consumed a diet of higher legume content. Calculated intakes of DM, CP and ME were similar for ewes grazing long drought pasture and willow fodder blocks (Table 3.9), and were almost double the calculated DM, CP and ME intakes from the study of Pitta et al.(2005) in 2003, whilst calculated CT intakes were much higher for ewes grazing fodder blocks. Reasons for the lower performance of ewes in willow fodder blocks, compared to the ewes in long drought pasture, are not clear. Future management of willow fodder blocks needs to keep the herbage under better control with lower herbage mass/ha, as was done for the regrowth in both Experiments 1 and 2. Whilst the OMD and ME

values for the willow eaten were high, this probably had a limited effect on animal production due to their low yield. This should increase in subsequent years with the increasing age of trees by a factor of 2.6/year (Douglas et al., 2003).

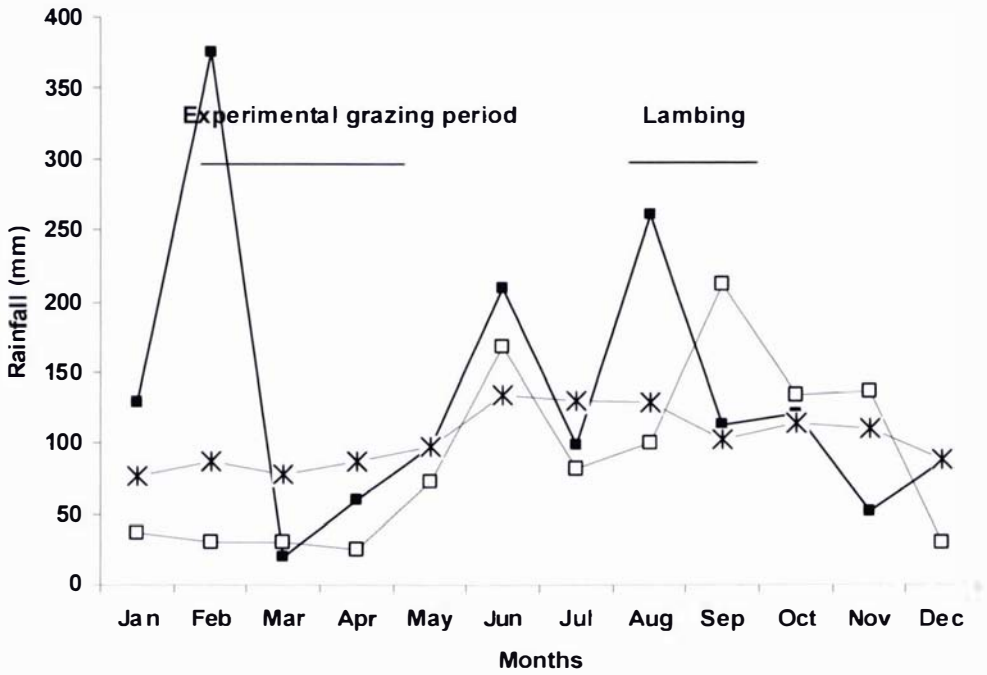

Figure 3.4 Pattern of rainfall recorded at Riverside Farm (Wairarapa) through out the years of 2003 (Pitta et al., 2005) and 2004 (this Experiment 1), compared with the long term average in the past 15 years. (□) 2003; (■) 2004; (*) long term average.

Although, ewes with full access to fodder blocks did not perform as well as ewes grazing long drought pasture during mating, full access to fodder blocks still provided a better feed supply than short drought pasture. Ewes grazing short drought pasture performed similarly to all other drought experiments (McWilliam 2004, Pitta et al., 2005) with low reproductive rate ($P<0.05$) and relatively high lamb mortality (20/100), despite 2004 not being a typical drought year (Figure 3.4). Higher than

Table 3.9 The effect of ewes grazing for 86 days during the late summer/autumn, including mating, on low quality control drought pastures (short and long) and willow fodder blocks on calculated dry matter intake (kg DM/ewe/day), calculated metabolisable energy (ME) intake (MJ ME/ewe/day), calculated crude protein (CP) intake (g/ewe/day) and calculated condensed tannin (CT) intake (g/ewe/day) and phenolic glycosides (g/ewe/day) (mean values with standard errors) (Experiment I)

	Control		Willow fodder blocks	
	Short drought pasture	**Long drought pasture**	**Herbage**	**Trees**
DM intake[a]	0.70±0.101	1.66±0.202	1.81±0.173	0.29±0.173
Total DM intake	0.7	1.66		2.1
CP intake[b]	109.9±19.44	238.2±30.08	204.7±32.81	26.0±3.46
Total CP intake	109.9	238.2		210.7
ME intake[c]	5.9±0.99	15.6±1.92	15.9±1.58	2.92±0.37
Total ME intake	5.9	15.6		18.8
CT intake[d]	1.31±0.07	3.40±0.262	6.50±1.570	11.10±1.195
Total CT intake	1.31	3.4		17.6
Phenolic glycosides[e]	3.11±0.683	15.29±1.983	9.28±2.257	10.59±1.765
Total Phenolic glycoside intake	3.11	15.29		19.9

[a] Estimated from pasture mass measurements before and after grazing;
[b] DM intake × CPconcentration in MJ/kg DM; [c] DM intake × ME concentration in g/kg DM
[d] DM intake × CT concentration in g/kg DM; [e] DM intake × PG concentration in g/kg DM

Table 3.10 The effect of grazing frequency per season upon the nutritive value of the diet selected by sheep grazing full access on fodder blocks

	No. of grazings	Closure of Willow fodder block [3]	Willow fodder block herbage			Trees		
			OMD	ME (MJ/ kg DM)	Total N (g/ kg DM)	OMD	ME (MJ/ kg DM)	Total N (g/ kg DM)
Pitta et al (2005)[1]	1	15 August 2002	0.54	8.0	18.0	0.72	10.7	15.5
This paper[1]								
Experiment I	1	7 October 2003	0.64	8.8	20.0	0.67	9.9	18.6
	2		0.74	10.6	38.2	0.57	8.5	16.3
Experiment II		7 October 2003						
	1		0.63	9.0	25.3	0.73	10.9	18.0
	2		0.66	9.4	33.4	0.80	11.9	35.3
*Diaz Lira (2005)[2]		15 October 2004						
	1		0.64	9.42	22.3	0.72	10.7	16.0
	2		0.67	9.83	24.2	0.68	10.2	16.2

* Diet select samples were not collected and hence nutritive data was not recorded but a third grazing was done
[1] Experimental grazing commenced mid February
[2] Experimental grazing commenced early December
[3] Exclusion of livestock at the time when tree growth commences in spring

normal late summer rainfall (Feb) in 2004 resulted in better control pasture nutritive value (Table 3.2) and lowered the ewe LW loss from typically 100 g/d to 40 g/d.

The benefit from trees in willow fodder blocks, relative to short and long drought pastures, is their unique concentrations of secondary compounds. Fodder block herbage was higher in CT concentration than control pastures, as also occurred in 2003 (Pitta et al., 2005). The main contributor in willow fodder block herbage is the likely growth of volunteer species of *L. pedunculatus*, containing high concentrations of CT (Figure 3.2). Herbage in both control pastures contained measurable concentrations of catechins and flavenoid monomers, but minimal concentrations of CT, suggesting that such pasture plants may lack the condensing enzymes to produce CT (Ramírez and Barry, 2005). For ewes grazing the fodder blocks, the trees provided approximately 0.12 and 0.15 of their calculated intakes of CP and ME respectively but 0.63 and 0.53 of their calculated intakes of CT and phenolic glycosides respectively.

Whilst ewes in full access to fodder blocks performed intermediate to long drought pasture and short drought pasture in conception rate and fecundity, this group recorded the highest lamb mortality at birth. Unusual weather conditions may also have influenced results of the present (2004) Experiment, where rainfall was higher than the long term average during both the experimental feeding period (including mating) and during lambing (Figure 3.4).

Full access to willow fodder blocks remains a useful supplement for ewes during mating in dry summer/autumns in NZ. Effective utilisation of willow fodder blocks is entirely dependent on proper and systematic grazing management and this

need to be developed further. A rotational grazing system was followed in this experiment, with ewes grazing the fodder blocks on primary growth during mating (i.e., summer/autumn) and grazing fodder blocks for a second time on regrowth. Delaying the closure of willow fodder blocks from further grazing by animals to mid October, compared with mid-August in the previous study, and using two grazings per season instead of one, increased the nutritional quality of the herbage (Table 3.10), and the dead matter content was reduced to 300 g/kg, compared to 600 g/kg for grazing once only (Pitta et al., 2005). These improvements in herbage nutritive value with increased frequency of grazing were achieved with no consistent change in tree nutritive value which, in most cases, exceeded that of fodder block herbage (Table 3.10).

3.6 CONCLUSIONS

Results of the present Experiments, and those of Pitta et al. (2005), suggest that grazing willow fodder blocks can be used to maintain ewes, and is a useful supplementary feed under drought conditions. In this application, use of willow fodder blocks should prevent reduction in reproductive performance, which would otherwise occur if ewes are mated whilst grazing short drought pasture (McWilliam et al., 2005). However for high levels of animal production in non-drought situations, willow fodder blocks may be inferior to a similar allowance of pasture only. Defining the grazing management of willow fodder blocks remains a priority for further research and others have shown that 2 or 3 grazings per season are needed to increase herbage nutritive value (Diaz Lira, 2005).

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CHAPTER 4.

EFFECT OF WILLOW SUPPLEMENTATION UPON PLASMA AMINO ACID CONCENTRATION IN EWES GRAZING DROUGHT PASTURES OF LOW NUTRITIVE VALUE

4.1 ABSTRACT

A grazing experiment was conducted for 10 weeks to study the effect of willow supplementation to ewes grazing drought pastures upon plasma amino acid concentration (μ mole/L) at Massey University's Riverside Farm, near Masteron, on the East Coast of New Zealand (NZ). Ewes of similar age and weight (59 kg) were assigned to two groups (n=7), with and without (control) supplementation of willow and Experimental grazing was carried for 10 weeks from early February until mid April, 2005. Live weight (LW) was recorded fortnightly and body condition score (BCS) was done monthly. Blood samples for the quantification of plasma amino acids were collected at 35 d and 70 d.

Both groups had a similar pre- grazing pasture mass (2000 kg DM/ha) and dead matter content (80%) with the diet selected containing 8.3 MJ ME/kg DM, typical of drought conditions. The willow was readily eaten, with intake averaging 0.26 kg DM/ewe/d. Willow had a superior nutritive value than short drought pasture and contained secondary compounds (CT; 41 g/kg DM). Both groups of ewes lost live weight at the rate of 50 g/d, which did not differ between treatments.

Plasma concentration of 3-MTH (88 vs 127 μ mole/L) at 35 d and non essential amino acids (NEAA; 1082 vs 1417 μ mole/L) at 35 d and (1155 vs 1324 μ mole/L) at 70d, were substantially lower ($P<0.05$) in willow supplemented ewes than control ewes. The concentration of branched chain amino acid (BCAA) and essential amino acid (EAA) were marginally higher ($P>0.05$) at both 35 and 70 d in supplemented ewes. It is concluded that the increased reproductive rate from willow supplementation in ewes grazing drought pasture as occurred in the previous

experiments might be partly explained by reduced body protein catabolism and increased plasma BCAA and EAA concentrations.

4.2 INTRODUCTION

Drought has become a common feature in East Coast areas of NZ and incurs losses of up to $ 14 per ewe at the farm level and approximately $250 million at the national level (McWilliam, 2004). Willows planted mainly for soil conservation on NZ farms (Hathaway 1986; Douglas et al., 1996) are now being used as supplementary forage, fed either as stem cuttings (Moore et al., 2003) or as fodder blocks (Douglas et al., 1996; Pitta et al., 2005). Feeding willow and poplar stem cuttings was beneficial to sheep (McWilliam et al., 2005) and to cattle (Moore et al., 2003) through reductions in their live weight losses and increases in lambing percentage in ewes. However the actual mechanism responsible for the enhanced production and reproduction is not known. A likely reason could be the presence of secondary compounds in willows viz., CT (38 g/kg DM) and PG (35 g/kg DM) (Pitta et al., 2007).

CT in forages are known to have beneficial effects in ruminant nutrition through binding to plant protein in the rumen, thus reducing microbial breakdown of plant protein and enhancing amino acid absorption from the small intestine (Min et al., 2003). PGs in Tagasaste (*Chamaecytisus proliferus)* were reported to have positive effects on rumen fermentation in vitro and increased rumen microbial populations (Edwards, 1999) due to use of the glucose component as a source of energy for microbial growth.

Objectives of the present investigation were to study the effect of feeding a supplement of willow stem cuttings to fistulated ewes grazing short drought pasture on the concentration of plasma amino acids, which is an index of protein absorbed

from the small intestines and to provide samples of rumen contents, which could be used in Chapters 5 and 6 to study the effect of willow supplementation upon aspects of rumen microbiology.

4.3 MATERIALS AND METHODS

4.3.1 Experimental design

A grazing study using 14 mixed age rumen fistulated Romney ewes was conducted at Massey University's Riverside Farm, near Masterton (New Zealand) on the North Island East Coast. Ewes grazing simulated short drought pastures (5-7 cm tall) with and without a supplement of willow stem cuttings (n = 7 /group). Both groups were offered 0.80 kg DM/ewe/d of dry, low quality pasture consistent with normal conditions in a drought. The supplemented group also received 1.40 kg/ewe/d, of fresh willow per day. Live weight (LW) and Body condition score (BCS) was recorded through out the experiment with samples for blood plasma taken at the mid point and at the end of the Experiment.

4.3.2 Ewes

Romney ewes of similar age, size and weight were prepared with rumen fistulae (63 mm internal diameter (id)) approximately 30 days prior to the start of the Experiment. All ewes were vaccinated with Salvexin ™ + B (Schering-Plough Animal Health Ltd., Upper Hutt, Wellington) before the experiment to prevent salmonella poisoning and were given Eweguard ™ (Fort Dodge New Zealand Ltd., Auckland) a combination 6-in-1 vaccine and anthelmintic drench.

The animals were fistulated and cared for 15 days at AgResearch in accordance with AgResearch's Animal Welfare guidelines and the experiment was approved by the

Agresearch Animal Ethics Committee. The ewes were then transported to Riverside Farm, Massey University, and grazed on normal pasture for 15 days before they were assigned to treatments.

4.3.3. Forages

4.3.3.1. Drought pasture

Drought conditions were simulated with perennial ryegrass/white clover pasture, grown on very shallow stony soil, as described in Chapter 3. Pasture was allowed to grow, mature and develop seed heads, thus decreasing the quality of the sward, and then grazed with non-experimental cows and ewes to reduce pasture mass to approximately 2000 kg DM/ha. The pasture thus achieved was high in dead matter content (50-70%) and low in nutritive value, typical of drought conditions.

4.3.3.2 Grazing management

Both treatment groups were moved in weekly breaks (i.e., experimental areas) and were shifted to a new break on the same day, using front and back electric fences. Pre grazing pasture mass was measured and plot area (ha) for each break was calculated using Equation 1:

$$\frac{\text{(no of ewes) x (daily DM allowance) x (no. of days/break)}}{\text{Initial pasture mass (kg DM/ha} - 500)} \quad (1)$$

In the above equation, the figure of 500 kgDM/ha was deducted, as this is as low as sheep are able to graze. Water was provided ad libitum to all groups from moveable water troughs. A feed allowance of 0.80 kg DM/ewe/d is below the

maintenance energy level for ewes grazing low quality perennial ryegrass/white clover pasture, as would typically occur in a drought.

4.3.3.3. Willow supply

Willow (Salix matsudana × alba, cultivar Tangoio) trimmings were delivered daily from the GreaterWellington Regional Council's Akura Nursery, near Masterton. The small stems (basal diameter < 15 mm) of willow were cut every two days from coppiced trees and stored in a room at 4°C to reduce dehydration and weight loss. Willow tree fodder, approximately 8.5 kg fresh weight of cultivar Tangoio, was weighed and fed daily to ewes in the willow supplemented treatment.

4.3.4. Forage measurements

4.3.4.1. Pasture

Pre- and post-grazing herbage mass was determined immediately before and after grazing each break, by cutting to ground level, eight random quadrats (590mm×295 mm), per treatment group per break, washed and then dried at 80°C for 18– 24 h. Six exclusion cages (approximately 1.4m×0.9 m) were placed in each break before grazing. Diet selected samples were hand-plucked from the exclusion cages, after grazing, to simulate the pasture diet consumed by the ewes. These samples were stored at -20°C for later nutrient analysis of the diet selected. Samples for pasture dead matter content were collected before grazing each break.

4.3.4.2. Willow

Willow fodder offered was weighed daily and samples were collected twice weekly, from fodder offered to willow supplemented group, then cut into 2 cm lengths and used to determine the DM content of the feed offered. The willow residue was

collected and weighed after the sheep had grazed each break and samples collected to determine DM content. Thus, the total amount of willow (kg DM) consumed could be calculated for each break. Diet selected samples for willow supplemented treatment were also pruned daily from the willow fodder on offer at a diameter that was consistent with the diameter consumed by the fistulated ewes, 3–5 mm, and cut into 2 cm lengths. The daily samples were pooled for each break and stored at -20°C for later nutrient analysis. The diameter of willow stem eaten was determined, at the end of grazing each break, by collecting 75 stems /treatment group and measuring the diameter eaten with electronic callipers (Mitutoyo Corp., Japan).

4.3.5. Animal measurements

Mean initial ewe LW and body condition score (BCS) was similar between the two groups with the control, and willow supplementation weighing 58.9 and 59.0 kg, with a BCS of 3.2 and 3.0 units, respectively. Ewes were weighed fortnightly using electronic scales (Tru-test, Auckland, New Zealand) during the period of supplementation and scored for body condition monthly from 1 to 5 (Jefferies, 1961).

Blood samples (5-10 ml) were taken from the jugular vein of all sheep from both treatments at 35 d (mid-way) and at 70 d (end) of the Experiment. The blood samples were collected into vacutainers with Na-EDTA (Becton Dickinson, USA) as an anticoagulant, placed on ice and centrifuged (3200 ***g*** for 20 min at 4 °C) to obtain plasma for plasma amino acid analysis, which was stored at - 20°C.

4.3.6 Laboratory Analyses

Willow and pasture diet select samples were freeze-dried and ground to pass a 1 mm diameter sieve. Total nitrogen (N) concentration was determined using the

Dumas method (Leco Corporation, USA 1994) and Organic Matter (OM) by ashing samples for 16 h at 550°C. NDF was determined by the detergent procedures of Robertson and Van Soest (1981) and Van Soest et al. (1991), with alpha amylase added (BDH, Poole, UK) during extraction and values expressed with residual ash. Sodium sulphite was not added. *In vitro* OM digestibility (OMD) was determined by the enzymatic method of Roughan and Holland (1977), using separate standard curves prepared from *in vivo* values for forages and from willow grazed by sheep. The ME in the diet select samples was calculated as 16.3 x digestible organic matter / 100g DM (DOMD; Drew and Fennessy 1980).

Willow samples were analysed for acetone/water-extractable, protein-bound and fibre-bound condensed tannin (CT) fractions, using the butanol-HCL colorimetric procedure (Terrill et al., 1992); total CT concentration was calculated by summing the three fractions. All CT concentrations were determined using CT extracted from *Lotus pedunculatus* as a reference standard (Jackson et al., 1996).

The plasma amino acid concentrations were analysed as per the method detailed in the user's manual of PCX 3100, Post-column derivatization instrument for amino acid analysis (Pickering Laboratories, CA, USA). Lithium citrate buffer (500µL, 0.24M) containing 5% sulfosalicylic acid (SSA) (w/v) was added to 500 µL of plasma and placed on ice for 15 min to deproteinize the plasma. pH was adjusted to 1.5 – 2 with 10 µL of 5.88M LiOH (lithium hydroxide), centrifuged and filtered through 13mm, 0.2 µm cellulose acetate filter to obtain a clear deproteinised supernatant, which was injected into the HPLC to effect the separation and quantification of the plasma amino acids.

All amino acids were analysed using a HPLC (Shimadzu LC10Ai, Japan) with a PCX 3100 post column reaction module (Pickering, CA, USA) using the Ninhydrin method. A lithium based ion-exchange resin column (3 mm ID (internal diameter) and 150 mm long) and a guard column (2mm ID and 20 mm long) were used (Pickering, CA, USA).The column was maintained at 37°C with a flow rate of 0.3 mL/min. The eluted amino acids were detected using UV-Vis Detector (SPD-10AV Shimadzu, Japan) and absorbance was measured at 440 nm for proline and hydroxyl proline (secondary amines) and 570 nm for all other amino acids (primary amines).

4.3.7 Statistical Analyses

Mean and standard errors for each of the variables describing chemical composition of the diet selected in each of the treatments were obtained from the GLM procedure of SAS (2003). Repeated data of individual LW and BCS of the ewes were analysed using the MIXED procedure of SAS (2003) fitting a mixed model including fixed effects of treatment and day of measurement.

The LW change and BCS change were analysed using the MIXED procedure of SAS (2003) with a linear model that considered effect of treatment. Plasma amino acid concentrations viz., BCAA, EAA, NEAA and 3-MTH were analysed using the PROC MIXED of SAS (2003) fitting a linear model including fixed effect of treatment and day of measurement. Regression equations for changes in DM, diameter of chewed willow and DM intake of willow over time for the willow supplemented group were estimated using the GLM procedure in SAS (2003).

4.4 RESULTS

Pre-grazing and post- grazing pasture mass and chemical composition of the diet selected for short drought pasture was similar in both groups, with mean values of 2075 kg DM/ha, 1500 kg DM/ha and 8.3 MJ ME/kg DM respectively. Pre-grazing dead matter content was approximately 80% (Table 4.1). Willow diet was superior in nutritive value to drought pasture, with higher N (23.4 vs 18.6 g/kg DM), lower NDF (376.4 vs 632.0 g/kg DM) and higher ME concentration (10.1 vs 8.4 MJ ME/kg DM). The willow diet selected contained 41 g/kg DM of CT (Table 4.1).

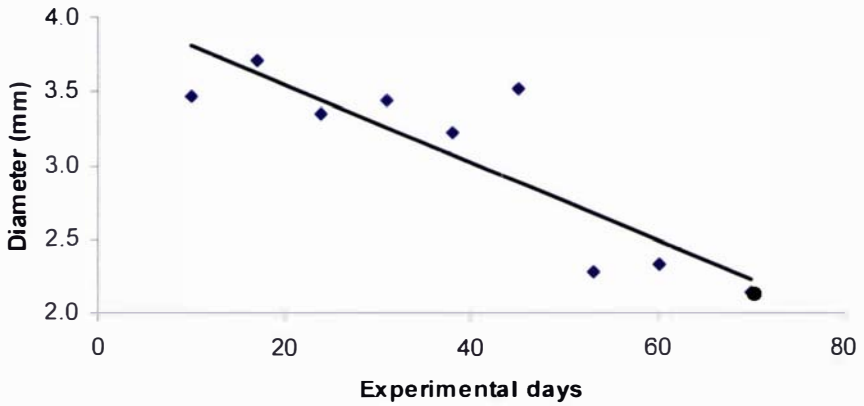

Figure 4.1. Change in diameter of willow chewed down (D; mm) with time by ewes in the willow supplemented group

D (mm) = 4.07 - 0.026 days (SE: 0.253, 0.0059; P *** ***) (2)

The diameter (D; mm) of willow chewed by ewes decreased (Fig 4.1; Equation 2; $P<0.001$) with time. However, the willow DM intake by ewes did not change with time and the mean value was 0.26 kg DM/ewe/d (SE: 0.051).

Table 4.1 Pasture mass, chemical composition of the diet selected and change in live weight (LWC) and body condition score (BCS) in ewes when grazing drought pasture with and without supplementation with willow during the experimental period (mean values with standard errors)

	Control	Supplemented	
	drought pasture	drought pasture	willow tree[1]
Pasture mass (kg DM/ha)			
Pre grazing	2076.5±186.37	2075.1±190.50	
Post grazing	1452.6±184.60	1542.4±156.36	
Pregrazing dead matter content (%)	82.9±4.25	80.2±6.40	
Chemical composition (g/kg DM)			
N	18.9 ● 2.64	18.6 ± 2.63	23.4 ± 0.21
[2] NDF	631.4 ± 52.29	632.3 ±44.69	376.4 ±3.80
[3]CT			40.8 ± 1.97
[4]OMD	0.57 ± 0.023	0.57 ± 0.020	0.68 ± 0.005
[5]DOMD	0.51 ± 0.019	0.52 ± 0.016	0.62 ± 0.005
[6]ME (MJ/kg DM)	8.31 ± 0.316	8.40 ± 0.268	10.11 ± 0.089
Animal performance			
LWC (g/d)	-57.96 ± 3.346	-50.00 ± 3.615	
BCS (units)	-0.143 ± 0.1687	-0.168 ± 0.1822	

[1] willow offered 1.4 kg fresh/ewe/day [2]NDF: Neutral Detergent Fibre; [3]CT: condensed tannins; [4]OMD: OM digestibility *in vitro*; [5]DOMD: Digestible OM in the DM *in vitro*; [6]ME: Metabolisable energy.

