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Improving the utilisation of home grown forage legumes by high yielding dairy cows

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Declaration

I declare that this work in this thesis is original and was composed by myself. None of this work has been presented in any previous application for any degree or qualification. I have acknowledged all sources of information used in this thesis by means of references.

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Publications

Campbell, C.E.A., Huntington, J.A. and Sinclair, L.A. (2016) The effect of white or coloured flower forage pea silage as a replacement for grass silage on the performance and whole tract digestibility of high yielding dairy cows. *Proceedings of the British Society of Animal Science*, Vol 7, page 121

Campbell, C.E.A., Huntington, J.A. and Sinclair, L.A. (2016) Replacing grass silage with forage pea silages in the diet of dairy cows on milk fatty acid profile. *67th Annual Meeting of the European Federation of Animal Science* Vol 22, page 270

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Abstract

In recent years, the interest in the use of home grown protein sources for the dairy industry has increased due to fluctuations in cost and availability of soyabean meal (SBM). Forage legumes have been of particular interest due to their high crude protein content, ranging between 170 to 220 g/kg DM. Protein in forage legumes is degraded rapidly within the rumen, however a high yielding dairy cow requires high levels of by-pass protein to maintain milk yield. Tannins are phenolic compounds that can bind to forage protein in the silo or rumen, providing protection from microbial breakdown, before dissociating in the small intestine, increasing the amount of by-pass protein available from the forage legume. Studies were therefore conducted to evaluate the effects of tannins, either naturally occurring or added to forage legumes at ensiling, on the nutritive value, nitrogen utilisation and performance of high yielding dairy cows. In the first study, the effect of the addition of condensed or hydrolysable tannins at inclusion rates of 0, 25, 50 or 75 g/kg DM at ensiling on the nutritional value of forage legumes was investigated. Forage source influenced all nutritional values including DM, CP, ash, NDF and ammonia nitrogen content, whilst forage DM content increased from 329 to 364 g/kg as inclusion of tannin increased from 0 to 75 g/kg DM. Addition of tannins influenced forage pH with forages supplemented with condensed tannins having a higher pH than those supplemented with hydrolysable tannins. Forage pea silages have varying levels of naturally occurring condensed tannins and also a high crude protein content and the second study investigated the effect of the inclusions of forage pea silages differing in tannin content on dairy cow performance. Dairy cows fed pea silage containing low levels of condensed tannin had the lowest dry matter intake whilst milk yield was highest in cows fed grass silage. Similarly, milk protein was highest in cows fed grass silage, but there was no effect of treatment on milk fat content. Milk fat content of C18:2 *n*-6 was highest in cows when fed grass silage ($P < 0.001$), whilst C18:3 *n*-3 was highest in cows fed pea silage containing high levels of tannin. In comparison to forage pea silages, lucerne and red clover silages have limited amounts of naturally occurring tannins but also have a high content of crude protein. The addition of hydrolysable tannins at ensiling can bind to the forage protein in the silo improving the availability of by-pass protein from the forage, therefore the third study investigated the addition of hydrolysable tannins at 25 g/kg DM to lucerne and red

clover silage on the performance of lactating dairy cows. The inclusion of tannin had no effect on dairy cow performance, however forage source influenced dry matter intake with cows fed lucerne silage having the highest intake ($P < 0.05$), similarly, OM and N intake was highest in cows fed lucerne silage. In both dairy cow studies, DM, N and OM digestibility were not significantly affected by forage type or the presence of tannins. In conclusion, any commercial advantage from feeding forage legumes to high yielding dairy cows will be based on savings in N from fertiliser or dietary protein rather than animal performance, whilst tannins will have little effect on milk performance.

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List of Abbreviations

AIA	Acid insoluble ash
ANOVA	Analysis of variance
CP	Crude protein
CT	Condensed tannin
d	Day
DM	Dry matter
FA	Fatty acid
FAME	Fatty acid methyl esters
g	Gram
GC	Gas chromatography
h	Hour
HT	Hydrolysable tannin
Kg	Kilogram
ml	Millilitres
mg	Milligram
ME	Metabolisable energy
MP	Metabolisable protein
N	Nitrogen
NDF	Neutral detergent fibre
NH_3-N	Ammonia nitrogen
NPN	Non-protein nitrogen
OM	Organic matter
P	Probability
PPO	Polyphenol oxidase
PUFA	Polyunsaturated fatty acids
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
s.e.d	Standard error of the difference
SBM	Soyabean meal
TMR	Total mix ration
TN	Total nitrogen

Chapter 1 Literature review

1.1 Introduction

In the past 10 years, prices of soya bean meal (SBM; *Glycine max*) have fluctuated due to an increase in global demand from different industries including the ruminant industry (Figure 1). The volatility in cost and availability of SBM has produced a greater interest in the use of home grown protein sources by the UK ruminant industry. Forage legumes have an ability to fix nitrogen and grow well in temperate regions like the UK, therefore offer a potential home grown source of protein for dairy cows. Forage legumes contain a high level of rumen degrabable protein however, high yielding dairy cows require high levels of rumen undegradable (by-pass) protein to improve and maintain high milk yields of above 35 kg per day. Forage legumes are unable to provide the high by-pass protein requirements of a high yielding dairy cow. Therefore, research of ways to reduce rapid degradation of protein from forage legumes in the rumen is necessary to make home grown forage legumes a viable source of protein to the UK dairy industry.

1.2 Use of purchased protein in UK dairy industry

Supplementary protein sources, such as SBM or rapeseed meal (*Brassica napus)*, contribute a large part to a ruminant's diet (Wilkinson, 2011). Price and availability of SBM to the ruminant market is dependent on the demands and use of SBM as the main source of protein for the monogastric agricultural industry in the UK (Wilkinson, 2011). Increased global demand for these supplementary protein sources and associated fluctuations in availability and price has resulted in greater interest in the utilisation of home grown, protein-rich forage sources for ruminants (Hart *et al*., 2011). Over the past 10 years, price of SBM has ranged between £200 and £400 per tonne, peaking in September 2012 and lowest in October 2008 (Figure 1). From September 2012, the price per tonne decreased but peaked rapidly in July 2016 at £335 per tonne; in March 2018 the price per tonne was £334.

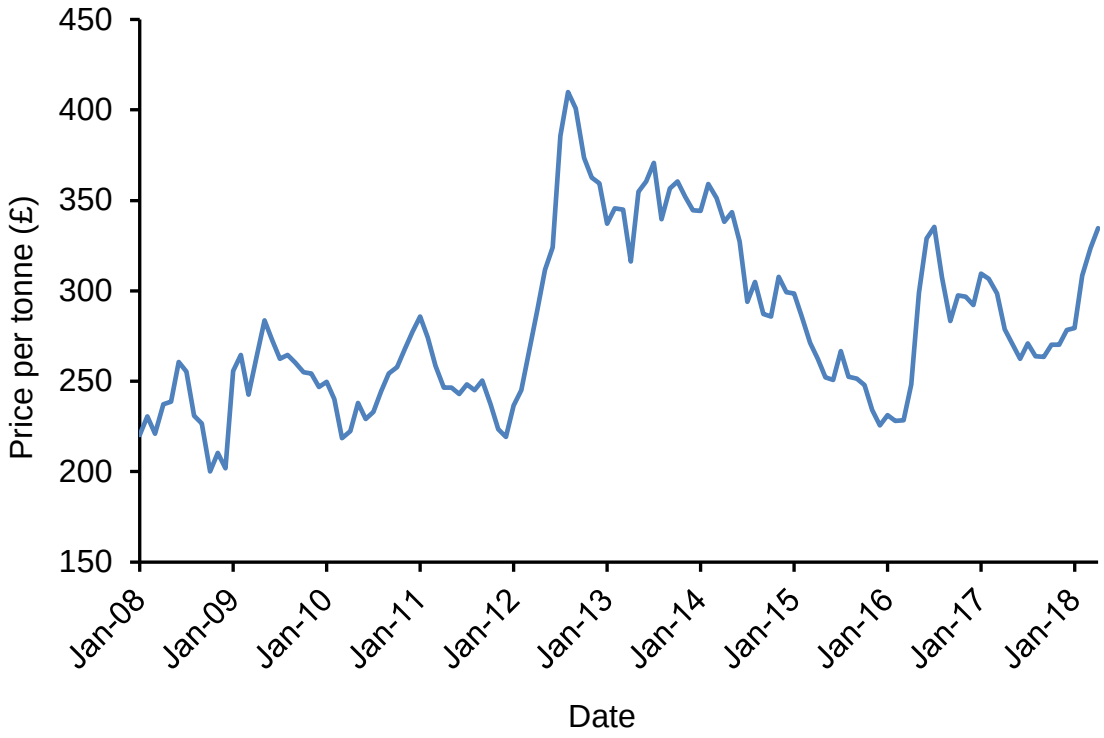


Figure 1 The market price per tonne (£) of soya bean meal (SBM) since January 2008

1.3 Digestion and metabolism of forage legumes by dairy cows

Forage legumes have been studied intensively as a forage source for ruminants (Broderick *et al.,* 2001; Moorby *et al*., 2009; Hart *et al.,* 2011; Arndt *et al*., 2015). Studies have considered the nutritive value of the silage, intake, rumen fermentation, whole tract digestion, milk production and composition, and efficiency of nitrogen use (Hoffman *et al*., 1998; Sinclair *et al*., 2009; Leduc *et al*., 2017; Broderick *et al.,* 2000).

1.3.1 Nutritive value

For alternative forages, like forage peas, lucerne and red clover, to be successful in the dairy industry, the nutritive value must be compared to conventional forages, like grass, to ensure their viability (Mustafa *et al*., 2002). The nutritive value of forage legumes and grass silage are summarised in Table 1. Forage legumes can provide a higher level of crude protein (CP) and starch than traditional grass silages (Mustafa *et al*., 2000). In traditional rations cereal crops, such as maize or whole crop wheat, are incorporated in the diet to provide fermentable carbohydrate, however some legume silages can contain higher levels of starch

reducing the need for large quantities of cereal crops (Mustafa *et al*., 2000). Forage pea silages can produce high DM contents of between 300 and 400 g/kg when harvested at growth stage 206 (Fraser *et al.,* 2001). In a study conducted by Sinclair *et al*. (2009), forage pea silages had a DM content that was 107 g/kg higher than grass silage at a mean value of 351 g/kg. Likewise, Hart *et al*. (2011) reported a DM content of 324 g/kg for pea silages, however the grass silage had a higher dry matter content of 403 g/kg.

Similarly, lucerne and red clover silage can have high DM contents. Broderick *et al.* (2001) reported a DM content of 520 and 358 g/kg for lucerne and red clover silage, respectively. However, the DM content of these silages can be variable with Broderick *et al*. (2000) reporting values of 392 and 448 g/kg for lucerne and red clover silage, respectively. Grass silage tends to have a lower or similar dry matter content to forage legumes with a range of 249 – 375 g/kg (Moorby *et al*., 2009; Dewhurst *et al*., 2003; Vanhatalo *et al*., 2009). It is known that timing of mowing and wilting is critical to the final DM content of the silage; dry, warm and windy weather conditions can increase the DM of the crop by wilting and removing water accumulated from rain and dew (Wilkinson, 2005).

A key nutritive value difference between forage legumes and grass silages is CP content with forage legumes ranging between 177 and 228 g/kg DM, whereas grass silage tends to be lower, ranging between 111 and 206 g/kg DM (Table 1). Sinclair *et al*. (2009) and Hart *et al.* (2011) reported values of between 177 and 197 g/kg DM for white and coloured pea silages, respectively. In contrast, grass silage was lower, ranging between 119 and 121 g/kg DM in the study of Hart *et al*. (2011) and Sinclair *et al*. (2009). Similarly, Fraser *et al*. (2001) observed high CP values of 228, 200 and 204 g/kg DM for forage pea silage harvested at 10, 12 and 14 weeks post sowing, respectively. Even though, CP content declined as harvest date post sowing increased for the forage pea silages, levels did not fall below 200 g/kg DM, which is higher than grass silage.

Broderick *et al.* (2001) conducted two studies that included red clover and lucerne silage that was harvested in two separate years. The CP content for both forages varied between 179 g/kg and 217 g/kg DM, with the red clover silage having a lower CP than lucerne silage in both years, averaging 185 and 204 g/kg DM, respectively. In contrast, in a previous study by Broderick *et al*. (2000) the

difference in CP content between red clover and lucerne silage was 36 g/kg DM compared to the 19 g/kg DM difference reported by Broderick *et al*. (2001). These slight differences in CP content are in agreement with Hymes-Fecht *et al*. (2013) who reported a 39 g/kg DM difference between red clover and lucerne silage.

In a study conducted by Hoffman *et al*. (1998), lucerne silage had a slightly higher CP content of 202 g/kg DM whereas grass silage was slightly lower at 184 g/kg DM. Similarly, Moorby *et al*. (2009) reported that grass silage had a CP content of 206 g/kg DM whereas red clover silage was comparable at 194 g/kg DM. Maturity at harvest can affect the CP content of grass silage, and in Hoffman *et al.* (1998) the grass silage was a late summer cut which may have contributed to the high CP content observed. In a study conducted by Vanhatalo *et al*. (2009), grass and red clover silages were harvested at two maturity levels, early (early heading = grass; preflowering = red clover) and late (heading = grass; early flowering). For both forages, CP content declined from early to late harvest, with grass silage declining from 134 to 111 g/kg DM and red clover declining from 212 to 181 g/kg DM.

Some forage legumes have a high starch content in comparison to grass silage therefore and consequently, there is an opportunity to reduce the reliance on cereal crops in the rations of dairy cows. Starch levels in lucerne and red clover silage are low, and comparable to grass silage, Halmemies-Beauchet-Filleau *et al*. (2014) observed a higher starch content of red clover silage at 16.3 g/kg DM compared to a starch content of 10.3 g/kg DM in grass silage. Starch content of lucerne silage tends to be higher than red clover silage and Benchaar *et al*. (2007) reported it at 176 g/kg DM. In contrast, starch content of forage peas and beans are higher and were variable when measured by Sinclair *et al*. (2009) and Hart *et al*. (2011). Sinclair *et al*. (2009) reported a higher starch content of 87 g/kg DM in white pea silage whereas red pea silage was 29 g/kg DM lower at 58 g/kg DM. In contrast, Hart *et al*. (2011) reported white pea silage having a lower starch content of 63 g/kg DM while red pea silage was 10 g/kg DM higher. Starch content of pea silage can be affected by the age at which the crop is harvested, and Fraser *et al*. (2001) observed an increase in starch content from 19.2 to 124.9 g/kg DM from 10 to 14 weeks post sowing. Starch content can also vary between species of pea silage, with Mustafa *et al*. (2002) reporting a difference between Lenca, Carneval and Delta at 68, 92 and 103 g/kg DM, respectively. Therefore, it can be concluded that forage legumes such as peas or beans have a higher starch content than

grass silage although maturity and variety of the legume can influence starch content.

Table 1 Nutritive value of forage legumes and grass silages

Silage	DM, g/kg	CP, g/kg DM	Ash, g/kg DM	NDF, g/kg DM	Starch, g/kg DM	Reference
Pea						Mustafa *et al*. (2002)
Lenca (coloured)	265	179	93	427	68	
Carnival (coloured)	280	205	68	317	92	
Delta (coloured)	274	190	100	333	103	
Pea						Sinclair *et al*. (2009)
Racer (coloured)	338	189	--	256	58	
Croma (white)	365	177	--	252	87	
Grass	244	121	--	537	1	
Red clover	233	200	103	339	16.3	Halmemies-Beauchet-Filleau
Grass	236	156	87.2	529	10.3	*et al*. (2014)
Red clover						
Early	214	212	102	375	--	Vanhatalo *et al*. (2009)
Late	212	181	93	463	--	
Grass						
Early	249	134	86	500	--	
Late	257	111	75	570	--	
Lucerne	590	164	--	393	176	Benchaar *et al*. (2007)
Maize	515	155	--	375	250	
Lucerne	406	183	110	375	--	Sinclair *et al*. (2015)
Grass	353	127	94	528	--	
Grass	160	22.1[1]	--	441	--	Lee *et al*. (2009)
Red clover	139	32.6[1]	--	309	--	

[1]Total N

1.3.2 Intake

Voluntary intake by dairy cows depends upon the forage physical and chemical characteristics, with one of the most important characteristic of a forage being its fibre content (Bertilsson and Murphy, 2003). The rate at which feed is cleared from the rumen can affect dry matter (DM) intake, an important factor in this clearance is the particle size (less than 2mm) of feed after chewing (Waghorn *et al*., 1989). A summary of the effects of forage legume inclusion on DM intake is provided in Table 2. In a study conducted by Moorby *et al*. (2009) red clover silage partially or totally replaced grass silage in the diet of dairy cows, with DM intake increasing with the inclusion of red clover silage. The difference in intake of grass and red clover silage in the study of Moorby *et al*. (2009) was suggested to be due to the difference in particle size. Particle size of forages affects the rumen fill rates leading to a change in feed intake by the cow (Dewhurst *et al*., 2003). Red clover is known to have a smaller particle size than grass silage, therefore allowing a larger feed intake. In a study by Hymes-Fecht *et al*. (2013) dairy cows fed red clover silage had a larger DM intake of 25.6 kg/d whilst cows fed lucerne silage had a lower DM intake of 24.7 kg/d. Waghorn *et al*. (1989) investigated the breakdown of fresh lucerne and ryegrass in the rumen, and reported that lucerne particles decreased in size at a faster rate than ryegrass. It was therefore concluded that lucerne passed through the rumen more rapidly allowing more protein from the feed to escape degradation in the rumen and reach the intestines to be digested than ryegrass. Waghorn *et al*. (1989) also concluded that for particles to move through the reticulo-rumen freely, the optimal size is 2 mm or less. Dewhurst *et al*. (2003a) also concluded that particles of 2 mm or less of forage legumes would move through the reticulo-rumen freely increasing rumen outflow. Rumen outflow was highest in cows fed lucerne silage, and lowest in cows fed grass silage (Dewhurst *et al*. 2003a).

In a study conducted by Mustafa *et al*. (2000), dairy cows were offered pea, barley or lucerne silage, at a 50:50 forage: concentrate ratio (DM basis), and reported there was no effect on DM intake which were 28.6, 27.5 and 26.0 kg/d for pea, lucerne and barley silage, respectively. In contrast, Adesogan *et al*. (2004) reported that forage DM intake of cows offered pea-wheat silage was 18.3 kg/d which averaged 3.65 kg/d more than cows offered grass silage with DM intake of 16.5kg/d. The difference in DM intake in the study by Adesogan *et al*. (2004) was

suggested to be due to characteristics of peas in intercrop silage that improved intake. A high starch content and high CP content are characteristics that are considered to improve intakes of dairy cows (Adesogan *et al*., 2004). In two separate studies, Broderick *et al*., (2000) and Broderick *et al*., (2001), replaced lucerne silage with red clover silage and reported that DM intake was lower in dairy cows that had been fed red clover silage. Hymes-Fecht *et al*. (2013) investigated the effects of birdsfoot trefoil silage with varying levels (8 – 16 g/kg DM) of condensed tannin as a replacement for red clover and lucerne silage. Forage legume source had no effect on DM intake of the dairy cows and it was concluded that the condensed tannins were not at a concentration to cause a depression in intake.

1.3.3 Rumen Fermentation

Ruminants are animals with a specialised digestive tract containing four stomach compartments, reticulum, rumen, omasum and abomasum (McDonald *et al*., 2011) A ruminant's feed will undergo extensive fermentation within the rumen prior to digestion in the abomasum and small intestine (Russell *et al*., 1992). The fermentation process is important as it will have a significant impact on the animal's performance (Russell *et al*., 1992). The rumen is an anaerobic environment with a diverse microbial population including bacteria, fungi, protozoa, methanogenic archaea and bacteriophages in varying numbers (Morgavi *et al*., 2010). The activity and presence of these microbes determine the quality of fermentation of feedstuffs that occurs in the rumen (Russell *et al*., 1992). The microbes benefit from the fermentation of different components of the feed, with carbohydrate fermentation providing an energy source for the microbes (Russell *et al*., 1992). The degradation of feed as seen in Figure 2 begins with polymers of protein and carbohydrate undergoing microbial hydrolysis by primary anaerobic fermenters to form monomers (Morgavi *et al*., 2010). Primary fermenters are capable of degrading protein and carbohydrate polymers to volatile fatty acids (VFA) and microbial protein without the assistance of secondary fermenters. However, primary anaerobic fermenters and secondary fermenters that are not capable of degradation of polymers are the microbes involved in the fermentation of monomers to CO_2, H_2, microbial protein and VFAs; acetate, propionate and butyrate (Figure 2; Morgavi *et al*., 2010). The animal is able to utilise these VFAs as an energy source, lactic acid can be produced in the rumen when fermentation

becomes rapid (Russell *et al*., 1992). Following fermentation, propionate can be converted into glucose via gluconeogenesis in the liver; butyrate is mainly used as an energy source by the digestive tract (Dijkstra *et al*., 2012; Vlaeminck *et al*., 2006). Acetate is absorbed across the rumen wall and may be used as energy source or is available for synthesis into milk fatty acids (Dijkstra *et al*., 2012; Vlaeminck *et al*., 2006).

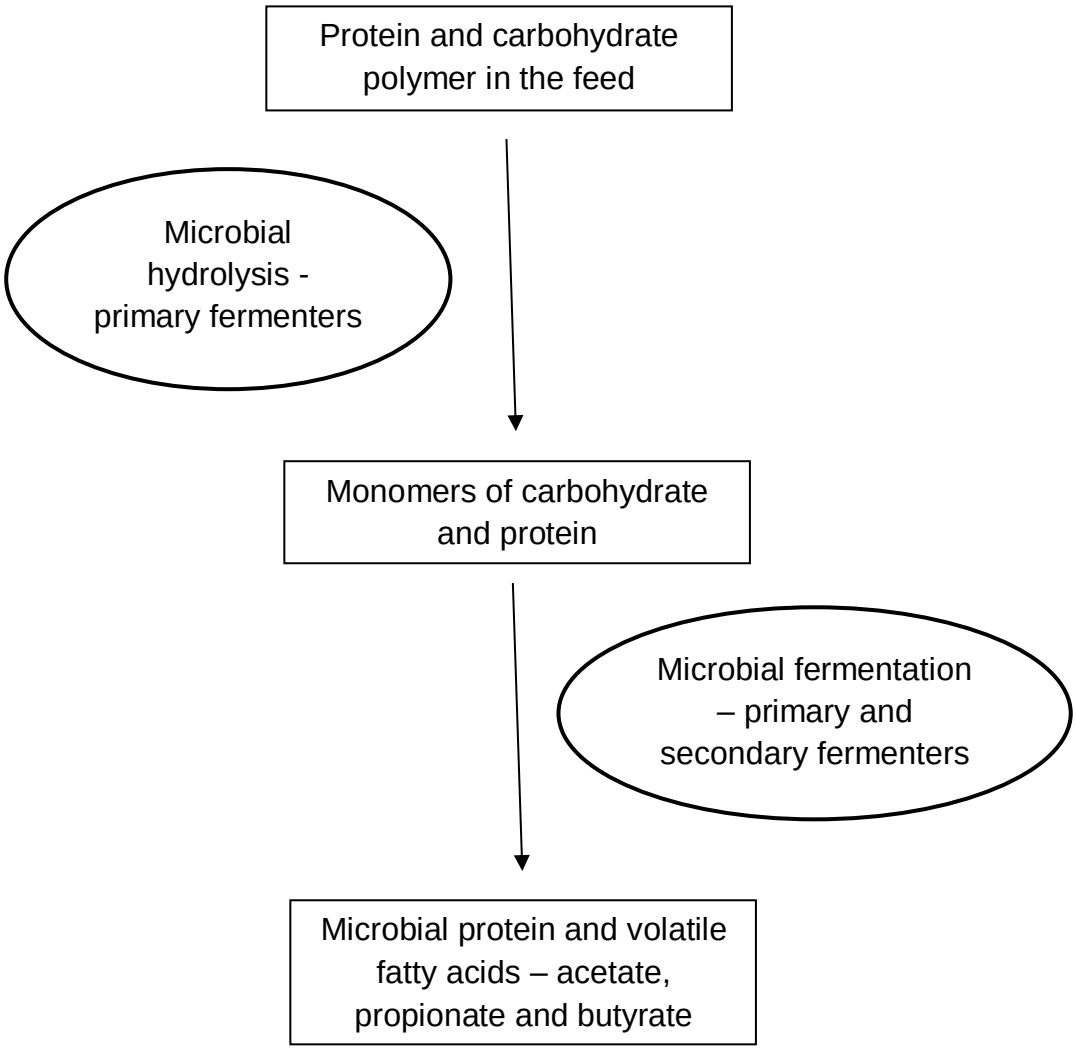


Figure 2 The microbial fermentation of carbohydrate and protein in the rumen. Adapted from Morgavi *et al*. (2010).

Rumen pH is normally between pH 5.0 to 7.0, however it can vary depending on the time of day and production of VFAs or lactic acid (Dijkstra *et al*., 2012). Following the ingestion of feed, rumen pH will fall due to the breakdown of carbohydrates to volatile fatty acids, and the accumulation of VFAs cause the decline in pH (Dijkstra *et al*., 2012). Rumen pH will rise as volatile fatty acids are

cleared from the rumen, however an accumulation of lactic acid will cause rumen pH to fall (Dijkstra *et al*., 2012). Leduc *et al*. (2017) observed a rumen pH of 6.53 in dairy cows fed lucerne silage but a higher pH of 6.69 in dairy cows fed red clover silage, and it was concluded this difference in pH was due to the higher fibre content at 300 g/kg DM for the red clover silage. This increase in rumen pH was associated with a decrease in propionic acid concentration in cows fed red clover silage by 10 mM. In contrast, Vanhatalo *et al*. (2009) observed no difference in rumen pH when dairy cows were fed grass or red clover silage at pH 6.42 or pH 6.38, respectively. However, cows fed red clover silage had a higher overall concentration of volatile fatty acids in the rumen at 117 mmol/L compared to cows fed grass silage in which volatile fatty acid concentration was 105 mmol/L. This lack of change in pH and difference in volatile fatty acid concentration was suggested to be due to the lower buffering capacity and substrates in grass silage being less fermentable than in red clover silage.

In a study by Arndt *et al.* (2015) when the inclusion rate of lucerne silage in the diet increased from 20% to 80% there was no change in rumen pH which averaged pH 6.48. Similarly, there was no difference in the overall volatile fatty acid concentration as the rate of inclusion of lucerne silage increased, however levels of acetate increased while levels of propionate decreased as the inclusion of lucerne silage increased from 20% to 80%. Arndt *et al*. (2015) concluded these changes in acetate and propionate concentrations may have resulted from the lower amounts of maize silage starch available in the high inclusion diets. In a study conducted by Adesogan *et al*. (2004) two varieties of pea silage (Magnus and Setchey) were intercropped with wheat, and rumen pH was highest in cows fed grass silage (pH 6.69) whereas cows fed pea-wheat intercrop silage had a lower pH (pH 6.62). However, total volatile fatty acid concentration was highest in cows fed grass and Setchey-wheat silage at 79.5 and 76.4 mmol/L, respectively. Total volatile fatty acid concentration was lowest in the Magnus-wheat silage at 67.4 mmol/L, and it was suggested these differences were due to differences in starch content of the silages as the breakdown of starch has a large impact on the volatile fatty acids that accumulate in the rumen.

1.3.4 Whole tract digestion

Whole tract digestibility of DM, CP, OM and NDF of feed can provide information about how efficiently each component of the feed has been digested by the ruminant. When pea (Magnus and Setchey) silages were intercropped with wheat in the study of Adesogan *et al*. (2004), DM, CP and OM digestibility was higher than in cows fed grass silage. Grass silage is degraded at a slower rate than pea silages in the rumen hence the lower digestibility of DM and OM in cows fed grass silage (Salawu *et al*., 2002a). Digestibility of NDF was higher in the cows fed Magnus-wheat silage than cows fed Setchey-wheat pea silage at 0.653 and 0.564 g/g, respectively. Adesogan *et al*. (2004) suggested this difference in NDF digestibility between the two pea – wheat silages was due to the higher pea to wheat ratio in the Magnus-wheat silage therefore a higher ratio of silage that can be degraded rapidly.

Previous studies have compared the differences in digestibility of grass silage to red clover or lucerne silage in a dairy cow's diet. Halmemies-Beauchet-Filleau *et al.* (2014) reported a linear decline in DM, OM, NDF and N whole tract digestibility as the inclusion rate of red clover to grass silage increased from 0, 33, 67 to 100%. Reductions in whole tract digestibility of DM, OM, NDF and N can indicate that the availability of nutrients in the rumen and small intestine are lowered (Halmemies-Beauchet-Filleau *et al*., 2014). The lower N digestibility in red clover silage may indicate a higher concentration of amino acids available from the forage protein supply rather than the microbial protein supply in the omasum, however these forage amino acids are not as readily absorbed (Halmemies-Beauchet-Filleau *et al*., 2014). In contrast, Vanhatalo *et al*. (2009) reported no change in total tract OM digestibility between cows fed red clover silage or grass silage at 74.4 or 73.4 %, respectively. Despite no change due to silage type, the age of the silage at cutting caused a decline in total tract OM digestibility from 75.6 to 72.1 % for early to late harvesting, respectively (Vanhatalo *et al*., 2009). Total tract N digestibility was lower in cows fed grass silage by 4.35 % compared to cows fed red clover silage in a study by Vanhatalo *et al*. (2009) study, although rumen N digestibility was lower in cows fed red clover silage (69.6% v 64.3%; grass v red clover silage). Similar to total tract OM digestibility, N digestibility was reduced by the maturity of the silage for both grass and red clover silages, averaging 70.6 and 68.5 % for early and late harvesting, respectively (Vanhatalo

et al., 2009). Vanhatalo *et al*. (2009) concluded that the lower N digestibility in the rumen was influenced by the greater flow of non-ammonia N (NAN) from the rumen to the small intestine.

Broderick *et al*. (2001) compared the apparent whole tract digestibility of cows fed either red clover or lucerne silage. Cows fed red clover silage had a higher whole tract digestibility for DM, OM, N and NDF compared to cows fed lucerne silage. The difference in NDF digestibility was suggested to be due to the different maturities of the silages with red clover silage being harvested as an early cut. Broderick *et al*. (2001) concluded that the higher N digestibility in red clover silage was due to an increase in N intake but there was, also 40 g/kg DM more soya bean meal included in the diet containing red clover silage, and consequently the availability of by-pass protein contained in SBM may be more readily degradable than protein in red clover silage which could have confounded these results.

Digestibility of DM, OM and CP were 3.2, 3.0 and 5.2 % lower, respectively, when cows were fed lucerne silage compared to cows fed grass silage in a study conducted by Hoffman *et al*. (1998), although digestibility of NDF was similar between the two forages. Hoffman *et al*. (1998) suggested these differences in DM, OM and CP digestibility may be attributable to the difference observed in rate of passage, with cows fed lucerne silage having a quicker rate of passage by 0.81 %/h. Arndt *et al*. (2015) observed no effect of an increase in lucerne to maize silage ratio from 20:80 to 80:20 on DM, OM and CP total tract digestibility averaging 70.2, 72.6 and 73.0 %, respectively. Although, NDF total tract digestibility increased linearly from 35.5 to 48.1% as lucerne silage inclusion rate increased from 20 to 80 %, respectively. Arndt *et al*. (2015) concluded that NDF present in lucerne silage was more digestible in the rumen than NDF in maize silage.

In contrast, Sinclair *et al*. (2015) reported changes in DM, OM, N and NDF whole tract digestibility with different inclusion rates of lucerne silage replacing grass and maize silage in the diet of dairy cows. As the inclusion rate of lucerne in the diet increased from 0 to 60 %, there was a decline in DM and OM digestibility from 0.712 to 0.655 kg/kg and 0.728 to 0.673 kg/kg, respectively. Similarly, a reduction from 0.712 to 0.673 kg/kg was observed in N digestibility as lucerne silage increased in the diet. Sinclair *et al*. (2015) suggested this change in N digestibility

may be influenced by protein post rumen degradation being less in lucerne than SBM. Sinclair *et al.* (2015) reported contrasting results for NDF digestibility with a decline when lucerne silage replaced grass silage from 0.631 to 0.582 kg/kg, agreeing with the previous study of Hoffman *et al*. (1998). However, Sinclair *et al*. (2015) reported no change in NDF digestibility (0.519 kg/kg) when lucerne silage replaced maize silage (40:60 v 60:40 DM basis). These varying effects of NDF digestibility between Arndt *et al*. (2015) and Sinclair *et al*. (2015) may be attributable to the higher inclusion rate of 80% lucerne silage and lower NDF intake by 2.41 kg/day of lucerne silage in the study of Arndt *et al*. (2015).

1.3.5 Milk production and composition

A number of studies have examined the effects of different legume silages on milk yield and composition, and are summarised in Table 2. Broderick *et al*. (2001) reported that replacing lucerne silage with red clover silage alone did not affect milk yield; however there was an increase in yield when a mixture of red clover-maize silage replaced lucerne silage. Cows fed diet containing red clover-maize silage (80:20 DM basis) also had an increase in milk protein yield, which averaged 0.04 kg per day more than in cows fed the lucerne-maize silage (80:20 DM basis) (Broderick *et al*., 2001). It was, however, suggested that this increase may have been due to the addition of soyabean meal to the diet improving rumen degraded protein and rumen undegraded protein content. In contrast, in a study by Moorby *et al*. (2009) dairy cows fed red clover silage produced a higher milk yield than those fed grass silage which may be due to the increased DM intake. Milk fat content declined with increasing red clover, as milk yield increased (Moorby *et al*., 2009). Moorby *et al*. (2009) concluded that milk fat was diluted in milk from cows fed this diet as there was no dietary treatment effect on milk fat yield. An important factor in milk fat content is body weight change of the cow, with an increase in milk fat content is associated with body weight loss (Broderick *et al*., 2000). The dairy cows in the study of Moorby *et al*. (2009) lost the greatest body weight of -0.25 kg per day when fed red clover silage, but was associated with the lowest milk fat content. In contrast, Broderick *et al*. (2000) reported that when dairy cows gained weight, there was a reduction in milk fat content. The deposition of fat into milk comes from four pathways in the body of a dairy cow, *de novo* synthesis in the mammary glands, uptake from the diet via blood, biohydrogenation in the rumen and the fat stores within the body (Stoop *et al*., 2009). Therefore, it could be

suggested that cows will utilise the fat in the feed to improve body fat stores rather than improving the milk fat content.

The reduction in milk protein content observed as the inclusion of red clover in the diet increased in the study of Moorby *et al*. (2009; Table 2) was suggested to be due to the grass silage having a high crude protein (CP) content of 206 g/kg DM compared to 194 g/kg DM for the red clover silage. Also, grass silage fed cows had a higher apparent efficiency of synthesis of milk protein than red clover fed cows. It was suggested that the amount of microbial protein reaching the small intestine was increased by grass silage causing a higher protein content in milk (Moorby *et al*., 2009). Grass silage has a higher water soluble carbohydrates but lower N content than the legume silages and the ratio of these two factors are important in the conversion of feed N to microbial N (Merry *et al*., 2006). It was shown by Merry *et al*. (2006) that the amount of microbial N from the rumen reaching the duodenum can be improved in dairy cows when both red clover and grass silage are included in the diet. Therefore, both these studies (Merry *et al*., 2006 and Moorby *et al*., 2009) suggest that for a high protein content of over 30 g/kg in milk, feeding a mixture of grass (33%) and red clover silage (66%) is better than feeding these silages individually to dairy cows.

Previous studies (Adesogan *et al*., 2004; Salawu *et al*., 2002b) have investigated the use of bi-crops in dairy cows. Legume silages can be used as part of a bi-crop with several cereals such as barley and wheat (Adesogan *et al*., 2004). Wheat has a high degradability of starch within the rumen and legumes are thought to be able to complement wheat by improving milk performance of cows (Salawu *et al*., 2002b). Dairy cows fed a pea-wheat bi-crop in a study conducted by Salawu *et al*. (2002b) had milk yields which averaged 22.3 kg/d, improving milk yield by 1.57 kg/d compared to those fed grass silage. There was however a reduction in milk fat content from 51.4 to 47.5 g/kg for cows fed the pea-wheat bi-crop Salawu *et al*. (2002b) which was suggested to be due to the lower DM intake. In a study conducted by Salawu *et al*. (2002b), growth stage of the crops at harvest may have been a contributing factor to milk fat content which was higher in all cows fed both pea crops that had been harvested after 13 weeks than cows that had received crops that had been harvested after 15 weeks. In contrast, Adesogan *et al*. (2004) reported no differences in milk yield or constituents when cows were fed pea-wheat bi-crop silage compared to grass silage. Comparing the bi-crops from

both studies (Salawu *et al*., 2002b; Adesogan *et al*., 2004) DM digestibility was 95 g/kg lower in the study of Salawu *et al*. (2002b) than in Aesogan *et al*. (2004). Aesogan *et al*. (2004) concluded that these factors may have contributed to the differences seen in milk fat content. Bi-crops, however, are difficult to compare with whole crops and whether effects seen in studies are due to the individual legume crop or not.

Table 2 Milk yield and composition of dairy cows fed leguminous silages.

Silage	Inclusion rate	DM intake, kg/d	Milk yield, kg/d	Milk fat, g/kg	Milk protein, g/kg	Milk lactose, g/kg	Reference
Red clover	100	19.0	26.1	35.5	29.3	45.8	Moorby *et al.* (2009)
Grass	100	16.7	25.2	38.0	30.8	45.8	
Lucerne	71.1	21.9	32.5	36.0	29.1	48.2	Broderick *et al.* (2000)
Red clover	70.5	20.7	31.2	33.8	28.8	48.3	
Lucerne	60.5	23.5	30.4	43.0	33.3	46.7	Broderick *et al.* (2001)
Red clover	60.2	21.8	30.4	40.6	33.0	47.6	
Lucerne + maize	48.3	23.8	30.3	43.3	33.9	46.9	
Red clover + maize	48.5	22.8	31.7	40.8	33.6	47.8	
Pea	50	28.6	45.2	36.5	30.3	45.0	Mustafa *et al.* (2000)
Lucerne	50	27.5	45.3	33.6	31.6	44.8	
Barley	50	26.0	43.2	36.5	30.8	45.1	
Pea/Wheat	100	18.3	22.4	42.9	31.6	46.8	Adesogan *et al.* (2004)
Grass	100	16.5	22.3	40.9	31.8	46.6	
Low tannin pea	50	20.5	24.6	43.8	34.6	45.5	Sinclair *et al.* (2009)
High tannin pea	50	20.3	22.9	43.5	35.8	45.7	
Grass	50	18.8	23.7	43.4	35.6	45.3	
Lucerne	59.4	24.7	30.9	37.4	31.9	46.9	Hymes- Fecht *et al*. (2013)
Red clover	50.5	25.6	30.8	37.2	30.6	47.3	
Low CT[1] birdsfoot trefoil	60.5	24.7	33.5	35.7	31.9	46.5	
Normal CT[1] birdsfoot trefoil	60.3	24.0	34.6	38.6	31.4	47.1	
High CT[1] birdsfoot trefoil	60.7	25.2	35.4	37.4	32.1	47.1	

[1]CT = condensed tannin

In more recent years, research has focussed on evaluating legumes, in particular peas, as a whole crop rather than as part of a bi-crop. In a study conducted by Sinclair *et al.* (2009), forage peas of either low (47.3 g/kg DM) or high (92.7 g/kg DM) condensed tannin content were included in the diet to investigate the effect on animal performance compared to a diet containing grass silage and whole crop wheat silage. It was reported that there was no change in milk yield or composition, and that both pea silages could replace soyabean meal at the rate of 1.1 kg/day/cow in the diet. In contrast, in a study conducted by Mustafa *et al*. (2000), pea silage was compared to lucerne and barley silage with all diets being isonitrogenous. Forage type had no effect on milk yield, however there were some differences in milk composition; in cows fed lucerne silage, milk fat content was lower at 33.6 g/kg compared to 36.5 g/kg for both pea and barley silage. Milk protein content was higher at 31.6 g/kg for cows fed lucerne silage compared to 30.3 g/kg for cows fed the pea silage. In the study conducted by Mustafa *et al*. (2000), the CP of the pea silage was lower at 170 g/kg DM and therefore may have contributed to the lower milk protein content. It was concluded by Mustafa *et al*. (2000) that lucerne and barley silage could be totally replaced by pea silage without affecting milk yield.

1.3.6 Efficiency of nitrogen use

Efficiency of nitrogen by lactating dairy cows is determined by the conversion of feed N to milk N, but in ruminant's N efficiency is generally low varying between 15 and 40 % (Calsamiglia *et al*., 2010). Nitrogen efficiency can indicate the flow of microbial protein from the rumen to the small intestine, relating to the nitrogen content of the feed with high efficiencies indicating a high flow rate (Moorby *et al*., 2009). Efficiency of N can be impacted by the ensiling process due to the extensive breakdown of protein to NPN (Schulz *et al*., 2018). Red clover silage tends to produce a higher N efficiency when fed to dairy cows compared to grass silage, due to the less extensive proteolysis that occurs during the ensiling process and higher CP content in red clover silage (Schulz *et al*., 2018). Dewhurst *et al*. (2003) observed a lower N efficiency of 21% in cows fed red clover silage compared to 25.6% in cows fed grass silage however lucerne silage had a lower N efficiency of 18%. Lucerne silage tends to undergo extensive proteolysis therefore the availability of true protein is lower than grass or red clover silage. However, when N intake is considered, in the study by Dewhurst *et al*. (2003), cows fed

lucerne silage had the highest intake at 778 g/day whilst cows fed grass silage had the lowest N intake at 507 g/day. Dewhurst *et al*. (2003) suggested the low N efficiency but high N intake of lucerne silage may be due to an imbalance of indigestible fibre and rapidly available N. In a study conducted by Arndt *et al*. (2015), it was observed that as the amount of lucerne silage in the diet increased from 20 to 80%, N efficiency decreased from 30.7 to 24.1 g/100g, respectively. Although, there was a linear increase in N intake, fecal N and urinary N, therefore Arndt *et al*. (2015) suggested that these changes in N efficiency may be due to the reduced CP available and higher losses of N in urine and faeces.

Similarly, Broderick *et al*. (2001) observed a higher N efficiency of 0.250 kg milk N/kg feed N when dairy cows were offered red clover silage compared to an N efficiency of 0.233 kg milk N/kg feed N for cows fed lucerne silage. These differences in N efficiency between red clover and lucerne silage may be due to the lower NPN content that is found in red clover silage following proteolysis in the silo (Schulz *et al*., 2018). It is noted that the CP content of the lucerne silage was higher at 191 g/kg DM compared to 179 g/kg DM for the red clover silage in Broderick *et al*. (2001) study, however due to the extensive proteolysis occurring within in the silo, the CP content in the lucerne silage may be higher in RDP increasing N losses in the urine and faeces. Therefore, the higher N efficiency in cows fed red clover silage may be due to higher levels of RUP which becomes available for digestion in the small intestine and conversion to milk N. In a study conducted by Vanhatalo *et al*. (2009), cows fed grass silage had an N efficiency that was 6% higher at 29% than cows fed red clover silage. Nitrogen efficiency of grass silage used in Dewhurst *et al*. (2003) and Vanhatalo *et al*. (2009) studies differed by 3.4%, and it was higher in Vanhatalo *et al*. (2009) study, which could be attributable to the difference in CP content of the grass silages. In Vanhatalo *et al*. (2009) study, the grass silage was 46.5 g/kg DM lower in CP content than Dewhurst *et al*. (2003) which would suggest that there were higher urinary and faecal N losses in the Dewhurst *et al*. (2003) study. Therefore, crude protein of silage plays an important role in the conversion of feed N to milk N and N efficiency.

Nitrogen efficiency was not improved by the inclusion of pea silage when Salawu *et al*. (2002b) fed a pea-wheat intercrop to dairy cows, with N efficiency averaging

21.6% which was 2.4% lower than in cows fed grass silage. In comparison, Sinclair *et al*. (2009) fed whole crop pea silage and reported that cows fed grass silage had an N efficiency of 0.295 g/g which was higher than the 0.241 g/g observed in cows fed whole crop pea silage. Nitrogen efficiency can be effected by different factors including milk yield and change in body weight therefore when comparing results from studies care must be taken (Sinclair *et al*., 2009). High levels of RUP in the feed can impact the N efficiency of forages because microbes will breakdown higher proportions of RDP to ammonia losses across the rumen wall, therefore the MP supply for the dairy cow is met by the supply of RUP in the feed (Lee, 2014). Therefore, the conversion of feed N to milk N is increased resulting in improved N efficiency (Lee, 2014).

1.4 Tannins

Tannins can be classified as either condensed or hydrolysable (Scalbert, 1991). Condensed tannins have a higher molecular weight of up to 20 000 g/mol compared to hydrolysable tannins that have a molecular weight varying between 500 to 3000 g/mol (Cieslak *et al*., 2013). Condensed and hydrolysable tannins are extracted from different plants. For example, tropical plants produce more condensed tannins such as quebracho (*Schinopsis marginata)* and mimosa (*Acacia mearnsii*), while temperate plants produce more hydrolysable tannins such as chestnut (*Castanea sativa* L.) tannins (Tabacco *et al*., 2006). In plant cells tannins can be found in various areas. For instance vacuoles can contain large quantities of tannins while in the epidermis and trichomes of leaves, tannins are more concentrated (Aerts *et al*., 1999).

