Harper Adams University

A Thesis Submitted for the Degree of Doctor of Philosophy at

Harper Adams University

Harper Adams University

EFFECT OF SUPPLEMENTAL TANNIN ON SILAGE QUALITY AND ANIMAL PERFORMANCE

By

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(BSc & MSc)

A thesis submitted in fulfilment of the requirements for the award of the degree of Doctor of Philosophy by Harper Adams University.

June 2015

Declaration

This thesis has been composed by myself and has not been accepted in any application for any qualification or degree. The work in this thesis has been a record of work carried out by myself and all sources of information have been acknowledged by means of references.

Vahel Jaladet Taha

Abstract

Tannin has the ability to reversibly complex with forage proteins. A series of experiments were conducted to test the effect of supplementary chestnut hydrolysable tannin (HT) on a variety of forages to reduce protein degradability.

Experiment 1 investigated the effect of additional HT to different forages on ensiling characteristics and rumen degradability. Tannin was applied prior ensiling at either 40g kg^{-1}FW high tannin (HiT), or 20g kg^{-1}FW low tannin (LT). An inoculant treatment (*L. plantarum*) 10^6 $CFUg^{-1}$FW (Inoc) as a positive control and untreated (W) as a negative control. Water addition was unified by adding 0.5 l kg^{-1}FW and forages ensiled for 100d. Addition of tannin reduced ($P<0.01$) silage NH_3-N concentration (42, 49, 56 and 60g kg^{-1}TN for HiT, LT, Inoc and W respectively). Tannin supplementation was found to reduce ($P<0.05$) DM and CP effective rumen degradability

Experiment 2, evaluated the effect of additional HT (30g kg^{-1}DN) to ryegrass either at ensiling or feeding on lamb performance. Five experimental treatments were prepared: fresh grass supplemented with HT (30g kg^{-1}DM) at ensiling (GET), untreated ryegrass silage (G), ryegrass silage treated with inoculate (G+I), G plus additional tannin (30g kg^{-1}DM) at feeding (G+T) and G+I plus additional tannin (30g kg^{-1} DM) at feeding (G+I+T). Supplementation with tannin had no effect on lamb performance, however it reduced rumen NH_3-N concentration in diets receiving tannin (0.14, 0.19, 0.17, 0.17 and 0.14g l^{-1} GET G, G+T, G+I and G+I+T, respectively).

In experiment 3, lucerne silage was treated with four levels of HT (0, 25, 50 and 75 g kg^{-1}DM) and offered *ad libitum* to single bearing ewes in late pregnancy and early lactation. Additional 25 and 50 g kg^{-1}DM HT were found to increase ($P<0.05$) milk yield (2.5, 2.8, 2.6 and 2.4 L $d^{-1}$for 0, 25, 50 and 75g kg^{-1}DM respectively).

In conclusion HT reduced protein degradability inside the silage silo and animal rumen, plus increased milk yield was observed.

Statement of publications and conferences attended

The following conference paper have been presented by the author.

V.J. Taha, R.G. Wilkinson, D. Davies and J.A. Huntington.2013. Effect of supplemental tannin on *in situ* protein degradability in ensiled forages and prediction using a laboratory based protein fractionation technique. WCAP 11, October, 2013, Beijing, China.

V.J. Taha, J.A. Huntington, R.G. Wilkinson and D. Davies.2014. Effect of silage additive (tannin or inoculate) on protein degradability of legume and grass silage. Proceeding of the British Society of Animal Science, Annual conference. April 2014, Vol. 5 part 1 Pp 079. ISSN 2040-4700.

V.J. Taha, J.A. Huntington, R.G. Wilkinson and D. Davies.2014 Effect of tannin or inoculum as silage additives on silage quality and rumen fermentation kinetics. July, 2014. J. Anim. Sci. Vol 92- E-Suppl. J Dairy Sci. Vol. 97, E-Suppl.1 Pp 325-326.

V.J. Taha, R.G. Wilkinson, D. Davies and J.A. Huntington. 2015. Effect of supplementary tannin on fed intake and digestibility in ewes offered lucerne silage during late pregnancy and early lactation. British Society of Animal Science, Annual conference. April, 2015. Vol 6 part2 .Pp 160. ISSN 2040-4700.

V.J. Taha, R.G. Wilkinson, D. Davies and J.A. Huntington. 2015. Effect of supplemental tannin (chestnut) at feeding on rumen pH and protozoa number *in vivo*. British Society of Animal Science, Annual conference. April, 2015. Vol 6 part 2. Pp 160. ISSN 2040-4700.

Acknowledgments

First of all I would like to express my great appreciation to my director of study Dr. James A. Huntington for his guidance, support, encouragement, extreme patience and the most important, his friendship throughout my PhD study.

Also I would like to thanks my supervisory team Drs. Dave Davies and Robert G. Wilkinson for all their help, guidance, suggestions and criticisms.

I would like to acknowledge the Ministry of Higher Education and Scientific Research of Kurdistan Region Government (KRG) for funding my study and Harper Adams University for giving me this opportunity to study here.

I would like to thank all the staff of the sheep unit especially Giles Vince and Nicola Naylor, also I like to thank Dr. Trisha Toop, Dr. Marie Kirby and all the staff of Princess Margret laboratories especially Dr. Victoria Talbot and Mr. Amjad Ali.

The international student service staff especially Mrs. Sarah Estibeiro for her language support and Miss Jo Rawlinson for all her help.

To all postgraduate students for their help and friendship especially Jamal Khurshid, Nyaz Sulaiman, Sherwan Sefdeen, Stephen Mansbridge, Ghazwan Mahmood, Michele Farali, Robert Fillingham and Terry Ansah for all their huge help any time I needed them.

Finally, I have to remember the huge support, patience and help of my family including my wife Kasar, my daughters: Wan, Jeen and Shaven, my Mother Fawzia and my sibling Haliz and Bewar, thank you very much for your support and help.

Thanks a lot again to all of you.

Vahel

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LIST OF ABBREVIATIONS

AA	Amino acid
ADF	Acid detergent fibre
ADG	Average daily gain
BW	Body weight
CH_4	Methane
CHO	Carbohydrate
CO_2	Carbon dioxide
CP	Crude protein
CT	Condensed tannin
DM	Dry matter
ED	Effective degradability
EE	Ether extract
Fraction A	Soluble protein
Fraction a	Immediately soluble
Fraction b	Insoluble but potentially degradable
Fraction B1	Quickly soluble protein
Fraction B2	Intermediate soluble protein
Fraction B3	Slowly soluble protein
Fraction C	Un-digestible protein
Fraction c	Rate of degradation
g	Gram
GE	Gross energy
HT	Hydrolysable tannin
kg	Kilogram
LW	Liveweight
MCP	Microbial protein
MJ	Mega joule
N	Nitrogen
NDF	Neutral detergent fibre
NH_3-N	Ammonia nitrogen
NPN	Non-protein nitrogen
OM	Organic matter
P	Probability
PEO	Plant-derived essential oils
PPO	Poly phenol oxidase
RDP	Rumen degradable protein
SBM	Soybean meal
SED	Standard error of the difference
t	Tonne
TN	Total nitrogen
UDP	Undegradable dietary protein
VFA	Volatile fatty acids

CHAPTER 1. Literature review

1.1 Introduction

Microbial protein (MCP) is the main protein source in ruminant nutrition (AFRC, 1992) and contributes over 60 % of amino acids (AA) supplied to the small intestine (Bach *et al.*, 2005). However, additional dietary protein supply to the small intestine is necessary with high production animals (Howie *et al.*, 1996).

Plant by-products especially oilseed meals are considered the most important protein sources in ruminant diets (McKevith, 2005), due to their high crude protein (CP) content (170-700 g kg^{-1}DM) and relatively high biological value (AFRC, 1993). However, increasing the use of oilseed meals especially soybean meal (SBM) in non-ruminant animal nutrition (pig and poultry) has led to an increase in their demand (Jezierny *et al.*, 2010). USDA (2015) reported that the global oilseed cultivated area increased from 197.4 million ha in 2001 to 263.1 million ha in 2014, with an increase in their production from 326.8 to 532.8 million tonnes (t) in the same time period.

In the UK most SBM is imported due to unsuitable weather condition for cultivating soya bean (Wilkins and Jones, 2000). Entec (1998) reported that rapeseed is the main oilseed cultivated in the UK with a CP content of (400 g kg^{-1}DM) after oil extraction (AFRC, 1993). DEFRA (2014) reported that the UK production of oilseed rape increased by approximately 53 % from 2000 to 2014. Although the global production of oilseed plants has increased, the high demand has led to increases in prices (USDA, 2015). The price of SBM, rapeseed meal, maize gluten and brewers' grain increased by approximately 140, 80, 75 and 100 % from 2005 until 2014 (FARM BRIEF, 2014), which has

encouraged ruminant farmers to utilize alternative sources of home grown protein (pasture and forages) in ruminant nutrition.

Protein requirements of ruminants could be supplied by pasture and forage using good management and cultivation systems (Wilkins and Jones, 2000). Hart (2005) reported that a significant reduction in feed cost could be achieved by replacing SBM with grass and leguminous silage in ruminant nutrition (dairy cows) without compromising performance. However, forage protein is highly degradable in the rumen (700-800 g kg^{-1}DM; AFRC, 1993) and could result in an oversupply of dietary rumen degradable protein, without satisfying metabolisable protein (MP) requirements. In addition, there may be an energy cost associated with the excretion of excess ammonia (NH_3) of 0.8 MJ ME 100 g^{-1} NH_3 (Sinclair *et al.*, 2014). Reducing the rumen protein degradability of ensiled crops could improve the efficiency of utilization of dietary protein and carbohydrates by improving the synchrony of nutrient supply in the rumen (Sinclair *et al.*, 1993). Supplementing ruminant diets with plant secondary compounds such as tannins is one of the methods among several techniques that has been used to reduce protein degradability in the rumen (Grabber and Coblentz, 2009).

Tannins are described as water soluble polyphenolic compound with high molecular weight, that are found in various plant (Piluzza *et al.*, 2014). In general tannins can be divided into two main groups, condensed tannins (CT) and hydrolysable tannins (HT) (Lorenz, 2011). Tannins have been shown to create a reversible bonds with different compounds including proteins, carbohydrates and minerals (McSweeney *et al.*, 2001) with the ability of both types to create a tannin-protein complex. The dietary tannin-protein complex

is considered to be stable at a rumen pH (6-7). However, as the post ruminal pH reduces in the abomasum (pH< 3.5) or rises in the small intestine (pH> 7), protein is released from this complex facilitating digestion of dietary protein in the small intestine (Frutos *et al.*, 2004). McMahon *et al.* (2000) reported that the absorption of dietary AA in the small intestine was increased when the diet contained tannin. Makkar (2003) suggested that the use of <50 g kg^{-1}DM of tannin as a silage additive could increase protein utilisation and enhance animal performance. In addition, improvements of silage quality (low pH and NH_3-N) have been reported when tannin was incorporated at the point of ensiling (Salawu *et al.*, 1999; Tabacco *et al.*, 2006). Condensed tannins are the most widely observed tannin in nature, especially in forages and hence, are the more commonly used tannin in ruminant nutrition (Naumann *et al.*, 2013). There is a paucity of published literature with regard to the use of HT as silage additives or rumen modifiers on animal performance and little available data with regarding the optimal dose of HT without being toxic to the animal (Katiki *et al.*, 2013).

The aim of this study was to test the hypothesis that:

- Chestnut HT supplementation to different forages at ensiling will reduce protein hydrolysis during ensiling.
- Supplementing grass and leguminous silages with chestnut HT, will increase rumen bypass protein and enhance ruminant performance.
- Inclusion of chestnut HT will alter rumen fermentation.

1.2 Protein digestion in the rumen.

The rumen could be described as a large anaerobic fermentation chamber containing diverse types and numbers of microorganisms as detailed in Table 1.1 (Lean *et al.*, 2014).

Table 1. 1 Numbers and function of rumen microorganisms.

Rumen microbes	Number	Functions
Bacteria	10^{10} cells ml^{-1}	Ferment and degrade diets
Protozoa	10^{5}-10^{6} cells ml^{-1}	Ferment and degrade diets, engulf bacteria and starch particles
Archaea	10^{7}-10^{9} cells ml^{-1}	Hydrogen metabolism
Fungi	10^{3}-10^{5} zoospores ml^{-1}	Fibre degradation using cellulolytic enzymes
Bacteriophages	10^{8}-10^{9} cells ml^{-1}	Infect bacteria

(Lean *et al.*, 2014)

Rumen microbes are capable of converting low quality dietary feed into volatile fatty acids (VFA), microbially degraded dietary protein and microbial protein (MCP) as shown in Figure 1.1 (Bach *et al.*, 2005). Bahrami-Yekdangi *et al.* (2014) suggested that most dietary CP is either converted to microbial amino acids (AA) which is the primary source of AA to ruminant animals, or used as a source of energy, with the final products being VFA and carbon dioxide (CO_2). However, high protein degradation in the rumen could lead to digestive disorders which may result in death of the animal and/ or could have negative influences on the environment due to greenhouse gases emissions such as methane (CH_4) and CO_2 and financial loss (Zhang and Yu, 2012).

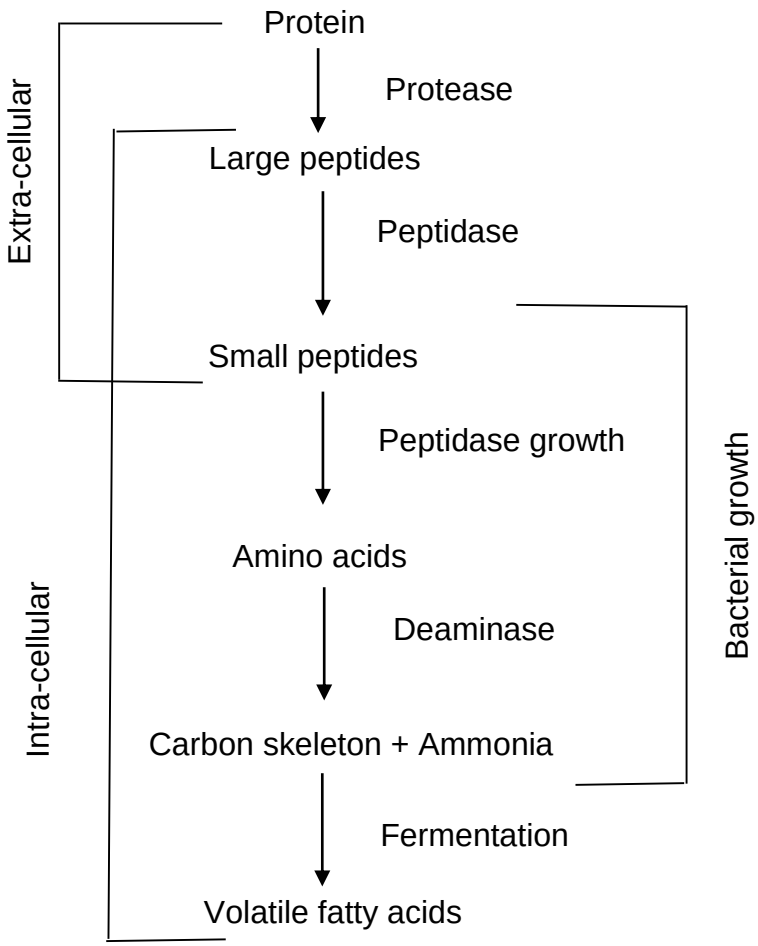

Figure 1.1 Dietary protein degradation and fermentation in the rumen (adapted from Leng and Nolan, 1984)

Plant enzymes released from the plant cells during harvest and ensiling or during mastication include protease enzymes which are responsible for initiating peptide bond breakdown in plant proteins (Brodrerick *et al.*, 1991; Givens and Rulquin, 2004). As a result both protein solubility and rumen digestibility increase. In addition, plant proteases have been found to increase silage protein solubility from 200 up to 400 g kg^{-1}DM depending on the wilting period, environmental temperature and additives at ensiling (Givens and Rulquin, 2004).

Several studies (Tamminga, 1979; Bach *et al.*, 2005; Firkins *et al.*, 2007) have reviewed the process of protein digestibility in the rumen. They suggested that both rumen bacteria and protozoa play a major role in dietary protein digestion in the rumen. Craig *et al.*(1987) showed that more than 70 % of rumen microbes have the ability to attach to dietary feeds and approximately half of these microorganisms have proteolytic activity. Figure 1.2 presents a schematic diagram showing the sequential degradation of dietary protein (Brodrerick *et al.*, 1991).

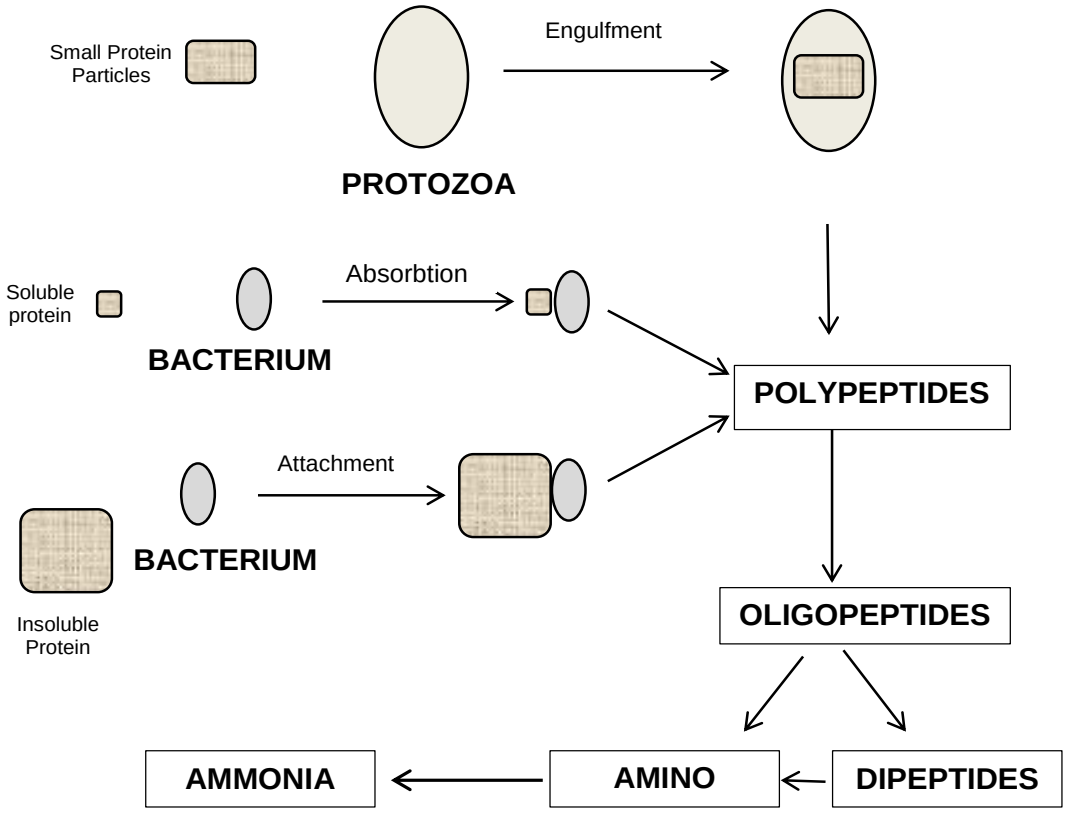

Figure 1.2 Mechanism of actions of digestion dietary protein by rumen microorganism (Brodrerick *et al.*, 1991).

Microbial biomass in the rumen consists of bacteria, protozoa and fungi (Tamminga, 1979). Rumen bacteria generally constitute the most microbial biomass (10^9-10^{10} cell ml^{-1}) in the rumen with over 200 species. Most rumen bacteria species (with the exception of some cellulolytic species) have the ability to digest dietary protein in the rumen (Theodorou and France, 2005). Rumen bacteria are considered the main microorganisms responsible for protein digestion in the rumen (Nugent and Mangan, 1981).

Rumen protozoa include over 100 species with a microbial biomass which could reach 40 % in some animals (10^5-10^6 cell ml^{-1}) (Theodorou and France, 2005). Hobson *et al.* (1982) explained that rumen protozoa have a lesser role in dietary protein digestion in the rumen than rumen bacteria, and most protozoal nitrogen and AA requirements are derived by engulfing other rumen microbes. Theodorou and France (2005) reported that the function of rumen protozoa is not completely clear due to their sensitivity to surviving in *in vitro* studies. The rumen fungi population ranges from 10^3-10^5 cell ml^{-1} with approximately 8 % rumen microbes biomass, consisting of a unique group of cellulolytic microorganisms, without any important roles in protein digestion in the rumen (Theodorou and France, 2005).

Rumen proteolytic bacteria attach to feed particles and start to secrete proteolytic enzymes (Lean *et al.*, 2014). Bacterial proteolytic enzymes are responsible for degrading the extra cellular protein which are usually associated with the plant cell wall (Allison, 1970; Kopencny and Wallace, 1982).

In the early stages of bacterial attachment to protein particles, the bacterial cell secrets proteolytic enzymes (Broderick *et al.*, 1991). Subsequently, free AA and short peptides might transfer into the bacteria cell (absorbed by rumen bacteria), the short peptide units may be further degraded to free AA which are then incorporated into bacterial protein synthesis (Buxton, 1991). In addition free AA inside bacteria cells may be further degraded by deamination and provide a source of energy to the bacteria, as shown in Figure 1.3. Bach *et al.* (2005) and Peng *et al.*(2014) stated that the extent of protein degradation depends on the availability of fermentable energy which is usually produced by degradation of carbohydrates. Hence, if fermentable energy is limited, free AA could be used as an energy source by the rumen microbes (Bach *et al.*, 2005).

Protozoa are larger than bacteria and have the ability to engulf bacteria and small feed particles (Lean *et al.*, 2014). Digestion of dietary and bacterial protein occurs inside the protozoal cells and free AA produced during digestion of the bacteria are used to form new protozoal AA, peptides and proteins; unused AA may be secreted into rumen fluid again (Theodorou and France, 2005).

Tamminga (1979) reported that dietary protein degradability was limited by the proteolytic process. However, Russell and Sniffen (1984) suggested that microbial uptake of peptides rather than AA was the rate limiting step of protein digestibility in the rumen. Brodrerick *et al.* (1991) and Cardozo *et al.* (2004) suggested that protein degradation and deamination may have an important role in controlling protein digestion in the rumen. They found that the

accumulation of peptides and AA in the rumen fluid peaked within 2-4 hours post feeding.

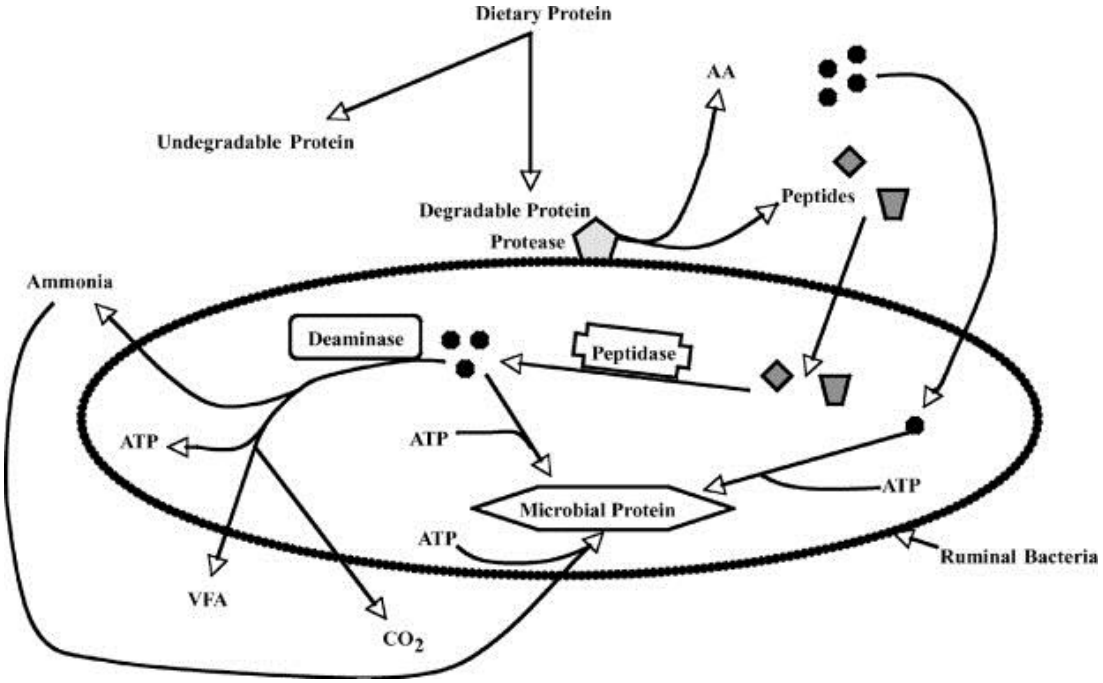

Figure 1.3 Schematic representation of protein degradation and fate of end products in the rumen bacteria.◆: peptides, ●: free amino acids, (Bach *et al.*, 2005)

1.2.1 Factors affecting protein digestion in the rumen:

Factors affecting protein digestibility have been extensively reviewed (Tamminga, 1979; Van Straalen and Tamminga, 1990; Bach *et al.*, 2005). The main factors could be summarized as:

- Feed factors
- Rumen factors

1.2.1.1 Feed factors

Protein type, solubility, structure and pre feeding process could be considered as the major factors affecting digestibility of protein in the rumen (Doiron *et al.*,

2009; Theodoridou and Yu, 2013). Protein digestion in the rumen may also be affected by plant cells protease enzymes (Theodoridou and Yu, 2013) eating behaviour and weather conditions (Samadi and Yu, 2011; Peng *et al.*, 2014). Approximately 75 % of forage N is in the form of true protein, while the rest is considered to be non-protein nitrogen (NPN). The ratio of true protein to NPN in forage crops was found to vary according to forage type, age, time of harvesting, dry matter and fertilizer application (Tamminga, 1986; Givens and Rulquin, 2004).

Wohlt *et al.* (1976) showed that most protein in ruminant diets consists of one or a combination of the following protein types: albumins, globulins, prolamins and glutenlins. Albumins and globulins are considered as soluble, while glutelins and prolamins are less soluble protein. The rate and extent of protein digestion depends on the relative proportions of different protein types (Romano-Ely *et al.*, 2006). Van Straalen and Tamminga (1990) suggested that all of the soluble and approximately half of insoluble protein are degradable in the rumen depending on feed type and outflow rate from the rumen. However, Givens and Rulquin (2004) reported that not all the soluble protein counted as degradable protein; part of the soluble protein (~100 g kg^{-1}total protein (TP)) could escape directly into the lower gut. Peng *et al.* (2014) studied protein solubility, rumen degradability and intestinal digestibility of eight feedstuffs used in ruminants' diets (lentil, peas, canola meal, expeller meal, dried corn, SBM, meat meal and blood meal). They (Peng *et al.,* 2014) found significant variation between the different feedstuffs in CP content (221-967 g kg^{-1}DM), water soluble CP (*in vitro*) (105-703 g kg^{-1}CP), buffer soluble CP (*in vitro*), and total digestible protein (198-783 g kg^{-1}CP), and hypothesized that the

observed differences related to different intrinsic molecular structures within different feedstuffs.

The structure of the protein, the bond types that join AA together and the presence of bonds between and within AA chain (tertiary and quaternary) have a major effect on protein solubility (Bach *et al.*, 2005). Although albumins are soluble and highly degradable proteins, some kinds of albumins have disulphide bonds which make them more slowly degradable in the rumen fluid (Stern *et al.*, 2006). In addition, the types of peptide bonds may affect the way in which they are hydrolysed in the rumen (Yang and Russell, 1992). For example, Lys-Ala dipeptide bonds (amide bond between lysine and alanine) are 5 times more degradable than Lys-Pro (amide bond between lysine and proline) dipeptide bonds. Similarly, dipeptides bonds comprising of Met-Ala (amide bond between methionine and alanine) are 2.5 times more degradable than dipeptides comprised of Pro-Met bonds (amide bond between proline and methionine). Moreover, the size of proteins in terms of the number of AA that compose these proteins, plus side chains or extensive cross-linking "disulphide bonds" in their structure, can have an effect on digestion. Increasing the number of AA and the side chains would be more resistant to rumen degradation. For example keratin in feather and hair protein (Velle *et al.*, 1998).

1.2.1.2 Rumen factors

Rumen factors are complex and interrelated (Dijkstra *et al.*, 2012). However, the varied factors that affect rumen protein digestibility will be discussed independently.

Rodriguez *et al.* (2008) found that increasing dry matter intake (DMI) from 40 to 80 g DM^{-1}kg empty body weight ($kg^{0.75}$) reduced apparent effective degradability (ED) by approximately 14.8, 11, 7, 13 and 17 % for corn grain, SBM, sunflower meal, vetch-oat hay and lentil straw respectively, while DMI has no effect on the soluble and insoluble fraction in the rumen (Rodriguez *et al.*, 2008).

Several studies (Lindberg, 1982; Andrade-Montemayor *et al.*, 2009) emphasized that feed intake has a major effect on rumen retention time of dietary ingredients. Protein digestion depends on rumen outflow rate into the small intestine (Warner *et al.*,2013). Bach *et al.* (2005) concluded that increasing the retention time of dietary protein in the rumen increased protein digestibility because of increased exposure to rumen microbial activity. Other factors affecting rumen outflow rate include animal species, diet ingredients (Hartnell and Satter, 1979; Warner *et al.*, 2013), diet particle size and physiological state of the animal (Huhtanen *et al.*, 2007). A study by Kramer *et al.* (2013) supports the evidence that rumen outflow rate could be affected by external factors (diet type including protein type and proportion and availability of energy) and/or internal factors (particle size, rumen liquidity, specific gravity and animal physiology). Tamminga *et al.* (1979) studied the effect of DMI on rumen outflow rate, and used three dairy cows fitted with rumen fistula and small intestinal re-entrant cannula. Cows were fed meadow hay and ground and pelleted concentrates with two levels of DMI 8.6 and 12.9 kg d^{-1}. Tamminga *et al.* (1979) found that degradability was reduced from 74 % to 58 % when DMI increased from 8.6 to 12.9 kg d^{-1} respectively. However, other studies have observed small or no effect of increased DMI on protein

degradation (Miller, 1973; Hartnell and Satter, 1979; Varga and Prigge, 1982 and McAllan; Smith, 1983).

Optimum rumen fluid pH for forage degradation has been cited (Franzolin *et al.*, 2010; Dijkstra *et al.*, 2012) to be in the range of pH 6.0- 7.0. Rumen pH was affected by forage type, feed intake, animal species and water intake (Franzolin *et al.*, 2010). Rumen pH is related to fermentation rate and VFA production (Dijkstra *et al.*, 2012), especially if the VFA production cannot be buffered by sufficient saliva secretion (Plaizier *et al.*, 2008) when animals consume diets containing a high percentage of highly fermentable carbohydrate. In addition, a rumen pH of 5.0-5.6 was considered to be an indication of sub-acute rumen acidosis (SARA), while less than pH 5.0 was considered to be acidosis (Krause and Oetzel, 2006).

A low rumen pH has been shown to have a negative effect on feed intake, rumen microbial activity and nutrient degradation (Krause and Oetzel, 2006; Enemark, 2008). Dijkstra *et al.* (2012) observed that the optimum pH for cellulolytic microorganisms was pH 6.2-6.7, if rumen pH drops below pH 6.0 cellulose degradation would reduce. Calsamiglia *et al.* (2008) found a reduction in fibre degradation (*in vitro*) when the pH of the solution was reduced from pH 7 to 4.9. Protein degradation was reduced when the pH was less than 6.0, due to the sensitivity of proteolytic bacteria to the acidic environment (Bach *et al.*, 2005). Cardozo *et al.* (2002) studied the effect of pH manipulation (*in vitro*) on the protein degradability of two high protein level diets (forage vs. concentrate), and demonstrated that protein degradation was reduced when the pH of a continuous culture fluid was reduced from 7.0 to 4.9. Lana *et al.* (1998) also observed a reduced NH_3-N concentration in

continuous culture fluid when the pH was reduced from pH 6.5 to 5.7. Strobel and Russell (1986) found that microbial growth reduced significantly (up to 50 %) when rumen pH was reduced to 5.7, concluding that bacteria would use most of the available energy to resist the acidic condition instead of using it in bacterial growth and activity. Rumen microbes may stop degrading structural carbohydrate (CHO) when the rumen pH falls below 5.8 (Storm and Ørskov, 1983; Pitt *et al.*, 1996).

Theodorou and France (2005) reported that rumen protozoa are more sensitive than bacteria to the changes in rumen pH, and they cannot survive if the pH rises above pH 8 or falls below pH 5. Dehority (2005) also found a reduction in protozoa number when the pH decreases to pH 5.4.

Rumen proteolytic activity has been found to be affected by the presence of non-proteolytic enzymes such as amylase. Bach *et al.* (2005) suggested that different rumen enzymes may have associative effects. Similarly, research by Assoumani *et al.* (1992) and Tomankova and Kopecny (1995) reported that protein degradation was enhanced in the presence of amylase or cellulase. Tomankova and Kopecny (1995) used three different types of protease enzymes (papain, bromelain and pronase) as enzymatic methods for measuring protein degradation of thirteen different ruminant feedstuffs. The results showed that protein degradation was greater when 0.5 mg of alpha-amylase enzyme was added to the solution, especially with cereals. Tomankova and Kopecny (1995) went on to conclude that starch could be one of the main limiting factors affecting the availability of protein to rumen microbes.

Kohn and Allen (1995) studied the degradation of SBM protein. The study showed that incubating SBM (*in vitro,* 16 h at 39^{o}C) with a combination of protease and cellulase enzymes resulted in higher protein degradation compared to the addition of the individual enzymes or control (no enzyme) (51.2, 42.4, 32.9 and 40.9 %, combination, protease, cellulose, control respectively). However, the addition of alpha-amylase was not found to exert an effect. This suggested that the lack of response to additional alpha-amylase was due to sufficient inherent amylase activity within the cultures. Endres and Stern (1993) stated that plant proteins are trapped with other nutrients such as fibres, therefore, preventing proteolytic bacteria from degrading proteins. Other enzymes may be required to release proteins for subsequent digestion. Thus, for maximizing protein degradation a combination of several proteolytic and non-proteolytic microbes may be needed.

1.2.2 Methods of estimating protein degradability in the rumen.

1.2. 2.1 In vivo techniques for estimating protein degradability

Assessment of dietary rumen protein degradability (*in vivo*) has been reported by Van Straalen and Tamminga, (1990) and Brodrerick *et al.* (1991). This method involved the use of animal fitted with abomasum or proximal duodenum cannula and the digestate samples were removed directly from either the proximal duodenum or abomasum (Brodrerick *et al.*, 1991; Robinson *et al.*, 1992; Gosselink *et al.*, 2004b). In addition, the technique has been refined further by including a cannula fitted in the ileum and corrected for MCP (where by the MCP content of rumen digestate was determined and subtracted from total digestate CP to facilitate the measurement of true dietary CP

content) (Gosselink *et al.*, 2004a). Rumen degradable protein was then estimated by the differences between total dietary protein and duodenal digesta protein after the MCP was subtracted from the digesta protein (Brodrerick *et al.*, 1991).

Robinson *et al.* (1992) reported that estimating carbohydrate degradability using the *in vivo* method could provide good information; however, estimating protein degradation would be more complicated due to contamination with rumen microbial nitrogen and endogenous animal nitrogen (mostly nitrogen involved in enzymes) secretion. Therefore, this technique could be a good method for estimating the total rumen protein supply to the small intestine which would be a mix of dietary protein, microbial and endogenous N, rather than estimating dietary undegradable protein.

In addition, this technique was prone to error arising from problems associated with acquisition of a representative sample and the time of sample collection, plus estimation of microbial and endogenous nitrogen contamination (Van Straalen and Tamminga, 1990). Robinson *et al.* (1992) suggested that using double duodenal cannula (T-shape) would provide an accurate estimation of digesta flow, however, using two piece duodenal cannula could have a negative influence on animal physiology.

For estimating rumen degradability (*in vivo)* a number of indigestible markers have been used including internal markers (such as lignin, indigestible fibre and acid insoluble ash) or external markers such as (Chromium cobalt complex, chromic oxide complex, lanthanum, ytterbium and Dysprosium). In addition, Robinson *et al.* (1992) reported that researchers have used several

microbial markers in order to estimate microbial nitrogen contamination, such as nucleic acids (RNA and DNA) unique bacteria and protozoal AA (DAPA and amino ethyl-phosphoric acid (AEPA) for bacteria and protozoa, respectively), protozoa unique phospholipid (phosphatidylcholine (PC)), radioisotopes (S^{35} and P^{32}) and heavy isotopes (N^{15}).

Gosselink *et al.* (2004a) estimated rumen escape protein *in vivo* for 11 different forages using six wether sheep fitted with rumen, duodenum and ileum cannulas, a double marker technique was used as described by Remond *et al.* (2003) with two different microbial markers (N^{15} from duodenal digesta flow plus urinary purine derivatives). Gosselink *et al.* (2004a) noted a 31 % difference between the 2 marker techniques for estimating rumen escape protein of all forages on their study. They (Gosselink *et al.*, 2004a) concluded that the error in estimation was due to estimated endogenous contamination (Gosselink *et al.*, 2004a). This method has several limitations, including maintaining the cannula requires experience, high level of animal care and time (Robinson *et al.*, 1992).

1.2.2.2 In situ technique

The *in situ* nylon bag method has been extensively reviewed by Huntington and Givens (1995) and Nocek (1988). Huntington and Givens (1995) reported that historically, Quin *et al.* (1938) were the first to discuss the possibility of measuring rumen degradability of animal feeds by placing samples of feedstuff in indigestible porous bags and incubating them inside the rumen via cannula. Nocek (1988) suggested that the *in situ* determination involves the use of animals fitted with rumen cannula. In this method samples of feedstuff (pre-

recorded weight) are placed in the nylon bags (with standard pore size) and incubated in the rumen via cannula for different incubation times. The incubated nylon bags are then removed from the rumen, washed and dried and the incubated feed disappearances measured (Nocek, 1988).

Huntington and Givens (1995) reported that using the *in situ* technique the following parameters could be estimated: the immediately soluble fraction “a”, insoluble but degradable fraction “b”, the rate of degradation fraction “c”, lag time, the effective rumen degradation (ED), rumen degradable protein (RDP) and undegradable dietary proline (UDP) according to the model published by McDonald (1981).

AFRC (1992) published a standardised method that has been the widely accepted for calculating rumen degradability for feed evaluation. The *in situ* technique has been shown to provide an accurate estimate of rumen degradability compared to *in vivo* determination (r^2=0.8-0.92) (AFRC, 1992; Huntington and Givens, 1995). Huntington and Givens (1995) reported that fewer animals are required for the estimation of *in situ* rumen protein degradability, however this technique is expensive, labour intensive and necessitates the use of surgically modified animals (Shannak *et al.*, 2000; Kirchhof *et al.*, 2010; Edmunds *et al.*, 2012). Furthermore, this technique has some limitations including microbial contamination which have been described by Lanzas (2007). Varvikko and Lindberg (1985) suggested that incubated samples in the rumen should be corrected for microbial N contamination. Hart (2005) reported that estimating protein degradability *in situ* for samples with low N concentration such as straw would result in an underestimation of 1285% after only 24 h incubation in the rumen due to microbial protein

contamination, while estimating CP degradability of feedstuff with relatively high CP content (120-250 g kg^{-1}DM) the under estimation would be no more than 0.7 % after 24 h incubating in the rumen.

1.2.2.3 In vitro technique

In vitro techniques for the estimation of protein degradability that do not require the use of surgically modified animals have been developed by Broderick (1987), Sniffen *et al.* (1992) and Licitra *et al.* (1996). Feedstuff protein can be divided into three fractions according to protein solubility in various chemical solutions to provide a measurement of NPN, true protein and un-available protein (Van Soest *et al.*,1981). Estimation of protein degradability using enzymatic breakdown or solubility has been developed by Sniffen *et al.* (1992) and Licitra *et al.* (1996). The Cornell Net Carbohydrate and Protein System (CNCPS) published by Sniffen *et al.* (1992) based the estimation of dietary protein degradability on these three fractions, where NPN is fraction A, true protein is fraction B and bound true protein is fraction C. True protein is further sub divided into: quickly (B1), intermediate (B2) and slowly (B3) soluble proteins, based on their solubility in the rumen (Van Soest *et al.*, 1981).

Fraction A and B1 are soluble in buffer solution, where B1 is measured as trichloroacetic acid (TCA)-precipitate. Fraction B2 is buffer insoluble protein but soluble in neutral detergent solution. Fraction B3 is insoluble in neutral detergent solution but soluble in acid detergent solution (protein associated with cell wall which is slowly degradable in the rumen) and fraction C is protein which is insoluble in acid detergent solution, and counted as indigestible protein (protein associated with lignin, tannin and Maillard products), (Sniffen

et al., 1992). Several studies (Shannak *et al.*, 2000; Gosselink *et al.*, 2004a; Edmunds *et al.*, 2012) found a close linear relationship (r^2=0.5-0.9) between protein fractionation using the CNCPS and *in situ* technique.

Sample preparation could be a source of error for estimating protein degradability using the CNCPS protein fractionation technique due to the formation of Mailliard complexes (Kirchhof *et al.*, 2010). Abdalla *et al.* (1988) observed an increase in approximately 45 % in the slowly degradable (B3) and indigestible protein (C) fraction, and a decrease of about 20 % in the NPN and rapidly degradable protein fraction (A) when using oven dried samples (55^{o}C), compared to freeze dried samples. Deinum and Maassen (1994) found that fraction B3 and C nearly increased by 100 % in lucerne and ryegrass when they prepared their samples at 30^{o}C compared to freeze dried preparation. Kirchhof *et al.* (2010), Lorenz *et al.* (2010) and Edmunds *et al.* (2012) recommended using freeze dried samples when CP solubility was measured using the CNCPS protein fractionation technique. In addition, Shannak *et al.* (2000) reported that the use of the CNCPS protein fractionation technique published by Sniffen *et al.* (1992) did not provide any equations for estimating UDP.

Shannak *et al.* (2000) and Kirchhof (2007) developed regression equations for predicting UDP using the CNCPS protein fractionation technique at rumen outflow rates of 0.02, 0.05 and 0.08 h^{-1}. However, these equations have a heavy reliance on the B1 fraction, and are complicated. In addition different equations are required for estimating protein degradability at different rumen outflow rates. Creation of a simple single general equation of estimating UDP using the CNCPS protein fractionation technique would be of great benefit.

1.3 Protein supply to the small intestine

1.3.1 Protein supply from rumen microbes

It has been reported (Storm and Ørskov, 1983; Clark *et al.*, 1992) that 35-85% "60 % on average" (depending on the consumed diet) of AA reaching the small intestine are from rumen microbial origin. AFRC (1992) reported that microbial protein should be considered as one of the most important protein sources for ruminants. In addition, the possession of rumen microbes gives ruminants the ability to use low quality diets such as fibre and NPN to convert it to MCP with high biological value that could then be used for milk production and muscle growth (Dewhurst *et al.*, 2000). McAllan *et al.* (1994) found that MCP synthesis was 31.8 and 49.0 g microbial N kg^{-1} rumen degraded OM of grass and concentrate respectively. NRC (2001) reported that legume silage has lower MCP synthesis compared to maize silage, grass silage and cereal silages (19.5, 48.4, 30.1 and 35.9 g N kg^{-1} degraded OM, respectively).

Saro *et al.* (2014) found that feeding rumen cannulated sheep different types of forage hay (lucerne hay vs. grass hay) had a significant effect on rumen fermentation, and the number and species of rumen microbes. Similarly, Givens and Rulquin (2004) reported that MCP synthesis ranged from 30-40 g microbial N kg^{-1} degraded OM in the rumen. They went on to show that the efficiency of MCP synthesis was increased when the ratio of concentrate: forage in the diet was increased (Givens and Rulquin, 2004).

Block (2006) found that the AA profile of MCP and milk protein were more similar than the AA profiles of dietary protein (Table 1.2).

Table 1.2 Comparison between some essential AA of milk, bacteria, canola meal, fish, blood and corn meals.

Item	Milk	Rumen Bacterial	g kg^{-1}AA Canola meal	Fish meal	Blood meal	Corn meal
Arginine	**34**	**51**	50	57	41	33
Lysine	**75**	**79**	51	75	88	20
Threonine	**44**	**58**	47	43	43	35
Isoleucine	**58**	**57**	32	27	15	38
Valine	**63**	**62**	40	33	75	43
Histidine	**26**	**20**	20	20	63	19
Leucine	**83**	**81**	78	70	128	181
Phenylalanine	**46**	**51**	41	38	66	66
Methionine	**25**	**26**	19	30	11	26

Adapted from (Block, 2006)

Gamage *et al.* (2012) reported that 0.75 of MCP was true protein and 0.25 nucleic acids. They went on to suggest that the digestibility of MCP (true protein) that reached to small intestine was 0.85, hence 0.64 of total MCP that reached small intestine was absorbed by the animal (Gamage *et al.*, 2012). Although MCP contributes over two thirds of the protein flowing to the small intestine, additional dietary protein supply may be required especially for highly productive animals (Howie *et al.*, 1996).

1.3.2 Protein supply from dietary sources

1.3.2.1 Animal by-product sources.

Animal by-products are a useful dietary protein source (500-900 g kg^{-1}DM) with good AA profile including essential AA and source of vitamins plus trace elements (NRC, 1998). However, the use of animal by-products in ruminant nutrition has been banned across European Union since 2001 (European Commission directive, 999/2001) as a result of the diagnosis of bovine spongiform encephalopathy in the UK and some other European countries.

1.3.2.2 Plant sources

Ruminant dietary protein form plant sources could include protein provided from plant by-product such as food and drink manufacturers, cereals, whole crops and forages (including pastures). Most of the plant by-products derived from food and drink industries have a high CP content. For example maize gluten meal has a protein content of 600-700 g kg^{-1}DM, brewer's grains from both alcoholic and non-alcoholic drinks 170-320 g CP kg^{-1}DM and oil seed meals 400-500 g CP kg^{-1}DM (AFRC, 1993).

Demand for high quality plant protein such as SBM has led to increased production globally. USDA (2015) reported that the global oilseed cultivated area had increased from 197.4 million hectare (ha) in 2001 to 263.1 million ha in 2014, with an increase in production from 326.8 to 532.8 million tonnes (t) in the same time period. Although the global production of oilseed plants has increased, the high demand has led to increases in prices from 323 \$US t^{-1} in 2001 to 592 \$US t^{-1} in 2014 (USDA, 2015).

Soybean meal (CP 350-550 g kg^{-1}DM) was noted to be the predominant protein source used in livestock diets (USDA, 2002). Willis (2003) concurred with these findings, reporting that SBM was the most widely used dietary protein source for livestock (ruminant and non-ruminant) around the world. Cutrignelli *et al.* (2001) also reported that in beef feeding SBM may be considered the major source of supplemental dietary protein. USDA (2015) reported that imported SBM in the EU has increased from 16 million t in 2000 to over 25 million t in 2014. Other by-products may be used as a source of

dietary protein including sunflower meal, sesame meal, cottonseed meal, linseed meal, groundnut meal and rapeseed meal (USDA, 2002).

Oilseed protein content and quality has been shown to vary according to a variety of factors including cultivation, harvesting and extraction methods (Willis, 2003). Entec (1998) reported that meal from oilseed rape processing was the main by-product used for ruminant nutrition in the UK. Rapeseed meal produced in the UK has been reported to have a CP content of 400 g kg^{-1}DM after oil extraction (AFRC, 1993). DEFRA (2014) reported that the UK production of oilseed rape increased by approximately 53 % from 2000 to 2014 (the production was 1.157 million t in 2000 and 2.5 million t in 2014). Increased yield was a result of both an increase in cultivated area (more than 40 % between 2000 and 2014 from 402000 ha in 2000 to 674000 ha in 2014) and yield t ha^{-1} (~25 % between average yield, 2.9 t ha^{-1} in 2000 while in 2014 the average yield had increased to 4 t ha^{-1}).

Wilkins and Jones (2000) have shown that beans and peas are traditional crops grown in the UK, ranking 3rd and 4th respectively in legume grain produced globally after soybean and peanuts (Vidal-Valverde *et al.*, 2003). However, in the UK beans and peas represented only 0.5 % of ruminant dietary protein because they have predominantly been used in human nutrition (Wilkins and Jones, 2000). Beans and peas grains are characterised as having a relatively high amount of energy due to their starch content (~430 and 500 g kg^{-1}DM, respectively) rather than CP (Cutrignelli *et al.*, 2001). In addition legume CP is characterised as being highly degradable in the rumen with a low supply of RUP in the small intestine (Wilkins and Jones, 2000).

Furthermore, Cutrignelli *et al.* (2001) stated that large parts of legume grains contain anti-nutritional factors (trypsin inhibitors, lecithin, saponin, phytase and tannin) which may have a negative impact on microbial protein synthesis in the rumen and digestion in the lower tract. Jezierny *et al.* (2010) showed that grain legumes, especially peas and beans, contain plant secondary metabolism compound. For example coloured flowered peas and beans have up to 35.5 g kg^{-1}DM condensed tannin, 25.5-39.3 g kg^{-1} saponin, trypsin inhibitor (0.2-3.9 and 0.2-2.9 trypsin inhibitor unit for faba beans and peas respectively). The chemical composition and AA profile of some feedstuff by-products are presented in Table 1.3 and 1.4.

Table 1.3 The chemical composition for some plant-by product meals.

Item	Plant by-products meals and some grain legumes				
	Soybean*	Rapeseed**	Bean***	Pea***	Canola*
DM ($g\ kg^{-1}$)	920	899	881	874	928
Composition ($g\ kg^{-1}$DM)					
OM	935	923	962	967	918
CP	474	402	308	249	373
RDP ($g\ kg^{-1}$CP)	628				694
RUP ($g\ kg^{-1}$CP)	372				305
NDF	180	295	140	111	335
ADF	93	206	119	79	187
Fat	30	21	15	20	33

* : Bahrami-Yekdangi *et al.*, 2014; **: HGCA, 2003; ***: Jezierny *et al.*, 2011

Jezierny *et al.* (2010) reported that the high percentage and biological value of CP of oilseed meals is associated with the AA profile content, and has increased the demand for their use in livestock nutrition, especially non-ruminant animals. Therefore, FARM BRIEF (2014) reported that the prices of plant by-products have been increasing. The price of SBM, rapeseed meal,

maize gluten and brewers' grain increased by approximately 140, 80, 75 and 100 % from 2005 until 2014 (FARM BRIEF, 2014). Increases in the prices of plant by-products have encouraged ruminant farmers to find alternative cheap home grown protein as a source of ruminant dietary protein such as forages.

Table 1.4 Crude protein and AA profile for some plant by-products.

	Plant by-products meals and some grain legumes				
Item	Soybean**	Rapeseed**	Bean*	Pea*	Lupinus*
CP $_{(g\ kg^{-1}\ DM)}$	541	350	301	246	361
AA $_{(g\ kg^{-1}DM)}$					
Methionine	6.9	7.2	2.2	2.2	2.0
Cysteine	6.9	2.1	3.5	3.5	4.8
Lysine	33.4	21.9	18.4	17.3	16.3
Threonine	20.5	16.5	10.5	9.1	11.9
Isoleucine	25.2	14.8	11.8	10.0	14.2
Valine	28.8	18.7	13.3	11.4	13.6
Tryptophan	7.4 *	4.7	2.6	2.2	3.0
Arginine	39.0	21.5	26.4	21.0	38.0
Histidine	15.7	11.2	7.8	6.1	9.7
Leucine	40.4	25.1	21.4	17.4	24.1
Phenylalanine	26.9	15.4	12.6	11.7	13.6
Glycine	21.7	18.1	12.2	10.6	14.3

*: Degussa (2006), **: HGCA (2003)

Protein requirements of ruminants could be supplied by pasture and forage with good management and cultivation systems (Merry *et al.*, 2001). However, forage protein quality may compromise AA supply to the small intestine (Wilkins and Jones, 2000). The use of different dietary protein source in the UK for ruminants are shown in Table 1.5.

Wilkins and Jones (2000) stated that grassland provides approximately 72 % of dietary protein to ruminant diets in the UK. Entec, (1998) reported that UK climatic conditions result in grassland providing the main and cheapest source of dietary protein for ruminant diets.

Table 1.5 Supply of dietary protein sources in the UK in 1995.

Source	'000 t annum^{-1}	Proportion %
Grassland feeds	4150	72
Cereals	800	14
Oilseeds	567	9
Maize gluten	175	3
Beans and peas	21	0.2

(Wilkins and Jones, 2000)

DEFRA (2014) reported that approximately 18.4 million hectares was the total agricultural land utilized in the UK in 2014 (Table 1.6).

Table 1.6 Utilized agriculture area in the UK in 2014.

Utilized agriculture area	'000, hectares
Permanent grassland and rough grazing	9710
Common rough grazing	1200
Temporary grass	1385
Cereals	3172
Oilseeds	691
Other crops	717
Other land of agriculture holdings	1210

(DEFRA, 2014)

DEFRA (2014) reported an increase of approximately one million t^{-1} annum^{-1} in the use of grass, clover and mixed grass-clover swards for the production of silage in England from 1992 to 2003. Arable crops such as wheat, barley and maize have also increased by approximately 187,000 tons^{-1} annum^{-1} over the same time period (DEFRA, 2014). Use of arable forage crops require greater protein supplementation, because of their high starch (~300 g kg^{-1}DM) content and generally low CP content (~110 g kg^{-1}DM) compared to grass and legume silages (Anil, 1998). Wilkins and Jones (2000) showed that most

forage protein is highly degradable with a low fermentable energy supply, which could limit rumen microbial synthesis.

Mustafa *et al.* (2000) and Tabacco *et al.* (2006) highlighted that forage legumes could also be an important protein source in ruminant nutrition. Hart *et al.*, (2012) concluded that SBM could be replaced with whole crop pea silage (44-100 % of SBM) in ruminant diets, with a significant reduction in feed costs.

Lucerne, red and white clover, lotus, sainfon, whole crop beans and peas are the main legumes forages cultivated in the UK (Wilkins and Jones, 2000). These forages are characterized by high DM yields (6-11 t ha^{-1}) and CP yields (1-2 t ha^{-1}) (Entec, 1998). Knaus *et al.* (1998) reported that lucerne was found to be the main forage produced in USA, Canada, Argentina, Italy and China (the utilization of lucerne was either for hay or silage with less being fed fresh or grazing). Lucerne is generally characterized by a high DM yield (12 t DM^{-1} ha^{-1}annum) with up to 200 g kg^{-1}DM of CP with a range 130-320 g kg^{-1}DM between different species (Knaus *et al.*, 1998). The chemical composition of some forage silages are presented in Table 1.7.

Entec (1998) reviewed the suitability of different forages used in the UK for ruminant nutrition (Table 1.8). The report showed that grass was the main forage produced in the UK (Table 1.8). However, large amounts (~200 kg^{-1}ha) of N fertiliser are required to increase grass DM and CP yield (Chadwick, 2004). It has been reported (EBLEX, 2011) that increasing the level of N fertilization (0, 50, 100, 150 or 200 kg N ha^{-1}) led to a linearly increase grass yield (3, 5, 8, 12 and 14 t DM $year^{-1}$ respectively).

Table 1.7 Chemical composition of some forage silages cultivated in the UK.

Item	Forage silages (g kg^{-1}DM)						
	GS*	LS**	RCS**	WCPS*	KS+	WCWS*	WCBS++
DM ($g\ kg^{-1}$)	244	356	317	365	187	402	237
OM	918	926	917	921	985	957	
CP	121	179	170	177	4.3	82	173
pH	4.1	4.5	3.9	4.1	206	3.8	
NH_3 ($g\ kg^{-1}$TN)	144	105	56	138	72	173	
NDF	537	519	449	252	436	338	566
Fat	23			13		12	13
ME($MJ\ kg^{-1}$DM)	11.4	10.0	10.2	11.8	10.2	11.6+	7.3

*: Sinclair *et al.* (2009) **: Speijers *et al.* (2005), +: Marley *et al.* (2007), ++: Entec (1998); GS: grass silage; LS: lucerne silage; RSC: red clover silage; WCPS: whole crop pea silage; KS: kale silage; WCWS: whole crop wheat silage; WCBS: whole crop bean silage.

Grass and legume CP content have been shown to be highly rumen degradable (Wilkins and Jones, 2000) and could result in an oversupply of dietary RDP at the expense of metabolisable protein (MP) (McDonald *et al.*, 1991). AFRC (1993) reported that a range of 700-800 g kg^{-1}DM of forage CP was degradable in the rumen. Compromised MP may have resulted from high rumen NH_3-N concentrations that exceed the capacity of the rumen microorganism to synthesize MCP (Ulyatt *et al.*, 1976). In addition, Sinclair *et al.* (2014) reported that there is an energy cost associated with excretion of excess NH_3-N of 0.8 MJ ME 100 $g^{-1}NH_3$. Wilkins and Jones (2000) stated that only 5-20 % of the consumed CP by animals could be converted to animal products such as milk and meat. Sinclair *et al.* (1993) suggested that reducing rumen protein degradability of ensiled crops could improve the efficiency of utilization of dietary protein and carbohydrates by improving the synchrony of dietary nutrient in the rumen.

Table 1.8 Dry matter and CP production from different forage crops cultivated in the UK, and their suitability for grazing, ensiling and hay production.

	CP g kg^{-1}DM	Yield (t ha^{-1})		Evaluation of forage crops			
		DM	CP	Agronomy	Grazing	Silage	Hay
Grass (grazed/ensiled)	170	11.6	2	Good	Good	Good	Moderate
Lucerne	194	12	2.3	Poor	Poor	Moderate	Poor
White clover	160	7.6	1.2	Moderate	Moderate	Poor	Poor
Alsike clover	180	5.5	1.0	Moderate	Poor	Moderate	Not suitable
Red clover	180	12	2.2	Moderate	Poor	Poor	Not suitable
Lotus	190	6.5	1.2	-----	-----	-----	-----
Kale	164	5.5	0.9	Good	Moderate	Poor	Not suitable
Sainfoin	190	8.4	1.6	Moderate	Moderate	Poor	Poor
Forage pea	190	7	1.3	Moderate	Not suitable	Moderate	Not suitable
Forage bean (winter Sown)	170	8	1.4	Moderate	Not suitable	Moderate	Not suitable
Sunflower/ Maize silage	100	9.6	1.0	Moderate	Not suitable	Moderate	Not suitable

Adapted from Entec (1998)

1.4 Methods of reducing rumen protein degradability in the rumen:

1.4.1 Physical methods

Weather conditions at harvest could affect the type of N available in the resulting forage, such that wet weather conditions at harvest would be responsible for washout of forage soluble N (Tamminga, 1979). Furthermore, hot weather conditions (>25^{o}C, McGee, 2004) at harvest were found to reduce rumen protein degradability as a result of inhibited plant protease activity and reduced protein solubility (Tamminga, 1979). Feed processing (pelleting, steam rolling and extrusion) has also been shown to reduce rumen protein degradability due to the effect of heat and/or shear forces on the protein molecules contained within the feed material (Doiron *et al.*, 2009; Samadi and Yu, 2011; Gamage *et al.*, 2012; Peng *et al.*, 2014).

Heat treatment of dietary ingredients (especially cereals) was the earliest method used to reduce protein degradability (Delgado-Andrade *et al.*, 2004). The principle of protein protection by the use of heat treatment has been described (Samadi and Yu, 2011) as the “Maillard reaction” between protein and reducing sugar of the feedstuff, resulting in a complex resistant to both rumen degradation and intestinal digestion. The amino group of protein molecule reacts with a carboxyl group of glucose and fructose molecules under high temperatures (>25^{o}C), the Schiff’s base formed at the initial reaction, and then the Amadori rearrangement of the Schiff’s bases to the 1-dexoy,2-ketosyl, further heating premelanoidin and melanodin polymers occur which are characterized by a brown colour (Hurrell and Finot, 1985) (Figure 1.4.)

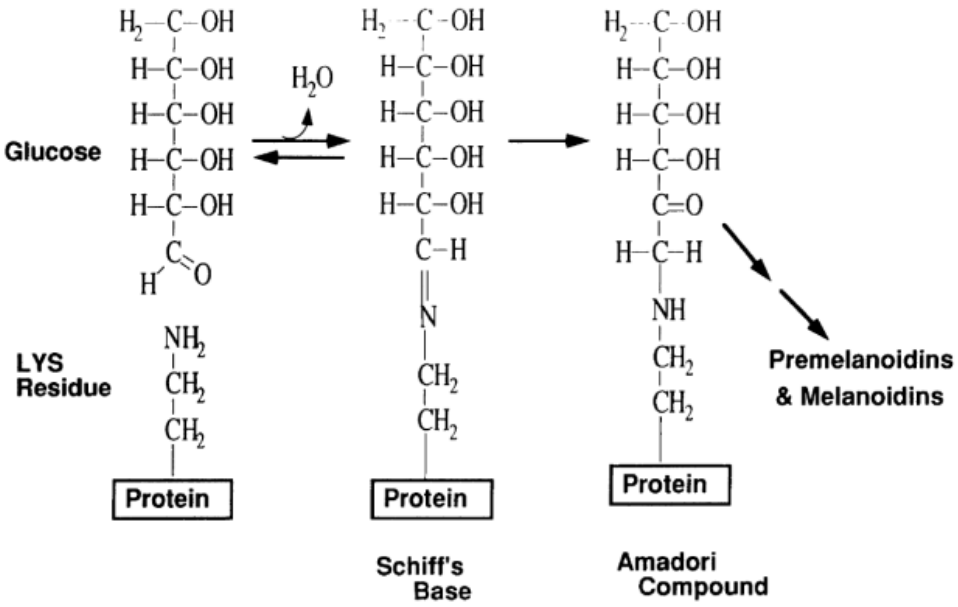

Figure 1.4 Maillard reactions between protein and glucose molecules under heating condition (Hurrell and Finot, 1985)

Broderick and Craig (1980) observed reduced rumen *in vitro* protein degradation when they incubated cottonseed meal that had been heat treated in an autoclave temperature (121^{o}C) for 0, 5, 10, 15, 30, 60, 90, 120, 180 and 240 minutes. The results showed a linear response to the duration of heating and reduced protein degradability. Additionally when cottonseed meal was heated for more than 60 minutes (min) un-digestible protein complexes were formed (Broderick and Craig, 1980). Peng *et al.* (2014) studied the effect of heating (120^{o}C for 60 min) using two procedures; dry (oven dry) and moist heat (autoclave) on the chemical structure of camelina seeds. Their results (Peng *et al.*, 2014) showed that heating reduced CP effective degradability (ED) *in situ* by approximately 23 and 11 % for autoclave heating and dry heating respectively compared to raw seeds. In addition, the results demonstrated that moist heat significantly changed the protein structure (alpha-helix and beta sheet ratio) of camelina seeds compared to dry heat or raw seeds. Peng *et al.* (2014) concluded that treating camelina

seeds with moist heat temperatures (120^{o}C autoclaved) changed the CP chemical profile, rumen degradability and molecular structure, digestive behaviour and increased RUP supply. Similar results were found by Doiron *et al.* (2009), Samadi and Yu (2011) and Theodorou and Yu (2013). However, the optimum time and temperature regimen for reduced rumen protein degradation and intestinal digestion for optimum protein utilisation have yet to be confirmed. Repetto *et al.* (2005) noticed that wilting reduced the DM and CP degradability of seven Uruguayan farm forages compared to fresh samples (ED DM: wilted= 54.25 %, fresh= 56.29 %) and (CP ED: wilted= 68.03 %, fresh= 71.65). Similar results were found by Kamalak *et al.* (2005) who observed that wilting forage (400 g kg^{-1}) reduced the *in situ* DM fraction ”a” by 3 % and increased DM fraction ”b” by 3.9 % compared to fresh forage, without any effect on ED. In addition CP degradability showed a significant reduction in fraction “a” and ED (27 and 46 % respectively) and increased fraction “b” by approximately 7 %. Kamalak *et al.* (2005) concluded that the effect of heating was to reduce N solubility.

