Assessing the potential of Flax (*Phormium tenax; Harakeke*) for utilisation by dairy cows

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ABSTRACT

This thesis is composed of a series of studies which assess the nutritional value of New Zealand flax (*Phormium Tenex; Harakeke*) for grazing ruminants. Flax samples of leaf sections from the top (FT) and bottom (FB) of the plant were evaluated using laboratory analyses and *in vitro* rumen fermentation and compared with predominant pastures forages in New Zealand, such as perennial ryegrass (NRG), chicory (NGHI), lucerne (NLUC), and white clover (NWC).

The FT contained high concentrations of neutral detergent fibre (NDF) and acid detergent fibre (ADF), 67.6 and 53.2% of the dry matter (DM), respectively. Similarly, FB contained high levels of NDF and ADF 56.5 and 45.0% of the DM; respectively, compared with other forages (NRG, NCHI, NLUC, and NWC), which ranged between 24.9- 47.6% of the DM for NDF and 11.6-22.1% of the DM for ADF. Crude protein (CP) levels in FT and FB in contrast were lower (6.0 and 2.4% of the DM, respectively) compared with the other forages (NRG, NCHI, NLUC, and NWC), which had a CP range of 21-31.6% of the DM.

An *in vitro* fermentation method measured gas production over 24 hours, and ammonia (NH_3) and volatile fatty acid (VFA) concentrations at 2, 4 and 8 h. For *in vitro* gas production two experiments were conducted (Experiments 5 and 6) and values were lower in Experiment 5 than Experiment 6, and lower than expected. However, in both experiments accumulated gas production for FT and FB were lower than the other forages at all time points. Cumulated gas production for FT, FB, NRG, NCHI, NLUC, and NWC was 38.50, 71.16, 84.12, 102.84, 76.66, and 85.48 ml gDM^{-1}, respectively. In experiment 6, accumulated gas production for FT and FB was also the lowest at 100.61, and 142.90 ml gDM^{-1}, respectively. This compared with 180.76, 195.73, 166.83, and 181.09 ml gDM^{-1} for the other forages (NRG, NCHI, NLUC, and NWC, respectively). In both experiments FB had 50-70% greater gas production than FT samples. Therefore, samples from the base of the Flax should be considered for further investigation in terms of gas production.

Ammonia (NH_3) concentration and VFA yield were determined at 2, 4, and 8 h of incubation. The *in vitro* net NH_3 production (mmol NH_3 $molN^{-1}$) of FT (0.2), and FB (-4.4), were the lowest compared with NRG (3.1). For VFA yield, total VFAs (tVFA) produced (ml gDM^{-1}) from NRG was 120.03, which was higher than that of FT (40.35) and FB (36.11) after 8 h of incubation. For FT, FB, and NRG the percentage of acetate was, 70.2, 63.5, and 62.6%, respectively;

propionate was 13.2, 13.7, and 23.2%, respectively; and butyrate was 13.7, 19.3, and 12.3% respectively

In conclusion, New Zealand flax leaf sections had lower *in vitro* fermentation characteristics, and cumulative gas production than standard New Zealand forages. This study concluded that flax leaf sections were lower-quality forages when compared with New Zealand forages in terms of nutritive quality, *in vitro* fermentation, and gas production. However, the high NDF and ADF and low CP content of New Zealand flax samples suggests it could be fed as a supplement to forages for dry grazing ruminants without major problems.

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LIST OF ABBREVIATIONS

AA	Amino acid
A:P	Acetate: Propionate ratio
NDF	Neutral detergent fibre
ADF	Acid detergent fibre
ADP	Acid detergent protein
B	Flax butt
B	Boron
Ca	Calcium
CF	Crude fibre
CH_4	Methane
CHO	Soluble carbohydrate
Co	Cobalt
CO_2	Carbon dioxide
CP	Crude protein
CT	Condensed tannin
Cu	Copper
DM	Dry matter
DOM	Digestibility organic matter
E	Effective degradation
EE	Ether extract
F	Stripped fibre
FB	Flax bottom section
Fe	Iron
FT	Flax top section
G	Stripped green matter
GP	Gas production
IENICN	The Interactive European Network for Industrial Crops and their Applications
IVDMD	In vitro dry matter digestibility
K	Potassium
$K\%h^{-1}$	Fractional degradation rate
L	Flax leaf
L, h^{-1}	Lag time
LWG	Live weight gain

MAFSFF	Ministry of Agriculture and Forestry Sustainable Farming Fund
ME	Metabolisable energy
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
N	Nitrogen
Na	Sodium
NCHI	New chicory
NDF	Neutral detergent fibre
NDIP	Neutral detergent insoluble protein
NIRS	Near-infrared spectroscopy
NLUC	New lucerne
NPN	Non-protein nitrogen
NRG	New ryegrass
NWC	New white clover
NZ	New Zealand
OCHI	Old chicory
OMD	Organic matter digestibility
ORG-WC	Old ryegrass/ white clover
OWC	Old white clover
P	Phosphorus
PDM	Potential degradable DM
RC	Red clover
RUP	Rumen undegraded crude protein
S	Sulphur
Se	Selenium
SP	Soluble protein
SSS	Soluble sugars and starch
THC	Tetrahydrocannabinol
VFA	Volatile fatty acid
WC	White clover
Zn	Zinc

CHAPTER 1

1.1. INTRODUCTION

In New Zealand (NZ) most of the pasture species are perennial, and pastures are predominantly composed of perennial ryegrass and white clover (Kemp, 1999). The production of pasture is seasonal with growth being limited by environmental conditions (McKenzie et al., 1999). The main parameters affecting pasture growth in NZ are temperature in winter and moisture in summer (Holmes et al., 2002). Pasture growth rates increase to a peak during spring and reduce to a minimum during winter, with differences between regions. On average, 14% of the annual pasture production occurs in the winter, 32% occurs in spring, 33% in summer and 21% of pasture production occurs in autumn (Valentine and Kemp, 2007).

When pasture is used as the sole component of the diet in pasture-based farming, this can introduce several constraints such as limiting animal intake and production, and supplements can be used as a tool to manage those constraints. Generally, the utilisation of supplements aims either to improve overall animal intake and milk production or to maintain performance during periods of pasture shortage (McGilloway and Mayne, 2002). However, supplements are more expensive than pasture (on a DM basis) and their use may not be profitable if pasture is replaced in an attempt to improve the composition of the cow's diet (Holmes and Roche, 2007).

Recently, there has been increasing interest in alternative protein feeds that can be locally produced, as well as a growing concern over the environmental impacts of pasture production techniques (Karlsson et al., 2009). Over feeding of protein is costly for farmers as excess protein in the diet will not be utilised by animals and discharged in the urine as wastage. It also results in losses of nitrogen (N) to the environment, resulting in damage to the ecosystems and human health significantly, which contribute to ground water and surface water pollution.

There has been a resurgence in research interest into the potential of New Zealand native flax (*phomium tenax*), to produce bioactive products, high fashion fibres for clothing, and eco-composites for building. The harvesting and processing of New Zealand flax generates by-products such as leaves, butts, thrash stripping fibre and the associated green matter stripping. The green stripping has potential as a stock feed as it has similar nutritive value (10.1 MJ ME $kgDM^{-1}$ and 15% crude protein (CP) on a DM basis) to that of mature perennial ryegrass leaves

(Litherland et al., 2005). Hence, if the flax industry is to expand, a market must be found for the residual by-products or green waste.

1.1.1. Objectives

The objectives of this research are, to determine the chemical composition of flax leaf and the digestion and fermentation changes when they are incubated using *in vitro* methods. A secondary aim is then to compare the digestibility of New Zealand flax leaf to common New Zealand pasture species and utilise these results to model the nutritive potential of flax leaf as feed for dairy cows.

CHAPTER 2

2.1. LITERATURE REVIEW

This literature review will give an explanation of the types of products produced from flax leaf and an overview of the results from scientific studies with a focus on the nutritive value of flax leaf for ruminant feed. It will discuss species, reproduction, harvesting, and the uses of the flax leaf in ruminants' feeds and other potential uses. In addition, the review will also cover the chemical composition of flax leaf *in situ* and following *in vitro* and *in vivo* digestibility.

2.1.1. New Zealand flax (*Phormium tenax; Harakeke*)

New Zealand flax (*Phormium tenax; Harakeke*), is a native plant (Figure 2.1), which was named flax by Europeans traders because it resembled the flax found in the other parts of the world. Maori, who used it first for hunting and fishing (Maoris twisted, plaited, and wove the fibre from flax leaves to create a wide range of items such as fishing nets and traps for hunting), and as a house material and medicine. Different parts of the plant are medicinal (e.g., gel and nectar) and were applied to boils and wounds and even used for toothache. Root juice used as a disinfectant and flax leaves used for binding broken bones (Wehi and Clarkson, 2007; Jones, 2003). The New Zealand native flax is called Harakeke by Maori and can grow on lower land or swamp, coastal areas, or mountains. Flax has been traded since 1828 when 22 tons was sold in Sydney (Hector, 1872). Flax was describing as NZ's first true processing/manufacturing raw material, with exported fibre averaging 20,000 tons pa^{-1} and the harvest 200,000 tons pa^{-1} of green leaf between 1900 and 1920 (Pool and Boyce,1949).

Figure 2.1. New Zealand flax *www.bing.com/images*

New Zealand Bonded Felts continued as a profitable operation, supplied from the Moutua Estate, until the factory was destroyed by fire in 1985 and industrial flax production in New Zealand came to an end (Harris and Heenan,1992). The Interactive European Network for Industrial Crops and their Applications (IENICA) reported that the communication between the main participants in fibre industry, farmers, seed vendors, research bodies, primary and secondary processors and industrial users need improvement to establishment of a flax plantation (IENICA, 2000).

Flax can tolerate a variety of environmental factors which is encouraging for farmers who want to invest in the natural fibre industry. However, an expanded fibre industry with its huge competition from other different fibre sources, makes it difficult for farmers to invest, so ways are needed to make the flax industry attractive to farmers who are interested in growing flax commercially (McGruddy, 2006).

The New Zealand Ministry of Agriculture and Forestry Sustainable Farming Fund (MAFSFF) have funded work looking at the integration of New Zealand flax into land management in New Zealand. However, significant government and financial support will be required, and cultivation practices will need to be investigated (Cruthers et al., 2009).

2.1.1.1. Flax Species

New Zealand has two species of Flax, *Phormium Tenax* (Harakeke) and *Phormium Cookianum* (Warariki). *P. Tenax,* the most common species known as harakeke or swamp flax, has stiff leaves which are often broad and can grow up to three meters, flowers which are usually red and seed pods that grow upright. *P. Cookianum,* known as wharariki or mountain flax, grows along exposed coastlines on mountain slopes. It has leaves that are narrower and shorter and usually grow up then arch downward, flowers which are usually yellow, or a lighter green and seed pods which are droopy, twisted and hang down and become thin and papery with age. *P.Cookianum* has two forms depending on the place it grows. The southern mountain form (with stiff leaves and short, thick capsules) grows along the Southern Alps and extends south to Stewart Island and north to the Tararuas. The second form of *P. Cookianum,* is found in the northern lowlands (with flaccid leaves and dangling capsules) and grows from the Manawatu gorge northwards mainly on damp cliffs of soft mudstone (Wardle, 1979; Jones, 2003). Each form has different characteristics which are valuable for commercial use.

2.1.1.2. Reproduction

Flax can reproduce both sexually (pollinated seed) and asexually (fans). After sexual reproduction (pollinated seed), flax seeds require a period of chilling to remove dormancy and this method is usually used in large scale propagation, but it has significant volume limitations and cost implications, and needs cool moist condition and storage for 5 months. After undergoing this chilling process, flax seeds germinate within 12 days (Craig and Stewart, 1988; Hector, 1889; Wehi and Clarkson, 2007).

Asexual reproduction occurs via fans. A fan is the basic unit of the flax and occurs as a sheath (set of leaves) of up to 10 leaves (Figure 2.2 A), with the two youngest or baby leaves (Rito) sheathed in the centre surrounded by its parents (awhi rito or matua) and then grandparents (tupuna) (Figure 2.2 B) (Hector, 1889; Mackay et al., 2002).

(A) 

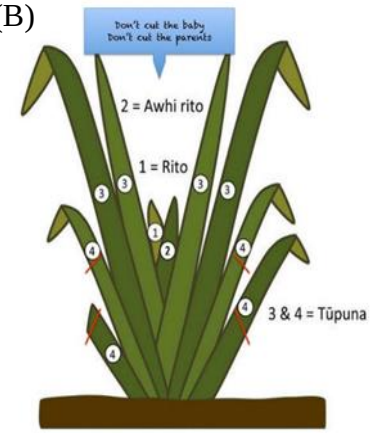
(B) 

Figure 2.2. (A) Flax heath (Manaaki Whenua, land care research) *https://www.landcarersearch.co.nz/resources/collections/harakeke/establishing-a-pa*; (B) Flax fan shows the rito, awhi rito and tupuna leaves (*www.bing.com/images*).

2.1.1.3. Flax requirements for establishment

Flax researchers agree on the requirements of flax for fertiliser, well-drained soil and the critical importance of the water table which should be 30 cm below the growing plant. Vigorous and healthy flax are achieved by planting in good fertile land which is not too wet and not too dry which explains the strong growth of flax on the edge of running streams (Hector, 1889).

New Zealand flax is a native plant which has a lower soil nutrient requirement than introduced pasture and crops because it is adapted to the soil with low fertility (He Korero Korari, 2004). Flax has none of the risks (environmental tolerance) and cost of these crops and can grow from one end of New Zealand to the other (easy to propagate and is cheap to establish). Soil has a high phosphorus content and the use of potassium manures in conjunction with phosphate gives a definite increase in NZ flax growth over phosphate alone (Rigg and Waston, 1945; Harris et al., 2005). So, it is a common Maori practice of returning unwanted leaf material to the base of the plant which may indeed have beneficial effects to returned nutrient to the plants (Wehi and Clakson, 2007). Harris et al, (2005) reported that phosphate deficiency limits flax growth. Flax yield can be between 35-40 tons $acre^{-1}$ provided selected lines are used, with some lines yielding up to 60 tons. According to Critchfield (1951), on the average 30 tone of green leaf $acre^{-1}$ is considered a good yield, though yield above 40 ton are known (approximately 8 tons of green leaf are required for 1 ton of fibre).

Planting flax requires spaces to be left between plants which improves air movement and prevents build-up of scale and fungal disease and allows trimming, harvesting and other maintenance. It is been reported by Landcare Research that there should be 3 m between each clump. (Hector, 1889), recommended that there is 8 foot spacing between rows or 6ft × 4ft 6inches, 8ft × 4ft or 6ft × 6ft (Figure 2.3).

Figure 2.3. (Manaaki Whenua, Land Care research)
https://www.landcarersearch.co.nz/resources/collections/harakeke/establishing-a-pa

Flax as a native plant can be found in different environments because it is tolerant to many factors such as frost, drought, and wind and salt spray. In 1986, the National Water and Soil Conservation Authority described flax as an excellent species for stabilizing stream banks and for planting in gully systems where there is seasonal water logging and no seasonally dry windswept hill sides. Flax, like other plants, can be affected by some diseases; one of which is yellow leaf, which may occur for several different reasons. Insect damage with discrimination of the "windower" or flax looper moth (*Orthoclydon praefectata*), the flax notcher moth (*Tmeolphota steropastis*), scale including *Poliapsis* and *Pseudaulacapsis* species, and flax mealy bug (*Balanococcus diminutus*), the fungal diseases Phormium leaf mould (*Periconiella phormii*) and Phormium leaf spot (*Glomerella phacidiomorpha* and *Kirramyces phormii*), and yellow leaf disease caused by phytoplasma (Boyce and Newhook,1953; Boyce, 1958; Scheele,1997; He Korero korari, 2004).

The trimming and cleaning of all dead leaves (maintenance) is one of the most important actions needed to keep flax healthy, so this needs to be done at specific times of the year to avoid negative effects on the flax plant. Maori avoid cutting flax leaves from the first appearance of the flowering stalk until its death (spring and summer) so that the fibre is of better quality and more easily stripped during the autumn and winter months (Scheele, 2004). However, weavers prefer to harvest leaves in summer (after the pods have developed), autumn and early winter when the leaves are regarded as easier to work, and muka (strong fibre obtained from the leaves by scraping the green flesh away) is more easily extracted. Cutting can be done any time during the year (September and October) except in late autumn/early winter (May and June) to avoid any frost damage (Harris et al., 2005).

The principal product from flax has historically been fibre, and it is most likely that it will be the fibre which drives new applications in the future. Nevertheless, all parts of the plant have traditionally been valued and utilized, and there is considerable merit in developing secondary products and utilising by-products as a part of whole plant management cycle. Traditional and industrial applications include fibre, gel, seed oil and green stripping.