1.4.1 Condensed tannins

Condensed tannins are heterogenous compounds that are also known as proanthyocyanides (Waghorn, 2008). Condensed tannins are secondary plant metabolites containing polyhydroxy-flavan-3-ol oligomers and polymers (McMahon *et al*., 2000) as seen in Figure 3. Carbon-carbon bonds occur between flavanol subunits, linking oligomers and polymers (Schofield *et al*., 2001). These carbon bonds are stable during hydrolysis (Reed, 1995). Chain lengths of flavanol units in polymers of condensed tannins that occur in temperate forages can vary from two to over twenty (Waghorn, 2008).

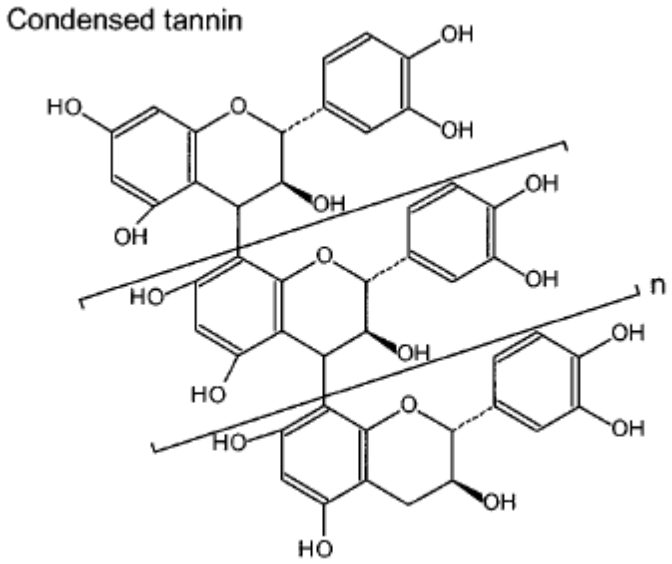


Figure 3 Chemical structure of condensed tannin containing three flavanol-3-ol subunits. After McMahon *et al*. (2000).

The palatability of a plant containing condensed tannins can be affected by the growth stage, due to tannins varying in concentration with age of the plant (McMahon *et al*., 2000). Condensed tannins can form complexes with proteins, fatty acids and minerals in certain conditions depending on pH, and in ruminants this binding of protein occurs in the rumen at pH 4.5 – 7.0, with normal rumen pH being 6.5 – 7.0 (McMahon *et al*., 2000). The protein complexes are stable within the rumen and will pass freely through to the abomasum and small intestine where dissociation occurs (Tabacco *et al*., 2006). In some cases, if the protein is covalently bound to the condensed tannin, this complex may not separate and will pass out in the faeces (McMahon *et al*., 2000).

1.4.2 Hydrolysable tannins

Hydrolysable tannins, can be classified as two esters of core molecules, either gallic acid or ellagic acid (Figure 4; McMahon *et al*., 2000). Gallic acid and ellagic acid are formed during hydrolysis of gallotannins and ellagitannins respectively, with glucose also being a product in both cases (Mueller-Harvey, 2001). In water, hydrolysable tannins are more soluble than condensed tannins (Reed, 1995). In acids and bases, hydrolysable tannins are very soluble, with certain enzymes

causing the same effect (Kumar and Vaithiyanathan, 1990). For both gallotannins and ellagitannins, the precursor for these structures is pentagalloylglucose (Mueller-Harvey, 2001). The main structure of gallotannins consists of gallic acid units that surround a polyol compound like glucose. A depside bond occurs in the structure between two gallic acid units (Mueller-Harvey, 2001). This bond occurs between the acid group of the gallic acid unit joining and the phenolic group of the gallic acid unit of the gallotannin (Mueller-Harvey, 2001). In ellagitannins, oxidative reactions occur between the gallic acid units to form large and complex tannins (Mueller-Harvey, 2001). Ellagitannins have intramolecular biphenyl linkages that cause hydrolysable tannins to be unyielding structurally, whereas gallotannins lack these linkages and are therefore more adaptable (Deaville *et al*., 2007). Unlike, condensed tannins that are not degraded by microbes in the rumen, hydrolysable tannins can be attacked by certain microbes (Makkar, 2003). The phenolic hydroxyl groups within hydrolysable tannins can also be methylated by certain rumen microbes causing a reduction in the tannin's protein binding activity (Makkar, 2003).

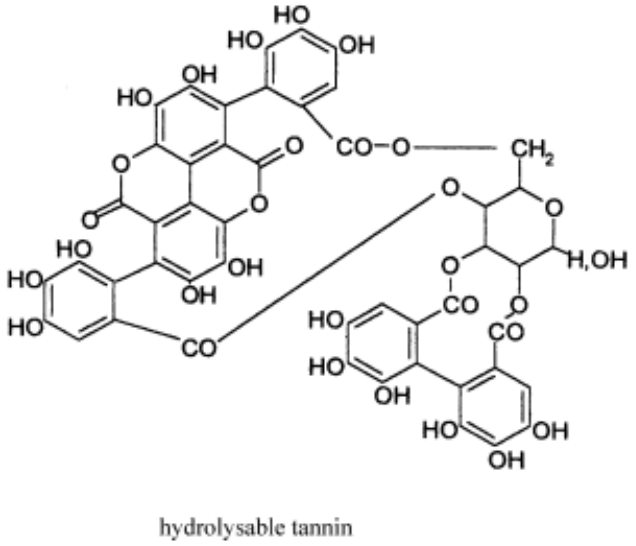


Figure 4 Chemical structure of hydrolysable tannin. After McSweeney *et al*. (2001).

1.4.3 Effects of tannins on ensiling

The effects of both condensed and hydrolysable tannins have been studied to observe their effects on ensiling, intake, rumen fermentation, animal performance and digestion. The ability of tannins to bind with protein can provide protection from proteolysis in the silo and microbial attack in the rumen therefore improving the flow of non-ammonia nitrogen to the small intestine (Ahnert *et al*., 2015).

Protein degradation during the ensiling process has been shown to be reduced when condensed tannins were added to grass silage as seen by the increase in total N from 25.9 to 26.7 g/kg DM when condensed tannins are included at a rate of 0 to 50 g/kg DM, respectively (Salawu *et al*., 1999; Table 3). In a similar study, hydrolysable tannins were added to lucerne silage at four different rates 0, 20, 40 and 60 g/kg DM at ensiling (Tabacco *et al*., 2006; Table 3). Following a 120 day conservation period, Tabacco *et al*. (2006) observed a reduction in non-protein nitrogen (NPN) content of the silage from 75.9 to 64.6 % total N as inclusion rate increased from 0 to 60 g/kg DM hydrolysable tannin. Similarly, in a study conducted by Deaville *et al*. (2010) the addition of hydrolysable tannin at 74.3 g/kg DM to grass silage had a higher total N content of 22.5 g/kg DM compared to 20.9 g/kg DM when condensed tannin was added to grass silage at a rate of 76.1 g/kg DM. Therefore, it can be concluded that the addition of condensed or hydrolysable tannins at ensiling can reduce the proteolysis of protein in silage during the conservation period.

Table 3 Effects of addition of tannins to silage at ensiling on fermentation and chemical characteristics

Silage	Tannin type	Inclusion rate, g/kg DM	DM, g/kg	Total N, g/kg DM	pH	NH_3-N, g/kg TN	Lactic acid, g/kg DM	Reference
Grass	Condensed	0	183	25.9	4.9	97	27.5	Salawu *et al*. (1999)
		5	180	26.2	5.6	116	1.8	
		50	186	26.7	5.4	85	11.6	
Grass	Control	0	240	20.5	3.8	11.1	130	Deaville *et al*. (2010)
	Condensed	76.1	251	20.9	3.8	11.0	110	
	Hydrolysable	74.3	254	22.5	3.9	11.7	104	
Lucerne	Hydrolysable	0	318	35.0	4.5	128	32.8	Tabacco *et al*. (2006)
		20	327	35.4	4.0	114	50.1	
		40	338	35.9	4.4	100	34.4	
		60	338	35.3	4.3	96	36.6	
High Pea/Wheat[1]	Condensed	0	287	36.0	4.10	135	63.7	Adesogan and Salawu, (2002)
		16	295	34.7	4.20	96.8	54.1	
Low Pea/Wheat[2]	Condensed	0	306	31.6	4.58	125	49.7	
		16	320	31.4	4.49	92.7	42.2	

[1]High pea/ wheat silage ratio 3:1

[2]Low pea/wheat silage ratio 1:3

1.4.4 Effects of tannins on intake

The addition of tannins to ruminant's diets have had a varying effect on DM intake of cows and sheep. Dschaak *et al*. (2011) observed a decline of 1.8 kg/d in DM intake in dairy cows when lucerne hay was supplemented with 30 g/kg DM of condensed tannins (Table 4). Similarly, Aguerre *et al*. (2016) observed a linear decline in DM intake from 25.5 to 23.4 kg/d in cows fed lucerne silage supplemented with a mixture of condensed and hydrolysable tannin (CT:HT = 33:66) at inclusion rates 0, 4.5, 9.0 and 18 g/kg DM. In contrast, Deaville *et al*. (2010) observed an improved DM intake of 109 g/day when condensed tannins replaced hydrolysable tannins at 75 g/kg DM to grass silage when fed to wether sheep. However, when the condensed tannins were added at feeding there was an increase in DM intake of 69 g/day compared to when the same tannins were added at ensiling (Deaville *et al*., 2010). There was no effect of tannin addition method on the DM intake for grass silage with hydrolysable tannins therefore suggesting that hydrolysable tannins can be added at ensiling or feed out without affecting DM intake (Deaville *et al*., 2010). Gerlach *et al*. (2018) reported no change in DM intake of dairy cows when condensed tannin was included at feed out in a grass and maize silage based diet at inclusion rates of 10 and 30 g/kg DM. Tannins have been associated with influencing the palatability of silages fed to ruminants however the inconsistency of DM intakes when varying levels of tannins have been fed make it difficult to draw a conclusion.

1.4.5 Effects of tannins on rumen pH

Previous studies have shown no effect of tannins on ruminal pH (Table 4), although studies have shown differing effects on rumen volatile fatty acid concentration. Augerre *et al*. (2016) reported no change in rumen pH when dairy cows were fed an increasing rate of a mix of condensed and hydrolysable tannins (ratio = 33:66, respectively), with rumen pH averaging pH 6.43 across the four inclusion rates of 0, 4.5, 9.0 and 18 g/kg DM. Similarly, Dschaak *et al*. (2011) observed no effect on rumen pH when dairy cows were fed lucerne hay supplemented with condensed tannins, with pH averaging pH 6.38 and pH 6.41 for no tannin and added tannins, respectively. Benchaar *et al*. (2008) also reported no effect of supplementation of condensed tannins at 150 g/d at feed out in a grass and maize silage based TMR fed to dairy cows on rumen pH which was pH 6.68

and pH 6.66 for control and tannins added, respectively. Considering the variation in levels of tannin supplemented to the diet and different species, rumen pH has remained unaffected in previous studies suggesting tannins can be included in the diet of a ruminant without any effect on the pH of the rumen.

1.4.6 Effects of tannins on rumen VFAs

Rumen volatile fatty acid concentrations, however, have been affected in some, but not all previous studies, for example, Benchaar *et al*. (2008) reported no effect of condensed (quebracho) tannins on total VFA concentration or individual VFA concentration. Similarly, Augerre *et al*. (2016) observed no changes in total VFA concentration or acetate, propionate and butyrate concentrations when a mix of hydrolysable (chestnut) and condensed (quebracho) tannins were included in the diet of dairy cows. In contrast, Dschaak *et al*. (2011) observed a decline in total VFA concentration but this was coupled with a decline in DM intake when condensed (quebracho) tannins were included in the diet. Interestingly, condensed tannins added to the diet containing a higher forage content (59:41 forage:concentrate; DM basis) increased concentrations of acetate, propionate and butyrate by 1.6, 1.6 and 1.0 mol/100 mol, respectively. However, in the diet containing a lower forage content (41:59 forage:concentrate; DM basis), condensed tannins had no effect on acetate, propionate or butyrate concentration which averaged 58.4, 25.0 or 13.3 mol/100 mol, respectively (Dschaak *et al*., 2011). Dschaak *et al*. (2011) concluded that lucerne hay supplemented with condensed tannins fed at a high forage to concentrate ratio could influence rumen fermentation. Although, these inconsistencies in rumen fermentation could be linked to the different species of condensed and hydrolysable tannins or inclusion rates of the tannins or the interaction between the tannin and diet, it is difficult to draw a conclusion on the effects of tannin on rumen fermentation.

Table 4 Milk yield and composition of dairy cows fed silage supplemented with condensed (CT) or hydrolysable tannins (HT).

Tannin Type	Tannin Species	Inclusion Rate, g/kg DM	DM Intake, kg/d	Milk yield, kg/d	Milk fat, g/kg	Milk protein, g/kg	Milk lactose, g/kg	Reference
Condensed	Quebracho	0	27.6	35.1	36.7	30.9	48.6	Dschaak *et al*. (2011)
		30	25.8	35.5	36.5	30.7	48.4	
Condensed	*A. mearnsii*	0	22.5	36.7	37.7	33.2	--	Gerlach *et al*. (2018)
		10	23.0	36.7	38.8	33.5	--	
		30	22.5	35.1	38.0	32.2	--	
Hydrolysable	Chestnut	0	14.8	29.5	49.4	31.5	45.1	Liu *et al*. (2013)
		10	15.1	30.3	47.0	33.1	46.8	
Hydrolysable/ Condensed[1]	Chestnut/ Quebracho	0	25.5	40.6	35.8	28.7	48.5	Augerre *et al*. (2016)
		4.5	25.4	40.8	36.0	29.2	48.6	
		9.0	24.5	40.3	35.1	28.6	48.7	
		18	23.4	40.3	35.7	28.3	49.0	

[1]Tannin extract = one third chestnut (CT) and two thirds quebracho (HT)

1.4.7 Effects of tannins on animal performance and digestion

Variations in the effects of added tannins on animal performance may be influenced by the different inclusion rates and species of tannins used in the different studies (Table 4). Augerre *et al*. (2016) and Dschaak *et al*. (2011) both reported no change in milk yield when dairy cows were offered condensed or hydrolysable tannins. Milk composition was also not changed by the inclusion of tannin in the diet in the study of Dschaak *et al*. (2011). However, Augerre *et al*. (2016) reported that milk fat and lactose content remained unchanged but milk protein yield declined linearly from 1.20 to 1.15 kg/d as tannin level increased from 0 to 18 g/kg DM, respectively. Liu *et al.* (2013), observed no changes in milk fat, protein or lactose content of milk from dairy cows fed a diet supplemented with 10 g/kg DM of hydrolysable tannins from 3 weeks pre-calving to 3 weeks post-calving. Similar to Augerre *et al*. (2016); Gerlach *et al*. (2018) observed no effect of condensed tannins on milk yield or fat content but reported a decline in milk protein yield and N efficiency in the 3rd period of the study when diets were supplemented with 30 g/kg DM condensed tannins. Gerlach *et al*. (2018) concluded that even though tannins dissociate from proteins in the small intestine, not all the protein will be digested. Therefore, the decline in milk protein yield may be due to a reduced absorption of amino acids in the small intestine thus reducing the supply of amino acids available for protein synthesis in the mammary gland.

Whole tract digestibility of DM, OM, CP and NDF were not affected by the inclusion of condensed tannins in a diet containing lucerne hay (Dschaak *et al*., 2011). In contrast, Gerlach *et al*. (2018) observed a decline in OM digestibility in dairy cows fed a diet containing 30 g/kg of condensed tannins. Likewise, Augerre *et al*. (2016) reported a linear decline in DM, OM, CP and NDF digestibility as the inclusion rate of a mixture of hydrolysable and condensed tannin increased in the diet. Crude protein digestibility had the greatest reduction (Augerre *et al*., 2016) which was to be expected due to the strong bond tannins have with protein compared to other components of the feed (Gerlach *et al*., 2018). The rate of clearance of digested feed from the rumen affects whole tract digestibility, and the decline in NDF digestibility would suggest a slow clearance of fibre which would explain the decline in DM intake in the study of Augerre *et al*. (2016). Similarly, Deaville *et al*. (2010) reported that the addition of hydrolysable or condensed tannin reduced DM and OM digestibility in comparison to the untreated grass

silage. The addition of condensed tannin produced the largest decline in DM and OM digestibility, Deaville *et al*. (2010) suggested that the condensed tannins were binding with the plant cell wall components forming protein complexes thereby reducing microbial degradation of the plant in the rumen. The results from previous studies on the effects of both condensed and hydrolysable tannins on ensiling, intake, rumen fermentation, animal performance and digestion have been inconsistent therefore it is difficult to draw a conclusion about whether the inclusion of tannins in a ruminant's diet is beneficial in reducing protein degradability in the rumen whilst improving RUP and MP supply to the dairy cow.

1.4.8 Effects of tannins on milk fatty acid profile

Milk fat is a triglyceride composed of one glycerol molecule and three fatty acids. Fatty acids are long chains of carbon atoms containing a carboxyl acid group at the end. Fatty acids (FAs) can be saturated or unsaturated depending on the type of bonds within the chain, saturated FAs contain only single bonds between carbon atoms whereas in unsaturated fatty acids contain a mixture of single and double bonds between carbon atoms (McDonald *et al*., 2011). The number of carbon atoms in the FA chain determines the length and type of FA, long chain FAs have more than 12 carbon atoms, medium chain FAs have between 8 to 12 carbon atoms whilst short chain FAs have less than 8 carbon atoms (McDonald *et al*., 2011). Milk fatty acids are derived in two main ways, either by *de novo* synthesis in the mammary gland or uptake from the diet in the blood (Mansbridge and Blake, 1997). Further to these two pathways, milk FAs can also be derived from biohydrogenation in the rumen and body stores of fat (Stoop *et al*., 2009). In milk, FAs of chain length less than C16 tend to be produced by *de novo* synthesis, whilst FAs of chain length more than C16 tend to be taken up from the diet (Mansbridge and Blake, 1997).

In milk fat, saturated FAs are the main constituent accounting for up to 75 g/100g of the total fatty acids. The polyunsaturated fatty acid (PUFA) content of milk is 4 g/100g with linoleic (C18:2 *n*-6) and α-linolenic (C18:3 *n*-3) being the main FAs present (Mansbridge and Blake, 1997). PUFAs are typically found in high concentrations in grass, however occur in low concentrations in milk or meat products of ruminants fed grass or grass silage (Jenkins *et al*., 2008). In the rumen, lipids will undergo lipolysis by microbes which releases the fatty acids from the glycerol molecule (Jenkins *et al*., 2008). Following lipolysis, these free PUFAs

will undergo biohydrogenation which is the transformation of PUFAs to saturated FAs, both linoleic and α-linolenic acids are converted to C18:0 during this process (Jenkins *et al*., 2008; Figure 5). Diet and nutrition of a ruminant can alter the microbial population within the rumen, altering lipolysis and biohydrogenation of dietary lipids, causing a change in both composition and fat content of the milk (Bauman and Griinari, 2003). Therefore, there is potential for the diet of ruminants to be altered to improve the concentrations of PUFAs in the milk by providing protection of the PUFAs from biohydrogenation or changing the biohydrogenation pathway.

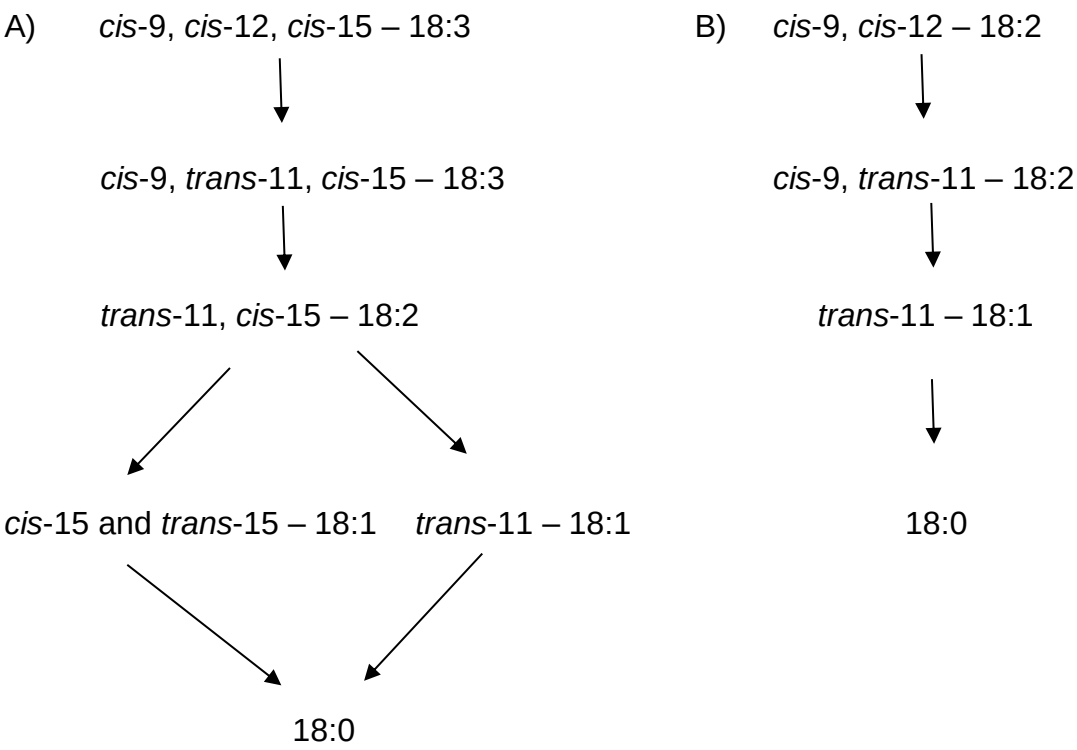


Figure 5 The biohydrogenation pathways of α-linolenic (C18:3 *n*-3; A) and linoleic (C18:2 *n*-6; B) acids. After Jenkins *et al*. (2008) and adapted from Harfoot and Hazlewood, (1988).

The addition of tannins to the diet of dairy cows has had varying effects on the milk fatty acid profile. A previous study by Benchaar and Chouinard, (2009) observed no change in individual milk fatty acid concentrations of lactating dairy cows when 150 g/d condensed tannin was added to grass silage based TMR. In a study conducted by Turner *et al*. (2005), dairy cows grazed either a ryegrass-based pasture or birdsfoot trefoil which contains naturally occurring condensed tannins, although the study did not measure the tannin content. It was observed that there was a decrease in the saturated fatty acid C18:0 concentration in milk of cows fed

birdsfoot trefoil. Concentrations of linoleic (C18:2 *n*-6) and α-linolenic (C18:3 *n*-3) acids were higher, along with concentrations of omega-3 fatty acids being significantly higher in milk from cows fed birdsfoot trefoil compared to cows fed ryegrass pasture. Therefore, Turner *et al*. (2005) concluded that the condensed tannins present in birdsfoot trefoil altered the biohydrogenation occurring in the rumen therefore affecting the milk fatty acid profile. Similarly, Dschaak *et al*. (2011) observed minor effects of the addition of 30 g/kg DM condensed tannin to a lucerne hay and maize silage based TMR on milk fatty acid profile of dairy cows. Although, the addition of condensed tannin did increase the concentrations of α-linolenic (C18:3 *n*-3) acid and C20:1 by 0.04 g/100g in dairy cows. Dschaak *et al*. (2011) suggested these changes in these fatty acids were caused by the tannins hindering the microbes in the rumen therefore changing the biohydrogenation pathways. The effect of condensed tannins at a rate of 79 g/kg DM on biohydrogenation in the rumen has been studied in continuous cultures in vitro by Khiaosa-Ard *et al*. (2009) who observed an accumulation of C18:1 *trans*-11 due to the condensed tannins preventing the final step of the biohydrogenation pathway. Therefore, it can be concluded that the presence of tannins in the diet of lactating dairy cows have the potential to alter the biohydrogenation pathway thereby influencing the milk fatty acid profile.

1.5 Degradability of proteins in the rumen

Protein available in forages and concentrates can be classified as two types of protein, rumen degradable protein (RDP) and rumen undegradable protein (RUP) (Bach *et al*., 2005). Rumen degradable protein is composed of two components; true protein and non-protein nitrogen (NPN) (Bach *et al*., 2005). The amount of RDP and RUP available from forages or concentrates is variable (Broderick, 1995). The digestion of RDP and RUP in the ruminant is shown in Figure 6.

Rumen degradable protein will undergo degradation in the rumen by microbes, producing microbial protein and ammonia (Reynal and Broderick, 2005). Approximately 50% of protein reaching the small intestine will be made up from microbial protein in a high yielding dairy cow (Schwab and Broderick, 2017). Microbial protein synthesis is dependent on the amount of energy and protein available from the feed as energy is required for growth of the microbes (Clark *et al*., 1992). Bacteria form an attachment with protein in the feed and the activity of

these microorganisms degrade protein to peptides, amino acids and ammonia-nitrogen (Bach *et al*., 2005). For successful degradation of protein, many different species of bacteria will ferment the protein due to the large number of bonds (Bach *et al*., 2005). The type of protein and action of microbes play an important role in the degree of degradation; when this is rapid, large quantities of protein will be degraded however rumen microbes are often unable to fully utilise the available amino acids and ammonia (Broderick., 1995; Bach *et al*., 2005). Although, the degree of degradation will depend on the availability of fermentable metabolisable energy and the balance between RDP and RUP in the diet (Bach *et al*., 2005). Therefore, a loss of ammonia occurs, resulting in it being absorbed across the rumen epithelium into the blood to be transported to the liver (Broderick., 1995; Bach *et al*., 2005). In the liver, ammonia is converted into urea prior to being excreted via urine (Broderick and Albrecht., 1997). Urea can be transported back to the rumen to provide a nitrogen source for microbes when RDP levels are low (Lapierre and Lobley, 2001). Recycling of urea to the rumen can also have the potential to improve N efficiency and lower the excretion of N, therefore reducing the amount of N being released into the environment (Mutsvangwa *et al*., 2016). Mutsvangwa *et al.* (2016) investigated the effects of reducing dietary CP (149 vs. 175 g/kg DM) and RDP (63 vs 69% of CP) fed to dairy cows on urea-N recycling. It was reported that urea-N entry rate into the rumen increased when dietary CP and RDP were reduced as there were lower concentrations of ammonia in the rumen therefore microbial protein supply remained constant. Lower excretions of N in urine were also observed due to the recycling of urea-N, therefore improving N efficiency (Mutsvangwa *et al*., 2016).

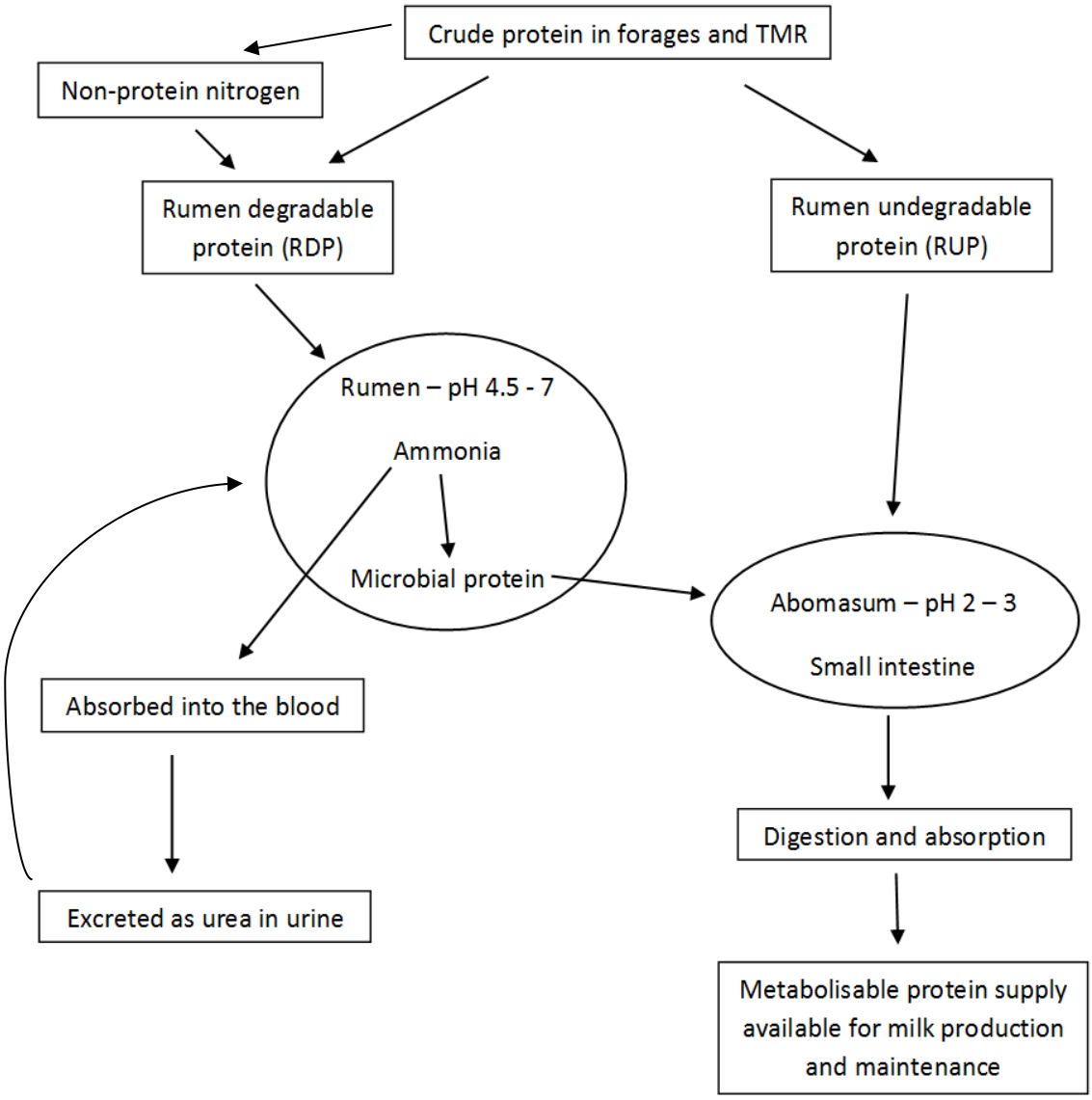


Figure 6 The digestion of protein within a ruminant. Adapted from McDonald *et al*. (2011).

Protein that escapes microbial degradation in the rumen, rumen undegradable protein (RUP), and passes through the rumen to the abomasum prior to digestion and absorption in the small intestine (Allison and Garnsworthy, 2002). Metabolisable protein (MP) is a combination of RUP and microbial protein that has become available in the small intestine following digestion in the rumen (Van Emon *et al*., 2014). In the small intestine, RUP undergoes digestion by enzymes to release amino acids that are available for absorption (Bohnert *et al*., 2002). Amino acids and peptides are absorbed across the small intestinal wall; excess amino acids will be converted to urea in the liver and excreted in the urine (Bohnert *et al*., 2002). The availability of individual amino acids from MP determines whether the

ruminant can utilise them; microbial protein will supply the majority of amino acids however methionine, lysine, and histidine can be lacking in RUP depending on the feed source (Johansen *et al*., 2018). Milk production can be compromised when these amino acids are limited or lacking even if MP supply is optimal (Johansen *et al*., 2018). Therefore, amino acids from RUP should harmonise with the microbial protein supply of amino acids (Korhonen *et al*., 2002). Soybean meal (SBM) is a source of RUP which is used in the dairy industry, and can supply a respectable amount of histidine and lysine however methionine levels are low (Korhonen *et al*., 2002). However, microbial protein supply can provide methionine therefore SBM and microbial protein can provide the essential amino acids that are required during milk production (Korhonen *et al*., 2002).

Dairy cows in early or peak lactation have the highest requirement for RUP as microbial protein supply alone is unable to meet the cow's MP requirement (Dunlap *et al*., 2000). Microbial protein can met the MP requirements of lower yielding dairy cows but microbial protein supply cannot provide all the MP a dairy cow producing 50 kg/d of milk requires, and therefore there must be an adequate supply of by-pass (RUP) protein to meet the cow's MP requirement (Dunlap *et al*., 2000).

1.5.1 Measurement of dietary RDP and RUP

Ruminal degradability of protein can be measured using the *in situ* technique and many studies have studied protein degradability in forage legumes using this technique. The *in situ* technique provides degradation parameters including the soluble fraction, the potential rumen degradable fraction, and the rate of degradation (Figure 7; Ørskov and McDonald., 1979). In a study conducted by Mustafa *et al*., (2000) pea, barley and lucerne silage was compared for protein degradability. All three silages had a soluble CP fraction that was above 69.2 % of CP with pea and lucerne silage being highest at 78.5 % of CP. In contrast to the high soluble fraction, the slowly degradable CP fraction was below 20 % for all three silages, with pea and barley silage being the lowest at 15.4 %. The rate of degradation is related to the slowly degradable fraction, and Mustafa *et al*. (2000) concluded that the high ruminal CP degradability was influenced by the fast rate of degradation observed in pea silages.

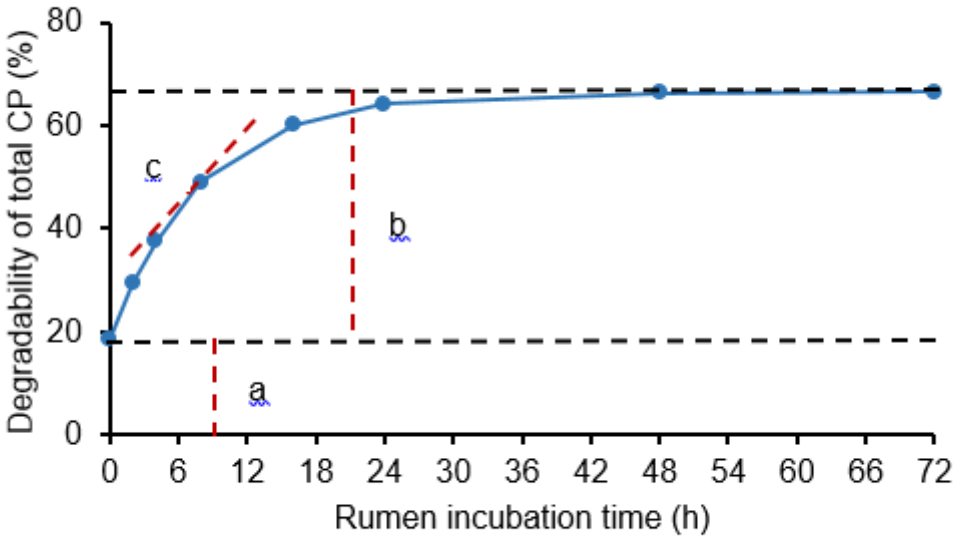


Figure 7 The degradation curve of protein in the rumen over 72 hours. Where a = soluble fraction, b = potential rumen degradable fraction and c = rate of degradation. Adapted from Chamberlain and Wilkinson, (1996).

In a later study, Mustafa *et al*. (2002) compared the rumen degradability three varieties of pea silage, Lenca, Carneval and Delta. Delta pea silage had the highest soluble fraction at 82.8 % whilst Lenca pea silage was 13.7 % lower. The slowly degradable CP fraction varied between the three pea silages, however the rate at which the slowly degradable CP fraction was degraded was similar and rapid averaging 11.8 %/h. Therefore, ruminal degradability was high for the three pea silages, results were similar to Mustafa *et al.* (2000). Differences in rumen degradable CP between pea silages is influenced by the CP content of the silage and Mustafa *et al.* (2002), reported that Carneval had the highest CP content of 205 g/kg DM and Lenca the lowest at 179 g/kg DM. In a study conducted by Merry *et al*. (2006), ruminal degradability of N was 9.5 % higher in grass silage compared to red clover silage. Merry *et al.* (2006) suggested the lower N degradability in red clover silage was due to the activity of polyphenol oxidase (PPO) enzyme by binding to the protein thereby reducing degradability in the rumen.

1.6 Forage peas

Forage peas (*Pisum sativum*) are forage legumes that have previously been grown in the UK only for seed production but more recently have been ensiled for winter feed for dairy cows (Fraser *et al*., 2001). Spring sown forage peas have a short growing season of between 12 and 18 weeks with a high DM yield and protein content, and have the ability to fix nitrogen therefore requiring no nitrogen fertilisers (Fraser *et al*., 2001).

1.6.1 Growth and establishment

Forage peas require free-draining, sandy soils with a pH between 6 and 7 for successful plant growth (Frame, 2005). In the UK, seeds are most often sown in the spring when soil temperatures have reached between 7 and 8°C, and are drilled at a depth of between 3 and 5 cm. Seed rate is dependent on seed size and whether the crop will be whole crop or as part of a bi-crop (Frame, 2005; Knott, 1987). In studies examining forage peas as part of a bi-crop, seed rate varied between 117 – 188 kg per hectare (Adesogan *et al*., 2004) and 134 – 230 kg per hectare (Salawu *et al*., 2002b; Adesogan *et al*., 2002). When seeds are sown for a whole crop, seed rate varied between 127 – 245 kg per hectare (Fraser *et al*., 2001; Sinclair *et al*., 2009; Mustafa *et al*., 2002).

The growth of forage peas can be described in four stages; germination and emergence, vegetative, reproductive and senescence stages which are summarised in Table 5. During the first stages of germination, the seed swells as water is taken up, a tap root will then begin to grow and can grow to depths of over 1 m (Knott, 1987 and Frame, 2005). At the same time, a shoot will begin to emerge and will form the main stem of the plant (Knott, 1987). The vegetative stage involves the growth of nodes on the main stem, with the number of nodes varying between species; leaves will develop and unfold on each node (Knott, 1987).

Table 5 The growth stages of forage pea

Stage	Development stage
Germination and emergence stage	
000	Dry seed
001	Seed is swollen due to uptake of water
002	Radicle visible
003	Shoot visible
004	Emergence
Vegetative stage	
101	First node
102	Second node
103	Third node
Reproductive stage	
201	Enclosed flower buds
202	Visible flower buds
203	First open flower
204	Pod set
205	Flat pod
206	Pod swollen with small seeds
207	Green seeds fill the pod
208	Green wrinkled pod
209	Yellow wrinkled pod
210	Dry and brown pods with hard seeds
Senescence stage	
301	Lower brown pods, middle yellow pods and upper green pods
302	Lower and middle brown pods, upper yellow pods
303	All pods are brown with hard seeds

Adapted from Knott, (1987)

During the reproductive stage, flower buds begin to develop on the stems and eventually the flowers will open. Flower colour varies between species with either pink, purple or white flowers (Knott, 1987). In forage peas, condensed tannins occur naturally with flower colour indicating the level of tannin present, with white flowers indicating low levels while coloured flowers indicate high levels (Wang *et al*., 1998). Hart *et al.* (2011) and Sinclair *et al.* (2009) reported condensed tannin levels of 47.3 and 92.7 g/kg DM for white and coloured forage pea silage,

respectively. Once flowers open, pods begin to develop with small seeds and as the pods fill out, the seeds become green in colour (Knott, 1987). Gradually the pods become wrinkled and change colour from green to yellow, and eventually the pods and seeds dry out, again changing colour to brown (Knott, 1987). The senescence stage describes the changes in pod colour across the whole plant (Table 5).

1.6.2 Harvesting, ensiling and nutritive value

A number of studies have examined the effects of wilting and ensiling on the nutritive value of forage peas; and are summarised in Table 6. In forage pea silage, there are a number of factors that can affect overall quality of the silage, including plant maturity, wilting, drop in pH and protein degradation (Cavallarin *et al*., 2006). In a study conducted by Fraser *et al.* (2001), forage pea silage was harvested on three dates, 10, 12 and 14 weeks post sowing which represented growth stages 204, 205 and 207, respectively (Knott., 1987). At harvest, DM content was similar for weeks 10 and 12 averaging 153 g/kg whereas by week 14 DM content had risen to 206 g/kg. However, following 24 hours of wilting, Fraser *et al.* (2001) observed a similar DM content of 331 g/kg for the three different harvest dates. After 24 hours wilting and 90 days of ensiling, crude protein content was 45 and 27 g/kg DM higher in silage harvested at 10 weeks post sowing, respectively, compared to 12 and 14 weeks. Pea silage had the highest DM yield of 6172 kg/ha when harvest occurred 12 weeks post sowing compared to yields of 5593 and 5596 kg/ha for harvest occurring 10 and 14 weeks post sowing, respectively (Fraser *et al*., 2001). Fraser *et al.* (2001) concluded that forage peas should be harvested at growth stage 205, which corresponds to 12 weeks post sowing, to achieve good DM yields and nutritive value of the ensiled forage.

In a study conducted by Mustafa *et al*. (2002) the fermentation profile of three pea silages; Lenca, Carneval and Delta, were compared over the ensiling process at 0, 2, 4, 8, 16 and 70 days. A drop in pH to below 4 was observed during the first eight days of fermentation with a rapid drop in the first two days to pH 4.5 (Mustafa *et al*., 2002). In all three pea silages, from day 8 to 70, pH continued to remain below pH 4 indicating good preservation of the silages. Similarly, Mustafa and Seguin, (2003) observed a drop in pH to below pH 4 in the first 8 days followed by a steady decline to 45 days post ensiling. The rapid drop in pH to below 4 in pea

silage during the first two days post ensiling is essential for preservation of the crop, and Mustafa and Seguin, (2003) observed lactic acid concentration rising from day 0 to day 16 before stabilising until the silos were opened on day 45. These observations of pH and lactic acid concentration show that forage peas can be ensiled and preserved to the same quality as grass and other forage legumes.

Table 6 Chemical and fermentation characteristics of forage pea silage

Forage pea silage species	Growth stage at harvest	Wilting time, h	No. of days ensiling	DM, g/kg	CP, g/kg DM	pH	Lactic acid, g/kg DM	Reference
Magnus	204	24	90	268	228	4.71	42.5	Fraser *et al.* (2001)
	205	24	90	297	200	4.02	52.7	
	207	24	90	280	204	4.08	50.6	
Timo	204	96	103	498	301	4.90	47.7	Rondahl *et al.* (2011)
	206	68	103	421	285	4.30	59.1	
	207	165	103	489	289	4.40	50.0	
Capella	204	96	103	447	270	4.50	65.8	
	206	48	103	436	243	4.30	51.4	
	207	65	103	466	221	4.30	40.6	
Alembo	*	0	145	423	25.7 (TN)	4.88	23.1	Borreani *et al.* (2009)
	*	6	145	573	24.5 (TN)	5.13	10.5	
Croma	206	30	*	365	177	4.10	*	Sinclair *et al.* (2009)
Racer	206	30	*	338	189	4.10	*	
Croma	206	36	*	328	184	3.8	*	Hart *et al*. (2011)
Racer	206	36	*	320	197	4.1	*	

TN: total nitrogen

In several studies, research has focussed on feeding pea silage as part of a bi-crop with a cereal forage (Adesogan *et al*., 2004; Salawu *et al*., 2001). Salawu *et al.* (2001) investigated the effects of growth stage at harvest, inclusion rate of pea in the crop and variety of pea (magnus and setchey) on chemical composition of the silage. Silages were harvested over a range of growth stages from 103 to 209 which represented weeks 7 to 15 post sowing, over a period of two years. In both years and varieties, DM content at harvest increased from 125 to 333 g/kg over weeks 7 to 15 post sowing, respectively. When inclusion rate of pea silage to wheat was 3:1 and the silage was harvested at 15 weeks post sowing, crude protein content was 14 g/kg DM higher than silage sown with a ratio of 2:1 pea:wheat and harvested at 13 weeks post sowing. Salawu *et al.* (2001) concluded that when pea silage was intercropped with wheat, the optimum growth stage to harvest was 209 when the pods were yellow and wrinkled, while a higher pea inclusion rate improved the crude protein content of the bi-crop due to the high protein content of peas.

1.6.3 Milk performance

Adesogan *et al.* (2004) examined the performance of dairy cows fed a pea-wheat silage with Magnus and Setchy pea varieties sown with wheat at a ratio of 4:1, and harvested 14 weeks post sowing and wilted (Table 6). A grass silage (with either 4 or 8 kg/d concentrate) diet was used as the control, whilst pea-wheat silages were fed *ad libitum* with 4 kg/d concentrate. Adesogan *et al.* (2004) observed cows fed pea-wheat silages had forage DM intakes that were 3.5 kg/d more than cows fed grass silage. Milk yields were also 3.2 kg/d higher in cows fed setchy pea-wheat silage while being similar to grass silage when fed with 8 kg/d of concentrate, averaging 24.2 kg/d.

Salawu *et al.* (2002b) investigated different inclusion rates of pea silage in an intercrop with wheat in dairy cows, with Magnus peas sown with wheat to establish inclusion rates of 75:25 and 25:75 (pea:wheat). Both silages were harvested at 13 and 15 weeks post sowing at growth stages 206 (full pod) and 209 (yellow wrinkled pods), respectively, and grass silage was used as a control, with all silages fed with 6 kg/d of concentrate, and a second grass silage diet with 9 kg/d concentrate. Salawu *et al.* (2002b) observed higher forage DM intakes in cows fed pea-wheat silages averaging 10.6, 11.1 and 8.7 kg/d for high pea-wheat,

low pea-wheat and grass silage, respectively. However, total DM intakes were similar between the pea-wheat silages and grass silage when fed with 9 kg/d concentrate, averaging 16.0 and 16.3 kg per day, respectively. Salawu *et al.* (2002b) reported that cows fed pea-wheat silage spent 249 minutes per day at the feeders whereas cows fed grass silage spent 211 minutes per day. Similarly, the number of meals eaten by cows fed pea-wheat silages was higher than cows fed grass silage averaging 13.3 and 9.4 meals per day, respectively. From these two studies, it can be concluded that bi-crops containing pea silage can improve DM intake and milk yield of dairy cows compared to grass silage, or replace concentrates and result in a similar performance.

