Novel genetic engineering technology which increases leaf lipid content modifies the ensiling properties of perennial ryegrass

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Abstract

A novel strategy to increase the metabolisable energy (ME) yield of pastures has been the development of a genetic engineering technology which increases the leaf lipid content and biomass production of perennial ryegrass (PRG). Outdoor plot/feeding trials of genetically engineered crops are prohibited under the current New Zealand (NZ) regulatory framework. However, this high lipid PRG may become available to farmers and eventually be used to make silage, which could fulfill an important role as a high ME, inexpensive, supplementary feed for livestock. Ensiling preserves a crop's nutrients at a high moisture content and at a low pH, by microbial fermentation of plant sugars into lactic acid under anaerobic conditions.

In a preliminary investigation into the ensiling biochemistry of this high lipid PRG, glasshouse-grown materials were wilted and inoculated, and then ensiled on a miniature scale. A series of method development ensiling experiments revealed that non-transgenic PRG grown in glasshouse conditions during the NZ spring/summer was very difficult to ensile naturally, due to its low water soluble sugar to buffering capacity ratio. In order to generate well-preserved silage in the main experiment, glucose was added (post-harvest) to a non-transgenic PRG genotype (WT) and two transgenic PRG genotypes containing 'medium' and 'high' leaf lipid levels (ML and HL).

The HL plants produced 51% more dry biomass than WT during the regrowth period. Pre-ensiled HL had 31% higher fatty acid content, 70% higher nitrate content and a 17% lower water soluble sugar to crude protein ratio than WT. ML was intermediate. The glasshouse growth environment resulted in an atypical overall PRG nutritional composition. WT, ML and HL underwent a similar fermentation, and nutrients were well-preserved. The nutritional differences in the ensiled material largely reflected those in their fresh counterparts, although a longer wilt caused greater overall digestible nutrient losses in HL. In an *in vitro* rumen incubation experiment the fatty acids in HL silage exhibited less complete biohydrogenation than in fresh and ensiled WT. Experiments using a range of high lipid PRG lines grown in a range of environments will be needed to validate these results.

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List of abbreviations

AA; acetic acid
ACCase; acetyl-coA carboxylase
ACP; acyl carrier protein
ADF; acid detergent fibre
a_w; water activity
BA; butyric acid
BC; buffering capacity
BH; biohydrogenation
cfu/g; colony forming units per gram
CLA; conjugated linoleic acid
CO_2; carbon dioxide
CP; crude protein
DAC; days after cutting
DAG; diacylglycerol
DAS; days after sowing
DGAT; diacylglycerol acyl transferase
DGAT1; diacylglycerol O-acyltransferase 1
DM; dry matter
DMD; dry matter digestibility
DOMD; dry organic matter digestibility
DW; dry weight
ER; endoplasmic reticulum
FA; fatty acid
FAME; fatty acid methyl ester
FFA; free fatty acids
GE; gross energy
HL; high lipid
iWUE; intrinsic water use efficiency
LA; lactic acid
LAB; lactic acid bacteria
LD; lipid droplet
ME; metabolisable energy
MJ; Mega joules
ML; medium lipid
N; nitrogen
NDF; neutral detergent fibre
NH_3; ammonia
NO_3^-; nitrate
NPN; non-protein nitrogen
NSC; non-structural carbohydrate
NZ; New Zealand
OMD; organic matter digestibility
PAR; photosynthetically active radiation
PC2; physical containment level 2
PRG; perennial ryegrass
PUFA; polyunsaturated fatty acid
Rubisco; ribulose-1, 5-bisphosphate carboxylase
scVFA; short chain volatile fatty acid
TAG; triacylglycerol
VA; vaccenic acid
VFA; volatile fatty acid
VOC; volatile organic compound
WAC; weeks after cutting
WSC; water soluble carbohydrates
WT; wild type
16:0; palmitic acid
16:1; palmitoleic acid
18:0; stearic acid
18:1; oleic acid
18:2; linoleic acid
18:3; linolenic acid

CHAPTER 1. INTRODUCTION

The initiative to generate high metabolisable energy crops by engineering higher lipid contents into leaves came from the Plant Biotechnology group at AgResearch Ltd. Molecular work has progressed over the last 15 years, using model species and agronomically-important species as substrates. Winichayakul et al. (2013) describe a novel technology in which leaf lipids are elevated to high and stable levels in *Arabidopsis thaliana*. This technology holds significant potential for application in various agricultural and industrial fields (Roberts et al., 2015). When expressed in perennial ryegrass (*Lolium perenne*, PRG) it has generated plants with increased biomass accumulation rates and with a range of leaf lipid concentrations, up to approximately double 'normal' PRG leaf lipids (6-7% dry matter). Significant progress has been made in characterizing the multitude of altered features in these high lipid PRG plants. However, due to the current regulatory environment in New Zealand, this has been limited to glasshouse trials, *in vitro* experiments and modelling exercises. Therefore, the suitability of this high lipid PRG for adoption into the pastoral system remains largely unknown.

Ensiling is a widespread practice in pastoral agriculture. In New Zealand, surplus spring pasture is harvested and stored as silage and then fed out during feed shortages. In contrast, silage makes up the base diet of lactating dairy cows in many foreign farming systems. In a future scenario where a version of this high lipid PRG becomes available to farmers, this new PRG could eventually be used to make silage. However, the ensiling process itself could be modified by changes in the plant due to expression of the high lipid technology. The aim of this work was to investigate whether this high lipid PRG could be successfully ensiled, and whether the accumulated lipids could 'survive' the silage making process and subsequently improve the fatty acid profile of ruminant-derived products. Important features regarding the interpretation of this work were that the experiments were limited to using small quantities of glasshouse-grown PRG, small scale model silos and *in vitro* rumen incubation methods.

CHAPTER 2. LITERATURE REVIEW

2.1 Principles of ensiling

Ensiling is a means of forage conservation that has increasingly gained popularity over haymaking on account of its superior nutrient preservation, and because it is less dependent on the weather and the need to harvest at a late stage of regrowth (Woolford & Pahlow, 1998). The purpose of making silage (ensiling) is to preserve as much of a crop's nutrients as possible at a high moisture content. Preservation is primarily achieved by the anaerobic fermentation of the crop's soluble sugars by lactic acid bacteria (LAB). This type of fermentation lowers the crop pH until it is inhibitory for the activity of plant enzymes and undesirable microorganisms, thus preventing further nutrient losses. This section describes the objectives and basic biochemistry of the various phases of the ensiling process.

2.1.1 Objectives of ensiling

The primary requirement of silage making is to generate and maintain anaerobic conditions for the crop. These conditions inhibit aerobic microbial activity, and allow the proliferation of a succession of anaerobic microbes, most importantly the LAB. The LAB compete for plant nutrients, converting them to a range of end-products, predominantly lactic acid (LA). As the concentration of LA rises and the pH declines, a secondary essential requirement of silage making is achieved-a critical pH is reached at which the activity of undesirable anaerobic microbes is suppressed (McDonald et al., 1991). Crops ensiled at a higher dry matter (DM) content rely less on the fermentation process and more on reduced water activity for a preservative effect (Weissbach, 1996).

2.1.2 The phases of successful ensiling

Conceptually, the ensiling process can be divided into phases (Rooke & Hatfield, 2003). These differ in their duration depending on management practices and the biochemistry of the crop.

Aerobic phase

This phase proceeds from the time when the crop is cut, until the silo is sealed and the residual oxygen is used up by plant and microbial respiration. For the duration of this phase, continued metabolic activity negatively alters the nutritional composition of the crop. Minimizing the duration of the aerobic phase is desirable, however field-wilting a crop to a higher DM content before ensiling is a common practice in many regions (Muck et al., 2003). Therefore, depending on wilting practices and the prevailing climate, the aerobic phase may last several days. Following cutting and wilting, the crop is typically chopped to 2-

4 cm lengths and then transported and thoroughly compacted before the silo is sealed or wrapped. Assuming the silage is well-sealed, the residual oxygen remaining in the silo is rapidity depleted by plant respiration (McDonald et al., 1991).

Fermentation phase

This phase begins when anaerobic conditions commence and is characterized by a decline in the crop's pH, until a stable pH is reached. Anaerobic microbes including LAB, enterobacteria and clostridia rapidly proliferate and compete for nutrients which are increasingly exuded from collapsing plant cells (Winters et al., 1987). Plant water soluble carbohydrates (WSCs) are converted into a range of neutral and acidic end products. The predominant fermentative pathway during successful ensiling will be the conversion of glucose to LA, which is the strongest of the silage organic acids (with the lowest pKa) (Rooke & Hatfield, 2003). This pathway leads to the most efficient acidification and is also highly efficient in terms of energy/DM conservation. As the fermentation phase progresses, the acid-tolerant LAB will dominate and consolidate the pH decline (Pahlow et al., 2003). If WSCs are non-limiting, growth suppression of enterobacteria, clostridia and yeasts will be achieved.

Stable phase

This phase begins when the pH approaches the level required to suppress LAB growth. The critical pH at which this occurs depends on silage DM content, and is in the range of 3.8-4.5 for 'low DM' silages. Fermentative activity slows, microbial populations decline and some species enter a state of dormancy. Very little microbial or plant enzymatic activity occurs, and so the nutrient composition of the crop remains relatively constant. Very slow acid hydrolysis of cell walls may provide acid-tolerant yeasts with WSC substrate to convert into ethanol (Kung Jr & Der Bedrosian, 2010). Some degradation of protein fractions may continue. This phase can last indefinitely.

Feed-out phase

When the silo is opened, the face exposed to air inevitably undergoes aerobic deterioration before feeding, resulting in nutrient losses and heat production. The porosity of the silage will determine the extent of air infiltration. A succession of aerobic microorganisms will develop and multiply as fermentation end-products and residual sugars are metabolized and the pH of the silage rises (Lindgren et al., 1985).

2.1.3 Poorly preserved silage

Where crop factors, post-harvest management or ensiling conditions are not optimal, the microbial and biochemical processes during ensiling may differ from those described above. 'Success', in the context of ensiling requires that the pH and/or concentration of fermentation acids suppresses the growth of undesirable microbial groups, particularly the clostridial bacterial species and the enterobacteria. In a WSC-limited fermentation, growth suppression of these microbial groups will not occur and the pH fall that has occurred will be reversed, rather than becoming stabilized, due (usually) to secondary fermentation of LA to butyric acid (BA) by clostridia. The production of BA involves a condensation reaction between two LA molecules and the net effect is the conversion of a glucose molecule into a single BA molecule. BA is a far weaker acid than LA, therefore this pathway increases the silage pH (Rooke & Hatfield, 2003) and causes substantial energy/DM losses, which have subsequent negative effects on animal intake and performance. Chemical indicators of clostridial secondary fermentation include a high and unstable pH, a high BA concentration, a high acetic acid (AA) concentration, a low LA concentration and a high proportion of total nitrogen (N) as ammonia (NH_3) (McDonald et al., 1991).

2.2 Silage microbiology and LAB inoculants

The silage fermentation process involves microbial conversion of plant sugars (also known as WSCs) into neutral and acidic end products at a low moisture content. The microbial population size and taxonomic composition on the standing crop (Table 2.1) undergoes large changes during the transition into a fermented product stored at a low pH under anaerobic conditions.

Table 2.1 *Typical (pre-ensiled) population counts of epiphytic bacterial and fungal groups (Pahlow et al., 2003).*

Group	Population count (cfu/g crop)
Total aerobic bacteria	>10,000,000
Lactic acid bacteria	10-1,000,000
Enterobacteria	1000,-1,000,000
Yeasts and yeast-like fungi	1000-1,00,000
Molds	1000-10,000
Clostridia (endospores)	100-1,000
Bacilli (endospores)	100-1,000
Acetic acid bacteria	100-1,000
Propionic acid bacteria	10-1,000

cfu = colony forming unit

Facultative heterofermentative LAB inoculants are the dominant silage additives in practice. LAB inoculants increase the probability that the activity of other silage microorganisms will be minimized in the early stages of the fermentation, thereby increasing the rate of pH decline and a shifting the fermentation towards LA and away from AA, and improving WSC utilization and nutrient preservation (Muck, 2010). In some cases these effects have translated into improved performance of animals fed these silages. This section describes the important ecological features of the major microbial groups in the context of ensiling, followed by a brief review of the effects of LAB inoculant addition on the silage fermentation.

2.2.1 Lactic acid bacteria

The term lactic acid bacteria (LAB) covers a number of facultative anaerobic bacterial genera. They all principally ferment simple WSCs to one or both isomers of LA. Other fermentation products include AA, ethanol and CO_2. In the context of ensiling, the literature groups LAB into the three groups (listed below) based on their fermentative activity rather than their taxonomic/evolutionary origins;

1) Obligate homofermentative; ferment only hexose sugars to two LA molecules.
2) Facultative heterofermentative; ferment hexoses to two LA molecules and pentoses to other end products.

3) Obligate heterofermentative; ferment hexoses and pentoses to a range of end products.

The former two groups are most relevant to the ensiling process (Pahlow et al., 2003). Epiphytic LAB are found at a wide range of population densities on the surface of leaves (Table 2.1) and tend to be present in greater numbers at the base of leaves and on senescent leaves. Interestingly, detectable numbers (using traditional methods) can increase by a factor of 100 or more as a result of chopping the forage crop (Muck, 1989). This is the result of resuscitation of previously unculturable LAB by plant juices released by chopping, rather than population growth (Pahlow et al., 2003). LAB have a pH optima of 5.0-6.0 and temperature optima of 25-40°C. They are more tolerant of a low pH and low water activity (a_w) than other silage microbes. They consolidate the pH decline in silage, reaching peak numbers of approximately 10^{10} colony forming units per gram (cfu/g) crop. It is common for LAB to rapidly become the dominant microbial population under anaerobic, moist conditions containing sugars and complex N sources (Teuber, 1993).

2.2.2 Enterobacteria

Enterobacteria are a diverse group of facultative anaerobes. They often outnumber LAB on the standing crop (Table 2.1), and are overall the second most numerous bacterial group during ensiling after LAB (Pahlow et al., 2003). In the early fermentative phase a succession of enterobacteria proliferate (Heron et al., 1993), and total population counts can reach 10^8-10^{10} cfu/g crop (McDonald et al., 1991). During this period, they are the LAB's principal competitor for fermentable substrate (Pahlow et al., 2003). Enterobacteria ferment plant WSCs and sometimes proteins into a range of end products, primarily AA (Pahlow et al., 2003). Enterobacteria also play an important role in silage in that they are primarily responsible for the reduction of nitrate to nitrogen oxides including nitrite and nitric oxide; compounds which are toxic to clostridia (Spoelstra, 1985) and humans (McDonald et al., 1991). Because enterobacteria population counts peak before LAB counts (and before the stable phase), clostridial inhibition by the production of nitrogen oxides occurs predominately before inhibition by acidification (Spoelstra, 1985). Regardless, the persistence of enterobacteria in silage is generally undesirable and a decline in enterobacteria counts is a good indicator of silage quality (Pahlow et al., 2003).

2.2.3 Clostridia

Clostridial bacteria are obligate anaerobes. The influence of clostridia on silage quality is negative in all aspects. Clostridia can multiply early in the fermentative phase of ensiling, although their negative effect on silage quality primarily occurs after LAB have stopped actively growing (Muck, 2010). Like the LAB,

clostridia are grouped according to the compounds that they ferment in the context of ensiling (Pahlow et al., 2003). However unlike LAB, they are also classified by their ability to multiply at different pH ranges as indicated below;

1) Proteolytic clostridia; ferment amino acids primarily to NH_3, amines and CO_2. Also ferment carbohydrates. pH range >5.0
2) *Clostridium butyricum*; ferment carbohydrates primarily to BA and AA. pH range >4.5
3) *Clostridium tyrobutyricum*; ferment carbohydrates and LA primarily to BA and AA. pH range >4.2

C. tyrobutyricum, on account of its acid tolerance and ability to degrade LA, frequently triggers a loss of stability in silage. If the pH required to suppress *C. tyrobutyricum* growth is reached, then silage is described as 'anaerobically stable' (Weissbach, 1996). Secondary fermentation of LA to BA will then increase the pH, allowing *C. butyricum*, then proteolytic clostridia, and/or a succession of other undesirable microorganisms to become active. Prediction of clostridial development is based on a number of interactive factors, mainly those that modify the relative growth rates of LAB and clostridia (Leibensperger & Pitt, 1987). A low crop nitrate concentration and a high temperature *in silo* will favour clostridial growth (McDonald et al., 1991). Factors that favour rapid LAB growth include a high crop water soluble carbohydrate to buffering capacity ratio (WSC:BC, Chapter 2.6), a high DM (up until approximately 40% DM), high numbers of epiphytic LAB and low levels of soil contamination of the crop (Buxton & O'Kiely, 2003). If conditions favour clostridia, they may eventually come to dominate during ensiling, regardless of initial clostridial spore counts (Leibensperger & Pitt, 1987).

2.2.4 Yeasts and other aerobic spoilage microbes

Yeasts and moulds are of low significance during the fermentation phase, but play a major role in deterioration of silage once exposed to air. The yeast composition of silages varies widely and is influenced to a large extent by the degree of air ingress into the silage stack (Pahlow et al., 2003). Some yeast species grow anaerobically and counts may be as high as 10^7 cfu/g crop during the early stable phase of ensiling. Acid tolerant strains may continue to ferment excess sugars or LA (primarily to ethanol and CO_2) after LAB have stopped growing (Pahlow et al., 2003). Yeasts often initiate aerobic deterioration on account of their acid-tolerance and ability to assimilate LA of certain strains. The bacilli and clostridia will then exit dormancy and play a role in further advancing the aerobic spoilage of silage. The acetic acid bacteria may also initiate or further advance aerobic spoilage. Moulds are absent from the inner mass of stable silage, and instead grow on aerated surface areas of silage. They may produce mycotoxins.

2.2.5 Commercial LAB inoculants

Various commercial products exist containing (at least) one selected facultative heterofermentative LAB strain, and sometimes other strains of microorganisms which improve the fermentation and/or the aerobic stability of silage during the feed out phase (Limin et al., 2003). The facultative heterofermentative bacterium *Lactobacillus plantarum* is the most commonly used species for improving fermentation. However even with excellent silage making practices, LAB inoculants may have no positive effect on the fermentation if 1) the existing LAB population count on the standing crop vastly exceeds the quantity in the applied inoculant e.g. (Cherney et al., 2006) or 2) if WSC levels in the pre-ensiled crop are severely limited e.g. (Seale et al., 1986). In some trials, LAB-inoculated silages have been shown to improve the performance of animals relative to un-inoculated controls, although the mechanism by which this occurs is not always clear (Kung Jr & Muck, 1997). LAB-inoculated silages may improve nutrient utilization by the animal, which may be related to the ability of rumen microorganisms to ferment LA, but not other volatile fatty acids (VFAs) (Muck, 2010).

2.3 Fermentation analyses

The term 'silage quality' is often used in a way that does not directly relate to the nutritional value of the ensiled feed to an animal ('nutritional quality'). Instead it refers to a combination of chemical factors related to ensiling; the pH of the crop, and the concentrations of fermentation end-products ('silage quality parameters') which together indicate how efficiently the nutrients in the pre-ensiled crop have been preserved during the fermentation and storage phases of ensiling (Table 2.2) (Naoki & Yuji, 2008) and the palatability of the silage to livestock (Cherney & Cherney, 2003). Fermentation analyses are ideally performed once the silage has reached the stable phase, at which point the crop's nutritional characteristics will be similar to what the animal later ingests. They can usually be related to crop's WSC:BC ratio and its DM content at ensiling. Fermentation analyses give an indication of the types of microbial species that dominated the fermentation, and may complement microbiological enumeration data and silage gas/effluent data. Further, certain fermentation products influence the aerobic stability of silage during the feed out phase and influence silage palatability and animal intake. Despite extensive research into understanding the relationships between silage quality parameters and animal performance, there is no one group of parameters that can satisfactorily explain the feeding value of all silages (Cherney & Cherney, 2003).

Table 2.2 *Typical range for fermentation end products in 30-35% dry matter grass silage.*

Chemical factor	Typical range
pH	4.3-4.7
Lactic acid	6-10%
Acetic acid	1-3%
Propionic acid	<0.1%
Butyric acid	0.5-1.0%
Ethanol	0.5-1.0%
NH_3: N	0.08-0.12

Typical range of common fermentation end products of grasses successfully ensiled at 30-35% dry matter. These values, reproduced from an industry report (Kung & Shaver, 2001) are in general agreement with values from 'well-preserved' silage in the literature (Cherney & Cherney, 2003), however lactic acid and total acid concentration may be significantly lower for low buffering capacity grasses.

2.3.1 Terminology regarding different silage fermentations

The literature on ensiling uses a range of subjective terms to describe the type of fermentations that a crop has undergone. This terminology ultimately makes reference to the 'goal' of maximizing animal performance. The literature generally refers to one of two of the most common endpoints in the fermentation profile of silages. A rapid LA fermentation leading to a low and stable pH is described as 'normal', 'acceptable', 'typical' or 'successful' because it results in efficient crop nutrient preservation and

high animal intake and therefore best delivers the goal of maximizing animal performance. Conversely, a clostridial (BA) fermentation (or any fermentation where LA is not the predominate acid) is described as 'unsuccessful', 'low quality', 'unusual', or 'atypical'.

2.3.2 pH

The most important measure of high moisture content silage quality is pH. When forages are ensiled at a higher DM (>40%, sometimes termed 'haylage') the fermentation becomes restricted and the extent of pH decline is reduced and the pH (and fermentation acid concentrations) are unreliable silage quality parameters (Weissbach, 1996). In 'low DM silages' (<40%), maximum nutrient preservation requires a rapid pH decline to a low and stable level. Low DM silages will have a stable pH of approximately 4.0 (range 3.8-4.5), after 4 to 8 days of anaerobic fermentation (Table 2.2). Although a low pH has been the traditional measure of ensiling success, it provides little information about the extent/type of fermentation that has occurred i.e. two silages with the same pH could differ in fermentation acid profiles. Measurements of pH can be made directly on silage effluent, or on water extracts of macerated fresh plant material, usually with a fixed 1: 10 ratio of plant material to water (Cherney & Cherney, 2003). In an experimental setting, pH is often measured at intervals throughout the fermentation process in order to track the fermentation dynamics, while in practice it is typically measured once, during the stable phase. A prolonged pH decline is undesirable, as it increases the window of opportunity for enterobacteria and clostridia to multiply (McDonald et al., 1991). The pH will decline slowly in very low DM silages, and in crops with a high BC, especially if epiphytic LAB numbers are low. If silage pH initially decreases and then increases, this indicates that the quantity of WSC substrate for LA production was insufficient to overcome the intrinsic buffering capacity (BC, Chapter 2.6) of the crop, and therefore reach the critical pH for generating well-preserved silage. The resulting pH incline is always indicative of undesirable secondary fermentation of LA (Leibensperger & Pitt, 1987).

2.3.4 Common fermentation end products

Lactic acid (LA) should be the predominant acid in silage. An industry report states that silage should contain 65-70% of fermentation acids as LA (Kung & Shaver, 2001) (Table 1.1). LA is less volatile than other silage acids and is analyzed separately. Other common silage acids are acetic acid (AA) and butyric acid (BA). A high level of AA production most commonly indicates the activity of enterobacteria. AA is also produced by some clostridial and LAB species. AA production has some positive effects on silage quality, for example by enhancing aerobic stability (Pahlow et al., 2003). However, AA production diverts WSC

substrate away from potential LA production and is less efficient in terms of DM and energy conservation. A ratio of LA to AA less than 2:1 is considered acceptable (Kung & Shaver, 2001) (Table 2.2). BA is a product of clostridial activity. Depending on its concentration, BA may indicate a slow or substrate-limited fermentation, or an absence of nitrates from the pre-ensiled crop. Ethanol is the primary neutral fermentation product in silage and is produced mainly by acid-tolerant yeast species. On account of its high energy density, ethanol production does not reduce the feeding value of silages, however it does not contribute to acidification. Rarer fermentation end-products that are not frequently measured include propionic acid, butane-diol, mannitol, amines, amides, and volatile N compounds other than NH_3. Mycotoxins and anti-quality compounds may exist in silage, and can have a significant negative effect on animal intake and performance.

2.3.5 Silage protein preservation

In well-preserved silages, plant enzyme proteolysis will predominate and silage N will mainly be in the form of peptides and amino acids. Most LAB species are non-proteolytic, and so contribute little to further breakdown of silage N compounds. Enterobacteria can further degrade the N fraction by deaminating amino acids, producing ammonia (NH_3) and other compounds of low nutritional value. Proteolytic clostridia have the ability to degrade various amino acids through a number of fermentation pathways, producing organic acids, NH_3, amines and CO_2. NH_3 is often the only volatile protein breakdown product in silage and therefore the NH_3: N ratio is a common feature of fermentation analyses. A NH_3: N ≤ 0.1 is considered acceptable (Table 2.2).

2.4 Moisture content and wilting

The moisture content of a crop at ensiling is a pivotal factor determining the type and the extent of the silage fermentation, and the conditions in the silo. High moisture content (low DM) crops rely on the fermentation process for inhibiting undesirable microbial activity. Higher DM silages achieve a preservative effect increasingly through reduced water activity. Ensiling a crop at a very high moisture content increases the probability of undesirable secondary fermentation of LA and increases the production of silage effluent (McDonald et al., 1991). Therefore, wilting is a common post-harvest practice before ensiling. In regions where the climate is favourable, most farmers will mow a forage crop and leave it to wilt in the field, before chopping, compacting and ensiling. Although greater DM losses occur during an extended aerobic phase, effluent losses from the silage stack are reduced. Due to the well-documented positive effect on the silage fermentation, wilting to intermediate DM contents (25-40%) result in the lower average nutrient losses throughout the ensiling process (McGechan, 1989). In tropical or damp climates wilting is impractical, and so many crops are ensiled directly (Muck et al., 2003).

2.4.1 Moisture content and water activity

Moisture content exerts its effect on silage biochemistry through its impact on water activity (a_w) which is a measure of the content of solutes in water and their effect on the chemical activity of water. The a_w of pure water is 1 and decreases with increasing solute content. The relationship between DM (d) and a_w, described by Greenhill (1964) is given below;

$$a_w = 1-bd/(1-d)$$

Crop a_w is reduced when water leaves the crop during wilting, and this influences plant and microbial metabolic processes in the silo. Farmers and scientists use DM content measurements to predict fermentation outcomes, due to its relative ease of measurement. However, a crop of a given DM may have a range of a_w values, depending on plant cell solute composition and concentration. This range (determined by the constant b in the above equation) differs between crops and growth environments. For grasses b is typically 0.02 to 0.04 (Greenhill, 1964).

2.4.2 Reduction of moisture content by wilting

Crops that are field wilted are harvested and laid to dry in a ‘swath’ (Photograph 2.1) in the field until the estimated target DM for ensiling is approached. In order to minimize nutrient losses, a rapid wilt is

desirable. Plant factors influence drying rates, however environmental conditions are the most important factors in determining the necessary duration of the wilt.

Photograph 2.1 *A recently cut ryegrass-clover sward being field-wilted in a swath prior to ensiling on a New Zealand (Manawatu) dairy farm.*

2.4.3 Effects of reduced water activity on silage microbiology

Species and strains of microorganisms differ in their ability to multiply under various abiotic conditions. One or a combination of the following factors; low pH, high fermentation acid concentrations, and low a_w, are most often limiting for microbial growth in silages. Of particular importance in silage is the minimum pH at which certain microbial groups cease to grow, a value which varies directly with a_w (Figure 2.1).

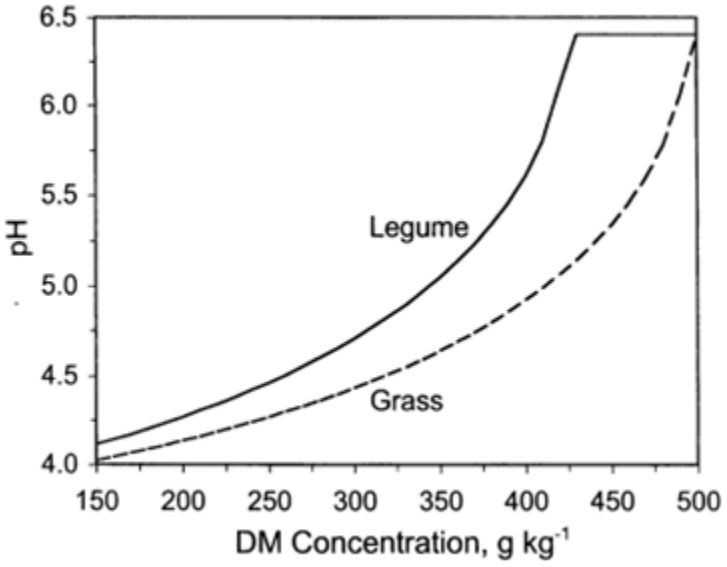
Lactic acid bacteria

Of the main fermentative bacterial groups in silage, LAB are most tolerant of low a_w. LAB also possess relatively high tolerance of low pH. Pure culture studies and laboratory-scale grass silage experiments have shown that rates of LAB growth (and therefore LA production and pH decline), slow at DM contents above 40% (ryegrass a_w =0.98) and cease at approximately 70% (a_w =0.93) (Muck et al., 2003). Individual LAB strains vary in their tolerance of low moisture contents. Therefore, while the minimum potential pH of silage is primarily determined by the crop's sugar to buffering capacity ratio, the osmotolerance of the dominant LAB strains present determine the 'stable phase' pH if sugars are not limiting.

Clostridia

Suppressing clostridial growth in silage is particularly important for successful preservation (McDonald et al., 1991). If growth suppression of clostridia can be achieved, then silages are defined as 'anaerobically stable' (Weissbach, 1996). Clostridia are less tolerant to low a_w than LAB. Therefore the critical pH value at which anaerobic stability occurs increases with decreasing a_w. Various models have been described which predict clostridial development based on pre-ensiling characteristics e.g. (Figure 2.1). Wilting to a higher DM content provides a means of selective inhibition of clostridial activity and is a highly effective post-harvest strategy employed for 'improving' the fermentation (Haigh, 1996).

Figure 2.1 *The relationship between dry matter (DM) concentration and the pH required for anaerobic stability as proposed by (Leibensperger & Pitt, 1987) for two crops which possess a different water activity (a_w), for a given DM concentration. On account of their lower a_w legume silages require a smaller pH decline in order to inhibit clostridial growth.*



2.5 Factors affecting plant water-soluble carbohydrates

Water-soluble carbohydrates (WSCs) are the main substrate for LA production by LAB in silage. Together with the intrinsic buffering capacity and a_w of the crop, the WSC concentration determines the potential extent of LA production, pH decline and therefore success of nutrient preservation of a crop. Due to a number of interactive effects that influence immediate concentrations of various plant carbohydrate pools, it is difficult to estimate the WSC concentration of a crop at a point in time. For example the impact of plant maturity on WSC concentrations is often overshadowed by recent weather effects (Buxton & O'Kiely, 2003). This section describes the major factors influencing WSCs in PRG and summarizes the literature on glucose addition to silages.

2.5.1 Water soluble carbohydrates in perennial ryegrasses

Within the non-structural carbohydrate (NSC) pool only small, simple, WSCs are readily fermented by LAB to yield LA (Rooke & Hatfield, 2003). The abundant WSCs in temperate C3 grass species include glucose, fructose and sucrose and fructans. The concentrations of simple mono- and oilgo- saccharides are low and variable, and on their own are often insufficient for ensiling when a crop is harvested. However, the more abundant reserve polysaccharides, which are mainly fructans (rather than starch) in temperate C3 grass species, are readily degraded by plant enzymes (and some microbial species) yielding additional simple sugars for fermentation (Rooke & Hatfield, 2003). White (1973), state that WSC concentrations in PRG range between 74-314g/kgDM, with a mean of 181g/kgDM. WSC concentrations in the ryegrass pseudostem are higher than in the leaf, because fructans primarily concentrate there, acting as a reserve of energy for regrowth following defoliation (Fulkerson & Donaghy, 2001; McDonald et al., 1991). For most purposes in relation to ensiling, overall WSC is measured. Measurement involves an extraction step, which separates them from other carbohydrate pools based on their solubility in hot or cold water. Although a given extraction method may not directly reflect the availability of WSCs to silage microorganisms, WSC concentration is the single most useful measure of the ensiling potential of a crop (Rooke & Hatfield, 2003).