Fibre

The Maori people have utilised flax fibre for hundreds of years, extracting it from leaves with a mussel shell and preparing it for weaving or plaiting by a slow and laborious process of scraping, washing, beating, and bleaching. Flax fibre occupies an intermediate position

between the lower value hard fibre (e.g., sisal and manila) and the higher value soft fibre (e.g., linen flax). New Zealand flax has traditionally been positioned commercially as a hard fibre in respect to its uses. In the early years of flax processing in NZ, a few companies were interested in the principle of exploring NZ flax fibre, but the machine-stripped fibre samples were too coarse to be of any commercial use. So, developing a new processing platform was a critical precursor to exploring an expanded range of clothing and textile applications (Hector, 1872; Cruthers et al., 2009). Today in New Zealand there is no large-scale flax fibre processing industry.

Gel

Flax gel (pia-harakeke) is an exudate found in the leaf butt. It is an excellent glue and is antiseptic, so it has long been used for burns, skin conditions (e.g., eczema) and wound healing. Work has also been undertaken assessing flax gel as a standardised thickener for cosmetics (Sims and Newman, 2006). The viscosity of pia harakeke is due to a polysaccharide, identified as an acidic xylan (Tauwhare et al., 2006).

Seed Oil

The world's major seed oils (e.g., soy, rapeseed, sunflower, and cotton) are mostly grown as intensive chemical monocultures with genetic modification increasingly employed as a tool to manipulate crop attributes. Worldwide demand for vegetable oil is increasing as mineral oils/petrochemicals are being depleted, and there is growing interest in the nutrition/health benefits of flax oil. New Zealand flax seed oil is rich in the omega-6 fatty acid linoleic acid (as distinct from linen flax seed oil which is rich in omega-3 linolenic acid). New Zealand flax seed can yield up to 29% oil that consists of 5-16% oleic acid, 75-89% linoleic acid, 3-11% palmitic acid, and 1-3% stearic acid (Morice, 1962). Flax seed oil could be a major by-product from a *phormium* plantation and be positioned as a premium, edible vegetable oil (comparable to sunflower and safflower, and superior to rape seed and soya been), with the seed meal potentially suitable for stock feed (Fergus, 1976 cited by McGruddy, 2006).

2.1.1.4. Using flax as a ruminant feed

Dairy farmers have reported that cows who have access to flax bushes on farm never suffer from bloat, and when green stripping (a flax by-product) is offered to horses mixed with oats they eat it greedily again with no digestive upset (Hector, 1889).

Anecdotally, farmers also have reported that calves that suck flax leaves appear very clean and have no scours (Mclntoch,1959). Flax green stripping appears to have potential as a supplement for pasture-fed animals which provides 4-9% CP, 30-49% ADF, 39-73% NDF, 6-14% CHO and 41-70% OMD, especially on NZ pasture which contains high protein and low carbohydrate in winter (poor quality pasture) and may improve digestion. Flax by-products can be used as a primary or secondary resource for ruminant feed (Litherland et al., 2005).

Benefits in addition to use as feed, may extend to sheep farms. Flax can be used to provide shelter, especially in lambing paddocks (Barry et al., 2002). Studies suggest 10-25% of lambs die within 3 days of birth but offering shelter by using flax may keep lambs dry and protect them from wind (Hector, 1889).

2.1.1.5. Flax chemical composition

The chemical composition of flax and by-products can be affected by treatment or time of harvesting during the year (Tables 2.1 and 2.2). The digestible organic matter of flax straw (bottom section) was increased by 72% by ammonisation and *in vitro* and *in vivo* digestibility studies indicated that ammoniated flax straw is similar in digestibility to medium quality hay. Treatment with ammonia (NH_3) also causes changes in nutritive value for the flax (Table 2.3), with crude protein (CP) levels 2.9 times higher after treatment. Also, the digestible organic matter increased *in vitro* by 31% and 72% *in vivo* (Mann et al., 1988).

Table 2.1. Difference in flax quality in different times of the year during feeding of Sambar deer in a Flax Swamp in the Manawatu, New Zealand (Kelton and Skipworth, 1987).

Flax	**April**	**August**	**November**	**February**
CP%	5.8	6.4	6.7	6.5
Energy Joules g^{-1}	17982	18812	17769	17832
ADF%	34.7	21.9	23.9	26.2
DM%	85.5	84.9	86.5	84.9

Table 2.2. Digestible organic matter (DOM) *in vitro* and *in vivo* digestibility and the digestibility of acid detergent fibre (ADF) and neutral detergent fibre (NDF) (%) of ammoniated (NH_3) and untreated flax straw (Mann et al., 1988).

Flax straw	**DOM (%)**		**Digestibility (%)**	
Treatment	*In vitro*	*In vivo*	ADF	NDF
None	35.2	33.9	9.9	- 0.6
NH_3	46.3	58.4	43.9	37.9

Table 2.3. Nutrition composition of flax straw pre- and post-treated with ammonia (NH_3) (% of DM) (Mann et al., 1988).

Treatment	**CP**	**ADF**	**NDF**	**ADL**
None	2.8	58.7	73.8	10.6
NH_3	8.1	57.4	71.9	10.5

Litherland et al. (2005), compared the nutritive value of the New Zealand flax (*P. tenax*) by-products (stripped green matter, leaf, butt, and stripped fibre) to white clover (Table 2.4).

Table 2.4. Chemical composition of flax by-products (% DM) as determined by wet chemistry and volatile fatty acid (VFA) production from *in vitro* incubation after 8 h.

Forage	ADF	NDF	CHO	CP	lipid	Starch	OMD	Kinetic data						VFA		
								A	B	P	K	L	E	Acetate	Propionate	Butyrate
G	30	39	14	9	1.9	3.6	70	40	41	81	11	2.1	63	44	33	21
B	40	58	11	4	0.8	2.1	48	27	44	71	6	2.5	43	43	33	22
L	49	73	6	5	0.9	0.33	41	21	36	56	6	1.9	35	51	29	16
F	49	69	6	6	1.1	0.76	43	16	34	51	3	0.1	27	49	28	19
WC	16	21	8	28	2.9	1.4	78	53	43	96	13	0.0	82	40	38	15

Stripped green matter (G), butt (B), leaf (L), and stripped fibre (F), acid detergent fibre (ADF), neutral detergent fibre (NDF), soluble carbohydrate (CHO), crude protein (CP), organic matter digestibility (OMD), kinetic data (DM%) defined as soluble DM (A), degradable insoluble DM (B), potential degradable DM (P), fractional degradation rate (K,% h^{-1}), lag time (L, h^{-1}) and effective degradability (E), compared to white clover (WC) (Litherland et al., 2005).

Only 30% DM of stripped fibre was digested after 24 h. The potential degradability of the stripped fibre was 51%, however this required retention in the rumen for 72 h. The fractional DM degradation rates of the fibrous flax by-products (e.g., leaf, butt, and stripped fibre) were approximately half that of the fractional DM degradation rates for stripped green matter and white clover (Table 2.4).

Following 24 h incubation *in sacco*, 96% DM of the white clover had been digested, compared with 76, 52 and 46% DM of the stripped green matter, leaf, and butt respectively. While flax fibre was poorly degradable, flax CP was readily degradable. After 24 h in the rumen, ADF degradability was 93, 45, 33, 24 and 3% DM; NDF degradability was 95, 57, 33, 30 and 9% DM; and CP degradability was 98, 88, 88, 87 and 81% DM for white clover, stripped green matter, butt, leaf and stripped fibre, respectively (Figure 2.4).

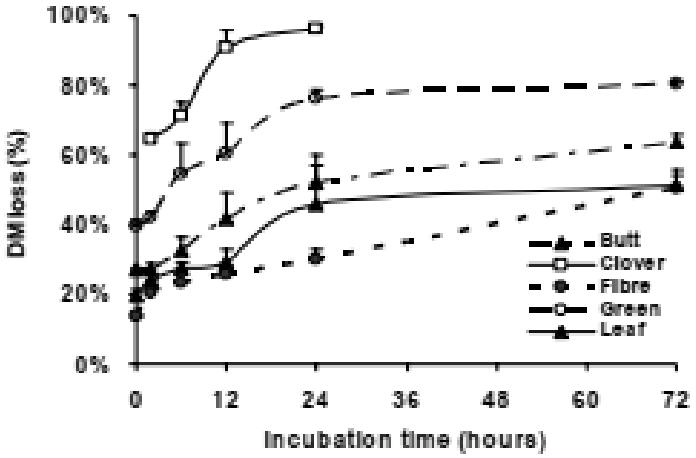


Figure 2.4. *In sacco* dry matter degradation curves of white clover and New Zealand native flax by-products: leaf, stripped green matter, fibre, and butt (Litherland et al., 2005).

In vitro incubation showed that up to 39% of white clover plant nitrogen was released as ammonia over 24 h. On the other hand, the incubated flax by-products produced ammonia for different lengths of time; with the butt producing it for 2 h, the stripping fibre and leaf for 4 h and the stripped green matter for 8 h (Figure 2.5).

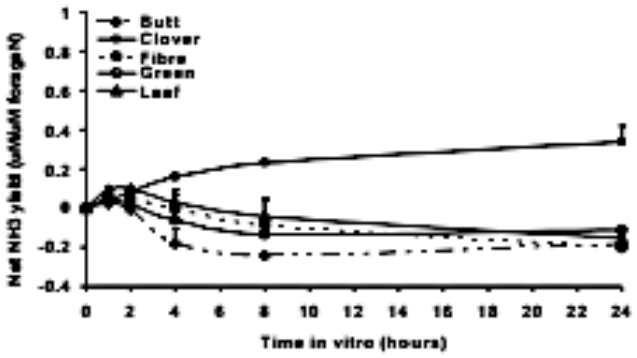


Figure 2.5. Net ammonia (NH_3) released from *in vitro* incubation of white clover and New Zealand native flax by-products: leaf, stripping green matter, butt, and stripped fibre (Litherland et al., 2005).

For white clover 21% DM was used for the production of volatile fatty acid (VFA) by 8 h which was higher than the 14% DM used for VFA production from stripped green matter. The fibrous flax by-products converted 9-10% of the DM to VFA. Acetate production by leaf and stripped fibre digestion represented a greater proportion ($P<0.05$) of the total VFA production than the other flax components (Table 2.5) (Litherland et al., 2005).

Table 2.5. Volatile fatty acid (VFA) production from *in vitro* incubation of white clover (WC), New Zealand native flax; stripped green matter (G), leaf (L), butt (B) and stripped fibre (F) for 8 h (Litherland et al., 2005).

	WC	**G**	**L**	**B**	**F**	**P <**
Yield of VFA (mg gDM^{-1})	210 ^{a}	141^{b}	101 ^{c}	101 ^{c}	90 ^{c}	0.001
Acetate %	40 ^{b}	44 ^{b}	51 ^{a}	43 ^{b}	49 ^{a}	0.001
Propionate %	38 ^{a}	33 ^{a}	29 ^{ab}	33 ^{a}	28 ^{b}	0.01
Butyrate %	15 ^{b}	21 ^{a}	16 ^{b}	22 ^{a}	19 ^{ab}	0.05
Minor VFA%	7 ^{a}	3 ^{c}	5 ^{b}	2 ^{c}	4 ^{bc}	0.001
Acetate: Propionate	1.0 ^{b}	1.35 ^{b}	1.8 ^{a}	1.3 ^{b}	1.8 ^{a}	0.01

Flax by-products can be used as a supplement during winter and autumn with pasture to achieve maintenance feeding levels. Maintenance feeding could be achieved following replacement of 24% of winter pasture by flax leaves and with all other flax by-products fed at less than 10%. (Table 2.6) (Litherland et al., 2005).

Table 2.6. The amount (kg DM hd^{-1} day^{-1}) for each of the leaf (L), butt (B) and stripping fibre (F) required to meet maintenance requirements alongside winter and autumn pasture (Litherland et al., 2005).

Flax by-product	**Intake (kg DM hd^{-1} day^{-1})**			
	Winter pasture	Flax	Autumn pasture	Flax
L	5.5	5.8	7.8	2.3
B	6.7	4.1	8.3	1.8
F	8.5	1.5	8.5	1.5

2.1.2. New Zealand Pasture

Composite ryegrass and clover pastures (Table 2.6) are the predominant pasture in New Zealand. The quality of the pasture is determined by such components such as the proportions of legume and grass, proportions of green and dead matter, and proportions of leaf and stem. Shorter pasture with less dead matter and more leaf than stem will therefore have higher feed value by virtue of higher levels of soluble carbohydrate and crude protein, and less structural carbohydrate and lignin. Pasture with high dead matter content reduces cow intake. The management of grazed pasture therefore requires a compromise between quality and quantity.

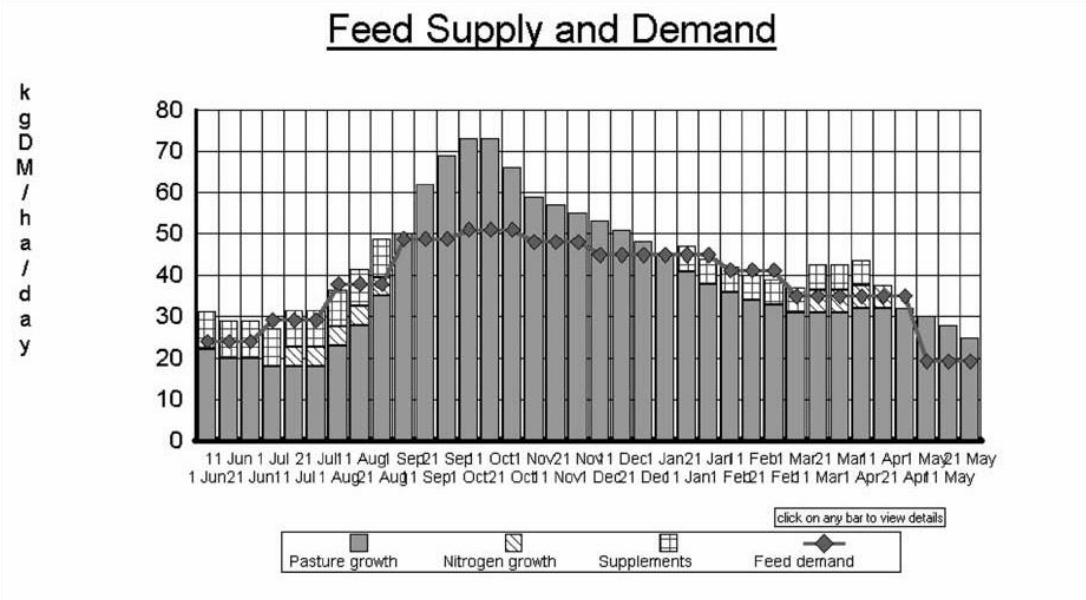


Figure 2.6. Example of a feed budget for a Waikato farm (200 ha), milking 600 cows using the Feed4profit Feed plan computer software (Verkerk, 2003).

Figure 2.6 shows that there are periods of the year (June, July, August and January, February, and March) when feed demand exceeds supply. The activity that incorporates all the above aspects of pasture and nutritional management is known as a feed budget. Excess pasture can then be harvested for silage at a time that pasture quality needs to be maintained, while feed deficits can be predicted and filled by supplements. In this way cow production and body condition is maintained at an appropriate level, and pasture covers on the farm can be controlled to the desirable level. The most common supplement used is pasture silage that is harvested during periods when pasture cover exceeds cow demand or cereal silage (maize, wheat or barley) or by-products from some horticultural industries (e.g., apples, potatoes, carrots Palm kernel extract, sunflower meal, and pumpkin meal) (Verkerk, 2003; Valentine and Kemp, 2007; Vlaicu et al., 2018).

Table 2.7. The chemical composition of typical New Zealand pasture (white clover and perennial ryegrass) (g $kgDM^{-1}$) (Hammond et al., 2011).

Composition	White Clover	Perennial Ryegrass
Dry matter	166	172
Organic matter	905	864
Crude protein	255	192
Lipid	24	31
NDF	276	444
ADF	183	230
Cross energy MJ $kgDM^{-1}$	18.4	17.6

NDF, neutral detergent fibre; ADF, acid detergent fibre

2.1.2.1. Forage Maturity

The effect of forage maturity on digestion and animal performance usually occurs via changes in plant morphology and in cell wall components, which affect dry matter intake and digestibility (Van Soest, 1994).

Maturity is the most important factor affecting pasture quality. From a nutrition perspective, forage quality relates to the feeding value or the ability of ruminants to utilise feed for production (e.g., milk, meat, wool). Forage quality continually changes as it matures. As plant cell wall content increases, indigestible lignin accumulates and in late spring, grass maturity changes so rapidly that it is possible to measure a significant decline in forage quality every 2 or 3 days. This change occurs in both the proportions of leaf, steam, and chemical composition of these structures. Both the extent and the rate of change in components are important and farmers face considerable challenges when they manage pasture to ensure a high nutrient intake for high producing animals (Chaves et al., 2006).

Grass maturation has a major impact upon dairy cow productivity because grass dominant pasture is unable to provide enough nutrients to match the genetic merit of New Zealand dairy

cows. This is evidenced by low milk production compared to cows fed concentrate diets and anoestrus coinciding with pasture maturation in some situations (Verkerk, 2003).