Amino acids especially methionine, cysteine and tryptophan were highly reactive in Maillard reactions and sensitive to thermal treatment resulting in their destruction (Hurrell and Finot, 1985; Delgado-Andrade *et al.*, 2004). However, reduced rumen degradation of other single AA (lysine, asparagine and glutamine) may be achieved by thermal treatment, to create an isopeptide cross-link. Brodrerick *et al.* (1991) concluded that heat treatment of individual AA could be used to increase their supply to the small intestine. Heat treatment of feedstuffs may be used to protect them from rumen degradation, however heating beyond 120^{o}C may form un-digestible end products that are of little use to the animal (Brodrerick *et al.*, 1991).

Encapsulation of dietary protein or individual AA (Kamalak *et al.*, 2005) with resistant compounds such as ruminally protected lipid (Lynch *et al.*, 1987), calcium soaps (Sklan, 1989) and slowly degraded proteins (feather, blood and meat meals) (Loerch *et al.*, 1983; Church *et al.*, 1982) have been also used to protected protein in the rumen. Protective coating (encapsulation) provided a physical barrier to the rumen microbial population (Brodrerick *et al.*, 1991). Sklan (1989) found that SBM and whey powder treated with palm oil (in order to protect dietary protein reduced rumen degradability (*in vitro*) by 81 and 82 % respectively. However, Bayourthe *et al.* (1993) found that supplementation of chopped ryegrass with different levels (0, 50, 100, and 200 g kg^{-1}) of animal fat or vegetable oil (as encapsulation method) had no effect on *in situ* DM degradability.

1.4.2 Chemical methods

Historically, chemical compounds (especially formic acids (FA) and formaldehyde) have been used as silage additives in order to control undesirable reactions and improve protein utilization (Chalmers *et al.*, 1954). The mode of action of FA at ensiling has been related to reduced CP solubility (Lorenz and Uden, 2011), inhibited plant and bacterial enzyme activity and reduced plant and microbial respiratory activity (Winters *et al.*, 2000). Vagnoni *et al.* (1997) observed a reduction in enzymatic proteolytic kinetic activity when lucerne forage was treated with FA (215 mequiv. kg^{-1}FW) or sulphuric acid (147 mequiv. kg^{-1} FW). Salawu *et al.* (2001) used FA as a silage additive with pea/wheat bi-crops. The results showed a lower concentration of lactic and acetic acid compared with the untreated group, however, there were no significant effects of FA on soluble N or NH_3-N (Salawu *et al.*, 2001). Similarly, Winters *et al.* (2000) noticed that ryegrass

treated with FA or a bacterial (lactic acid bacteria) inoculant reduced protein degradation in-silo compared with the untreated silage. Winters *et al.* (2000) reported that the main benefit of using FA as a silage additive was to rapidly drop herbage pH, thus inhibiting proteolytic enzymes for both plant and microorganisms which are responsible for increasing N solubility and NH_3-N concentration inside the silo. However, mineral acids and some organic acids have been prohibited (European Commission directive, 2003) for use as silage additives due to their toxic and corrosive nature (EFSA, 2012)

Formaldehyde is a chemical compound that reacts with diet ingredients especially protein, and was used as a dietary supplement to reduced protein degradation in the rumen (Mangan *et al.*, 1980). The reactions between formaldehyde and dietary protein occur in the rumen at pH 6-7 and that post ruminal pH changes in the abomasum (pH< 3.5) and the small intestine (pH>7), would release protein from these complexes, facilitating digestion of dietary protein in the small intestine (Brodrerick *et al.*, 1991). Merry and Davies (1999) suggested that a significant improvement of animal performance has been reported when formaldehyde was used as a silage additive. Brodrerick *et al.* (1991) reported that the use of formaldehyde was reduced because of handling and health effects on animals and consumers.

1.4.3 Plant secondary compound.

Plant secondary compounds are a group of chemical compounds that do not have a major role in the main plant processes such as photosynthesis or growth, but could have a role in the plant immune systems (Patra and Saxena, 2009; 2010). It has been reported (Gladine *et al.*, 2007; Giannenas *et al.*, 2011) that plant

secondary compounds such as polyphenol oxidase enzymes (PPO), tannin or saponins could have both direct and indirect effects on protein degradation in the rumen.

Polyphenol oxidase (PPO) is known as plant endogenous copper-containing enzymes (Mayer, 2006). These plant enzymes have the ability to stimulate plant mono-phenol into o-diphenols which can be further oxidized to p and o-quinones (Winters *et al.*, 2008). It has been reported (Lee *et al.*, 2004) that some plants produce PPO as a defence line mechanism against wounding and/ or pathogens. Polyphenol oxidase enzymes can be found in active or inactive form. The inactive from of PPO can be reactivated when treated with some chemical compound such and alcohols, fatty acids, adjusted pH and some other enzymes (protease) (Mayer, 2006). Winters *et al.* (2008) reported that fruits and vegetable browning during storage could be largely due to the reactions of PPO enzymes generated quinones compounds which are highly reactive compounds with nucleophilic sites of plant proteins and phenols.

The results of these reactions are a compound which is a highly oxidized compound and the final products would be a cross-linked protein polymers (Kroll *et al.*, 2000). These final products have been found to be resistant to protease enzymes protecting plant protein from enzymatic degradation either during the ensiling process and/or rumen fermentation (Kroll and Rawel, 2001). Red clover, is known as one of the main forages that produce PPO enzymes (Lee *et al.*, 2004), which could be the reason that red clover protein were found to be less degradable inside silage clamps (Winters and Minchin, 2002) and/or the rumen (Broderick and Albrecht, 1997) compared to some other legumes silages.

Saponins are known as a plant secondary compound found in many plants, which are responsible for the formation of a stable foam in different liquids. Generally saponins consist of different compounds including glycosylated steroids, steroid alkaloids and triterpenoids (Wina *et al.*, 2005). Saponin has indirect effects on protein degradation in the rumen due to its negative effect on rumen protozoa. Wina *et al.* (2005) reported that supplementation with saponin resulted in defaunation of the rumen; with consequential reduction on rumen fermentation including NH_3, VFA concentration and methane emissions.

It has been reported (Giannenas *et al.*, 2011) that dietary inclusion of plant-derived essential oils (PEO) improved nutrient utilization and ruminant performance. Calsamiglia *et al.* (2007) suggested that PEO reduces microbial activity in the rumen, especially CH_4 emission and inhibits the deamination process within bacterial cells. Similar to saponins, PEO has an indirect effect on protein degradation in the rumen due to its negative effects on rumen microbes, especially rumen bacteria (Calsamiglia *et al.*, 2007). Giannenas *et al.* (2011) tested the effect of different levels of PEO (0, 50, 100 and 150 mg^{-1}kg of concentrated diet) on daily milk yield, milk composition and some rumen fluid characteristics (pH, NH_3-N, VFA, total bacteria and protozoa numbers) of eight ewes in late pregnancy period. The results showed that treating concentrated diets with PEO increased linearly the average milk yield (1.56, 1.68, 1.87 and 2.12 l^{-1}d for 0, 50, 100 and 150 mg^{-1}kg respectively) and a linear reduction in milk urea and somatic cells was noticed when the level of PEO was increased (Giannenas *et al.*, 2011). In addition, the number of rumen microbes and the concentration of urea, acetate and propionate in the rumen liquid have been affected negatively by PEO (Giannenas *et al.*, 2011). However, Gladine *et al.*

(2007) suggested that consuming a PEO in the long term that rumen microbes would adapt to the PEO's effect.

Frutos *et al.* (2004), Mueller-Harvey (2006) and Waghorn (2008) have all discussed the potential for tannins to reversibly bind with different compounds such as protein and carbohydrate to form complex undegradable molecules. Jones and Mangan (1977) showed that the mode of action of tannin is similar to formaldehyde, but less aggressive. Piluzza *et al.* (2014) reported that using tannin as a growth promoter for ruminant nutrition would be more useful than synthetic compounds (such as formaldehyde) because tannins are a natural plant compounds and could provide a cheaper and a safer method for ruminant feeding. Some studies (Bhatta *et al.*, 2009; Deaville *et al.*, 2010; Hymes-Fecht *et al.*, 2013) have shown that using tannins as a silage additive and/or in ruminant nutrition could improve silage quality, maximizing microbial protein synthesis in the rumen, improving animal digestion and performance.

1.5 Tannins and their effect on ruminant nutrition

Tannins have been described as poly-phenolic, oligomeric compounds, that are found in different plant species (forage crops especially in legumes, shrubs and forage trees) and in different plant components (leaves, stem, fruits and seeds) (Patra and Saxena, 2010; Piluzza *et al.*, 2014). Approximately 80 % of woody and 15 % of forages species could have one or more types of tannin in their structures (Mueller-Harvey, 2006). Giner-Chavez (1996) reported that tannins were found widely in plants indigenous to hot climates (tropical, arid and semi-arid regions). However, tannins are also present in plant species found in Atlantic and Mediterranean regions such as legumes (*Luthyrus, Medicago, Lotus, Cytisus, Trifolium* and *Onobrychis*), Ericaceae (*Erica, Vaccinium* and *Calluna*) and Fagaceae (*Qurecus* and *Castanea*) (Terrill *et al.*,1992; Hervas *et al.*, 2003; Frutos *et al.*, 2004; Minho *et al.*, 2008; Sinclair *et al.*, 2009). Tannins have been found in plant cell walls or vacuoles throughout the plant (Lamy *et al.*, 2011). Furthermore, tannins are generally water-soluble, with high molecular weights (up to 20,000 Dalton units) according to their types and source (Naumann *et al.*, 2013). Makkar (2003), Mueller-Harvey (2006) and Shete *et al.* (2011) reported that the free phenolic groups (hydroxyl and/or carboxyl) were the active biochemical groups in all tannins and that these active sites are found in the phenolic groups and aromatic rings of different tannin types. Tannins have been shown to create reversible bonds with different compounds including proteins, carbohydrates and minerals (McSweeney *et al.*, 2001) via the active sites available on different tannin types.

Barry (1989) and Waterman and Mole (1994) suggested that tannins could have a role in the plant immune system. This could include protecting the plant from pathogens, UV light (UV-absorption by poly-phenol active groups), allelopathyic functions against plant parasites and protect the plant from herbivores animals. In addition, tannins may also be involved in flower pollination by bearing anthocyanidins (Schofield *et al.*, 2001) and energy and N conservation (Waghorn, 2008).

In general tannins can be divided into two main groups, condensed tannins (CT) and hydrolysable tannins (HT), although natural tannins may contain a combination of the two groups rather than existing single tannin species (Waghorn, 2008; Lamy *et al.*, 2011). Condensed tannins can be found in legume forages such beans and peas, while HT are often appear in fruit pods, tree leaves and wood such as chestnut and oak, but are rarely found in forages (Min *et al.*, 2003). Haslam (1981) showed that both CT and HT can be found in the same plant, and usually CT is present in gymnosperms and angiosperms, while HT is found in di-cotyledons only. The variations in the types and concentration of tannin found in plants may depend on genetic, environmental temperature, geographical region soil fertility, seasonal factors and plant stress (McMahon *et al.*, 2000). Hot weather conditions (high temperature, extreme sunlight and water stress) and poor soil quality can increase tannin concentration (Van Soest, 1994). All tannin types have been shown to be able to create a tannin-protein complex (Lorenz, 2011).

1.5.1 Types of tannin

1.5.1.1 Condensed tannin

Condensed tannins are the most widely observed tannin in nature, with a molecule weight of 1000-20000 Da, and chain length of 2,20-flavanol units (Waghorn, 2008; Naumann *et al.*, 2013). Structurally CT consist of flavan-3-ol or flavan-3,4-diol polymers bound by carbon-carbon or carbon-oxygen-carbon bonds (McMahon *et al.*, 2000). Schofield *et al.* (2001) extensively reviewed the molecular structures, main reactions and methods of determination of CT. The type of R group (H or OH) found in CT (Figure 1.5) may have a major effect on CT reactivity (Schofield *et al.*, 2001). The position and ratio of the OH group in the prodephinidin group in the structure can increase the ability of CT to react with other molecules (such as protein) to form complexes (Aerts *et al.*, 1999)

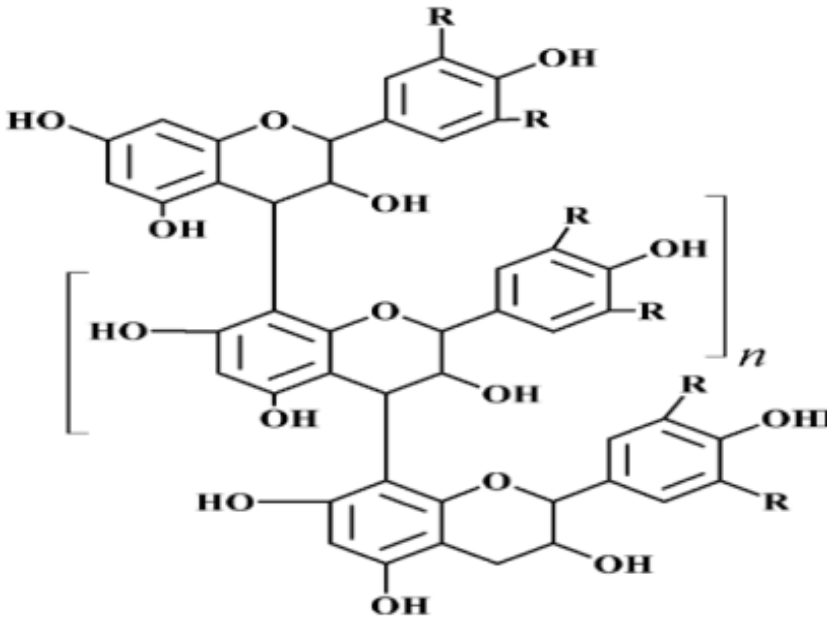

Figure 1.5 Structure of condensed tannin (Schofield *et al.*, 2001).

The presence of CT in ruminant nutrition could lead to better utilization of dietary protein leading to a broad range of animal performance responses including increased liveweight gain, wool, milk production plus improved animal fertility, welfare and health (Waghorn, 2008; Naumann *et al.*, 2013). Katiki *et al.* (2013) reported that the beneficial effect of CT on ruminant nutrition has been extensively studied in the last decade.

1.5.1.2 Hydrolysable tannins

Hydrolysable tannins can be found in oak, acacia, eucalyptus, chestnut and different browsing and tree leaves (Waghorn and McNabb, 2003). Hydrolysable tannins consisted mostly of gallic and/or hexahydroxydiphenic acid building blocks that bind by esterification with hexo-sugasr (Waghorn, 2008; Patra and Saxena, 2010, Figure1.6). The structural, analysis and method of extraction of HT have been extensively reviewed by Mueller-Harvey (2001).

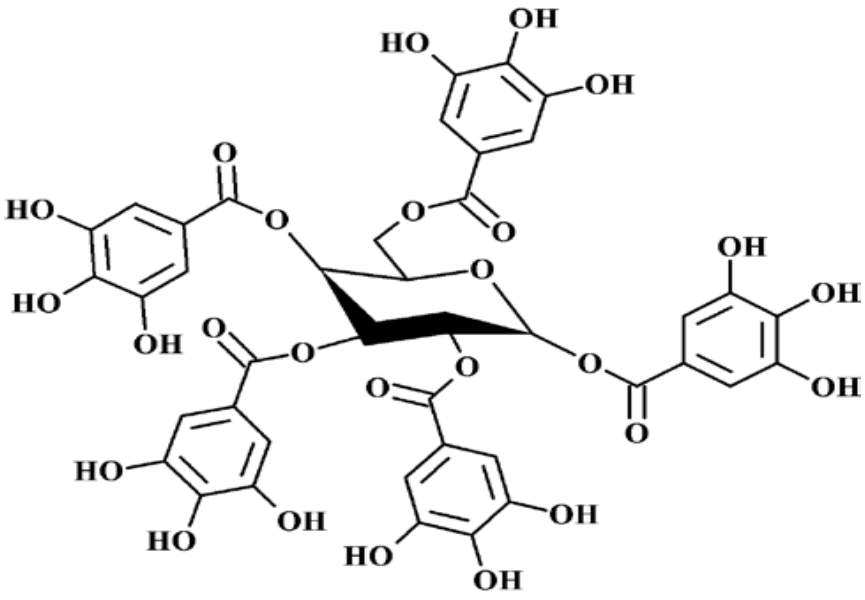

Figure 1.6 The structure of hydrolysable tannin from chestnut wood (Mueller-Harvey, 2001).

The molecular weight of HT is relatively low (500-3000 Da) depending on plant species, location within the plant and concentration of tannin (Mueller-Harvey, 2001). Hydrolysable tannins can be broken down (hydrolysed) by heating in weak acids, whilst CT can only be hydrolysed by strong acids (McMahon *et al.*, 2000). Mueller-Harvey (2001) emphasised that similar to CT, many kinds of HT can be found in nature due to the oxidation of aromatic rings or gallic acids in HT structures. Hydrolysable tannins could potentially be toxic to animals especially when fed in high quantities (>150 g HT kg^{-1}DM) which could lead to lesions of the liver and kidney and cause death of the animal (Waghorn, 2008). Katiki *et al.* (2013) suggested that less research in to the benefits of CT has been conducted possibly due to its perceived toxicity. Despite the potential toxicity of HT to ruminant animals, dietary supplementation has been shown to improve silage quality (when used as silage additives), manipulate rumen fermentation/ degradation and improve animal performance (Frutos *et al.*, 2004). However, there is a paucity of published literature with regard to HT as a silage additives or rumen modifier. also there have been few published results on the effect of tannin on animal performance, and the optimal doses of HT without being toxic to the animal (Katiki *et al.*, 2013).

Hydrolysable tannins and CT generally have the same ability to complex with protein (Lamy *et al.*, 2011; Patra and Saxena, 2011). However, there is some evidence that rumen microbes can degrade HT while CT may escape rumen degradation (Makkar, 2003). These findings are supported by the results from Perez-Maldonado and Norton (1996) and Terrill *et al.* (1994) who observed approximately 15-25 % of consumed CT were excreted in faeces, when they fed a group of ewes ration containing CT.

The complex between feed protein and quebracho CT (tannin extracted from quebracho trees *Luciano malo*) is weak that only some kinds of tannins have the ability to increase UDP such as *Dichrostachys cinerea* Sainfoin*, Lotus corniculatus and L. leucocephela* (Mueller-Harvey, 2006). Bhatta *et al.* (2000) reported an increase in body weight gain of dairy cows in mid lactation (222 to 325 g d^{-1}) when they added 75 g of tamarind seed husks kg^{-1}DM (140 g of CT kg^{-1}DM). Bhatta *et al.* (2000) also noticed an increased in faecal nitrogen and decreased in urinary nitrogen. Mueller-Harvey (2006) reported that quebracho CT could react with endogenous proteins rather than dietary protein which would cause a dramatic reduction of protein absorption in the small intestine. It may be postulated that non hydrolysed HT may also complex with endogenous and free AA (from protein digestion) in a similar way to that suggested by Mueller-Harvey (2006). However, Śliwiński *et al.* (2002) and Lavrencic and Levar (2006) showed that using low doses of HT <30 g kg^{-1}DM resulted in improved animal performance and silage quality. Śliwiński *et al.*, (2002) observed a reduced rumen NH_3-N concentration (~21 %) when 2.5 g kg^{-1}DM HT was fed to dairy cows. The reduced rumen NH_3-N concentration observed by Śliwiński *et al.* (2002) suggests that rumen escape protein may be elevated, which might raise protein absorption in the small intestine, whilst Zimmer and Cordesse (1996) found that dry matter digestibility was reduced by approximately 6 % in sheep and 3 % in goat when the diet is sprayed with high doses of chestnut HT (80 g kg^{-1}DM).

1.5.2 Mode of action of tannins:

Tannin has been shown to be able to complex with protein (mainly) carbohydrate (polysaccharide), minerals, alkaloids and nucleic acids (Patra and Saxena,

2010). Kumar and Singh (1984) explained that the bond between tannin and protein molecules could be one or more of the following types:

- Hydrogen bond, between the oxygen of the amide group in the protein structure and hydroxyls radical of the tannin structure.
- Hydrophobic bond, between hydrophobic rings of protein and the aromatic ring of tannin.
- Ionic bond, between cationic site of protein and phenolat ion of tannin.
- Covalent bond, between condensations of phenolic groups of tannin with the nucleophilic group of the protein molecules through polyphenol oxidation.

Tannin-protein complexes are probably stable at pH 5.6-7.0, however, at other pH values the hydrogen bonds would breakdown and the complex would be dissociated (McSweeney *et al.*, 2001; Makkar, 2003; Waghorn, 2008).

The post ruminal pH shifts in the abomasum pH 2.3-4.1 (Wheeler and Noller, 1977) release protein from this complex that can then be digested in the small intestine making the protein available for digestion and absorption in the animal body. Therefore, the ability of microbial enzymes to degrade dietary protein would be reduced (Mueller-Harvey, 2006) and an increase in UDP would occur (Figure 1.7). This reaction would affect animal nutrition negatively or positively, depending on the type and the amount of dietary tannin (Frutos *et al.*, 2004). Makkar, (2003) reported that not all tannin-protein complexes could dissociate post rumen; complexes could be reversible or irreversible depending on the nature of the tannin, and the degree of affinity of the bonds. There was more than 78 % of tannin protein complex digestible in the small intestine (McSweeney *et al.*, 2001). Piluzza *et al.* (2014) reported that it is still unclear as to the fate of

tannin in the digestive tract, especially post ruminally. Similarly, there is uncertainty as to whether tannin binds with endogenous protein or may rebind with feed protein again.

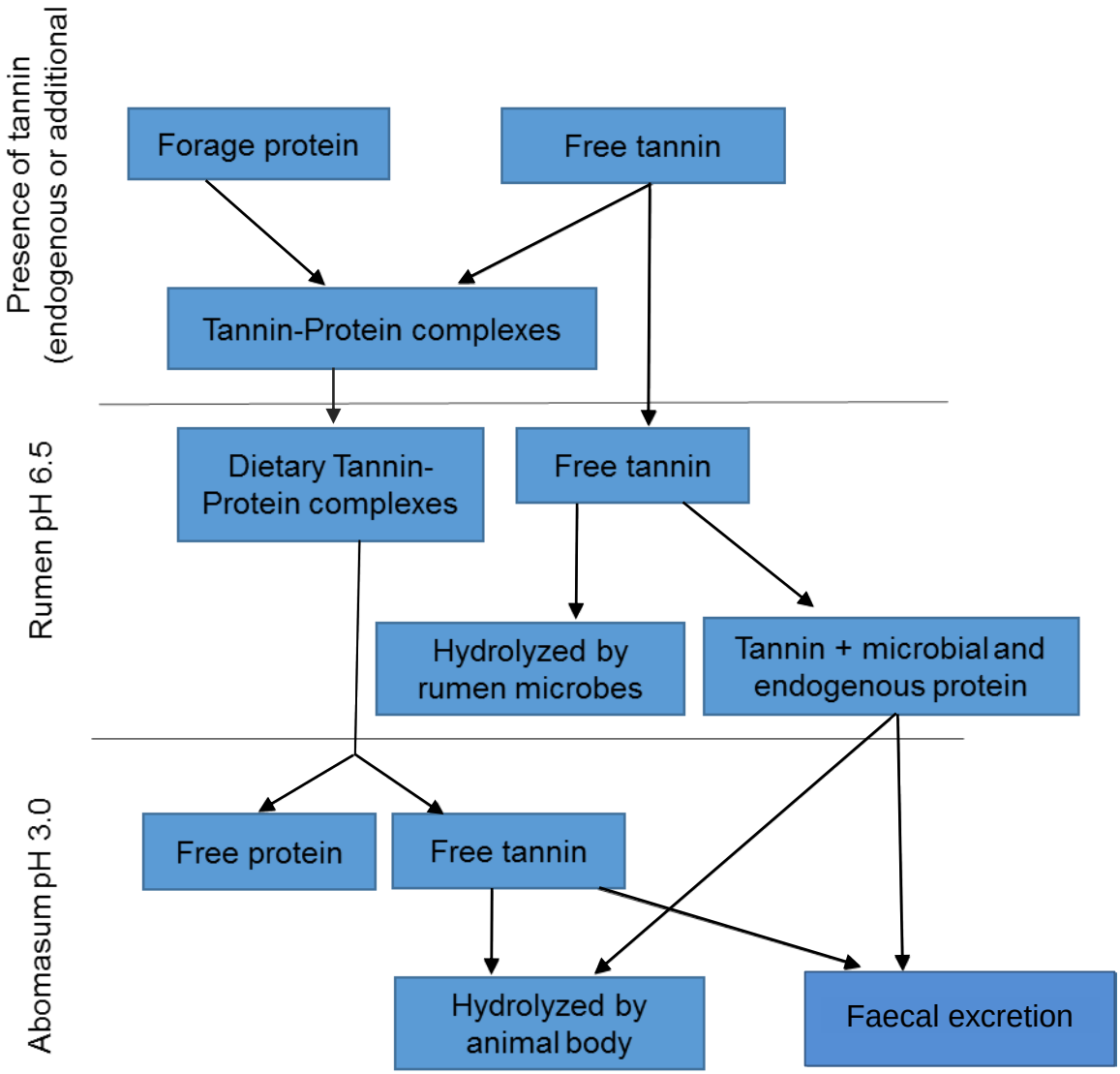

Figure 1.7 The reaction between tannin and forage protein at ensiling and inside animal body (adapted from Hart, 2005).

1.5.3 The effect of tannin on ruminant nutrition

Tannins have been described as anti-nutritional compounds which have a negative effect on nutrient utilization, palatability, digestibility, VFA and kidney and liver damages (Lamy *et al.*, 2011). The anti-nutritional effects of tannin depend on tannin concentration, tannin types, animal species, physiological status of the animal and feed composition (Schofield *et al.*, 2001; Makkar, 2003). However, recently some studies (Bhatta *et al.*, 2009; Deaville *et al.*, 2010; Hymes-Fecht *et al.*, 2013) have shown that using relatively low levels of tannin (<50 g kg^{-1}DM) as a silage additive and/or in ruminant nutrition could improve silage quality, maximizing microbial protein synthesis in the rumen, improving animal digestion and performance. Depressed rumen fibre digestion and DMI was also cited by Piluzza *et al.* (2014) but they concluded that dietary inclusion of tannin at low levels could be beneficial to animal performance but longer term studies would be necessary to be able to interpret the effect of HT interactions with dietary components. The effect of tannin on ruminant nutrition could be summarized in the following categories:

- Effect of tannins on animal health
- Tannins as silage additives and their effect on silage composition
- Effect of tannins on rumen fermentation.
- Effect of tannins on animal performance

1.5.3.1 Effect of tannins on animal health

Bloat is a nutritional disorder seen in ruminants resulting from the formation of stable foam in the rumen, possibly due to high solubility of forage protein and could lead to the death (Wang *et al.*, 2012). Bloat is commonly observed when

ruminant animals graze young lush forage, particularly legumes, such as lucerne and clover (Piluzza *et al.*, 2014). Makkar (2003), Frutos *et al.* (2004) and Mueller-Harvey (2006) reported that animals grazing on forages containing trace levels (1-2 g kg^{-1}DM) of tannin (endogenous or supplanted) were protect from bloat. Mueller-Harvey, (2006) suggested 2 potential modes of action of an anti-bloat effect of tannin: (1) an inhibition in activity of slime-producing ruminal bacteria; (2) disruption of proteins involved in creating frothy foam in the rumen.

Frutos *et al.* (2004) and Waghorn (2008) reported that lambs grazing on forages containing tannins such as *Lotus sp.* and birdsfoot trefoil were shown to have more tolerance to alimentary tract parasites such as nematode Spp. Dietary tannins could disrupt the life cycle of nematodes resulting in reduced nematode egg production (Minho *et al.*, 2008). Minho *et al.* (2008) studied the effect of feeding CT (1.6 g kg^{-1}LW) extracted from *Acacia molissima* on days 1, 2, 31, 32 and 60 in sheep fed grass. Prior to the start of the experiment all sheep were confirmed to be infected with *Haemonchus contortus* or *Trichistringylus colubriformis* (average of 1000, 1200 faecal eggs respectively). The results showed that weekly faecal eggs was reduced (~65 %) when CT was fed compared with untreated groups. In addition, a reduction of approximate ~64% of abomasal worm burden in sheep supplemented with CT were observed (Minho *et al.,* 2008). Dawson *et al.* (2011) studied the effect of quebracho CT fed at levels (0, 80 or 100 g Kg^{-1}DM) in pelleted concentrate. The results showed that tannin level did not have any effect on nematode faecal egg counts, abomasal worm burden (*Teladorsagia circumcincta*) or small intestine worms (*Trichostrongylus vitrines*).

1.5.3.2 Tannins as silage additives and their effect on silage composition

It has been reported (Salawu *et al.*, 1999; Makkar, 2003; Mueller-Harvey, 2006) that a reduction in forage N hydrolysis was observed within the clamp when tannin was used as a silage additive. Supplementary tannin at ensiling may have inhibited plant protease enzymes due to the creation of an enzyme-tannin complex (Salawu *et al.*, 1999; Lorenz *et al.*, 2011). Tabacco *et al.* (2006) investigated the impact of chestnut (HT) on the endogenous plant microbe population. A reduction of the endogenous microbial population was noted which could be related to the formation of a tannin-microbes (cell membrane) complexes due to effects on cell membrane permeability (Tabacco *et al.*, 2006). Reduced plant cell wall degradation was observed by Salawu *et al.* (1999), who noted that tannin could bind with plant cell wall. Lorenz *et al.* (2010) and Perez-Maldonado and Norton (1996) reported that at pH~4 the tannin-protein complex decoupled. However, it should also be noted that at this pH endogenous plant enzymes and microbial activity are also inhibited (Merry and Davies, 1999) such that no further degradation of plant protein is likely to occur. The effects of tannin inclusion at the point of ensiling are summarised in Table 1.9

Table 1.9 Effect of different types and dosage of different tannins on silage proximate analysis (DM: dry matter, CP; crude protein, SolN; soluble N, NDF: neutral detergent fibre, NH_3: ammonia).

References	Source	Dosage*	Types	Occur	Additives	DM	CP	SolN	NDF	NH_3	pH
Salawu *et al.* (1999)	Mimosa	5,50	CT	ST		=		-(50)		+(5),-(50)	+
	Myrabolam	5,50	HT	ST		+		-(50)		+(5) ,-(50)	+
	Quebracho	5,50	CT	ST		=		-(50)		+(5),-(50)	+
Salawu *et al.* (1999)	Mimosa	50	CT	ST	± 12.5g FA	+		-	-	-	=
	Quebracho	50	CT	ST	± 12.5g FA	+		-		-	=
Lavrencic and Levar (2006)	Chestnut	0, 3, 15, 30	HT	ST		=	-(>15)	-	- (3)	-	-(3)
Tabacco *et al.* (2006)	Chestnut	0, 20, 40, 60	HT	ST		=	=	- (20)	- (>40)	- (>20)	
Grabber and Coblentz (2009)	Birdfoot trifold	5, 9, 15	CT	ET		-(15)		- (>9)	+(15)	- (>9)	
De Oliveira *et al.* (2009)	Sorghum	0.2, 1	CT	ET	± PEG	=	+ without PEG			- without PEG	+ with PEG
Deaville *et al.*, (2010)	Mimosa	50	CT	ST		+	=		+	=	=
	Chestnut	50	HT	ST		+	=		+	=	=
Hymes-Fecht *et al.* (2013)											

*: g kg^{-1} DM, CT: condensed tannin, HT: hydrolysable tannin, ET: plant endogenous tannin, ST: supplemented tannin, = no significant differences, +: increased significantly, -: decreased significantly, PEG: polyethylene glycol (PEG is highly reacted with tannins and reduce their activity), FA: formic acid, number in parentheses refer to source or dose level of tannin.

Table 1.9 shows that tannins (endogenous or supplemented) reduced soluble N and NH_3 inside the silage clamp, with slight effects on DM, CP and NDF concentration, however, some studies (Lavrencic and Levar, 2006; Tabacco *et al.*, 2006) found that supplementing tannin (30-50 g kg^{-1}DM) increased DM and reduced CP and NDF. However, their (Lavrencic and Levar, 2006; Tabacco *et al.*, 2006) results did not correct for the additional DM added (30-50 g tannin kg^{-1} DM) which could account for the increased DM, CP and NDF content. Polyethylene glycol (PEG) is a chemical compound which is high reactive with different types of tannin, and is often used by farmers and researchers to reduce the negative effect of plant endogenous tannin in ruminant nutrition.

Deaville *et al.* (2010) supplemented ryegrass at ensiling or at feeding with mimosa (CT) and chestnut (HT) tannin (55.6 and 55.0 g kg^{-1}DM, respectively). The results showed that both tannin types increased forage DM compared to the untreated silage which could be due to the inclusion of high DM of the tannin (~75 g kg^{-1}DM commercial mimosa and chestnut tannin). In addition, a reduction of fermentation acids (especially lactic acid) was observed with slight effects of both tannin types on NH_3-N. Deaville *et al.* (2010) explained that endogenous tannin (found within the plant cell) would influence initial protein degradation by plant enzymes but not necessarily microbial breakdown, as compared to ensiled forage. Where additional tannin reduced clamp NPN (P<0.05) but not NH_3-N (P>0.05), there may be no free tannin molecules available to bind with microbial protein compared with supplemented tannin (Mueller-Harvey, 2006).

De Oliveira *et al.* (2009) found that supplemented PEG reduced (P<0.05) silage CP by approximately 7-20 % when sorghum forages (containing two levels of endogenous CT: 1, 0.2 g kg^{-1}DM) were treated with PEG prior ensiling. Similarly,

Lavrencic and Levar (2006) observed an elevated concentration of true protein in silages containing >15 g kg^{-1}DM chestnut (HT). However, their findings suggested that increasing the level of tannin inclusion reduced CP (P<0.05), soluble N (P<0.05) and NH_3-N (P<0.05) but their results were not corrected for the additional DM added due to treatment. Salawu *et al.* (1999) noted that ryegrass treated with additional tannin (mimosa, myrabolam or quebracho) resulted in silage with an elevated pH at days 7 and 32 post ensiling (~8-15 %). In addition, the study showed that using tannin at 50 g kg^{-1}DM had a greater effect of reducing soluble nitrogen (-22 %) and NH_3 (-27 %) compared to the addition at 5 g kg^{-1}DM or untreated control (Salawu *et al.*, 1999). Tabacco *et al.* (2006) added different levels of chestnut (HT) (0, 20, 40 and 60 g kg^{-1}DM) to lucerne forage at ensiling and after 120 days of fermentation they observed a linear reduction in NH_3-N (12.8, 11.4, 10.0, 9.6 % total N) and NPN (75.9, 72.9, 66.1 and 64.6 % total N) for (0, 20, 40 and 60 g kg-1DM, respectively) and increased lactic acid compared to control silage (P<0.05). Rumen degradability parameters were also found to be linearly affected by increased tannin addition. The immediately soluble fraction “a” was reduced (842, 821, 776 and 747g kg^{-1} DM), the insoluble but potentially degradable fraction “b” increased (102, 118, 154 and 184 g kg^{-1}DM) and the resulting CP $ED_{0.06}$ reduced (897, 888, 860 and 852 g kg^{-1}DM) at tannin inclusion rates of 0, 20, 40 and 60 g kg^{-1}DM, respectively (Tabacco *et al.*, 2006).

1.5.3.3 Effect of tannins on rumen fermentation

Mueller-Harvey (2006), Patra and Saxena (2011) and Tan *et al.* (2011) have suggested that the addition of tannin to ruminant diets has a negative impact on

rumen fermentation (reduction of NH_3-N, VFA concentration and microbial population numbers in the rumen fluid) in addition to tannin's effect on reducing CH_4 and CO_2 emissions. Similar effects have been observed by other authors and their findings are summarised in Table 1.10.

Min *et al.* (2002) studied the effect of *Lotus corniculatus* CT (32 g kg^{-1}DM) in a changeover design. Sheep were fed freshly mowed ryegrass and white clover without tannin for 12 days (d), followed by an experimental period of 18 days where *L. corniculatus* was fed as the sole diet. One group of six sheep received 100g^{-1} d of PEG to reduce the CT effect, and then the sheep were grazed outdoor for 12 days.

Inclusion of *L. corniculatus* to the diet was found to reduce rumen proteolytic bacteria, concentration of rumen NH_3-N and soluble N, *Clostridium proteolasticum, Eubacterium sp., Streptococcus bovis* and *Butyrivibrio fibrisolvens* were found to reduce after 8-120 h from the inclusion of *L. corniculatus* (Min *et al.*, 2002). However, when the *L. coniculatus* was fed with PEG the total number of bacteria were higher than sheep fed *L corniculatus* only. Tan *et al.*, (2011) observed that the addition of *Leucaena* CT at 0, 20, 30, 40, 50 or 60 mgg^{-1}DM dried grass effected both the total *in vitro* gas production and the composition of the gas produced. A linear reduction in total gas production (86.4 to 49.8 ml g^{-1}DM degraded), CH_4 (17.2 to 11.0 %) and CO_2 (71.4 to 62.6 %) was found as tannin level increased (Tan *et al.*, 2011). In addition, Tan *et al.*, (2011) observed a linear reduction of total methanogens (24-95 %), total protozoa (18.8-30 %) and total VFA (17-22 %) as level of tannin addition increased. Only a small effect (P=0.46) on rumen pH was observed (7.12-7.14) (Tan *et al.*, 2011). The

negative effect of tannin on rumen microorganisms resulted in a reduction in rumen fermentation (Min *et al.*, 2003).

Hervas *et al.* (2003) studied the effect of different levels of quebracho CT (0, 0.5, 1.5 and 3 g kg^{-1}LW which was equivalent to 0, 28, 83 and 166 g kg^{-1}DM) on rumen fermentation of sixteen rumen fistulated sheep. Tannin (CT) treatments were pumped directly into the rumen immediately before morning feeding, for 21 days and sheep were offered lucerne hay at 1.2 X maintenance requirements. Animals fed the highest level of infused tannin (3 g kg^{-1}LW) were removed from the experiment after 5-6 days as they stopped eating.

Tannin (CT) infused at lower concentrations <3 g kg^{-1}LW showed that treating ewes with1.5 or 0.5 g kg^{-1} LW CT did not have any effect on rumen fluid fermentation (pH, NH_3, VFA, acetic acid, propionic acid or butyric acid) compared to untreated ewes, but there was a tendency for a reduction in rumen pH after 1 and 3 h post feeding and NH_3 concentration 3 h post feeding in ewes treated with 1.5 g kg^{-1}LW (Hervas *et al.*, 2003). Getachew *et al.* (2008) found that tannin type (gallic acid (HT), tannic acids (CT) and quebracho (CT)) at different inclusion levels (0, 20, 40 and 60 g kg^{-1}DM) had different effect on gas production kinetics. The results showed that quebracho CT supplemented at 40 and 60 g kg^{-1}DM and tannic acids supplemented at 60 g kg^{-1}DM reduced(P<0.05) rumen NH_3 concentration, but gallic acid was found to have a lesser effect (Getachew *et al.,* 2008). Additional gallic acid at all levels of inclusion increased the predicted total gas production (asymptote) but the rate of gas production was reduced only with supplemented 60 g kg^{-1}DM (P<0.05).

In a second experiment Getachew *et al.* (2008) studied the effect of quebracho tannin, tannic acid and gallic acid at higher concentrations (0, 50, 100 and 150 g kg^{-1}DM). Quebracho tannin was found to reduce linearly (P<0.001) both the rate and predicted total gas production, but the addition of gallic acid and tannic acid were found to increase (P<0.001) the predicted total gas production (Getachew *et al.*, 2008). In addition, rumen CP degradability was found to reduce by supplemented quebracho tannin (13, 30 and 36 %) and tannic acid (14, 25 and 48 %) compared to the control, with a greater effect (P<0.001 at all rate of inclusion) on NH_3-N concentration than the gallic acid treatment (P<0.05 at 150 g kg^{-1}DM only). Getachew *et al.* (2008) concluded that the variation between different tannin types on gas production kinetics might be due to the chemical structure of each tannin.

Similar results were found by Pellikaan *et al.* (2011), who mixed 100 g kg^{-1}DM of three types of CT (grape seed, quebracho or green tea) or four types of HT (chestnut, myrabolan, tara or valonea) with lucerne hay as a substrate control with or without PEG (0.5 g^{-1}DM) to study the effect of different tannin types on gas production and composition. The results showed that both tannin groups (CT, HT) had no effect on total gas production, but reduced CH_4 concentration (P<0.05), total VFA (P=0.007), pH (P<0.001) and NH_3 (P<0.001), whilst when the PEG was added to the mixture the NH_3 significantly (P<0.001) increased in both tannin groups, but the VFA and pH increased only in CT (P=0.056 and P=0.028 respectively) (Pellikaan *et al.*, 2011). It was also noticed that addition of PEG seems to have a greater effect with CT compared to HT (Pellikaan *et al.*, 2011).

The results published by Pellikaan *et al.* (2011) and Getachew *et al.* (2008) show that CT had greater effects on rumen fermentation than HT which could be due

to CT having a higher molecular weight. In addition rumen microbes have the ability to hydrolyse HT but not CT (Mueller-Harvey, 2006). Woodward *et al.* (2004) found that CH_4 emission could be reduced (~31 %) when grazed dairy cows were supplemented with *L. corniculatus* (26.2g kg^{-1}DM CT). Similarly, Puchala *et al.* (2005) divided 24 Angora goat does in to two equal groups, and each group was fed sericea lespedeza (17.7 g kg^{-1}DM CT) or crabgrass/tall fescue forage (0.5 g kg^{-1} DM). Methane production (quantity per day) was found to be significantly reduced ($P<0.001$) (7.4 vs. 10.6 g d^{-1} for 17.7 and 0.5 g kg^{-1}CT, respectively). Naumann *et al.* (2013) reported that the negative effect of tannin on rumen CH_4 emission could be due to the direct effects of inhibiting the growth and activity of rumen methanogens microbes and/or indirect effects by the complexing with fibre and reducing fibre degradation in the rumen.

Table 1.10 Nutritional effect of different types and dosage of tannin on rumen fermentation.

References	Source	Dosage*	Types	Occur	Add.	Asses	NH_3	VFA	CH_4	G.P.	T.P	pH
Min *et al.* (2002)	Birdfoot trefoil	32	CT	ET	± PEG	*in vitro*	- without PEG	=			- without PEG	=
Hervas *et al.* (2003)	Quebracho	0, 28, 83, 116	CT	ST		*in vivo*	- (83)	=		- (83)		-
Tavendale *et al.* (2005)	*L. peduncultus* Lucerne	68	CT	ET	± PEG	*in vitro*	-(L.P.) without PEG	-(L.P.) without PEG				=
Puchala *et al.* (2005)	Sericea	17.7	CT	ET		*in vivo*	- (Seri.)	=	- (Seri.)			
	Crabgrass	0.5	CT	ET								
Bhatta *et al.* (2009)	Mimosa	0, 4.6, 9.3, 13.9, 18.6, 23.2	T	ST		*in vitro*	-	- (23.3)	-	- (23.2)	- (>9.3)	=
Alipour and Rouzbehan (2010)	Grape pomace	0, 15, 30, 45, 60	CT	ST		*in vitro*	-			-		
Tan *et al.*, (2011)	*Leucaena leucocepala*	0,20, 30, 40, 50, 60	CT	ST ST		*in vitro*		-	-	-	-	=
Williams *et al.* (2011)	Lucerne	5.17		ST		*in vitro*	- Cicer & Sainfoin	=				- Cicer
	Cicer milkvetch	4.49		ST								
	Birdfoot trefoil1	7.68	CT	ST								
	Birdfoot trefoil2	9.9		ST								
	Sainfoin	48.5		ST								

References	Source	Dosage*	Types	Occur	Add.	Asses	NH_3	VFA	CH_4	G.P.	T.P	pH
Pellikaan *et al.* (2011)	Green tea	100	CT	ET	± PEG	*in vitro*	- without PEG	- without PEG	- without PEG	=		- without PEG
	Quebracho	100	CT	ET								
	Vitis uinifera	100	CT	ET								
	Caesa lipinia	100	HT	ET								
	Chestnut	100	HT	ET								
	Valoena	100	HT	ET								
	Myrobolam	100	HT	ET								
Aghamohamadi *et al.* (2014)	Oak acorn	0, 6.2, 18.3	T	ST	± PEG	*in vitro*	- without PEG	- without PEG	- (18.3)	=	- without PEG	=
Dentinho *et al.* (2014)	*Cistus ladanifer L.*	1.4, 13.6, 22.8	CT	ST		*in vivo*	- linearly					=

* g kg^{-1} DM, Add: additives, Asses.: assessments, G.P: gas production T.P: total protozoa, CT: condensed tannin, HT: hydrolysable tannin, ET: plant endogenous tannin, ST: supplemented tannin, = no significant differences, +: increased significantly, -: decreased significantly number in parentheses refer to source or dose level of tannin, PEG: polyethylene glycol (PEG is highly reacted with tannins and reduce their activity).

Table 1.10 shows that the presence of tannin resulted in reduced NH_3-N, which could be due to formation of a tannin complex with dietary protein (Mueller- Waghorn, 2008; Muller-Harvey, 2006 and Makkar, 2003). The results also showed that tannins had a negative effect on rumen VFA and CH_4 emissions which could be due to an antimicrobial effect of tannin on rumen microbial population especially methanogenic microorganisms (Piluzza *et al.*, 2014; and Patra and Saxena, 2010). Furthermore, the effect of tannin was reduced when PEG was supplemented with either endogenous or supplemented tannin (Pellikaan *et al.*, 2011; Tavendale *et al.*, 2005 and Min *et al.*, 2002). However, rumen pH was found to be more stable and did not affected by tannins (either endogenous or supplemented) in most studies reported in Table 1.10.

1.5.3.4 Effect of tannins on animal performance

Dietary tannin concentration, type and molecular weight, have a major effect on DMI and animal performance (Piluzza *et al.*, 2014; Frutos *et al.*, 2004). Makkar (2003), Min *et al.* (2003) and Muller-Harvey (2006) reported that DMI would be negatively affected when the ruminant diets contain >50 g kg^{-1}DM of tannin. Barry and McNabb (1999) found that DMI was reduced when a group of sheep were grazed on swards of *Lotus pedunculatus* (>50 g kg^{-1}DM CT), whilst DMI recovered when the same flock was grazed on a *Lotus corniculatus* (35-44 g kg^{-1}DM CT) sward. Zhu *et al.* (1992) observed that DMI was reduced dramatically when they fed a group of sheep a diet containing 8 g kg^{-1} liveweight (LW) tannic acid. Similar results were found by Hervas *et al.* (2003) who found that sheep stopped eating after 5-6 days when 166 g kg^{-1}DM CT

was directly infused into the rumen, however intakes remained at control levels when sheep were supplemented with either 28 or 83 g kg^{-1}DM CT.

Hervas *et al.* (2003) concluded that high tannin (CT) levels were affecting the physiological status of the animal resulting in reduced intake. Dschaak *et al.*, (2011) observed reduced DMI in dairy cows when 30 g kg^{-1}DM CT was added to a forage containing either high or low CP. However, tannin had no effect on diet digestibility, milk yield or composition, however a reduction of milk urea was observed (~20%) (Dschaak *et al.*, 2011). Alonso-Diaz *et al.* (2009) and Lamy *et al.* (2011) suggested that there are special types of protein in ruminant saliva called “proline” which has the ability to react with dietary tannin and which may as a consequence reduce the negative impact of dietary tannin on palatability.

Salivary proline-rich protein may differ in different ruminant species, physiological state of the animal and geographical region. For example, the concentration and activity of proline in goat and deer in tropical regions may be higher than cattle and sheep, hence deer and goat are more resistant to tannin rich diet (Mueller-Harvey, 2006). There are three theories that have been reported by Frutos *et al.* (2004) that could explain the negative effect of tannins on DMI: (1) taste receptors in the animals mouth could be sensitive to the reaction between tannin and salivary protein, resulting in an astringent sensation to the brain, (2) formation of a tannin-protein complex slows digestion resulting in a sensation of satiety at the brain, (3) negative effect of tannin on rumen microorganisms resulting in reduced digestion. A fourth mode of action was postulated by Hervas *et al.* (2003) who suggested that infused tannin could have direct physiological effects.

De Oliveira *et al.* (2007) found no effect of feeding sorghum silage with two levels of CT (1.0 and 0.2 g kg^{-1}DM) on DMI of eight steers. Similarly, Krueger *et al.* (2010) found that supplemented either chestnut HT or mimosa CT (14.9 g kg^{-1}DM) to concentrate diet (high grain diet ME=11.7 MJ kg^{-1} , CP 132 g kg^{-1}) had no effect (*P*>0.05) on DMI of thirty-six crossbred steers fed for 42 days.

Puchala *et al.* (2005) observed an increase in DMI and DM digestibility when a group of goats (12 does) was fed *Sericea lespedoza* (17.7 g kg^{-1}DM CT) compared to feeding another group fed Crabgrass (0.5 g kg^{-1}DM CT). Similarly, Sinclair *et al.* (2009) found that DMI significantly increased (*P*<0.05) when whole crop peas silage with higher endogenous tannin levels (93.1 or 47.2 g kg^{-1}DM CT) was fed to Holstein dairy cows. Deaville *et al.* (2010) found an increased in DMI (4.8 %) in sheep when they supplemented with 55 g kg^{-1} DM of chestnut (HT) to grass silage either at ensiling or at feeding, while DMI was reduced (-11.2 %) when they used the same amount of mimosa (CT) as silage additives at ensiling or at feeding.

Toral *et al.* (2011) supplemented 14 lactating ewes with 10 g kg^{-1}DM of a commercially available tannin (mixture of CT and HT) for 28 days to study the effects of tannins on DMI, milk yield, composition and fatty acids. The results showed that supplemented tannin did not have any effect on DMI, milk yield and composition and milk fatty acids compared to the untreated diet (Toral *et al.*, 2011). Similarly, Buccioni *et al.* (2015) found no effect of supplemented HT or CT (52.8 g kg^{-1}DM) on DMI, milk yield or composition in ewes fed 800g $head^{-1}$ d^{-1} of concentrate diet and *ad libitum* chopped grass hay. However, milk fatty acid profile was affected by additional tannin; supplemented chestnuts

HT were found to increased linoleic acid (2.77 %), vaccenic acid (7.07 %) and stearic acid (8.71 %) and reduced rumenic acid (1.88 %) and saturated fatty acids (0.47 %), while supplemented quebracho CT was found to increase linoleic acid (9.23 %), vaccenic acid (13.88 %) and rumenic acid (24.24 %) and reduced stearic acid (11.45 %) and saturated fatty acids (3.38 %).

Liu *et al.* (2011) added three levels of chestnut HT (0, 10 or 30 g kg^{-1}DM) with two levels of coconut oil (0 or 25 g kg^{-1}DM) in the concentrate portion of a diet fed to growing lambs. The results showed that either level of tannin or coconut oil had no effects on animal performance in terms of DMI, average daily liveweight gain (ADG) or total liveweight gain (Liu *et al.*, 2011). Priolo *et al.* (2000) observed a reduction in ADG from 140 to 50 g d^{-1} when they fed a group of growing lambs a diet contain 25 g kg^{-1}DM *Ceratonia siliqua* (CT), while, Douglas *et al.* (1999) did not find any effect of CT on ADG when they fed a group of lambs diet (*H. coronarium*) containing 72 g kg^{-1}DM CT.

Hymes-Fecht *et al.* (2013) studied the effect of ensiled lucerne, red clover and three varieties of birdsfoot trefoil contain different levels of endogenous CT (2.3, 8.3, 12.1 and 15.7 g kg^{-1}DM), on DMI, body weight changes, milk yield and composition of 25 lactating cows in a Latin square design. The results showed that birdsfoot varieties contained 8.3-15.7 g of CT kg^{-1}DM tended (P=0.07) to reduce DMI, which was considered to be due to the high level of CT tannin. However, Hymes-Fecht *et al.* (2013) noticed that switching the cows diet from silage made from lucerne or red clover to birdsfoot (*L. corniculatus)* varieties resulted in increased milk yield (~8 %), but no differences was noted for the 3 varieties of birdsfoot trefoil (low, moderate and high in CT). Feeding birdsfoot trefoil silage was also found to improve mean N

efficiency from approximately 21.5 to 26.3 % (for lucerne and red clover as compared to birdsfoot trefoil silages respectively). Milk and urinary urea were also found to be reduced with increasing levels of dietary CT. Their results suggest that origin of tannin had a greater effect on animal performance than the level of dietary tannin inclusion. Thus, different plants have different types and concentration of tannin and they did not have a similar influence on animal performance (Piluzza *et al.*, 2014).

The effect of different dietary tannin levels and types of (either endogenous or supplemented) on DMI and ruminant performance are summarised in Table 1.11. Tannin source, type and level of supply may be considered the main factors that affect the bioactivity of tannins. Makkar (2003) concluded in his review that tannin source was the most important factor that could affect animal performance. Pellikaan *et al.* (2011) investigated the effect of 3 different CT; quebracho, grape seed and green tea. The results showed that treating lucerne hay with quebracho tannin had a much greater effect on total gas production and CH_4 emissions compared to grape seed and green tea tannin, even when supplemented at a much lower concentration in the diet. Similarly, Barry and McNabb (1999) observed that DMI was not affected when a group of sheep were fed *Lotus corniculatus* (35-45g kg^{-1}DM CT), while Priolo *et al.* (2000) found a reduction in DMI when a group of lambs were fed diets contain 25 g kg^{-1}DM *Ceratonia siliqua* (CT). The effect of tannin type was investigated by Deaville *et al.* (2010) who noticed that chestnut HT increased DMI whereas mimosa CT reduced DMI and DM digestibility when diets were supplemented at the same level of inclusion. Hymes-Fecht *et al.* (2013) and Solaiman *et al.* (2010) found no effect of endogenous tannin level on DMI or animal

performance. Bhatta *et al.* (2009) found that only the highest levels of tannin reduced rumen fermentation *in vitro*. However, there is a paucity of literature regarding the effect of different levels of supplemented HT either added to the fresh forage at ensiling or in the diet on ensilage profile / composition and animal performance.

Table 1.11 Effect of different tannin types and dosage on animal performance

References	Source	Dosage*	Types	Occur	Animal	DMI	WG	DMD	MY	MCo
Zimmer and Cordesse (1996)	Chestnut	110	HT	ST	Sheep & goat		=**	- (both)		
Bhatta *et al.* (2009)	(*Tamarindus indica*) seed husk	0.25, 75	Tan	ET	Cows	=			=	Pro. + (75)
Woodward *et al.* (2004)	Birdfoot trefoil Ryegrass	26.2 0	CT	ET	Cows	=			+	Pro. +
Ben Salem *et al.* (2005)	Acacia cyanophylla Oaten hay	59.6 0.5	CT CT	ET ET	Lambs	- (acacia)	- (acacia)	- (acacia)		
Puchala *et al.* (2005)	Sericea lespedeza Crabgrass	17.7 0.5	CT CT	ET ET	Goat	+	=**	+		
Sinclair *et al.* (2009)	Whole crop wheat Pea low Tan. Plea high Tan.	 47.2 93.1	 CT CT	 ET ET	Cows	+ (low/high tan)	=		=	=
Kruege *et al.* (2010)	Mimosa Chestnut	14.9 14.9	CT HT	ST ST	Steers	=	=			
Deaville *et al.* (2010)	Mimosa Chestnut	55.6 55.0	CT HT	ST ST	Wether lambs	+ (HT) -(CT)		= (HT) - (CT)		
Dschaak *et al.* (2011)	Quebracho	3	CT	S T	Cows	-		=	=	=

References	Source	Dosage*	Types	Occur	Animal	DMI	WG	DMD	MY	MCo
Solaiman *et al.* (2010)	*Lespedeza cuneata*	0, 0.72, 1.46, 2.22	CT	ET	Kids	=	=			
Toral *et al.* (2011)	Oenological	10	HT+CT	ST	Ewes	=	=		=	=
Cieslak *et al.* (2012)	*Vaccinium vitrs idaer L.*	10	Tan	ST	Cows			=	=	=
Hart *et al.* (2012)	Grass silage				Lambs	=	+	+(47.5)		
	Pea silage Low Tan	47.5	CT	ET						
	Pea silage high Tan.	92.3	CT	ET						
Toral *et al.* (2013)	Quebracho	20	CT	ST	Ewes	=	=		=	=
Hymes-Fecht *et al.* (2013)	Lucerne	0			Cows	=			+(CT)	Pro. & Lac +(CT)
	Red Clover	0								
	Birdfoot trefoil	8, 12, 16	CT	ET						
Liu *et al.* (2013)	Chestnut	10	HT	ST	Cows	=	=**		=	=

*: g kg^{-1} DM, **: weight changes, Add: additives, , CT: condensed tannin, HT: hydrolysable tannin, ET: plant endogenous tannin, ST: supplemented tannin, DMI: dry matter intake, WG: weight gain, DMD: dry matter digestibility, MY: milk yield, MCo: milk composition, = no significant differences , +: increased significantly, -: decreased significantly number in parentheses refer to source or dose level of tannin, Pro: Protein, Lac.: lactose .

Table 1.11 shows that in general addition of tannin to the diet of ruminants had little or no effect on animal performance. Additional tannin was found to reduce rumen protein degradability and increased UDP supply (Sinclair *et al.*, 2009). It may be postulated that these effects may be due to the protein-tannin complex inhibiting microbial activity (Makkar, 2003) plus a direct effect of tannin reducing protozoa and bacteria numbers (Min *et al.*, 2002; Bhatta *et al.*, 2009; Aghamohamadi *et al.*, 2014). An increased flow of dietary protein to the small intestine but decreased flow of MCP may result in little net change in amino acid availability to the small intestine which could explain the lack of effect in animal performance parameters.

1.6 Conclusion of the literature review.

Microbial protein contributes over 70 % of the protein reaching the small intestine in ruminant animals, however, additional dietary by-pass protein supply may be required especially for highly productive animals.

The high demands for plant derived proteins as an important source of dietary protein in livestock production has led to an increase in their prices globally, which has stimulated interest by ruminant farmers in the use of cheaper home grown forage protein forages. Home grown forages are potentially high in protein. However, this protein is highly degradable and has a low efficiency of utilisation. Protein efficiency could be enhanced by reducing protein degradability and increasing UDP supply. Tannins are naturally occurring compounds that could be used to reduce protein degradability. In general, tannins are divided into two types: condensed and hydrolysable tannin. Condensed tannins are the most widely observed tannins in the nature

especially in forages, thus CT have the most attention in the animal nutrition compared to HT. There is a paucity of information regarding the use of hydrolysable tannin as a diet supplementation to reduce protein degradability either at ensiling or in the animal rumen.

1.7 The hypothesis and objectives of the study.

The hypothesis to be evaluated in this study was that supplementary tannin to a variety of forage crops either prior to ensiling or before feeding could bind with forage protein and reduce protein degradability, increase dietary protein supply (UDP and MP) to the small intestine and enhance animal performance. The objectives of this study were to determine the potential effect of supplementary chestnut HT to bean, pea and grass forage at ensiling on silage chemical composition, rumen degradability *in situ*, protein fractionation using the CNCPS and pattern of fermentation. The second objective was to investigate the effect of method of chestnut HT inclusion (time of supplementation) either at ensiling or at feeding to ryegrass silage on lamb dry matter intake and performance. In addition the influence of additional tannin (both methods of inclusion) on diet whole tract digestibility, rumen DM, OM and CP degradability *in situ* and protein fractionation using the CNCPS. The third objective was to evaluate the effect of supplemented chestnut HT (different levels) on lucerne silage post opening silage silos on silage degradability *in situ*, protein fractionation using CNCPS and rumen fermentation, In addition the effect of chestnut HT levels on feed intake and performance of ewes in late pregnancy and early lactation was examined.