Table 7 summarises a number of studies that have examined the effect of pea silage as a bi-crop and a whole crop; all silages were harvested at either growth stage 206 (full pod) or 209 (yellow wrinkled pods). Sinclair *et al.* (2009) examined performance of dairy cows fed whole crop pea silage harvested at growth stage 206 and inclusion rates of 50 % forage DM. Dry matter intake of pea silage in the study of Sinclair *et al.* (2009) was 1.5 kg/d more than cows fed grass silage. Milk yields observed in Sinclair *et al.* (2009) were similar in cows fed pea or grass silage. From previous studies, pea silage has maintained DM intakes and milk yield as either a bi-crop or whole crop silage therefore pea silage, can replace grass silage in a lactating dairy cow diet.

Table 7 Effects of growth stage and inclusion rate of forage pea silages on performance of dairy cows

Forage pea silage species	Growth stage at harvest	Inclusion rate[1]	DM[2], g/kg	CP[2], g/kg	DM intake, kg/d	Milk yield, kg/d	Milk protein, g/kg	Reference
Magnus-wheat	209	100	322	177	17.2	20.8	31.5	Adesogan *et al.* (2004)
Setchey-wheat	209	100	289	166	19.3	24.0	31.7	
Grass 4	*	100	244	186	15.4	20.1	31.1	
Grass 8	*	100	244	186	17.5	24.5	32.4	
Magnus-wheat	206	75	227	184	15.4	21.9	30.4	Salawu *et al.* (2002b)
	206	25	223	165	16.1	21.8	30.7	
Magnus-wheat	209	75	251	187	16.3	23.4	30.4	
	209	25	259	166	16.5	22.4	31.0	
Grass 6	*	100	226	150	13.7	20.8	30.6	
Grass 9	*	100	226	150	16.3	23.7	31.7	
Grande (coloured pea)	206	50	*	170	28.6	45.2	30.3	Mustafa *et al.* (2000)
Lucerne	5	50	*	193	27.5	45.3	31.6	
Barley	*	50	*	101	26.0	43.2	30.8	
Croma (coloured pea)	206	50	365	177	20.5	24.6	34.5	Sinclair *et al.* (2009)
Racer (white pea)	206	50	338	189	20.2	22.9	35.7	
Grass	*	50	244	121	18.8	23.7	43.4	

[1]Inclusion rate as a % forage DM
[2]DM and CP content post ensiling
Grass 4 = grass silage with 4 kg/d concentrate
Grass 8 = grass silage with 8 kg/d concentrate
Grass 6 = grass silage with 6 kg/d concentrate
Grass 9 = grass silage with 9 kg/d concentrate
Magnus = coloured flower forage pea
Stetchey = white flower forage pea

1.7 Red clover and lucerne

Red clover (*Trifolium pratense*) and lucerne (*Medicago sativa*) are forage legumes that are grown in the temperate regions of Europe and United States (Moorby *et al*., 2016). As legumes, red clover and lucerne have the ability to fix nitrogen, therefore during growth and establishment stages there is no requirement for nitrogen fertilisers (Moorby *et al*., 2016). Both red clover and lucerne are perennial legumes that can be harvested between two and three times per year (Frame *et al*., 1998).

1.7.1 Growth and establishment

In a broad range of soil types except acidic, wet or shallow soils, red clover develops and establishes well whereas lucerne prefers well drained and deep soils due to having a tap root requiring depths of between 2 and 4 metres (Frame and Laidlaw, 2011; Frame *et al*., 1998). Red clover and lucerne require a soil pH of between 6.0 and 6.5 for successful plant growth, however red clover can be grown in soils with a lower pH of 5.0 to 6.0 (Frame, 2005). Seeds of forage legumes are small in size and therefore require sowing at a shallow depth to ensure germination can occur quickly as the seed has a small food reserve (Frame, 2005). Seeds for lucerne and red clover are best direct drilled at depths of less than 20 mm and 15 mm, respectively (Frame *et al*., 1998). For a monoculture, lucerne is sown at a seed rate of between 18 and 25 kg/ha whereas red clover has a lower seed rate of between 12 and 15 kg/ha (Frame and Laidlaw, 2011). Successful germination of lucerne and red clover occurs when the seeds are sown in the spring, from April through to July, when soil temperatures are above 7°C (Frame and Laidlaw, 2011).

In the early stages of germination of lucerne, cotyledons emerge first followed by a unifoliate leaf (Frame *et al*., 1998). An alternating pattern of trifoliate leaves develop and produce leaflets; at the base of these leaflets, buds form to produce further stems (Frame, 2005). At the base of the stems, an accumulation of buds form a crown (Frame *et al*., 1998). Following harvest of lucerne, stems are produced from the crown buds allowing the plant to regrow (Frame *et al*., 1998; Frame, 2005). Lucerne flowers are purple in colour and following pollination, seed pods develop in a spiral coil. As the seed pods mature, a colour change from green to brown occurs as summarised in Table 8.

Table 8 The stages of development of lucerne

Stage	Development stage
0	*Early vegetative*: stems less than 15 cm
1	*Mid vegetative*: plants are up to 50% height, stems 15 – 30 cm
2	*Late vegetative*: stems over 30 cm tall, bud development beginning
3	*Early bud*: One or two nodes with flower buds showing
4	*Late bud*: Three or more nodes with flower buds
5	*Early flower*: One open flower
6	*Late flower*: Two or more open flowers
7	*Early seed pod*: One to three green seed pods
8	*Late seed pod*: Four or more green seed pods
9	*Rip seed pod*: Seed pods, brown and mature

Adapted from Frame *et al*., 1998 and Frame, 2005

In the early stages of red clover germination, two cotyledons appear above ground while underground a radicle forms which will ultimately become the tap root for the plant reaching depths of up to 1 m (Frame *et al*., 1998; Frame, 2005). An unifoliate leaf develops between 5 and 10 days after germination, before trifoliate leaves form and develop into true leaves (Frame *et al*., 1998). Like lucerne, buds form a crown at the base of the plant, which develop into stems, and following defoliation or winter, stems develop from these buds allowing plant regrowth (Frame *et al*., 1998). The flower head of red clover is pink in colour, seed pods develop after pollination containing up to two seeds, and seed colour can vary between brown, yellow and purple (Frame, 2005; Frame *et al*., 1998). Red clover can remain as a productive ley for between 2 – 3 years whereas lucerne remains productive for longer with a ley lasting between 4 – 6 years (Frame, 2005).

1.7.2 Harvesting and ensiling

For lucerne and red clover to begin germinating or regrowth after winter dormancy, the soil temperature must reach 7°C, which is 2°C higher than for grass, therefore harvesting of lucerne and red clover will begin between 3 to 4 weeks after grass (Wilkinson, 2005). The ideal growth stage to cut lucerne and red clover are early bud and early flowering stage, respectively (Lampkin, 1990). There are three stages to the harvesting process, before the forage reaches the clamp; mowing, wilting and harvesting which can all influence the successfulness of the final silage (Wilkinson, 2005). Wilting is an important process for lucerne and red clover as

both plants can be sappy so should not be ensiled without wilting (Lampkin, 1990). However, the nutritive value of lucerne and red clover can be reduced during wilting particularly if the crop becomes too dry as leaves become brittle therefore shattering when the crop is harvested (McDonald *et al*., 2011). Harvesting of lucerne and red clover normally occurs between 24 and 48 hours after mowing depending on weather conditions at mowing and during wilting to ensure high nutritive value for the ensiling process (Wilkinson, 2005 and McDonald *et al*., 2011).

In the ensiling process, the elimination of oxygen from the freshly harvested crop is critical for the success of the silage (Merry *et al*., 2001; Wilkinson, 2005; and McDonald *et al*., 2011). Poor sealing of the silo can lead to nutrient losses as the crop respires and produces heat in the early stages of the ensiling process (Wilkinson, 2005). Rapid filling and complete sealing of the silo are therefore important to ensure anaerobic conditions (McDonald *et al*., 2011).

The next stage of the ensiling process is the fermentation stage which involves a drop in pH due to fermentation of lactic acid bacteria in the forage (Wilkinson, 2005; Sullivan and Hatfield, 2006). On the forage, lactic acid bacteria are present and begin fermenting the naturally occurring sugars, like glucose, to lactic acid when the forage is ensilaged (McDonald *et al*., 1991). If lactic acid production is low in the silo, other bacteria are able to grow, producing butyric acid that can degrade amino acids reducing the quality of the silage (McDonald *et al*., 1991). The rapid fall of pH to less than 5 and adequate quantities of naturally occurring sugars improves lactic acid production therefore eliminating the opportunity for detrimental bacteria to grow, improving overall quality of the silage (Owens *et al*., 1999). Lucerne silage tends to have a higher pH than red clover silage, and Owens *et al.* (1999) observed pH's of 4.1 to 5.0 for lucerne while red clover pH values were lower at 4.0 to 4.4. These higher pH values observed in lucerne silage are due to lower levels of naturally occurring sugars in the crop and a higher buffering capacity ("defined as the amount of acid needed to reduce forage pH from 6 to 4 per unit DM" (Owens *et al*., 1999)). Legumes tend to have a higher buffering capacity than grass, with lucerne having the highest (McDonald *et al*., 1991).

Protein in the forage is broken down to amino acids and small peptides during the ensiling process, and in legume silages protein breakdown is large (McDonald *et al*., 1991; Sullivan and Hatfield., 2006; Schulz *et al*., 2018). The rate at which the pH of the forage reaches pH 5 or less is vital in the extent of degradation of protein in the silo (Sullivan and Hatfield, 2006). If pH falls slowly to 5 or less, the quantity of protein degraded is large therefore reducing the quality of the silage (Sullivan and Hatfield, 2006; Owens *et al*., 1999). Red clover and lucerne have a high protein content, and in lucerne, between 44 and 87 % of the protein in the forage is converted to non-protein nitrogen (NPN), whereas in red clover only 7 – 40 % is degraded (Jones *et al*., 1995). Unlike forage peas, lucerne and red clover contain very small amounts of naturally occurring condensed tannins. Lucerne silage contains no naturally occurring tannins whilst there are minor levels (2.3 g/kg DM) present in red clover silage (Hymes-Fecht *et al*., 2013).

During the first year of a lucerne ley, the number of cuts of silage is half of what can be expected from the ley in the following years which can be up to four cuts (Lampkin, 1990). This difference in number of cuts is due to the slow establishment of lucerne following sowing. Red clover can be harvested up to three times in the first year of establishment and the following years of the ley (Lampkin, 1990).

1.7.3 Polyphenol oxidase (PPO)

Red clover contains an enzyme, polyphenol oxidase (PPO) that inhibits protein degradation by converting phenols into *o*-quinones which bind to the forage protein (Broderick *et al*., 2000; Hoffman *et al*., 1997; Jones *et al*., 1995). The PPO enzyme has been linked to the browning effect in ripening fruit and vegetables, whilst in the silo and rumen, PPO can initiate a decline in proteolysis and lipolysis (Lee *et al*., 2009b). A covalent bond forms between the amino acids and *o*-quinones to produce a protein polymer (Figure 8; Igarashi & Yasui, 1985; Lee *et al*., 2009a). The enzyme PPO is located in the chloroplast of the red clover plant and can be present in two forms; active and latent (Lee, 2014). Approximately 10% of PPO enzyme found in the red clover plant will be in the active form whilst 90% will be present in the latent form with both forms functioning at a neutral pH (Lee *et al*., 2009b). The latent form of PPO becomes activated during the ensiling process due to proteolytic activity and endogenous substances, therefore

increasing the amount of active PPO available to bind to protein and protect the protein from degradation in the silo (Lee *et al*., 2009b). Therefore, the presence of PPO in red clover silage reduces proteolysis in the silo by protecting the protein from plant proteases (Lee *et al*., 2009b). In the first two hours following cutting of the crop, there is an initial rise in PPO activity due to the activation of the latent form; as the *o*-quinones bind to the protein, PPO activity declines (Lee, 2014).

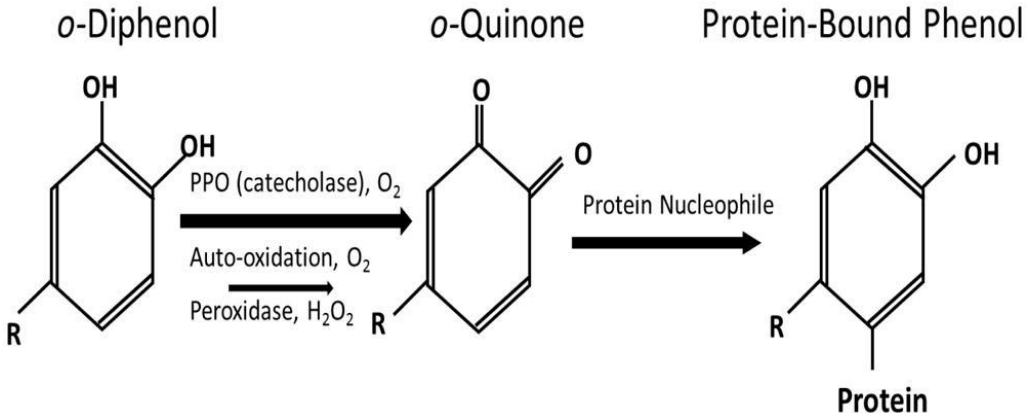


Figure 8 The reaction of the PPO enzyme where *o*-quinone binds with forage protein to form a protein-bound protein. After Lee, (2014) and modified from Lee *et al*., (2013).

The amount of PPO activity in red clover plants varies between plants and previous studies have investigated the effects of length of growth and how the red clover is treated following cutting. Table 9 summarises the activity of PPO enzyme in red clover plants in some previous studies (Lee *et al*., 2009a; Lee *et al*., 2009b). Conditioning the red clover plant decreased PPO activity by 135.9 U/g of DM, however protein-bound phenol increased by 5.02 g/kg DM in Lee *et al*. (2009b). In the study by Lee *et al*. (2009a), PPO activity in all three varieties of red clover decreased as regrowth length increased from 4 to 8 weeks. However, protein-bound phenol remained unchanged in both high and low PPO activity red clover plants as length of regrowth increased from 4 to 8 weeks, whereas in the wild type red clover protein-bound phenol decreased by 16 mg/g protein. The PPO enzyme requires oxygen to be active, therefore in the anaerobic conditions of the rumen, PPO enzyme becomes inactive (Lee, 2014). For the PPO enzyme to be effective in a dairy cow's diet, the protection of protein has to occur prior to ingestion in the silo (Lee, 2014). Protein that has become bound to *o*-quinones in the silo are protected from microbial attack, therefore this protein flows through the rumen and

becomes available for digestion as rumen undegradable protein in the small intestine (Lee, 2014). Consequently, the PPO enzyme present in red clover silage can reduce the degradation of forage protein in the silo and improve the availability of RUP to the small intestine. Lucerne does not contain polyphenol oxidase hence the difference between the quantities of protein degraded in red clover and lucerne.

Table 9 The polyphenol oxidase (PPO) activity, activated PPO and protein-bound phenol content of red clover plants

Forage	PPO activity[1]	Activated PPO (%)	Bound phenol (mg/g protein)	Reference
Grass (fresh)	17.2	100	1.72[3]	Lee *et al.* (2009b)
Red clover (fresh)	158	29.1	5.48[3]	
Conditioned red clover	22.1	100	10.5[3]	
HRC – 8 weeks[2]	471	81.2	18.6	Lee *et al*. (2009a)
HRC – 4 weeks[2]	1825	29.0	18.3	
LRC – 8 weeks[2]	157	33.3	10.2	
LRC – 4 weeks[2]	287	15.9	10.0	
WRC – 8 weeks[2]	159	100	61.2	
WRC – 4 weeks[2]	255	100	77.2	

[1]PPO activity measured as U/g of DM in Lee *et al*. (2009b) and as ΔOD/g DM/min in Lee *et al*. (2009a).
[2]Weeks of regrowth
[3]Bound phenol measured as g/kg DM
HRC = High PPO activity in fresh red clover
LRC = Low mutant PPO activity in fresh red clover
WRC = Wild red clover, frozen, thawed and wilted for 24 hours to activate latent PPO (Lee *et al*., 2009a)

1.7.4 Effects of PPO on milk fat

Generally, the presence of polyunsaturated fatty acids (PUFAs) in milk is greater when dairy cows are feed red clover silage compared to grass silages (Van Ranst *et al*., 2011). These changes in PUFAs have been suggested to be due to the presence of PPO in the red clover, providing protection to the lipids from metabolism within the rumen, allowing for a higher concentration of fatty acids in the milk (Lee *et* al., 2009b; Leduc *et al*., 2017). In a study conducted by Lee *et al*. (2009b), cows were offered either fresh grass or red clover, or conditioned red clover silage; milk fat was similar for all three treatments averaging 39.5 g/kg. However, there was a change in milk fatty acid profile, milk from cows fed either

diet containing red clover had higher levels of C18:2 *n*-6 and C18:3 *n*-3 than cows fed the grass diet. Lee *et al*. (2009b) concluded that the C18 PUFAs were being provided protection in the rumen by the presence of PPO in the red clover thereby elevating the concentrations of FAs found in the milk. Similarly, in a study conducted by Dewhurst *et al*. (2003), dairy cows were offered either grass or red clover silage, milk FA C16:0 was reduced in cows offered red clover silage whereas concentrations of C18:3 *n*-3 was higher compared to cows offered grass silage. Therefore, the recovery of C18:3 *n*-3 from the diet to the milk was higher by 4.6% in red clover silage. Dewhurst *et al*. (2003) suggested that the rumen biohydrogenation was reduced by the presence of red clover silage in the diet. Halmemies-Beauchet-Filleau *et al*. (2014) reported a linear decrease in short and medium-chain FAs as the amount of red clover silage increased in the diet from 0 to 100%, whereas there was a linear increase in concentrations of C18:2 *n*-6 and C18:3 *n*-3. In an earlier study, Halmemies-Beauchet-Filleau *et al*. (2013) observed a linear increase in the flow of C18:2 *n*-6 and C18:3 *n*-3 to the omasum of 24 to 32 g/d and 11 to 21 g/d, respectively, as red clover silage in the diet increased from 0 to 100%, therefore a reduction in lipolysis and biohydrogenation of these fatty acids was occurring within the rumen. Therefore, it can be concluded that the presence of PPO in red clover silage can provide protection of dietary PUFAs from lipolysis and biohydrogenation in the rumen, influencing the milk fatty acid profile of dairy cows.

1.7.5 Feeding value

A number of studies have examined feeding red clover and lucerne to dairy cows, and in particular studying stage of maturity, wilting, supplementation and different levels of forage within the diet as summarised in Table 10 and 11. Moorby *et al*. (2016), offered dairy cows third cut red clover silage at different inclusion rates of 10, 50 and 90 % of forage DM replacing maize silage. As red clover silage inclusion increased from 10 to 90 %, the DM and CP of the diet increased from 338 to 516 g/kg and 107 to 163 g/kg DM, respectively, while starch content of the diet decreased from 23.9 to 2.5 % DM. Dry matter intake and milk yields were improved by 1 and 1.4 kg/d, respectively, in dairy cows fed the 50:50 red clover to maize silage diet therefore, Moorby *et al.* (2016) concluded that an equal mixture was the ideal combination in maize silage based diets.

Table 10 Effects of cut of lucerne and red clover silage on performance of dairy cows

Forage	Cut	Inclusion rate[1]	DM, g/kg	CP, g/kg	DM intake, kg/d	Milk yield, kg/d	Reference
Lucerne	Third	60	412	220	24.7	30.9	Hymes-Fecht *et al.* (2013)
Red clover	Second	50	600	181	25.6	30.8	
Trial 1							Broderick *et al*. (2001)
Lucerne	First	60.5	520	191	25.5	32.0	
Red clover	First	59.8	358	179	23.0	32.7	
Trial 2							
Lucerne	First	60.5	366	217	23.5	30.4	
Red clover	First + second	60.7	289	191	21.8	30.4	
Lucerne	First	100	415	166	21.1	31.9	Hoffman *et al*. (1997)
	Second	100	423	171	20.2	30.5	
Red clover	First	100	421	187	21.4	32.1	
	Second	100	430	170	18.6	30.1	
Trial 1							Broderick *et al*. (2000)
Lucerne	Second + third	66 + 34	392	213	20.9	35.3	
Red clover	First + second	73 + 27	448	177	21.1	31.9	
Trial 2							
Lucerne	Second	100	392	213	20.0	29.4	
Red clover	First + second	55 + 45	448	177	19.3	29.9	
Trial 3							
Lucerne	Third	100	392	213	24.2	33.4	
Red clover	Second	100	448	177	21.6	31.8	

[1]Inclusion rate as a % forage DM

Moorby *et al.* (2009) compared different levels of red clover and grass silage in the diet of dairy cows. Cows were offered third cut red clover silage at rates of 0, 34, 66 and 100 % with grass silage included at 100, 66, 34 and 0 % respectively. The DM and CP content decreased as the proportion of red clover silage increased in the diet (Moorby *et al*., 2009). Dry matter intake increased linearly from 13.2 to 15.5 kg/d as red clover increased in the diet, however milk yield was highest at 26.5 kg/d in the diet containing 66 % red clover silage. From these two studies, it can be concluded that milk production is greatest when red clover silage is between 50 to 66 % of the overall forage ratio in grass silage based diets.

Sinclair *et al.* (2015), included lucerne at three rates, 20, 40 and 60 % of the forage DM with maize making up the remaining proportion (in the 20 % diet, grass and lucerne silage was included at 20%) and reported a decrease in DM intake when lucerne was included at 60 % of the forage DM. However milk yield was similar across all rates of inclusion averaging 40.9 kg/d for cows that were 61 days in milk. In a similar study, Arndt *et al.* (2015) examined four different rates of inclusion of lucerne in a maize based diet. Unlike Sinclair *et al.* (2015), Arndt *et al.,* (2015) reported no effect of inclusion level of lucerne on DM intake or milk yield which averaged 24.6 kg/d and 37.8 kg/d, respectively for dairy cows that were 136 days in milk. Milk production was higher in the study of Sinclair *et al.* (2015) which could be associated with the cut of lucerne silage as Hoffman *et al.* (1998) observed higher milk yields in cows (61 days in milk) fed lucerne silage when harvested early.

In a study conducted by Broderick *et al.* (2000), red clover and lucerne silage were compared as the sole forage in a total mixed ration fed to dairy cows. Lucerne was a mixture of second and third cut silage while red clover was a mixture of first and second cut silage. The DM content of lucerne silage was 56 g/kg lower than red clover silage, whereas CP content was 36 g/kg DM higher. Broderick *et al.* (2000) reported that DM intake and milk yield improved by 1.2 kg/d and 1.3 kg/d respectively, when cows were fed lucerne silage. In contrast, Broderick *et al.* (2001) observed higher DM intakes when lucerne replaced red clover silage but there was no difference in milk yield. The observations in study of Broderick *et al.* (2001) in DM intakes were suggested to be due to the 120 g/kg difference in DM content between the lucerne and red clover silage with lucerne being higher, whereas the CP content was only 19 g/kg DM lower in red clover silage.

Table 11 Effects of inclusion rate of lucerne and red clover silages on performance of dairy cows

Forage	Inclusion rate[1]	DM[2], g/kg	CP[2], g/kg	DM intake, kg/d	Milk yield, kg/d	Milk protein, kg/d	Reference
Lucerne	20	524	166	24.0	37.7	30.4	Arndt *et al.* (2015)
	40	536	170	24.7	38.3	30.0	
	60	550	175	25.0	37.9	29.8	
	80	564	180	24.8	37.3	29.0	
Lucerne	20	444	171	24.9	40.7	30.8	Sinclair *et al.* (2015)
	40	452	170	24.5	40.2	31.0	
	60	470	169	23.4	40.5	30.8	
Red clover	10	338	107	19.6	26.1	30.9	Moorby *et al.* (2016)
	50	419	144	20.5	27.3	30.6	
	90	516	163	19.5	25.7	29.2	
Red clover	0[3]	375	206	16.7	25.2	30.8	Moorby *et al.* (2009)
	34	359	205	17.8	26.1	30.7	
	66	339	197	18.3	26.5	30.6	
	100	322	194	19.0	26.1	29.3	

[1]Inclusion rate of the forage DM
[2]DM and CP content of the TMR
[3]100% grass silage

Hoffman *et al.* (1997) investigated the effects of early and late harvesting of lucerne and red clover silages when fed to dairy cows, over two years. In year 1, milk yields were similar in cows fed lucerne and red clover silage, however harvest time influenced milk yield, with silages harvested early, resulting in milk yields that were 3.5 kg/d higher in cows fed early lucerne and red clover silages. In contrast, in year 2, milk yields were 2.55 kg/d lower in cows fed lucerne silage, and time of harvest had no effect on milk yield. In both years, DM intake was similar in cows fed lucerne or red clover silage averaging 20.6 and 20.0 kg/d respectively (Hoffman *et al*., 1997) and it was concluded that forages harvested early had the potential for higher DM intake and suggested milk yield was supported by mobilisation of body fat reserves when late harvested forages were fed.

1.8 Gaps in current knowledge

Following an extensive review of the literature, it is clear that the gaps in the current knowledge of using forage legumes as silages for high yielding dairy cows are:-

- Forage peas and red clover contain varying levels of naturally occurring condensed tannins which have the potential to improve the availability of by-pass protein from forage legumes which tend to be high in RDP. Tannins can occur naturally or can be supplemented at ensiling. The addition of condensed or hydrolysable tannins at ensiling has primarily focussed on grass silage with observed increases in total N post ensiling. There have been very few studies investigating the effects of tannins on nutritive value using forage legumes like lucerne, red clover or forage peas. Therefore, this is a key area that needs to be researched further to investigate whether condensed or hydrolysable tannins have the ability to improve the availability of RUP during the ensiling process.
- Previously, forage peas have been fed to dairy cows as a bi-crop with wheat, in these studies milk yields tended to be higher than in cows fed grass silage. However, only a few studies have investigated forage peas as a whole crop pea silage in a maize silage based diet. These studies have focussed on low yielding dairy cows, and it was observed that DMI was higher in cows fed forage pea silages compared to cows fed grass silage. It was observed that milk yields remained similar for cows fed either grass or forage pea silages.

Therefore, research needs to investigate the effects of feeding forage pea silage to high yielding dairy cows on animal performance, digestibility and degradability.

- Many studies have compared red clover silage and lucerne silage in high yielding dairy cows, generally cows performed similarly on either silage. Although, cows fed red clover silage tended to have a higher N efficiency due to less extensive proteolysis in the silo allowing for a higher availability of RUP. The addition of tannins to lucerne or red clover silage is an area of research that has very few studies. Generally it is reported that there is no change in milk production when tannins are added to the diet at feed out. Previous studies have reported a decline in DMI when condensed tannins have been included at rates above 30 g/kg DM at feed out. Studies have not investigated the effects of hydrolysable tannins added at ensiling to red clover or lucerne silage on animal performance of high yielding dairy cows, therefore, this is another area that requires further research.

1.9 Conclusions

Global demand for soya bean meal has risen in recent years, thus influencing the cost and availability of SBM to the UK ruminant industry. This volatility in SBM and other purchased feed has resulted in greater interest in the utilisation of home grown protein sources in particular, forage legumes including lucerne, red clover and forage peas conserved as a silage. Forage legumes are crops that grow well in temperate regions like the UK and North America. Compared to traditional grass silage, forage legumes require less nitrogen fertiliser during growth due to their ability to fix nitrogen, therefore reducing costs to produce the silage. Forage legumes have high crude protein levels ranging between 170 to 220 g/kg DM, although this protein degrades rapidly within the rumen resulting in low amounts of rumen undegradable protein reaching the small intestine. Although, in red clover silage, the presence of PPO has the potential to improve the availability of RUP due to the binding with protein in the forage during the ensiling process, the protein becomes available for digestion in the small intestine. High yielding dairy cows require high levels of RUP to improve and maintain high milk yields. High levels of RUP can improve N efficiency in high yielding dairy cows, however forage legumes have low levels of RUP resulting in reduced N efficiency. Tannins are phenolic compounds that can be classified as condensed or hydrolysable, and can

occur naturally or be added to the forage legume at either ensiling or feed-out. Tannins can potentially reduce the amount of RDP that occurs in forage legumes as the tannins bind with the protein and form complexes at pH 4.5 – 7. These tannin-protein complexes can form during ensiling or within the rumen, and dissociate in the small intestine improving the amount of by-pass protein available for absorption in a high yielding dairy cow. Therefore, the potential to alter protein degradability of forage legumes by supplementation of tannins at ensiling or by naturally occurring tannins requires investigation in high yielding dairy cows.

1.10 Experimental hypothesis and objectives

The overall hypothesis of this thesis was that the utilisation of N from home grown forages can be improved by using naturally occurring condensed tannins in legumes or the addition of hydrolysable tannins at ensiling, reducing the requirement for purchased dietary protein and improving the performance of high yielding dairy cows.

The first objective of this study was to investigate the effects of condensed and hydrolysable tannins that occur naturally or are added to forage legumes on their nutritive value post ensiling. Following on from this, the second objective of this study was to investigate the effects of feeding forage pea silages with naturally occurring condensed tannins or lucerne and red clover silages supplemented with hydrolysable tannins at ensiling on the utilisation of nitrogen and performance of high yielding dairy cows.

Chapter 2 – General Material and Methods

2.1 Dry Matter (DM)

Forage and TMR samples were oven dried at 105°C for 24 h to a constant weight according to AOAC (2012; 943.01). Samples were cooled in a desiccator and weighed. Residues from the *in* situ degradation study and faecal samples were oven dried at 60°C for 48 h. A cyclone mill (Cyclotec, FOSS, Warrington, UK) with a 1 mm screen was used to mill all dried samples. Dry matter was calculated as follows:

$$\text{Dry matter (g/kg)} = \frac{(Dried\ sample\ weight\ (g))}{(Initial\ sample\ weight\ (g))} \times 1000 \qquad \text{Equation 2.1}$$

2.2 Crude protein (CP)

Nitrogen content of dried forage, TMR, faecal and residue *in situ* degradation samples was measured using a Leco FP-528 (Leco Corporation, St Joseph, MI) operating the Dumas method according to AOAC (2012; 990.03). Approximately 0.15 g forage, TMR and faecal samples; and 0.10 g of residue of *in situ* samples were weighed into aluminium foil trays and placed on to the Leco FP-528. Crude protein was calculated as follows:

$$\text{Crude protein (g/kg DM)} = \text{total nitrogen (g/kg)} \times 6.25 \qquad \text{Equation 2.2}$$

2.3 Organic matter (OM)

Organic matter was determined by weighing approximately 2 g of dried forage and TMR sample in to a pre-weighed porcelain crucible. Samples were placed in a muffle furnace (Carbolite AAF 1100, Hope Valley, England) at 550°C for 4 h, samples were cooled in a desiccator and weighed. Ash and organic matter was calculated as follows:

$$\text{Ash (g/kg DM)} = \frac{Ashed\ weight\ (g)}{Initial\ sample\ weight\ (g)} \times 1000 \qquad \text{Equation 2.3a}$$

$$\text{OM (g/kg DM)} = 1000 - \text{ash (g/kg DM)} \qquad \text{Equation 2.3b}$$

2.4 Neutral detergent fibre (NDF)

Neutral detergent fibre (NDF) was determined according to Van Soest *et al.* (1991). Approximately 0.5 g of dried forage, TMR or faecal sample was weighed

into a pre-weighed glass crucible (porosity 1, Soham Scientific, Ely, UK) and placed onto Fibertec apparatus (1020, FOSS, Warrington, UK). To each crucible, 25 ml of neutral detergent reagent (93 g of di-sodium ethylene diamine tetra-acetic acid dehydrate (EDTA) and 34 g of sodium tetraborate was dissolved in approximately 3 L of hot distilled water, 150 g of sodium dodecyl sulphate (SDS) and 50 ml of tri-ethlylene glycol was added. In a small beaker with approximately 500 ml of hot distilled water, 22.8 g of anhydrous disodium hydrogen phosphate was dissolved. The contents of the small beaker were added to the first solution and mixed together, the volume was made up to 5 L and the pH adjusted to lie between 6.9 and 7.1 with either 0.1 M NaOH or 0.1 M HCl) and 0.5 ml octan-1-ol was added.

Samples were boiled and digested for 30 minutes, a further 25 ml of neutral detergent reagent was added to each crucible along with 2 ml alpha amylase (2 g of alpha amylase (80 EU/mg, Sigma, Gillingham, UK) was dissolved in 90 ml distilled water and 10 ml tri-ethylene glycol). Samples were boiled and digested for a further 30 minutes, filtered and washed with 3 x 25 ml of hot distilled water (80°C) to remove the neutral detergent reagent. Following washing, 2 ml alpha amylase and 25 ml hot distilled water (80°C) was added to each sample and allowed to stand for 15 minutes. Samples were filtered and washed with 3 x 25 ml of hot distilled water (80°C), crucibles removed from the Fibretec apparatus and oven dried at 105°C overnight. The crucibles were cooled in a desiccator and weighed before being placed in a muffle furnace at 550°C for 16 h. Crucibles were cooled in a desiccator and reweighed.

$$\text{NDF (g/kg DM)} = \frac{Dried\ weight\ (g) - Ashed\ weight\ (g)}{Initial\ sample\ weight\ (g\ DM)} \times 1000 \qquad \text{Equation 2.4}$$

2.5 pH

Forage pH was determined using the method of MAFF, (1986) where water extract of the silage was determined using a Jenway 3505 pH probe and meter (Bibby Scientific Limited, Staffordshire, UK). The pH probe was calibrated daily using pH 4 and 7 buffers. Approximately, 50 g of forage was placed in a beaker and 125 ml distilled water added. Samples were stirred every 15 minutes for 1 h, and the pH determined. The pH probe was cleaned with distilled water between each sample.

2.6 Ammonia nitrogen

Forage ammonia nitrogen was determined using a method adapted from MAFF (1986) using an auto-titrator (FOSS 1030 auto-titrator, FOSS, Warrington, UK and Buchi Labortechnik AG CH-9230, Flawil, Switzerland). Approximately, 20 g fresh forage was weighed into a glass shaking bottle and 100 ml of distilled water added. Samples were shaken for 1 h on a laboratory shaker at 275 stokes per minute. After shaking, the water extract was filtered (150 mm Whatman number 1 filter paper) and 5 ml of filtrate transferred to a Kjeldahl digestion tube. To each sample, 6 ml magnesium oxide (17 g heavy magnesium oxide dissolved in 100 ml distilled water) was added prior to the sample being analysed by the auto-titrator.

$$\text{Ammonia nitrogen (g/kg DM)} = \frac{7\ x\ T\ x\ (120-(0.02\ x\ DM))}{10\ x\ DM} \qquad \text{Equation 2.5}$$

Where T was the titre reading – blank.

2.7 Feed fatty acids

Feed fatty acid (FA) content of forage and TMR samples were determined according to Jenkins, (2010). Approximately, 0.5 g of dried, ground forage sample was weighed into a 16x125 mm screw cap Pyrex culture tube, 1 ml of methyl C19:0 (100 mg C19:0 in 50 ml methanol, dissolved in a water bath) and 2 ml of sodium methoxide (1.75 ml methanol added to 0.2 ml 30% sodium methoxide solution) was added. Samples were vortexed lightly for 30 seconds, and incubated in a water bath at 50°C for 10 minutes. Samples were then removed from the water bath and allowed to cool for 5 minutes. To each sample, 3 ml of 5% methanolic HCl (on a magnetic stirrer in a fume cupboard, a conical flask in a beaker with ice ensuring no ice enters the conical flask. To the flask 100 ml of methanol was added and 10 ml acetyl chloride slowly added) was added. Samples were vortexed lightly for 30 seconds and incubated in a hot water bath at 80°C for 10 minutes. Samples were then removed from the water bath and cooled for 7 minutes. To each sample, 3 ml hexane and 10 ml of 6% K_2CO_3 (6 g K_2CO_3 in 100 ml distilled water) was added. Samples were then vortexed for 5 minutes and 1 g of anhydrous sodium sulphate added to each sample. If the sample was coloured then 0.5 g of charcoal was added or if it was highly coloured then 0.75 g of charcoal was added as the sample must be clear to be run through the GC. Samples were then centrifuged at 500 *g* for 5 minutes. A glass pipette was used to

collect the hexane layer containing FAME into a GC vial. Feed and milk FAME in hexane were analysed using a Hewlett-Packard 6890 gas chromatograph (GC) (Germany) fitted with a 100m CP Sil 88 column (CP Sil 88, Agilent Technologies, UK) as described by Lock *et al*. (2006). Alongside the column, the GC contained an automatic sampler and flame ionization detector. Firstly, an oven temperature of 70°C was reached and remained steady for 2 min before a gradual increase to 110°C by 8°C/min. The temperature of 110°C remained for 4 min before increasing to 170°C by 5°C/min and remained at this temperature for 10 min. Following this, a final increase to 225°C occurred by 4°C/min and this temperature was held for 15 min. A run time lasted 61.75 min followed by a 1 min post run at 70°C. Individual FAME standards (Sigma-Aldrich, UK) were used to identify peaks in feed and milk fatty acid.

Corrected total area = Total area – internal standard area Equation 2.6a

Fatty acid (FA) (g/kg DM) = $\frac{Total\ FA\ (mg)}{100}$ x FA area (g/100g) Equation 2.6b

2.8 Tannin content

Tannin content of forages was determined according to Makkar *et al*. (1993). Dried forage sample, 0.4 g, was weighed into a 50 ml tube and 20 ml of 70% aqueous acetone (30 ml distilled water was added to 70 ml acetone) added. Samples were centrifuged at 3000 *g* for 10 minutes at 4°C, the liquid tannin extract was collected into a clean tube and stored at 4°C.

2.8.1 Total Phenols

Fifty microlitres (50 µl) of tannin extract as prepared in section 2.8 was added to 950 µl of distilled water. To each sample, 1.25 ml of 20% sodium carbonate solution (50 g of sodium carbonate was dissolved in 250 ml of distilled water) was added. Then 0.5 ml of 1N folin ciocalteu (an equal volume of distilled water was added to 2N folin ciocalteu reagent, the resultant solution was kept at 4°C in a dark bottle) was added in a dark room. Samples were vortexed and kept in the dark for 40 minutes. Sample absorbance was measured on a spectrophotometer (Jenway 6305 Spectrophotometer, Bibby Scientific Ltd, Dunmon, Essex, UK) at 725 nm. Total phenol concentration was estimated from a calibration curve as a tannic acid (Sigma-Aldrich, UK) equivalent of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/ml.

2.8.2 Non tannin phenols

In a glass tube, 100 mg of polypyrrolidone (PVPP which binds to the tannins) was added to 1 ml of distilled water and 1 ml of tannin extract as prepared in section 2.8. A blank sample was prepared with 100 mg PVPP, 1 ml distilled water and 1 ml 70 % aqueous acetone. Samples were vortexed and placed in a fridge at 4°C. After 15 minutes, samples were vortexed and centrifuged at 3000 *g* for 10 minutes at 4°C. After centrifuging, there were two layers, a white bottom layer and a clear liquid top layer. Fifty microlitres (50 µl) of the supernatant was transferred into a tube and 950 µl distilled water added along with 1.25 ml of 20% sodium carbonate solution. In a dark room, 0.5 ml 1N folin coicalteu was added to each sample prior to being vortexed. Samples were then kept in dark conditions for 40 minutes and the optical density measured using a spectrophotometer at 725 nm. Non tannin phenol concentration was estimated from a calibration curve as a tannic acid equivalent of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/ml. Total tannin was calculated as follows:

Total tannin (g/kg DM) = Total phenols (g/kg) – Non-tannin phenols (g/kg) Eq. 2.7

2.8.3 Condensed tannin

In both a plastic and glass test tube, 0.3 ml of 70% aqueous acetone was added to 0.2 ml tannin extract as prepared in section 2.8. To each sample, 3 ml of butanol HCl (50 ml of concentrated HCl was added to 950 ml butanol) and 0.1 ml ferric reagent (16.6 ml concentrated HCl diluted with 20 ml distilled water and 2 g ferric ammonium sulphate was added and dissolved. The volume was made up to 100 ml with distilled water) was added. All samples were vortexed, and a glass marble used to cover the mouth of the glass tubes and then boiled at 97°C to 100°C for 1 h. The plastic tubes (blanks) were left at room temperature for 1 h without heating. Glass tubes were removed from the water bath and cooled to room temperature. The optical density was measured at 550 nm on a spectrophotometer with the sample (glass tube) being measured against the blank (plastic tube). Condensed tannins were calculated as follows:

$$\text{CT (\%)} = \frac{Absorbance\ x\ 78.26\ x\ dilution\ factor}{Dry\ matter\ (\%)} \qquad \text{Equation 2.8}$$

2.9 Forage volatile fatty acids (VFAs)

Forage samples were sent to Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) for determination of fermentation acids, lactic, acetic, propionic and butyric acids using gas chromatography with FID detection.

2.10 Milk composition

Milk composition of fat, protein and lactose were measured on a Milkoscan minor spectrophotometer (Foss Ltd., Denmark) calibrated by the methods of AOAC (2012).

2.11 Milk fatty acids

Milk fatty acids were extracted according to Feng *et al*. (2004) in Experiment 2 and Hara and Radin, (1987) in Experiment 3. All samples were methylated according to Christie, (1982) modified from Chouinard *et al*. (1999).

2.11.1 Extraction (Experiment 2)

Milk samples were placed in a water bath at 40°C for 20 minutes, and shaken occasionally to disperse the milk fat. In a 50 ml conical plastic tube, 30 ml of fresh milk from the AM and PM were combined according to milk yield. Samples were then centrifuged at 17,800 *g* for 30 minutes at 4°C, and separated into two layers, fat cake and water bottom layer. For each sample, 1 g of fat cake was transferred in to a 2 ml Eppendorf and left at room temperature for 20 minutes. Samples were then centrifuged at 19,300 *g* for 30 minutes at room temperature using a micro centrifuge (Beckman, Avanti 30 Centrifuge, Harbor Boulevard, California). After centrifuging, the fat separated into three layers, a lipid top layer; a protein, fat and other water-insoluble solids and a water bottom layer. The lipid layer of each sample was carefully transferred to two 0.5 ml Eppendorf tubes. Samples were stored at -20°C until methylation.

2.11.2 Extraction (Experiment 3)

In a 50 ml conical plastic tube, 35 ml of fresh milk from the AM and PM milking's were combined according to milk yield. Samples were then centrifuged at 17,800 *g* for 30 minutes at 4°C. Approximately, 300 mg of the fat cake layer was transferred into a 16x150 extraction tube that was pre-rinsed with hexane. To each sample,

5.4 ml hexane:isopropanol (600 ml hexane added to 400 ml isopropanol (3:2) and 50 mg butylated hydroxytoluene (BHT) dissolved into the solution) was added, samples were vortexed for 30 seconds. To separate the hexane from the isopropanol, 3.6 ml sodium sulphate (1 g sodium sulphate anahydrous dissolved in 15 ml distilled water) was added to each sample. Samples were then vortexed for 30 seconds and allowed to stand before a further 30 seconds of vortexing. Samples were then left to stand and separate, if the sample had not separated well, samples were centrifuged at 2600 *g* for 5 minutes at 5°C. Sodium sulphate, 1g, was weighted into a new hexane pre-rinsed 16x150 extraction tube. The top hexane layer of each sample was transferred into the tube containing sodium sulphate, tubes were capped and left to stand for 30 minutes. After 30 minutes, the top hexane and milk lipid layer was transferred to a new 16x150 extraction tube pre-rinsed with hexane, and the tubes placed in a water bath at 40°C. The hexane was evaporated off with nitrogen to leave the milk lipid, which was transferred into a 0.5 ml eppendorf tube and stored at -20°C until methylation.

2.11.3 Methylation

Lipid samples were placed in an incubator at 40°C for 20 minutes until the lipid had melted, then 50 mg of lipid was weighed into a 16x150 extraction tube pre-rinsed with hexane. To each sample, 2 ml hexane and 40 µl methyl acetate was added. Samples were then vortexed for 30 seconds, 40 µl methylation reagent (1.75 ml methanol mixed with 0.4 ml 30% sodium methoxide solution, and used within 24 h) was added to each sample and vortexed for 2 minutes. Samples were left to stand for a further 8 minutes, with the reaction being complete 10 minutes after the addition of methylation reagent. To each sample, 60 µl termination reagent (a reagent bottle was placed in an oven at 105°C for 30 minutes to remove water and cooled in a desiccator. Oxalic acid was weighed (1g) into the bottle and the bottle placed in the oven again at 105°C for a further 30 minutes. After cooling in a desiccator, 30 ml diethyl ether was added. The reagent was stored in the dark and used within 2 weeks) was added and samples vortexed for 30 seconds. Approximately, 200 mg calcium chloride was added to each sample which were then vortexed and left to stand for 1 h. Samples were centrifuged at 2600 *g* for 30 minutes at 5°C and the top layer of FAME and hexane was transferred to GC vials. The FAME in hexane was analysed using a Hewlett-

Packard 6890 gas chromatograph (GC) fitted with a 100 m CP Sil 88 column as described in section 2.7.

2.12 Blood analysis

Blood samples were collected from the jugular vein via venepuncture into vacutainer tubes containing EDTA and fluoride/potassium oxalate. Blood samples were centrifuged at 1390 *g* for 10 minutes at 4°C to separate the plasma. All plasma samples were analysed for total protein (TP), urea, beta-hydroxybutryrate (BHB), glucose, albumin and non-esterified fatty acids (NEFAs). Plasma samples were analysed on a Cobas-Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK). Kits used for analysis were TP, Ref TP245; UREA, Ref UR221; RANBUT, Ref RB1008; GLUC-HK, Ref GU611; ALBUMIN, Ref AB362; and NEFA, Ref FA115, respectively.