Nutritive value declines because of changes in chemical composition. The principal changes are increased proportions of fibrous stem and decreased concentrations of leaf protein. This means that mature plants have higher proportions of fibre and lower proportion of protein in the dry matter. These changes reduce the amounts of amino acid (AA) available to ruminants and may increase the proportions of acetate: propionate available for absorption (Wilman and Asiegba, 1982).

2.1.2.2. New Zealand pasture species

Perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) are common permanent pastures in New Zealand. However, in dry regions, especially in summer and without irrigation, their herbage production and nutritive value are often limited, which can cause feed deficits and reduce animal productivity (Waller and Sale, 2001). As a result, farmers are becoming interested in alternative forages that have deeper root systems and/or greater drought tolerance. For example, multi-graze forages such as chicory (*Cichorium intybus* L.) narrow-leaved plantain (*Plantago lanceolata* L.), lucerne (Alfalfa) (*Medicago sativa L.*) and red clover (*Trifolium pratense* L.) which all have the potential to produce quantities of good quality feed during dry periods (Glassey et al., 2013).

2.1.2.2.1. Chicory (*Cichorium intybus* L.)

Chicory (*Cichorium intybus* L.; Figure 2.7) is a European native forage, found in many parts of Asia, Africa, and America. First recorded in New Zealand in 1867. It has a long, thick taproot and new shoots appear from the crown. It is a moderately persistent, leafy herb, which gives improving production in sheep, deer, and cattle. It produces high yields; on average of 18 tonnes of high-quality dry matter per hectare from spring through to late autumn, and is very palatable to all livestock. However, chicory requires rotational grazing (or cutting) for best results. In addition, it has tendency to bolt rapidly into flower during spring so it must be controlled by grazing to maintain quality, otherwise its tall stems become woody and unpalatable. Its growth accelerates in spring, peaks over summer and early autumn and declines in winter. According to Stewart (1996), chicory is digested by animals more rapidly than normal forage and animals fed on chicory showed higher weight and health performance.

Figure 2.7. Description of Chicory plant (www.bing.com/images)

2.1.2.2.2. Plantain (*Plantago lanceolata* L.)

Plantain (*Plantago lanceolata* L. Figure 2.8) is a perennial herb that performs well in a range of pastures, particularly in soils with low fertility. Plantain is also known as narrow leaf plantain or ribgrass and has a rosette growth form with a deep taproot. The leaves are long and wider in the middle and taper to a point at each end (Rumball et al., 1997). Plantain has several advantages as a forage, such as being highly palatable to livestock, establishing rapidly, being drought resistance, pest tolerant, and having a high mineral content (calcium, magnesium, sodium, phosphorus, zinc, copper, and cobalt). It is recommended to minimise seed head development through grazing as leafy plantain is of high quality but plants with seed heads have a reduced feed value (Stewart,1996).

Figure 2.8. Description of Plantain plant (www.bing.com/images)

2.1.2.2.3. Lucerne (Alfalfa) (*Medicago sativa* L.)

Lucerne (Alfalfa) (*Medicago sativa* L. Figure 2.9) is a perennial legume grown for grazing or haymaking. It is commonly grown in dryland pastures when traditional annual pastures become limited in both nutritive value and production, which can lead to a reduction in livestock performance (Thomas et al., 2010). Its taproots can extract water deep from the soil, making it more tolerant to drought than other pasture plants and it can increase soil organic matter, improve soil structure, and build up nitrogen reserves in topsoil (Gault et al., 1995).

Figure 2.9. Lucerne (Alfalfa) (*Medicago sativa* L.)

2.1.2.2.4. White clove (*Trifolium repens* L.) and Red clover (*Trifolium pratense* L.)

White clover (WC; *Trifolium repens* L.) and red clover (RC; *Trifolium pratense* L. Figure 2.10), are the most well-known forage legumes for their significant benefits in reproduction for livestock. Both possess high protein contents and high digestibility that lead to high intake (10-35%) due to low resistance to chewing because of less cell wall and lower length/width ration of fibres (Caradus et al., 1995).

(A) (B)

Figure 2.10. (A) White clover (*Trifolium repens* L.) and (B) red clover (*Trifolium pratense* L.)

In general, clover has the ability to fix nitrogen from the atmosphere. It is estimated that white clover can fix up to 600 kg ha^{-1} $year^{-1}$ while red clover can fix up to 400 kg ha^{-1} $year^{-1}$ in symbiosis with root nodule bacteria (rhizobia) allowing a reduction in the use of N fertilizer (Peoples and Baldock, 2001; Taylor and Quesenberry ,1996). White clover is classified into four different varieties based on leaf size including: small, medium, large, and very large leaf. Small leaf is considered suitable for hard sheep grazing, medium leaf is used under rotation grazing and large or very large leaf size clovers are used mainly for cattle grazing or conservation (Ledgard et al., 1999). Red clover is classified by ploidy level and flowering date. Early flowering gives two similar conservation cuts and subsequent lower yield cuts while late flowering provides a greater proportion of yield at the first cut and is considered as more grazing tolerant and persistent than early flowering cultivars (Black et al., 2009). Ploidy level (e.g., diploid and tetraploid), is breeding for improved crop cultivars to better sustain livelihood and increase the economic return to farmers. Red clover is an out crossing, insect pollinated natural diploid species, but breeding has resulted in tetraploid red clover with higher forage yield, better persistence, and increased resistance to biotic and abiotic stress (Boelt et al., 2015).

2.1.3. Nutritive Value of the New Zealand pasture species

The nutritive value of New Zealand pasture species has been thoroughly evaluated in a wide range of trials. The nutritive value of the pasture species is a measure of available nutrients that are required by ruminants. Nutrients include nitrogen, often expressed as crude protein (CP; nitrogen concentration × 6.25), neutral detergent fibre (NDF), acid detergent fibre (ADF),

and metabolizable energy (ME) (Table 2.8), and macronutrients and micronutrients (Table 2.9). Metabolisable energy is derived from the microbial and intestinal digestion and absorption of nutrients. It is an essential component of nutritive value as it is the most commonly used indicator of feed value in New Zealand farm systems (Waghorn and Clark, 2004).

Ruminant performance is limited largely by forage quality and the importance of forage analysis and digestibility has long been recognised. All feed evaluation attempts to identify the degree to which individual feedstuffs contribute to the nutritional requirement of the livestock. The ideal situation would be for each feed to be offered to the appropriate class livestock and then observations made of the production response obtained. Several methods are available for predicting nutritive value. Chemical analyses of feeds determine nutrition composition (Table 2.8), but do not predict animal performance. The *in vivo* direct method and the *in vitro* indirect method are both alternative procedures for estimating nutritive value and digestion kinetics (Corson et al., 1999)

In vitro methods of feed evaluation have numerous advantages over *in vivo* methods, as they are less expensive, less time-consuming and allow incubation conditions to be maintained more precisely than the *in vivo* methods. *In vivo* methods involve host animals and large amounts of forage and are expensive and time-consuming (Barnes, 1965; Losada et al., 2010).

In vitro methods can easily quantify the products of fermentation, gas, NH_3, VFA, and CH_4 when a foodstuff is incubated with buffered rumen fluid (Table 2.10). Volatile fatty acids produced by the fermentation of carbohydrates, include acetate, propionate and butyrate that can be used to generate energy (ATP) in intermediary metabolism. Ammonia production from protein fermentation is relatively small compared to carbohydrate fermentation (Dijkstra, 1994; Getachew et al., 1998). The amount of gas produced during incubation is measured to predict the extent and rate of feed digestion and quantify the chemical composition of feeds (Blümmel et al., 1997).

Table 2.8. Nutrition composition of New Zealand pasture species from different experiments (%DM); crude protein (CP); neutral detergent fibre (NDF); acid detergent fibre (ADF); metabolisable energy (ME).

Species/ References	Season	CP %	NDF %	ADF %	ME (MJ $kgDM^{-1}$)
Perennial Ryegrass					
Burke et al., 2000	-	15.5	48.7	-	-
Fulkerson et al., 2007	Summer	22.1	31.3	51.5	9.9
	Autumn	24.0	26.6	49.7	10.0
	Winter	24.3	23.2	48.9	11.4
	Spring	26.3	25.9	55.2	11.1
Gregorini et al., 2013	Summer and Autumn	18.8	45.5	29.6	10.5
Pain et al., 2015	Spring	12.9	43.0	23.2	10.4
	Summer	21.7	44.6	24.6	9.7
Waugh et al., 1998	Summer	20.0	41.0	30.2	11.9
	Autumn	24.3	48.5	25.8	9.7
Chicory					
Burke et al., 2000	-	19.3	23.8	-	-

Gregorini et al., 2013	Summer and Autumn	20.6	22.2	24.2	-
Hayes et al., 2010	Spring	21.8	38.3	23.6	11.5
	Summer	13.1	43.2	29.1	9.0
	Autumn	24.0	27.5	13.3	10.5
	Winter	16.6	26.7	17.7	10.7
Pain et al., 2015	Spring	12.7	24.5	18.0	11.0
	Summer	15.4	19.7	15.2	11.4
Schmidt et al., 2013	Summer	21.8	19.5	14.8	-
Waugh et al., 1998	Summer	22.5	25.5	19.7	12.8
	Autumn	22.9	18.9	18.9	11.4
Plantain					
Burke et al., 2000	-	24.7	28.3	-	-
Golding et al., 2011	Autumn	12.9	39.9	-	10.2
Gregorini et al., 2013	Summer and Autumn	20.4	28.1	26.2	11.5
Hayes et al., 2010	Spring	17.7	36.1	24.3	10.7
	Summer	10.7	36.5	22.7	11.9
	Autumn	20.4	49.0	33.4	8.2

	Winter	18.4	34.7	21.3	10.5
Pain et al., 2015	Spring	9.1	41.5	27.0	10.0
	Summer	17.7	34.0	23.6	10.7
Lucerne					
Bessa et al., 2005	-	15.6	48.1	35.4	-
Burke et al., 2000	-	29.9	29.5	-	-
Fulkerson et al., 2007	Summer	20.6	35.8	28.3	9.0
	Winter	30.1	47.2	24.6	9.3
	Spring	29.0	32.2	26.3	9.7
Hayes et al., 2010	Spring	22.3	36.9	24.0	10.4
	Summer	15.9	48.1	34.2	7.5
	Autumn	18.0	36.2	24.8	8.0
	Winter	27.5	29.3	20.3	11.3
Kaiser and Combs, 1989	Early vegetative (May)	26.7	36.1	25.7	-
	Late bud (May)	20.6	51.7	40.3	-
	Full bloom (Winter)	18.7	51.7	39.1	-
Robertson et al., 2015	Autumn	27.4	52.6	-	12.7
Schmidt et al., 2013	Summer	26.3	28.0	20.0	-

White clover					
Burke et al., 2000	-	26.9	25.6	-	-
Fulkerson et al., 2007	Summer	24.2	27.6	21.0	10.0
	Winter	29.8	29.1	19.3	10.5
	Spring	28.1	33.9	23.3	9.3
Lindsay et al., 2007	Summer	24.3	26.9	-	11.8
Red clover					
Burke et al., 2000	-	27.4	33.6	-	-
Fulkerson et al., 2007	Summer	24.2	39.5	26.2	9.2
	Winter	30.3	35.3	25.4	10.0
	Spring	28.9	41.2	37.5	9.5
Pain et al., 2015	Spring	22.5	26.4	17.2	10.9
	Summer	20.4	30.3	20.9	10.6

Table 2.9. Mineral composition of New Zealand pasture species.

Macronutrient composition (% of DM)	**P**	**K**	**S**	**Ca**	**Mg**	**Na**		
Harrington et al., 2006								
Perennial ryegrass	0.37	3.8	0.35	0.42	0.17	0.18		
White clover	0.35	2.8	0.21	1.19	0.24	0.21		
Chicory	0.66	3.8	0.63	1.18	0.39	0.59		
Plantain	0.48	2.0	0.53	1.77	0.25	0.62		
Scales et al., 1995								
Perennial ryegrass	0.36	2.55	0.28	0.66	-	0.08		
Chicory	0.34	3.64	0.39	1.49	-	0.21		
Lucerne	0.36	2.48	0.27	1.28	-	0.06		
Micronutrient composition ($mgkg^{-1}$)	**Fe**	**Mn**	**Cu**	**Zn**	**B**	**Co**	**Se**	**Mo**
Harrington et al., 2006								
Perennial ryegrass	151	99	7.9	22.0	19.0	0.193	0.023	0.640
White clover	109	55	8.6	22.0	28.7	0.173	0.073	0.223
Chicory	167	161	18.6	57.7	38.3	0.273	0.043	0.420
Plantain	182	109	15.1	37.7	23.3	0.360	0.053	0.270

Table 2.10. *In vitro* fermentation parameters after 24h incubation of selected New Zealand pasture species.

Species /References	Country	pH	Gp	tVFA	NH_3 mg L^{-1}	A:P	A%	P%	B%
Perennial ryegrass									
Banik et al., 2013	Australia								
7 weeks after sowing		6.11	104 kPa	110 mmol L^{-1}	536	2.78	-	-	-
11weeks after sowing		6.32	96 kPa	96 mmol L^{-1}	324	2.92	-	-	-
Muetzel et al., 2014	New Zealand	-	171 ml $gDMi^{-1}$	-	-	-	65.1	23.2	8.3
Navarro-Villa et al., 2011	Ireland	6.60	178 ml $gDMi^{-1}$	45 mmol L^{-1}	-	2.85	65.6	22.4	7.1
Purcell et al., 2011	Ireland	6.65	173 ml $gDMi^{-1}$	49.9 mmol L^{-1}	-	2.9	66	22.8	7.7
Purcell et al.,2012	Ireland	-	155 ml $gDMi^{-1}$	4.7 mmol $gDMi^{-1}$	-	3.17	65.9	20.9	8.3

Sun et al., 2011	New Zealand	-	241 ml $gDMi^{-1}$	-	-	-	-	-	-
White clover			-				-	-	-
Banik et al., 2013	Australia								
7 weeks after sowing		5.66	108 kPa	114 mmol L^{-1}	430	2.85	-	-	-
11weeks after sowing		6.17	106 kPa	101 mmol L^{-1}	280	2.85	-	-	-
Muetzel et al., 2014	New Zealand	-	173.7 ml $gDMi^{-1}$	-	-	-	64.1	24.2	7.6
Purcell et al.,2012	Ireland	-	169 ml $gDMi^{-1}$	5.4 mmol $gDMi^{-1}$	-	2.80	62.4	23.1	8.7
Chicory									
Durmic et al., 2016	Australia	-	395 ml $gDMi^{-1}$	116 mmol L^{-1}	229.6	3.4	70	20	10
Muetzel et al., 2014	New Zealand	-	161.3 ml $gDMi^{-1}$	-	-	-	71.1	19.5	6.5

Sun et al., 2011	New Zealand	-	244 ml $gDMi^{-1}$	-	-	-	-	-	-
<u>Lucerne (alfalfa)</u>									
Banik et al., 2013	Australia								
7 weeks after sowing		6.13	105 kPa	108 mmol L^{-1}	437	3.08	-	-	-
11weeks after sowing		6.28	100 kPa	94 mmol L^{-1}	340	2.98	-	-	-
Muetzel et al., 2014	New Zealand	-	137.1 ml $gDMi^{-1}$	-	-	-	68.8	21.7	7
<u>Plantain</u>									
Durmic et al., 2016	Australia	-	399 ml $gDMi^{-1}$	123 mmol L^{-1}	177	3.5	69	19.5	11.5
<u>Red clover</u>									
Banik et al., 2013	Australia	6.15	-	96 mmol L^{-1}	242	3.42	-	-	-

GP, gas production; tVFA, total volatile fatty acid; and A:P, Acetate: propionate ratio; kPa, kilopascal; DMi, dry matter incubation.; A, acetate; P, propionate; B, butyrate.

2.1.4. Herbage production

New Zealand permanent pasture of perennial ryegrass (*Loloium Perenne* L.) is limited in quality and quantity under dry weather conditions. Therefore, it is necessary to consider alternative forages that higher nutritive value and production in summer and autumn (Powell et al., 2007). However, several studies have shown that alternative pasture species are highly productive and capable of giving excellent animal performance (Table 2.11)

Table 2.11. Herbage production obtained for perennial ryegrass, chicory, plantain, white and red clover from different studies.

Pasture species	**Yield (tDM ha^{-1})**	**References**
Perennial ryegrass	10-25	Kemp,1999
	9.3-11.2	Kerr et al., 2012
Chicory	8.5	Rumball, 1986
	15-18	Matthews et al., 1990
	13-20	Brown and Moot, 2004
	9-14	Li and Kemp 2005; Powell et al., 2007
Plantain	20	Stewart, 1996
	17	Powell et al., 2007
Dec. 2010 - May 2011	11.5-16.1	Lee et al., 2015
June 2011-May 2012	9.8-13.7	
Red clover	11-15	Hyslop, 1999
	11.5-17	Brown et al., 2005
Lucerne	20	Brown et al., 2005

2.1.5. Animal Performance

One of the most important factors to consider in digestion is chewing which causes the rupture of between 50-80% of plant cell walls depending on the forage type. This enables microbial access to the cell contents and reduces the feed particle size to enable passage through the digestive system and leads to an improved digestion process. The approximate size of particles able to leave the rumen is 1.0 mm for sheep and 2.0 mm for cattle, which means less chewing of forage and faster digestion leading to an increase in daily intakes (Waghorn and Clark, 2004).