2.5.2 Flux of plant non-structural carbohydrates

The concentration of various classes of NSCs is in a constant state of change, which reflects the dynamic processes of anabolism and catabolism of carbohydrate pools and their highly mobile nature. Factors influencing the relative rates of photosynthesis and respiration ('sink' and 'source') determine net NSC accumulation in the short term (Fulkerson & Donaghy, 2001). During the process of photosynthesis the

plant forms simple NSCs, which may be combined into larger reserve polysaccharide molecules (fructans in PRG). Respiration continually consumes a proportion of the NSCs generated by photosynthesis. If immediately available NSCs are insufficient for respiration, then reserve polysaccharides are degraded and utilized.

2.5.3 Major factors affecting WSC levels in PRG

The growth environment and the way that a grass crop is managed are reflected in changes in plant growth and development, and in nutritional changes. NSCs, more than any other plant nutritional component, fluctuate due to changes in their growth environment (White, 1973). The magnitude of the effects of individual environmental factors on NSC concentrations in PRG are well characterized in the literature. The following section briefly reviews the major factors influencing WSC accumulation in PRG. Because this section focuses on PRG (with regard to ensiling), and most PRG NSCs are WSCs, the term WSC is used in place of NSC from herein.

Irradiance

Photosynthesis increases with the amount of intercepted photosynthetically active radiation (PAR), which increases with solar or artificial irradiation. An increase in solar radiation increases the rate of photosynthesis, and therefore the production of WSCs. Artificial shading or periods of cloudy weather severely reduce WSC levels (Hight et al., 1968; McDonald et al., 1991; Woledge, 1977). The interactive effect between irradiance levels and temperature on plant growth is also important because WSC concentrations usually decrease during rapid growth (Fulkerson & Donaghy, 2001). Hunt and Halligan (1981), found that PRG plants grown in spaced pots approached light saturation at low levels of irradiance (2-4 MJ m^2 daily PAR) when grown at temperatures of 7-20°C. Plants grown at 30-33°C approached light saturation at higher levels of irradiance (8.6 MJ m^2 daily PAR).

Temperature

Plant WSC concentrations typically only increase when the temperature is below the optimum for plant growth (Buxton & O'Kiely, 2003). Plant respiration increases exponentially with temperature (Fulkerson & Donaghy, 2001) up until approximately 40°C. The rate of photosynthesis increases with (day) temperature up until the optimum temperature for photosynthesis and growth, which is approximately 25-30°C for temperate C3 grasses such as PRG (cultivar 'Grasslands Ruanui') (Hunt & Halligan, 1981), and providing that other factors (light, carbon dioxide, and water) are not limiting (Woledge & Parsons, 1986). An increase in day temperature above the species optimum for photosynthesis and growth will increase

the rate of respiration, but not photosynthesis, leading to a reduction in WSC concentrations. Increasing the night temperature increases only respiration, and can therefore severely reduce net WSC accumulation (Fulkerson & Donaghy, 2001; White, 1973). Increasing temperatures also influences WSC concentrations by promoting stem development over leaf development (Buxton & O'Kiely, 2003).

Nitrogen fertilizer application

The energy requirements for plant growth are derived from available WSCs. Therefore any factor that reduces growth (without affecting photosynthesis) will initially increase WSC concentrations (Buxton & O'Kiely, 2003), while factors that promote growth/alleviate stress will decrease WSC concentrations in the short term. Increasing the rate of N fertilizer application indirectly reduces the concentration of WSCs by increasing plant growth (McDonald et al., 1991). WSC concentrations of N-fertilized grasses may also be reduced by a dilution effect due to increases in protein, nitrate (Harrison et al., 2003) and fatty acid content (Glasser et al., 2013). A recent example or a reduction in WSCs in PRG due to a high N fertilization rate was recorded by King et al. (2013).

Defoliation and regrowth

WSC concentrations in ryegrass follow a U-shaped curve during the regrowth period. Immediately after defoliation, energy reserves (fructans) in the remnant pseudostem are mobilized and used to fuel rapid refoliation and thus restore photosynthetic capacity (Fulkerson & Donaghy, 2001). This causes a steep reduction in total WSCs. As photosynthetic capacity is recovered, WSC production eventually meets the demands for growth, and a period of fructan and net total WSC accumulation in the pseudo stem can begin (Fulkerson et al., 1994; Hume, 1991). As the regrowth advances further, the concentration of reserve WSCs also increases due to the increasing pseudostem to leaf ratio (McDonald et al., 1991).

Diurnal fluctuation and seasonal trends

WSCs fluctuate on a diurnal basis, increasing throughout the day and decreasing during the night e.g. (Greenfield & Smith, 1974; Waite & Boyd, 1953). The difference between night and day peaks depends upon solar radiation intensity and the day/night temperature. Seasonal patterns in WSC levels are largely explained by the effects of season on solar radiation and temperature and by plant developmental stage (Pollock & Cairns, 1991). In general, WSC concentrations are maximized when growth is slow during cold weather with clear skies, and minimized during periods of rapid growth with warm weather and overcast skies (Bathurst & Mitchell, 1958). The transition into a reproductive state is accompanied by an increase in WSCs, due to an increasing proportion of (true) stem which contains high concentrations of fructans

(Buxton & O'Kiely, 2003). Fructans form an accumulation gradient from the base to the apex of the stem (Pollock & Cairns, 1991).

2.5.4 The effect of glucose addition on the silage fermentation

In practice, where WSC levels are anticipated to be low and/or ensiling conditions are challenging, fermentable carbohydrates in the form of molasses are added to a crop at a rate of 20-40kg/tonne prior to ensiling (Limin et al., 2003). Molasses addition at this rate effectively increases concentration of carbohydrates in the crop by 1-2% DM. Although simple hexose sugars such as glucose are not added to silage crops in practice, several studies have examined the effect of adding glucose on silage fermentations (table 2.3). Generally, adding glucose in a sufficient quantity can promote a rapid LA fermentation in silage crops with a low WSC:BC ratio. In crops where existing WSCs are sufficient, glucose addition has no positive effect on silage fermentation or nutrient preservation. An excess of glucose has no major detrimental effect on silage quality, as measured by fermentation parameters, although it often results in an increase the production of ethanol. This indicates that excess glucose may stimulate the growth of acid-tolerant yeasts, which will reduce silage stability on exposure to air.

Table 2.3 *The impact of adding glucose on the silage fermentation.*

Author	Crop, and ensiling characteristics	Quantity of sugar added	Effect on glucose addition on silage fermentation
Ohyama et al., 1975	Unwilted Italian ryegrass DM; 13.6% WSC; 93g/kgDM	Glucose at a rate of 1-2% of fresh weight, with or without commercial inoculant	Only the combined addition of glucose and commercial inoculant produced consistently well-preserved silage (with high LA and low BA content).
Seale et al., 1986	Lucerne; DM; 23.6% WSC; 49g/kgDM. (High buffering capacity crop)	42.4g/kgDM of glucose or fructose, with or without two different commercial inoculants.	Both inoculants and sugars had a similar positive effect on pH decline and lactic acid accumulation, but were needed in combination in order to produce well-preserved silage. Sugar addition increased ethanol content and reduced BA content of lucerne silage.
Heron et al., 1988	Unwilted ryegrass; DM; 15.0% WSC; 180g/kgDM	700g/L glucose solution applied at 28 ml/kg of fresh material.	Glucose did not improve the silage fermentation or reduce degradation of the protein fraction, but resulted in more ethanol being produced.
Chamberlain, 1989	Wilted ryegrass DM; 20.3% WSC; 144g/kgDM	Glucose and xylose at a rate of 0, 25, 35 & 45g/kg fresh material.	The amount of ethanol produced increased and the amount of lactic acid produced decreased as the rate of glucose added increased. No major change in pH or BA as a result of sugar addition.
Zhang et al., 2010	Guinea grass; DM; 29.0% WSC; 45.1g/kgDM BC; 31.2 (g.LA/kgDM)	Glucose added at a rate of 5, 10, 20, and 40 g/kg DM and ensiled at 20, 30 and 40°C.	Addition of glucose at 40 g/kg DM had the most beneficial effect on silage fermentation (increased lactic acid, reduced BA) at 20 and 40°C. Lower quantities of glucose reduced silage quality, especially at 30°C.

WSC = water soluble carbohydrates, BC = buffering capacity, DM = dry matter, LA = lactic acid, BA = butyric acid

2.6 Buffering capacity and the WSC:BC ratio

The intrinsic buffering capacity (BC) of plant tissues describes their ability to resist a change in pH. BC can be measured in order to determine the minimum quantity of fermentation acids required to lower the pH of a crop to a sufficiently low level to ensure anaerobic preservation. This information can subsequently be used to estimate the minimum quantity of fermentable substrate required to reach this critical pH (which varies with a_w). A low BC is desirable for silage fermentation as it minimizes the amount of LA that needs to be produced. The BC of plant material over the pH range of 4.0-6.0 is most relevant in the context of ensiling, although a reduced range will be relevant for higher DM silages. BC measurements of aqueous plant material macerates are made over this range using acids and bases as titrants, most commonly using the method described by Playne and McDonald (1966). The use of LA instead of either dilute HCl or NaOH as the titrant will generate higher BC measurements, and will more closely reflect pH buffering dynamics in the resulting silage, because LA itself has buffering activity over the pH range of 4.0-6.0 (Playne & McDonald, 1966), while HCl or NaOH do not. However in many cases it is desirable to use a titrant that does not buffer over the pH range of interest and hence these dilute strong acids and bases are frequently used. The units for measuring BC are expressed interchangeably as Milliequivalents or grams of LA required to shift the pH of 1 kg of crop DM between 4.0 and 6.0 (Meq kg DM^{-1} or gLA kg DM^{-1}).

2.6.1 Factors affecting buffering capacity

The buffering capacity of PRG ranges from 257-558 Meq kg DM^{-1}, with a mean of 380 Meq kg DM^{-1} (McDonald et al., 1991). Therefore, approximately 30-80g LA are required to be produced for a typical ryegrass silage fermentation (Cherney & Cherney, 2003). Leaves have a higher BC than stem due to higher protein and anion concentrations, and so conditions encouraging vegetative growth increase BC. The two most important factors affecting BC for a given crop are maturity and soil fertility (in particular nitrogen fertilizer use) (Tremblay et al., 2005). As plants mature, the ratio of cell contents to cell wall declines, in part due to a reduction in the stem to leaf ratio, which reduces buffering capacity. In permanent, mixed species pastures undergoing a transition into a reproductive state, the BC declined from 450 Meq kg DM^{-1}, by approximately 2.5 Meq kg DM^{-1} per day, however the BC of vegetative regrowth stayed relatively constant (Muck et al., 1991). An average daily decline of 3.2 Meq kg DM^{-1} per day across five pasture species under different N input regimes was reported by King et al. (2012). The rate of decline in BC slows during regrowth (King et al., 2013).

2.6.2 The WSC:BC ratio

The WSC:BC ratio of a crop is considered a useful indicator of its ensiling potential (Buxton & O'Kiely, 2003; Leibensperger & Pitt, 1987) however this ratio is not equally useful for predicting ensiling outcomes across all species/environments (Tremblay et al., 2005). The fermentation of a hexose molecule to two LA molecules has a 100% DM conversion efficiency (Rooke & Hatfield, 2003). This means that a WSC:BC of 1 (where BC is measured in LA required to shift a kg of DM between pH 4 to 6) would be sufficient for generating well-preserved silage, if this was the only fermentation pathway active. In reality, pentoses also constitute plant WSCs, and therefore there is a degree of heterofermentative activity from LAB. Further, competing microorganisms convert WSCs and LA into weaker acids and neutral end-products. Therefore a WSC:BC ratio far in excess of 1 is desirable, particularly for low DM silages. WSC:BC measurements/estimates inform best-practice decisions regarding silage making practices, for example wilt duration, or the use of preservation aids such as bacterial inoculants or inorganic acids. Clovers have low levels of WSCs and high BC relative to temperate C3 grasses and are therefore ensiled at higher DM (Buxton & O'Kiely, 2003). Given that ryegrasses usually contain abundant WSCs, preservation aids are less likely to be necessary for ryegrasses than for other pasture grasses (King et al., 2013).

2.6.3 Nitrogen fertilizer and the WSC:BC ratio

Inorganic N fertilizer is used to increase herbage yields, however it can have a detrimental effect on the nutritional and ensiling characteristics of forage. With regard to ensiling, it is well documented that N fertilizer application results in an increase in herbage crude protein (CP), nitrates and BC and a reduction in WSC and DM, generally making the crop more difficult to ensile, especially soon after application (Harrison et al., 2003). In five pasture grass species King et al. (2013) showed that high N input following defoliation increased BC significantly during the early stages of regrowth. The BC of ryegrass pastures decreased from approximately 450 Meq kg DM^{-1} after 2 weeks regrowth to 250 Meq kg DM^{-1} after 10 weeks regrowth in the 0 kg N ha treatment, and from and 550 to 270 Meq kg DM^{-1} in the 120 kg N ha^{-1} treatment. The BC of mixed pasture grasses increased by 27% due to increasing N application rate from 0 to 120 kg N ha^{-1} (O'Kiely et al., 1997). The BC of pasture species Timothy (*Phleum pratense*) harvested at a range of developmental stages, sites and years increased by 19% with increasing N application rate from 0 to 180 kg N ha^{-1}, although there was wide variation among site-years (Tremblay et al., 2005). It is important to note that an unsuccessful ryegrass silage fermentation due to a high BC (for example due to excessive N fertilizer use) will often only occur where conditions are already challenging for ensiling (e.g. if the crop is harvested in overcast conditions) (King et al., 2013). The negative impact of N application on

ensilability (WSC:BC ratio) is exacerbated further by reducing sugar concentrations (O'Kiely et al., 1997; Tremblay et al., 2005).

2.7 Laboratory-scale silages

Small-scale laboratory silos are accepted as a suitable model for farm-scale ensiling. They are used for rapid, cost-effective evaluation of the effects of numerous experimental treatments on silage fermentations. The ideal ensiling conditions that lab silos provide are difficult to achieve in practice, however these conditions allow controlled repeatable comparisons between silage fermentations to be made.

A range of laboratory and pilot scale silo types have been used, with capacities ranging from 50g to several tonnes (McDonald et al., 1991). These include fixed volume vessels such as boiling tubes, glass jars, and plastic pipes, all fitted with sealing devices. Historically, the glass tube has been most widely used laboratory silo (McDonald et al., 1991), however vacuum packing in plastic bags has become a popular method in recent times (Johnson et al., 2005). The basic criteria for any laboratory silo is the exclusion of air, and thus maintenance of anaerobic conditions. Other desirable features of a laboratory silo that mimic the conditions of a farm silo may include; high herbage density, light exclusion, and a means of escape for effluent or gasses produced in the silo. There are inherent problems with any type of laboratory silo, and caution is advised in extrapolating data from the lab scale to farm scale silages (Cherney et al., 2004).

Only a few studies have compared fermentations in different types of laboratory silo, or compared laboratory silo fermentations with pilot scale or farm scale silo fermentations. Wilson and Wilkins (1972), compared 100g test tube silages with 6 kg and 1000 kg plastic bag silages of 18 grass and 8 legume species. They found good replication between the 100g test tubes, and close correlation in fermentation parameters between the test tube and plastic bag silages. O'Kiely and Wilson (1991), observed that ryegrass plastic pipe silos fitted with effluent and gas release devices were a better model than test tube silos for farm-scale clamp silos. The general process for ensiling was the same between silo types, however they discovered the potential for significant fermentation x silo type interactions when comparing treatments. Such interactions were observed recently in a comparison of vacuum packed and glass jar silages (Hoedtke & Zeyner, 2011).

2.7.1 Vacuum packing versus fixed volume vessel silages

There are several drawbacks to using fixed volume vessels as small model silages. Packing and unpacking vessels is labour and time consuming. Depending on the operator, variability in packing densities can arise within a vessel and between replicates, which affects the speed of fermentation and pH decline. In a

laboratory setting, vessel packing aims to achieve densities within the range of those found in a farm silo stack (0.32-0.64g cm^{-3}) (Johnson et al., 2005). Even packing of samples becomes particularly difficult with heterogeneous plant material (e.g. containing stems or large structures). Chopping can help in this regard, however fine chopping may not reflect typical on-farm practices.

Two recent studies have explored the use of vacuum bags as model silo vessels and have confirmed that vacuum packing herbage in plastic bags is a convenient ensiling method that avoids the shortcomings of fixed volume vessels. Johnson et al. (2005), ensiled chopped, wilted PRG and red clover (*Trifolium pratense*), with a DM of 28.0% and 25.0%, respectively. They applied commercial inoculant to half of the plant material before ensiling 100g replicates (wilted material) in heat sealed polyethylene vacuum bags or boiling tubes fitted with rubber bungs. Desired packing densities were achieved by varying the length of time spent under vacuum. A roughly comparable herbage density to that in the boiling tubes (0.625g cm^{-3}) was identified and used for comparison to the boiling tubes. LA accumulation and pH decline was more rapid at higher packing densities, but was very similar for glass tube and vacuum packed silos. After 35 days, LA and pH were similar for both silo types at all packing density treatments. Effluent production was greater at higher vacuum settings, due to crushing of the plant material.

Differences exist between conditions in sealed fixed volume vessels and vacuum packed bags. Vacuum packed bags leave very little residual oxygen and so anaerobic conditions are achieved more rapidly. While it is easier to achieve similar packing densities between vacuum packed silo replicates at the beginning of ensiling, the volume of vacuum bags can increase significantly during the fermentation phase, due to fermentation gas production. The resulting loosening of the herbage is not observed in rigid walled vessels such as glass tube. In the above experiment, the gradual loosening of herbage did not affect fermentation parameters (Johnson et al., 2005). Vacuum bags may differ in their permeability to oxygen and carbon dioxide, however Ashbell et al. (2001) confirmed that there were no significant differences in fermentation and nutritional parameters in silages made with a number of different vacuum bag types with different permeability to these gases.

Hoedtke and Zeyner (2011), compared glass jar and vacuum packed silages for both fresh and wilted PRG (15.1 and 28.6% DM respectively) and remoistened coarsely ground rye grain, using the silage fermentation profile and aerobic stability as criteria of fermentation quality. Polyethylene bags were air-evacuated in order to achieve the desired packing density of the plant material (equivalent to the glass jar). An attempt was made to constrain herbage within vacuum bags, by first placing plant material in an inner bag, wrapping adhesive tape tightly around this bag, puncturing it with a disinfected needle, and

then placing into larger vacuum bags which were air-evacuated. This meant that the plant materials were held at high packing density for the duration of their fermentation. The authors argued that these silos more accurately modelled the high density storage conditions of a crop in a farm-scale silo. As judged by fermentation parameters, the two ensiling methods were broadly comparable (Hoedtke & Zeyner, 2011). Mean pH, AA, propionic acid and ethanol content across the three crops (with or without a commercial inoculant) did not differ significantly, while LA content was higher and BA content lower, in the vacuum bag silages (Hoedtke & Zeyner, 2011). These results suggested that the material in the double bagged vacuum packed silages may have undergone a better fermentation than the material in the glass jars. Regardless of ensiling method, all of the non-inoculated ryegrass silages failed to reach the pH required for successful preservation, as indicated by a rise in pH and decrease in LA between day 8 and 49 and 90 of the fermentation.

2.7.2 Silage sample size

Few studies have addressed how much plant material is sufficient in order for small-scale modelling exercises to be useful. A theoretical lower limit will depend on how closely the researcher wishes to mimic a certain farm scale silo, and on experimental design. It will also depend on which measurements are made in order to evaluate the success of ensiling. Smaller quantities may be adequate if factors important to the fermentation process are controlled as accurately as possible.

An early silage experiment using glass tubes reported using 25g quantities of finely chopped, well mixed ryegrass (Allen et al., 1937). They found that with rare exceptions, there was consistency between replicates in chemical and bacteriological data. It is frequently quoted that 50g is the lowest quantity used for laboratory silages (McDonald et al., 1991). Vacuum packing as an ensiling method offers greater flexibility in modifying sample size, but this method has not yet been employed for quantities less than 100g to the author's knowledge. Techniques for further 'scaling down' of the fermentation process have also been described by Johnson et al. (2004).

The effect of silage sample size on corn (*Zea mays*) fermentation in vacuum packed bags was studied by (Cherney et al., 2004), who concluded that 200g is the smallest useful sample size. In this study, 50g corn silos differed in their fermentation profile from 200-600g silage sample sizes, with higher LA, AA and NH_3:N, and were deemed non-representative of typical corn silage (Cherney et al., 2004). Fermentation in the 100g sample size also differed from the larger sample sizes, but to a lesser extent. The reason for this was not apparent, but the authors suggest that it was unlikely due to sub-sampling from the fresh

corn samples. Other reports indicate that achieving a representative ratio of plant organs (ear:stover ratio) is difficult with corn. Interestingly, pH and the LA to AA ratio were consistent across all silage sample sizes.

Due to their lower WSC:BC ratio, perennial grasses are more difficult to ensile than corn. However, it is easier to obtain small representative samples of grasses due to their small stems and tillers, and lack of significant grain component. Hoedtke and Zeyner (2011), advocated a minimum 400g sample size for double bagged vacuum packed grasses. Using non-wilted tall fescue (*Festuca arundinacea)*, Cherney et al. (2006) found no difference in pH after 30 days of fermentation between ensiled 250g and 500g samples across two different harvest and chopping treatments. A quantity of 100g has been used for vacuum packed grass, with low variation among replicates (Johnson et al., 2005), although no comparison was made between these silages and larger sample sizes.

Fermentation analyses and silage size

An important question related to the discussion of adequate silage sample size is 'which measurements are being made in order to assess the type of fermentation that has occurred?' When sampling from the face of a silage stack, large variation exists in, for example, DM and LA concentration, compared to pH and total nitrogen. Minimum sample numbers vary depending on the silage constituents chosen for measurement (Haslemore & Holland, 1981). It logically follows that when analyzing laboratory silages, smaller silage sample sizes may be satisfactory to measure may be sufficient for measuring certain parameters, such as pH.

2.8 Expression of 'high lipid' technology in Arabidopsis and perennial ryegrass

A novel approach to increasing the nutritional value of agronomically-important pasture species has been the development of PRG lines with synthetically elevated levels of leaf lipids, by the Plant Biotechnology group at AgResearch Ltd. There are proven productive, environmental and consumer-health advantages of feeding livestock diets with higher lipids. However, traditional plant breeding methods offer only limited scope for improving the lipid content of important pasture species (Hegarty et al., 2013; Smith et al., 1997). Winichayakul et al. (2013), describe a novel technology which utilizes genetic engineering methods to generate elevated lipid contents and increased biomass accumulation in the leaves and roots of the model plant species Arabidopsis (*Arabidopsis. Thaliana*). The technology has now been expressed in a range of C3 crop species, and holds significant potential for application in agricultural and industrial fields. This section provides some background on the science behind this innovation, and gives an account of the proposed mechanisms by which the technology described by Winichayakul et al. (2013), has enhanced lipid levels and biomass accumulation in *A. thaliana*. It then describes the expression of the technology in PRG under green tissue-specific promoters, which form the plant material used for experimental work in this thesis.

2.8.1 Leaf and seed lipids; natural roles

A typical leaf lipid profile reflects the structural roles of lipids in these organs. It consists predominately of polar galacto- and phospholipids, which make up plasma and organellular membranes. Neutral lipids including triacylglycerol (TAG) are incapable of integrating into membranes, and instead form the hydrophobic core of short-lived lipid droplets (LDs or oil bodies); dynamic energy storage organelles with a phospholipid monolayer embedded with proteins. Lipid storage is not a function of leaves, and so TAG exists in small quantities and only as a short-term intermediate for membrane turnover and remodeling (Chapman et al., 2013). Conversely, seeds specialize in energy storage and accumulate a significant proportion of their weight as TAG, stored within LDs which are protected by the seed-expressed oleosin protein.

2.8.2 Plant fatty acid and lipid biosynthesis

Fatty acid (FA) synthesis occurs in the plastid. It involves a series of repeated additions of two carbon units to an elongating fatty acid chain. The ultimate source of carbon is plant carbohydrates derived from photosynthesis. Briefly; glucose is converted into two three-carbon pyruvate molecules via glycolysis.

Pyruvate is then converted into acetyl-CoA by the pyruvate dehydrogenase complex, releasing a CO_2 molecule in the process. Acetyl-CoA is converted to Malonyl-CoA by the highly regulated acetyl-CoA carboxylase enzyme (ACCase), which is the committed step of fatty acid synthesis. The fatty acid synthase complex transfers the Malonyl moiety to an acyl carrier protein (ACP) and uses Malonyl-CoA as the primary substrate for FA chain elongation, which proceeds via repeated condensation reactions between acetyl-CoA and Malonyl-ACP.

When FA elongation is terminated a proportion of FAs are exported from the plastid to the endoplasmic reticulum (ER) (via the cytosol), where they are converted to acyl-coAs. Lipid biosynthesis proceeds via sequential esterification reactions between glycerol-3-phosphate and acyl chains (the CoA molecules are recycled) or other chemical groups. The intermediate diacylglycerol (DAG) is used as a precursor for membrane lipid synthesis. Alternatively it is used in the final and only committed step in the TAG biosynthesis pathway; addition of a third fatty acid to a diacylglycerol molecule. This step is catalyzed, predominately, by the Diacylglycerol O-acyltransferase 1 (DGAT1) enzyme, and is a limiting factor in TAG synthesis. Overexpression of the DGAT1 enzyme increases TAG assembly *in planta*, however the resulting increase in plant leaf lipid content is transient, because the plant cell catabolizes the 'unnecessary' accumulated TAG via the action of TAG lipases, followed by FA recycling in the ER and beta-oxidation of FAs in the peroxisome (Winichayakul et al., 2008).

2.8.3 Natural roles and topology of oleosin proteins

Oleosins are small plant proteins that occur naturally within seeds and pollen. They embed into the phospholipid outer layer of LDs, encapsulating them and protecting against lipase entry and inhibiting LD coalescence. Oleosin proteins consist of a conserved hydrophobic central domain which extends into hydrophobic core of LDs, and N and C-terminal amphipathic 'arms' which sit on the surface of the LD and are exposed to the cytoplasm. The length and overall sequence of these 'arms' are comparatively diverse, suggesting that their role in providing LD integrity is not dependent on a particular amino acid sequence. There are no reported cases of natural existence of cysteine residues in oleosin's amphipathic arms (pers. comm, Nick Roberts)

2.8.4 Strategies for engineering TAG accumulation into non-seed tissues

On account of its high energy density, engineering higher levels of the neutral lipid TAG into non-seed plant tissues is recognized as a promising strategy for meeting future energy and food demands (Napier et al., 2014). However until recently, efforts have failed to yield industrially relevant increases in plant

TAG content. A number of stages of plant TAG metabolism have been identified as potential engineering targets by research groups. Vanhercke et al. (2014b), divide current engineering strategies into three broad categories; 1) increasing fatty acid synthesis, 2) increasing TAG assembly and 3) reducing lipid turnover. In order to convey the property of long-term TAG accumulation to a tissue, requires that the rate of TAG catabolism relative to synthesis must be reduced. It has been recognized that in order for engineering efforts to be successful, a 'multi-gene approach' rather than the 'single gene approach' may be necessary. The more successful recent strategies have manipulated two or more aspects of TAG metabolism, which has been achieved by co-expressing gene pairs or groups.

2.8.5 Co-expression of DGAT1 and Cys-oleosin in Arabidopsis

The critical innovation reported in Winichayakul et al. (2013) leading to long-term TAG accumulation in *A. thaliana* leaves and roots was the constitutive expression of an oleosin protein engineered to contain cysteine residues at strategic locations in its amphipathic arms (Cys-oleosin). When Co-expressed with the Arabidopsis DGAT1 gene (both under the control of the CaMV 35S promoter), this unique protein conferred enhanced stability to LDs in vegetative tissues, thereby slowing TAG catabolism. It is proposed that the diversion of FAs into stable LDS led to an ongoing demand for *de novo* FA synthesis.

Winichayakul et al. (2013) engineered between one and seven cysteine residues along the amphipathic arms of oleosin proteins isolated form *Sesame indicum*. Amino acid substitutions were at positions that maximized the potential to form disulphide bonds, and minimized the disruption of interactions between the arms and the LD surface. *A. thaliana* lines co-expressing DGAT1 and various Cys-oleosin configurations showed different degrees of lipid accumulation. Plant lines expressing DGAT1 and oleosin with three cysteine residues introduced into each arm (termed D1o3-3 plants), accumulated the highest lipid levels without a negative impact on growth phenotype (Winichayakul et al., 2013).

The engineered oleosin in D1o3-3 plants was found to correctly target the surface of LDs. This number and configuration of cysteine residues resulted in the formation of inter-oleosin disulfide bonds between the arms of neighboring oleosins on the surface of LDs, thereby crosslinking them. LDs containing crosslinked engineered oleosin proteins showed a high level of resistance to cysteine-protease degradation, and partial resistance to serine-proteases compared to the native oleosin protein *in vitro*. The authors propose that this protease resistance conferred greater stability to LDs *in planta* by slowing the entry of TAG lipases into LDs, thereby extending LD lifespan. This in turn allowed greater quantities of TAG to accumulate in the leaves and roots (Winichayakul et al., 2013).

Changes to FA and TAG content and FA composition in D1o3-3 Arabidopsis

Control plants; consisting of transformed plant lines containing empty vector constructs, and transformed lines expressing DGAT1 with and without the native oleosin protein, contained similar levels of leaf FA content to non-transformed wild type (WT) plants. FA content in WT peaked at 14 days after sowing (DAS) at 5% dry weight (DW) and then fell to 3.8% DW at 21 DAS and then to 3.7% DW at 35 DAS.

After preliminary screening for lipid content, homozygous progeny of the original D1o3-3 plants became the main transgenic lines for comparison to WT. D1o3-3 plants contained higher FA content at 14 DAS (6.7% DW), 21 DAS (7.0% DW) and 35 DAS (7.0% DW) than WT. FA content in D1o3-3 plants did not peak until 49 DAS, and at full senescence was 340% greater than WT. Approximately 65% of the *additiona*l leaf FA in D1o3-3 plants was present as TAG (2.1% DW) while the remainder was polar glycerolipids. In the roots, all of the accumulated FA was present as TAG. D1o3-3 leaf TAG levels did not decrease until 63 DAS. Confocal microscopy confirmed the presence of accumulated LDs in D1o3-3 leaves.

In addition to accumulating higher FA content, D1o3-3 plants were found to have an altered leaf and root FA profile, with an increase in proportions of 18:1 and 18:2, and a concomitant decrease in 16:0, 16:1, 16:3, and 18:3, relative to WT. Additionally, very long chain FAs were detected in the D1o3-3 plants. Differences in the FA profile were reflected in the profile of the TAG fraction of D1o3-3 plants.

CO_2 assimilation and Biomass increase mechanism in D1o3-3 Arabidopsis

An interesting finding reported by (Winichayakul et al., 2013) was that the co-expression of DGAT1 with Cys-oleosin in *A. thaliana* gave plants a 24% increase in their CO_2 assimilation rate per unit of leaf area, and an approximate 50% increase in biomass at 25 DAS, relative to WT. This was not observed in plants expressing DGAT1 and the native oleosin. A positive correlation between FA and TAG accumulation and CO_2 assimilation rate was found among independent D1o3-3 lines.