2.13 Acid insoluble ash (AIA)

Acid insoluble ash of faecal and TMR samples were determined according to Van Keulen and Young, (1977). Approximately 5 g of dried, ground faecal or TMR sample was weighed in to a pre-weighed porcelain crucible and ashed in a muffle furnace at 550°C for 4 hours. Samples were then cooled in a desiccator and re-weighed. Each sample was transferred to a digestion tube with 10 ml of 2M hydrochloric acid (HCl) used to wash out the porcelain crucible in to the digestion tube. A further 90 ml of 2M HCl was added to each sample, samples were heated to 175°C and boiled for 10 minutes. Following boiling, samples were allowed to cool and then filtered through ashless filter paper (150 mm Whatman number 1 filter paper). Samples were washed with hot (80°C) distilled water to remove excess acid. Each sample in the filter paper was placed into a porcelain crucible and ashed in a muffle furnace at 550°C for 4 hours. Samples were then cooled in a desiccator and re-weighed. Feed and faecal AIA were calculated as follows:

$$\text{AIA (\%)} = \frac{Second\ Ashed\ weight\ (g)}{Initial\ sample\ weight\ (g)} \times 100 \qquad \text{Equation 2.9a}$$

Digestibility was calculated as:

$$\text{Digestibility (kg/kg)} = 1 - \frac{(Feed\ AIA)}{(Faecal\ AIA)} \qquad \text{Equation 2.9b}$$

2.14 *In situ* degradation characteristics

Three mature wethers sheep fitted with a permanent rumen cannulae and with a mean live weight of 76.5 kg were used. The sheep were group housed on straw under continuous lighting with free access to clean water. The sheep were offered a basal diet of haylage and concentrate (Ram Master Coarse Mix, Wynnstay, Shropshire, UK; Table 12) providing a forage to concentrate ratio of 60:40 DM basis. Concentrate feed was offered in two equal meals at 0830 and 1630 h.

Table 12 Composition of Ram Master Coarse Mix

Ingredient	Quantity (g/kg DM)
Protein pellets[1]	250
Barley cooked/rolled	200
Sugar beet	150
Molasses	80
Wheat feed	60
Peas micronized	50
Maize micronized	50
Soybeans micronized	50
Soya hipro	60
Oat feed	35
Soypass	25

[1]Protein pellets contained 50% rape meal, 25% sunflower meal, 20% palm kernel and 5% molasses

Approximately 8 g of fresh forage was weighed into synthetic in situ bags with a pore size of 42 µm with a rounded base to prevent lodging of the sample. Each bag was sealed by threading the neck of the bag through a brass curtain ring; the neck was then folded over and secured by a rubber band. Each bag was connected by the brass curtain ring to a stainless steel clip on a 30 cm nylon cord attached to the cannula cap. Bags were inserted 30 minutes post feeding and incubated in the rumen for a period of either 2, 4, 8, 16, 24, 48 or 72 h with each forage incubated in duplicate in each animal. Following rumen incubation, bags were placed in a bucket of cold water to prevent further microbial action, and then washed on a cold rinse cycle of a commercial washing machine. For each forage, a set of bags were washed without rumen incubation to determine the zero hour time point. Following washing, all bags were dried at 60°C for 48 h to a constant

weight and the residue bulked for each time point for each sheep prior to analysis for DM and CP.

Chapter 3 – Experiment 1: The effect of condensed and hydrolysable tannins at varying rates of inclusion on the nutritional value of legume silages

3.1 Background

In recent years, due to their high protein content and nitrogen fixing abilities, legumes have become of greater interest for ensiling as a winter forage and protein source for dairy cows (Schulz *et al*., 2017). During the wilting period and ensiling process, forage legumes undergo extensive proteolysis of protein to non-protein nitrogen (NPN), reducing the amount of true protein available in the conserved forage (Tabacco *et al*., 2006). Condensed and hydrolysable tannins are a group of secondary plant metabolites that have an ability to bind with protein to form a pH dependent complex structure (Mueller-Harvey, 2006). These protein-tannin structures can form during the ensiling process protecting protein from plant enzyme hydrolysis (Tabacco *et al*., 2006; Salawu *et al*., 1999). Plant proteases activity is highest at pH 6 – 7; tannins bind with forage protein between pH 4.5 and 7 therefore are able to reduce the extent of protein degradation in the silo (Henderson, 1993; Salawu *et al*., 1999). In a study conducted by Tabacco *et al.* (2006), lucerne silage was supplemented with chestnut (hydrolysable) tannins at four inclusion rates, 0, 20, 40 and 60 g/kg DM at ensiling. The NPN levels decreased from 75.9 % of total N to 62.6 % of total N as inclusion rate of tannin increased from 0 to 60 g/kg DM, respectively, suggesting protection of the protein during the ensiling process.

Forage legumes have varying amounts of naturally occurring tannins. Lucerne silage contains no naturally occurring tannins whilst there are minor levels (2.3 g/kg DM) present in red clover silage (Hymes-Fecht *et al*., 2013). Condensed tannins occur naturally in forage peas with flower colour indicating the level of tannin present, white flowers indicate low levels while coloured flowers indicate high levels (Wang *et al*., 1998). Hart *et al.* (2011) and Sinclair *et al.* (2009) reported condensed tannin levels of 47.3 and 92.7 g/kg DM for white and coloured forage pea silage, respectively. There is little information on the effects of tannins on ensiling properties therefore, the study objectives were to determine the effects of the addition of different inclusion rates of condensed and hydrolysable tannins

at ensiling on the nutritional value of grass, lucerne, red clover, red pea and white pea silages.

3.2 Materials and Methods

3.2.1 Forages, tannin types and tannin inclusion rates

The five forages, two tannin types and four tannin inclusion rates used to make mini silos in this study are shown in Table 13. Each forage received treatment of the two tannin types at four tannin inclusion rates, with each forage sample being replicated by four mini silos. Five forages; lucerne (*Medicago sativa*), red clover (*Trifolium pratense*), grass (*Lolium multiflorum*), white pea (*Pisum sativum; Montara*) and red pea (*Pisum sativum; Daytona*) were harvested during June and July 2014. Both pea silages were mown at approximately 1000 h on the 3rd of July 2014 when the crop had reached growth stage 206 (pod swollen with small seeds) (Knott, 1987). A front mounted drum mower without a conditioner was used with a stubble of 10 cm. The peas were wilted for approximately 30 hours prior to being picked up by a precision chop, self-propelled forage harvester with an additive added (Axcool Gold, Biotal Ltd, Pontprenna, Cardiff; 4 l/tonne. Axcool Gold is an inoculant containing the bacteria *L. buchneri* 40788). The grass silage was a second cut ley of predominantly rye grass and white clover, wilted for approximately 24 hours and harvested on 25th June 2014 using a precision chop, self-propelled forage harvester with an additive added (Axcool Gold, Biotal Ltd, Pontprenna, Cardiff; 4 l/tonne). The lucerne silage was a first cut ley, wilted for 24 hours and harvested on 1st June 2014 using a precision chop, self-propelled forage harvester with an additive added (Axcool Gold, Biotal Ltd, Pontprenna, Cardiff; 4 l/tonne). The red clover silage was a second cut ley, wilted for 24 hours and harvested on 12th June 2014 using a forage wagon with an additive added (Axcool Gold, Biotal Ltd, Pontprenna, Cardiff; 4 l/tonne).

Table 13 The forages, tannin types and tannin inclusion rates used for producing the mini silos.

Forage	Tannin Type	Tannin Inclusion Rate (g/kg DM)
Grass	Condensed	0
Lucerne	Hydrolysable	25
Red clover		50
Red pea		75
White pea		

Two tannin types; chestnut hydrolysable tannins (Inovitec Ltd, Cheshire, UK) and quebracho condensed tannins (Inovitec Ltd, Cheshire, UK) were added to the five forages as a liquid (tannins were mixed with water as a 30 % solution based on DM weight) at four inclusion rates; 0, 25, 50 and 75 g/kg DM before ensiling. Pipes of length 33 cm and diameter 14 cm attached to a wooden base were lined with two plastic bags. Approximately 1 kg of fresh silage was placed in the plastic bags and compressed down. Bags were then sealed with silage tape, sand placed on top and the mini silo sealed with silage tape. Mini silos were stored at ambient temperature and opened 128 ± 7 days following harvest. Following opening, the mini silos were sub-sampled and stored at -20ºC until subsequent analysis.

3.2.2 Chemical analysis

Forage samples were analysed for DM (943.01), crude protein (990.03), and ash (942.05) according to AOAC (2012) as described in Chapter 2. The NDF content was determined according to Van Soest *et al*. (1991) as described in section 2.4. Crude protein was determined using a Leco FP-528 (Leco Corporation, St Joseph, MI). Forage pH was determined using a method by MAFF, (1986) using a Jenway 3505 pH meter (Bibby Scientific Limited, Staffordshire, UK). Ammonia nitrogen was determined using a method adapted from MAFF, (1986) using an auto-titrator (FOSS 1030 auto-titrator, FOSS, Warrington, UK and Buchi Labortechnik AG CH-9230, Flawil, Switzerland) as described in section 2.6.

3.2.3 Statistical analysis

Data for DM, CP, ash, NDF, NH_3-N and pH were evaluated by ANOVA in a factorial plus control design using Genstat Release 18 (VSN International Ltd).

Treatment degrees of freedom were split into main effects of forage (Silage), tannin (Tannin), tannin inclusion rate (Level) and their interactions (Silage x Tannin, Silage x Level, Tannin x Level, and Silage x Tannin x Level). In all cases, data were checked for normality and homogeneity of variance by visual assessment of the residual plots. No adjustments were required and data are presented as means with an s.e.d of the means.

3.3 Results

The interaction between silage, tannin and level of tannin did not have an effect ($P > 0.05$) on mean DM, ash, NDF, pH or NH_3-N (Table 14a, b & c). However, there was a silage, tannin and level of tannin interaction ($P < 0.001$) between these variables with regards to crude protein content that averaged 191, 175, 106, 162 and 192 g/kg for grass, lucerne, red clover, red pea and white pea silage, respectively.

Type of silage had an effect ($P < 0.001$) on the DM content which was highest in red pea silage and lowest in lucerne silage with grass, red clover and white pea silage being intermediate. Tannin type had no effect ($P > 0.05$) on DM content with silage treated with condensed and hydrolysable tannin having a similar dry matter content which averaged 356 g/kg. Dry matter content increased ($P < 0.001$) when the inclusion rate of tannin increased from 0 to 75 g/kg DM. There was an interaction between silage and tannin type with DM content being higher ($P < 0.001$) when condensed tannin was added to grass and red clover silage, and when hydrolysable tannin was added to lucerne and red pea silage. For white pea silage, DM content was similar when condensed or hydrolysable tannin was added but higher than the untreated silage. There was no interaction ($P > 0.05$) between silage type and level of tannin on DM content (Figure 9 and Table 14).

Table 14a Chemical composition (g/kg DM) of grass, lucerne, red clover, red pea and white pea silages ensiled with condensed (CT) or hydrolysable (HT) tannin at varying levels of 0, 25, 50 or 75 g/kg DM.

		Treatment										
		Grass		Lucerne		Red clover		Red pea		White pea		
n		28		28		28		28		28		
	Level	CT	HT	CT	HT	CT	HT	CT	HT	CT	HT	s.e.d
DM, g/kg	0	288	288	276	276	349	349	384	384	346	346	18.4
	25	327	328	258	292	388	372	360	396	375	379	
	50	398	311	281	298	400	403	389	392	381	379	
	75	352	331	297	297	401	362	401	399	399	398	
CP	0	198	198	179	179	110	110	161	161	195	195	5.67
	25	177	186	170	175	106	103	164	158	190	193	
	50	202	172	167	173	107	100	159	163	185	192	
	75	194	167	167	178	99.2	99.2	167	165	186	187	
Ash	0	135	135	104	104	78.5	78.5	229	229	135	135	27.9
	25	124	129	110	95.9	70.3	72.7	250	277	149	138	
	50	132	121	101	137	71.7	67.6	246	228	139	133	
	75	117	114	98.2	105	69.3	69.6	215	213	119	136	
NDF	0	520	520	533	533	612	612	396	396	391	391	70.9
	25	505	491	526	516	614	625	405	385	454	402	
	50	487	491	527	493	593	608	399	406	411	420	
	75	476	431	503	504	581	637	374	421	442	402	

Number of replicates for each forage and tannin type per level = 4

Table 14b Chemical composition of grass, lucerne, red clover, red pea and white pea silages ensiled with condensed or hydrolysable tannin at varying levels of 0, 25, 50 or 75 g/kg DM.

		Treatment										
		Grass		Lucerne		Red clover		Red pea		White pea		
n		28		28		28		28		28		
	Level	CT	HT	CT	HT	CT	HT	CT	HT	CT	HT	s.e.d
pH	0	4.66	4.66	5.90	5.90	4.67	4.67	6.40	6.40	4.63	4.63	0.513
	25	4.65	4.76	6.58	5.73	4.73	4.26	6.66	5.57	4.49	4.59	
	50	5.15	4.63	6.27	5.51	4.50	4.22	5.72	6.39	4.82	4.73	
	75	5.16	4.60	5.78	6.04	4.45	4.54	5.85	4.99	4.84	4.64	
NH_3-N, g/kg total N	0	53.4	53.4	179	179	34.6	34.6	130	130	80.4	80.4	31.7
	25	36.2	84.0	251	151	15.0	22.5	125	114	48.5	84.4	
	50	43.0	53.4	193	148	13.9	19.5	134	114	95.9	56.3	
	75	72.7	44.1	177	134	16.1	17.1	101	114	59.9	64.7	

Number of replicates for forage and tannin type per level = 4

Table 14c P values for the chemical composition of grass, lucerne, red clover, red pea and white pea silage ensiled with condensed or hydrolysable tannin at varying levels of 0, 25, 50 or 75 g/kg DM.

	P values			Interactions			
	Silage	Tannin	Level	Silage.Tannin	Silage.Level	Tannin.Level	Silage.Tannin.Level
DM	<0.001	0.828	<0.001	<0.001	0.054	0.005	0.329
CP	<0.001	0.173	0.704	<0.001	0.271	0.235	<0.001
Ash	<0.001	0.622	<0.001	0.945	<0.001	0.203	0.170
NDF	<0.001	0.656	0.410	0.302	0.931	0.619	0.776
pH	<0.001	0.027	0.734	0.884	0.696	0.729	0.210
NH_3-N	<0.001	0.193	0.433	0.042	0.869	0.792	0.450

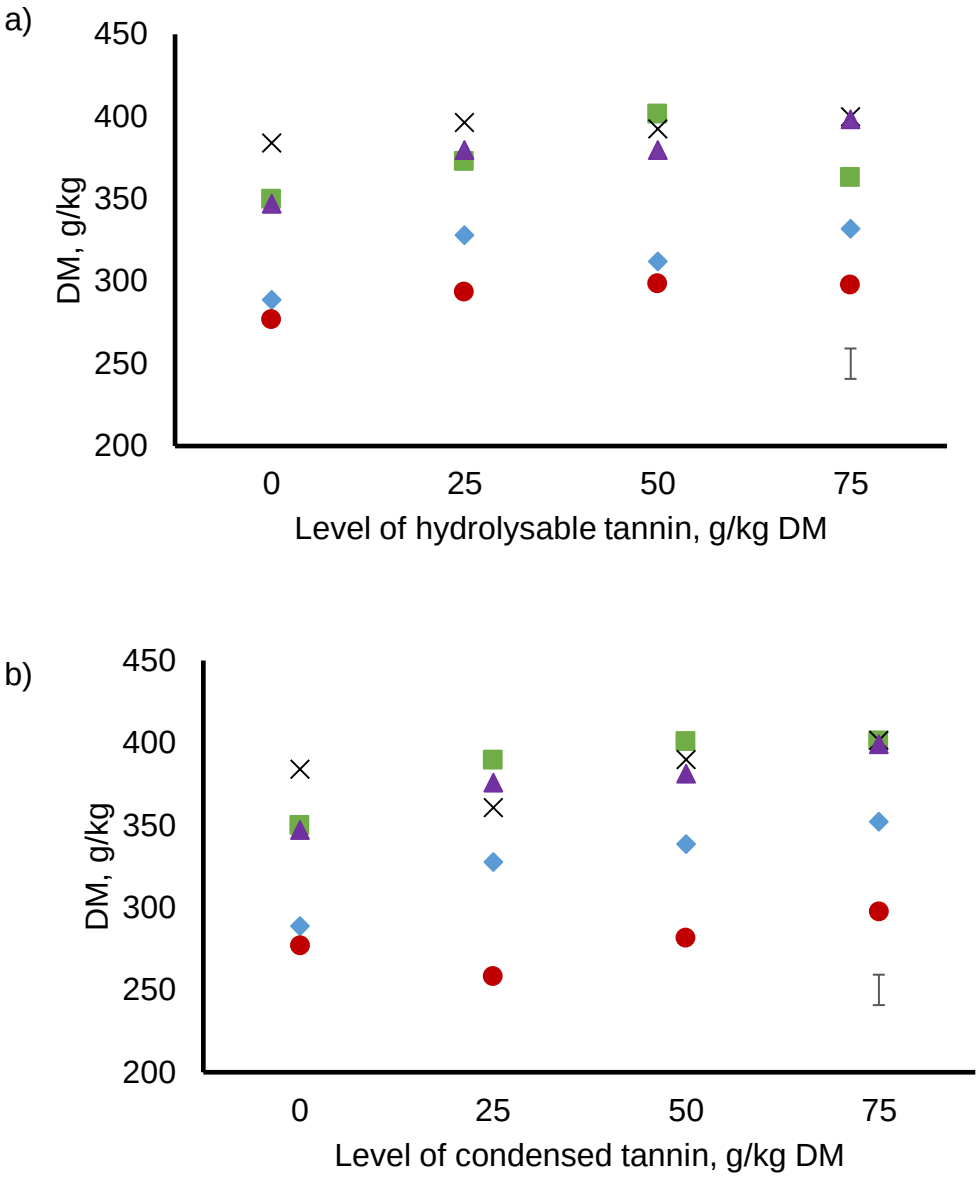


Figure 9 Dry matter content of mini silos that contained grass silage (♦; n = 28), lucerne silage (●; n = 28), red clover silage (■; n = 28), red pea silage (×; n = 28) or white pea silage (▲; n = 28). Error bars indicate pooled s.e.d.

Crude protein content was highest ($P < 0.001$) in white pea and grass silage, whilst being lowest in red clover silage with lucerne and red pea silage being intermediates (Table 14). Tannin type had no effect ($P > 0.05$) on crude protein content and averaged 169, 163 and 161 g/kg DM for no tannin, condensed and hydrolysable tannin, respectively. Rate of inclusion of tannin also had no effect ($P > 0.05$) on crude protein content which averaged 169, 162, 162 and 161 g/kg for 0, 25, 50 and 75 g/kg DM, respectively. There was an interaction between silage and tannin type with crude protein content being higher ($P < 0.001$) in grass, lucerne,

red clover and white pea silage when no tannin was added and marginally higher when condensed tannins were added to red pea silage. Crude protein content was not affected (P > 0.05) by silage type as tannin level increased from 0 to 75 g/kg DM (Figure 10).

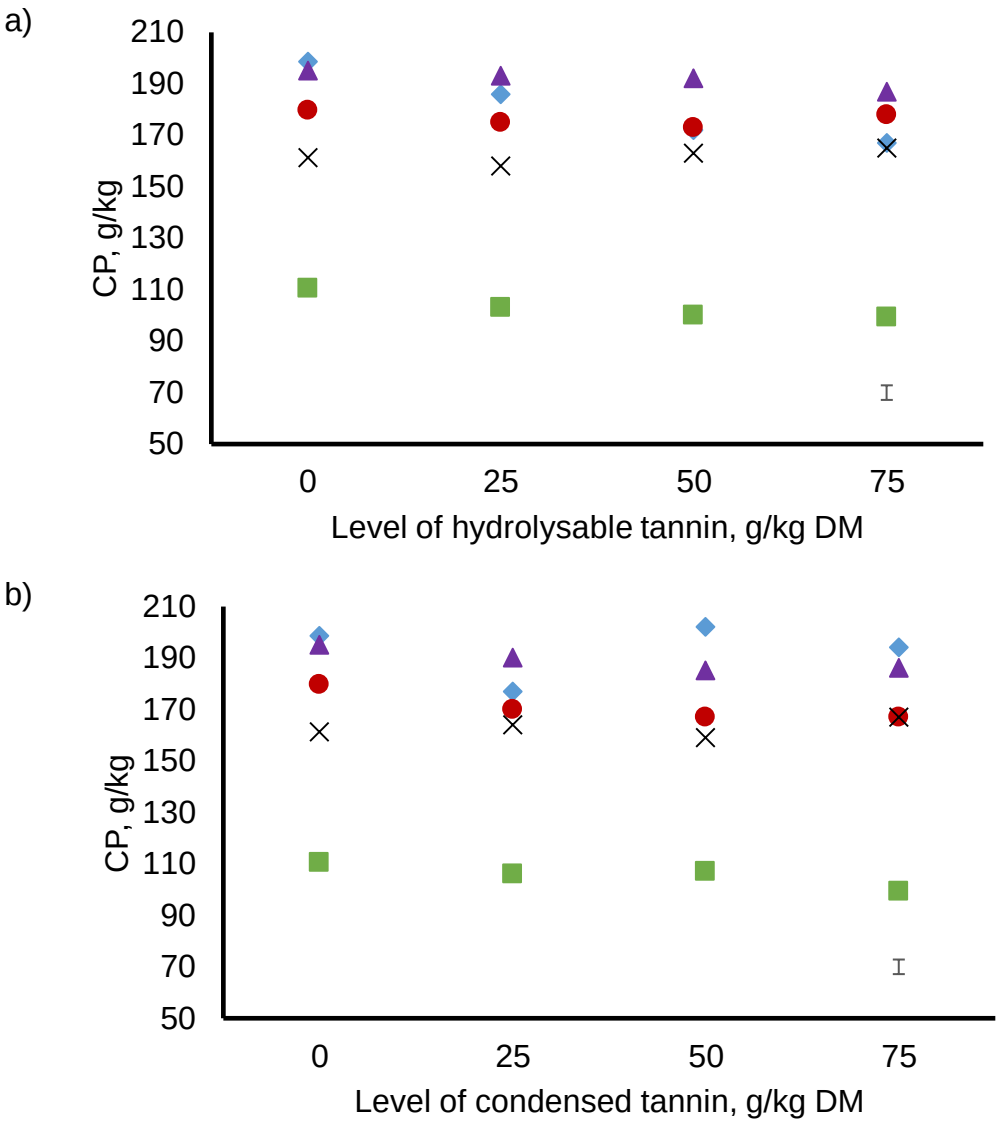
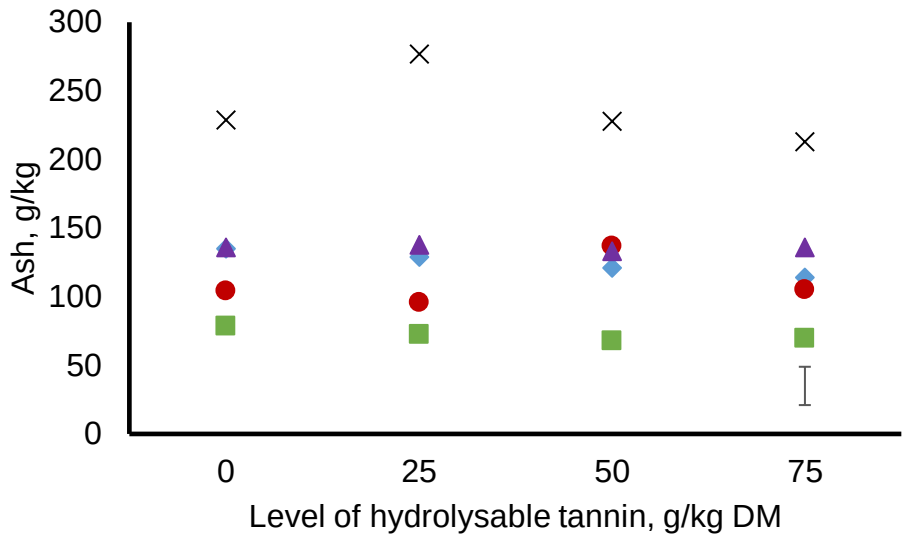


Figure 10 Crude protein content of mini silos that contained grass silage (♦; n = 28), lucerne silage (●; n = 28), red clover silage (■; n = 28), red pea silage (×; n = 28) or white pea silage (▲; n = 28). Error bars indicate pooled s.e.d.

Ash content was affected (P < 0.001) by silage type and averaged 129, 106, 74, 233 and 135 g/kg DM for grass, lucerne, red clover, red pea and white pea silage, respectively (Table 14). Tannin type had no effect (P > 0.05) on ash content and averaged 136, 134 and 136 g/kg for no tannin, condensed tannin and hydrolysable tannin respectively. Ash content increased (P < 0.001) from 136 to 142 g/kg at 0 to 25 g/kg DM inclusion rate before decreasing to 134 and 126 g/kg for 50 and 75

g/kg DM inclusion rate of tannin. There was an interaction (P < 0.05) between silage type and tannin level on ash content (Figure 11), which increased at an inclusion rate of 25 g/kg DM in the red pea silage before declining, but not in the other treatments.

a)



b)

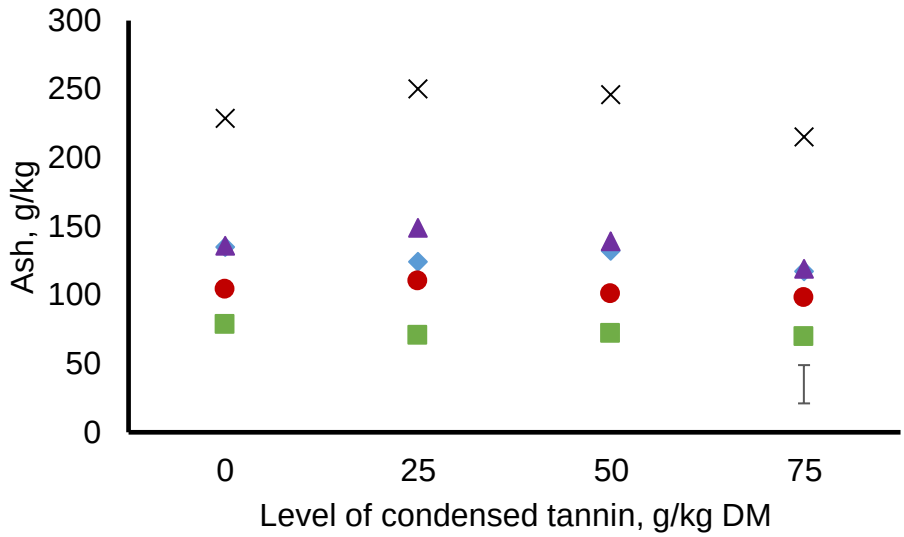


Figure 11 Ash content of mini silos that contained grass silage (♦; n = 28), lucerne silage (●; n = 28), red clover silage (■; n = 28), red pea silage (×; n = 28) or white pea silage (▲; n = 28). Error bars indicate pooled s.e.d.

Silage type had an effect (P < 0.001) on NDF content with a mean value of 500, 522, 610, 397 and 406 g/kg DM for grass, lucerne, red clover, red pea and white pea silage, respectively (Table 14 and Figure 12). Tannin type had no effect (P > 0.05) on NDF content and averaged 490, 486 and 482 g/kg for no tannin,

condensed and hydrolysable tannin, respectively. Inclusion rate of tannin also had no effect (P < 0.05) on NDF content.

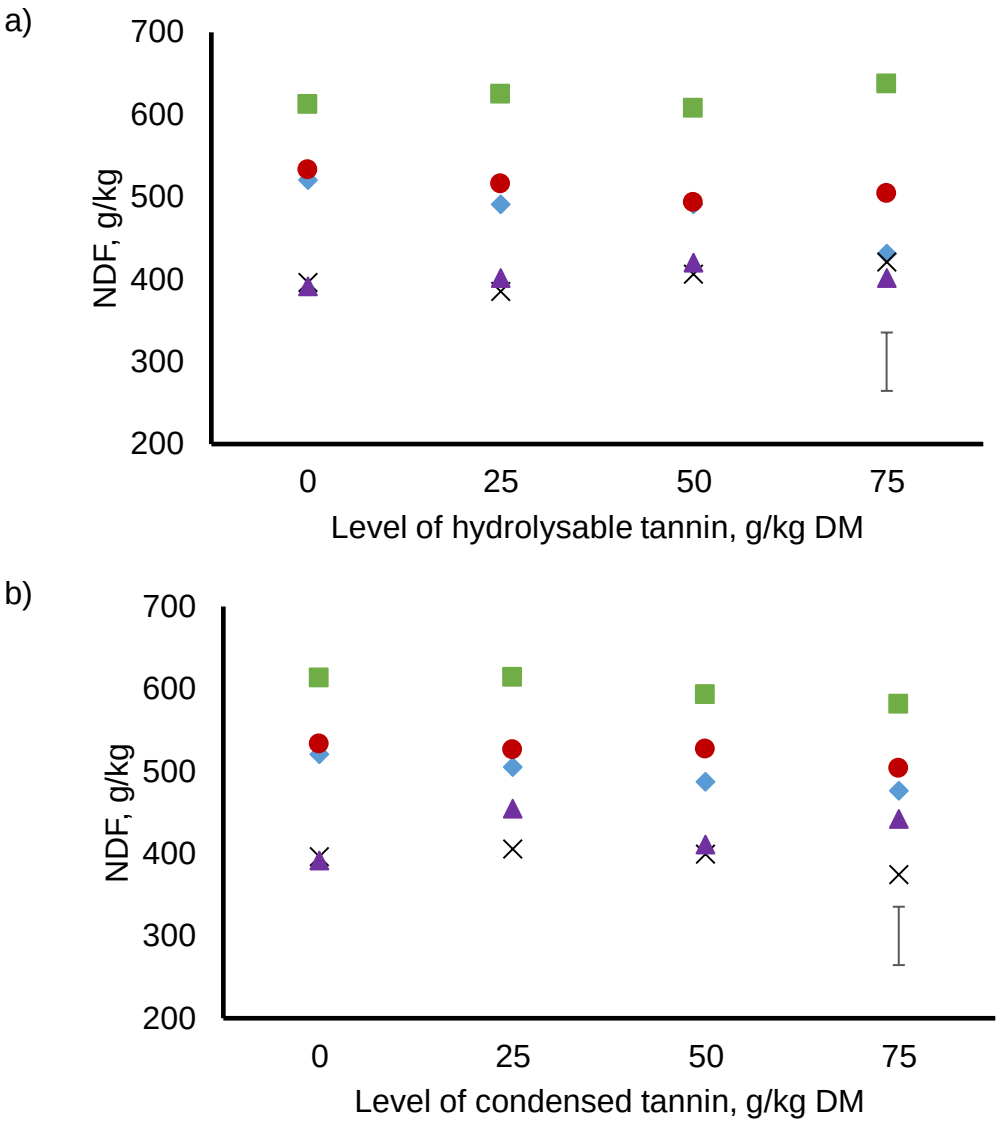


Figure 12 Neutral detergent fibre content of mini silos that contained grass silage (♦; n = 28), lucerne silage (●; n = 28), red clover silage (■; n = 28), red pea silage (×; n = 28) or white pea silage (▲; n = 28). Error bars indicate pooled s.e.d.

Silage pH was affected (P < 0.001) by silage type, with red pea silage having the highest pH of 6.13 while red clover had the lowest pH of 4.56 and grass, lucerne and white pea silage being intermediate (Table 14). Tannin type had an effect (P = 0.027) with condensed tannin having the highest at pH 5.31 while hydrolysable tannin had the lowest at pH 5.01 with no tannin being the intermediate at 5.25. There was no effect of the level of the tannin in the silage on pH. There was also no (P > 0.05) interaction between silage and tannin type; or tannin type and level of tannin; or silage type and level of tannin on pH (Figure 13).

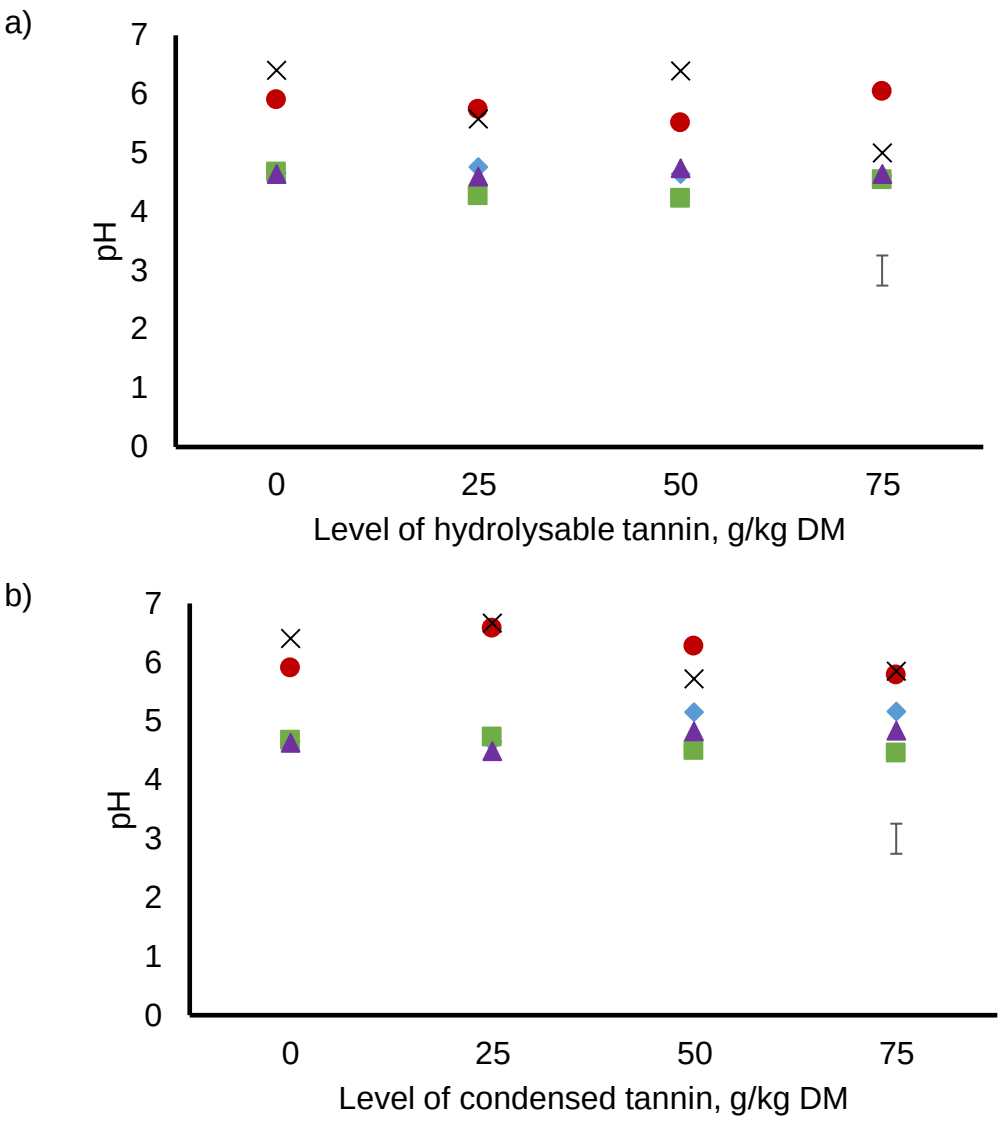


Figure 13 Forage pH of mini silos that contained grass silage (♦; n = 28), lucerne silage (●; n = 28), red clover silage (■; n = 28), red pea silage (×; n = 28) or white pea silage (▲; n = 28). Error bars indicate pooled s.e.d.

Ammonia nitrogen was affected ($P < 0.001$) by silage type, with red clover silage having the lowest content at 26.0 g/kg total N while lucerne silage was 151.8 g/kg total N higher at 177.8 g/kg total N, with grass, white pea and red pea silage being intermediate at 54.5, 74.3 and 123.7 g/kg total N, respectively. Tannin type and level had no effect ($P > 0.05$) on ammonia nitrogen content. There was no ($P > 0.05$) interaction between silage type and level of tannin on ammonia nitrogen (Figure 14).

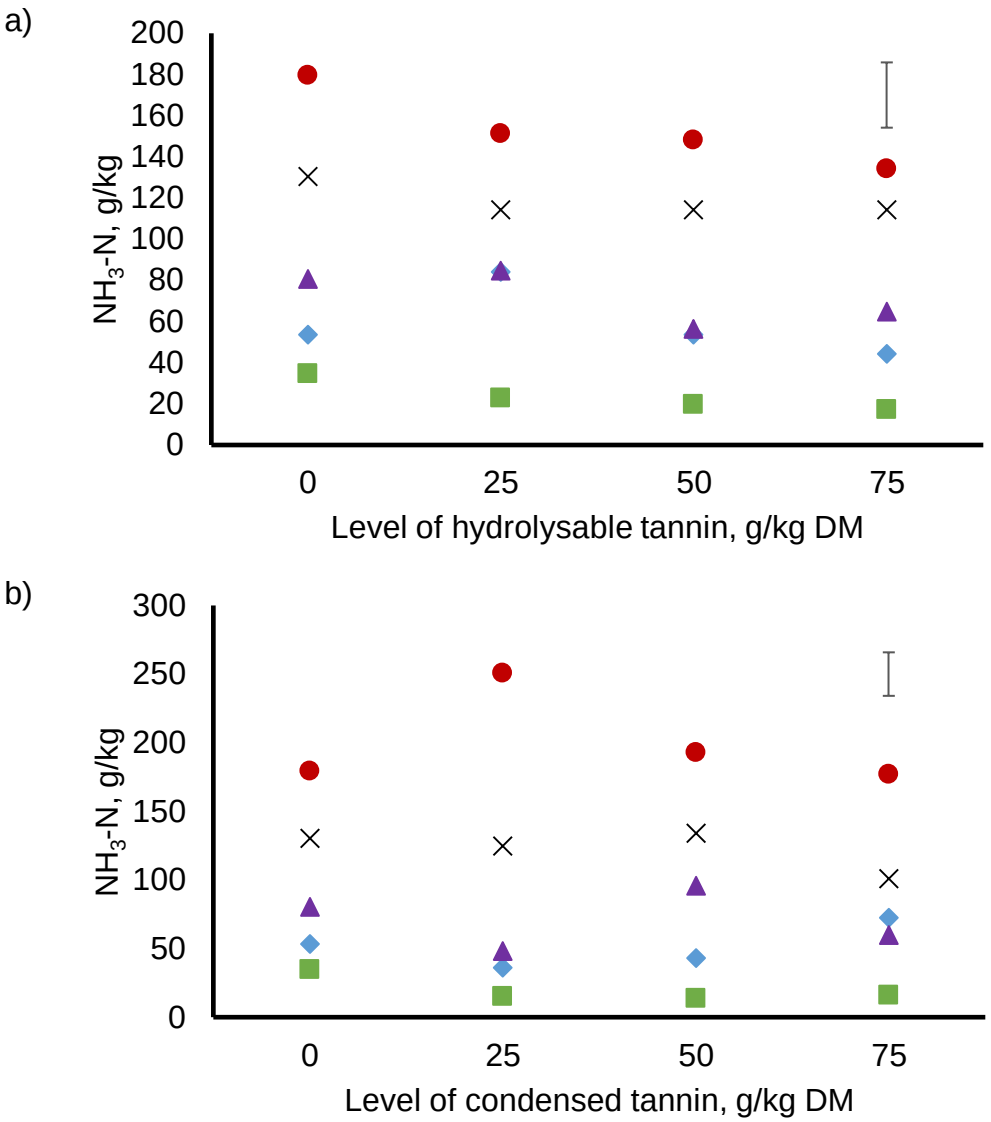


Figure 14 Ammonia nitrogen of mini silos that contained grass silage (♦; n = 28), lucerne silage (●; n = 28), red clover silage (■; n = 28), red pea silage (×; n = 28) or white pea silage (▲; n = 28). Error bars indicate pooled s.e.d.

3.4 Discussion

This was the first study to investigate the addition of different inclusion rates of condensed and hydrolysable tannins at ensiling to grass, lucerne, red clover, red pea and white pea silages. The effects on the fermentation and nutritional value post ensiling were compared when tannins were added to the silage. The DM content of all the forages in the current study had a range between 281 – 387 g/kg, with red pea silage having the highest DM and lucerne silage the lowest. The DM content of the white pea silage was consistent with Sinclair *et al.* (2009), however, the red pea silage in the current study had a DM content that was 49 g/kg higher than Sinclair *et al.* (2009). In contrast, Hart *et al.* (2011) reported a lower and similar DM contents for both silages compared to the current study and Sinclair *et al.* (2009). Differences in DM content of pea silages may reflect the different species of pea silage used in the studies and the length of the wilting period. In both Sinclair *et al.* (2009) and Hart *et al.* (2011), the pea species Croma (white flowers) and Racer (coloured flowers) were used, whereas in the current study Montara (white flowers) and Daytona (coloured flowers) were used. Wilting period may have influenced the differences in DM content observed between the three studies, in the current study and Sinclair *et al*. (2009) both pea silages were wilted for 30 hours whereas in Hart *et al*. (2011) pea silages were wilted for 36 hours. Weather conditions at the time of wilting and harvesting may have influenced the DM content, however this was not recorded in the current study, or Sinclair *et al*., (2009) or Hart *et al*., (2011).

The grass and red clover silages had DM contents that were similar to Dewhurst *et al.* (2003). In the current study, lucerne had a DM content that was considerably lower than values reported in previous studies (Dewhurst *et al.,* 2003; Sinclair *et al.,* 2015; Aguerre *et al*., 2016; Broderick *et al*., 2017). Hoffman *et al.* (1997) used first cut lucerne silage and wilted for 36 hours and reported a similar DM content to the current study. This similarity of DM content between studies may reflect the cut of silage and wilting time. In the current study, lucerne was first cut with a wilting time of 24 hours whereas in the previous studies (Dewhurst *et al.,* 2003; Sinclair *et al.,* 2015; Broderick *et al*., 2017), the silages were a mixture of cuts (1st to 4th cut) and had a range of wilting times between 24 and 48 hours. Wilting time is important for the removal of water from the silage prior to ensiling to increase the DM content (Wilkinson, 2005).

Dry matter content of the silages used in the current study increased as the inclusion rate of tannin increased. Similarly, Deaville *et al.* (2010) observed an increase in DM content of grass silage when condensed and hydrolysable tannins were added at ensiling at a rate of 75 g/kg DM. These changes in DM content of silage may reflect the high DM content of the tannins. In contrast, Tabacco *et al.* (2006) reported no difference in DM content when hydrolysable tannins were added at varying inclusion rates from 0 to 60 g/kg DM to lucerne silage at ensiling. In the current study, grass and red clover silage DM was higher when condensed tannins were added, and lucerne and red pea silage DM was higher when hydrolysable tannins were added, whereas white pea silage was unaffected. In contrast, Deaville *et al.* (2010) observed no differences in DM content when condensed or hydrolysable tannin were added to grass silage at ensiling. The differences observed in the current study also may reflect the different DM contents of the silage before either type of tannin was added to the silage.

The CP content of the five forages used in the current study varied between 106 and 192 g/kg DM, with red clover silage having the lowest and white pea silage the highest CP content. The grass silage used in the current study had a particular high CP content compared to previous studies, (Dewhurst *et al*., 2003; Sinclair *et al*., 2015; Vanhatalo *et al*., 2009) who reported values of between 111 and 140 g/kg DM, which is approximately 79 g/kg DM lower than that observed by the current study. In the current study, the grass silage was predominantly ryegrass however it did contain some white clover which has a higher CP content (Dewhurst *et al.,* 2003) so could influence the CP content of the grass silage. In a study conducted by Dewhurst *et al.* (2003), it was found that the CP content of white clover silage ranged from 212 to 261 g/kg DM, whilst grass silage ranged from 139 to 140 g/kg DM showing the difference in CP content between these silages. When grass and white clover silage were mixed as a 50:50 (DM basis), the CP content was 183 g/kg DM showing how the high CP content of white clover silage influenced the overall CP content of the mixed silage (Dewhurst *et al*., 2003). Therefore, it can be concluded that the presence of white clover in the grass silage of the current study was influencing the overall CP content.

The CP content of the red clover silage used in the current study is lower than values reported in some previous studies (Halmemies-Beauchet-Filleau *et al*., 2014; Moorby *et al*., 2009; Hymes-Fecht *et al*., 2013). Values reported in previous

studies fell in a range between 181 and 200 g/kg DM, which is up to 94 g/kg DM higher than the CP content of the red clover silage used in the current study. Forage legumes tend to have a delicate leaf in comparison to grasses, therefore leaf shatter can occur if the forage becomes too dry during the wilting process and the leaves shatter during harvesting (Wilkinson, 2005). Leaf shatter in red clover can reduce the CP content of red clover silage (Wilkinson, 2005) and therefore, in the current study, the low CP content may be due to leaf shatter occurring. The high NDF content of the red clover silage may indicate the maturity of the plant, it was found by (Vanhatalo *et al*., 2009) that NDF content of red clover silage increased from 375 to 463 g.kg DM as the age of the plant increased. Therefore, it can be suggested that the red clover used in the current study was an older plant than previous studies. Lucerne silage in the current study had CP content values were comparable to previous studies by Sinclair *et al.* (2015) and Hoffman *et al.* (1997) whereas the pea silages had CP content that was consistent with Mustafa *et al.* (2002). In contrast, Sinclair *et al.* (2009) and Hart *et al.* (2011) reported higher CP values in the red pea silage than the white pea silages which was in contrast to the current study.