Table 4.2 Plasma concentration of amino acids (μ mole/L) in fistulated ewes grazing short drought pasture with (supplemented) and without (control) supplementation of willow for 35 and 70 days. Mean values with pooled standard error (SEM)

	35 days		70 days		
Aminoacids	Control	Willow	Control	Willow	SEM
Valine*	163.0	191.4	218.8	226.4	13.03
Isoleucine*	65.5	70.9	87.9	83.2	4.73
Leucine*	99.1	100.2	122.8	122.6	6.55
Tyrosine$^{\Psi}$	45.1	53.2	76.1	74.4	5.49
Phenylalanine*	40.2	41.7	57.3	59.0	4.44
Histidine*	49.3	57.2	59.9	64.4	3.46
Lysine*	114.3	143.3	193.0	185.9	18.28
Arginine*	93.7	116.5	188.5	167.9	13.27
Threonine*	98.2	111.4	130.0	152.5	11.51
Methionine*	16.8	16.5	26.7	25.4	2.10
Serine$^{\Psi}$	160.7	112.6	144.0	131.9	11.96
Glutamine$^{\Psi}$	176.9	184.7	143.2	128.3	15.08
Proline$^{\Psi}$	59.8	78.8	90.0	92.8	10.21
Glycine$^{\Psi}$	763.6	514.6	608.3	566.8	38.51
Alanine$^{\Psi}$	256.1	191.3	294.0	222.1	17.68
BCAA[1]	327.7	362.5	411.0	432.2	23.54
EAA[2]	785.2	902.4	1092.2	1161.6	71.62
NEAA[3]	1417.1[a]	1082.0[b]	1324.5[a]	1155.8[b]	46.00
3-MTH[4]	126.6[a]	88.5[b]	57.0	51.2	9.34

[a, b] within a row differ significantly ($P<0.05$); [1] Branched-chain amino acids (valine, leucine and iso-leucine); [2*] Essential amino acids (including BCAA); [3Ψ] Non-essential amino acids;[4] 3-MTH - 3 methyl histidine

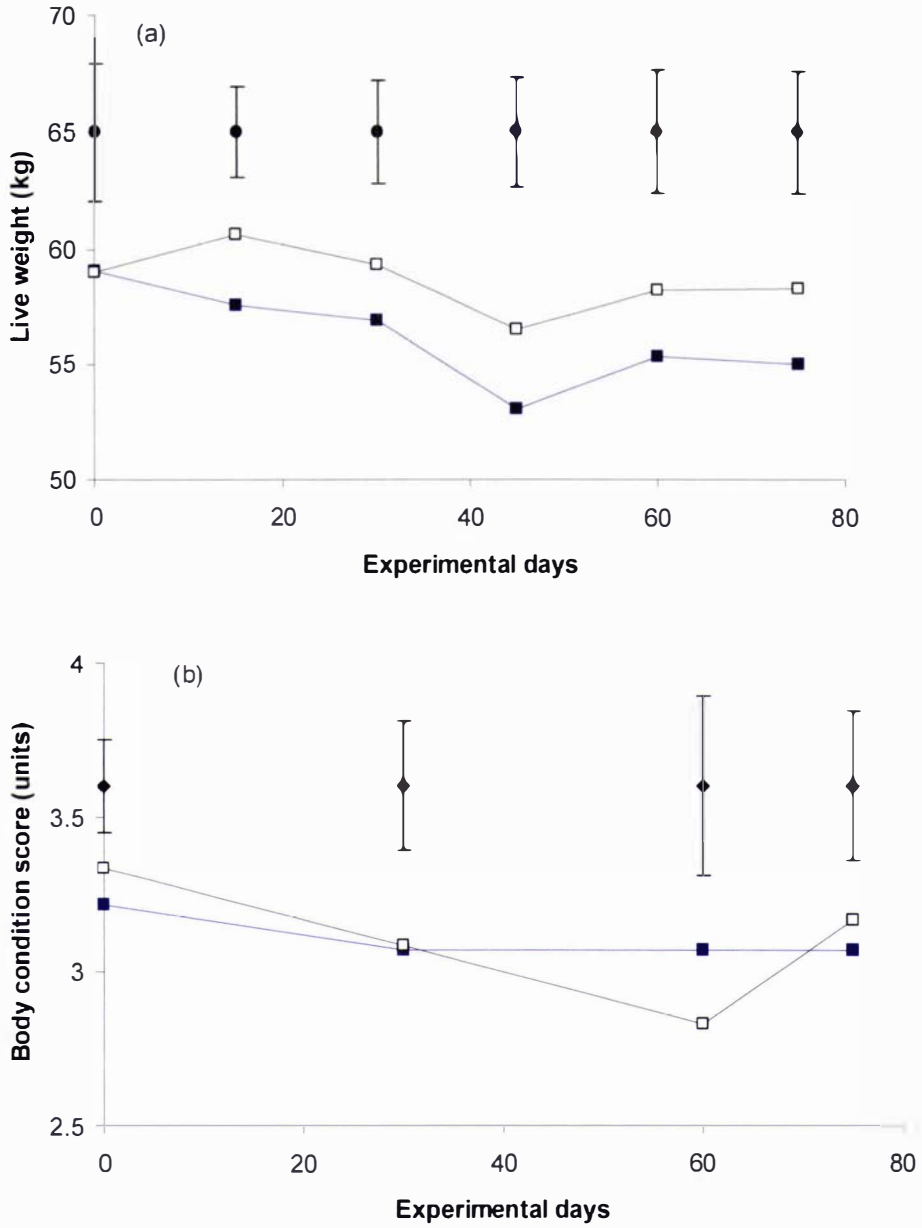

Figure 4.2 Changes in (a) mean ewe live weight and (b) body condition score in ewes grazed on short drought pastures with (supplemented) and without (control) supplementation of willow stem cuttings. (□) supplemented; (■) control; (I) indicates pooled standard error

Ewes in both groups lost similar amounts of LW (Table 4.1), which was not significantly different between groups. LW loss (Figure 4.2a) was greater earlier in the Experiment than later in the Experiment. Changes in BCS were minimal.

Plasma concentration (μ mole/L) of BCAA and EAA in willow supplemented ewes were higher than for control ewes at both 35 d and 70 d sampling time (Table 4.2), but these effects did not attain significance ($P>0.05$). Plasma concentration of NEAA (μ mole/L) were lower in willow supplemented ewes than control ewes ($P<0.05$), at both 35 d and 70 d.

Plasma concentration of 3-MTH was lower ($P<0.05$) in the willow supplemented group (88.5 μ mole/L) when compared to control (126.6 μ mole/L) at 35 d (Table 4.2). The concentration of 3-MTH reduced in both treatments to 57 μ mole/L in control and 50μ mole/L in willow supplemented group by 70d, with the difference being non significant ($P>0.05$).

4.5 DISCUSSION

The pasture grazed was typical of drought conditions (McWilliam et al., 2005), with a high dead matter content and a low ME concentration, resulting in live weight loss in grazing sheep. Whilst willow supplementation had no effect upon live weight loss, it did substantially reduce plasma NEAA and 3-MTH concentrations, indicating reduced degradation of body protein in ewes grazing low quality drought pastures.

A likely reason for the elevated plasma concentration of NEAA in control ewes is increased rate of body protein degradation, as indicated by the presence of 3-MTH at higher concentrations at 35d ($P<0.05$). The amino acid 3-MTH is present only in the actin and myosin structural proteins in the muscle and cannot be re-utilised in protein synthesis but is eliminated in urine (Harris and Milne, 1980). High concentration of 3-MTH in plasma and urine of ewes is an indicator of muscle protein breakdown (Harris and Milne, 1978). Its higher concentration in the plasma of control ewes (127 - 57 μ mole/L) than the normal range of 49 - 23 μ mole/L (Harris and Milne, 1980) indicates increased rate of body protein degradation in these ewes fed drought pasture.

Plasma concentrations of BCAA and EAA were higher for ewes in willow supplemented compared to control ewes at both 35d and 70d, however the difference failed to reach statistical significance. To detect these treatment differences in plasma concentrations of BCAA and EAA at the 5% level of probability with a power of 80%, it has been calculated that the number of ewes required per treatment should be increased to 24 in future studies. Increased concentrations of plasma BCAA and EAA in ewes fed *Lotus* spp was attributed to the presence of CT in *Lotus corniculatus* (Min et al., 1999).

Greater rates of live weight loss were observed earlier than later in this study (Fig 4.2a) and this was also true for plasma 3-MTH concentration (Table 4.2). Whatever the reason for these time trends, it is unlikely to be due to the amount of forage tree consumed, which did not change with time even though the diameter eaten declined with time.

It is concluded that the increase in reproductive rate from willow supplementation of ewes grazing drought pasture during mating (McWilliam, 2004) is associated with reduced plasma concentration of NEAA and 3-MTH and possibly increased plasma concentration of BCAA and EAA. Also, the reduced concentrations of 3-MTH at 35d in willow supplemented ewes is an indication of reduction in the catabolism of muscle protein and appears to be the reason for the reduction in their live weight loss.

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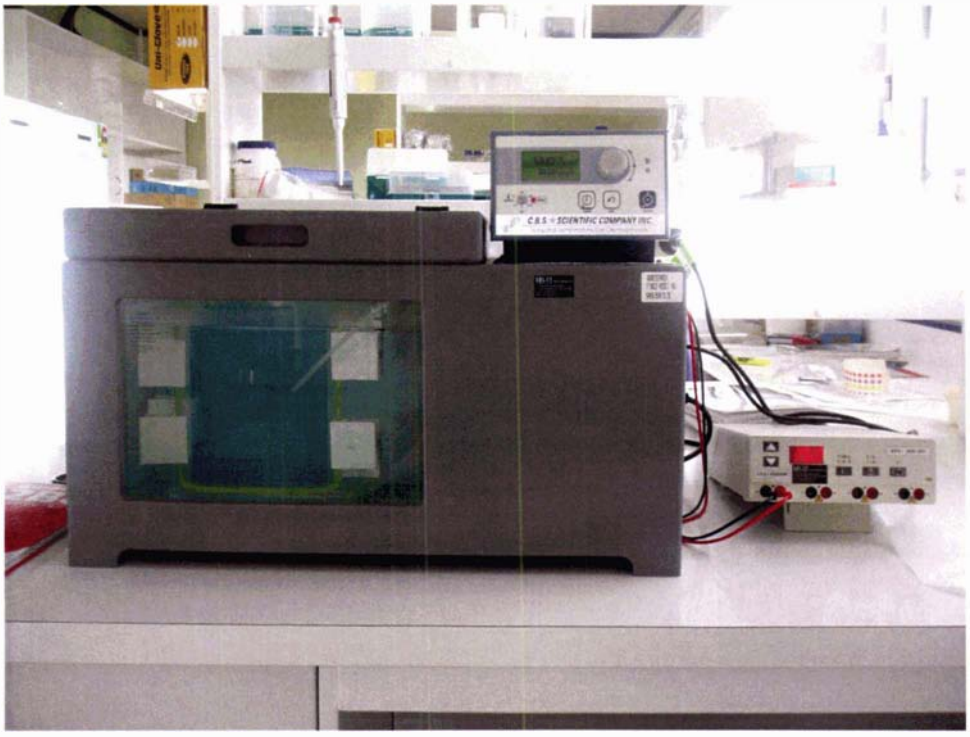
CHAPTER 5.

COMPARISON OF RUMEN MICROBIAL PROFILES USING PCR-DGGE IN FISTULATED SHEEP GRAZING DROUGHT PASTURE WITH AND WITHOUT SUPPLEMENTATION OF WILLOW



5.1 ABSTRACT

Willow trees are used as supplementary feed to ruminants during dry summer and autumn months in New Zealand and are reported to cause an increase in reproductive rate and decreased live weight losses compared to animals grazed solely on drought pasture. However, the effect of this feeding practice on bacteria in the rumen is not known. An animal trial was conducted to study the effects on the rumen bacteria of feeding willow supplements to fistulated sheep grazing low quality drought pastures. Rumen bacterial populations were examined using a denaturing gradient gel electrophoresis (DGGE) technique.

DGGE analysis of the V3 regions of DNA extracted from samples of rumen contents showed a discernable difference in banding patterns between treatment groups which progressively developed over a 10 week feeding period. However, phylogenetic analysis of the DNA sequences retrieved from the DGGE bands did not cluster by treatment group, indicating that willow supplementation does not induce shifts to completely different bacterial populations but rather selects for related sub-populations of organisms already present in the rumen. The changes in the rumen bacterial populations is attributed to the ability of these bacteria to metabolise secondary compounds in willow such as phenolic glycosides and flavanoid monomers and their ability to resist the inhibitory effects of CTs.

5.2 INTRODUCTION

Willow and poplar trees were originally planted in NZ for animal shelter and soil conservation purposes (Charlton et al., 2003) and are now being used as supplementary feed to livestock during dry summer and autumn in the East Coast regions of the North Island (Moore et al., 2003; McWilliam, 2004). McWilliam (2004) reported an increase in reproductive rate of 20% units and reduced live weight loss in willow supplemented ewes grazing low quality drought pasture compared to ewes that were grazed solely on drought pasture. A direct mechanism for the enhanced reproductive rate in ewes is the simple increase in DM (dry matter), ME (metabolisable energy) and CP (crude protein) intakes. A further mechanism for the increased ewe performance is attributed to the higher concentrations of secondary compounds found in willow stems in the form of CT (38g/kg DM), PG (35g/kg DM) and FM (14g/kg DM) compared to those in drought pasture (CT; 2.6g/kg DM, PG; 1.7 g/kg DM, and FM; 2.2 g/kg DM).

Ewes adapted to willow supplementation over a period of 10 wks, showed a consistent increase in DM intake (McWilliam, 2004; Pitta et al., 2005, 2007) and a progressive increase in diameter of the willow chewed from 3 mm to 5 mm. The increased performance in livestock when supplemented with willow has been reported in field production experiments but no work has been done on aspects of rumen microbiology. Therefore, the present work was conducted to study the rumen microbial populations during willow feeding and to compare the rumen microbial profiles over time using DGGE, a molecular typing technique which is useful for following changes in microbial populations in gastrointestinal environments.

5.3 MATERIALS AND METHODS

5.3.1 Grazing trial

A grazing study using 14 mixed age fistulated ewes was conducted at Massey University's Riverside Farm, Masterton, New Zealand. The ewes (Romney, of similar age, size and weight, 7 animals per treatment group) were prepared with rumen fistulae (63 mm internal diameter (id) in accordance with AgResearch's Animal Welfare guidelines and approved by the Animal Ethics Committee) approximately 30 days prior to the start of the experiment. All ewes were vaccinated with Salvexin ™ + B (Schering-Plough Animal Health Ltd., Upper Hutt, Wellington, NZ) and were given Eweguard ™ (Fort Dodge New Zealand Ltd., Auckland, NZ), a combination 6-in-1 vaccine and anthelmintic drench. The ewes were grazed on normal pasture for 15 days before they were assigned to treatment groups. During the experiment the animals grazed simulated short drought pastures of low nutritive value and containing >50 % dead matter, with (supplemented) or without (control) willow stem cuttings as supplemental feed. The willow stems were offered daily over 70d from 9 February 2005 (late summer) to 18 April (autumn) 2005. Short drought pasture was prepared by grazing the pasture with non experimental animals, to a pre-grazing pasture mass of 1200-1400 kg DM/ha and a sward height of 5-7 cm, which is typical of drought conditions in the area of the experiment. Both groups were offered approximately 0.80 kg DM/ewe/d of the short drought pasture using weekly break feeding. The supplemented group received 1.40 kg/ewe/d of fresh willow while the control group was offered no willow.

5.3.2 Rumen sampling

Weighed samples of rumen contents for the molecular study were taken fortnightly over 10 weeks, approximately 9am before feeding the sheep and immediately transported to the laboratory, freeze-dried, ground and stored at -72°C.

5.3.3 Molecular study

5.3.3.1 DNA extraction

Extraction of DNA from the freeze-dried ground rumen samples was performed using modified cetyl-trimethyl ammonium bromide (CTAB) method as previously described (Min et al., 2002). Briefly, the method consisted of homogenising the rumen sample with 2 × CTAB-lyzing buffer followed by 1× CTAB-lyzing buffer. The samples were shaken for 5 min, then physically disrupted using a Mini-beadbeater (Biospec Products Ltd.) at maximum speed for two intervals of 2 min each, with a 1-min incubation on ice between each treatment. The bead-beaten mixture was centrifuged at 12 000 × *g* for 10 min at 20°C and the upper aqueous phase was recovered. The interface layer was re-extracted with 1× CTAB buffer and the resulting upper aqueous phase pooled with the first aqueous phase. Proteinase K was added to a final concentration of 50 μg /ml, and the mixture was incubated for 1 h at 60°C. A final chloroform – isoamyl alcohol (24:1; wt/vol) extraction was performed before the nucleic acids were precipitated with an equal volume of CTAB–precipitation buffer (50 mM Tris–hydrochloride, 10 mM EDTA, and 1% (wt/vol) CTAB). The precipitate was recovered by centrifugation at 8000 × *g* for 10 min at room temperature. The pellet was dispersed with a pipette tip and washed three times with 500 μL of cold 0.4 M NaCl. The DNA pellet was dissolved in 100 μL of 2.5 M ammonium acetate at 50°C. RNase A was added to a final concentration of 1 mg /ml and the tubes incubated at 37°C for 30 min. RNase A was removed by phenol–chloroform – isoamyl alcohol (25:24:1, vol/vol/vol) extraction

followed by ethanol precipitation and centrifugation at 12 000 × *g* for 20 min at 4°C. The air-dried DNA pellet was resuspended in 100 μL of sterile water and stored at –20°C until amplification by PCR.

5.3.3.2 PCR amplification of 16S rRNA gene V3 region

The primers, annealing temperatures and PCR protocol used in this study were previously described in Yu and Morrison (2004). The hyper-variable region (V3) of the *16S* rRNA gene was amplified using forward primer 357f*a* CCT ACG GGA GGC AGC AG (*E.coli* numbering 341–357) and reverse primer 518r ATT ACC GCG GCT GCT GG (518–534). All PCR amplifications were performed using a PTC-100 thermocycler (MJ Research, Waltham, Mass.) in 50-μl volumes containing 1× PCR buffer (20 mM Tris-HCl [pH 8.4] and 50 mM KCl), 200 μM deoxynucleoside triphosphates, 500 nM of each primer, 1.75 mM $MgCl_2$, and 1.25 U of Platinum *Taq* DNA polymerase (Invitrogen Corporation, Carlsbad, CA, USA), which allows for hot-start PCR. After an initial denaturation at 94°C for 5 min, 10 cycles of touchdown PCR were performed which consist of : denaturation at 94°C for 30 s, annealing at 61°C for 30 s with a 0.5°C/cycle decrement at a temperature 5°C above the annealing temperature at 56°C and extension at 72°C for 1 min, followed by 25 cycles of regular PCR (94°C for 30 s, 30 s at 56°C, and 72°C for 1 min and a final extension step for 20 min at 72°C). Negative controls, containing all the components except DNA templates, were included in parallel reactions. The final PCR product is approximately 200bp in length, and these were visualised after PCR by electrophoretic separation of 3-5μl aliquots of each PCR reaction in 1.5% (wt/vol) agarose gels.

5.3.3.3 Cleaning of PCR product

PCR products were cleaned to remove ssDNA remaining from the PCR reaction using Mung Bean nuclease (New England Biolabs, Ipswich, MA, USA) as described by Simpson et al. (1999). The reaction mixture contained 15 μl PCR product, 3 μl Mung Bean buffer, 1.5 μl Mung Bean nuclease (diluted 1:50 in nuclease dilution buffer), and high purity deionized H_2O to 30 μl. The mixture was incubated at 37°C for 10 min. After incubation, 10 μl of DGGE gel loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol (wt/vol) in H_2O) was added to stop the reaction. The mixture was then stored at -20°C until analysis by DGGE.

5.3.3.4 Denaturing gradient gel electrophoresis (DGGE)

DGGE is an electrophoretic separation method which relies on differences in the melting behaviour of double stranded DNA fragments (Fisher and Lermann, 1979). The electrophoresis takes place in a vertical polyacrylamide gel in a gradient of denaturants at elevated temperature (60°C). Hyper-variable region V3 of the 16*S* rRNA gene was amplified using PCR and separated in 6.5% (wt/vol) polyacrylamide gels with 1 × TAE buffer (20 mM Tris–acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na EDTA) with 25–60% linear gradients of denaturant (100% denaturant corresponds to 7 M urea and 40% deionized formamide).

DGGE was performed with a TTGE/DGGE system (Model TTGE-2401; C.B.S. Scientific Company, Inc, Del Mar, CA, USA). The conditions used to run DGGE in this study for separating rumen microbial communities were previously described in Yu and Morrison (2004) which are discussed below. The gels were 1.5 mm thick, cast between glass plates with 6.5% polyacrylamide and were run at 60°C at 150 V for 6.5 h. The best separation of V3 DNA's was achieved with denaturant concentration

ranging from 25 to 60% in 6.5% polyacrylamide gel. The composition of the lowest and highest denaturant are presented in Table 5.1

Table 5.1 Chemical composition of the lowest (25%) and highest (60%) denaturing solutions used to make the denaturing gradient

Chemical	**25%**	**60 %**
7M Urea	2.6 g	6.3 g
40% Formamide	2.5 ml	6.5 ml
6.5% polyacrylamide	4.05 ml	4.05 ml
50% TAE	0.5 ml	0.5 ml
Distilled water	15.35 ml	7.65 ml

Polyacrylamide gels were formed between two glass plates separated by 1.5mm spacers and held together with a gasket in a casting stand. Both the low and high denaturant solutions were prepared, vortexed for 5mins and kept at 4°C for degassing. Before casting the gel, 95µl of 10% ammonium persulphate (APS), 95 µl of D-code dye and 55 µl of TEMED (N,N,N,N,-Tetramethyl ethyl diamine) were added to each of the denaturant solution. A gradient of denaturant was formed with a gradient maker and the flow rate from the gradient maker to the gel casting assembly was regulated with a peristaltic pump. A comb (1mm thickness) was inserted into the top of the gel and a small amount of butanol mixed in water was added just above the comb for better well formation. The gels were allowed to stand for approximately 30 min for polymerisation.

Prior to loading the samples onto the gel, 1× TAE (24 L) was used to fill the DGGE tank and allowed to equilibrate to 60^0C. The glass plate assembly was loaded into the DGGE apparatus and the wells of the gel were flushed with 1× TAE to remove excess urea. Amplified V3 DNA fragments were loaded into the wells at 15 to 20µl per lane. The voltage was set at 150V and the gel was typically run for 6.5 h or until the dye reached the bottom of the gel.

After completion of electrophoresis, gels were transferred carefully to the staining container and stained with SYBR GOLD (10,000X conc in DMSO; 3 µl stain diluted in 15 ml water. Gels were kept on a shaker for 10 min and destained in fresh water for 5 min. Gels were photographed using a Gel Logic 200 imaging system (GL 200, Eastman Kodak Company, Rochester, NY).

5.3.3.5 DGGE Standard DNAs

Rumen microbes with a wide range of G+C % content were selected to construct a DNA standard to act as a marker for DGGE. *Methanobrevibacter ruminantium*, strain M1 (DSM 1098, 24% G+C), *S. bovis* strain JB1 (26-37% G+C), *Clostridium perfringens* (40% G+C), *Megasphaera elsedenii* strain T81 (53.6% G+C) *and Bifidobacterium animalis* DSM 20104 (64% G+C) were grown and their DNA extracted. The 16*S* rRNA gene V3 region of each DNA was amplified individually as described previously. Each amplified V3 region was analysed separately by DGGE to determine their relative migration within the gel. Subsequently, equal amounts of each amplified V3 region were mixed and run in a single DGGE lane to provide a ladder of DNA standards. These standard DNA's were used as a reference to normalise bands between the DGGE gels.

5.3.3.6 Analysis of DGGE DNA bands

The digital images of the DGGE gels were analyzed to detect DNA bands using the band-searching algorithm of BioNumerics software (BioSystematica, Tavistock, Devon, UK). After normalization of the gels relative to internal standards, only those bands with a peak height intensity exceeding 2.0% of the strongest band in each lane were included in further analyses. A similarity matrix (Pearson's coefficient) was calculated from the densitometric curves.

5.3.3.7 Sequencing DNA from DGGE bands

DGGE bands that were either common or unique to both control and willow supplemented rumen DNA samples were manually excised using a sterile scalpel blade and transferred into a 1.5 ml Eppendorf tube with 50 µl of sterile water and frozen at -20°C. The frozen gel slices were allowed to thaw at room temperature and after a brief spin for 1 min in a bench-top micro centrifuge at 11,000 rpm. The supernatant was recovered and used as a template for PCR's using the primers 357F (no GC clamp) and 518R. The products of the PCR were cloned using the TOPO TA cloning kit (Invitrogen, Auckland, NZ) and the ligated products were transferred into competent *Escherichia coli* DH5α cells. Insert-containing clones were picked and grown, plasmid DNA extracted and used for sequencing. Sequencing was done using BigDye™ Terminator Cycle Sequencing version 3.1 with Ready Reaction Sequencing kit according to the manufacturer's instructions (Applied Biosystems, CA, USA) The primer M13f (0.1 IM) was used for the sequencing and products were analysed using a 310 Genetic Analyzer (Applied Biosystems, CA, USA).

5.3.4 Feed Analyses

Pre-grazing and post-grazing pasture mass measurements and diet select samples of pasture and willow were collected as described in previous Chapters. Concentrations of total nitrogen, NDF, *in vitro* organic matter digestibility (OMD), ME and CTs present in the diet selected samples of pasture and willow samples were measured as described in Pitta et al. (2005, 2007). Willow and pasture samples and rumen samples collected at 0, 2, 4, 6, 8 and 10 weeks were analysed for salicin, PGs and FMs using high-performance liquid chromatography (HPLC). Samples were processed following the method of Meier et al. (1988). Briefly the HPLC analysis involved the elution of 10 µL sub-samples on a 150 mm × 2.1 mm Reverse Phase (C18)

Phenomenex Luna column (Phenomenex, North Shore, NZ) in a Shimadzu LC-MS QP8000 α equipped with a Shimadzu SPD-M10A VP PDA detector (Shimadzu, Kyoto, Japan). The elution solvents were 0.1% formic acid in water (Buffer A) and 0.1% formic acid in acetonitrile (Buffer B), with the following gradient: initial concentration 5% Buffer B to 5 min; 10% Buffer B to 10 min; 17% Buffer B at 25 min; 23% Buffer B at 30 min; 30% Buffer B at 40 min; 97% Buffer B at 48 min and 97% Buffer B at 53 min; at a flow rate of 0.2 ml/min. Concentrations of PGs and FMs were estimated by integration of chromatographic peaks detected at 275 nm relative to that for resorcinol as the internal standard.

5.4 RESULTS

5.4.1 Feed Analysis

Ewes in both control and willow-supplemented treatments grazed a low quality pasture with a pre-grazing pasture mass of approximately 2000 kg DM/ha and a dead matter content of 80%. The analysis of feed composition (Table 5.2) showed a low nutritive value for short drought pasture compared to the supplementary willow. Plant phenolic compounds (CT, PGs and FMs) were present at very high concentrations in willow compared to drought pasture. Salicin, PGs and FMs concentrations (Figure 5.1) in the samples of rumen contents from willow-supplemented ewes reached a maximum at the end of Week 2 and thereafter declined to a level similar to that seen in control animals.

Table 5.2 Pasture mass, chemical composition (primary and secondary compounds)of the diet selected and change in live weight (LWC) and body condition score (BCS) in ewes when grazing drought pasture with and without supplementation with willow during the experimental period (mean values with standard errors)

	Control	Willow	
	drought pasture	drought pasture	willow stems[1]
Pasture mass (kg DM/ha)			
Pre grazing	2076.5±186.37	2075.1±190.50	
Post grazing	1452.6±184.60	1542.4±156.36	
Pregrazing dead matter content (%)	82.9±4.25	80.2±6.40	
Chemical composition (g/kg DM)[†]			
N	18.9 ± 2.64	18.6 ± 2.63	23.4 ± 0.21
[2] aNDF	631.4 ± 52.29	632.3 ±44.69	376.4 ±3.80
[3]OMD	0.57 ± 0.023	0.57 ± 0.020	0.68 ± 0.005
[4]DOMD	0.51 ± 0.019	0.52 ± 0.016	0.62 ± 0.005
[5]ME (MJ/kg DM)	8.31 ± 0.316	8.40 ± 0.268	10.11 ± 0.089
Secondary compounds (g/kg DM)			
[6]CT			40.8 ± 1.97
Rutin	0.05±0.043		1.08±0.518
[7]FM	0.65±0.128		32.56±10.191
[8]CA	0.17±0.103		2.87±1.495
Salicin	ND		1.24±0.666
[9]PG	2.36±1.440		21.57±10.020
Animal performance			
LWC (g/d)	-57.96 ± 3.346	-50.00 ± 3.615	
BCS (units)	-0.143 ± 0.1687	-0.168 ± 0.1822	

[1] willow offered 1.4 kg fresh/ewe/day
[2]aNDF: Neutral Detergent Fibre; [3]OMD: OM digestibility *in vitro*; [4]DOMD: Digestible OM in the DM *in vitro*; [5]ME: Metabolisable energy; [6]CT: condensed tannins; [7]FM: total flavanoid monomers; [8]CA: chlorogenic acid; [8]PG: other phenolic glycosides.
Note: [†] secondary compound values for both drought pastures is assumed to be the same.