The mechanism by which the authors proposed to explain these changes in D1o3-3 plants is as follows; expression of DGAT1 with Cys-oleosin leads to the formation of stable LDS *in planta*. This lipid protection mechanism acts to prevent a continuing catabolic cycle of FA and TAG biosynthesis, followed by TAG hydrolysis and FA Beta-oxidation. Incorporation of TAG into Cys-oleosin-protected LDs generates a constant demand for elevated *de novo* FA biosynthesis, which are needed for cell membrane maintenance requirements (Winichayakul et al., 2013). Elevated *de novo* FA biosynthesis increases the conversion of pyruvate to Acetyl-CoA (and subsequently Malonyl CoA, the primary substrate for FA synthesis). A carbon dioxide molecule is released during this conversion, which is available for re-fixation by ribulose-1, 5-

bisphosphate carboxylase (rubisco) (without the Calvin cycle) (Schwender et al., 2004). Elevated *de novo* FA biosynthesis therefore increases the partial pressure of CO_2 relative to O_2 in the chloroplast, which reduces rubisco's oxygenase activity (photorespiration) and increases carbon dioxide assimilation. This results in an increase in carbon use efficiency, which is expected to increase biomass production in organisms using the C3 photosynthetic pathway (Durrett et al., 2008). The changes in D1o3-3 plants are the only reported instance of an increase in carbon assimilation and plant biomass production due to a metabolic engineering effort aimed at increasing TAG accumulation.

2.8.6 Co-expression of DGAT1 and Cys-oleosin in PRG.

A promising application of the high lipid technology in livestock agriculture is its expression in the C3 pasture species perennial ryegrass (*Lolium. perenne*, PRG). Multiple independent transformation events have produced D1o3-3 PRG plants (derived from the cultivar 'Impact') containing a single or multiple copies of the D1o3-3 genetic constructs under the transcriptional control of green tissue-specific promoters at a single genetic loci (hemizygous transformants, pers. comm, Kim Richardson) (Appendix VII). In addition to the increased lipid and biomass accumulation recorded in D1o3-3 Arabidopsis plants, a number of additional changes in D1o3-3 PRG have been recorded, which reveal how expression of the high lipid technology may affect the agronomic performance of this species.

Green tissue-specific promoters

The accumulation of significant quantities of TAG in D1o3-3 Arabidopsis roots (6.5% DW) showed that constitutive expression of DGAT1 and Cys-oleosin (under the control of the CaMV 35S promoter) can create a significant carbon sink in non-photosynthetic tissues. Given that the leaves are the harvested component of ryegrass plants, storage of TAG in the roots is of no agronomic benefit. Further, photosynthate directed below-ground for FA synthesis is not available for above-ground growth. Therefore, a sensible development of the high lipid technology in PRG has been its expression under light-regulated 'green tissue-specific' rice (*Orytz sativa*) ribulose-1, 5-bisphosphate carboxylase small subunit promoter and chlorophyll *a/b* binding protein promoter sequences (Appendix VII).

Sustained expression under grazing simulation

Under repeated mechanical defoliation, with non-limiting nutrient and water supply, D1o3-3 PRG plants show elevated lipid contents and altered fatty acid profiles compared to WT plants grown in the same conditions. These changes are present for a typical ryegrass regrowth interval (3-4 weeks). The stability of the high lipid trait under these conditions reveals that the potential benefits of the high lipid technology

may be sustained in the long-term in a grazing (or cut and carry) pastoral farming scenario. However, analysis of these plants has been limited to small-scale experiments in containment glasshouse facilities.

Changes to FA and TAG content and FA composition in D1o3-3 PRG

Transformed plant lines containing empty vector constructs (VC) and non-transformed wild type plants (WT) had similar total leaf FA content; approximately 3.5% DW, 3 weeks after cutting (WAC). Total leaf FA content of independent hemizygous D1o3-3 PRG lines ranged from 23-100% higher than WT plants; approximately 4-7% DW, 3 WAC. Total FA content in the roots of D1o3-3 PRG lines was marginally higher than WT. As in D1o3-3 Arabidopsis, D1o3-3 PRG total leaf FA content showed a positive correlation with TAG content and with increased proportions of 18:1 and 18:2 (with a concomitant decrease in 16:0, 16:1, 18:0 and 18:3), relative to WT. Hemizygous D1o3-3 PRG lines have been grouped into low (4-5% DW), medium (5-6% DW) and high lipid (6-7% DW).

CO_2 assimilation and biomass increase in D1o3-3 PRG

As in D1o3-3 Arabidopsis, a positive correlation was found between FA and TAG accumulation and the CO_2 assimilation rate per unit of leaf area among independent D1o3-3 PRG lines (up to 20% more than WT). However, the leaf and root biomass accumulation advantage (over WT) peaked at approximately 50% in plants with medium levels of the lipid accumulation trait expression (5-6% DW), while the high lipid plants (6 -7%DW) incurred a growth disadvantage, relative to WT.

Additional changes in D1o3-3 PRG

Among independent D1o3-3 PRG lines, the CO_2 assimilation rate correlates with reduced stomatal aperture, reduced stomatal conductance (up to 10% lower than WT), increased iWUE (up to 35% higher than WT), reduced levels of rubisco, and increased levels of chlorophyll. Many of the recorded differences between D1o3-3 and WT PRG are characteristic of the changes seen when C3 plants are grown in high CO_2 environments. Therefore these changes may be a consequence of the speculated increase in plastidial CO_2 concentration as a result of elevated *de novo* FA biosynthesis in these plants. Detailed physiological analysis and isotopic labelling experiments are expected to elucidate the precise mechanisms by which these changes in D1o3-3 plants occur.

2.9 Fatty acid and lipid metabolism during wilting and fermentation

Agronomic practices that impact the delivery of beneficial fatty acids from forages into ruminant-derived products have received recent research interest (Shingfield et al., 2013). Ensiled PRG provides a large proportion of the fatty acids in ruminant diets in many farming systems. In this context, research on fatty acid and lipid metabolism in silage has been driven by the frequently observed phenomenon that animal products from ruminants fed silage-based diets contain lower levels of poly-unsaturated fatty acids (fatty acids containing two or more double bonds, PUFAs) than from animals fed fresh forage (Dewhurst et al., 2006). This is due, to a large extent, to PUFA losses during field wilting (Dewhurst et al., 2003b). Other factors unrelated to ensiling *per se* may be important; for example silage-based feed systems often allow longer regrowth intervals before harvesting than grazing systems, and FA content declines with maturity (Khan et al., 2012). Additionally, the physiochemical nature of ensiled forages and metabolism of the lipid component during anaerobic fermentation may be important factors for rumen biohydrogenation. Two major processes affect lipids and fatty acids during the overall ensiling process. Extensive losses of PUFAs during the aerobic field-wilting phase reduce the concentration and alter the composition of fatty acids. During anaerobic fermentation, fatty acid composition is largely unaffected, however lipolysis of membrane lipids is extensive and so the majority of fatty acids in silage occur as free fatty acids. This section discusses fatty acid and lipid metabolism events during the wilting and fermentative phases of the ensiling process.

2.9.1 Fatty acid and lipid metabolism during wilting

When plant tissues are cut or wounded, stress pathways induce lipase activity, which hydrolyze FAs from membrane lipids. Lipolysis is a prerequisite for the activity of lipoxygenases, which preferentially catalyze the deoxygenation of PUFAs (Feussner & Wasternack, 2002). Free deoxygenated PUFAs are substrates for the production of a range of volatile components including signalling molecules, anti-feedant and anti-microbial compounds. The rate at which lipolysis and oxidation occurs can be studied by measuring the emission of volatile organic compounds (VOCs). VOC emissions increase in response to cutting and drying, which are integral to wilting (Dewhurst et al., 2003b).

Studies measuring forage fatty acid concentrations typically focus on the five FAs; palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3), which together account for 95% of forage FAs. In forage grasses, the concentration of 18:3 is often emphasized, as it usually accounts for over half of total FA, and is more sensitive to metabolic changes than the FAs with a higher

degree of saturation (Glasser et al., 2013). Because enzymes in the lipoxygenase pathway preferentially act upon PUFAs, losses of 18:3 during wilting are greatest in absolute and relative terms. Wilted silages are proportionately lower in 18:3 than their fresh counterparts and a concomitant increase in 18:0 and 16:0 can be expected (Dewhurst & King, 1998; Khan et al., 2011). Relative losses of each FA can vary widely; perhaps due to variation in the intrinsic susceptibility of plant tissues to oxidation events e.g. different levels of activation of anti-oxidant pathways (Van Ranst et al., 2009b).

Variation in total fatty acid losses during wilting

Total FA losses and relative 18:3 losses increase with the duration of the wilt. However, studies employing rapid wilting procedures (<24 hours) have reported a wide range of magnitudes of FA losses. A short wilt of the pasture species Timothy had no consistent effect on crude fat content or fatty acid composition (Arvidsson et al., 2009). Extensive drying of fresh grass in a 50°C oven had no effect on FA concentration and composition, only on lipid fractions (Fievez et al., 2003). Conversely, rapid wilting to a high DM concentration has resulted in marked reductions in PUFAs and overall FA concentration (Boufaïed et al., 2003; Elgersma et al., 2003). The wilt conditions and extent of moisture reduction varied widely in these studies (Table 2.4).

Table 2.4 *The impact of different wilting procedures on total fatty acid (FA) concentration in perennial ryegrass (Lolium perrene, PRG) and Timothy (Phleum pratense).*

Author	Wilt duration	Wilt conditions	Fresh DM-Wilted DM (%)	Forage type	FA fresh (gFA/kgDM)	FA wilted (gFA/kgDM)
Dewhurst & King, 1998	68 hours	Lab bench	15.4-23.3	PRG x3 cultivars	24.6	17.5
Boufaïed et al., 2003	'a few hours'	Field	23.0-40.0	Timothy	19.3	16.66
Elgersma et al., 2003	24 hours	'Air-dried'	20.0-70.0	PRG; x 6 cultivars	29.1	19.5
Chow et al., 2004	52 hours	Field	-	PRG (cv. 'Barnhem')	13.0	11.6

FA = fatty acid, DM = dry matter, PRG = perennial ryegrass

Main factors affecting total fatty acid losses during wilting

In addition to plant factors, the highly variable responses in FA losses during wilting could be explained by environmental factors during the wilt such as temperature, humidity and light exposure or by the extent of damage to plant tissues (Van Ranst et al., 2009a). In a comprehensive set of wilting experiments Khan et al. (2011) compared the effects of each of these factors on FA concentration and composition of mature summer PRG regrowth. PRG was wilted to three approximate DM contents, both in the field and in controlled climate chambers. Across all treatments, they recorded a large reduction in FA concentration during the extended initial wilting period (2-3 days) which was required to increase DM content to 42.5%. This decrease was mainly associated with a decrease in 18:3, with a concomitant change in the FA profile. Further wilting, to 52.5 and 62.5% DM caused no further significant change to FA content and composition. These results agree with those from Dewhurst and King (1998) (Table 2.4), where an extended wilt led to a marked decline in FA concentration. Increasing the wilt temperature for a given wilt duration caused a greater reduction in FA concentration (Khan et al., 2011). The effects of bruising and irradiance were comparatively minor. In an early study, FA losses from PRG were minimized when dried to a high DM content at 100°C for a short time, compared to lower temperatures and longer wilting durations (Czerkawski, 1967), however this temperature would have rapidly aborted all enzymatic activity, which would never occur in practice. Increasing temperatures up to 40-50°C will increase most plant enzymatic activity, and so may increase the activity of the lipoxygenase pathway or other FA degradation pathways. However if a high temperature causes a more rapid reduction in moisture content, then metabolic activity and therefore FA degradation will slow and then cease due to reduced a_w. Khan et al. (2011), concluded that from a practical perspective, FA stability in silage could usually be improved by minimizing wilt duration.

Lipid metabolism during wilting

Lipolysis; the splitting of ester bonds from glycerol-based lipids, results in the formation of free fatty acids (FFAs). Stress-activated plant lipases become active during the aerobic wilting phase, and preliminary lipolysis occurs during this phase (as a precursor to lipoxygenase activity). However during the wilt, the proportion of FFAs may fall due to oxidation and further breakdown of newly-formed FFAs. The proportion of neutral lipids (DAG + TAG) increases during wilting due to; 1) hydrolysis of ester linkages of polar membrane lipids by galactosidases and phospholipases 2) stress-induced DGAT activity, which causes FFAs (mainly from membrane lipids) to be re-esterified into the neutral fraction (Lee et al., 2006; Van Ranst et al., 2009a). The overall shift is predominately from the membrane lipid fraction to the neutral lipid

fraction. The extent of this shift depends upon the extent of PUFA oxidation and the balance of activity between different lipases and DGAT enzymes. Neutral lipids can make up a significant proportion of lipids in the eventual silage product e.g. (Van Ranst et al., 2009b) because unlike membrane lipids, they are stable during the fermentative phase.

2.9.2 Lipid and fatty acid metabolism during fermentation

Overall, forage lipids and fatty acids undergo much smaller changes during anaerobic fermentation than the carbohydrate or protein components. Regardless, lipid and fatty acid metabolism during the ensiling process in PRG and other pasture species has been thoroughly researched in recent years. FAs undergo only small changes during fermentation. Lipolysis *in silo* is an extensive process and so the majority of fatty acids in silage are un-esterified, whereas in fresh forage they occur in the membrane lipid fraction. A reduction in the degree of *in silo* lipolysis has been related to improved delivery of PUFAs into animal products. Significant recent research has focused (mainly in red clover) on exploring endogenous plant factors as a potential means of preventing extensive lipolysis *in silo* (Buccioni et al., 2012).

Fatty acid preservation during anaerobic fermentation

A meta-analysis performed by Glasser et al. (2013) combined data from studies from 1970 onward on the effects of various factors (including forage conservation) on forage fat and fatty acid concentration and composition, from a range of species. Pooled data for forage grasses and legumes showed the effect of ensiling at three different DM ranges; non-wilted (16.8-24.5 %DM), wilted (21.2-43.2 %DM) and haylage (48.8-70.0 %DM). The mean difference in total FA concentration between fresh forages and silages revealed a small, non-significant reduction in FA concentration due to ensiling in wilted silages and haylage (-0.71 and -0.41g/kgDM respectively). Mean losses of 18:3 were proportionally greater than losses of other FAs. Given that these changes were measured across the entire ensiling process, FA losses due to field wilting and losses due to anaerobic fermentation/storage were not separated. On the other hand, non-wilted silages had a slightly higher FA concentration than their fresh counterparts (+1.99g/kgDM), and the proportion of 18:3 did not change. The increase in FA concentration with the absence of a wilting phase, suggests that on average, changes in fatty acid content and composition during the anaerobic fermentation and storage phases are small (Glasser et al., 2013). Losses of other soluble or volatile DM constituents during fermentation (which are often higher in low DM silages) probably explain the slight increase in FA concentration.

Additives affect the fermentation but not FA concentration and composition

Silage additives can have a large impact on the type and the extent of silage fermentations. For example LAB inoculants increase the rate and extent of LA production and pH decline, while formic acid restricts fermentative activity by creating an inhibitory environment for anaerobic microorganisms. Providing that normal silage making techniques are employed, the FA concentration and composition of unwilted silages usually reflects the composition of the fresh crop (Glasser et al., 2013). They are largely unaffected by the use of various silage additives, which suggests that FAs are relatively stable in a well-sealed silo, independent of the type of fermentation that occurs (Arvidsson et al., 2009; Dewhurst & King, 1998; Van Ranst et al., 2009a).

Lipid metabolism during fermentation

In silo lipolysis results in an accumulation of FFAs and a concomitant reduction in membrane lipids during fermentation. Various authors have used different definitions of '*in silo* lipolysis' in their work. '*In silo* lipolysis' is sometimes stated as the percentage change in the concentration of total esterified lipids due to both wilting and ensiling e.g. (Elgersma et al., 2003; Ribeiro Alves Lourenço et al., 2005). In other cases, it is stated as the percentage change in the concentration of membrane lipids specifically, using wilted material as the starting point for calculations. If the former definition is used then the stated value for '*In silo* lipolysis' is lower because formation of TAG during wilting will mask the reduction in the concentration of membrane lipids during the fermentation.

Lipolysis of forage membrane lipids during fermentation is a very extensive process e.g. (Ribeiro Alves Lourenço et al., 2005; Steele & Noble, 1984; Van Ranst et al., 2013). Van Ranst et al. (2009b) recorded lipolysis of membrane lipids between 85% and 95% in ryegrass silages, across several cutting dates. In contrast, the proportion of neutral lipids often increases during the fermentation e.g. (Chow et al., 2004; Van Ranst et al., 2009b). Evidently, the lipases that are active *in silo* predominantly target polar lipids. The percentage of FFAs in ryegrass silages ranges from 31.5% (Van Ranst et al., 2009b) to 65.2% (Chow et al., 2004). However, the wide range of methods of extraction of lipid fractions used by various workers casts some doubt upon the exact proportions measured (Chow et al., 2004; Glasser et al., 2013).

Most *In silo* lipolysis of membrane lipids is mainly a result of continued plant lipase activity and occurs very early in the fermentation phase. For example, in red clover silages (wilted for 24 hours and inoculated with LAB), 70.6% of membrane lipids underwent lipolysis within 1 day of ensiling, and lipolysis had reached

only 86.8% by day 60 of ensiling (Van Ranst et al., 2010). However, these silages did not attain the pH required for successful preservation, which would indicate differences in microbial ecology and therefore possibly lipid metabolism compared to pH-stable silages. Generally speaking, microbial lipases may become increasingly important in lipid metabolism as microbial populations expand and plant cells die.

Factors influencing the extent of lipolysis in perennial ryegrass

Van Ranst et al. (2009a) provided evidence that lipolysis of membrane lipids in ryegrass silages may be reduced by restricting the fermentation by either wilting to a high DM content or through the use of a formic acid additive. Ryegrass wilted to approximately 30, 40 and 50% DM underwent lipolysis of membrane lipids to an extent of 69.1, 50.4 and 38.7%, respectively, after 8 weeks fermentation. The pH of these silages was, in all cases, above the threshold for successful preservation. This trend was not observed in a later study with ryegrass (Van Ranst et al., 2009b) or with red or white clover (*Trifolium repens*). Crops wilted to approximately 30% DM underwent lower lipolysis when treated with formic acid (Van Ranst et al., 2009a), and higher lipolysis when treated with a buffering solution, suggesting that a longer fermentation phase (and continued plant enzymatic activity) increases lipolysis.

2.10 Conclusion

Ensiling is an intriguing potential application of the D1o3-3 technology in perennial ryegrass (termed high lipid PRG from herein) because TAG, which is significantly elevated in these plants and accounts for the majority of their increased ME content, is relatively stable throughout the ensiling process compared to the other major nutritional components of PRG (WSCs, protein and polar lipids). Therefore, it would be expected that successfully ensiled high lipid PRG should make high lipid silage. Further, if the increased biomass accumulation rates in high lipid PRG (recorded in the glasshouse) are realized outdoors, then this may present farmers with a surplus of pasture cover. This could translate into a hypothetical 'use-it or-lose-it' scenario, where the animal performance benefits of feeding elevated lipids in the diet become increasingly diluted by the negative overall effect of advancing plant maturity on nutritional quality. In such a scenario, ensiling would be a useful option for preserving high lipid PRG for later use. Finally, uncharacterized metabolic changes in high lipid PRG may influence factors related to its pre-ensiling characteristics, fermentation pattern and/or ensiled nutritional value. If such changes were of a large magnitude, then changes to best-practice silage making or animal feeding recommendations with high lipid PRG silage would be necessary.

2.11 Experimental objectives and hypothesis

The primary objective of this thesis was to investigate the impact that the high lipid technology described in Winichayakul et al. (2013), had on aspects of the ensiling biochemistry of perennial ryegrass, and subsequently to assess the nutritional properties of the ensiled material as a ruminant feed. An important related aim was to achieve a rapid lactic acid fermentation of the plant material, to ensure that the nutritional properties of the ensiled material reflected those of well-preserved silage made under typical outdoor conditions. It was hypothesized that successfully ensiled high lipid PRG would make high lipid silage.

CHAPTER 3. METHOD DEVELOPMENT

3.1 Layout of the experimental work

The practical work in this thesis was divided into two phases. In the first phase, a series of 'method development' small-scale ensiling experiments (Experiments 1-4) were performed using the available leafy regrowth from *either* high lipid transgenic or non-transgenic PRG plants. These plants were grown in two glasshouse environments; a physical containment level 2 (PC2) containment glasshouse and a non-containment glasshouse, (both at AgResearch Ltd. Palmerston North). This phase focused on designing a repeatable 'high quality' silage-making protocol with small quantities of glasshouse-grown PRG (50 and 20g wilted), using paddock-grown PRG silage for comparison (Appendix IV). In the second phase, a 'transgenic versus wild type silage comparison' experiment was performed (Experiment 5) using a 'medium' and a 'high' lipid transgenic genotype and a non-transgenic control genotype grown in the PC2 containment glasshouse. This phase involved a detailed comparison of the ensiling process and nutritional properties of the material generated in experiment 5.

3.1.1 Plant material and glasshouses

In the non-containment glasshouse, 40 well-established PRG nursery plants; consisting of vegetative clones of the IMP566 genotype were inherited from the AgResearch Grasslands transformation laboratory, moved into the glasshouse and propagated in 3 litre pots with 8-9 month controlled-release osmocote potting mix*[1]. The IMP566 genotype is derived from the commercially available cultivar 'Grasslands Impact', and was selected by the Plant Biotechnology group for its amenability to the transformation procedures used by the laboratory (superior tissue culture characteristics; pers. comm, Kim Richardson). The IMP566 genotype was considered to be an ideal candidate for developing a small-scale ensiling protocol with glasshouse-grown PRG, leading up to the 'transgenic versus wild type silage comparison' (Experiment 5) because; 1) IMP566 is the background 'pre-transformed' genotype for the high lipid transgenic genotypes used in the Experiment 5 and 2) IMP566 is the 'non-transformed' genotype used as a control genotype for comparison to the transgenic genotypes in Experiment 5.

In the containment glasshouse, 6 to 12 PRG plants from each of three genotypes were inherited from the AgResearch Grasslands transformation laboratory and propagated in 3 litre pots with 8-9 month osmocote controlled-release potting mix*[1]. The plants from the two transgenic genotypes; ODR3704 ('high lipid') and ODR5203 ('medium lipid'), consisted of vegetative clones derived from independent IMP566 transformation events that resulted in the successful incorporation of D1o3-3 constructs (Chapter 2.8.5)

under green tissue-specific promoters, into their genome (Appendix VII). The 'control genotype' consisted of the non-transformed IMP566 genotype (referred to as 'wild type', WT in Experiment 5). Therefore, the transgenic genotypes differed genetically from the 'control genotype' only in the presence or absence of the D1o3-3 constructs. The two transgenic genotypes differed in the position and possibly copy number of the D1o3-3 constructs in the genome (pers. comm, Kim Richardson).

*[1] pH = 5.7, NO_3^-N = 27mg/L, NH_4^+-N = 14mg/L P = 3mg/L, K = 109 mg/L, S = 85 mg/L, Ca = 61 mg/L, Mg = 16 mg/L, Na = 33 mg/L, Fe = 21.8 mg/L, Mn = 8.0 mg/L, Zn = 2.28 mg/L, Cu = 1.03 mg/L, B = 0.07 mg/L, released over 8-9 months.

3.1.2 Method development phase

A substantial amount of the method development phase was based around modifying and optimizing post-harvest treatments of glasshouse-grown PRG in order to ensure that a rapid LA fermentation occurred. Paddock-grown PRG (Appendix IV) was ensiled alongside the glasshouse-grown PRG and was used as a 'reference point' to other ensiling experiments in the literature (where silage is made from PRG grown outdoors). Chemical analyses during the method development phase focused exclusively on measuring parameters related to the pre-ensiling characteristics of material (WSC, BC) and on parameters related to the fermentation pattern of the material (pH, fermentation acids).

Although it was recognized that the plant material and the abiotic environment in the two glasshouses differed (Appendix I), Experiments 1-4 provided critical information about the *gross* differences in the ensiling characteristics of glasshouse-grown and outdoor-grown PRG. These experiments provided data to optimize the post-harvest treatments for Experiment 5. The post-harvest treatments included a rapid wilt to an accurate DM content, and accurate application of a LAB inoculant and later glucose to the (wilted) pre-ensiled PRG. The development of each of these post-harvest treatments are described in detail in this chapter. Alongside this work, the number of plants in the containment glasshouse was increased using sequential vegetative propagations (Appendix III) in order to generate a sufficient quantity of material for Experiment 5.

3.1.3 Transgenic versus wild type silage comparison

This phase focused on comparing the high lipid transgenic genotypes to the WT genotype using an ensiling protocol developed in the method development phase. Comparisons were made in the following areas; 1) pre-ensiling characteristics, 2) fermentation pattern 3) efficiency of nutrient preservation during ensiling and 4) performance in an *in vitro* rumen incubation.

3.2 Small-scale ensiling

In all ensiling experiments, the amount of plant material available was severely limited due to working in glasshouse facilities. The quantities of wilted plant material used for individual vacuum bag silage packets (termed silos from herein) were 20g for measuring silage pH at intervals throughout the fermentations and 40-50g for detailed 'silage quality' and nutritional analysis. Both quantities are lower than has been previously reported in the literature for vacuum bag silos. The sizes chosen were a compromise between the amount of plant material available, modelling accuracy and the need to generate technical replicates for fermentation analyses over time. Due to the small size and number of silos, it was necessary to ensure careful pre- and post-harvest treatment of the material in order to ensure a minimal amount of variation in the material entering each silo. This was achieved through;

- An adequate model silo design.
- The use clonal PRG plants (in Experiment 5), randomized in space and shuffled around the glasshouse bench regularly during regrowth to minimize positional effects.
- Harvesting homogenous leafy tissue, and largely excluding stem and dead material from silos.
- Pooling all the harvested regrowth from a given genotype/population*[2] and then thoroughly mixing the entire mass at key time points post-harvest.
- The use of a commercial LAB inoculant to reduce the probability of unusual fermentations due to variation in natural or introduced microflora on leaf surfaces.
- Using controlled rapid wilting procedures, to ensure a consistent DM between PRG materials within an experiment.
- Even and accurate application of additives (commercial inoculant and later glucose).

*[2] the terms genotype and population are both used because some experiments involved comparing the ensiling characteristics of a PRG genotype with a PRG population (Appendix IV).

3.2.1 Model silo design

A double-bag model silo was used for Experiments 1-5. The design followed the method described in Hoedtke and Zeyner (2011) for vacuum packed plastic bag silages, with minor modifications. The desired amount of wilted and chopped PRG (+/- 0.5g), with or without additives, was placed in an ordinary plastic bag (Photograph 3.1, top left), and compressed by hand into one corner of the bag. The top of this bag was twisted and cut (Photograph 3.1, top right) and then the open end was taped down. The constrained herbage was further compressed and shaped by hand and then rubber bands were used to generate fairly uniform and spherical packets under compression (Photograph 3.1, bottom left). One rubber band was

used per 20g silo and two rubber bands per 50g silo. The bags were punctured with a disinfected needle to allow gas and effluent escape. The bags were placed into a 20 x 15 cm, 130 micron thick vacuum bag (Munro, Australia), from which air was evacuated with a vacuum packer (V.350, LAVA vacuum package, Bad Saulgau, Germany) (Photograph 3.1, bottom right). Using manual settings, the vacuum was allowed to reach the 50mbar maximum vacuum setting and was then left at this level of vacuum for 10 seconds (for 20g silos) or 20 seconds (for 50g silos), before triple heat sealing.

Photograph 3.1 *Steps for ensiling perennial ryegrass by double bagging and vacuum packing*.

Advantages of silo design

Generating these double bagged spherical silos was considered to be advantageous because herbage was held in a single mass at a high density by the rubber bands for the duration of their fermentation, even when production of silo gases or effluent led to a loosening of the outer vacuum bag. Therefore compression was considered to represent packing density in a silage stack. Further, the shape minimized the surface area to volume ratio of the herbage within the silos.

3.2.2 Preliminary silo assessment

Prior to the major method development ensiling experiments (Experiments 1-4), the model silo was tested using paddock-grown perennial ryegrass. The model silo design was assessed primarily by pH readings of water extracts of ensiled material (as described in Chapter 3.6.5) and also by visual observation and volume measurements. Model silos of a given herbage weight were visually similar in size and in shape. Herbage was held at an approximate density of 0.5g.cm^{-3} (measured using the water displacement method) immediately after vacuum packing, a density comparable with stack silo densities recorded in the literature (0.32-0.64g cm^{-3}) (Muck & Holmes, 2000; Muck et al., 2004). Accurate measurements of herbage density were not possible after this, due to gas production from fermentation. However, the inner bags could be seen to retain their shape and approximate volume throughout ensiling e.g. (Photograph 3.2).

Photograph 3.2 *Preliminary silo assessment; 50g paddock-grown perennial ryegrass silage packets.*

Paddock-grown perennial ryegrass silage pH curve

The pH of water extracts of silage made from 50g and 20g paddock-grown PRG, wilted to approximately 30% DM, double-bagged, and vacuum-packed (with and without LAB inoculant) showed that well-preserved silage could be made using this method. Very low variation in pH occurred between duplicate silos, especially for inoculated material (Table 3.1). The data also indicated that the reduction in pH over time was similar for 20g and 50g paddock-grown PRG silage, providing that the same post-harvest treatments were applied and that care was taken in mixing plant material thoroughly at key post-harvest time points.

Table 3.1 *pH of double-bagged, vacuum packed silos made from 20g and 50g wilted paddock-grown perennial ryegrass, with and without commercial inoculant. Values represent means ± SD (n = 2). Day 0 pH was estimated from the literature (Playne & McDonald, 1966).*

Days of fermentation	20g		50g	
	+LAB	-LAB	+ LAB	-LAB
0	6.50	6.50	6.50	6.50
2	4.14 ± 0.01	6.31 ± 0.03	4.12 ± 0.07	6.20 ± 0.1
4	4.00 ± 0.07	4.85 ± 0.01	3.99 ± 0.01	4.95 ± 0.07
8	3.97 ± 0.07	4.55	3.96 ± 0.07	4.70 ± 0.06
30	3.95 ± 0.07	4.25 ± 0.03	3.93 ± 0.07	-

20g: Twenty gram silo, 50g: Fifty gram silo, + LAB: *L. plantarum* inoculant applied, -LAB: No *L. plantarum* inoculant applied.

The following sections provide justification for the post-harvest treatments which were sequentially introduced during the method development phase (Experiments 1-4) in order to generate well-preserved silage from glasshouse-grown PRG. These post-harvest treatments, and the ensiling experiment in which they were introduced, were; LAB inoculant addition (Experiment 1), a controlled, rapid wilt (Experiment 1), and glucose addition (Experiment 4). Protocols for each post-harvest treatment are described.

3.3 Addition of a commercial LAB inoculant

Uncertainty existed about the taxonomic composition of PRG epiphytic microflora in the glasshouse environment. It was speculated that the population composition might vary from the established typical counts reported in the literature (Table 2.1) for the reasons listed below;

1) **Abiotic growth environment;** epiphytic microorganisms are naturally found in high numbers on the lower parts of leaves and stems in order to avoid UV radiation and desiccation. The low light, high humidity and high temperature conditions in the glasshouses relative to outdoors (Appendix I, Appendix II) were expected to favour the growth of most epiphytic microorganisms. However, the microorganisms relevant to ensiling that predominated on leaf surfaces were unknown.
2) **Spaced potted plants;** The amount of bare soil around a plant and the proximity of harvested material to the soil are regarded as important in the context of silage microbiology because the extent of contact between harvested organs and soil microorganisms negatively affects the fermentation pattern (Leibensperger & Pitt, 1987; Pahlow et al., 2003). Given the use of individual potted plants in this work, these effects were potentially larger than in the majority of ensiling experiments, where grass is grown in a sward and therefore plant exposure to the soil surface is low.