There was no significant effect of tannin type or rate of inclusion on CP content of the silages in the current study. Similarly, Tabacco *et al.* (2006) reported no effect of increasing hydrolysable tannin in lucerne silage on total N content. Deaville *et al.* (2010) also reported no change in N content when grass was supplemented with either condensed or hydrolysable tannins at 75 g/kg DM. In the silo, condensed and hydrolysable tannins have the ability to bind with proteins to form a tannin-protein complex, this binding occurs when pH is between pH 4.5 – 7.0 (Salawu *et* al., 1999). The tannins provide protection of the protein from the extensive proteolysis that occurs in forage legumes during the ensiling process. Due to the lack of change in CP content with the addition of tannins to the silages at ensiling, in the current study, this would suggest that the tannins did not influence proteolysis in the silages. It can be concluded from these studies and the current study that tannins contain little N, and therefore the inclusion of either condensed or hydrolysable tannin does not significantly alter the CP content of silages.

There was an effect of silage source on ash content with red pea silage having the highest value at 233 g/kg DM, and red clover silage the lowest at 74.4 g/kg DM.

Ash content of lucerne silage was comparable to Sinclair *et al.* (2015), whilst the grass silage used in the current study was higher by 35 g/kg DM. Halmemies-Beauchet-Filleau *et al.* (2014) and Broderick *et al.* (2000) reported similar ash contents of red clover silage with a mean value of 105 g/kg DM, which was 30.6 g/kg DM higher than the current study. The ash content of both pea silages were higher than values reported by Mustafa *et al.* (2002), and may reflect the different varieties used between studies. In the current study, Montara (white flowers) and Daytona (coloured flowers) were used whereas Mustafa *et al.* (2002) used three varieties, Lenca, Carneval and Delta. The inclusion of tannin increased the ash content between 0 and 25 g/kg DM, however there was a decrease in ash content from 25 to 75 g/kg DM of tannin inclusion. Ash content was not significantly affected by the type of tannin, which was consistent with the observations by Deaville *et al.* (2010) when condensed or hydrolysable tannin was added to lucerne silage at a rate of 75 g/kg DM.

Silage source had an effect on the NDF content in the current study, with values ranging from 397 to 610 g/kg DM. The NDF content of the pea silages were higher than that reported by Hart *et al.* (2011) and Sinclair *et al.* (2009). Different varieties of plant depend on different structures to remain upright, with the varieties used in the current study using the stem and therefore having a higher fibre level than pea varieties used by Hart *et al.* (2011) and Sinclair *et al.* (2009) that relied upon the tendrils to become tangled (Hart *et al*., 2011). However, the NDF values observed in the current study are comparable to Fraser *et al.* (2001). The fibre content of the grass silage used was similar to previous studies (Vanhatalo *et al*., 2009; Dewhurst *et al*., 2003), whereas the fibre levels of lucerne and red clover silage were higher in the current study than previous studies (Broderick *et al*., 2000; Broderick *et al*., 2001; Hymes-Fecht *et al*., 2013). Tannin type and rate of inclusion had no significant effect on the fibre content of the silages.

The pH of the five silages used in the current study ranged between pH 4.5 and pH 6.1, with red clover, white pea and grass silages having a pH below 5 which would suggest that these silages were well fermented and preserved. However, lucerne and red pea silage had a pH above pH 5 which would suggest these silages had poorer fermentation therefore poor preservation. However, pH was not measured at the start of ensiling or over the course of ensiling so it is unclear when the silages reached these pH levels or how quickly the decline in pH

occurred. In contrast, Hart *et al.* (2011) and Sinclair *et al.* (2009) observed pH similar values for white pea and red pea silages which were in the range of pH 3.8 to 4.1. Mustafa *et al.* (2002) concluded that the optimal pH range for successful prevention of proteolysis in pea silage was between pH 4.5 and pH 5.9, and in the current study, white pea silage fell within the range at pH 4.6, however the red pea silage was slightly outwith the range at pH 6.1. Red clover silage pH values were comparable with Hoffman *et al.* (1997) and Broderick *et al.* (2000) indicating the silage was well preserved. In contrast, the lucerne silage in the current study was higher than that observed by previous studies (Hoffman *et al*., 1997; Broderick *et al*., 2000; Sinclair *et al*., 2015) potentially indicating poorer fermentation occurred in the present study. Grass silage in the current study had a slightly higher pH than that reported in previous studies (Vanhatalo *et al*., 2009; Dewhurst *et al*., 2003; Moorby *et al*., 2009), however the pH was below pH 5 therefore indicating that successful prevention of proteolysis had occurred and the silage was well preserved.

In the current study, forage pH was affected by the type of tannin, with silage that had been supplemented with condensed tannin having a higher pH than silage supplemented with hydrolysable tannin. In contrast, Deaville *et al.* (2010) reported no difference in forage pH when condensed or hydrolysable tannin was added to grass silage at ensiling. The difference observed in pH between condensed and hydrolysable tannin may reflect the different sources of silage used in the current study. Forage pH was not affected by inclusion rate of tannins in the current study, in contrast Tabacco *et al.* (2006) observed a decrease in pH when hydrolysable tannin supplemented lucerne silage at 20 g/kg DM. In silage that contained 10 g/kg DM tannin, lactic acid content was higher and acetic acid content was lower than the other inclusion rates. However, when hydrolysable tannin was included at 0, 40 and 60 g/kg DM there was no difference in the lucerne pH in the study of Tabacco *et al.* (2006).

Ammonia nitrogen content varied between the five silages and ranged from 26.0 to 177.8 g/kg TN, with red clover silage having the lowest and lucerne silage the highest. Both Hart *et al.* (2011) and Sinclair *et al.* (2009) reported ammonia nitrogen values that were higher by 22 and 62 g/kg TN for red pea and white pea silage, respectively, compared to the current study. Similar to the current study, Broderick *et al.* (2000) and Hymes-Fecht *et al.* (2013) reported that red clover

silage had a lower ammonia nitrogen content than lucerne silage. However, in the current study, there was a 151 g/kg TN difference between red clover and lucerne silage whereas, in the previous studies (Broderick *et al*., 2000; Hymes-Fecht *et al*., 2013) there was a smaller difference of 22.1 g/kg TN. These lower values for red clover silage may be due to the presence of PPO which binds with the protein, protecting the protein for proteolysis. For grass silage, the ammonia nitrogen content in the current study was comparable but slightly lower by 10 g/kg TN to Vanhatalo *et al.* (2009). Further measurements that could be conducted on the silages to assess quality and preservation would be fermentation acids, lactic, acetic, propionic and butyric acids. Hymes-Fecht *et al*. (2013) observed higher levels of lactic acid in red clover silage at 7.27 g/kg DM compared with 5.83 g/kg DM in lucerne silage, whereas acetic acid was higher in lucerne silage by 0.89 g/kg DM. Hymes-Fecht *et al*. (2013) suggested that lactic acid is important for influencing the final pH of silage and therefore quality, whilst acetic acid influences the aerobic stability of the silage. Therefore, further work is required to investigate the quality and preservation of these forage legumes with the determination of fermentation acids.

Tannin type and rate of inclusion of tannin supplemented to the silages had no significant effect on the ammonia nitrogen content. Similarly, Deaville *et al.* (2010) reported no change in ammonia nitrogen when condensed and hydrolysable tannins were added to grass silage at a rate of 75 g/kg DM. In contrast, Tabacco *et al.* (2006) reported a decrease in ammonia nitrogen as hydrolysable tannin increased from 0 to 60 g/kg DM in lucerne silage. Variety of lucerne may have influenced the difference in ammonia nitrogen, with Daisy used in the current study and Equipe used in Tabacco *et al.* (2006). The extent of proteolysis that occurs in the silo influences the NPN levels which will impact the ammonia nitrogen levels, and different varieties undergo varying amounts of proteolysis. The results from the current study would suggest that neither the condensed or hydrolysable tannins at the varying levels were binding with the protein in the forage to form tannin-protein complexes, therefore there was no influence on the proteolysis within the silo.

3.5 Conclusions

The results in the present study show that forage source influences chemical composition following ensiling. The inclusion of either condensed or hydrolysable tannin did not influence DM, CP, ash, NDF or ammonia nitrogen content of the silage. However silages supplemented with condensed tannins had a higher forage pH than silages supplemented with hydrolysable tannins. Dry matter content of the silages increased with the inclusion rate of tannin from 0 to 75 g/kg DM while ash content increased from 0 to 25 g/kg DM, but then decreased as the inclusion of tannin increased. Crude protein, NDF, pH and ammonia nitrogen were unaffected by the inclusion rate of tannin. Therefore, the use of tannins as an additive at ensiling may not improve the utilisation of N from home grown forage legumes as the tannins did not provide protection to the protein so the amount of RUP available in the forages will not have been improved.

Chapter 4 – Experiment 2: Effect of forage pea silages on performance of high yielding dairy cows

4.1 Background

Within forage legumes, peas (*Pisium sativum*) are of particular interest due to their high protein content, with values of between 170 to 220 g/kg DM being reported (Mustafa *et al*., 2000; Hart *et al*., 2011). In Experiment 1, the forage pea silages indicated high crude protein values of 162 and 192 g/kg DM for red pea and white pea silage, respectively. Forage legumes can complement the lower protein levels found in cereal based forages such as maize or whole-crop wheat silages (Sinclair *et al*., 2009). Wheat has a high degradability of protein and starch within the rumen and legumes are thought to be able to complement wheat by improving N retention and milk performance of cows (Salawu *et al*., 2002b). Adesogan *et al*. (2004) found that the inclusion of a pea-wheat intercrop increased the levels of odd chain fatty acids in milk of dairy cows, indicating improvement of microbial synthesis within the rumen. In a study conducted by Salawu *et al*. (2002b), polyunsaturated fatty acids in the milk of dairy cows were lower when fed a pea-wheat intercrop silage compared to grass silage, however the effect of a pure pea silage on milk fatty acid composition is unknown.

Protein in forage legumes, like forage peas, is rapidly degraded within the rumen, resulting in poor utilisation and low rumen undegradable (by-pass) protein available in the small intestine (Sinclair *et al*., 2009). Wastage of nitrogen by the dairy cow can be reduced by improving the efficiency of protein utilisation particularly by reducing rumen degradability (Bach *et al*., 2005). Additionally, high yielding dairy cows have a greater requirement for rumen undegradable protein to meet their requirements than lower yielding animals (McDonald *et al*., 2011).

Tannins are defined as any phenolic compound of high molecular weight containing reactive phenolic hydroxyl or carboxyl groups that enable them to complex with protein, minerals and other macromolecules (Reed, 1995). Tannins can be classified as either condensed or hydrolysable with each being found in different plants (Scalbert, 1991). Protein from forages bind to tannins to form a complex; these complexes are pH dependent (Aerts *et al*., 1999) and in ruminants this binding occurs in the rumen at pH 4.5 – 7.0, normal rumen pH is 6.5 – 7.0

(McMahon *et al*., 2000). These tannin-protein complexes can enable the protection of the protein from microbial attack, and dissociate at pH 2 – 3 found in the abomasum allowing the protein to reach the small intestine where absorption occurs (Fraser *et al*., 2001). In some cases, if the protein is covalently bound to the condensed tannin, this complex may not separate and will pass out in the faeces reducing the degradability of the protein (McMahon *et al*., 2000). It is therefore possible to alter protein degradability, in the rumen by adding tannins to the forage crop either at ensiling or feed-out. Condensed tannins that are found naturally within the vacuoles and epidermis of the leaves in certain legumes, such as forage peas, offer a practical and easy way of reducing the rumen degradability of protein found in legumes.

Previous studies that have investigated the effect of condensed tannins on animal performance have focussed on growing lambs or lower yielding dairy cows in late lactation (Hart *et* al., 2011; Sinclair *et al.,* 2009). In contrast, higher yielding dairy cows have a greater requirement of by-pass protein (Thomas, 2002) and therefore the benefits of condensed tannins in forage peas may be more apparent.
Following on from Experiment 1, the red and white pea silages containing naturally occurring condensed tannins and high CP content were used to investigate the effects of condensed tannins in high yielding dairy cows. The study objectives were to determine the effect of the inclusion of forage peas differing in their tannin content in the diet of high yielding dairy cows on performance, diet digestibility, milk fatty acid content and N efficiency (conversion of feed N to milk N).

4.2 Materials and Methods

Ethical approval for the current study was granted by Harper Adams University Ethics Committee, and all procedures involving animals were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

4.2.1 Animals

Fifteen multiparous Holstein-Friesian and three Brown Swiss cross dairy cows that were in early lactation 100 ± 23 days in milk, yielding 44 ± 2.3 kg of milk per day, weighing 638 ± 30.4 kg and an average body condition score of 2.88 ± 0.16 were used in a 3x3 Latin square design with 3 periods, each of 4 weeks duration. Cows were blocked and allocated to one of three treatments according to breed, and milk yield in the week prior to the study. The three Brown Swiss cross dairy cows were allocated first to ensure each treatment had one Brown Swiss cross dairy cow. Following that allocation, the Holstein-Friesian dairy cows were allocated by milk yield. The experiment began on 9th February 2015 and cows remained on study for 12 weeks.

4.2.2 Forages

In spring 2014, two 1 ha plots of forage peas silages were established. The field was ploughed, sub-soiled and harrowed prior to drilling. Two varieties of pea, Daytona, red flower and Montara, white flower, were sown at approximately 240 kg/ha in accordance with the breeder's specification in mid-April using a pneumatic seed drill. Daytona is a high tannin coloured pea variety while Montara is a low tannin white pea variety (Wang *et al*., 1998). Both pea crops received cyanazine and pendimethalin herbicides at pre-emergence. Post emergence, a mixture of cyanazine and MCPA and MCPB herbicides were applied along with the insecticide cypermethrin.

Both pea silages were mown at approximately 10:00 h on the 3rd July 2014 when the crop had reached growth stage 206 when pods are swollen with seeds (Knott, 1987). A front mounted drum mower without a conditioner was used and a stubble of 10 cm was left. The peas were wilted for approximately 30 hours prior to being picked up by a precision chop, self-propelled forage harvester and ensiled in a roofed, concrete clamp with an additive (Axcool Gold, Biotal Ltd, Pontprenna, Cardiff; 4 l/tonne; Axcool Gold is an inoculant containing the bacteria *L. buchneri*

40788). Clamps were filled quickly, rolled well and sealed with 2 separate sheets. Both clamps were weighed down with large straw bales. The grass silage was a second cut ley of predominantly rye grass and white clover, wilted for approximately 24 hours and harvested on 25th June 2014 using a precision chop, self-propelled forage harvester, and ensiled in a roofed, concrete clamp with additive (Axcool Gold, Biotal Ltd, Pontprenna, Cardiff; 4 l/tonne). The maize silage was harvested using a precision chop, self-propelled forage harvester in October 2014 at approximately 300 g DM/kg and was ensiled in a concrete clamp without an additive.

4.2.3 Diets

Cows were allocated to one of three dietary treatments for each of the 3 periods of 4 weeks duration. Each four week period included 21 days of adaption and 7 days of sampling. Dietary treatments were composed of 55:45 forage to concentrates (DM basis) and the forage proportions varied on a DM basis as follows:

Control (C): 34:66 grass to maize silage

White pea (W): 34:66 white pea to maize silage

Red pea (R): 34:66 red pea to maize silage

All diets had maize silage included to represent the diets fed to commercial UK dairy cow herds. All diets were formulated to be isonitrogenous and isoenergetic and to produce approximately 40 kg of milk per day according to Thomas, (2004). All diets were fed as a total mixed ration (TMR; Table 15), with the forages and concentrates mixed using a Hi-Spec MixMax 10 diet forage mixer (Hi-Spec Engineering Ltd, County Carlow, Ireland) calibrated to ±1 kg. The diets were fed through Insentec roughage intake feeders (RIC feeders, Insentec B.V., Marknesse, The Netherlands) calibrated to ±0.1 kg (Sinclair *et al*., 2007). Cows were identified by a transponder collar and an automatic animal identification fitted to the feeders. Each diet was offered at the rate of 1.05 of *ad libitum* daily intake at 0800 h, with refusals collected three times a week.

Table 15 Diet composition (g/kg DM) for control (C) diet based on grass and maize silage, white (W) diet (white pea and maize silage), or red (R) diet (red pea and maize silage).

	C	W	R
Maize silage	370	373	372
Grass silage	183	---	---
White pea silage	---	177	---
Red pea silage	---	---	179
Soya hulls	63	67	67
Rapeseed meal	75.7	76.5	76.2
Wheat distillers grain	75.7	76.5	76.2
Palm kernel	22.6	22.9	22.7
Soya bean meal	39	25	24
Wheat	72	77.5	77.5
Sugar beet pulp	72	77.5	77.5
Protected fat	20	20	20
Urea	---	1	1
Mins/vits[1]	6	6	6
Predicted composition			
Forage: concentration (kg/kg DM)	0.55	0.55	0.55
Metabolisable energy (MJ/kg DM)	12.5	12.4	12.4
MPE (g/kg DM)[2]	108	108*	112*
MPN (g/kg DM)[3]	116	116	118

[1]Minerals/vitamins premix (KW Feeds, Market Drayton, Shropshire). Major minerals: (per kg/DM) calcium iodate anhydrous, 625 ppm; sodium selenite, 66 ppm. Trace minerals: coated granule cobalt, 154 ppm; copper sulphate pentahydrate, 3000 ppm; manganese oxide, 6452 ppm; zinc oxide, 4167 ppm; zinc chelate of glycine hydrate, 11,538 ppm. Vitamins: (per kg/DM): Vitamin A, 1,000,000 IU; Vitamin D3, 300,000 IU; Vitamin E, 3,000 IU; Vitamin B_{12}, 2,500 mcg; Biotin, 135 mg.

[2]MPE: metabolisable protein-rumen energy limited

[3]MPN: metabolisable protein-rumen nitrogen limited

*predicted using the degradability co-efficients of Sinclair *et al*., (2009).

The sugar beet pulp/wheat mix and soya bean meal were purchased from S & C Feeds Ltd (Stone, Staffordshire). The protein mix and soya hulls were purchased from KW Feeds (Market Drayton, Shropshire). Megalac (protected fat) was purchased from Volac (KW Feeds, Market Drayton, Shropshire) and urea prills were purchased from YARA Rumisan (KW Feeds, Market Drayton, Shropshire).

4.2.4 Housing

All cows were housed in an area containing Super Comfort foam mat cubicles and cubicles were bedded with sawdust twice weekly, limed weekly and scraped automatically six times daily. All cows had continual access to water and were milked twice daily at approximately 0600 and 1600 h in a 40 point internal rotary parlour.

4.2.5 Experimental Routine

Forage samples were collected once a week; a sub-sample was oven dried at 105°C and the ratio of pea to maize silage and grass to maize silage adjusted according to produce the desired ratio. During the final week of each period, samples of each TMR and forage were collected daily, and stored at -20°C for subsequent analysis.

Milk yield was recorded twice daily during the final week of each period with samples collected on four occasions (twice in the morning and twice in the afternoon) for analysis of fat, protein and lactose. An additional two samples (once in the morning and once in the afternoon) were collected for milk FA analysis.

Twelve cows were randomly allocated to blood sampling, including the three Brown Swiss cross dairy cows. Blood samples were collected from 12 cows (4 per treatment) from the jugular vein via venepuncture into vacutainer tubes containing EDTA and fluoride/potassium oxalate. Samples were collected over two days in the final week of each period at 07:00, 09:00, 11:00 and 13:00 h, placed on ice immediately prior to separation of the plasma via centrifuging at 1390 *g* for 10 minutes at 4°C. Plasma was stored at -20°C until further analysis.

During the final week of each period, faecal grab samples were collected 5 consecutive days at 08:00 and 14:00 h to determine whole tract digestibility. Samples were frozen at -20°C prior to bulking and subsequent analysis. All cows were body condition scored using the method by Ferguson *et al* (1994) and weighed at 10:30 h in the week prior to the start of the study, and on the final day of the final week. All cows were transitioned onto the next diet the following day after the final day of sampling week.

4.2.6 Chemical analysis

Forage and TMR samples during the sampling week were bulked between days for each period. Sub-samples were analysed for DM (943.01), crude protein (990.03), and ash (942.05) according to AOAC (2012) as described in chapter 2. The NDF content was determined according to Van Soest *et al*. (1991) as described in section 2.4. Crude protein was determined using a Leco FP-528 (Leco Corporation, St Joseph, MI). Sub-samples of TMR were analysed for acid insoluble ash (Van Keulan and Young, 1977) as described in section 2.13. Forage sub-samples were analysed for pH. Forage pH was determined using a method by MAFF (1986) where water extract of the silage was determined using a Jenway 3505 pH meter (Bibby Scientific Limited, Staffordshire, UK) as described in section 2.5. Tannin content of the pea forages were determined according to Makkar *et al*. (1993) as described in section 2.8. Forage and TMR FAs were determined according to Jenkins, (2010) as described in section 2.7. Forage samples were sent to Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) for determination of fermentation acids, lactic, acetic, propionic and butyric acids.

Milk samples were analysed for fat, protein and lactose on a Milkoscan minor spectrophotometer (Foss Ltd., Denmark) calibrated by the methods of AOAC, (2012). Milk FA extraction was conducted according to Feng *et al*. (2004), methylation of milk FA was conducted according to Chouinard *et al*. (1999) as described in section 2.11. The FAME in hexane were analysed using a Hewlett-Packard 6890 gas chromatograph (GC) fitted with a 100m CP Sil 88 column as described by Lock *et al*. (2006).

Blood samples were analysed for total protein (TP), urea, beta-hydroxybutryrate (BHB), glucose, albumin and non-esterified fatty acids (NEFAs). Blood samples were analysed on a Cobas-Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK). Kits used for analysis were TP, Ref TP245; UREA, Ref UR221; RANBUT, Ref RB1008; GLUC-HK, Ref GU611; ALBUMIN, Ref AB362; and NEFA, Ref FA115.

Faecal samples were bulked between days and sampling times (AM and PM) for each period and analysed for DM, OM and acid insoluble ash (Van Keulan and Young, 1977). Nitrogen and protein content were determined using a Leco FP-528

(Leco Corporation, St Joseph, MI) according to AOAC (2012) (990.03) and NDF was determined according to Van Soest *et al*. (1991).

The *in situ* residues of the forages were analysed for DM and CP as described in Chapter 2 materials and methods, sections 2.1 and 2.2, respectively. Crude protein being determined using a Leco FP-528 (Leco Corporation, St Joseph, MI).

4.2.7 Statistical analysis

All data were analysed within GenStat Release 18 (VSN International Ltd). Data for milk yield, body weight, body condition score and faecal parameters were evaluated by ANOVA as a Latin square design. Data for blood metabolites were analysed using repeated measures ANOVA. In all cases, data were checked for normality and homogeneity of variance by visual assessment of the residual plots. No adjustments were required. Data are presented as means with an s.e.d, with post hoc analysis using Tukey's test at a 5% level of significance.

The kinetics of rumen DM and CP degradability were calculated from Ørskov and McDonald (1979) as:

$$p = a + b\ (1 - e^{-ct})$$

where p = potential degradability at time t, a = soluble fraction, b = potentially rumen degradable fraction, c = degradation rate of fraction b per hour and t = incubation time (h). Effective degradability was calculated from Ørskov and McDonald, (1979) using the following equation:

$$ED = a + (b*c)/(c+k)$$

where k = rumen outflow rate of 0.08 h^{-1}. Data were analysed using GenStat 18 (VSN International Ltd, Oxford, UK).

4.3 Results

4.3.1 Feed Analysis

The DM content of all four silages was above 300 g/kg, with white pea silage having the lowest DM while the red pea silage was 56 g/kg higher, with grass and maize silages being intermediate (Table 16). For both crude protein and ash content, the maize silage had the lowest and red pea silage had the highest content, while grass and white silage had intermediate values. Grass silage had the highest pH, while maize silage was 0.46 units lower. The mean NDF content was 432 g/kg DM, and was highest in grass silage and lowest in red pea silage. Tannin content was highest in white pea silage whilst red pea silage had a lower content. White pea and red pea silages had the highest ammonia nitrogen content with a mean value of 67.5 g/kg TN, and the grass and maize silages had the lowest ammonia nitrogen content with a mean value of 42.1 g/kg TN.

Total fatty acid content of the silages was highest in grass silage and lowest in maize silage while white pea and red pea silages had intermediate values that had a mean value of 16.2 g/kg DM. White pea silage had the lowest content of C16:0 fatty acid while grass silage had a content 1.21 g/kg DM higher, with maize and red pea silage being intermediates with a mean value of 2.10 g/kg DM. In contrast, C18:0 was highest in red pea silage, lowest in grass and maize silage with a mean value of 0.27 g/kg DM while white pea silage had an intermediate value. Maize silage had the highest content of fatty acid C18:1 *c*9, while white pea silage had the lowest content, with red pea and grass silages being intermediates with a mean value of 0.53 g/kg DM. Maize silage had the highest content of C18:2 *c*9 *c*12, while white pea silage had the lowest content, with red pea and grass silage being intermediates with a mean value of 1.86 g/kg DM. In contrast, red pea and white pea silage had the same amount of C18:3 *c*9 *c*12 *c*15, while grass silage was higher and maize silage was lower.

Lactic acid content was highest in red pea silage, maize silage had the lowest lactic acid content whilst grass and white pea silage were intermediates with a lactic acid content of a mean value of 75.1 g/kg. Maize silage had the lowest acetic acid content, and white pea silage had the highest content. Grass and red pea silage had intermediate acetic acid contents. Grass and white pea silage had intermediate values for propionic acid content, while maize silage had the highest

propionic acid content and red pea silage had the lowest content. Butyric acid was highest in maize, white pea and red pea silage with a mean value of 0.2 g/kg while grass silage had a lower content.

In the TMR, the diet containing the red pea silage (R) had the highest dry matter content with the diet containing white pea silage (W) had the lowest dry matter content (Table 17). The mean crude protein content was 165 g/kg DM, and was highest in the diet containing grass silage (C), and lowest in the W diet. Both the control and white pea diet had the same ash content, while red pea diet was 5.4 g/kg DM higher. The NDF content was highest in the C diet and lowest in the W diet with the R diet being the intermediate. Total fatty acid content was highest in the C diet and lowest in the R diet with the W diet being the intermediate. The C diet had the highest amount of C16:0, while the W diet had the lowest and the R diet had an intermediate value. Similarly, the C18:0 content was lowest in the W diet and highest in the C diet, while the R diet had an intermediate value. The C18:1 *c*9 content was lowest in the W diet and highest in the C diet with the R diet being the intermediate value. The C diet had the highest content of C18:2 *c*9 *c*12 and the W diet had the lowest content, with the R diet being the intermediate value. Similarly, the W diet had the lowest amount of C18:3 *c*9 *c*12 *c*15 and the C diet had the highest, while the R diet was intermediate.

Table 16 Chemical composition (g/kg DM) of forages for the diets that contained (forage DM basis) 36:64 grass : maize (C); 36:64 white pea : maize silage (W); and 36:64 red pea : maize silage (R).

	Forages				
	Maize silage	Grass silage	White pea silage	Red pea silage	s.e.d
n	3	3	3	3	
DM, g/kg	365	376	333	389	7.55
CP	82.5	185	195	208	10.4
Ash	38.6	117	117	129	10.9
NDF	429	499	417	386	8.85
pH	4.17	4.63	4.34	4.53	0.078
NH_3-N (g/kg TN)	42.2	42.0	67.8	67.2	4.21
Fermentation acids (g/kg)					
Lactic acid	2.4	51.8	98.5	102	--
Acetic acid	1.7	17.1	44.1	33.5	--
Propionic acid	0.3	0.2	0.2	0.1	--
Butyric acid	0.2	0.1	0.2	0.2	--
Fatty acids (g/kg DM)					
C16:0	2.15	3.04	1.83	2.05	0.19
C18:0	0.28	0.25	0.37	0.43	0.03
C18:1 *c*9	2.12	0.73	0.28	0.33	0.25
18:2 *c*9 *c*12	3.20	2.10	1.34	1.62	0.30
18:3 *c*9 *c*12 *c*15	0.47	5.09	1.76	1.76	0.52
Total fatty acids	11.6	21.6	15.5	16.9	1.30
Condensed tannin content (g/kg DM)	--	--	67.2	58.6	2.74

Table 17 Chemical composition (g/kg DM) of diets that contained (forage DM basis) 36:64 grass : maize (C); 36:64 white pea : maize silage (W); and 36:64 red pea : maize silage (R).

	Dietary Treatments			
	C	W	R	s.e.d
n	3	3	3	
DM, g/kg	451	444	464	3.48
CP	169	164	162	2.64
Ash	74.5	74.5	79.9	9.45
NDF	458	426	431	5.03
Fatty acids (g/kg DM)				
C16:0	10.2	7.01	7.41	0.93
C18:0	0.84	0.69	0.76	0.06
C18:1 *c*9	5.54	3.99	4.20	0.39
18:2 *c*9 *c*12	4.71	3.56	4.03	0.20
18:3 *c*9 *c*12 *c*15	0.94	0.44	0.48	0.09
Total fatty acids	32.0	24.4	23.9	2.22

4.3.2 Dry matter degradation characteristics

The soluble DM fraction (a) was lowest (P<0.001) in the red pea silage while grass and white pea silage had a similar soluble fraction (Table 18 and Figure 15).

Table 18 Mean *in situ* DM degradability co-efficient (%) of grass (G), white pea (WP) and red pea (RP) silages.

	G	WP	RP	s.e.d	P value
n	3	3	3		
a	36.9^{b}	34.4^{b}	18.6^{a}	0.921	<0.001
b	46.4^{b}	36.4^{a}	47.8^{b}	0.739	<0.001
a+b	83.4^{c}	70.9^{b}	66.4^{a}	0.523	<0.001
c	0.0617^{a}	0.109^{b}	0.126^{b}	0.0048	<0.001
ED	62.4^{c}	56.3^{a}	59.2^{b}	0.898	0.001

^{a,b,c} Means within a row with a different superscript differ (P < 0.05)
Where a = soluble fraction, b = potentially rumen degradable fraction,
c = degradation rate of fraction b per hour and ED = effective degradability

In contrast, grass and red pea silage had a similar potential degradable DM fraction (b) with white pea silage being lower (P<0.05). Grass silage had the

highest (P<0.05) extent of degradation (a+b) with red pea silage the lowest and white pea silage being the intermediate. Red pea silage had the fastest (P<0.001) rate of degradation (c) while grass silage had the slowest rate and white pea being intermediate. Effective degradability (ED) was highest (P = 0.001) in grass silage and lowest in white pea silage.

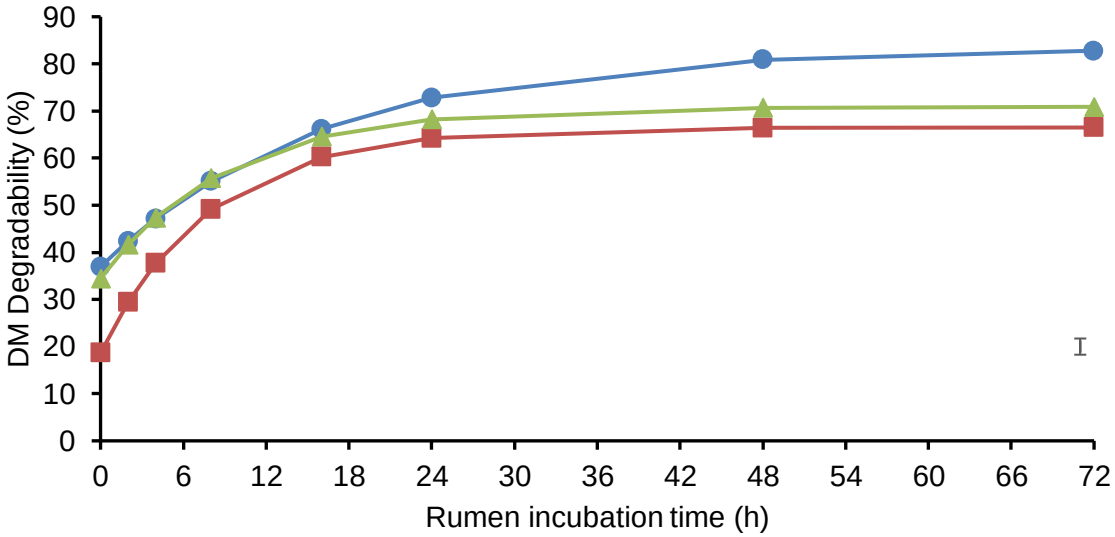


Figure 15 *In situ* DM degradation of grass (G ●; n = 24), white pea (WP ▲; n = 24) and red pea (RP ■; n = 24) silages. Error bar indicates pooled s.e.d.

4.3.3 Crude protein degradation characteristics

The soluble CP fraction (a) was similar for grass and white pea silage with red pea silage being lower (Table 19 and Figure 16).

Table 19 Mean *in situ* CP degradability co-efficients (%) of grass (C), white pea (WP) and red pea (RP) silages.

	C	WP	RP	s.e.d	P value
n	3	3	3		
a	51.9^{b}	51.0^{b}	39.9^{a}	0.773	<0.001
b	32.8^{ab}	30.2^{a}	39.7^{b}	1.06	<0.001
a+b	84.7^{b}	81.2^{ab}	79.7^{a}	0.650	<0.001
c	0.140^{a}	0.509^{b}	0.386^{ab}	0.113	0.044
ED	72.7^{a}	76.8^{b}	72.6^{a}	0.866	0.005

^{a,b} Means within a row with a different superscript differ (P < 0.05)
Where a = soluble fraction, b = potentially rumen degradable fraction,
c = degradation rate of fraction b per hour and ED = effective degradability

The potentially degradable CP fraction (b) was highest (P < 0.001) in red pea silage whereas grass silage and white pea were lower and similar. Red and white pea silages had a similar extent of degradation (a+b) however grass silage had a higher value (P < 0.001). Grass silage had the fastest (P < 0.05) rate of degradation (c) while white pea silage had the slowest rate, with the red pea silage being intermediate. Effective degradability (ED) was highest (P < 0.05) in white pea silage while grass and red pea silage were lower with a similar ED.

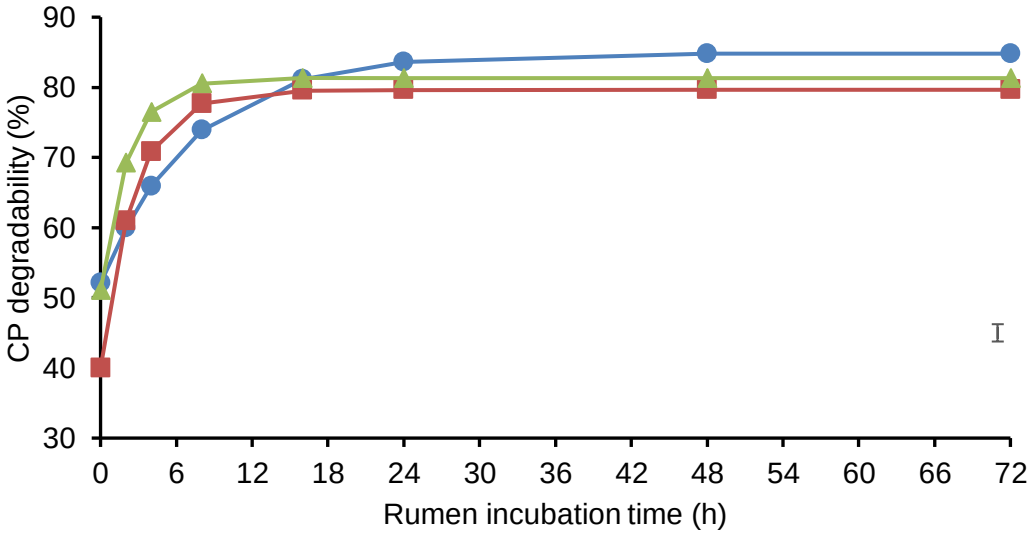


Figure 16 *In situ* CP degradation of grass (G ●; n = 24), white pea (WP ▲; n = 24) and red pea (RP ■; n = 24) silages. Error bar indicates pooled s.e.d.

4.3.4 Animal performance

Total DM intake averaged 20.8 kg/day and was lower (P = 0.002) in cows fed W than C, with R being intermediate (Table 20). Milk yield averaged 39.0 kg/day and was highest (P = 0.005) in cows when fed the C diet than W or R diets, which did not differ. There were no dietary effects (P > 0.05) on milk fat content which averaged 38.3 g/kg, but diet had an effect (P = 0.045) on milk protein content which was highest in cows fed C diet and lowest in cows fed R diet. Milk lactose content averaged 45.4 g/kg and was lowest (P = 0.026) in cows when fed C diet. Mean liveweight was 642 kg and was higher in the C diet (P = 0.026) than W diet, with R diet being intermediate. In contrast, dietary treatment had no effect (P > 0.05) on body condition score or live weight change which averaged 2.87 and 0.28 kg/day respectively. Diet had a minor effect on N efficiency which was slightly lower in cows fed W diet and higher in cows fed C and R diets.

Table 20 Milk performance, live weight and body condition score of dairy cows fed diets that contained (forage DM basis) 36:64 grass : maize (C); 36:64 white pea : maize silage (W); and 36:64 red pea : maize silage (R).

	C	W	R	s.e.d	P value
n	18	18	18		
DM intake, kg/d	21.4[b]	19.9[a]	20.7[ab]	0.37	0.002
Milk yield, kg/d	40.6[b]	38.4[a]	38.3[a]	0.75	0.005
Milk fat, g/kg	37.5	39.6	37.9	0.12	0.165
Milk protein, g/kg	30.8[b]	30.2[ab]	30.1[a]	2.64	0.045
Milk lactose, g/kg	45.2[a]	45.5[ab]	45.6[b]	1.64	0.026
Live weight, kg	647[b]	637[a]	643[ab]	3.33	0.026
Body Condition	2.90	2.86	2.86	0.06	0.731
Live weight change[1], kg/d	0.53	0.06	0.24	0.205	0.088

[1] Over 28-d period.
[a,b] Means within a row with different superscript differ (P < 0.05).

4.3.5 Milk fatty acid concentrations

Diet had no effect (P > 0.05) on milk FA C4:0 – C18:0; C18:1 *n*-9, C18:1 *t*-10, C18:1 *t*-12, *c*-9, *t*-11 conjugated linoleic acid (CLA), *t*-10, *c*-12 CLA, or C20:0 (Table 21). In contrast, C16:0 was higher (P = 0.006) in cows when fed W diet whereas C17:0 was highest in cows when fed either W or R diets. Cows fed C diet had the highest proportion of C18:2 *n*-6 (P < 0.001) and C20:3 *n*-3 (P = 0.049) whilst C18:3 *n*-3 was higher (P = 0.001) in cows when fed R diet whilst cows fed C and W diets had a similar content. Diet had no effect on FA with chain lengths of less than or greater than C16. Saturated, MUFA and PUFA fatty acids were also not affected by diet (P > 0.05). Fatty acids of chain C16:0 and C16:1 were higher (P = 0.005) in cows when receiving the diet containing white pea silage.

Table 21 Milk fatty acid profile of dairy cows fed diets that contained (forage DM basis) 40:60 grass to maize silage (C); 40:60 white pea silage to maize silage (W) and 40:60 red pea silage to maize silage (R).

Item	C	W	R	s.e.d	P value
n	18	18	18		
Fatty acid (g/100g)					
4:0	2.19	2.23	2.22	0.050	0.673
6:0	1.58	1.61	1.56	0.033	0.325
8:0	1.01	1.00	0.98	0.024	0.325
10:0	2.34	2.29	2.21	0.069	0.188
12:0	2.84	2.73	2.67	0.076	0.089
14:0	9.64	9.56	9.34	0.163	0.173
14:1	0.96	0.94	0.87	0.050	0.260
15:0	0.84	0.85	0.83	0.027	0.747
16:0	28.8[a]	30.4[b]	29.1[a]	0.474	0.006
16:1	0.43	0.41	0.36	0.030	0.142
17:0	0.43[a]	0.47[b]	0.47[b]	0.009	<0.001
18:0	7.77	7.70	7.74	0.209	0.940
18:1*n*-9	22.1	21.4	21.7	0.460	0.320
18:2*n*-6	0.52[b]	0.40[a]	0.41[a]	0.021	<0.001
18:3*n*-3	0.12[ab]	0.12[a]	0.13[b]	0.025	0.001
*c*9, *t*11 CLA	0.75	0.73	0.79	0.032	0.235
*t*10, *c*12 CLA	0.069	0.067	0.069	0.002	0.504
20:0	0.11	0.10	0.11	0.000	0.210
20:5*n*-3	0.07[b]	0.04[a]	0.06[ab]	0.012	0.049
Summation					
<C16	21.4	21.2	20.7	0.380	0.171
C16 + C16:1	29.3[a]	30.9[b]	29.6[a]	0.470	0.005
>C16	39.7	38.4	39.3	0.803	0.187
SFA	57.8	59.1	57.4	0.818	0.114
MUFA	28.6	27.3	27.9	0.644	0.140
PUFA	4.18	3.99	4.19	0.104	0.115

[a,b] Means within a row with different superscript differ (P < 0.05).
SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid

4.3.6 Blood metabolites

Mean total protein, albumin, glucose, BHB and NEFAs, were not affected by diet (P > 0.05) averaging 70.2 g/l, 36.3 g/l, 3.04 mmol/l, 0.587 mmol/l and 0.170 mmol/l, respectively (Table 22). Mean plasma urea concentration was lower (P < 0.001) in cows receiving C diet than W or R diets which did not differ.

Table 22 Mean blood plasma metabolites for dairy cows fed diets; grass and maize silage (C), white pea and maize silage (W), or red pea and maize silage (R).

	C	W	R	s.e.d	P value
n	144	144	144		
Total protein, g/l	70.48	70.54	69.67	2.262	0.912
Albumin, g/l	36.50	36.27	36.21	0.803	0.931
Glucose, mmol/l	3.064	3.114	2.944	0.179	0.623
Urea, mmol/l	4.10[a]	5.34[b]	5.43[b]	0.301	<0.001
BHB[1], mmol/l	0.558	0.603	0.601	0.069	0.769
NEFA[2], mmol/l	0.171	0.193	0.146	0.038	0.484

[1] β-hydroxy butyrate
[2] Non-Esterified Fatty Acids
[a,b] Means within a row with different superscript differ (P < 0.05).

Diet had no effect (P > 0.05) on the mean concentration of total protein at any of the time points (Table 23) and averaged 69.8, 69.9, 71.8 and 69.4 g/L for 07:00, 09:00, 11:00 and 13:00 hours, respectively (Figure 17).

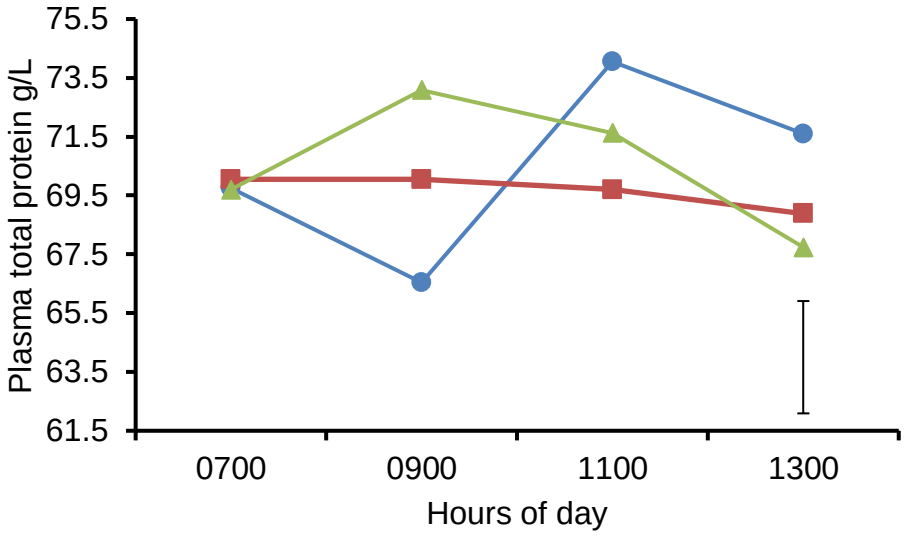


Figure 17 Plasma total protein in dairy cows fed diets that contained grass silage (C ●; n = 12); white pea silage (W ▲; n = 12); or red pea silage (R ■; n = 12). Error bar indicates pooled s.e.d.

Table 23 Blood plasma metabolites over time for dairy cows fed diets containing grass (C), white pea (W) or red pea (R) silage.