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1 Dry matter (DM)

Concentrate, fresh and ensiled forage samples were oven dried (Binder, Tuttlingen, Germany) at 104^{o}C 16 h or freeze dried (Edwards Modulyo freeze dryer, Sussex, UK). Residues from *in situ*, protein fractionation using the CNCPS, faeces and gas production kinetic determination were dried at 60^{o}C. Dried samples were milled through a 1 mm screen (lab analysis) or a 3 mm Screen (for the *in situ* experiments) using a cyclone mill (Cyclotec, FOSS, Warrington, UK). Dry matter (DM) was measured as g kg^{-1} and calculated as:

$$\text{DM g kg}^{-1} = \frac{\text{dried sample weight(g)}}{\text{initial sample weight (g)}} \text{ X } 1000 \qquad \text{Eq.2.1}$$

2.2 Organic matter (OM)

Organic matter was determined by AOAC (2000). Approximately 2 g of dried, ground (1 mm screen) sample was accurately weighed into a pre-weighed porcelain crucible and heated overnight at 550°C in a muffle furnace (Gallenkamp muffle furnace, Size 3, GAFSE 620, Gallenkamp, Loughborough, UK). Samples were then cooled in a desiccator and reweighed.

$$\text{Ash g kg}^{-1}\text{DM} = \frac{\text{weight of ash(g)}}{\text{initial sample weight (g)}} \text{ x } 1000 \qquad \text{Eq. 2.2a}$$

$$\text{OM g kg}^{-1}\text{DM} = 1000 - \text{ash weight (g)}. \qquad \text{Eq. 2.2b}$$

2.3 Crude protein (CP)

Oven dried samples (concentrate, fresh and ensiled forages) and the residue from *in situ* and protein fractionation using the CNCPS experiments were analysed for nitrogen concentration. Approximately 0.15 g of dried sample was accurately weighed into aluminium foil trays to determine N concentration using a C/N analyzer (type FP-528, LECO Instruments, St. Joseph, MI, USA) operating the Dumas method (AOAC, 2000).

$$CP\ g\ kg^{-1}DM = total\ nitrogen\ (g\ kg^{-1}DM) \times 6.25 \qquad \text{Eq. 2.3}$$

2.4 Ammonia nitrogen (NH_3-N)

Ammonia nitrogen was measured according to MAFF (1986) using a Buchi AutoKjeldahl Unit K-370 (BUCHI LABORTECHNIK AG CH-9230, FLAWIL, SWITZERLAND). Samples of silage were accurately weighed (~20 g) into 250 ml bottles. Deionized water (100 ml) was added and all bottles shaken for 60 minutes. The extracted solution was then filtered through a 120 mm Whatman number 45, filter paper. For rumen fluid and gas production kinetic solutions, defrosted samples were placed in 50 ml test tubes and centrifuged at 3000 g at 4^{o}C for 15 min (Rotina 46R Hettich Zentrifugen, Germany); the supernatant was used for NH_3-N determination. Exactly 5 ml of filtrated or 2 ml of centrifuged solution was pipetted into a 250 ml kjeldahl digestion tube and 6 ml of magnesium hydroxide solution (17 g of ignited heavy MgO_2 (VWR, Lutterworth, UK), and suspended in 100 ml of distilled water) was added. The mixed solutions were then steam distilled using an auto-titrator (FOSS 1030 auto-titrator, FOSS, Warrington, UK) and the liberated ammonia bubbled

through 25 ml of receiver solution. Receiver solution was prepared by dissolving 50 g of boric acid in 5 L of distilled water, 50 ml of bromocresol green (100 mg in 100 ml methanol), 35 ml of methyl red solution (100 mg in 100 ml methanol), and 1.5 ml of 0.1 M sodium hydroxide solution added. The receiver solution was then back titrated using 0.01 M hydrochloric acid (VWR, UK) as the titrant. NH_3-N was determined as:

Ammonia N of silage samples:

$$NH_3\text{-N g kg}^{-1}\text{DM} = \frac{7 \text{ x T x } (120 - 0.02 \text{ DM})}{10 \text{ x DM}} \quad \text{Eq.2.4a}$$

The NH_3-N is often expressed as NH_3-N g kg^{-1} total nitrogen (TN) content.

$$NH_3\text{-N g kg}^{-1}\text{TN} = \frac{\text{Ammonia (g kg/DM)}}{\text{total nitrogen}} \text{ x } 1000 \quad \text{Eq.2.4b}$$

The NH_3-N in rumen effluent or supernatant gas production kinetic solution samples:

$$NH_3\text{-N g L}^{-1} = \frac{\text{Sample titre (ml)} - \text{Blank titre (ml)}}{\text{weight of sample distilled (g)}} \text{ x } 0.014 \text{ x } 0.01 \text{ x } 1000 \quad \text{Eq.2.4c}$$

Where T is titre reading (corrected for method blank), DM is the dry matter of the sample (g kg^{-1}) and TN is total nitrogen

2.5 pH

The pH of silage samples were determined by the method of MAFF (1986) using a pH probe (Jenway, Stone, Staffordshire) with daily calibration using pH 4 and pH 7 buffers. Approximately 50 g fresh silage was accurately weighed

into a 250 ml beaker and 100 ml distilled water added. The beaker was swirled for 30 seconds every 15 min for 1 h. The solution was then filtered through Whatman No. 1 filter paper and the pH determined. For different liquid samples (either rumen fluid or media) the pH of the liquid was determined directly. The probe was washed in distilled water between every sample. The pH of lambs' meat was measured using a pH meter (Testo 205 pH/temperature meter Lenzkirch, Germany).

2.6 Neutral detergent fibre (NDF)

Neutral detergent fibre (NDF) content of fresh, ensiled forages, concentrate and faeces samples were determined according to Van Soest *et al.* (1991) using Fibertec apparatus (1020, FOSS, Warrington, UK). Approximately 0.4-0.6 g of the sample was accurately weighed into a pre-weighed glass crucible (porosity 1, Soham Scientific, Ely, UK) and placed into the apparatus. Exactly 25 ml of neutral detergent reagent (93 g of disodium ethylene diamine tetra-acetae dihydrate and 34 g of sodium borate were dissolved in 3 L of deionised water using gentle heat. To this solution 150 g of sodium lauryl sulphate and 50 ml of tri-ethlylene glycol were added. In a separate beaker 22.8 g of anhydrous disodium hydrogen phosphate was dissolved in approximately 500 ml of deionised water. The two solutions were then mixed and diluted to 5 L using deionised water. The pH was adjusted to lie between 6.9 and 7.1 using either 0.1 M NaOH or 0.1 M HCl) and 0.5 ml of octan-1-ol (FOSS, Warrington, UK) was added to each sample to reduce foam. Samples were boiled for 30 min, after which an additional 25 ml of cold neutral detergent reagent and 2 ml of α-amylase (2.8 g of stable alpha-amylase E.C.3.2.1.1. from *Bacillus subtilis*

(Sigma, Gillingham, UK) dissolved in mixed of 10 ml of 2-ethlylene glycol and 90 ml of distilled water and) was added. Samples were boiled for a further 30 min, drained and then washed with 3 x 30 ml of hot (80°C) water under vacuum to remove the NDF reagent. To each sample, 25 ml of hot (80°C) water and 2 ml of alpha-amylase solution was added. After 15 min the samples were drained and then washed under vacuum, with 3 x 25 ml of hot (80°C) water. Crucibles were removed from the apparatus and dried at 104°C overnight. After cooling in a desiccator, crucibles were weighed and then placed in a muffle furnace at 550°C for 4 h. Crucibles were then allowed to cool in a desiccator to room temperature and reweighed.

$$\text{NDF g kg}^{-1}\text{DM} = \frac{\text{residue weight (g)} - \text{ash weight (g)}}{\text{sample weight (g)}} \text{ X1000} \qquad \text{Eq.2.6}$$

2.7 Acid detergent fibre (ADF):

Acid detergent fibre content of fresh, silage forage and concentrated diet samples were determined according to (Van Soest *et al.*, 1991) using Fibertec apparatus (1020, FOSS, Warrington, UK). Approximately 1 g of dried sample was weighed into pre-weighed glass crucible (porosity 1, Soham Scientific, Ely, UK) and placed into the apparatus. Exactly 100 ml of acid detergent reagent (20 g of solid CTAB (cetyl trimethylammonium brmide) dissolved in 1 L of 1 M sulphuric acid) was added and boiled for 60 min. The crucibles were drained and then washed under vacuum, with 3 x 25 ml of hot (80°C) water. Crucibles were then removed from the apparatus and dried at 104°C overnight. After cooling in a desiccator, crucibles were weighed and then

placed in a muffle furnace at 550°C for 4 h. Crucibles were then allowed to cool in a desiccator to room temperature and reweighed.

$$\text{ADF g kg}^{-1}\text{DM} = \frac{\text{residue weight (g)} - \text{ash weight(g)}}{\text{sample weight (g)}} \text{ X 1000} \qquad \text{Eq. 2.7}$$

2.8 Ether extract (EE):

Ether extract of fresh and ensiled samples were determined according to a solvent method of FOSS (1987) using a Soxtec system (HT 1043 extraction apparatus, FOSS, Warrington, UK). Approximately 1 g of dried sample was accurately weighed into cellulose extraction thimble (Whatman, Maidstone, UK), and then the top of the thimble was plugged with cotton wool. Samples were boiled at 40-50^{o}C in 25 ml of petroleum ether (Analar, VWR, Lutterworth, UK) for half an hour. Thimbles were then rinsed for 30 min and the petroleum ether was evaporated, and the ether extract was measured as:

$$\text{EE g kg}^{-1}\text{DM} = \frac{\text{Fat weight (g)}}{\text{sampleweight (g)}} \text{ X1000} \qquad \text{Eq. 2.8}$$

2.9 Gross energy (GE):

An adiabatic bomb calorimeter (Parr 6200 Instrument Company, Moline, IL, 61265, USA) was used to determine the gross energy of the silage samples. Samples were pelleted using a 2811 Parr pellet press then accurately weighed and placed into a crucible. A 10 cm fuse wire was inserted through the holes of the bomb; care was taken to ensure the wire did not contact the sample. The apparatus was assembled, filled with O_2 and placed in a bucket containing 2 L of water and wires were connected. The bomb calorimeter measured the

samples energy content by burning it with O_2 under enclosed conditions at a constant volume. The energy produced was measured as MJ kg^{-1}DM.

2.10 Tannins

Hydrolysable tannins in silage samples were measured according to Makkar *et al.* (2007). Tannin extract from silages were prepared by weighing 0.4 g of sample (oven dried and ground through a 1 mm screen) into 50 ml tubes. Twenty ml of 700 ml L^{-1} aqueous acetone added was added and samples centrifuged for 10 min at 3000 g, 4^{o}C. The supernatant was collected into clean tubes and stored at 4^{o}C for the tannin extraction. In order to measure HT, total phenol, total tannin and condensed tannin were measured as follows:

2.10.1 Total phenol

Total phenol was determined using Folin-Ciocalteu (Makkar *et al.*, 1993), fifty µl of tannin extract (as prepared in Section 2.10) are placed in glass test tube, whereupon deionised water was added to make the volume made up to 1.0 ml. To this solution of 2.5 ml 20 % sodium carbonate solution (50 g dissolved in 250 ml deionized water) and 0.5 ml of Folin-Ciocalteu reagent 1 N (commercially available Folin Ciocalteu reagent (2N) was diluted with an equal volume of deionized water) were added. The test tube was mixed well using vortex and kept for 40 min at room temperature. Optical density was taken at 725 nm in spectrophotometer (Beckman, DU640 spectrophotometer, Fullerton, CA) in a 1 cm^3 cuvette and the concentration estimated from the standard curve. Total phenol was estimated as tannic acid (Sigma, Gillingham, UK) equivalent from the calibration in standard (tannic acid) solution (0, 10,

20, 40, 50, 60, 70, 80, 90 and 100 mg ml^{-1}) and the results expressed as total phenols, g kg^{-1} DM.

2.10.2 Total tannin

Total tannin was measured by precipitating tannins with polyvinyl polypyrrolidone (PVPP, Sigma, Gillingham, UK), which binds tannins. Exactly 0.2 g of PVPP was placed in a test tube and dissolved in 2.0 ml distilled water. To this solution 2.0 ml of tannin extract was added, mixed and kept in a refrigerator for 15 min at 4^{o}C. The mixture was then centrifuged at 3000 g for 10 min at 4^{o}C. The optical density of the supernatant which contained non tannic phenols (PVPP bounds to the tannin) was taken at 725 nm in a spectrophotometer (Beckman, DU640 spectrophotometer, Fullerton, CA). Total tannin was then measured by measuring the total phone of the supernatant as mentioned in Section 2.10.1 and expressed then content of the non-tannins phenolic on a dry matter basis. From the above results the tannin content of the samples were calculated as:

Total tannin g kg^{-1}DM=Total phenols (g) – Non-tannic phenols (g) Eq. 2.10a

2.10.3 Hydrolysable tannin

Hydrolysable tannin was measured by the differences between total tannin (measured in 2.10.2.) and condensed tannin (using the butanol-HCl method, according to Porter *et al.* (1985)). For the butanol-HCl method 0.5 ml of tannins extract was taken in a test tube in triplicate. To this 3.0 ml butanol HCl reagent ((butanol- HCl, 95:5 v/v): 950 ml n-butanol was mixed with 50 ml of concentrated HCl) and 0.1 ml of 2 % ferric reagent (16.6 ml concentrated HCl

diluted to 100 ml with distilled water to make 2N HCl and 2.0 g ferric ammonium sulphate was added and dissolved). The two solutions were combined and mixed by vortex. The mouth of the tubes were covered with a glass marble and then boiled for 60 minutes at a temperature of 97 to 100^{o}C.

Similarly a blank was prepared for each sample but without the heating process. The tube was cooled to room temperature and the reading was taken at 550 nm using spectrophotometer (Beckman, DU640 spectrophotometer, Fullerton, CA). Hydrolysable tannin was calculated as below:

HT g kg^{-1} DM = Total tannin (g) – condensed tannin (g). Eq. 2.10b

2.11 Protein fractionations using the CNCPS:

Freeze dried silage samples (milled through a 1 mm screen) were analysed for protein fractions using the *in vitro* protein fractionation technique based on the Cornell Net Carbohydrate and Protein System (CNCPS, Sniffen *et al.*, 1992) as described by Licitra *et al.* (1996). Protein solubility in different chemical reagents are summarized as:

2.11.1 Non-protein nitrogen (NPN):

Non-protein nitrogen determination depends on the reaction between true protein parts of nitrogen with suitable precipitant, filtration and measuring the non-protein nitrogen (soluble parts) (Sniffen *et al.*, 1992). Tungstic or trichloroacetic acid (TCA) are the most widely precipitant used for protein participation (Sniffen *et al.*, 1992). Approximately 0.5 g of ground freeze dried silage samples were accurately weighed in to a 100 ml beaker; 50 ml of deionised water was added and allowed to stand for 30 min, 10 ml of 10%

TCA solution (the TCA solution was prepared by dissolving 10 g of TCA in to a 100 ml of deionised water) was added, then left to stand for 30 min. The residue was filtered using Whatman 54 and moved to a hot oven at 60^{o}C for 48 h. The dried weight was recorded and the CP of the dried residue measured using a LECO-N machine as described in Section 2.3. The NPN was calculated as:

True protein g kg^{-1}CP = residual CP (g) x DM solubility % Eq. 2.11a

Non-protein N g kg^{-1}CP = sample CP (g) – true protein (g) Eq. 2.11b

Were CP is crude protein (g kg^{-1}DM) DM solubility is the DM losses of the residual samples inside the solution.

2.11.2 Buffer soluble protein.

Buffer soluble protein is known as true protein that is soluble in buffer at rumen pH (Sniffen *et al.*, 1992). Buffer soluble protein was determined by weighing approximately 0.5 g of ground freeze dried samples in 100 ml beaker. To this, 50 ml of borate-phosphate buffer pH 6.7 (12.2 g of mono sodium phosphate, 8.91 g of sodium tetraborate and 100 ml of tertiary butyl alcohol mixed and made up to 1 L using deionised waster; the pH was adjusted using 1M HCl) and 1ml of sodium azide 10 % (10 g of sodium azide was dissolved in 100 ml of deionised water, freshly prepared) was added. The solution was left to stand for 3h. The residue was filtered using Whatman 54 and moved to a hot oven at 60^{o}C for 48 h. the dried weight recorded and the CP of the dried residue was measured using LECO-N machine as described in Section 2.3. The buffer soluble N calculated as shown:

Buffer insoluble CP g kg^{-1}CP= residual CP (g) x DM solubility % x Eq. 2.11c

Buffer soluble CP g kg^{-1}CP = sample CP - buffer insoluble CP (g) Eq. 2.11d

Were CP is crude protein (g kg^{-1}DM) DM solubility is the DM losses of the residual samples inside the solution.

2.11.3 Neutral detergent insoluble protein (NDIP):

Neutral detergent insoluble protein is known as the CP associated with neutral fibre (Sniffen *et al.*, 1992) and their determination is similar to NDF determination as described in Section 2.6, however after the residue was dried in a hot oven at 105^{o}C, the dried weight was recorded and the CP of the dried residue was measured using LECO-N machine as described in Section 2.3. The neutral detergent insoluble N was calculated as:

NDIP g kg^{-1}CP = residual CP (g) x DM solubility % Eq. 2.11e

Neutral detergent soluble N g kg^{-1}CP = sample CP - NDIP Eq. 2.11f

Were CP is crude protein (g kg^{-1}DM) DM solubility is the DM losses of the residual samples inside the solution.

2.11.4 Acid detergent insoluble protein (ADIP):

Acid detergent insoluble protein is considered as indigestible protein (Sniffen *et al.*, 1992). These kinds of proteins are usually attached with lignin fibres which the animal cannot digest (McDonald *et al.*, 2011). The method of measuring acid detergent insoluble protein, is similar to ADF as described in Section 2.7, however after the residue has been dried in a hot oven, crucibles

were moved to the LECO-machine in order to measure its N content as described in Section 2.3. The acid detergent insoluble N was calculated as:

ADIP g kg^{-1}CP = residual CP (g) x DM solubility % Eq. 2.11g

Acid detergent soluble N g kg^{-1}CP = sample CP – ADIP Eq.2.12h

Were CP is crude protein (g kg^{-1}DM) DM solubility is the DM losses of the residual samples inside the solution.

The protein fractionation of forage protein using the CNCPS technique can be fractioned in to: A, B1, B2, B3 and C (Table 2.1). Fraction A is non-protein nitrogen, fraction B is true protein which can be sub divided. B1 is rapidly degradable protein in the rumen which is buffer soluble N, pH 6.8 at room temperature, B2 is intermediate degradable N, which is neutral detergent soluble N, B3 is slowly degradable in the rumen and is insoluble in neutral detergent solution but soluble in acid detergent solution and fraction C is unavailable protein which is insoluble in acid detergent solution.

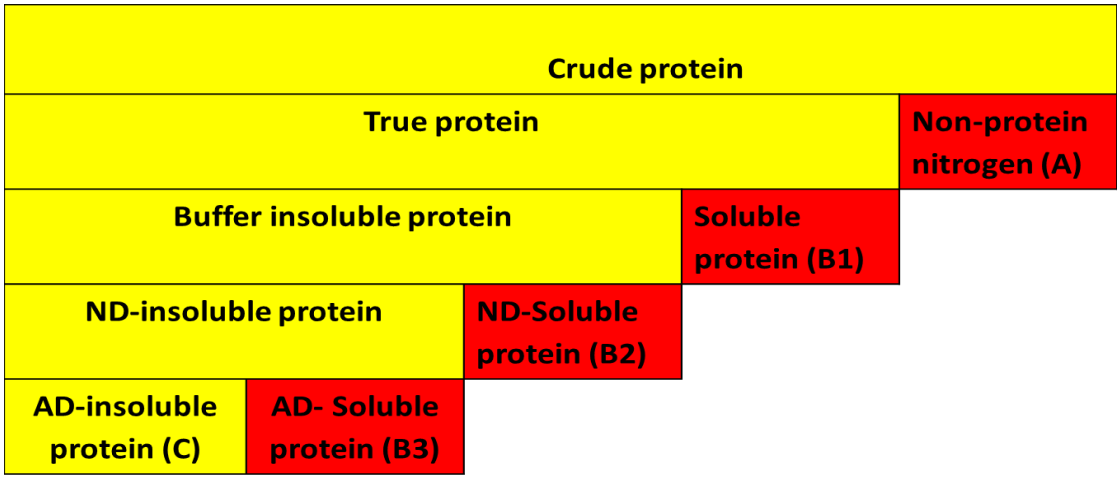

Figure 2.1 Feedstuff crude protein fractionation (Sniffen *et al.*, 1992)

The undegradable dietary protein at outflow rate of 0.05 h^{-1} (UDP5) was calculated according to Kirchhof (2007) as follows

$$UDP5 = 321.9023 + (0.1676 \times ADIP) + (-0.0022 \times (CP \times (A+B1))) + (0.0001 \times (CP \times C^2)) \quad \text{Eq. 2.11i}$$

Where A, B1, B2, B3 and C are described above, ADIP described 2.11.4.

2.12 *In situ* technique:

Approximately 20-25 g of fresh material (experiment 1a) or 5-6 g of freeze dried samples milled through a 3 mm screen (experiments 2a and 3a) were accurately weighed into mono filamentous polyester fibre bags (*in situ* nylon bags) (23 x 9 cm and aperture size 43 x 43 µm) (quadruplicate) and recorded. The round bottoms of the bags were sealed by passing the neck of the bag through a brass curtain ring (18 mm diameter), folding the neck of the bag against itself, and securing it with an elastic band.

Four bags were connected to a stainless steel clip, which was attached to the cannular cap by 30 cm length of nylon cord. Each treatment sample was incubated in the rumen for 4, 8, 16, 24, 48 and 72 h using a complete exchange method (Paine *et al.*, 1982). After the incubation period the bags were placed in cold water to remove rumen remains, then the bags were washed using a washing machine with cold water for 45 min. A standard from each treatment (quadruplicate) bag with zero hours (without rumen incubation) were washed also in order to know the washing losses. Post washing, the bags were dried in an oven at 60^{o}C for 48h and weighed.

Four mature, ruminally fistulated wethers (80 kg ± 10) were used for the *in situ* experiments. The wethers were kept as a group feeding and offered a concentrate diet (500 g head^{-1} meal^{-1}) in two equal meals at 900 and 1600 (850 g kg dry matter and 922, 160, 32, 88 g kg^{-1}DM for OM, CP, crude oil and crude fibre, respectively, plus selenium 0.5 mg kg^{-1}DM and 8000, 1500 and 100 IU kg-1DM for vitamin A, D3 and E respectively) at 1.1 x maintenance requirements according to AFRC (1993) with free access to clean water and straw.

The *in situ* residual were milled and analysed for CP and OM.

The DM, CP and OM degradability of all treatments were fitted without zero value using the equation suggested by McDonald (1981), were this model incorporates an estimation of lag phase prior to the commencement of degradation.

$$p= a+b\ (1-\exp^{-c\ (t-t_0)}). \qquad \text{Eq.2.12a}$$

Where p is the disappearance at time t, “a” is the immediately soluble fraction, “b” is the potentially degradable fraction and “c” is the constant rate of the degradation of “b”, t is the time in hours and t_0 is the lag time. Effective degradability (ED) was calculated using the equation of Ørskov and McDonald (1979) and r is the rumen outflow rate of 0.05 (ED5) as shown:

$$ED5\ (g\ kg^{-1}DM) = a+ ((b\ X\ c)/(c + r))\ X1-\exp^{-(c+r)\ t} \qquad \text{Eq.2.12b}$$

The undegradable dietary protein (UDP) for the *in situ technique* at outflow rate 0.05 (UDP5) was calculated as

$$UDP5\ (g\ kg^{-1}DM) = 1000-ED \qquad \text{Eq. 2.12c}$$

The non-linear *in situ* variables a, b and c were estimated using curve fit of SigmaPlot 12 (Systat Software Inc., London UK), for each treatment within each sheep

The calculation of dietary protein parameters were measured according to AFRC (1993):equations as follows:

Quickly degradable protein (QDP) g kg^{-1}DM= a x CP Eq. 2.12 d

Slowly degradable protein (SDP) g kg^{-1}DM = {(b x c)/ (c + r)} x CP Eq. 2.12e

Effective rumen degradability (ERDP) g kg^{-1}DM = 0.8 (SDP) + (QDP)

Eq. 2.12f

Undegradable dietary protein (UDP) g kg^{-1}DM = CP- (QDP+SDP) Eq. 2.12g

Digestible undegradable protein (DUP) g kg^{-1}DM = 0.9 (UDP) - (ADIP)

Eq. 2.12h

Metabolisable protein (MP) g d^{-1}= 0.6375 (MCP) +DUP Eq. 2.12i

Microbial protein (MCP) g d^{-1} = EDRP g d^{-1} Eq. 2.12j

Where a is immediately soluble protein, CP is crude protein, b is insoluble but potentially degradable protein, c is the rate of degradation, r is outflow rate (r measured as 0.05 h^{-1} for adult sheep), ADIP is acid detergent indigestible protein.

2.13 Blood analysis:

A cobas-Mira (Mira plus ABX Diagnostics) blood analyser machine was used for blood plasma analysis. Plasma samples were analysed for: glucose

(Randox Laboratories kit, GL1611)(experiment 3a only), total protein (Randox Laboratories kit, TP245), urea (Randox Laboratories kit, UR221) and beta hydroxybutyrate (Randox Laboratories kit, RB1008).

2.14 Digestibility

Acid insoluble ash was used as indirect marker to measure feed digestibility in sheep according to Van Keulen and Young (1977). Approximately 5 g of dried ground sample (in duplicate) of either feed or faeces were accurately weighed in a pre-weighed furnace crucible. The crucible was transferred to a hot oven 135^{o}C for 2 h, cooled in desiccator and re-weighed, and then the crucible moved to a muffle furnace (Gallenkamp muffle furnace, Size 3, GAFSE 620, Gallenkamp, Loughborough, UK) and heated at 550^{o}C for 4 h. The exact weight of the ash was recorded and transferred into a 600 ml Berzelius beaker. Exactly 100 ml of HCl 2N was added and the mixture boiled for 5 min. The hot hydrolysed solution was filtered using Whatman No. 41 (Fisher Scientific Ltd, Leicestershire, UK) and washed free of acid with hot deionised water (~80^{o}C). The filter paper with the residue was then transferred to in to the crucibles and ashed at 550^{o}C for 4 h in a muffle furnace. After cooling the crucibles were reweighed and the acid insoluble ash (AIA) calculated as

$$\text{AIA} = \frac{(\text{weight of crucible (g)} + \text{ash} - \text{weight of crucible (g)})}{(\text{weight of dry smaple (g)})} \text{ X } 100$$

Eq. 2.14a

Digestibility (kg kg^{-1}) of the DM was calculated from the following equation

Digestion coefficient (kg kg^{-1}) of DM=1000-1000x $\frac{\text{DM inicator in feed (kg)}}{\text{DM indicator in faecal (kg)}}$

Eq.2.14b

The faecal DM output (g d^{-1}) for each ewe was also calculated as:

Faecal DM output (g d^{-1}) = DMI (kg) – (DMI (kg) x digestibility (kg kg^{-1}))

Eq.2.14c

The organic matter, CP and NDF digestibility were also calculated by knowing the DMI, faecal output, diet and faecal analysis for OM, CP and NDF.

2.15 Volatile fatty acids (VFA)

Rumen fluid samples were analysed for volatile fatty acids using gas chromatography (GC). After samples were collected, the samples were centrifuged (Beckman, Avanti^{TM} 30 centrifuge, Harbor Boulevard, California) at 3000 g for 15 min and stored at -20^{o} C. Prior to analysing the samples for VFA, the samples were defrosted at 4^{o}C for 24 h, vortexed (FB 15013 Topix$^{®}$, Fisher Scientific Ltd, Leicestershire, UK) for 30 sec, and prepared for VFA analysis according to the method published by Cruwys *et al.* (2002). One ml of homogenised rumen sample was pipetted into a 22.3 ml headspace GC vial using a large tip. To this 1 ml of sodium bisulphate, 1 ml of distilled water and 0.1 ml internal standard (2-Ethylbutyric acid) were added. The vials were then tightly capped and vortexed for 1 min to mix thoroughly. The samples were left to stand for a further for 10 min.

The samples were run on a headspace gas chromatograph an Agilent Technologies 7820A GC system (Agilent, UK). The system consisted of a

headspace sample unit and an auto Agilent GS sampler (Hewlett Packard HP, 7694) equipped with a 122-3232: 250^{o}C: 30m x 250 µm x 0.25 µm (Back SSZ Inlet N2) column.

The headspace sample controlled the optimized vial thermostatting time for 30 min, vial pressurisation time of 3 min and sample injection period of 0.10 min. The initial temperature of the column was 60^{o}C which was held for 2 min, after that the temperature was raised to 190^{o}C by increasing rate 10^{o}C min^{-1}, held for one minute followed by increasing the temperature to 220^{o}C with the increased rate of 40^{o}C min^{-1} and was held at this temperature for 2 minutes. The flow rate of hydrogen carrier gas was 1.9079 ml min^{-1} with column head pressure 19 psi. The temperature at injection was set at 250^{o}C. The injection ratio was operated as a split flow of 5.0 m min^{-1} at 200^{o}C.

A calibration standered containing all of the VFA (acetic, propionic, butyric, iso-butytic, valeric, iso-valeric and caproate) was run before and between samples to ensure constant reading were achieved. Measurement were obtained in mg ml^{-1} and converted to mmol ml^{-1} as using the following equation:

$$\text{Individual VFA (mmol ml}^{-1}\text{)} = \frac{\text{concnetration of individual VFA (mg)}}{\text{moliculare weight of the individual VFA}}$$

Eq. 2.15

CHAPTER 3, Experiment 1: Evaluation of chestnut HT as a silage additive mixed with pea, bean and ryegrass at ensiling.

3.1 Introduction

Tannin has been reported (Salawu *et al.*, 1999; Mueller-Harvey, 2006) to reduce forage protein degradation of ensiled forage during the fermentation period inside the silage clamp, hence improve the quality of the final silage (reducing NH_3-N and VFA concentration). The reduction of protein degradation could be due to different reasons: firstly tannin might create a complex with the protein part of plant protease enzymes inhibiting their activity (Lorenz *et al.*, 2010). Secondly tannins have a negative impact on endogenous plant microbes which have an important role in protein degradation inside the silage clamp (Tabacco *et al.*, 2006), and finally tannin might bind with the plant cell wall to make them resistant to the proteolysis process inside the silage clamp (Salawu *et al.*, 1999). Several studies (Salawu *et al.*, 1999; Lavrencic and Levar, 2006; Tabacco *et al.*, 2006, De Oliveira *et al.*, 2009; Grabber and Coblentz, 2009) have found that either endogenous or supplemented tannin reduced ammonia nitrogen (NH_3-N) in silage which gives an indication that tannin decreases protein hydrolysis. However, there is a paucity of data regarding the use of chestnut hydrolysable tannin (HT) on silage additive in different forages (especially ryegrass silage) in order to reduce protein degradation either inside silage silo or animal rumen. In addition, chestnut HT is commercially available (often use in wine industry) and considered as nontoxic tannin to ruminants (Deville *et al.*, 2010).

The objective of this study was to investigate the potential effects of chestnut tannin (HT) supplementation (two levels of inclusion) to bean, pea and ryegrass forages at ensiling on the chemical composition of the silage, rumen degradability *in situ* and pattern of fermentation.

3.2 Materials and methods

3.2.1 Experimental design and silage making

The experiment was conducted using three different forages (bean, pea and ryegrass) treated with four additives (two levels of chestnut HT, inoculate and water). The main experimental design was a 3 x 4 factorial design.

Whole crop pea (white flower Magnus, *Pisum sativum*), bean (field bean, *Vicia faba*) and ryegrass (perennial ryegrass mix sward, second cut) forages were harvested at Harper Adams University on the 12th July 2011. Forages were wilted for 48 h, chopped using a forage harvester (Jaguar 870-840 forage harvester, UK) and ensiled in 25 kg experimental silos. Each forage was treated with one of 4 additives; 40g^{-1}kg FW chestnut HT (Thomas Ware & Sons Ltd, Bristol, UK) [HiT], 20 g^{-1}kg FW tannin chestnut HT (Thomas Ware & Sons Ltd, Bristol, UK) [LT], an inoculant (10^{6} colony forming unit homofermentative *Lactobacillus plantarum* g^{-1}) [Inoc] as a positive control, or water [W] as a negative control. According to the manufacturer chestnut HT had an actual tannin content of 750 g kg^{-1}DM (with a mix of the following tannins: castalagin, vescalagin, castalin and vescalin, with a proportion of 530, 350, 30 and 80 g kg^{-1}).

Additives were mixed manually with 100 kg of each forage types. To ensure consistency, water was applied to all treatments at a rate of 1 $L^{-1}t$ FW. A subsample from each fresh forage was taken and stored at -20^{o}C prior to further analysis. Each experimental silo, lined with a plastic bag was filled with approximately 25 kg of fresh treatment and consolidated well. The neck of the plastic bag liner was then sealed using silage tape and approximately 4 kg of sand was place on the top of each silo. Three replications per treatment (12 treatments) were made, with the total number being 36 silage silos. Silos were ensiled for 100 days prior to being opened and subsamples stored (-20˚C) prior to further analysis.

Table 3.1 Proximate analysis of fresh forage samples.

Forage	Bean*	Pea*	Ryegrass
DM $_{(g\ kg^{-1})}$	235	318	345
OM $_{(g\ kg^{-1}DM).}$	899	922	888
CP $_{(g\ kg^{-1}DM).}$	150	148	174
NDF $_{(g\ kg^{-1}DM).}$	365	351	496
ADF $_{(g\ kg^{-1}DM).}$	276	271	254
EE $_{(g\ kg^{-1}DM).}$	9	11	21
GE $_{(\ MJ\ kg^{-1}DM)}$	16.8	17.1	17.2

DM: dry matter, OM: organic matter, CP: crude protein, NDF: neutral detergent fibre, ADF: acid detergent fibre, EE: ether extract and GE: gross energy. *: whole crop.

Samples of fresh forages were defrosted slowly at 2-4ºC, oven dried at 104ºC and milled through a 1 mm screen and analysed for organic matter (OM), crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), ether extract (EE) and gross energy (GE), all the methods are described in Chapter 2 (Sections: 2.1, 2.2, 2.3, 2.6, 2.7, 2.8 and 2.9 respectively). The proximate analyses of the fresh forages are shown in Table 3.1.

3.2.2 Effect of chestnut HT supplementation on the chemical composition of the final silage.

Silage samples produced in Section 3.2.1 were defrosted slowly at 2-4ºC, and analysed for NH_3-N and pH (in duplicate) as described in Chapter 2 (Sections 2.4 and 2.5 respectively). The silage samples were then oven dried and milled through a 1 mm screen and analysed for DM, OM, CP, NDF, ADF, EE, GE and HT (in duplicate) by the methods are described in Chapter 2 (Sections: 2.1, 2.2, 2.3, 2.6, 2.7, 2.8, 2.9 and 2.10, respectively).

3.2.3 Experiment 1 a: Effects of supplemental chestnut HT at ensiling to different forages on *in situ* rumen degradability and protein fractionation using the CNCPS.

3.2.3.1 *In situ* technique

Samples of the silages prepared in Section 3.2.1 were used for the determination of *in situ* rumen degradability (in quadruplicate), as described in Chapter 2 Section 2.12. Approximately 20-25 g of fresh, un-chopped silage sample of each treatment was accurately weighed individually into pre labelled, precision woven, mono filamentous nylon bag, the weight was recorded and the bags then incubated inside the animal's rumen as described

in Chapter 2 Section 2.12. Four mature wether (80 kg ± 10) fitted with permanent rumen cannula were used for the *in situ* experiment, where each wether received bags of all treatments for the same incubation time period. The wethers were kept as a group for feeding and offered a concentrate diet (Section 2.12). Dry matter and CP degradation profiles were measured for all treatments including: the immediately soluble fraction “a”, insoluble but potentially degradable fraction “b”, the total potential degradable fraction “a+b”, the rate of degradation fraction “c”, lag time and the effective degradability at outflow rate 0.05 h^{-1} (ED5) according to the procedure described by McDonald (1981).

3.2.3.2 Protein fractionation technique using the CNCPS

Silage samples from each silage silo prepared in Section 3.2.1 were freeze dried (Edwards Modulyo freeze dryer, Sussex, UK) and milled through a 1 mm screen, and then the samples were analysed (in duplicate) for protein fractions using the CNCPS technique according to Sniffen *et al.* (1992) and recommendations published by Licitra *et al.* (1996), as described in Chapter 2, Section 2.11. The protein content of the forage silages were fractioned into: non-protein nitrogen (fraction A), rapidly degradable protein (fraction B1), midrate degradable protein (fraction B2), slowly degradable protein (fraction B3) and indigestible protein (fraction C). In addition, the undegradable dietary protein (UDP5) at outflow rate 0.05 h^{-1} was calculated according to the equation reported by Kirchhof (2007) as described in Chapter 2 Section 2.11.

3.2.4 Experiment 1 b: Effect of chestnut HT supplementation on *in vitro* rumen fermentation kinetics

Kinetics of rumen fermentation was estimated using the gas production technique described by Theodorou *et al.* (1994). The rumen fluid was collected from four wethers fitted with permanent rumen cannula, 4 hours post feeding and transported to the lab in pre-warmed thermos flasks. The wethers were kept as a group for feeding and offered a concentrate diet (Section 2.12). Rumen fluid was then strained through four layers of muslin into a pre-warmed (39^{o}C) conical flask under a constant stream of gaseous CO_2.

Two types of media were used: nitrogen sufficient media as described by Huntington and Givens (1998) and nitrogen deficient media as described by Getachew *et al.* (2000). Exactly 750 ml of stained fluid was added to 4250 ml of each media to form 150 ml rumen fluid L^{-1} media. Each solution was kept at 39^{o}C under anaerobic conditions until dispensed into the 250 ml fermentation vessels. Media was made the day prior to rumen fluid collection and autoclaved (at 120˚C for 30 min) then stored in a fridge temperature under a constant stream of CO_2. Approximately 2 g of oven dried (60^{o}C) and milled (1mm screen) silage samples (prepared in Section 3.2.1) for each treatment (in duplicate) were accurately weighed into a 250 ml screw topped bottle (Schott) and stored in an incubator at 39^{o}C. Four blank bottles were also incubated in each run. Exactly 200 ml of the mixed rumen fluid buffer solution was added into each bottle using a peristaltic pump dispenser and the bottle was sealed using a lid fitted with a syringe needle and a 3-way tap (Figure 3.1).

After sealing all bottles, the needle tap was closed and the bottles incubated at 39^{o}C for 72 h. The gas pressure of the bottles were measured using a manual pressure transducer (T443; Bailey and MacKay Ltd., Birmingham UK) at 2, 4, 6, 8, 12, 16, 20, 24, 32, 40, 48, 60 and 72 h post inoculation. After 72 h the lids were removed from all bottles which were directly chilled at -20^{o}C for 30 min to inhibit microbial activity. After chilling the pH of the solution was recorded and 20 ml of liquid sample was collected and stored at -20^{o}C for analysis of NH_3-N. Solid residues were collected by filtering under vacuum using a glass crucible (50 ml, porosity P1, Pyrex). Crucibles were dried at 60^{o}C for 48 h and reweighed to calculate DM loss. The DM residues were then ashed at 500^{o}C for 12 h and the final weight recorded to determine OM loss. This method was repeated four times.

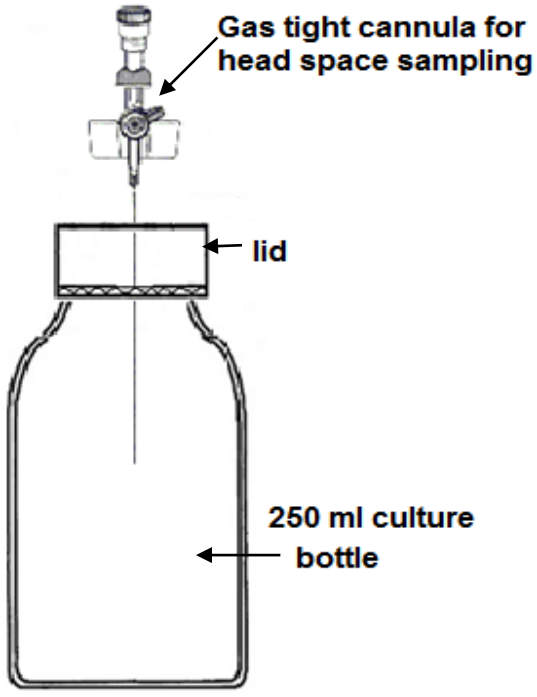

Figure 3.1 Rumen fermentation kinetics (*in vitro*) culture bottle

The *in vitro* DM and OM degradability (disappearance) was corrected for the blank and calculated according to the following equation:

$$\textit{In vitro}\ \text{digestibility of X (g kg}^{-1}\text{DM)} = \frac{\text{X in} - \text{X out}}{\text{X in}} \qquad \text{Eq.3.1}$$

Were X is DM or OM

The gas production pressure (kPa) data were corrected for the substrate blank and transferred to volume (ml) according to Purcell *et al.*, (2011) as:

$$\text{Gas production (ml)} = \frac{\text{Vh}}{\text{Pa}} \text{ X Pt} \qquad \text{Eq.3.2}$$

Where Vh is bottle headspace volume (107.55 ml), Pa is atmospheric pressure (101.4 kPa) and Pt the gas pressure of a transducer (kPa).

3.2.6 Statistical analysis

All measured parameters were statistically analysed using an ANOVA procedure of Genstat (GenStat version 15, VSN International Ltd, UK). Proximate analysis of silage samples (three replication per treatment, n=36) were analysed as factorial 3 x 4 (forage types x silage additives) the forage types were: bean, pea and ryegrass. The silage additives were: water (negative control), inoculate (positive control) low tannin (20 g kg^{-1}FW) and high tannin (40 g kg^{-1}FW).

The non-linear *in situ* degradability for DM and CP parameters were fitted with zero hour according to the equation of McDonald (1981), using the curve fit of SigmaPlot 12 (Systat Software Inc., London UK) for each treatments and wether, for estimating fractions “a, b and c”, lag time and ED. The *in situ* DM and CP degradability parameters (four replication per treatment, n=48) and protein fractionation using the CNCPS parameters (three replication per treatment, n=36) were then analysed as factorial 3x4 (forage types x silage additives) were the forage types and silage additives as mentioned above.

The non-linear rumen fermentation kinetic *in vitro* parameters of each individual bottle and incubation times, fractions “b and c” were estimated using curve fit of SigmaPlot 12 (Systat Software Inc., London UK). The rumen fermentation kinetic *in vitro* parameters were analysed as factorial 2x3x4 designed (media x forages x silage additives), where media was N-sufficient and N-deficient, forage types and silage additives as mentioned above. The gas production kinetic study was conducted four times, n=96.

3.3 Results

3.3.1 Effect of chestnut HT supplementation on the chemical composition of the final silage.

All forages were well fermented as indicated by the low pH (3.8-4.3) and NH_3-N levels (40-66 g kg^{-1}TN). The NH_3-N values were significantly different between forages (56.5, 55.8 and 42.1 g kg^{-1}TN for BS, PS and GS respectively) and additives (41.6, 48.8, 55.7 and 60.2 g kg^{-1}TN for HiT, LT, Inoc and W respectively). The forage, additive interaction for the NH_3-N determination was found to be significant (*P*=0.024). High tannin addition significantly reduced the NH_3-N (~14-30 %) compared to the other additives (Table 3.2).

Ensiled samples of pea, bean and ryegrass crops were found to be different for all proximate analyses, except GE (Table 3.2). Ryegrass silage (GS) had the highest DM, CP, NDF and EE compared to pea silage (PS) and bean silage (BS). Acid detergent fibre was found to be highest in BS compered to PS and GS. Moreover, the proximate analysis of all forage silages were unaffected by supplemented tannin. Ensiling reduced the DM of all forages by approximately 10-20 %, while the proportion of ADF and NDF increased in the leguminous silages and reduced in GS (Table 3.1. and 3.2.).

Table 3.2 Effect of supplemented chestnut HT or inoculate at ensiling on chemical composition of bean, pea and ryegrass silages.

Forages	Bean silage				Pea silage				Ryegrass silage				SED.			*Probability*		
Additives	W	Inoc	LT	HiT	W	Inoc	LT	HiT	W	Inoc	LT	HiT	Add	For.	Inte	Add	For	Inte
pH	3.88	3.90	3.91	3.93	4.01	4.03	4.03	3.99	4.33	4.23	4.29	4.24	0.022	0.025	0.044	0.69	<0.01	0.32
NH_3-$N_{(g\ kg^{-1}TN)}$	65.8	61.5	50.9	48.1	65.7	62.8	55.1	39.6	48.4	42.6	40.2	37.1	1.71	1.97	3.42	<0.01	<0.01	0.02
DM $_{(g\ kg^{-1})}$	221	206	205	203	257	255	256	253	302	303	302	303	4.0	3.5	7.0	0.38	<0.01	0.54
OM $_{(g\ kg^{-1}DM)}$	898	892	891	895	919	913	915	921	879	877	878	880	3.0	2.6	5.3	0.27	<0.01	0.97
CP $_{(g\ kg^{-1}DM)}$	151	154	155	155	149	148	148	141	176	177	170	173	2.8	2.5	4.9	0.72	<0.01	0.50
$NDF_{(g\ kg^{-1}DM)}$	392	398	399	395	370	370	368	363	460	475	489	487	8.0	6.9	13.8	0.52	<0.01	0.66
$ADF_{(g\ kg^{-1}DM)}$	323	317	344	335	295	319	312	310	277	299	279	263	8.8	7.7	15.3	0.36	<0.01	0.011
EE $_{(g\ kg^{-1}DM)}$	9	10	8	8	13	12	12	10	29	28	28	30	0.9	0.7	1.5	0.47	<0.01	0.12
$GE_{(g\ kg^{-1}DM)}$	16.8	16.9	16.9	16.8	17.1	17.0	16.9	17.7	17.0	17.4	18.1	17.4	0.27	0.31	0.53	0.08	0.68	0.51
$HT_{(g\ kg^{-1}DM)}$	-	-	81	162	-	-	61	122	-	-	65	130	-	-	-	-	-	-

W: water (negative control), Inoc: inoculate (positive control), LT: low tannin level, HiT: high tannin level. For.: forages, Add: additives, inte: interaction between additives x forages; NH_3-N: ammonia nitrogen. DM: dray matter, OM: organic matter, CP: crude protein; NDF: natural detergent fibre; ADF: acid detergent fibre; EE: ether extract; GT: gross energy, HT: hydrolysable tannin.

3.3.2 Experiment 1 a: Effects of supplemental chestnut HT at ensiling to different forages on *in situ* rumen degradability and protein fractionation using the CNCPS.

3.3.2.1 In situ rumen degradability

The *in situ* DM degradability results are shown in Table 3.3. Ryegrass silage was found to be more degradable compared to BS and PS (Figure 3.2.). The immediately soluble DM fraction "a" results showed that GS had the highest value compared to BS and PS (344, 334 and 300g kg^{-1}DM respectively, $P<0.01$).

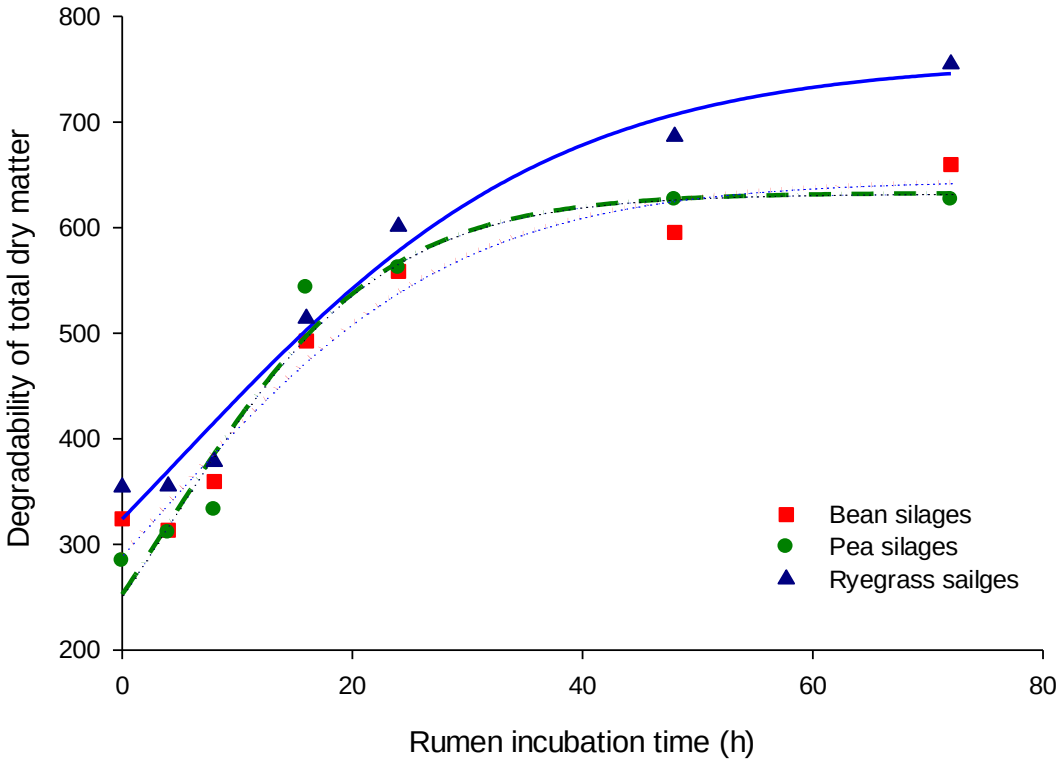

Figure 3.2 Effect of supplemented chestnut HT (two levels) or inoculate to different forages at ensiling on *in situ* DM degradability (g kg^{-1}D) with different forage types.

Similarly, GS had the highest (P<0.01) insoluble but potentially degradable fraction “b” compared to BS and PS (477, 410 and 437g kg^{-1}DM, respectively), hence, the total potentially degradable fraction “a+b” were highest in GS compare to the leguminous silages (Table 3.3).

The rate of degradation fraction “c” in GS was lower by 41 % compared to leguminous silages. As a consequence GS had the highest DM ED at an outflow rate of 0.05 h^{-1} compared to BS and PS (ED5: 874, 781 and 674 g kg^{-1}DM, respectively P<0.01, Table 3.3).

Treated silages with tannin (both levels) or inoculate (positive control) were found to reduce (P<0.01) *in situ* DM fraction “a” (17, 9.2 and 8.3 %) and DM fraction “a+b” (14.5, 6.0 and 3.4 %) for HiT, LT and Inoc, respectively, compared to treating the silages with water only. Additives had no effect (P=0.57) on the insoluble but potentially degradable fraction “b” (Figure 3.3).

The rate of degradation fraction “c” results showed that HiT had the slowest (P<0.01) rate of degradation (0.022) compared to W (0.026), Inoc (0.029) or LT (0.027). Furthermore, HiT reduced (P<0.001) DM ED5 compared to other additives (692, 784,803 and 827 g kg^{-1}DM, for HiT, LT, Inoc and W, respectively).

The dry matter fraction “a” results showed that there was a significant interaction between forage types and additives (Table 3.3). Treated GS with either tannin levels (HiT or LT) had a higher effect reducing fraction “a” compared to PS and BS. In addition, treating BS with HiT reduced ED5 by approximately (25 %), while the reduction was only 9.5 and 13.2 % in GS and PS, respectively (Table 3.3).

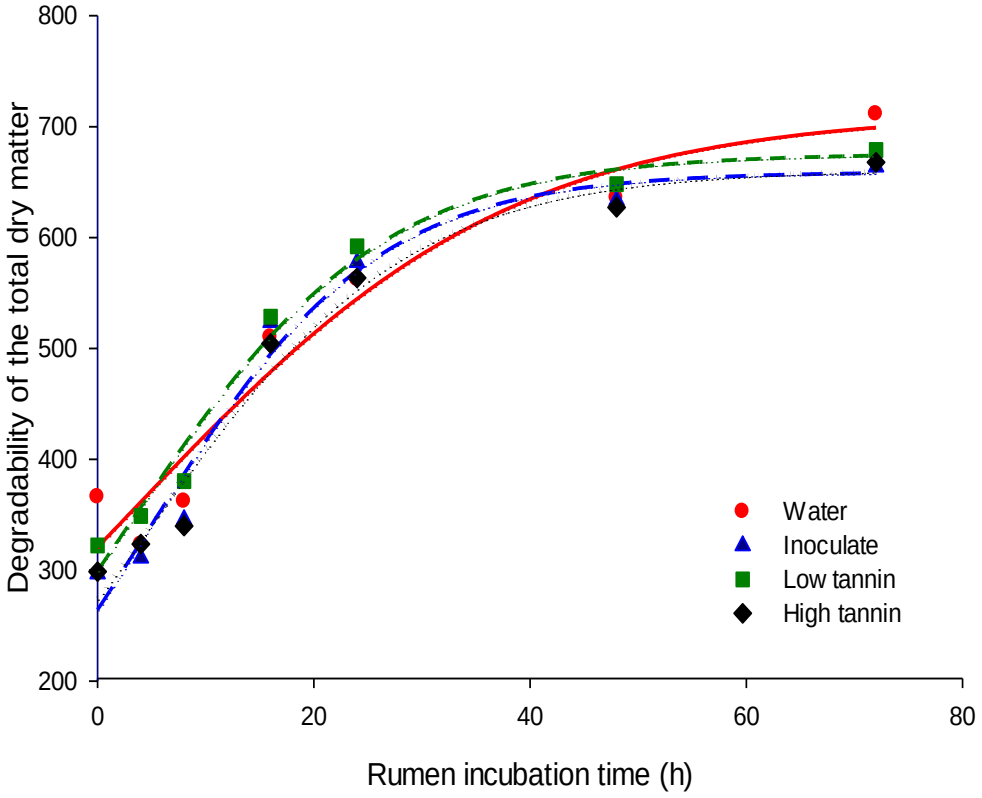

Figure 3.3 Effect of supplemented chestnut HT (two levels) or inoculate to different forages at ensiling on *in situ* DM degradability (g kg^{-1}D) with different silage additives.

The *in situ* CP degradability results are shown in Table 3.4. Pea silage had a higher (P<0.05) CP fraction "a" compared to BS and GS (460, 436 and 425 g kg^{-1}DM respectively). Ryegrass silage was found to have a higher (P<0.01) CP fraction "b" compared to bean and pea silages (436, 335 and 344 g kg^{-1} DM for GS, BS and PS respectively). The total potentially degradable CP fraction "a+b" was found to be highest (P<0.01) in GS (861 g kg^{-1}DM) compared to PS (408 g kg^{-1}DM) and BS (770 g kg^{-1}DM) (Figure 3.4). Ryegrass silage had the highest ED5 compared to leguminous silage (Table 3.4).

Table 3.3 Effect of supplemented chestnut HT or inoculater to different forages at ensiling on *in situ* DM degradability.

Forages	Bean silage				Pea silage				Ryegrass silage				SED			*Probability*		
Additives	W	Inoc	LT	HiT	W	Inoc	LT	HiT	W	Inoc	LT	HiT	Add	For	Inte	Add	For	Inte
a ($g\ kg^{-1}DM$)	355	330	335	315	326	299	312	260	388	351	321	312	7.1	6.2	12.3	<0.01	<0.01	0.03
b ($g\ kg^{-1}DM$)	404	417	400	418	449	441	423	436	491	489	475	450	14.6	12.7	25.4	0.57	<0.01	0.79
a+b ($g\ kg^{-1}DM$)	760	748	735	734	775	741	736	696	880	840	779	763	13.2	11.4	22.8	<0.01	<0.01	0.16
c	0.03	0.03	0.04	0.02	0.3	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.002	0.002	0.004	0.01	<0.01	0.27
Lag (h^{-1})	0.51	0.85	0.97	2.51	0.39	0.71	0.92	1.24	0.49	0.84	0.97	1.16	0.180	0.156	0.31	<0.01	0.03	0.03
ED5 ($h\text{-}^{1}$)	853	865	775	632	732	659	670	635	895	906	884	809	22.5	19.5	39.0	<0.01	<0.01	0.01

W: water (negative control), Inoc: inoculate (positive control), LT: low tannin level, HiT: high tannin level. For.: forages, Add: additives, inte: interaction between additives x forages, a: immediately soluble, b: insoluble but potentially degradable, a+b: the total potential degradable c: the rate of degradation, Lag: lag time, ED5 effective degradability at outflow rate 0.05.

Treating fresh forage with tannin at ensiling (both levels) significantly ($P < 0.01$) reduced CP fraction “a” compared to Inoc and W (396, 416, 469 and 481 g kg^{-1}DM for HiT, LT, Inoc and W respectively). The effect of supplemented tannin (20 and 40 g kg^{-1}FW) on CP fraction “a” showed a greater effect ($P<0.01$) in PS (21-26 %) compared to BS (7-8.5 %) and GS (10-17.5 %). Additive had no effect ($P=0.41$) on CP fraction “b”, whereas the total potential degradable fraction “a+b” was reduced ($P<0.001$) (12.1-8.5 %) when tannin (both rates of inclusion) was used as a silage additive (Figure 3.5).

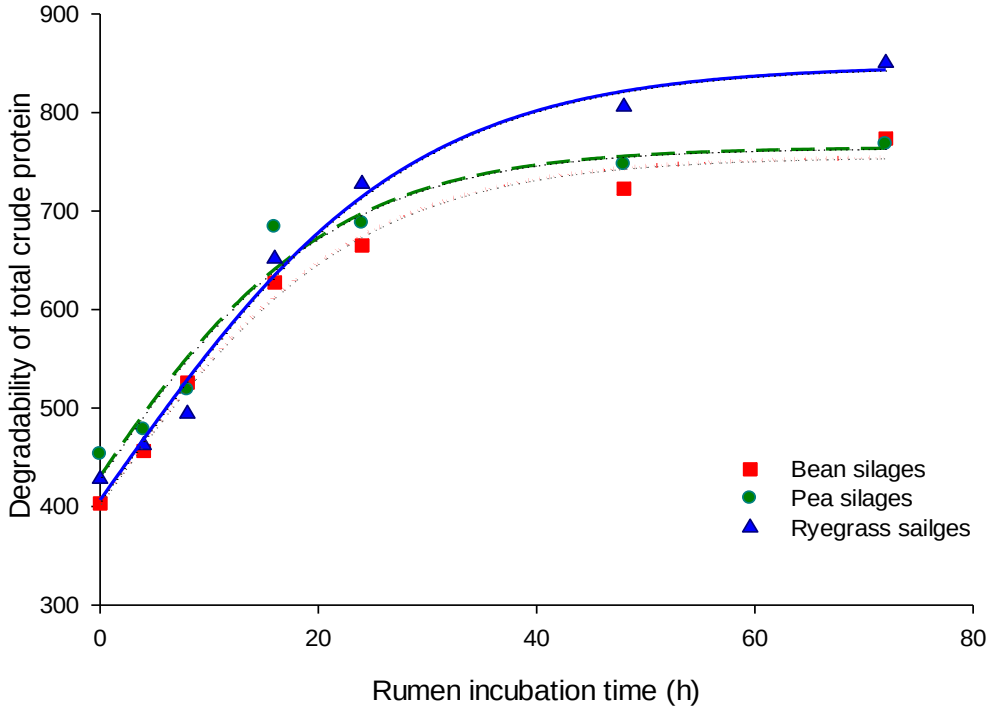

Figure 3.4 Effect of supplemented chestnut HT (two levels) or inoculate to different forages at ensiling on *in situ* CP degradability (g kg^{-1}D) with different forage types.

Supplemented high tannin level reduced CP ED5 in all silage forages, while low tannin level reduced CP ED5 in leguminous silages only. In addition, treated forage with inoculated bacteria (positive control) was found to reduce

CP ED5 in BS compared to negative control with no effect in PS or GS (Table 3.4).

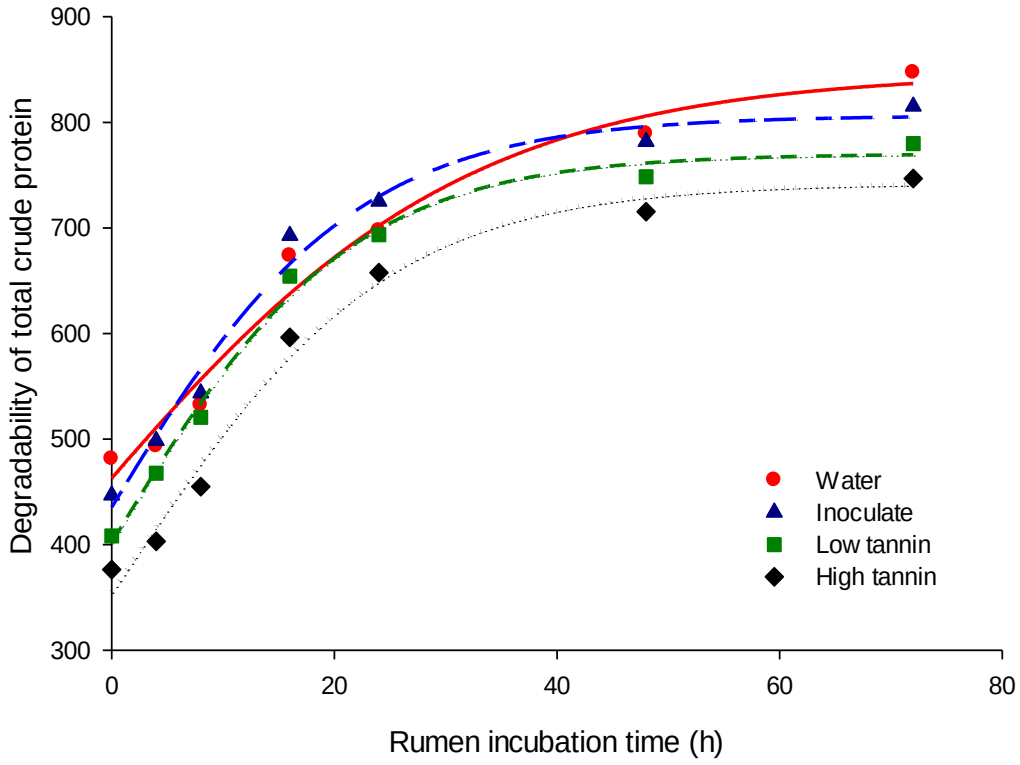

Figure 3.5 Effect of supplemented chestnut HT (two levels) or inoculate to different forages at ensiling on *in situ* CP degradability (g $kg^{-1}D$) with different silage additives.

Table 3.4 Effect of supplemented chestnut HT or inoculate to different forages at ensiling on *in situ* CP degradability.