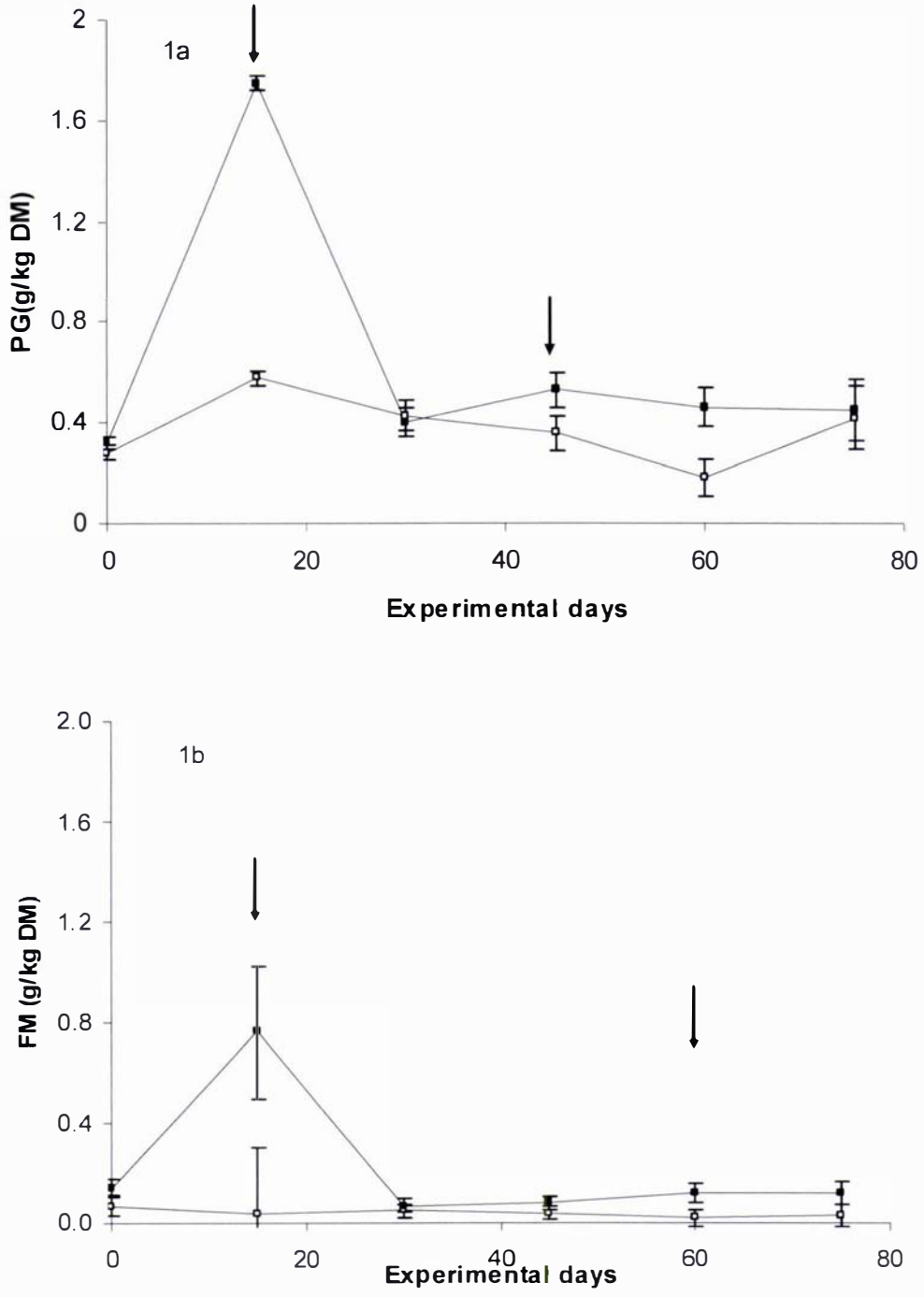


Figure 5.1 Change in concentration (g/kg DM) of (a) phenolic glycosides (PGs) and (b)flavanoid monomers (FMs) in the rumen samples of willow supplemented ewes (■) and control ewes (□) with time. (I) indicate pooled standard error (↓) indicate significance ($P<0.05$)

5.4.2 DGGE analysis of bacterial population changes during diet adaptation

To investigate changes in rumen bacterial populations during diet adaptation, DNA extracted from rumen samples collected from control and willow-supplemented ewes during the 10 week experimental period were analysed using DGGE separation of PCR-amplified hyper-variable V3 regions of bacterial 16*S* rRNA genes.

The banding patterns observed from each sample was normalised relative to internal standards, the banding similarity between samples was analysed using Pearson's Coefficient. The results show a progressive change from banding patterns being indistinguishable between control and willow-supplemented in Week 0 through to distinctly different patterns by Week 10 (Figure 5.2). More bands were common between the willow-supplemented and unsupplemented groups than were different. Only one unique band was found exclusively in the willow-supplemented group at the Week 2 sampling point. More unique bands appeared in the willow-supplemented group over the course of the supplementation period so that by Week 10 their DGGE banding pattern was significantly different. The bands uniquely present in the willow supplemented group and the bands common between the treatment groups were excised, cloned and DNA sequenced. A DGGE gel showing both unique and common bands that have been excised is shown (Figure 5.3). The cloned sequences were used to construct a phylogenetic tree showing the relationship of these retrieved sequences to 16*S* rRNA gene V3 variable region sequences of characterised rumen bacteria (Figure 5.4). The details of the excised bands and their cloned sequences with their similarity to other reports are presented (Table 5.3). Many of the sequences both common and unique to willow supplemented animals showed closest similarity to sequences from 16*S* rRNA gene clone libraries generated from DNA extracted of rumen contents of Holstein heifers fed a concentrate-ryegrass diet (Tajima et al., 2007).

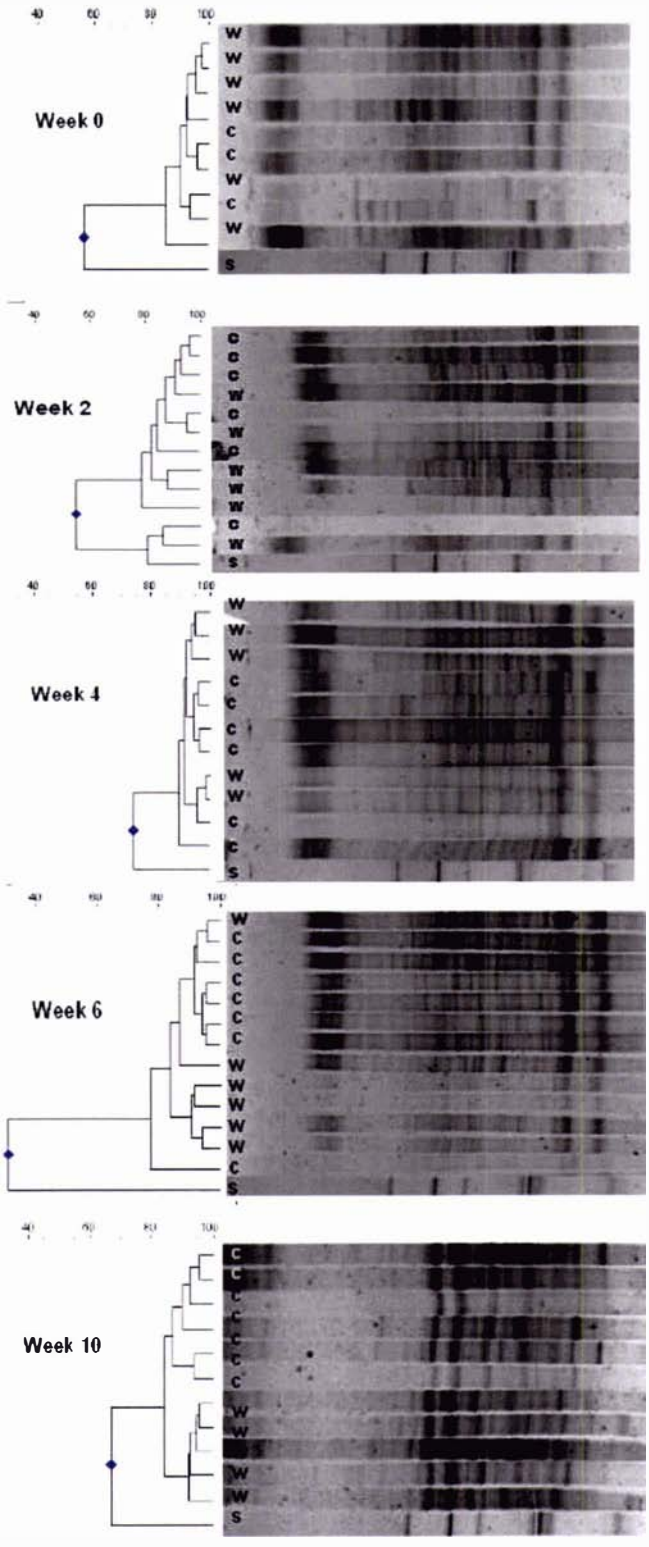


Figure 5.2 DGGE gels of the PCR amplified, hyper-variable region (V3) of 16*S* DNA from rumen samples in control and willow supplemented ewes collected at weeks (0, 2, 4, 6 and 10) along with their similarity matrices (Pearson's Coefficient).

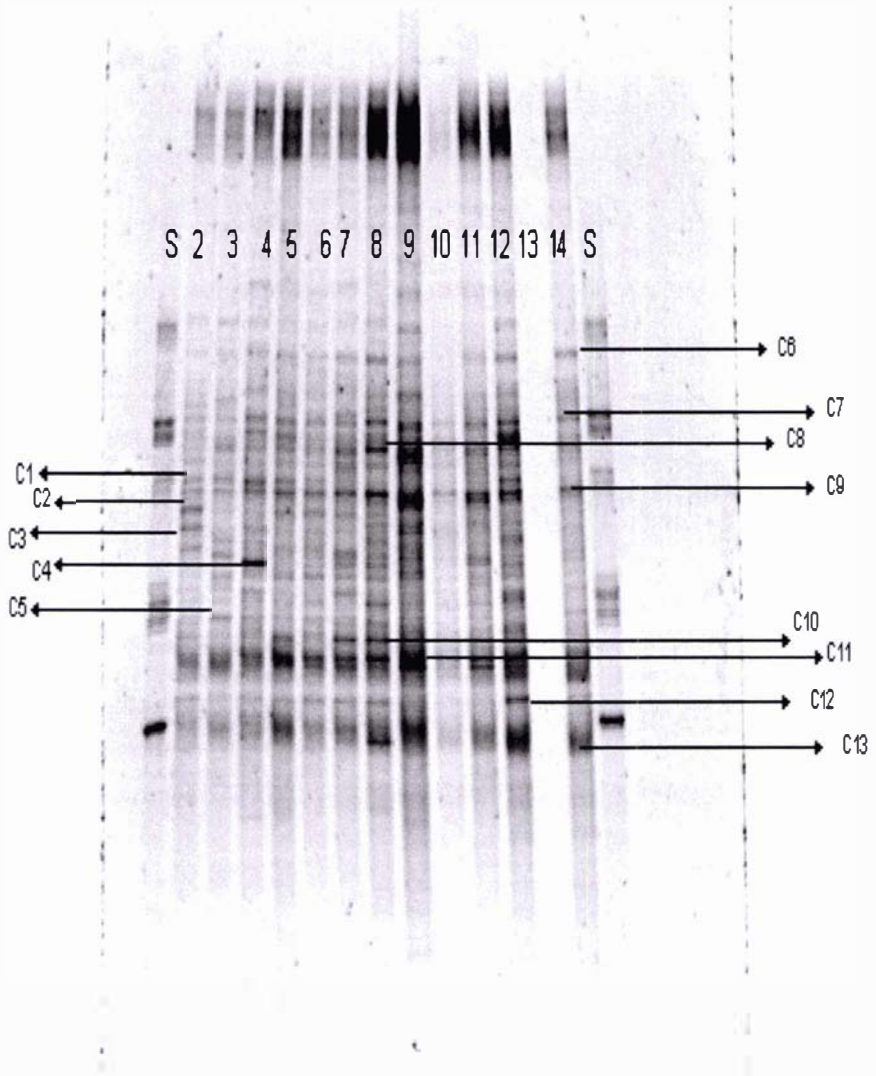
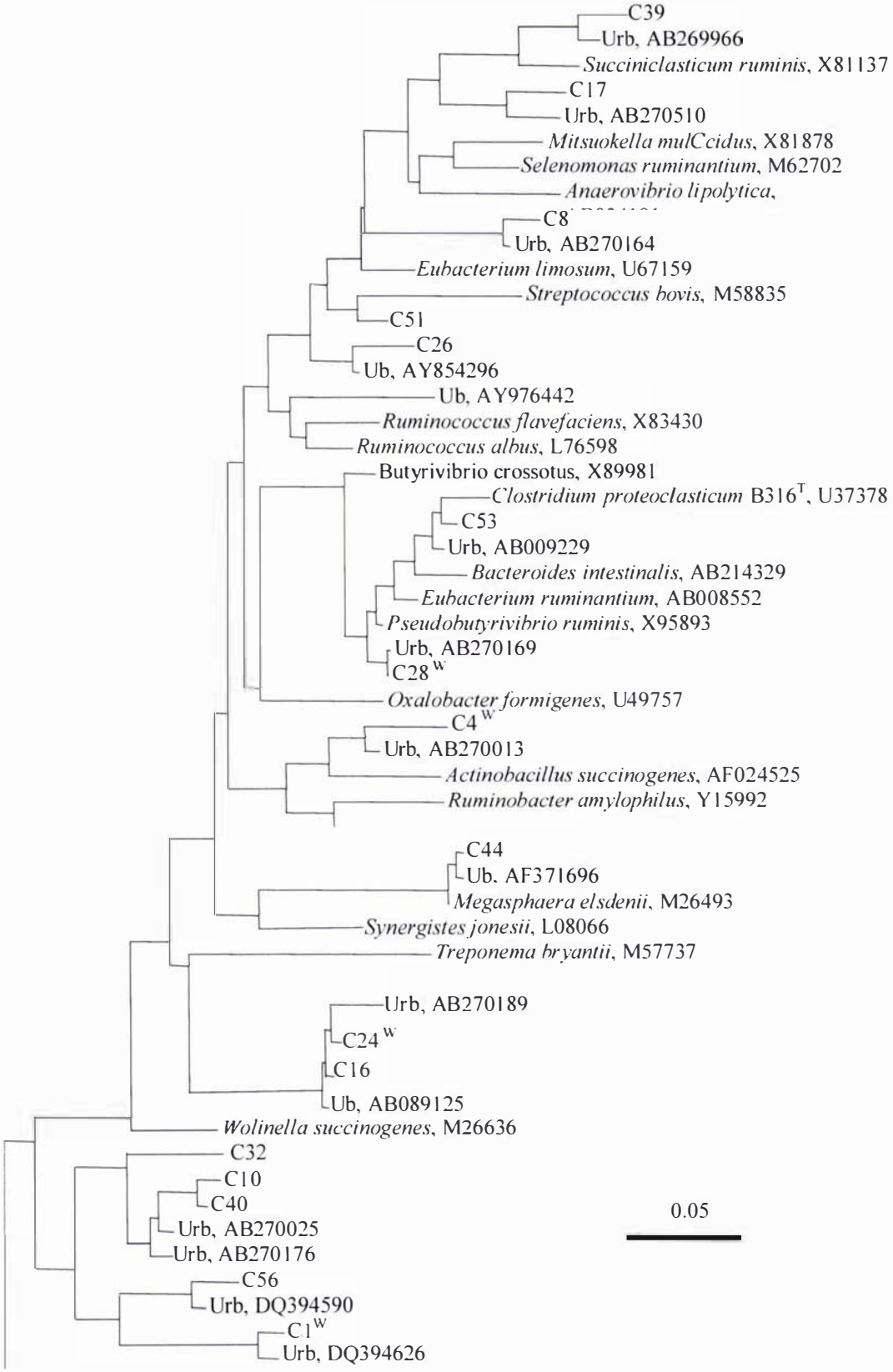


Figure 5.3 A DGGE gel (Week 4) of the PCR amplified, hyper-variable region (V3) of 16*S* rRNA genes from rumen samples collected from willow-supplemented animals (lanes 2-7) and control animals (lanes 8-14). Lanes labelled with S contain standard DNAs. The bands marked from C1-C5 are unique to willow-supplemented animals and bands marked C6-C13 are common to both control and willow-supplemented animals were excised and selected for sequencing.


C39
Urb, AB269966
Succiniclasticum ruminis, X81137
C17
Urb, AB270510
Mitsuokella mulCcidus, X81878
Selenomonas ruminantium, M62702
Anaerovibrio lipolytica,
C8
Urb, AB270164
Eubacterium limosum, U67159
Streptococcus bovis, M58835
C51
C26
Ub, AY854296
Ub, AY976442
Ruminococcus flavefaciens, X83430
Ruminococcus albus, L76598
Butyrivibrio crossotus, X89981
Clostridium proteoclasticum B316T, U37378
C53
Urb, AB009229
Bacteroides intestinalis, AB214329
Eubacterium ruminantium, AB008552
Pseudobutyrivibrio ruminis, X95893
Urb, AB270169
C28W
Oxalobacter formigenes, U49757
C4W
Urb, AB270013
Actinobacillus succinogenes, AF024525
Ruminobacter amylophilus, Y15992
C44
Ub, AF371696
Megasphaera elsdenii, M26493
Synergistes jonesii, L08066
Treponema bryantii, M57737
Urb, AB270189
C24W
C16
Ub, AB089125
Wolinella succinogenes, M26636
C32
C10
C40
Urb, AB270025
Urb, AB270176
C56
Urb, DQ394590
C1W
Urb, DQ394626
0.05

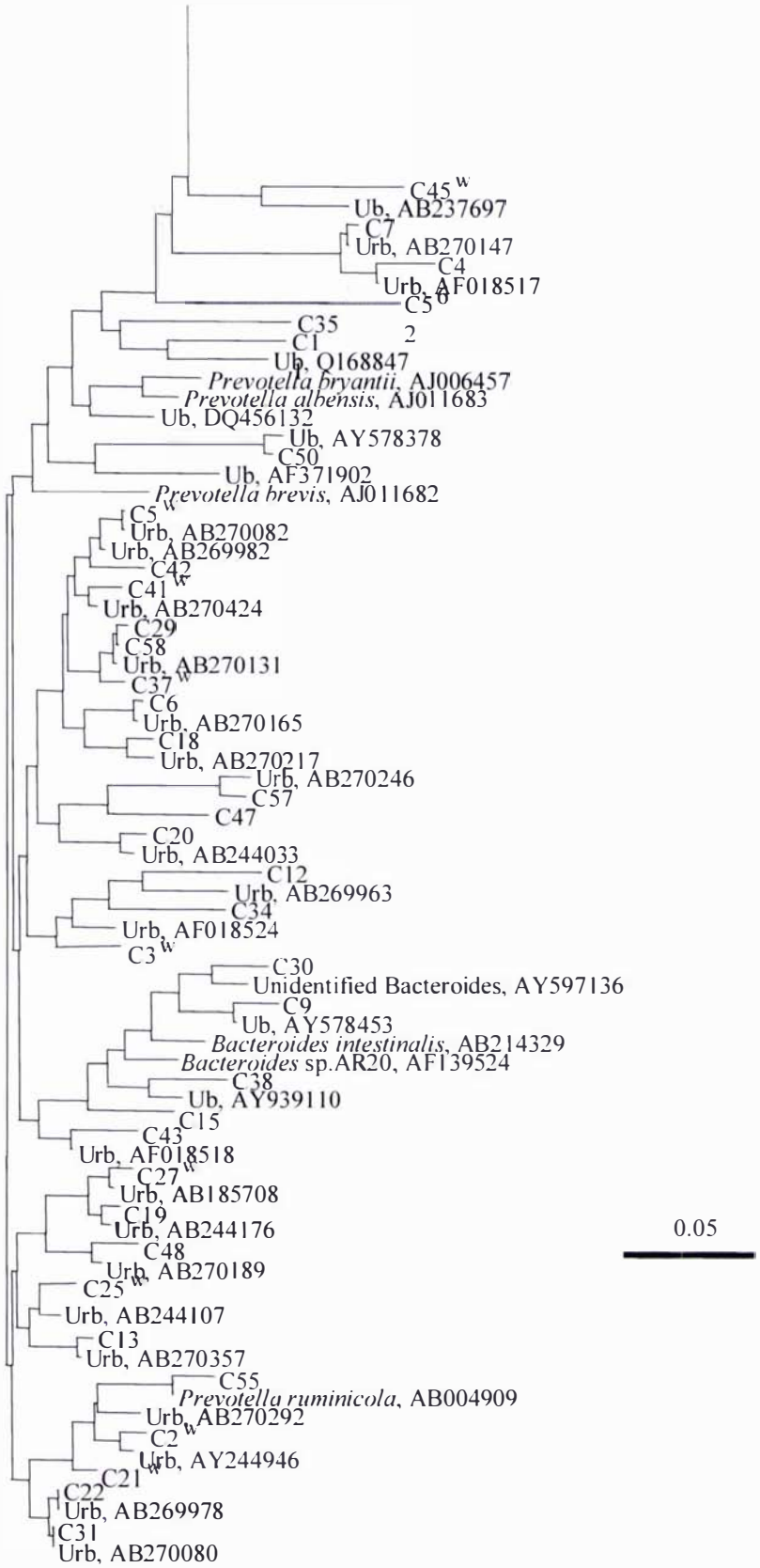


Figure 5.4 A phylogenetic tree constructed using the 16*S* rRNA gene V3 cloned sequences retrieved from the DNA bands excised from DGGE gels.

[w]: Clones unique to willow treatment group, Urb: unidentified rumen bacterium; Ub: unidentified bacterium.

Table 5.3. Clone sequence information

Clone	DGGE Band Position	Closest relative	Similarity (%)	Clone	Source	Feed	Reference
C1[w]	47.1	urb DQ394626	96	SR14	Svalbard deer	Lush tundra vegetation	Sundset et al. (2007)
C2[w]	50	urb AY244946	97	BE32	Cow	NR	Shin et al (unpublished)
C3[w]	52.5	urb AB244107	96	T20H80B03	Holstein heifers	NR	Tajima et al.(unpublished)
C4[w]	56.5	urb AB270013	94	T20H60F91	Holstein heifers	Concentrate and ryegrass based diets	Tajima et al. (2007)
C5[w]	60.58	urb AB270082	99	T33H60F3	Holstein heifers	Concentrate and ryegrass based diets	Tajima et al. (2007)
C21[w]	37	urb AB270292	97	T33H80A10	Holstein heifers	Concentrate and ryegrass based diets	Tajima et al. (2007)
C27[w]	53	urb AB185708	98	U28-E10	Cattle	Sudan grass hay and concentrate	Ozutsumi et al. (2005)
C28[w]	63.4	urb AB270169	99	T28H80D05	Holstein heifers	Concentrate and ryegrass based diets	Tajima et al. (2007)
C37[w]	56.4	urb AB270131	98	T33H60F61	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C41[w]	62.1	urb AB270424	98	T28H60SF06	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C45[w]	49.5	ub AB237697	91	HDBW-WB34	Deep surface water	NR	Shimizu et al. (2006)
C6	36	urb AB270165	99	T28H80D03	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C7	41	urb AB270147	99	T33H60F89	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C8	44	urb AB270164	97	T28H80E03	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C10	59.8	urb AB270025	95	T28H60F10	Holstein heifers	NR	Tajima et al. (unpublished)
C11	63.8	ub DQ168847	85	J3	Anaerobic sludge	NR	Kim et al. (unpublished)
C13	68	urb AB270357	98	T20H60SH10	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C15	35	ub EF071477	94	M0035_049	Humans	NR	Florin et al (unpublished)
C16	40	ub AB089125	95	Rs-J47	Termites	NR	Hongoh et al. (2003)
C19	55	urb AB244176	97	T20H80H10	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C26	52	ub AY854296	97	Thompsons8	African ruminants	wild vegetation	Nelson et al. (2003)
C29	67	urb AB270131	98	T33H60F61	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C30	39.3	ub AY597136	95	TNBo1-10	Cattle faeces	NR	Layton et al. (2006)
C31	45.1	urb AB270080	99	T33H60F1	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C32	45.1	urb AB270176	91	T28H80D07	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C34	49.1	urb AF018524	94	clone 39-30	Cattle	haylage, corn silage and concentrate	Whitford et al. (1998)
C35	52.1	DQ456132	91	CFT114G9	Turkey	NR	Scupham et al. (2007)
C38	58.6	ub AY939110	95	PS250L2.8F_F05	Big horn sheep	NR	Safaee et al. (unpublished)

Table 5.3 continued

C39	60	urb AB269966	98	T20H60F29	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C40	60	urb AB270025	95	T28H60F10	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C47	43.1	ub AY939206	96	PO72NL1C06	Big horn sheep	-	Safaee et al. (unpublished)
C48	47.3	urb AB 270189	97	T28H80F11	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C51	60.83	ub AY976442	91	LZ39	Humans	-	Eckburg et al. (2005)
C52	62.3	ub AF371902	86	p-1922-s962-3	cattle	-	Roe et al. (unpublished)
C55[Ψ]		urb AB270020	97	T28H60F3	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C56[Ψ]		urb DQ394590	95	NP18	Svalbard deer	Lush tundra vegetation	Sundset et al. (2007)
C57[Ψ]		urb AB270246	97	T33H80D05	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)
C58[Ψ]		urb AB270131	97	T33H60F61	Holstein heifers	concentrate and ryegrass based diets	Tajima et al. (2007)

C55-C58 from wk 0; C45-C53 from wk 2; C1-C13 from wk 4; C30-C44 from wk 6; C15-C20 from wk 8 and C21-C29 from wk 10.
[w] denotes the cloned sequences retrieved from the bands that were unique to willow supplemented animals; [Ψ] The band position for these clones was not recorded.
urb: unidentified rumen bacterium; ub: unidentified bacterium
NR: not reported.

5.5 DISCUSSION

Supplemental feeding of willow cuttings to ewes grazing drought pasture reduces live weight loss (McWilliam, 2004; Pitta et al., 2005, 2007) due to an increase in dry matter intake and metabolisable energy available. The effect of willow supplementation on the microbiology of the rumen is largely unexplored as *Salix* species are not commonly fed to ruminant livestock. This study showed that willow supplementation had significant effects on rumen microbial profiles in the animals examined in this study. The DGGE analysis of the V3 regions of microbial 16*S* rRNAs revealed a discernable difference in banding patterns between treatment groups after a 10 week feeding period. Previous experiments with supplemental feeding of willow over 10 weeks (McWilliam, 2004; Pitta et al., 2005, 2007) have shown that ewes have an increase in DM intake and consume willow stems up to 5 mm in diameter compared to control animals. An adaptation period of 16 days is normally allowed for ruminants to become adapted to a new diet (Matejovsky and Sanson, 1995). In this study the willow supplement made up only approximately one third of the diet and a degree of diet selection among the willow-supplemented animals was possible within the first few weeks. However, after animals became accustomed to feeding on willow cuttings, they would routinely eat all of the leaves and thin stems, eventually eating stems up to a diameter of 5 mm. Thus it is likely that an extended adaptation period was required to allow the bacteria in the rumen to adapt to the changing diet. Clearly the supplemental willow diet changed conditions in the rumen sufficiently to observe a transient ruminal accumulation of PG's and FM's after 2 weeks, and a significant shift in rumen bacterial populations after 10 weeks. The decreasing concentrations of CT, PG and FM in the rumen after week 2 could have induced the shift in rumen bacterial populations of

willow supplemented animals at low level and increased gradually with time which was picked by DGGE at week 10.