		C	W	R				
n	Time	12	12	12	s.e.d	Treatment	Time	TxT
Total protein, g/l	0700	69.7	69.7	70.0	3.82	0.912	0.559	0.420
	0900	66.5	73.0	70.0				
	1100	74.0	71.6	69.7				
	1300	71.6	67.7	68.8				
Albumin, g/l	0700	36.6	37.1	36.8	1.51	0.931	0.533	0.894
	0900	36.1	36.1	35.1				
	1100	36.9	35.7	36.7				
	1300	36.2	36.2	36.0				
Glucose, mmol/l	0700	3.08	3.26	3.26	0.226	0.623	0.013	0.398
	0900	3.28	3.21	2.95				
	1100	3.01	2.98	2.77				
	1300	2.88	2.99	2.78				
Urea, mmol/l	0700	3.56	4.78	4.78	0.400	<0.001	<0.001	0.507
	0900	3.66	5.28	5.28				
	1100	4.60	5.38	5.38				
	1300	4.57	5.88	5.88				
BHB[1], mmol/l	0700	0.379	0.471	0.408	0.103	0.769	<0.001	0.628
	0900	0.325	0.471	0.471				
	1100	0.725	0.708	0.754				
	1300	0.804	0.763	0.771				
NEFA[2], mmol/l	0700	0.216	0.212	0.179	0.0487	0.484	<0.001	0.939
	0900	0.229	0.241	0.197				
	1100	0.109	0.155	0.100				
	1300	0.129	0.162	0.108				

[1] β-hydroxy butyrate
[2] Non-Esterified Fatty Acids

Plasma albumin concentrations at different time points were unaffected (P > 0.05) by diet and averaged 36.9, 35.8, 36.4 and 36.2 g/L for 07:00, 09:00, 11:00 and 13:00 hours respectively (Figure 18 and Table 23).

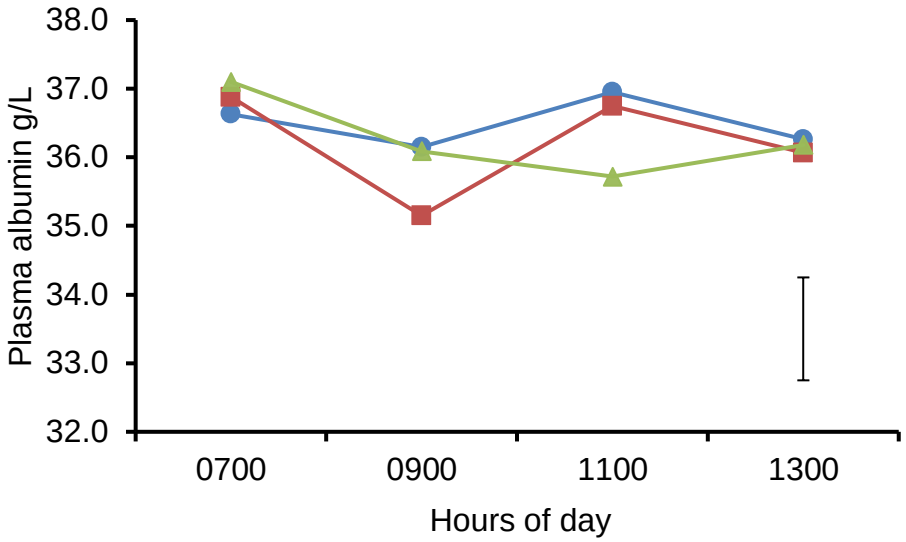


Figure 18 Plasma albumin in dairy cows fed diets that contained grass silage (C ●; n = 12); white pea silage (W ▲; n = 12); or red pea silage (R ■; n = 12). Error bar indicates pooled s.e.d.

The type of diet had no effect (P > 0.05) on plasma glucose levels, however over time there was a decrease (P = 0.013) in plasma glucose concentrations averaging 3.20, 3.15, 2.92 and 2.88 mmol/L for 07:00, 09:00, 11:00 and 13:00 hours respectively (Figure 19 and Table 23).

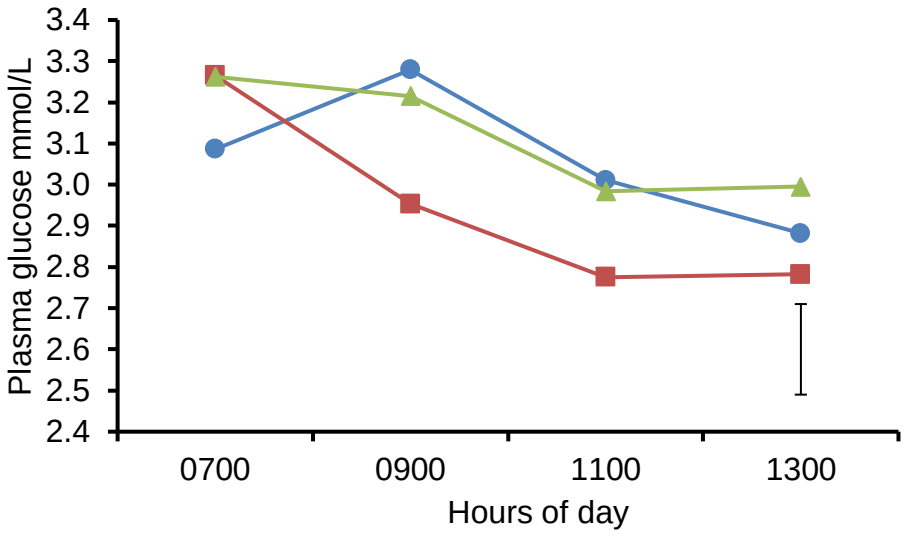


Figure 19 Plasma glucose in dairy cows fed diets that contained grass silage (C ●; n = 12); white pea silage (W ▲; n = 12); or red pea silage (R ■; n = 12). Error bar indicates pooled s.e.d.

Plasma urea concentrations increased post feeding (P < 0.001) with cows receiving W and R being consistently higher concentrations than those fed C at all time points which averaged 4.41, 4.68, 5.30 and 5.43 mmol/L for 07:00, 09:00, 11:00 and 13:00 hours respectively (Figure 20 and Table 23).

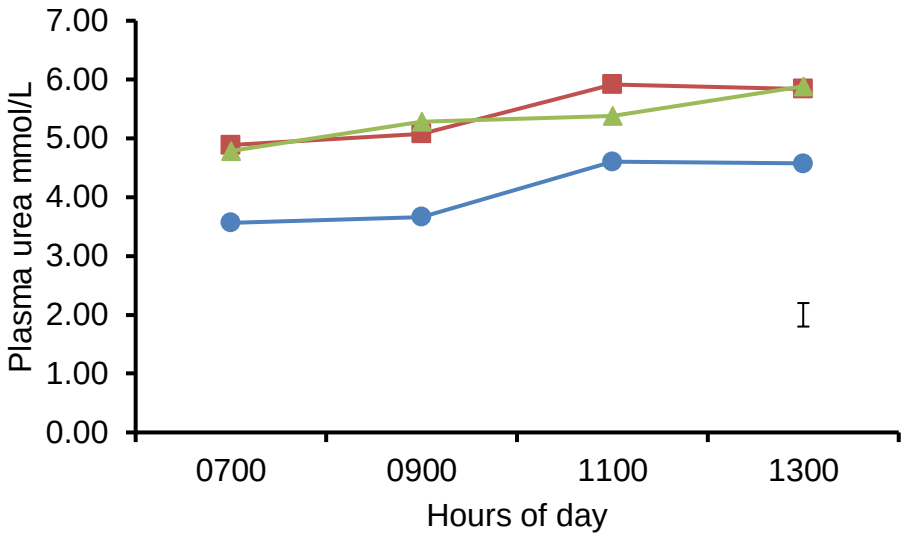


Figure 20 Plasma urea in dairy cows fed diets that contained grass silage (C ●; n = 12); white pea silage (W ▲; n =12); or red pea silage (R ■; n = 12). Error bar indicates pooled s.e.d.

Plasma BHB concentrations increased with time (P < 0.001) being lowest at 07:00 h increasing to 11:00 h before plateauing at 13:00 h (Figure 21 and Table 23).

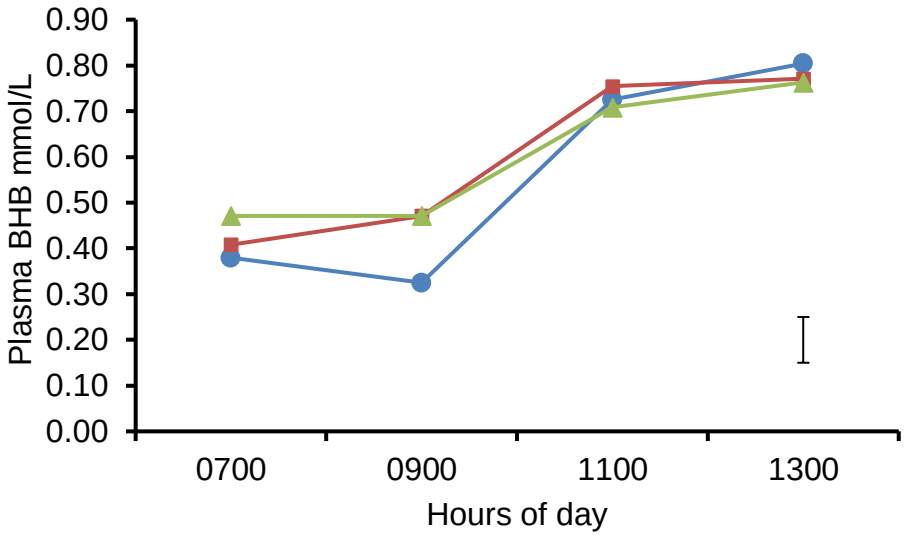


Figure 21 Plasma BHB in dairy cows fed diets that contained grass silage (C ●; n = 12); white pea silage (W ▲; n = 12); or red pea silage (R ■; n = 12). Error bar indicates pooled s.e.d.

Plasma NEFA concentrations increased post feeding before decreasing to 11:00 h prior to plateauing at 13:00 h (P < 0.001) and averaged 0.203, 0.223, 0.121 and 0.133 mmol/L for 07:00, 09:00, 11:00 and 13:00 hours respectively (Figure 22 and Table 23).

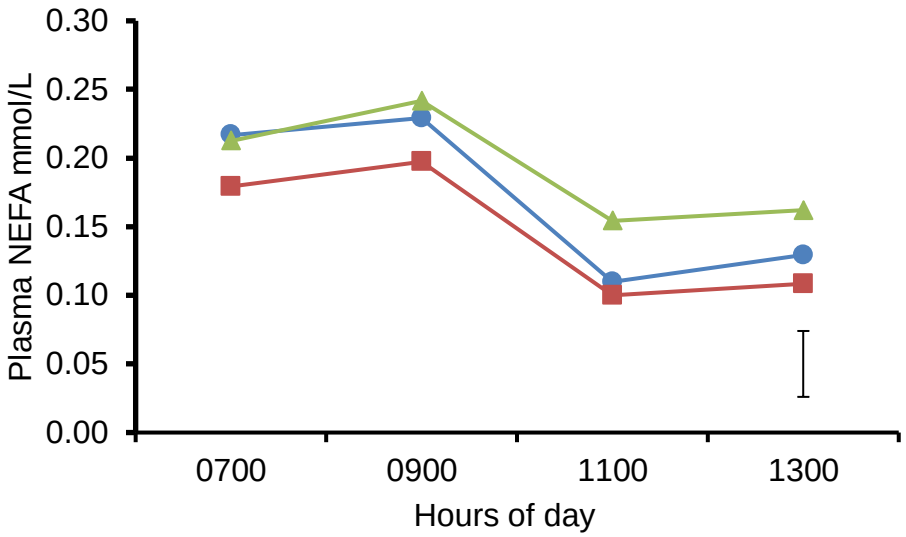


Figure 22 Plasma NEFA in dairy cows fed diets that contained grass silage (C ●; n = 12); white pea silage (W ▲; n = 12); or red pea silage (R ■; n = 12). Error bar indicates pooled s.e.d.

4.3.7 Whole tract digestibility

Dry matter intake was highest (P = 0.002) in cows when fed C diet and lowest in W diet with cows receiving R diet having an intermediate intake (Table 24). There was no dietary effect on faecal DM output and similarly, DM digestibility was unaffected (P > 0.05) by diet. Organic matter intake was lowest (P = 0.001) in cows when receiving W diet and highest when receiving C diet. There was no effect of diet (P > 0.05) on faecal OM digestibility or output. Nitrogen intake was affected (P = 0.001) by diet being 39 g/d higher in cows when fed C diet. Diet had no effect (P > 0.05) on N faecal output or digestibility. Intake of NDF was highest (P < 0.001) in cows fed C diet and lowest in cows receiving the W diet. In contrast, neither faecal NDF digestibility nor output was affected by diet (P > 0.05).

Table 24 Digestibility of DM, OM, and N in dairy cows fed grass silage (C); white pea silage (W) or red pea silage (R).

	C	W	R	s.e.d	P value
n	18	18	18		
Dry matter, kg/d					
Intake	21.4[b]	19.9[a]	20.7[ab]	0.360	0.004
Faecal output	7.11	6.47	6.64	0.471	0.390
Digestibility, kg/kg	0.665	0.679	0.678	0.022	0.776
Organic matter, kg/d					
Intake	19.8[b]	18.4[a]	19.1[ab]	0.332	0.001
Faecal output	6.31	5.65	5.78	0.423	0.269
Digestibility, kg/kg	0.679	0.696	0.697	0.021	0.655
Nitrogen, g/d					
Intake	587[b]	548[a]	560[a]	9.79	0.001
Faecal output	220	198	208	14.4	0.341
Digestibility, kg/kg	0.624	0.641	0.627	0.025	0.787
NDF, kg/d					
Intake	9.79[c]	8.48[a]	8.95[b]	0.160	<0.001
Faecal output	3.91	3.31	3.57	0.302	0.153
Digestibility, kg/kg	0.599	0.613	0.601	0.031	0.896
N efficiency (kg milk N/kg feed N)[1]	0.33[a]	0.32[a]	0.33[a]	0.0056	0.045

[a,b] Means within a row with different superscript differ (P < 0.05).

[1] Milk N = milk protein (g/day)/6.38

4.4 Discussion

4.4.1 Feed analysis

To the authors knowledge this is the first study to investigate the replacement of grass silage with white or red pea silage in maize silage based diets as dietary treatments to compare effects on dairy cow performance, milk quality and apparent digestibility in high yielding cows. The pea varieties in the current study, Daytona and Montara, were selected for their condensed tannin content based on their flower colour. Peas with coloured flowers contain high condensed tannin content while peas with white flowers contain low condensed tannin pea content (Wang *et al*., 1998). The peas were harvested, after a wilting period of approximately 30 hours, at growth stage 206 (Knott, 1987). Fraser *et al*. (2001) concluded that it was important that peas were harvested before lodging occurred in the crop, and that growth stage 206 gave the highest DM and CP yield. Both pea silages had a similar chemical composition; however the red pea silage had a higher DM content than the white pea silage. The DM content of the forage peas in the current study differed to a previous study by Sinclair *et al*. (2009) in which DM content was 32 g/kg higher in white pea silage which may reflect the different varieties of peas used. The CP content of the pea silages used in the current study were slightly higher than observed in Sinclair *et al*. (2009). Crude protein content of grass silage in the current study was considerably higher than reported for grass silages in the UK (Yan and Agnew, 2004). The difference in CP content of grass silage in the current study and Sinclair *et al*. (2015) may be attributed to the stage of harvest as the current study; the grass silage was harvested from a second cut ley of predominantly ryegrass compared to a first cut in Sinclair *et al*. (2015). A second possibility for the high crude protein content of the grass silage was the ley contained white clover which generally has a CP content of between 210 to 260 g/kg DM (Dewhurst *et al*., 2003), therefore increasing the overall CP content of the grass silage. In contrast, the CP content of the grass silage was comparable to Moorby *et al*. (2009), although it is unclear whether the ryegrass sward was first or second cut. Generally, all silages were well preserved and fermented with lactic acid content above 40 g/kg. The maize silage had a very low lactic acid content, although the acetic, propionic and butyric acid levels were all below 2 g/kg DM indicating that the silage was well preserved. Similarly, Sinclair *et al*. (2015) reported a low lactic acid content of 19 g/kg DM and concentrations of

less than 5 g/kg DM for the other fermentation acids. The tannin content of the forage peas in the current study differed to previous studies by Hart *et al*. (2011) and Sinclair *et al*. (2009) in which tannin content was 45.4 g/kg DM higher in red pea silage, this may reflect the different varieties of peas. However, these differences may reflect the sensitivity of the method as there was an intra-assay CV of 7.1% indicating a small presence of variation between the samples. Therefore, the forage pea silages in the current study, had similar levels of condensed tannins which does not adhere to the expected tannin levels present with different flower colours.

4.4.2 Degradation characteristics

Dietary treatment had an effect on the soluble DM and CP fraction (a) where red pea silage had lower content than grass and white pea silage. In contrast, Mustafa *et al.* (2000) reported that Grande pea silage had a higher soluble DM fraction than lucerne and barley silage however the soluble CP fraction was similar for all three silages. The potential (b) degradable CP fraction was highest in red pea silage whereas rate of degradation was faster in grass silage. In contrast, Sinclair *et al*. (2009) observed a higher potential degradable N fraction in grass silage with red pea silage having the lowest content. Sinclair *et al*. (2009) concluded that protein degradability in the rumen was reduced by the presence of condensed tannins that occur naturally within forage pea silage. Mustafa *et al*. (2000) reported that pea silage had a similar potential degradable CP fraction to lucerne silage but lower than barley silage. However, rate of degradation was similar for pea, lucerne and barley silage suggesting that protein in pea silage is degraded at the same rate as lucerne and barley silage in the rumen. Although, in the current study, protein was degraded the quickest in grass silage, therefore suggesting that the condensed tannins in the pea silage slowed the rate of protein degradation in the rumen. Effective CP degradability, in the current study, was highest in white pea silage which was in agreement with Sinclair *et al*. (2009).

4.4.3 Animal performance

Neither the white nor red pea silage TMR improved DM intake of mid-lactation cows, with cows fed the grass silage having the highest DM intake. All silages were fed as a mixture with maize silage and concentrates. In previous studies, (Adesogan *et al*., 2004; Salawu *et al*., 2002b), pea-wheat silages have been reported to improve DM intake compared to feeding grass silage as the only silage

in a TMR diet. Similarly, Mustafa *et al*. (2000), reported an improvement in DM intake of cows when pea silage replaced barley silage in a TMR based diet. The difference in intake reported in the current study may be attributed to the differences seen in the CP content of the silages. Broderick, (2003) and Sinclair *et al*. (2015), reported that when dietary protein was increased that there was a similar improvement in DM intake.

The increase in DM intake in cows when fed the grass silage was reflected in milk yield. Cows fed white or red pea silage had a similar yield, however these were lower than cows when fed the grass silage. In previous studies (Salawu *et al*., 2002b; Adesogan *et al*., 2004; Sinclair *et al*., 2009) milk yield was lower than in the current study which may reflect stage of lactation with days in milk averaging 66, 77 and 194 days in milk, respectively. In contrast, Mustafa *et al*. (2000) reported similar milk yields to the current study and found no effect of pea silage on the milk yield of cows in early lactation.

There was no significant effect of dietary treatment on milk fat content whereas milk protein content was higher in dairy cows when fed grass silage. Two studies (Salawu *et al.*, 2002b; and Adesogan *et al.*, 2004) fed pea-wheat bi-crops to dairy cows, milk fat content was lower in cows fed pea-wheat bi-crops compared to cows fed grass silage in Salawu *et al*. (2002b) study whereas Adesogan *et al*. (2004) reported no effects on milk fat content. The differences observed in these two studies may be attributed to the higher starch concentration and DM digestibility reported by Adesogan *et al*. (2004) compared to Salawu *et al*. (2002b). The increase in milk protein observed in cows fed grass silage in the current study may reflect the higher CP content observed; however the CP content was similar to the white and red pea silage diets. This response may suggest that more metabolisable protein was reaching the small intestine for digestion in cows fed grass silage. Mustafa *et al*. (2000) observed a higher milk fat content but a lower milk protein content in cows fed pea silage and concentrates in a 50:50 forage:concentrate ratio. Unlike milk fat and protein content, milk lactose content was higher in cows when fed either of the pea silages compared to grass silage. The responses observed in milk composition in the current study, may have been different if the inclusion rate of pea silage had been higher. Nitrogen efficiency was slightly lower in cows fed white pea silage, although cows fed grass or red pea silage had the same N efficiency. Protein levels were balanced across all three

treatments to be isonitrogenous, therefore there should be the same amount of N available at intake to be converted to milk N. However, in the current study, cows fed grass silage had a higher DMI, therefore N intake was higher in cows fed grass silage. Sinclair *et al*. (2009) observed slightly higher N efficiency in cows fed red pea silage; however, these differences between the pea silages were small, similar to the current study. N intake was similar for both pea silages averaging 0.53 kg, although cows fed grass silage had a higher N efficiency than both pea silages but N intake was lower averaging 0.45 kg. From these studies, it can be concluded that N efficiency does not tend to be improved by the inclusion of white or red pea silage however N efficiency may be improved if the inclusion rate of pea silage in the diet was higher.

4.4.4 Milk FA composition and blood metabolites

Dietary treatment had no effect on the majority of the FAs, milk fat content in C16:0 was in higher concentrations in cows fed white pea silage, whereas C17:0 was at similar concentration in cows fed white or red pea silages compared to the grass silage. Odd chain fatty acids are normally derived from *de novo* synthesis in the mammary glands; however this response of C17:0 was comparable with Adesogan *et al*. (2004) who suggested that microbial synthesis within the rumen may have been improved by inclusion of pea-wheat bi-crop silage. Therefore, in the current study, the presence of pea silage in the diet may have increased microbial synthesis in the rumen. Although in the current study, FAs of chain length less than C16, indicating *de novo* synthesis in the mammary glands and more than C16, indicating uptake from the diet, were not affected by dietary treatment. This response would suggest that microbial synthesis was not increased in the rumen by the diet. The concentration of α-linolenic acid (C18:3 *n*-3) was highest in cows when fed the red pea silage. In contrast, Adesogan *et al*. (2004) found no effect of pea-wheat intercrop on milk concentration of α-linolenic acid. Cows fed the grass silage had a higher concentration of C18:2 *n*-6 than cows fed the white or red silages.

There was no effect of dietary treatment on blood metabolites in the current study except plasma urea, with cows fed white or red pea silage having higher levels which increased during the day. These increased urea values are comparable with Sinclair *et al*. (2009) and Salawu *et al*. (2002b) in which cows fed pea silage had higher urea values and indicate a greater rumen CP degradability. This would

suggest that more nitrogen is leaving the rumen as ammonia which will be taken up by the blood to be transported to the liver to be converted into urea prior to excretion via urine (Broderick and Albrecht, 1997; Bach *et al*., 2005). Plasma NEFA is an indicator of energy status of the dairy cow (Cozzi *et al*., 2011), and in the current study plasma NEFA levels decreased post feeding, however there was no effect of dietary treatment. It is known that plasma NEFA levels can change according to feeding times (Topps and Thompson, 1984) and therefore sampling time can have an effect on NEFA levels, which is why, cows in the current study were sampled at varying times from feeding. Plasma BHB levels in the current study were below 0.9 mmol/l indicating that the cows had a positive energy balance and were past their peak in milk production (Topps and Thompson, 1984).

4.4.5 Apparent whole-tract digestibility

Digestibility of DM, OM, NDF and N were not affected by dietary treatment in the current study. Dschaak *et al*. (2011) observed no differences in DM, OM and NDF digestibility when supplementing the diet of dairy cows with quebracho condensed tannin extract at a rate of 3% DM. In contrast, Adesogan *et al*. (2004) observed a higher digestibility of DM and OM in cows fed pea-wheat intercrop compared to those fed grass silage. Adesogan *et al*. (2004) suggested this increase in digestibility was due to a rapid degradation of peas within the rumen. In the current study, DM and OM digestibility of both pea silages was similar to the grass silage suggesting that the pea silages degraded at the same rate as the grass silage in the rumen. In intercrop silages, one crop will be harvested at the incorrect maturity stage due to the two crops maturing at different rates (Anil *et al*., 1998). In Adesogan *et al*. (2004) the crops were harvested at 14 weeks post sowing whereas in the present study, both whole crop peas were harvested at 12 weeks post sowing therefore the peas in Adesogan *et al*. (2004) study may have been harvested past the optimum maturity stage.

4.5 Conclusion

The results of the present study show that the total replacement of grass silage with white or red pea silage in a maize silage based TMR reduces dry matter intake, milk yield and had no effect on diet digestibility. The inclusion of white or red pea silage did not improve milk fat content or milk protein content compared to dairy cows fed grass silage. Plasma urea levels were increased by the inclusion of white and red pea silage. Nitrogen digestibility and intake were higher in dairy cows fed grass silage compared to dairy cows fed white or red pea silage. The soluble DM and CP fraction were lowest in red pea silage, and the potential CP degradable fraction was highest in red pea silage. The rate of degradation was fastest in grass silage and slowest in white pea silage. Effective degradability of protein was highest in white pea silage. Therefore, any commercial advantage from feeding forage peas to high yielding dairy cows will be based on savings in N from fertiliser or dietary protein.

Chapter 5 – Experiment 3: Effect of the addition of hydrolysable tannins to lucerne and red clover silages on the performance of high yielding dairy cows

5.1 Background

Similar to the forage peas used in Experiment 2, red clover and lucerne have a high protein content and have been used in previous studies as a source of protein for dairy cows. Studies in the United States comparing grass, lucerne, and red clover silages have reported that feeding lucerne silage increases DM intake, milk yield, milk fat and protein levels (Broderick *et al*. 2005), although relatively few studies have compared the forages directly. Broderick *et al*. (2000) compared lucerne and red clover silage as the sole forage fed with concentrates in a TMR for lactating dairy cows. It was observed that DM intake, milk yield and milk composition were all higher in cows that were fed lucerne silage. Although, feeding red clover silage improved body weight gain and apparent digestibility of DM, OM and NDF, N digestibility was higher in lucerne silage. However, Broderick *et al*. (2001) observed improved N efficiency and apparent digestibility of DM, OM and NDF when cows were fed red clover silage, although DM intake was the only performance factor to be increased by lucerne silage. Due to the lack of studies and varying results, it is therefore difficult to draw a conclusion about the direct effects of lucerne and red clover silage on the performance of high yielding dairy cows.

Hydrolysable tannins have the ability to bind to proteins in the silo at pH 4.5 – 7.0 and can reduce the extensive proteolysis of proteins that occurs (Salawu *et al*., 1999). Therefore, protecting the protein in the silage and improving the amount of RUP available to the ruminant at feed out (Salawu *et al*., 1999). In Experiment 1 (Chapter 3), the addition of hydrolysable tannin to lucerne and red clover silage at four inclusion levels had no influence on the crude protein content of silage. Therefore, it can be suggested from this experiment that the addition of hydrolysable tannins at ensiling to lucerne or red clover silage did not reduce the extent of proteolysis of protein during the ensiling process. Similar to condensed tannins, hydrolysable tannins will dissociate with the protein in the abomasum at pH 2.0 – 3.0 allowing the protein to be digested and absorbed across the small intestinal wall (Mueller-Harvey, 2006). Previous studies have investigated the

effects of inclusion of hydrolysable tannins in a dairy cow diet however have yielded inconsistent results. Dschaak *et al*. (2011) reported a lower DM intake but no effect on milk yield when hydrolysable tannins were included in the diet of dairy cows at 30 g/kg DM. Similarly, Liu *et al*. (2013) observed no changes in DM intake or milk yield when hydrolysable tannin was included at 10 g/kg DM in a postpartum diet of a dairy cow. Previous studies that have investigated the effect of supplementation of condensed or hydrolysable tannins on animal performance have focussed on tannins being added to the diet at feed out (Dschaak *et* al., 2011; Liu *et al.,* 2013). In Experiment 2 (Chapter 4), the presence of naturally occurring condensed tannins in forage peas reduced DMI and milk yields in comparison to cows fed grass silage. However, overall cow performance was similar when fed either white or red pea silage suggesting the presence of condensed tannins during the ensiling process had no effect on animal performance. A few studies have compared lucerne and red clover silage on animal performance (Broderick *et al*., 2000; Broderick *et al*., 2001) but there is little information on the effects of lucerne and red clover silage supplemented with hydrolysable tannins at ensiling on animal performance therefore the benefits of hydrolysable tannins may be more apparent. Therefore, the study objectives were to determine the effects of lucerne and red clover silages with the addition of tannins at ensiling in the diet of high yielding dairy cows on performance, N efficiency, milk fatty acid content and diet digestibility.

5.2 Materials and Methods

Ethical approval for the current study was granted by Harper Adams University Ethics Committee, and all procedures involving animals were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

5.2.1 Forages

Lucerne (*vr.* Daisy) was harvested using a forage wagon on 1st July 2015, wilted for 48 hours and ensiled in two roofed, concrete clamp with an additive (Axcool Gold, Biotal, Cardiff, UK; 4l/t) or in mini-silos. The red clover (*vr.* Corvus) was harvested using a forage wagon on 21st July 2015, wilted for 48 hours and ensiled in two roofed, concrete clamp with an additive (Axcool Gold. Biotal, Cardiff, UK; 4l/t) or in mini-silos. Chestnut hydrolysable tannins (*Castanea sativa mill;* Provit Sp. Zo. O., Poland) were added at ensiling to one clamp of lucerne and red clover silage at a rate of 25 g/kg DM. The rate of 25 g/kg DM was determined from a previous study conducted at Harper Adams University. It was observed that milk yield in Suffolk cross ewes was improved when hydrolysable tannin was added to lucerne silage at feed out at 25 g/kg DM compared to no supplementation (Taha, 2015). However, Taha, 2015 observed reduced milk yields when hydrolysable tannins were included at higher rates of 50 or 75 g/kg DM, but DM intake was unaffected by inclusion of tannin. Hydrolysable tannin was mixed with water as a 30% solution based on DM weight of each silage and added to the lucerne and red clover at ensiling. The same volume of water was added to the control clamps of lucerne and red clover forage. Either water or hydrolysable tannin solution were mixed with each forage in a Hi-Spec MixMax 10 forage mixer (Hi-Spec Engineering Ltd, County Carlow, Ireland) for 5 minutes. Following mixing, forages were added to their appropriate clamp. Maize (*vr.* Adept) was harvested in October 2015 using a self-propelled forage harvester at approximately 300 g/kg DM and ensiled in a concrete clamp without additive.

5.2.2 Experiment 3a – Ensiling characteristics

In Experiment 3a, mini silos were produced for the four forages described in section 5.2.1 to investigate the effect of the supplementation of hydrolysable tannins on DM, pH and NH_3-N during the ensiling process from day 1 to 120. For all forages, the silos were opened on days 1, 4, 7, 10, 48, 90 and 120 days for DM

and pH, whereas NH_3-N analysis was completed on all forages for days 1, 4, 7, 10, 48 and 120 days, day 90 was not analysed due to time constraints. Chemical composition (DM, CP, ash, NDF, pH & NH_3-N) of the four forages was investigated in mini silos opened on day 120.

5.2.2.1 Mini silo production

Twenty eight mini silos were made for each of the four forages, lucerne (LCS), lucerne plus tannin (LTS), red clover (RCS) and red clover plus tannin (RTS) silage and were conserved in pipes of length 33 cm and diameter 14 cm attached to a wooden base lined with two plastic bags. Approximately 1 kg of fresh silage was placed in plastic bags and compressed. Bags were then sealed with silage tape, sand placed on top and the mini silo sealed with silage tape. Mini silos were stored at ambient temperature and opened at 1, 4, 7, 10, 48, 90 and 120 days following harvest. For each time point, four mini silos of each forage were opened and each silo was analysed to ensure adequate replication. Following opening, the mini silo samples were stored at -20ºC until subsequent analysis.

5.2.2.2 Chemical analysis

Mini silos opened on day 120 were analysed for DM (943.01), CP (990.03), and ash (942.05) according to AOAC, (2012) as described in chapter 2 and also for NDF content according to Van Soest *et al*. (1991), with pH determined according to MAFF, (1986) using a Jenway 3505 pH meter (Bibby Scientific Limited, Staffordshire, UK). Ammonia – nitrogen's were determined on day 1, 4, 7, 48 and 120 silos using a method adapted from MAFF, (1986) using an auto-titrator (FOSS 1030 auto-titrator, FOSS, Warrington, UK and Buchi Labortechnik AG CH-9230, Flawil, Switzerland) as described in section 2.6. Dry matter content and pH were determined on the mini silo samples that were opened on days 1, 4, 7, 10, 48, 90 and 120 days post-harvest.

5.2.2.3 Statistical analysis

All data were analysed using GenStat Release 18 (VSN International Ltd). Data for the ensiling parameters were evaluated by analysis of variance as a 2x2 factorial design. Treatment degrees of freedom were split into main effects of forage (forage), tannin (Tannin) and their interaction (F x T). Data for DM, pH and NH_3-N of mini silos opened on day 1, 4, 7, 10, 48, 90 and 120 were analysed using

repeated measures model. Treatment degrees of freedom were split into main effects of treatment (Treatment), day (Day) and their interactions (Treatment, Treatment x Day). In all cases, data were checked for normality and homogeneity of variance and no adjustments were required. Data are presented as means and s.e.d of means.

5.2.3 Experiment 3b – Performance of dairy cows fed lucerne and red clover silages

In Experiment 3b, twelve high yielding dairy cows in early lactation were fed either lucerne, lucerne plus tannin, red clover or red clover plus tannin silage in a 4x4 Latin square design over 16 weeks. Animal performance, diet digestibility, milk fatty acid content and N efficiency was investigated.

5.2.3.1 Animals

Twelve multiparous Holstein-Friesian dairy cows, which were in mid lactation (88 ± 26.9 days in milk; mean ± standard error of difference), yielding 41 ± 3.7 kg of milk per day, weighing 634 ± 51.8 kg and body condition score of 2.9 ± 0.16 at the beginning of the study were used. Cows were blocked and allocated to one of four treatments according to milk yield and days in milk in the week prior to the study. The experiment began on 11th January 2016 and cows remained on study for 16 weeks.

5.2.3.2 Diets

Cows were allocated to one of four dietary treatments for each of four periods of four weeks. Each four week period included 21 days of adaptation and 7 days of sampling. Dietary treatments were composed of 55:45 forage to concentrates (DM basis) and the forage proportions varied on a DM basis as follows:

Lucerne control (LC):	36:64 lucerne to maize silage
Lucerne + tannin (LT):	36:64 lucerne plus tannins to maize silage
Red clover control (RC):	36:64 red clover to maize silage
Red clover + tannin (RT):	36:64 red clover plus tannins to maize silage

All diets were formulated to be isonitrogenous and isoenergetic to produce approximately 40 kg of milk per day according to Thomas, (2004). A Hi-Spec MixMax 10 diet forage mixer (Hi-Spec Engineering Ltd, County Carlow, Ireland) calibrated to ±1 kg, was used to mix the forages and concentrates which were fed as a total mixed ration (TMR; Table 25). The diets were fed in Insentec roughage intake feeders (RIC feeders, Insentec B.V., Marknesse, The Netherlands) calibrated to ±0.1 kg (Sinclair *et al*., 2007). Cows were identified by a transponder collar and an automatic animal identification fitted to the feeders. Each diet was offered at the rate of 1.05 of *ad libitum* daily at 0800 h, and refusals were collected three times a week. Following the final day of each sampling week, all cows were changed over onto their next diet for their next period. Diet change over did not involve a gradual change due to the diets being formulated to be isonitrogenous and isoenergetic with the only difference between the four diets being the type of experimental forage fed to the cows.

Table 25 Diet composition (g/kg DM) for lucerne control (LC) diet based on lucerne and maize silage, lucerne tannin (LT) diet based on lucerne with tannin and maize silage, red clover control (RC) diet based on red clover and maize silage, or red clover tannin (RT) diet based on red clover with tannin and maize silage.

	LC	LT	RC	RT
Maize silage	355	355	356	356
Lucerne silage	197	--	--	--
Lucerne silage + tannin	--	197	--	--
Red clover silage	--	--	198	--
Red clover silage + tannin	--	--	--	198
Protein blend				
Rapeseed meal	68	68	71	71
Molasses	6	6	6	6
Wheat distillers grain	68	68	71	71
Palm kernel	19	19	20	20
Wheat	68	68	60	60
Sugar beet pulp	68	68	60	60
Soya hulls	103	103	104	104
Megalac	15	15	15	15
Soya bean meal	28	28	32	32
Urea	--	--	2	2
Mins/Vits[1]	5	5	5	5
Predicted composition				
Forage:concentration (kg/kg DM)	0.55	0.55	0.55	0.55
Metabolisable energy (MJ/kg DM)	11.7	11.7	11.9	11.9
MPE (g/kg DM)[2]	102	102	101	101
MPN (g/kg DM)[3]	112	112	112	112

[1]Minerals/vitamins premix (KW Feeds, Market Drayton, Shropshire). Major minerals: (per kg/DM) calcium iodate anhydrous, 625 ppm; sodium selenite, 66 ppm. Trace minerals: coated granule cobalt, 154 ppm; copper sulphate pentahydrate, 3000 ppm; manganese oxide, 6452 ppm; zinc oxide, 4167 ppm; zinc chelate of glycine hydrate, 11,538 ppm. Vitamins: (per kg/DM): Vitamin A, 1,000,000 IU; Vitamin D3, 300,000 IU; Vitamin E, 3,000 IU; Vitamin B_{12}, 2,500 mcg; Biotin, 135 mg.

[2]MPE: metabolisable protein-rumen energy limited

[3]MPN: metabolisable protein-rumen nitrogen limited

The sugar beet pulp/wheat mix and soya bean meal were purchased from S & C Feeds Ltd (Stone, Staffordshire). The protein blend (rapeseed meal, molasses, wheat distillers grain and palm kernel) and soya hulls were purchased from KW Feeds (Market Drayton, Shropshire). Megalac was purchased from Volac (KW

Feeds, Market Drayton, Shropshire) and urea prills were purchased from YARA Rumisan (KW Feeds, Market Drayton, Shropshire).

5.2.3.3 Housing

Cows were housed in an area containing cubicles fitted with Super Comfort foam mats. Cubicles were bedded with sawdust twice weekly limed weekly. Automatic scrapers were used to scrape the cubicles on six occasions during the day. All cows had continual access to water.

5.2.3.4 Experimental routine

Forage samples were collected once a week; a sub-sample was oven dried at 105°C and the ratio of lucerne to maize silage and red clover to maize silage adjusted to produce the desired ratio. During the final week of each period, samples of each TMR and forage were collected daily, and stored at -20°C for subsequent analysis.

All cows were milked twice daily at approximately 0600 and 1600 h in a 40 point internal rotary parlour. Milk yield was recorded at each milking during the final week of each period. Milk samples were collected on four occasions during the final week of each period (twice in the morning and twice in the afternoon) for analysis of fat, protein and lactose. Milk samples were also collected for milk fatty acid analysis on two occasions (once in the morning and once in the afternoon).

Blood samples were collected from all 12 cows (3 per treatment) from the jugular vein via venepuncture into vacutainer tubes containing EDTA and fluoride/potassium oxalate. Samples were collected over two days in the final week of each period at 0700, 0900, 1100 and 1300 h, placed on ice immediately prior to separation of the plasma via centrifuging at 1390 *g* for 15 minutes at 4°C. Plasma was stored at -20°C until further analysis.

During the final week of each period, faecal grab samples were collected on 5 consecutive days at 0800 and 1400 h to determine whole tract digestibility. Samples were frozen at -20°C prior to bulking and subsequent analysis. All cows were body condition scored using the method by Ferguson *et al* (1994) and weighed in the week prior to the start of the study, and on the final day of each period at 1100 h.

5.2.3.5 Chemical analysis

Forage and TMR samples during the sampling week were bulked between days for each period. Sub-samples of the forages and TMR were analysed for DM (943.01), crude protein (990.03), and ash (942.05) according to AOAC, (2012) as described in chapter 2. Sub-samples of TMR were analysed for acid insoluble ash (Van Keulan and Young, 1977). Forage sub-samples were analysed for pH as described in section 2.5. Crude protein was determined using a Leco FP-528 (Leco Corporation, St Joseph, MI) and NDF content was determined according to van Soest *et al*. (1991). Forage pH was determined using a method by MAFF, (1986) where water extract of the silage was determined using a Jenway 3505 pH meter (Bibby Scientific Limited, Staffordshire, UK). Tannin content of the pea forages were determined according to Makkar *et al*. (1993) as described in section 2.8. Forage and TMR FAs were determined according to Jenkins, (2010) as described in section 2.7. Forage samples were sent to Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) for determination of fermentation acids, lactic, acetic, propionic and butyric acids.

Milk samples were analysed for fat, protein and lactose on a Milkoscan minor spectrophotometer (Foss Ltd., Denmark) calibrated by the methods of AOAC, (2012). Milk FA extraction was conducted according to Feng *et al*. (2004), with methylation of FA conducted according to Chouinard *et al*. (1999) as described in section 2.11. The FAME in hexane were analysed using a Hewlett-Packard 6890 gas chromatograph (GC) fitted with a 100m CP Sil 88 column as described by Lock *et al*. (2006).

Blood samples were analysed for total protein (TP), urea, beta-hydroxybutryrate (BHB), glucose, and albumin. Blood samples were analysed on a Cobas-Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK). Kits used for analysis were TP, Ref TP245; UREA, Ref UR221; RANBUT, Ref RB1008; GLUC-HK, Ref GU611; and ALBUMIN, Ref AB362.

Faecal samples were bulked between days and sampling times for each period. Samples were analysed for DM, OM, and acid insoluble ash (Van Keulan and Young, 1977). Nitrogen was determined using a Leco FP-528 (Leco Corporation, St Joseph, MI).

In situ dry matter and crude protein degradation of the lucerne and red clover silages were determined as described in section 2.14. Crude protein was determined using a Leco FP-528 (Leco Corporation, St Joseph, MI).

5.2.3.6 Statistical analysis

All data were analysed using GenStat Release 18 (VSN International Ltd). All data for milk yield parameters, body weight, body condition score and faecal parameters were evaluated by analysis of variance as a 2x2 factorial design. Treatment degrees of freedom were split into main effects of forage (Forage), tannin (Tannin) and their interaction (F x T). Data for blood metabolites were analysed using repeated measures model. Treatment degrees of freedom were split into main effects of diet (Treatment), time (Time) and their interactions (Treatment, Treatment x Time). In all cases, data were checked for normality and homogeneity of variance by assessment of the residual plots. No adjustments were required. Data are presented as means with an s.e.d, with post hoc analysis using Tukey's test at a 5% level of significance.

The kinetics of rumen DM and CP degradability were calculated from Ørskov and McDonald, (1979) as:

$$p = a + b\ (1 - e^{-ct})$$

where p = potential degradability at time t, a = soluble fraction, b = potentially rumen degradable fraction, c = degradation rate of fraction b per hour and t = incubation time (h). Effective degradability was calculated from Ørskov and McDonald, (1979) using the following equation:

$$ED = a + (b*c)/(c+k)$$

where k = rumen outflow rate of 0.08 h^{-1}. Data was analysed using GenStat 18 (VSN International Ltd, Oxford, UK).

5.3 Results

5.3.1 Experiment 3a – Ensiling characteristics

5.3.1.1 Fermentation profile from day 1 to 120

Addition of tannin had no effect (P > 0.05) on the fermentation profile of the mini silos from day 0 to 120 as seen in Table 26 and 27, although forage type had an effect (P < 0.05).

Table 26 Mean chemical composition of mini silos during ensiling containing lucerne silage (LCS), lucerne plus tannin silage (LTS), red clover silage (RCS) and red clover plus tannin silage (RTS).

	Treatment					P value		
	LCS	LTS	RCS	RTS	s.e.d	Forage	Tannin	FxT
n	28	28	28	28				
DM, g/kg	457	459	207	215	5.42	<0.001	0.162	0.382
pH	5.07	5.11	4.76	4.91	0.106	<0.001	0.215	0.465
NH_3-N, g/kg TN	16.9	14.2	28.9	26.3	0.141	0.004	0.626	0.479

Table 27 Dry matter, pH and NH_3-N from day 1 to 120 of the mini silos containing lucerne silage (LCS), lucerne plus tannin (LTS), red clover silage (RCS) and red clover plus tannin silage (RTS).

		Treatment					P value		
		LCS	LTS	RCS	RTS	s.e.d	Treatment	Day	TxD
n	Day	28	28	28	28				
DM, g/kg	1	457	467	219	226				
	4	437	466	216	226				
	7	469	449	206	212				
	10	468	456	218	215	13.4	0.579	0.015	0.243
	48	457	470	201	209				
	90	431	474	199	214				
	120	451	438	188	206				
pH	1	5.68	5.57	5.38	5.32				
	4	5.23	5.34	4.61	4.68				
	7	5.04	5.04	4.51	4.63				
	10	4.96	4.92	4.41	4.72	0.196	0.273	<0.001	0.765
	48	4.69	4.68	4.56	4.85				
	90	4.73	5.08	4.65	4.87				
	120	5.04	5.06	5.21	5.58				
NH_3-N, g/kg TN	1	3.26	4.22	13.3	12.4				
	4	8.38	5.65	18.0	17.2				
	7	8.28	7.86	20.2	21.3	4.45	0.982	<0.001	0.508
	48	31.1	18.6	34.6	29.2				
	120	33.5	34.8	58.3	51.2				

Dry matter content averaged 335 g/kg and was higher (P < 0.001) in lucerne silage. Dry matter content decreased (P < 0.001) with the mean values of 347 and 321 g/kg on days 1 and 120 post-harvest, respectively (Figure 23).