Forages	Bean silage				Pea silage				Ryegrass silage				SED			*Probability*		
Additives	W	Inoc	LT	HiT	W	Inoc	LT	HiT	W	Inoc	LT	HiT	Add	For.	Inte	Add	for	Inte
a ($g\ kg^{-1}DM$)	479	436	429	369	518	531	407	382	445	435	410	409	6.1	5.2	10.5	<0.01	<0.01	<0.01
b ($g\ kg^{-1}DM$)	335	333	332	336	344	342	356	334	446	437	427	413	11.6	10.1	20.1	0.41	<0.01	0.52
a+b ($g\ kg^{-1}DM$)	814	769	762	733	863	873	763	716	909	873	838	822	12.6	10.9	21.8	<0.01	<0.01	<0.01
c	0.02	0.03	0.03	0.02	0.03	0.02	0.03	0.03	0.02	0.03	0.02	0.02	0.003	0.003	0.006	0.19	0.13	0.11
Lag (h^{-1})	0.41	0.86	1.40	2.10	0.40	0.81	1.06	1.41	0.48	0.85	0.90	1.27	0.279	0.241	0.482	<0.01	0.38	0.86
ED5 (h^{-1})	845	751	771	661	855	854	734	705	884	852	842	809	14.8	12.8	25.7	<0.01	<0.01	0.02

W: water (negative control), Inoc: inoculate (positive control), LT: low tannin level, HiT: high tannin level. For.: forages, Add: additives, inte: interaction between additives x forages, a: immediately soluble, b: insoluble but potentially degradable, a+b: the total potential degradable c: the rate of degradation, Lag: lag time (h^{-1}), ED5: effective degradability at outflow rate 5%(h^{-1})

3.3.3.2 Protein fractionation using the CNCPS.

Bean silages had the highest non-protein N fraction A (P=0.06) and neutral soluble protein fraction B2 (P<0.001), and lowest (P<0.001) buffer soluble N fraction B1 compared to GS and PS (Table 3.5 and Figure 3.6), whereas, GS was found to have the highest (P<0.001) acid soluble protein fraction B3 and the lowest (P=0.014) acid insoluble protein fraction C compared to BS and PS (B3: 114, 60 and 58 g kg^{-1}CP and C: 66, 86 and 89 g kg^{-1}CP for GS, BS and PS, respectively). In addition, GS had the highest protein solubility in acid detergent reagent compared to PS and BS (Figure 3.6). Approximately 935g kg^{-1}CP of ryegrass silage was soluble in CNCPS solutions compared to 910 and 914 g kg^{-1}CP in PS and BS, respectively.

Supplemented chestnut HT (both levels of inclusion) or inoculate (positive control) reduced (P<0.01) fraction A compared to the negative control silages (535, 500, 404 and 384 g kg^{-1}CP for W, Inoc, LT and HiT, respectively). Fraction A in leguminous silages were seen to be more affected by supplemented tannin (both rates of inclusion) compared to GS, the reduction rate being approximately 26-29 % in BS and 30-37 % in PS compared to only 9-16 % in GS (Table 3.5).

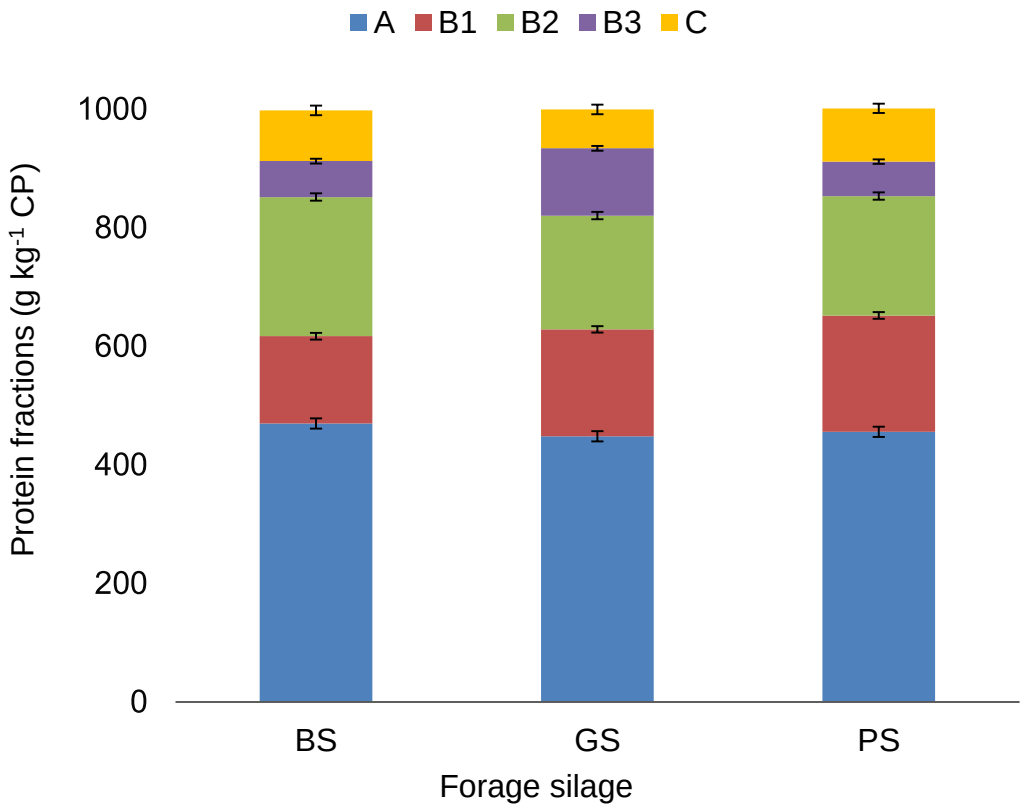

Figure 3.6 Effect of supplemented chestnut HT (two levels) or inoculate to different forages at ensiling on protein fractionation using the CNCPS (forage types). BS: bean silage, GS: ryegrass silage, PS: pea silage, A: non protein nitrogen, B1: buffer soluble protein, B2: natural, soluble protein, B3 acid soluble protein and C: acid insoluble protein.

Treating forage silages with tannin or inoculate at ensiling increased fraction B1 (209, 197, 156 and 131 g kg^{-1}CP for HT, LT, Inoc and W respectively) especially in PS.

Both tannin levels increased (P<0.01) fraction B2 compared to the negative control silages (232, 224, 193 and 188 g kg^{-1}CP for HiT, LT, Inoc and W, respectively). Both tannin levels increased (P<0.05) fraction B2 only in BS and PS, although a slight increase (P>0.05) was noticed in GS (Table 3.5).

Treating forages with HiT or LT significantly (P<0.05) reduced fraction B3 compared to the Inoc and W (73, 72.2, 83 and 81 g kg^{-1}CP, respectively); the

increment was found only in GS while a slight increase was observed in the leguminous silages (Table 3.5). Supplemented tannin at ensiling increased significantly the fraction-C compared to Inoc and W (Table 3.5 and Figure 3.7).

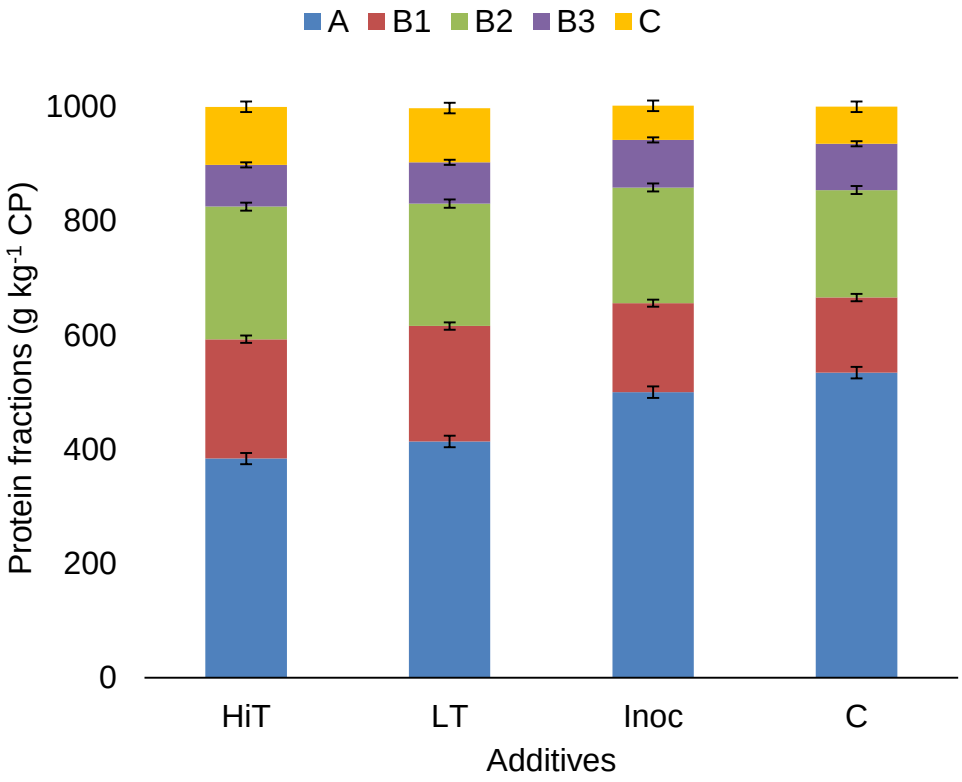

Figure 3.7 Effect of supplemented chestnut HT (two levels) or inoculate to different forages at ensiling on protein fractionation using the CNCPS (forage types). HiT: high tannin, LT: low tannin: Inoc: inoculate, W: water, A: non protein nitrogen, B1: buffer soluble protein, B2: natural, soluble protein, B3 acid soluble protein and C: acid insoluble protein.

Leguminous silages had a higher (P<0.01) UDP5 compared to GS (Table 3.5). Supplemented forages with either levels of chestnut HT were found to increase UDP5 by approximately 22-55 % compared to both the negative and positive controls.

Table 3.5 Effect of supplemented chestnut HT or inoculate at ensiling to different forages on protein freactionation using the CNCPS.

Forages	Bean silage				Pea silage				Ryegrass silage				SED			*Probability*		
Additives	W	Inoc	LT	HiT	W	Inoc	LT	HiT	W	Inoc	LT	HiT	Add	For.	Inte	Add	for	Inte
A ($g\ kg^{-1}DM$)	561	506	410	398	560	524	484	351	483	471	432	404	10.0	8.7	17.3	<0.01	0.06	<0.01
B1 ($g\ kg^{-1}DM$)	103	123	185	174	151	156	188	220	140	188	219	233	6.4	5.5	11.0	<0.01	<0.01	0.03
B2 ($g\ kg^{-1}DM$)	206	225	253	255	174	171	221	239	184	183	198	203	7.1	6.2	12.3	<0.01	<0.01	0.02
B3 ($g\ kg^{-1}DM$)	58	61	60	63	56	53	59	65	131	135	97	91	4.6	4.0	7.9	0.05	<0.01	0.04
C ($g\ kg^{-1}DM$)	72	57	103	110	70	69	108	113	51	55	75	82	9.3	8.0	16.1	<0.01	0.01	0.89
RUP5 ($g\ kg^{-1}DM$)	228	229	279	293	243	248	274	279	197	189	265	273	14.2	12.3	24.5	<0.01	0.04	0.73

W: water (negative control), Inoc: inoculate (positive control), LT: low tannin level, HiT: high tannin level. For.: forages, Add: additives, inte: interaction between additives x forages, A: (fraction A) non-protein nitrogen, B1: (fraction B1) buffer soluble N, B2: (fraction B2) neutral soluble N, B3: (fraction B3) acid soluble N, C: (fraction C) acid insoluble N, RUP5 rumen ungradable protein at outflow rate 0.05 h^{-1}.

3.3.4 Experiment 1 b: Effect of chestnut HT supplementation on *in vitro* rumen fermentation kinetics

The gas production kinetics results showed that incubating dried silage samples at 39ºC for 72 h in N-sufficient media had a higher (P<0.01) asymptote gas production, NH_3-N and pH compared to N-deficient (asymptote: 352 vs. 321 ml g^{-1}DM, NH_3-N 0.2 vs. 0.13 g L^{-1} and pH: 6.47 vs. 6.44, for N-sufficient and N-deficient media respectively), while different media had no effect (P>0.05) on total gas production, time dependent rate (c) (Figure 3.8), DM or OM disappearances (Table 3.6).

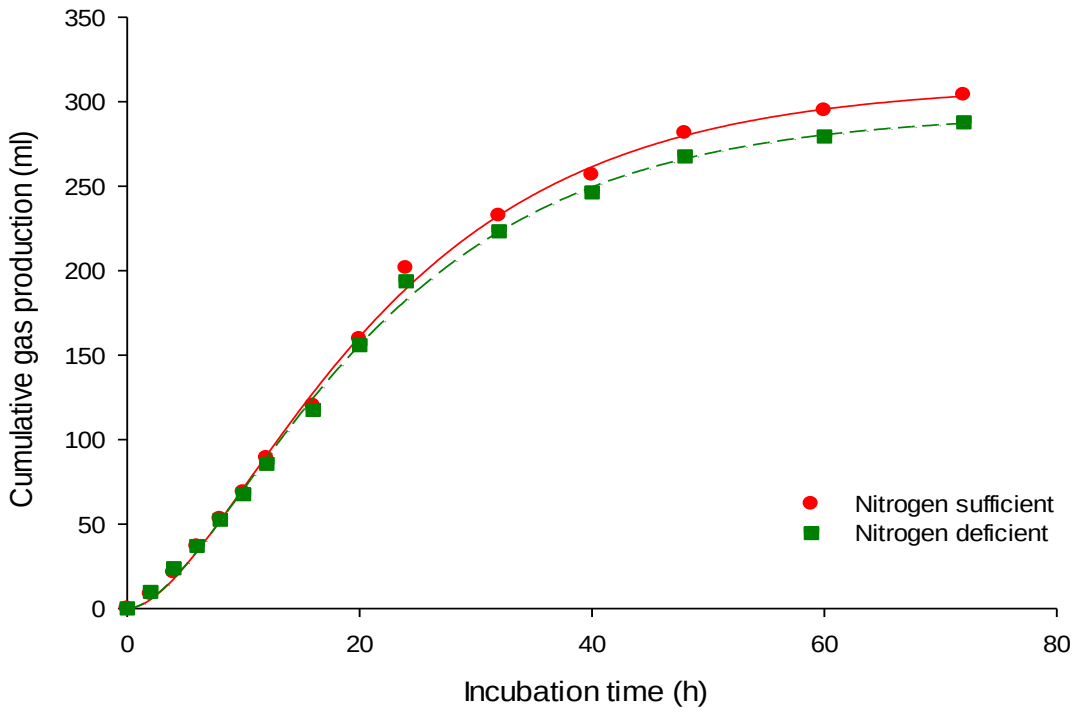

Figure 3.8 Effect of supplemented chestnut HT (two levels) or inoculate to different forages at ensiling on cumulate gas production (ml g^{-1}DM) with different media.

Leguminous silages (BS and PS) behaved similarly in the whole experiment and did not have any significant difference in any measured parameters. However, both leguminous silages were found to have a lower asymptote gas production, DM and OM degradability and faster time dependent rate compared to GS (Table 3.6), whereas, there was no difference between forage types on the total gas production (Figure 3.9)

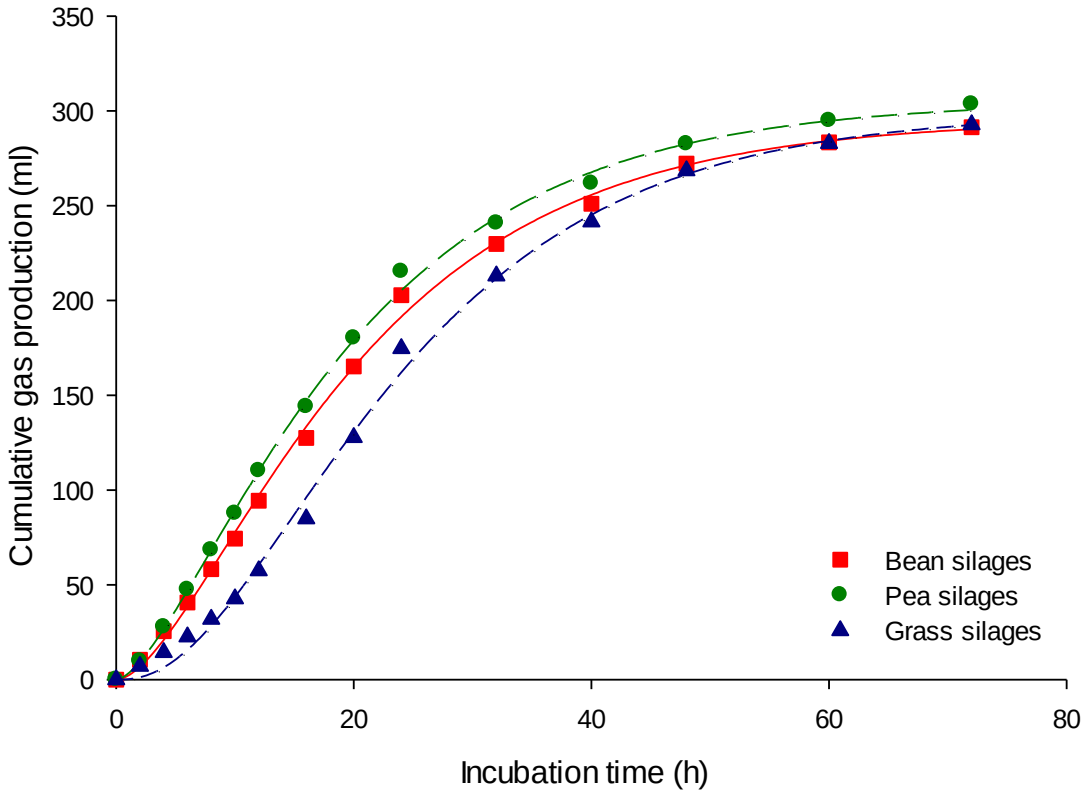

Figure 3.9 Effect supplemented chestnut HT (two levels) or inoculate to different forages at ensiling on cumulate gas production (ml g^{-1}DM) with different forage silages.

Supplemented tannin (both levels of inclusion) reduced (*P*<0.001) the total gas production (16-24 %), asymptote gas production "b" (13.3-16.8 %) and time dependent rate "c" (14.7-22.2 %) compared to the negative control silages (Figure 3.10). Furthermore, a significant reduction (*P*<0.001) of DM and OM

disappearance was observed when either tannin levels (HiT or LT) was used as a silage additive (DM: 641, 624, 583 and 532, OM: 661, 650, 605 and 568 g kg^{-1}DM for W, Inoc, LT and HiT, respectively). Treating silages with either tannin or inoculate was found to increase (P=0.006) pH (6.5, 6.48, 6.44 and 6.39, for W, Inoc, LT and HiT, respectively) and reduce (P<0.01) NH_3-N (0.12, 0.15, 0.18 and 0.22 g L^{-1}, for W, Inoc, LT and HiT, respectively) compared with treating silage with water only. There was no significant effect of the interaction between media, forage types and silage additives in all studied traits except the pH of the liquid. Treated forages especially PS with either tannin or inoculate increased the pH of the liquid compared to negative control (water) especially when N-deficient media was used (Table 3.6).

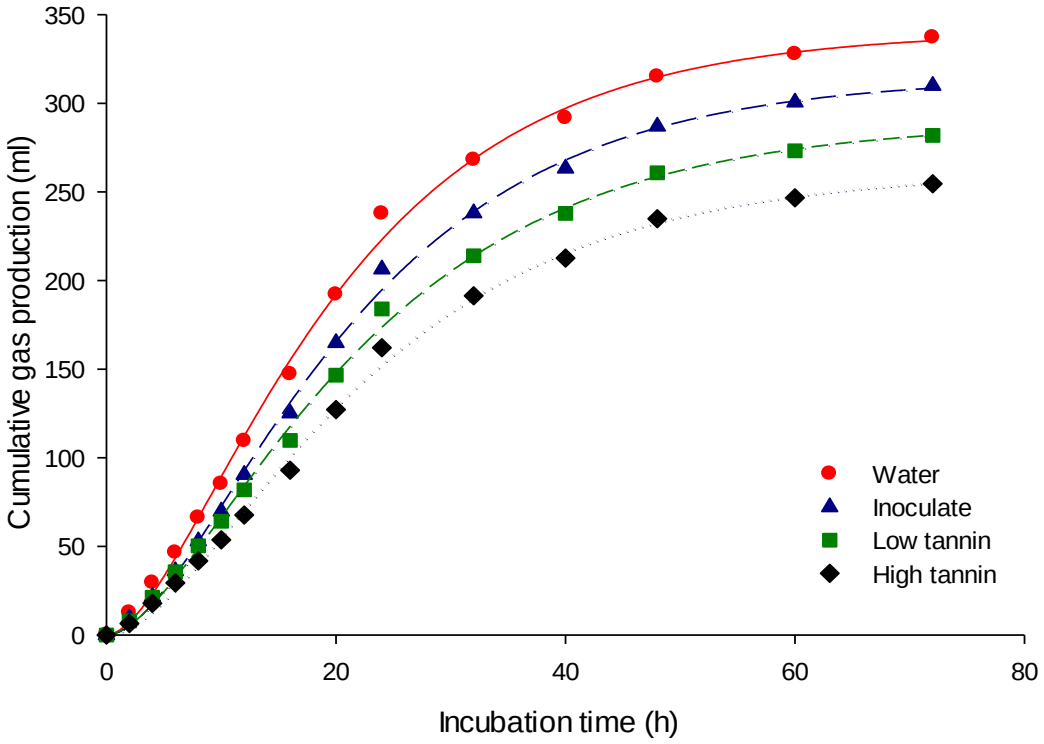

Figure 3.10 Effect of supplemented chestnut HT (two levels) or inoculate to different forages at ensiling on cumulate gas production (ml g^{-1}DM) with different silage additives.

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Table 3.6 Effect of supplemented chestnut HT (two levels) or inculate to different forage incubated in different media (N-sufficient or N-deficient) on gas production kinetics parameters.

Med.	For	Add.	b	c	DM	OM	TG	pH	NH_3-N
Nitrogen sufficient	BS	W	365	46	613	626	357	6.42	0.26
		Inoc	373	47	577	622	296	6.51	0.25
		LT	316	42	592	544	261	6.56	0.21
		HiT	294	36	506	536	232	6.53	0.14
	PS	W	363	50	577	638	331	6.44	0.28
		Inoc	343	46	589	620	306	6.43	0.24
		LT	324	43	535	612	298	6.48	0.20
		HiT	295	38	495	577	252	6.43	0.15
	GS	W	391	33	717	717	323	6.44	0.30
		Inoc	405	28	710	724	289	6.44	0.17
		LT	391	29	627	666	268	6.50	0.14
		HiT	377	26	597	606	251	6.44	0.12
Nitrogen deficient	BS	W	373	50	624	640	332	6.34	0.15
		Inoc	318	47	582	610	312	6.47	0.14
		LT	291	41	545	560	286	6.52	0.13
		HiT	275	38	409	444	256	6.46	0.10
	PS	W	339	44	608	646	344	6.30	0.21
		Inoc	344	43	579	609	351	6.34	0.18
		LT	285	40	528	584	295	6.39	0.15
		HiT	281	36	548	576	263	6.73	0.10
	GS	W	381	35	707	702	335	6.41	0.14
		Inoc	340	25	705	718	298	6.44	0.12
		LT	313	26	669	666	296	6.42	0.09
		HiT	315	26	637	668	278	6.42	0.09
Probability	P	Media	<0.01	0.27	0.99	0.65	0.204	<0.01	<0.01
		Treat.	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
		Forage	<0.01	<0.01	<0.01	<0.01	0.574	<0.01	<0.01
	s.e.d	Media	8.3	1.18	11.4	12.9	11.7	0.010	0.01
		Treat.	117.3	1.68	16.1	18.3	16.6	0.015	0.013
		Forage	101.6	1.47	13.9	15.8	14.4	0.013	0.012

W: water (negative control), Inoc: inoculate (positive control), LT: low tannin level, HiT: high tannin level, Med: media, For: forages, Add: additives, inter: interaction between additives x forages, c time dependent rate x 10^{-3}, DM: dry matter disappearance (g kg^{-1}DM), OM organic matter disappearance (g kg^{-1}DM). b: asymptote gas production (ml g^{-1}DM), TG: total gas production (ml g^{-1}DM); NH_3-N: ammonia nitrogen g L^{-1}.

3.4. Discussion

3.4.1 Effect of chestnut HT supplementation on the chemical composition of final silage.

The dry matter and proximate analysis of the bean, pea and ryegrass silages used in the current study were within the normal range for the forages cultivated in the UK (AFRC, 1993; Salawu *et al.*, 1999; Hart *et al.*, 2012) with a variation between beans, peas and ryegrass (Table 3.1). Fresh ryegrass had the highest DM, CP, NDF and EE and lowest OM and ADF compared to leguminous forages, which could be due to species differentiation and/or cultivation managements such as soil fertilization, irrigation and forage maturity at harvesting. Fraser *et al.* (2001) reported that peas respond better to wilting than beans, which could be due to peas having thinner stems compared to beans, thus increasing the surface area to volume.

Lactic acid bacteria are the common commercial silage additives used in the UK (Salawu *et al.*, 2001). Silage inoculants have been developed for their ability to promote a beneficial fermentation that maximizes the nutritive value of the silage for ruminant animals (Merry and Davies, 1999). Wilkinson and Davies (2013) reported that supplemented inoculated bacteria to forage at ensiling would cause a rapid reduction of the silage pH in order to prevent the growth of undesirable microorganisms such as clostridia, in addition it reduces DM losses and for these reasons, the used of inoculated bacteria as a silage additive in the present study was as a positive control.

In the current study the DM losses for BS, PS and GS during the ensiling period was approximately 6, 19 and 12 % respectively. No silage effluent nor visible mould growth was observed when the silos were opened. Therefore, the silage DM losses was not affected by effluent losses. Moreover, the silages were kept in plastic bags and the integrity of the bags would inhibited effluent escape. Mayne and Gordon (1986) suggested that DM losses could occur in three ways: effluent escape, surface waste and invisible losses (the invisible losses could include: fermentation characteristic and plant respiration during fermentation period especially the initial fermentation phase). Wilting the pre ensiled forage reduced effluent and invisible losses of DM up to 50 % (Lorenz and Udén, 2010). In the current study forages were wilted for 48 h prior to silage making increasing the forage DM. In addition, silage DM losses could be affected by the activity of some microorganisms in the silage clamp such as enterobacteria (Tabacco *et al.*, 2006).

All treatments were considered to have low pH and NH_3-N values (Table 3.2). McDonald and Edwards (1976) classified silages into 5 categories according to fermentation process: chemically restricted, wilted, butyrate, acetate and lactate. Based on their (McDonald and Edwards, 1976) classification, the silages produced in the current study could be classified as lactate silage which are characterized by a low pH (3.7-4.3) and NH_3-N (<120 g kg^{-1} TN). The low silage pH, perhaps due to rapid anaerobic acidification of forage water soluble carbohydrate, would result in good quality silage (Davies *et al.*, 1998; Wilkinson and Davies, 2013). In addition, rapid acidification of the silage silo would reduce protein hydrolysis hence decrease NH_3-N concentration (Wilkinson and Davies, 2013). Deaville *et al.* (2010) reported that low NH_3-N

concentrations produced inside the silage silo is one of the indicators of a well fermented silage. The concentration of NH_3-N in the current experiments was lower than reported by Hart (2005) who observed that NH_3-N concentration was 76.8-81.7 g kg^{-1}TN for pea and bean silages, respectively.

Two levels (20 or 40 g kg^{-1}FW) of chestnut HT were supplemented to bean pea and grass forages at ensiling based on forage fresh weight, however, and due to the variation in forage DM, the supplemented tannin g kg^{-1}DM were varied between the forages, thus it would be more useful to treat forages with additional tannin based on forage DM. The results from the silage proximate analysis were corrected for additional DM of tannin that was added at ensiling (HT and LT; 40 and 20 g kg^{-1}FW, respectively, which was equivalent to 162 and 81 g kg^{-1}DM in BS; 122 and 61 g kg^{-1}DM in PS and 130 and 65 g kg^{-1}DM in GS respectively). A similar approach was reported by Salawu *et al.* (1999) and Deaville *et al.* (2010). Deaville *et al.* (2010) found that treating ryegrass silage with mimosa and chestnut tannin (74.8 g kg^{-1}DM) either at ensiling or at feeding increased the silage DM compared to untreated silage, corresponding to the addition of tannin DM. The chemical composition of the ensiled forages showed that ryegrass silage had the highest pH, DM, CP, NDF and EE and lowest NH_3-N and ADF compared to PS and BS, which could perhaps be due to the differences between the species, cultivation system and the maturity at harvesting.

The majority of research has examined the effect of endogenous forage tannin (especially CT) on forage quality and protein protection in ruminant nutrition (Makkar, 2003; Tabacco *et al.*, 2006). A paucity of information is available regarding the potential use of tannin (particularly hydrolysable tannin) as silage

additives and their effect on forage protein during the fermentation period (Katiki *et al.*, 2013), hence in the current experiment, chestnut HT was used as a silage additive (at ensiling) to study their effect on the final silage for different forages.

Silage additives had no effect (P>0.05) on any of the proximate analysis except NH_3-N which was reduced by additional tannin to all forage types, most probably due to the formation of tannin-protein complex (Makkar, 2003; Mueller-Harvey, 2006), inhibiting plant protease (Salawu *et al.*, 1999) and/or reducing the microbial activity in herbage (Ohshima and McDonald, 1978).

Tabacco *et al.* (2006) stated that tannins could considered to be an anti-microbial compound, which could affect chemically and biologically the micro flora inside the silo. Similar results have been reported by Tabacco *et al.* (2006) who found that additional chestnut HT (20, 40 and 60 g kg^{-1}DM) in lucerne silage reduced (P<0.01) the NH_3-N value compared to the control. De Oliveira *et al.* (2009) also observed a reduction in NH_3-N in the absence of PEG in high and low tannin sorghum silage compared with adding PEG (which reacts with tannin), reducing its activity. Salawu *et al.* (1999) also found that after 7 days of ensiling, the control silage had a significantly higher NH_3-N compared to treated silages with different types of tannin (mimosa and quebracho tannin), formaldehyde and formic acid. Salawu *et al.* (1999) stated that one of the main reason for adding tannin at ensiling is to reduce forage protein hydrolysis during the fermentation period. Reduction in CP hydrolysis would increase rumen by-pass protein (Muller-Harvey, 2006). Ammonia nitrogen concentration in all forage silages treated with inoculate (positive control) was found to be lower than the negative control. Inoculates inhibited the activity of

undesirable microorganisms' in the silo (Davies *et al.*, 1998) and could rapidly reduce the pH of the silo as a result of fermentation processes.

3.4.2 Experiment 1 a: Effects of supplemental chestnut HT at ensiling to different forages on *in situ* rumen degradability and protein fractionation using the CNCPS.

3.4.2.1 In situ rumen degradability

AFRC, (1992) recommended using 5 g DM with 3 mm milled dried sample and/or 15-20 g FW with approximately 1 cm chopped fresh silage inside polyester bags for the determination of rumen degradability using the *in situ* technique. However, in the current study 20-25 g fresh silage was placed in the *in situ* nylon bags in the same form as feeding animals without any milling or chopping. Hence, samples did not receive any mechanical mastication, thus decreasing the homogenous substrate and surface area to volume ratio compared to dried ground samples.

It has been reported (Hart, 2005) that when forage samples are dried and milled through a 2 mm screen, approximately 50 % of the ground material can pass through 0.5 mm sieve, which could lead to increased water soluble losses compared to fresh samples. Huntington and Givens (1998) also reported that using milled samples would increase small particle losses and increase the rate of degradation of the samples. Similar approaches have been done by Sinclair *et al.* (2009), who also used un-chopped fresh silage samples for the *in situ* DM and N degradability. However, using un-chopped fresh silage could result in the underestimation of degradability and reduce the uniformity of the

sample, thus increasing the replications necessary to reduce the variation between the samples.

The results of the *in situ* CP degradability in the present study were not corrected for microbial protein contamination. Varvikko and Lindberg (1985) suggested that incubated samples in the rumen should be corrected for microbial N contamination. Hart (2005) reported that estimating protein degradability *in situ* for samples with low N concentration such as straw would result in an underestimation of 1285 % after only 24h incubation in the rumen due to microbial protein contamination, while estimating CP degradability of feedstuffs with a relatively high CP content (120-250 g kg^{-1}DM) the under estimation would be no more than 0.7 % after 24 h incubation in the rumen. The crude protein content of the samples used in the current study was 141-177 g kg^{-1}DM, thus minimal underestimation (microbial N contamination) of CP degradability was expected, and hence the results were not corrected for MCP contamination.

In the current work, the CP fraction “a” for all treatments ranged from 400-491 g kg^{-1}DM, respectively. These values are slightly lower than the values published by AFRC (1993) (423-715 g kg^{-1}DM) or NRC (2001) (573 g kg^{-1}DM), for ryegrass and leguminous silages. This might be due to using 20-25 g un-chopped fresh silage per bag here. Huntington and Givens (1995) reported that processing feeds before rumen incubation within bags might affect the *in situ* rumen degradability results.

Adding tannin significantly reduced the DM and CP fraction “a” (Table 3.3 and 3.4). These results are in agreement with those reported by several studies

(Tabacco *et al.*, 2006; Sinclair *et al.*, 2009; Alipour and Rouzbehan, 2010; Coblentz and Grabber, 2013), reported that the presence of tannin reduced *in situ* DM or CP degradability, probably due to complexing tannin with forage proteins or fibre reducing their solubility in the rumen (Makkar, 2003; Mueller-Harvey, 2006) and/ or the negative effect of tannin on rumen microbes (McSweeney *et al.,* 201). Kumar and Singh (1984) suggested that tannin-protein complexes would reduce *in situ* CP fraction "a" and increase fraction "b"; and overall reduce ED CP and increase UDP. Salawu *et al.* (2001) observed that using quebracho CT as a silage additive reduced CP fraction "a", ED and fraction "c" and increased CP fraction "b". Sinclair *et al.* (2009) found that high tannin level pea silage had a significantly lower CP fraction "a" compared to low tannin level pea silage. Furthermore, Coblentz and Grabber (2013) showed that increasing the level of CT reduced the CP fraction "a" in different alfalfa hay and silages. However, Messman *et al.* (1996) found that different proportions of tannin in leguminous silage had no effect on fraction "a". Azuhnwi *et al.* (2012) also found that fraction "a" was not affected by sainfoin varieties which contained different amounts of tannin.

The insoluble but potentially degradable CP fraction "b" ranged from 332-446 g kg^{-1}DM. These values are in agreement with those reported by AFRC (1993) (190-530 g kg^{-1} DM) and NRC (2001) (350 g kg^{-1}DM) for different ryegrass and leguminous silages. Treating forage silages with either tannin or inoculate had no effect on fraction "b" compared to silages treated with water only. The rate of degradation fraction "c" was not affected by either additives or forage types. Similar results had been published by several studies (Poncet and Remond, 2002; Tabacco *et al.*, 2006; Sinclair *et al.*, 2009). The effective rumen CP

degradability at outflow rate 0.05 h^{-1} ranged from 661-884 g kg^{-1}DM; values in agreement with those reported by AFRC (1993) (750-780 g kg^{-1}DM) for ryegrass and leguminous silages. Tannin reduced (P<0.05) the CP ED5 compared to both control groups. These results agreed with the result published by Alipour and Rouzbehan (2010) who found that CP ED at outflow rate 0.05 of SBM CP reduced linearly (630, 573, 511, 502 and 480) when tannin level was increased (0, 15, 30, 45 and 60 g kg^{-1}DM respectively). In contrast, Poncet and Remond (2002) observed that any tannin had no effect on fraction “b”, fraction “c” and ED when they added a level of chestnut tannin (20 and 30 g kg^{-1}DM) to pea seeds compared with the untreated diet.

3.4.2.2 Protein fractionation using the CNCPS.

In the current study, firstly a hot dry oven (104^{o}C) process was used for sample preparation. However, fractions A and B1 were lower and fraction C higher compared to previous studies (Licitra *et al.*, 1999; Edmunds *et al.*, 2012; Heendeniya *et al.*, 2012; Coblentz and Grabber, 2013). Thereafter, the samples were freeze dried and milled through a 1 mm screen and used for CP fractionation using the CNCPS, similarly to the study published by Kirchhof *et al.* (2010) and Edmunds *et al.* (2012). In a comparison between the results obtained from oven or freeze dried method, fraction A and B1 were increased by 60-70 % and fraction C was reduced by 100 % in all silage samples dried using the freeze dryer. This variation was perhaps due to using a high temperature (104^{o}C) in the drying process causing protein denaturation and Maillard reactions, resulting in protein-carbohydrate complexes which are resistant to chemical solutions (Lanzas, 2007). Abdalla *et al.* (1988) observed

an increase in approximately 45 % of fraction B3 and C, and decrease of about 20 % in fraction A and B1 when they used an oven dried sample (55^{o}C), compared to freeze dried samples. Deinum and Maassen (1994) found that fraction B3 and C nearly increased by 100 % in lucerne and ryegrass when they prepared their samples at 30^{o}C compared to freeze dried preparation. Therefore, the silage samples in the current study were dried using a freeze dryer.

The methodology of protein fractionation published by CNCPS (Sniffen *et al.*, 1992), explained that after rinsing samples in the chemical solutions, the filtered residue is directly transferred to Kjeldahl apparatuses in order to measure N concentration of the residual samples. However, in the present experiment, N concentration was measured using a LECO-N machine based on dried samples, thus the filtered residue was moved to a hot oven (60^{o}C for 48 h) and the DM losses were calculated and multiplied with the N concentration of the residual samples.

Ryegrass silage had the lowest fraction A and C and highest fraction B1, B2 and B3 compared to leguminous silages (Figure 3.6). These differences might be due to the differences between species and/or maturity stage at harvesting. Kirchhof (2007) reported that fraction A was increased with increasing maturity. Hymes-Fecht *et al.* (2013) reported that the rate of pH decline also has an effect on fraction A and B1; if the pH decreased quickly during fermentation low amounts of forage protein will be converted to NPN in the herbage. In the current study, GS had the highest pH compared to PS and BS (Table 3.6). Hart (2005) reported that leguminous forages had a higher buffering capacity compared to ryegrass silage and the reduction of herbage

pH to reach <4.5 could take approximately 96 h, whereas in ryegrass can take only 36 h. Therefore, the activity of protease enzymes could take a longer time in leguminous silage, thus having a higher proportion of NPN compared to ryegrass silages. Guo *et al.* (2008) reported that in leguminous silages the proteolysis process converted most of B1 to A fraction during the ensiling period.

Chestnut HT supplementation (both HiT and LT) reduced fraction A (23 and 17 %) and fraction B3 (12 and 11.8 %) and increased fraction B1 (34 and 50 %), fraction B2 (20 and 19 %) and fraction C (68 and 48 %) respectively compared to the negative control silages. This was probably due to the tannin effect inhibiting proteolytic enzymes during the fermentation period. In addition, tannin could bind with macromolecules of feedstuff making them less soluble in chemical solutions (Albrecht and Muck, 1991; Lorenz and Udén, 2010). Kirchhof *et al.* (2010) found a negative relationship between tannin concentration and fractions A and B1. Grabber and Coblentz (2009) also found that tannin concentrations shift CP solubility from fraction A and B1 to fraction B2 and C when they used different conservation methods on lucerne, red clover and three verities of birdsfoot trefoil (low, moderate and high tannin) silages in two seasons.

3.4.4 Experiment 1 b: Effect of chestnut HT supplementation on *in vitro* rumen fermentation kinetics

Two types of media (N-sufficient or deficient media) were used in order to determine if the N provided by the media had any effect on microbial activity or diet would provide enough N requirements for the microbes. The results

showed that using N-sufficient or deficient media did not have a significant effect on most of the studied parameters, which could indicate that forage N was available for the microbes during 72 h of incubation, even when using a high level of tannin (40 g kg^{-1}FW). Using nitrogen sufficient media resulted in a higher NH_3 and pH compared to N-deficient media. With respect to NH_3 the original media (N-sufficient) was prepared using ammonia sulphate as a source of N, while no source of N was used in the N deficient media. With respect to pH, although there was a significant difference between both media, the pH variation was only 0.03 which could be due to using a high replication (96) per media, thus no biological differences in pH values were observed.

The results of the asymptote and total gas production of the samples used in the current study showed that all forages were considered as highly fermentable (Table 3.6). Cone *et al.* (1999) reported that the maximum gas production of ryegrass silages could reach 208 ml g^{-1}OM, while it could get up to 246 ml g^{-1}OM in maize silages.

The silage samples used in the gas production kinetics were oven dried (60^{o}C for 48h) and milled in a 1mm screen. Lowman *et al.* (2002) studied the effect of drying methods (hot oven, microwave and freeze) compared to fresh grass samples. Their results showed that fresh grass samples had a significantly lower asymptote gas production compared to dried samples, while the lag time was increased in the hot oven and microwave methods and reduced in the freeze drying method compared to fresh grass samples. Using hot temperature in sample drying could change the chemical structure of the plant cells due to the Maillard reaction, making plant cells less available to rumen microbes (Lowman *et al.*, 2002). This could be the reason for the relatively high lag time

in the samples of the current study especially in GS (1.58 h) which was higher than reported by Hart (2005) and Alipour and Rouzbehan (2010).

Ryegrass silage had a higher asymptote gas production compared to BS and PS (364, 326 and 322 ml g^{-1}DM, respectively), which might be due to the higher N concentration in GS compared to the leguminous silages. Getachew *et al.* (2000) reported that increasing the CP content of the feedstuff would affect rumen fermentation as NH_3-N is the main source for microbial N in the rumen. The results observed in the current study agreed with those published by Hart (2005), who observed that the asymptote gas production was 303 and 294 ml g^{-1}DM for PS and BS respectively.

Supplementation with either tannin level depressed the asymptote and total gas production, which might as previously suggested, be due to tannin complexing with macromolecules of the feedstuff making them less available to the rumen bacteria. Furthermore, tannin could have a direct effect on rumen microbes (Getachew *et al.*, 2000), and/ or indirect effect via tannins reacting with bacterial enzymes (Jones *et al.*, 1994), which is supported by a lower DM and OM degradability during fermentation compared to Inoc and W. Waghorn (2008) reported that tannins bind with dietary protein in the rumen and reduced protein degradation and increased by-pass protein supplied to the small intestine. The current results are in agreement with the results published by McSweeney *et al.* (2001), Hervas *et al.* (2003) and Alipour and Rouzbehan (2010). However, Getachew *et al.* (2008) reported that supplemented tannic and gallic acid reduced total gas production but increased potential gas production. They (Getachew *et al.*, 2008) explained that these differences

were due to the variation between tannin levels and types and their effects on rumen fermentation.

Treating forages with tannin or inoculate reduced NH_3-N concentration *in vitro* which was probably due to the additives reducing proteolysis as explained previously. Furthermore, the results of the final liquid pH showed that there were a significant difference between media, forages, additives and an interaction between them. However, the pH value range was 6.35-6.73 with a variation of only 0.38 (CV=0.7) for 288 samples.

3.5. Conclusion

It may be concluded from the current experiment that chestnut HT supplementation at ensiling to different forages reduced forage protein hydrolysis during the fermentation period. Silage samples treated with HiT had the lowest NH_3-N of concentration compared to the other treatment (41.6, 48.8, 55.7 and 60.2 g kg^{-1}TN for HiT, LT, Inoc and W respectively). Moreover, supplemented tannin to different forages at ensiling were found to reduce the proportion of NPN and increase forage true protein inside the silage clamp. These results indicate that using tannin as a silage additive could alter the quality of the final silage. The (*in situ*) rumen degradability of BS, PS and GS were reduced when forage supplemented with chestnut HT as ensiling. In addition, tannin supplementation (especially HiT) were found to reduce rumen fermentation *in vitro* (experiment 1 b). Collectively, these results suggest that feeding ruminant silage treated with chestnut HT may increase rumen bypass protein.

Two levels (20 or 40 g kg^{-1}FW) of chestnut HT were supplemented to bean pea and grass forages at ensiling based on forage fresh weight, however, and due to the variation in forage DM, the supplemented tannin g kg^{-1}DM varied between the forages, thus it would be more useful to treat forages with additional tannin based on forage DM.

CHAPTER 4 Experiment 2: Effect of supplemented chestnut HT either at ensiling or at feeding on feed intake, growth and carcass characteristics of growing lambs.

4.1 Introduction

Results obtained from experiment 1 showed that using chestnut HT as a silage additive at ensiling with whole crop bean, pea or ryegrass reduced (P<0.05) NH_3-N production in the silage during the fermentation period. In addition, supplemented tannin was found to reduce ruminal DM and CP degradability (*in situ*) and *in vitro* total gas production kinetics, which gives an indication that feeding ruminants silages treated with tannin could reduce rumen degradability and increase by-pass protein, and hence enhance ruminant performance. However, tannin has often been described as an anti-nutritional compound which has a negative effect on nutrient utilisation, dry matter intake and digestibility (Frutos *et al.*, 2004; Patra and Saxena, 2010 and Lamy *et al.*, 2011). Schofield *et al.* (2001) and Makkar (2003) suggested that the anti-nutritional effects of tannin could depended on tannin concentration, source, type, animal species, physiological status of the animal and feed composition. Tabacco *et al.* (2006) and Deaville *et al.* (2010) reported that using relatively low levels of tannin (<50 g kg^{-1}DM) in ruminant nutrition might increase microbial protein synthesis in the rumen, improve animal digestion and performance. Several studies (Sinclair *et al.*, 2009; Deaville *et al.*, 2010 and Hart *et al.*, 2012) have suggested that complexing tannin with protein would reduce protein degradability in the rumen and increase undegradable protein, thus enhance animal performance.

It has been reported that the colour and flavour of red meat is affected by the oxidation process (Luciano *et al.*, 2009). Oxidation of lipids would perhaps develop meat off-flavour and oxidation of myoglobin would result in discoloration of the meat (Gray *et al.*, 1996). Luciano *et al.* (2009) reported that there is a possibility that both colour and lipid oxidation are linked together. Feeding ruminants anti-oxidant compounds such as vitamin E or polyphenols such as tannins, have been shown to delay oxidation of lipid and discoloration (Baron *et al.*, 2002).

The objectives of this experiment were to evaluate the effect of supplementing with tannin (30g kg^{-1}DM) at ensiling or at feeding on lamb dry matter intake (DMI), liveweight gain, diet whole tract digestibility, carcass characteristics and meat quality. In addition the influence of additional tannin either at ensiling or after opening the silo on rumen DM, OM and CP degradability (*in situ*) and protein fractionation using the CNCPS was tested.

4.2 Materials and methods

4.2.1 Silage production and experimental design:

Approximately 20 t from each of ryegrass (perennial ryegrass mix sword) and lucerne (Daisy *Medicago sativa*) forages (second cut) were used for silage making. Forages were mowed on the 12th and 18th of July 2012 for ryegrass and lucerne respectively, and left in the field for 48 h to wilt. Silage clamps were cleaned well and sheeted with a double layer of plastic sheet one day before silage making. Both forages were chopped using a forage harvester (Jaguar 870-840 forage harvester, UK) and transferred from the field into

silage clamp on the 14th and 20th of July 2012 respectively, and treated with one of 3 additives; 30 g kg^{-1}DM chestnut HT (Thomas Ware & Sons Bristol, according to the manufacturer chestnut HT had an actual tannin content of 750 g kg^{-1}DM with a mix of the following tannins: castalagin, vescalagin, castalin and vescalin in the proportion 530, 350, 30 and 85 g kg^{-1}), an inoculant (10^6 colony forming unit, homofermentative *Lactobacillus plantarum* g^{-1}) as a positive control, or water as a negative control. Additives were mixed with the forages individually using a mixer (Super 10 MIXMAX. HISPEC Ltd, Carlow, Republic of Ireland) with 500 kg capacity. To ensure consistency, water was applied to all treatments at a rate of 1 L^{-1}t FW. To facilitate accurate tannin supplementation at ensiling, forage DM was measured using a forage drier machine (METTLER TOLEDO-HB43-S Halongen, Columbus, OH, USA). A subsample from each treatment was taken and stored at -20°C prior to further analysis. The clamps were filled rapidly and manually pressed by feet. Clamps were sealed with three layers of plastic sheet. Big square bales of straw were used to add weight to each clamp in order to prevent oxygen invasion. Each treatment was left for 100 days to ensile. Samples from each clamp were taken on the 12th of January 2013 using a sample spear (silage corer) and stored at -20°C, to await proximate analysis. Silage samples were analysed for: DM, pH, NH_3-N, OM, CP, NDF, ADF and EE as described in Chapter 2. Sections: 2.1, 2.5, 2.4, 2.2, 2.3, 2.6, 2.7, 28 and 2.11 respectively.

The study was designed as a factorial 2 x 3 (forage types x silage additives), the forage types were: ryegrass and lucerne, the silage additives were: tannin, inoculate and water. However, when the silage silos were opened on 10th of May 2013. A white mould was observed throughout all of the lucerne clamps

rendering them void for use. The study was redesigned as a factorial plus control experiment. Five experimental treatments were prepared from the three ryegrass silage as follows:

- Ryegrass silage (negative control) (G).
- Ryegrass forage treated with chestnut HT at ensiling (GET).
- Ryegrass silage treated with inoculum (positive control) (G+I).
- G + supplemented (30 g kg^{-1}DM) chestnut HT after opening the silo (G+T).
- G+I + supplemented (30 g kg^{-1}DM) chestnut HT after opening the silo (G+I+T).

4.2.2 Experiment 2 a: Effect of supplemented chestnut HT at either ensiling or after opening the silos to ryegrass on *in situ* rumen degradability and protein fractionation using the CNCPS.

4.2.2.1 In situ degradability

Samples of ryegrass silage treatments (G, GET, G+T, G+I and G+I+T) as prepared in Section 4.2.1 were freeze dried, milled through a 3 mm screen and used for *in situ* DM, OM and CP degradability according to AFRC (1992) guidelines (in quadruplicate) as described in Chapter 2 Section 2.12. Approximately 5 g of freeze dried silage sample of each treatment was accurately weighed individually into pre labelled, precision woven, mono filamentous nylon bags, the weight was recorded and the bags then incubated inside an animal's rumen as described in Chapter 2 Section 2.12. Four mature wether (80 kg ± 10 kg) sheep fitted with permanent rumen cannula were used for the *in situ* experiment, where each wether received bags of all treatments

for the same incubation time period. The wethers were kept as a group for feeding and offered a concentrate diet (Section 2.12). Dry matter, OM and CP degradation profiles were measured for all treatments including: the immediately soluble fraction “a”, insoluble but potentially degradable fraction “b”, the total potential degradable fraction “a+b”, the rate of degradation fraction “c”, lag time and the effective degradability at outflow rate 0.05 h^{-1} (ED5).

4.2.2.2 Protein fractionation technique using the CNCPS

Silage samples (G, GET, G+T, G+I and G+I+T) as prepared in Section 4.2.1 were freeze dried and milled through a 1 mm screen, and then the samples were analysed (in triplicate) for protein fractions using the CNCPS technique according to Sniffen *et al.* (1992) using recommendations published by Licitra *et al.* (1996), as described in the Chapter 2 Section 2.11. The protein content of the silages were fractioned into: non-protein nitrogen (fraction A), rapidly degradable protein (fraction B1), midrate degradable protein (fraction B2), slowly degradable protein (fraction B3) and indigestible protein (fraction C). In addition, the undegradable dietary protein (UDP5) at outflow rate 0.05 h^{-1} was calculated according to the equation reported by Kirchhof (2007) as described in Chapter 2 Section 2.11.

4.2.3 Experiment 2 b: Effect of chestnut HT supplemented at either ensiling or at feeding on dry matter intake of ryegrass silage, lamb growth and carcass characteristics.

4.2.3.1 Lambs and experimental diet formulation

Forty single Suffolk cross lambs (20 wether, average liveweight 29.5 kg ± 2.5 and 20 female average liveweight 29.2 kg ± 2.0) were used in this experiment. The experiment was designed as factorial plus control design. Lambs were blocked by liveweight and sex. Blocks were randomly allocated to receive one of the 5 experimental forage treatments (G, GET, G+I, G+T and G+I+T) were prepared as described in Section 4.2.1, with 8 lambs per treatment. Lambs were kept on a wood shaving bed and fed in individual pens (2 m^2) with free access to clean water. The diet was formulated to meet the requirements of growing Suffolk cross breed lambs (body weight 29 kg) according to AFRC (1993). The diet consisted of two parts: concentrate and silage. Lambs were offered 215 g DM of concentrate diet every day in the morning meal, which consisted of barley, sunflower extract, palm kernel, wheat feed pellets, molasses, soybean meal un-molassed, rapeseed meal, limestone, vegetable oil, ammonium chloride, vitamins and minerals (226, 181, 179, 179, 100, 42.7, 30, 26, 15.7, 7.5, 5, 4, 4 g kg^{-1} respectively), the vitamins consisted of vitamin A, D3 and E (10000, 2400 and 30 IU kg^{-1} respectively), and the minerals consisted of magnesium, copper and selenium (11.2 and 0.339 mg^{-1}kg respectively). The chemical composition of the concentrate diet is presented in Table 4.1.

Silage treatments were weighed out every day and offered (*ad libitum*) in two equal meals in the morning and afternoon at a rate of approximately 1.5 kg for each meal. The silages were offered in a 10 L capacity wooden bucket by placing the bucket into each pen. Refusals were weighed back twice a week. There were no concentrate refusals on any day for any lamb.

Samples of each treatment (concentrate, silages and refusals) were taken (Tuesday and Friday every week for 6 weeks) and stored frozen at -20^{o}C to await proximate analysis. Concentrate and offered silage samples (G, GET and G+I) were analysed (in triplicate) according to AOAC (2000) for DM, pH, NH_3-N, OM, CP, NDF, ADF and EE as described in Chapter 2 general materials and methods, Sections: 2.1, 2.5, 2.4, 2.2, 2.3, 2.6, 2.7, and 2.8 respectively. In addition, metabolisable energy (ME) and metabolisable protein (MP) of the silage samples were estimated using near infra-red reflectance spectroscopy (NIR) (RUMENCO, Staffordshire, UK), while for the concentrates the ME and MP was calculated according to McDonald *et al.* (2011). Proximate analysis results of the silages and concentrate are shown in Table 4.1.

The experiment was started on the 13th of May 2013 with one week as an adaptation period to introduce the lambs to the silage and on the 20th of May 2013 the experimental period commenced and continued for 42 days, with the lambs being slaughtered on the 2nd of July in a local abattoir.

Metabolisable dietary protein requirement for growing Suffolk lambs with average daily liveweight gain 0.2 kg was calculated according to the AFRC (1993) equations as follows:

$MP_{requirement}$ (g d^{-1}) = $MP_{maintenance}$ + $MP_{growth + wool}$ Eq. 3.1

$MP_{maintenance}$ (g d^{-1}) = 2.19 x $W^{0.75}$ Eq.3.2

MP $castrated_{growth + wool}$ (g d^{-1}) = ΔW (334-2.54W + 0.022W^2) +11.5 Eq.3.3

MP $female_{growth + wool}$ (g d^{-1}) = ΔW (325 -4.03W + 0.036W^2) +11.5 Eq.3.3

Were MP is metabolisable protein, ΔW is change in body weight (kg), W is liveweight.

In addition the dietary metabolisable protein was calculated according to the information obtained from the proximate analysis of the silage treatments, experiment 2a and lambs DMI.

Table 4.1 Proximate analysis of concentrate diet and ryegrass silage treated with tannin at ensiling, inoculated with bacteria or water.

Analysis	GET	G+I	G	Concentrate
DM ($g\ kg^{-1}$)	244	247	245	880
pH	3.8	4.0	3.7	-
NH_3N($g\ kg^{-1}$TN)	18	21	22	-
CP ($g\ kg^{-1}$DM)	168	165	166	180
OM ($g\ kg^{-1}$DM)	906	907	904	920
NDF ($g\ kg^{-1}$DM)	433	445	434	111
ADF ($g\ kg^{-1}$DM)	278	277	280	
EE ($g\ kg^{-1}$DM)	26	28	27	50
ME($MJ\ kg^{-1}$DM)	11.4*	10.9*	11.9*	11.2**
MP ($g\ kg^{-1}$DM)	100*	99*	102*	110**

DM: dry matter, NH_3-N: ammonia nitrogen, CP: crude protein, OM: organic matter, NDF: neutral detergent fibre, ADF: acid detergent fibre, EE: ether extract, ME: metabolisable energy, MP: metabolisable protein, GET: supplemented tannin at ensiling, G+I ryegrass silage treated with inoculate, G: ryegrass silage treated with water, *: calculated using NIR, **: estimated according to McDonald *et al.* (2011)

4.2.3.2 Lamb growth

Lambs were weighed at 11:00 on Tuesday of each week during the experimental period using portable calf scales (IAE Leek, Staffordshire, UK). The scale was calibrated prior to use using standard weights.

4.2.3.3 Metabolic profile

Blood samples were collected from each lamb four times during the experimental period on days 0, 15, 30, and 38 days. Blood samples (10 ml) were taken via jugular venepuncture at 11:30 (2.5 hours post feeding) on Monday once every two weeks, into lithium heparin vacutainers tubes (for total protein, urea and BHB analysis) and into potassium oxalate vacutainers tubes (for glucose analysis) (Bioscience Int. Plc., Bridgend, UK). Blood samples were centrifuged at 3000 g for 15 min at 4^{o}C, and the plasma transferred into 2 ml tubes and stored at -20^{o} C for further analysis. Frozen plasma samples were defrosted in the fridge and analysed for urea, total protein and beta-hydroxybutyrate (BHB) for those preserved with lithium heparin, and for glucose for those samples preserved with potassium oxalate. Samples were analysed as described in Chapter 2 general materials and methods Section 2.13.

4.2.3.4 Diet whole tract digestibility

Diet DM digestibility was measured using acid insoluble ash (as an indirect marker for measuring feed digestibility). Acid insoluble ash was determined based on the method published by Van Keulen and Young (1977) as described in Chapter 2, the general material and methods (Section 2.14). Approximately

50 g of fresh faecal samples were collected into a plastic pots directly from the anus of each of the 40 lambs every day at 16:00 for 5 days during week 4 of the experiment. Faecal samples were dried in an oven dryer at 60^{o}C for 48 h and then stored at -20^{o}C. Twenty g of dry faeces samples for each lamb for each of the 5 days were bulked and milled through a 1 mm screen and 5 g were used for measuring DM digestibility in duplicate as described in Chapter 2 Section 2.14.

4.2.3.5 Lamb slaughter

Six weeks post feeding, lambs were weighed and removed from their pens at 16:00 on the 1st of July 2013 and group housed overnight with free access to clean water. The following morning the lambs were sent to a commercial abattoir (Approved Design Slaughterhouse. Walsall, West Midland, UK). Lambs were slaughtered, rumen fluid samples were taken from each lamb into 50 ml pots and stored on ice in a polystyrene box. Rumen pH was measured 2 h post slaughtering as described in Chapter 2 the general materials and methods section 2.5, and stored at -20^{o}C to prior analysis for NH_3-N and VFA (Sections 2.4 and 2.15, respectively). Lamb carcasses were weighed twice, 30 min post slaughter as hot carcass weight and 24 h post slaughter as chilled carcass weight (carcasses were kept in a chiller 4^{o}C for 24 h).

4.2.3.6 Carcass measurements

Prior to hygiene inspection the dimensional measurements of the carcasses (body length, barrel width, chest depth, and gigot depth and gigot width) were recorded according to Brown and Williams (1979) (Figure 4.1.) Carcasses were then halved longitudinally with a band saw and the left side was collected

for carcass characteristics and meat parameters. The left half was then quartered between the penultimate and rib 12 using a knife. The subcutaneous fat thickness (at rib 12) was measured using a set of metal callipers, and the eye muscle area was obtained by tracing the eye muscle at rib 12 upon acetate paper; later the papers; were scanned and the muscle area measured using an Image Analyser Pro Plus 4.1 software (Media Cybernetics Inc., PA 15086, USA) . Dressing percentage was calculated by dividing the hot carcass weight by the final live weight. 100 g of loin muscle of each lamb was stored at 4^{o}C for meat colour and rancidity measurements.

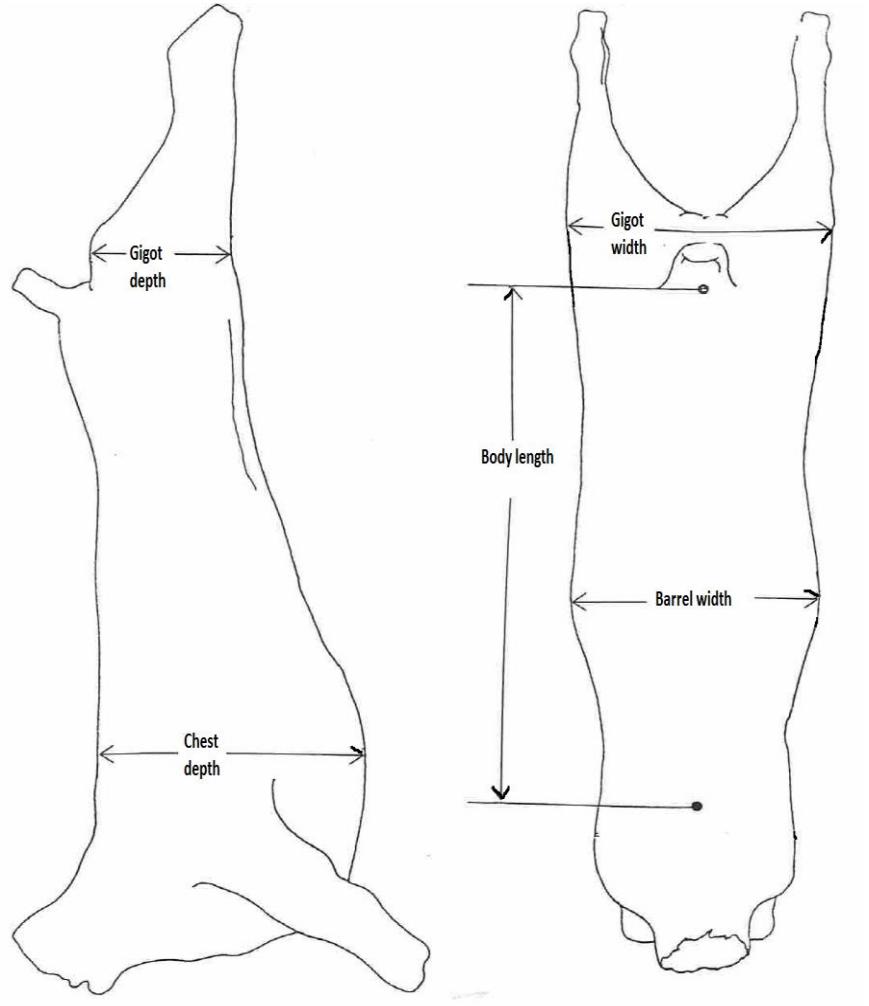

Figure 4.1 Illustration of dimensional measurements taken on lamb carcass, adapted from Brown and Williams (1979)

4.2.3.7 Meat colour and rancidity

Meat colour parameters of the chilled loin muscle (stored at 4^{o}C) was measured 3 days post slaughter. A Minolta colour meter (model CR-400, Konica Minolta Sensing Inc., Osaka, JAPAN) was used to measure meat colour coordinates including: lightness (*L**), yellowness (*b**) and redness (*a**), The Minolta colour meter contained a head with 0.8 cm diameter, a measuring surface and a diffused illumination viewing. The measurements were made using the D65 illuminate and 2^{o} standard observed. The camera was first calibrated with a white calibration plate at the beginning of measurements. The lens of the camera was allowed to touch the surface of the meat during the measurement, and a double reading was made for each sample.