Cows grazing on chicory or plantain have reduced ruminative chews per rumination bolus by approximately 9% and 6% respectively, compared with those grazing on ryegrass (chew min^{-1} of rumination time per bolus) (Gregorini et al., 2013). Nutrient detergent fibre (NDF) content of chicory and plantain is 2 and 1.6 times less than that of ryegrass which significantly reduces the chew min^{-1} and reduces of forage particle size (Gregorini et al., 2009).

Livestock performance such as daily weight gain, wool, meat, and milk production in terms of both quality and quantity is a measure of production. Animals grazing on chicory (*Cichorium Intybus* L.), plantain (*Plantago Lanceolata* L.), lucerne (Alfalfa) (*Medicago sativa* L.), red clover (*Trifolium pratense* L.) and white clover (*Trifolium repens* L.) have significantly improved liveweight, greater dry matter intake and milk production (fat + protein) (Minneé et al., 2017)

Nutritive value of New Zealand pasture species can be affected by the time of the year (Table 2.12) (Kusmartono et al., 1996; Waugh et al., 1998).

Table 2.12. Effect of season on nutrient levels for ryegrass/ white clover and chicory.

Nutrition value/ reference	Ryegrass/white clover	Chicory
Kusmartono et al.,1996		
% Organic matter digestibility		
Summer	76.5	86.4
Autumn	77.6	85.8
Winter	85.2	-
Spring	84.8	86.5
Metabolizable energy (MJ $kgDM^{-1}$)		
Summer	11.1	12.0
Autumn	11.1	11.8
Winter	11.4	-
Spring	12.1	12.1
Protein (g $kgDM^{-1}$)		
Summer	215	201
Autumn	268	199
Winter	253	-
Spring	226	194
Waugh et al., 1998		
January % DM		
DM	26.3	8.6
NDF	41.0	25.5
ADF	30.2	19.7
CP	20.0	22.5
IVD	76.2	83.4
ME (MJ $kgDM^{-1}$)	11.9	12.8
March % DM		
DM	22.8	7.5
NDF	48.5	19.8
ADF	25.8	18.9
CP	24.3	22.9
IVD	64.9	76.4
ME (MJ $kgDM^{-1}$)	9.7	11.4

2.1.5.1. Sheep performance

It is estimated that New Zealand lamb average daily growth rates are 150g day^{-1} on traditional New Zealand pastures. However, lambs fed on forage species such as chicory, lucerne, red and white clover show increased liveweight gains of up to 300g day^{-1}, Which results in a reduction in time taken to reach slaughter weight (Lindsay et al., 2007; Speijers et al., 2004).

Kenyon et al. (2010) reported that lambs born to ewes fed on forage species were heavier and had better body condition scores than those born to ewes fed on ryegrass. During lactation ewes showed between 17 and 25% higher milk yield and improved milk quality (CP 5.0g $100g^{-1}$ vs. 4.8g $100g^{-1}$, and casein 4.0g $100g^{-1}$ vs. 3.8g $100g^{-1}$: forage vs. ryegrass) (Hutton et al., 2011).

Ewes grazed on lucerne showed a significant 10 kg improvement in liveweight compared to ewes grazed on senescent pastures (containing annual grasses [barley grass (*Hordeum leporinum*) and brome grass (*Bromus spp.*)]). In addition, body condition scores were higher on lucerne (3.7±0.04) vs. old pasture (2.7±0.03) and 30% more lambs were born per ewe on lucerne then old pasture (Robertson et al., 2015).

There are several isoflavones - particularly formononetin, daidzein, genistein and biochanin A in red clover, which can exert oestrogenic effects within the animal disrupting reproductive cycles as well as impairing fertility in sheep (Newton and Betts, 1968). So, grazing of red clover or feeding of red clover pellets or silage prior to and during mating should be avoided (Thomson, 1975).

Greasy wool production in sheep can be improved by grazing on different pasture species compared with perennial ryegrass (Table 2.13). Wool production (greasy fleece weight) is higher on white clover than chicory, plantain, and ryegrass (Fraser and Rowarth ,1996).

Table 2.13. Effect of pasture species on greasy wool production (kg) over 83 days (year 1) and 120 days (years 2 and 3) in summer/ early autumn (Fraser and Rowarth, 1996).

Year	Chicory	Plantain	White clover	Ryegrass
One	1.62±0.03	1.48±0.02	1.79±0.04	1.42±0.04
Two	2.15±0.06	1.61±0.05	2.48±0.07	1.69±0.04
Three	2.54±0.07	1.79±0.09	2.69±0.07	1.69±0.08

2.1.5.2. Deer performance

New Zealand deer farmers aim to achieve a carcass weight of 50-65 kg by 1 year of age grazing traditional New Zealand perennial ryegrass/white clover pasture (Kusmarton et al., 1996).

It was reported that the carcass weight for red deer and hybrid stags after weaning at 12 months of age was 63.2 and 73.0 kg respectively when grazed on chicory, while 56.6 and 57.0 kg respectively when grazed on perennial ryegrass/white clover (Kusmarton et al., 1996). Other studies reported the weights to be 56.0 and 59.3 kg respectively when grazed on chicory and 48.6 and 53.3 kg respectively when grazed on pasture (Min et al., 1997). Deer grazed on chicory showed a 56%, 26% and 15% higher voluntary feed intake than deer grazed on perennial ryegrass-based pasture during summer, autumn, and spring respectively (Kusmarton et al., 1996).

Similarly, relative to perennial ryegrass/white clover pasture, deer grazed on swards of red clover or chicory showed a 20% increase in liveweight gain during summer (pre-weaning), compared to 26-47% during autumn and 10-14% during spring (Barry et al., 2002).

Alternative forage crops can also help grazing animals maintain necessary trace elements. For example, deer grazing on plantain and chicory can enhance their levels of copper, vitamin B_{12} and selenium (Hoskin et al., 2006). Chicory also provides higher copper concentrations than plantain (Barry et al., 2001). In ranking forage herbs for use in venison production system, growth rates of young deer were greater for chicory, followed by plantain, with the lowest for ryegrass (Hoskin et al., 2006).

2.1.5.3. Cow performance

Alternative forage crops play a role in controlling internal parasites in all livestock because of their condensed tannin (CT) content. However, cows grazed on chicory have been found to have a bitter taint to their milk, so it is recommended to restrict the grazing time on chicory after morning milking for 2 h per day, which reduces chicory intake to 25% of total daily dry matter intake (Barry, 1998). Tainted compounds in the milk of chicory fed cows include dihydrolactucin, tetrahydrolactucin and hydroxypenyacetic acid (Visser, 1992).

Condensed tannins cause a significant increase in milk protein yields by increasing AA absorption in the small intestine by 62%. In addition, tannins cause a reduction in rumen protein degradation and increase AA supply for absorption. The ideal CT concentration for

ruminant animal nutrition is 20-40g $kgDM^{-1}$ (Waghorn et al., 1990; Waghorn and McNabb, 2003).

In autumn, it was observed that the combined response of milk fat and protein in cattle grazing on chicory and ryegrass is higher than cattle grazing on ryegrass only (0.93 vs. 0.87 $kgDM^{-1}$ eaten). Moreover, milk production is significantly greater in cattle grazing on a chicory and white clover sward than when grazing ryegrass (18.8 vs. 9.8 L cow^{-1} day^{-1}). It was reported that addition of 40% of these herbs to the daily intake had no negative effects on cattle health during summer (Chapman et al., 2008; Minneé et al., 2017). Another advantage of CT is the control of internal parasites. Secondary compounds which are found in a range of forages (Table 2.14), are organic compounds which are not strictly essential to the plant in roles such as growth and reproduction but show promise for controlling internal parasites and ability to protect protein from rumen degradation (Waghorn et al.,1990; Barry et al., 2002).

Condensed tannins can reduce *in vitro* motility of both lungworm and gastrointestinal nematode larvae which improves the ability of young animals to overcome internal parasites infection (Barry et al., 2002). Furthermore, lambs grazing herbs containing CT have shown an 12% increase in liveweight and wool production (Wang et al., 1996).

According to Marley et al. (2003), *Nematodirus* and *Trichostrongylus* are internal parasites that cause a significant loss in production in livestock, reducing growth rates by up to 40%. However, it was reported that grazing on chicory and plantain reduces the risk of these parasites by reducing faecal egg counts by 42% (Table 2.15) (Judson et al., 2009).

Table 2.14. Condensed tannin and secondary compounds contained in a range of forage (Barry et al., 2002).

Forage	**Condensed tannin (g kgDM^{-1})**	**Other known secondary compounds**
Legumes		
Red clover (*Trifolium pratense* L.)	1.7	Iso-flavones (7-14 g kgDM^{-1})
Lucerne (*Medicago sativa* L.)	0.5	0
Grasses		
Perennial ryegrass (*Plantago Lanceolata* L.)	1.8	Endophyte alkaloids (12-30 mg kgDM^{-1})
Herbs		
Chicory *(Cichorium Intybus* L.)	4.2	Sesquiterpene lactones (3.6 g kgDM^{-1})
Plantain (*Plantago Lanceolata* L.)	14	Iridoid glycosides (30g kg DM^{-1})

Table 2.15. Liveweight gain (LWG) g day^{-1} of lambs grazing on different pasture species in New Zealand from different experiments.

Forage species	**LWG g day^{-1}**	**References**
Perennial ryegrass	227	Cruickshank, 1986
	104	Young et al., 1994
	158	Scales et al., 1995
	121	Fraser and Rowarth, 1996
	201	Marley et al., 2003
	184	Fraser et al.,2004
White clover	320	Cruickshank, 1986
	226	Fraser et al., 1988
	282	Marley et al., 2003
	269	Lindsay et al., 2007
Red clover	292	Marley et al., 2003
	305	Fraser et al., 2004
Lucerne	146	Young et al., 1994
	236	Scales et al., 1995
	210	Marley et al., 2003
	243	Fraser et al., 2004
Plantain	222	Moorhead et al., 2002

2.1.6. Techniques to measure nutritional value of ruminant feed

Food is the most important source of energy for ruminants, which is required for maintenance and production (milk, meat, and wool). Therefore, knowledge is required about quality and quantity of the nutrients supplied. Methods that can accurately determine nutrient composition of feed can therefore maximise production (Tamminga and Williams, 1998).

2.1.6.1. In vitro digestion methods

In vitro techniques that digest feedstuffs using anaerobic fermentation in the presence of a buffered rumen liquor (inoculum) have been used to measure DM and can also be applied to measure other nutrients such as protein (Weiss, 1994). The development of suitable buffers made it possible to enhance the length of *in vitro* incubation and quantify the fermentation products, NH_3 and VFA. The buffers also maintain the pH at around 6.7 which is ideal for the growth of cellulolytic microorganisms and the maximisation of fibre digestion (McDougall, 1948).

Several different *in vitro* systems have been developed, but all must have the following criteria for evaluation: maintain a normal microbiological population; maintain a normal digestion rate and predict *in vivo* results.

Tilly and Terry (1963) developed a two-stage in *vitro* incubation technique. It required a 48-h incubation of a finely ground sample in buffered rumen fluid under anaerobic conditions, followed by another 48 h of digestion with pepsin acid solution. Then the insoluble residue was filtered, dried, and combusted for DM estimation. Inoculum has a significant effect on the accuracy of *in vitro* DM disappearance (IVDMD). Host animals can be affected by a number of factors which impact IVDMD values such as: animal to animal variation, species of host animal, feed management and diet fed to the host animal. *In vitro* organic matter disappearance can be significantly affected even when all animals have been fed the same diet (Ayres, 1991); however, Engels and van der Merwe (1967) reported that the host animal had no effect on *in vitro* disappearance. In contrast, Nelson et al. (1972) reported that in some situations, host animal was a significant source of variation.

Crude protein (CP) is considered an important factor. In general, when both the diet fed to the host animal and the sample had low concentrations of CP, variation among host animals was greater than when diets fed to host animals and samples contained higher CP concentrations.

The diet fed to ruminants affects the microbiological population and chemical environment within the rumen and this will influence IVDMD. Feeding host animals, a relatively concentrated diet (barley) leads to reduced IVDMD of alfalfa hay and of the basal diet as compared to host animals fed alfalfa hay (Calder, 1970).

Type of forage fed to host animals when the diet is composed predominantly of forage does not influence the results greatly, with the exception of low protein forages. Generally, when forage fed to host animals has less than 100g kg^{-1} of CP (DM basis) the IVDMD is low (Nelson et al. 1972). Species and quality of forage fed to host animals influence the accuracy of *in vitro* values more than they influence the results. However, when rumen fluid collected from animals fed alfalfa (a high CP forage) is used, *in vitro* DM and fibre digestibility values are higher than when ruminal fluid from animals fed grasses or low-quality roughages is used (Bezeau, 1965; Nelson et al., 1972). As forage type fed to host animals affects IVDMD values, samples should be analysed using inoculum from host animals that have been fed a common diet. The forage fed to the host animal should be common in the local area so that similar feed can be obtained over time (Weiss,1994).

Species of host animal can affect the accuracy of IVDMD values, but it does not appear to have a significant effect on the results. Ruminal fluid collected from sheep produces different IVDMD values compared to ruminal fluid collected from cattle, however the animal species effect is not consistent. In general, large quantities of ruminal fluid are easier to obtain from cattle than from sheep. This difference may make rumen fistulated cattle the host animal of choice for commercial laboratories. The host animal should be the same species as the target animal (Horton et al.,1980).

The time ruminal fluid is collected relative to feeding, should also be kept constant. The activities of most fibrolytic enzymes peak 8 to 12 h post feeding (Williams, 1988); however, Ayres (1991) reported IVDMD values decrease when inoculum is collected more than 16 to 18 h post-feeding.

2.1.6.1.1. Batch Culture

Batch culture is the simplest and most commonly used *in vitro* fermentation method and measures the amount of ammonia produced from the feed incubated with rumen fluid. Numerous parameters such as nature of the substrate, the incubation time and the buffer used may affect this measurement. The amount of ammonia produced depends on the *in vitro*

incubation time. Microbial activity rapidly decreases due to the difficulty in maintaining anaerobic conditions for an extended period. The accumulation of end-products of degradation or the diminution of pH are common issues, so a pH buffer solution is usually added to the batches. Artificial saliva is commonly used as a buffer, with a pH of 6.0 required to maintain fermentation, but if the pH falls, the amount of gas produced will decreased (Michalet-Dorean and Ould-Bah, 1992).

2.1.6.1.2. Continuous Culture

In continuous culture systems or chemostats, there is a regular addition of buffer and nutrients and continual removal of fermentation products allowing steady-state conditions to be reached, which allows the establishment of a stable microbial population that can be maintained for a long period of time (López, 2005). These systems can measure fermentation parameters, DM degradation, output of end products and microbial protein synthesis. However, these systems are expensive and require some time to achieve the steady state condition (López, 2005).

2.1.6.1.3. Production of fermentation

In vitro techniques can easily quantify the products of fermentation (NH_3 and VFA). When a foodstuff is incubated with buffered rumen fluid *in vitro*, carbohydrates are fermented, and what is produced is basically acetate, propionate, and butyrate. Gas production from protein fermentation is relatively small when compared to carbohydrate fermentation (Getachew et al., 1998).

The measurement of VFA production during the fermentation process can be indicative of the nutritional value of feed tested, as VFA provides more than 50% of the digestible energy intake of ruminants. Of the VFAs (acetate, propionate, and butyrate), propionate is a major contributor of gluconeogenesis, while acetate and butyrate converted to β-hydroxybutyrate are lipogenic precursors (Harvatine et al., 2009). The amount of gas produced during fermentation of carbohydrate is related to the proportion of the carbohydrate going through each of the fermentation pathways described in Table 2.16 (below) for glucose. One gram of glucose results in 267 ml of gas when only propionic acid is produced, 400 ml of gas when only butyric acid is produced, and 533 ml gas when only acetic acid is formed (Cone, 1994).

Table 2.16. Direct and indirect gas production from glucose fermented to different acidic endpoints.

Gas production from substrate
Glucose + $2H_2O \rightarrow$ 2 acetic acid + $2CO_2$ + 8H
Glucose + 8H $\rightarrow$ 2 propionic acid + $2H_2O$
Glucose ◥ 1 butyric acid + $2CO_2$ + 4H
8H + CO_2 ◥ CH_4 + $2H_2O$

Another common use of the *in vitro* methods is to measure protein degradation by the concentration of NH_3 present during the *in vitro* incubation of a feed with rumen fluid. Rumen microorganisms degrade the dietary protein to AAs and peptides, which are further degraded to produce NH_3 and carbon, which can contribute to the VFA pool in the rumen (Waghorn et al., 2004). Some parameters such as the nature of the substrate, incubation time and buffer addition can affect the measurement of NH_3 during the incubation (Michalet-Doreau and Ould-Bah, 1992). The microbial activity in the rumen fluid is a great source of variation in *in vitro* methods and can vary due to the time of collections, species and diet of the donor animal and variations between animals within species (Kitessa et al., 1999).