Further, it was anticipated that effects on silage microbiology might result from the post-harvest treatments employed and/or the use of very small scale silos;

3) **Sample size;** because ensiling is a solid-state fermentation, it relies on the natural distribution of (non- motile) LAB on leaf surfaces. Conditions on a standing crop are non-uniform, and so successful fermentation relies to some extent upon redistribution of LAB during the harvesting and chopping procedure (Pahlow et al., 2003). The use of small scale silos can generate atypical fermentations if measures are not taken to ensure that a sufficient number of LAB are present on the pre-ensiled crop (McDonald et al., 1991).
4) **The 'chopper inoculation' phenomenon**; Pahlow et al. (2003) described the observation that detectable LAB numbers increase dramatically due to the release of plant juices during chopping. This effect is increased by the degree of mechanical disturbance of plant tissues during harvesting and chopping (Muck, 1989). Given that scissors were used to chop the plant material in these experiments (rather than mechanical equipment as is used by industry), the extent of LAB resuscitation during harvest was likely much lower.
5) **Wilting effects**; placing plant material in an oven at 35-45°C inevitably affects populations of epiphytic microflora. The use of different ovens/wilting methods in order to equate the DM content of PRG

genotypes/populations in a timely manner before ensiling (Chapter 3.4) may have generated unwanted variation in epiphytic microflora among treatments within experiments.

3.3.1 Commercial inoculant application protocol

Accurate application of a LAB inoculant ('Ecosyl MTD/1 100', Batch # 184046, 08/2014, Volac International Ltd, West Glamorgan, Wales) was employed as a strategy to overcome the probability of atypical fermentation patterns due to unforeseen variability in epiphytic microflora in the glasshouse environment. Although this LAB inoculant was used in Experiments 1-5, an accurate method of delivery of the suspended inoculant to the pre-ensiled PRG was not *fully* developed until Experiment 3. Preliminary experiments verified that the bottle used throughout contained sufficient numbers of LAB to increase the rate of silage pH decline when applied at the recommended application rate (Table 3.1). The true quantity of LAB in the preparation was not determined, however the method of delivery of suspended inoculum solution was accurate. Therefore, although estimated rates of LAB delivery may have been inaccurate, they were similar for treatments within an experiment (and between experiments).

The manufacturers' estimation was $1.54x10^{11}$ cfu LAB/g freeze-dried preparation. The instructions given were to suspend the contents of the bottle (containing approximately 500g freeze-dried granulated inoculant) in 100L of water, and apply this formulation (approximately 5g/L) at 1L per tonne of forage. According to the manufacturer, this would guarantee at least 10^6 live *Lactobacillus plantarum* per g of forage. In order to increase the accuracy of delivery of LAB to small quantities of plant material, a more dilute formulation was used (0.125g/L instead of 5g/L). Application of the inoculant at this dilution rate decreased the effective DM content of the pre-ensiled plant material by approximately 1%. The following protocol was used in Experiments 3-5 to make up an inoculant solution and apply it to wilted, chopped plant material;

1) Approximately 0.5g of Ecosyl MTD1 freeze-dried commercial LAB inoculant ($1.54x10^{11}$ cfu/g) was suspended in 1 litre of milli-Q water in order to make up a stock solution.
2) A 4x dilution of this solution was made up by combining 1 part stock solution with 3 parts milli-Q water.
3) The solution was poured into a small atomizer (Photograph 3.3). The atomizer containing the solution was calibrated using scales. The atomizer that was used expelled approximately 1g inoculant solution with 8 full lever compressions. Chopped, wilted plant material was spread thinly and evenly, 25g at a time into a clean container on a set of digital scales (Photograph 3.3).

The inoculant solution was applied (using the atomizer) at a rate of 8 sprays per 25g plant material, as a fine mist, as evenly as possible between each layer of plant material. The scales were re-zeroed before each addition of plant material or inoculant to record the actual rate of application per unit weight of plant material. Afterwards, the entire contents of the container was thoroughly mixed before vacuum packing.

Photograph 3.3 *Layered, chopped and wilted perennial ryegrass during the inoculation procedure. The small atomiser bottles were used for inoculation in Experiments 2-5.*

3.4 Controlled rapid wilt

PRG was rapidly wilted in ovens at 35-45°C to a target DM content between 25 and 40% before ensiling. This DM content range was considered appropriate for several reasons;

1. **Industry practice;** wilting pasture grasses to between 25 and 40% DM before ensiling is a common practice in New Zealand and other countries where the climate is favourable (Muck et al., 2003). Silage within this approximate DM range is generally termed 'wilted silage'. Higher DM content silage is termed 'haylage'.
2. **Fermentation 'quality';** reducing crop moisture content favours LAB growth over clostridial growth (Leibensperger & Pitt, 1987). Wilting is a cheap, effective and popular means of reducing the probability of undesirable fermentative activity and thus nutrient losses (Haigh, 1996).
3. **To assess the stability of lipids in the high lipid PRG during wilting;** Oxidation of polyunsaturated fatty acids during the aerobic wilting phase is a major source of fatty acid losses during forage conservation (Dewhurst et al., 2003b). Therefore, a 'post-wilt' quantitative FA analysis was performed in order to compare the stability of fatty acids during wilting in the transgenic and control genotypes.
4. **To equate DM content between treatments;** the DM content of freshly harvested PRG in Experiments 1-5 varied widely. Wilting to a DM content well above the fresh DM content allowed an assessment of the fermentation pattern of various PRG genotypes/populations at a given, accurate DM content. Other studies have reported confounded treatment differences in ensiled crops due to inaccurate wilting procedures (and therefore different DM contents) between crops at ensiling (Van Ranst et al., 2009b).
5. **To minimize effluent production;** in the model silos that were used herbage density was high. With non-wilted or 20-25% DM wilted silage, crushing of plant material led to effluent production (Photograph 3.5, bottom left). The silos used provided no means of effluent escape (as would occur in a silage stack) and so increasing silage DM to an extent where effluent production was minimized better suited the model silo design.

Practical difficulties regarding accurate wilting

Achieving a target DM content proved difficult in the early method development phase. This difficulty has been expressed by other workers (Cherney et al., 2006; Van Ranst et al., 2009b). Preliminary work indicated that the rate of moisture loss during laboratory bench wilting was slow and variable so an oven wilt at 35-45°C was employed for Experiments 1-5. It was found that the most reliable method of wilting

to a precise target DM content was to regularly reweigh samples of a known fresh mass, following DM content determination of a subsample from that fresh mass (see below). Although PRG was wilted in Experiments 1-5, a high accuracy wilting method was not *fully* developed until Experiment 4.

3.4.1 Wilting protocols

The wilting method employed (target DM content, oven model, temperature, and containers used) varied depending on the experiment and on the physical containment requirements of the plant material (Photograph 3.4). The accuracy of the wilt increased throughout the experiments (Table 3.2). For ensiling experiments with containment glasshouse material (Experiments 2, 4, 5), oven space was severely limited, and so the individual PRG genotypes had to be wilted separately in two small ovens (Photograph 3.4).

Photograph 3.4 *Two wilting procedures*. **Left & center;** *transgenic perennial ryegrass being wilted in separate ovens within bags or plastic trays in the containment glasshouse ovens (Experiment 4).* **Right;** *non-containment glasshouse-grown perennial ryegrass being wilted in steel trays (Experiment 3).*

Table 3.2 *Wilting accuracy achieved during Experiments 1-5.*

Experiment	Target DM content	Minimum DM content of a treatment	Maximum DM content of a treatment
1	25%	20.4%	22.7%
2	30%	29.0%	32.3%
3	40%	40.1%	40.2%[1]
4	34%	34.1%	34.2%
5	36%	35.5%	36.6%

[1] this wilted DM was calculated after the reapplication of water spray.
DM = dry matter

For Experiments 2-5, 3 x 20g subsamples were removed from the mixed mass of each pooled PRG genotype/population immediately after harvesting, and placed in aluminium trays in an oven at 80°C for 4-5 hours, or until a consistent weight was reached. The average DM content of the fresh material was calculated and then used to determine the appropriate extent of wilting. In experiments 4 & 5 an automated spreadsheet for these purposes (Appendix VI). The aim was always to equate the DM content

of all the PRG genotypes/populations within an experiment, before ensiling. With experience using this method, the wilted DM could usually be achieved to within 1% point of the DM target for that particular experiment (between 25 and 40% DM, Table 3.2). Material was wilted in steel trays, plastic trays and plastic perforated bags (Photograph 3.4). The oven temperature used was manipulated over the range of 35-45°C in order to increase or decrease the wilting rate for convenience. Although the wilting methods employed were not identical (between treatments within experiments or between experiments), the wilting methods were all similar in that; 1) all wilting periods were quite short (6-18 hours) compared to field wilting and 2) all were at a fairly high temperature. DM losses during wilting were assumed to be negligible.

3.5 Addition of glucose

Repeated ensiling experiments (1-3) using LAB-inoculated glasshouse-grown PRG indicated that this material was difficult to successfully ensile over the normal silage DM content range. However, pH and fermentation end product measurements of silages made from PRG grown outdoors in Experiments 1 & 3 verified that the vacuum packing technique employed and the commercial inoculant application procedure were adequate for making well-preserved silage. Henceforth, the difficulty in making well-preserved, stable silage from glasshouse-grown PRG was attributed to its low WSC:BC.

3.5.1 Glasshouse-grown perennial ryegrass pre-ensiling characteristics

Buffering capacity

In order to maximize plant biomass accumulation before each ensiling experiment, N fertilizer was regularly applied to potted plants. Fertilizer ('Thrive all-purpose soluble'*[3], Yates, Australia) was applied at the manufacturer's recommended rate fortnightly during each regrowth period (usually once after cutting and then again 2 weeks later). Due to the very fertile growth conditions, it was predicted that the BC of the glasshouse-grown PRG would be fairly high relative to PRG grown outdoors harvested at a similar stage of maturity. In Experiments 1-5, glasshouse-grown PRG was harvested after 3-5 weeks of regrowth (range 22-33 days). It was therefore predicted that the BC at harvest would range between approximately 45-65gLA/kgDM (Chapter 2.6.3).

*[3] Formula (w/w); N = 25%, P = 5%, K = 8.8%, S = 4.6%, Mg = 0.5%, Fe = 0.18%, Mn = 0.01%, B = 0.005%, Cu = 0.005%, Zn = 0.004%, Mo = 0.00%. Approximately 9g dissolved into 4.5 litres of water, applied at approximately 300ml per potted plant.

Water soluble carbohydrates

Measurements of WSC contents of pre-ensiled fresh material provided evidence that the abiotic environment in the two glasshouses was unfavourable for WSC accumulation (Tables 3.5 and 3.7). The combination of a very low WSC content and fairly high BC meant that all glasshouse-grown PRG had a WSC:BC ratio well below 2 at the time of harvest; a value below that of typical outdoor-grown PRG (King et al., 2013; McDonald et al., 1991). Given that well-preserved silage could not be made over the typical silage DM content range, fermentable sugars in the form of glucose were applied to wilted material in Experiments 4 & 5.

3.5.2 Glucose application protocol

A solution of 1 part analytical grade D+ glucose to 2 parts milli-Q water was made up and this solution was poured into a small atomizer. The atomizer containing the solution was calibrated using scales. The atomizer expelled approximately 2.4g glucose solution with 16 full lever compressions. Chopped, wilted, inoculated plant material was layered 25g at a time into a container on a set of scales. The sugar solution was applied (using the atomizer) as a fine mist, as evenly as possible between each layer of plant material. The scales were re-zeroed before each addition of plant material or liquid to record the actual rate of application per unit weight of plant material. Afterwards, the entire contents of the container were thoroughly mixed before ensiling. Using this method, and a measurement of the wilted DM% of the PRG, the desired quantity of glucose could be increased to within 0.1% of the target (% of DM) increase.

Quantity of glucose applied

Glucose was applied to the pre-ensiled material at a rate of 5% and 7.5% DM, in Experiments 4 & 5, respectively. The chosen application rate was based on an informed estimation of the quantity of WSCs required to support a LA fermentation, given an estimate of the WSC:BC of the plant material being ensiled (data which was not available at the time). Prior measurements of the WSC content of glasshouse-grown PRG after a regrowth interval of approximately 1 month were in the range of 80-105g/kgDM. Measurements of glasshouse-grown PRG BC were in the range of 45-65gLA/kgDM. Therefore it was anticipated that the WSC:BC was between 1.2 and 2.3. The decision to apply glucose at 50g/kgDM and 75g/kgDM in Experiment 4 & 5 respectively was expected to increase the WSC content to near the literature mean for outdoor-grown PRG; 181g/kgDM (White, 1973) and increase WSC:BC to at least 2.0. The added glucose was expected to be efficiently converted into LA, given it's the simultaneous application with the LAB inoculant to the exterior of the leaves. Excess glucose was applied in Experiment 5 given the particularly difficult conditions for WSC accumulation during this regrowth period (Table 4.6).

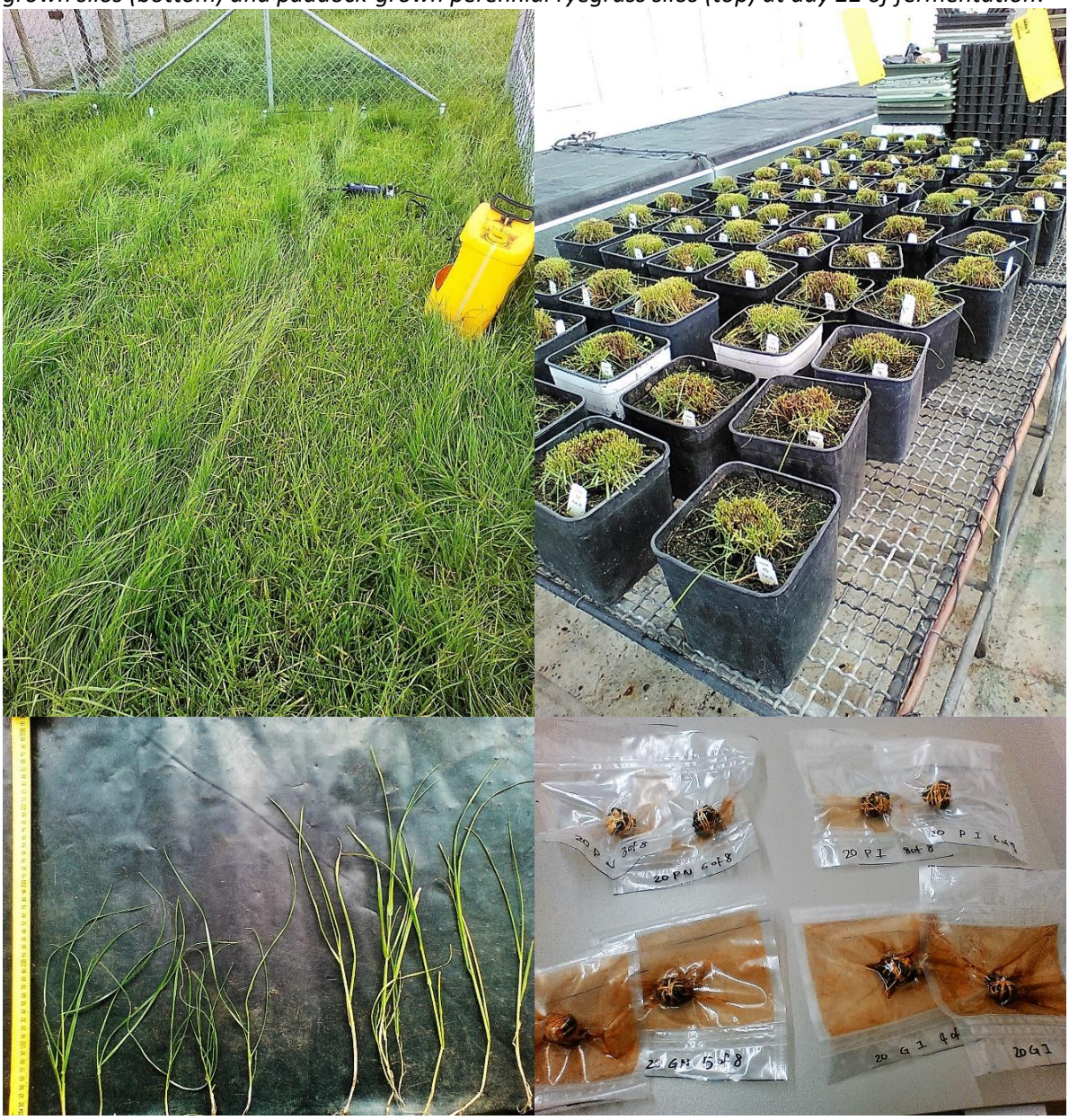
The following section describes, in order, the 4 experiments which investigated the ensiling biochemistry of glasshouse-grown PRG. The detailed protocols for; LAB inoculant addition, controlled wilting and glucose addition (described above) were developed *during* these experiments.

3.6 Ensiling experiments 1-4 materials and methods

3.6.1 Experiment 1

Objectives: The aim of this ensiling experiment was primarily to compare the fermentation pattern of silage made from (non-containment) glasshouse-grown and paddock-grown PRG wilted to 20% DM. Further, the effect of two silage sample sizes; 20 and 50 g, and the effect of using a commercial LAB inoculant on the silage fermentation were explored.

Photograph 3.5; Top left; *section of paddock during harvest.* **Top right;** *glasshouse plants after cutting.* **Bottom left;** *5 x glasshouse tillers (left) and 5 x paddock tillers (right).* **Bottom right;** *20g glasshouse-grown silos (bottom) and paddock-grown perennial ryegrass silos (top) at day 22 of fermentation*.



Harvest: The 33 days of vegetative regrowth that accumulated between the dates 25/09/2015 and 27/10/2015 from paddock-grown PRG and (non-containment) glasshouse-grown potted PRG was harvested with electric shears between 10:00 am and 12:00 pm. A total of 72 potted plants and 4 m^2 of paddock-grown PRG yielded approximately 2.8kg and 1.2 kg material respectively. Cutting height was adjusted for each plant/section of paddock in order to avoid harvesting excessive dead material and stubble. Therefore, the cut height from the soil in the paddock (17.7cm, range 10-20 cm) (Photograph 3.5, top left) was higher and more variable than in the glasshouse (6.5 cm, range 5-8cm) (Photograph 3.5, top right). The difference in cut height was necessary due to the different distribution of plant organs between the two PRG materials, which clearly developed differently in these two vastly different environments (Photograph 3.5, bottom left). This allowed for a physiologically relevant comparison between the ensiling characteristics of the *leaf* material of paddock-grown and glasshouse-grown PRG. After thorough mixing of the harvested mass, representative subsamples were removed for DM determination (Chapter 3.4.1). Dry weight analysis of these subsamples showed that 93% and 94% of the harvested material was leaf material from paddock-grown PRG and glasshouse-grown PRG respectively. The remainder was sheath and dead material.

Post-harvest treatments: Material was placed in steel trays in an oven at 37.5 °C for 5 hours. During this time, the paddock-grown PRG DM increased from 17.7% to 22.7% and the glasshouse-grown PRG DM increased from 15.2% to 20.4%. The material was chopped with scissors to 2-4 cm lengths, mixed in large containers and then divided into inoculated and non-inoculated treatments. Inoculation differed from the more precise protocol described in Chapter 3.3; a 5 x dilution of the stock solution was used, and was applied at a rate of 3 mls per 50g of plant material with a larger spray bottle (approximately 924,000 cfu/g crop). Further, actual rates of dry inoculant applied by weight were not recorded. After inoculation, material was again thoroughly mixed.

Ensiling, storage and analysis: Materials were divided into 50g and 20g groups and ensiled as described previously in this chapter. Silos were stored in a controlled temperature room in darkness at 17 °C. A large volume of effluent was released from all glasshouse PRG silos in the first week of fermentation (Photograph 3.5, bottom right). The outer bag of the silos were observed to swell to different degrees due to silo gas production. Duplicate 20g silos were opened and pH analyzed on days 8, 22 and 86 of fermentation, and the fermentation end product profile of silages were analyzed on day 22 (Chapter 3.6.5).

3.6.2 Experiment 2

Objectives: The aim of this ensiling experiment was to observe the fermentation pattern of silage made from glasshouse-grown, LAB-inoculated, medium lipid transgenic PRG *accurately* wilted to 30% DM. pH analysis of 20g silos was performed during the early fermentation phase of ensiling.

Harvest: The 22 days of regrowth that accumulated between 20/10/2015 and 12/11/2015 from the transgenic 'medium lipid' genotype in the containment glasshouse was harvested with scissors at 8:00 pm. Again, the cut height from the soil was adjusted for each plant in order to avoid harvesting dead material, and averaged 6.7 cm (range 5.5-9 cm). After thorough mixing of the harvested mass, subsamples were removed for DM determination (Chapter 3.4.1). Dry weight analysis of these subsamples showed that >90% of the harvested material was leaf.

Post-harvest treatments: Material was placed in perforated plastic bags in a small forced draught oven at 40°C for 6 hours. During this period the DM content increased from 20.5% to 33.4%. Material was then chopped to 2-4 cm lengths and mixed. Inoculation proceeded as described in Chapter 3.3, although actual rates of inoculant application by weight were not recorded. Following inoculant spray application, the effective DM content decreased to 32.1%. After inoculation, material was again thoroughly mixed.

Ensiling, storage and analysis: 10 x 20g silos were generated and stored in darkness at 17°C. Duplicate silos were opened and pH analyzed on days 2, 4, 8, 14, 22 of the fermentation. Gas and effluent production were observed to be low compared to glasshouse material from Experiment 1.

3.6.3 Experiment 3

Objectives: The aim of this ensiling experiment was to compare the pre-ensiling characteristics and the fermentation pattern of silage made from *accurately* LAB-inoculated (non-containment) glasshouse-grown and paddock-grown PRG *accurately* wilted to 40% DM. Further, the effect of two silage sample sizes (20 and 50 g) on the fermentation were again explored after the more precise wilting and inoculation procedures.

Photograph 3.6 Experiment 3 harvest. **Left;** *paddock-grown perennial ryegrass during harvest.* **Right;** *two glasshouse-grown perennial ryegrass plants prior to harvest..*

Harvest: The 30 days of regrowth that accumulated between the dates 02/11/2015 and 3/12/2015 from both paddock-grown PRG and (non-containment) glasshouse-grown potted PRG was harvested with electric shears between 3:00 and 5:00 pm. A total of 43 potted PRG plants and 6 m^2 of paddock-grown PRG were harvested. The paddock-grown PRG from this regrowth was visibly stressed compared with the regrowth in Experiment 1. This was presumably a result of cutting to <10cm on 27/10/2015 and 02/11/2015 and/or soil nutrient deficiencies (Photograph 3.6, left). The cut height from the soil in the paddock (9.2cm, range 6-12 cm) was higher and more variable than in the glasshouse (6.5 cm, range 5-8cm). The difference in cut height was justified for the same reasons as in Experiment 1. The glasshouse plants were undergoing a reproductive transition and differed widely in their appearance and developmental stage (Photograph 3.6, right). After thorough mixing of the harvested mass, subsamples were removed in triplicate for DM determination and a single subsample was removed for fresh chemical analysis (stored at -20°C). Dry weight analysis of these subsamples showed that 87% and 86% of the material ensiled was leaf material from paddock-grown PRG and glasshouse-grown PRG, respectively. The majority of the remaining proportion of the material was reproductive tillers.

Post-harvest treatments: Material was placed in steel trays in an oven at 35°C overnight. During this time, the paddock-grown PRG DM content increased from 21.7% to 46.3% and the glasshouse PRG DM content increased from 20.9% to 41.9%. Water spray was reapplied evenly to the over-dried paddock-grown PRG (with the same model atomizer used to apply the inoculant) in order to bring the effective DM down to approximately 42%. The material was chopped with scissors to 2-4 cm lengths and mixed. Inoculation proceeded as described in Chapter 3.3 with actual rates of inoculant application by weight recorded (Table 3.3). Following inoculant spray application, the effective DM content had decreased to an estimated 40.1% and 40.2%, for the paddock-grown PRG and glasshouse-grown PRG respectively. After inoculation, material was again thoroughly mixed.

Table 3.3 *Average rate of LAB application by weight to perennial ryegrass in Experiment 3*

	Grass added (g)	Liquid spray (g)	cfu LAB/g PRG
Target	25	0.0001	800,000
Paddock	25.26	0.00102	788029
Glasshouse	25.48	0.00108	824190

LAB = *L. plantarum*, cfu = colony forming units, PRG = perennial ryegrass

Ensiling, storage and analysis: Material was divided into 50g and 20g groups and ensiled. Silos were stored in a controlled temperature room in darkness at 17°C. Effluent and gas production from these silos was observed to be minimal. Duplicate 20g silos were opened and pH analyzed on days 3, 6, 18, 40 and 72 of fermentation, and the fermentation end product profile of silages was analyzed on day 40. Fresh subsamples were stored for several weeks at -20°C for analysis of pre-harvest pH, WSC and BC (Chapter 3.6.5).

3.6.4 Experiment 4

Objectives: The aim of this ensiling experiment was to compare the pre-ensiling characteristics and the fermentation pattern of silage made from *accurately* LAB-inoculated, containment glasshouse-grown wild type, medium lipid and high lipid (transgenic) PRG *accurately* wilted to 34% DM. Additionally, the effect of adding glucose at a rate of 5% DM to the pre-ensiled PRG was explored. Only a limited number of the planned analyses were actually performed due to a major error in the silage-making procedure. Briefly; glucose was dissolved directly into the LAB inoculant solution, at a sufficiently high concentration (approximately 2M) to induce osmotic stress on these microorganisms prior to ensiling. Although the silage reached a low pH, this error led to a prolonged and uneven fermentation between PRG genotypes (Appendix XIII), which would have subsequently affected any silage nutritional comparison during the stable phase (analyses were not performed).

3.6.5 Chemical analyses

Buffering capacity

Buffering capacity (BC) was quantified using the methodology described by Playne and McDonald (1966). BC measurements were made using 5g fresh pre-ensiled plant material (stored at -20 °C) homogenized in a kitchen blender (George Foreman, 21820 AU) with 50 ml deionized water. The homogenized plant material was filtered and the water extract was weighed, transferred into a plastic vessel with a magnetic stirrer and titrated to pH 3.0 with 0.1M HCl in order to release the bicarbonate ion as carbon dioxide. The water extract was then titrated up to pH 6.0 with 0.1M NaOH. BC was calculated using the Milliequivalents of NaOH required to raise the pH of the water extract from 4.0 (±0.02) to 6.0 (±0.03) after correction for the titration value of a water blank. A calibrated pH meter (PHM2200, F-69627, Radiometer analytical, Lyon, France) was used for measuring the pH of the extract as NaOH in a burette was gradually added to the extract, with automatic mixing equipment (Photograph 3.7). Before each set of BC measurements, 0.1 NaOH was titrated against a primary standard in order to allow accurate comparisons of plant BC from different experiments. The BC values of samples were determined as a mean of duplicate assays.

Photograph 3.7 *Buffering capacity measurement equipment.*

Water soluble carbohydrates

Water soluble carbohydrates (WSCs) were extracted and quantified using the method described by Parsons et al., (2004). To extract the WSCSs a 25mg sample of freeze-dried plant material was mixed with 80% ethanol in a 2ml screw cap tube and incubated in an Eppendorf Thermo Mixer (12,000rmp, 65°C for

30 min). After each extraction the homogenate was centrifuged (13,000rpm, 15min) and the supernatant removed. This supernatant contained fructose, glucose, sucrose and fructans of a low degree of polymerization (DP) (referred to as the low molecular weight or LMW fraction). Fructans of a high DP (high molecular weight or HMW fraction) were extracted by twice mixing the remaining insoluble residue with 1ml of water and centrifuging (13,000rpm, 15min), then removing the supernatant. WSCs were analyzed in LMW and HMW fractions by the anthrone method (Jermyn, 1956). The assay used a standard 96-well microtiter plate with a Spectramax plate reader and Softmax Pro 3.0 software (Molecular Devices, Sunnyvale, CA). An aliquot of 3µl or 10µl of extracts were diluted to 50µl by 50mM KH_2PO_4 / K_2HPO_4 (pH: 7.0) and reacted with 250µl 1.25% anthrone in a mixture of H_2SO_4 and EtOH (6:4 (V:V)). The mixtures were agitated on the plate reader by using its mixing function and then incubated at 65^{o}C for 25 min. The blue-green color produced from the reaction between fructose and anthrone reagent was read at 620nm. LMW WSCs were calibrated against a series of known concentration of sucrose and HMW WSCs were standardized using inulin standards. All samples and standards were determined as a mean value of duplicate assays.

pH analysis

Silage quality was primarily determined by measuring the pH of silage water extracts at intervals along the fermentations. In Experiments 1-5, duplicate silos from each treatment and time point were pH analyzed. A standardized silo sampling and water extract preparation method was used throughout; sealed silos were cut open and then stored at -20°C until processed. Silos were allowed to defrost for 1-2 hours before the contents were emptied into a large clean vessel and mixed thoroughly (ensuring any effluent was distributed throughout the ensiled mass) (Photograph 3.8). A subsample of 5g of silage was removed for pH analysis. For day zero pH analysis a 5g fresh subsample was used. Water extracts were prepared by homogenizing plant material with room temperature milli-Q water in a kitchen blender, in a 1: 10 ratio of plant material to water, followed by a filtration step (Cherney & Cherney, 2003). In all cases the pH meter (PHM2200, F-69627, Radiometer analytical, Lyon, France) was calibrated before each set of readings.

Photograph 3.8 *Silage subsampling protocol.* **Left;** *open silage packet defrosting*. **Right;** *mixing packet contents prior to subsampling.*

Fermentation end products analysis

Silage fermentation end products were quantified by the Internationally Accredited New Zealand-approved Nutrition laboratory at Massey University (Palmerston North). In Experiments 1, 3 and 5 the analyses were performed on days 22, 40 and 45 days of fermentation, respectively. Common silage volatile fatty acids (VFAs); acetic acid (AA), propionic acid (PA) and butyric acid (BA) were measured (Shimadzu GC17A). Initial silage LA measurements from Experiments 3 and 5 using the D LA rapid assay kit (Megazyme Ltd.) were repeated using the D/L LA rapid assay kit (Megazyme Ltd.) on account of the late realization that initial measurements were only of the D isomer of LA. Measurements from Experiment 1 could not be repeated to include data on both LA isomers. Ethanol was not quantified. The stated g/kgDM concentration of silage fermentation end-products (wet analyses) used the post-wilt (oven) DM content measurement for calculations rather than the silage (freeze-dried) DM content measurement. This was considered to be advantageous because silage ethanol (a volatile) was not measured, but may have constituted a large proportion of the fermentation profile. Therefore the silage DM content could not be accurately corrected for all volatile losses during the freeze drying process.

It should be acknowledged that microbial enumeration was considered unnecessary in these experiments. The application of a commercial LAB inoculant pre-ensiling (as advised by McDonald et al. (1991) for small scale laboratory silages) likely led to active LAB population counts far in excess of competing microbial species. Aerobic stability, another important measure of silage quality (Keady & O'kiely, 1996) was not measured due to the limited quantity of material.

3.6.6 Statistical analysis

Statistical analysis was only conducted for Experiments 1 & 3 because Experiment 2 did not involve any comparison and because an error occurred in the silage-making procedure for Experiment 4.

Experiment 1

Using the open source R 3.2.0 software, separate two-way analysis of variance (ANOVA) tests were performed at multiple time points during the fermentation in order to investigate statistically significant differences in silage pH due to the two factors; 'Material' (Glasshouse and Paddock),'Inoculation' (+LAB and –LAB) and their interaction. A three-way ANOVA test was performed in order to investigate differences in the concentration of fermentation products on day 22 of the fermentation due to the three factors; 'Material' (Glasshouse and Paddock),'inoculation' (+LAB and –LAB) and 'Silage sample size' (50 and 20 grams) and their respective interactions. Significance levels were set at $p<0.05$. Where significant differences were detected, comparisons of treatment means were further investigated using Tukey's honestly significant differences (HSD) test.