The extent of lipid oxidation in raw meat was calculated by measuring 2-thiobarbituric acid reactive substance (TBARS) according to the method described by Buege and Aust (1978). Raw chopped (1 mm screen miller) meat (0.5 g) from the chilled loin muscle (3 days post slaughter) was mixed with 2.5 ml of stock solution (3.75 g L^{-1} thiobarbiruic acid, 150 g L^{-1} trichloroacetic acid and 0.25 N HCl) in test tubes. The tubes were heated for 10 min in a boiling water bath (>95^{o}C) until a pink colour appeared; tubes were then directly cooled in running tap water. Sample tubes were centrifuged at 3000 g for 10 min, the supernatant transferred to a spectrophotometer and the absorbance measured at 532 nm. Background measures of tubes containing all chemicals without samples were also taken. A standard curve was prepared using 1,1,3,3, tetra-ethoxyprpane at concentrations of 0.1-0.6 mg ml^{-1}

4.2.4 Statistical analyses

All measured parameters were analysed using an ANOVA procedure of GenStat (GenStat version 15, VSN international Ltd, UK). Chestnut HT supplemented at ensiling (GET) was compared to the mean of the other treatments factorial 2 x 2 (tannin supplemented prior feeding x inoculum). The non-linear *in situ* DM, OM and CP degradation were fitted with zero hour according to the model of McDonald (1981) using the curve fit of SigmaPlot 12 (System Software Inc., London, UK) for each parameter and each wether, the calculation included: fraction “a, b, c and lag time”. The *in situ* DM, OM and CP parameters (four replication per treatment, n=20) and protein fractionation using the CNCPS method (three replications per treatment, n=15) parameters were then analysed as factorial plus control design.

The lamb growth study was analysed by ANOVA as a factorial plus control design (as above) using measurement procedure of GenStat version 15 (VSN International Ltd, UK.). Lamb average daily liveweight gain (ADG) was calculated using Microsoft Excel. Feed efficiency was measured by dividing the total DMI by total gain (kg kg^{-1}). Blood plasma parameters (BHB, total protein, glucose and urea) concentration for week 0 was used as a covariate for the other weeks 2, 4 and 6.

4.3 Results

4.3.1 Experiment 2a: Effect of supplemented chestnut HT at either ensiling or after opening the silos to ryegrass on *in situ* rumen degradability and protein fractionation using the CNCP.

4.3.1.1 In situ rumen degradability

Supplemented tannin at ensiling (GET) was found to reduce (P<0.01) the immediately soluble fraction “a” compared to the mean of other treatments (417 vs 425g kg^{-1}DM, respectively). In addition, G (negative control) had the lowest faction “a” compared to positive control or supplemented tannin after opening the silage silos (414, 429 and 521 and 435 g kg^{-1}DM, for G+I, G+T and G+I+T respectively). The insoluble but potentially degradable DM fraction “b” was found to be reduced (P<0.001) in GET compared to the mean of the other treatments (330 vs 370 g kg^{-1} DM respectively) (Table 4.2). Similarly, supplementation with tannin after opening the silos (G+T or G+I+T) reduced fraction “b” compared to G or G+I (Figure 4.2). Consequently, the total potential degradable fraction “a+b” was found to be lowest in the presence of both tannin inclusion methods (747 826, 772, 816 and 764g kg^{-1}DM for GET, G, G+T, G+I and G+I+T, respectively). Furthermore, GET, G+T or G+I+T increased lag time by approximately 40% compared to G or G+I (Table 4.2). The effective rumen degradability (ED5) was found to be reduced (P=0.01) by supplementing with tannin after the silage silos had been opened, while additional tannin at ensiling trended to reduce (P=0.07) ED5 (Table 4.2). Supplemented tannin at ensiling increased (P=0.06) the rate of degradation

fraction “c” compared to the mean of the other treatments (0.06 vs 0.05 for GET and mean of the other treatments respectively).

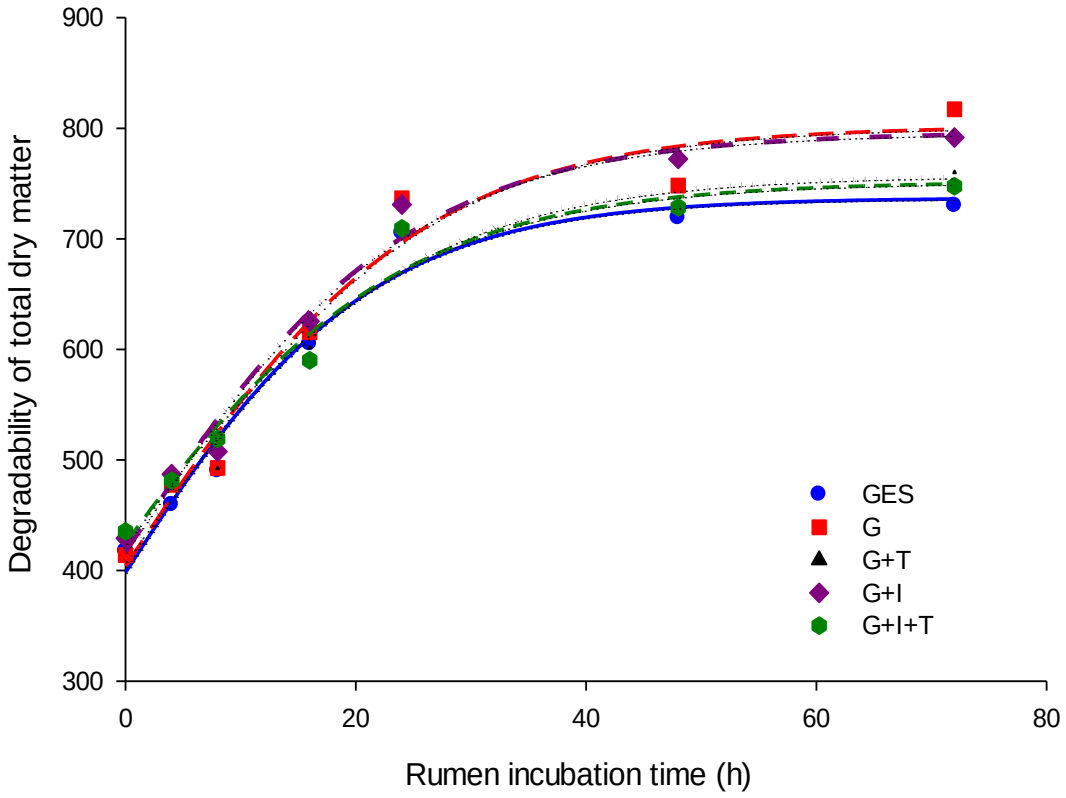

Figure 4.2 Effects of supplemented chestnut HT to ryegrass silage either at ensiling or after opening the silage silos on *in situ* DM degradability. GET ryegrass silage + tannin at ensiling, G: ryegrass silages, G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T ryegrass silage + inoculate + tannin at feeding.

Crude protein fraction “a” was reduced (P<0.01) in GET compared with the mean of the other treatments (712 vs 735g kg^{-1}DM respectively) (Table 4.3). Supplemented tannin after opening the silos was found to reduce (P=0.002) fraction “b” (190, 153 and 228 g kg^{-1} DM, for G+T, G+I+T and G respectively). Figure 4.3 shows that GET had a lower (P= 0.003) CP fraction “a+b” compared to the means of the other treatments (887 and 926 g kg^{-1} DM respectively).

Table 4.2 Effects of supplemented chestnut HT or inoculate to ryegrass silage either at ensiling or after opening the silage silos on *in situ* DM degradability.

	Treatments					SED			*Probability*		
	GET	G	G+T	G+I	G+I+T	GST	TF	Inoc	GST	TF	Inoc
a $_{(g\ kg^{-1}DM)}$	417	414	421	429	435	1.6	1.7	1.7	<0.01	<0.01	<0.01
b $_{(g\ kg^{-1}DM)}$	330	412	351	387	329	7.3	8.0	8.0	<0.01	<0.01	<0.01
a+b $_{(g\ kg^{-1}DM)}$	747	826	772	816	764	8.4	9.2	9.2	<0.01	<0.01	0.26
c	0.06	0.05	0.05	0.05	0.05	0.004	0.005	0.005	0.06	0.47	0.61
Lag $_{(h^{-1})}$	0.69	0.30	0.62	0.30	0.59	0.093	0.102	0.102	0.02	<0.01	0.86
ED5 $_{(h^{-1})}$	598	612	600	624	603	6.4	7.0	7.0	0.07	0.01	0.20

GET: ryegrass silage + tannin at ensiling G: ryegrass silages, , G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +tannin at feeding, a: the immediately soluble fraction-a, b: the in soluble but potentially degradable fraction-b, a+b: the total potential degradable, c: the rate of degradation, lag: lag time, ED5: the effective rumen degradability at outflow rate 5% h^{-1} , GST effect of tannin supplemented at ensiling, TF effect of tannin supplemented after opening the silos, Inoc: effect of inoculation (positive control).

In addition, supplementation with tannin after opening the silage clamps was found to reduce (*P*<0.01) the CP fraction “a+b” and increased (*P*=0.007) the lag time compared to both control groups (Figure 4.3). The effective rumen degradability at outflow rate 0.05 h^{-1} was reduced from 830 to 798 g kg^{-1}DM when ryegrass silage was treated with 30g kg^{-1}DM chestnut HT at ensiling compared to the mean of the other treatments. Similarly, supplemented tannin after opening the silos was also found to reduce (*P*=0.04) CP ED5 compared to the negative and positive control groups (Table 4.3).

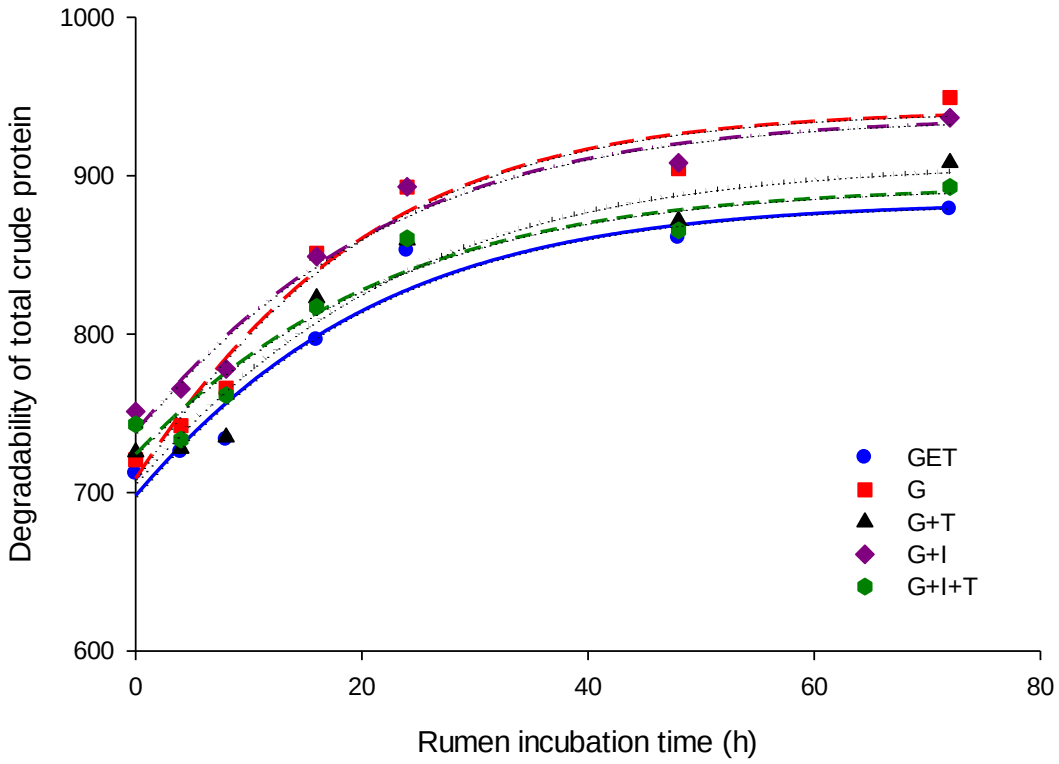

Figure 4.3 Effects of supplemented chestnut HT or inoclutoin to ryegrass silage either at ensiling or after opening the silage silos on *in situ* CP degradability. GET: ryegrass silage + tannin at ensiling, G: ryegrass silages, G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +tannin

Supplemented tannin at ensiling was found to reduce (*P*=0.001) the OM fraction “a” compared with the mean of the other treatments (395 vs 405g kg^{-}

^{1}DM, respectively), Treated ryegrass silage with 30g kg^{-1}DM tannin after opening the silage clamps (G+T or G+I+T) was found to reduce *in situ* OM parameters including: fraction “b”, fraction “a+b” (Figure 4.4) and ED5 and increase lag time compared with the negative control (Table 4.4).

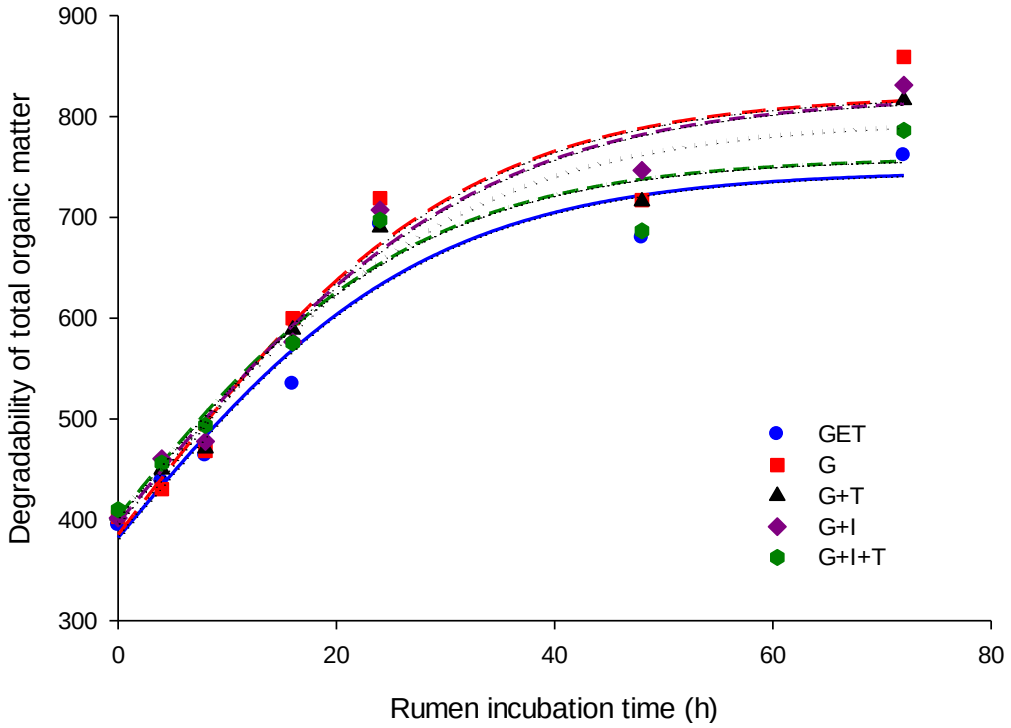

Figure 4.4 Effects of supplemented chestnut HT or inoculation to ryegrass silage either at ensiling or after opening the silage silos on *in situ* OM degradability. GET ryegrass silage + tannin at ensiling, G: ryegrass silages, G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T ryegrass silage + inoculate + tannin at feeding.

Table 4.3 Effects of supplemented chestnut HT or inoculate to ryegrass silage either at ensiling or after opening the silage silos on *in situ*.CP degradability.

	Treatments					SED			*Probability*		
	GET	G	G+T	G+I	G+I+T	GST	TF	Inoc	GST	TF	Inoc
a ($g\ kg^{-1}DM$)	712	721	726	751	743	5.1	5.6	5.6	<0.01	0.72	<0.01
b($g\ kg^{-1}DM$)	175	228	190	192	153	10.9	12.0	12.0	0.18	<0.01	<0.01
a+b ($g\ kg^{-1}DM$)	887	948	916	943	896	10.8	11.9	11.9	<0.01	<0.01	0.22
c	0.05	0.05	0.04	0.05	0.06	0.015	0.016	0.016	0.63	0.32	0.28
Lag (h^{-1})	1.04	0.49	1.32	0.62	1.24	0.259	0.283	0.283	0.64	<0.01	0.92
ED5 (h^{-1})	798	834	812	842	831	8.4	9.2	9.2	<0.01	0.04	0.08

GET: ryegrass silage + tannin at ensiling G: ryegrass silages, , G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +tannin at feeding, a: the immediately soluble fraction-a, b: the in soluble but potentially degradable fraction-b, a+b: the total potential degradable, c: the rate of degradation, lag: lag time, ED5: the effective rumen degradability at outflow rate 5%, GST effect of tannin supplemented at ensiling, TF effect of tannin supplemented after opening the silos, Inoc: effect of inoculation (positive control).

Table 4.4 Effects of supplemented chestnut HT or inoculate to ryegrass silage either at ensiling or after opening the silage silos on *in situ* OM degradability.

	Treatments					SED			*Probability*		
	GET	G	G+T	G+I	G+I+T	GST	TF	Inoc	GST	TF	Inoc
a $_{(g\ kg^{-1}DM)}$	395	403	406	402	410	2.6	2.8	2.8	<0.01	0.03	0.55
b $_{(g\ kg^{-1}DM)}$	379	480	437	456	377	28.4	31.1	31.1	0.06	0.03	0.12
a+b $_{(g\ kg^{-1}DM)}$	774	883	843	858	787	28.8	31.6	31.6	0.03	0.05	0.14
c	0.042	0.035	0.036	0.036	0.043	0.0051	0.0055	0.0055	0.35	0.42	0.43
Lag $_{(h^{-1})}$	0.74	0.35	0.73	0.18	0.57	0.113	0.124	0.124	0.03	<0.01	0.12
ED5 $_{(h^{-1})}$	564	598	583	589	581	6.6	7.2	7.2	<0.01	0.07	0.35

GET: ryegrass silage + tannin at ensiling G: ryegrass silages, , G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +tannin at feeding, a: the immediately soluble fraction-a, b: the in soluble but potentially degradable fraction-b, a+b: the total potential degradable, c: the rate of degradation, lag: lag time, ED5: the effective rumen degradability at outflow rate 5% , GST effect of tannin supplemented at ensiling, TF effect of tannin supplemented after opening the silos, Inoc: effect of inoculation (positive control).

4.3.1.2 Protein fractionation using the CNCPS

Non-protein nitrogen (fraction A) was reduced (*P*= 0.07) when tannin was added at ensiling compared with the mean of the other treatments (Table 4.5). In addition, the slowly degradable nitrogen (fraction B3) was reduced (*P*=0.03) in GET compared with the mean of the other treatments (78 vs 89 g kg^{-1}CP respectively). The indigestible protein (fraction C) was increased by approximately 22% when tannin was added at ensiling compared to the other treatments (Figure 4.5). Moreover, treating ryegrass silage with tannin (the mean of both methods of inclusion) was found to increase fraction C by approximately 9.6 and 21.3% compared with the positive and negative control (G+I and G respectively) (Table 4.5). Rumen undegradable protein at outflow rate 5% h^{-1} was found to increase when tannin was added at ensiling compared to the mean of the other treatments. Furthermore, treated ryegrass forage with inoculated bacteria was found to increase UDP5 compared to negative control group (Table 4.5)

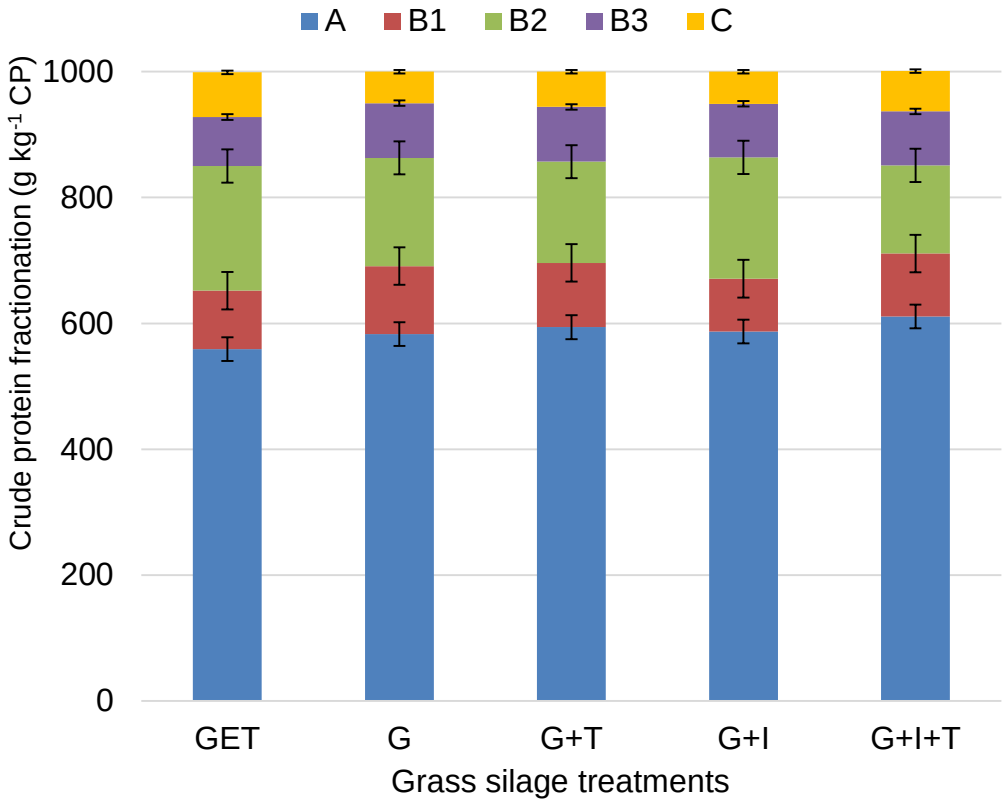

Figure 4.5 Effects of supplemented chestnut HT or inculation to ryegrass silage either at ensiling or after opening the silage silos on CNCPS. GET: ryegrass sailge + tannin at ensiling, G: ryegrass silages, G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +tannin at feeding. A: non protein nitrogen, B1: buffer soluble protein, B2: neutral detergent soluble protein, B3 acid detergent soluble protein, C: acid detergent insoluble protein

Table 4.5 Effects of supplemented chestnut HT or inoculate to ryegrass silage either at ensiling or after opening the silage silos on CP fractionation using the CNCPS.

	Treatments					SED			*Probability*		
	GET	G	G+T	G+I	G+I+T	GST	TF	Inoc	GST	TF	Inoc
A (g kg^{-1}DM)	559	583	594	587	611	17.4	19.0	19.0	0.07	0.29	0.51
B (g kg^{-1}DM)	369	367	360	361	325	17.1	18.8	18.8	0.36	0.19	0.22
B1 (g kg^{-1}DM)	93	108	102	84	100	27.2	29.8	29.8	0.83	0.82	0.60
B2 (g kg^{-1}DM)	198	172	161	193	140	24.1	26.4	26.4	0.21	0.16	0.99
B3 (g kg^{-1}DM)	78	87	87	85	86	4.0	4.4	4.4	0.03	0.15	0.09
C (g kg^{-1}DM)	71	50	56	51	64	2.6	2.9	2.9	<0.001	0.003	0.09
RUP5 (g kg^{-1}DM)	168	111	106	121	129	6.3	6.9	6.9	<0.01	0.83	0.02

GET: ryegrass silage + tannin at ensiling G: ryegrass silages, G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +tannin at feeding, A: non-protein nitrogen g kg^{-1}CP, B:degradable protein g kg^{-1}CP, B1: rapidly degradable protein, B2: intermediate degradable protein, B3: slowly degradable protein, C indigestible protein, RUP5: rumen undegradable protein outflow rate of 5% h^{-1}, GST effect of tannin supplemented at ensiling, TF effect of tannin supplemented after opening the silos, effect of inoculation (positive control).

4.3.2 Experiment 2 b: Effect of chestnut HT supplemented either at ensiling or at feeding on dry matter intake of ryegrass silage, lamb growth and carcass characteristics.

4.3.2.1 Effect of supplemented tannin on DMI, digestibility and growth rate

Supplemented tannin (30g kg^{-1}DM) at ensiling (GET) had no effect (*P*>0.05) on forage DMI, total DMI, forage MP intake, total MP intake, digestibility, average daily liveweight gain (ADG) or feed efficiency compared with the mean of the other treatments (Table 4.6). Tannin supplemented at feeding G+T and G+I+T also had no effect (*P*>0.05) on lamb growth parameters compared to both control groups (Table 4.6). However, G trended to reduced (*P*=0.1) DM digestibility compared with GET and G+I (0.83, 0.79, 0.81, 0.83 and 0.81 for GET, G, G+T, G+I and G+I+T, respectively). The lambs' liveweight gain during the experiential period is shown in Figure 4.6.

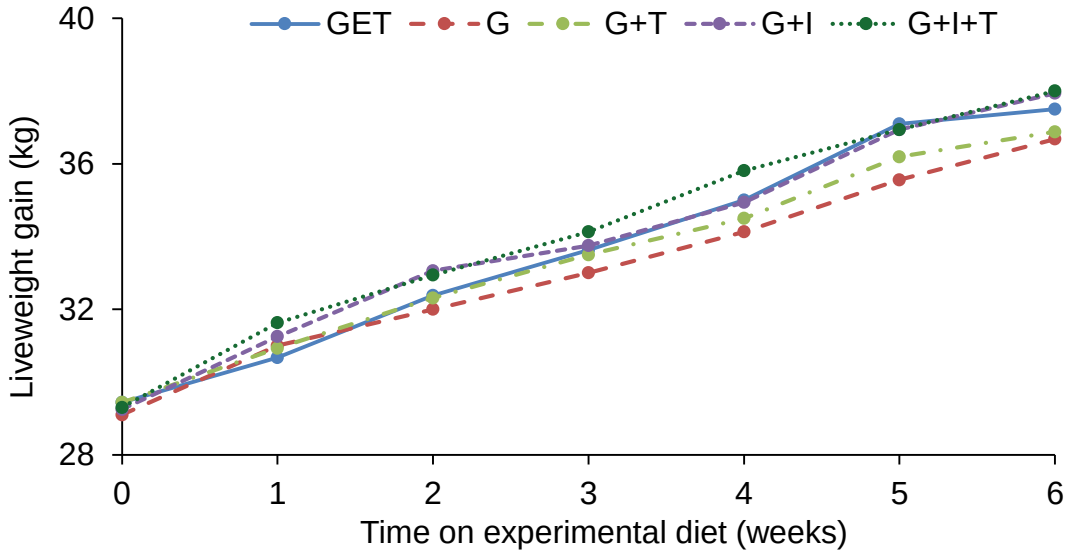

Figure 4.6 Effects of supplemented chestnut HT or inoculation on lamb liveweight gain (kg) GET: ryegrass silage +HT at ensiling, G: ryegrass silages, G+T: ryegrass silage +HT at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +HT at feeding. (SED, tannin at ensiling =1.06, tannin at feeding =0.98, inoculate =0.98, Time= 0.20)

Table 4.6 Effect of supplmented chestnut HT or inoculate to ryegrass silage either at ensiling or at feeding on lambs dry matter intake, dietary metabolisable protein, digestibility, total gain and feed efficiency.

	Treatments					SED			Probability		
	GET	G	G+T	G+I	G+I+T	GST	TF	Inoc	GST	TF	Inoc
F-DDMI ($g\ d^{-1}$)	699	701	684	713	699	19.8	21.7	21.7	0.93	0.38	0.43
T-DDMI ($g\ d^{-1}$)	914	916	899	928	914	19.8	21.7	21.7	0.93	0.38	0.43
E-DDMI ($g\ d^{-1} BW^{0.75}$)	50.3	51.4	49.8	50.4	51.4	1.25	1.37	1.37	0.72	0.26	0.81
F-MP ($g\ d^{-1}$)	72	70	70	71	72	1.8	2.0	2.0	0.81	0.16	0.27
T-MP ($g\ d^{-1}$)	96	94	93	94	96	1.8	2.0	2.0	0.81	0.16	0.27
Digestibility ($kg\ kg^{-1}$)	0.83	0.79	0.81	0.83	0.81	0.016	0.018	0.018	0.26	0.86	0.184
Initial W (kg)	29.4	29.1	29.4	29.3	29.3	0.61	0.67	0.67	0.78	0.69	0.96
Final W (kg)	37.5	36.7	36.9	37.9	37.9	1.09	1.19	1.19	0.89	0.92	0.24
ADG ($g\ d^{-1}$)	197	204	181	212	210	17.0	18.6	18.6	0.71	0.43	0.20
Total gain (kg)	8.1	7.6	7.5	8.7	8.6	0.78	0.86	0.86	0.96	0.86	0.10
FE ($kg\ DM\ kg^{-1}$ gain)	4.6	6.3	5.2	4.5	4.7	0.99	1.09	1.09	0.58	0.60	0.21

Ryegrass silage + tannin at ensiling G: ryegrass silages, , G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +tannin at feeding, F-DDMI: daily forage dry matter intake, T-DDMI: daily total dry matter intake, E-DDI: total dry matter intake based on empty body weight, ADG: average daily gain, FE: feed efficiency, Initial W: initial weight Final W: final weight, F-MP: metabolisable protein from forages, T-MP: total metabolisable protein, GST effect of tannin supplemented at ensiling, TF effect of tannin supplemented after opening the silos, effect of inoculation (positive control).

4.3.2.2 Metabolic profile

Tannin supplementation (30g kg^{-1}DM) at ensiling (GET) was found to increase (*P*=0.05) total blood plasma protein concentration compared with the mean of the other treatments (63.3 vs 58.9 mg ml^{-1} for GET and mean of the other treatment respectively). The highest blood plasma protein values were measured in the last 2 weeks of the experiment (total blood plasma protein means were 79.1 and 66.2 mg ml^{-1} for GET and mean of other treatment respectively). Supplementation with tannin at feeding (G+T and G+I+T) was found to increase (*P*=0.1) total blood plasma protein concentration during the whole experimental period. However, the effect of the additional tannin was significant (*P*=0.02) during the last two weeks compared to G and G+I (full duration of blood plasma means: 60.2, 58.0 and 56.1, week 6: 68.8, 63.6 and 61.0 mg ml^{-1} for mean of (G+T and G+I+T), G+I and G, respectively, Figure 4.7).

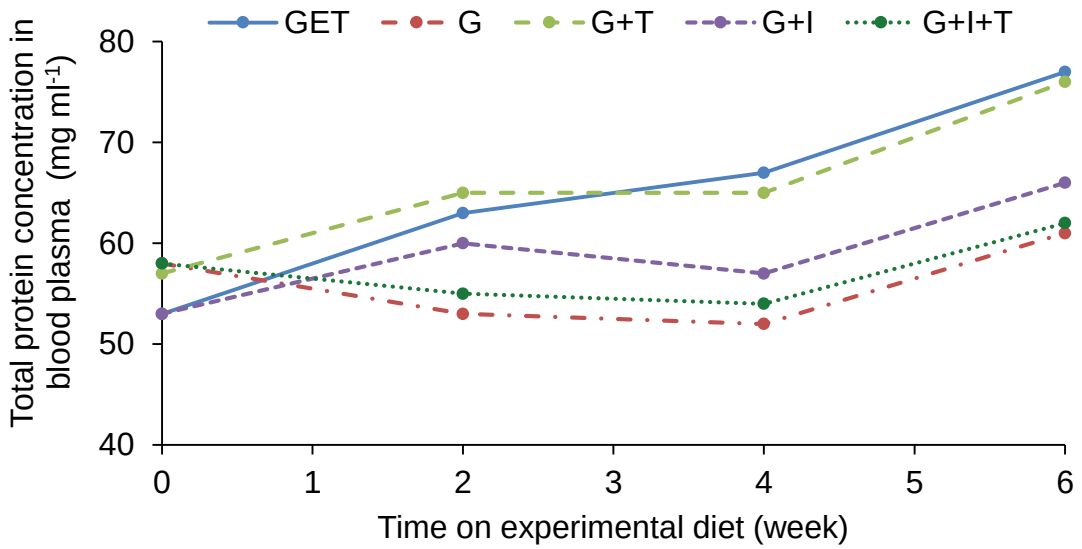

Figure 4.7 Effects of supplemented chestnut HT or inoculation on blood plasma protein concentration in lambs' fed: GET: ryegrass silage +HT at ensiling, G: ryegrass silages, G+T: ryegrass silage +HT at feeding, G+I: ryegrass + Inoc a protein concentration in lambs' fed: GET: ryegrass silage +HT. (SED, tannin at ensiling=4.60, tannin at feeding =2.53, inoculate=2.53, Time= 2.60).

Tannin supplemented at ensiling (GET) did not have an effect (P>0.05) on blood plasma urea concentration compared to the mean of the other treatments. In contrast, G+T and G+I+T were found to reduce (P=0.005) urea concentration in the last week of the study from 6.8 mmol l^{-1} (in both control groups) to 5.8 mmol l^{-1} (for supplemented tannin at feeding) as shown in Figure 4.8.

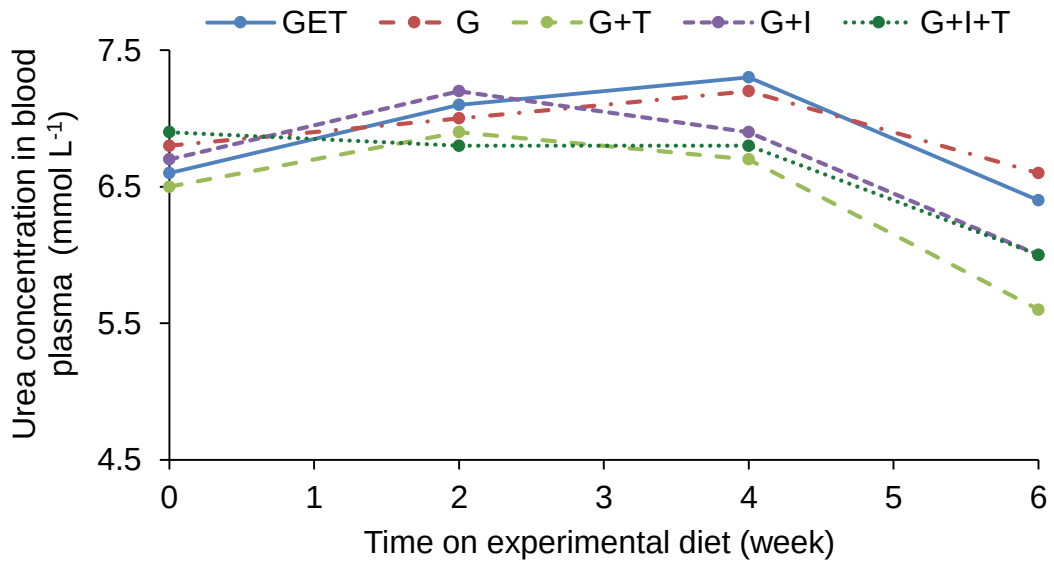

Figure 4.8 Effects of supplemented chestnut HT or inoculation on blood plasma urea concentration in lambs' fed: GET: ryegrass silage +HT at ensiling, G: ryegrass silages, G+T: ryegrass silage +HT at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +HT at feeding. (SED, tannin at ensiling =0.58, tannin at feeding =0.41, inoculate =0.41, Time= 0.30)

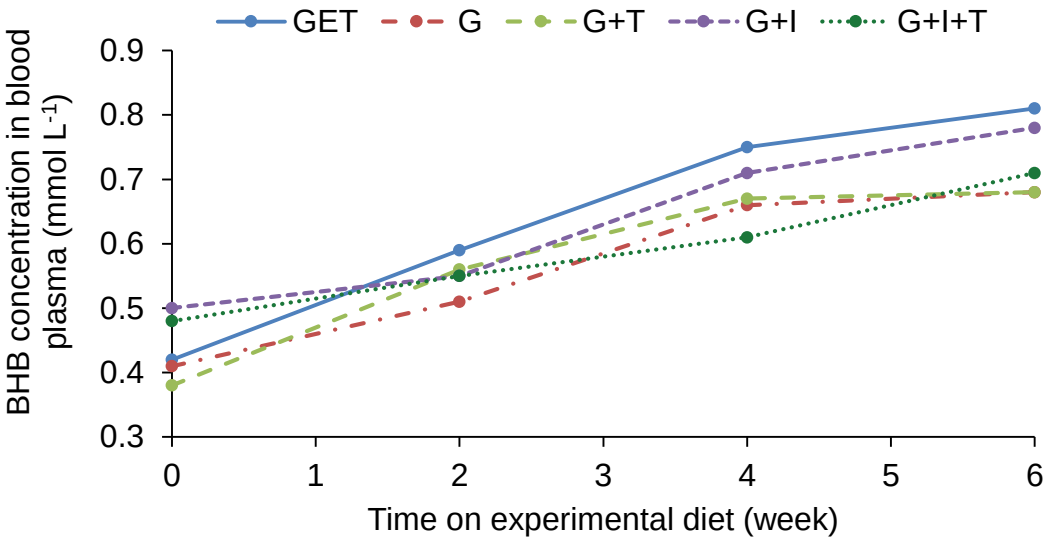

Figure 4.9 Effects of supplemented chestnut HT or inoculation on BHB concentration in blood plasma lambs fed: GET: ryegrass silage + tannin at ensiling, G: ryegrass silages, G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +tannin at feed (SED, tannin at ensiling=0.042, tannin at feeding=0.046, inoculate control=0.047, Time=0.035).

Tannin supplementation either at ensiling or at feeding had no effect (*P*>0.05) on beta-hydroxybutyrate (BHB) (Figure 4.9) or blood glucose (Figure 4.10).

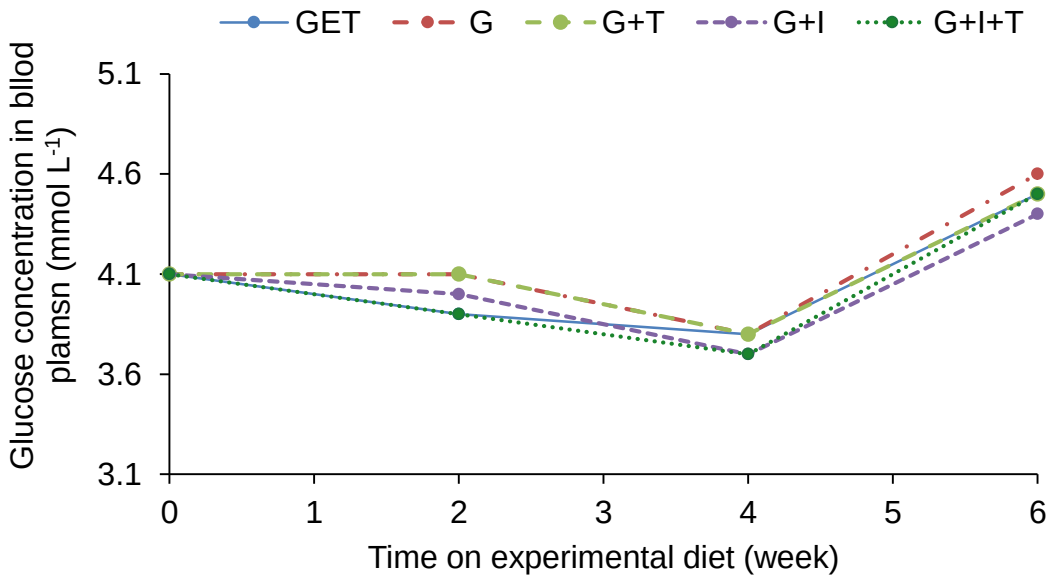

Figure 4.10 Effect of supplanted chestnut HT or inoculation on glucose concentration in blood plasma lambs fed: GET: ryegrass silage + tannin at ensiling, G: ryegrass silages, G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +tannin at fee. (SED, tannin at ensiling= 0.138, tannin at feeding=0.152, inoculate=0.152, Time=0.098)

4.3.2.3 Lamb slaughter and carcass parameters

Table 4.8 shows that GET had no effect (*P*>0.05) on any studied slaughter parameter. However, there was a trend (*P*=0.1) for animal fed GET to have an increase in barrel width compared to the mean of the other treatments (22.1 vs 21.6 cm, GET, mean of all other treatments, respectively). Furthermore, tannin addition at feeding did not have a significant effect on slaughter traits compared to both control groups (G or G+I). However, back fat thickness trended to be lower for lambs fed the G+T treatment (*P*=0.07) (9.8, 9.9 and

10.1 mm for tannin at feeding, positive and negative controls respectively Table 4.7).

4.3.2.4. Meat colour and rancidity.

Feeding lambs GET trended to reduce (P=0.06) meat lightness (L) compared to the other treatments (44.0, 45.1, 45.3, 45.7 and 45.8 for GET, G, G+T, G+I and G+I+T respectively) while the other silage treatments did not have an effect (P>0.05) on other meat colour parameters (Table 4.7). In addition, feeding lambs ryegrass silage treated with chestnut HT either at ensiling or at feeding had no effect (P>0.05) on meat rancidity (TBARS) as shown in Table 4.7.

Table 4.7 Effects of supplemented chestnut HT or inoculate to ryegrass silage either at ensiling or at feeding on slaughter trait, carcass characteristics, meat rancidity and meat colour.

	Treatments					SED			*Probability*		
	GET	G	G+T	G+I	G+I+T	GST	TF	Inoc	GST	TF	Inoc
HCW (kg)	16.1	15.7	15.4	16.1	16	0.48	0.52	0.52	0.55	0.66	0.24
Dressing%	42.6	42.8	41.7	42.3	42.3	0.76	0.83	0.83	0.46	0.41	0.93
CCW (kg)	15.6	15.1	14.9	15.5	15.6	0.47	0.51	0.51	0.48	0.89	0.19
BFT (cm)	10.0	10.1	9.8	10.0	9.8	0.16	0.18	0.18	0.55	0.07	0.81
EMA (cm^2)	16.7	15.9	13.7	15.4	15.4	1.40	1.54	1.54	0.26	0.37	0.64
BW (cm)	22.1	21.9	21.3	21.5	21.6	0.35	0.38	0.38	0.10	0.42	0.98
BL(cm)	54.0	54.1	54.8	54.4	54.4	0.57	0.62	0.62	0.47	0.54	0.90
BD (cm)	24.0	23.8	23.8	23.9	23.5	0.39	0.43	0.43	0.48	0.59	0.86
GW (cm)	21.8	21.6	21.5	21.3	21.4	0.39	0.43	0.43	0.43	0.98	0.47
Meat pH	5.60	5.66	5.64	5.65	5.62	0.027	0.030	0.030	0.14	0.26	0.57
L	44.0	45.1	45.3	45.7	45.8	0.81	0.88	0.88	0.06	0.93	0.38
a	14.1	14.4	15.0	13.8	14.1	0.57	0.63	0.63	0.74	0.38	0.17
b	10.9	11.1	11.3	11.5	11.2	0.53	0.59	0.59	0.45	0.93	0.75
TBARS ($mg\ kg^{-1}$meat FW)	18.1	21.6	15.6	17.8	18.39	1.87	2.05	2.05	0.90	0.12	0.77

GET: ryegrass silage +HT at ensiling G: ryegrass silages, G+T: ryegrass silage + HT at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate + HT at feeding, HCW: hot carcass weight, CCW: child carcass weight, BFT: back fat thickness, EMA: eye muscle area, BW: barrel width, BL: body length, BD: body depth, GW: gigot width. *L*: lightness, *a*: redness, *b*: yellowness, TABRS: lipid oxidation (2-thiobarbituric acid), GST effect of tannin supplemented at ensiling, TF effect of tannin supplemented after opening the silos, Inoc: inoculation.

4.3.2.5 Rumen fermentation

The results of the rumen fluid parameters are presented in Table 4.9. Tannin inclusion at ensiling (GET) was found to reduce (*P*=0.03) NH_3-N concentration compared to the mean of the other treatments (0.14 and 0.17 g L^{-1} respectively). In addition, supplementation with tannin at feeding (G+T or G+I+T) was also found to reduce (*P*=0.03) NH_3-N concentration compared to both positive and negative control groups (0.15, 0.19 and 0.17 g L^{-1} for the mean of (G+T and G+T+I vs G+I and G, respectively) (Table 4.3). There was a trend (*P*=0.07) for tannin supplementation at ensiling to reduce rumen pH compared to the mean of other treatments (6.60 and 6.73, respectively) (Table 4.8), whereas, supplemented tannin at feeding had no effect on rumen pH. The molar concentration or the proportion of the total VFA or individual VFA of the rumen fluid was not affected by feeding lambs ryegrass silage treated with chestnut tannin either at ensiling or at feeding (Table 4.8).

Table 4.8 Effects of supplemented chestnut HT to ryegrass silage either at ensiling or at feeding on lambs' rumen characteristics at slaughter.

	Treatments					SED			Probability		
	GET	G	G+T	G+I	G+I+T	GST	TF	Inoc	GST	TF	Inoc
pH	6.6	6.7	6.7	6.7	6.8	0.06	0.06	0.06	0.07	0.61	0.15
NH_3-N ($g\ L^{-1}$)	0.14	0.19	0.17	0.17	0.14	0.013	0.014	0.014	0.03	0.03	0.04
tVFA ($mmol\ L^{-1}$)	151.1	158.5	139.9	145.2	143.2	10.51	11.52	11.52	0.68	0.28	0.60
Acetic ($mmol\ L^{-1}$)	112.4	120.9	110.0	111.8	108.7	6.64	7.27	7.24	0.94	0.25	0.38
Butyric ($mmol\ L^{-1}$)	5.9	6.6	5.5	6.1	6.5	0.64	0.70	0.70	0.71	0.59	0.67
Propionic ($mmol\ L^{-1}$)	22.4	22.7	20.5	23.0	23.6	2.54	2.78	2.78	0.97	0.73	0.45
Isobutyric ($mmol\ L^{-1}$)	1.3	1.6	0.9	1.4	1.4	0.23	0.25	0.25	0.83	0.11	0.44
Valeric ($mmol\ L^{-1}$)	0.9	1.1	0.8	0.9	1.1	0.12	0.13	0.13	0.74	0.76	0.88
Isovaleric ($mmol\ L^{-1}$)	1.5	1.5	1.5	1.6	1.5	0.23	0.26	0.26	0.84	0.81	0.95

GET: ryegrass silage + tannin at ensiling G: ryegrass silages, G+T: ryegrass silage +tannin at feeding, G+I: ryegrass + inoculated, G+I+T: ryegrass silage + inoculate +tannin at feeding, NH3: ammonia nitrogen, tVFA: total volatile fatty acid, GST effect of tannin supplemented at ensiling, TF effect of tannin supplemented after opening the silos, Inoc: effect of inoculation (positive control).

4.4 Discussion

4.4.1 Silage production

When ryegrass and lucerne silage silos were opened, all ryegrass silage treatments were shown to be well fermented, as indicated by the low pH (3.7-4) and NH_3-N (20-22 g kg^{-1}TN) value, with no visible mould growth being observed. However, a clearly visible white mould was noticed in every corner of the lucerne silage clamps, which indicated aerobic deterioration. During lucerne silage making and after the lucerne had been mowed on 18th of July 2012 and left in the field 48 h for wilting, the weather condition during these two days was dry and the temperature reached 30^{o}C, as a result of that the DM of lucerne became approximately 600 g kg^{-1} at ensiling. In addition, the forage chop size was 5-10 cm, and the herbage air exclusion process was by pressing feet; all these factors could increase the risk of air contamination. Wilkinson and Davies (2013) reported that ensiling of high DM forage (>500 g kg^{-1}) could reduce the availability of water for bacteria (including fermentation bacteria) which could inhibit their growth and increase the growth of yeasts and moulds. In addition, a lack of good ensiling practise, especially excluding air from the silage silos would put the forage at risk of aerobic deterioration and increase the chance of developing a large fungal population, thus compromising the aerobic stability and mycotoxin content of the silage (Wilkinson and Davies, 2013).

4.4.2 Experiment 2 a: Effect of supplemented chestnut HT either at ensiling or after opening the silos to ryegrass on *in situ* rumen degradability and protein fractionation using the CNCP.

4.4.2.1 In situ rumen degradability

Tannin supplementation by either method of inclusion was found to reduce the *in situ* DM, CP and OM degradability including “a, b” fractions and ED5 and increasing lag time compared to the mean of the other treatments (Table 4.2-4.4). Frutos *et al.* (2004) reported that the most commonly known effect of tannins in ruminant nutrition is their influence on reducing rumen degradability, especially protein degradation. Makkar (2003) suggested that the formation of tannin-protein complexes which is characterized by resisting the activity of rumen microbes, plus tannin could have a direct harmful effect on rumen microorganisms (Mueller-Harvey, 2006) and/or tannin could inhibit proteolytic enzymes (Frutos *et al.*, 2004) which would be the main reason for reducing rumen degradability. Sinclair *et al.* (2009) found that whole crop pea silage (coloured flower) which contained high levels of CT (93.1 g kg^{-1}DM) had a lower *in situ* fraction-a, fraction a+b and higher digestible undegradable protein compared to whole crop pea silage (white flowers) containing low levels of CT (47.3 g kg^{-1}DM). Messman *et al.* (1996) found that tannin levels reduced the protein degradation and increased the protein remaining in the rumen after 12 h rumen incubation *in situ,* with no effect on fraction “a”, when the protein degradability of different forage legumes containing different types and levels of tannin have been measured (*in situ*). Alipour and Rouzbehan (2010) and Coblentz and Grabber (2013) also found that using forages rich in tannin

reduced rumen degradability (*in situ*). Salawu *et al.*, (2001) observed a reduction in fraction “a” and ED and increase in RUP when they used quebracho CT as a silage additive at ensiling to grass forage.

When comparing the methods of inclusion, additional tannin at ensiling had more of an effect in reducing rumen degradability compared to additional tannin after opening the silage silos. The differences noted between the methods of tannin addition may be related to the time taken for the formation of the tannin protein complex (Deville *et al.*, 2010). Moreover, there is a possibility that some of the supplemented tannins after opening the silos were dissolved in rumen liquid without reacting with forage protein, which could had an effect on rumen microbial activity (Salawu *et al.*, 2001).

4.4.2.2 Protein fractionation using the CNCPS

The CNCPS protein fractionation technique showed that additional tannin at ensiling reduced protein degradability and increased acid insoluble detergent nitrogen fraction C, which may perhaps be due to the reaction between tannin and forage protein. Similar to the *in situ* results, the reduction in protein solubility was greater in tannin supplementation at ensiling compared to incorporating at feeding (Table 4.8), which could be related to the time take for complexing tannin with forage protein (Deville *et al.*, 2010). Salawu *et al.* (1999) and Tabacco *et al.* (2006) observed that exogenously supplemented tannin reduced NPN of ensiled grass and lucerne, but did not necessarily reduce NH_3 concentration, while Deaville *et al.* (2010) reported that endogenous tannins may have a direct effect on inhibiting plant protease with a small effect on microorganisms inside the silage clamps. Makkar and Becker

(1997) found that *in vitro* protein degradability of faba beans with coloured flowers (high tannin level) was significantly higher compared to faba beans with white flowers.

4.4.3 Experiment 2 b: Effect of chestnut HT supplemented either at ensiling or at feeding on dry matter intake of ryegrass silage on lamb growth and carcass characteristics.

4.4.3.1 Animal performance

Mean ryegrass silage intake in the current study was 50.2 g DM kg^{-1} $BW^{0.75}$ d^{-1} which was higher than reported by Fitzgrerald (1996), Speijers *et al.* (2005) and Kruege *et al.* (2010) who observed an intake of 26.2, 43.4 and 26.5 g DM $kg^{-1}BW^{0.75}d^{-1}$, respectively. The high forage DMI could be due to the fact that lambs used in the current study were lighter than used in the other studies, thus, DMI was higher where expressed as units of DMI to empty body wright ($BW^{0.75}$).

Ryegrass silage was treated with chestnut HT at a rate of 30 g HT kg^{-1} either at ensiling or at feeding. Lambs consumed approximately 21 g d^{-1} of HT (mean forage DMI was 699 g d^{-1}) which was equivalent to 1.57 g HT $kg^{-1}BW^{0.75}$ d^{-1}. Although lambs ate a relatively high level of tannin, there was no significant influence on forage DMI either based on actual intake or on $BW^{0.75}$. Similar results were observed by De Oliveira *et al.* (2007) and Krueger *et al.* (2010) who found that feeding diets rich in tannin did not reduce forage palatability. Similarly, Toral *et al.* (2011) also found that supplementing 10 g of a mix of HT and CT kg^{-1}DM to lactating ewes' diet had no effect on DMI and animal performance. In contrast, Hervas *et al.* (2003) found that feed intake was

completely stopped after 5-6 days when 3 g of CT kg^{-1}body weight (which equivalent to 166 g kg^{-1}DM) was directly infused into the rumen via a rumen cannula in four mature sheep. Hervas *et al.* (2003) suggested that high tannin levels could have a negative impact on the physiological status of the animal, decreasing DMI. Dschaak *et al.* (2011) reported that supplementing 30 g quebracho CT kg^{-1} DM to dairy cows' diet reduced DM, OM, CP and NDF intake, with no effect on DM, OM, CP and NDF digestibility. Ben Salem *et al.* (2005) found that feeding Barbarine lambs acacia rich in CT (59.6 g kg^{-1}DM) reduced DMI and growth rate in the first 6 days, however, the intake and growth rate recovered after continued feeding for 24 days. In contrast, Puchala *et al.* (2005) and Sinclair *et al.*, (2009) noticed an increase in ruminant DMI when offered forages rich in tannin. The variation in the effect of tannin on DMI between different studies may perhaps be due to the variation between tannin source, types and levels that have been used. Makkar (2003) suggested the source and type of tannins would have more impact than tannin levels on DMI.

Frutos *et al.* (2004) reported that there are three theories that could explain the negative effect of tannins on DMI: (1) taste receptors in the animal's mouth could be sensitive to the reaction between tannin and salivary protein, resulting in an astringent sensation to the brain, (2) formation of a tannin-protein complex that slows digestion resulting in a sensation of satiety at the brain, (3) negative effect of tannin on rumen microorganisms resulting in reduced digestion. Makkar (2003) reported that tannins could decrease microbial attachment to feed particles in the rumen. A fourth mode of action was postulated by Hervas *et al.* (2003) who suggested that infused tannin could have a direct physiological effect on the animal and can be harmful or toxic,

resulting in kidney and liver lesions and even animal death. Krueger *et al.* (2010) reported that herbivorous mammals produced special types of protein in their saliva called proline rich salivary protein which is highly reactive with dietary tannin to reduce tannin's effect. Mueller-Harvey (2006) and Piluzza *et al.*, (2014) suggested that the reaction between proline-rich salivary protein and tannin would be responsible for the astringent taste in the animals mouth, thus the DMI could be reduced. Makkar (2003) explained that there are no such proteins (proline) in cattle and sheep saliva, hence no reaction or astringent test would occur in cattle and sheep. Priolo *et al.* (2000) showed that tannin from different sources could be more astringent. For example CT in croup pulp seems to provide a more astringent taste in the animals mouth than other tannins, thus feeding ruminants small amounts of this types of tannin (>25 g kg^{-1}DM) would have a great reduction in DMI ~37 % (Priolo *et al.*, 2000), whereas, supplementing 55.1 g chestnut HT kg^{-1}DM to lucerne silage did not have an effect on DMI (Deaville *et al.*, 2010).

In the current study the diet DM digestibility was measured using AIA with an average of 0.81 kg kg^{-1} with no effect (P>0.05) of additional tannin (Table 4.6). These results agree with the results reported by Hart *et al.* (2012) who found that different tannin levels did not have a significant impact on DM digestibility when they fed lambs whole crop pea silage containing different levels of CT. Deaville *et al.* (2010) also observed that there was no significant effect of supplementing 55.1 g chestnut HT kg^{-1}DM to grass silage on DM and OM digestibility while an additional 55.6 g of mimosa CT kg^{-1}DM was found to reduce (*P*<0.01) DM and OM digestibility. Makkar (2003) suggested that not all tannin protein complexes could dissociate in the abomasum, or complexes

could be reversible in the small intestine depending on the tannin source, types and concentration. Mueller-Harvey (2006) and Piluzza *et al.* (2014) reported that the fate of tannins in the digestive track are still unclear especially post ruminally and it is uncertain whether tannin binds with endogenous protein or it rebinds with feed protein again. In a report published by Cornell University (2014) it was suggested that HT could be hydrolysed in mild acids while CT can resist acids hydrolysis. As a result HT may be hydrolysed in the abomasum and not show a negative influence on digestibility.

The average daily gain and feed efficiency of the lambs used in the current study was approximately 200.8 g d^{-1} and 5.06 kg kg^{-1}, respectively, with no significant difference between treatments, which could be due to that the lambs were fed the experimental treatments for a relatively short time (6 weeks). In addition, in the current study chestnut HT was used, and chestnut HT is characterised as being a less aggressive tannin compared to some other types such as quebracho or mimosa tannin (Deaville *et al.*, 2010; Pellikaan *et al.*, 2011). Makkar (2003) reported that the availability of tannin (low levels) could depend on type and tannin sources, which could modulate rumen fermentation and increase microbial protein synthesis as well as reducing protein degradability and increasing AA supply to the lower gut. Increasing those two sources of AA supply to the small intestine could lead to an increase in animal performance in the form of producing higher milk, meat and wool, reducing CH_4, CO_2 and NH_3 emissions plus nitrogen excretion to the soil via urine, thus reducing environmental pollution.

Metabolisable protein requirements for Suffolk lambs (either castrated or female) was calculated according to the equations published by AFRC (1993)

as described in Chapter 4 Section 4.2.3.1. The initial liveweight was approximate 29 kg and the final liveweight was approximately 37.5 kg, with an average daily liveweight gain approximate 0.2 kg, thus the MP requirements for both castrated and female lambs was approximate 95 and 88 g d^{-1} respectively. Metabolisable protein supplied from dietary treatments used in the current study was approximately 97 g d^{-1} which covered the MP requirements for castrated and female lambs, with no differences between experimental treatments (Table 4.6), which may be one of the reasons that no differences in lambs liveweight gain or performance were noticed.

4.4.3.2 Metabolic profile

The mean blood plasma analysis results for BHB, glucose, urea and total protein was 0.59, 4.09, 6.59 mmol L^{-1} and 59.75 mg ml^{-1} respectively. The results showed that blood parameters were within the normal physiological condition of the lambs 2-3 h post morning feed as reported by Hart (2005). Additional tannin at ensiling or at feeding were found to reduce plasma urea concentration by approximately 5 and 14 % respectively, compared with positive control (G+I) or negative control (G). Lewis (1957) reported that plasma urea N are highly correlated with rumen ammonia concentration, and as mentioned previously, in the current study tannin supplementation reduced ammonia concentration in the rumen. Therefore, these results concur with the reduced rumen ammonia concentration observed when tannin was fed. The results showed that supplemental tannin was found to increase total protein in blood plasma in the last week of the experiment, which could indicate that tannin improved protein utilization in the animal body, but the experiment

needed to be longer to establish a longer term trend. In contrast, additional tannin at either ensiling or at feeding did not affect BHB (Figure 4.9) or glucose (Figure 4.10) concentration in the blood. These results coincide with no effect of tannin on rumen VFA (Table 4.8).

4.4.3.3 Meat colour and rancidity

In the current study, three groups of lambs were fed ryegrass silage treated with tannin either at ensiling or prior to feeding, however tannin supplementation (both methods of inclusion) did not have any significant effect on meat colour or lipid oxidation. These results agree with those reported by Luciano *et al.* (2009), who fed a group of 7 lambs' a concentrated diet supplemented with 40.3 g kg^{-1} DM quebracho CT and found that tannin had no effect on fresh meat colour. However, Luciano *et al.* (2009) found that after the meat was stored for 14 days at a chilled temperature (4^{o} C), the meat colour and lipid oxidation was less in the lambs group that were fed tannins (the meat kept its original colour and lipid oxidation) compared to the meat from lambs fed the control diet. Therefore, tannin can behave as an anti-oxidant compound during the storage period. However, in the current study, the effect of the storage period on meat colour and lipid oxidation have not been studied.

4.4.3.4 Rumen fermentation

Rumen fluid was collected directly after the lambs were slaughtered, rumen pH was measured 2 h post slaughtering and stored at -20^{o}C for determining ammonia and VFA concentrations. Tannin supplementation at ensiling or at feeding had no effect (P>0.05) on rumen pH, total VFA and individual VFA

concentration, while rumen NH_3-N concentration was reduced (P<0.05) (Table 4.3). Makkar (2003) reported that complexing tannin with protein could reduce protein hydrolysis in the rumen and hence reduce NH_3-N production during rumen fermentation. Similar results were reported by Min *et al.* (2003) who found that feeding sheep *Lotus corniculatus* forage containing 32 g kg^{-1}DM CT reduced rumen ammonia and soluble N concentration. Pellikaan *et al.*, (2011) noticed that mixing 100 g kg^{-1}DM of three types of CT (grape seed, quebracho or green tea) or four types of HT (chestnut, myrabolan, tara or valonea) with lucerne hay reduced total VFA (P=0.007), pH (P<0.001) and NH_3 (P<0.001).

The lack of response to additional tannin on the VFA or pH of the rumen fluid in the current study may be due to lambs being kept in one group at 18:00 with free access to clean water only for one day prior to slaughter. Therefore, drinkable water plus salivary contamination could dilute VFA concentration and buffered rumen pH, thus it would be worth to study the effect of tannin supplementation on rumen pH and VFA concentration using cannulated animals. Krueger *et al.* (2010) observed that an additional 14.9 g of either chestnut HT or mimosa CT to concentrate diets fed to growing steers had no effect on rumen pH, NH_3-N concentration, total VFA nor molar proportion of acetate and propionate acids. Hervas *et al.* (2003) also did not find any significant differences in rumen pH or NH_3-N concentration for cannulated sheep when they infused three levels of quebracho CT (0, 28 and 83 g kg^{-1} DM) directly to the rumen. Therefore, the effect of tannin on rumen fermentation parameter seems to be related not only to the type but also to the source of the tannin used.