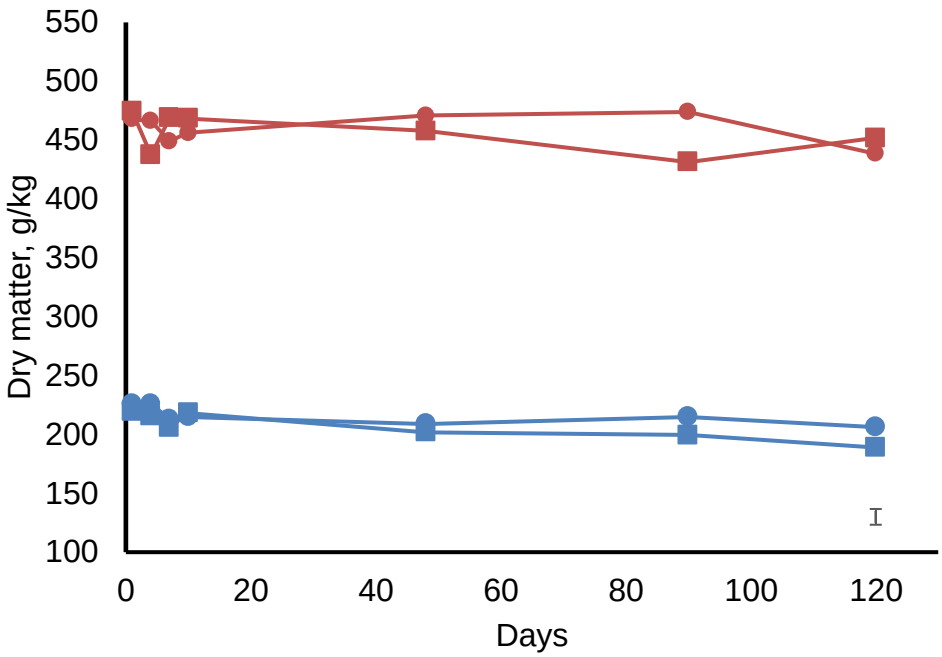


Figure 23 Dry matter content of mini silos that contained lucerne silage (■; n = 4); lucerne silage with tannins (●; n = 4); red clover silage (■; n = 4) and red clover silage with tannins (●; n = 4). Error bar indicates pooled s.e.d.

Forage pH was higher (P < 0.001) in lucerne silage with the mean values of pH 5.09 and pH 4.83 for lucerne and red clover silage, respectively. Over the course of ensiling, pH decreased (P < 0.001) from day 1 to day 48 averaging 5.49 and 4.69 respectively. From day 48 to day 120, pH increased from 4.69 to 5.22, respectively (Figure 24).

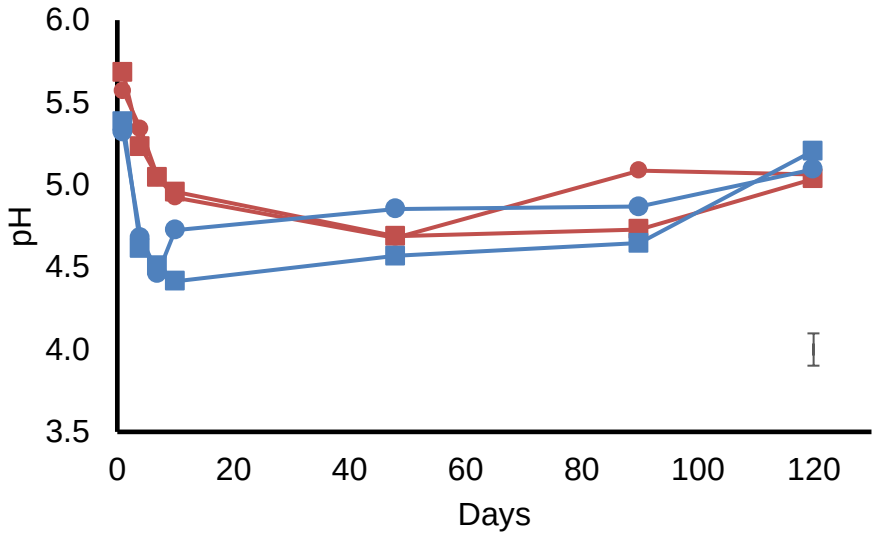


Figure 24 Forage pH of mini silos that contained lucerne silage (■; n = 4); lucerne silage with tannins (●; n = 4); red clover silage (■; n = 4) and red clover silage with tannins (●; n = 4). Error bar indicates pooled s.e.d.

Ammonia-nitrogen increased (P < 0.001) over the course of ensiling and had mean values of 8.32, 12.3, 14.4, 28.4 and 44.4 g/kg TN for day 1, 4, 7, 48 and 120, respectively (Figure 25).

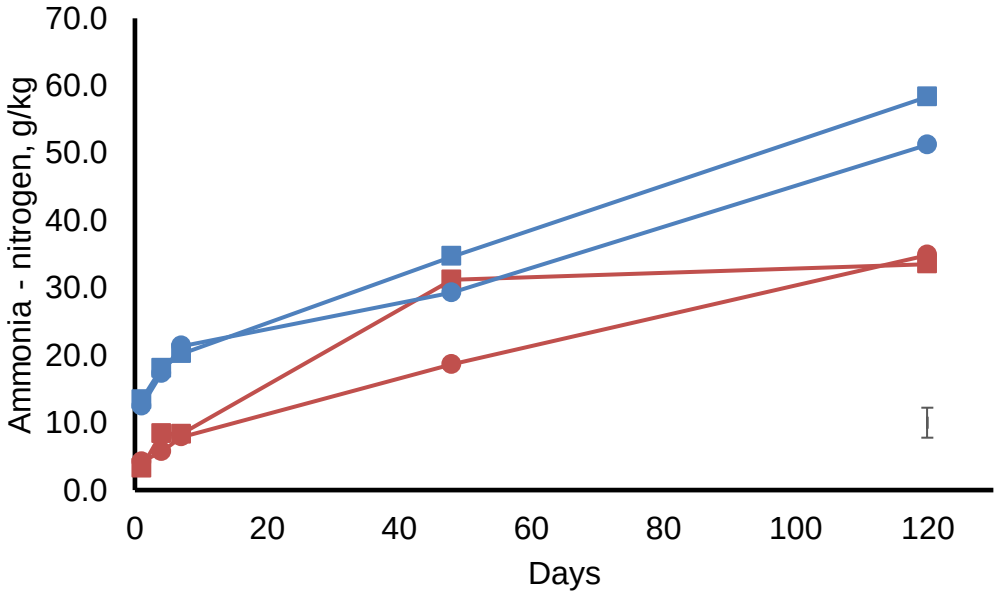


Figure 25 Ammonia - nitrogen of mini silos that contained lucerne silage (■; n = 4); lucerne silage with tannins (●; n = 4); red clover silage (■; n = 4) and red clover silage with tannins (●; n = 4). Error bars indicate s.e.d.

5.3.1.2 Chemical composition at day 120

Lucerne silage had the highest DM content whilst red clover silage averaged 263 g/kg lower (Table 28). Lucerne and lucerne plus tannin silage had the highest crude protein content of mean value 218 g/kg DM whilst red clover and red clover plus tannin silage had a mean value of 165 g/kg DM. Ash content of lucerne, lucerne plus tannin and red clover silage was similar and had a mean value of 153 g/kg DM, whereas red clover plus tannin had a lower ash content.

Table 28 Mean chemical composition of mini silos at day 120 containing lucerne silage (LCS), lucerne plus tannin silage (LTS), red clover silage (RCS) and red clover plus tannin silage (RTS).

	Lucerne	Lucerne plus tannin	Red clover	Red clover plus tannin	s.e.d
n	4	4	4	4	
DM, g/kg	451	438	188	206	32.4
CP, g/kg	223	213	170	161	0.747
Ash	104	101	102	88	3.93
NDF	405	397	495	476	12.4
pH	5.04	5.06	5.21	5.58	0.124
NH_3-N, g/kg TN	33.5	34.9	58.3	51.2	3.81

Red clover silage had the highest NDF content whilst lucerne plus tannin silage had the lowest NDF content with lucerne and red clover plus tannin silages being intermediates. Red clover and red clover plus tannin silage had the highest pH with a mean value of pH 5.39 while lucerne and lucerne plus tannin silage had the lowest pH which had a mean value of pH 5.05. Ammonia-nitrogen was highest in red clover silage while the other three silages had a mean value of 39.8 g/kg TN.

5.3.2. Experiment 3b - Performance of dairy cows fed lucerne and red clover silages with or without hydrolysable tannins

5.3.2.1 Feed analysis

The DM content of all five silages was above 200 g/kg, with red clover silage having the lowest DM while the lucerne silage was 241 g/kg higher, with maize, lucerne plus tannin and red clover plus tannin silages being intermediates (Table 29). For both crude protein and ash content, maize silage had the lowest content.

The lucerne and lucerne plus tannin silages had the highest CP and ash content while the two red clover silages had intermediate values. The two red clover silages had the highest NDF content and the two lucerne silages had the lowest content with maize silage being intermediate. Maize silage had the lowest pH while the other four silages had an average pH of 4.53. Red clover silages had the highest ammonia nitrogen content with a mean value of 68.2 g/kg TN, and the lucerne silages had the lowest ammonia nitrogen with a mean value of 38.6 g/kg TN with maize being the intermediate. Lucerne silage had the lowest tannin content whilst red clover plus tannin silage had the highest tannin content with lucerne plus tannin and red clover silages being intermediates.

Maize silage had the highest lactic acid content, red clover plus tannin silage had the lowest lactic acid content, while lucerne, lucerne plus tannin and red clover silages being intermediates (Table 29). Acetic acid was highest in red clover and red clover plus tannin silages averaging 53.2 g/kg while maize, lucerne and lucerne plus tannin silage was lower averaging 30.2 g/kg. Lucerne and lucerne plus tannin silage had the lowest propionic acid content averaging 0.6 g/kg while red clover and red clover plus tannin silages had the highest propionic acid content averaging 5.3 g/kg, with maize silage being the intermediate. Butyric acid was lowest in maize, lucerne and lucerne plus tannin silages averaging 0.3 g/kg while red clover and red clover plus tannin silages had a higher content averaging 22.4 g/kg. Total fatty acid content was highest in red clover plus tannin silage, while maize silage had the lowest. Lucerne, lucerne plus tannin and red clover silage were intermediates with a mean value of 18.3 g/kg DM. Lucerne silage had the highest C16:0 content, while red clover silage had the lowest content. Maize, lucerne plus tannin and red clover plus tannin silage were intermediates with a mean value of 2.75 g/kg DM. Maize silage had the lowest C18:0 content and lucerne silage had the highest content, while lucerne plus tannin, red clover and red clover plus tannin silage had intermediate values. Maize silage had the highest content of C18:1 *c*9 and lucerne silage plus tannin had the lowest content, whilst lucerne, red clover and red clover plus tannin silage were intermediates. Red clover plus tannin silage had the lowest content of C18:2 *n*-6 and lucerne silage had the highest content. Maize, lucerne plus tannin and red clover silage were intermediates with a mean value of 2.06 g/kg DM. Maize silage had the lowest content of C18:3 *n*-3 and lucerne silage had the highest content. Lucerne plus

tannin, red clover and red clover plus tannin silage were intermediates with a mean value of 2.59 g/kg DM.

Table 29 Chemical composition (g/kg DM) of maize, lucerne, lucerne plus tannin, red clover and red clover plus tannin silages fed to dairy cows.

	Maize silage	Lucerne silage	Lucerne plus tannin silage	Red clover silage	Red clover plus tannin silage	s.e.d
n	4	4	4	4	4	
DM, g/kg	266	456	452	215	217	17.4
CP	82.8	219	218	164	164	11.3
Ash	36.1	103	103	100	99.6	4.22
NDF	468	420	422	511	521	10.3
pH	3.72	4.52	4.54	4.50	4.57	0.086
NH_3-N (g/kg TN)	47.9	40.2	37.1	76.0	60.4	7.15
Fermentation acids (g/kg)						
Lactic acid	131.6	68.3	64.1	65.2	61.8	--
Acetic acid	29.6	29.0	32.1	51.9	54.5	--
Propionic acid	2.0	0.3	0.9	5.6	5.1	--
Butyric acid	0.4	0.1	0.4	24.6	20.2	--
Fatty acids (g/kg DM)						
C16:0	2.65	3.16	2.81	2.41	2.80	0.14
C18:0	0.25	0.41	0.36	0.35	0.37	0.017
C18:1 *c*9	1.81	0.27	0.24	0.30	0.37	0.14
18:2 *c*9 *c*12	2.31	2.39	2.20	1.50	1.49	0.11
18:3 *c*9 *c*12 *c*15	0.33	2.89	2.85	2.73	2.23	0.23
Total fatty acids	12.0	20.5	16.2	18.2	29.0	2.07
Tannin content, g/kg DM	--	16.2	22.0	23.5	25.1	1.45

Table 30 Chemical composition (g/kg DM) of diets fed to dairy cows containing lucerne and maize silage (LC); lucerne plus tannin and maize silage (LT), red clover and maize silage (RC) and red clover plus tannin and maize silage (RT).

	LC	LT	RC	RT	s.e.d
n	4	4	4	4	
DM, g/kg	406	415	328	325	7.72
CP	177	176	172	172	0.97
Ash	72.2	74.0	71.9	72.7	0.45
NDF	406	416	449	445	5.15
Fatty acids (g/kg DM)					
C16:0	6.43	5.84	6.86	6.19	0.32
C18:0	0.64	0.60	0.65	0.61	0.024
C18:1 *c*9	3.74	3.38	4.10	3.93	0.23
18:2 *c*9 *c*12	4.97	4.41	4.58	5.11	0.21
18:3 *c*9 *c*12 *c*15	1.43	1.40	1.09	1.28	0.064
Total fatty acids	24.7	21.5	26.8	24.9	1.09

The TMR of the diets containing lucerne silage had the highest average DM content of 410 g/kg while the diets containing red clover silage had a lower DM content of 326 g/kg (Table 30). Crude protein content was similar for the four diets averaging 174 g/kg DM. The NDF content of all four diets was above 400 g/kg, with diets containing red clover silage having higher NDF values than diets containing lucerne silage.

Total fatty acid content was highest in diets containing red clover silage and lowest in diets containing lucerne plus tannin silage, whilst diets containing lucerne and red clover plus tannin silage had intermediate values. Diets containing lucerne plus tannin silage had the lowest content of C16:0 and highest in diets containing red clover silage, diets containing lucerne and red clover plus tannin silage had intermediate values. The content of C18:0 was highest in diets containing red clover silage and lowest in diets containing lucerne plus tannin silage. Diets containing red clover silage had the highest content of C18:1 *c*9 whilst diets containing lucerne plus tannin silage had the lowest content, whereas diets containing lucerne and red clover plus tannin silage had intermediate values. The content of C18:2 *n*-6 was highest in diets containing red clover plus tannin silage and the lowest content was in diets containing red clover silage, whilst diets containing lucerne and lucerne plus tannin silage had intermediate values. Diets containing lucerne silage had the highest content of C18:3 *n*-3 and diets containing red clover silage had the lowest content. Diets containing lucerne plus tannin and red clover plus tannin silage had intermediate content with a mean value of 1.34 g/kg DM.

5.3.2.2 Dry matter degradation characteristics

The soluble DM fraction (a) was highest ($P<0.001$) in the lucerne silage and lowest in the red clover silage (Table 31 and Figure 26). Lucerne silage supplemented with tannin had a lower soluble DM fraction than lucerne silage without tannin. However, supplementation of tannin to the red clover silage had no effect on the soluble DM fraction.

Table 31 Effect of supplemented chestnut hydrolysable tannin to lucerne and red clover silages on *in situ* DM degradability co-efficient (%).

	Treatment							
	Lucerne		Red Clover			P value		
	-Tannin	+Tannin	-Tannin	+Tannin	s.e.d	Forage	Tannin	FxT
n	3	3	3	3				
a	24.1[c]	20.9[b]	10.2[a]	10.3[a]	0.431	<0.001	<0.001	<0.001
b	51.4[a]	53.2[a]	60.2[b]	57.7[b]	1.063	<0.001	0.622	0.020
a+b	75.5	74.1	70.5	68.0	1.075	<0.001	0.035	0.508
c	0.0817	0.0876	0.0587	0.0679	0.0027	<0.001	0.004	0.419
ED	50.1[b]	48.7[b]	35.7[a]	36.8[a]	0.728	<0.001	0.791	0.044

[a,b] Means within a row with a different superscript differ (P < 0.05)
Where a = soluble fraction, b = potentially rumen degradable fraction,
c = degradation rate of fraction b per hour and ED = effective degradability

Red clover silage had a higher (P < 0.001) potential degradable fraction (b) than lucerne silage. There was an interaction (P < 0.05) between forage source and tannin addition on the potentially degradable fraction, which was lower when tannin was added to red clover silage but not lucerne silage. The extent of DM degradation (a+b) was lowest (P < 0.001) in the red clover silage and highest in the lucerne silage. The inclusion of tannin reduced (P < 0.05) the extent of degradation in both the lucerne and red clover silages. Lucerne silage had a higher (P < 0.001) rate of degradation (c) than red clover silage. The addition of tannin to red clover and lucerne silage increased (P < 0.05) the rate of degradation. Effective degradability (ED) was highest (P < 0.001) in lucerne silage and lowest in red clover silage. The addition of tannin had no effect (P > 0.05) on effective degradability.

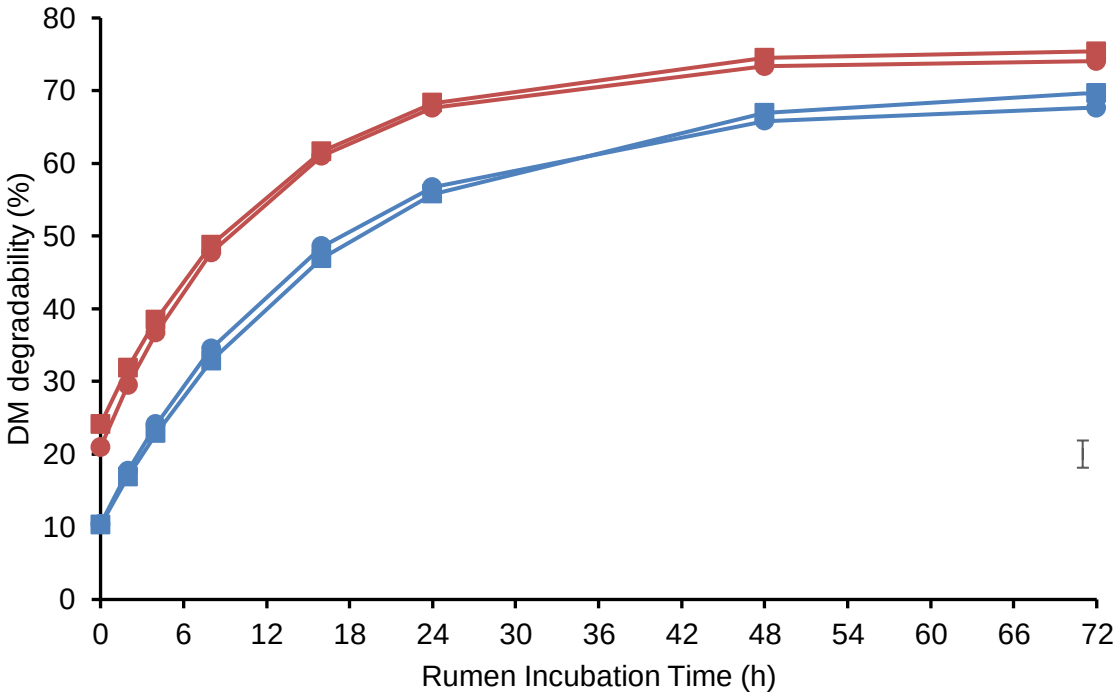


Figure 26 *In situ* DM degradation of lucerne silage (■; n = 3); lucerne silage with tannins (●; n = 3); red clover silage (■; n = 3) and red clover silage with tannins (●; n = 3). Error bar indicates pooled s.e.d.

5.3.2.3 Crude protein degradation characteristics

Lucerne silage had a higher (P < 0.001) soluble CP fraction (a) than red clover silage (Table 32 and Figure 27). The soluble CP fraction was also higher (P < 0.05) when lucerne and red clover silage were supplemented with hydrolysable tannins.

Table 32 Effect of supplemented chestnut hydrolysable tannin in lucerne and red clover silages on *in situ* CP degradability co-efficient (%).

	Treatment							
	Lucerne		Red Clover			P value		
	-Tannin	+Tannin	-Tannin	+Tannin	s.e.d	Forage	Tannin	FxT
n	3	3	3	3				
a	30.0	35.3	16.8	25.5	2.72	<0.001	0.007	0.409
b	54.8	49.7	58.2	47.4	4.11	0.854	0.026	0.355
a+b	84.8	85.1	75.0	72.9	1.74	<0.001	0.479	0.368
c	0.185^{b}	0.121^{ab}	0.0702^{a}	0.0721^{a}	0.0102	<0.001	0.003	0.002
ED	68.2^{c}	65.1^{c}	44.0^{a}	48.0^{b}	1.24	<0.001	0.615	0.004

^{a,b,c} Means within a row with a different superscript differ (P < 0.05)
Where a = soluble fraction, b = potentially rumen degradable fraction,
c = degradation rate of fraction b per hour and ED = effective degradability

There was no effect (P > 0.05) of forage type on the potential degradable fraction (b), however the potential degradable fraction was lower (P < 0.05) when lucerne or red clover silages were supplemented with hydrolysable tannins. Lucerne silage had a higher (P < 0.001) extent of degradation (a+b) than red clover silage. There was no effect (P > 0.05) of tannin inclusion on the extent of degradation. Rate of degradation was higher (P < 0.001) in red clover silage than lucerne silage. Rate of degradation was also higher in lucerne silage that was supplemented with hydrolysable tannins (P < 0.001). Effective degradability (ED) was higher (P < 0.001) in lucerne silage and also higher (P < 0.05) in red clover silage that was supplemented with hydrolysable tannins.

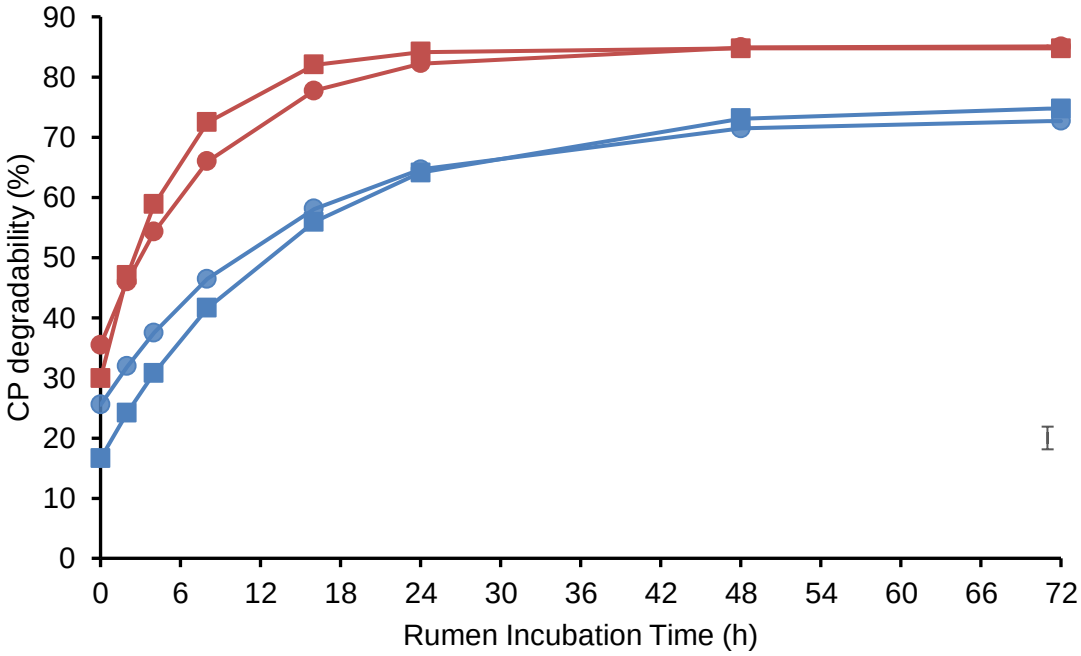


Figure 27 *In situ* CP degradation of lucerne silage (■; n = 3); lucerne silage with tannins (●; n = 3); red clover silage (■; n = 3) and red clover silage with tannins (●; n =3). Error bar indicates pooled s.e.d.

5.3.2.4 Animal performance

Total DM intake had a mean value of 20.9 kg/d and was higher (P = 0.009) in cows fed diets containing lucerne silage (Table 29). There was no effects of forage source or tannin inclusion (P > 0.05) on milk fat, milk protein or milk lactose content and had mean values of 41.5, 33.7 and 48.9 g/kg respectively. Forage source or tannin inclusion also had no effect (P > 0.05) on fat yield, protein yield or lactose yield which had mean values of 1.57, 1.28 and 1.85 kg/d respectively.

Similarly, there was no dietary effect ($P > 0.05$) on live weight, body condition or live weight change which had mean values of 652 kg, 2.89 and 11.1 kg/d respectively. Nitrogen efficiency had a mean value 0.36 kg milk N/kg feed N and was 0.045 kg milk N/kg feed N higher ($P < 0.001$) in cows fed diets containing red clover silage than lucerne silage.

Table 33 Milk performance, live weight and body condition score of cows fed diets that contained (36:64 forage DM basis) lucerne and maize silage; lucerne plus tannin and maize silage, red clover and maize silage and red clover plus tannin and maize silage.

	Treatment							
	Lucerne		Red Clover			P value		
	-Tannin	+Tannin	-Tannin	+Tannin	s.e.d	Forage	Tannin	F x T
n	12	12	12	12				
DM Intake, kg/d	21.3	22.9	20.1	19.6	1.15	0.009	0.533	0.206
Milk yield, kg/d	38.2	38.5	38.6	37.2	2.24	0.781	0.739	0.606
Milk fat, g/kg	40.7	42.2	40.9	42.4	1.98	0.900	0.297	0.972
Fat yield, kg/d	1.54	1.61	1.57	1.56	0.08	0.859	0.710	0.520
Milk protein, g/kg	34.1	34.5	33.1	33.4	0.96	0.142	0.673	0.899
Protein yield, kg/d	1.30	1.31	1.28	1.23	0.07	0.341	0.798	0.541
Milk lactose, g/kg	49.1	48.7	49.0	48.8	0.51	0.965	0.520	0.819
Lactose yield, kg/d	1.87	1.87	1.88	1.81	0.10	0.758	0.626	0.612
Live weight, kg	644	660	657	650	25.8	0.942	0.799	0.518
Body Condition	2.89	2.84	2.90	2.93	0.15	0.673	0.918	0.700
LW change[1], kg/d	0.16	0.54	0.44	0.48	0.36	0.673	0.404	0.510
N-efficiency (kg milk N/kg feed N)[2]	0.35	0.33	0.38	0.39	0.016	<0.001	0.476	0.414

[1] Over 28-d period

[2]N-milk = milk protein (g/day)/6.38

5.3.2.5 Milk fatty acid concentrations

Dietary treatment had no effect (P > 0.05) on milk fat content of C6:0 – C10:0; C14:0, C16:0, C16:1, C18:0, C18:1 *n*-9, C18:1 c-9, C18:2 *n*-6t or C20:0 (Table 34). In contrast, C4:0 was higher (P = 0.002) in cows when fed red clover silages, whereas C12:0 (P < 0.001) and C15:0 (P < 0.001) were higher in cows when fed lucerne silage.

Table 34 Milk fatty acid profile of dairy cows fed diets that contained (36:64 forage DM basis) lucerne and maize silage (LC); lucerne plus tannin and maize silage (LT), red clover and maize silage (RC) and red clover plus tannin and maize silage (RT).

Item	LC	LT	RC	RT	s.e.d	P value		
n	12	12	12	12		Forage	Tannin	FxT
Fatty acid (g/100g)								
4:0	2.33	2.37	2.52	2.43	0.053	0.002	0.524	0.112
6:0	1.72	1.75	1.79	1.75	0.041	0.276	0.794	0.232
8:0	1.20	1.14	1.12	1.12	0.063	0.248	0.457	0.526
10:0	2.67	2.77	2.62	2.61	0.088	0.105	0.487	0.386
12:0	3.45	3.57	3.34	3.32	0.117	0.040	0.553	0.423
14:0	11.1	11.3	11.1	11.1	0.198	0.431	0.457	0.388
15:0	1.05	1.05	0.91	0.94	0.038	<0.001	0.676	0.594
16:0	33.3	33.5	33.6	33.6	0.469	0.605	0.898	0.809
16:1	0.469	0.469	0.489	0.496	0.036	0.377	0.883	0.886
17:0	0.526	0.521	0.491	0.491	0.013	0.020	0.777	0.784
18:0	8.24	8.21	8.45	8.50	0.345	0.302	0.972	0.871
18:1*n*-9	19.5	18.8	19.2	19.0	0.488	0.945	0.228	0.423
18:2*n*-6	0.383	0.394	0.420	0.415	0.011	<0.001	0.693	0.285
18:3*n*-3	0.100	0.095	0.107	0.106	0.0028	<0.001	0.128	0.292
*c*9, *t*11 CLA	0.522	0.575	0.604	0.574	0.052	0.283	0.765	0.270
*t*10, *c*12 CLA	0.050	0.051	0.055	0.057	0.0037	0.055	0.593	0.847
20:0	0.103	0.104	0.118	0.108	0.0085	0.123	0.422	0.373
22:0	0.108	0.123	0.103	0.114	0.0048	0.040	<0.001	0.658
Summation								
<C16	23.7	24.3	23.6	23.5	0.481	0.154	0.557	0.311
C16 + C16:1	34.9	34.9	35.0	34.9	0.474	0.683	0.969	0.889
>C16	31.9	31.2	31.8	31.7	0.751	0.718	0.541	0.603
SFA	66.0	66.7	66.4	66.2	0.571	0.814	0.524	0.330
MUFA	21.3	20.5	20.9	20.8	0.514	0.908	0.201	0.382
PUFA	3.11	3.23	3.12	3.16	0.057	0.489	0.072	0.333

SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid

Cows fed lucerne silage had the highest amount of C17:0 (P = 0.020) and C22:0 (P = 0.040) whereas those fed red clover silage had the highest milk fat content of C18:2 *n*-6 (P > 0.001) and C18:3 *n*-3 (P < 0.001). Tannin inclusion had an effect on milk fat content of C22:0 which was higher (P < 0.001) in cows fed hydrolysable tannins. Neither forage type nor addition of tannin had no effect (P > 0.05) on FA with chain length less than or greater than C16, or C16 and C16:1 (P > 0.05). Saturated, MUFA and PUFA fatty acids were also not affected by dietary treatment (P > 0.05).

5.3.2.6 Plasma metabolites concentrations

For all blood metabolites measured, diet did not have an effect (P > 0.05; Table 35).

Table 35 Mean blood plasma metabolites for dairy cows fed diets that contained (36:64 forage DM basis) lucerne and maize silage; lucerne plus tannin and maize silage, red clover and maize silage and red clover plus tannin and maize silage.

	Treatment							
	Lucerne		Red Clover			P value		
	-Tannin	+Tannin	-Tannin	+Tannin	s.e.d	Forage	Tannin	FxT
n	192	192	192	192				
Total protein, g/l	75.1	75.3	75.9	74.3	1.298	0.929	0.474	0.328
Albumin, g/l	35.5	35.8	36.1	35.9	0.476	0.347	0.823	0.468
Glucose, mmol/l	3.24	3.20	3.22	3.25	0.072	0.835	0.910	0.426
Urea, mmol/l	4.79	4.64	4.59	4.44	0.159	0.081	0.190	0.996
BHB[1], mmol/l	0.574	0.602	0.657	0.626	0.043	0.079	0.966	0.330

[1] β-hydroxy butyrate

Table 36 Blood plasma metabolites over four time points for dairy cows fed diets that contained (36:64 forage DM basis) lucerne and maize silage; lucerne plus tannin and maize silage; red clover and maize silage; and red clover plus tannin and maize silage.

		Treatment							
		Lucerne		Red Clover			P value		
		-Tannin	+Tannin	-Tannin	+Tannin	s.e.d	Treatment	Time	TxT
n	Time	12	12	12	12				
Total protein, g/l	0700	74.4	76.0	76.7	74.8	2.595	0.900	0.316	0.008
	0900	76.4	74.7	70.8	74.7				
	1100	74.6	76.2	77.2	74.8				
	1300	74.9	74.4	78.9	73.0				
Albumin, g/l	0700	35.1	35.3	36.0	35.8	0.953	0.880	0.309	0.252
	0900	36.3	35.7	35.0	35.8				
	1100	35.3	36.5	36.4	36.5				
	1300	35.3	35.9	36.8	35.6				
Glucose, mmol/l	0700	3.36	3.31	3.25	3.28	0.143	0.931	<0.001	0.940
	0900	3.44	3.37	3.65	3.53				
	1100	3.16	3.08	3.12	3.09				
	1300	3.00	3.02	3.10	3.08				
Urea, mmol/l	0700	4.33	4.25	4.05	3.93	0.319	0.632	<0.001	0.878
	0900	4.58	4.57	4.45	4.35				
	1100	4.82	4.61	4.82	4.55				
	1300	5.42	5.12	5.05	4.93				
BHB[1], mmol/l	0700	0.492	0.500	0.510	0.518	0.086	0.513	<0.001	0.512
	0900	0.404	0.414	0.440	0.364				
	1100	0.612	0.715	0.830	0.737				
	1300	0.787	0.791	0.850	0.909				

[1] β-hydroxy butyrate

For all blood metabolites measured, diet did not have an effect (P > 0.05; Table 36), and there were no interactions between diet and time except for total protein (P < 0.05). Plasma albumin did not change during the day, (P > 0.05) with mean values of 35.5, 35.7, 36.2 and 35.9 g/L for 07:00, 09:00, 11:00 and 13:00 hours respectively (Figure 28).

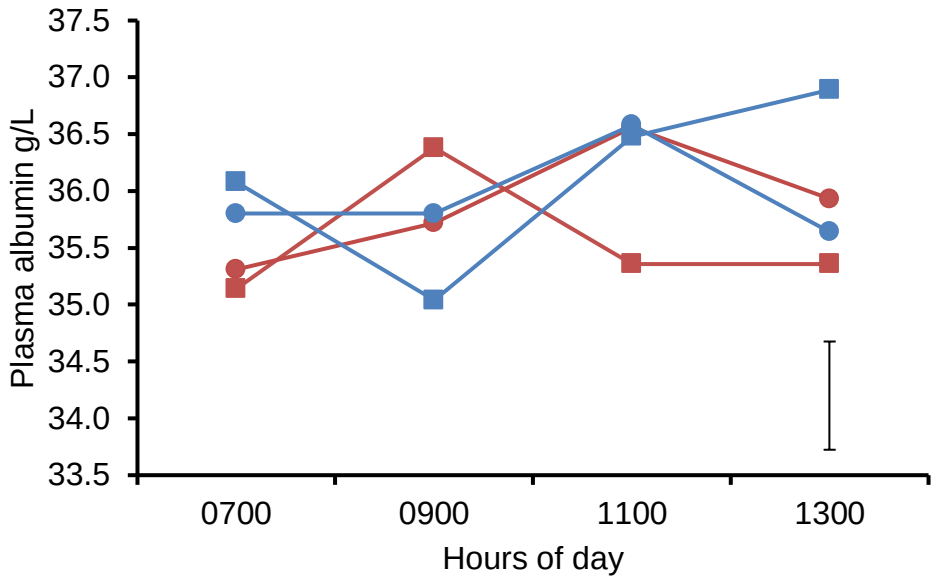


Figure 28 Plasma albumin in dairy cows fed diets that contained lucerne silage (■; n = 12); lucerne silage with tannins (●; n =12); red clover silage (■; n =12) and red clover silage with tannins (●; n =12). Error bar indicates pooled s.e.d.

Plasma BHB decreased (P < 0.001) from 07:00 h to 09:00 h before increasing to 13:00 h (Figure 29 and Table 36), but there was no effect of dietary treatment (P > 0.05).

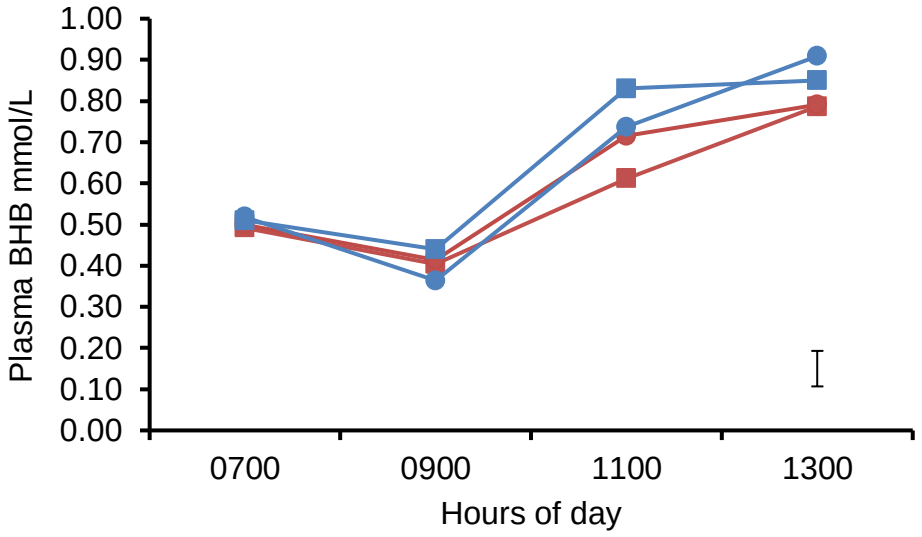


Figure 29 Plasma BHB in dairy cows fed diets that contained lucerne silage (■; n = 12); lucerne silage with tannins (●; n = 12); red clover silage (■; n =12) and red clover silage with tannins (●; n = 12). Error bar indicates pooled s.e.d.

Plasma glucose increased (P < 0.001) from 07:00 h to 09:00 h before decreasing to 13:00 h (Figure 30 and Table 36) but there was no effect of dietary treatment.

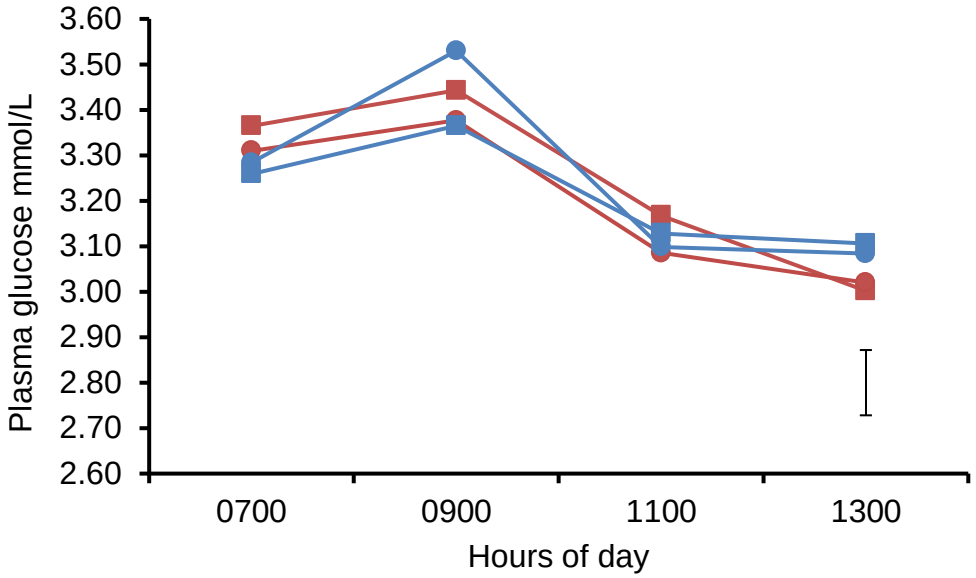


Figure 30 Plasma glucose in dairy cows fed diets that contained lucerne silage (■; n = 12); lucerne silage with tannins (●; n = 12); red clover silage (■; n = 12) and red clover silage with tannins (●; n =12). Error bar indicates pooled s.e.d.

Plasma urea increased (P < 0.001) with time from 07:00 h to 13:00 h (Figure 31 and Table 36) but there was no effect of dietary treatment.

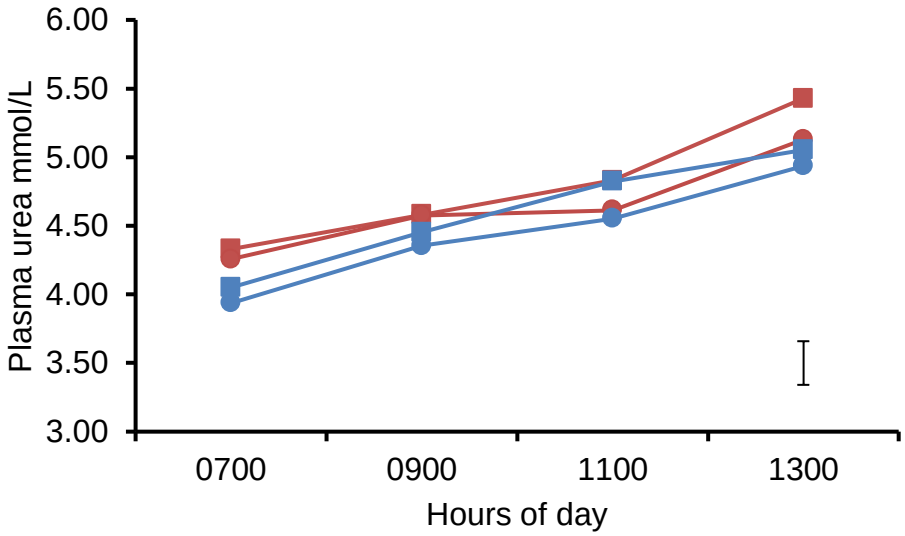


Figure 31 Plasma urea in dairy cows fed diets that contained lucerne silage (■; n = 12); lucerne silage with tannins (●; n = 12); red clover silage (■; n = 12) and red clover silage with tannins (●; n = 12). Error bar indicates pooled s.e.d.

Plasma total protein did not change during the day, (P > 0.05) with mean values of 75.5, 74.1, 75.7 and 75.3 g/L for 07:00, 09:00, 11:00 and 13:00 h respectively (Figure 32 and Table 36) and there was no effect of dietary treatment.

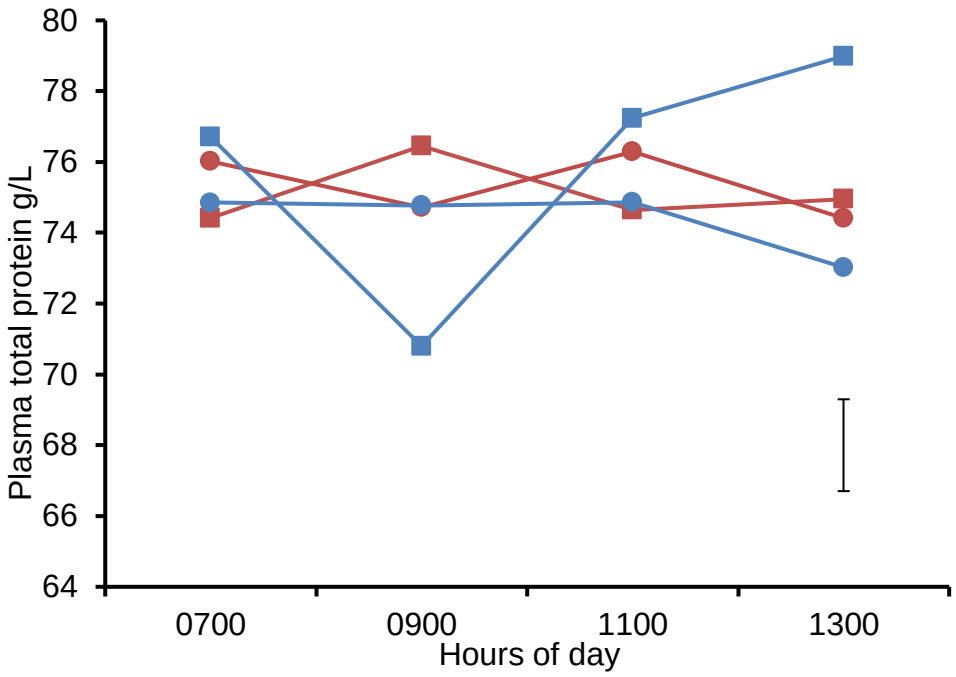


Figure 32 Plasma total protein in dairy cows fed diets that contained lucerne silage (■; n = 12); lucerne silage with tannins (●; n = 12); red clover silage (■; n = 12) and red clover silage with tannins (●; n = 12). Error bar indicates pooled s.e.d.

5.3.2.7 Whole tract digestibility

Dry matter intake was highest (P = 0.009) in cows fed diets containing lucerne silage and lowest in cows fed diets containing red clover silage (Table 37). Tannin inclusion had no effect (P > 0.05) on dry matter intake. Similarly, faecal DM output was highest (P = 0.041) in cows fed diets containing lucerne silage. However, there was no effect (P > 0.05) of tannin inclusion on faecal DM output. Both organic matter (P = 0.004) and nitrogen (P <0.001) intake was highest in cows fed diets containing lucerne silage with mean values of 20.61 kg/d and 582 g/d respectively. Tannin inclusion had no effect (P > 0.05) on faecal OM output or nitrogen output. Dietary treatments had no effect (P > 0.05) on DM, OM or nitrogen digestibility and had mean values of 0.678, 0.688 and 0.613 kg/kg respectively.

Table 37 Digestibility of DM, OM, and N in dairy cows fed diets containing (36:64 forage DM basis) lucerne and maize silage, lucerne plus tannin and maize silage, red clover and maize silage and red clover plus tannin and maize silage.