Although the DGGE banding patterns differed significantly between the treatment groups after 10 weeks, phylogenetic analysis of the band sequences did not cluster these sequences by treatment group. This indicates that, although the 16*S* rRNA gene sequences of the rumen bacteria in each animal treatment group were different enough to be separated by DGGE, they still fall within similar phylogenetic groups. The majority of sequences from bands both unique to the willow-supplemented animals (7 of 11, 64%) and common between the treatments (17 of 27, 63%) fell within the *Bacteroides-Prevotella* group of organisms. This group is more tightly clustered than the remainder of the sequences which span a wide range of bacterial genera. The retrieval of clones closely related to the *Bacteroides-Prevotella* group is common among rumen bacterial 16*S* rRNA gene libraries. For example, many of the clones in the present study (15 of 27, 56%) including most of the band sequences unique to willow-supplemented ewes (7 of 11, 64%) were most similar to 16*S* rRNA sequences retrieved from gene libraries constructed from DNA extracted from the rumen contents of Holstein heifers (Tajima et al., 2007). They found that the majority of their clone sequences were most closely related to bacterial species within the phyla *Bacteroidetes* and *Firmicutes*. Similarly the study of bacterial diversity in dairy cattle by Whitford et al. (1998), found that members of the *Bacteroides-Prevotella* group were prominent in their libraries. A more recent study on 16*S* rRNA gene libraries of bacterial populations in the rumen of Svalbard reindeer located in different geographic locations found that their clone sequences were dominated by species within the *Firmicutes* (*Clostridiales*) and *Bacteroidales* (*Bacteroides-Prevotella*) but which were not closely related to any cultured rumen bacteria. Only one of the retrieved clone sequences was

sufficiently similar to *R. flavefaciens* to be considered to represent a bacterium currently in culture (Sundset et al., 2007). This work is of particular relevance to the present study as the reindeer were reported to eat willow as part of their winter diet. In fact, two of the clones found in the present study (C1 and C56) that cluster between *Wolinella succinogenes* and the *Bacteroides-Prevotella* group were most closely related to clones retrieved from the reindeer study (Sundset et al., 2007). Similarly, only 2 sequences from the present study (Clones 51 and 55) are closely related to organisms (*S. bovis* and *P. ruminicola* respectively) currently held in culture collections. Several of the clone sequences from this study clustered with sequences retrieved from non-ruminant animals or water sources. However, the low level of sequence similarity observed in these cases indicates that the rumen clones probably represent organisms that are only distantly related.

Those bacteria present in the willow-supplemented animals are presumably selected either by having the metabolic capability to utilise willow compounds as substrates for growth (such as PGs), or by being able to avoid the effects of inhibitory compounds such as CTs or FMs (Lowry et al., 1996). Sundset et al. (2007) also reported that secondary metabolites were degraded by rumen microbes present in reindeer. The Norwegian reindeer in their study had a high proportion of their winter diet made up of lichens which contain a variety of secondary compounds. One of these compounds, the antibiotic usnic acid, is known to decrease in vitro digestibility in sheep, but enhance digestibility in the reindeer. This was taken as evidence that reindeer have rumen microbial populations adapted to metabolise usnic acid and allow rumen fermentation to proceed. The shift in rumen bacterial populations on the willow-supplemented diet in the present study probably relate to the ability of certain bacteria to utilise secondary metabolites of willow or avoid their inhibitory effects. Comparison

of drought pasture composition with that of willow shows the largest differences were due to the presence of high levels of plant phenolics in willow. CTs are present at around 40 g/kg DM of DM while the PGs and FMs account for 1 to 30 g/kg DM. Although the actual CT composition of the willow used in the present study (*S. matsudana* x *S. alba*) was not determined, it is known that the CT in other *Salix* species ranges in the procyanidin:prodelphinidin ratio ranges from 34:66 to 83:17 and their average chain lengths range from 1380 to 4200 molecular weight (Ayres et al., 1997). These characteristics are similar to those of CT from *L. corniculatus* and *L. pedunculatus* species thus one might expect that the willow CT would have similar effects on bacteria in the rumen. The PGs (salicylates) from willow are known to contain a backbone of salicin (2-*O*-β-D glucoside of salicyl alcohol) linked to a variety of substituents such as benzyl, acetyl or hydroxycyclohexenone (Lindroth and Pajutee, 1987, Julkunen-Tiitto, 1989) and salicin and salicortin are the most commonly encountered PGs in willow species. Salicin is readily fermented in the rumen by a wide range of bacteria (Ogimoto and Imai, 1981). When ingested by ruminants, PG are likely to be broken down into their constituent parts by rumen microbes which then utilise the released glycoside for growth (Lowry et al., 1996). The released phenolic compounds may also be further metabolised by rumen microbes (Lowry et al., 1996). Similarly FMs such as quercetin, can be metabolised through to acetate and phenylacetic acid (Krumholz and Bryant, 1986). The phenolic compounds released can also cause inhibitory effects in the rumen depending on the concentration and structure of the phenolic compound. Free phenol is known to have antimicrobial effects and in the rumen this is likely to lead to decreased microbial activity and DM intake (Sivaswamy and Mahadevan, 1986). Thus, the secondary compounds encountered in the rumen of the willow-supplemented sheep are likely to have modulated the microbial

populations via a combination of enhancement of bacterial growth of those able to utilise or resist these compounds, or via the inhibition of those bacteria sensitive to their toxic effects.

In summary, the observations in this study demonstrate a significant change in rumen bacterial populations during the supplemental feeding of willow. These changes are correlated with the disappearance of PGs and FMs from the rumen and support the idea that specialist bacteria are selected to metabolise or resist these compounds. However, the types of bacteria selected in the willow-supplemented animals clustered within the same phylogenetic groupings of bacteria from control animals, suggesting selection of sub-populations of related organism with specialised metabolic capability. Future work should concentrate on culturing these organisms and defining their differences to identify the specific metabolic capabilities or detoxification mechanisms that permit these bacteria to predominate during willow supplementation.

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CHAPTER 6

EFFECT OF WILLOW SUPPLEMENTATION TO EWES GRAZING DROUGHT PASTURE UPON RUMEN BACTERIA

6.1 ABSTRACT

Willow supplementation to ewes during dry summer conditions on NZ farms, resulted in a consistent increase in reproductive rate of sheep and reduced their live weight losses compared to sheep that grazed on low quality drought pasture only. This study was undertaken to study the effect on rumen microbes of ewes grazing drought pastures with and with out willow supplementation using microbial cultivation techniques.

This cultivation study involved enumeration, isolation and purification of bacterial colonies on complete carbohydrate, Salicin, Xylan, Cellulose and Willow media, followed by full characterisation of a representative set of pure bacterial cultures. Animals supplemented with willow had a lower count of rumen bacteria on different media and the majority of isolates characterised from both Salicin and Xylan media, had 16*S* rRNA gene sequences most closely related to species from the *Pseudobutyrivibrio* genus. Most of the isolates from Willow medium were retrieved from willow-supplemented sheep and clustered as two distinct groups. One group was made up mainly of organisms not usually associated with the rumen and probably represented non-resident organisms that are passing through the rumen. The other group of bacteria were most closely related to species of the *Olsenella* genus and were able to metabolise plant secondary compounds within willow. Most of these bacteria did not grow on a range of carbohydrates and grew only very slowly on hydrolysed protein.

6.2 INTRODUCTION

Willows (*Salix spp*) were originally planted in NZ for animal shelter and soil conservation purposes (Charlton et al., 2003) but now willow cuttings are commonly used as an alternate supplement to drought pasture during dry summers (McCabe and Barry, 1988). The feeding of willow and poplar stem cuttings has been shown to be beneficial to sheep (McWilliam et al., 2005a and b) and to cattle (Moore et al., 2003) through reductions in live weight losses and increases in lambing percentage in ewes when compared to low quality drought pasture. The actual mechanism responsible for the enhanced production and reproduction by supplementary willow feeding is not understood, and neither is the effect of willow on microbial fermentation in the rumen. It is known that the nutritive value of willow is consistently superior to low quality drought pasture with a higher ME value of approximately 10.5 MJ/ kg DM (McWilliam et al., 2005a and b) compared to 8 MJ/kg DM for drought pasture. A special feature of willow is the presence of secondary compounds such as CTs (38 g/kg DM) and PGs (35 g/kg DM) (Pitta et al., 2007). CTs are known to either inhibit or stimulate animal production depending on the type and concentration of CTs involved via direct or indirect effects on rumen bacteria, (Lowry et al., 1996; McAllister et al., 1993). They are also known to alter the growth and protein degrading ability of rumen microbes (Jones et al., 1994; Molan et al., 2001). PGs such as salicin, are readily fermented by a wide range of rumen bacteria (Ogimoto and Imai, 1981). However, the effects of secondary compounds from willow on the fermentation in the rumen and their influence on ruminal bacterial populations are poorly understood. To gain a better understanding of the types of rumen bacteria found when willow cuttings are included in the diet, we sampled rumen contents of

sheep grazing on a drought pasture diet with or without willow-supplementation. Here we describe the isolation of several both common and specialised bacterial species from willow-supplemented animals and investigate their breakdown of willow secondary compounds.

6.3 MATERIALS AND METHODS

6.3.1 Bacterial strains and media

The medium for the routine growth of anaerobic bacteria was Complete Carbohydrate medium (CC, Leedle and Hespell, 1980). Four different variants of CC medium were used in which the carbohydrates in CC medium were replaced with either salicin, xylan, cellulose or freeze-dried and ground willow at 0.5% (wt/vol). Both liquid broth and agar (for roll tubes) versions of the media were prepared anaerobically, dispensed into Hungate tubes and autoclaved (Attwood et al., 1996; Attwood et al., 1998). The bacterial strains used in this study were isolated from Salicin (S1, S2, S5, S7 and S10), Xylan (X3, X4, X5, X6, X9, X10 and X15a) and Willow (W2, W3, W5, W6, W7, W8, W10, W12, W17, W18 and W20) media.

6.3.2 Animals, willow supplementation and rumen sampling

The Experimental design including preparation of drought pasture and the animals with their dietary allowance was described in the previous Chapter 5. Samples of rumen contents were taken at week 5 (half-way through the experiment) and week 10 (end of the experiment) from all experimental ewes. Samples were collected by removing the fistula bung and retrieving a total of approximately 250 ml of rumen contents from several different locations within the reticulo-rumen using a pair of blunt forceps. The combined sample was placed in glass screw-cap Schott bottles, preflushed with CO_2, and immediately transported to the laboratory.

6.3.3 Enumeration, Isolation and purification of bacterial colonies

Sub samples of sheep rumen contents were taken from each sample bottle and squeezed through two layers of cheese cloth into a Erlenmeyer flask flushed with CO_2. One ml of the filtered rumen contents sample was inoculated into 9 ml of Anaerobic Dilution Buffer (Bryant and Burkey, 1953) and then triplicate serial dilutions were carried out in molten agar roll tubes of the appropriate media held at 42 °C. The tubes were gently mixed and rolled under a stream of cold water until the agar solidified around the inside of the tubes. Roll tubes were incubated at 39°C and bacterial colonies were counted using a low power stereomicroscope after 24 hrs for CC, Salicin, and Xylan roll tubes and after 7 days for Cellulose roll tubes. Colony counts on Willow agar roll tubes were not done but colonies were picked after incubation at 39°C for 24-48h. A minimum of three representative individual bacterial colonies from each animal within each medium were selected from the highest serial dilution. Purification of bacterial isolates was accomplished by repeated cycles of picking individual colonies from serial dilutions in roll tubes, re-inoculation into broths and re-dilution through roll tubes until pure. The purity of bacterial cultures was confirmed by microscopic examination of wet mounts and Gram stained cultures.

6.3.4 Phenotypic characterisation of bacteria

Bacterial isolates were characterised by morphological analysis (light and electron microscopy), Gram stain (Doetsch, 1981), substrate utilisation, biochemical tests, and by end products of fermentation. Cell motility was determined by observation of fresh cultures under phase-contrast microscopy and the presence of flagella was confirmed by electron microscopy. Substrate utilisation tests were performed using CC medium lacking any carbon source, and individual substrates

were added at the following final concentrations (wt/vol): arabinose, 0.5%; cellobiose, 1.0%; dulcitol, 1.0%; fructose, 1.0%; galactose, 1.0%; glucose, 1.0%; glycerol, 1.0%; lactate, 0.77%; lactose, 1.0%; maltose, 1.0%; melezitose, 0.5%; melibiose, 0.5%; mannitol, 1.0%; mannose, 1.0%; raffinose, 1.0%; rhamnose, 1.0%; ribose, 1.0%; salicin, 1.0%; sorbitol, 1.0%; sucrose, 1.0%; trehalose, 0.5%; and xylose, 1.0%. Bacterial growth was determined by change in Optical Density (OD) at 600 nm as measured in a Ultraspec 6110 pro UV-Vis spectrophotometer. Biochemical tests were carried out as previously described (Holdeman and Moore, 1972) on overnight cultures grown in Willow medium broths. End products of fermentation were analyzed using a nitroterephthalic acid-modified polyethylene glycol column (DB-FFAP; 30 m by 0.53 mm by 1.0 μm film thickness; J & W Scientific, Folsom, CA) attached to a Hewlett-Packard 6890 series gas chromatography system (Attwood et al., 1996). Electron microscopy was used for bacterial cell size measurements and observation of fine structures. Freshly grown cultures were collected by low speed centrifugation (3,000 x g for 10 min at room temperature) and washed twice in distilled water. The washed cells were negatively stained with 1% phosphotungstic acid and mounted on Formvar-coated copper grids prior to observation under a Philips model 201C Electron Microscope.

6.3.5 Extraction of DNA, sequencing and phylogenetic analysis

The extraction of DNA from pure cultures was performed using a phenol-chloroform extraction method (Saito and Miura, 1963). 16*S* rRNA genes were Polymerase Chain Reaction (PCR) amplified using universal ribosomal DNA (rDNA) primers (fD1 and rD1; Lane, 1991) with genomic DNAs as templates. PCR mixtures contained 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1 μM of each primer, and 0.5 U of *Taq* DNA

polymerase (GIBCO BRL, Auckland, New Zealand). PCR reactions were in a final volume of 20 μL, sealed in a capillary tip, and thermocycled using a Corbett FTS-1 Capillary Thermal Sequencer (Corbett Research, Sydney, Australia). PCR amplification conditions were denaturation at 94°C for 3 min, followed by 6 cycles of 94°C for 30 s, 56°C for 15 s, 72°C for 30 s; 25 cycles of 94°C for 15 s, 56°C for 5 s, and 72°C for 30 s; and a final cycle of 72°C for 3 min (Reilly and Attwood, 1998). After PCR, 3 μL aliquots were separated by electrophoresis in 1% agarose gels (wt/vol), stained with ethidium bromide, and visualized by UV transillumination. The 16*S* rRNA PCR products were sequenced with 16*S* rDNA primers fD1, rD1, F3, R5 and R2 (Lane, 1991) and the sequencing products analysed using a model 373A automated sequencer (Applied Biosystems, Foster City, Calif.). The 16*S* rDNA sequence runs from each bacterial isolate were aligned using Contig software (Megalign, Vector NTI, Bethesda, Maryland, USA) to produce full length 16*S* rRNA sequences. Closely related 16*S* rDNA sequences were obtained from Genbank and the Ribosomal Database Project (Olsen et al., 1992) and were aligned with the new bacterial 16*S* rDNA sequences to construct similarity matrixes. A single phylogenetic tree was constructed using approximately 1,100 unambiguous base pairs of 16*S* rRNA gene sequences in ARB software (Ludwig et al., 2004)

6.3.6 Analysis of secondary compounds

Willow culture samples before inoculation and after incubation were analysed for salicin and the concentration of PGs and FMs using HPLC as described in the previous Chapter 5.

6.4 RESULTS

6.4.1 Enumeration

Rumen samples collected from animals at weeks 5 and 10 were serially diluted and inoculated into different media to enumerate different classes of bacteria able to utilise different plant fibre and secondary compounds. The bacterial colony counts are presented in Table 6.1.

Table 6.1 Bacterial colony counts ($\times 10^9$) at Weeks 5 and 10 in control and willow-supplemented ewes.

	Week 5		Week 10	
Medium	Control	Willow	Control	Willow
CC	2.46[a]	1.00[b]	13.8	8.40
Salicin	2.39[a]	1.23[b]	4.20	2.80
Xylan	2.33	1.66	13.50[a]	24.50[b]
Cellulose	1.38	1.84	21.50[a]	5.40[b]

[a, b] Means within rows with different superscripts differ significantly ($P < 0.05$)

In general the number of bacteria retrieved from all types of media were lower in willow-supplemented animals, the exceptions being a slightly higher count on Cellulose in Week 5 and higher counts on Xylan in Week 10. Bacterial counts at Week 10 were higher than those at Week 5 probably due the increased pasture mass that was made available for the animals during the last two weeks of the animal trial due to low animal condition score.

6.4.2 Bacterial isolation and characterisation of bacterial cultures

A total of 44 bacterial colonies were picked from the highest dilution of Salicin and Xylan roll tubes and purified. DNA was extracted from each of these

pure cultures, their 16*S* rRNA genes amplified and a single sequencing reaction from the 5' end of the gene was carried out producing around 500 to 700 bp of sequence. Analysis of these short 16*S* rRNA sequences was used to give a preliminary indication of isolate identity. Based on these preliminary identifications, 12 representative isolates (7 from Xylan medium and 5 from Salicin medium) were selected for further phenotypic characterisation and full 16*S* rDNA sequencing.

The bacterial isolates from Salicin medium were all Gram positive and fell into 3 groups. All isolates on Salicin medium, except S10, were from willow-supplemented animals. Isolates S1 and S2 were similar to each other and *S. bovis* JB1 in their phenotypic characteristics and carbon source utilisation (Tables 6.2 and 6.3). Isolates S5 and S10 were identical phenotypically and both strains were lipase positive, formed spores (subterminal), were motile with peritrichous flagella and have characteristics similar to those reported for *Clostridium sporogenes*. Isolate S7 was a curved, motile rod which produced acetate, propionate and butyrate as end products of fermentation. The bacteria isolated on Xylan media (Table 6.2) were all Gram negative, curved rods with the exception of isolate X10 which was a Gram positive, coccus. All isolates from Xylan medium, apart from X10 and X15a, were derived from rumen samples from willow-supplemented animals. All of the remaining isolates from Xylan medium produced butyrate as a major end product of fermentation with the exception of X15a, in which acetate was the main product and butyrate was a minor product of fermentation. Substrate utilisation patterns of isolates X4, X5, X6 and X9 were similar while isolates X3 and X15a differed noticeably (Table 6.3).

Table 6.2 Phenotypic characteristics of representative bacterial isolates on Salicin, and Xylan media

Strains	Characteristics												Biochemical tests	Fermentation end products
	Gram stain	Size µm		Shape	Mot.	Colony characteristics						Temperature			
		L	W			size	form	optical	margin	surface	elevation	25°C	45°C		
S1[w]	+	0.65	0.58	Cocci	-	M	R	O-Cen	T	S	F	+	+	-	A, P, b
S2[w]	+	0.51	0.58	Cocci	-	M	R	O-Cen	T	S	F	+	+	-	A, P, b
S5[w]	+			thick rods	+							-	-	Lipase +	A, p, b, ib
S7[w]	+	1.35	0.54	c.rods	+	M	R	T	T	S	C	-	+	-	A, P, B, ib, v
S10[c]	+			thick rods	+							-	-	Lipase +	A, B, p, ib
X3[w]	-	2.59	0.64	c.rods	-	M	R	O	E	F	F	-	-	-	A, B, p, ib
X4[w]	-	1.72	0.61	c.rods	-	M	R	O	E	U	C	-	-	-	B, A, p, ib
X5[w]	-	1.44	0.40	c.rods	-	M	R	O	E	U	C	-	-	-	B, A, p, ib
X6[w]	-	1.00	0.29	c.rods	-	M	R	O	E	F	RA	-	-	-	B, ib, p
X9[w]	-	1.50	0.51	c.rods	-	M	R	O	E	F	RA	-	-	-	B, a , p
X10[c]	+	0.85	0.75	Cocci	+	M	R	O	T	S	C	+	+	-	A, P, b, ib
X15a[c]	-	2.00	0.31	Thin c.rods	-	L	R	O	E	S	F	-	-	-	A, p, b

Motility (Mot): +, motile; -, non-motile. Colony characteristics: size: M, medium; L, large. Form: R, round; Optical: O, opaque; T, transparent; O-Cen, opaque in the centre. Margin: E, entire; T, Transparent; Surface: S, smooth, U, umbonate; F, flat. Elevation: F, flat; C, convex; RA, raised. Temperature: -, no growth ; w, slight growth between 0.015 to 0.3 OD; +, moderate growth >0.3 to 1 OD; ++, heavy growth > 1 OD. Biochemical tests: Tested for H_2S, Indole, Lipase, Lecithinase, Catalase and Urease, only positive reactions are indicated End products: A and a, acetate; P and p, propionate; B and b, butyrate; ib, isobutyrate; v, valerate. Capitalised or lower case letters indicate major or minor products of fermentation respectively. [w] Bacterial isolates from willow supplemented ewes [C] Bacterial isolates from control ewes

Table 6.3 Substrate utilisation tests of bacterial isolates from Salicin and Xylan media

	S1 [w]	S2 [w]	S5 [w]	S7 [w]	S10 [c]	*S. bovis*	X3 [w]	X4 [w]	X5 [w]	X6 [w]	X9 [w]	X10 [c]	X15a [c]	*B. fibrisolvens*
Glucose	++	++	++	++	++	++	++	++	++	++	++	++	+	+
Fructose	++	++	w	++	w	++	+	++	++	++	++	++	+	+
Galactose	++	++	w	++	w	++	+	++	++	++	++	++	+	+
Mannose	++	++	w	++	w	++	+	++	++	++	++	++	-	-
Rhamnose	-	-	w	-	w	-	-	+	w	w	W	-	-	-
Ribose	-	-	w	-	w	-	-	w	w	w	-	-	-	-
Xylose	-	-	w	-	w	-	+	++	++	++	++	w	+	+
Maltose	++	++	++	++	++	++	+	++	++	++	++	++	+	+
Cellobiose	++	++	w	++	w	++	+	++	++	++	++	++	+	+
Lactose	++	++	w	w	w	++	w	w	w	w	W	++	-	-
Sucrose	++	++	w	++	w	++	+	++	++	++	++	++	+	+
Mannitol	-	-	+	-	+	-	-	++	++	++	++	-	-	-
Glycerol	-	-	+	-	+	-	w	+	+	w	W	-	-	-
Raffinose	++	++	w	-	w	++	+	++	++	++	++	++	-	-
Lactate	+	+	-	-	-	+	-	w	w	w	-	-	-	-
Arabinose	-	-	w	-	w	-	++	++	++	++	++	-	+	+
Trehalose	++	-	++	++	++	++	w	++	++	+	++	++	-	-
Melibiose	++	++	w	-	w	++	+	+	+	+	+	++	-	-
Melezitose	-	-	w	-	w	-	-	++	++	++	++	w	-	-
Sorbitol	-	-	+	-	+	-	-	+	w	w	W	w	-	-
Dulcitol	-	-	w	-	w	-	-	w	-	-	-	w	-	-
Salicin	++	++	+	++	+	++	+	++	++	++	++	++	+	+

Growth: no growth (-); slight growth (w) at 0.015 to 0.3 OD; moderate growth (+) at >0.3 to 1 OD; heavy growth (++) at > 1 OD.
[w] Bacterial isolates from willow supplemented ewes [c] Bacterial isolates from control ewes

A total of 25 bacterial colonies were isolated from the highest dilution tubes in Willow medium and based on their colony morphology, cell morphology as observed by phase-contrast microscopy, and analysis of the 5' region of 16S rRNA gene sequences, 11 representative isolates were selected for full phenotypic characterisation (Table 6.4; Figure 6.1). Most of the isolates were Gram positive; while isolates W2, W3 and W8 isolates were Gram negative. Strains had varying morphology ranging from very short rods, cocci, coccobacilli and irregular shapes in chains.

Isolate W5 was a motile, Gram-positive, thick rod, which produced acetate, butyrate and propionate as its main fermentation products. Its substrate utilisation patterns were similar to those seen in *C. butyricum*. All strains were negative to the biochemical assays but some isolates produced traces of hydrogen in the head space when grown in Willow medium. Most isolates produced acetate as the major end product of fermentation, with propionate as the second most common end product. Isolate W3 was the exception, producing butyrate as the main end product followed by acetate (Table 6.4). Substrate utilisation tests showed that isolates W2, W3 and W8 failed to grow on any of the sugars tested, isolates W6, W10, W7, W17 and W18 had similar utilisation patterns using a few of the commonly fermented sugars while isolates W5, W12 and W20 each used a broad range of sugars but had unique utilisation patterns (Table 6.5). The absence or poor utilisation of sugars in some of the isolates suggested that carbohydrates may not be their main substrates. We therefore tested the growth of each of the isolates on 1% trypticase in the absence of other added carbon sources. Isolates W2 and W3 exhibited moderate growth on trypticase alone, while the remainder of isolates exhibited weak or no growth.