4.5 Conclusion

The results obtained from experiment 2a showed that ryegrass silage treated with 30 g kg^{-1}DM chestnut HT either at ensiling or after opening the silage silos reduced *in situ* DM, CP and OM degradability parameters which perhaps due to the complexing tannin with macro molecules of the silage. In addition, a decrease of protein solubility and increase in undegradable protein and acid insoluble fraction using CNCPS method were obtained, which give an indication that feeding ruminants ryegrass silage treated with chestnut HT (30 g kg^{-1}DM) could enhance their performance. However, experimental treatments had no effect on voluntary feed intake or lamb performance, which may have related to a short experimental period (6 weeks). Also the MP supply was sufficient for the requirements of growing lambs, with no differences in MP supply between all experimental treatments.

Feeding ruminants with higher MP requirements (lactating ewes) forage silage treated with different levels of chestnut HT for longer periods (10-12 weeks) would be important, to understand whether tannin would have a negative effect on DMI, increased MP supply to the small intestine and thus enhance animal performance.

CHAPTER 5 Experiment 3: Effect of supplementary chestnut HT on ewes feed intake and performance during late pregnancy and early lactation and rumen fermentation and degradability in sheep fed lucerne silage

5.1 Introduction

The results of experiment 2 showed that supplementation with chestnut HT (30g kg^{-1}DM) either at ensiling or at feeding reduced protein degradability and increased rumen undegradable protein (experiment 2 a), but no effect on lamb performance was observed (experiment 2 b), which may have been related more to the duration of the lamb growth study (42 d) and also supplying a similar dietary MP for all treatments. A further study to investigate the effect of level of tannin inclusion on animals requiring a greater MP supply might prove a more sensitive scenario to observe the effect of tannin that on animal performance. Tabacco *et al.* (2006) found a linear reduction in protein effective rumen degradability and increase in intestinal protein digestibility (*in situ*) when they used different levels (0, 20, 40 and 60 g kg^{-1}DM) of chestnut HT as an additive to lucerne silage. In addition, Hymes-Fecht *et al.* (2013) found that feeding dairy cows different varieties of birdsfoot trefoil silages containing different levels of CT (8, 12 and 16 g kg^{-1}DM) led to an increased milk yield (33.5, 34.6 and 35.4 kg d^{-1}, respectively). Therefore, experiment 3 was designed to study the effect of supplementing different levels (0, 25, 50 and 75 g kg^{-1}DM) of chestnut HT to lucerne silage on (1) silage DM, CP and OM degradability (*in situ* and protein fractionation using the CNCPS), (2) on rumen fermentation parameters (pH, NH_3 and VFA concentrations and protozoa numbers) *in vivo*, and (3) feed intake, digestibility, growth rate, body condition score, lamb

birth weight and growth rate, blood analysis, milk yield and composition of ewes offered lucerne silage (*ad libitum*).

5.2 Material and methods

5.2.1 Silage production

Approximately 20 tons of second cut lucerne forage (Daisy, *Medicago sativa*) was mowed on 10th of July 2013 and left in the field for 48h to wilt. The silage clamp was cleaned well and sheeted with a double layer using a plastic sheet (silage sheet) one day prior to silage making. Lucerne was chopped using a forage harvester (Jaguar 870-840 forage harvester, UK) and transferred from the field into the silage clamp on the 12th of July 2013 without any additives. The clamp was filled rapidly and rolled using a tractor and then sealed with three layers of plastic sheet. Big square bales of straw were used to add weight to the clamp in order to prevent oxygen invasion, and the clamp was left for more than 100 days to ensile. On the 5th of January 2014 the silo was opened and samples from different places of the clamp taken using a silage corer and stored at -20^{o}C, for substrate proximate analysis.

After opening the silage clamp, lucerne silage was treated manually with one of four different levels of chestnut HT (0, 25, 50 or 75 g kg^{-1}DM). The chestnut tannin was commercially available (Thomas Ware and Sons Ltd. Bristol, UK), the actual amount of tannin content was 750 g kg^{-1}DM, with a mix of the following tannins: castalagin, vescalagin, castalin and vescalin, in the proportions 530, 350, 30 and 80 g kg^{-1}). The experimental treatments were used in experiments 3a, 3b and 3c

5.2.2 Experiment 3 a: Effect of supplemental chestnut HT (levels) on lucerne silage degradability.

The experiment was designed to study the effect of supplementing different levels (0, 25, 50 and 75 g kg^{-1}DM) of chestnut HT to lucerne silage (prepared in Section 5.2.1) after opening the silo on rumen *in situ* degradability and protein fractionation using the CNCPS, as a complete randomized design. Treated lucerne silage was freeze dried and milled either through either a 3 mm screen (for *in situ* method) or a 1 mm screen (for protein fractionation using the CNCPS method) as follows.

5.2.2.1 In situ degradability

Approximately 5 g of freeze dried (milled through a 3 mm screen) silage sample of each treatment was accurately weighed individually into a pre labelled, precision woven, mono filamentous nylon bag, the weight was recorded and the bags were then incubated inside the animal's rumen according to AFRC (1992) as described in Chapter 2 Section 2.12. Four mature wethers (80 kg ± 10 kg) fitted with permanent rumen cannula were used for the *in situ* experiment, where each wether received bags of all treatments for the same incubation time period. The wethers were kept as a group for feeding and offered a concentrate diet (Section 2.12). Dry matter, OM and CP degradation profiles were measured for all treatments including: the immediately soluble fraction "a", insoluble but potentially degradable fraction "b", the total potential degradable fraction "a+b", the rate of degradation fraction "c", lag time and the effective degradability at outflow rate 0.05 h^{-1} (ED5).

The calculation of dietary protein parameters were also measured according to AFRC (1993) equations as mentioned in Chapter 2 the general materials and methods Section 2.12. The parameters includes: quickly degradable protein

(QDP), slowly degradable protein (SDP), effective rumen dietary protein (ERDP), undegradable dietary protein (UDP), digestible undegradable protein (DUP) and metabolisable protein (MP).

5.2.2.2 Protein fractionation CNCPS

Approximately 0.5 g of freeze dried (milled through a 1 mm screen) of silage samples prepared in Section 5.2.1 (the experimental treatments) were accurately weighed and used for protein fractionation using the CNCPS method according to Sniffen *et al.* (1992) as described in Chapter 2, the general material and methods (Section 2.11). The measurements included the non-protein nitrogen fraction A, degradable protein fraction B which was sub-divided into: rapidly degradable protein fraction B1, intermediate degradable protein fraction B2 and slowly degradable protein fraction B3, the indigestible protein fraction C and UDP outflow rate 0.05 h^{-1}, of each silage treatment measured in triplicate.

5.2.3 Experiment 3 b: Effect of supplemented chestnut HT (different levels) to lucerne silage on rumen fermentation *in vivo*.

5.2.3.1 Experimental routine

The experiment was designed to study the effect of supplemented different levels (0, 25, 50 and 75 g kg^{-1}DM) of chestnut HT to lucerne silage (prepared in Section 5.2.1) prior to feeding on rumen fermentation parameters and protozoa numbers (*in vivo*) at different times during 12 h, as a complete randomized design. Four mature wethers (80 ± 10 kg) fitted with permanent rumen cannula were used in this study. Wethers were group housed and acclimated to the lucerne silage (prepared in Section 5.2.1) for 10 days as a basal diet at 1.1 x requirement for

maintenance (AFRC, 1993). Post adaptation period the wethers were housed individually and offered lucerne silage once every day at 09:00 h. Water and straw were available *ad libitum* and the experiment was conducted for 4 weeks. Each week wethers were offered lucerne silage manually supplemented with one of the chestnut HT levels (0, 25, 50 and 75 g kg^{-1}DM) for weeks 1, 2, 3 and 4 respectively. On the final day of each week, 200 ml of rumen fluid $wether^{-1}$ was collected manually via the rumen cannula at 6 times at: 0900, 1100, 1300, 1500, 1700 and 1900 h. The pH of the rumen fluid was measured directly as described in Chapter 2 the general materials and methods (Section 2.5). A subsample, of rumen fluid (50 ml) from each collection time was stored at -20^{o}C for subsequent NH_3-N, and VFA analysis. Additionally, a 20 ml aliquot of rumen fluid was mixed with 20 ml of formalin (18.5 % concentration of formaldehyde) and two drops of brilliant green dye (mix of 2 g of brilliant green dye and 2 ml of glacial acetic acid diluted in 100 ml with distilled water) added. This second rumen fluid sample was allowed to stain for 16 h. The fixed solutions were transferred to a refrigerated cabinet at 4^{o}C to await protozoal number determination.

5.2.3.2 Chemical analysis of the rumen fluid

Frozen rumen fluid samples were defrosted at 4^{o}C and centrifuged (15 min 3000 g at 4^{o}C) and the samples were used for NH_3-N and VFA analysis as described in Chapter 2 Sections 2.4 and 2.15 respectively.

5.2.3.3. Rumen fluid protozoa counting

Rumen protozoa numbers were determined as described by Dehority (1984). One ml of rumen fluid solution (mixed of rumen fluid with formalin and brilliant green dye) was taken and mixed well with 9 ml of 30 % glycerol solution (the dilution

became 1:20). One ml of final solution was pipetted into a Sedgewick-Rafter counting chamber (OD 02C00415 PYSER-SGI, Edenbridge, UK) by a wide-orifice pipette and the chamber transferred to a microscope and single cell protozoa were counted at a magnification of X100 using a counting grid with 0.5 mm square in the eyepiece. Protozoal counts were expressed as cells ml^{-1} of rumen fluid.

5.2.4 Experiment 3 c: Effect of supplemented chestnut HT (different levels) on feed intake and ewes' performance during late pregnancy and early lactation.

The experiment was designed to study the effect of supplementation with different levels (0, 25, 50 and 75 g kg^{-1}DM) of chestnut HT to lucerne silage (prepared in Section 5.2.1) prior to feeding on ewes' performance as a completely randomized block design. Forty single bearing (Suffolk x Mule) ewes (average liveweight: 83.9 ± 5.2 kg) were used, six weeks pre-partum. Ewes were fed lucerne silage for the first two weeks (group feeding) as an adaptation period. Four weeks pre-partum ewes were weighed and body condition scored then divided in to 10 blocks (4 ewes per block) based on their parity, liveweight and body condition score. Ewes were then housed individually in pens (2 m^2 with wood shaving bed) on the 17th of January 2014 which was the first day of the experiment.

5.2.4.1 Experimental diet formulation

A diet was formulated to meet the requirements of single bearing ewes (average body weight 83.9 kg) producing three litres of milk based on AFRC (1993) guidelines. The diet consisted of two parts: concentrate and silage (lucerne silage). The concentrate consisted of wheat, maize gluten, wheat feed, rape meal, palm kernel, molasses, distillers grains, sugar beet pulp, sunflower, hipro soya,

limestone, ewe minerals and fat (200, 150, 150, 150, 150, 70, 50, 20, 20, 10, 12.5, 12.5 and 5 g kg^{-1}), the proximate analysis of the concentrate diet is presented in Table 5.1. In the first 14 days of the experiment, the ewes were offered 300 g ewe^{-1} d^{-1} of the concentrate; then increased to 400 g ewe^{-1} d^{-1} during 15-21 days of the experiment and then increased to 500 g ewe^{-1} d^{-1} for the remaining days of the experiment.

Lucerne silage was weighed out every day and offered to ewes individually twice a day in the morning and afternoon meals (*ad libitum*) at a rate of approximately 2 kg $meal^{-1}$. Silage refusals were weighed back twice a week. Chestnut HT was mixed with the lucerne silage manually prior to feeding. Four different levels of tannin were used in this study: 0, 25, 50 and 75 g kg^{-1}DM. Each ewe within the same block received one of the treatments. Ewes had free access to clean water. Samples of each treatment (concentrate, silage and refusal) were taken (Tuesday and Friday every week for 10 weeks) and stored at -20^{o}C to await proximate analysis.

Defrosted silage samples were analysed for pH and NH_3-N as described in Chapter 2 Sections 2.5 and 2.4 respectively. Silage and concentrate samples were oven dried (60^{o} C for 48h) and milled through a 1 mm screen and analysed for DM, OM, CP, NDF, ADF and EE as described in Chapter 2 the general materials and methods Sections 2.1, 2.2, 2.3, 2.6, 2.7 and 2.8 respectively. The dietary MP for lucerne silage was calculated according to AFRC (1993) equations as described in Chapter 2 Section 2.12, while, the ME was estimated according to McDonald *et al.* (2011). The metabolisable protein and energy for concentrate diet were calculated according to the standard MP and ME of raw materials as described by

McDonald *et al.* (2011). The proximate analyses of the silage and concentrate diet are shown in Table 5.1.

Table 5.1 Proximate analysis of lucerne silage and concentrated diet offered to ewes.

Analysis	Concentrate	Lucerne silage
DM $_{(g\ kg^{-1})}$	860	482
pH		5.1
$NH_3N_{(g\ kg^{-1}TN)}$		72.1
OM $_{(g\ kg^{-1}DM)}$	920	822
CP $_{(g\ kg^{-1}DM)}$	160	195
NDF $_{(g\ kg^{-1}DM)}$	92	424
ADF $_{(g\ kg^{-1}DM)}$		231
EE $_{(g\ kg^{-1}DM)}$	48	16
ME $_{(MJ\ kg^{-1}DM)}$	10.7*	8.5*
MP $_{(g\ kg^{-1}DM)}$	130*	117

*: calculated from publish data of raw materials (McDonald *et al.,* 2011)

Silage refusal samples were oven dried, milled (1 mm screen) and analysed for DM, CP and NDF. There was no concentrate refusal during the experiment for any of the ewes.

The metabolisable protein (MP) requirements for ewes was calculated according to AFRC (1993) equations as follows:

$$MP_{requirements}\ (g\ d^{-1}) = MP_{maintenance} + MP_{growth} + MP_{wool} + MP_{milk} \quad \text{Eq. 5.1}$$

$$MP_{maintenance\ +\ wool}\ (g\ d^{-1}) = 2.1875 \times W^{0.75} + 20.4 \quad \text{Eq. 5.2}$$

$$MP_{growth}\ (g\ d^{-1}) = \Delta W \times (156.1 - 1.94W + 0.0173W^2) \quad \text{Eq.5.3}$$

$$MP_{milk}\ (g\ d^{-1}) = 74.9 \times \text{milk yield}\ (kg\ d^{-1}) \quad \text{Eq. 5.5}$$

Where MP is metabolisable protein W is liveweight (kg), ΔW change is liveweight (kg).

5.2.4.2 Measurement of liveweight change and body condition scoring

Ewes were weighed and body condition scored at 11:00 every Friday for 10 weeks using portable calf scales (IAE, Leek, Staffordshire, UK), the scale was calibrated prior to use using standard weights. The body condition score of the ewes were measured manually by the same person throughout the experiment according to MLC (1988) guidelines at the same time as ewes were weighed.

5.2.4.3 Metabolic profile

Ewes were blood sampled via jugular venepuncture at 11:30 (2.5 hours post feeding) on Monday once every two weeks, into lithium heparin vacutainer tubes (10 ml) for total protein and urea analysis and into potassium oxalate vacutainer tubes (10 ml) for glucose analysis (Bioscience Int. Plc., Bridgend, UK). Blood samples were then centrifuged at 3000 g for 15 minutes at 4^{o}C, and the plasma transferred into small tubes (2 ml) and stored at -20^{o}C for further analysis. Samples were analysed as described in Chapter 2 Section 2.13.

5.2.4.4 Measurement of diet digestibility

Diet DM, OM, CP and NDF component digestibility was measured using acid insoluble ash as described by Van Keulen and Young (1977). Approximately 50 g of fresh faecal sample was collected directly from the anus of each of the 40 ewes every day at 16:00 h for 6 days in two different periods (3 weeks' pre lambing and 5 weeks' post lambing). Faecal samples were kept in small plastic pots and directly dried at 60^{o}C for 48 h every day. Dried faecal samples (20g) for each ewe in each

6 day collection period were bulked and milled through a 1 mm screen and 5 g were used for DM digestibility in duplicate as described in Chapter 2, the general materials and methods Section 2.14.

5.2.4.5 Measurement of lamb growth

The ewes lambed within one week between 10th-16th of February 2014, the birth weight of the lambs was recorded. A colostrum sample (100 ml) was manually collected from each ewe directly after lambing and stored at -20^{o}C to await analysis. Ewes milk yield for the first 3 days of lactation was estimated according to the method described by MLC (1988) guidelines, where the lamb was weighed every 12 h post lambing for 72 h. Milk samples (100 ml) were taken 24, 48 and 72 h post lambing and stored at -20^{o}C to assess milk composition. Subsequently lambs were weighed every Tuesday at 11:00 for six weeks using portable calf scales (IAE, Leek, Staffordshire, UK) the scales were calibrated prior to use, using standard weights.

5.2.4.6 Milk yield and composition

Ewes were milked 2, 4 and 6 weeks post lambing, using a double oxytocin injection method. Ewes were injected with 1 ml of oxytocin (Oxytocin-S, solution for injection, Intervet UK Let, Walton Manor, UK) to simulate milk let down and milked using a mechanical milking machine (Bronzoni, Motori Eletirici Ramuseto- Italy). The ewes were separated from their lambs for 4 hours, then the ewes were injected with oxytocin (1 ml) and milked again. The milk yield within four hours was measured and multiplied by 6 in order to calculate 24 h milk yield. Milk samples (100 ml) for each ewe were stored at -20^{o}C for subsequent determination of milk composition. Milk and colostrum samples were defrosted in the fridge and

analysed for fat, lactose, protein, ash and total solid. Milk samples were analysed using a MilkoScan Minor (Foss UK Ltd., Warrington, UK) calibrated by the methods of AOAC (2000). Colostrum samples were dried at 60^{o}C for 48 h and milled through a 1 mm screen. Dried colostrum samples were analysed for ash (2 g) and CP(0.15 g) as described in Chapter 2 Sections 2.2 and 2.3, while for fat analysis; samples were analysed based on the Gerber method (International Dairy Federation, 1991). Defrosted colostrum samples were left to stand on the bench at room temperature for 3-5 min to allow air bubbles to rise, then 5 ml of each sample was diluted with 20 ml deionized water and mixed well. Ten ml of sulphuric acid (95 %) was placed in the butyrometer tube, to this 10.94 ml of diluted colostrum and 1 ml of amyl alcohol were added. The tube was then shaken several times until no white particles could be seen, and was then immediately centrifuged at 1100 g for 5 min. The butyrometer tubes were removed from the centrifuge and placed in a water bath at 65^{o} C for 10 min, then fat scale was read at eye level. The lactose percentage in colostrum was calculated as follows:

Lactose (g kg^{-1}) = TS -Ash -CP -fat Eq.5.6

Were TS is milk total solid, CP is milk crude protein.

5.2.5 Statistical analysis

All measured parameters were statically analysed using an ANOVA procedure of Genstat (GenStat version 15, VSN International Ltd, UK). Experiment 3 a, the non-linear *in situ* degradability for DM, CP and OM parameters were fitted with zero hour according to the equation of McDonald (1981), using curve fit SigmaPlot 12 (Systat Software Inc., London UK) for each treatment and wether, for estimating fractions “a, b and c”, lag time and ED. The *in situ* (four replication per treatments,

n=16) and protein fractionation using the CNCPS (three replication per treatment, n=12) parameters were analysed as a complete randomised design (dose response) ANOVA, results were analysed using both linear and quadratic regression.

Experiment 3 b, the rumen fermentation parameters (four replication per treatments, n=16) were analysed as a complete randomised design (dose response) ANOVA with the effect of time post feeding (repeated measurement), results were analysed using both linear and quadratic regression.

Experiment 3 c: ewes study (ten replication per treatments, n=10) were analysed as a complete randomized block design (dose response) ANOVA. In addition the time effect (repeated measurement) were studied for parameters: ewe's liveweight, body condition scoring, blood analysis measurement and lamb liveweight gain. Ewes' weight and body condition scoring plus lambs average daily liveweight gain were calculated using Microsoft Excel. Blood plasma parameters (total protein, glucose and urea) concentration for week 0 was used as a covariate for the other weeks and analysed as repeated measurements analysis. The results were analysed using both linear and quadratic regression.

5.3 Results

5.3.1 Experiment 3 a: Effect of supplemental chestnut HT (different levels) on lucerne silage degradability.

5.3.1.1 In situ degradability

The DM degradability results are presented in Table 5.2. The addition of tannin to lucerne silage was found to linearly reduce the immediately soluble fraction "a" value (P<0.05) as the level of supplemented tannin increased (Figure 5.1). In addition the 50 and 75 g kg^{-1}DM chestnut HT treatment levels were found to reduce (P<0.001) the total potential degradable fraction "a+b" (Figure 5.1), and the effective degradability at outflow rate of 0.05 h^{-1} by approximately by 6.5 and 11 % compared to the other treatments.

Table 5.2 Effect of chestnut tannin supplemented at different levels on *in situ* DM degradability.

Parameters	Supplemented tannin g kg^{-1}DM				SED	*Probability*	
	0	25	50	75		Lin.	Quad.
a (g kg^{-1}DM)	471^{a}	444^{b}	422^{c}	410^{d}	4.5	<0.001	0.04
b (g kg^{-1}DM)	374	372	358	350	17.5	0.14	0.78
a+b (g kg^{-1}DM)	845^{a}	817^{a}	780^{b}	759^{b}	16.5	<0.001	0.75
c	0.050^{a}	0.056^{a}	0.043^{ab}	0.034^{b}	0.0079	<0.001	0.75
Lag (h^{-1})	0.75	0.65	0.63	0.77	0.025	0.96	0.52
ED5 (h^{-1})	657^{a}	641^{a}	584^{b}	546^{c}	12.2	<0.01	0.24

Means with different letters within each row differ significantly (P<0.05), a: immediately soluble, b: insoluble but potentially degradable, a+b: total degradable, c rate of degradation, Lag: lag time ED5: effective degradability at outflow rate 0.05 h^{-1}, Lin: linear, Quad: quadratic.

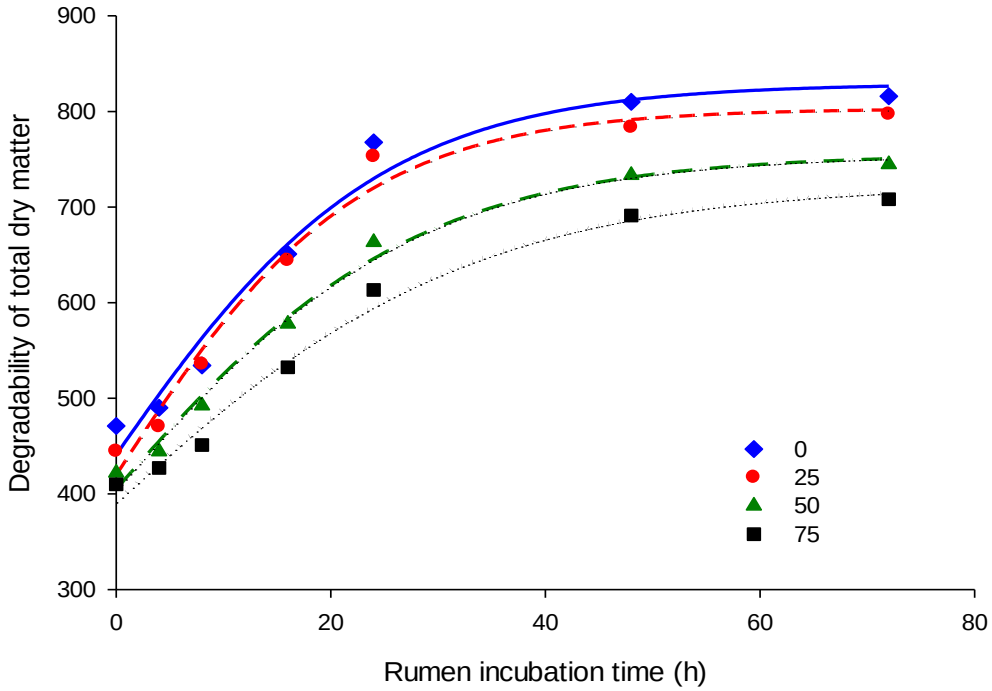

Figure 5.1 Effect of chestnut tannin supplemented at different levels on *in situ* DM degradability. 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

Increasing supplementary tannin levels (0, 25, 50 and 75 g kg^{-1} DM) was found to linearly reduce CP fraction “a”, fraction “a+b” and ED5 and increase the lag time (Figure 5.2, Table 5.3). Moreover, the addition of 50 and 75 g kg^{-1}DM HT reduced the rate of degradation CP fraction “c” compared to 0 and 25 g HT (Table 5.3). Predicted dietary protein evaluations are presented in Table 5.4. All parameters were calculated at an outflow rate of 0.05 h^{-1}. Supplemented tannin levels reduced the quickly degradable protein (QDP), slowly degradable protein (SDP) and effective rumen degradable protein (ERDP). Increasing tannin level was found to increase the undegradable dietary protein (UDP) and digestible undegradable dietary protein (DUP).

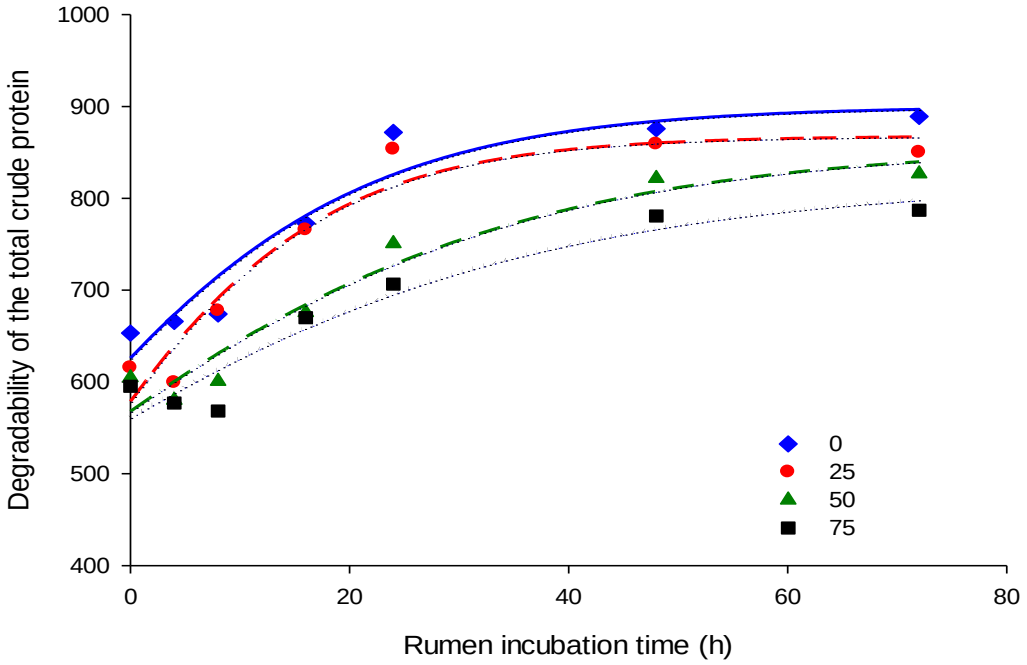

Figure 5.2 Effect of chestnut tannin supplemented at different levels on *in situ* CP degradability. 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

Table 5.3 Effect of chestnut tannin supplemented at different levels on *in situ* CP degradability.

Parameters	Supplemented tannin g kg^{-1}DM				SED	*Probability*	
	0	25	50	75		Lin.	Quad.
a (g kg^{-1}DM)	653^{a}	615^{b}	605^{bc}	595^{c}	9.3	<0.001	0.05
b (g kg^{-1}DM)	255	262	270	240	10.7	0.29	0.31
a+b (g kg^{-1}DM)	908^{a}	877^{b}	875^{b}	835^{c}	15.2	<0.001	0.66
c	0.052^{a}	0.051^{a}	0.035^{b}	0.025^{b}	0.0083	0.002	0.05
Lag (h^{-1})	0.92^{a}	0.11^{ab}	1.64^{b}	2.37^{c}	0.484	0.008	0.42
ED5 (h^{-1})	780^{a}	763^{b}	715^{c}	670^{d}	6.1	<0.001	0.25

Means with different letters within each row differ significantly (P<0.05), a: immediately soluble, b: insoluble but potentially degradable, a+b: total degradable, c rate of degradation, Lag: lag time, ED5: effective degradability at outflow rate 0.05 h^{-1}, Lin: linear, Quad: quadratic.

Table 5.4 Effect of chestnut tannin supplemented at different levels on dietary protein parameters of lucerne silage at rumen outflow rate 0.05 h^{-1}.

Parameters	Supplemented tannin g kg^{-1}DM				SED	*Probability*	
	0	25	50	75		Lin.	Quad.
QDP5$_{(g\ kg^{-1}DM)}$	128^{a}	121^{b}	119^{bc}	117^{c}	1.8	<0.001	0.05
SDP5 $_{(g\ kg^{-1}DM)}$	28^{a}	25^{b}	22^{c}	15^{d}	1.3	<0.001	0.07
ERDP5 $_{(g\ kg^{-1}DM)}$	151^{a}	140^{b}	136^{b}	129^{c}	2.4	<0.001	0.35
UDP5 $_{(g\ kg^{-1}DM)}$	40^{s}	51^{b}	56^{b}	64^{c}	2.6	<0.001	0.47
DUP5 $_{(g\ kg^{-1}DM)}$	28^{a}	37^{b}	40^{b}	45^{c}	2.3	<0.001	0.34

Means with different litters within each row differ significantly (P<0.05), QDP: quickly degradable protein, SDP: slowly degradable protein, ERDP: effective rumen degradable protein, UDP: undegradable protein, DUP: digestible undegradable dietary protein, Lin: linear, Quad: quadratic.

Addition of HT at 50 and 75 g kg^{-1}DM was found to reduce (P<0.05) OM fraction “a”, fraction “b”, fraction “a+b” and fraction “c” but had no effect on lag time as shown in Table 5.5 and Figure 5.3. Effective Degradability (ED5) was significantly reduced by the higher levels of tannin addition (Table 5.5).

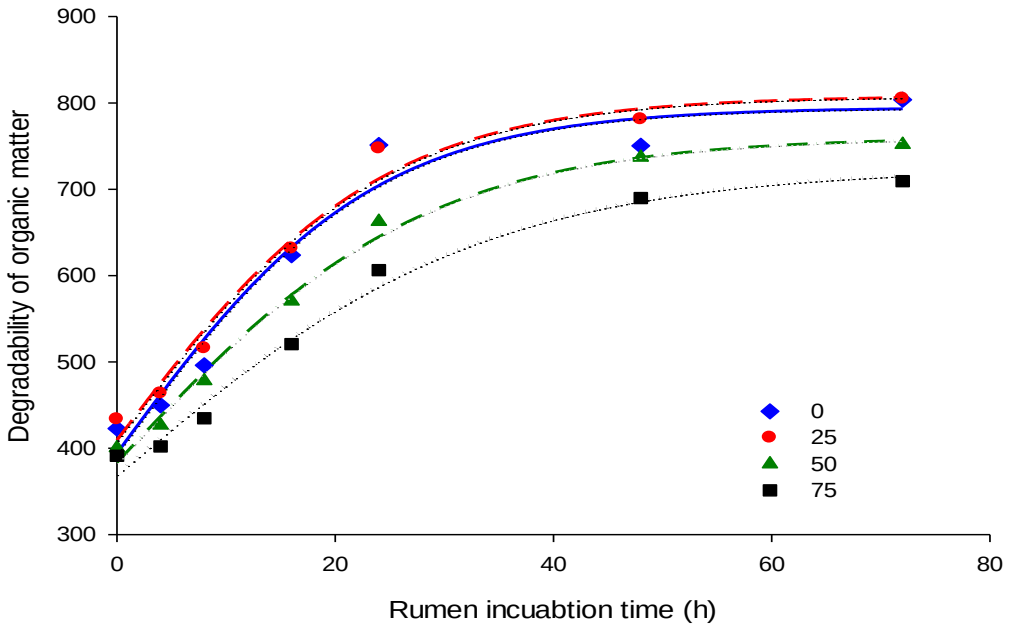

Figure 5.3 Effect of chestnut tannin supplemented at different levels on *in situ* OM degradability. 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

Table 5.5 Effect of chestnut tannin supplemented at different levels on *In situ* OM degradability.

Parameters	Supplemented tannin g kg^{-1}DM				SED	*Probability*	
	0	25	50	75		Lin.	Quad.
a ($g\ kg^{-1}DM$)	423^{a}	433^{a}	400^{b}	391^{b}	5.1	<0.001	0.02
b ($g\ kg^{-1}DM$)	404^{a}	388^{b}	389^{b}	363^{c}	12.1	0.008	0.62
a+b ($g\ kg^{-1}DM$)	827^{a}	821^{a}	789^{b}	755^{b}	12.9	<0.001	0.15
c	0.051^{a}	0.052^{a}	0.042^{ab}	0.031^{b}	0.0075	0.01	0.32
Lag (h^{-1})	0.54	0.64	0.67	0.82	0.186	0.61	0.27
ED5 (h^{-1})	578^{a}	585^{a}	533^{b}	495^{c}	11.8	<0.001	0.02

Means with different letters within each row differ significantly (*P*<0.05), a: immediately soluble, b: insoluble but potentially degradable, a+b: total degradable, c rate of degradation, Lag: lag time, ED5: effective degradability at outflow rate 0.05, Lin: linear, Quad: quadratic.

5.3.1.2 Protein degradability using the CNCPS

The addition of tannin (25, 50, 75 g kg^{-1} DM) was found to reduce (*P*<0.05) non protein nitrogen (fraction A) by approximately 18, 19 and 22 % respectively, while the buffer soluble N (fraction B1) increased by about 46, 52 and 62 % respectively. High tannin level (75 g kg^{-1}DM) was found to reduce (*P*<0.05) the neutral detergent soluble N fraction (B2), and the addition of 25 g kg^{-1}DM had the highest acid detergent soluble fraction (B3) compared to the other treatments (Table 5.6). Increasing the level of supplemented tannin was found to linearly increase acid detergent insoluble protein fraction C (Figure 5.4) with no effect on RUP at an outflow rate of 0.05 h^{-1} (111, 118, 125 and 126 g kg^{-1} CP for 0, 25, 50 and 75 g kg^{-1} DM, respectively).

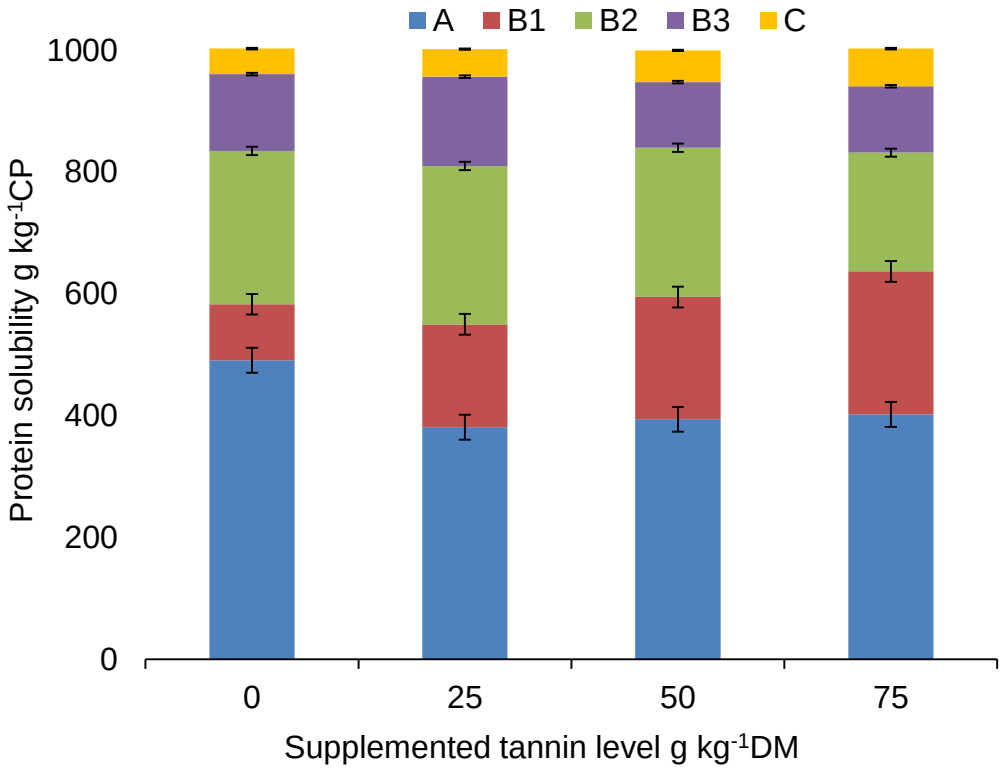

Figure 5.4 Effect of chestnut tannin supplemented at different levels of chestnut tannin on protein fractionation using the CNCPS (g kg^{-1}CP). A is non protein nitrogen, B1: buffer soluble protein, B2: neutral soluble protein, B3: acid soluble protein and C: acid insoluble protein.

Table 5.6 Effect of chestnut tannin supplemented at different levels on protein fractionation using the CNCPS.

Parameters	Supplemented tannin g kg^{-1}DM				SED	*Probability*	
	0	25	50	75		Lin.	Quad.
A (g kg^{-1}CP)	487^{b}	381^{a}	394^{a}	392^{a}	20.4	0.002	0.002
B1 (g kg^{-1}CP)	94^{c}	169^{b}	201^{b}	243^{a}	17.0	<0.001	0.150
B2 (g kg^{-1}CP)	252^{a}	260^{a}	245^{a}	195^{b}	7.8	<0.001	0.005
B3 (g kg^{-1}CP)	126^{b}	147^{a}	108^{c}	109^{c}	2.2	<0.001	0.005
C (g kg^{-1}CP)	42^{d}	45^{c}	52^{b}	62^{a}	1.2	<0.001	0.004
UDP5 (g kg^{-1}CP)	111	118	125	125	6.2	0.151	0.460

Means with different letters within each row differ significantly (P<0.05), A: non protein nitrogen, B1 rapidly degradable protein, B2 moderate degradable protein, B3 slowly degradable protein, C: un-digestible protein, UDP5: undegradable dietary protein at outflow rate 0.05, Lin: linear, Quad: quadratic.

5.3.2 Experiment 3 b: Effect of supplemental chestnut HT (different levels) to lucerne silage on rumen fermentation *in vivo*

5.3.2.1 Ammonia concentration

Rumen ammonia concentration was found to reduce linearly (P<0.001) as supplemented tannin level increased (0.33, 0.29, 0.23 and 0.2 g L^{-1} for 0, 25, 50 and 75 g kg^{-1}DM added chestnut HT respectively). Peak rumen ammonia concentration was observed 4 h post feeding (Figure 5.5)

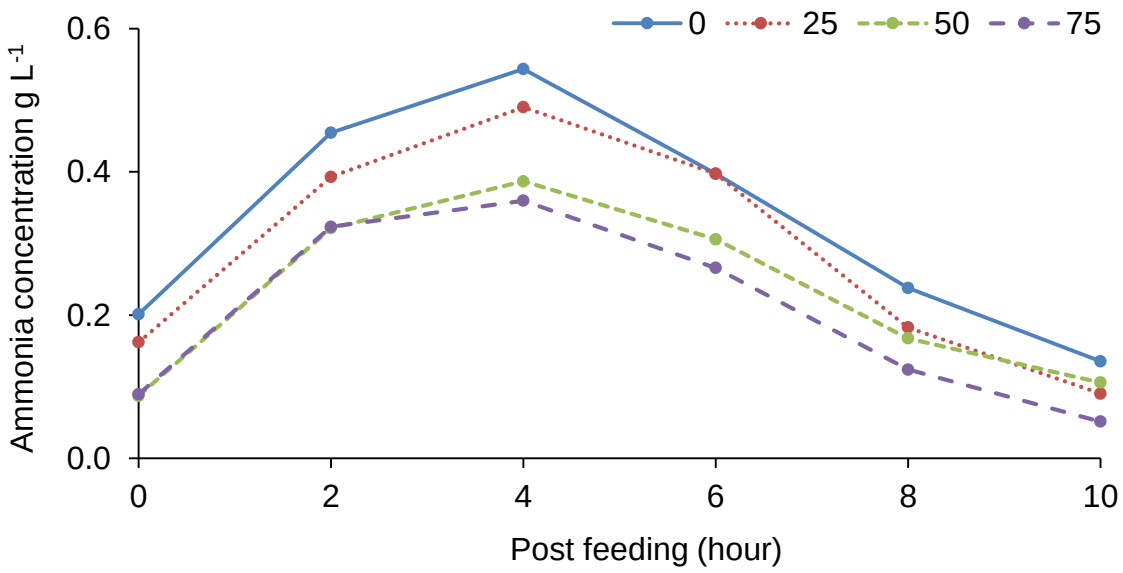

Figure 5.5 Effect of chestnut tannin supplemented at different levels on rumen ammonia concentration (treatment= 4, P-values: level=<0.001, time=<0.001, , SED values: level=0.021, time=0.012), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

5.3.2.2 Assessment of rumen protozoa population density

Rumen protozoa counts were found to linearly reduce with increasing level of tannin inclusion (P<0.05). Mean protozoa numbers reduced by approximately 23.3, 33.8 and 63.4 % for 25, 50 and 75 g kg^{-1} DM HT compared to the control

group. Maximal protozoa numbers were observed 2h post feeding for all treatments (Figure 5.6)

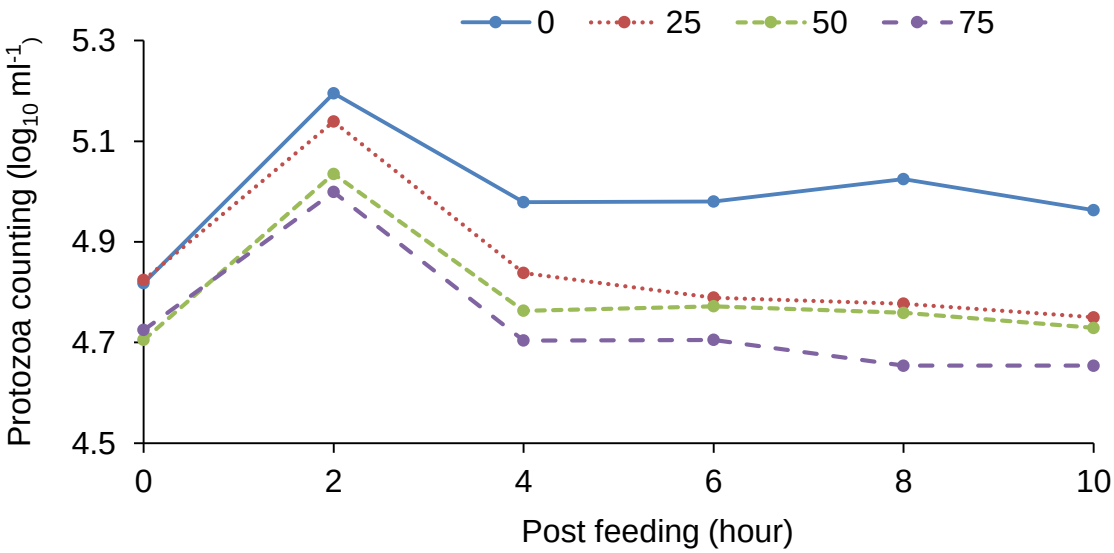

Figure 5.6 Effect of chestnut tannin supplemented at different levels on total protozoa number (log_{10}) in the rumen fluid. (treatment= 4, *P*-values: level=0.03, time=<0.001, SED values: level=0.075, time=0.035), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

5.3.2.3 Rumen pH

Dietary supplementation with tannin increased (P<0.05) rumen pH before the morning meal (6.75, 7.01, 719 and 7.13 for 0, 25, 50 and 75 g kg^{-1}DM respectively), but, there was no significant differences between treatments at 2, 4, 6 and 8 hours post feeding (Figure 5.7).

5.3.2.4 Rumen VFA

Tannin supplementation had no effect on the total molar VFA concentration (Figure 5.8). Individual VFA were not affected by the inclusion of dietary tannin with the exception of butyrate and isovalerate which were linearly reduced with increasing level of tannin (*P*<0.05) as shown in Table 5.7.

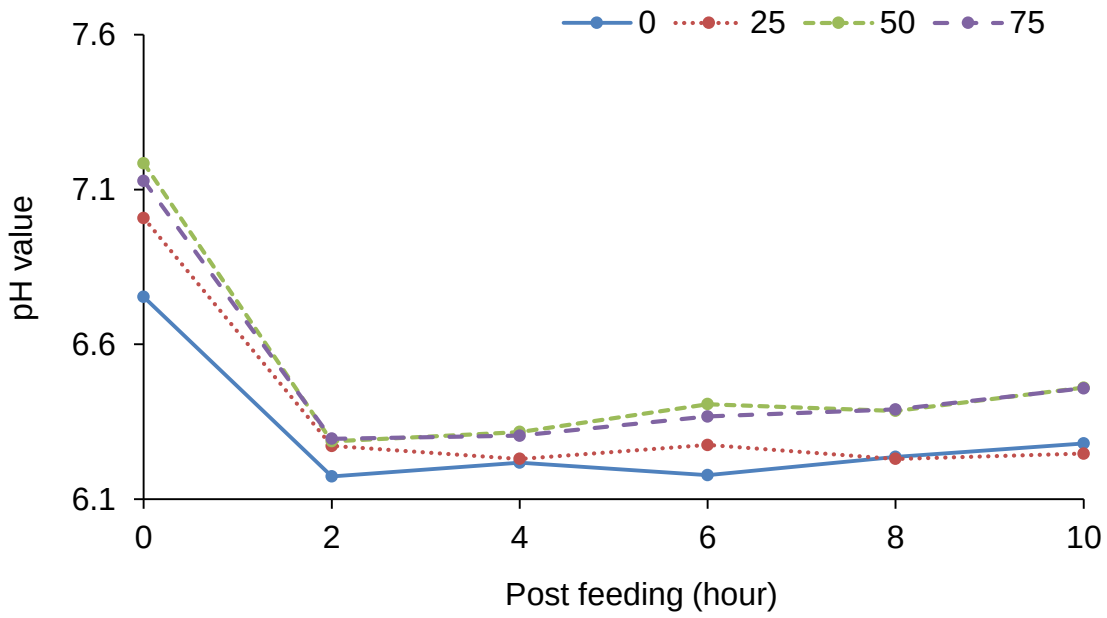

Figure 5.7 Effect of chestnut tannin supplemented at different levels on rumen fluid pH (treatment= 4, *P*-values: level=0.03, time=<0.001, SED values: level=0.061, time=0.041). 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

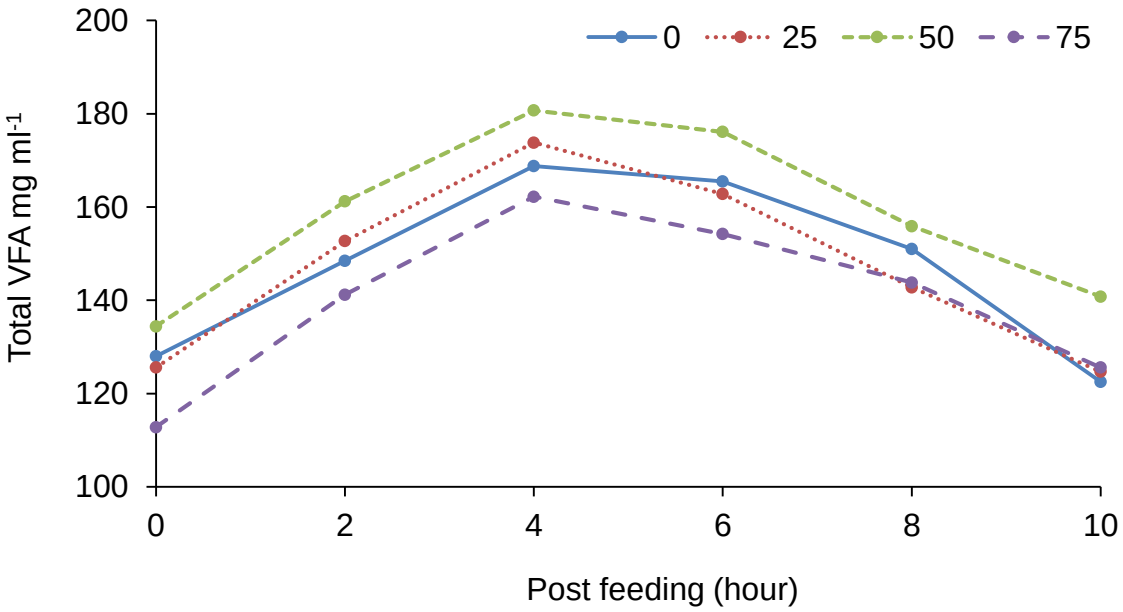

Figure 5.8 Effect of chestnut tannin supplemented at different levels on molar concentration of VFA total. (treatment= 4, *P*-values: level=0.45, time=<0.001, SED values: level=13.5, time=10.4). 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

Table 5.7 Effect of chestnut tannin supplemented at different levels on molar concentration of rumen total and individual volatile fatty acids

	Supplemented tannin g kg^{-1}DM				SED			*Probability*		
	0	25	50	75	Tannin	Time	Tan. x T.	Tannin	Time	Tan. x T.
tVFA (mole L^{-1})	140.7	143	163.2	141.8	13.49	10.43	23.33	0.33	0.004	0.74
Acetic (mole L^{-1})	107.7	110.2	118.5	102.8	6.7	3.45	9.2	0.18	<0.001	0.54
Propionic (mole L^{-1})	26.5	23.4	28.9	23.6	2.45	1.35	3.48	0.13	<0.001	0.22
Isobutyric (mole L^{-1})	2.5	3.2	3.1	2.5	0.61	0.37	0.91	0.35	0.03	0.12
Butyric (mole L^{-1})	11.1	7.4	8.9	6.9	1.08	0.81	1.83	0.01	<0.001	0.85
Isovaleric (mole L^{-1})	3.6	3.1	2.3	2.2	0.42	0.58	1.15	0.01	0.01	0.09
Valeric (mole L^{-1})	1.9	1.7	1.7	2.1	0.25	0.21	0.46	0.37	<0.001	0.18
Caproate (mole L^{-1})	1.1	1.1	1.0	1.0	0.11	0.1	0.22	0.81	<0.001	0.12
Ace: Prop	2.9	3.3	3.1	3.1	0.17	0.14	0.31	0.23	<0.001	0.15
Ace + Prop: But	4.0	4.4	3.9	4.1	0.22	0.16	0.37	0.14	<0.001	0.11

Ace: prop: acetate propionate ratio, Ace + Prop: But: acetate + propionate, butyrate ratio. Tan x T: interaction between tannin levels and time.

5.3.3 Experiment 3 c: Effect of supplemented chestnut HT (different levels) on feed intake and ewe performance during late pregnancy and early lactation

5.3.3.1 Dry matter intake

The average DMI (forage and total) was 2.06 and 2.44 kg d^{-1} respectively. The addition of chestnut HT to lucerne silage had no effect on forage or total DMI at any level of inclusion (Table 5.8). In addition, tannin supplementation had no effect (P>0.05) on crude protein intake (CPI).

Table 5.8 Effect of chestnut tannin supplemented at different levels on dry matter intake and ewes performance.

	Supplemented tannin g kg^{-1}DM				SED	*Probability*	
	0	25	50	75		Lin	Quad
F-DMI ($kg\ d^{-1}$)	2.02	2.10	2.01	2.13	0.092	0.47	0.42
T-DMI ($kg\ d^{-1}$)	2.41	2.49	2.40	2.51	0.092	0.47	0.42
F-CPI ($kg\ d^{-1}$)	0.39	0.41	0.39	0.41	0.017	0.49	0.42
T-CPI ($kg\ d^{-1}$)	0.45	0.47	0.45	0.47	0.017	0.49	0.42
Pre-partum							
LW change ($kg\ d^{-1}$)	0.35	0.27	0.24	0.37	0.082	0.29	0.07
CS changes	-0.07	0.10	-0.03	-0.11	0.177	0.62	0.28
Post-partum							
LW changes ($kg\ d^{-1}$)	-0.26	-0.23	-0.22	-0.22	0.050	0.61	0.74
CS changes	-0.08	-0.09	-0.12	-0.10	0.157	0.89	0.91

F-DMI: forage dry matter intake, T-DMI: total dry matter intake, F-CPI: forage crude protein intake, T-CPI: total crude protein intake, LW: live weight, CS: body condition score (body condition unit). Lin: linear, Quad: quadratic.

5.3.3.2 Measurement of ewe liveweight change and body condition score

The average weight and body condition score of ewes at the beginning of the experiment was 83.90 kg and 3.5 respectively. Average body weight increased on average to 91.26 kg pre partum with no significant difference observed between treatments. Twelve hours post lambing the ewes' weight was reduced on average by 9.8 kg compared to the previous weighing. During the lactation period the body weight reduced slightly in all treatments with no significant effect of treatments, as shown in Figure 5.9.

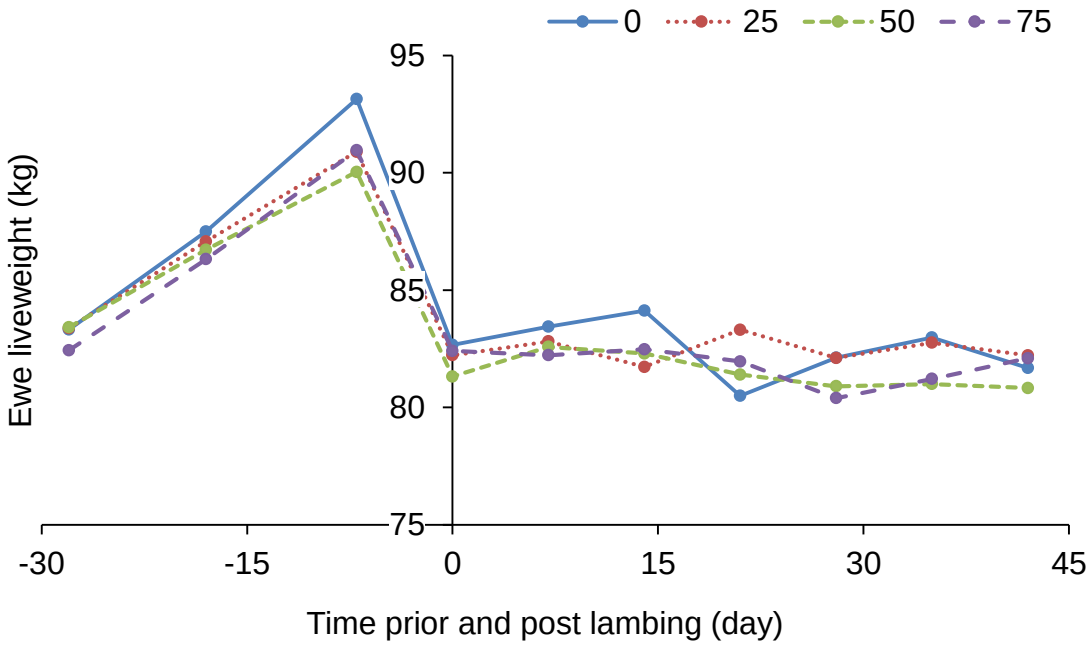

Figure 5.9 Effect of chestnut tannin supplemented at different levels on ewe liveweight change in late pregnancy and early lactation period (treatment= 10, *P*-values: level=0.91 time=<0.001, SED values: level=2.39, time=0.75), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

Body condition score was found to reduce throughout the duration of the experiment, but there was no effect (*P*>0.05) of treatment on body condition score as shown in Figure 5.10 and Table 5.8.

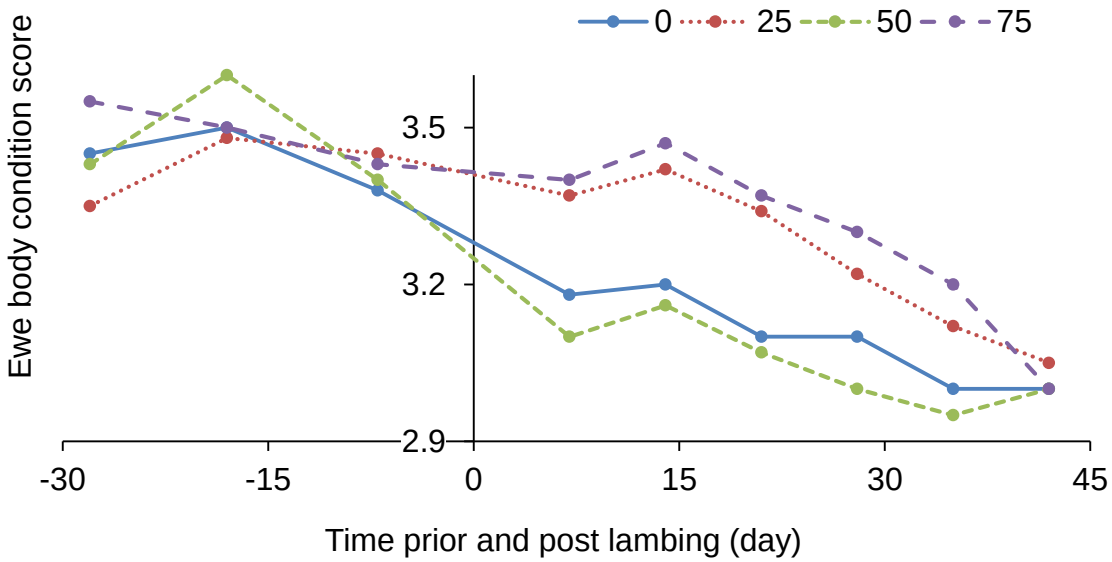

Figure 5.10 Effect of chestnut tannin supplemented at different levels on ewe body condition scoring changes in late pregnancy and early lactation period (treatment= 10, *P*-values: level=0.47 time=<0.001, SED values: level=0.15, time=0.06), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

5.3.3.3 Metabolic profile

Chestnut HT supplemented at different levels (0, 25, 50 and 75 g kg^{-1}DM) to lucerne silage had no effect on mean blood plasma concentration for total protein, urea and glucose (Table 5.9), with no interaction between time and supplemented tannin levels.

Table 5.9 Effect of chestnut tannin supplemented at different levels on blood plasma parameters of ewes' pre and post-partum.

	Supplemented tannin g kg^{-1}DM				SED			*Probability*		
Trait	0	25	50	75	Tannin	Time	Inter	Tannin	Time	Inter
TP (mg ml^{-1})	56.9	57.7	56.9	56.6	0.75	1.01	1.67	0.72	<0.001	0.36
Ur (mmol L^{-1})	7.9	7.8	7.8	7.9	0.28	0.27	0.57	0.88	0.06	0.44
Gl (mmol L^{-1})	3.8	3.7	3.8	3.7	0.09	0.07	0.18	0.35	0.05	0.31

TP: total protein, Ur: urea, Gl: glucose, Inter: interaction between tannin and time.

Average blood plasma total protein was 58.8 mg ml^{-1} across all treatment at the beginning of the experiment and slightly reduced during the experiment to 55.6 mg ml^{-1} at 6 weeks post-partum (Figure 5.11).

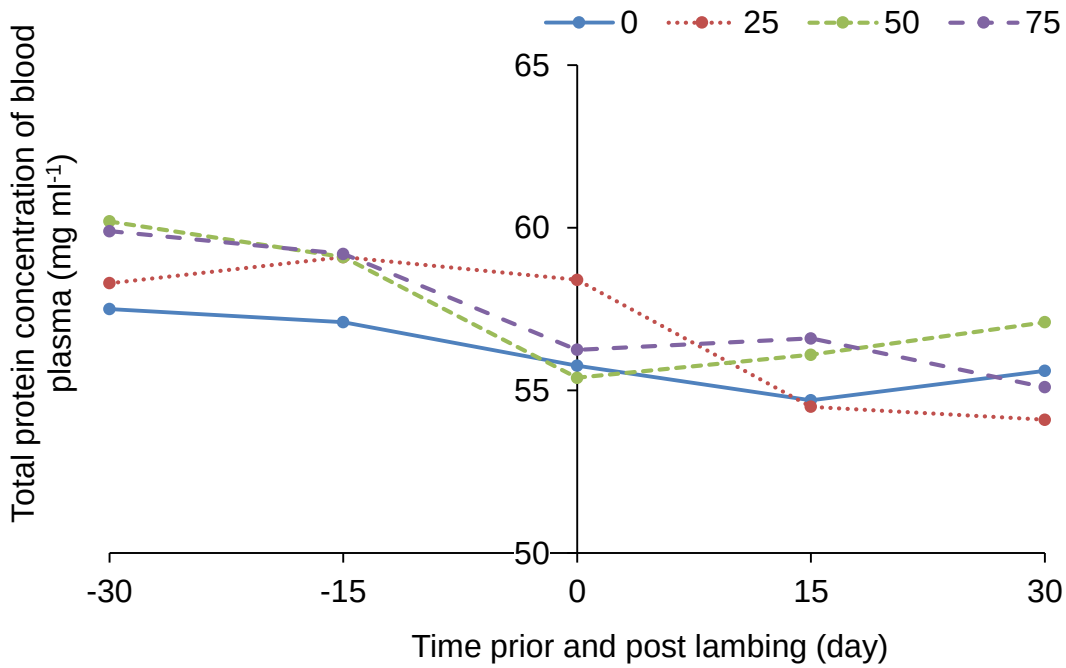

Figure 5.11 Effect of chestnut tannin supplemented at different levels on ewe blood plasma total protein concentration in late pregnancy and early lactation period (treatment= 10, *P*-values: level=0.72, time=<0.001, SED values: level=1.01, time=0.76), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

Minimum blood plasma urea concentration was observed at lambing. Treated lucerne silage with different levels of chestnut HT did not have any effect (*P*>0.05) on blood plasma urea concentrations during the experimental period as shown in Figure 5.12.

Mean blood glucose concentration across treatments was found to increase during the experimental period from 3.6 mmol L^{-1} during the first week of the experiment to reach 3.9 mmol L^{-1} by week 10. Dietary addition of tannin did not affect blood plasma glucose concentration as shown in Figure 5.13.