Experiment 3

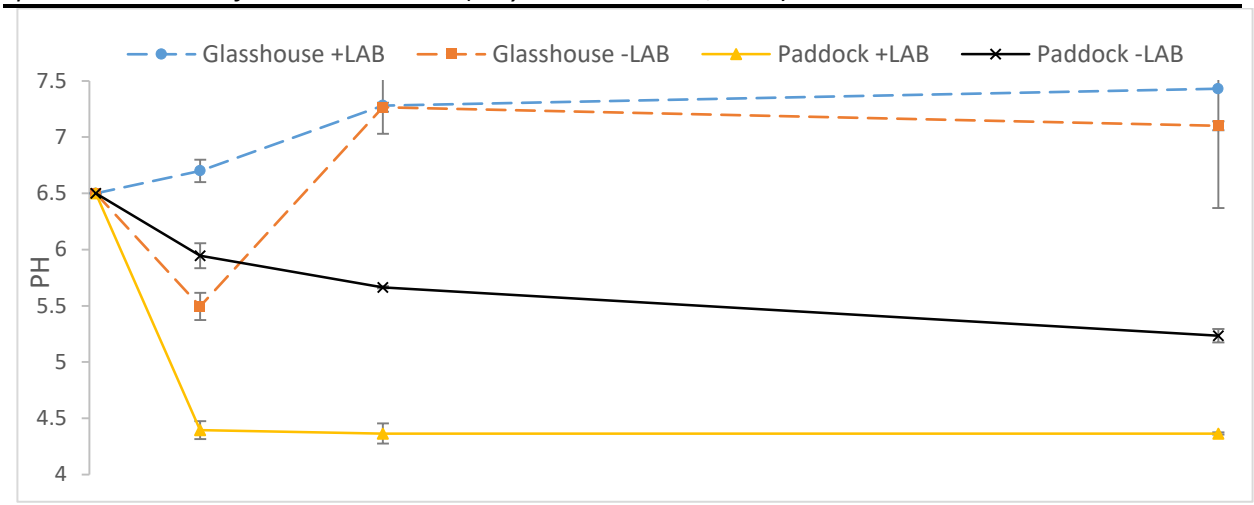
Using the R 3.2.0 software, separate two-tailed t-tests were performed at multiple time points during the fermentation in order to investigate statistically significant differences in silage pH due to 'Material' (Glasshouse and Paddock). A two-way ANOVA test was performed in order to investigate differences in the concentration of fermentation products on day 40 of the fermentation due to the factors; 'Material' (Glasshouse and Paddock), 'Silage sample size' (50 and 20 grams). Significance levels were set at $p<0.05$. Where significant differences were detected, comparisons of treatment means were further investigated using Tukey's HSD test.

3.7 Ensiling experiments 1-4 results

3.7.1 Experiment 1

Figure 3.1 shows the change in pH over time of glasshouse-grown and paddock-grown PRG which was wilted to approximately 20% DM and vacuum packed in order to make silage (termed glasshouse silage and paddock silage from herein). The pH of glasshouse silage with and without inoculant was >7.0 at 22 days of fermentation. The pH of non-inoculated paddock silage decreased at each time point to <5.25 at 86 days of fermentation. The pH of inoculated paddock silage was <4.5 at 8 days of fermentation, and remained <4.5 at 86 days of fermentation. The effect of 'Material' on pH was significant at each time point, while the effect of 'Inoculant' was significant at day 22 of fermentation only. The interaction between 'Material' and 'Inoculant' was significant at day 8 and 22 of fermentation. Pairwise comparisons of the means at each time point (not shown in Figure 3.1) showed that the pH of inoculated paddock silage was significantly lower than the pH of non-inoculated paddock silage at each time point, while the pH of the inoculated and non-inoculated glasshouse silage was not significantly different at any time point. Standard deviation in the data was low (<0.3 of a pH unit), with the exception of the '86 days of fermentation glasshouse silage +LAB' data point.

Figure 3.1 *pH of glasshouse and paddock perennial ryegrass silage, wilted to 20% dry matter, with and without inoculant, at intervals during the fermentation. Values represent means ± SD (n = 2). Time zero pH was estimated from the literature (Playne & McDonald, 1966).*



M	***	***	**
I	NS	**	NS
M x I	***	**	NS

'Material' = M; (Glasshouse and Paddock), 'inoculation' = I; (+LAB and -LAB), and their interaction M x I. LAB = *L. plantarum,* *** = p<0.001, ** = p<0.01, * = p<0.05, NS = non-significant, calculated by separate ANOVA at each time point.

At day 22 of the fermentation, glasshouse silage with and without inoculant contained <0.1g/kgDM D LA and >6g/kgDM AA. The effect of 'Material', 'Inoculant' and their interaction on D LA content were significant and D LA content corresponded inversely with the pH of the silage (Table 3.4). The factor 'Inoculant' did not affect silage AA content. The factor 'Silage sample size' did not affect any fermentation end products in paddock silage. Silage propionic acid and BA contents were negligible (Table 3.4). Pairwise comparisons of the means (not shown in Table 3.4) showed that D LA and AA content did not differ between inoculated and non-inoculated glasshouse silage. Glasshouse silage with and without inoculant contained significantly lower D LA and significantly higher AA than paddock silage with and without inoculant. Inoculated paddock silage contained significantly higher D LA than non-inoculated paddock silage for both the 50g silage sample size and the 20g silage sample size.

Table 3.4 *Fermentation end products at day 22 of the fermentation of glasshouse and paddock perennial ryegrass silage, wilted to 20% dry matter, with and without inoculant. Values represent means ± SD (n = 2). The pH values are presented as a reference point and represent measurements from different silage packets to those shown in Figure 3.1.*

Material	Silage sample size	Inoculation	pH	D Lactic acid (g/kgDM)	Acetic Acid (g/kgDM)	Propionic acid (g/kgDM)	Butyric Acid (g/kgDM)
Glasshouse	**50g**	**+LAB**	7.40 ± 0.00	0.04 ± 0.00	6.36 ± 0.43	0.15 ± 0.07	0.07 ± 0.03
		-LAB	7.60 ± 0.14	0.08 ± 0.01	7.31 ± 0.97	0.01 ± 0.00	0.05 ± 0.00
Paddock	**50 g**	**+LAB**	4.45 ± 0.07	2.56 ± 0.02	2.43 ± 0.14	0.09 ± 0.00	0.04 ± 0.00
		-LAB	5.70 ± 0.14	0.58 ± 0.17	2.19 ± 0.05	0.09 ± 0.00	0.07 ± 0.03
	20 g	**+LAB**	4.50 ± 0.00	2.58 ± 0.03	2.23 ± 0.60	0.09 ± 0.00	0.04 ± 0.00
		-LAB	5.75 ± 0.07	0.44 ± 0.22	2.73 ± 0.08	0.09 ± 0.00	0.09 ± 0.00
M				***	***	NS	NS
S				NS	NS	NS	NS
I				***	NS	NS	NS
M x I				***	NS	NS	*
S x I				NS	NS	NS	NS

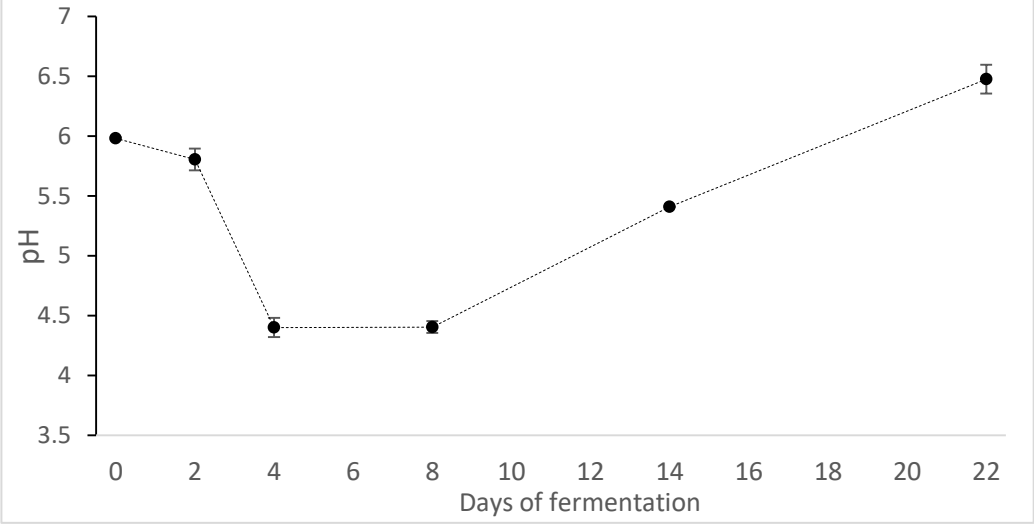
'Material' = M; (Glasshouse and Paddock), 'Inoculation' = I; (+LAB and -LAB), 'Silage sample size' = S; (50g and 20g), and their respective interactions M x I and S x I.

*** = p<0.001, ** = p<0.01, * = p<0.05, NS = non-significant, calculated by ANOVA.

3.7.2 Experiment 2

The pH of silage made from transgenic 'medium lipid' glasshouse-grown PRG wilted to 32% DM and inoculated, decreased from 5.8 to 4.4 between 2 and 4 days of fermentation, before increasing to >6 at 22 days of fermentation (Figure 3.2).

Figure 3.2 *pH of transgenic containment glasshouse-grown perennial ryegrass silage, wilted to 32% dry matter and inoculated, at intervals during the fermentation. Values represent means ± SD (n = 2).*



3.7.3 Experiment 3

After 30 days regrowth the total WSC content of glasshouse-grown PRG was <105g/kgDM; approximately half that of paddock-grown PRG (Table 3.5). The buffering capacity (BC) of glasshouse-grown PRG was approximately double that of paddock-grown PRG. The overall effect was an approximate four-fold difference in the WSC:BC ratio.

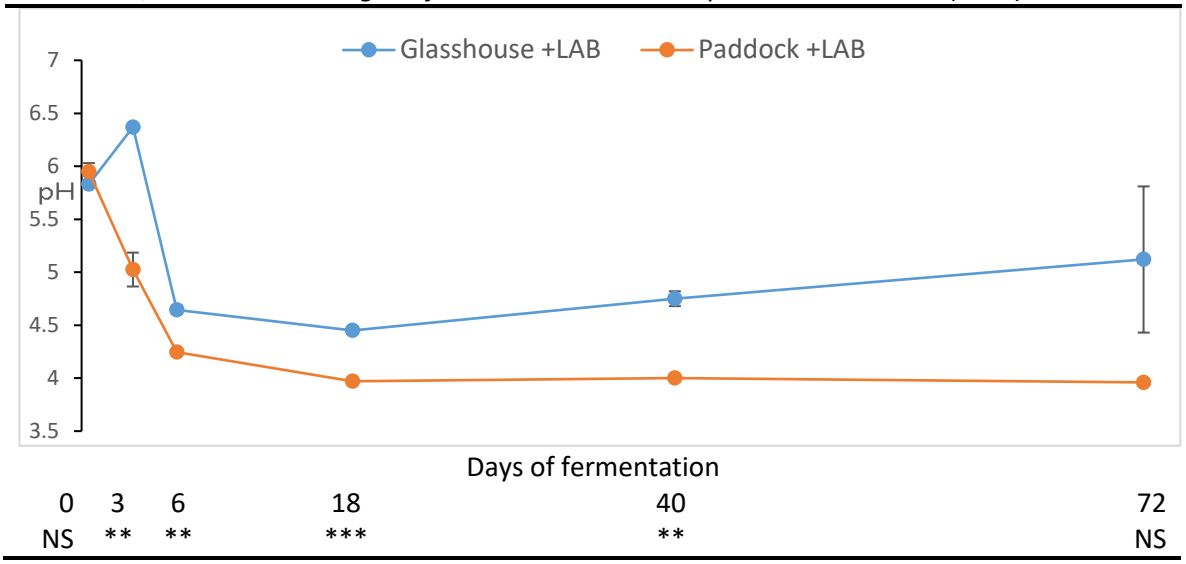
Table 3.5 *Pre-ensiling characteristics of glasshouse-grown and paddock-grown perennial ryegrass after 30 days regrowth.*

	Glasshouse-grown PRG	Paddock-grown PRG
Low MW sugars (g/kgDM)	85	134
High MW sugars (g/kgDM)	19	91
BC (gLA/kgDM)	63	35
WSC:BC	1.64	6.41

PRG = perennial ryegrass, MW = molecular weight, DM = Dry matter, LA = lactic acid, BC = buffering capacity, WSC = Water soluble carbohydrates

Figure 3.3 shows the change in pH over time of glasshouse-grown and paddock-grown PRG which was wilted to approximately 40% DM, LAB-inoculated and made into silage (termed glasshouse silage and paddock silage). Glasshouse silage pH was significantly higher than paddock silage pH at 3, 6, 18 and 40 days of fermentation. The pH of the fresh material (day 0 in figure 3.3) did not differ. Glasshouse silage pH decreased rapidly to approximately 4.5 at day 6 of the fermentation, and then increased to >5 at day 72 of the fermentation. Paddock silage pH decreased rapidly to approximately 4 at day 6 of the fermentation, and remained stable until the final pH measurement at day 72 of the fermentation. Standard deviation in the data was low, with the exception of the '72 days of fermentation glasshouse silage' data point.

Figure 3.3. *pH of glasshouse and paddock perennial ryegrass silage, wilted to 40% dry matter and inoculated, at intervals during the fermentation. Values represent means ± SD (n = 2).*



*** = p<0.001, ** = p<0.01, * = p<0.05, NS = non-significant, calculated by separate t-tests at each time point.

At day 40 of the fermentation, glasshouse silage contained significantly higher LA and AA than paddock silage (Table 3.6). Propionic acid and BA were not detected. The factor 'Silage sample size' had no effect on any fermentation end product contents in glasshouse silage.

Table 3.6 *Fermentation end products at day 40 of the fermentation of glasshouse and paddock perennial ryegrass silage, wilted to 40% dry matter and inoculated. Values represent means ± SD (n = 2). The pH values are presented as a reference point and represent measurements from different silage packets to those shown in Figure 3.3.*

Material	Silage sample size	pH	Lactic acid (g/kgDM)	Acetic acid (g/kgDM)	Propionic acid (g/kgDM)	Butyric acid (g/kgDM)
Paddock	50 g	4.00 ± 0.00	0.99 ± 0.02	0.37 ± 0.07	ND	ND
Glasshouse	50 g	4.80 ± 0.00	2.16 ± 0.04	3.42 ± 0.09	ND	ND
	20 g	4.75 ± 0.07	2.10 ± 0.05	3.11 ± 0.60	ND	ND
M			***	**		
S			NS	NS		

'Material' = M; (Glasshouse and Paddock), 'Silage sample size' = S; (50g and 20g)
*** = p<0.001, ** = p<0.01, * = p<0.05, NS = non-significant, calculated by ANOVA.

3.7.4 Experiment 4

After 28 days regrowth in the containment glasshouse the wild type, medium lipid and high lipid PRG genotypes had <105g/kgDM total WSCs. The high lipid genotype contained higher FA and nitrate content and lower WSC content than both the medium lipid and wild type genotypes (Table 3.7).

Table 3.7 *Pre-ensiled nutritional characteristics of perennial ryegrass grown in the containment glasshouse; wild type, medium lipid and high lipid plants after 28 days regrowth.*

	Wild type	Medium lipid	High lipid
Fatty acids (g/kgDM)	33.3	35.4	46.7
Low MW sugars (g/kgDM)	87.1	80.8	71.8
High MW sugars (g/kgDM)	16.4	19.2	11.2
Nitrates (g/kgDM)	12.8	13.1	18.4

MW= molecular weight

The change in silage pH over time from the material generated in experiment 4 are presented in Appendix VIII.

3.8 Ensiling experiments 1-4 discussion

The aim of the method development phase was to design a repeatable 'high quality' silage making protocol, as assessed by the fermentation pattern, using very small quantities of glasshouse-grown PRG. PRG is regarded as a forage species well-suited to ensiling as a means of forage conservation, because despite a high degree of variability, the concentration of WSCs in ryegrasses are typically in excess of the requirements to support a LA fermentation (Harrison et al., 2003). In this work, silage made from glasshouse-grown PRG failed to reach the pH required for successful nutrient preservation (approximately 3.8-4.5 for low DM silages) in the majority of cases. Given the use of a robust small-scale silo design and inoculation procedure in these experiments, the difficulty in generating quality silage was attributed to plant factors determined by the low light and high day/night temperatures in the glasshouses.

3.8.1 Pre-harvest factors influencing glasshouse ensiling

Abiotic growth conditions in the two glasshouses during each of the regrowth periods for Experiments 1 -5 are presented in Appendix I. An investigation of temperature conditions in the glasshouses compared to outdoors in October 2015 showed that the non-containment glasshouse had an average temperature of 18.5°C (range 5.5-39.5°C), while the containment glasshouse had an average temperature of 22.2°C (range 18.0-39.5°C). Therefore the non-containment glasshouse and containment glasshouse, respectively, had an average temperature of 6.4 and 10.1°C higher than the ambient outdoor environment during this period. The containment glasshouse also had an average night temperature 7.1°C higher than the non-containment glasshouse during this period.

Light conditions in both glasshouses were lower than the ambient exterior, but were not measured during the method development phase. A retrospective measurement in March 2016 revealed that the photosynthetically active radiation (PAR) available to glasshouse plants was approximately 50-70% lower than outdoors (Appendix I). Average daily solar radiation (outdoors) increased from 15.7 MJ/m^2/day during the regrowth period for Experiment 1 (October) to 20.1 MJ/m^2/day during Experiment 4 (November-December). Therefore it was expected that the amount of PAR available to plants also increased. However, a concomitant increase in average outdoor temperature was observed (11.7 to 14.9 from Experiment 1 to 4) and this was reflected in higher average temperatures in the glasshouses (Appendix I). Day temperatures in the range of 35-42°C in the glasshouses became more common in the later ensiling experiments. Together, the combination of the high day/night temperatures and low light conditions in the glasshouses relative to outdoors likely resulted in high rates of plant respiration relative

to photosynthesis and were therefore extremely unfavourable conditions for WSC accumulation (Fulkerson & Donaghy, 2001). Glasshouse plants were regularly watered and fertilized, and exhibited strong vegetative regrowth before the majority of the ensiling experiments. These rapid growth rates likely further diluted plant WSCs. The very fertile growth conditions likely explained the high BC of the glasshouse-grown PRG, relative to PRG harvested at a similar stage of regrowth from outdoors. To conclude; the glasshouse conditions were extremely unfavourable for ensiling because they resulted in an unusually low WSC:BC ratio for PRG.

3.8.2 Fermentation analyses criteria for successful ensiling

The data from pH curves and fermentation end product profiles were used to assess the type of fermentation that occurred in each ensiling experiment. Three criteria were defined for 'well-preserved' silage; 1) pH stability, as assessed by a pH <4.5 at three time points in the fermentation pH, 2) a LA: AA ratio greater than 2:1 during the 'stable phase' and 3) a concentration of BA lower than 5% DM (Kung & Shaver, 2001). It should be noted that in Experiments 1 & 3 the concentrations of LA were lower than anticipated for well-preserved stable silage and that BA concentrations were lower than anticipated for destabilized silage, based on the typical ranges in the literature (Cherney & Cherney, 2003). In the case of LA, this observation was also based on the pre-ensiled BC measurements of the material, which suggested that at least 60 g/kgDM LA were required to be produced in order to make well-preserved silage from glasshouse-grown PRG. Further, ethanol was not quantified. Ethanol may have been a significant fermentation end-product, especially if yeast or yeast-like fungi were active in the fermentations.

3.8.3 Success of post-harvest strategies

Post-harvest treatments were employed in order to overcome the challenging pre-harvest plant factors for successful ensiling; in particular the low WSC:BC ratio which compromised the development of a low, stable pH and a high LA concentration expected in well-preserved silage. The application of a commercial LAB inoculant decreased pH and increased LA concentration in silage made from paddock-grown PRG (Table 3.1, Figure 3.1) but not from glasshouse-grown PRG (Figure 3.1). There was also some evidence that LAB inoculation reduced the standard deviation in silage pH (Table 3.1, Figure 3.1) and LA concentration (Table 3.4) between replicate silos, at a given time point in the fermentation.

It was anticipated that increasing the pre-ensiled glasshouse-grown PRG DM content across sequential experiments (1-3) from approximately 20% to 30% to 40% might improve 'silage quality' (Haigh, 1996; Weissbach, 1996). This strategy was successful to some extent in that the silage pH was lower after 3-4

weeks of fermentation with each incremental increase in DM (in separate glasshouse ensiling experiments) (Figures 3.1, 3.2, 3.3). Further, glasshouse silage wilted to 40% DM contained high total LA (but also high AA) at day 40 of the fermentation (Table 3.6), while glasshouse silage wilted to 20% DM contained a negligible concentration of D LA (and higher AA) DM at day 22 of the fermentation (Table 3.4). This would most likely indicate that lower rates of secondary fermentation of LA occurred in the higher DM glasshouse silage. Regardless, wilting to the highest DM content for 'wilted silage' (approximately 40% DM, Experiment 3) failed to reliably generate well-preserved glasshouse PRG silage (as assessed by a low and *stable* pH over time, Figure 3.3). Interestingly, this was the case despite the glasshouse-grown PRG from experiment 3 having a 'fermentation coefficient' of 53 (calculated as DM% + 8 x WSC:BC); a value greater than the 'critical value' of 45 for generating anaerobically stable silage (Weissbach et al., 1974).

Only the combination of wilting, applying a LAB inoculant and adding glucose to the pre-ensiled plant material succeeded in generating high quality silage from glasshouse-grown PRG. However it is acknowledged that following the addition of glucose, the extent to which wilting and inoculating were necessary for generating quality silage was not explored.

3.8.4 Comparisons with paddock-grown perennial ryegrass silages

It was recognized that PRG from a section of paddock (cultivar Trojan/NEA2, agriseeds, described in Appendix IV) was not an ideal comparison to the clonal IMP566 PRG plants in the glasshouses. However, differences in WSC levels between even elite high sugar ryegrasses (bred to accumulate WSCs) and controls may be large under low growth temperatures (in the field), but disappear at 20/20°C day/night temperatures, similar to those in the glasshouse (Parsons et al., 2004). This could be taken as indirect evidence that using *any* PRG cultivar in the paddock would have had only a minor effect on the magnitude of difference observed in WSC levels between glasshouse-grown and paddock-grown PRG. No studies on the effect of PRG cultivar upon ryegrass BC were found, but were anticipated to be small compared to environmental factors such as soil fertility. The effect of growing PRG in a sward on tiller development and on the WSC:BC was acknowledged and was partially corrected for by adjusting the cutting height from the soil in order to ensure that leaf blade made up the majority of the material of both paddock and glasshouse silage.

In conclusion, paddock sward PRG was considered to be a useful 'positive control' and reference point to other ensiling experiments in the literature. It provided critical information about the ensiling

characteristics of similar plant materials grown in a vastly different environment to the glasshouse, while controlling important factors (such as regrowth interval and silage making protocols).

3.8.5 Experimental design and silage sample size

The large difference in WSC:BC between glasshouse-grown and paddock-grown PRG and the simple design of these ensiling experiments explained why treatment differences in pH and fermentation end-products were often statistically significant despite a low number of silo replicates being generated. All of the harvested material from each PRG genotype/population was pooled before ensiling in Experiments 1 and 3. This meant there were effectively no biological replicates in these experiments, only technical replicates. The material entering replicate silos was therefore very similar, and the variation in pH and LA concentration between replicate silos at a given time point was minimized. However, variation in pH was occasionally very high in silages which did not reach the pH required for successful preservation (Figures 3.1, 3.3).

Using the model silo design described in Hoedtke and Zeyner (2011), with minor modifications, two separate experiments indicated that there were no significant differences in the fermentation pattern between 20g and 50g silage sample size, for either paddock-grown PRG (Table 3.4) or glasshouse-grown PRG (Table 3.6). To the author's knowledge, 20g is the smallest silage sample size that has been used for any type of model silo, and seemed to be adequate for the simple experimental design used here. Larger scale experiments will need to be conducted to verify whether this silage sample size is suitable for modelling silage made from PRG grown in the field. In general, sampling error in laboratory silo studies is inversely proportional to the ratio of the silage sample size to the total mass harvested (e.g. field or treatment) (Cherney & Cherney, 2003). Reducing sampling error can be achieved either by increasing the size of the experimental silo, or generating replicates and pooling or averaging analytical means. On the other hand, many controllable factors determine sampling accuracy; i.e. whether a laboratory silo of a given size will reflect the attributes of the whole mass harvested (if it was ensiled). The most important factors are; the level of heterogeneity in the plant material harvested, consistency of packing technique by the operator, the density distribution of natural microflora or applied inoculant, and variation in DM content after wilting. In an experimental setting, variation in any of these factors will determine variability in fermentation parameters between replicate silos. This in turn will determine whether treatment differences can be detected. In this work, particular care was taken to control all of these factors. The repeatable, high accuracy protocols for glasshouse-grown PRG silage-making were used in Experiment 5 to investigate the effect of the high lipid technology on the ensiling biochemistry of PRG.

CHAPTER 4. TRANSGENIC VERSUS WILD TYPE SILAGE COMPARISON

4.1 Experiment 5 material and methods

Objectives: The aim of this ensiling experiment was to compare the pre-ensiling characteristics, fermentation pattern and nutritional composition of silage made from containment glasshouse-grown IMP566 ('wild type', WT), medium lipid (ML) and high lipid (HL) transgenic perennial ryegrass following an *accurate* wilt to 37.5% DM, LAB inoculation and glucose addition at a rate of 7.5% DM.

Harvest: The 30 days regrowth that accumulated between 14/12/2015 and 13/01/2016 from containment glasshouse-grown WT, ML and HL PRG was harvested with scissors between 7:00 am and 2:00 pm. WT plants were visibly more stressed than the ML and HL plants (Photograph 4.1). The four most visibly stressed WT plants were excluded. Prior to harvest, developing inflorescences were removed for compliance reasons. Respectively, a total of 18, 23 and 24 WT, ML and HL plants were harvested. The cut height from the soil averaged 8.35 cm (range 7-9.5 cm). A 5g subsample was removed from each plant for a biomass experiment (Chapter 4.1.1) and the remaining material from all the plants of a genotype were pooled. After thorough mixing of the pooled mass, subsamples were removed in triplicate for DM determination (Chapter 3.4) and a single 50g subsample was removed for fresh nutritional analysis (placed in liquid nitrogen). Dry weight analysis showed that 90, 96 and 98% the material ensiled was leaf from WT, ML and HL respectively. The remaining material was reproductive tillers.

Post-harvest treatments: Material was placed in plastic trays or perforated bags for 4-8 hours in one of two ovens at 35-45°C. During this period, the DM content of WT increased from 19.9% to 36.6%, the DM content of ML increased from 18.6% to 35.5%, and the DM content of HL increased from 17.2% to 36.6%. The wilted material was thoroughly mixed and 10g subsamples were taken for nutritional analysis (placed in liquid nitrogen). The material was chopped with scissors to 2-4 cm lengths and mixed. Inoculation and glucose application proceeded as described in Chapter 3.3 and 3.4 respectively, and actual rates of application by weight were recorded (Table 4.1). Afterwards, the material was again thoroughly mixed.

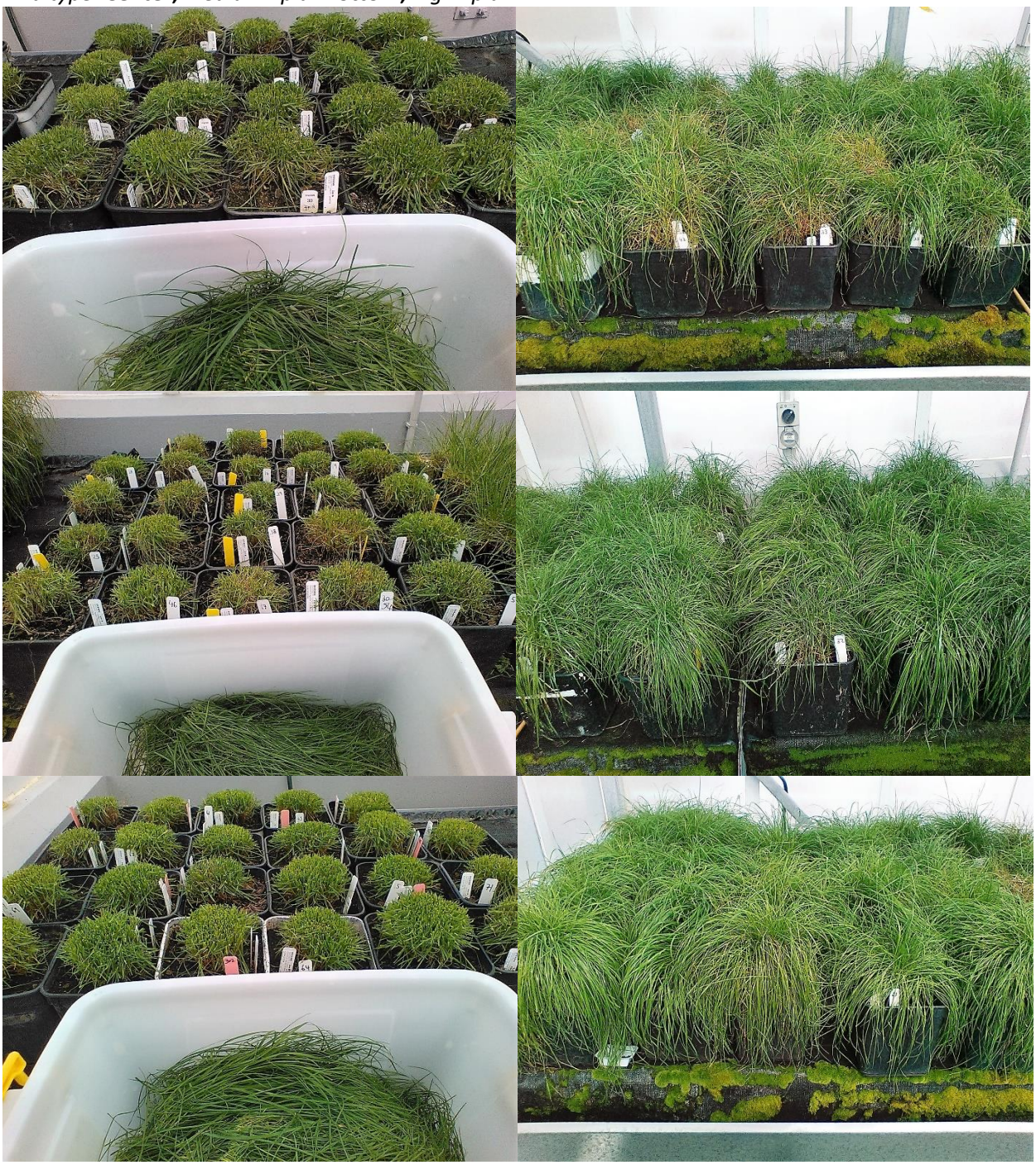
Table 4.1 *Average rates of LAB and glucose application by weight to perennial ryegrass in experiment 5.*

	PRG added (g)	Glucose solution spray (g)	Liquid inoculant spray (g)	Sugar increase (as a % of DM)	cfu LAB/g PRG
Target	25	2.25	1	7.5	800,000
Wild type	25.06	2.41	1.02	7.4	845,391
Medium lipid	25.73	2.45	1.09	7.4	880,525
High lipid	25.65	2.45	1.04	7.5	842,994

DM = Dry matter, cfu = colony forming units, LAB = L. *plantarum,* PRG = perennial ryegrass

Ensiling, storage and analysis: Material was divided into quantities of 40g and 20g and ensiled. Silos were stored in a controlled temperature room in the dark at 17°C. Duplicate 20g silos were opened and pH analyzed on days 3, 6, 22 and 45 of the fermentation, and the fermentation end product profile and nutritional composition of 40g silages were analyzed on day 45 of the fermentation. Silages were visually similar, although effluent production was visually reduced in ML silages compared to HL and WT silages (Photograph 4.2).