Table 6.4 Phenotypic characteristics of bacterial isolates from rumen samples of willow-supplemented [W] and control [C] ewes

Type	W2[W]	W3[W]	W6[W]	W8[W]	W10[W]	W5[W]	W7[W]	W12[C]	W17[C]	W18[C]	W 20[C]
Sizes(μ) (lt)	1.52	1.05	0.83	0.90	0.99	1.30	0.53	0.85		1.09	0.94
width(μ)	0.44	0.77	0.62	0.47	0.58	0.62	0.58	0.52		0.90	0.58
Gram's stain	-	-	+	-	+	+	+	+	+	+	+
Morphology	Rods	short rods in chains	coccobacilli in chains	irregular shapes	rods in chains	thick rods	chains of rods	Short rods	chains of rods	rods in chains	Cocci (diplo, tetrads)
Wet mount	motile			motile	non motile	motile	motile		non motile	non motile	non motile
Colony morphology											
a. Size	medium	medium	medium	very small	small		medium	Medium	medium	medium	small
b. Form	umbonate	umbonate	circular	round	round		round	Circular	round	round	circular
c. Optical	Opaque	Opaque	Opaque	Transparent	centre-opaque		opaque in centre	Opaque	Opaque in centre	Opaque	Opaque
d. Margin	entire/glossy	entire/glossy	entire	round	transparent		transparent	Entire	transparent	entire	entire
e. Surface	smooth	smooth	smooth	smooth	smooth		smooth	Smooth	smooth	smooth	smooth
f. Color	white in centre	white	colorless	colorless	colorless		colorless	Creamy	colorless	colorless	colorless
g. Elevation	convex	convex	flat	flat	convex		convex	Convex	convex	flat	convex
Temperature											
a. 25°C	-	-	-	-	-	-	-	+	-	-	+
b. 45°C	-	+	+	-	+	-	-	++	-	+	-
Biochemical tests											
a. H_2S	-	-	-	-	-	-	-	-	-	-	-
b. Indole	-	-	-	-	-	-	-	-	-	-	-
c. Lipase	-	-	-	-	-	-	-	-	-	-	-
d. Lecithinase	-	-	-	-	-	-	-	-	-	-	-
e. Catalase	-	-	-	-	-	-	-	-	-	-	-
f. Urease	-	-	-	-	-	-	-	-	-	-	W (pH 7.2)
Head space	-	0.49 ml	-	-	0.5 ml	-		-		0.63ml	0.53 ml
End products (mM)											
Acetate	4.60	4.11	6.43	9.01	4.87	8.83	2.99	13.55	45.99	9.12	7.60
Propionate	0.87	1.93	1.60	2.18	2.37	3.68	2.60	4.89	9.64	2.89	2.78
iso-butyrate	0.41	1.65	1.00	1.12	0.86	1.51	0.83	1.85	1.37	0.29	0.82
Butyrate	0.42	6.30	3.08	2.00	1.11	4.19	2.54	4.43	5.11	0.97	2.36
iso-valerate	0.15	0.32	0.32	0.23	0.13	0.35	0.18	0.32	0.21	0.15	0.20
Valerate	0.17	2.99	1.53	0.42	0.42	1.34	0.22	0.57	0.94	0.28	0.61

[W] Bacterial isolates from willow supplemented ewes [C] Bacterial isolates from control ewes

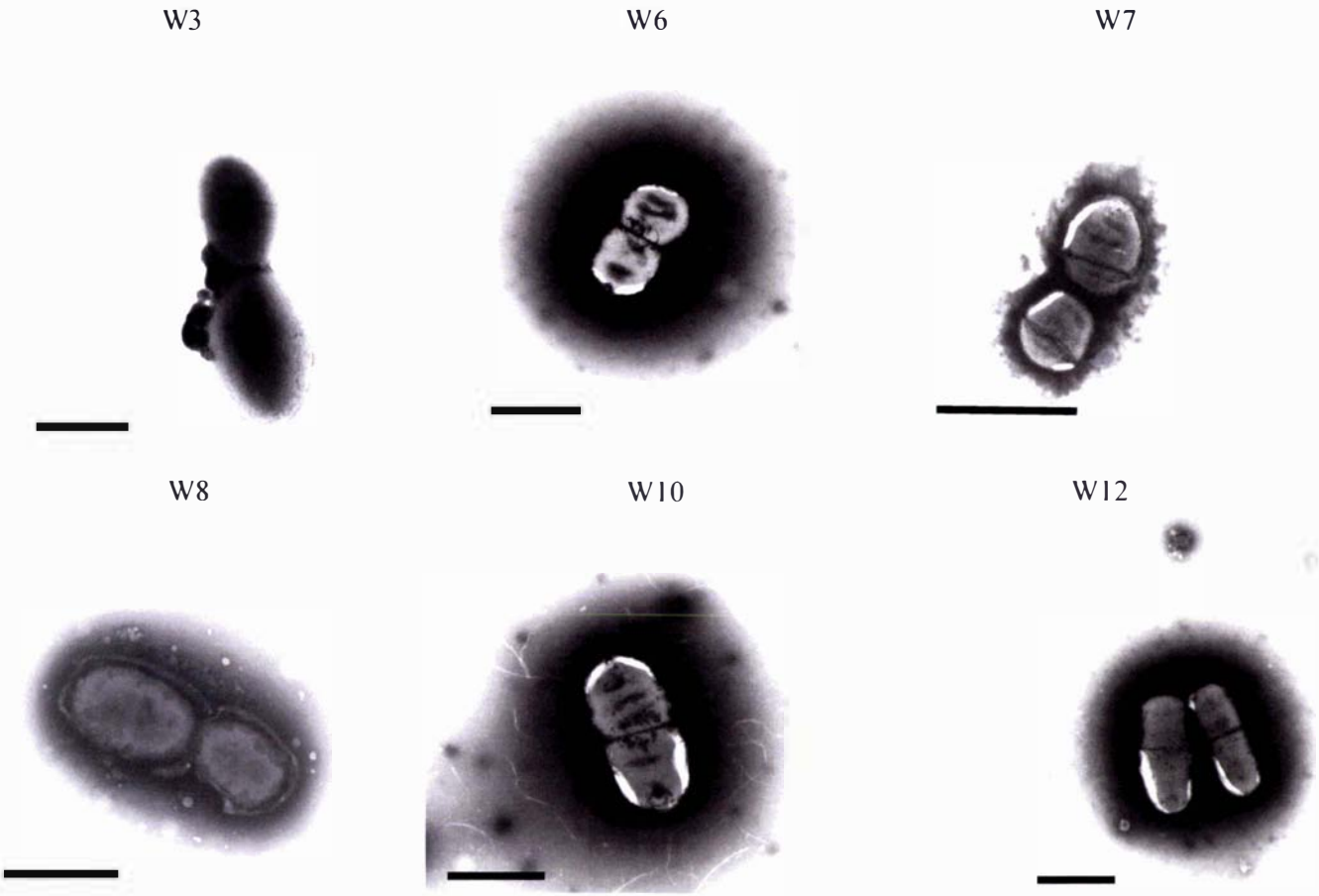


Figure 6.1 Electron micrographs of the negatively stained bacterial cells isolated on Willow enrichment media (—— indicate 1 μm).

Table 6.5 Substrate utilisation tests of Willow bacterial isolates from willow supplemented [w] and control [c] ewes

Substrate	W2[w]	W3[w]	W6[w]	W8[w]	W10[w]	W5[w]	W7[w]	W12[c]	W17[c]	W18[c]	W20[c]
Glucose	-	-	w	-	w	+	+	++	+	+	++
Fructose	-	-	+	-	w	+	++	++	+	+	++
Galactose	-	-	-	-	-	+	-	+	-	-	++
Mannose	-	-	-	-	w	+	+	w	+	w	-
Rhamnose	-	-	-	-	-	-	-	+	-	-	-
Ribose	-	-	-	-	-	-	-	+	-	+	-
Xylose	-	-	-	-	-	-	-	++	-	-	-
Maltose	-	-	+	-	+	++	+	++	+	+	++
Cellobiose	-	-	-	-	-	++	-	++	-	-	-
Lactose	-	-	-	-	-	++	-	++	-	-	++
Sucrose	-	-	+	-	w	++	+	++	+	+	+
Mannitol	-	-	-	-	-	-	-	++	w	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	++	-	++	-	-	-
Lactate	-	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	++	-	-	-
Trehalose	-	-	+	-	+	-	+	++	+	+	-
Melibiose	-	-	-	-	-	++	-	++	-	-	-
Melezitose	-	-	-	-	-	-	-	++	-	-	-
Sorbitol	-	-	-	-	-	-	-	++	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	w	-	-	++	-	++	-	-	-
Trypticase alone	+	+	w	-	w	-	w	w	w	w	-

6.4.3 Utilisation of components in willow

The isolation of the above organisms on Willow medium suggests that they are able to utilise component of willow for growth. Therefore the utilisation of willow components was measured by analysing the composition of the Willow medium before inoculation and after growth of each isolate. Most of the isolates utilised at least one of the components of willow, while isolate W3 was unusual in that it was a net producer of each of the willow components measured. (Figure 6.2). The components of willow were separated using reverse phase chromatography and peaks were detected using UV spectroscopy (Figure 6.3). Peaks corresponding to common PGs (salicin and salicortin) and FMs (rutin and quercetin) were detected along with several other unidentified peaks. The unidentified peaks were classified as either PGs other than salicin, or as FMs other than rutin.

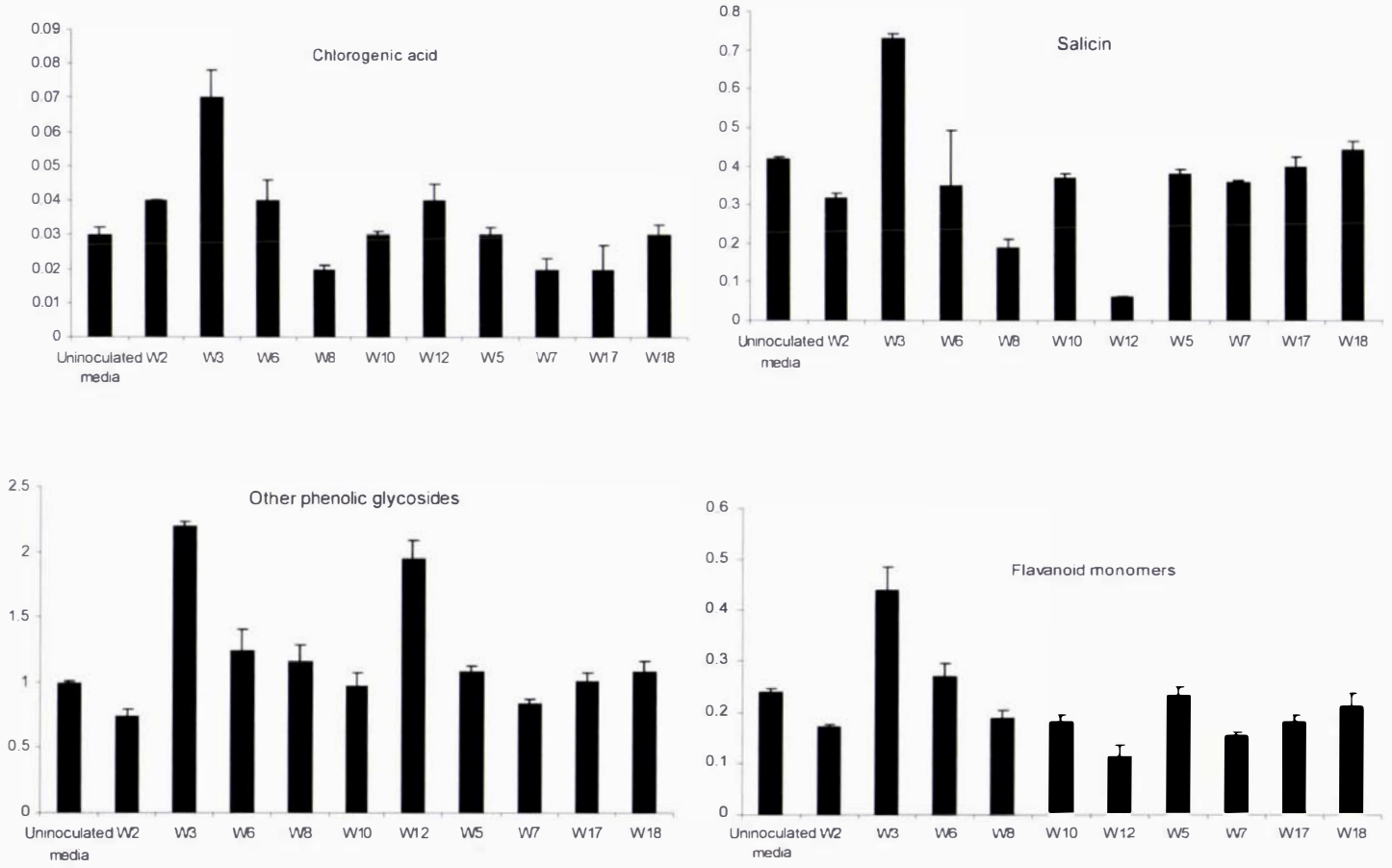
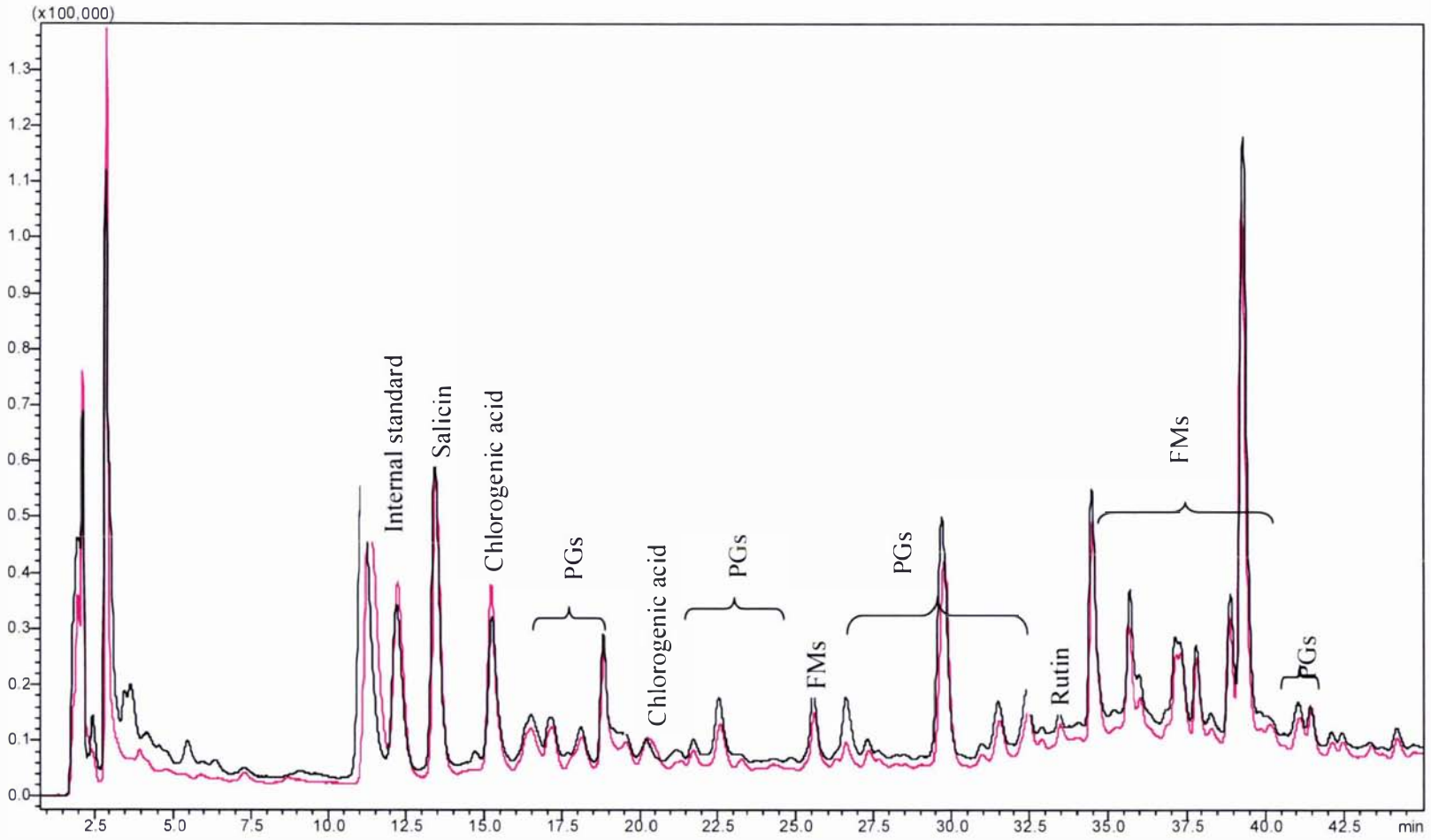
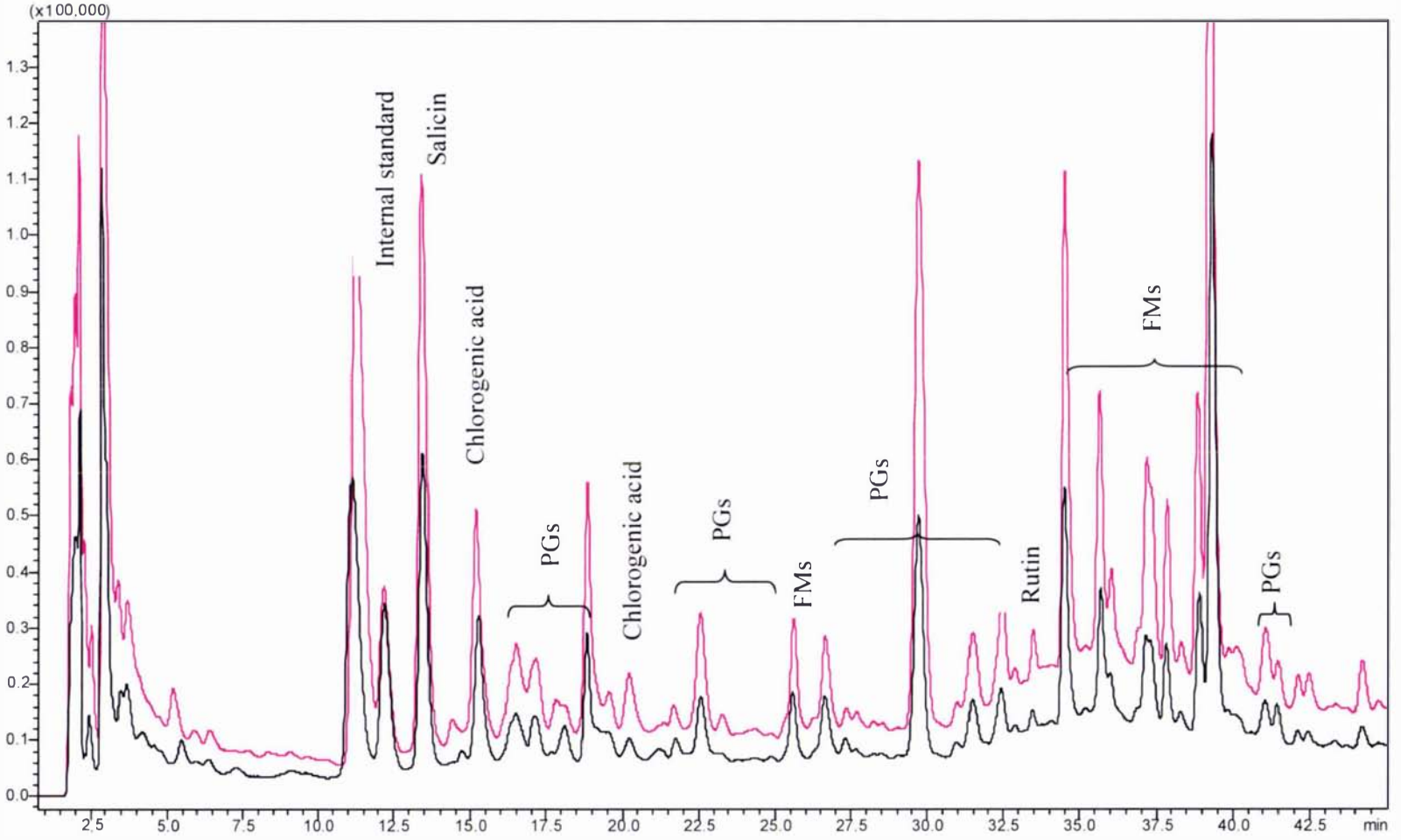


Figure 6.2 Utilisation patterns of secondary compounds (g/kg DM) in uninoculated media and bacterial cultures grown on Willow media after growth isolated from both willow supplemented (W2, W3, W5, W6, W7, W8 and W10) and control (W12, W17 and W18) ewes

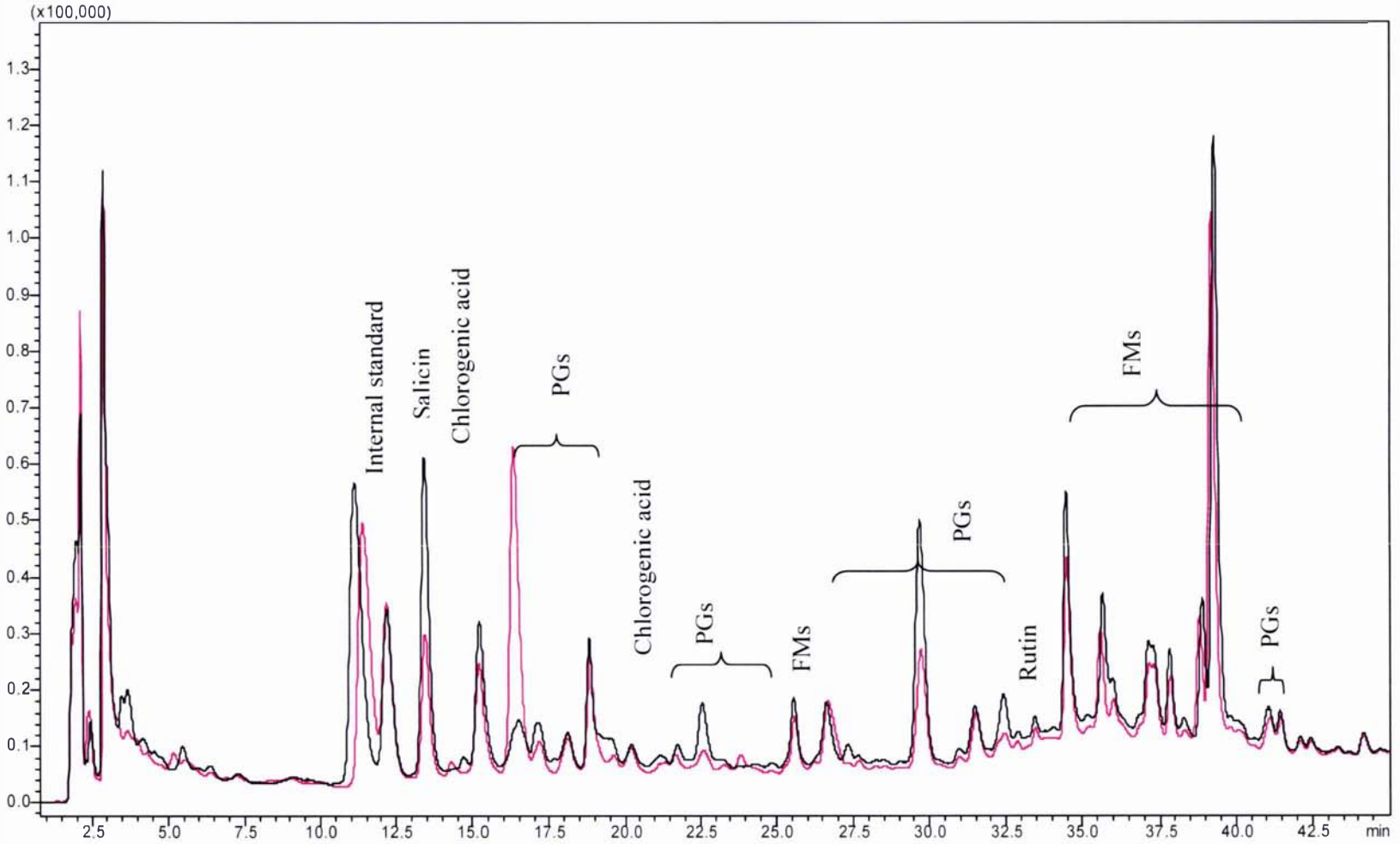
a. Isolate W2


(x100,000)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Internal standard
Salicin
Chlorogenic acid
PGs
Chlorogenic acid
PGs
FMs
PGs
Rutin
FMs
PGs
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
32.5
35.0
37.5
40.0
42.5
min

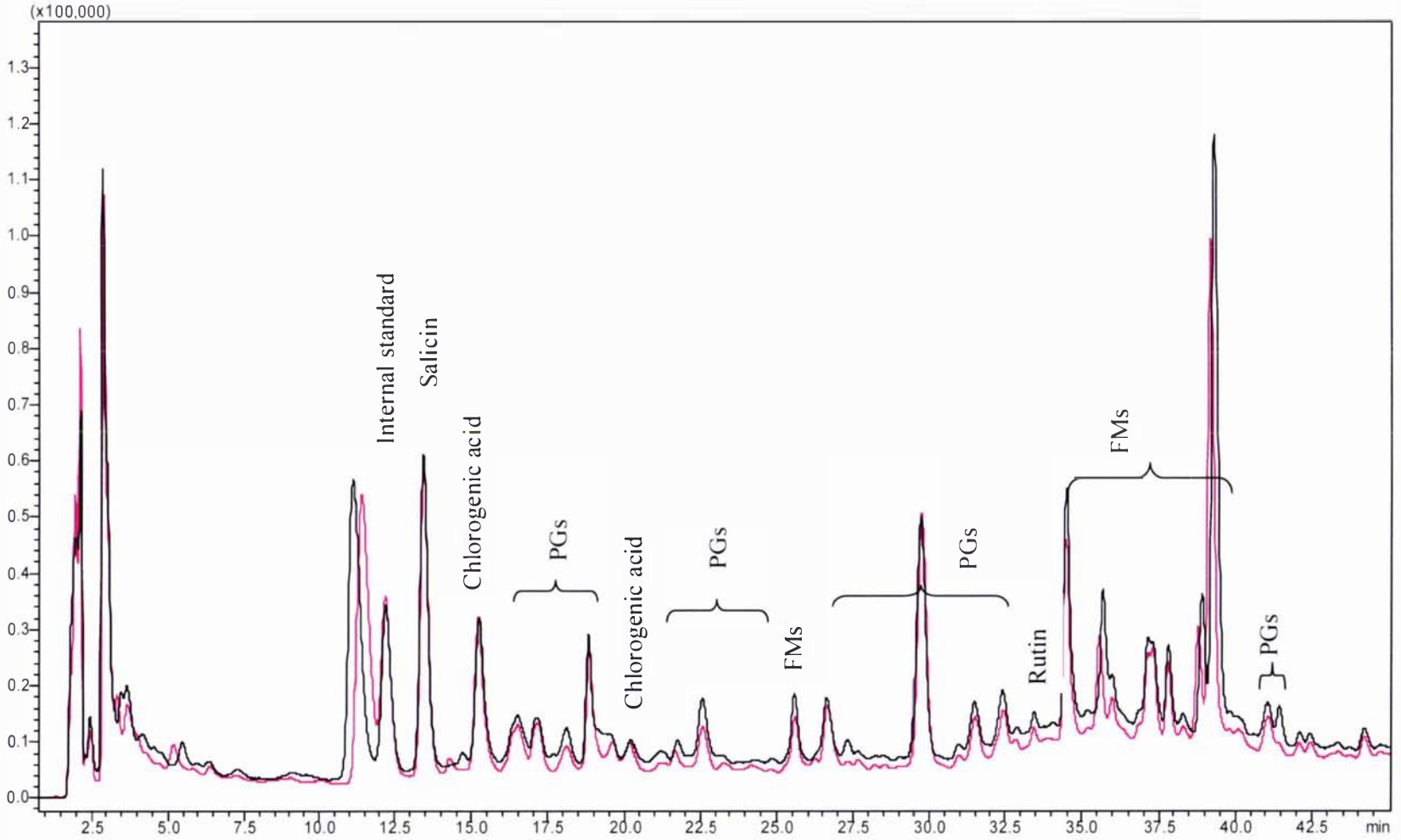
b. Isolate W3


(x100,000)
Internal standard
Salicin
Chlorogenic acid
PGs
Chlorogenic acid
PGs
FMs
PGs
Rutin
FMs
PGs
min

c. Isolate W8


(x100,000)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Internal standard
Salicin
Chlorogenic acid
PGs
Chlorogenic acid
PGs
FMs
PGs
Rutin
FMs
PGs
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
32.5
35.0
37.5
40.0
42.5
min

d. Isolate W10



e. IsolateW12

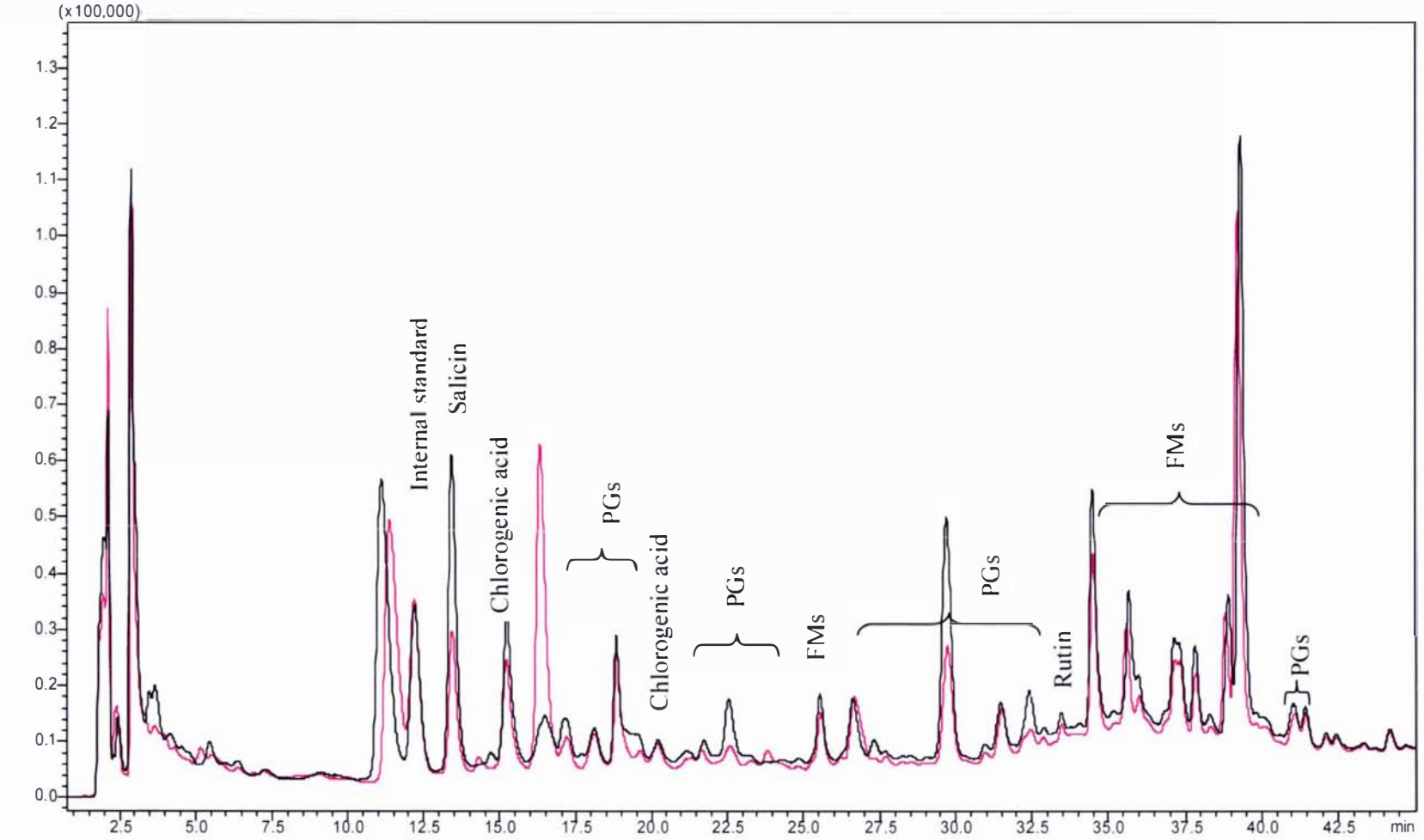


Figure 6.3 HPLC chromatograms indicating the elution of peaks before inoculation (—)and after growth (—) of Willow isolates