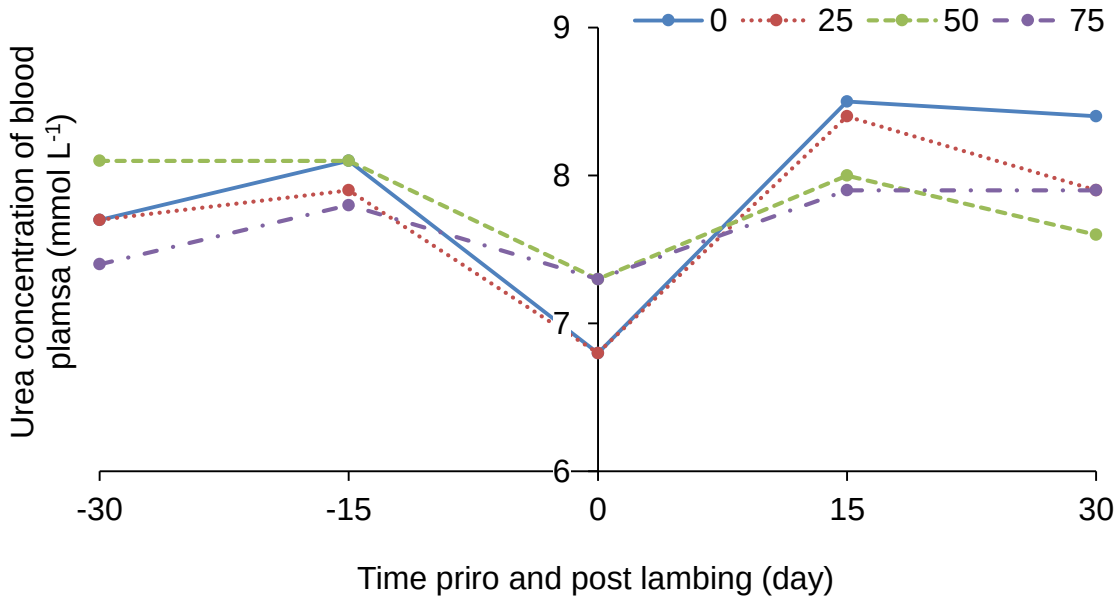

Figure 5.12 Effect of chestnut tannin supplemented at different levels on ewe blood plasma urea concentration in late pregnancy and early lactation period (treatment= 10, *P*-values: level=0.88, time=0.06, SED values: level=0.27, time=0.28), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

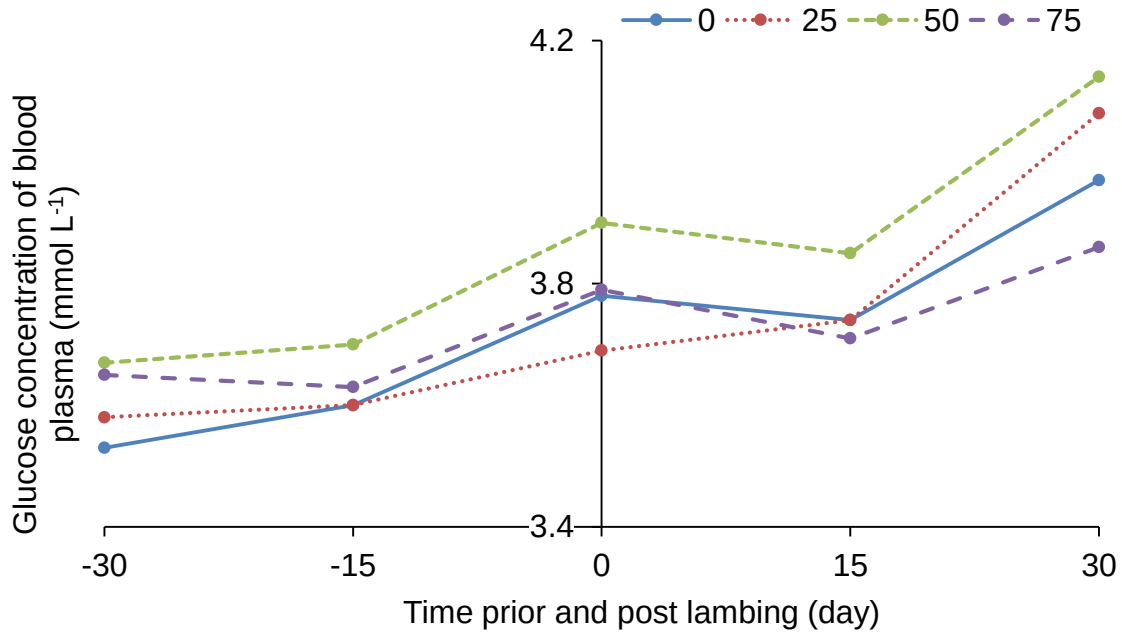

Figure 5.13 Effect of chestnut tannin supplemented at different levels on ewe blood plasma glucose concentration in late pregnancy and early lactation period (treatment= 10, *P*-values: level=0.35, time=0.05, SED values: level=0.07, time=0.09), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

5.3.3.4 Diet digestibility.

Diet digestibility results are shown in Table 5.10. Supplementation with chestnut HT had no effect on DM, OM and NDF digestibility pre lambing. However, chestnut HT supplementation at 75 g kg^{-1}DM significantly reduced (*P*=0.03) CP digestibility pre lambing. In addition, chestnut HT supplementation at 75 g kg^{-1}DM was found to reduce (*P*<0.05) OM, CP and NDF digestibility post lambing. Supplementation with HT at 50 and 75 g kg^{-1} DM was found to increase (*P*<0.01) faecal nitrogen concentration either pre or post-partum. Tannin treatment was not found to affect faecal OM or NDF content (Table 5.10).

5.3.3.5 Milk yield and composition

The average milk yield across all treatments was 2.17 2.75, 2.8 and 2.5 L d^{-1} in weeks 0, 2, 4 and 6 post lambing respectively. Level of tannin inclusion had no effect (*P*>0.05) on colostrum yield or composition (Table 5.11). A significant effect of tannin treatment on milk yield was observed. Mean 24 h milk yield values for the 0, 25, 50 and 75 g kg^{-1}DM HT treatments were 2.2, 2.3, 2.2 and 2.1 L d^{-1} respectively. Addition of 25 g kg^{-1}DM HT was found to have the highest peak yield as shown in Figure 5.14, and the addition of HT at 50 g kg^{-1}DM was also found to have a greater yield than the 75 g kg^{-1}DM treatment.

Supplementation with HT at 75 g kg^{-1} DM significantly reduced the daily milk total solids yield by 16 and 11 % compared to the 25 and 50 g kg^{-1}DM treatments respectively, as shown in Table 5.10. Tannin level had no effect on colostrum and milk composition, however supplementation of 75 g kg^{-1}DM

chestnut HT significantly reduced milk component yield including total solids, fat, protein and lactose (Table 5.11).

Table 5.10 Effect of chestnut tannin supplemented at different levels on diet digestibility and faecal analysis of ewes in late pregnancy and early lactation period.

	Supplemented tannin g kg^{-1}DM				SED	*Probability*	
	0	25	50	75		Lin	Quad
Diet digestibility ($kg\ kg^{-1}$)							
Pre-partum							
DM	0.56	0.55	0.54	0.57	0.072	0.81	0.62
OM	0.79	0.79	0.79	0.75	0.016	0.51	0.08
CP	0.79^{a}	0.78^{a}	0.76^{a}	0.73^{b}	0.017	0.02	0.03
NDF	0.75	0.76	0.73	0.76	0.019	0.95	0.31
Post-partum							
DM	0.61	0.63	0.66	0.63	0.054	0.27	0.07
OM	0.77^{a}	0.77^{a}	0.76^{a}	0.71^{b}	0.023	0.31	0.05
CP	0.76^{a}	0.75^{a}	0.73^{a}	0.68^{b}	0.023	0.05	0.04
NDF	0.73^{a}	0.74^{a}	0.74^{a}	0.66^{b}	0.027	0.65	0.05
Faecal analysis ($g\ kg^{-1}DM$)							
Pre-partum							
OM	809	811	812	815	5.8	0.15	0.54
CP	181^{a}	187^{a}	203^{b}	205^{b}	4.1	<0.001	0.58
NDF	450	443	442	444	9.8	0.53	0.78
Post-partum							
OM	817	822	821	824	5.9	0.14	0.61
CP	190^{a}	197^{ab}	202^{bc}	209^{c}	3.9	<0.001	0.94
NDF	443	435	441	437	7.1	0.65	0.28

Means with different letters within each row differ significantly (P<0.05), DM: dry matter, OM: organic matter, CP: crude protein, NDF: neutral detergent fibre. Means with different litters within each row are differ significantly (P<0.05), Lin: linear, Quad: quadratic.

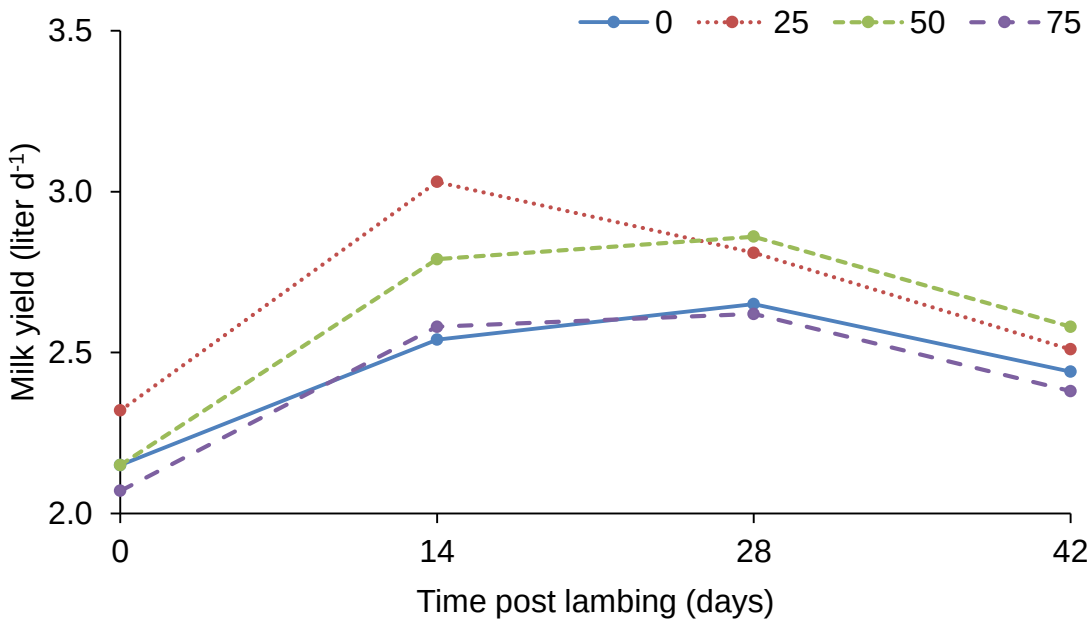

Figure 5.14 Effect of chestnut tannin supplemented at different levels on ewe milk yield at 0, 14, 28 and 42 days post lambing (treatment= 10, *P*-values: level=0.01, time=<0.01,SED values: level=0.09, time=0.08), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

Maximum daily milk total solid yield was observed 14 days post lambing. Ewes fed lucerne silage supplemented with 25 or 50 g kg^{-1}DM chestnut HT had the highest milk total solid yield compared with the 0 or 75 g HT treatments (Table 5.10 and Figure 5.15). Milk fat yield was found to reduce significantly in ewes offered the lucerne silage treated with 75 g kg^{-1}DM compared to the other treatment (Figure 5.16).

Table 5.11 Effect of chestnut tannin supplemented at different levels to lucerne silage on means of milk yield, composition and component of ewes during lactation period.

	Supplemented tannin g kg^{-1}DM				SED	*Probability*	
	0	25	50	75		Lin	Quad
Milk yield ($L\ d^{-1}$)							
Lambing	2.2	2.3	2.2	2.2	0.22	0.41	0.54
Day 14	2.5^{b}	3.0^{a}	2.8^{ab}	2.8^{ab}	0.16	0.82	0.05
Day 28	2.7	2.8	2.9	2.8	0.15	0.19	0.01
Day 42	2.4	2.5	2.6	2.3	0.12	0.76	0.12
Mean	2.5^{bc}	2.7^{a}	2.6^{ab}	2.4^{c}	0.09	0.29	0.02
Colostrum composition ($g\ kg^{-1}$)							
TS	337	313	337	323	33.5	0.66	0.64
Fat	115	105	103	108	22.9	0.71	0.65
Lactose	61	53	54	52	7.3	0.15	0.12
Protein	150	145	171	154	19.1	0.53	0.67
Milk composition ($g\ kg^{-1}$)							
TS	202	212	206	201	6.0	0.58	0.08
Fat	73	76	75	71	3.9	0.51	0.23
Lactose	48^{c}	53^{a}	50^{b}	49^{bc}	1.4	0.93	0.02
Protein	49	49	49	50	0.8	0.47	0.27
Milk yield ($g\ d^{-1}$)							
TS	490^{bc}	559^{a}	530^{ab}	471^{c}	24.0	0.27	<0.001
Fat	176^{ab}	199^{a}	192^{a}	165^{b}	11.8	0.31	0.005
Lactose	121^{bc}	130^{a}	128^{ab}	118^{c}	4.4	0.50	0.004
Protein	117^{b}	139^{a}	126^{b}	115^{b}	6.1	0.34	<0.001

Means with different letters within each row differ significantly (P<0.05), TS: total solid, means not sharing a common superscript in the same row differ significantly (P<0.05).

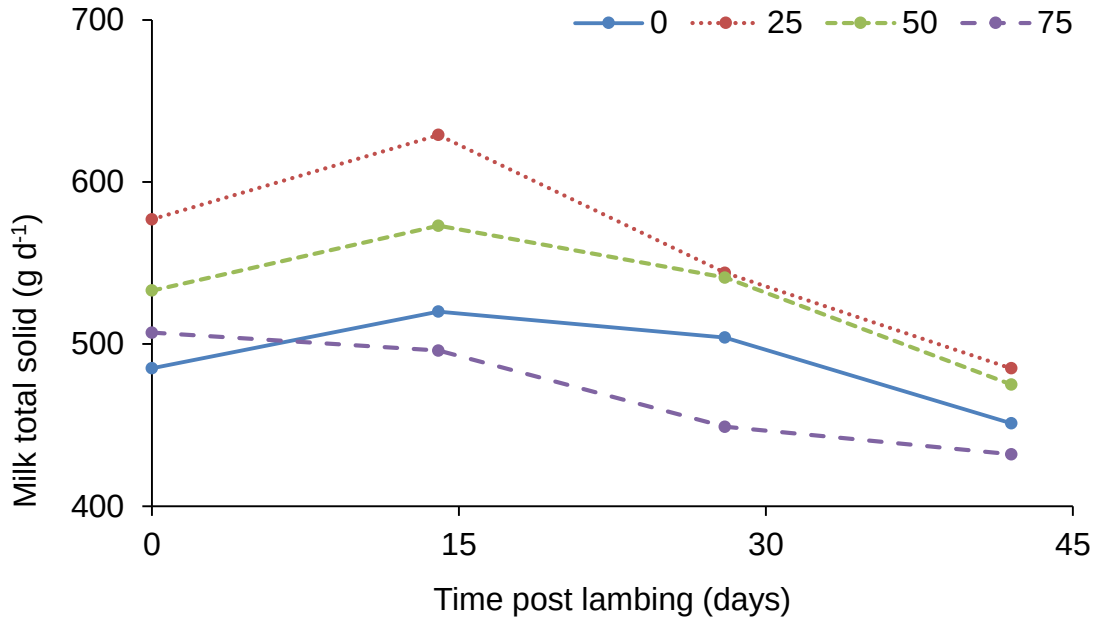

Figure 5.15 Effect of chestnut tannin supplemented at different levels on ewe milk total solid yield at 0, 14, 28 and 42 days post lambing (treatment= 10, *P*-values: level=0.04, time=0.001, SED values: level=24.1, time=20.6), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

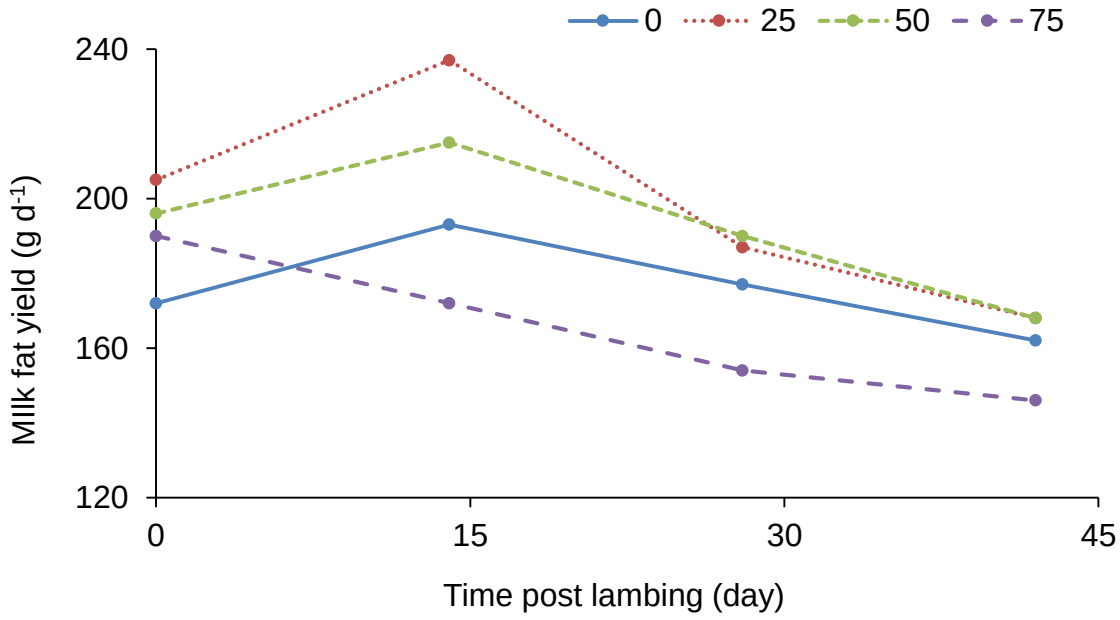

Figure 5.16 Effect of chestnut tannin supplemented at different levels on ewe milk fat yield at 0, 14, 28 and 42 days post lambing (treatment= 10, *P*-values: level=0.03, time=0.002, SED values: level=11.9, time=10.0), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

Figure 5.17 shows that milk lactose yield was in highest when ewes offered lucerne silage supplemented with 25 g kg^{-1}DM compared with the other treatments. Treating lucerne silage with 75 g kg^{-1}DM chestnut HT significantly reduced milk protein yield compared with the other treatments (Figure 5.18)

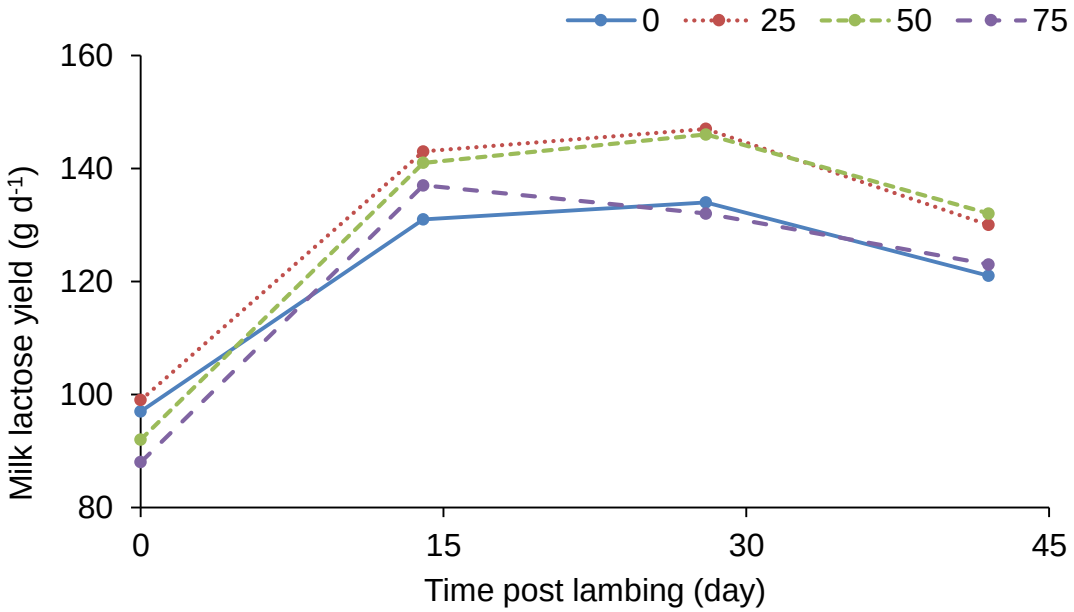

Figure 5.17 Effect of chestnut tannin supplemented at different levels on ewe milk lactose yield at 0, 14, 28 and 42 days post lambing (treatment= 10, *P*-values: level=0.05, time=<0.001, SED values: level=4.5, time=3.8), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

5.3.3.6 Lamb birth weight and liveweight

Dietary supplementation with HT at any level had no effect on lamb birth weight (P>0.05).The average lamb birth weight for all treatments was 6.24 kg. Lamb average daily liveweight gain (ADG) from birth until 6 weeks of age was 0.40, 0.39, 0.41 and 0.37 kg d^{-1} for 0, 25, 50 and 75 g kg^{-1}DM of supplemented tannin, respectively. Treatment had no effect on average liveweight (*P*= 0.355) during the experimental period, as shown in Figure 5.19.

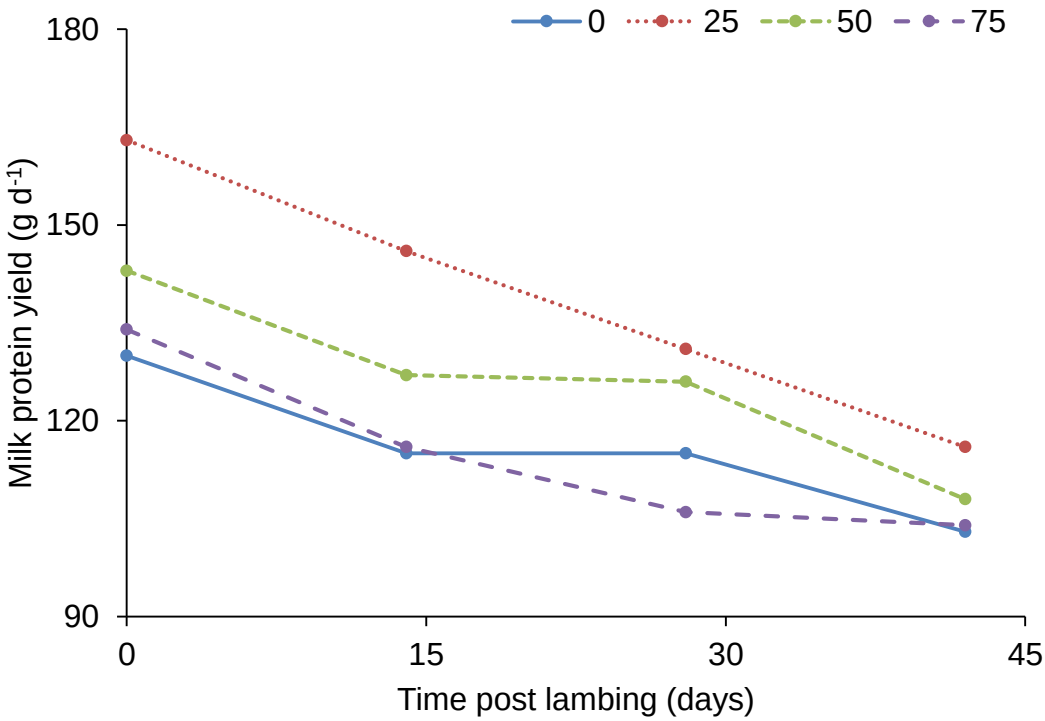

Figure 5.18 Effect of chestnut tannin supplemented at different levels on ewes' milk protein yield at 0, 14, 28 and 42 days post lambing (n treatment= 10, *P*-values: level=0.001, time=<0.001, SED values: level=6.1, time=4.8), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

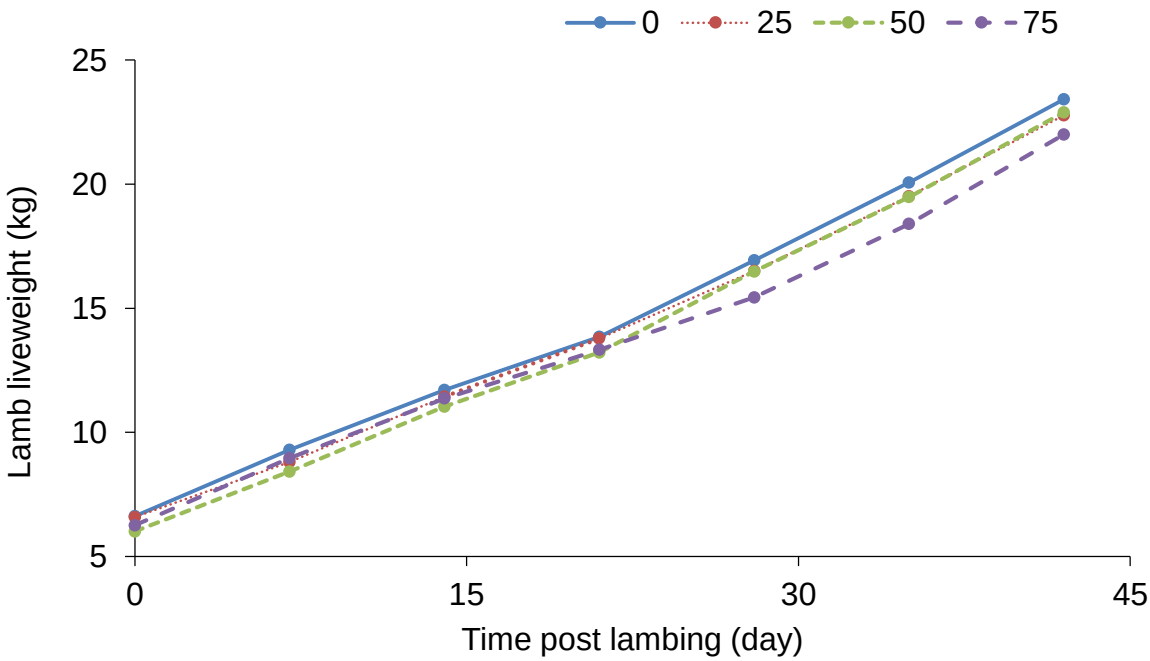

Figure 5.19 Effect of chestnut tannin supplemented at different levels on lamb liveweight change (treatment= 10, *P*-values: level=0.62, time=<0.001, SED values: level=0.67, time=0.32), 0, 25, 50 or 75 are the levels of supplemented tannin (g kg^{-1}DM) to lucerne silage.

5.4 Discussion

5.4.1 Experiment 3a: Effect of supplemental chestnut HT (different levels) on lucerne silage degradability.

The immediately soluble fraction “a” of DM, CP and OM determined by the *in situ* was found to be linearly reduced when the levels of supplemented chestnut HT were increased (Figures 5.1-3) with no effect on fraction “b”. In addition, a reduction of protein solubility fraction A determined via the CNCPS and an increase in fraction B1 was observed (Figure 5.4). This could indicate that complexing tannin with plant protein has the ability to shift protein solubility from the immediately soluble to buffer soluble protein (Edmunds *et al.*, 2012). This might be due to the tannin inhibiting proteolytic enzymes during fermentation in addition to tannin binding with macromolecules of the feedstuffs making them less soluble in chemical solutions (Albrecht and Muck, 1991; Lorenz *et al.*, 2010).These results agreed with the results reported by Tabacco *et al.*(2006) who found that using different levels (0, 20, 40 and 60 g kg^{-1}DM) of chestnut HT linearly reduced *in situ* CP fraction “a” and increased CP fraction “b”. Kirchhof *et al.* (2010) found a negative relationship between tannin concentration and fractions A and B1. Grabber and Coblentz, (2009) also found that tannin concentration shifted CP solubility from fraction A and B1 to fraction B2 and C when they used different conservation methods for lucerne, red clover and three types of birdsfoot trefoil (low tannin, moderate tannin and high tannin) silages in two seasons. Poncet and Remond (2002) found that supplementing 0, 20 or 30 g of chestnut HT kg^{-1}DM into milled pea

seed slightly reduced N solubility from 730, 710 and 690 g kg^{-1}TN, respectively.

Kumar and Vaithiyanathan (1990) reviewed the potential formation of tannin-protein complexes to reduce fraction “a” and ED and increase rumen bypass protein. Hervas *et al.* (2003) concluded that the main effect of tannin (CT) was on the immediately soluble fraction “a”. Salawu *et al.* (2001) observed that using quebracho CT as a silage additive reduced CP fraction “a”, ED and fraction “c” and increased CP fraction “b”. In the current study, treated lucerne silage with different levels of chestnut tannin had no effect on DM, CP and OM fraction “b”, however, the total potential fraction “a+b” result was found to reduce linearly when tannin levels were increased. The effective DM, CP and OM rumen degradability at outflow rate 0.05 h^{-1} were found to reduce linearly by increasing tannin levels. Alipour and Rouzbehan (2010) also found that the results of CP effective degradability 0.05 h^{-1} of soybean meal was reduced linearly when supplemented tannin was increased (630, 573, 511, 502 and 480 g kg^{-1}DM for 0, 15, 30, 45 and 60 g kg^{-1}DM tannin levels, respectively).

Calculating rumen dietary protein parameters were conducted using the UK metabolisable protein systems (AFRC, 1993). The results indicate that a consequence of increasing additional tannin levels to lucerne silage led to an increase in rumen bypass protein. Quickly degradable protein, slowly degradable protein and effective rumen degradable protein calculated at outflow rate 0.05 h^{-1} were reduced linearly when the level of additional tannin was increased (Table 5.4). Moreover, both the rumen undegradable protein and the digestible undegradable dietary protein were increased linearly by increasing tannin supplementation. The mean value of the ERDP and DUP

results calculated in the current study (151 and 29 g kg^{-1} DM respectively) are slightly higher than reported by AFRC (1993) for lucerne silage in the UK which could due to the lucerne silage used here containing a high CP (195 g kg^{-1} DM). The ERDP supplied by untreated lucerne silage represent 0.58 g g^{-1} of that supplied by soybean meal. Treating lucerne silage with different levels (25, 50 and 75 g kg^{-1}DM) of chestnut HT reduced the ERDP and increased the UDP and DUP supplied to the small intestine. Reduced ERDP supply may have a consequences for MCP synthesis. Supplementing chestnut HT to lucerne silage was also found to slightly increase MP compared to control silage. Table 5.12 compares the dietary protein supply by lucerne silage treated with different chestnut HT levels and soybean meal (SBM).

Table 5.12 Protein supply of lucerne silage treated with different levels of chestnut HT, soybean meal at outflow rate 0.05 h^{-1}.

	Supplemented tannin g kg^{-1}DM (Current study)				SBM (AFRC,1993)
	0	25	50	75	
CP $_{(g\ kg^{-1}DM)}$	195	195	195	195	497
ERDP $_{(g\ kg^{-1}DM)}$	151	140	136	129	260
UDP $_{(g\ kg^{-1}DM)}$	40	51	56	63	217
DUP $_{(g\ kg^{-1}DM)}$	28	37	40	45	193
MP $_{(g\ kg^{-1}DM)}$	124	126	126	127	359

SBM: soya bean meal, CP: crude protein, ERDP: effective rumen degradable protein, UDP: undegradable dietary protein, DUP: digestible undegradable protein.

The digestible undegradable protein supplied was 28 g kg DM^{-1}. Whereas when lucerne silage was treated with different levels of chestnut HT (25, 50 and 75 g kg^{-1}DM) the DUP supplied was increased to 37, 40 and 45 g kg^{-1} DM

respectively, due to the tannin's effect on protecting dietary protein from rumen microbes. The digestible undegradable protein supplied by SBM is 193 g kg^{-1} DM (AFRC, 1993). Hence, treated lucerne silage with chestnut HT could reduce the need of using SBM by approximately 47, 62 and 88 g of soybean meal for 25, 50 and 75 g kg^{-1} DM chestnut HT, respectively. However, tannin supplementation to lucerne silage reduced ERDP (especially when lucerne silage was supplemented with 75 g kg^{-1}DM), and therefore could have an influence on microbial protein synthesis, hence a slight increase was noticed in MP when lucerne was treated with different levels of chestnut HT (Table 5.12).

5.4.2 Experiment 3 b: Effect of supplemented chestnut HT (different levels) to lucerne silage on rumen fermentation *in vivo*.

The results of the rumen ammonia concentrations showed that increasing the level of supplemented tannin reduced NH_3-N concentration in the rumen fluid (Figure 5.5). These findings are supported by the results obtained from the *in situ* and protein fractionation using the CNCPS (experiment 3 a) which showed that supplementary tannin significantly reduced protein degradability. Rumen NH_3-N is the main product of protein degradation in the rumen (Bahrami-Yekdangi *et al.*, 2014), thus reducing the NH_3-N concentration would indicate that protein degradation was reduced (Patra and Saxena, 2011). Frutos *et al.* (2004) suggested that reducing protein degradability would be the main well known influence of tannin. Similar to the results of the current study, Pellikaan *et al.* (2011) found that different types of tannin had a negative effect on NH_3-

N concentration, when 100 g kg^{-1}DM of three types of CT (grape seed, quebracho or green tea) or four types of HT (chestnut, myrabolan, tara or valonea) were supplemented to lucerne hay and incubated in a gas production kinetic study *in vitro*. Hymes-Fecht *et al.*(2013) found that feeding dairy cows three different varieties of birdsfoot trefoil rich in tannin (8.3, 12.1 and 15.7 g CT kg^{-1}DM) significantly reduced rumen NH_3-N concentration compared with feeding cows lucerne or red clover forages. However, Toral *et al.* (2011) found that supplementing 10 g kg^{-1}DM of mixed HT and CT to a concentrate diet of dairy ewes had no effect on rumen ammonia concentration.

Increasing tannin supplementation was found to linearly reduce rumen total protozoal number (Figure 5.6). McSweeney *et al.* (2001) reported that dietary tannin could bind with the microbial cell membrane and decrease its permeability, which would likely reduce nutrient exchange into the microbial cell, hence the growth of the microbes would be retarded. These results are in agreement with the results reported by Salawu *et al.* (1999) who found that supplementing 50 g of quebracho CT to a grass-barley diet significantly reduced total protozoa numbers in sheep. Similarly Tan *et al.* (2011) observed a linear reduction 18.8-30 % in total protozoal numbers *in vitro* when different levels (0, 10, 15, 20, 25 and 30 mg) of CT from *Leucaena* were incubated with 500 mg of dried grass. Aghamohamadi *et al.* (2014) also found that feeding adult sheep a concentrate supplemented with 100 or 300 g kg^{-1}DM acorn reduced total protozal numbers (*in vivo*).

Supplementary tannin had no effect on total VFA or the molar concentration of most individual VFA except the reduction of butyrate and isovalerate (Table 5.7), which could be related to the tannin's effect on protozoa. Theodorou and

France (2005) reported that rumen protozoa are responsible for producing butyrate and isovalerate, therefore any negative effect on rumen protozoal population would reflect negatively on the concentration of these VFA. Tannin level was found to increase rumen pH before the morning meal but had no effect at 0, 2, 6 and 8 h post feeding. Rumen pH is heavily depended on carbohydrates fermentation and the production of VFA. Hervas *et al.* (2003) found that different level of CT (0, 28 and 83 g kg^{-1}DM) infused directly into the rumen had no effect on rumen pH and VFA.

5.4.3 Experiment 3 c: effect of supplemented chestnut HT (different levels) on feed intake and ewes' performance during late pregnancy and early lactation.

5.4.3.1 Dry matter and protein intake.

Ewes were offered lucerne silage supplemented with different levels of chestnut HT (0, 25, 50 and 75 g kg^{-1}DM) at feeding. The average forage DMI was 2.07 kg during the experimental period. The ewes' tannin consumption was approximately 52, 100 and 160 g d^{-1} for 25, 50 and 75 g kg^{-1}DM additional tannin groups, which is equivalent to 1.91, 3.65 and 5.81 g HT kg^{-1} $BW^{0.75}$ respectively.

Deaville *et al.*(2010) found that the DMI was not affected when adult sheep were offered 1.63 g chestnut HT kg^{-1} $BW^{0.75}$, whereas mimosa CT had a negative effect on DMI when the sheep offered the same amount. The reason for selecting such a high tannin level in the current study was to understand the effect that a high level of additional chestnut tannin would have on DMI. However, no significant differences between treatments on DMI or CPI were

noticed during the experimental period (Table 5.8). Dschaak *et al.*(2011) found that treated forage diet with 30 g kg^{-1}DM quebracho CT significantly reduced DMI in dairy cows. Makkar (2003) and Frutos *et al.*(2004) reported that increasing tannin levels, especially CT (either endogenous or external tannin) at <50 g kg^{-1} DM would probably have a negative effect on palatability.

Several studies (Barry and McNabb, 1999; Hervas *et al.*, 2003; Ben Salem *et al.*, 2005; Dschaak *et al.*, 2011) found that voluntary feed intake was reduced when ruminants were offered diets containing high levels of CT >50 g kg^{-1} DM. They went on to show that consuming high levels (>50 g kg $^{-1}$DM) of tannin could cause a physiological problem to the animal. Mueller-Harvey (2006) reported that hydrolysable tannin would hydrolyse inside in the rumen or abomasum which increase polyphenol compound concentrations in the blood causing liver and kidney lesions. However, Deaville *et al.*(2010) suggested that chestnut HT are non-toxic compounds and were traditionally used against diarrhoea. In addition, the biological activity of chestnut HT appears to be less aggressive when compared to other HT (myrabolam and oak extraction) or CT (mimosa, quebracho or grape pomace) (Tabacco *et al.*, 2006; Deaville *et al.*, 2010). The results obtained from this experiment for the voluntary feed intake agree with those reported by Krueger *et al.*(2010), Liu *et al.*(2011), Hymes-Fecht *et al.*(2013) and Toral *et al.*(2013) who found that tannin had no effect on DMI. In contrast, Dschaak *et al.*(2011) found that intake parameters (DM, OM, CP and NDF) were reduced when 30 g kg^{-1}DM of quebracho CT was added to cannulated lactating cows diets in a Latin square design with no effect of tannin on digestibility.

5.4.3.2. Metabolisable protein balance

The average metabolisable protein requirements for lactating ewes (3 L d^{-1}) with average body liveweight 83.5 kg was 296 g d^{-1} (Section 5.2.4.1). The daily DMI was 2.05 and 0.43 kg DM d^{-1} forage and concentrate diet respectively, which provide ewes with 242 and 56 g d^{-1} MP respectively, thus the offered diet covered the ewes MP requirements.

Table 5.13 shows the effect of supplement levels on the dietary CP. Increasing the tannin level was found to reduce ERDP and increased UDP and DUP. AFRC (1993) reported that MCP synthesis equated to the ERDP of the diet. It may be postulated that additional tannin reduced MCP synthesis but elevated DUP supply so that MP supply remained constant.

Table 5.13 Effect of supplemented different levels of chestnut HT to lucerne silage on ewes' dietary protein parameter.

	Supplemented tannin g kg^{-1}DM			
	0	25	50	75
ERDP $_{(g\ d^{-1})}$	304	294	275	258
UDP $_{(g\ d^{-1})}$	80	107	113	126
DUP $_{(g\ d^{-1})}$	56	77	80	90
F-MP $_{(g\ d^{-1})}$	249	264	255	254
T-MP $_{(g\ d^{-1})}$	299	314	305	304

ERDP: effective rumen dietary protein, UDP: undegradable dietary protein, DUP: digestible undegradable protein, F-MP: forage metabolisable protein, T-MP: total metabolisable protein.

Although supplemented tannin reduce ERDP, treated lucerne silage with either tannin levels (25, 50 or 75 g kg^{-1}DM) were found to increase MP by 15, 6 or 5 g d^{-1} respectively compared to control silage due to an increase in UDP.

Feeding ewes 435 g DM d^{-1} concentrate provided 50 g d^{-1} MP, thus treated lucerne silage with tannin would reduce the need of using concentrate in the diet by approximately 130, 52 or 43 g DM d^{-1} respectively. The highest level of forage MP was found when 25 g kg^{-1}DM of chestnut HT was supplemented due to the effect of both ERDP and DUP compared to 0, 50 or 75 g kg^{-1}DM. Crude protein digestibility was reduced (P<0.05) when lucerne silage was treated with high tannin level (75 g kg^{-1}DM), thus treated lucerne silage with 25 g kg^{-1}DM will provide a higher MP which could decrease the diet cost. A further study on estimating the influence of tannin on MCP synthesis could be required.

5.4.3.2 Diet digestibility and blood metabolites

In the current study, the 75 g kg^{-1}DM HT treatment reduced OM, CP, and NDF diet digestibility post-partum by 7.8, 8.8 and 10.8% respectively. This effect may related to the reformation of the tannin complex in the small intestine rendering the reformed tannin digestate complex indigestible. This theory is supported by Makkar (2003) who suggested that not all tannin protein complexes dissociate in the abomasum and/or complexes could be reversible in the small intestine depending on the tannin source, type and concentration, which is probably the reason for increasing faecal nitrogen concentration in the current study (Table 5.10). Zimmer and Cordesse (1996) suggested that free un-complexed dietary tannin could complex directly with digestive enzymes thereby reducing enzyme activity and hence digestibility. Mueller-Harvey (2006) and Piluzza *et al.* (2014) reported that the post rumen fate of tannin in the digestive tract is still unclear and that there is potential for tannin

to bind with endogenous protein (such as digestive enzymes) or for it to recomplexed with feed protein. Similarly to the results of the current study, Zimmer and Cordesse (1996) observed that supplementing 110 g kg^{-1}DM chestnut HT to hay significantly reduced DM and OM digestibility by approximately 8.8 and 4.7% in sheep and goats, respectively. Ben Salem *et al.* (2005) noted a reduced DM digestibility in lambs grazing a tannin rich (59.6 g kg^{-1}DM) acacia browse compared to lambs group fed tannin free hay; thereafter the digestibility of both groups were increased after 24 days. Deaville *et al.* (2010) also observed that additional 55.6 g mimosa CT kg^{-1}DM was found to reduced (P<0.01) DM and OM digestibility, whereas no significant effect of supplemented 55.1 g chestnut HT kg^{-1} DM to lucerne silage on DM and OM digestibility was noticed.

In the current study, feeding ewes lucerne silage treated with different chestnut HT had no significant differences on blood plasma parameters (Figures 5.11-5.14, Table 5.9). These results are in agreement with those observed in several studies (Buccioni *et al.*, 2015; Hymes-Fecht *et al.*, 2013; Sinclair *et al.*, 2009; Woodward *et al.*, 2002) who found that feeding adult sheep or cow a diet rich in tannin (CT or HT) (either endogenous or external tannin) had no effects on plasma blood analyses.

5.4.3.3 Milk yield, colostrum and milk composition

The milk yield in the current study was measured at 0, 14, 28 and 42 day's post-partum and averaged 2.17, 2.73, 2.68 and 2.48 L d^{-1} respectively. Supplementing lucerne silage with 25 g kg^{-1}DM chestnut HT was found to enhance milk yield by 8 % compared to the control group. An elevated milk

yield of by 5.7 % was also found when lucerne silage was treated with 50 g kg^{-1}DM. These results are supported by the finding of Hymes-Fecht *et al.* (2013), who found that switching dairy cows from lucerne silage to one of three different varieties of birdsfoot trefoil silage (containing 8.3, 12.1 or 15.7 g kg^{-1} DM CT) increased milk yield by approximately 8%, although there was no difference in silage DMI. Woodward *et al.* (2002) also found that feeding dairy cows' Lotus and Sulla silages (containing 33.7 and 16.3 g kg^{-1}DM CT respectively) produced more milk (32 and 16% respectively) compared to cows' grazed restricted pasture (control). Woodward *et al.* (2002) suggested that the high milk yield would probably due to the combination between the high nutritive value of Lotus and Sulla silage and the activity of CT. Turner *et al.* (2005) found that milk yield was increased by approximately 13 and 27 % when dairy cows were fed fresh lotus forage rich in CT compared to supplemented lotus with PEG (50 g kg^{-1}DM) or ryegrass, with no differences in milk composition. Dschaak *et al.* (2011) observed that treating high or low DM forage with 30 g kg^{-1}DM quebracho CT in dairy cows had no effect on milk yield and composition. Similarly, Toral *et al.* (2011 and 2013) found that tannin supplementation had no effect on milk yield in dairy ewes.

Buccioni *et al.* (2015) fed a group of eighteen Comisana ewes grass hay treated with either 84.5, 52.8 or 52.8 g kg^{-1}DM of soybean oil, chestnut HT or quebracho CT, respectively. Buccioni *et al.* (2015) noticed that there were no differences between diets with any additives on milk yield and composition, except milk casein which was increased when ewes were fed grass hay treated with chestnut HT. In the current study, treating lucerne silage with different levels of chestnut tannin had no effect on colostrum or milk

composition (Table 5.11). These results agree with those reported by Turner *et al.* (2005), Toral *et al.*(2011, 2013), Hymes-Fecht (2013) and Buccioni *et al.* (2015). However, and due to the significant differences in milk yield between treatments, the highest milk total solid, fat, lactose and protein were recorded in ewes fed lucerne silage treated with 25 g kg^{-1}DM chestnut HT, and the lowest milk components were ewes fed lucerne silage treated with 75 g kg^{-1} DM chestnut HT.

5.5 Conclusion

The increased levels (0, 25, 50 or 75 g kg^{-1}DM) of chestnut HT supplemented to lucerne silage resulted in a linear reduction of QDP (12, 121, 119 or 117g kg^{-1}DM), ERDP (151, 140, 136 or129 117 g kg^{-1}DM), increased UDP (40, 51, 56, 64 g kg^{-1}DM), DUP (28, 37, 40 or 45 g kg^{-1}DM) and MP (124, 126, 126 or 127 g kg^{-1}DM) content. It may be postulated from these results that dietary CP was protected in the rumen from rumen microbial activity.

Rumen fluid NH_3-N concentration was found to be reduced linearly when the level of additional tannin was increased (0.33, 0.29, 0.23 or 0.2 g L-1). In addition the rumen total protozoa number also linearly reduced when supplemented tannin was increased, which could indicate that tannins did not only reduced protein degradation but also have a negative effect on rumen protozoa.

Results obtained from ewe study showed that additional HT levels did not affect ewes' voluntary feed intake. Supplementary lucerne silage with either tannin levels (25, 50 or 75 g kg^{-1}DM) were found to increase MP by 15, 6 or 5

g d^{-1} respectively compared to control silage which could reduce the need of using concentrate diet by approximately 30, 12 or 9% respectively. However, crude protein digestibility was reduced (P<0.05) and faecal N was increased when lucerne silage was treated with high tannin level (75 g kg^{-1} DM). Treated lucerne silage with 25-50 g kg^{-1}DM will proved higher MP which could decrease the diet cost. Additional 25 or 50 g kg^{-1}DM were found to increase milk yield and milk component especially in peak lactation.

CHAPTER 6 General Discussion

A series of experiments was carried out to examine the effect of supplementary chestnut hydrolysable tannin (HT) when either added prior to ensiling or added just before feeding on silage quality, forage degradability, rumen function and animal performance. Results from all experimental chapters found that the addition of tannin to the forage reduced the immediately soluble fraction and increased the supply of undegradable dietary protein to the small intestine. In the final experiment the increased supply of digestible UDP to the small intestine resulted in an increase in ewe milk yield in early lactation.

Chestnut HT was used in all experiments for the following reasons:

- The paucity of published data on the effect of using HT as a method of protein protection in ruminant nutrition compared to condensed tannin (Katiki *et al.*, 2013).
- Chestnut HT is considered to be non-toxic to the animal and has traditionally been used as a treatment against diarrhoea (Deaville *et al.*, 2010). In contrast most other HT are hydrolysed in the rumen leading to an increase in polyphenol compounds in the animals blood that could lead to liver and kidney lesions (Waghorn, 2008 and Mueller-Harvey, 2006).
- Chestnut HT are commercially available as a powder tannin in the UK, and are often used in the wine industry.

In the current study chestnut HT were applied to a variety of forage crop at different rates either prior to ensiling or before feeding as presented in Table 6.1.

Table 6.1 The methods and the level of additional chestnut HT that were added to different forage based on fresh weight (FW) and dry matter weight (DM).

Silage	DM ($g\ kg^{-1}$)	Method of inclusion	Level ($g\ kg^{-1}$FW)	Level ($g\ kg^{-1}$DM)	Experiment
Bean	235	Ensiling	20	85	One
Bean	235	Ensiling	40	170	One
Pea	318	Ensiling	20	63.5	One
Pea	318	Ensiling	40	127	One
Grass	345	Ensiling	20	58	One
Grass	345	Ensiling	40	116	One
Grass	245	Ensiling/ prior feeding	7.5	30	Two
Lucerne	480	Prior feeding	12	25	Three
Lucerne	480	Prior feeding	24	50	Three
Lucerne	480	Prior feeding	36	75	Three

6.1 Effect of supplemented tannin at ensiling on silage quality.

One of the objectives of the current study was to investigate the potential effects of additional chestnut HT at ensiling on silage quality. Tannin was supplemented at ensiling in experiment 1 and 2 to pea, bean and grass forages as shown in Table 6.1. The proximate analysis results (Tables 3.2. and 4.1) provide evidence that tannin supplementation prior to ensiling reduced forage ammonia nitrogen (NH_3-N) concentration in the final silage

suggesting that plant protein hydrolysis during the ensiling process had been reduced. These results are supported by the findings of Salawu *et al.* (1999), Tabacco *et al.* (2006) and Deaville *et al.*(2010) who similarly observed a reduction in NH_3-N concentration of silages when treated with different levels of tannin (either HT or CT). The hydrolysis of plant proteins predominantly in the chloroplasts commences after harvesting due to the release of plant protease within the plant cells (Theodoridou and Yu, 2013) and/or the activity of proteolytic bacteria and other microorganisms which colonise the plants external surface (Bach *et al.*, 2005). Kopencny and Wallace (1982) reported that the optimum pH of proteolysis enzymes is pH 7-5.5. Protein hydrolysis reduces when the pH of the silage clamp drops pH <5 inhibiting microbial activity (Wilkinson and Davies, 2013). The addition of tannin prior to ensiling provides an opportunity for the tannin to create a tannin complex with either the plant enzymes, plant cell walls and/or bacterial cell membranes inhibiting proteolytic activity. It was reported (Mueller-Harvey, 2006; Patra and Saxena, 2011) that the tannin-protein complex is pH sensitive, such that at a pH <4 the hydrogenic bond between the tannin and protein dissociates. Thereby tannin would be un-associated in the final ensiled forage and conferring no effect at this point. However crop proteins in ensiled forages with pH 4 would not require protection since both plant protease and proteolytic bacteria are not active in pH 4 as mentioned previously. Therefore, future work might consider how to measure / differentiate between free and complexed tannin, and then go on to investigate the association / disassociation of different tannin at a variety of different pH's.

The effects of tannin supplementation on reducing NH_3-N concentration was higher in leguminous silages (experiment 1) compared to grass silage (experiments 1 and 2) as shown in Table 3.2 and 4.1. These differences might be explained by the time of pH reduction during the ensiling process, plus the types and structure of the proteins. Davies *et al.* (1998) found that the pH of untreated grass reached pH 4.5 in less than 36 h leading to an inhibition of plant protease enzymes and undesirable silage microorganisms such as proteolytic bacteria. Whereas in the leguminous silages, the reduction in silage pH took longer due to their higher buffering capacity compared to grass silage (McDonald and Henderson, 1962). For example Hart (2005) observed that leguminous silages could take 96 h before the pH of the silo become pH 4.5. Consequently the addition of tannin to leguminous forages at ensiling may have a higher protein protection effect during the fermentation period as compared to grass forages.

In situ rumen degradability results plus the results from protein fractionation using the CNCPS technique showed that supplementary tannin at ensiling was found to have more of an effect on reducing protein degradation and increasing rumen undegradable protein supply compared to the addition of tannin to the diet prior to feeding (experiments 1 a, 2 a and 3 a). It is suggested that supplementary tannin at ensiling had a longer time to react with forage protein during the ensiling period, which facilitated the binding of tannin to plant protein. Whereas it is possible that some of the tannin molecules supplemented at feeding, dissolved directly either in the rumen liquid (*in situ*) or chemical solution (protein fractionation CNCPS) before reacting with the forage protein.

6.2 Effect of supplementary chestnut HT on voluntary feed intake, rumen fermentation and animal performance.

6.2.1 Rumen fermentation.

In situ, CNCPS and *in vitro* gas production results all showed that the addition of tannin to the diet either before ensiling or prior to feeding reduced the CP degradability of the forage. This may be due as previously discussed, to the most widely reported effect of tannins being on rumen fermentation and protein degradability in the rumen (Frutos *et al.*, 2004). Moreover, supplementary tannins were found to reduce quickly degradable and EDRP and increase UDP and MP supplied to the lower gut which could be the reason for increasing milk yield in experiment 3 c. In addition, results obtained from experiment 3 b showed that additional tannin prior to feeding reduced total protozoa number. McSweeney *et al.* (2001) reported that dietary tannin could bind with microbial cell membranes and decrease its permeability, which is likely to reduce nutrient exchange into the microbial cell, hence the growth of the microbes would be retarded. These results could explain the results from experiment 1 b, when it was found that supplementary tannin at ensiling reduced the total and asymptotic gas production kinetics *in vitro.* Rumen NH_3-N concentration results obtained throughout the current experiments showed that additional tannin either at ensiling or prior feeding reduced NH_3-N (experiments 1 b, 2 b and 3 b). These results could be the reason for decreased blood plasma urea in lambs in the growth study (experiment 2 b). Bahrami-Yekdangi *et al.* (2014) reported that NH_3-N would be the final product of dietary protein degradation in the rumen.

6.2.2 Animal performance

Tannin form part of the plants immune system to protect it from ingestion by herbivorous animals (Waghorn, 2008) and are often described as anti-nutritional compounds (Mueller-Harvey, 2006). It has been reported (Frutos *et al.*, 2004) that consumption of tannins have a negative effect on voluntary feed intake especially when fed at >50 g kg^{-1}DM of CT. However, in the current study, supplementation with chestnut HT had no effect on voluntary feed intake in either the lamb growth (experiment 2 b) or adult sheep (experiment 3 b and c) studies. Table 1.11 showed different effects of tannin on voluntary feed intake in ruminants. The variation between these results suggested that researchers used tannin from different source, types and levels. According to the literature, the source of tannin would be the most important factor that could influence voluntary feed intake, followed by tannin type and then tannin level. In addition, results from the current study showed that tannin level had no effect on feed intake even when high levels (75 g kg^{-1}DM) of chestnut HT was supplemented.

Diet digestibility was estimated for both the lamb growth and ewe studies using acid insoluble ash as an endogenous marker according to the technique described by Van Keulen and Young (1977). Results from experiment 3 c showed that supplementation of tannin prior to feeding at 75 g kg^{-1}DM had a negative effect on OM, CP and NDF digestibility. Ewes offered lucerne silage treated with 75 g kg^{-1} DM chestnut HT, consumed approximately 150 g d^{-1} chestnut HT. Hence part of the consumed tannin protein complex or free tannin could escape post abomasum and rebind either with digesta protein, microbial protein and/or digestible enzymes (Makkar, 2003), resulting in a

reduction in diet digestibility and increased faecal N. The fates of tannin (especially HT) post ruminally are still unclear (Piluzza *et al.*, 2014). Supplementary tannin at either ensiling or prior feeding (experiment 2 b) had no effect on enhancing lamb performance nor meat quality, to the which may perhaps be due to that lambs offered diet content MP slightly higher MP requirements, with no differences between the experimental treatments regarding MP supplied (Table 4.6), in addition, the length of the study (6 weeks) also could had an effect. Lamb growth studies for longer periods (8-12 weeks) might observe a greater response to the tannin on lamb performance. This argument is supported by the results observed by Hart *et al.* (2012), who found that the final weight of lambs offered high or low tannin pea silage were greater compared to lambs offered grass silage in a growth study for 8 weeks. In experiment 3 c, an increase in ewes milk yield and component yield were observed when ewes were offered lucerne silage treated with 25-50 g kg^{-1}DM chestnut HT, which could be due to an increase in rumen bypass protein to the small intestine (Table 5.10) as a result of reduced CP degradability.

6.3. Prediction of rumen undegradable dietary protein using both *in suit* and protein fractionation CNCPS techniques.

The *in situ* rumen technique is considered the standard and the most widely acceptable method for estimating rumen degradability (AFRC, 1993; NRC, 2001). However, it is expensive, labour extensive and requires the use of animals fitted with permanent cannula (Edmunds *et al.*, 2012). The protein fractionation in the CNCPS technique could be an alternative method for estimating protein solubility, rumen degradable and rumen undegradable

protein (Kirchhof *et al.*, 2010). For calculating UDP using the protein fractionation technique, Shannak *et al.* (2000) and Kirchhof (2007) evaluated a series of equations for estimating UDP at outflow rates 8, 5 and 2% h^{-1}:

$UDP_{0.02}$= 204.3207+ (1.0753 x C) + (-0.0014 x (CP x (A+B1))) Eq. 6.1

$UDP_{0.05}$= 321.9023 + (0.1676 x PADF) + (-0.0022 x (CP x (A+B1))) + (0.0001 x (CP x C^2)) Eq. 6.2

$UDP_{0.08}$= 285.5459 + (1.2143 x C) + (0.0005 x (PNDF x B2)) + (-110.1740 x (A+B1)/PNDF)) Eq.6.3

Where UDP is undegradable dietary protein, A is non protein nitrogen, B1 is buffer soluble protein, B2 is neutral detergent soluble protein and B3 is acid soluble detergent protein, PNDF and PADF are the residual protein content after boiling in neutral or acid detergent solution respectively (Licitra et al, 1996). However, different equations are required for estimating protein degradability at different rumen outflow rate, in addition these equations have a heavy reliance on the A and B1 fractions and seem to be complicated equations.

In the current study data from experiment 2 a and 3 a (10 silage treatments in quadruplicate) were undertaken to develop a simple equation for estimating rumen effective protein degradability at different outflow rates. The data were used for measuring protein effective degradability (ED) at outflow rate 2, 5 and 8 % h^{-1} using both *in situ* and protein fractionation techniques using the new equation. The new equation were estimated using GenStat 15 as follows:

$ED_{(g\ kg^{-1}CP)} = 498 + (-1192.8 \times k) + (0.411 \times A) + (1.33 \times B2) + (-0.0133 \times (B1 \times B2))$ Eq6.4

Were, k is rumen outflow rate ED, A, B1, B2, B3 and C are mentioned above.

$UDP_{(g\ kg^{-1}CP)} = 1000 - ED_{(g\ kg^{-1}CP)}$ Eq. 6.5

The new equation for protein fractionation method were used and compared with the results conducted from *in situ*. Linear regression (GenStat 15) was used to compare the results from both methods (Figures 6.1, 6.2 and 6.3).

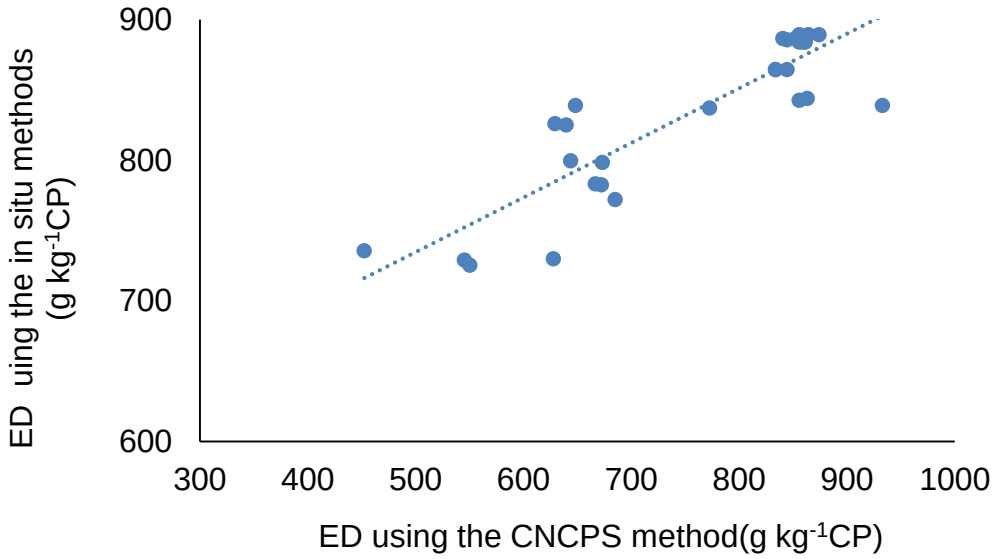

Figure 6. 1 Linear regression of effective degradability (ED) at outflow rate 2% h^{-1} calculated by *in situ* technique (Y-axis) and *in vitro* protein fractionation using the CNCPS (X-axis).

The protein ED from the *in situ* method was used as standard values. The correlation (r^2) were used to describe the relationships between the results from both techniques.

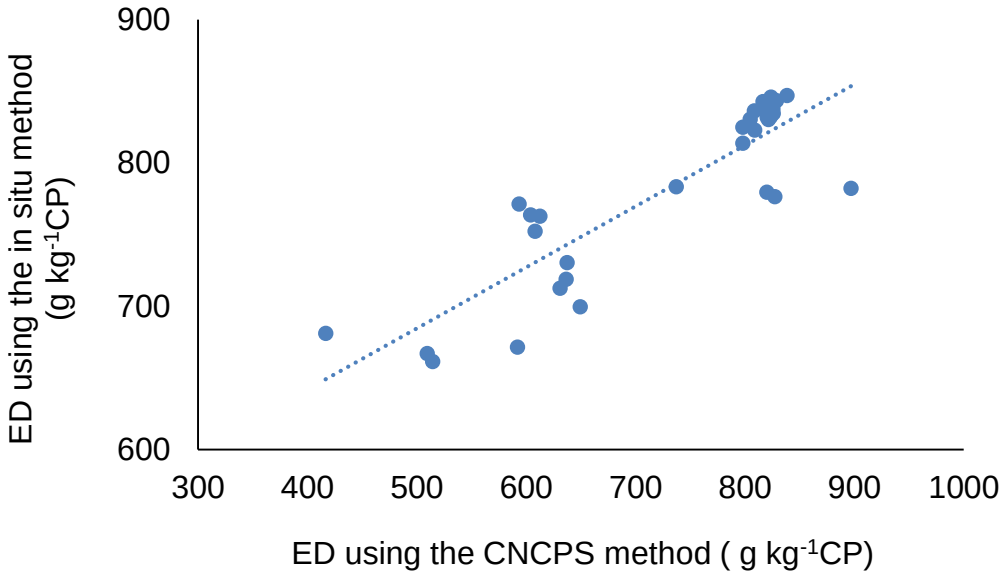

Figure 6. 2 Linear regression of effective degradability (ED) at outflow rate 5% h^{-1} calculated by *in situ* technique (Y-axis) and *in vitro* protein fractionation using the CNCPS (X-axis).