Photograph 4.1 Left; *perennial ryegrass plants at the beginning of the regrowth for experiment 5 (14/12/2015).* **Right;** *plants at the end of the regrowth, immediately before harvest (13/01/2016)* **Top;** *wild type*. **Center;** *medium lipid.* **Bottom;** *high lipid.*



4.1.1 Biomass experiment

On December 14th 2015, 20 WT, 23 ML, and 24 HL plants were cut back to approximately 8 cm. The plants were well established in their pots (Photograph 4.1) following a series of regrowths and vegetative propagations from the original small number of plants inherited in August 2015 (Appendix III). WT, ML and HL, respectively, yielded an average of 57g, 24g and 50g fresh matter per plant during the regrowth period ending 14th December 2015. The plants were spatially randomized on the glasshouse bench and regularly shuffled around the bench in order to minimize positional effects during the regrowth. Fertilizer ('Thrive all-purpose soluble', Yates, Australia) was applied to plants at the recommended rate twice during the regrowth. On the 12th January 2016, prior to harvest, the small proportion of developing reproductive material was removed for compliance reasons. Plants were cut with scissors 8-9 cm from the soil surface (mean 8.35 cm, range 7-9.5cm) and the fresh weight of each plant was recorded. A random 5g (± 0.05g) subsample was weighed and placed in a paper bag in an oven at 80°C. Once dried to a consistent weight (5-6 hours), the dry weight of this subsample was recorded. DM content and dry biomass were calculated from these values.

4.1.2 Subsampling for chemical analyses

Fresh and wilted PRG subsamples were submerged in liquid nitrogen and then stored at -80°C before being freeze-dried and further processed*[4]. 20g silos for pH analysis at intervals along the fermentation were stored at -20°C then processed at AgResearch Ltd Palmerston North. 6 x 40g silos (from each genotype) were opened on day 45 of the fermentation and the contents of individual silos were mixed. A 10g subsample of the silage was submerged in liquid N and stored at -80°C, then freeze-dried before being further processed at AgResearch Ltd Palmerston North for FA, Nitrates, and the *in vitro* rumen incubation. The remaining contents of duplicate silos (from each genotype) were pooled, mixed, vacuum packed and then transported on ice to the Massey University Nutrition Laboratory (Palmerston North). The samples were then further subdivided for dry nutritional analyses; dry matter (DM), water soluble carbohydrate (WSC), crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), Lignin, Ash, dry matter digestibility (DMD), dry organic matter digestibility (DOMD), organic matter digestibility (OMD) and gross energy (GE) and for wet analysis; lactic acid (LA) acetic acid (AA), propionic acid (PA), butyric acid (BA) and ammonia (NH_3) and then stored at -80°C until further processed.

*[4] Note that 'fresh' material was frozen immediately after harvest and 'ensiled' material was frozen after being ensiled. Both fresh and ensiled material were then chemically analyzed in either a dry (freeze dried) and wet (not freeze dried) state.

4.1.3 Chemical analyses

Wet analyses

For the fresh, wet material, buffering capacity (BC) was determined as described in Chapter 3.6.5. For the wet, ensiled material, pH and fermentation end product concentrations (LA, AA, PA, and BA) were determined as described in Chapter 3.6.5. Silage NH_3 was determined on wet samples by colorimetric method (phenol-nitroprusside) by the Internationally Accredited New Zealand-approved Nutrition laboratory at Massey University (Palmerston North). The stated g/kgDM concentration of silage fermentation end-products (wet analyses) used the post-wilt (oven) DM content measurement for calculations rather than the silage (freeze-dried) DM content measurement. This was considered to be advantageous because silage ethanol (a volatile) was not measured, but may have constituted a large proportion of the day 45 fermentation profile. Therefore the silage DM content could not be accurately corrected for all volatile losses during the freeze drying process (Haigh, 1996).

Dry analyses

WSC (Nelson/Blumenkrantz), CP (Dumas method, AOAC 968.06), NDF/ADF/Lignin (Tecator Fibertec, AOAC 2002.04) DM/Ash (AOAC 930.15/925.10), DMD/DOMD/OMD (Roughan & Holland, 1977) and GE (bomb calorimeter) were determined on freeze-dried and ground samples by the Internationally Accredited New Zealand-approved Nutrition laboratory at Massey University (Palmerston North). FAs were determined by quantitative fatty acid methyl ester analysis (FAMEs) on freeze-dried and ground samples at AgResearch Ltd Palmerston North. Following addition of a C:15 internal standard, FAs were extracted and methylated in hot methanolic HCl and quantified by GC-MS (Browse et al., 1986). Nitrates were determined by colorimetric nitration of salicylic acid assay upon freeze-dried and ground samples at AgResearch Ltd Palmerston North. Nitrates were extracted in water and reacted with 5% w/v Salicyclic acid in concentrated H_2SO_4. The yellow color produced by the nitration reaction was read at 410 nm. Samples were calibrated against a series of known concentrations of potassium nitrate (Cataldo et al., 1975). The stated g/kgDM values for all silage nutritional components were not corrected for the addition of glucose to the pre-ensiled PRG or for the loss of volatiles during freeze drying.

4.1.4 In vitro rumen incubation experiment

A 0.35g sample of fresh and ensiled WT and HL PRG were incubated in rumen fluid over 24 hours using the *in vitro* incubation system described by Muetzel et al. (2014). ML PRG was not used in the incubation. Freeze-dried and ground material from both freshly harvested and 3 silos made from WT and HL PRG were weighed into duplicate 60 ml glass incubation bottles with rumen fluid from a single cow. Two independent *in vitro* incubation runs were performed. Rumen fluid was squeezed into a pre-warmed insulated flask, filtered through a layer of cheesecloth and mixed with a pre-warmed buffer described by Mould et al. (2005) under continuous CO_2 flushing. After equilibrating the solution with CO_2, a 35 ml aliquot of rumen fluid-buffer mixture was added to the bottles containing the samples. The bottles were closed with a rubber stopper and placed in an incubator at 39°C under agitation and connected to a gas measurement system via a 23g needle. At 0, 1, 3, 7, 12 and 24 hours, sampling bottles were removed from the incubator and shaken by hand before removing 3 ml of sample suspended in rumen fluid for short chain and long chain FA analysis. A precise volume (0.9 mL) of fluid was removed and stored at -80°C then freeze-dried and analyzed for long-chain FAs (FAMEs). The remaining approximately 2ml was centrifuged at 20,000 g for 10 min. An aliquot of 0.9 ml of supernatant was collected, acidified with 0.1 ml of internal standard and stored at -20°C for analysis of short-chain volatile fatty acids (scVFAs) using the method described by Attwood et al. (1998). Total gas production was recorded automatically in the second *in vitro* incubation run using the system described by Muetzel et al. (2014).

4.1.5 Statistical procedures

Using the open source R 3.2.0 software, one-way analysis of variance (ANOVA) tests were performed in order to investigate differences between WT, ML and HL biomass production, silage fermentation end-products, nutritional components and silage pH at each time point of the fermentation. The biomass production data violated the assumption of equal sample variances required for the ANOVA test, and so the results were checked with a permutation test. Separate two-way ANOVA tests were performed at each sampling time point in the rumen incubation in order to investigate differences in short chain and long chain FAs due to the two factors; 'Material' (HL and WT) (ML was not used in the incubation), 'Ensiling' (Fresh and Ensiled) and their interaction. In all cases, significance levels were set at $p<0.05$. Where significant differences were detected, pairwise comparisons of means were performed using Tukey's HSD test.

4.2 Experiment 5 results

4.2.1 Pre-ensiling characteristics

Fresh nutritional properties

On a g/kgDM basis, differences in individual nutritional components between the three genotypes were small (≤16g/kgDM, Table 4.2), however percentage differences were large for some nutritional components. HL and ML, respectively, had 31% and 15% higher FA content and 11% and 1% lower WSC content than WT. HL and ML, respectively, had 5% and 6% higher CP content and 70% and 40% higher nitrate content than WT. All genotypes had low WSC content, high CP, BC, ash and nitrate content and an associated low WSC:CP and WSC:BC.

Table 4.2 *Pre-ensiled nutritional properties of wild type, medium lipid and high lipid perennial ryegrass after 30 days regrowth. Values represent measurements of a single subsample of the pooled and mixed plants from each genotype.*

	Wild type	Medium lipid	High lipid
FA (g/kgDM)	32	37	42
WSC (g/kgDM)	81	80	72
BC (gLA/kgDM)	52.4	58.5	50.5
WSC:BC	1.54	1.37	1.43
CP (g/kgDM)	281	295	297
WSC:CP	0.29	0.27	0.24
NO_3^- (g/kgDM)	10	14	17
NDF (g/kgDM)	386	391	393
ADF (g/kgDM)	216	215	221
Lignin (g/kgDM)	36	30	28
Ash (g/kgDM)	123	122	127
DMD (%DM)	73.8	75.0	74.7
DOMD (%DM)	68.1	69.4	68.8
OMD (%DM)	76.5	77.9	77.5
GE (kJ/g)	18.6	18.8	18.5

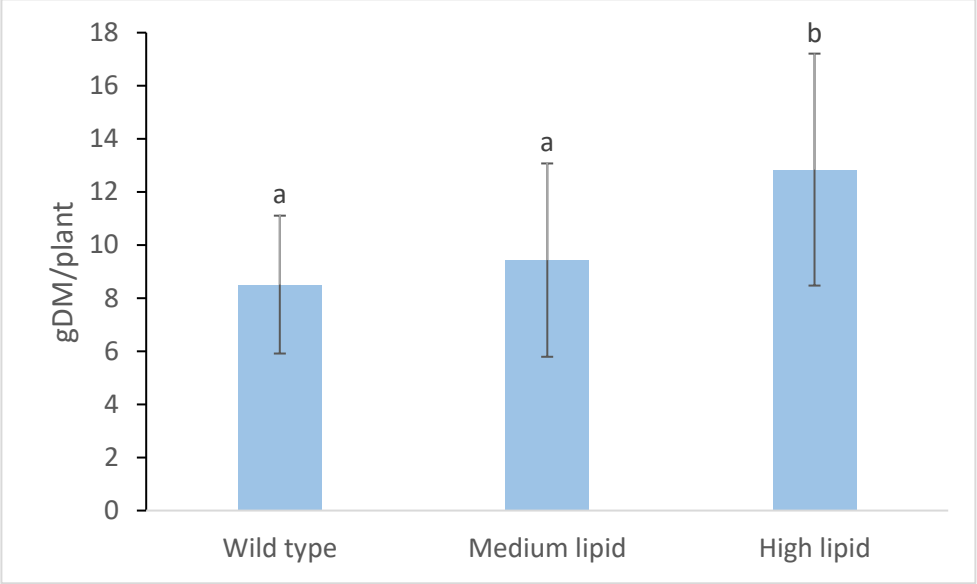
FA = fatty acids, DM = dry matter, WSC = water soluble carbohydrates, BC = buffering capacity, LA = lactic acid, CP = crude protein, NO_3^- = nitrates, NDF = neutral detergent fiber, ADF = acid detergent fiber, DMD = dry matter digestibility, DOMD = Dry organic matter digestibility, OMD= organic matter digestibility, GE = Gross energy.

Biomass production

Average fresh biomass production (Figure 4.1, below) from the HL plants was significantly greater than from the ML plants and WT plants (Tukey's HSD test, data not shown). Average DM content was significantly lower from the HL plants (17.2 ± 1.7, n = 24) than from the ML plants (19.5 ± 1.9, n = 23) and WT plants (20.0 ± 1.6, n = 18), which did not differ significantly (Tukey's HSD test). Average dry biomass production from the HL plants (calculated as fresh biomass production x DM content for each plant) was

significantly greater than from the ML and WT plants (36% and 51% greater than ML and WT respectively) (Figure 4.1). Fresh and dry biomass production was significantly more variable among HL plants than among ML and WT plants.

Figure 4.1 *Dry biomass production from wild type, medium lipid and high lipid perennial ryegrass plants after 30 days regrowth. Bars represent means in grams of dry matter ± SD (n = 18, 23, 24 for wild type, medium lipid and high lipid respectively).*

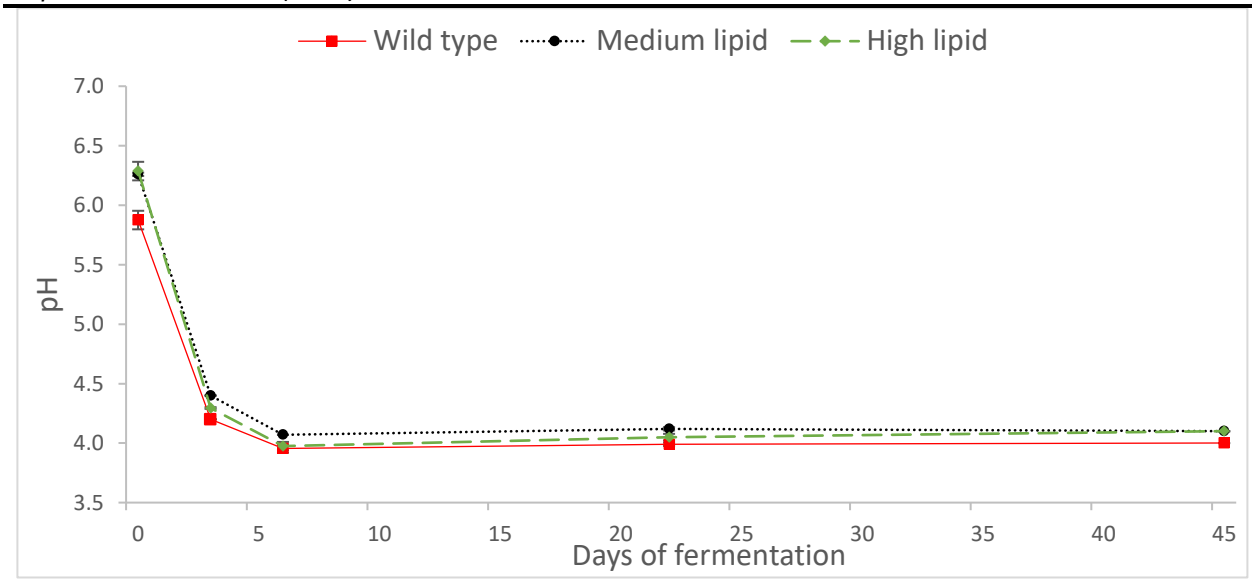


Different letters indicate statistically significant differences between means, calculated using Tukey's HSD test ($p<0.05$)
gDM = grams of dry matter

4.2.2 Fermentation pattern

When WT, ML and HL PRG were wilted to 37.5% DM, LAB-inoculated, had glucose added at 7.5% DM and were then vacuum packed, the pH of the resulting silage decreased rapidly to approximately 4.0 within 6 days of fermentation, and then remained stable until the final pH measurement at day 45 of the fermentation (Figure 4.2). Differences in WT, ML and HL silage pH from day 6 of the fermentation onwards, were small (≤0.13 pH unit) but sometimes significant (data not shown).

Figure 4.2 *pH of wild type, medium lipid and high lipid perennial ryegrass silage, wilted to 37.5% dry matter and inoculated, with glucose added at 7.5% DM, at intervals during the fermentation. Values represent means ± SD (n = 2).*



At day 45 of the fermentation, WT, ML and HL silage had a similar LA content of approximately 25g/kgDM and AA content <10g/kgDM (Table 4.3). Silage propionic acid and BA contents were negligible and NH_3 was not detected. Fermentation end product concentrations for HL, ML and WT silage did not differ significantly.

Table 4.3 *Fermentation end products at day 45 of the fermentation of wild type, medium lipid and high lipid silage, wilted to 37.5% dry matter and inoculated, with glucose added at 7.5% DM. Values represent means in g/kgDM ± SD (n = 3).*

	LA	AA	PA	BA	NH_3: N
Wild type	24.5 ± 0.4	7.2 ± 0.6	<0.01	<0.01	ND
Medium lipid	25.0 ± 0.3	7.9 ± 1.9	<0.01	<0.01	ND
High Lipid	25.2 ± 1.0	9.1 ± 1.8	<0.01	0.01 ± 0.02	ND

LA = lactic acid, AA = acetic acid, PA = propionic acid, PA = propionic acid, BA = butyric acid, NH_3: N = ammonia: nitrogen

4.2.3 Nutrient preservation

Photograph 4.2 *Perennial ryegrass silage packets at day 1 of fermentation.* **Left;** *wild type.* **Center;** *medium lipid.* **Right;** *high lipid.*

The nutritional composition of WT, ML and HL silage generally reflected that of the freshly harvested material (Table 4.2), although the percentage decrease from their fresh counterparts differed for some nutritional components (Table 4.4). HL silage had significantly higher FA, CP, nitrate, ash and significantly lower WSC content than WT silage. ML silage nutritional composition was intermediate between HL and WT. A greater reduction in digestibility, with a concomitant increase in insoluble DM components (FA, NDF, ADF, Lignin) occurred due to wilting and ensiling HL and (to a lesser extent) ML, compared to WT.

Table 4.4 *Nutritional composition of wild type, medium lipid and high lipid perennial ryegrass silage, and percentage decrease in nutritional components from the freshly harvested material. Values represent means ± SD (n = 3 for all nutritional components with the exception of; n=2 nitrates, and n = 6 fatty acids).*

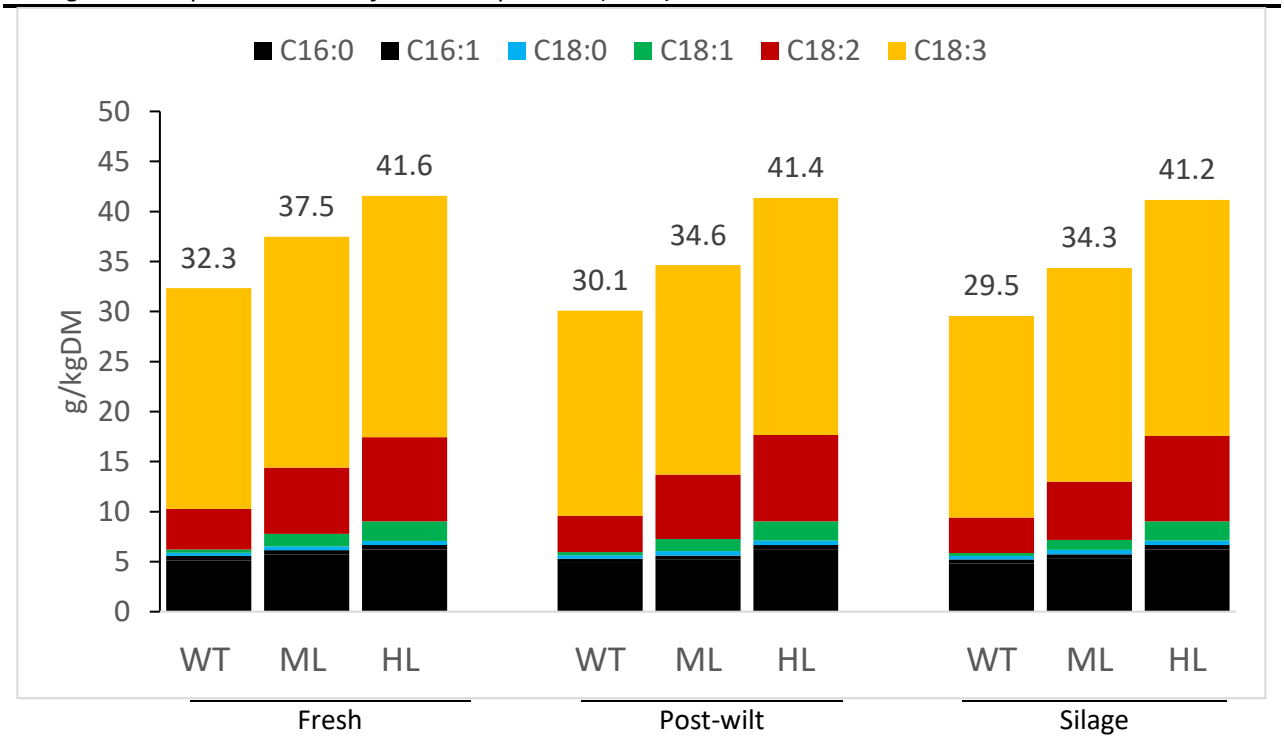
	Wild type	-Δ%	Medium lipid	-Δ%	High lipid	-Δ%
DM(%FW)	38.8a ± 0.39	-3	39.7b ± 0.27	-10	39.2ab ± 0.33	-1
FA (g/kgDM)	30a ± 1.2	9	35b ± 0.7	9	41c ± 0.7	2
WSC (g/kgDM)	46a ± 1.3	43	33b ± 1.1	58	33b ± 5.0	54
CP (g/kgDM)	280a ± 3.3	0	294b ± 2.9	0	291b ± 5.1	2
NO_3^- (g/kgDM)	8a ± 0.5	20	12b ± 0.0	19	15c ± 0.2	13
NDF (g/kgDM)	412a ± 3.3	-7	435b ± 3.5	-11	486c ± 8.8	-24
ADF (g/kgDM)	232a ± 3.9	-7	239ab ± 8.2	-11	256b ± 11.3	-16
Lignin (g/kgDM)	32 ± 4.0	12	28 ± 8.3	6	40 ± 11.3	-44
Ash (g/kgDM)	115a ± 1.1	6	119b ± 0.6	3	119b ± 0.6	6
DMD (%DM)	72.0a ± 0.15	2	72.1a ± 0.45	4	70.3b ± 0.06	6
DOMD (%DM)	66.3a ± 0.11	3	66.2a ± 0.50	5	64.2b ± 0.01	7
OMD (%DM)	74.2a ± 0.15	3	74.3a ± 0.55	5	72.1b ± 0.06	7
GE (kJ/g)	19.1 ± 0.06	-3	19.1 ± 0.01	-2	19.3 ± 0.11	-4

1. -Δ% = percentage decrease due to wilting and ensiling for 45 days
2. Different letters indicate statistically significant differences between means, calculated using Tukey's HSD test ($p<0.05$)
3. DM = dry matter, FA = fatty acids, WSC = water soluble carbohydrates, CP = crude protein, NO_3^- = nitrates, NDF = neutral detergent fiber, ADF = acid detergent fiber, DMD = dry matter digestibility, DOMD = Dry organic matter digestibility, OMD= organic matter digestibility, GE = Gross energy.

Fatty acids

Changes in the FA profile during the ensiling procedure were very small and therefore the silage FA profiles closely reflected the fresh and post-wilt FA profiles. HL silage had significantly higher total FA content and higher proportions of 18:1 and 18:2 than WT silage, and a significantly lower proportions of all other major FAs (n = 6, data not shown). The ML FA profile was intermediate between HL and WT. During wilting, total FA content decreased less in HL (0.5%) than WT (7%) and ML (8%) (Figure 4.3). The decrease in total FA content due to ensiling for 45 days was small (0.5-2%).

Figure 4.3 *Changes in fatty acid content and composition in wild type, medium lipid and high lipid perennial ryegrass during wilting to 37.5% dry matter and then ensiling for 45 days. 'Fresh' and 'Post-wilt' bars represent measurements of a single subsample of the pooled and mixed plants from each genotype. 'Silage' bars represent means from multiple silos (n = 6).*



WT = Wild type, ML = Medium lipid, HL = High lipid

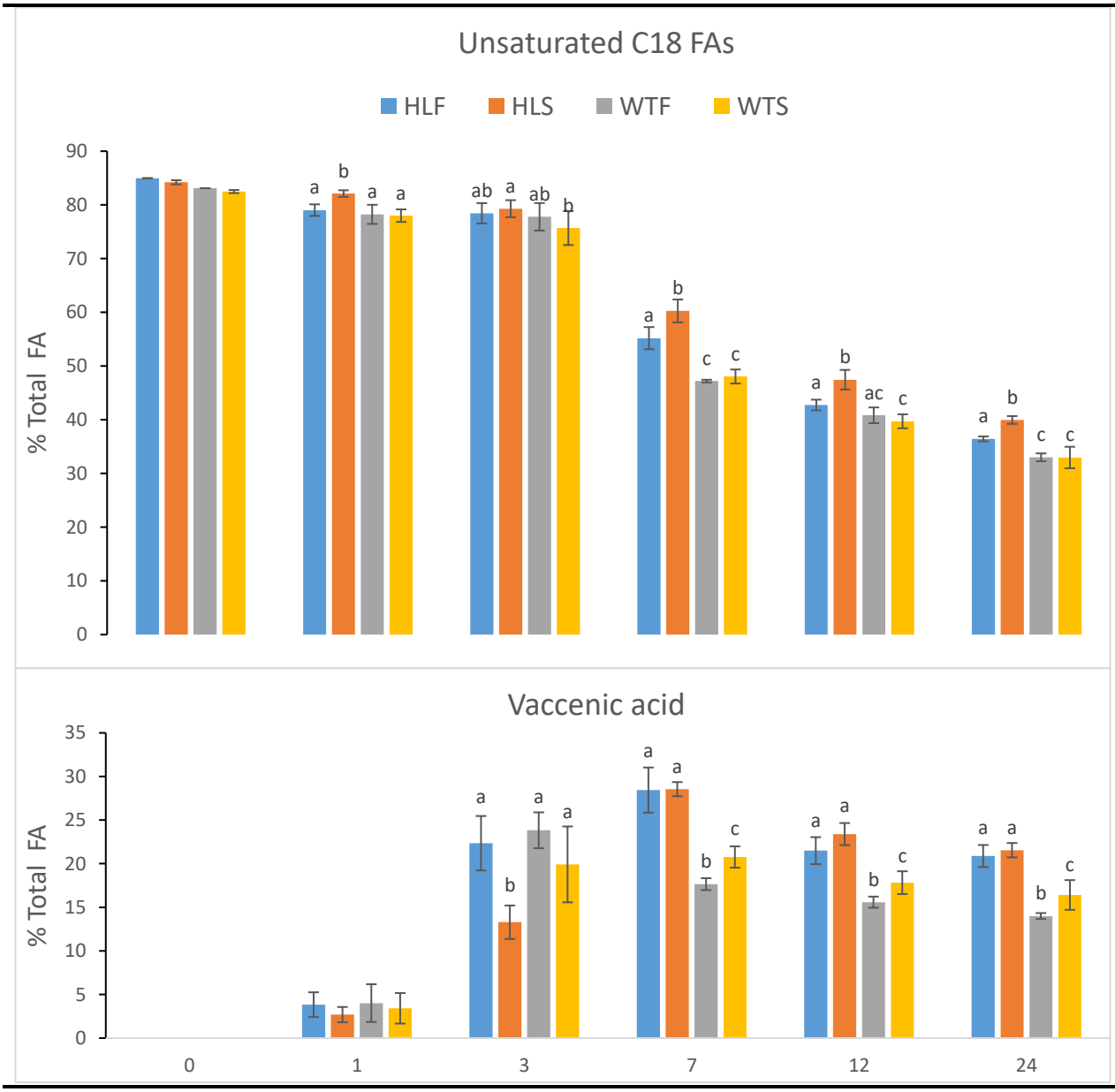
4.2.4 In vitro rumen incubation

Biohydrogenation

Changes in the degree of FA saturation (biohydrogenation) in fresh (non-ensiled) and ensiled HL and WT PRG during an *in vitro* rumen incubation were complex. In total, 7 parameters related to rumen biohydrogenation were compared at 5 time points during the incubation; 16:0, 18:0, vaccenic acid (VA), and conjugated linoleic acid (CLA) as a percentage of total FA, 18:2 and 18:3 as a percentage of their pre-incubated concentration, and total FA as a percentage of DM (35 comparisons in total). Most of these data are presented in Appendix V. Statistically significant differences detected by separate ANOVA at each sampling time point were equally common for 'Ensiling' (Fresh and Ensiled, 23 out of 35 comparisons), and 'Material' (WT and HL, 23 out of 35 comparisons) while 'Ensiling x Material' interactions also common (17 out of 35 comparisons).

Figure 4.4 a) simplifies the data by showing the change in total unsaturated 18-carbon (C18) FAs (the sum of 18:3, 18:2, 18:1, CLA and VA, as a percentage of total FA). Figure 4.4 b) shows the change in vaccenic acid as a percentage of total FA. HL silage had significantly higher unsaturated C18 FA content than fresh and ensiled WT, and also fresh HL, throughout the incubation. Unsaturated C18 FA content did not differ between fresh and ensiled WT. Differences in unsaturated C18 FA content were often reflected in differences in VA. Fresh and ensiled HL contained higher VA content than fresh and ensiled WT from 7 hours of incubation onward. WT silage contained higher VA than WT fresh in the later hours of the incubation.

Figure 4.4 *Changes in the fatty acid (FA) profile of fresh and ensiled wild type and high lipid perennial ryegrass during a 24 hour in vitro rumen incubation. Bars represent means ± SD from two independent incubations and multiple silos (n=3). a) Unsaturated C18 FAs as a % of total FAs, b) Vaccenic acid as a % total FAs.*

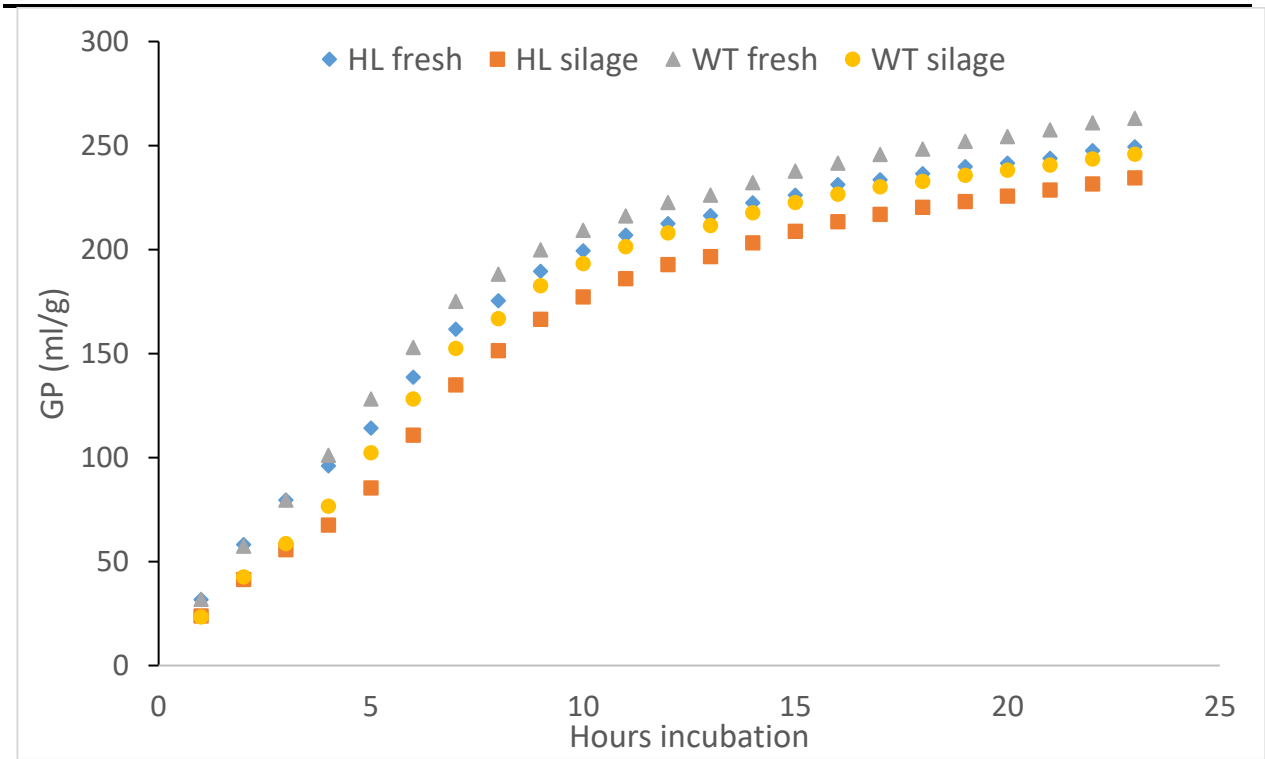


HLF = High lipid fresh, HLS = High lipid silage, WTF = Wild type fresh, WTS = Wild type silage, FA = fatty acid, C18 = 18-carbon
Different letters indicate statistically significant differences between means, calculated using Tukey's HSD test ($p<0.05$)

Gas production

Total fermentation gas production from WT was consistently higher than from HL and was higher from fresh than from ensiled material (Figure 4.5). The percentage difference in gas produced between ensiled HL and ensiled WT was greater than between fresh HL and fresh WT, between 4 and 20 hours of the incubation.