6.4.4 Phylogenetic analysis

DNA was extracted from each of the isolates and their 16*S* rRNA genes were PCR amplified and sequenced. These sequences were used to construct a phylogenetic tree (Figure 6.4). In this study, sequences that were 97% similar were considered same species and 93-96.9% similarity in their sequences were considered as same genus (Bond et al., 1995). Phylogenetic analysis confirmed this identification with S1 and S2 showing 99.2% and 99.4% 16*S* rDNA sequence similarity respectively to *S. bovis* ATCC 33317, a strain isolated from cow dung. 16*S* ssDNA sequence analysis indicates S5 is closely related to *C. sporogenes* (99.2% similarity) while isolate S10 is more distantly related (97.2 % similarity). 16*S* rDNA sequence analysis suggests isolate S7 belongs within the *Pseudobutyrivbrio* genus but was distinguishable from *P. ruminis* (94.3% similarity) or *P. xylanivorans* (93.5% similarity) and had the closest resemblance to isolates X3 and X15a that were isolated on Xylan medium (97.5 and 98.5% similarity respectively) from this study as described below. Isolate X10 had characteristics very similar to isolates S1 and S2 and *S. bovis* JB1 and 16*S* rDNA sequence comparison confirmed it was closely related to these organisms (99.6%, 99.7% and 99.5% similarity respectively). Phylogenetic analyses indicated that isolate X4 is closely related to *P. ruminis* (97.4% similarity) and isolates X3, X6, X9 and X15a from Xylan medium (97.1%, 98.5%, 97.9% and 97.7% similarity respectively). Isolates X3, X6, X9 and X15a clustered within the *Pseudobutyrivibrio* genus, where they show greater similarity to *P. ruminis* (98.6%, 98.7%, 98.7% and 99.3% similarity respectively) than *P. xylanivorans* (97.5%, 97.7%, 97.7% and 98.2% similarity respectively). Isolate X5 appears distinct from both *P. xylanivorans* and *P. ruminis*, and shows greatest similarity to isolate X15a (97.2% similarity). Although isolate X5 is located adjacent to isolate S7 in the tree, the phylogenetic distance between them is large as their 16*S* rRNA gene sequences show only 94.4% similarity.

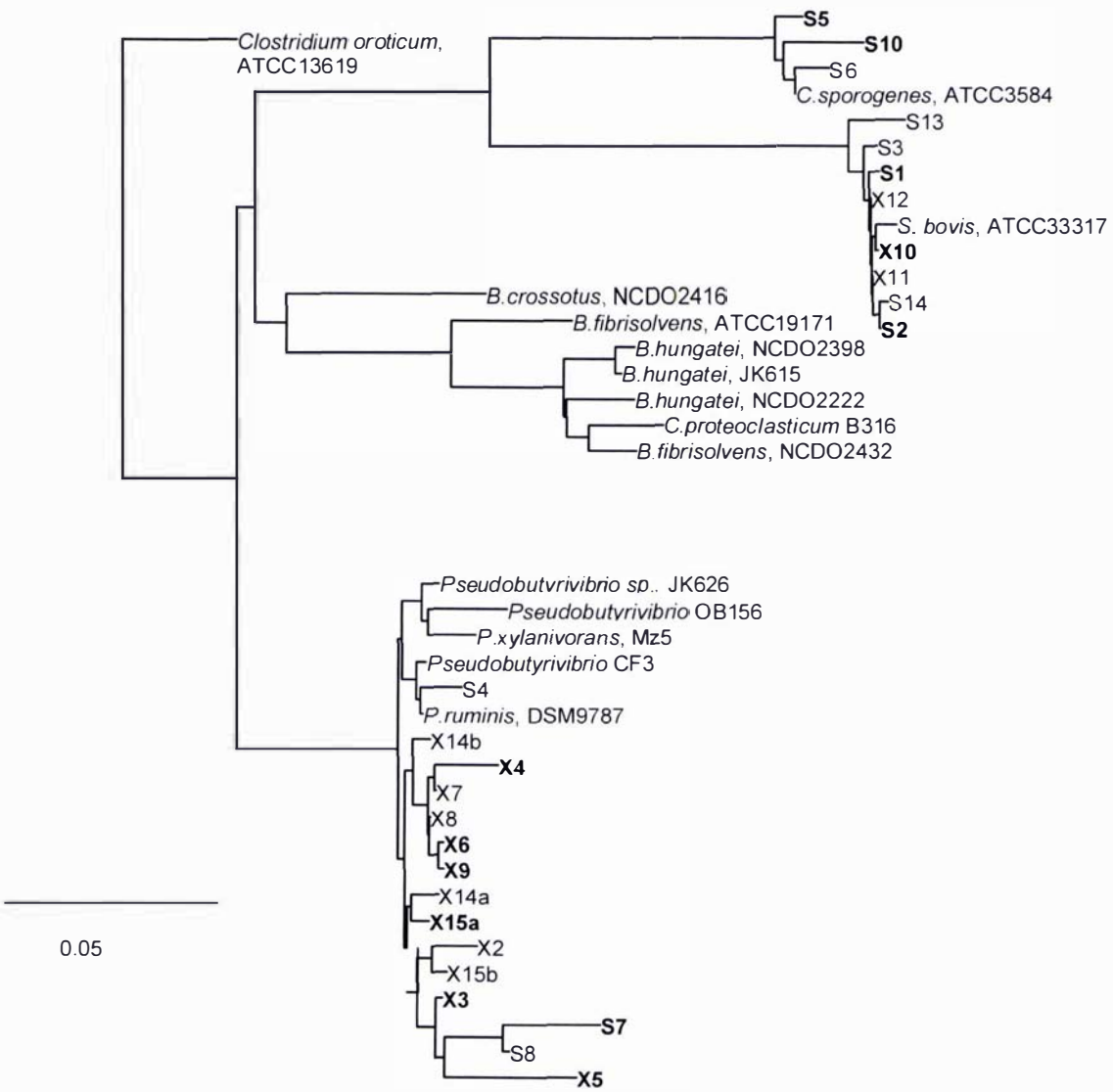


Figure 6.4 A phylogenetic tree constructed using the 16*S* rRNA gene sequences from the isolates on Salicin (S) and Xylan (X) media. The isolates in bold font were selected as representative isolates for phenotypic characterisation. 0.05 scale bar equals 5% difference in nucleotide sequence.

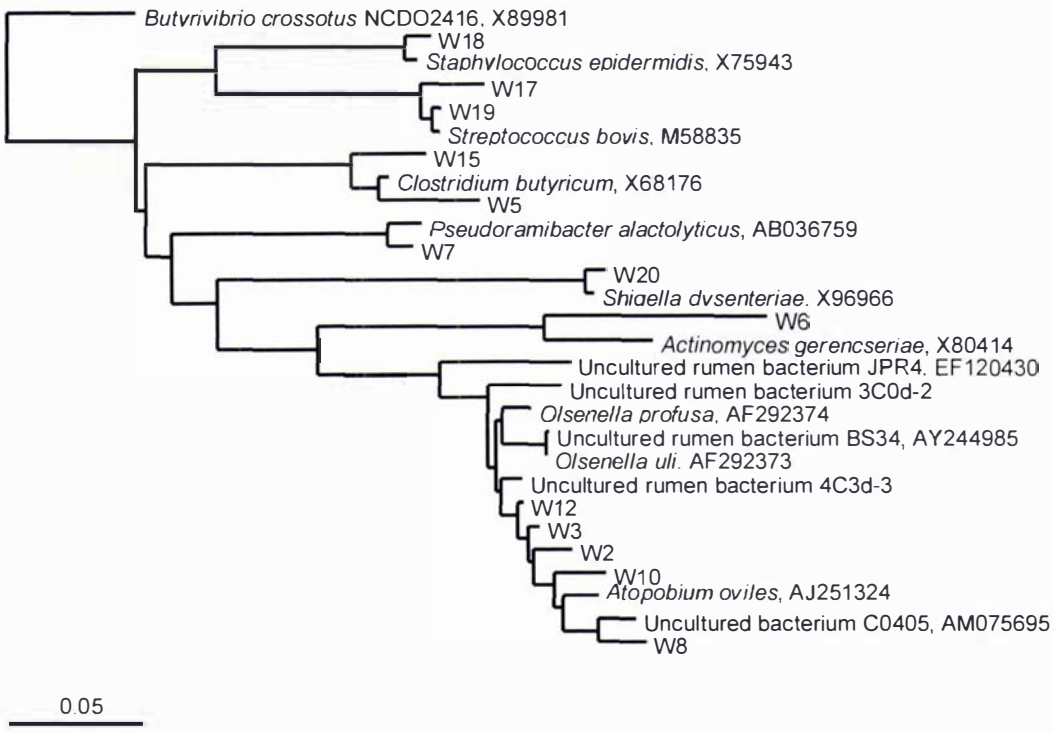


Figure 6.5 A phylogenetic tree constructed using the 16*S* rRNA gene sequences from the isolates on Willow (W) medium. 0.05 scale bar equals 5% difference in nucleotide sequence.

The bacterial isolates retrieved from Willow medium represented a wide range of bacterial types (Figure 6.5). Isolate W17 was most similar to *Streptococcus bovis* although the level of similarity (96.4%) suggests that it is not a close association. Isolate W18 was similar to *Staphylococcus epidermis* (98.1% similarity), W5 was similar to *Clostridium butyricum* (96.6% similarity), W7 was similar to *Pseudoramibacter alactolyticus* (97.9% similarity), W20 was closely related to *Shigella dysenteriae* (98.9% similarity) while the closest relative of isolate W6 was *Actinomyces gerencseriae* (89.8% similarity). Isolates W2,

W3, W8, W10, and W12 formed a separate cluster with *Olsenella uli* (95.2%, 96.5%, 93.1%, 94.4%, and 96.9% similarities to *O. uli* respectively).

6.5 DISCUSSION

Supplemental feeding of willow cuttings to ewes grazing drought pasture reduces live weight loss (McWilliam et al., 2005a and b; Pitta et al., 2005, 2007) due to an increase in dry matter intake and metabolisable energy available. The effect of willow supplementation on the microbiology of the rumen is largely unexplored as *Salix* species are not commonly fed to ruminant livestock. There is one report on seasonal changes in the ruminal microflora of Svalbard reindeer (Orpin et al., 1985) which browse on *Salix* species, in addition to mosses and herbs, during the winter months. The study showed that culturable bacteria decreased dramatically in the winter months compared to the summer but that the percentage of fibre-degrading organisms increased dramatically in the winter. It also found that *Butyrivibrio fibrisolvens* dominated the organisms isolated in both seasons and that *B. fibrisolvens* was the main cellulolytic and xylanolytic organism. The *Butyrivibrio* genus has since been amended to include the new species, *B. hungatei* and many *B. fibrisolvens* strains have been reclassified within a new genus, *Pseudobutyrivibrio*, which contains 2 species, *P. ruminis* and *P. xylanivorans* (Kopecny et al., 2003).

In the current study results similar to Svalbard reindeer study were observed with the number of culturable bacteria retrieved from all types of media being generally lower in willow-supplemented animals. The overall decrease in numbers of culturable bacteria in willow-supplemented animals is probably due to the high levels of secondary compounds present in the willow which are known to have antimicrobial effects. CTs and phenolic compounds released from PGs and FMs present in willow can affect rumen bacterial populations. The unusually high counts on Xylan medium in willow-supplemented animals at Week 10 may be due to an increase in *Butyrivibrio-Pseudobutyrivibrio* species able to use

xylan. The majority (7/12) of bacterial isolates characterised from the Salicin and Xylan media belonged to the *Pseudobutyrivibrio* genus, 6 of which were isolated from willow-supplemented animals. Most of these isolates were retrieved from Xylan medium and were able to utilise a wide range of sugars, including cellobiose, xylose and arabinose, the main sugars that make up the plant structural polymers, cellulose and hemicellulose. This indicates that these bacteria are adapted to use the breakdown products of cellulose and hemicellulose which supports their importance in the degradation of plant structural polymers. The 16*S* rDNA sequences of these isolates cluster within the *Pseudobutyrivibrio* genus, and isolates X3, X4, X6, X9 and X15a show sufficient similarity to *P. ruminis* to be included in this species. The phenotypic characteristics of these isolates are generally consistent with those reported for *P. ruminis* (van Gylswyk et al., 1996) except for some minor differences in the fermentation of lactose, trehalose, mannitol, glycerol and sorbitol. It is interesting to note that isolate X15a had a substrate utilisation pattern more similar to *B. fibrisolvens* $D1^T$ but its 16*S* rRNA gene sequence indicates it is more closely related to *P. ruminis* (99.3% similarity). The phenotypic characteristics of isolate X5 are very similar to those of the isolates X4, X6 and X9 and phylogenetic analysis places isolate X5 within the *Pseudobutyrivibrio* genus. However its level of similarity suggests it is sufficiently different from *P. ruminis* and *P. xylanivorans* to be considered a separate species. Likewise, isolate S7 belongs within the *Pseudobutyrivibrio* but separate from the currently defined species and also from isolate X5. The phenotypic characteristics of isolate S7 also differ to the other *P-ruminis*-like isolates in regard to xylose, mannitol, glycerol, raffinose, arabinose, melibiose, melezitose, sorbitol and dulcitol fermentation, although not within the currently defined species. Both isolates X5 and S7 probably represent new species of *Pseudobutyrivibrio*, but their description as separate species awaits the isolation of additional strains from the rumen with similar characteristics.

The remaining isolates obtained from Xylan and Salicin medium were similar to *S. bovis* or *C. sporogenes*. *S. bovis* is commonly isolated from rumen contents and is thought to be an opportunist in the rumen environment. It can utilise a wide range of sugars, is a facultative anaerobe and has a high growth rate, presumably so that it can quickly take advantage of changing conditions in the rumen (Stewart et al., 1997). However these characteristics also make it one of the more readily cultivated organisms from the rumen, so that it is often retrieved in cultivation experiments. *C. sporogenes* has been isolated from the rumen previously (Attwood et al., 2006) where it was implicated in skatole production. Clostridia are not considered to be significant inhabitants of the rumen (Stewart et al., 1997) and are probably only passing through the rumen transiently. *C. sporogenes* has been isolated from soils and animal faeces (Shreeve and Edwin 1974; Smith 1975), suggesting that it or its spores can survive in environments where grazing animals would ingest them and can also remain viable after passage through the digestive tract of animals.

The current study also isolated a number of unusual bacteria using a medium in which the sole carbon source was freeze-dried willow which they were able to degrade or extract some portion of the freeze-dried willow to support bacterial growth. Isolate W18 was identified as a strain of *Staphylococcus epidermis* a facultative anaerobe usually found on the skin of humans and animals, while isolate W5 was similar to *Clostridium butyricum,* a strict anaerobe found in a wide range of anaerobic environments. Isolate W7 was characterised as a strain of *Pseudoramibacter alactolyticus*, formerly known as *Eubacterium alactolyticum* isolated from oral cavities of periodontal cases (Willems and Collins, 1996). Isolate W20 was closely related to *Shigella dysenteriae* a facultative anaerobic, Gram negative rod which causes shigellosis (bacillary dysentery) in humans. Isolate W6 was distantly related to *Actinomyces gerencseriae* which is usually found in the periodontal flora of humans.

Isolates W2, W3, W8, W10, and W12 clustered separately in the phylogenetic analysis and fall within the *Olsenella* genus which has cultivated relatives from the human oral cavity. They were most closely related to *Olsenella uli*, a Gram positive rod-shaped bacterium originally isolated from the sub-gingival crevice of patients suffering periodontitis and acute necrotising ulcerative gingivitis (Dewhirst et al., 2001). When the genus was initially described, it included two species, *O. uli* and *O. profusa* which had 16*S* rRNA gene sequences which were similar to two sequences from a bovine rumen clone library (Tajima et al., 2000) and similar to a partial 16*S* rRNA gene sequence (GenBank AJ251324) from a hyper ammonia-producing rumen bacterial isolate, A2 "Atopobium oviles" retrieved from the ovine rumen (Eschenlauer et al., 2002). In the human oral cavity, *Olsenella* appear to have a role in carbohydrate fermentation and possibly a role in periodontitis (Dewhirst et al., 2001). Isolates W2, W3 and W8 were unable to ferment any of the sugars tested including the commercially available salicin, the main phenolic glycoside from willow. However, isolates W2 and W3 were able to use trypticase alone for growth indicating they are capable of metabolising peptides, while isolate W8 is not. Analysis of the components of Willow medium after growth indicated that isolate W2 and W8 were able to use FMs and PGs, including a small portion of the PG fraction identified as salicin. However, W3 did not utilise any of the willow secondary compounds measured and in fact released more secondary compounds from the Willow medium than were present in the uninoculated medium. This suggests that W3 degrades components of Willow medium that are not measured by our analysis, and in doing so has released additional willow secondary compounds. Isolates W10 and W12 were able to ferment carbohydrates, although W10 grew only weakly on a small number of sugars and both grew weakly when trypticase was sole substrate. Both isolates used FMs but did not generally use PGs, although W12 used salicin. The ability of these isolates to use components of willow may explain similar

reports (Edwards, 1999) of utilisation of plant secondary metabolites on rumen fermentation. The study observed that glucosidic forms of the flavones, apigenin and luteolin, from the fodder shrub Tagasaste (*Chamaecytisus proliferus*) had a positive effect on *in vitro* rumen fermentation through increased gas production, while the aglycone forms of these flavones failed to show these effects.

In summary, isolates on conventional media containing xylan and salicin as carbon sources were bacteria that are commonly isolated from the rumen while bacteria isolated from Willow medium were unusual organisms, not often, or ever, seen previously in rumen samples. Most of the isolates from the Salicin and Xylan media originated from willow-supplemented animals and belonged to the *Pseudobutyrivibrio* group of rumen bacteria. These bacteria are known to be important in the breakdown of hemicellulose and their isolation on the highly lignified willow supplemented, drought pasture is expected. The general ability of *Butyrivibrio-Pseudobutyrivibrio* to withstand the inhibitory effects of secondary plant compounds (Min et al., 2005) has also probably enhanced their populations in willow supplemented animals, despite the general drop in bacterial numbers in this treatment group. The isolates recovered from the Willow medium appear to fall into two distinct groups: those that are unlikely to reside permanently in the rumen but which are able to use carbohydrates released from breakdown of plant material, and a closely related group of specialised bacteria which appear to be adapted to use plant secondary compounds for growth. These specialised bacteria show an ability to use particular (or in some cases unidentified) components of willow, most commonly PGs or FMs. The retrieval of several isolates with these characteristics from willow-supplemented animals suggests they may play an important role in the ruminal metabolism of secondary compounds. The fact that one of these organisms (isolate W12) was cultured from a control animal suggests that they are also present in the rumen in the absence of a willow diet, but probably at low numbers. However,

as indicated in the previous chapter, these isolates were not present among the groups of bacteria identified by DGGE analysis as being unique to the willow supplemented animals. Therefore, further work using molecular detection techniques will be required to establish the numbers of these organisms present in the rumen on willow diets and the extent to which these organisms contribute to plant secondary compound metabolism.

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CHAPTER 7.
GENERAL DISCUSSION

7.0 INTRODUCTION

Climatic predictions indicate that summer/autumn droughts will be more frequent and severe in the East Coast regions of NZ in the near future (Korte and Rhodes., 1993). A loss of 14$/ewe/annum at the individual farm level has been reported during drought (McWilliam, 2004). One economic solution to combat drought is to use willow and poplar trees which were originally planted in these areas for soil conservation, by cutting small stems and feeding them to grazing livestock. Supplementing willow and poplar stem cutting has become widespread in hill country areas of the East Coast of NZ over the past decade, although this process involves labor and time (Charlton et al., 2003; Moore et al., 2003; McWilliam, 2004). McWilliam (2004) conducted a series of three experiments in 2001-2003 at Massey University's Riverside Farm near Masterton in Wairarapa on supplementation of willow and poplar stem cuttings to mating ewes grazing drought pasture upon their production and reproduction. The author reported a reduction in ewe live-weight loss and an increase in scanning and weaning percentages by 16 and 20 % units respectively.

Willow stem cuttings can be planted as fodder blocks in areas of the farm that have low productivity (low lying, wet and swampy), turning them into productive grazing areas whilst also saving labor and time in cutting and supplementing willow and poplar stems (Douglas et al., 2003). This thesis is a continuation from the above experiments to evaluate the use of willow fodder blocks at Massey University's Riverside Farm from 2003-2006, as a feed source for grazing sheep during drought. From the grazing experiments in 2003 and 2004, it was concluded that willow fodder blocks are a good supplement for mating ewes during dry summer conditions and the year round management of these willow fodder

blocks is crucial to effectively utilise them as a supplementary feed. The 2005 short grazing experiment showed that supplementing willow to fistulated ewes grazing short drought pasture reduced tissue protein breakdown that occurs when ewes loose live-weight during a drought. The microbiological and molecular study of rumen samples resulted in the isolation and characterisation of novel microbes which grow only on willow and no other carbohydrate source and showed that mixed rumen micro-organisms can degrade FMs and PGs.

7.1 DROUGHT

Scenarios of future climate change for NZ differ between regions but indicate increasing drought risk across the country, including drought prone eastern regions (Mullan et al., 2001; Wratt et al., 2003). Predictions of the frequency of droughts in NZ have been done by the National Institute for Water and Atmospheric Research (NIWA).

7.1.1 Defining the severity of drought in NZ

In response to the weather predictions and drought warnings, NIWA (2005) conducted a study to quantify the effects of drought risk by taking into consideration the amount of rainfall and potential evapotranspiration. Data was obtained from January, 1973 until 2003 December at NIWA (Tait et al., 2005). From these studies, NIWA (2005) have concluded the following.

- Drought is caused by a number of climatic factors, including how much rain falls, how high the temperatures are, and how much wind the country experiences.
- Potential evapotranspiration deficit (PED) was used as a measure of drought. This measure incorporates all three of the above climatic factors. Accumulated PED (measured in mm) is the amount of water that would need to be added to a crop over a year to prevent loss of production due to water shortage. For pastures not receiving irrigation, an increase in accumulated PED of 30 mm corresponds to approximately one week more of pasture moisture deficit (reduced grass growth).
- Accumulated PED is calculated over a July to June 'growing year' from daily information stored in NIWA's climatic database. A PED between 200 mm to 400

mm indicates very dry conditions, a PED of >400 mm could result in a drought and >600 mm corresponds to a severe drought.

- The driest parts of the country (Gisborne, Hawkes Bay, Wairarapa, Marlborough, most of coastal Canterbury, and inland Otago) experience annual water deficits in the 300-500mm range. Most of this occurs during the summer/autumn months of Feb-April.
- The incidence of drought varies from year to year. El Nino tends to bring drier conditions to the northeast of both the North Island and the South Island. La Nina can also bring drought to the eastern South Island. PED exceeded 600 mm in the eastern NZ in the severe drought year of 1997/98, which coincided with a strong El Nino in the tropical Pacific. PED levels of 200, 400 and 600mm indicate the severity of drought when the total area of the country was plotted against years (Fig 7.1). For an annual PED accumulation of 400mm or more, there were 9 years out of the 31-year historic record, where more than 11% of the country was "in drought". These years, in decreasing order of severity, were: 1997/98, 1972/73, 1977/78, 2000/01, 1982/83 and 1988/89, 2002/03, 1981/82 and 1984/85. Severe drought covering 10% of the country occurred in 1997 and covering approximately 2.5 % of the country in 1998/2000 and 1977/78 (Figure 7.1).

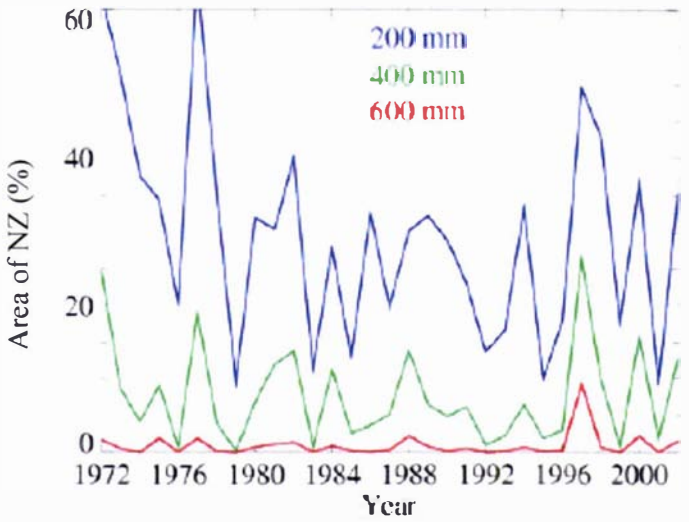

Figure 7.1 The areas of NZ which experienced drought during 1972-2003 with July-June PED accumulation exceeding specified threshold (> 200 mm represent dry conditions; > 400 mm is defined as drought; > 600 mm is defined as severe drought) have been plotted against time (NIWA, 2005).

7.1.2 Prediction of future drought risk under scenarios of climate change

NIWA (2005) predict the following future occurrence of droughts in NZ.

- Under all our climate change scenarios, average annual PED increases across virtually the entire country (that is, it gets drier), except for the west coast of the South Island by the 2030s. Average annual PED increases even more by the 2080s.
- The risk of drought (extreme PED) increases in most eastern parts of the country, areas which are already drought-prone.
- Under all climate change scenarios, a 1-in-20 year severe drought in eastern regions becomes more common in future. By the 2080s, the frequency of a current 1-in- 20 year PED increases between two and more than fourfold, depending on the

scenario. That is, a severe drought that currently occurs once in 20 years on average could become a 1-in-10 year, or even a 1-in-5 year, event in that same area in the future.