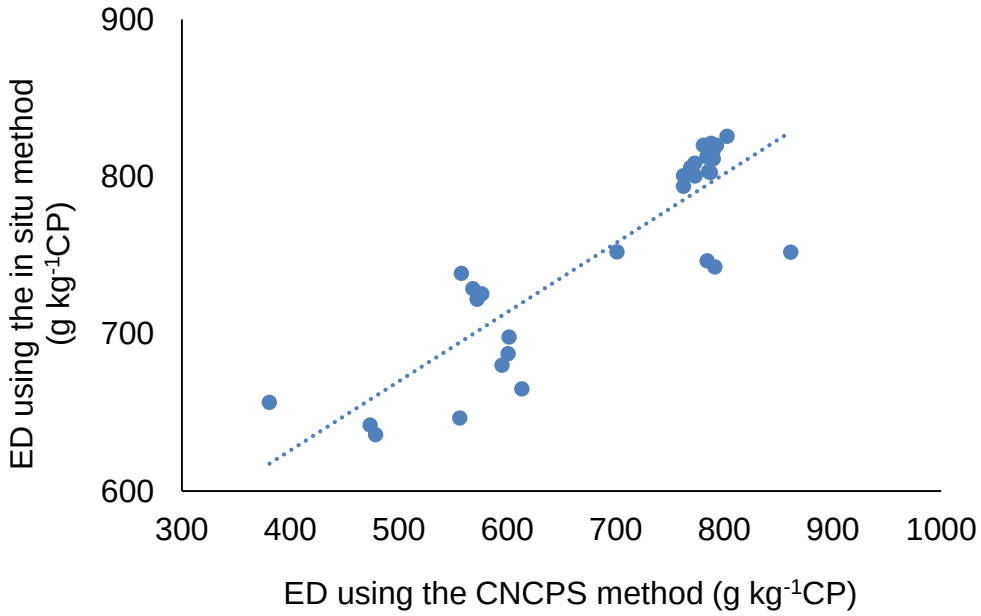

Figure 6. 3 Linear regression of effective degradability (ED) at outflow rate 8% h^{-1} calculated by *in situ* technique (Y-axis) and *in vitro* protein fractionation using the CNCPS (X-axis).

A linear and significant (P<0.001) relationship was observed at all outflow rate using protein ED estimated from *in situ* (dependent variable) and protein

fraction method (new equation), with a relatively high r^2 (0.75-0.78). Table 6.2 shows the linear regression parameters for each outflow rate.

Table 6. 2 Linear regression parameters of ED for grass and lucerne silage samples calculated from protein fractionation method for three rumen outflow rate (2, 5 and 8% h^{-1}), regressed against ED estimated using *in situ* method.

Parameters	Slop ± SE	Protein fractionation (X) ± SE	r^2	SE of OE	*P*
In situ 2	541 ± 30	0.39 ± 0.04	0.77	26	<0.001
In situ 5	471 ± 32	0.43 ± 0.04	0.76	30	<0.001
In situ 8	450 ± 33	0.44 ± 0.05	0.75	32	<0.001

SE: standard error, SE of OE: standard error of observation

Although a strong relationship was observed between protein ED estimated *in situ* and the new equation from protein fractionation methods, few silage samples were used. The validity and strength of this approach could be further improved with more variety and number of diet samples. The equation will also have to be tested before it can be applied as a final equation for estimating ED and UDP using the protein fractionation techniques, but could lead to a reduced the use of fistulated animals.

6.4 Recommendations and future work

Results obtained from this study recommend using chestnut HT as a silage additive at ensiling due to its effect of reducing forage protein hydrolysis. The best level of additional tannin recommended in the current study is 25-50 g kg^{-1}DM. Application of the tannin prior to ensiling has been shown to be beneficial but the practical element of how to add the tannin will need to addressed. Potential methods of application could be dissolving the tannin in water and applying to the crop as it is forage harvested. However the optimum

tannin concentration and practicality of this approach would require further investigation

Further work is required to study the behaviour and fate of chestnut HT inside different parts of the digestive tract both *in vivo* and/or *in vitro*. In addition, developing a method for measuring tannin-protein complexes to determine where the dissociation of tannin protein complex starts and finishes, and whether tannin rebinds with digesta or endogenous protein post ruminally. to understand the fate of tannin inside animal's body. Further work with dairy animals would be required to study the effect of tannin supplementation on milk fatty acid profile.

CHAPTER 7 General conclusion

Results obtained from the current study support the hypothesis: that supplementary chestnut hydrolysable tannin (HT) can be added to a variety of forage crops at ensiling or before feeding to bind with forage protein to reduce rumen protein degradability. The main results obtained in the current study are summarized as:

- Supplementary tannin to grass and leguminous forages at ensiling reduced protein hydrolysis and ammonia concentration in the resulting silage.
- Tannin supplemented at either ensiling or prior feeding was found to reduce *in situ* rumen degradation and ERDP and increase UDP and MP (experiment 3 c) supplied to the small intestine, and in addition reduced protein solubility (protein fractionation; CNCPS).
- Chestnut HT had no effect on ewe or lamb dry matter intake.
- Tannin inhibited rumen fermentation by reducing gas production kinetics (*in vitro*), total protozoa number and ammonia nitrogen concentration in the rumen with no effect on rumen pH or the molar concentration of total and major individual VFA (*in vivo*).
- Using a high level of tannin supplementation (75 g kg^{-1}DM) had a negative effect on diet digestibility in late pregnancy and early lactation ewes.
- Increase in milk yield and milk component yield were obtained when ewes were offered lucerne silage treated with 25-50 g kg^{-1}DM of chestnut HT.

CHAPTER 8. References

Abdalla, H. O., D. G. Fox, and P. J. Van Soest. 1988. An Evaluation of Methods for Preserving Fresh Forage Samples Before Protein Fraction Determinations. J. Anim. Sci. 66:2646–2649.

Aerts, R. J., T. N. Barry, and W. C. McNabb. 1999. Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages. Agric. Ecosyst. Environ. 75:1–12.

AFRC. 1992. Technical committee on responses to nutrients. Report No. 9 Nutritive requrment of ruminant animals: protein. Nutr. Abstr. Rev. Enviroment 62:788–835.

AFRC. 1993. Energy and Protein Requirments of Ruminants . Oxen, UK CAB Int.

Aghamohamadi, N., F. Hozhabri, and D. Alipour. 2014. Effect of oak acorn (Quercus persica) on ruminal fermentation of sheep. Small Rumin. Res. 120:42–50.

Albrecht, K. A., and R. E. Muck. 1991. Proteolysis in ensiled forage legumes that vary in tannin concentration. Crop Sci. 31:464–469.

Alipour,D and Y., Rouzbehan, 2010. Effects of several levels of extracted tannin from grape pomace on intestinal digestibility of soybean meal. Livest. Sci. 128:87–91.

Allison, M. J. 1970. Nitrogen Metabolism by ruminal microorganisms. In Physiology Digestion and Metabolism in the Ruminant. A.T. Phillipson , ed. Oriel Press. Newcastle upon Tyne, Engl.Pp.:456–463.

Alonso-Diaz, M.A., J. F. J.Torres-Acosta, C. A.Sandoval-Castro and H. Hoste 2009. Tannins in tropical tree fodders fed to small ruminants: A friendly foe? Small Rumin. Res. 89:164–173.

Andrade-Montemayor, H., F. Alegría-Ríos, M. Pacheco-López, H. Aguilar-Borjas, J. L. O. Villegas-Díaz, R. Basurto-Gutierrez, H. Jimenez-Severiano, and H. R. Vera-Ávila. 2009. Effect of dtu roasting on composition, digestibility and degradability of fiber fractions of mesquite pods (*Prosopis laevigata*) as feed supplement in goats. Trop. Subtrop. Agroecosystems 11:237–243.

AOAC. 2000. Official methods of analysis of AOAC international; 17th Edidtion. Editor William Horwitz, Publisher AOAC Intl 2000.

Assoumani, M. B., F. Vedeau, L. Jacquot, and C. J. Sniffen. 1992. Refinement of an enzymatic method for estimating the theoretical degradability of proteins in feedstuffs for ruminants. Anim. Feed Sci. Technol. 39:357–368.

Azuhnwi Thomann, B. ,Arrigo, Y., Boller, B., Hess, H. D., Kreuzer, M., and Dohme-Meier, F., B. N. 2012. Ruminal dry matter and crude protein degradation kinetics of five sainfoin (*Onobrychis viciifolia Scop*) accessions differing in condensed tannin content and obtained from different harvests. Anim. Feed Sci. Technol. 177:135–143.

Bach, A., S. Calsamiglia, and M. D. Stern. 2005. Nitrogen Metabolism in the Rumen. J. Dairy Sci. 88, Supple:E9–E21.

Bahrami-Yekdangi, H., M. Khorvash, G. R. Ghorbani, M. Alikhani, R. Jahanian, and E. Kamalian. 2014. Effects of decreasing metabolizable protein and rumen-undegradable protein on milk production and composition and blood metabolites of Holstein dairy cows in early lactation . J. Dairy Sci. 97:3707–3714.

Baron, C. P., L. H. Skibsted, and H. J. Andersen. 2002. Concentration Effects in Myoglobin-Catalyzed Peroxidation of Linoleate. J. Agric. Food Chem. 50:883–888.

Barry, T. N., and W. C. McNabb. 1999. The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. Br. J. Nutr. 81:263–272.

Barry, T. N. 1989. Condensed tannins: their role in ruminant protein and carbohydrate digestion and possible effects upon the rumen ecosystem. In: The role of protozoa and fungi in ruminant digestion. J.V. Nolan, R.A. Leng and D.I. Demeyer.Perambul Books, Armidale, Australia. Pp:153-169.

Bayourthe, C., R. Moncoulon, and M. Vernay. 1993. Effect of Protein-Protected Fat on Ruminal and Total Nutrient Digestibility of Sheep Diets. J. Anim. Sci. 71:1026–1031.

Bhatta, R., U. Krishnamoorthy, and F. Mohammed. 2000. Effect of feeding tamarind (*Tamarindus indica*) seed husk as a source of tannin on dry matter intake, digestibility of nutrients and production performance of crossbred dairy cows in mid-lactation. Anim. Feed Sci. Technol. 83:67–74.

Bhatta, R., Y. Uyeno, K. Tajima, A. Takenaka, Y. Yabumoto, I. Nonaka, O. Enishi, and M. Kurihara. 2009. Difference in the nature of tannins on *in vitro* ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J. Dairy Sci. 92:5512–5522.

Ben Salem, H., A. Nefzaoui, H. P. S. Makkar, H. Hochlef, I. Ben Salem, and L. Ben Salem. 2005. Effect of early experience and adaptation period on voluntary intake, digestion, and growth in Barbarine lambs given tannin-containing (*Acacia cyanophylla* Lindl. foliage) or tannin-free (oaten hay) diets. Anim. Feed Sci. Technol. 122:59–77.

Block, E. 2006. Rumen microbial protein production: Are we missing an opportunity to improve dietary and economic effiecencies in protein nutrition of the high producing dairy cow? High Plains Dairy Conf.:33–46.

Broderick, G. A., and K. A. Albrecht. 1997. Ruminal *In vitro* Degradation of Protein in Tannin-Free and Tannin-Containing Forage Legume Species. Crop Sci. 37:1884–1891.

Broderick, G. A., and D. R. Buxton. 1991. Genetic variation in alfalfa for ruminal protein degradability. Can. J. Plant Sci. 71:755–760.

Broderick, G. A., and W. M. Craig. 1980. Effect of heat treatment on ruminal degradation and escape, and intestinal digestibility of cottonseed meal protein. . J. Nutr. 110:2381–2389.

Brodrerick, G. A., R. J. Wallace, and E. R. Orskov. 1991. Control of Rate and Extent of Protein Degradation. In: Physiological aspect pf digestion and metabolisum in ruminant. T. Tsuda, Y.Sasaki and R. Kawahima. Press Inc. London. UK.

Brown, A. J., and D. R. Williams. 1979. Momorandum No. 38. Sheep carcass evaluation measurements of composition using a standardized butchery methods. Agric. Res. Counc. Meat Res. Institute, Langford, Bristol.

Buccioni, A., M. Pauselli, C. Viti, S. Minieri, G. Pallara, V. Roscini, S. Rapaccini, M. T. Marinucci, P. Lupi, G. Conte, and M. Mele. 2015. Milk fatty acid composition, rumen microbial population, and animal performances in response to diets rich in linoleic acid supplemented with chestnut or quebracho tannins in dairy ewes. J Dairy Sci 98:1145–1156.

Buege, J. A., and S. D. Aust. 1978. Microsomal lipid peroxidation. Methods Enzymol. 52:302–310.

Calsamiglia, S., M. Busquet, P. W. Cardozo, L. Castillejos, and A. Ferret. 2007. Invited Review: Essential Oils as Modifiers of Rumen Microbial Fermentation. J. Dairy Sci. 90:2580–2595.

Calsamiglia, S., P. W. Cardozo, A. Ferret, and A. Bach. 2008. Changes in rumen microbial fermentation are due to a combined effect of type of diet and pH. J. Anim. Sci. 86:702–711.

Cardozo, P., S. Calsamiglia, and A. Ferret. 2002. Effect of pH on nutrients digestion and microbial fermentation in a dual flow continuous culture system fed high concentrate diet. J. Dairy Sci. 85:182 (Abstr.).

Cardozo, P. W., S. Calsamiglia, A. Ferret, and C. Kamel. 2004. Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. J Anim Sci 82:3230–3236.

Chadwick, L. 2004. The Farm Management Handbook 2004/2005. The UK refernces for farm business mangement. Scottish Agricultural College.

Chalmers, M. I., D. P. Cuthbertson, and R. L. M. Synge. 1954. Ruminal ammonia formation in relation to the protein requirement of sheep: I. Duodenal administration and heat processing as factors influencing fate of casein supplements. J. Agric. Sci. 44:254–262.

Church, D. C., D. A. Daugherty, and W. H. Kennick. 1982. Nutritional Evaluation of Feather and Hair Meals as Protein Sources for Ruminants. J. Anim. Sci. 54:337–344.

Cieslak Zmora, Pawel, Pers-Kamczyc, Emilia, Szumacher-Strabel, Malgorzata, A. 2012. Effects of tannins source (Vaccinium vitis idaea L.) on rumen microbial fermentation *in vivo*. Anim. Feed Sci. Technol. 176:102–106.

Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Microbial Protein Synthesis and Flows of Nitrogen Fractions to the Duodenum of Dairy Cows. J. Dairy Sci. 75:2304–2323.

Coblentz, W. K., and J. H. Grabber. 2013. *In situ* protein degradation of alfalfa and birdsfoot trefoil hays and silages as influenced by condensed tannin concentration. J. Dairy Sci. 96:3120–3137.

Cone, J. W., A. H. Van Gelder, I. A. Soliman, H. De Visser, and A. M. Van Vuuren. 1999. Different techniques to study rumen fermentation characteristics of maturing grass and grass silage. J. Dairy Sci. 82:957–66.

Cornell University. 2014. Tannins: fascinating but sometimes dangerous molecules. from interent. http//www.ansci.cornell.edu/plants/toxicagents/tannin.html access 10/05/2015.

Craig, W. M., G. A. k Broderic, and D. B. Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. J. Nutr. 117:56–62.

Cruwys, J. A., R. M. Dinsdale, F. R. Hawkes, and D. L. Hawkes. 2002. Development of a static headspace gas chromatographic procedure for the routine analysis of volatile fatty acids in wastewaters. J Chromatogr A 945:195–209.

Cutrignelli, M. I., S. Calabro, R. Tudisco, F. Infascelli, and V. Piccolo. 2001. Protein Soucres in Ruminant Nutrition. In: Recent tends for enhancing the diversity and quality of soybean products. D. Krezhove. In Tech. P.p:191-214.

Davies, D. R., R. J. Merry, A. P. Williams, E. L. Bakewell, D. K. Leemans, and J. K. S. Tweed. 1998. Proteolysis during ensilage of forages varying in soluble sugar content. J. Dairy Sci. 81:444–453.

Dawson, L.E.R., M.A. McCoy, H.W.J. Edgar,.and A.F. Carson. 2011. Effect of concentrate supplementation at pasture and inclusion of condensed tannins (Quebracho) in concentrates on lamb performance and faecal egg and worm counts. Livest. Sci. 135, 205-214.

Deaville, E. R., D. I. Givens, and I. Mueller-Harvey. 2010. Chestnut and mimosa tannin silages: Effects in sheep differ for apparent digestibility, nitrogen utilisation and losses. Anim. Feed Sci. Technol. 157:129–138.

DEFRA. 2014. Farming Sttistics. From. https://www.gov.uk/government/organisations/department-for-environment-food-rural-affairs/about/statistics. Accessed 27/10/2014.

Degussa. 2006. The amino acid composition of feedstuff. In: 5th rev. ed. Vol. Degussa AG. Feed additives, Hanau, Germany.

Dehority, B. A. 1984. Evaluation of subsampling and fixation procedures used for counting rumen protozoa. Appl. Environ. Microbiol. 48:182–185.

Dehority, B. A. 2005. Effect of pH on viability of entodinium caudatum, entodinium exiguum, epidinium caudatum, and ophryoscolex purkynjei *in vitro*. J. Eukaryot. Microbiol. 52:339–342.

Deinum and Maassen, A., B. 1994. Effects of drying temperature on chemical composition and *in vitro* digestibility of forages. Anim. Feed Sci. Technol. 46:75–86.

Delgado-Andrade, C., I. Seiquer, R. Nieto, and M. P. Navarro. 2004. Effects of heated glucose–lysine and glucose–methionine model-systems on mineral solubility. Food Chem. 87:329–337.

Dentinho, M. T. P., A. T. Belo, and R. J. B. Bessa. 2014. Digestion, ruminal fermentation and microbial nitrogen supply in sheep fed soybean meal treated with Cistus ladanifer L. tannins. Small Rumin. Res. 119:57–64.

De Oliveira B. G., T. T. Berchiell, M. S. Pedreira, O. Primavesi, R. Frighetto, M. A. Lima. 2007. Effect of tannin levels in sorghum silage and concentrate supplementation on apparent digestibility and methane emission in beef cattle. Anim. Feed Sci. Technol. 135:236–248.

De Oliveira B. G., T. T. Berchiell, R. A. Reis, , M. E. Vechetini, M. S. Pedreira. 2009. Fermentative characteristics and aerobic stability of sorghum silages containing different tannin levels. Anim. Feed Sci. Technol. 154:1–8.

Dewhurst, R., D.0 Davies, and R. Merry. 2000. Microbial protein supply from the rumen. Anim. Feed Sci. Technol. 85:1–21.

Dijkstra, J., J. L. Ellis, E. Kebreab, A. B. Strathe, S. Lopez, J. France, and A. Bannink. 2012. Ruminal pH regulation and nutritional consequences of low pH. Anim. Feed Sci. Technol. 172:22–33.

Doiron, K., P. Yu, J. J. McKinnon, and D. A. Christensen. 2009. Heat-induced protein structure and subfractions in relation to protein degradation kinetics and intestinal availability in dairy cattle. J. Dairy Sci. 92:3319–3330.

Douglas, G. B., W. Stienzen, G. C. Waghorn, and A. G. Foote. 1999. effect of condensed tannins in birdsfoot trefoil (*Lotus corniculatus*) and sulla (*Hedysarum coronarium*) on body weight, carcass foot depth and wool growth of lambs in New Zealand. New Zeal. J. Agric. Res. 42:55–64.

Dschaak, C. M., C. M. Williams, M. S. Holt, J. S. Eun, A. J. Young, and B. R. Min. 2011. Effects of supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk production of lactating dairy cows. J. Dairy Sci. 94:2508–2519.

EBLEX, 2011. Making grass silage for better results. From: http://beefandlamb.ahdb.org.uk/wp/wp-content/uploads/2013/06/ Manual-5-makinggrasssilageforbetterreturns070211.pdf. Access 13/09/2015

Edmunds, B., K. H. Sudekum, H. Spiekers, and F. J. Schwarz. 2012. Estimating ruminal crude protein degradation of forages using *in situ* and *in vitro* techniques. Anim. Feed Sci. Technol. 175:95–105.

EFSA. 2012. Guidance for the preparation of dossiers for zootechnical additives. EFSA panel on additives and products of substances used in animal feed (FEEDAP). Parma, Italy. EFSA J.10:2536. From: http://www.efsa.europa.eu/en/search/doc/2536.pdf. Access 04/05/2015.

Endres, M. I., and M. D. Stern. 1993. Effect of pH and diets containing various levels of lignosulfonate-treated soybean meal on microbial fermentation in continuous culture. J. Dairy Sci. 76:177–185.

Enemark, J. M. D. 2008. The monitoring, prevention and treatment of sub-acute ruminal acidosis (SARA): a review. Vet. J. 176:32–43.

Entec. 1998. Home growth protein for animal feeds. A technical report to Ministry of Agriculture Fisheries and Food, Scottish Office Agriculture, Environment and Fisheries Department, Milk Development Council, Meat and Livestock Commission.

Eryavuz, A., and B. A. Dehority. 2004. Effect of Yucca schidigera extract on the concentration of rumen microorganisms in sheep. Anim. Feed Sci. Technol. 117:215–222.

European Commission directive. 2001. EC directive, 999/2001. Regulation (EC) No. 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. Off. J. Eur. Communities: L 147/1–L 147/40.

European Commission directive. 2003. EC directive, 1831/2003. Regulation (EC) No 1831/2003 of the Eurpean Parliament and of the council of 22 September 2003 on additives for use in animal nutrition. Off. J. Eur. Communities:L268/29–L268–43.

FARM, and BRIEF. 2014. UK feed prices. From: http://www.dairyco.org.uk/market-information/farm-expenses/feed-prices/uk-feed-prices/#.VJRT4aAI84. Access 19/12/2014.

Firkins, J. L., Z. Yu, and M. Morrison. 2007. Ruminal Nitrogen Metabolism: Perspectives for Integration of Microbiology and Nutrition for Dairy. J. Dairy Sci. 90, Supple:E1–E16.

Fitzgerald, J. J. 1996. Grass silage as a basic feed for store lambs. 2. Effect of harvesting system and chop length of grass silage on silage intake and performance of store lambs. Grass Forage Sci. 51:378–388.

FOSS. 1987. Determination of total crude fat in plant materails and feeding stuff with the soxtec hydrolyzing system. Appl. note 92/:87.

Franzolin, R., F. P. Rosales, and W. V. Bã´. Soares. 2010. Effects of dietary energy and nitrogen supplements on rumen fermentation and protozoa population in buffalo and zebu cattle. Rev. Bras. Zootec. 39:549–555.

Frutos, P., G. Hervas, F. J. Giráldez, and A. R. Mantecón. 2004. Review. Tannins and ruminant nutrition. Spn. J. of Agric. Res. 2:191-202

Gamage, I. H., A. Jonker, D. A. Christensen, and P. Yu. 2012. Metabolic characteristics of proteins and biomolecular spectroscopic profiles in different batches of feedstock (wheat) and their co-products (wheat distillers dried grains with solubles) from the same bioethanol processing plant. J. Dairy Sci. 95:6695–6715.

Getachew, G., H. P. S. Makkar, and K. Becker. 2000. Tannins in Tropical Browses: Effects on *in vitro* microbial fermentation and microbial protein synthesis in media containing different amounts of nitrogen. J. Agric. Food Chem. 48:3581–3588.

Getachew, G., W. Pittroff, D. H. Putnam, A. Dandekar, S. Goyal, and E. J. DePeters. 2008. The influence of addition of gallic acid, tannic acid, or quebracho tannins to alfalfa hay on *in vitro* rumen fermentation and microbial protein synthesis. Anim. Feed Sci. Technol. 140:444–461.

Giannenas, I., J. Skoufos, C. Giannakopoulos, M. Wiemann, O. Gortzi, S. Lalas, and I. Kyriazakis. 2011. Effects of essential oils on milk production, milk composition, and rumen microbiota in Chios dairy ewes. J. Dairy Sci. 94:5569–77.

Giner-Chavez, B. I. 1996. Condensed tannin in tropical forages. Ph.D. Thesis. Cornell University, Ithaca, NY, USA.

Givens, D. I., and H. Rulquin. 2004. Utilisation by ruminants of nitrogen compounds in silage-based diets. Anim. Feed Sci. Technol. 114:1–18.

Gladine, C., E. Rock, C. Morand, D. Bauchart, and D. Durand. 2007. Bioavailability and antioxidant capacity of plant extracts rich in polyphenols, given as a single acute dose, in sheep made highly susceptible to lipoperoxidation. Br. J. Nutr. 98:691–701.

Goering and Van Soest, P.J., H. K. 1979. Forage fibre analysis. In: United States Department of Agriculture Handbook. Pp. 379.

Gosselink, J. M. J., J. P. Dulphy, C. Poncet, J. Aufrere, S. Tamminga, and J. W. Cone. 2004a. Rumen escape nitrogen from forages in sheep: comparison of *in situ* and *in vitro* techniques using *in vivo* data. Anim. Feed Sci. Technol. 116:35–51.

Gosselink, J. M. J., J. P. Dulphy, C. Poncet, M. Jailler, S. Tamminga, and J. W. Cone. 2004b. Prediction of forage digestibility in ruminants using *in situ* and *in vitro* techniques. Anim. Feed Sci. Technol. 115:227–246.

Grabber, J. H., and W. K. Coblentz. 2009. Polyphenol, conditioning, and conservation effects on protein fractions and degradability in forage legumes all rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including p. Crop Sci. 49:1511–1522.

Gray, J. I., E. A. Gomaa, and D. J. Buckley. 1996. Oxidative quality and shelf life of meats. Meat Sci. 43:111–123.

Guo, X. S., D. J. Undersander and D. K. Combs. 2008. Effect of Lactobacillus inoculants and forage dry matter on the fermentation and aerobic stability of ensiled mixed-crop tall fescue and meadow fescue. J. Dairy Sci. 96:1735–1744.

Hart, K. J., L. A. Sinclair, R. G. Wilkinson, and J. A. Huntington. 2012. Effect of whole-crop pea (Pisum sativum L.) silages differing in condensed tannin content as a substitute for grass silage and soybean meal on the performance, metabolism, and carcass characteristics of lambs. J. Anim. Sci. 89:3663–3676.

Hart, K. J. 2005. Evaluation of Ensiled Whole-crop Legumes for Ruminants. Ph.D. thesis. Harper Adams University College. Shorpshire, UK.

Hartnell, G. F., and L. D. Satter. 1979. Determination of rumen fill, retention time and ruminal turnover rates of ingesta at different stages of lactation in dairy cows. J. Anim. Sci. 48:381–392.

Haslam, E. 1981. Vegetable Tannins. The biochemistry of plants. Vol. 7. Academic Press, New York. Pp.: 527-554.

Heendeniya, R. G., D. A. Christensen, D. D. Maenz, J. J. McKinnon, and P. Yu. 2012. Protein fractionation byproduct from canola meal for dairy cattle. J. Dairy Sci. 95:4488–4500.

Hervas, G., P. Frutos, F. Javier Giraldez, A. R. Mantecón, and M. C. Del Pino. 2003. Effect of different doses of quebracho tannins extract on rumen fermentation in ewes. Anim. Feed Sci. Technol. 109:65–78.

HGCA. 2003. The nutrative value for livestock of UK oilseed rape and rapeseed meal. Research review No OS14.

Hobson, P. N., R. J. Wallace, and M. P. Bryant. 1982. Microbial Ecology and Activities in the Rumen: Part I. Crit. Rev. Microbiol. 9:165–225.

Howie, S. A., S. Calsamiglia, and M. D. Stern. 1996. Variation in ruminal degradation and intestinal digestion of animal byproduct proteins. Anim. Feed Sci. Technol. 63:1–7.

Hristov, A. N., and S. G. Sandev. 1998. Proteolysis and rumen degradability of protein in alfalfa preserved as silage, wilted silage or hay. Anim. Feed Sci. Technol. 72:175–181.

Huhtanen, P., M. Rinne, and J. Nousiainen. 2007. Evaluation of the factors affecting silage intake of dairy cows: a revision of the relative silage dry-matter intake index. Animal 1:758-770.

Huntington J. A. and D. I. Givens. 1995. The *in situ* technique for the studying the rumen degradation of feeds: A review of procedure. Nutr. Abstr. Rev. Enviroment 65:63–93.

Huntington J. A. and D. I. Givens. 1998. The effect of host diet on the gas production profile of hay and high-temperature dried grass. Anim. Sci. 67:59–64.

Hurrell, R. F., and R. A. Finot. 1985. Effect of food processing on protein digestubility and amino acid availability. In: Digesibility and amino acid availability in aereal and oilseeds. J.W.Finley and D.T. Hopkins, American Association of Cereal Chemists, St. Paul, Minn. Pp.233-258.

Hymes-Fecht U. C., G. A. Broderick, R. E. Muck and J. H. Grabber. 2013. Replacing alfalfa or red clover silage with birdsfoot trefoil silage in total mixed rations increases production of lactating dairy cows1. J. Dairy Sci. 96:460–469.

International Dairy Federation. 1991. Fat content in milk—Butyrometric method. Standard 152. Brussels, Belgium.

Jezierny, D., R. Mosenthin, and E. Bauer. 2010. The use of grain legumes as a protein source in pig nutrition: A review. Anim. Feed Sci. Technol. 157:111–128.

Jezierny, D., R. Mosenthin, N. Sauer, S. Roth, H. P. Piepho, M. Rademacher, and M. Eklund. 2011. Chemical composition and standardised ileal digestibilities of crude protein and amino acids in grain legumes for growing pigs. Livest. Sci. 138:229–243.

Jones, B. A. and J.L. Mangan. 1977. Complexes of the condensed tannins of sainfoin with fraction 1 leaf and with sub-maximally mucoprotein, and their reversal by polyethylene glycol and pH. J. Sci. Food Agric. 28:126–136.

Jones G. A., T.A. McAllister, A. D. Muir, and K. J. Cheng. 1994. Effects of sainfoin (*Onobrychis viciifolia Scop*.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria . Appl. Enviromental Microbiol. 60:1374–1378.

Kamalak, A., O. Canbolat, Y. Gurbuz, and O. Ozay. 2005. Protected protein and amino acids in ruminant nutrition. J. Sci. Eng. 8:84–88.

Katiki, L. M., J. F. S. Ferreira, J. M. Gonzalez, A. M. Zajac, D. S. Lindsay, A. C. S. Chagas, and A. F. T. Amarante. 2013. Anthelmintic effect of plant extracts containing condensed and hydrolyzable tannins on Caenorhabditis elegans, and their antioxidant capacity. Vet. Parasitol. 192:218–227.

Kirchhof S., I. Esner, M. Gierus, and K.H. Südekum. 2010. Variation in the contents of crude protein fractions of different forage legumes during the spring growth . Grass Forage Sci. 65:376–382.

Kirchhof, S. 2007. Research into the kinetics of ruminal *in situ* nutrient degradation of grassland forage from Alpine regions harvested at different stages of maturity and of maize silage and hay - a contribution towards improvements of ration formulation for dairy cows. Ph.D. thesis. Christian-Albrechts-Universität Kiel, Germany. Cited by Edmunds *et al.* (2012)

Knaus, W. F., D. H. Beermann, T. F. Robinson, D. G. Fox, and K. D. Finnerty. 1998. Effects of a dietary mixture of meat and bone meal, feather meal, blood meal, and fish meal on nitrogen utilization in finishing Holstein steers. J. Anim. Sci. 76:1481–1487.

Kohn, R. A., and M. S. Allen. 1995. Effect of plant maturity and preservation method on *in vitro* protein degradation of forages. J. Dairy Sci. 78:1544–1551.

Kopencny, J., and R. J. Wallace. 1982. Cellular location and some properties of proteolytic enzymes of rumen bacteria. Appl. Enviromental Microbiol. 43:1026–1033.

Kramer, M., P. Lund, and M. R. Weisbjerg. 2013. Rumen passage kinetics of forage- and concentrate-derived fiber in dairy cows. J. Dairy Sci. 96:3163–3176.

Krause, K. M., and G. R. Oetzel. 2006. Understanding and preventing subacute ruminal acidosis in dairy herds: A review. Anim. Feed Sci. Technol. 126:215–236.

Kroll, J., and H. M. Rawel. 2001. Reactions of Plant Phenols with Myoglobin: Influence of Chemical Structure of the Phenolic Compounds. J. Food Sci. 66:48–58.

Kroll, J., H. M. Rawel, and N. Seidelmann. 2000. Physicochemical properties and susceptibility to proteolytic digestion of myoglobin-pohenol derivatives. J. Agric. Food Chem. 48:1580–1587.

Kruege, W.K., H. Gutierrez-Banuelos, G.E. Carstensa,b, B.R. Min, W.E. Pinchakd, R.R. Gomezb, R.C. Anderson, N.A. Kruegerc, T.D.A. Forbese. 2010. Effects of dietary tannin source on performance, feed efficiency, ruminal fermentation, and carcass and non-carcass traits in steers fed a high-grain diet. Anim. Feed Sci. Technol. 159:1–9.

Kumar, R., and M. Singh. 1984. Tannins: Their Advers Role in Ruminant Nutrition. J. Agric. Food Chem. 32:447–453.

Kumar, R., and S. Vaithiyanathan. 1990. Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. Anim. Feed Sci. Technol. 30:21–38.

Lamy, E., H. Rawel, F. J. Schweigert, F. Capela e Silva, A. Ferreira, A. R. Costa, C. Antunes, A. M. Almeida, A. V. Coelho, and E. Sales-Baptista. 2011. The Effect of Tannins on Mediterranean Ruminant Ingestive Behavior: The Role of the Oral Cavity. Molecules 16:2766–2784.

Lana, R. P., J. B. Russell, and M. E. Van Amburg. 1998. The role of pH in regulating ruminal growth in the rumen. J. Anim. Sci. 76:2190–2196.

Lanzas, C. 2007. Models to predict ruminal carbohydrate and nitrogen supply and nitrogen excretion in cattle. Cornell University, Ithaca, USA. Pp. 84-115.

Lanzas L.O., Seo, S. and Fox, D.G., A. T. 2007. Evaluation of protein fractionation systems used in formulating rations for dairy cattle. J. Dairy Sci. 90:507–521.

Lavrencic, A., and A. Levar. 2006. Effect of tannin on grass silage composition. KRMIVA 48:87–93.

Lean, I. J., H. M. Golder, and M. B. Hall. 2014. Feeding, evaluating, and controlling rumen function. Vet. Clin. North Am. Food Anim. Pract. 30:539–575.

Lee, M. R. F., A. L. Winters, N. D. Scollan, R. J. Dewhurst, M. K. Theodorou, and F. R. Minchin. 2004. Plant-mediated lipolysis and proteolysis in red clover with different polyphenol oxidase activities. J. Sci. Food Agric. 84:1639–1645.

Leng, R. A., and J. V Nolan. 1984. Nitrogen metabolism in the rumen. J. Dairy Sci. 67:1072–1089.

Lewis, D. 1957. Blood-urea concentration in relation to protein utilization in the ruminant. J. Agric. Sci. 48:438–446.

Licitra Hernandez, T. M., Van Soest, P. J., G. 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57:347–358.

Licitra, G., P. J. Van Soest, I. Schadt, S. Carpino and C. J. Sniffen. 1999. Influence of the concentration of the protease from Streptomyces griseus relative to ruminal protein degradability. Anim. Feed Sci. Technol. 77:99–113.

Lindberg, J. E. 1982. Ruminal flow rate of soya-bean meal, rapeseed meal and cottonseed meal in cows fed at maintenance and at three times maintenance. J. Agric. Sci. 98:689–691.

Liu, H., V. Vaddella, and D. Zhou. 2011. Effects of chestnut tannins and coconut oil on growth performance, methane emission, ruminal fermentation, and microbial populations in sheep. J. Dairy Sci. 94:6069–6077.

Liu, H. W., D. W. Zhou, and K. Li. 2013. Effects of chestnut tannins on performance and antioxidative status of transition dairy cows. J. Dairy Sci. 96:5901–5907.

Loerch, S. C., L. L. Berger, S. D. Plegge, and G. C. Fahey. 1983. Digestibility and rumen escape of soybean meal, blood meal, meat and bone meal and dehydrated alfalfa nitrogen. J. Anim. Sci. 57:1037–1047.

Lorenz, M.M, T. Eriksson and P. Udén. 2010. Effect of wilting, silage additive, PEG treatment and tannin content on the distribution of N between different fractions after ensiling of three different sainfoin (Onobrychis viciifolia) varieties. Grass Forage Sci. 65 :175–184.

Lorenz, M. M., L. Karlsson, M. Hetta and P. Udén. 2011. Recycling of microbial N and estimation of protein degradation by *in vitro* gas production. Anim. Feed Sci. Technol. 170:111-116.

Lorenz, M. M., and P. Uden. 2011. Influence of formic acid and dry matter on protein degradation in the tanniniferous legume sainfoin. Anim. Feed Sci. Technol. 164:217–224.

Lorenz, M. M. 2011. Sainfoin tannins and their impact on protein degradation during silage and rumen fermentation and testing of novel techniques. Ph.D. thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Lowman, R. S., M. K. Theodorou, and D. Cuddeford. 2002. The effect of sample processing on gas production profiles obtained using the pressure transducer technique. Anim. Feed Sci. Technol. 97:221–237.

Luciano, G., F. J. Monahan, V. Vasta, L. Biondi, M. Lanza, and A. Priolo. 2009. Dietary tannins improve lamb meat colour stability. Meat Sci. 81:120–125.

Lynch, G. L., L. L. Berger, and J. R. Fahey. 1987. Effects of ethanol, heat and lipid treatment of soybean meal on nitrogin utilization by ruminants. J. Dairy Sci. 70:91–97.

MAFF. 1986. The analysis of agriculture materail; 3rd Edition. London, HMSO.

Makkar, H. P. S., and K. Becker. 1997. Degradation of quillaja saponins by mixed culture of rumen microbes. Lett. Appl. Microbiol. 25:243–245.

Makkar, H. P. S., M. Blümmel, N. K. Borowy, and K. Becker. 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric. 61:161–165.

Makkar, H. P. S., P. Siddhuraju, and K. Becker. 2007. Plant Secondary Metabolites. Humana Press Inc. LLC, Totoawa, New Jersey, USA. p. 67-91.

Makkar, H. P. S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rumin. Res. 49:241–256.

Mangan, J. L., D. J. Jordan, J. West, and P. J. Webb. 1980. Protection of leaf protein of lucerne (*Medicago sativa*) against degradation in the rumen by treatment with formaldehyde and glutaraldehyde. J. Agric. Sci. 95:603–617.

Marley, C.L., R. Fychan, M.D. Sanderson, and R. Jones. 2007. Effect of feeding different ensiled forages on the productiveity and nutrient-use efficiency of finishing lambs. Grass Forage Sci. 62:1-12.

Mayer, A. M. 2006. Polyphenol oxidases in plants and fungi: Going places? A review. Phytochemistry 67:2318–2331.

Mayne, C. S., and F. J. GORDON. 1986. Effect of harvesting system on nutrient losses during silage making. 2. In-silo losses. Grass Forage Sci. 41:341–351.

McAllan, A. B., and R. H. Smith. 1983. Estimation of flows of organic matter and nitrogen components in postruminal digesta and effects of level of dietary intake and physical form of protein supplement on such estimates. Br. J. Nutr. 49:119–127.

McAllan, A. B., J. D. Sutton, D. E. Beever, and D. J. Napper. 1994. Rumen fermentation characteristics and duodenal nutrient flow in lactating cows receiving two types of grass silage with two levels of concentrates. Anim. Feed Sci. Technol. 46:277–291.

McDonald, I.M. 1981. A revised model for the estimation of protein degradability in rumen. J. Agric. Sci. (Cambridge). 96:251-252.

McDonald, P., and R. . Edwards. 1976. The influence of conservation Methods on digestion and utilization of forages by rumnants. Proc. Nutr. Soc. 33:201–211.

McDonald, P., R.A. Edwards, J.F.D. Greenhalgh, C.A. Morgan, L.A. Sinclair, and R.G. Wilkinson. 2011. Animal nutrition. Seventh Edition, Ashord Colour Press Ltd., Gosport, UK. Pp. 641-645.

McDonald, P., and A. R. Henderson. 1962. Buffering capacity of herbag samples as the factor in ensiling. J. Sci. Food Agric. 13:395–400.

McMahon, L. R., T. A. McAllister, B. P. Berg, W. Majak, S. N. Acharya, J. D. Popp, B. E. Coulman, Y. Wang, and K. J. Cheng. 2000. A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. Can. J. Plant Sci. 80:469–485.

McSweeney, C. S., B. Palmer, D. M. McNeill, and D. O. Krause. 2001. Microbial interactions with tannins: nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91:83–93.

Merry and Davies D.R., R. J. 1999. Propionibacteria and their role in the biological control of aerobic spoilage in silage. 2nd International Symposium on Propionibacteria: 79:149–164.

Merry, R. J., R. Jones, and M. K. Theodorou. 2001. Alternative forages--back to the future. Biologist (London). 48:30–34.

Messman, M. A., W.P. Weiss and K.A. Albrecht. 1996. *In situ* disappearance of individual proteins and nitrogen from legume forages containing varying amounts of tannins . J. Dairy Sci. 79:1430–1435.

Miller, E. L. 1973. Evaluation of foods as sources of nitrogen and amino acids. Proc Nutr Soc 32:79–84.

Min, B. R., G. T. Attwood, K. Reilly, W. Sun, J. S. Peters, T. N. Barry, and W. C. McNabb. 2002. *Lotus corniculatus* condensed tannins decrease *in vivo* populations of proteolytic bacteria and affect nitrogen metabolism in the rumen of sheep. Can. J. Microbiol. 48:911–921.

Min, B. R., T. N. Barry, G. T. Attwood, and W. C. McNabb. 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Anim. Feed Sci. Technol. 106:3–19.

Minho, A. P., I. C. S. Bueno, H. Louvandini, F. Jackson, S. M. Gennari, and A. L. Abdalla. 2008. Effect of Acacia molissima tannin extract on the control of gastrointestinal parasites in sheep. Anim. Feed Sci. Technol. 147:172–181.

MLC. 1988. Sheep imporvement Services: Feeding the Ewe. 3rd ed. Meat and Livestock Commission, Milton Keynes.

Mueller-Harvey, I. 2001. Analysis of hydrolysable tannins. Anim. Feed Sci. Technol. 91:3–20.

Mueller-Harvey, I. 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86:2010–2037.

Mustafa Christensen, D. A., McKinnon, J. J., A. F. 2000. Effects of Pea, Barley, and Alfalfa Silage on Ruminal Nutrient Degradability and Performance of Dairy Cows. J. Dairy Sci. 83:2859–2865.

Naumann, H. D., L. O. Tedeschi, J. P. Muir, B. D. Lambert, and M. M. Kothmann. 2013. Effect of molecular weight of condensed tannins from warm-season perennial legumes on ruminal methane production inÂ vitro. Biochem. Syst. Ecol. 50:154–162.

Nocek, J. E. 1988. *In situ* and Other Methods to Estimate Ruminal Protein and Energy Digestibility: A Review. J. Dairy Sci. 71:2051–2069.

NRC. 1998. Nutrient Requirements of Swine: Tenth Revised Edition.

NRC. 2001. Nutrient Requirements of Dairy Cattle.

Nugent, J. H. A., and J. L. Mangan. 1981. Characteristics of the rumen proteolysis of fraction 1(18S) leaf protein from lucerne (Medicago sativa L.). Br. J. Nutr. 46:39–58.

Ohshima P., and M. McDonald. 1978. A review of the changes in nitrogenous compounds of herbage during ensling. Sci. Food Agric. 29:497–505.

Ørskov E. R. and I. McDonald. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. J. Agric. Sci. 92:499–503.

Paine, C. A., R. Crawshaw, and W. P. Barber. 1982. A complete exchange method for the *in sacco* estimation of rumen degradability on a rotine basis. In Forage protein in ruminant animal production. Occasional publication No. 6. British Society of Animal Production. D.G. Thompson, D.E. Beever and R.G. Gunn,.

Patra, A. K., and J. Saxena. 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71:1198–1222.

Patra, A. K., and J. Saxena. 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J. Sci. Food Agric. 91:24–37.

Patra, A., and J. Saxena. 2009. Dietary phytochemicals as rumen modifiers: a review of the effects on microbial populations. Antonie Van Leeuwenhoek 96:363–375.

Pellikaan, W. F., E. Stringano, J. Leenaars, D. J. G. M. Bongers, S. van L. Schuppen, J. Plant, and I. Mueller-Harvey. 2011. Evaluating effects of tannins on extent and rate of *in vitro* gas and CH4 production using an automated pressure evaluation system (APES). Anim. Feed Sci. Technol. 166-167:377–390.

Peng, Q., N. A. Khan, Z. Wang, and P. Yu. 2014. Relationship of feeds protein structural makeup in common Prairie feeds with protein solubility, *in situ* ruminal degradation and intestinal digestibility. Anim. Feed Sci. Technol. 194:58–70.

Peng, Q., N. A. Khan, Z. Wang, and P. Yu. 2014. Moist and dry heating-induced changes in protein molecular structure, protein subfractions, and nutrient profiles in camelina seeds. J. Dairy Sci. 97:446–457.

Perez-Maldonado, R. A., and B. W. Norton. 1996. The effects of condensed tannins from Desmodium intortum and Calliandra calothyrsus on protein and carbohydrate digestion in sheep and goats. Br. J. Nutr. 76:515–533.

Piluzza, G., L. Sulas, and S. Bullitta. 2014. Tannins in forage plants and their role in animal husbandry and environmental sustainability: a review. Grass Forage Sci. 69:32–48.

Pitt, R. E., J. S. Van Kessel, D. G. Fox, A. N. Pell, M. C. Barry, and P. J. Van Soest. 1996. Prediction of ruminal volatile fatty acids and pH within the net carbohydrate and protein system. J. Anim. Sci. 74:226–244.

Plaizier, J. C., D. O. Krause, G. N. Gozho, and B. W. McBride. 2008. Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. Vet. J. 176:21–31.

Poncet C. and D. Remond. 2002. Rumen digestion and intestinal nutrient flows in sheep consuming pea seeds: the effect of extrusion or chestnut tannin addition . Anim. Res. 51:201–216.

Porter, L. J., L. N. Hrstich, and B. G. Chan. 1985. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 25:223–230.

Priolo, A., G.C. Waghorn, M. Lanza, L. Biondi, and P. Pennisi. 2000. Polythylene glycol as a means of reducing the impact of condensed tannins in carob pulp: Effects on lamb growth performance and meat quality. J. Anim. Sci. 78:810–816.

Puchala, R., B. R. Min, A. L. Goetsch, and T. Sahlu. 2005. The effect of a condensed tannin-containing forage on methane emission by goats1. J. Anim. Sci. 83:182–186.

Purcell, P. J., M. O'Brien, T. M. Boland, M. O'Donovan, and P. O'Kiely. 2011. Impacts of herbage mass and sward allowance of perennial ryegrass sampled throughout the growing season on *in vitro* rumen methane production. Anim. Feed Sci. Technol. 166-167:405–411.

Quin, J. L., J. G. Van der Wath, and S. Myburgin. 1938. Studies on the alimentary tract of merino sheep in south africa. 4. Description of experimental technique. J. Vet. Sci. Anim. Ind. 11:341–360.

Remond, D., M. P. Le Guen, and C. Poncet. 2003. Degradation in the rumen and nutritional value of lupin (*Lupinus albus L.*) seed proteins effect of extrusion. Anim. Feed Sci. Technol. 105:55–70.

Repetto, J. L., C. Cajarville, J. D. Alessandro, A. Curbelo, C. Soto, and D. Garin. 2005. Effect of wilting and ensiling on ruminal degradability of temperate grass and legume mixtures. Anim. Res. 54:73–80.

Robinson, P. H., E. K. Okine, and J. J. Kennelly. 1992. Measurement of Protein Digestion in Ruminant: In Modren Methods in Protein Nutrtion and Metabolism. S. Nissen. Academic Press, Inc. London, UK. Pp. 121-144.

Rodriguez, C. A., J. Gonzalez, M. R. Alvir, and R. Caballero. 2008. Effects of feed intake on *in situ* rumen microbial contamination and degradation of feeds. Livest. Sci. 116:108–117.

Romano-Ely, B. C., M. K. Todd, M. J. Saunders, and T. S. Laurent. 2006. Effect of an isocaloric carbohydrate-protein-antioxidant drink on cycling performance. Med. Sci. Sports Exerc. 38:1608–1616.

Russell, J. B., and C. J. Sniffed. 1984. Effect of carbon-4 and carbon-5 volatile fatty acids on growth of mixed rumen bacteria *in vitro*. J. Dairy Sci. 67:987–994.

Salawu, M. B., T. Acamovic, C. S. Stewart, T. Hvelplund, and M. R. Weisbjerg. 1999. The use of tannins as silage additives: effects on silage composition and mobile bag disappearance of dry matter and protein. Anim. Feed Sci. Technol. 82:243–259.

Salawu, M. B., E. H. Warren, and A. T. Adesogan. 2001. Fermentation characteristics, aerobic stability and ruminal degradation of ensiled pea/wheat bi-crop forages treated with two microbial inoculants, formic acid or quebracho tannins. J. Sci. Food Agric. 81:1263–1268.

Samadi, and P. Yu. 2011. Dry and moist heating-induced changes in protein molecular structure, protein subfraction, and nutrient profiles in soybeans. J. Dairy Sci. 94:6092–6102.

Saro, C., M. J. Ranilla, M. L. Tejido, and M. D. Carro. 2014. Influence of forage type in the diet of sheep on rumen microbiota and fermentation characteristics. Livest. Sci. 160:52–59.

Schofield, P., D. M. Mbugua, and A. N. Pell. 2001. Analysis of condensed tannins: a review. Anim. Feed Sci. Technol. 91:21–40.

Shannak, S., K. H. Sudekum, and A. Susenbeth. 2000. Estimating ruminal crude protein degradation with *in situ* and chemical fractionation procedures. Anim. Feed Sci. Technol. 85:195–214.

Shete, S. M., S. K. Tomar, S. K. Sirohi, and B. Singh. 2011. Plant secondary metabolites as rumen fermentation modifiers. a review. Agric. Rev. 32:1–13.

Sinclair, K. D., P. C. Garnsworthy, G. E. Mann, and L. A. Sinclair. 2014. Reducing dietary protein in dairy cow diets: implications for nitrogen utilization, milk production, welfare and fertility. Animal 8:262–74.

Sinclair, L.A., P.C. Garnsworthy, J. R. Newbold, and P.J. Buttery.1993. Effect of synchronizing the rate of dietary energy and nitrogen release on rumen fermentation and microbial protein synthesis in sheep. J. Agric Sci. (Cambridge).120: 251-263.

Sinclair, L. A., K. J. Hart, R. G. Wilkinson, and J. A. Huntington. 2009. Effects of inclusion of whole-crop pea silages differing in their tannin content on the performance of dairy cows fed high or low protein concentrates. Livest. Sci. 124:306–313.

Sklan, D. 1989. *in vitro* and *in vivo* rumen protection of proteins coated with calcium soaps of long-chain fatty acids. J. Agric. Sci. 112:79–83.

Śliwiński, B. J., C. R. Soliva, A. Machmüller, and M. Kreuzer. 2002. Efficacy of plant extracts rich in secondary constituents to modify rumen fermentation. Anim. Feed Sci. Technol. 101:101–114.

Sniffen C. J., J.D. O'Connor, P. J. Van Soest, D. G. Fox, and J. B. Russell. 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. J. Anim. Sci. 70:3562–3577.

Solaiman, S., J. Thomas, Y. Dupre, B. R. Min, N. Gurung, T. H. Terrill, and G. F. W. Haenlein. 2010. Effect of feeding sericea lespedeza (*Lespedeza cuneata*) on growth performance, blood metabolites, and carcass characteristics of Kiko crossbred male kids. Small Rumin. Res. 93:149–156.

Speijers, M. H. M., M. D. Fraser, V. J. Theobald, and W. Haresign. 2005. Effects of ensiled forage legumes on performance of store finishing lambs. Anim. Feed Sci. Technol. 120:203–216.

Stern, M. D., A. Bach, and S. Calsamiglia. 2006. New Concepts in Protein Nutrition of Ruminants. In: 21st Annual Southwest Nutrition & Management Conference. Vol. 45-66.

Storm, E., and E. R. Orskov. 1983. The nutritive value of rumen micro-organisms in ruminants. 1. Large-scale isolation and chemical composition of rumen micro-organisms. Br. J. Nutr. 50:463–470.

Strobel, H. J., and J. B. Russell. 1986. Effect of pH and energy spilling on bacterial protein synthesis by carbohydrate-limited cultures of mixed rumen bacteria. J. Dairy Sci. 69:2941–2947.

Tabacco, E., G. Borreani, G. M. Crovetto, G. Galassi, D. Colombo, and L. Cavallarin. 2006. Effect of Chestnut Tannin on Fermentation Quality, Proteolysis, and Protein Rumen Degradability of Alfalfa Silage. J. Dairy Sci. 89:4736–4746.

Tamminga, S., C. J. Van Der Koelen, and A. M. Van Vuuren. 1979. Effect of the level of feed intake on nitrogen entering the small intestine of dairy cows. Livest. Prod. Sci. 6:255–262.

Tamminga, S. 1979. Protein degradation in the forestomachs of ruminants. J. Anim. Sci. 49:1615–1630.

Tamminga, S. 1986. Utilization of naturally occurring NPN-compounds by ruminants. Arch Tierernahr 36:169–175.

Tan, H. Y., C. C. Sieo, N. Abdullah, J. B. Liang, X. D. Huang, and Y. W. Ho. 2011. Effects of condensed tannins from Leucaena on methane production, rumen fermentation and populations of methanogens and protozoa *in vitro*. Anim. Feed Sci. Technol. 169:185–193.

Tavendale, M. H., L. P. Meagher, D. Pacheco, N. Walker, G. T. Attwood, and S. Sivakumaran. 2005. Methane production from *in vitro* rumen incubations with *Lotus pedunculatus* and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. Anim. Feed Sci. Technol. 123-124, :403–419.

Terrill, T. H., G. B. Douglas, A. G. Foote, R. W. Purchas, G. F. Wilson, and T. N. Barry. 1992. Effect of condensed tannins upon body growth, wool growth and rumen metabolism in sheep grazing sulla (*Hedysarum coronarium*) and perennial pasture. J. Agric. Sci. 119:265–273.

Terrill, T. H., G. C. Waghorn, D. J. Woolley, W. C. Mcnabb, and T. N. Barry. 1994. Assay and digestion of C-labelled condensed tannins in the gastrointestinal tract of sheep. Br. J. Nutr. 72:467–477.

Theodoridou, K., and P. Yu. 2013. Application potential of ATR-FT/IR molecular spectroscopy in animal nutrition. In: Revelation of protein molecular structures of canola meal and presscake, as affected by heat-processing methods, in relationship with their protein digestive behavior and utili. J. Agric. Food Chem. 61:5449–5458.

Theodorou, M. K. ., and J. France. 2005. Rumen mircoorganisms and their interaction, In: Qantitative Aspects of Ruminant Digestion and Metabolism. J. Dijkstra, J.M. Fobes and J. France. CABI Publishing. Oxford. UK. Pp.207-228

Theodorou, M. K., B. A. Williams, M. S. Dhanoa, A. B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48:185–197.

Tomankova, O., and J. Kopecny. 1995. Prediction of feed protein degradation in the rumen with bromelain. Anim. Feed Sci. Technol. 53:71–80.

Toral, P. G., G. Hervas, A. Belenguer, E. Bichi, and P. Frutos. 2013. Effect of the inclusion of quebracho tannins in a diet rich in linoleic acid on milk fatty acid composition in dairy ewes. J. Dairy Sci. 96:431–439.

Toral, P. G., G. Hervas, E. Bichi, A. Belenguer, and P. Frutos. 2011. Tannins as feed additives to modulate ruminal biohydrogenation: Effects on animal performance, milk fatty acid composition and ruminal fermentation in dairy ewes fed a diet containing sunflower oil. Anim. Feed Sci. Technol. 164:199–206.

Turner, S. A., G. C. Waghorn, L. S. Woodward, and N. A. Thomson. 2005. Condensed tannins in birdsfoot trefoil (*Lotus corniculatus*) affect the detailed composition of milk from dairy cows. New Zeal. Soc. Anim. Prod. 65:283–288.

Ulyatt M.J., J.A. Lancashire and W.T.Jones. 1976. The nutritive value of legumes. Proc. New Zeal. Soc. Anim. Prod. 38:107–118.

USDA. 2015. Oilseeds: World Markets and Trade. From https://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf. Access :15/04/2015.

Van Keulen, J., and B. A. Young. 1977. Evaluation of Acid-Insoluble Ash as a Natural Marker in Ruminant Digestibility Studies. J. Anim. Sci. 44:282–287.

Van Soest, P. J. 1994. Nutritional Ecology of the Ruminant. In: Cornell University Press, Ithaca, NY. Vol. Chaps 3 an. Pp. 22–39,108–121.

Van Soest P.J., C.J. Sniffen, D.R. Mertens, D.G. Fox, P.H. Robinson and U.C. Kirshnamoorthy.1981. A net protein systems for cattle: The rumen submodel for nitrogen. In: Protein Requirement for Cattle: Proceedings of an International Symposium. F.N. Owen (Ed.). Oklahoma State Univ. Stillater MP 109:285.

Van Straalen, W. M., and S. Tamminga. 1990. Protein degradation of ruminant diets. In: Feedstuff Evaluation. J. Wiseman and D. J. A. Cole, editors. Butterworth-Heinemann. Pp. 55–72.

Varga, G. A., and E. C. Prigge. 1982. Influence of forage species and level of intake on ruminal turnover rates. J. Anim. Sci. 55:1498–1504.

Varvikko, T., and J. E. Lindberg. 1985. Estimation of microbial nitrogen in nylon-bag residues by feed 15N dilution. Br. J. Nutr. 54:473–481.

Velle, W., T. I. Kanui, A. Aulie, and O. V Sjaastad. 1998. Ruminal escape and apparent degradation of amino acids administered intraruminally in mixtures to cows. J. Dairy Sci. 81:3231–3238.

Vidal-Valverde, C., J. Frias, A. Hernandez, P. J. Martin-Alvarez, I. Sierra, C. Rodriguez, I. Blazquez, and G. Vicente. 2003. Assessment of nutritional compounds and antinutritional factors in pea (Pisum sativum) seeds. J. Sci. Food Agric. 83:298–306.

Waghorn, G. C., and W. C. McNabb. 2003. Consequences of plant phenolic compounds for productivity and health of ruminants. Proc. Nutr. Soc. 62:383–392.

Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production-progress and challenges. Anim. Feed Sci. Technol. 147:116–139.

Wang, Y., W. Majak, and T.A. McAllister. 2012. Frothy bloat in ruminants: Cause, occurrence, and mitigation strategies. Anim. Feed Sci. Technol. 172: 103-114.

Warner, D., J. Dijkstra, W. H. Hendriks, and W. F. Pellikaan. 2013. Passage kinetics of 13C-labeled corn silage components through the gastrointestinal tract of dairy cows. J. Dairy Sci. 96:5844–5858.

Waterman, P. G., and S. Mole. 1994. Methods in ecology: Analysis of phenolic plant metabolites. Blackwell Science Publications, Oxford, UK.

Wheeler, W. E., and C. H. Noller. 1977. Gastointestinal tract pH and starch in feces pf ruminants. J. Anim. Sci. 44:131–135.

Wilkins, R. J., and R. Jones. 2000. Alternative home-grown protein sources for ruminants in the United Kingdom. Anim. Feed Sci. Technol. 85:23–32.

Wilkinson, J. M., and D. R. Davies. 2013. The aerobic stability of silage: key findings and recent developments. Grass Forage Sci. 68:1–19.

Williams, C. M., J. S. Eun, J. W. MacAdam, A. J. Young, V. Fellner, and B. R. Min. 2011. Effects of forage legumes containing condensed tannins on methane and ammonia production in continuous cultures of mixed ruminal microorganisms. Anim. Feed Sci. Technol. 166-167:364–372.

Willis, S. 2003. The use of soybean meal and full fat soybean meal bye the animal feed Industry. In: 12th Australian soybean conference. p. 1–8.

Wina, E., S. Muetzel, and K. Becker. 2005. The impact of saponins or saponin-containing plant materials on ruminant production--a review. J. Agric. Food Chem. 53:8093–8105.

Winters, A. L., J. E. Cockburn, M. S. Dhanoa, and R. J. Merry. 2000. Effects of lactic acid bacteria in inoculants on changes in amino acid composition during ensilage of sterile and non-sterile ryegrass. J. Appl. Microbiol. 89:442–452.

Winters, A. L., F. R. Minchin, T. P. T. Michaelson-Yeates, M. R. F. Lee, and P. Morris. 2008. Latent and active polyphenol oxidase (PPO) in red clover (*Trifolium pratense*) and use of a low PPO mutant to study the role of PPO in proteolysis reduction. J. Agric. Food Chem. 56:2817–2824.

Winters, A. L., and F. R. Minchin. 2002. The effect of PPO on the protein content of ensiled red clover. In: Proceeding of the XIIIth international silage conference. L.M. Gechie and C. Thomas. Auchincruive, Scotland. Pp.84-85.

Wohlt, J. E., C. J. Sniffen, W. H. Hoover, L. L. Johnson, and C. K. Walker. 1976. Nitrogen metabolism and wethers as affected by dietary protein solubility and amino acid profile. J. Anim. Sci. 42:1280–1289.

Woodward, S., A. Chaves, G. C. Waghorn, and P. G. Laboyrie. 2002. Supplementing pasture-fed dairy cows with pasture silage, maize silage, Lotus silage or sulla silage in summer-does it increase production? Proc.87–91.

Woodward, S. L., G. C. Waghorn, and P. G. Laboyrie. 2004. condensed tannins in birdsfoot trefoil (*Lotus corniculatus*) reduce methane emission from dairy cows. Proc. New Zeal. Soc. Anim. Prod. 64:160–164.

Yang, C. M., and J. B. Russell. 1992. Resistance of proline-containing peptides to ruminal degradation *in vitro*. Appl Env. Microbiol 58:3954–3958.

Zhang, X., and P. Yu. 2012. Molecular basis of protein structure in combined feeds (hulless barley with bioethanol coproduct of wheat dried distillers grains with solubles) in relation to protein rumen degradation kinetics and intestinal availability in dairy cattle. J. Dairy Sci. 95:3363–3379.

Zhu, J., L. J. Filippich, and M. T. ALsalam. 1992. Tannic acid intoxication in sheep and mice. Res. Vet. Sci. 53:280–892.

Zimmer, N., and R. Cordesse. 1996. Digestibility and ruminal digestion of non-nitrogenous compounds in adult sheep and goats: Effects of chestnut tannins. Anim. Feed Sci. Technol. 61:259–273.