Figure 4.5 *Total gas production from fresh and ensiled wild type and high lipid perennial ryegrass during a 24 hour in vitro rumen incubation. Points represent means from a single incubation and multiple silos (n=3).*



HL = High lipid, WT = Wild type, GP = Gas produced

S*hort chain volatile fatty acids*

Total scVFA, and the molar proportion of rumen fluid acetate, propionate, butyrate, and other scVFAs were compared at 5 time points during the *in vitro* rumen incubation of fresh and ensiled HL and WT PRG (25 comparisons in total). Statistically significant differences detected by separate ANOVA at each sampling time point were most common for 'Ensiling' (Fresh and Ensiled, 19 out of 25 comparisons), followed by 'Material' (WT and HL, 17 out of 25 comparisons) while interactions between 'Ensiling' and 'Material' were less common (4 out of 25 comparisons). Total scVFA production was higher for fresh material early in the incubation but similar for all fresh and ensiled material later in the incubation (Table 4.5). Ensiling generally shifted the rumen fermentation towards butyrate and to a lesser extent propionate, and away from acetate. The HL technology shifted the fermentation towards acetate and away from butyrate, and also away from 'other scVFAs' (mainly valerate). The molar proportion of propionate was similar for HL and WT.

Table 4.5 *Total scVFA, and the molar proportion of rumen fluid acetate, propionate, butyrate, and other scVFAs from fresh and ensiled wild type and high lipid perennial ryegrass during a 24 hour in vitro rumen incubation. Values represent means ± SD from two independent incubations and multiple silage packets (n=3).*

	Hours incubated				
Total scVFA (mmol/gDW)	**1**	**3**	**7**	**12**	**24**
HLF	1.0a ± 0.2	3.3a ± 0.4	6.3 ± 0.5	8.0ab ± 0.5	9.5 ± 0.6
HLS	0.7b ± 0.1	2.3b ± 0.1	5.9 ± 0.4	7.7a ± 0.4	9.7 ± 0.5
WTF	1.1a ± 0.3	3.3a ± 0.4	6.3 ± 0.6	8.0ab ± 0.6	9.7 ± 0.6
WTS	0.8b ± 0.1	2.5b ± 0.1	6.1 ± 0.1	8.2b ± 0.1	10.1 ± 0.3
Acetate (% scVFAs)					
HLF	78.2 ± 2.2	69.5ab ± 0.7	69.6a ± 1.1	68.1a ± 1.1	66.3a ± 0.8
HLS	75.2 ± 1.4	70.5a ± 1.1	61.5b ± 1.7	62.0b ± 1.2	61.2b ± 0.7
WTF	76.1 ± 4.2	68.9b ± 0.4	67.3a ± 0.5	66.1c ± 0.4	65.1c ± 0.4
WTS	74.4 ± 3.4	68.3b ± 0.6	57.7c ± 1.3	57.8d ± 0.8	57.8d ± 0.3
Propionate (% scVFAs)					
HLF	18.5 ± 3.6	24.3a ± 0.8	21.5a ± 0.1	21.0a ± 0.4	20.2ab ± 0.4
HLS	22.3 ± 2.1	21.5b ± 1.2	22.7a ± 1.5	22.1a ± 1.4	21.0ab ± 1.4
WTF	19.5 ± 4.3	24.2a ± 0.1	21.7a ± 0.2	20.8a ± 0.2	19.6a ± 0.1
WTS	21.8 ± 2.9	22.3b ± 1.1	24.4b ± 1.0	23.3b ± 0.7	21.5b ± 0.9
Butyrate (% scVFAs)					
HLF	2.9 ± 1.4	4.3a ± 0.4	5.4a ± 0.6	6.6a ± 0.5	7.9a ± 0.4
HLS	2.1 ± 1.7	6.4b ± 0.3	13.6b ± 0.4	12.9b ± 0.4	12.8b ± 0.5
WTF	3.4 ± 0.4	4.6a ± 0.1	7.0c ± 0.2	7.6a ± 0.1	8.5a ± 0.3
WTS	2.9 ± 1.1	7.4c ± 1.1	15.1d ± 0.1	14.3c ± 0.9	13.8c ± 0.6
Other (% scVFAs)					
HLF	0.4a ± 0.2	1.9 ± 0.4	3.5ac ± 0.5	4.3a ± 0.5	5.7a ± 0.6
HLS	0.4a ± 0.4	1.7 ± 0.2	2.2b ± 0.4	3.1b ± 0.3	5.0b ± 0.2
WTF	1.1b ± 0.1	2.3 ± 0.4	4.1a ± 0.5	5.5a ± 0.6	6.9c ± 0.2
WTS	0.9b ± 0.3	1.8 ± 0.6	2.8c ± 0.5	4.6a ± 1.0	6.9c ± 0.1

HLF = High lipid fresh, HLS = High lipid silage, WTF = Wild type fresh, WTS = Wild type silage, scVFAs = short chain volatile fatty acids

Different letters indicate statistically significant differences between means, calculated using Tukey's HSD test ($p<0.05$).

4.3 Experiment 5 discussion

Experiment 5 investigated the effect of the high lipid technology (Chapter 2.8) on aspects of the ensiling biochemistry of perennial ryegrass (PRG), and subsequently assessed the nutritional properties of the ensiled material as a ruminant feed. For these purposes, two independent transgenic PRG genotypes hemizygous for the D1o3-3 constructs (Chapter 2.8.5), termed medium lipid (ML) and high lipid (HL), and a non-transgenic control (WT) were grown for 30 days (in summer glasshouse conditions). Plants were harvested, wilted to 37.5% DM, LAB-inoculated, had glucose added at 7.5% DM, and were then vacuum packed in order to make well-preserved silage with a pH of approximately 4.0. The nutritional properties of the ensiled material and performance in an *in vitro* rumen incubation were then analyzed.

4.3.1 Interpretation of data from experiments 5

To interpret the data from Experiment 5 and then accurately comment on the suitability of high lipid PRG for silage making would require a complete understanding of the major differences between typical silage from a farm and the material generated here. Working with small quantities of plant material within containment glasshouse facilities imposed limitations on the physical scale and the design of this experiment. The artificial nature of the silage-making procedures (e.g. oven wilt, glucose addition) and the processing of the materials before the *in vitro* rumen incubation arguably reduce the relevance of the data in a wider agronomic context. However, perhaps the most important factor to consider when interpreting the data from this experiment was the pre-harvest glasshouse growth environment. It was anticipated that the glasshouse environment had a large (mainly negative) impact on the pre-ensiled nutritional properties of the PRG. This was related to the high day/night temperatures and the low levels of incident photosynthetically active radiation in the glasshouse, compared with a field environment.

Regardless of these implications, some of the recorded features of the high lipid PRG allowed some speculation about how it might perform as a silage crop outdoors. Several metabolic consequences of elevated *de novo* FA synthesis may affect the pre-ensiling characteristics and/or the nutritional value of silage made from high lipid PRG in a future scenario where this technology may be commercialized.

4.3.2 The fermentation pattern did not differ between WT, ML and HL silage

WT, ML and HL PRG were converted into well-preserved silage as indicated by 1) pH stability <4.5 at three time points in the fermentation pH 2) a LA: AA ratio greater than 2:1 during the stable phase and 3) a concentration of BA lower than 5% DM (Buxton & O'Kiely, 2003). The WSC:BC of all three genotypes was lower than that of the glasshouse-grown WT PRG used in Experiment 3, which confirmed that the addition

of glucose was necessary for generating well-preserved silage. The reduction in pH to a low and stable level (approximately 4.0) by day 6 of the fermentation (Figure 4.2) indicated that a rapid LA fermentation occurred and that low levels of undesirable microbial fermentative activity occurred from this point onward (McDonald et al., 1991). Differences in the stable phase pH were sometimes statistically significant, but given that these differences were small they were unlikely to be of major biological significance. The LA and AA contents at day 45 of the fermentation did not differ significantly between WT, ML and HL silage. Silages contained negligible BA and no NH_3, which indicated that clostridial activity was minimized and that silage protein was very well-preserved (Table 4.3). Replicate silos, (which were technical replicates only, Chapter 3.8.5) exhibited low variation in fermentation parameters, indicating that the post-harvest processing of the material was accurate. It should be noted the LA content of silages (approximately 25g/kgDM) was lower than is typically reported for well-preserved PRG silage in the literature (Cherney & Cherney, 2003). Further, the pre-ensiled BC measurements of the material suggested that approximately 50-60g/kgDM LA were required to be produced in order to make well-preserved silage from glasshouse-grown PRG. The reason for the low LA content measured in these silages was not clear.

4.3.3 Nutrients were well-preserved in WT, ML and HL silage

The nutrients in WT, ML and HL PRG were successfully preserved during wilting and 45 days of ensiling, as indicated by a small percentage reductions (<10%) for all important nutrients compared to their fresh (pre-wilted) counterparts (Table 4.4). The reduction in total FA content due to ensiling was small (0.5-2%) in accordance with most examples in the literature (Glasser et al., 2013), although losses of 8% and 7% occurred in ML and WT, respectively, during wilting (Figure 4.3). The concentration of residual WSCs was significantly higher in WT silage than ML and HL silage, possibly on account of its higher pre-ensiled WSC:BC (Table 4.2). CP content was significantly higher in ML and HL silage than in WT silage and this difference closely reflected the difference between the fresh materials, because the change in CP due to wilting and ensiling was very low in all three genotypes. NDF content was significantly higher in ML and HL silage than in WT silage, and HL silage had significantly lower overall digestibility than WT and ML silage, despite having a higher fresh digestibility than WT (Table 4.4).

4.3.4 Wilt duration influenced nutrient preservation

The greater reduction in digestibility and the concomitant increase in cell wall components that occurred in HL and ML compared to WT during wilting and ensiling, indicated that greater overall losses of digestible

nutrients occurred in HL and in (to a lesser extent) ML than in WT (Table 4.4) (McDonald et al., 1991). Given that the fermentation pattern of the material was very similar, these differences were unlikely to have arisen during anaerobic fermentation. Instead, they probably originated during the uneven wilt conditions required to increase the DM content of the three genotypes to 37.5%. However, the post-wilt nutritional composition was not determined (with the exception of FA content/composition), and so this could not be verified. WT, ML and HL were wilted separately in one of two ovens at 35-45°C for different lengths of time. Oven space was a bottleneck in post-harvest operations, and the HL material took longer to wilt to 37.5% DM (8 hours) on account of its higher moisture content and its greater pooled bulk (c.f. ML and WT) (Figure 4.1). A longer wilt will result in greater respiratory losses of nutrients (McDonald et al., 1991). Future experiments should, wherever possible, avoid the use of different wilt durations/conditions in order to achieve a consistent wilted DM content between treatments before ensiling.

4.3.5 Glasshouse conditions influenced differences in pre-ensiled HL and WT total FA content

The regrowth period for Experiment 5 was characterized by high day temperatures in the glasshouse, particularly in the fortnight before harvest (Table 4.6). The average temperature was near the optimum for temperate grasses (Hunt & Halligan, 1981), but was much higher than would be expected outdoors. Day temperatures over 40°C were frequently reached, which likely imposed considerable stress upon the PRG (Wehner & Watschke, 1981). HL and ML showed fewer visual signs of stress than WT (Photograph 4.1).

Table 4.6 *Containment glasshouse temperature data 27/12/2015-12/01/2016 (°C).*

Average temperature	Day temperature	Night temperature	Max temperature	Min temperature	Day RH	Night RH
24.5	27.6	19.4	42.5	17.0	52.8	81.9

Day temperature was calculated as the average of data logger readings made every 10 minutes, between 5:45 am and 8:45 pm.

The total FA content of ensiled HL (41g/kgDM) was higher than has been reported for ensiled PRG in the literature, or fresh PRG, with the exception of Palladino et al. (2009). Total FA content of fresh WT (32g/kgDM) was also substantially higher than the average for ryegrass after a 30 day regrowth interval (19.4g/kgDM). It is expected that FA content will decrease during the regrowth according to the formula below, devised by Glasser et al. (2013). These authors performed a meta-analysis on 'factors influencing forage FA content' and determined the average effect of growth interval on ryegrass FA content.

total FA (g/kgDM) = 32.1-0.42(days regrowth)

Therefore, Experiment 5 growth conditions were fairly favourable for a high FA content in PRG compared to many outdoor environments. This was likely because of the high levels of fertilizer N applied, which encourages the growth of vegetative organs that are rich in membrane lipids. However, the difference in total FA content between fresh HL; 42g/kgDM and WT; 32g/kgDM, 30 days after cutting (DAC) was reduced compared to the previous regrowth trial (Experiment 4); (HL ;47g/kgDM and WT; 33g/kgDM 28 DAC) (Table 3.7) and compared to previous measurements from this genotype (pers. comm, Somrutai Winichayakul) although WT FA content was similar in the two regrowth trials. It should be noted that the 'HL' line used in this experiment, had a fatty acid content similar to D1o3-3 PRG lines termed 'low' lipid (Chapter 2.8.5) in other experiments by the plant biotechnology group (pers. comm, Somrutai Winichayakul). The smaller difference in fresh HL and WT total FA content in Experiment 5 might have resulted from the frequent periods of heat stress imposed on plants during this regrowth (Table 4.6). Interestingly, these conditions did not reduce the apparent biomass production advantage for HL relative to WT (Figure 4.1). Future experiments could investigate the effect of growth temperature on the triacylglycerol (TAG) accumulation trait in high lipid PRG.

4.3.6 Differences in fatty acid losses during wilting

During wilting, the decrease in total FA content in HL was proportionally smaller than for ML and WT (Figure 4.3). Given that HL was wilted for the longest duration, the smaller reduction in FA content could indicate that the HL FAs were more stable over the wilting period, although an intermediate effect between HL and WT was not observed in ML. The reductions in FA content were calculated from measurements of a single subsample from the pooled and mixed plants from each crop, pre and post-wilting, and so could not be statistically assessed. It is also possible that this effect was a result of greater respiratory losses of other DM components in HL compared to ML and WT during wilting, which could have masked a decrease in FA content expressed on a g/kgDM basis.

The potential importance of wilting for HL perennial ryegrass ensiling

The beneficial effects of wilting on ensiling (improved fermentation and reduced silage effluent losses) conflict with the losses of beneficial PUFAs incurred during the extended aerobic phase (Dewhurst & King, 1998). Degradation of PUFAs during field wilting causes the second largest losses of PUFAs from livestock farming (after ruminal biohydrogenation), which ultimately reduces the human health value of ruminant-derived products (Shingfield et al., 2013). This has particular relevance in the context of high lipid PRG ensiling (or haymaking) because high lipid PRG has elevated levels of PUFAs, and therefore could be

subjected to greater absolute losses of PUFAs than other ryegrasses, for a given wilt duration. The TAG that accumulates within high lipid PRG is protected from lipase entry *in planta* by the Cys-oleosin protein (Winichayakul et al., 2013). Lipolysis is the first step of the lipoxygenase pathway which ends in the loss of PUFAs during wilting (Dewhurst et al., 2003b). This reveals the possibility that high lipid PRG TAG may be protected from degradation via the lipoxygenase pathway, and therefore PUFA loss may be retarded during the wilting phase. Future experiments should investigate the stability of high lipid PRG FAs during an extended wilt.

4.3.7 High lipid plants accumulated more biomass

HL PRG plants produced significantly more biomass than ML and WT during the regrowth period for Experiment 5. Biomass measurements were made on plants of different sizes at the beginning of the regrowth and with different recent propagation histories. Further, there was a large degree of variation in biomass production within each genotype that could not be controlled (Figure 4.1). Therefore this data should not be interpreted as independent evidence for increased biomass accumulation in high lipid PRG. However, the 51% dry biomass production advantage in HL over WT strongly indicated that the proposed mechanism by which increased *de novo* FA synthesis increases CO_2 assimilation (and biomass production) in high lipid C3 plants (Chapter 2.8.5) was functioning in the HL genotype during this regrowth period, and that this probably influenced its nutritional properties.

A faster growing PRG could influence ensiling practices

It is conceivable that a faster growing PRG with altered nutritional properties may require changes to best-practice silage-making practices in a future scenario where high lipid PRG is grown outdoors. Firstly, with regard to the FA component, the regrowth interval explains most of the variation in total FA content (and composition) in PRG (Glasser et al., 2013; Khan et al., 2012). The rate that high lipid PRG FA content changes over an extended vegetative regrowth period (>4 weeks) has not been recorded, but could be important in the context of high lipid PRG ensiling, because i) silage-based livestock farming systems tend to allow a longer regrowth interval (before cutting) than grazing systems (Buxton & O'Kiely, 2003), and ii) in a New Zealand context, the time elapsed between paddock closure and cutting for silage is highly variable (Howse et al., 1996). In Arabidopsis, the percentage difference in total FA content between high lipid and WT increased until senescence (Winichayakul et al., 2013). A similar trend should be tested for in high lipid PRG.

Future experiments could also explore seed head development in high lipid PRG. In a New Zealand context, the timing of spring silage production around PRG seed head production is important because of the negative impact of seed head formation on PRG nutritional value (and FA content (Dewhurst et al., 2002)). Best practice guidelines indicate that PRG should be cut for silage during the short window when digestible dry matter yield (DDM) peaks. The later in spring that a paddock is closed for conservation (silage or haymaking), the earlier in the regrowth seed heads emerge. In effect, the maximum potential DDM yield (and total FA yield) falls rapidly when paddock closure is delayed by a short time (Browse et al., 1981). An altered spring reproductive transition may occur in high lipid PRG (Maw et al., 2014; Winichayakul et al., 2013) which would influence how its digestibility changes with time and thus shift the optimal harvest date for maximizing DDM yield from high lipid PRG. However, peak high lipid PRG FA yield and DDM yield may not occur simultaneously. Further, measures such as digestibility may not adequately describe the nutritional quality of PRG, given its different composition of digestible nutrients. Characterizing the changes in high lipid PRG biomass production and nutritional composition over time could be important for realizing the potential animal performance benefits of ensiled high lipid PRG.

4.3.8 Increased lipid accumulation may alter other nutritional components of perennial ryegrass

Enhancing the content of one forage nutritional component causes changes in other nutritional components. Such changes can be due to a simple dilution effect or may have a physiological basis (Cosgrove et al., 2009). There have been limited analysis of high lipid PRG nutritional composition, however the expression of the high lipid constructs results in substantially altered C3 plant metabolism (Chapter 2.8.6, Winichayakul et al., 2013) which indicates that physiologically-based changes could be important. In Experiment 5, the differences in fresh HL, ML and WT fibre components were small, but there were interesting differences WSCs and CP.

Water soluble carbohydrates

In Experiments 4 & 5, respectively, fresh HL had 20% and 11% lower WSC content than WT (Tables 3.7 and 4.2). ML also had lower WSC content than WT in both experiments, but the difference was smaller (3% and 1%). These percentage differences were calculated from measurements of single subsample from the pooled and mixed plants from each genotype and so could not be statistically assessed. In Experiment 5, the differences in fresh WSC were reflected in the significantly lower residual WSC content of HL and ML silage compared to WT silage (Table 4.4). However, the different BC values and the different wilt procedures employed (Table 4.2) may also have influenced silage residual WSC content. As with FA

content, the difference in fresh WSC content between HL, ML and WT was reduced in Experiment 5 compared to Experiment 4, probably as a result of the heat stress imposed on plants during this regrowth (Table 4.6).

In 'conventionally-bred' PRG, there is only a small trade-off between lipid and WSC content. Cosgrove et al. (2009), regressed WSC content against lipid content for 220 PRG samples from 3 ryegrass varieties and found a weak negative relationship between WSC and lipid, which agreed with the results of Palladino et al. (2009), and could be explained by a dilution effect alone. However, the mechanism by which lipid content is increased in high lipid PRG could impact carbon metabolism more dramatically, by shifting carbon allocation away from reserve carbohydrate accumulation, as occurred in the recent successful effort to engineer higher TAG contents into leaves (Vanhercke et al., 2014a). Further, the increased energy requirements for faster growth could prevent WSCs from accumulating (Fulkerson & Donaghy, 2001) in high lipid PRG by rapidly using up new photosynthate. However, considering the metabolic roles of plant WSCs and the sensitivity of PRG WSCs levels to the growth environment (Chapter 2.5), there is particular uncertainty about whether these differences would translate to the outdoors. Future experiments should determine whether the increased FA/lipid synthesis and increased biomass accumulation effects the WSC content and general carbon metabolism of high lipid PRG.

Crude protein

In Experiment 5, fresh crude protien (CP) was 5-6% higher in ML and HL than in WT, and this difference was reflected in the significantly higher CP content of HL and ML silage (Table 4.4). Fresh ML and HL also had a lower WSC:CP than WT. Nitrate-N is not included in the calculation of CP (N x 6.25), so the increased nitrate content in ML and HL (Table 4.2) could not explain the higher CP recorded in Experiment 5. Forage CP content exhibits a positive correlation with total FA content and 18:3, because a large proportion of forage FA content is 18:3 which occurs in in the same location as a large proprotion of protein; in the chloroplast membrane within photosynthetically active tissues (Buccioni et al., 2012). Consequently FA and CP are typically high in the early vegetation stage, under high N conditions, which encourage leafy growth.

High lipid PRG have elevated chlorophyll content, and reduced levels of rubisco, which is speculated to be a result of the rebalancing of photosynthetic componentry in order to efficiently utilize the additional CO_2 available to these plants (pers. comm, Somrutai Winichayakul). A shift in the relative abundance of these major photosynthetic enzymes reveals that high lipid CP might be distributed differently across N and

(true) protein fractions. Such changes could affect proteolysis and microbial protein uptake in the rumen and ultimately the supply of protein to the animal (Bach et al., 2005). In silage, CP is regarded as a particularly poor measure of protein quality because of the variations in proteolysis and further breakdown of N compounds that occur during wilting and fermentation, which subsequently determines the effiency of protein utilization by the animal (Cherney & Cherney, 2003; Harrison et al., 2003). Future experiments could investigate, in more detail, the changes in N and protein fractions in fresh and ensiled high lipid PRG.

4.3.9 Glasshouse growth conditions resulted in atypical perennial ryegrass nutritional properties

Method development ensiling experiments revealed that glasshouse-grown PRG had a much lower WSC: BC than similar material grown in a paddock (Table 3.5). This explained the initial difficulty in converting it into well-preserved silage. The effect of the glasshouse environment on the broader nutritional composition of PRG were not measured. In Experiment 5 the use of a non-transgenic WT control for comparison to the ML and HL genotypes was useful in this regard because it (presumably) separated most 'glasshouse effects' from those which resulted from expression of the high lipid technology in PRG. Comparisons could then be made between the WT genotype to PRG grown in the field (from the literature), in order to estimate how the glasshouse conditions affected the overall nutritional composition of the material. Even so, it cannot be assumed that all of the recorded differences between HL, ML and WT would translate to the outdoors. The potential for unanticipated genotype x environment interactions (Lee et al., 2012; Parsons et al., 2011) seems especially important given the presently limited understanding metabolism of plants expressing the high lipid constructs.

In general, the PRG from Experiment 5 had low WSCs, high FA, CP, ash, nitrates and a high BC (Table 4.2) compared to outdoor-grown sward PRG harvested after a similar growth interval e.g. (King et al., 2012; Palladino et al., 2009). These nutritional features were characteristic of PRG grown under high N input regimes (Keady & O'kiely, 1996), and were probably exaggerated by the high temperature, low light glasshouse conditions (Appendix I) which resulted in the very low WSC contents. All PRG material had a low WSC:BC (1.37-1.54) and WSC:CP (0.24-0.29), which indicated that they were unsuitable for ensiling, and would be a nutritionally unbalanced feed for ruminants.

In low WSC:CP feeds, peptides, in place of carbohydrates, are used as the primary energy source to support early growth of rumen microbial populations, rather than being used for microbial protein synthesis. This occurance (proteolysis) happens before the major source of energy in forage is released

via cellulolysis by fibrolytic microorgansims (Kingston-Smith & Theodorou, 2000). Proteolysis in the hours after feeding generates large quantities of ammonia in the rumen which must be excreted in the urine; a process which imposes a metabolic load on the animal and pollutes the environment (Parsons et al., 2011). A forage WSC:CP in the range of 0.7-2.0 has been identified as a breeding target, because an increase above 0.7 reduces N loss as urinary ammonia (increased nitrogen use efficiency) (Edwards et al., 2007). In Experiment 5, HL, ML and WT had a WSC:CP well below the ideal range (Edwards et al., 2007). Although this did not impact the success of ensiling, it illustrated the extent to which the glasshouse growth environment distorted the nutrtitional composition of the PRG. This may have subsequently had an impact on *in vitro* rumen incubation fermentation parameters. Future nutritional analysis of high lipid PRG will be more insightful if, wherever possible, growth conditions can better reflect the outdoors.

4.3.10 Elevated HL nitrate content could influence silage fermentation and ruminant performance

Fresh HL and ML, respectively, contained 70% and 40% higher nitrate content than WT (Table 4.2). A similar difference was recorded in the previous regrowth trial (Experiment 4, Table 3.7). In both regrowth trials, the fresh nitrate content of all three genotypes was above the 'high risk of toxicity' threshold for ruminants (>1% dietary DM) (Bruning-Fann & Kaneene, 1993). The high plant nitrate levels were attributed to the high levels of N fertilizer application prior to harvest (Buxton & O'Kiely, 2003) and the glasshouse growth conditions (Table 4.6, Appendix I) which were very different to what would occur outdoors. Plant nitrates accumulate when the rate of nitrate uptake by the roots exceeds the rate of assimilation into ammonia and subsequently organic N. The reason that nitrates accumulate in the high lipid plants is presently unknown, but may be related to the hypothesis that nitrate assimilation in C3 plants is dependent upon photorespiration (Bloom et al., 2012; Rachmilevitch et al., 2004); a process which is speculated to be reduced in the high lipid plants (Winichayakul et al., 2013). It is unclear whether the higher nitrate content in the high lipid PRG would translate to the outdoors.

Nitrate reduction silage

In Experiment 5 the nitrate content of WT, ML and HL decreased 20%, 19% and 13%, respectively, during wilting and 45 days ensiling, meaning that all silages except WT still contained a 'high risk' nitrate level (<1% DM) (Table 4.4). Nitrate content decreases during wilting and anaerobic fermentation, and ensiling has been proposed as a strategy to reduce animal nitrate intake from pastures (Bolan & Kemp, 2003). The extent of nitrate reduction in silage depends on the type of fermentation that occurs. Nitrate reduction is reduced when measures are taken to encourage a rapid LA fermentation (McDonald et al., 1991) because

LAB do not metabolize nitrate, and their rapid proliferation minimizes the activity of the enterobacteria, which are the primary nitrate-reducing microorganisms in silage (Spoelstra, 1985). The residual nitrate content of high DM silages are high because a low a_w limits all microbial activity. Conversely, poorly preserved, low DM silages rarely contain any nitrate (Spoelstra, 1985). In Experiment 5 a rapid LA fermentation occurred (Figure 4.2), because of the addition of a LAB inoculant and glucose to the surface of the leaves. This provided an excess of fermentable substrate for LAB to convert into LA as soon as anaerobic conditions commenced, and explained the small decrease in nitrate content in all silages. Future experiments should measure the fresh nitrate content of independent high lipid PRG lines under different N input regimes, and could additionally assess the potential for a 'natural' fermentation (without additives) to reduce the nitrate content of high lipid PRG.

Nitrates reduce silage butyric acid content

Nitrates exert their influence on the silage fermentation pattern via their effect on silage microbial ecology. When silage microorganisms (primarily enterobacteria) reduce nitrate to ammonia, the intermediate nitrite accumulates, which in turn inhibits clostridial growth early in the fermentation phase (Spoelstra, 1985). Therefore, in addition to a crop's WSC:BC and DM content, a minimum quantity of nitrate is required to ensure that butyric acid (BA) does not develop in silage (Iv, 2001). While high N growth conditions generally reduce the probability of successful ensiling by increasing crop BC and lowering WSC and DM (Keady & O'kiely, 1996), they also increase crop nitrate content which will have a positive effect on the fermentation pattern by reducing BA production, providing that WSCs are not limiting (King et al., 2013). This could explain why no BA was detected in destabilized silages in Experiments 1 and 3 (Tables 3.4 and 3.6). It also suggests that if the high nitrate levels in high lipid PRG occur outdoors, then successfully ensiled high lipid PRG may contain low BA.

Nitrates and the rumen

High dietary nitrate levels can negatively influence animal performance (reduced feed intake, lower weight gain, and in extreme cases death) when rumen microbes reduce nitrate to nitrite; an intermediate to ammonia which can accumulate to high levels in the rumen. Nitrite is absorbed through the rumen wall and binds to the Fe^{2+} form of haemoglobin in the blood, converting it into Fe^{3+} (methaemoglobin), which cannot bind oxygen (Bolan & Kemp, 2003). Despite its toxicity potential, there is an increasing evidence that established 'toxicity thresholds' can be shifted upward (allowing more nitrates to be fed) if animals are gradually acclimatized to higher dietary nitrate levels (Lee & Beauchemin, 2014). Nitrates also reduce

enteric methane emissions by acting as an alternative hydrogen sink (to methane) in the rumen (Van Zijderveld et al., 2010). Nitrate reduction to ammonia is a more energetically favourable pathway than the reduction of CO_2 to methane and according to the stoichiometry of nitrate reduction, every mole of nitrate converted into ammonia reduces methane production by 1 mole (and increases CO_2 by 1 mole). Importantly, both *in vitro* and *in vivo* experiments show that nitrates consistently and persistently lower enteric methane production over time (Lee & Beauchemin, 2014).

4.3.11 High lipid silage exhibited less complete biohydrogenation in an in vitro rumen incubation

Microbial biohydrogenation (BH) of FAs is an extensive process in the rumen, and is the primary explanation for the apparent anomaly that meat and dairy products are a major source of saturated fats in the human diet, while forage FAs (which are the main feed for ruminants) are predominantly PUFAs (18:2 and 18:3). Two sequential processes are involved in BH; lipolysis of esterified lipids, followed by saturation of the double bonds on the free fatty acyl chain (Harfoot & Hazlewood, 1997). The former process is rate limiting for the first steps of BH, although complete saturation of plant PUFAs is additionally limited by the slow conversion from vaccenic acid (VA) to 18:0. There are well-established human health benefits of certain FAs which are found in high levels in ruminant products (Shingfield et al., 2013) and the BH intermediates conjugated linoleic acid (CLA) and VA have been targeted as desirable FAs flowing from the rumen into animal tissues (Lourenço et al., 2010). Accordingly, various efforts have been made to manipulate and/or reduce complete BH of FAs in the rumen. Dietary lipid supplementation (McKain et al., 2010) or lipid encapsulation technologies (Jenkins & Bridges, 2007) are effective for these purposes, however they are also costly. There is increasing scientific focus upon 'feedstuff endogenous factors' which reduce BH (Buccioni et al., 2012).