- The areas where drought risk is projected to increase significantly include parts of North Otago, Canterbury, Marlborough, Wairarapa, Hawkes Bay, Gisborne, Bay of Plenty, and Northland.
- Because all the scenarios predict increased PED accumulation over the course of a year, drought periods are likely to 'expand' into spring and autumn more often than currently. In most severe (medium-high) scenario, the drying of pasture in spring is advanced by about a month in the 2080s in dry eastern regions, compared to the current climate.

Thus, using current models of climate change, all scenarios indicate that both the frequency and severity of drought is likely to increase in NZ, especially in the dry East Coast regions of both Islands, and that two new areas (Bay of Plenty and Northland) will have a higher risk of developing drought.

7.1.3 Quality of pastures during drought

McCabe and Barry (1988) defined a typical drought pasture as being of low quality with high dead matter content and McWilliam et al. (2005a and b) defined a pregrazing pasture mass of approximately 1200-1500 kg DM/ha with a sward height (5-7 cm tall) to be short drought pasture. In this study, short drought pasture was simulated in 2003, and 2004 (Table 7.1), which was very similar to the pasture simulated by McWilliam (2004).

The pasture mass was low with a high dead matter content (>50%), a low nutritive value of < 8 MJ ME/kg DM and a high fibre content (600g NDF/kg DM).

In addition to the short drought pasture, long drought pasture was simulated and defined in this study. Pre-grazing pasture mass (>4000 kg DM/ha) with a sward height of (> 30cm) and a considerable amount of accumulated dead matter content (30–60 %) is defined as a long drought pasture (Table 7.1). Long drought pasture was similar to short drought pasture in composition during 2003 (a drought year), but in 2004 (a non drought year) was of higher nutritive value. Both short and long drought pastures contained low concentrations of the secondary compounds (i.e., CTs, FMs and PGs). In both years drought pastures were simulated by using the areas of the farm with the shallowest stony soils, allowing the pasture to reach maturity with the development of seed heads, and then grazing to the desired height with non experimental stock. Using these procedures, drought pastures could be produced with a high degree of reproducibility.

7.1.4 Concentration of other secondary compounds in short drought pastures

Zearalenone (oestrogenic mycotoxin), and common trichothecene mycotoxins like Nivalenol (NIV) and deoxynivalenon (DON) are produced by *Fusarium sp* fungi in NZ pasture (Lauren et al., 1988,1992). These compounds are often produced during dry summer and autumn under NZ grazing systems (Towers 1997) and were also reported by McWilliam, (2004; Table 7.2). The concentrations of these compounds recorded in 2003 were three to four fold greater than the previous years, which was associated with greater live weight loss of approximately 150g/d and reduced fecundity when grazed by ewes at the time of mating.

Table 7.1 Comparison of herbage mass and nutritive value of long and short drought pastures simulated in this study during the 2003 and 2004 grazing experiments

	2003				2004			
	short drought pasture	long drought pasture	Willow fodder block		short drought pasture	long drought pasture	Willow fodder block	
			Willow pasture	Willow tree			Willow pasture	Willow tree
Herbage mass								
Pre-grazing (kg DM/ha)	1639	3776	5206	549	1486	4256	5724	814
Pre-grazing dead matter content (%)	66.2	63.5	50.6		56.9	26.9	30.6	
Nutritive value (g/kg DM)								
Total N	22.7	16.0	18	15.5	24.2	25.5	20	13.6
NDF	588.1	648.7	587	370.4	550.7	460.5	512	417.4
DOMD	0.47	0.45	0.5	0.66	0.50	0.59	0.59	0.67
ME(MJ/kg DM)	7.7	7.4	8.0	10.7	8.2	9.6	8.8	9.9
Condensed Tannins	2.6	2.4	5.0	30.1	1.9	2.0	3.6	38.3
Flavanoid monomers	2.2	2.0	3.3	13.5	5.2	10.2	4.2	14.2
Phenolic glycosides	1.7	1.9	1.9	14.5	4.4	9.2	5.1	36.5

(Pitta et al., 2005, 2007)

Table 7.2 Concentration of other secondary compounds (g/kg DM) in short drought pasture simulated in the 2001-2003 year long grazing experiments

	2001	2002	2003
Zearalenone	0.58	0.16	1.51
Nivalenol	0.05	0.05	0.19
Deoxy-nivalenol	0.10	0.06	0.26
Total Trichothecenes	0.15	0.11	0.45

(McWilliam, 2004)

7.2 WILLOW FODDER BLOCKS

7.2.1 Contribution of willow tree in fodder block

Willow trees in the fodder block at Massey University's Riverside Farm were 2 to 3 years old in 2003, when the grazing experiment started. Willow tree's contribution (550 kg DM/ha) was only 10% of total biomass yields from willow fodder block in 2003, but increased to 20% by 2004 (814 kg DM/ha). The nutritive value of edible willow tree (Table 7.1) was consistently superior to both drought pastures (long and short) in experiments conducted in 2003 and 2004. Nutritive value of the willow was similar to that reported by McWilliam et al., (2005a and b), with a digestibility of 0.66 and ME of approximately 10-11 MJ/kg DM. An interesting finding in these two experiments is the presence of secondary compounds in edible willow in very high concentrations; CT (30-40 g/kg DM), FM (14 g/kg DM) and PG (15-30 g/kg DM) similar to the findings reported by McWilliam et al. (2005a and b).

7.2.2 Contribution of willow pasture in fodder block

The sites selected to establish willow fodder blocks at Riverside Farm were rush infested areas, containing no edible forage in the undeveloped state. During the development, the process of mowing the rushes, spraying and ripping the ground followed by plantation of willow poles, resulted in a drier area (more evapotranspiration from willow trees), which supported the growth of a pasture from volunteer plant species including grasses, legumes and herbs (Pitta et al., 2005). Willow pasture masses were higher (approximately 5000 kg DM/ha) contributing (approximately 90%) of total biomass yields at the start of experiment 1, but a significant amount of this mass was not consumed with a high dead matter content of 50% (Table 7.1). The quality of willow pasture was greatly improved with the development of an improved management strategy (3 grazings per season) that reduced the dead matter content to 30% at the start of experiment 2 in 2004, although the pasture masses remained high (5000kg DM/ha). The nutritive value of willow pasture was similar to that of long drought pasture (Table 7.1), with the exception of the concentrations of CTs being double that of control drought pastures.

7.2.3 Dietary allowance and access to willow fodder blocks

Douglas et al. (1996; 2003) reported that the amount of edible biomass harvested from a young willow tree by livestock is approximately 50%. In the 2003 experimental grazing, two treatment groups (n=100) with restricted access and full access to willow fodder blocks were compared to control drought pastures. In restricted access, ewes had (75%) of dietary allowance (0.8 kg/ewe/d) from short drought pasture and 25% (0.4 kg/ewe/d) from willow fodder blocks whilst in the full access group, ewes were kept on

fodder blocks all the time (fenced) at an allowance of 2.0 kg/ewe/d and had no access to outside pasture. Performance of ewes with restricted access to willow fodder blocks was similar to those grazing control drought pastures and lower ($P<0.05$) than ewes with full access (Pitta et al., 2005). From the 2003 grazing experiment, it was concluded that restricted access to willow fodder blocks was uneconomical and labor intensive and therefore this group was eliminated in the 2004 experiment. Ewes with full access to willow fodder blocks were consistent in their higher reproductive performance in both 2003 and 2004 experiments at an allowance of 2.0 kg DM/ewe/d.

7.2.4 Grazing management of willow fodder blocks

Willow fodder block was considered as a tree system when the programme first started, but with the unexpected growth of pasture cover underneath the trees, it turned out to be tree/pasture system. Weaknesses observed in one experiment were minimised in the following year until a better management system was developed over 4 years to efficiently utilise willow fodder blocks as a supplementary feed with minimal investment of labor; this management system for fodder blocks at Riverside Farm is detailed below.

7.2.4.1 Weaknesses in grazing willow fodder blocks

- There is an inherent mis-match in the grazing systems required to control herbage on the one hand and trees on the other. Herbage requires a short grazing interval of 3-6 weeks, whilst trees require a longer interval of at least 7-10 weeks to regenerate.

- Waiting for the trees to reach an ideal stage results in pasture mass getting too high, resulting in high dead matter content and low nutritive value such as occurred in 2003.
- The principal cause was rapid growth of volunteer grasses in spring that went rapidly into the reproductive phase. Because of the presence of the trees the herbage in the fodder blocks could not be controlled by mechanical topping.
- If the grazing interval was too long, then the trees got too tall for sheep to browse. Therefore more than one grazing during the tree growing season was needed.

7.2.4.2 Grazing plan

Based on the above findings, a grazing system was developed over the last 4 years of experimentation at Riverside Farm with three grazings of the fodder blocks during the tree growing season, followed by continuous grazing in the non tree growing season to control the grasses (mid may to early Oct). Major aspects are as follows.

- Exclude all livestock and close up willow fodder blocks from grazing, in early October (spring) to protect developing tree leaf buds.
- Browse the trees in December, February and March/April, allowing about 7-8 weeks between grazing. Each fodder block has been grazed for 7-10 days, in this rotation.
- Graze hard with ewes in mid May, to eat down the tree stems and to remove stemmy pasture. Manual topping may be needed to reduce tree height.
- Graze lightly over winter and set stock over lambing (September-early October).

7.2.4.3 Improvement in quality in willow fodder blocks

With the progressive implementation of this grazing plan at Riverside Farm in managing willow fodder blocks, the following improvements were noticed (Table 7.3) over a 4 year period.

- Pasture masses were reduced in 2006, compared with the initial experiment in 2003, and herbage dead matter content was reduced.
- The percentage of legumes in willow pasture increased with increased grazings and was higher in willow fodder blocks than in control drought pastures (Table 7.3).
- The nutritive value of willow fodder block herbage improved with increased grazing frequency, especially for regrowth. The presence of *Lotus pedunculatus* containing CT was noted in the willow pasture and this developed over time; it appeared to be suited to the grazing system that was developed.
- The nutritive value of edible willow remained high and was unaffected by increased grazing frequency. Increased grazing gave better control of tree height.

Table 7.3 Effect of increasing the number of grazings per season upon herbage mass and botanical composition in willow browse blocks from 2003 to 2006

	2003[1]		2004[2]		2004-2005[3]		2006[4]	
	Herbage	**Tree**	**Herbage**	**Tree**	**Herbage**	**Tree**	**Herbage**	**Tree**
Grazings per season	1		2		3		3	
Primary growth								
Pre grazing mass	5206	549	5724	814	5074	562	Light grazing was done with non experimental sheep in December, 2005 before the experiment commenced	
Post grazing mass	2958	370	3605	470	3545	262		
Botanical composition (%)								
Dead matter content	50.6		30.6		9.5			
Grasses			57.8		49.5			
Legumes			24.0 (12.7)[5]		19.4 (19.0)[5]			
Second growth								
Pre grazing mass			3369	226	4213	775	4542	698
Post grazing mass			1333	101	3169	236	3445	417
Botanical composition (%)								
Dead matter content	Not grazed		15.0		23.7		22.2	
Grasses			81.9		30.1		68.4	
Legumes			12.2 (8.8)[5]		37.1 (24.0)[5]		17.4 (6.6)[5]	
Third growth								
Pre grazing mass							3139	367
Post grazing mass					Light grazing was done with experimental lambs and no experimental data was collected		2079	287
Botanical composition (%)	Not grazed		Not grazed					
Dead matter content							20.3	
Grasses							77.6	
Legumes							9.5 (6.0)[5]	

[1] This study – experiment 1 (2003); [2] This study – experiment 1 (2004); [3] Diazlira (2005) – experiment conducted in 2004-2005; [4] Musonda (2007)- experiment conducted in 2006; [5] Value for control drought pasture in the same study.

7.3 EFFECT OF TREE FODDER SUPPLEMENTATION ON EWE PERFORMANCE

7.3.1 Increase in ewe reproductive rate

Willow stem cuttings were supplemented to ewes grazing drought pastures during mating in 2002, 2003 (McWilliam et al., 2005a and b) and ewes grazed willow fodder blocks in 2003-2004 (Pitta et al., 2005) as an alternate to drought pasture during mating. Both studies reported improvements in reproductive performance of ewes in the form of higher conception rates and more multiple births as compared to ewes mated on short drought pasture (Table 7.4), through increases in both conception rate and multiple births in ewes (fecundity). Trees comprised approximately 30% of the dry matter intake in the supplementation trials and 11-16 % of the dry matter intake when ewes grazed the willow fodder blocks.

7.3.1.1 Reasons for the enhanced reproductive rate

One direct mechanism for the increased ewe reproductive performance when supplemented with willow or when grazed willow fodder block is explained by the increase in DM intake. In Table 7.5 ewe DM intakes during the period of supplementation have been calculated using estimates of pasture and tree mass made before and after grazing. Over a 5 year experimentation with short drought pasture at Riverside Farm (McWilliam et al., 2005 a & b; this study), it was concluded that DM intake by ewes grazing short drought pasture is low and reasonably reproducable with an average of approximately 0.6 kg/ewe/d. At this rate of DM intake, ewes were observed to loose LW of approximately 100g/d, which is typical of drought. Willow supplementation or grazing willow fodder blocks by ewes resulted in an increase in

DM intake relative to short drought pasture. This increased DM intake was close to a maintenance level in ewes (1 kg/ewe/d) supplemented with willow stem cuttings and slightly above maintenance for ewes grazing willow fodder blocks. This was evident by the pattern of LW change in ewes where LW loss was reduced in ewes supplemented with willow stem cuttings compared to ewes grazing drought pasture (Table 7.5) and was reduced still further when grazing willow fodder blocks. Ewes grazing the latter were close to maintenance.

The increases in reproductive rate of ewes (>15%) when grazed willow fodder blocks during mating (Table 7.4) were similar to the results reported by McWilliam (2004) and were much higher than the marginal increase in the calculated reproductive rate of about 4 to 5% that could have resulted from an increase in 1.5% unit for each additional megajoule of digestible energy consumed (Smith, 1985). Lindsay (1976) observed that ovulation rate increased with an increase in protein intake without the associated change in LW. Smith (1985) reported that a threshold of 125g/ewe/d in CP intake is necessary to achieve an increased ovulation rate, independent of the increases caused by increases in ME intake. Ewes grazing short drought pasture in various experiments from 2001 to 2004 (Table 7.6) had low intakes of CP (average 76g/ewe/d) which is substantially lower than 125 g/ewe/d, which further explains the low reproductive performance of ewes grazing short drought pasture. The levels of CP intake in 3 of the 5 experiments summarised were augmented to levels that were ≥ 125 g/ewe/d with willow supplementation or grazing willow fodder blocks and substantial increases occurred in the other two experiments (Table 7.6). Therefore increased levels of CP intake might have a significant

contribution in increasing the reproductive rate when ewes received supplementation of willow cuttings or grazing willow fodder blocks.

Providing supplementary willow or grazing willow fodder blocks notably increased the calculated intake of CT (11g/d) compared with the averages for ewes grazing short drought pasture (1g/d; Table 7.6). CT is known to bind plant protein which is pH dependent and stable between 3.5 to 7.5, reducing protein degradation in the rumen but dissociates in the abomasum at pH 2.5 to 3.5 (Barry and McNabb, 1999). CT is reported to be beneficial at low to medium concentrations by increasing the outflow of protein-N to the intestine (UDP) from the rumen relative to N intake and also increases the flow of EAA out of Abomasum by 50-53%. Thus the net absorption of EAA from the small intestine is increased by 59-63% (Barry and McNabb, 1999; Barry et al., 2001) in sheep fed the legume *Lotus corniculatus*. These increases in EAA absorption also depend upon the molecular weight and structure of the CT, which are unknown for willow CT. However, the increased CT intakes reported in this study (Table 7.6) by ewes with willow supplementation or grazing willow fodder blocks could have contributed to the increased reproductive performance of ewes by the above mechanism and increase the absorption of UDP.

The presence of phenolic compounds like PG and FM in higher concentrations in willow tree and their higher calculated intakes suggest their contribution to the increased performance in ewes supplemented with willow compared to ewes that grazed drought pasture. Lowry et al. (1996) indicated that phenolic compounds are esterified to sugars as PGs and are degraded during the process of digestion; stripping of glycosidic part from flavones present in *Tagasaste* when fed to sheep had a positive influence on rumen fermentation through increases in gas production whilst

also providing a carbohydrate source (Edwards, 1999). During a drought, cleavage of glycosidic part from PGs or phenolics themselves seem to be possible mechanisms (Lowry et al., 1993, 1996) to provide for energy to support rumen microbial fermentation. The contribution of PG in rumen metabolism will be discussed at a later stage in this Chapter.

Table 7.4 Comparison of willow stem cuttings as supplementary feed to ewes grazing drought pastures and grazing willow fodder blocks during mating (summer/autumn) upon ewe reproductive performance in a series of grazing experiments conducted at Massey University's Riverside Farm from 2002 until 2004

	Willow stem cuttings		Willow fodder blocks	
	2002 McWilliam et al (2005a)	**2003 McWilliam et al (2005b)**	**2003 Experiment 1**	**2004 Experiment 2**
Reproductive rate (%)				
Scanning	148	128	148	147
Lambing	148	127	137	134
Relative increase in reproductive rate (% units)[1]				
Scanning	16	0	24	12
Lambing	17	3	15	18
Conception rate	91.4 (91.4)[2]	97.0 (93.9)	95.0 (92.0)	96.0 (91.0)
Fecundity				
Singles	37.8 (56.1)	69.2 (67.0)	50.0 (66.0)	49.7 (53.3)
Twins	62.2 (43.9)	30.8 (33.0)	50.0 (37.0)	50.3 (46.7)

[1] Relative increase compared to ewes mated on short drought pasture

[2] Values in parenthesis indicate values recorded in short drought pasture from that year.

Table 7.5 Comparison of calculated intakes of DM (kg/d) by ewes and their live weight change in grazing experiments with willow stem cuttings and willow fodder blocks as supplementary feed to ewes grazing drought pastures during mating (summer/april) conducted at Massey University's Riverside Farm from 2001 until 2004

	Year	Control		Willow fodder blocks (pasture and trees)	Short drought pasture + willow/poplar supplementation
		Short drought pasture	Long drought pasture		
DM intake (kg/d)					
Experiment 1	2003	0.42	0.88	0.88	
Experiment 2	2004	0.70	1.66	2.10	
McWilliam (2004)	2001[1]	0.67			1.03
	2002	0.59			0.86
	2003	0.47			0.75
LW change (g/d)					
Experiment 1	2003	-101	-75	-41	
Experiment 2	2004	-17	54	-5	
McWilliam (2004)	2001[1]	-82			-67
	2002	-103			-86
	2003	-147			-96

[1] Experiment with poplar supplementation

Table 7.6 Comparison of calculated intakes (g/d) of CP, CT and PG in grazing experiments with willow stem cuttings and willow fodder blocks as supplementary feed to ewes grazing drought pastures during mating (summer/april) conducted at Massey University's Riverside Farm from 2001 until 2004

	Year	Control		Willow fodder blocks (pasture and trees)	Short drought pasture + willow/poplar supplementation
		Short drought pasture	Long drought pasture		
CP intake (g/d)					
Experiment 1	2003	60	89	97	
Experiment 2	2004	110	238	211	
McWilliam (2004)	2001[1]	71			132
	2002	91			137
	2003	47			91
CT intake (g/d)					
Experiment 1	2003	1.1	1.9	6.3	
Experiment 2	2004	1.3	3.4	17.6	
McWilliam (2004)	2001[1]	1.1			2.5
	2002	0.9			14.0
	2003	0.7			7.6
PG intake (g/d)					
Experiment 1	2003	0.67	1.7	2.6	
Experiment 2	2004	3.1	15.3	19.9	
McWilliam (2004)	2002	1.3			2.7
	2003	0.7			1.1

[1] Experiment with poplar supplementation

7.3.2 Increase in plasma amino acid concentrations

Relative to ewes mated on perennial ryegrass/white clover pasture, Min et al. (1999) found that ewes mated on CT- containing *Lotus corniculatus* had increased plasma concentrations of EAA and BCAA. Heightened plasma concentrations of EAA and BCAA have been associated with increased OR. Similar changes were induced by willow supplementation in experiment 3, notably at 35 days, but the changes were of reduced magnitude to those found by Min et al. (1999).

The concentrations of NEAA (Table 7.7) were higher in control ewes that grazed drought pasture in experiment 3, compared to pasture fed ewes reported by Min et al. (1999) that were gaining LW. This is explained by increased rate of body protein degradation in ewes loosing weight in a drought, as also indicated by the presence of 3-MTH (discussed in Chapter 4) in higher concentrations at 35d in the plasma of control ewes. As plasma concentrations of both NEAA and 3-MTH was reduced by willow supplementation, it was concluded that muscle protein breakdown under drought is reduced by willow supplementation.

Collectively, the information on plasma amino acid concentrations support the deductions made on CP intake and suggests that one of the mechanisms for the increased reproductive rate in ewes given willow supplementation or grazing willow fodder blocks, relative to ewes grazing short drought pasture, is increased absorption of limiting amino acids.

Table 7.7 Comparison of plasma amino acid concentrations (μ mole/L) in fistulated ewes grazing short drought pasture, with and without willow supplmentation (experiment 3), with that of ewes grazing *Lotus corniculatus* and ryegrass/white clover pasture (Min et al., 1999).

	This study				Min et al (1999)	
	35 days		70 days			
	Drought pasture	Willow supplemented	Drought pasture	Willow supplemented	Pasture	*Lotus corniculatus*
Diet composition						
Total N (g/kg DM)	18.9	23.4	18.9	23.4	41.5	29.0
CT (g/kg DM)	ND	40.8	ND	40.8	1.1	23.1
Plasma amino concentrations (μ mole/L)						
n	6	6	6	6	18	18
BCAA	327.7	362.5	411.0	432.0	241	379
EAA	785.2	902.4	1092.2	1161.6	742	1128
NEAA	1417.1	1082.0	1324.5	1155.8	819	1091
3 Methyl histidine	126.6	88.5	57.0	51.2	ND	ND

ND: Not determined

7.4 ASPECTS OF RUMEN MICROBIOLOGY

7.4.1 DGGE study

In Chapter 5, rumen samples from willow-supplemented and control ewes grazing drought pastures were analysed using DGGE and revealed several interesting points. Over the 10 week grazing period there was a gradual formation of two distinct clusters of DGGE banding patterns of bacterial 16*S* rRNA gene V3 sequences within each treatment group. However, phylogenetic analysis of the DGGE bands unique to the willow-supplemented group and common bands between the willow-supplemented group and the control group showed that the sequences did not cluster by treatment group. The DGGE technique is able to resolve DNA fragments with single base-pair differences and it appears that this technique has detected a subtle shift in the rumen bacterial populations during supplementary willow feeding. However, the bacteria detected within the willow supplemented group are still phylogenetically similar to those bacteria seen in the control group. This indicates that supplementary willow feeding has selected for sub populations of bacteria that are usually present in the rumen under drought conditions. These subpopulations are likely to be adapted to be able to metabolise PGs or FMs for growth or able to avoid the antimicrobial effects of plant secondary compounds found in willow. Most of the sequences detected by DGGE clustered in *Bacteroides-Prevotella* group of organisms. This is similar to a previous report (Avgustin, 2001) in which *Prevotella* species represented a dominant microbial population in the rumen of cattle and are typically present as an important inhabitant in the gastrointestinal tract, oral cavity and genital areas of animals and man. The genus *Prevotella* is part of the Cytophaga-Flexibacter-Bacteroides (CFB) group which, based on 16*S* rRNA gene library sequence data

(Edwards et al., 2004), forms a major group of bacteria in the rumen. *Prevotella* spp were also reported to resist the antimicrobial properties of CTs (McSweeney et al. 2001) and formed the majority of the retrieved DGGE sequences identified in the present study irrespective of the treatment.

7.4.2 Culture study

The culture study in Chapter 6, showed lower bacterial numbers in the willow-supplemented animals and the majority of the bacteria cultured were related to species from the *Pseudobutyrivibrio* genus. The work on Svalbard reindeer (Orpin et al., 1985) is the only other study in which the effect of grazing *Salix* species on ruminal microflora has been reported and they also found that bacterial numbers decreased during willow feeding and that *Butyrivibrio* species were prominent among the organisms cultivated. This indicates that a *Salix* diet has pronounced, mainly negative, effects on the rumen microflora, and provides a selection for those organisms able to deal with the secondary compounds found in *Salix* species. The ability of the rumen microflora to adapt to different animal diets is well documented and it is this feature which makes the rumen able to detoxify plant material that is otherwise toxic to the animal. The best documented case is *Synergistes jonesi* which is able to detoxify the toxic amino acid, mimosine, within the tropical plant Leucaena (Hammond, 1995). It is likely that supplemental feeding of willow selects those rumen organisms best able to degrade and detoxify the PGs and FMs within the plant and to avoid the inhibitory effects of CTs.

7.4.3 Comparision of DGGE and Culture study

While the DGGE study obtained sequences predominantly from the *Bacteroides-Prevotella* group, no organisms from this group were retrieved from the

cultivation study. Similarly, only 2 clones from the DGGE study fell within the *Pseudobutyrivibrio* cluster that was most commonly retrieved in the cultivation study and neither of these clone sequences was very similar to the new *Pseudobutyrivbrio* isolates from this study or to previously cultivated *Butyrivibrio* or *Pseudobutyrivibrio* species. One clone from the DGGE study clustered with *S. bovis*, but again the level of sequence similarity was poor. These observations indicate that the bacterial diversity retrieved via culture techniques is a poor representation of the actual diversity present in these animals as measured by DGGE. The failure of culture techniques to retrieve all or even the majority of organisms from the environment is well known and is often referred to as the "Great Plate Count Anomaly" (Staley and Konopka, 1985). This phenomenon is particularly evident in the rumen where it is estimated that, at best, only 10% of the microbes have been cultured (Edwards et al., 2004). Therefore, the culturable bacteria represent only a small proportion of those present in the rumen which explains why only 2 out of the 52 clones (3.5%) characterised from the DGGE sequences show any similarity to 16*S* rRNA gene sequences from previously cultivated bacteria. There are several hypotheses why current culture techniques do not allow the growth of the majority of bacteria present in natural environments. One problem is that the cultivation media used do not exactly mimic the environment from which the organisms originate and therefore do not provide the correct conditions to allow growth. Alternatively, the physiological state of the organisms in the rumen may preclude them from cultivation in vitro. The organisms that can be cultivated from rumen samples are those that are best able to grow on the chosen medium under the growth conditions employed. Because these

media and conditions are unlike the actual conditions in the rumen, the organisms retrieved are not representative of the important bacterial groups.

The wide variation in substrate utilisation of the *Olsenella*-like isolates in this study is puzzling. Previous characterisation of *O. uli* strains has noted a degree of variability in their substrate utilisation (Olsen et al., 1991; Dewhirst et al., 2001). This may be due to individual strains within a species population losing the ability to transport or ferment certain substrates that are not critical to survival of that strain under the prevailing environmental conditions. Thus over time, the metabolic capacity residing within a species population may be quite varied. If changes occur in the microbial environment, then only those strains of the species best suited to the new conditions would be able to survive, thus selecting for a related, but metabolically different strain. It is interesting that the changes in rumen bacterial populations observed by DGGE analysis in the Chapter 5 indicate selection of closely related organisms that have the ability to grow on, or avoid the toxic effects of, secondary compounds in willow. It may be possible that phenotypic variability within a bacterial species, as seen in the variable substrate utilisation patterns of the *Olsenella*-like isolates, could explain the shift in bacterial populations when the animals were fed a willow diet.