	Treatment							
	Lucerne		Red Clover			P value		
	-Tannin	+Tannin	-Tannin	+Tannin	s.e.d	Forage	Tannin	FxT
n	12	12	12	12				
Dry matter, kg/d								
Intake	21.4	22.9	20.2	19.6	1.15	0.009	0.533	0.206
Faecal output	6.78	8.14	6.32	6.10	0.837	0.041	0.341	0.189
Digestibility, kg/kg kg/kg	0.683	0.647	0.687	0.695	0.031	0.250	0.521	0.317
Organic matter, kg/d								
Intake	19.8	21.3	18.8	17.8	1.06	0.004	0.774	0.101
Faecal output	6.08	7.32	5.69	5.47	0.768	0.046	0.353	0.189
Digestibility, kg/kg	0.695	0.660	0.699	0.699	0.0309	0.333	0.421	0.430
Nitrogen, g/d								
Intake	564	601	499	480	29.0	<0.001	0.674	0.177
Faecal output	204	245	191	192	24.6	0.066	0.220	0.253
Digestibility, kg/kg	0.640	0.594	0.617	0.604	0.036	0.803	0.260	0.531

5.4 Discussion

5.4.1 Feed analysis

This is the first study to investigate the addition of hydrolysable tannins to lucerne and red clover silages in a maize silage based diet as dietary treatments to compare effects on animal performance, milk quality and apparent digestibility in high yielding dairy cows. The dry matter content of all the forages in the current study fell between ranges of 215 – 456 g/kg, however, the lucerne silages had the largest DM content while red clover silages were 238 g/kg lower. The DM content of lucerne and red clover silages differed to previous studies by Hymes – Fecht *et al.* (2013) and Broderick *et al*. (2000) in which the DM content averaged 52 g/kg lower for lucerne silage and 308 g/kg higher for red clover silage than the silages in the current study. In the study of Broderick *et al*. (2000), both the lucerne and red clover silages were a mixture of first, second and third cuts whereas in the current study both silages were second cut. Similarly Hymes – Fecht *et al*. (2013), also fed second cut lucerne and red clover silages, however the difference in DM content may reflect the different varieties of lucerne and red clover used. In the current study, varieties Daisy (lucerne) and Corvus (red clover) were used whereas Hymes - Fecht *et al*. (2013) used Forecast 1001 and Rebound 4.2 (lucerne) while the red clover variety was Marathon. Alternatively, wilting time and weather conditions may have influenced the DM content between studies.

The crude protein content of lucerne and red clover silages in the current study were similar to values observed in previous studies (Broderick *et al*., 2001; Broderick *et al*., 2000 and Hoffman *et al*., 1997), with red clover silage having a lower CP content. For all the forages, pH values were below pH 4.6 suggesting the forages had fermented, which was reflected by the rapid drop in pH between days 1 and 7 post ensiling. The lactic acid content of the lucerne silages indicated good fermentation and preservation as the content was above 60 g/kg DM, similar to previous study by Dewhurst *et al*. (2003). However, in the current study, the red clover silages had high levels of butyric acid and ammonia nitrogen indicating poor fermentation and preservation. The poorer quality of red clover silage may have been influenced by the DM content at ensiling and weather conditions during the wilting period as there were periods of rainfall prior to harvesting. Fibre levels

observed in the current study were higher in the red clover than the lucerne silage, but were similar to that reported by Hymes - Fecht *et al*. (2013).

Tannin content in the current study for lucerne and red clover silage without tannin had values of 16.2 and 23.5 g/kg DM. Hymes-Fecht *et al*. (2013) reported lucerne silage to have no naturally occurring tannins while red clover silage had a tannin content of 2.3 g/kg DM. These variations in tannin content between the current study and Hymes-Fecht *et al*. (2013) may reflect the sensitivity of the methodology used as there was an intra-assay CV of 22.5%. This high CV indicates the presence of a large variation between samples suggesting that the tannin content was not consistent throughout the clamps of silage. This intra-assay CV may also indicate that the method used to determine tannin content in the current study had poor sensitivity for the level of tannins present in the silages. Although, the addition of tannins did increase the tannin content of lucerne and red clover silage slightly by 5.8 and 1.6 g/kg DM, respectively, this was lower than expected, as hydrolysable tannins were added at a rate of 25 g/kg DM. It may indicate that the tannins were not fully homogenised throughout the clamps, resulting in variable samples which may be attributable to the application and mixing method of the tannin to the forage prior to ensiling. The reliability of these results require further assessment, the current method used was the butanol-HCl assay. An alternative method that could be used to investigate tannin levels in lucerne and red clover silage is the acidified vanillin assay. However, a previous study (Broadhurst and Jones, 1978) investigated the effects of temperature and light on the sensitivity of the acidified vanillin assay and observed varying results. It was observed that both light and temperature influenced the assay and therefore impacted on the tannin concentration measured. As time increased, it was observed that there was a decline in absorbance when the reaction was exposed to light. However, this decline could be prevented by completing the reaction in the dark with the samples remaining stable for up to 60 minutes. The ambient temperature also affected the rate of the reaction. Where the assay was incubated at a temperature of 25°C it took 12.5 minutes to reach maximum absorbance, whereas at lower temperatures of 20°C and 15°C it took longer (Broadhurst and Jones, 1978). Therefore, temperature and light were potentially affecting the butanol-HCl assay used in the current study thus impacting the estimation of tannin concentrations. This would suggest that the butanol-HCl assay needs to be further investigated to optimise

the temperature and light to enhance the sensitivity of the technique. Therefore, it can be concluded that multiple variables have potentially influenced the tannin concentrations observed in this experiment.

5.4.2 Degradation characteristics

Forage had an effect on the soluble DM fraction (a) where lucerne silage had higher content, and the potential degradable fraction (b), where red clover silage had higher content. The soluble protein fraction (a) was increased by the supplementation of tannins in the current study. In contrast, Tabacco *et al.* (2006) reported a decrease in the soluble protein fraction as the inclusion rate of hydrolysable tannin increased from 0, 20, 40 to 60 g/kg DM in lucerne silage. Rate of degradation (c) for DM and CP was higher in lucerne than red clover silage which is consistent with Coblentz *et al.* (1998) and Tabacco *et al.* (2006). These observations are similar to that reported by Broderick *et al.* (2004) when protein degradation was conducted in vitro, with lucerne silage having a faster rate of degradation compared to red clover silage. Effective degradation was also affected by dietary treatment for DM and CP with lucerne silage being higher than red clover silage and for DM, supplementation of tannin increased the effective degradation of red clover silage but not lucerne silage. In contrast, Tabacco *et al.* (2006) reported a decrease in effective protein degradability as the supplementation of hydrolysable tannin increased in lucerne silage. Tabacco *et al.* (2006) concluded that the addition of tannins could reduce the high rumen degradable protein that is associated with forage legumes like lucerne and red clover silages, although the results from the current study, where hydrolysable tannin was included at 25 g/kg DM does not support this.

5.4.3 Animal performance

In the mid-lactation dairy cows used in the current study, DM intake was improved by the inclusion of lucerne silage in a maize based diet. In previous studies (Hoffman *et al*., 1998; and Dewhurst *et al*., 2003) improvements in DM intake have been reported when cows were fed lucerne silage compared to feeding grass silage alone. In contrast, in studies (Arndt *et al*., 2015 and Sinclair *et al*., 2015) comparing different inclusion rates of lucerne silage in a maize silage based TMR, DM intake did not differ between treatments except in Sinclair *et al*. (2015) who reported a drop in DM intake when lucerne silage was included in the TMR at a

rate of 60 % forage DM. Dry matter intakes have been shown to be improved by red clover silage when fed as a sole forage or at high inclusion rate in a grass based diet (Moorby *et al*., 2009; and Bertilsson and Murphy., 2003). Similar to the current study, Broderick *et al*. (2000) reported a higher DM intake 1.2 kg/d in cows offered lucerne silage over red clover silage. Broderick *et al*. (2000) concluded this difference in DM intake may be attributable to the higher CP content of 36 g/kg DM in the diets containing lucerne silage. However in the current study, the differences in DM intake may be attributable to the quality of the red clover silage which had high levels of butyric acid and ammonia nitrogen indicating poor fermentation and preservation. In addition, the high difference in DM content of the lucerne and red clover silages (238 g/kg DM) may have influenced DM intake.

Although DM intake was improved by lucerne silage, cows fed lucerne and red clover silages had a similar milk yield. Similarly, Broderick *et al*. (2000) and Hymes – Fecht *et al*. (2013) reported no effect on milk yield when cows were offered lucerne or red clover silage. In contrast, Hoffman *et al.* (1998) reported increased milk yields of 1.6 kg/d when comparing lucerne silage to grass silage. Previous studies have reported improvements in milk yield when red clover silage was compared with grass silage (Moorby *et al*., 2009); or as part of maize based diet (Moorby *et al.,* 2016). The similarity in milk yields in the current study may be attributed to the similar crude protein content of all four treatment diets.

There was no effect of dietary treatment on milk fat, milk protein or milk lactose content in the current study. Arndt *et al*. (2015) also reported no effect of dietary treatment on milk fat or lactose content. However a decrease in milk protein was reported as lucerne silage in the diet increased from 20 to 80 % forage DM. Similarly, Moorby *et al*. (2009) and Moorby *et al.* (2016) reported a decline in milk protein content as the amount of red clover silage in the diet increased from 0 to 100 % forage DM. These changes in milk protein content as the amount of legume silage in the diet increases may reflect the high rumen degradable protein that is present in legume silages, therefore reducing the amount of metabolisable protein available for digestion in the small intestine. The responses in milk composition observed in the current study may have differed if the inclusion rate of hydrolysable tannins had been higher allowing an increase in rumen undegradable protein being available in the small intestine. However, in a previous study, Taha, (2015) fed hydrolysable tannin at four inclusion rates (0, 25, 50 or 75 g/kg DM) to

lactating ewes and found that there was no effect on milk protein suggesting that there was no increase in availability of RUP in the small intestine when tannin was added to the diet. Similarly, Lui *et al*. (2013) found that the inclusion of hydrolysable tannins at 10 g/kg DM in the diet of early lactating dairy cows had no effect on milk protein. Therefore, the current study used an inclusion rate of 25 g/kg DM of hydrolysable tannins as milk yields were highest in ewes fed 25 g/kg DM hydrolysable tannin in the study by Taha, (2015). The inclusion of tannins however, had no effect on N efficiency in the current study, however forage source had a significant effect with N efficiency improved in cows fed red clover compared to lucerne silages principally due to the lower DMI in red clover fed cows. The higher N efficiency with red clover silage may be related to the presence of PPO binding with the protein (Lee, 2014), increasing the amount of RUP available for digestion in the small intestine. The lack of an effect of tannin on N efficiency is consistent with Dschaak *et al.* (2011) when lucerne hay was supplemented with 30 g/kg DM of condensed tannin. In contrast, Gerlach *et al.* (2018) reported a decline in N efficiency when grass silage was supplemented with condensed tannins at a rate of 30 g/kg DM. Dschaak *et al.* (2011) suggested that these observations in N efficiency may be due to no change in milk protein yield which is consistent with the current study.

5.4.4 Milk FA composition and blood metabolites

Inclusion of tannins had no effect on milk FA composition, however forage source altered a number of FAs. Milk fat content of C4:0, C18:2 *n*-6 and C18:3 *n*-3 were all higher in milk from cows fed red clover compared to lucerne silage. The changes in linoleic acid (C18:2 *n*-6) and α-linolenic acid (C18:3 *n*-3) are consistent with previous studies where red clover silage has been compared to grass silage (Halmemies-Beauchet-Filleau *et al*., 2014; Dewhurst *et al*., 2003; Vanhatalo *et al*., 2007). Moorby *et al.* (2009) suggested that changes in linoleic acid and α-linolenic acid in milk may be a result of lowered biohydrogenation of forage fatty acids in the rumen possibly influenced by the polyphenol oxidase (PPO) enzyme system that is present in red clover silage. Similar to the current study, Lee *et al*. (2009) observed an increase in milk fat content of C18:2 *n*-6 and C18:3 *n*-3 in cows fed fresh red clover compared to cows fed fresh grass. Lee *et al*. (2009) suggested that the linoleic acid and α-linolenic acid are provided protection from rumen metabolism by the presence and binding of PPO in the red clover. Lucerne silage

improved milk fatty acids C12:0, C15:0, C17:0 and C22:0 compared to cows fed red clover silage. Similarly, Leduc *et al*. (2017) reported increased levels in milk fatty acid concentrations of C12:0, C15:0 and C17:0 in cows fed lucerne silage compared to red clover silage as part of a TMR based diet. Leduc *et al*. (2017) suggested these increases in FA content of C15:0 and C17:0 may be due to higher concentrations of propionate and valerate available in the rumen for elongation during rumen fermentation by the microorganisms to form C15:0 and C17:0. Similarly, Sinclair *et al*. (2015) observed an increase in concentration of C17:0 from 0.48 to 0.51 g/100g when lucerne silage was increased in the diet of dairy cows from 20 to 60%, respectively. However, in contrast to the current study, Sinclair *et al*. (2015) observed an increase in C18:2 *n*-6 and C18:3 *n*-3 as lucerne in the diet increased, suggesting that biohydrogenation was reduced in the rumen. Dietary treatment had no effect on fatty acids of chain length more than C16, indicating uptake from the diet or less than C16, indicating *de novo* synthesis, in the mammary gland. Supplementation with tannins had no effect on milk fatty acid content in the current study. In contrast, Dschaak *et al.* (2011) observed increases in milk fat content of 18:3 *n*-3 and 20:1 when lucerne hay was supplemented with condensed tannins and suggested these changes were caused by the tannins impeding microbes in the rumen therefore changing biohydrogenation pathways. In the current study, it was observed that there was an increase in C22:0 when tannin was added to both lucerne and red clover; however, it is unlikely that the tannins brought about an increase in FA C22:0 as there was no measurable difference in the tannin levels of the lucerne or red clover silage with supplemented tannins. Therefore, the increase in FA C22:0 may due to an artefact in the FAME and hexane sample that was observed by the GC. Apart from this observation, in the current study, the inclusion of hydrolysable tannin had no effect on the milk FA profile. It remains to be determined whether inclusion of tannins at levels higher than 25 g/kg DM significantly effects on milk FA profile observed.

Dietary treatment had no significant effects on blood metabolites in the current study. Dewhurst *et al.* (2003) observed an increase in plasma urea when cows were fed lucerne silage compared to red clover silage, however crude protein content of the diets varied by 30 g/kg DM. Similarly, Broderick *et al.* (2000) observed increases in plasma urea in cows fed lucerne silage when crude protein was not balanced across treatments. However, in the current study, no significant

effects were observed in plasma urea concentrations. All four diets were formulated to have a similar CP, MPE and MPN content. However, the silage analyses indicate that CP was higher in the lucerne silage. Also the DM intake was higher by 2.2 kg/d in cows fed lucerne silage, so N intakes were 93 g/d higher. Plasma BHB levels lower than 0.9 mmol/l indicate a cow is post peak lactation and has a positive energy balance (Topps and Thompson, 1984); all plasma BHB levels observed in the current study were lower than 0.9 mmol/l.

5.4.5 Apparent whole-tract digestibility

Digestibility of DM, OM and N were not affected by dietary treatment in the current study. Similarly, when lucerne hay was supplemented with condensed tannins, Dschaak *et al.* (2011) observed no differences in DM, OM or CP digestibility. In contrast, Hymes-Fecht *et al.* (2013) observed increases in DM and OM digestibility when red clover silage was offered to dairy cows compared to lucerne silage, however nitrogen digestibility was similar for cows fed lucerne and red clover silage. Similarly, Broderick *et al.* (2000) and Broderick *et al.* (2001) reported higher DM and OM digestibility when cows were offered red clover than lucerne silage suggesting that red clover silage is degraded more rapidly than lucerne silage. However, the observations in the current study would suggest that all diets were degraded at a similar rate in the rumen. The higher DM intakes by cows fed lucerne silage in the current study was reflected in OM and N intakes; in contrast, Hoffman *et al.* (1997) observed no difference in CP intake when lucerne and red clover silages were offered to dairy cows. Hoffman *et al.* (1998) observed improved DM and CP intakes when dairy cows were offered lucerne silage compared to grass silage and these differences were related to the higher rate of passage observed in cows fed lucerne silage. In the current study, it may be suggested that the lucerne silage had a higher rate of passage than red clover silage, although rumen outflow rate was not determined. However, the main suggestion for these differences would be the higher DM intake response in cows fed lucerne silage.

5.5 Conclusions

In conclusion, dry matter intake was higher in cows fed lucerne silage. Forage type and tannin inclusion did not improve milk yield, milk fat or milk protein content. Blood metabolites were unaffected by type of forage or tannin inclusion. Cows fed lucerne silage had higher organic matter and nitrogen intake, however dietary treatment did not affect DM, OM or nitrogen digestibility. Cows fed red clover silage had higher levels of milk FAs C18:2 *n*-6 and C18:3 *n*-3, however inclusion of tannins had no overall effect on milk FA composition. Forage type had an effect on soluble DM fraction and potential DM degradable fraction being higher in lucerne and red clover silage, respectively. Lucerne silage had a faster rate of degradation for DM and CP than red clover silage. Inclusion of tannin increased soluble CP fraction and effective degradability in red clover silage. Overall, a concern with the current study is the apparent lack of effect of tannin application on the tannin concentrations reported in the silages. Therefore, the lack of a change in in cow performance in response to the inclusion of tannins may be related to the low rate of application of tannins.

Chapter 6 General discussion

6.1 Introduction

Over the past 10 years, there has been a greater interest in home grown protein sources in the UK dairy industry. Previous studies have focussed their investigations on forage legumes as an alternative to grass silage, whereas very few studies have compared the different forage legumes that can be grown in the UK. Similarly, a few studies have investigated the use of tannins as a method of improving RUP within forage legumes, however in these studies the tannins have been supplemented to the diet at feed out rather than at ensiling. Therefore, the current studies are the first study to investigate forage legumes containing naturally occurring tannins or the addition of tannins at ensiling on animal performance of high yielding dairy cows.

6.2 The effect of condensed tannins on nutritive value and dairy cow performance

Nutritive value of forage legumes post ensiling varies between species, thereby having the potential to influence the performance of a high yielding dairy cow. Condensed tannins are found naturally within forage peas and occur at varying levels depending on the flower colour, peas which have coloured flowers tend to have higher levels than peas with white flowers (Wang *et al*., 1998). Previously, it has been shown that forage peas have high CP contents of between 170 to 220 g/kg DM (Mustafa *et al*., 2000; Hart *et al*., 2011) with the current study reporting values of 161 and 195 g/kg DM in experiment 1, whilst in experiment 2, CP levels of 208 and 195 g/kg DM were reported for red pea and white pea silage, respectively. However, in forage legumes this protein tends to be degraded rapidly within in the rumen resulting in low quantities of RUP available in the small intestine (Sinclair *et al*., 2009). Tannins have the ability to form a tannin-protein complex at pH 4.5 – 7.0 in either the silo or rumen, therefore improving the availability of RUP in the small intestine (McMahon *et al*., 2000). The microbial protein in a high yielding dairy cow is not sufficient to supply the cow's MP requirement, therefore the cow requires RUP in the diet to meet these requirements (McDonald *et al*., 2011). The addition of condensed tannins in experiment 1, had no effect on CP content of forage pea silages suggesting that there was no reduction in proteolysis in the silo therefore there was no increase in the amount of RUP available. Similar to experiment 1, Adesogan and Salawu,

(2002) observed no change in total N when 16 g/kg DM condensed tannins were added to a pea-wheat bi-crop at ensiling. Although, ammonia-nitrogen content was reduced, with a content of 130 g/kg total N for silage with no tannin and 94.7 g/kg total N for silage that had 16 g/kg DM condensed tannin added. Although in the current study, the addition of condensed tannins to forage peas (Chapter 4) had no change on ammonia-nitrogen content. Therefore, it can be concluded that the addition of condensed tannins to forage pea silages has very little influence on nutritive value or availability of RUP. Consequently, experiment 2 investigated the effects of the natural levels of condensed tannin in forage pea silages on dairy cow performance.

In experiment 2, the total replacement of grass silage with either red or white pea silage reduced DM intake and milk yield which may be attributed to the difference in CP content of the forages however had no effect on diet digestibility. In a previous study conducted by Sinclair *et al*. (2009), it was found that the inclusion of pea silage in the diet of dairy cows improved DM intakes compared to cows fed grass silage, although milk yields were unaffected. These differences may be attributable to the amount of forage pea silage included in the diet, in experiment 2 forage pea silages were included at 0.4 of the forage DM whereas in Sinclair *et al*. (2009) the forage pea silages were included at 0.5 of the forage DM. Sinclair *et al*. (2009) concluded that the lack of change in milk yield may be due to the dairy cow utilising the nutrients from the forage pea silages for her body stores rather than milk production although there was no BCS change observed. In the current study, there was no change in BCS which averaged 2.87 suggesting that the cow was not utilising the nutrients from the forage pea silages for her body stores.

Since completion of experiment 2, more recent research (Broderick *et al*., 2017) investigated the effects of increasing levels of naturally occurring condensed tannins from 5.1 to 16.6 g/kg DM in birdsfoot trefoil silage on performance of high yielding dairy cows. These levels of condensed tannins in the birdsfoot trefoil silages were much lower than in the forage pea silages of experiment 2 which averaged 62.9 g/kg DM. In two trials conducted by Broderick *et al*. (2017), DM intake and milk yield averaged the highest at 26.8 and 41.3 kg/d, respectively, in cows fed birdsfoot trefoil silage containing 10 g/kg DM condensed tannins. In contrast, Hymes-Fecht *et al*. (2013) found that milk yield was highest in cows fed birdsfoot trefoil silage containing the highest amount of condensed tannin at 15.7

g/kg DM although DM intake was unaffected. These contradicting results from previous studies and the current study make it unclear what the optimal level of naturally occurring condensed tannins in forage legumes would be for optimising DM intake and milk production in dairy cows. It has been suggested (Hymes-Fecht *et al.*, 2013 and Gerlach *et al*., 2018) that levels of higher than 30 g/kg DM of condensed tannins in the diet can have a detrimental effect on DM intake and milk yield, therefore in the current study, this may be the reason there was a reduced DM intake and milk yield compared to cows fed grass silage. However, these higher levels of condensed tannin were used in the current study due to a previous study by Sinclair *et al*. (2009) observing improved DM intakes with forage pea silage containing condensed tannin at levels higher than 30 g/kg DM.

Savings can be made by the reduction of the use of imported dietary protein with the use of forage legumes in the diet of high yielding dairy cows due to their high crude protein content. In experiment 2, there was a saving in soyabean meal of approximately 0.4 kg/cow/day when cows were fed forage pea silages, this would be a significant saving for a commercial dairy cow herd. For a 100 cow herd with a lactation period of 305 days this saving would equate to a 12.2 tonne reduction in SBM which would be an overall cost saving of £3891 at £319 per tonne. Therefore, the overall conclusion from experiment 2 and the previous published studies would suggest that any commercial advantage from feeding forage legumes containing condensed tannins to high yielding dairy cows will be based on savings in N from fertiliser or dietary protein (Sinclair *et al*., 2009).

6.3 The effect of hydrolysable tannins on nutritive value and dairy cow performance

Forage legumes, lucerne and red clover, contain either no or low levels of naturally occurring tannins, respectively. In these forage legumes, there is no protection of the protein from tannins in the silo or rumen although red clover silage contains the enzyme, polyphenol oxidase, which inhibits protein degradation by the binding of *o*-quinones with forage protein (Broderick *et al*., 2000; Hoffman *et al*., 1997; Jones *et al*., 1995). Polyphenol oxidase provides protection to the protein in red clover silage in both the silo and rumen, with the protein becoming available in the small intestine (Lee, 2014). Therefore, experiment 1 and 3 investigated the effects of the addition of hydrolysable tannins to lucerne and red clover silages on

nutritive value and dairy cow performance. Similar to the forage peas used in experiment 1 and 2, lucerne and red clover silages have been reported to have high CP content with values varying between 166 to 220 g/kg DM in previous studies (Broderick *et al*., 2000; Broderick *et al*., 2001 and Broderick *et al*., 2005). In the current study, CP values of 179 and 110 g/kg DM were reported in experiment 1, whilst in experiment 3, CP levels of 219 and 164 g/kg DM were reported for lucerne and red clover silage, respectively. The differences in CP content between the two experiments may be due to the different years and fields that the forages were grown in, similarly weather conditions at wilting and harvest may have influenced the CP content. In both experiments, the addition of hydrolysable tannins to the silages did not affect CP content suggesting that proteolysis was not reduced in the silo. Similarly, Tabacco *et al*. (2006) observed no effect of the addition of hydrolysable tannins on the total N content of lucerne silage. Therefore, from these studies, it can be concluded that hydrolysable tannins do not provide protection to the protein in the silo. The protein in the red clover silage may have been provided protection from the polyphenol oxidase; however this was not measured in the current study, although ammonia-nitrogen content was higher in the red clover silages suggesting protection of nitrogen. Overall, it can be concluded that the addition of hydrolysable tannins to lucerne or red clover silage had very little influence on the nutritive value. In a previous study, Taha, (2015) observed reduced milk yields in Suffolk cross ewes when hydrolysable tannins were added to lucerne silage at feed out at levels of 50 or 75 g/kg DM, however there was an increase in milk yield when the sheep were fed 25 g/kg DM. Following results from this study and experiment 1, experiment 3 investigated the effect of the addition of hydrolysable tannin at 25 g/kg DM at ensiling in lucerne and red clover silage on the performance of high yielding dairy cows.

In experiment 3, DM intakes were influenced by forage type rather than inclusion of tannin, with cows fed lucerne silage having higher DM intakes. Milk yield, milk fat and protein content were not significantly affected by forage type or tannin inclusion. There are few studies that have compared lucerne and red clover silage, however the results shown in experiment 3 are agreeable with Broderick *et al*. (2000) who showed a 1.2 kg/d increase in DM intake in cows fed lucerne silage. Differences in DM intake in the current study and Broderick *et al*. (2000) may be

attributable to the variation in DM and CP content of lucerne and red clover silage in which CP varied by 54.5 and 36 g/kg DM for the current study and Broderick *et al*. (2000), respectively. Thus had these variables had been similar, DM intake may have been similar, however lucerne silage tends to have a higher CP content than red clover silage as seen in previous studies (Hymes-Fecht *et al*., 2013; Dewhurst *et al*., 2003; Broderick *et al*., 2001).

The findings of experiment 3 are in agreement with a recent meta-analysis conducted by Johansen *et al*. (2018). Dairy cows fed lucerne silage had higher DM intakes compared to dairy cows fed red clover silage, however there was no difference in milk yield or BCS suggesting that the cow was not utilising the nutrients. Although, the current study observed no significant effect of forage type on milk protein which was slightly lower in cows fed red clover silage, Johansen *et al*. (2018) reported that dairy cows fed red clover silage had a lower milk protein content than cows fed lucerne or grass silage. In cows fed red clover silage, the expected milk protein levels would be higher due to the presence of PPO that binds to the forage protein preventing breakdown in the rumen thereby causing a shift of RDP to RUP, therefore there is an availability of amino acids in the small intestine (Lee, 2014). However, data from experiment 3, and Johansen *et al*. (2018) show this isn't the case, there was no effect of forage type on milk protein suggesting that the presence of PPO in the red clover silage did not improve availability of amino acids in the small intestine. Therefore, the current study suggests that the lucerne and red clover silage were providing the same quantities of amino acids in the small intestine.

Since the completion of experiment 3, more recent research (Gerlach *et al*., 2018) has shown that the inclusion of condensed tannins at varying rates from 10 to 30 g/kg DM had no influence on DM intake, milk yield or energy corrected milk. Inclusion rates in experiment 3 and Gerlach *et al*. (2018) are below 30 g/kg DM which has been suggested to be too low to influence DM intake or milk yield when fed to dairy cows. However, in experiment 2 the natural levels of condensed tannin at 67.2 and 58.6 g/kg DM for white and red pea silage, respectively, observed reduced DM intakes and milk yield. A concern for both experiments is the tannin levels measured within the forage legumes used in the current study. In experiment 2, the red pea silage would be expected to have higher levels of condensed tannin than the white pea silage whilst in experiment 3, the inclusion of

25 g/kg DM hydrolysable tannin to lucerne and red clover silage is not apparent. In the current study, the method used to determine tannin content was according to Makkar *et al*. (1993), the similar levels observed may be due to the sensitivity of the method. A previous study by Broadhurst and Jones, (1978) investigated how the acidified vanillin assay reacted to different temperatures and levels of light. It was observed that light levels influenced the stability of the absorbance with the assay whilst temperature influenced the rate of the reaction to reach maximum absorbance. It is clear from this previous study that tannin determination is sensitive to temperature and light levels, therefore the assay in the current study requires optimisation for temperature and light levels. In experiment 2 and 3, the butanol-HCl assay used to determine tannin content was conducted in the dark, however, the lab was not temperature controlled, and therefore the contradicting results observed may be attributable to the lab conditions. In experiment 3, the apparently similarity in tannin content between the non-tannin silage and tannin supplemented silage may be attributable to the application process of the tannin. Thus, it can be concluded that there are a number of possible variables which may have influenced the tannin content of the forage legume silages used in experiment 2 and 3. Therefore, the overall conclusion from previous studies and experiment 3 would suggest that the inclusion of tannins at ensiling are an ineffective method for the improvement of nitrogen use and milk performance in dairy cows.

6.4 Influence of forage legumes and tannins on milk fatty acid profile

In experiment 2 and 3, the composition of milk FAs were measured to investigate whether the inclusion of forage legumes and tannins in the diet of dairy cows altered the milk fatty acid profile. It is known that different forages provide varying concentrations of FAs and the PUFAs present in the forage will undergo biohydrogenation in the rumen to form saturated FAs, in particular C18:0 (Jenkins *et al*., 2008). The presence of tannins in the forage or silage have been shown to alter the pathway of biohydrogenation (Turner *et al*., 2005; Khiaosa-Ard *et al*., 2009) whilst the presence of PPO in red clover silage has been shown to provide protection to the PUFAs (Lee *et* al., 2009b; Leduc *et al*., 2017). Therefore, it was important to include these measurements within the current studies to determine if there were any changes in milk FA profile of dairy cows fed forage legumes. In experiment 2, there were minor changes to the milk FA profile when dairy cows

were fed either red or white pea silage, C16:0 was higher in cows fed white pea silage whereas C18:3 *n*-3 was higher in cows fed red pea silage. In both white and red pea silages the concentrations of C18:3 *n*-3 were the same at 1.76 g/kg DM, however the grass silage had a higher concentration at 5.09 g/kg DM. Similarly, previous studies, (Turner *et al*., 2005; Dschaak *et al*., 2011), observed higher levels of C18:3 *n*-3 with the presence of condensed tannins in either birdsfoot trefoil or lucerne hay, respectively. It was suggested that the condensed tannins were altering the pathway of biohydrogenation, thereby reducing the amount of PUFAs being transformed into C18:0 and improving the concentrations of these PUFAs in the milk. In the current study, it could be suggested that the condensed tannins present in red pea silage were altering the biohydrogenation pathway in the rumen but not in cows fed the white pea silage. The low levels of C18:3 *n*-3 present in the milk of dairy cows fed grass silage would suggest that the PUFAs were undergoing biohydrogenation in the rumen. In contrast, cows fed grass silage in the current study had higher concentrations of C18:2 *n*-6 than the cows fed either white or red pea silage, these differences may be due to the higher levels present in the grass silage.

In experiment 3, forage type influenced the milk fatty acid profile of dairy cows rather than the presence of tannins in the diet. Cows fed red clover silage had higher concentrations of both C18:2 *n*-6 and C18:3 *n*-3 PUFAs compared to cows fed lucerne silage. The presence of these fatty acids within the silage were lower in the red clover silage which would suggest that there was a reduction in biohydrogenation in the rumen of cows fed red clover silage. These results are consistent with previous studies (Dewhurst *et al*., 2003; Vanhatalo *et al*., 2007) when red clover silage was compared with grass silage. It has been suggested by Lee *et al*. (2009) that these changes in milk FA profile of dairy cows fed red clover silage are due to the presence of PPO in the red clover silage. The PPO is able to provide protection to the PUFAs from metabolism and biohydrogenation in the rumen by binding to the FAs, therefore there will be higher concentrations present in the milk. It can be concluded in the current study that the presence of PPO was having an influence on the milk FA profile in cows fed red clover silage. Overall, it can be concluded from the current studies that the milk FA profile of dairy cows can be influenced by the forage type and the presence of either tannins or PPO.

6.5 Nitrogen efficiency in dairy cows fed forage legumes

An objective of both dairy cow studies (Chapter 4 and 5) was to investigate the effect of forage legumes with naturally occurring or supplemented tannins on nitrogen efficiency. Nitrogen efficiency is the conversion of feed N to milk N (Calsamiglia *et al*., 2010), and in the current studies, cows fed either white pea or lucerne silage had the lowest N efficiency in their respective treatments, although N efficiency in the current studies were high and above 0.32 kg milk N/kg feed N. It has been shown that N efficiency increases as the amount of RDP decreases in the diet, suggesting that a higher amount of N is captured in the rumen efficiently (Calsamiglia *et al*., 2010). In contrast, in a study by Broderick *et al*. (2001) N efficiency was lower and had a mean value of 0.24 kg milk N/kg feed N in dairy cows fed either lucerne or red clover silage. In the current studies, the protein and metabolisable protein content of the diets were formulated to be similar, however there was a variation in N intake between treatment groups. Interpretation of the N efficiency results is limited due to this N intake variation, however when N efficiency and N intake results are considered together there was a negative relationship (P = 0.014; Figure 33). The addition of tannins to red clover or lucerne silage had no effect on N efficiency. However when total N intake was considered, a decline in N efficiency became apparent with the addition of hydrolysable tannins to lucerne silage. Although, there was an increase in N efficiency when total N intake was considered for red clover silage that had supplemented hydrolysable tannins. In a previous study, the effect of N intake on N efficiency has been shown by Dewhurst *et al*. (2003) who observed no effect on N efficiency following the increase of concentrate in the diet from 4 to 8 kg/d. However, when N intakes were considered, N efficiency showed a decline as the amount of concentrate in the diet declined (Dewhurst *et al*., 2003). In more recent research, Gerlach *et al*. (2018) supplemented grass silage with 30 g/kg DM of condensed tannins and observed a decline in N efficiency. It has been reported by Poppi and McLennan, (1995) that the presence of legumes in the diet can increase protein intake, although the intestinal supply of protein per unit of DMI does not always increase at the same rate due to losses in the rumen. Therefore, Poppi and McLennan, (1995) concluded that the availability of N at intake will influence the utilisation of the amino acids in the small intestines thereby influencing N efficiency. Therefore, these studies suggest that the relationship between N efficiency and N intake is

significant and more important than the N degradability, and requires further investigation.

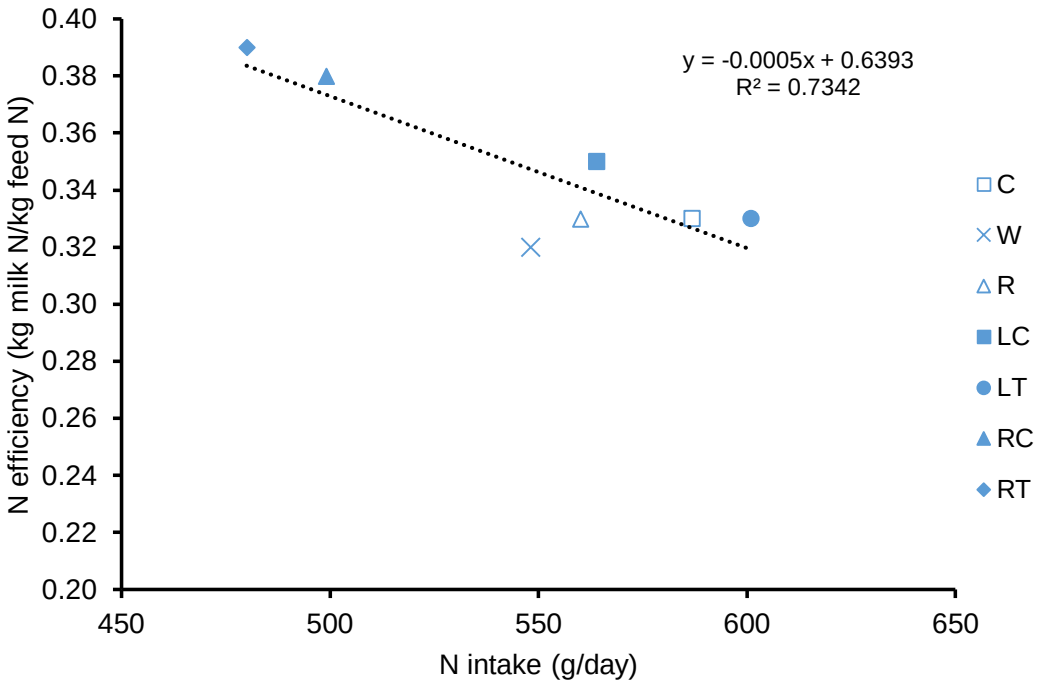


Figure 33 The nitrogen efficiency of forage treatments in Experiments 2 and 3 (C = grass silage, W = white pea silage, R = red pea silage, LC = lucerne silage, LT = lucerne plus tannin silage, RC = red clover silage and RT = red clover plus tannin silage).

6.6 Limitations of experimental design

Following completion of experiments 1, 2 and 3 it has become clear that there are aspects of the experiments which could have been altered to improve the reliability of the results. A key area that requires further investigation is the tannin assay methodology. Future studies are needed to ascertain the optimal temperature and light conditions to ensure the sensitivity of the method. Further to this, the application and mixing of the tannins to the forage needs consideration to ensure that the tannin is evenly applied to avoid differences in tannin concentrations throughout the clamp. In experiment 1, further consideration could be made to investigate the effectiveness of application of tannins before ensiling and various stages throughout the fermentation process to ascertain how the evenly the tannins have been applied.

In experiment 1 and 3, mini silos of each forage were produced to investigate how the addition of condensed or hydrolysable tannins affected nutritive value of the final silage. Additionally, experiment 3 investigated how DM, CP and pH altered over the course of ensiling from day 1 to 120. However, fermentation profile was not considered in the current study. Therefore, a potential measurement for both of these experiments would be to investigate the effect of tannins on fermentation profile over the course of ensiling.

Previous studies have considered varying levels of condensed or hydrolysable tannin in the diet of dairy cows, however experiment 3 only considered two levels of hydrolysable tannins at 0 or 25 g/kg DM. An alteration to the study design would be to consider a wider range of inclusion rates of hydrolysable tannin to lucerne and red clover silage ranging from 25 to 60 g/kg DM as Taha, (2015) observed decreased milk yields when 75 g/kg DM of hydrolysable tannins were fed to sheep. Investigating a wider range of inclusion rates would allow the study to potentially determine the optimal level of hydrolysable tannin to feed to dairy cows to improve performance. The optimal levels may vary between species of commercially used forages and potentially between types of tannin, therefore the proposed study may provide important information that is currently inconsistent from previous studies (Sinclair *et al*., 2009; Broderick *et al*., 2017; Tabacco *et al*., 2006; Gerlach *et al*., 2018).

In a meta-analysis by Johansen *et al*. (2018), it was concluded that dairy cows fed forage legumes can achieve higher DM intakes and milk yields than those fed diets containing grass silages. In experiment 3, it was observed that dairy cows fed lucerne silage had higher DM intakes, however a grass based diet was not included as part of this study therefore it is difficult to compare these studies. A change of design to experiment 3 may consider the inclusion of grass silage as a control diet as this silage is typically included in diets fed to dairy cows in the UK. However, in experiment 2, it was observed that dairy cows fed forage pea silages had a reduced DM intake and milk yield compared to dairy cows fed grass silage. In both experiment 2 and 3, the study design was a Latin square design therefore dairy cows received the diets for a period of 28 days which was considered a suitable adaptation period before data collection. To fully understand these varying results in DM intake and milk performance, the design of the experiments could involve a longer adaptation period or the forages could be fed over the course of a

lactation to understand the full effects of forage legumes on the full lactation performance of dairy cows. Potential further measurements to consider for a study scheduled over a full lactation would be the methane emissions of the dairy cows fed forage legumes with the inclusion of tannins. A previous study (Grainger *et al.,* 2009) fed condensed tannins at two rates, 163 and 244 g/day, to early lactating dairy cows grazing a ryegrass pasture. Methane emissions were reduced by both levels of condensed tannins in the diet with levels reduced by 14 and 29% for 163 and 244 g/day, respectively. Therefore, it would be interesting to investigate how tannins in forage legumes included as part of a maize-based diet may influence methane emissions of dairy cows.

6.7 Future research

The current study is the first study to investigate the use of home grown forages as potential options to reduce the requirement of purchased dietary protein whilst improving performance in high yielding dairy cows. Following the completion of three experiments, there are areas which have arisen as potential areas for future research in forage legumes and dairy cows. An area that the current studies did not investigate were the effects of condensed and hydrolysable tannins on the fermentation characteristics of the silage. Fermentation characteristics of silages are an important indicator of how the silage has fermented and preserved during the ensiling process. These characteristics indicate the quality of the silage thus have the potential to influence DM intake and milk yield of dairy cows. Therefore, future research is required to further demonstrate the effects of hydrolysable and condensed tannins on the fermentation profile of forage legumes.

In a commercial setting, the current method of application and mixing of tannins to silage is not viable so tannins would not be considered an option at ensiling. The similarities in tannin concentrations measured in silages with or without added tannin in experiment 3 in the current study, suggests that future research is required to determine whether / how tannins can be applied to the forage and mixed with the forage at ensiling to ensure an even spread of tannins throughout the clamp. Many studies have considered the use of tannins at feed out as the application can be through the mixer wagon at the mixing stage of the dietary ingredients of the TMR. Therefore, a comparison study between supplementation of tannins at ensiling or feed out may be a future study to investigate the optimal

way of adding tannins to the diet of high yielding dairy cows. Furthermore, to investigate the effectiveness of tannin application, further work is required on the butanol-HCl assay for determining tannin content in forages as it has been observed in Broadhurst and Jones, (1978) that the assay is sensitive to light and temperature thereby influencing the result.

The structure of condensed and hydrolysable tannins vary thus having the potential to influence the binding strategies of these tannins in forage legumes. These binding strategies can impact the digestion of feed and nutritive value of the feed that the ruminant is able to utilise (Gerlach *et al*., 2018). In feeding studies, the structure of tannins is often not studied therefore future research may provide a better knowledge and understanding of how tannin structure is influencing the effects of tannin on ruminant performance. There are many methods that can used when measuring condensed and hydrolysable tannins, these methods include a variety of chemical, biochemical or biological assays (Mueller-Harvey, 2001; Schofield *et al*., 2001). However, currently there is a lack of understanding of tannin structure making it difficult to recommend one particular method, therefore it has been suggested that a mixture of methods should be used until knowledge of tannin structure is improved allowing for specific analytical methods to be developed (Mueller-Harvey, 2001; Schofield *et al*., 2001).

The results of experiments 1 and 3 in the current study and from other published studies (Tabacco *et al*., 2006) suggest that future research is required to discover the optimum level of condensed or hydrolysable tannin added to forage legumes at ensiling to optimise dairy cow milk production and nitrogen use. Very few studies have investigated the effects of addition of tannins at ensiling due to the impractical methods of application limiting studies to focus on mini silos rather than commercial sized silos. Therefore, studies have focussed on the addition of tannins at feed out to investigate effects on cow performance. Experiment 3 and recent research by Gerlach *et al*. (2018) observed no effect of the inclusion of condensed or hydrolysable tannins up to 30 g/kg DM. It has been suggest that higher levels of condensed tannin above 30 g/kg DM have a detrimental effect on DM intake and milk yield which agrees with the results of experiment 2 on forage pea silages containing levels of condensed tannin averaging 62.9 g/kg DM. Therefore, results from the current study and previous studies suggest that future research needs to focus on discovering the optimal level of condensed or

hydrolysable tannin between 30 and 60 g/kg DM in the diet of high yielding dairy cows to determine what level has a beneficial effect on cow performance.

6.8 Conclusion

In conclusion, the presence of either naturally occurring or supplemented condensed or hydrolysable tannins has had varying effects on nutritive value of forage legumes and performance of high yielding dairy cows. Nutritive value of forage legumes was found to be determined by the species of forage whilst tannin type or inclusion have very little influence on nutritive value post ensiling. The inclusion of tannins at varying rates to forage legumes did not change CP contents suggesting that the quantity of RUP available in the silo or rumen remained unaltered. Dairy cows fed forage pea silages had reduced DM intakes and milk yields, whereas dairy cows fed lucerne silage had a higher DM intake than cows fed red clover silage although there was no forage effect on BCS. Milk yield was unaffected by forage type or inclusion of tannin in cows fed lucerne or red clover silage. Digestibility of DM, OM or N were unaffected by the inclusion of forage peas, lucerne and red clover silages in the diet of dairy cows. Similarly, the inclusion of condensed or hydrolysable tannin in the diet had no influence on digestibility. Therefore, the inclusion of condensed or hydrolysable tannins in the diet of high yielding dairy cows have no influence on performance, whereas the type of forage has a varied effect on performance. A commercial advantage of using home grown forage legumes would be savings in nitrogen from fertilisers. Forage legumes have no requirement of fertilisers during growth and establishment due to the presence of nitrogen fixing bacteria within the root nodules. Through this system, nitrogen can be transferred from the forage legume to the next crop in the crop rotation reducing the use of fertilisers. A second commercial advantage would be the reduction in use of purchased dietary protein by the high yielding dairy cow as forage legumes in the current study reduced the quantity of soyabean meal used in the diet. Therefore, requirement for purchased dietary protein will be reduced but performance of a high yielding dairy cow will not be improved by home grown forages, whilst tannins will have little effect on milk performance.

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