2.1.6.2. In Sacco Methods

The *in sacco* technique is also known as *in situ* incubation or the Terylene (Dacron) or nylon-bag technique. It is suitable for kinetic studies and has been used widely to evaluate the rate and extent of degradation in the rumen. Samples of dried and milled feed (small enough to pass through a 3mm screen) or wet minced samples (usually about 2 to 5g, depending on density), are weighed precisely, and placed in nylon bags (usually 10×17 cm). The tied-up bags are incubated in the rumen of sheep or cattle on an appropriate diet by suspending them from a rumen fistula. They are then withdrawn after various intervals of time, washed, and dried before the digestibility of DM, N and gross energy can be measured against time. Incubation often proceeds for a maximum period of 48 h and the choice of this time is influenced by *in vitro* digestibility methods of Tilley and Terry (1963) and Ørskov, (2000).

2.1.6.2.1. Bag incubation

Fabric type, size and uniformity of pores, and bag size can all affect *in situ* results. Silk bags have been used but have largely been replaced by synthetic fabrics. Nylon has a high concentration of N when compared to Dacron which may be a disadvantage when using *in situ* techniques to measure protein digestion. When using woven fabrics, the type of fabric probably has little effect on *in situ* results if the fabrics have similar pore sizes. Pore size has a significant effect on *in situ* results, it must be large enough to allow free exchange of fluid and microorganisms between the bag and the ruminal milieu, but small enough to prevent the efflux of undegraded feed particles or the entry of feed particles (Michalet-Doureau and Ould-Bah, 1992; López, 2005).

The ratio of sample size to bag surface area has a significant impact on *in situ* data. As sample size increases relative to surface area of the bag, rate, and extent of disappearance decreases. For woven fabric bags with pore sizes between 40 and 50µm, sample mass to bag surface area should be no greater than 10mg DM per cm^2. If the bags are overfilled with sample the mixing and soaking of bag contents with rumen fluid can be incomplete (Nocek and Kohn, 1988; Huntington and Givens, 1995).

2.1.6.2.2. Feed sample preparation

Homogeneity of the feed sample should also be considered when determining the particle size of the sample. To establish the feed particle size for *in sacco* measurements, it is necessary to measure the feed particle size after grinding through different screens and then choose the feed grinding for which the mean particle size is nearest to the size following mastication and present to the rumen. The feed particle size is determined by sieving either dry or wet sample through sets of screens and weighing the material retained on each screen. For concentrates, the decrease in the particle size during mastication is considered negligible, so that the particle size of feed introduced in the bags is the same as the concentrate and given in ground form to the animal. For forage, coarse grinding is necessary to reproduce the mean particle size of the feed bolus or of the rumen content of animals receiving forage. However, grinding samples through screens ranging from about 0.3 to 5 mm had minimal effect on *in situ* digestion (48h) (Michalet-Doureau and Ould-Bah, 1992).

The preparation of feed samples prior to grinding and *in situ* incubation can include drying by using an oven (60°C), freeze drying or no drying. However, these drying techniques had little effect on *in situ* disappearance of oesophageal samples. However, drying at temperatures, above 60°C has been shown to alter the chemical composition of samples and should be avoided (Playne et al., 1978).

2.1.6.3. In vivo methods

It its most basic form, digestibility is determined by measuring the quantity of food consumed and the quantity of faeces voided after an animal has had enough time to become accustomed to a diet (Van Soest, 1982). The determination of intake and digestibility of feedstuffs *in vivo* is time consuming, labour intensive, expensive, requires large quantities of feed and is unsuitable for large-scale feed evaluation. It also relies on the use of animals, therefore, is not appropriate as a means of routinely evaluating forage. However, it is needed as a technique to validate many of the *in vitro* procedures that have been developed, although it provides no information about the site of digestion the kinetics of digestion or the supply of nutrition to the animal (Rymer, 2000).

2.1.7. Conclusion

The grazing systems in New Zealand are affected by feed quality and availability, which consequently affects ruminant performance. Normally supplements are used as a tool to either to improve overall ruminant intake and production or to maintain performance during periods of pasture deficit. Supplements are more expensive than pasture and the use of alternative feeds has grown in importance recently, with dairy farmers in New Zealand feeding increasing quantities of supplements.

New Zealand flax has nutritive potential as a supplement to pasture fed animals. It is grown normally in New Zealand with less cost, lower soil nutrient requirement, more environmental tolerant compared with New Zealand grass species, this can reduce the cost and provide sufficient crop to cover the nutrition requirement for pasture fed animals during drought and winter time.

CHAPTER 3

IN VITRO RUMEN INCUBATION OF NEW ZEALAND FLAX AND SOME SELECTED KEY PASTURE SPECIES IN NEW ZEALAND

3.1. Abstract

In vitro gas production and rumen fermentation characteristics were measured to evaluate New Zealand flax top (FT) and bottom (FB) leaf sections in comparison with New Zealand grass species perennial ryegrass/white clover (ORG-WC), perennial ryegrass (NRG), chicory (NCHI), lucerne (NLUC), and white clover (NWC).

A gas test technique was performed using fistulated cow rumen fluid. Cumulative gas production was recorded after 2, 6, 12, and 24 h of incubation. Ammonia (NH_3) concentration and volatile fatty acids (VFA) concentration were determined after 2, 4, and 8 h of incubation. The CP, NDF, ADF% and ME (MJ $kgDM^{-1}$) were significantly different among forages. The FT and FB contain the highest level of NDF and ADF. The lowest fiber levels were observed in NCHI. The NWC showed the highest value for ME than all other forages. The maximum gas production was for NCHI followed by NWC, NRG, FB, and lowest FT. The fermentation end products either NH_3 concentration or total VFA varied significantly among the forages.

Flax leaf sections had lower *in vitro* fermentation characteristics, and cumulative gas production than New Zealand grass species used in this study. This study concluded that flax leaf sections was considered as low-quality foragers in comparison with New Zealand grass species according to nutritive quality and *in vitro* fermentation and gas production.

3.2. Introduction

New Zealand's pastoral system is largely based on ruminants grazing perennial ryegrass/white clover, as they grow well and generally provide good quality and quantity feed for ruminants. However, quality and/or quantity can become limited in summer and winter leading to impaired animal performance (Roche et al., 2009; Clark et al., 1997; Mackenzie et al., 1999).

Alternative forage/crop species are one option for New Zealand pastoral systems to maintain animal performance during those periods of pasture deficits. Chicory, plantain, lucerne, and red clover have attracted farmers' interest due to their drought tolerance and excellent yield and quality (Moorhead and Piggot, 2009; Minneé, et al., 2013). Supplementation with

conserved forages (hay or silage) and the use of industry by-products (e.g., Palm kernel extract, sunflower meal, and pumpkin meal) are also valid options (Valentine and Kemp, 2007; Vlaicu et al., 2018).

New Zealand flax is a native plant, which was named "flax" by European traders because it resembled true flax found in other parts of the world. New Zealand native flax (*Phormium tenax*), named Harakeke by Maori, can be grown on low land or swamp, coastal areas, or mountains. It can be used for fibre, gel, and oil production (Carr et al., 2005; Sims and Newman, 2006; Morice, 1962). The harvesting and processing of flax generates by-products that may have an important value as a supplement feed for ruminants (Litherland et al., 2005).

Flax can tolerate a variety of environmental conditions, which could make it a profitable investment for farmers interested in expanding the natural fibre industry. However, an expanded flax fibre industry faces huge competition from other different fibre sources and makes it currently unattractive to farmers. Therefore, there is a need to make the flax industry attractive to farmers who are interested in growing flax commercially (McGruddy, 2006).

The feeding value of forages can be measured using direct *in vivo* methods or indirect *in vitro* methods, as well as analysis of the forage composition. Typically, *in vivo* methods are expensive and time-consuming compared with *in vitro* methods (Losada et al., 2010; Barnes, 1965). The *in vitro* rumen incubation method can quantify the products of fermentation, NH_3 and VFA, when a foodstuff is incubated with a buffered rumen fluid solution. The fermentation of carbohydrate produces acetate, propionate, and butyrate, which are VFA, and can be used by the ruminant as an energy source. Ammonia production from protein fermentation is relatively small compared to carbohydrate fermentation (Getachew et al., 1998; Dijkstra, 1994). In addition, rumen fermentation by anaerobic microbes produces gases; carbon dioxide (CO_2), methane (CH_4) and traces of H_2.

The objective of this study was to determine the chemical composition and *in vitro* fermentation parameters of the top or bottom parts of the flax plant and compare it to ryegrass/white clover(mix), chicory, white clover and lucerne which are forages commonly used in pastoral systems in New Zealand. The ultimate aim is to determine the potential value of flax leaf as a feed for grazing ruminants.

3.3. Material and methods

All experiments were conducted between August to October 2020 at Massey University, Palmerston North, New Zealand. The experiments were carried out using the top (FT) and bottom (FB) sections of New Zealand flax (*Phormium tenax*) leaves and selected key New Zealand pasture species, which were used as "standards" to compare the results of flax against.

3.3.1. Sample collection and preparation

3.3.1.1. New Zealand flax (Phormium tenax)

Twelve flax leaves were randomly selected and harvested by hand from a mature flax bush in March 2020. The length of the leaves ranged from 865 to 2140 cm (Figure 3.1).

Flax leaves grow folded along their mid ribs, fused together at the base to form the stem, but opening to form a more or less flat leaf blade towards the tip. Therefore, each leaf was divided into two sections: a top section, which was identified as the open part of the leaf, and a bottom section, which was identified as the folded part of the leaf (Figure 3.2 and 3.3). Samples of all top and bottom sections were chopped into small pieces (1-2 cm) using scissors and kept separately (Figures 3.4 and 3.5). Once chopped about 200 g of wet material was stored in small bags and frozen at -20°C. Some fresh material was also used to estimate dry matter (DM).

Figure 3.1. A complete flax leaf showing the top and bottom sections

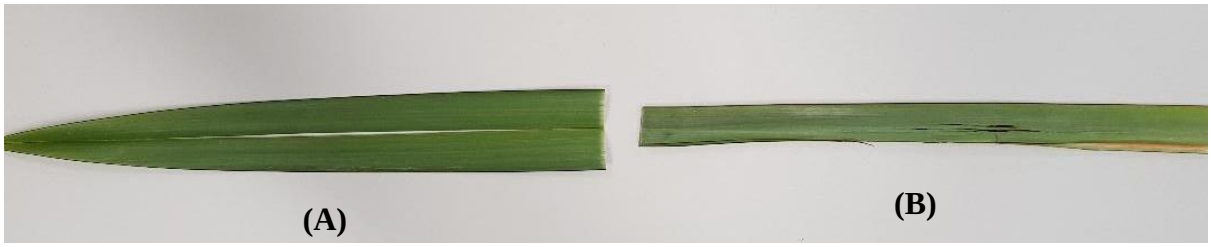


Figure 3.2. Flax leaf top (FT) section (A) and bottom (FB) section (B).

Figure 3.3. Cross-section of the cut ends of the flax top (FT) and bottom (FB) sections. (A) cross-section of the cut end of the flax top section, (B) cross-section of the end point of the flax top section and beginning of the flax bottom section.

Figure 3.4. (A) Flax top section chopped; (B) Flax bottom section chopped

3.3.1.2. Species used as standards

Old samples of Perennial ryegrass/white clover (*Lolium perenne* L./ *Trifolium repens* L.) (ORG-WC), old Chicory (OCHI), old White Clover (OWC), and new samples of pure Perennial ryegrass (*Lolium perenne* L) (NRG), new Lucerne (*Medicago sativa* L.) (NLUC), new Chicory (*Cichorium intybus* L.) (NCHI), and new White clover (*Trifolium repens* L.) (NWC) were used as standard forages.

The ORG-WC, OCHI, and OWC samples had been previously obtained from Massey University's Keeble farm, Palmerston North and the freeze-dried, ground plant material had been stored at room temperature in airtight containers. However, indications from initial pilot *in vitro* experiments indicated that the old samples had deteriorated during storage and were not going to be a very good standards with which to compare FT and FB, therefore new forages were collected and prepared to be used as comparative standard forages.

The NRG, NCHI, NWC and NLUC were available at Massey University farms and are common forage species used in New Zealand grazing systems, therefore were chosen to be used as comparative standards. These forages were collected randomly from various sites at Massey University. All forages were collected in the pre-grazing state and were harvested by

cutting the forages 5-10 cm above ground level with hand shears. Any weeds were picked out of the samples and approximately 200 g of pure sample wet material was placed in small bags and frozen at -20^{o}C until they were able to be freeze-dried and ground. Some of the fresh sample was also used to estimate DM percentage.

All frozen plant samples (NRG, NCHI, NWC and NLUC) were then freeze-dried at -20°C for 72 h (Cuddon, model FD18, Cuddon Ltd, Blenheim, New Zealand) and ground through a 1mm screen (Thomas grinding). Sub-samples of FT and FB were taken for wet chemistry and Near-infrared Spectroscopy (NIRS) analysis was conducted on the standard forages (NG, NLUC, NHIC and NWC). The remainder of the samples were used for the *in vitro* incubation. Wet chemistry had already been conducted on the ORG-WC. OWC and OCHI reported by (Ekanayake et al., 2019) and the entire sample was available for *in vitro* incubation.

3.3.2. Dry matter estimation (DM)

Triplicate sub-samples were randomly selected from the freshly collected and sorted FT, FB, NRG, NLUC, NCHI, and NWC samples. The initial weight (20g wet weight/ replicate) of each sample was recorded and then the samples were dried, using an oven drier (Unitherm Drier), at 75°C to a constant weight. Weights of the samples were recorded over a 48 h period. Dry matter percentage was then calculated.

3.3.3. Chemical and NIRS analysis**.**

A sub-sample of 20g freeze-dried ground (1mm) plant material was used for each of the chemical analysis and NIRS. The FT, FB and ORG-WC were analysed by wet chemistry, and the NRG, NCHIC NWC, and NLUC, were analysed by NIRS. Total nitrogen (N) was determined by combustion using a Leco analysis (Dumas procedure, AOAC, 2000; method 968.06), and subsequently the crude protein (CP) content was calculated by multiplying the N content by 6.25. Acid detergent fibre (ADF) and neutral detergent fibre (NDF) and lignin were analysed using a Tecator Fibertec System (Foss Fibretec, Hoganas, Sweden) by the detergent procedures of Robertson and van Soest (1981). Hot water- soluble carbohydrates (HWSC) were measured by the reducing sugar method (Nelson, 1944). Organic matter digestibility (OMD) and estimated ME were measured by following the Roughan and Holland (1977) method.

A sub-sample of 20g freeze-dried ground (1mm) plant material from NRG, NCHIC NWC, and NLUC were analysed by NIRS (Bruker MPA, Ettlingen, Germany) to determine the DM, ash, N, lipid, ADF, NDF, SSS, OMD and ME content of the sample.

3.3.4. *In vitro* fermentation

The *in vitro* fermentation was undertaken in a WiseCube shaking incubator (Model WIS-10, Songbook-gu, Seoul, South Korea) with a temperature control and rack that can hold up to 68 bottles. Forage samples were incubated for up to 24 h to measure gas production, NH_3 and VFA concentrations.

3.3.4.1. Rumen inoculum collection

Strict anaerobic techniques were used in all steps during the rumen fluid collection, transfer, and incubation periods. Rumen fluid inoculum (approximately 1000 ml) was collected before the morning feed from non-lactating rumen fistulated cows (average body weight 700-750 kg) from AgResearch, Grasslands, Palmerston North. The feeding protocol of the cow was as follows: the animal grazed pasture during the day (maintenance feeding level) and was fed hay in the afternoon (maintenance feeding level). Rumen fluid was collected via the rumen fistula and filtered by hand squeezing through double layers of cheesecloth into a pre-warmed 1000 ml thermos flask, then transported to the laboratory which was 5 minutes away. The thermos was purged with carbon dioxide (CO_2) for approximately 5 minutes to minimise the presence of oxygen prior to using it. The pH of the rumen fluid was measured by pH meter (Mettler Toledo) immediately after collection and sub-samples taken for NH_3 and VFA concentration measurement.

3.3.4.2. Medium solution preparation

One day prior to the incubation, an artificial saliva, McDougals buffer, was prepared (McDougals, 1948). The buffer, consisted of one litre of H_2O (distilled); sodium bicarbonate ($NaHCO_3$) 9.8 g L^{-1}; sodium phosphate dibasic (Na_2HPO_4) 3.6 g L^{-1}; sodium chloride (NaCl) 0.47 g L^{-1}; potassium chloride (KCL) 0.57 g L^{-1}; magnesium chloride ($MgCl_2$) anhydrous 0.06 g L^{-1}; and calcium chloride ($CaCl_2$) anhydrous 0.04 g L^{-1}. It was stirred overnight using a magnetic stirrer fitted with a hot plate.