7.4.4 Utilisation of plant secondary compounds

The *Olsenella*-like isolates as described in Chapter 6, W2, W3, W8, W10 and W12 were all isolated from willow supplemented ewes with the exception of W12. Substrate utilisation tests indicate W12 was capable of using most sugars and growth on trypticase, where as the other isolates collected did not grow on these substrates. However, these strains showed a range of preference to utilisation of various PGs

(including salicin) and FMs with the exception of W3 being a net producer of PGs and FMs. The concentrations of PGs and FMs in rumen samples as mentioned in Chapter 5 also indicate that there is a spike in their concentrations at week 2 and thereafter declined indicating their degradation in the rumen with time.

From this study, it is evident that willow supplementation has induced a change in rumen microbial populations so as to utilise the components of willow as a means of adaptation. In the future, it would be interesting to cultivate representatives from the predominant *Bacteroides-Prevotella* cluster to investigate the variability of their substrate utilisation patterns and to test the theory that some of them have the ability to metabolise or otherwise deal with secondary plant compounds.

7.5 ALTERNATIVE FORAGES FOR DROUGHT PASTURES

With both the frequency and severity of drought predicted to increase in East Coast regions of NZ, it is pertinent to discuss other forage options for use in these conditions. Forages that have good nutritive value and grow well in dryland areas of NZ include the herb chicory (*Chicorium intybus*), forage legumes like lotus (*Lotus corniculatus*) and lucerne (*Medicago sativa)*, and a leguminous tree Tagasaste (*Chamaecytisus palmensis*). The tree *Leucaena leucocephala* may also have possibilities for Northland, if this area starts to experience severe droughts predicted by NIWA (2005) in the next 50 years.

Chicory has a high nutritive value with concentrations of CP (123g/kg DM), ME (13.7 MJ/kg OM) and CT (1.7 g/kg DM) contents and was observed to be superior in feeding value for growing lambs and deer to perennial ryegrass, red clover and lucerne during dry summer/autumn conditions (Barry, 1998). Chicory grows well

in summer/autumn under dry conditions but remains dormant during winter. Barry (1998), defined a management plan including rotational grazing during summer/autumn and not grazing in winter to maintain the plant densities for up to 4 to 6 years (Fig 7.2). Chicory turns reproductive during summer; vegetative state is maintained by mechanical topping in later summer. Chicory is non bloating for cattle (Barry, 1998) and reduced internal parasite infections besides increasing animal productivity in deer compared to grazing perennial ryegrass-based pasture during autumn (Hoskin et al., 1999, 2003).

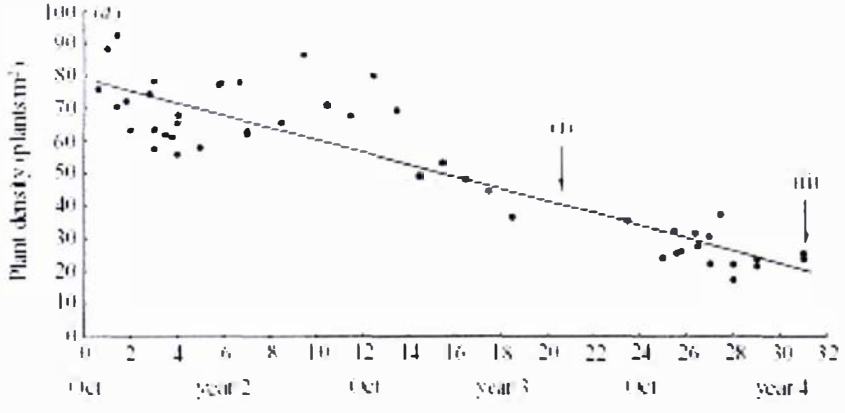

Figure 7.2 Plant density (plants/m2) versus plant age (month as unit) for chicory over 4 year period (Barry, 1998).

Ramírez-Restrepo et al., (2006) conducted an intensive study with lotus over 3 years (2000-2003) and reported a total herabage masses of 24.3 t DM/h for lotus and 24.1 t DM/h for dryland pasture, with the DM production from lotus exceeding dryland pasture during summer/autumn conditions in the first two years (8.5 Vs 7; 10.5 Vs 10) t DM/h and declining to 5.3 t DM/h in 3 year. Ramírez-Restrepo and Barry (2005) concluded that grazing on *Lotus corniculatus* during mating was associated with increases in reproductive rate in sheep, whilst grazing during spring

and summer was associated with increased milk production in both ewes and dairy cows, with reduced methane production and reduced dag formation in sheep.

Lucerne (*Medicago sativa)* is usually grown on flat land where it is either grazed or mechanically harvested for conservation, but it can also be grown on flat-rolling contour land in hill farming systems (McGowan et al., 2003). It produces a high quality feed during dry summers with an ME of 11.5 MJ/kg DM (Waghorn and Barry, 1987). McGowan et al. (2003) established different cultivars of Lucerne at Whatawhata and reported that Lucerne can be maintained for 5 years on hill country even with intensive sheep grazing. Average DM production was higher in summer compared with pasture. Rotational grazing for 7-10 days was suggested for priority stock (O'Connor and Vartha, 1968) whilst continuous grazing for 6 to 8 weeks on Lucerne was suggested for finishing stock to achieve higher LW gains at an allowance of 2.5- 4.0 kg DM/hd/d (Jagusch, 1982; Figure 7.3). Lucerne has lost popularity in NZ grazing systems in recent years, probably due to the bloat risk in grazing cattle (especially in spring) and to coumestan production (Smith et al., 1979) following attack by insects and virus and its effect in depressing ovulation in sheep (Smith et al., 1980). Lucerne is currently at the centre of gene transfer research to induce CT production (Ramírez-Restrepo and Barry, 2005); release of such transformed varieties of lucerne in the future may well remove some of these disadvantages and lead to its increased use in dryland farming.

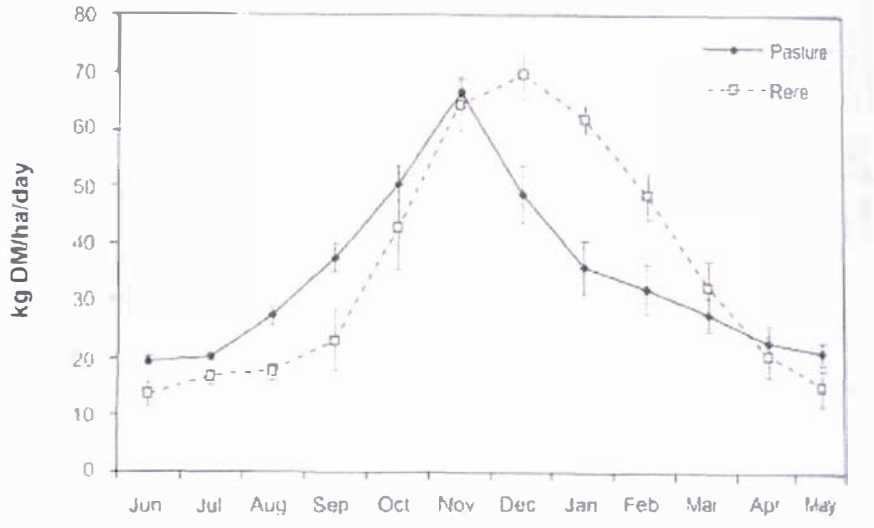

Figure 7.3 Mean monthly growth rates of pasture and Rere (Lucerne) grown on hill country (20° slope) at Whatawhata over five years (1982-87; McGowan et al., 2003).

Tagasaste (*Chamaecytisus palmensis*), also known as tree Lucerne, is a leguminous tree that grows in dry areas to a height of 5 -6 m and produces 12 t DM/ha/year including approximately 4-5 t leaf DM/ha/year (Radcliffe, 1985). Tagasaste leaf has a high CP content (164-260 g/kg DM) and digestibility (0.71) in the spring/summer season, compared to the declining digestibility values of pasture during late summer when it reaches maturity (0.8 to 0.65) and CP (330 to 70 g/kg DM; (Borens and Poppi, 1990). Similar to willow, Tagasaste leaf is retained on the plant for 3-5 months without much change in nutritive value; thus giving a flexibility in planning the grazing intervals and in defining management. Cut and supplementing stem cuttings when it is of high quality feed seems an option and Borens and Poppi (1990) demonstrated this in lambs; a growth rate of 95g/d was reported which was less than that of lambs grazing prairie grass and lucerne (approximately 200g/d).

Borens and Poppi (1990) reported a high digestibility and protein degradability, with 60% of protein disappearing in 4 h and 90% in 24 h with an average NAN absorption across the small intestine of 68%. The authors concluded that Tagasaste can be used as a supplement to low quality forage to increase ME intake and protein flow to the intestines.

Leucaena (*Leucaena leucocephala)* is a long lived, perennial forage tree legume of very high nutritive value for ruminant production in tropical areas of the world. Leucaena has a high CP content (150-400 g/kg DM) and low NDF of 150 - 300g/kg DM and an IVDMD of 0.42-0.7 (Dalzell et al., 1998) and a high CT of 60.3 g/kg DM (Jackson et al., 1996). In Queensland, Northern Australia, it is seeded into rows approximately 5-10m apart with buffel grass (*Cenchrus ciliaris)* planted in the inter-row to form a sustainable grass-legume pasture; once established , the tree pasture system remains productive for >40 years (Mullen et al., 2005). It can thrive well during the dry season, as it has a deep rooted system to exploit moisture beyond the reach of grasses. Beef production of 150 kg/ha/year was obtained with a mixed pasture of leucaena and pangola grass compared to 45-70 kg/ha/year on range pasture in the humid subtropical area of Argentina. Frosts of different intensities limit the growth but doesn't kill the plant (Goldfarb and Casco, 1998). However, leucaena has antinutritional factors like high CT and mimosine and the advent of psyllid insect during the 1980's and 90's resulted in lower yield and therefore lowered animal production. With new germplasm in leucaena (Shelton and Brewbaker, 1994) it was established that hybrids are more tolerant to psyllid insect attack and the problem of mimosine degradation has been solved through transferring 2, 3-dihydroxy pyridine degrading activity from the rumen of Hawaiian goats to the rumen of Queensland

cattle (Jones and Megarrity, 1986). Use of Leucaena for animal production has expanded in Northern Australia, as a result of these two innovations.

The north of NZ is generally considered to be too cold for the growth of Leucaena. However, under current NIWA climatic predictions, Northland will join NZ's drought belt in 50 year's time if climate change continues its present course. By that time, northern NZ may have a similar climate to what S Queensland enjoys today. Under this scenario use of Leucaena as a leguminous forage tree under drought may offer possibilities for Northland in the future.

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APPENDICES

Appendix 1 Similarity matrix of the phylogenetic tree (Figure 6.4) constructed using xylan isolates

	B.crossotus, NCDO2416	B.fibrisolvens, ATCC19171	B.fibrisolvens, NCDO2222	C.proteoclasticum B316	B.fibrisolvens, NCDO2432	B.hungatei, NCDO2398	B.hungatei, JK615	P.ruminis, DSM9787	Pseudobutyrivibrio sp. JK62	P.xylanivorans, Mz5	P.fibrisolvens, OB156	C.oroticum ATCC13619	C.sporogenes	Streptococcus bovis	B.fibrisolvens, CF3	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X14a	X14b	X15a	X15b
B.crossotus, NCDO2416																													
B.fibrisolvens, ATCC19171	0.89																												
B.fibrisolvens, NCDO2222	0.87	0.93																											
C.proteoclasticum B316	0.87	0.92	0.96																										
B.fibrisolvens, NCDO2432	0.88	0.93	0.97	0.97																									
B.hungatei, NCDO2398	0.87	0.93	0.97	0.96	0.97																								
B.hungatei, JK615	0.88	0.93	0.97	0.96	0.97	0.99																							
P.ruminis, DSM9787	0.90	0.88	0.87	0.87	0.87	0.87	0.87																						
Pseudobutyrivibrio sp. JK626	0.90	0.88	0.86	0.86	0.86	0.87	0.87	0.99																					
P.xylanivorans, Mz5	0.89	0.87	0.86	0.86	0.86	0.86	0.86	0.98	0.98																				
P.fibrisolvens, OB156	0.90	0.88	0.86	0.85	0.86	0.86	0.87	0.98	0.98	0.97																			
C.oroticum ATCC13619	0.89	0.87	0.87	0.86	0.86	0.87	0.87	0.91	0.90	0.89	0.88																		
C.sporogenes	0.79	0.79	0.79	0.78	0.79	0.79	0.80	0.81	0.81	0.80	0.79	0.82																	
Streptococcus bovis	0.78	0.75	0.75	0.74	0.75	0.75	0.76	0.79	0.78	0.78	0.76	0.77	0.81																
B.fibrisolvens, CF3	0.90	0.88	0.86	0.86	0.86	0.86	0.86	1.00	0.99	0.98	0.98	0.91	0.81	0.79															
X2	0.88	0.86	0.85	0.84	0.85	0.85	0.85	0.98	0.97	0.96	0.95	0.89	0.80	0.78	0.97														
X3	0.90	0.88	0.86	0.86	0.86	0.86	0.87	0.99	0.98	0.97	0.98	0.91	0.81	0.79	0.98	0.99													
X4	0.89	0.87	0.85	0.85	0.85	0.85	0.85	0.97	0.97	0.96	0.95	0.89	0.80	0.78	0.97	0.97	0.97												
X5	0.86	0.84	0.83	0.83	0.83	0.83	0.83	0.95	0.94	0.94	0.94	0.87	0.77	0.76	0.95	0.95	0.97	0.95											
X6	0.90	0.88	0.87	0.86	0.87	0.87	0.87	0.99	0.98	0.98	0.97	0.91	0.81	0.79	0.99	0.98	0.99	0.98	0.96										
X7	0.90	0.88	0.87	0.86	0.87	0.87	0.87	0.99	0.98	0.98	0.96	0.91	0.81	0.79	0.99	0.98	0.99	0.99	0.96	1.00									
X8	0.90	0.88	0.87	0.86	0.87	0.87	0.87	0.99	0.98	0.98	0.97	0.91	0.81	0.78	0.99	0.98	0.99	0.98	0.96	1.00	1.00								
X9	0.90	0.88	0.87	0.86	0.87	0.86	0.87	0.99	0.98	0.98	0.96	0.90	0.81	0.78	0.99	0.97	0.98	0.98	0.96	1.00	1.00	1.00							
X10	0.78	0.75	0.75	0.75	0.75	0.75	0.76	0.79	0.79	0.78	0.79	0.76	0.81	1.00	0.79	0.78	0.79	0.77	0.77	0.79	0.79	0.79	0.79						
X11	0.79	0.76	0.76	0.75	0.76	0.76	0.76	0.79	0.79	0.78	0.77	0.77	0.81	0.99	0.80	0.79	0.80	0.78	0.77	0.79	0.79	0.79	0.79	1.00					
X12	0.79	0.75	0.75	0.75	0.75	0.76	0.76	0.79	0.79	0.78	0.80	0.76	0.81	1.00	0.79	0.78	0.79	0.78	0.77	0.79	0.79	0.79	0.79	1.00					
X14a	0.89	0.87	0.86	0.86	0.86	0.86	0.86	0.99	0.98	0.97	0.97	0.90	0.80	0.78	0.99	0.97	0.99	0.97	0.95	0.98	0.99	0.99	0.99	0.79	0.79	0.79			
X14b	0.90	0.88	0.86	0.85	0.86	0.86	0.87	0.99	0.99	0.98	0.96	0.90	0.81	0.78	0.99	0.98	0.99	0.98	0.95	0.99	0.99	0.99	0.99	0.79	0.79	0.79	0.99		
X15a	0.91	0.89	0.87	0.87	0.87	0.87	0.87	0.99	0.99	0.98	0.99	0.92	0.82	0.80	0.99	1.00	0.99	0.98	0.97	1.00	1.00	1.00	1.00	0.81	0.81	0.81	1.00	1.00	
X15b	0.91	0.88	0.87	0.87	0.87	0.87	0.87	0.99	0.98	0.98	0.98	0.91	0.81	0.80	0.99	0.99	0.99	0.98	0.97	0.99	0.99	0.99	0.99	0.80	0.80	0.80	0.99	1.00	0.99

Appendix 2 Similarity matrix of the phylogenetic tree (Figure 6.4) constructed using salicin isolates

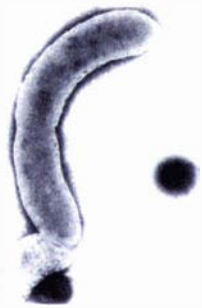
	B.crossotus, NCDO2416	B.fibrisolvens, ATCC19171	B.fibrisolvens, NCDO2222	C.proteoclasticum B316	B.fibrisolvens, NCDO2432	B.hungatei, NCDO2398	B.hungatei, JK615	P.ruminis, DSM9787	Pseudobutyrivibrio sp. JK62	P.xylanivorans, Mz5	P.fibrisolvens, OB156	C.oroticum ATCC13619	C.sporogenes	Streptococcus bovis	B.fibrisolvens, CF3	S1	S2	S3	S4	S5	S6	S7	S8	S10	S13
B.crossotus, NCDO2416																									
B.fibrisolvens, ATCC19171	0.89																								
B.fibrisolvens, NCDO2222	0.87	0.93																							
C.proteoclasticum B316	0.87	0.92	0.96																						
B.fibrisolvens, NCDO2432	0.88	0.93	0.97	0.97																					
B.hungatei, NCDO2398	0.87	0.93	0.97	0.96	0.97																				
B.hungatei, JK615	0.88	0.93	0.97	0.96	0.97	0.99																			
P.ruminis, DSM9787	0.90	0.88	0.87	0.87	0.87	0.87	0.87																		
Pseudobutyrivibrio sp. JK626	0.90	0.88	0.86	0.86	0.86	0.87	0.87	0.99																	
P.xylanivorans, Mz5	0.89	0.87	0.86	0.86	0.86	0.86	0.86	0.98	0.98																
P.fibrisolvens, OB156	0.90	0.88	0.86	0.85	0.86	0.86	0.87	0.98	0.98	0.97															
C.oroticum ATCC13619	0.89	0.87	0.87	0.86	0.86	0.87	0.87	0.91	0.90	0.89	0.88														
C.sporogenes	0.79	0.79	0.79	0.78	0.79	0.79	0.80	0.81	0.81	0.80	0.79	0.82													
Streptococcus bovis	0.78	0.75	0.75	0.74	0.75	0.75	0.76	0.79	0.78	0.78	0.76	0.77	0.81												
B.fibrisolvens, CF3	0.90	0.88	0.86	0.86	0.86	0.86	0.86	1.00	0.99	0.98	0.98	0.91	0.81	0.79											
S1	0.79	0.76	0.76	0.75	0.76	0.76	0.76	0.79	0.79	0.78	0.77	0.76	0.81	0.99	0.79										
S2	0.78	0.75	0.75	0.75	0.76	0.76	0.76	0.79	0.79	0.78	0.79	0.76	0.81	0.99	0.79	1.00									
S3	0.79	0.76	0.76	0.75	0.76	0.76	0.76	0.79	0.79	0.78	0.77	0.77	0.81	0.99	0.80	0.99	1.00								
S4	0.90	0.88	0.86	0.86	0.86	0.86	0.87	0.99	0.98	0.97	0.95	0.90	0.80	0.78	0.99	0.79	0.79	0.79							
S5	0.79	0.79	0.79	0.78	0.79	0.79	0.80	0.81	0.81	0.80	0.79	0.82	0.99	0.81	0.81	0.80	0.81	0.81	0.81						
S6	0.79	0.78	0.78	0.77	0.78	0.79	0.79	0.80	0.80	0.80	0.78	0.82	0.99	0.81	0.80	0.81	0.80	0.81	0.79	0.98					
S7	0.86	0.85	0.83	0.82	0.83	0.83	0.84	0.95	0.94	0.94	0.92	0.87	0.78	0.76	0.95	0.76	0.77	0.76	0.94	0.77	0.77				
S8	0.89	0.87	0.85	0.85	0.85	0.85	0.86	0.97	0.96	0.95	0.96	0.90	0.80	0.79	0.97	0.79	0.79	0.79	0.97	0.79	0.79	0.98			
S10	0.78	0.78	0.78	0.76	0.78	0.78	0.78	0.80	0.79	0.79	0.77	0.81	0.98	0.79	0.79	0.79	0.79	0.79	0.79	0.97	0.97	0.76	0.78		
S13	0.78	0.75	0.75	0.74	0.75	0.75	0.76	0.79	0.79	0.78	0.76	0.75	0.80	0.98	0.79	0.98	0.99	0.98	0.78	0.79	0.79	0.75	0.79	0.78	
S14	0.78	0.76	0.75	0.74	0.76	0.76	0.76	0.79	0.79	0.78	0.77	0.77	0.81	0.99	0.79	0.99	1.00	0.99	0.79	0.81	0.80	0.76	0.79	0.79	0.78

Appendix 3 Similarity matrix of the phylogenetic tree (Figure 6.5) constructed using willow isolates

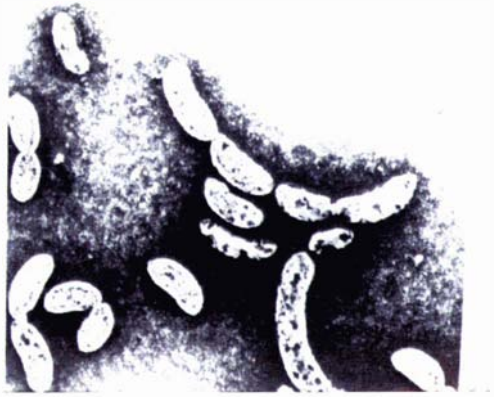
	W2	W3	W5	W6	W7	W8	W10	W12	W15	W17	W18	W19	W20	Atopobium oviles, AJ251324	Uncultured rumen bacterium 3C0d-2, AB034002	Uncultured rumen bacterium BS34, AY244985	Uncultured rumen bacterium JPR4, EF120430	Uncultured bacterium C0405, AM075695	Actinomyces gerencseriae, X80414	Olsenella uli, AF292373	Olsenella profusa, AF292374	Uncultured rumen bacterium 4C3d-3, AB034096	Shigella dysenteriae, X96966	Pseudoramibacter alactolyticus, AB036759	Clostridium butyricum, X68176	Staphylococcus epidermidis, X75943	Streptococcus bovis, M58835
W2																											
W3	0.976																										
W5	0.700	0.692																									
W6	0.715	0.716	0.655																								
W7	0.751	0.746	0.739	0.662																							
W8	0.943	0.941	0.679	0.711	0.713																						
W10	0.954	0.964	0.669	0.705	0.717	0.919																					
W12	0.978	0.987	0.692	0.717	0.752	0.943	0.962																				
W15	0.714	0.714	0.924	0.676	0.777	0.677	0.701	0.722																			
W17	0.693	0.675	0.746	0.648	0.752	0.663	0.649	0.672	0.736																		
W18	0.707	0.701	0.751	0.661	0.802	0.689	0.675	0.701	0.760	0.818																	
W19	0.703	0.696	0.756	0.647	0.784	0.661	0.668	0.696	0.768	0.973	0.821																
W20	0.702	0.700	0.665	0.621	0.725	0.669	0.671	0.703	0.686	0.683	0.722	0.720															
Atopobium oviles, AJ251324	0.983	0.998	0.730	0.748	0.752	0.998	0.994	0.999	0.734	0.694	0.729	0.698	0.730														
Uncultured rumen bacterium 3C0d-2, AB034002	0.933	0.945	0.689	0.722	0.751	0.904	0.920	0.954	0.724	0.681	0.701	0.704	0.710	0.962													
Uncultured rumen bacterium BS34, AY244985	0.949	0.958	0.688	0.708	0.757	0.916	0.930	0.967	0.711	0.685	0.700	0.710	0.707	0.973	0.952												
Uncultured rumen bacterium JPR4, EF120430	0.910	0.942	0.682	0.739	0.737	0.936	0.924	0.944	0.723	0.726	0.694	0.729	0.715	0.870	0.904	0.914											
Uncultured bacterium C0405, AM075695	0.987	0.995	0.769	0.774	0.765	0.995	0.989	0.995	0.765	0.721	0.751	0.721	0.766	0.996	0.960	0.965	0.650										
Actinomyces gerencseriae, X80414	0.761	0.764	0.703	0.868	0.710	0.751	0.749	0.765	0.723	0.693	0.718	0.694	0.665	0.780	0.766	0.761	0.758	0.810									
Olsenella uli, AF292373	0.949	0.964	0.692	0.708	0.762	0.922	0.937	0.968	0.710	0.693	0.700	0.716	0.711	0.973	0.952	1.000	0.914	0.965	0.761								
Olsenella profusa, AF292374	0.954	0.971	0.703	0.717	0.760	0.925	0.941	0.975	0.730	0.686	0.700	0.709	0.717	0.972	0.952	0.971	0.927	0.971	0.768	0.971							
Uncultured rumen bacterium 4C3d-3, AB034096	0.965	0.974	0.700	0.722	0.755	0.929	0.946	0.984	0.733	0.674	0.709	0.699	0.711	0.983	0.959	0.970	0.929	0.978	0.770	0.970	0.976						
Shigella dysenteriae, X96966	0.704	0.703	0.669	0.621	0.739	0.670	0.676	0.707	0.699	0.702	0.722	0.726	0.988	0.725	0.714	0.712	0.712	0.764	0.665	0.713	0.715	0.715					
Pseudoramibacter alactolyticus, AB036759	0.748	0.744	0.740	0.660	0.977	0.708	0.714	0.750	0.778	0.754	0.798	0.784	0.717	0.745	0.746	0.753	0.729	0.765	0.702	0.758	0.759	0.754	0.733				
Clostridium butyricum, X68176	0.729	0.731	0.955	0.674	0.792	0.695	0.708	0.735	0.950	0.766	0.775	0.798	0.708	0.741	0.736	0.740	0.727	0.767	0.727	0.742	0.749	0.746	0.725	0.725			
Staphylococcus epidermidis, X75943	0.707	0.715	0.748	0.652	0.812	0.672	0.682	0.714	0.771	0.812	0.985	0.829	0.738	0.725	0.713	0.713	0.694	0.744	0.706	0.713	0.715	0.720	0.738	0.738	0.809		
Streptococcus bovis, M58835	0.704	0.699	0.753	0.645	0.786	0.660	0.666	0.702	0.789	0.961	0.822	0.994	0.712	0.694	0.709	0.707	0.726	0.715	0.694	0.712	0.708	0.705	0.726	0.726	0.786	0.805	
Butyrivibrio crossotus NCDO2416, X89981	0.724	0.723	0.762	0.663	0.784	0.685	0.698	0.725	0.773	0.761	0.777	0.779	0.740	0.734	0.730	0.728	0.691	0.768	0.715	0.729	0.728	0.732	0.741	0.741	0.777	0.804	0.778

Appendix 4. Electron micrographs of the negatively stained bacterial cells isolated on Xylan media

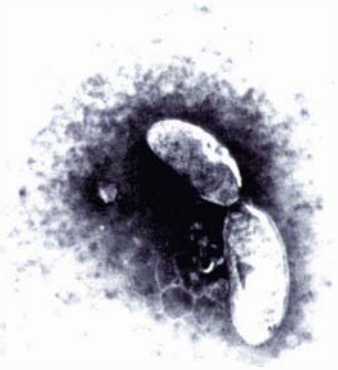
X3



X4



X5



X6

X15a