Feeding ensiled forages reduces the outflow of unsaturated FAs from the rumen, partly because silages contain high levels of free FAs (Steele & Noble, 1984), which are rapidly hydrogenated by rumen microorganisms. Endogenous plant factors that influence lipid metabolism *in silo* may offer opportunities to reduce this effect in silage (Van Ranst et al., 2009a). For example the efficiency of PUFA delivery from clover silages into ruminant products is often higher than from ryegrass silages e.g. (Dewhurst et al., 2003a). *In vitro* rumen incubation experiments (where the flow rate of digesta does not influence the extent of BH) indicate that these 'clover advantages' (in silage) have a chemical basis and may be related to the presence of plant lipase-inhibiting compounds (Van Ranst et al., 2013). In particular, it has been hypothesized that the activity of the polyphenol oxidase enzymes, which are high in clover, may inhibit lipolysis in silage and in the rumen, and therefore reduce rumen BH (Lee et al., 2004).

The quantity and class of lipids and FAs present in the diet influences microbial ecology and BH pathways and in the rumen (McKain et al., 2010). The leaves of high lipid PRG plants have higher total FA content and a higher proportion of 18:1 and 18:2 than WT. They accumulate a variable quantity of FAs in TAG (up to 35% DW compared to 5% DW in WT, pers. comm, Somrutai Winichayakul) which is protected from lipase entry *in planta* by the Cys-oleosin protein. The remaining additional FAs in these plants are located in the polar lipid fraction (Winichayakul et al., 2013). Early evidence from *in vitro* rumen incubation experiments show that the FAs in fresh (non-ensiled) high lipid PRG exhibit very different BH dynamics to WT PRG and may be protected (to some degree), from rumen BH (pers. comm, Somrutai Winichayakul). In the context of high lipid PRG ensiling, it is *possible* that if TAG protection from lipases (plant and microbial) is sustained *in silo*, then this might reduce the proportion of free FAs and therefore rumen BH in high lipid silage. This line of investigation could be pursued in future.

The HL silage generated in Experiment 5 maintained a 25% and 21% higher proportion of unsaturated C:18 FAs than WT silage after incubation in rumen fluid for 7 and 24 hours, respectively (Figure 4.4a). Many of the underlying changes in C:18 FA metabolism were complex, especially in the early hours of the incubation (Appendix V). 18:3 and 18:2 were rapidly hydrogenated, but the rate appeared to differ between substrates. Silage generally contained less 18:2 and more CLA and VA than fresh material at the later hours of incubation, although these differences were not always significant (data not shown). It should be acknowledged that the more sophisticated methods of analyzing biohydrogenation dynamics available were not employed in this preliminary work.

The BH intermediate VA accounted for much of the difference in overall C:18 FA saturation, and was 37% and 27% higher in HL silage than WT silage at 7 and 24 hours of the incubation, respectively (Table 4.4b). Together with the 37% and 33% higher total FA content in HL silage at these time points in the incubation (Appendix V, e), this suggests that substantial increases in the delivery of VA to animal tissues, and subsequently CLA to ruminant products (Griinari et al., 2000) may be possible with high lipid PRG silage. The often significant interactions between 'Material' and 'Ensiling' in parameters related to FA metabolism in the rumen indicated that the wilting and ensiling procedures influenced biohydrogenation in HL and WT silage differently (Appendix V). It was not clear whether these effects were due to the high lipid technology or uneven wilt conditions which were required to increase the DM content of the materials to 37.5%. Future experiments should avoid the use of different wilt durations/conditions before measuring the BH profile of HL silage because this will influence lipid metabolism during ensiling and subsequently BH of FAs in the rumen.

4.3.12 Ensiling and the high lipid technology altered the in vitro rumen fermentation pattern

Total gas production is an index of total fermentative activity in the rumen. Early gas production rates are higher in feeds with high levels of readily fermentable substrate (WSCs) (Lee et al., 2002) and this phenomenon was observable in Experiment 5 (Figure 4.5). Ensiling reduced gas production in both WT and HL, presumably due to nutrient losses during wilting and fermentation, and the conversion of plant WSCs (and the added glucose) into volatile components which would have been lost during freeze drying (Calabrò et al., 2005). A greater reduction in total gas production due to ensiling was observed in HL compared to WT, probably because of the greater losses of HL digestible nutrients, which were speculated to have occurred during wilting (Table 4.4). It should be acknowledged that the more sophisticated methods of analyzing gas production available were not employed in this preliminary work.

Volatile fatty acids (VFAs or scVFAs) are a product of microbial fermentation of plant nutrients in the rumen, and are absorbed through the rumen wall into the bloodstream to provide 40-70% of the animal's energy requirements. Total VFA production and the molar proportions of the three major VFAs; acetate, propionate and butyrate (VFA ratios) influence feed utilization, energy partitioning, milk composition and enteric methane production. Propionate is glucogenic and accounts for the majority of the glucose supply to the animal. Acetate and butyrate are non-glucogenic and are used as precursors for lipid synthesis (France & Dijkstra, 2005). A number of models attempt to relate rumen VFA ratios to nutritional composition of feeds, with varying degrees of success (Morvay et al., 2011). The acetate: propionate: butyrate ratio from dietary forages is approximately 70:20:10, while in high concentrate diets the proportion of propionate and/or butyrate is much higher. In Experiment 5, the HL and WT PRG material incubated in the *in vitro* system described by Muetzel et al. (2014) generally followed the 70:20:10 ratio, despite its rather atypical nutritional composition. Ensiling shifted the fermentation towards butyrate and (to a lesser extent) propionate, and away from acetate, while the HL technology shifted the fermentation away from butyrate and 'other scVFAs' and towards acetate (Table 4.5). Interactions between 'Material' and 'Ensiling' were rarely statistically significant, which suggested that the ensiling process had a similar overall effect on rumen VFA production for HL and WT.

Successfully ensiled forages typically increase the molar proportion of rumen propionate because propionate is the main end product of lactic acid (LA) fermentation by rumen microorganisms (Jalč et al., 2009; Sharp et al., 1994). However, variable losses of volatile silage components occur during the processing of ensiled samples before *in vitro* analyses, which can alter the rumen fermentation pattern (Alomar et al., 1999). The loss of silage alcohols, VFAs and ammonia during oven drying are almost

complete, while LA losses range from 1% in a 60°C oven over 48 hours to >35% in a 100°C oven over 16 hours (Porter & Murray, 2001). In Experiment 5, samples were freeze-dried (for >48 hours) and ground before the *in vitro* rumen incubation (for compliance reasons). To the author's knowledge, there is no published material on LA losses during freeze-drying. However, freeze-drying for 24 hours led to *total* DM losses from silage that were intermediate between oven drying at 70°C for 48 hours and 100°C for 24 hours (Aerts et al., 1974), suggesting that LA losses may be substantial (Calabrò et al., 2005). In Experiment 5, the potentially low LA content in the freeze-dried ensiled samples could explain why ensiling increased the molar proportion of butyrate more than propionate (Table 4.5). Acetate and butyrate are both non-glucogenic, and so the increase in rumen butyrate would be expected to impact animal performance less than an increase in propionate. Both non-glucogenic VFAs are associated with an increase in milk fat content (Dijkstra, 1994), however milk yield is strongly related to rumen butyrate concentration and to a lesser extent propionate, but not acetate (Seymour et al., 2005).

On the other hand, the high lipid technology increased the molar proportion of acetate and reduced butyrate compared to WT in both fresh and ensiled material (Table 4.5). In experiments where sheep and cattle are fed forages supplemented with plant oil or fish oil (which are rich in PUFAs), the effect is typically an increase in the molar proportion of propionate, with a concomitant reduction in butyrate and/or acetate (Broudiscou et al., 1994; Dong et al., 1997; Doreau & Chilliard, 1997; Sutton et al., 1983; Ueda et al., 2003). That the additional 31% (Table 4.2) and 37% (Table 4.4) total FA content in fresh and ensiled HL PRG compared to WT did not increase the molar proportion of rumen propionate in this *in vitro* rumen incubation experiment could indicate that these differences in FA content were not sufficiently large to induce such an effect. Other differences in HL nutritional composition might have masked any effects due to increased FA content. Carbohydrates are the main substrate for rumen fermentation, and proteins and the glycerol component of lipids are also fermented to various VFAs (France & Dijkstra, 2005). HL and WT PRG differed to a similar extent (9-16g/kgDM) in FA, CP and WSC content, and may have differed further in other unmeasured nutritional components. More detailed nutritional analyses will be required to how high lipid PRG influences the rumen fermentation.

CHAPTER 5. CONCLUSIONS

A promising application of the high lipid technology in livestock agriculture is its expression in the widely used pasture species perennial ryegrass (PRG). The elevated leaf lipid content and biomass accumulation traits in these plants could increase the energy yield from pastures, which could in turn improve animal performance and farm profitability. Field trials will determine how these traits translate to the outdoors, and how to best manage this novel technology in pastoral farming systems. In a future scenario where a version of this technology is available to farmers, ensiling could be a convenient option for preserving high lipid PRG, so that it can be later used as a high ME, inexpensive, supplementary feed. Ensiling preserves a crop's nutrients at a high moisture content and a low pH, by fermenting plant WSCs to LA under anaerobic conditions.

In this preliminary work, the influence of the high lipid technology on the ensiling biochemistry of glasshouse-grown PRG was investigated. Subsequently, the nutritional properties of the ensiled high lipid PRG were determined. Using high-precision ensiling procedures and unprecedentedly small model silages, it was found that the addition of glucose was necessary for glasshouse-grown PRG to undergo a rapid LA fermentation, as would be expected in well-preserved silage made under typical outdoor conditions.

Expression of the high lipid technology in PRG influenced plant metabolites that are important to the silage fermentation. Elevated FA synthesis and growth rates reduced WSC accumulation, while the speculated reduction in photorespiration increased nitrate accumulation, relative to a non-transgenic control genotype. Unrelated to ensiling, the high lipid technology increased PRG CP content. The fatty acid component of the high lipid PRG was well-preserved during anaerobic fermentation, however greater overall nutrient losses occurred in the high lipid PRG during the wilting phase. Less extensive microbial biohydrogenation of high lipid silage C18 fatty acids occurred during an *in vitro* rumen incubation experiment.

Only field trials and subsequent farm-scale ensiling and animal feeding experiments will confirm whether the anticipated animal performance benefits of feeding the high lipid PRG are present when it is ensiled. In the short term, further glasshouse experiments will investigate the stability of high lipid PRG FAs during an extended wilting phase. Subsequent *in vitro* rumen incubation experiments will determine the biohydrogenation dynamics of a number of wilted and ensiled high lipid PRG lines. The WSC and nitrate content of independent high lipid PRG lines grown in a range of abiotic environments will be determined. In-depth nutritional analysis of the high lipid PRG over an extended vegetative regrowth and during the

reproductive transition will provide insight into how this novel technology should be managed for grazing or ensiling purposes.

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APPENDICES

Appendix I Glasshouse abiotic growth environments

Air temperature (temp, °C) and relative humidity (RH) in the glasshouses, measured by data loggers kept at plant height.

I.I Non containment and containment glasshouses in October 2015

Non-containment glasshouse

Temp	Day temp	Night temp	Max temp	Min temp	Day RH	Night RH
18.5	22.7	13.1	39.5	5.5	65.3	85.6

Day temp and RH calculated as the average of half-hourly readings between the times of 6:30 am and 7:30 pm.

Containment glasshouse

Temp	Day temp	Night temp	Max temp	Min temp	Day RH	Night RH
22.2	24.6	20.2	39.5	18	61.1	75.8

Day temp and RH calculated as the average of half-hourly readings between the times of 6:30 am and 7:30 pm.

I.II Regrowths before ensiling experiments 1, 2 and 4

Experiment 1, non-containment glasshouse (29/09/2015-27/10/2015) [regrowth actually began 25/09/2015]

Temp	Max temp	Min temp	RH
18.61	39.5	5	73.62

Experiment 2, containment glasshouse (20/10/2015-12/11/2015)

Temp	Day temp	Night temp	Max temp	Min temp	Day RH	Night RH
22.9	25.1	20.8	34.5	16.5	59.2	74.5

Day temp calculated as the average of half-hourly readings between the times of 6:30 am and 7:30 pm.

Experiment 4, containment glasshouse (25/11/2015-15/12/2015) [regrowth actually began on 16/11/2015]

Temp	Max temp	Min temp	RH
21.8	38	16.5	78.9

I.III Retrospective measurement of PAR in the glasshouses compared to outdoors (March 2016).

In March 2016 (*after* Experiments 1-5) measurements of photosynthetically active radiation (PAR) at plant height on the glasshouse benches (and outdoors, adjacent to the glasshouses) were made with a light meter (Li-Cor, model LI-189, United States). 4-5 PAR measurements at each site were recorded and averaged, within 5-10 minutes of solar noon, on both a clear day and an overcast day. Large variation in PAR across the surface of the glasshouse bench was evident due to shading effects. There was little variation in PAR readings outdoors. PAR (measured in mol $m^{-2}s^{-1}$) in the two glasshouses were similar on both days, and approximately 50-70% of the recorded outdoor measurements.

21/03/2016, overcast day

Location	**Non-containment glasshouse**	**Outdoors**	**Containment glasshouse**
Time of measurement	1:29-1:31pm	1:26-1:28pm	1:24-1:25pm
Average PAR reading	267	660	322

30/03/2016, cloudless day

Location	**Non-containment glasshouse**	**Outdoors**	**Containment glasshouse**
Time	1:16-1:19pm	1:21-1:23pm	1:26-1:29pm
Average PAR reading	554	1570	548

Appendix II Climatic data

II.I Average daily climatic data during the regrowth period for Experiments 1-5

	Temperature mean (°C)	Temperature maximum (°C)	Temperature minimum (°C)	Rainfall (mm/day)	Sunshine hours	Solar Radiation (MJ/m^2)	RH (%)
Experiment 1	11.66	19.08	1.03	2.18	5.2	15.7	80.83
Experiment 2	12.54	22.21	2.64	2.40	4.7	17.5	79.25
Experiment 3	13.72	24.65	1.93	2.94	4.6	19.1	80.00
Experiment 4	14.94	24.65	6.39	3.09	4.7	20.1	80.28
Experiment 5	16.21	26.86	7.12	1.83	6.9	23.0	73.63

II.II Average daily climatic data for the 5 days before harvest for Experiments 1-5

	Date	Temperature (°C)	Sunshine hours	Solar radiation (MJ/m^2)
Experiment 1	22/10/2015	14.06	0.0	3.6
		13.81	1.3	11.1
		12.59	11.2	26.0
		12.96	7.0	21.4
	27/10/2015	13.04	7.2	21.4
Experiment 2	08/11/2015	12.69	4.6	23.6
		15.89	4.6	22.4
		15.48	1.1	15.9
		13.37	0.2	10.2
	12/11/2015	10.17	3.6	18.6
Experiment 3	28/11/2015	15.60	9.4	27.8
		18.25	7.4	25.1
		17.03	0.0	7.6
		17.43	0.0	12.3
	02/12/2015	17.86	0.1	13.8
Experiment 4	11/12/2015	16.47	6.0	25.9
		18.52	4.2	22.9
		15.34	9.5	28.8
		13.60	9.8	28.3
	15/12/2015	15.35	2.8	19.8
Experiment 5	09/12/2015	18.61	1.0	11.1
		16.76	5.5	23.3
		15.75	0.9	13.5
		16.56	11.4	30.1
	13/01/2016	16.09	0.6	11.4

II.III Trends in climatic data during Experiments 1-5

Meaningful interpretation of chemical and nutritional data from plants grown for sequential experiments can be complicated by the effect of seasonal weather patterns (whether experiments are run outdoors or in the glasshouse). Experiments 1-5 were run during spring/summer (the beginning of October 2015 until mid-January 2016). During this period there was a strong trend of increasing average daily outdoor temperature and solar radiation over time and a weak trend of increasing sunlight hours per day over time. These trends in outdoor weather patterns were reflected in the glasshouse (Appendix 1, Table 4.6). Together, the effects of increasing temperature and solar radiation from October to January, the effects of operating ensiling experiments between two glasshouses, and the effects of the reproductive transition that occurred in November and December, probably meant that no two regrowths used in Experiments 1-5 were fully comparable.

Appendix III Plant material vegetative propagation and cutting history

Date	Non-containment glasshouse	Containment glasshouse
14/08/2015	Received 40 established IMP566 (WT) plants (from nursery), cut to 5cm, and transplanted into 3L pots.	
17/08/2015		Received 9 WT, 6 ML and 6 HL plants (from glasshouse), cut to 5 cm, transplanted into 3L pots. ML split into 12 plants.
26/08/2015	All WT split into 80 plants and cut to 5cm.	
10/09/2015		Received an additional 14 WT and 12 ML plants (from glasshouse), transplanted into 3 L pots. HL split into 12 plants.
11/09/2015		All plants cut back to 5 cm.
25/09/2015	All plants cut back to 5 cm.	
04/10/2015		ML cut back to 5 cm.
19/10/2015		HL split into 24 plants. All plants cut to 5 cm.
27/10/2015	All plants cut to 5 cm for EXPERIMENT 1.	
02/11/2015	All plants cut to 5 cm.	
09/11/2015	27 WT plants moved into GH6.	All WT plants discarded and replaced with 27 WT plants from the Non-containment glasshouse.
12/11/2015		ML cut to 5cm for EXPERIMENT 2.
16/11/2015		All plants are cut to 5 cm.
02/12/2015	All plants cut to 5 cm for EXPERIMENT 3.	
15/12/2015		All plants cut to 8 cm for EXPERIMENT 4.
13/01/2016		All plants cut to 8 cm for EXPERIMENT 5.

HL = high lipid, ML = medium lipid, WT = 'Wild type' (IMP566)

Appendix IV Paddock section

IV.I Paddock section description

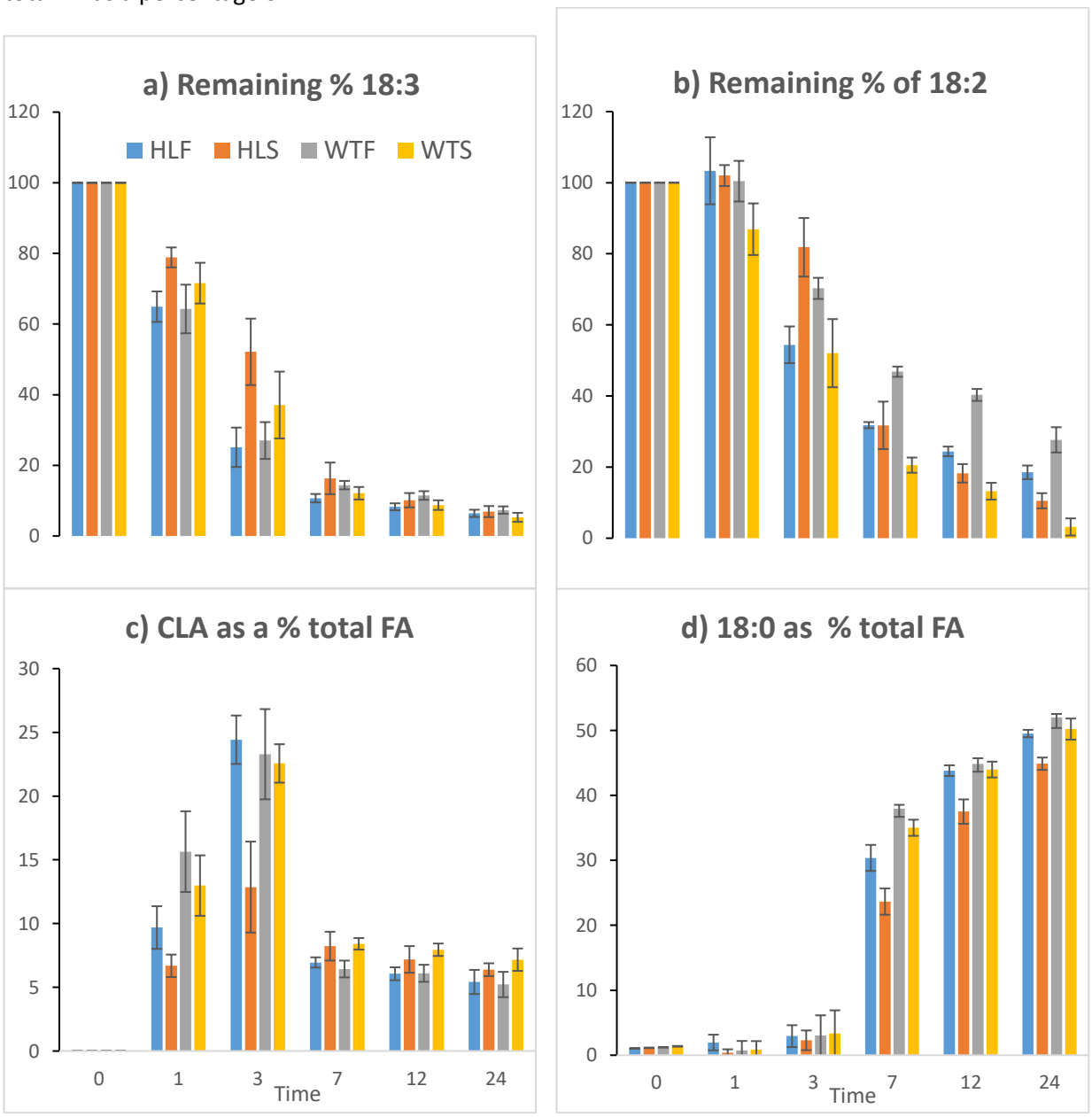
A 12m^2 section of sward ryegrass at the end of a paddock at AgResearch Palmerston North was cut to 6-7 cm with a lawnmower and fenced off from grazing sheep on 25/09/2015. The paddock was last grazed at 03/09/2015, although heavy rainfall prior meant that sheep were moved off to avoid treading damage before grazing was complete. The paddock was sown on 4/11/13 with Trojan NEA/2 Superstrike seed (Agriseeds Ltd., Christchurch New Zealand) and then over-sown on 7/5/14 after a dry summer.

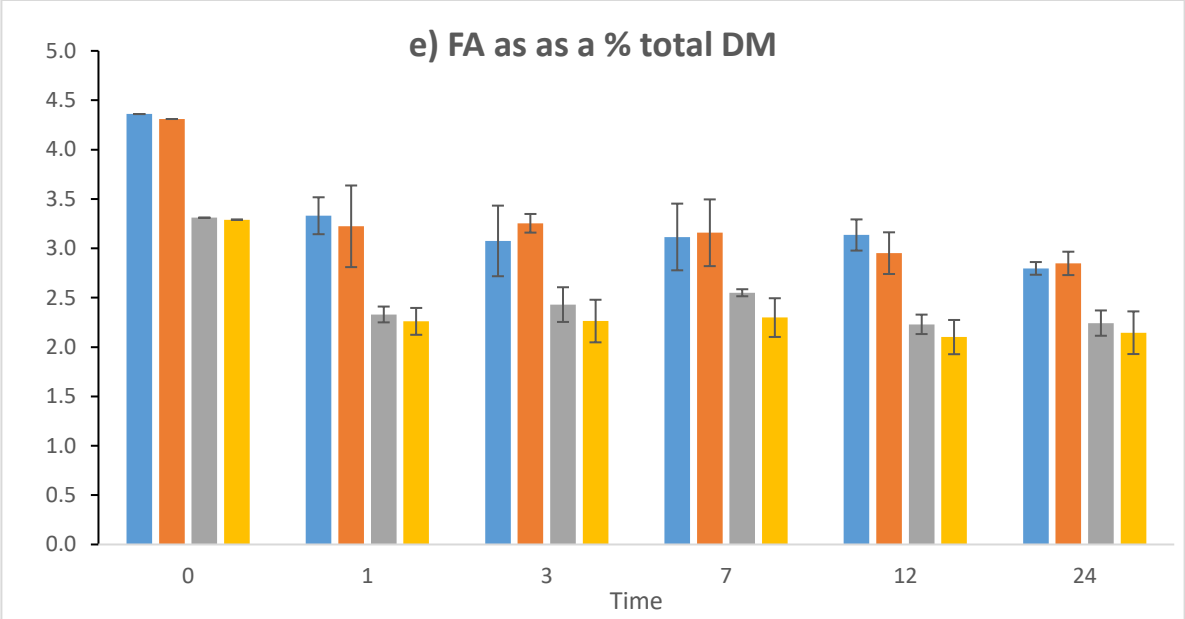
IV. II Trojan NEA/2 and Impact 566 comparison

Experiment 1 and 3 involved a comparison of the ensiling biochemistry of glasshouse-grown potted 'IMP566' PRG and outdoor-grown sward 'Trojan/NEA2' PRG. IMP566 is a single genotype selected from the cultivar 'Grasslands Impact' for its superior tissue culture characteristics (pers. comm, Kim Richardson). The cultivar 'Grasslands Impact' is a late-heading diploid PRG variety, derived from 'Grasslands Nui', and north-western Spanish germplasm (pers. comm, Tom Lyons). The cultivar 'Trojan NEA/2' is also a late-heading diploid PRG variety, derived from a reselection of the cultivar 'Tolosa' which came from germplasm sourced from north-western Spain in 1986 (Forde & Easton, 1986). Because the two PRG materials shared similar ancestry and were both late heading, they were considered to be a good candidates for a comparison glasshouse and outdoor PRG ensiling. The Trojan/NEA2 PRG contained a population of individuals while the glasshouse plants were vegetative clones. Therefore the material was pooled and thoroughly mixed after harvest in order remove most of the variation before ensiling.

Appendix V Biohydrogenation of fatty acids during in vitro rumen incubation

Changes in C18 FAs during a 24 hour *in vitro* rumen incubation of fresh HL, ensiled HL, fresh WT and ensiled WT PRG. a) 18:3 as a percentage of its pre-incubated concentration b) 18:2 as a percentage of its pre-incubated concentration c) CLA as a percentage of total FA d) 18:0 as a percentage of total FA e) total FA as a percentage of DM.





HLF = High lipid fresh, HLS = High lipid silage, WTF = Wild type fresh, WTS = Wild type silage, CLA = Conjugated linoleic acid

Appendix VI Example of automated spreadsheet for wilting calculations

Example of a spreadsheet template used in Experiment 4 & 5 to rapidly calculate PRG DM content during wilting. The 'Dry matter determination' table (top) was used to determine the average dry matter content of the pre-wilted fresh material, after drying to a consistent weight in an 80°C oven . This information was entered into the 'wilted weights' table along with the weight of the material being wilted in an oven at 35-45°C (in a tray) to calculate the real time DM content during wilting. Trays were removed from the oven when the plant material reached the target DM content. Here, a range of example values are entered into the 'Dry weight' column in the 'Dry matter determination' table and the 'WW' (wilted weight) column in the 'wilted weights' table, to show how the output values in the 'DM%' column change.

Dry matter determination

	Tray number	Tray weight	Fresh weight plus tray	Minus tray	Dry weight	DM proportion	Average
IMP566	1	5	25	20	4	0.2	
IMP566	2	5	25	20	4	0.2	
IMP566	3	5	25	20	4	0.2	0.2
ODR5203	4	5	25	20	3.5	0.175	
ODR5203	5	5	25	20	3.5	0.175	
ODR5203	6	5	25	20	3.5	0.175	0.175
ODR3704	7	5	25	20	3	0.15	
ODR3704	8	5	25	20	3	0.15	
ODR3704	9	5	25	20	3	0.15	0.15

Line	Target WW
IMP566	93.33333333
ODR5203	81.66666667
ODR3704	70

Wilted weights

	Number	Tray/bag weight	FW	MT	WW	WW - MT	DM%	Avg
IMP566	1	10	150	140	100	90	0.311111	
IMP566	2	10	150	140	99	89	0.314607	
IMP566	3	10	150	140	98	88	0.318182	
IMP566	4	10	150	140	97	87	0.321839	
IMP566	5	10	150	140	96	86	0.325581	
IMP566	6	10	150	140	95	85	0.329412	
IMP566	7	10	150	140	94	84	0.333333	0.322009
ODR5203	8	10	150	140	100	90	0.272222	
ODR5203	9	10	150	140	99	89	0.275281	
ODR5203	10	10	150	140	98	88	0.278409	
ODR5203	11	10	150	140	97	87	0.281609	
ODR5203	12	10	150	140	96	86	0.284884	
ODR5203	13	10	150	140	95	85	0.288235	
ODR5203	14	10	150	140	94	84	0.291667	0.281758
ODR3704	15	10	150	140	100	90	0.233333	
ODR3704	16	10	150	140	99	89	0.235955	
ODR3704	17	10	150	140	98	88	0.238636	
ODR3704	18	10	150	140	97	87	0.241379	
ODR3704	19	10	150	140	96	86	0.244186	
ODR3704	20	10	150	140	95	85	0.247059	
ODR3704	21	10	150	140	94	84	0.25	0.241507

IMP566 = Wild type, ODR5203 = Medium lipid, ODR3704 = High lipid, DM = Dry matter, FW = Fresh weight, MT = 'Minus tray', WW = wilted weight, WW-MT = 'wilted weight minus tray weight'

Appendix VII Construct design, transformation and regeneration procedure

Construct design

The Garden Nasturtium (*Tropaeolum majus*) DGAT1 peptide sequence with the single point mutation of serine at 197 amino acid sequence to alanine as described by Xu et al. (2008) linked with V5 epitope tag (GKPIPNPLLGLDST) at the C-terminal (DGAT1-V5), and the 15-kD Sesame (*Sesame indicum*) L-oleosin with three engineered cysteine residues on each N- and C-terminal amphipathic arms (Cys-oleosin) (Winichayakul et al., 2013) were custom synthesized by GeneART™ for expression in perennial ryegrass. Both DGAT1-V5 and Cys-oleosin coding sequences were optimized for expression in rice (*Orytz sativa*) and placed into the designed Gene Gun compatible construct. The resulting construct contained the DGAT1-V5 gene regulated by the rice ribulose-1, 5-bisphosphate carboxylase small subunit promoter back-to-back with the Cys-oleosin gene regulated by the rice chlorophyll *a/b* binding protein promoter.

Transformation, selection and regeneration procedure

Ryegrass plants expressing the construct described above were generated by micro-projectile bombardment (Gene Gun). Embryonic calli for transformation were induced from immature inflorescences. Floral tillers were harvested, surface sterilized in a sodium hypochlorite solution (4% available chlorine), dissected, and then cultured in the dark at 25°C for four to six weeks prior to transformation.

Plasmids were prepared using the Invitrogen Pure Link Hi Pure Plasmid Maxiprep 30 Kit with the concentration adjusted to 1 µg/µL. The plasmid pAcH1, which contains a hygromycin phosphotransferase gene expressed from the rice actin promoter, was used for 'selection' by conferring resistance to the antibiotic hygromycin to allow regrowth of transformed tissues. Plasmids containing the DGAT1-V5 and Cys-oleosin construct described above were mixed in a 1:1 molar ratio with pAcH1.

The Plasmid DNAs were coated onto M17 tungsten particles (1.0 µM diameter mean distribution) and transformed into target tissues using a Bio-Rad PDS-1000/He Biolistic Particle Delivery System using the settings recommended by the manufacturers. Following transformation the calli were transferred to a medium containing hygromycin and cultured in the dark for 4 weeks for the selection of transgenic events. Regeneration of whole plants from somatic embryos occurred on a shoot regeneration medium under a 16 hour photoperiod.

Transformed plants were transferred to a containment glasshouse environment for analysis by PCR to detect stable integration of both the hygromycin selection marker and the plasmid containing the DGAT1-V5 and Cys-oleosin construct. Multiple independent heterozygous ryegrass transformants were generated. Leaves from these plants were initially analyzed for total fatty acid content and recombinant DGAT1-V5 and Cys-oleosin.

Appendix VIII Experiment 4 silage pH at intervals during the fermentation

The pH of silage made from wild type, medium lipid and high lipid PRG with and without added glucose are presented below. All silages with added glucose reached a pH of approximately 4.4 at 24 days of fermentation, although the rate at which this pH was reached differed widely among the ensiled materials. Silages without glucose reached a pH >6 after 24 days of fermentation.

pH of wild type, medium lipid and high lipid perennial ryegrass silage, with and without glucose added at 5% DM, at intervals during the fermentation. Values for '+ glucose' silages represent means ± SD (n = 2). '- glucose' silage pH measurements were not replicated.