On the day of incubation, a cysteine sulphide reducing agent was prepared consisting of 48 ml H_2O (distilled); 135 mg L-cysteine hydrochloride (HCl); 315 mg sodium sulphide (NaS^{2-}); and 2 ml of 1 mol L^{-1} Sodium hydroxide (NaOH).

The total volumes of buffer and reducing agent required were dependent on the number and size of the incubation bottles required on the day of incubation and therefore amounts were adjusted accordingly.

3.3.4.3. In vitro Ammonia and volatile fatty acid

The *in vitro* study to measure NH_3 and VFA concentrations were conducted in two experiments (Experiment 1 and Experiment 2, Table 3.1) according to the methods of Burke et al. (2000). In Experiment 1, FT, FB, and the standard ORG-WC were incubated and in Experiment 2, FT, FB, and the standards ORG-WC and NRG were incubated.

In both Experiments 1 and 2, approximately 0.6g DM of each sample was weighed into 50 ml Schott bottles the day prior to the experiment. The forages FT, FB, ORG-WC and NRG were incubated in triplicate bottles at each sampling time point of 0, 2, 4, 8, 12, and 24 h.

On the day of the incubation, the bottles containing the samples were warmed to 39°C in the incubator for 90 minutes, gassed with CO_2, before 12 ml of artificial saliva, 0.5 ml of reducing agent and 3 ml of rumen fluid was added. Bottles with vented lids were screwed onto the Schott bottles before they were returned to the incubator where the temperature was maintained at 39°C and the rack set at 90 oscillations per minute.

The pH for each *in vitro* bottle at the respective collection times was recorded to monitor the fermentation. Additionally, sub-samples were taken from each *in vitro* bottle at the respective collection times 0, 2, 4, and 8 h for NH_3 and VFA determination. Ammonia sub-samples (1 ml) were acidified with 15µL of hydrochloric acid, and centrifuged (13,500 rpm) for 15 minutes, after which the supernatant was frozen at -20°C for NH_3 analyses. A sub-sample of 1.5 ml from each *in vitro* bottle, at each sampling point, and rumen fluid were frozen at -20°C for VFA analysis at each sampling point. Volatile fatty acid concentrations were determined using gas liquid chromatography. Sample was deproteinised using metaphosphoric acid. The supernatant was injected directly into gas chromatography (GC 17-A, capillary column Alltech ATTM-1000, 15m x 0.53mm ID, 1.00 um film) with hydrogen as the carrier gas, FID detector, and iso-caproic acid as an internal standard (Attwood et al., 1998). Ammonia concentrations were determined by the method of Chaney and Marbach (1962).

3.3.4.4. In vitro method development for gas production

An *in vitro* incubation method for gas production needed to be established. Therefore, a series of incubations were conducted to finalise the protocol (Table 1). These studies were conducted in August/September 2020 at Massey University, Palmerston North, New Zealand. The experiments were conducted by initially incubating FT, FB, and ORG-WC, and then NRG, NCHI, NWC, and NLUC. Table 3.1 illustrates the various experiments carried out, the timeline used, and the samples taken at each *in vitro* incubation conducted for gas production measurements and for NH_3 and VFA measurements.

Table 3.1. Summary of the experimental incubation procedures conducted throughout this thesis.

Experiments	Date	Cow ID	Incubation bottle volume (ml)	Incubation reagents	Feeds incubated[1]
Experiment 1: NH_3 and VFA production (using old standards)	12/08/2020	2	50	12 ml buffer, 3 ml rumen, and 0.5 ml reducing agent.	Triplicate bottles of FT, FB, and ORG-WC at 0, 2, 4, and 8 h only.
Experiment 2: NH_3 and VFA production (using old and new standards)	14/11/2020	6	50	12 ml buffer, 3 ml rumen, and 0.5 ml reducing agent.	Triplicate bottles of FT, FB, and NRG at 0, 2, 4, and 8 h only Duplicate bottles of ORG-WC at 2 and 4 h only
Experiment 3: Gas production method development (using old standards)	26/8/2020	2	160	12 ml buffer, 3 ml rumen, and 0.5 ml reducing agent.	Triplicate bottles of FT, FB, and ORG-WC
Experiment 4: Gas production method development (using old standards)	9/9/2020	1 and 2 mixed together	160	Treatment 1: (48 ml buffer, 12 ml rumen, and 2 ml reducing agent). Treatment 2: (12 ml buffer, 3 ml rumen, and 0.5 ml reducing agent).	Triplicate of FT, FB, and ORG-WC Duplicate bottles of OWC and OCHI (because of insufficient material).

Experiment 5: *In vitro* gas production (using new standards)	14/10/2020	2	160	48 ml buffer, 12 ml rumen, and 2 ml reducing agent	Triplicate bottles of FT, FB, and NRG, NCHI, NWC, and NLUC.
Experiment 6: *In vitro* gas production (using new standards)	21/10/2020	6 and 3 kept separate	160	48 ml buffer, 12 ml rumen, and 2 ml reducing agent.	Triplicate bottles of FT, FB, and NRG, NCHI, NWC, and NLUC.

[1] FT = flax top; FB = flax bottom; ORG-WC = old ryegrass/white clover; OWC = old white clover; OCHI = old chicory; NRG = new ryegrass; NCHI =new chicory; NWC = new white clover; NLUC = new lucerne.

Experiment 3

The forages FT, FB and ORG-WC were used in this experiment. An approximate 0.5g sample of each forage was weighed into 3 x 160 ml serum bottles (all serum bottles are rinsed and dried before used), before the bottles were warmed to 39°C in the incubator for 90 minutes. They were then gassed with CO_2 before 12 ml the artificial saliva, 0.5 ml of the reducing agent and 3 ml of the rumen fluid obtained from one fistulated cow was added. Bottles were capped with rubber seals (20 mm red rubber straight WAL) and crimped with an aluminium cap (20 mm ungrooved aluminium) with an aperture to allow needle insertion for gas removal and measurement. All bottles were returned to the incubator where the temperature was maintained at 39°C and the rack set at 90 oscillations per minute.

Experiment 4

In this experiment, rumen fluid was collected from two cows and mixed together before two incubation treatments using different volumes of buffer/rumen fluid were used according to the method description below.

Treatment 1:

The FT, FB, ORG-WC, OCHI and OWC were used in this experiment. Approximately 0.6g of each forage was weighed into 160 ml serum bottles for incubation. Triplicate bottles for FT, FB and ORG-WC, and duplicate bottles of OWC and OCHI (due to insufficient material for third bottle) were prepared. In one set of bottles, 48 ml McDougall's buffer, 12 ml rumen fluid, and 2 ml reducing agent was dispensed into each bottle. Triplicate bottles that contained no forage were also prepared and incubated to determine whether any forage material in the rumen fluid was contributing to gas production. Bottles were capped and sealed as described previously.

Treatment 2:

In this method the same forages were used as method A, however 12 ml McDougall's buffer, 3 ml rumen fluid and 0.5 ml reducing agent was dispensed into each bottle. Bottles were then capped and sealed as previously described.

On the day of incubation, all bottles were warmed to 39°C in the incubator, and the McDougall's buffer gassed with CO_2 for 90 minutes, followed by the addition of the liquids. All bottles were incubated at 39°C and the rack set at 90 oscillations per minute.

3.3.4.5. In vitro gas measurement experiments

Using the results of Experiments 3 and 4 and a review of literature of the methods used for measuring gas production, the final method was devised and used. Two experiments were conducted in October 2020 at Massey University, Palmerston North, New Zealand in a completely randomised design using FT, FB, NRG, NLUC, NCHI and NWC to measure gas production.

Experiment 5

Three replicates of each forage were prepared. Approximately 0.6g DM was weighed into 160 mL gas bottles for incubation the following day. Each bottle contained 48 ml McDougall's buffer, 12 ml of the rumen fluid collected from one non-lactating rumen fistulated cow, gassed with CO_2 and 2 ml reducing agents dispensed into them. Triplicate bottles that contained no forage were also prepared and incubated to determine whether any forage material in the rumen fluid was contributing to gas production. Bottles were capped with rubber stoppers sealed and crimped with aluminium cap as described previously. All bottles were randomly placed in the incubator.

Experiment 6

In this experiment, the bottles were prepared as described for experiment 5, but rumen fluid was collected from two different cows and kept separate.

3.3.4.6. Gas Measurement

For all gas measurement experiments, the gas accumulation in the headspace of each serum bottle was measured manually using a pressure transducer (Digital pressure meter, Greisinger electronic, Germany). A 20G 1 TW (0.9mm× 25mm) gauge needle attached to the pressure transducer was inserted through the butyl rubber stopper into the bottle and gas pressure in the headspace was recorded. After the reading was taken, the gas was released by inserting the needle (not attached to the pressure transducer) into the butyl rubber stopper and allowing the gas to release. The pressure in the bottle was checked to make sure it had all been released by re-measuring the gas pressure in the headspace.

It took between 15 and 20 minutes to get all bottles filled with buffer and rumen fluid. Immediately after the last bottle was sealed and crimped, the measurement of gas pressure in the bottles began. Gas measurements were taken every 20 minutes after the start of the

incubation for the first 1 h and then extended to every 30 minutes for second hour of the incubation and then at 1 h intervals up to 12 h incubation before one final measurement after 24 h incubation.

3.3.4.7. Calculations

All measured gas volumes were recorded in mbar and converted to gas volume (ml) and subsequently gas pressure (GP; calculated as ml gDM^{-1}) using the following two methods:

1. Lopez et al. (2007) method:
    - The gas production was calculated by the difference between the gas pressure in the bottle (mbar) minus the pressure in the bottles after gas released.
    - The difference was then converted into psi using the following equation, knowing that 1 mbar equals to 0.0145 psi

    (a) Gas pressure (psi) = GP (mbar) × 0.0145

- The gas pressure in psi is then converted into volume (ml) as follow:

    (b) Gas pressure (ml) = (160-62 ml) / (atmosphere GP (mbar)×0.0145) × GP psi

    Where; 160 ml = the volume capacity of the bottle and 62 ml = the volume of liquid added to the bottle which is 48 ml buffer, 12 ml rumen fluid and 2 ml reducing agent

    - Accumulated gas was calculated over the 24 h.
    - Total accumulated gas at each time point was converted into ml gDM^{-1} as follows:

    (c) Gas pressure (ml gDM^{-1}) = (GP ml)/ (g DM feed)

2. Developed calibration equations method in the current study:
    - A 160 ml serum gas bottle was used, and 62 ml water is dispensed. Considering the space occupied by the stopper, the calculated volume of the headspace was 98 ml. Bottles were capped with rubber seals (20 mm red rubber straight WAL) and crimped with an aluminium cap (20 mm ungrooved aluminium) with an aperture to allow needle insertion for gas measurement.

- A specific volume of air (starting from 10 ml initially and increasing to 100 ml) was injected in the bottle using a syringe.

- Air pressure (mbar) was measured for each air volume using a pressure transducer (Digital pressure meter).

- Gas was released after each gas pressure measure.

A calibration equation was calculated and simple linear regression analysis relating air volume (ml) and air pressure (mbar) values was carried out. The dependent variable "y" was air pressure (mbar), and the independent variable "x" was the air volume (ml), (Figure 3.5).

y = 9.3164x+ 5.6364

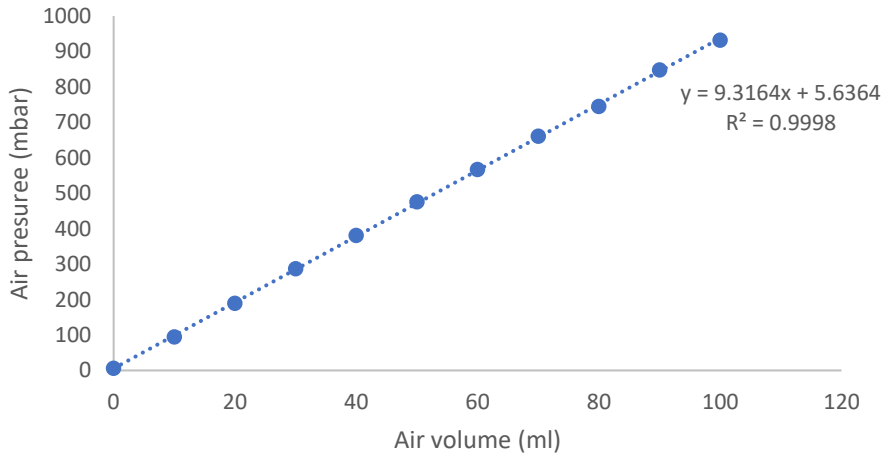


Figure 3.5. Regression of air pressure and volume measured in the current study.

Early calculation indicated that values were similar to those presented by Lopez et al. (2007).

Ammonia concentrations from *in vitro* incubations were corrected for concentrations in 0 h bottles and are expressed in terms of forage nitrogen (N) incubated. Whereas VFA concentrations (mg) were corrected for VFA concentrations of the rumen inoculum and are expressed in terms of forage DM incubated and proportions and ratios of non-glucogenic: glucogenic VFA are reported.

3.3.5 Statistical analysis

The data were analysed by one-way ANOVA (experiment 5) and by factorial design (experiments 1, 2, and 6) to determine the main effects (time, forage, and cow) and their interactions using the General Linear Models procedure of the SAS (version 9.4; 2015. SAS Institute Inc., Cary, NC). Significant differences between means were separated by Least Significant Difference test. Significance of effects was declared at $P \leq 0.05$.

3.4. Results

3.4.1. Chemical composition of forages

The chemical composition of the FT and FB sections, and ORG-WC, NRG, NCHI, NLUC, and NWC are presented in Table 3.2. The NDF and ADF concentration of the FT were 67.6% and 56.5 %, respectively, while in the FB they were 53.2 % and 45.0%, respectively. These values are higher than the other forages, which ranged from 24.6% (NCHI) to 47.6% (ORG-WC) for NDF and 11.6% (NCHI) to 22.1% (ORG-WC) for ADF. The CP of the flax was low at 6.0% for FT and 2.4% for FB. The CP concentration in the other forages ranged between 21.0% (NRG) and 31.6% (NLUC)

The OMD for FT and FB was the lowest of all the forages at 53.9% and 62.0%, respectively (Table 3.2). The NLUC had the highest OMD at 69%, while the other forages ranged between 82.9 (NRG) to 84.0% (NCHI). The estimation of ME for the FT was 8.0 MJ $kgDM^{-1}$ and for the FB was 9.2 MJ $kgDM^{-1}$ which was lower than ME estimated for the other forages, which ranged between 10.2 (NLUC) to 12.5 (NWC) MJ k DM^{-1}, respectively.

Table 3.2. Chemical composition, Dry matter (%), metabolisable energy (MJ $kgDM^{-1}$), and digestibility (%) of New Zealand flax (top and bottom sections), old ryegrass / white clover, new ryegrass, new chicory, new lucerne, and new white clover.

Forage	DM	Carbohydrate			NDF	ADF	Lignin	CP	Lipid	Ash	ME	Digestibility[3]		
		HWSC	Starch	SSS								DMD	DOM	OMD
FT[1]	43.3	9.4	0.2	9.6	67.6	56.5	10.0	6.0	3.2	3.5	8.0	52.0	49.8	53.9
FB[1]	30.5	22.4	0.1	22.5	53.2	45.0	4.8	2.4	1.8	3.8	9.2	59.7	57.7	62.0
Standard feed														
ORG/WC[1]	-	-	-	6.8	47.6	22.1	-	26.1	3.2	-	12.3			84.5
NRG[2]	17.5	-	-	13.9	44.5	20.1	-	21.0	4.1	9.6	12.0	-	-	82.9
NCHI[2]	11.6	-	-	16.8	24.6	11.6	-	21.6	5.6	11.8	12.3	-	-	84.0
NLUC[2]	21.1	-	-	-	32.1	19.5	-	31.6	3.5	10.1	10.2	-	-	69.0
NWC[2]	15.0	-	-	14.2	24.9	16.6	-	25.8	3.7	9.0	12.5	-	-	83.1

FT, flax top section; FB, flax bottom section; ORG/WC, old grass ryegrass/white clover; NRG, new ryegrass; NCHI, chicory; NLUC, lucerne; NWC, white clover; DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; HWSC, hot water-soluble carbohydrates; DMD, *in vitro* dry matter digestibility; DOM *in vitro* digestible organic matter in dry matter; OMD, *in vitro* organic matter digestibility; SSS, soluble sugars and starch; ME, metabolisable energy.

[1]Chemical analysis. [2] Near infrared spectroscopy (NIRS) analysis. [3]In vitro digestibility

3.4.2. *In vitro* rumen fermentation characteristics

3.4.2.1 Rumen fluid

The initial rumen fluid characteristics (pH, NH_3 and VFA) measured in all studies are presented in Table 3.3.

Table 3.3. Rumen fluid pH, ammonia (NH_3), and volatile fatty acid (VFA) from cows used for the *in vitro* incubation experiments.

Experiment	Cow ID	Rumen pH	Rumen NH_3 mmol L^{-1}	Rumen VFA mmol L^{-1}
In vitro NH_3 and VFA				
1	2	7.22	3.2	109
2	6	7.07	4.5	72
In vitro gas production				
5	2	6.86	4.8	106
6	6	6.92	5.1	103
	3	6.89	4.8	88

3.4.2.2. In vitro ammonia (NH_3) and volatile fatty acid (VFA) concentrations in experiment 1 and 2

Ammonia concentration (NH_3)

Experiment 1 data is not presented as there was 75% of the values were non-detectable.

The NH_3 concentration measured after 2, 4, and 8 h of incubation for FT, FB, NRG for experiment 2 is shown in Figure 3.6. There was a significant interaction ($P < 0.05$) between forages and incubation times (2, 4, and 8 h).

After 2 h of incubation, NH_3 concentration for FT was significantly higher than both NRG and FB (2.56 vs 1.12 and -0.49 mmol NH_3 mol N^{-1}). However, after 8 h of incubation the highest NH_3

concentration was observed for NRG, followed by FT and the lowest was for FB. At all incubation times (2, 4, and 8 h), the NH_3 concentration yield for FT was higher than FB.

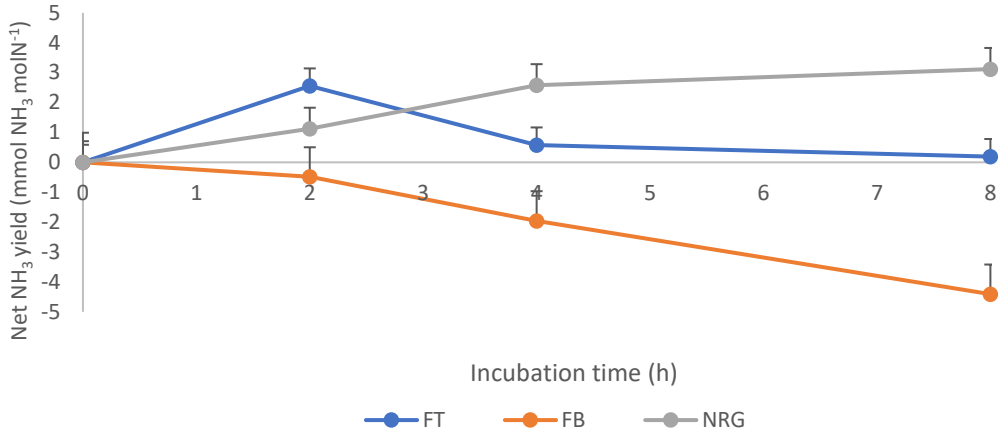


Figure 3.6. The net NH_3 yield (mmol NH_3 mol N^{-1}) for New Zealand flax top (FT) sections, flax bottom (FB) sections, and new ryegrass (NRG) incubated with rumen fluid for 0, 2, 4, and 8 h.

Volatile fatty acids (VFA)

Total VFA production (mg gDM^{-1}) and the concentration of different VFA was measured after 2, 4, and 8 h of *in vitro* incubation for FT, FB, and NRG and are presented in Tables 3.4. and 3.5. There was a significant interaction between forages and different incubation times (2, 4, and 8 h). The total VFA production at the end of the fermentation period was significantly higher ($P < 0.001$) for all forages (Table 3.4).

There was a significant interaction between incubation time x forages species in the production of individual VFAs (Figure 3.7).

During the entire incubation period acetate was the main VFA produced by all forages, followed by smaller quantities of propionate and butyrate (Figure 3.7). The yields of propionate and butyrate increased after 4 and 8 h, of the incubation, but acetate remained the main VFA produced by all forages (Table 3.5).

Acetate production represented the greatest proportion ($P < 0.01$) of the VFA products by NRG after 4 and 8 h of incubation with 28.30 and 75.12 mg gDM^{-1} produced respectively, when compared to 20.11 and 28.23 mg gDM^{-1} respectively for FT collected after the same incubation periods. The lowest acetate production was recorded for FB with 18.58 and 22.92 mg gDM^{-1} produced after 4 and 8 h of incubation. There was no significant difference in acetate production after 2 h of incubation between all forages.

Propionate concentrations were highest ($P < 0.01$) for NRG after 8 h of incubation (27.88 mg g DM^{-1}) compared to FT and FB (5.31and 4.93 mg gDM^{-1}) respectively. No differences were observed between 2 and 4 h of incubation for all forages.

Butyrate concentrations were highest ($P < 0.01$) for NRG (14.72 mg gDM^{-1}) after 8 h of incubation compared to FT and FB (5.54 and 6.96 mg gDM^{-1}) respectively. No differences were observed between 2 and 4 h of incubation for all forages.

The NRG had the highest VFA yield after 8 h incubation compared with FT and FB. There were substantial differences between forages in the rate of VFA production over 8 h, but not after 2 and 4 h incubation.

The acetate: propionate (A:P) ratio of the FT and FB was greater ($P < 0.01$) after 8 h incubation (5.34 and 4.65) respectively compared to the lowest NRG (2.69). No significant differences after 2 h were observed between FT and NRG compered to FB.

Table 3.4. Volatile fatty acid (VFA) production (mg gDM^{-1}) from New Zealand flax top (FT) sections, flax bottom (FB) sections, and new ryegrass (NRG) after *in vitro* incubation for 2, 4, and 8 h.

Forages	Incubation time (h)	Acetate	Propionate	Butyrate	Minor	tVFA	A:P	(A+B) :P
FT	2	15.57^{cde}	3.47^{de}	2.03^{e}	0.69^{c}	21.76^{ef}	4.42^{bc}	5.01^{b}
	4	20.11^{c}	4.71^{bcd}	3.65^{d}	1.14^{b}	29.57^{cde}	4.22^{bc}	5.00^{b}
	8	28.23^{b}	5.31^{b}	5.54^{c}	1.17^{b}	40.35^{b}	5.34^{a}	6.39^{a}
FB	2	11.87^{de}	3.57^{ced}	2.15^{e}	1.00^{bc}	18.60^{f}	3.32^{de}	3.93^{c}
	4	18.59^{cd}	4.77^{cbd}	4.17^{d}	0.99^{bc}	28.52^{de}	3.91^{cd}	4.78^{b}
	8	22.92^{bc}	4.93^{cb}	6.96^{b}	1.29^{b}	36.11^{cde}	4.65^{ab}	6.06^{a}
NRG	2	11.09^{e}	2.67^{e}	2.03^{e}	0.69^{c}	16.48^{f}	4.13^{bc}	4.89^{b}
	4	28.30^{b}	5.95^{b}	3.57^{d}	1.17^{b}	38.99^{bc}	4.76^{ab}	5.36^{b}
	8	75.12^{a}	27.88^{a}	14.72^{a}	2.30^{a}	120.03^{a}	2.69^{e}	3.22^{d}
SEM[1]		2.313	0.429	0.318	0.112	2.967	0.218	0.219
P-value								
Forages		<0.001	<0.001	<0.001	0.005	<0.001	0.003	0.001
Time		<0.001	<0.001	<0.001	<0.001	<0.001	0.188	0.021
Forages × Time		<0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001

[a-f] values with different superscripts within a column are significantly different at $P < 0.05$. tVFA, total Volatile Fatty Acid; A:P; Acetate to Propionate ratio; (A+B) :P, (Acetate + Butyrate): Propionate ratio.

[1] Standard error of mean.

Table 3.5. Total volatile fatty acid (tVFA; mg gDM^{-1}) production, and percentage of individual VFA produced after 2, 4, and 8 h of *in vitro* incubation for New Zealand flax top (FT) sections, flax bottom (FB) sections, and new ryegrass (NRG).

Forage	Incubation time (h)	tVFA (mg gDM^{-1})	Acetate (%)	Propionate (%)	Butyrate (%)	Minor (%)
FT	2	21.76	70.9	16.1	9.7	3.3
	4	29.57	67.7	16.1	12.5	3.7
	8	40.35	70.2	13.2	13.7	2.9
FB	2	18.60	63.8	19.2	11.6	5.5
	4	28.52	65.2	16.7	14.6	3.5
	8	36.11	63.5	13.7	19.3	3.6
NRG	2	16.48	67.2	16.3	12.3	4.2
	4	38.99	72.6	15.3	9.1	3.1
	8	120.03	62.6	23.2	12.3	1.9

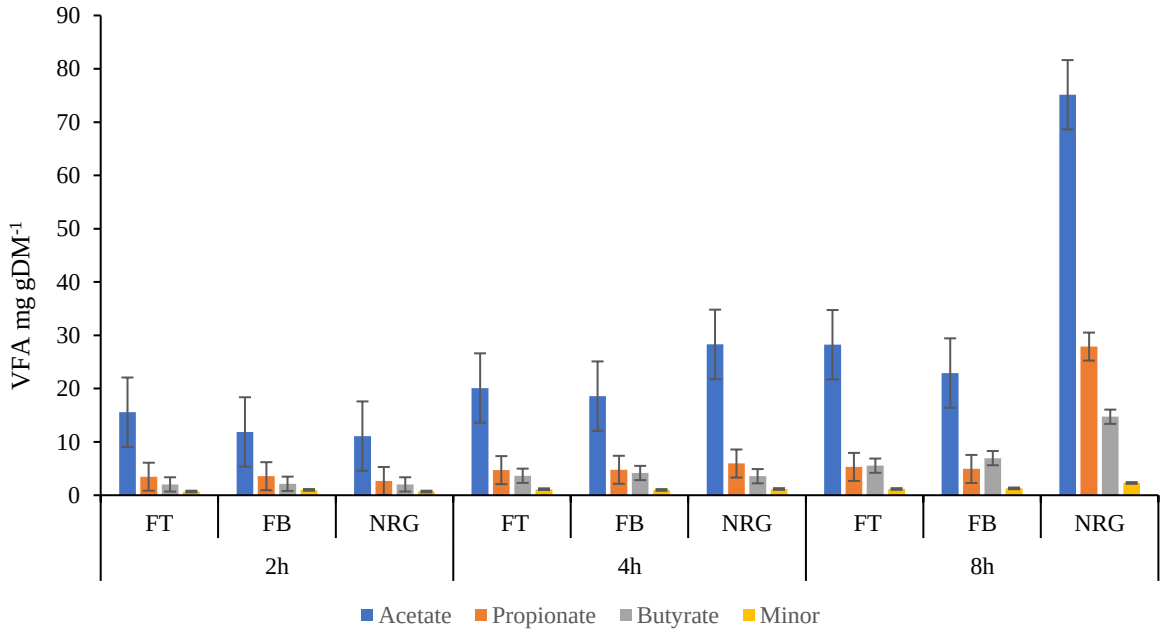


Figure 3.7. Individual VFA production (mg gDM^{-1}) after 2, 4, and 8 h of *in vitro* incubation for New Zealand flax top (FT) sections, flax bottom (FB) sections, and new ryegrass (NRG).

3.4.2.3. In vitro rumen gas production responses after 24 h incubation in Experiments 5 and 6.

The gas production (ml gDM^{-1}) from all forages are presented in Tables 3.6 and 3.8 for experiments 5 and 6 respectively. There was an interaction ($P < 0.05$) between forage total gas production and incubation time (2, 6, 12, and 24 h) in both experiments.

Gas production for all forages increased with time in both experiments (Figure 3.8 and 3.9). There was an increase ($P < 0.05$) in gas production up to 12 h, with no further significant increases between 12 and 24 h of the incubation for all forages. Overall, the gas production was lower in experiment 5 than in experiment 6 at all incubation times (2, 6, 12, and 24 h).

After 2, 4, 6 and 12 h incubation with rumen fluid in experiment 5 the gas production for FT, FB, NCHI, NLUC, NWC, and NRG was (100, 48, 45, 91, 63, and 36%), (76, 63, 29, 31, 55, and 45%), (60, 50, 50, 58, 53, and 50%), and (60, 48, 48, 54, 52, and 52%) respectively, which was lower than experiment 6. Due to this difference the results of experiments 5 and 6 are presented separately. However, despite the difference in gas production the ranking of the forages is similar.

Gas production for FT was the lowest over the entire 24 h incubation in both experiments 5 and 6 (38.50 and 100.61 ml gDM^{-1} respectively) compared with FB, NCHI, NLUC, NWC, and NRG (71.16,102.84, 76.66, 85.48, and 84.12 ml gDM^{-1} respectively in experiment 5), and (142.90, 195.73, 166.83, 181.09, and 180.76 ml gDM^{-1} respectively in experiment 6). The NCHI had the highest gas production at 2, 6, 12 and 24 h incubation. However, there was a trend at all incubation times where the gas production from FB was closer to the levels produced by the other forages than FT.

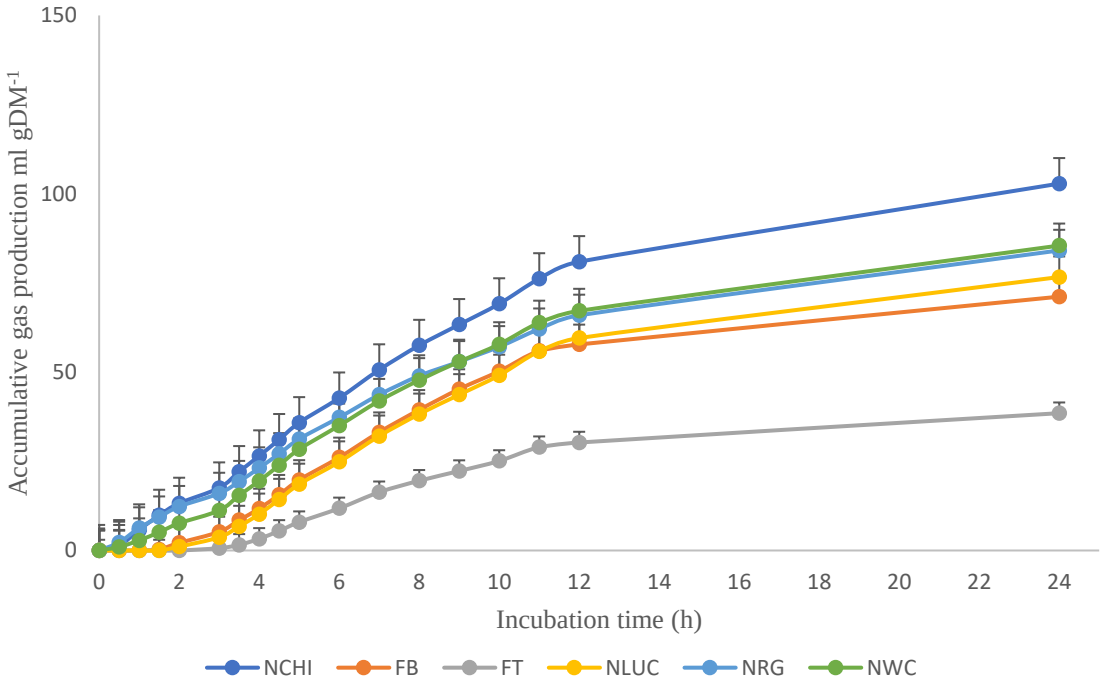


Figure 3.8. Accumulative gas production (ml gDM^{-1}) for New Zealand flax top (FT) sections, flax bottom (FB) sections, new ryegrass (NRG), new white clover (NWC), new chicory (NCHI) and new lucerne (NLUC) incubated for 24 h with rumen fluid (experiment 5).

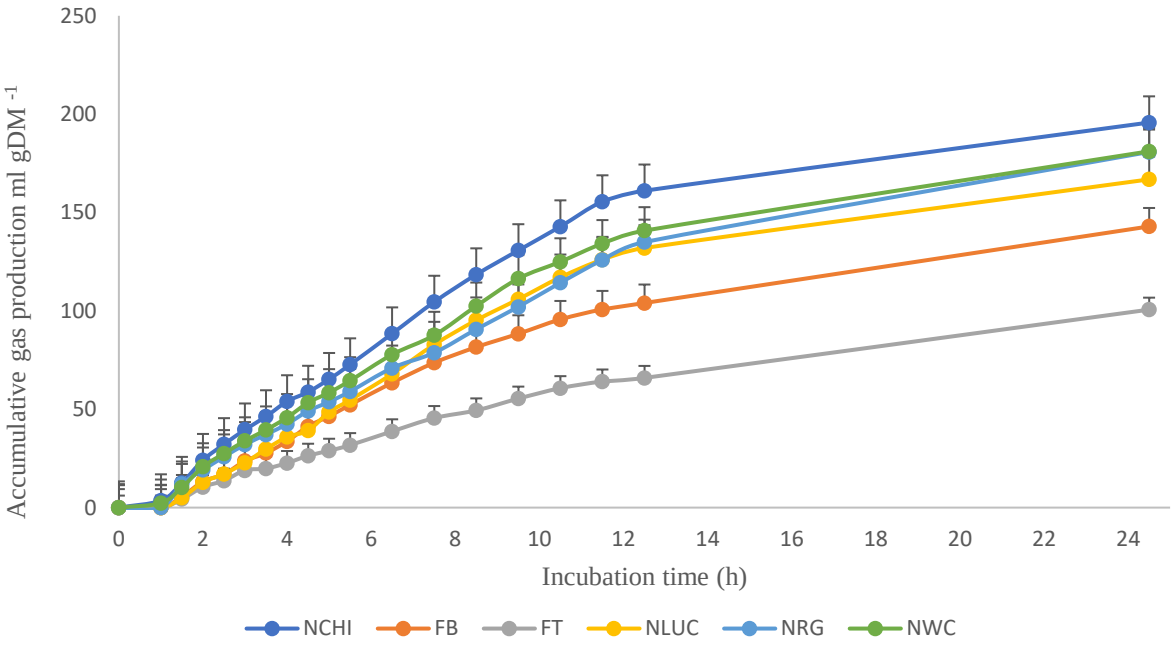


Figure 3.9. Accumulative gas production (ml gDM^{-1}) for New Zealand flax top (FT) sections, flax bottom (FB) sections, new ryegrass (NRG), new white clover (NWC), new chicory (NCHI) and new lucerne (NLUC) incubated for 24 h with rumen fluid (experiment 6).

Table 3.6. *In vitro* total gas production from New Zealand flax top (FT) sections, flax bottom (FB) sections, new chicory (NCHI), new lucerne (NLUC), new white clover (NWC) and new ryegrass (NRG) incubated for 24 h with rumen fluid (experiment 5).

Forage	Gas production ml gDM^{-1}			
	2 h	**6 h**	**12 h**	**24 h**
FT	0.00^{a}	11.81^{a}	30.27^{a}	38.50^{a}
FB	2.12^{a}	26.08^{b}	57.77^{b}	71.16^{b}
NCHI	13.23^{c}	42.74^{d}	80.96^{d}	102.84^{e}
NLUC	1.09^{a}	24.83^{b}	59.56^{b}	76.66^{c}
NWC	7.62^{b}	35.01^{c}	67.18^{c}	85.48^{d}
NRG	12.30^{c}	37.29^{c}	65.93^{c}	84.12^{d}
SEM[1]	1.831	1.831	1.831	1.831
P- value				
Species	<.0001			
Time	<.0001			
Species× time	<.0001			

Within a column values with different superscripts are significantly different at $P < 0.05$.

[1] Standard error of mean.

Table 3.7. The average gas production h^{-1} for New Zealand flax top (FT) sections, flax bottom (FB) sections, new chicory (NCHI), new lucerne (NLUC), new white clover (NWC) and new ryegrass (NRG) from 0-2 h, 2-6 h, 6-12 h and 12-24 h incubation in experiment 5.

Forage	Gas production ml gDM^{-1}			
	0-2 h	**2-6 h**	**6-12 h**	**12-24 h**
FT	0.00	2.95	7.01	5.70
FB	1.06	7.05	14.00	10.74
NCHI	6.61	19.90	20.62	15.32
NLUC	0.54	14.00	14.06	11.35
NWC	3.81	10.66	17.03	12.72
NRG	6.15	12.40	17.20	12.50

Table 3.8. *In vitro* total gas production after 24 h incubation with rumen fluid for New Zealand flax top (FT) sections, flax bottom (FB) sections, new chicory (NCHI), new lucerne (NLUC), new white clover (NWC) and new ryegrass (NRG) for experiment 6.

Forage	**Gas production ml gDM^{-1}**			
	2 h	**6 h**	**12 h**	**24 h**
FT	10.43^{a}	38.78^{a}	65.92^{a}	100.61^{a}
FB	13.24^{ab}	63.48^{b}	104.00^{b}	142.90^{b}
NCHI	24.11^{c}	88.44^{d}	161.06^{d}	195.73^{e}
NLUC	12.84^{ab}	67.96^{b}	131.95^{c}	166.83^{c}
NWC	20.79^{bc}	77.82^{c}	140.79^{c}	181.09^{d}
NRG	19.15^{bc}	70.97^{b}	134.98^{c}	180.76^{d}
SEM[1]	3.016	3.016	3.016	3.016
P- value				
Species	<0.001			
Time	<0.001			
Species × Time	<0.001			

Within a column values with different superscripts are significantly different at $P < 0.05$.

[1] Standard error of mean.

Table 3.9. The average gas production h^{-1} for New Zealand flax top (FT) sections, flax bottom (FB) sections, new chicory (NCHI), new lucerne (NLUC), new white clover (NWC) and new ryegrass (NRG) from 0-2 h, 2-6 h, 6-12 h and 12-24 h incubation in experiment 6.

Forage	Gas production ml gDM^{-1}			
	0-2 h	**2-6 h**	**6-12 h**	**12-24 h**
FT	5.22	12.30	17.45	13.88
FB	6.62	19.18	27.91	20.55
NCHI	12.05	28.14	41.60	29.73
NLUC	6.42	20.20	33.32	25.00
NWC	10.40	24.65	36.44	26.82
NRG	9.60	22.53	34.33	26.31

3.5. Discussion

This study aimed to produce the first comprehensive assessment of the nutritive value of the New Zealand flax leaf by comparing it with common New Zealand forage species and investigating the suitability of utilising it in dairy cow production systems in New Zealand.

3.5.1. Chemical composition of forages used for incubation

The chemical composition of the New Zealand forages used in this study were similar to previously reported ranges (Kaiser and Combs 1989; Waugh et al., 1998; Burke et al., 2000; Bessa et al., 2005; Fulkerson et al., 2007; Lindsay et al., 2007; Hayes et al., 2010; Gregorini et al., 2013; Schmidt et al., 2013; Robertson et al., 2015; and Pain et al., 2015).

The ORG-WC showed the highest amount of NDF (47.6 %) and ADF (22.1%). The NDF content was in line with previous studies that recorded a range of 23.2-48.7%, while the ADF level was slightly lower than the range recorded in the literature (23.2-51.5%). The CP of the ORG-WC in the current study was also within the range reported in the literature (12.9-26.3%). The ME (MJ $kgDM^{-1}$) of the ORG-WC was higher than that reported in other studies which ranged from 9.7-

11.9 (Waugh et al., 1998; Burke et al., 2000; Fulkerson et al., 2007; Gregorini et al., 2013; Pain et al., 2015).

For NCHI, the NDF and ADF contents in the current study were similar to values reported by other studies. The NDF was 24.6% in the current study which was within the reported range of 18.9-43.2%. However, the ADF content (11.6%) was lower than the reported range in previous studies (13.3-29.1%). Furthermore, the CP of the NCHI in the current study (21.6%) was within the range that other previous studies reported (13.1-24.0%). The ME (MJ $kgDM^{-1}$) content of the NCHI was also found to be within the range published in previous studies 9.0-12.8 MJ $kgDM^{-1}$ (Waugh et al., 1998; Burke et al., 2000; Hayes et al., 2010; Gregorini et al., 2013; Schmidt et al., 2013; Pain et al., 2015).

The NDF and ADF contents in NLUC for the current study (32.1% and 19.5%, respectively) were close to the range reported by previous studies for NDF (29.3-52.6%), and ADF (20.01- 40.3%). The CP content of the NLUC in the current study was higher (31.6%) than previous studies (15.6-30.1%). The ME (MJ $kgDM^{-1}$) content of the NLUC in the current study (10.2) was found to be within the range of previous studies (7.5-12.7) (Kaiser and Combs 1989; Burke et al., 2000; Bessa et al., 2005; Fulkerson et al., 2007; Hayes et al., 2010; Schmidt et al., 2013; Robertson et al., 2015).

In the present study, NWC contained 24.7% NDF. This value was within the NDF value range of 25.6-33.9% reported in the literature. However, NWC had 16.6% ADF, which was lower than the reported values (19.3-23.3%) in previous studies. For the CP in NWC, the current study showed similar values compared to previously published values (25.8% vs. 24.3-29.8%). The ME content of the NWC was 12.5 MJ $kgDM^{-1}$, which was higher than 9.3-11.8 MJ $kgDM^{-1}$, reported in previous publications (Burke et al., 2000; Fulkerson et al., 2007; Lindsay et al., 2007; Hayes et al., 2010).

In the current study, FT and FB recorded the highest amount of NDF and ADF, but lowest CP compared with other forages. The values reported here are similar to values of Litherland et al. (2005) who reported that flax leaf had the highest NDF (73% DM) and ADF (49% DM), and lowest CP (5% DM) compared with white clover.

Variations in the chemical composition are likely due to factors affecting the forage quality such as growing conditions (i.e., temperature and rainfall). Forage composition is significantly affected by growing conditions (Korte et al., 1987; Moller et al., 1996; Stevenson et al., 2003). Temperature and rainfall produce seasonally cyclical variation in growth rate, forage mass and forage quality. This often results in a leafy sward in spring, followed by stem and seed head formation in late spring, and a reduction in quality during summer when the growth rate is often restricted by low soil moisture availability (Lambert et al., 1983; Moller et al., 1996).

3.5.2. Rumen fluid

In the current study the rumen fluid pH ranged between 6.9 and 7.2 which is in line with a previous study, which recommended that a pH of between 6.4-7.0 was optimal for microbial activity (Roger et al., 1990). Furthermore, a previous study by Mouriño, et al. (2001) revealed that a pH between 6.0-6.3 impaired fibre breakdown by rumen microorganisms.

3.5.3. Ammonia concentration (NH_3)

Production of ammonia *in vitro* indicates the extent of wasteful protein degradation that occurs during digestion (Burke et al., 2000). The current study showed that overall NRG produced higher concentrations of NH_3 than FT and FB. The greater NH_3 production for the NRG could be due to the CP being more quickly and extensively degraded in the rumen compared to FT and FB (Van Vuuren et al., 1990), but also due to the fact there is more CP in NRG than the FT and FB. The current study showed that NRG contained a high concentration of CP and low ADF and NDF which resulted in greater DM degradation and fermentation and more proteolysis than FT and FB.

It is likely that the decline in NH_3 concentration for FT and FB after the first 2 h of the incubation could be related to the low CP content in both FT (6.0%) and FB (2.4%) compared to NRG (21.0%). Another contributing factor could also be a higher ratio of readily fermentable carbohydrates to structural carbohydrate providing an increased energy supply for microbial growth (Barry,1998). The ammonia disappearance with increased energy availability could be due to its incorporation into microbial protein (Raab et al.,1983). The net NH_3 production indicates the magnitude at which the protein degradation exceeds the capacity for microbial utilisation and the likelihood that plant N is insufficient for microbial growth (Chaves et al., 2006).

The NH_3 concentration for NRG after 8 h of incubation (3.4 mmol L^{-1}) was lower than the range of previous studies (6.0-8.9 mmol L^{-1}) (Chaves et al., 2006; Fleming et al.,2020). The current study showed that FT and FB had the lowest NH_3 concentration after 8 h of incubation compared with other forages, which agrees with Litherlandet al. (2005) who reported that the flax leaf had the lowest NH_3 concentration after 8 h of incubation compared with WC.

The variation in NH_3 concentration between current and previous studies is most likely due to the differences in the sample harvesting/collecting time and, pre-test diet fed to donor animals for rumen fluid collection (Chaves et al., 2006; Fleming et al.,2020).

3.5.4. Volatile fatty acids (VFA)

The tVFA production for NRG after 8 h incubation (120 mg gDM^{-1}) was less than 165 mg gDM^{-1} reported by Burke (2004) and greater than 83.46 mg gDM^{-1} reported by Chaves et al. (2006). Total VFA for FT and FB after 8 h incubation (40 and 36 mg gDM^{-1}, respectively) was less than 101 mg gDM^{-1} that was reported by Litherland et al. (2005).

Volatile fatty acids are produced in the rumen as the end products of the microbial fermentation and they represent the major energy source absorbed by ruminates, accounting for between 50 and 80% of total metabolizable energy supplied to ruminants. A reduction in their production is considered nutritionally unfavourable for animal production (Burke et al., 2000; Merchen, 2002; Busquet et al., 2006).

The lower amount of tVFA produced for FT and FB after 8 h incubation in the current study is probably due to the lack of rapidly degradable energy to accelerate the fermentation process in the incubations compared with NRG. Total VFA is considered a limiting energy factor in initial microbial growth, which is essential in the maintenance and development of the microbial biomass (Nocek and Russel, 1988).

The greater tVFA production from NRG during the first 8 h of the incubation may suggest that the microorganisms were more adapted to the environment because the rumen fluid obtained from cows fed a diet containing NRG. Weimer et al. (1999) reported that the diet has an influence on the rumen environment (microbial population and chemical environment), and consequently on the tVFA production. According to Huntington and Givens (1995) the rumen fluid used during the

in vitro incubation should be obtained from host animals fed diets similar to the test substrate. Zhu et al. (2018) reported that when dairy cows were transitioned from low grain to high grain diet the change in specific bacterial abundance resulted in changes in VFA profiles.

The variation in tVFA production may be due to the low CP concentrations in the FT and FB (6.0 and 2.4%, respectively), which could limit bacterial growth when compared to the higher CP levels in NRG (Chaves et al.,2006). This may subsequently contribute to reduced tVFA production.

Acetate was the main VFA produced during incubation in this study at all timepoints (2, 4, and 8 h), for NRG, FT and FB, which is in agreement with Dijkstra et al. (2005), who reported that acetate was the most abundant VFA produced in the rumen when forage-based diets are fed. After 8 hours of incubation FT and FB had only 13.2% propionate whereas NRG had 23%, and the A+B/P ratios were higher in FT and FB at 6.4 and 6.0 respectively compared to NRG which was only 3.2. Feeds with higher fibre content result in higher acetate and butyrate and relatively less propionate (Burke et al., 2006).

3.5.5. *In vitro* gas production

The *in vitro* gas production of the New Zealand forage species used in this study was in agreement with previous studies. The gas production in the present study for NCHI was 103-196 ml gDM^{-1} which was within the range reported by previous studies of 161-395 ml gDM^{-1} (Sun et al., 2011; Muetzel et al., 2014; Durmic et al., 2016). The gas production for NRG was 84-181 ml gDM^{-1} which was the highest gas production in the current study. This result was consistent with previous studies that reported gas production of 155-221 ml gDM^{-1}. Nevertheless, the lowest amounts of gas production recorded in the current study were below the range for previous studies (Navarro-villa et al., 2011; Purcell et al., 2011; Sun et al., 2011; Purcell et al., 2012; Muetzel et al., 2014).

The current study showed that the gas production from NLUC (77-167 ml gDM^{-1}) was higher to previous work (137 ml gDM^{-1}) (Muetzel et al., 2014). The gas production from NWC in the current study was 86-181 ml gDM^{-1} which was also equivalent to previous work (169-174 ml gDM^{-1}) (Purcell et al., 2012; Muetzel et al., 2014).

Gas production in FT was between 38.50-100.61 ml gDM^{-1} and the lowest compared to all the forages, whereas FB was comparable with the other forages at 71.16-142.90 ml gDM^{-1}. There has

been no gas production results for flax reported in the literature, therefore this information adds to the understanding of digestion and fermentation of flax when combined with the other attributes reported in this study.

Gas production for all forages initially increased rapidly, with time. Figures 3.12 and 3.13 showed no lag time, and variation between forages. The highest gas production was observed in NCHI. This is due to the relatively high soluble sugars and starch (SSS) content of the NCHI and the low cell wall content (NDF and ADF). Soluble sugars and starch are positively correlated with gas production, while NDF and ADF are negatively correlated with gas production, and FT and FB had higher ADF than any of the other forages. This may tend to reduce the microbial activity and therefore result in less gas production in FT and FB compared with other forages in this study. However, FB had more soluble carbohydrate than NCHI and FT, but due to the high cell wall content (NDF and ADF) compared to NCHI, this may cause a reduction in the microbial activity and subsequently limit *in vitro* degradation, resulting in lower gas production. These findings agree with previous research (Stefanon et al., 1996; Doane et al., 1997; Kamalak et al., 2002 and Sallam, 2005), who reported a significant relationship between short-chain fatty acids (SCFA) and gas production. When feed is incubated with buffered rumen fluid *in vitro*, the carbohydrate is fermented to SCFA, CO_2 and CH_4 and microbial cells. Gas production is the result of the fermentation of carbohydrates to the VFAs, acetate, propionate, and butyrate (Beuvink and Spoelstra, 1992; Blümmel et al., 1999). In this study forages, like NRG, that produced the greatest amount of gas also had higher tVFAs compared with FT that produced the least amount of gas and had the lowest tVFAs.

3.6. Conclusion

This study concluded that New Zealand forage species are digested and fermented better than flax leaf (top and bottom) sections. The bottom portion of the flax leaf was fermented better than the top portion in term of gas production and total VFA, but both parts of the flax plant do not have enough protein to support microbial growth.

Overall, supplementary feeding of New Zealand flax has limited use in the dairy industry for lactating dairy cows, due to its high NDF and ADF content. However, it may have potential for the dry grazing animal, but not as a sole feed.

Further research is required to investigate the presence of potential anti-nutritional factors in flax leaves, such as condensed tannins. Research into ways to improve the nutritional value of flax, for example by treating with its ammonia or bio fermentation for use as silage are also worthy of further investigation.

3.7. References

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