Effects of different forages on production of hydrogen sulphide in a rumen *in vitro* system

by

Katarina Häll-Larsson

Institutionen för husdjurens utfodring och vård

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A cooperation between SLU and IGER (the Institute of Grassland and Environmental Research)

Supervisors: Richard Dewhurst, IGER, UK
Jan Bertilsson, HUV, SLU

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1. Summary

The purpose of this project was to use a new *in vitro* method to estimate rumen protein degradation (proteolysis) in a series of fresh forages and silages. The new *in vitro* technique is less invasive than existing protein degradation techniques (nylon bag incubations in fistulated cows), and is easy to perform. The technique builds on the *in vitro* gas production technique and is based on the fact that sulphides- particularly hydrogen sulphide (H_2S)- are important products from rumen proteolysis.

Four experiments are included in this report and they were set up at IGER, Aberystwyth, U.K. One of the experiments included freeze dried silage and three were conducted with fresh herbage (grasses or legumes). One study looked at effects of different species and varieties of grass (Cocksfoot, Meadow Fescue, Ryegrass, Tall Fescue and Timothy) and the legume *Lotus spp*. Investigations of the effect of wilting time were made by studying ryegrass wilted for 48, 24, 12, 3 and 0 hours. The third study looked at the effects of silage additives. Untreated silage was compared to silage prepared with formic acid, and silages prepared with inoculants containing lactic acid bacteria ('Power Start' or 'Pioneer'). The work also included an investigation of the effects of tannin levels in *Lotus spp*. on the production of H_2S.

In each experiment, the plant material was incubated with rumen fluid for 12 hours. Measurements of H_2S production were made 2, 4, 6, 8 and 12 hours after the start of incubations. The amount of H_2S formed was also compared to the crude protein content of the plant material.

It was shown that *Lotus spp.* produced significantly lower levels of hydrogen sulphide than grasses. In the experiment where the effect of wilting duration was studied, there was no significant difference between samples when the total production of hydrogen sulphide was measured. However, when comparing the amount of H_2S formation to the crude protein content of the plant material, grass wilted for 48 hours had significantly lower H_2S production.

In the experiment comparing different silage additive treatments, the untreated silage had significantly lower production of H_2S than the other silages. When studying the effect of tannins, it was showed that *Lotus,* containing the highest levels of tannins, produced larger amounts of H_2S than the other varieties.

The *in vitro* gas production technique used in this work was easy to work with and some of the results were similar to the literature but unexpected differences were also observed. In the experiments studying the effect of inoculants in silage and the tannin content in *Lotus,* the results differed from the literature. Analysis of sulphides might not be a reliable measurement of protein degradation in the materials because of the reaction of H_2S with hydrogen cyanide produced when cyanogenic tissues are damaged. These results emphasise the importance of further research and evaluations to improve the accuracy of the *in vitro* H_2S production technique.

Sammanfattning

Syftet med detta arbete var att utveckla en *in vitro* metod för att undersöka proteinnedbrytning (protolys) i vommen. Målet är en metod som inte kräver försöksdjur men ändå är lätt att använda och ger säkra resultat. I försöket mättes mängden vätesulfid (H_2S) som bildas i vommen under fermentation av fodermaterial. Anledningen till att H_2S analyserades är baserat på resultat som visar att sulfider är viktiga produkter från protolys i vommen.

I försöket ingick fyra experiment, varav tre med färskt gräs och ett med ensilage. Alla experimenten utfördes vid IGER, Aberystwyth, Storbritannien. Det undersöktes vilken inverkan sorter och arter av växter har på bildningen av vätesulfid. I detta experiment jämfördes olika arter och sorter av gräs (hundäxing, rajgräs, rörsvingel, timotej och ängssvingel) samt ingick baljväxten kärringtand. Påverkan av torkningstid av gräs undersöktes då vätesulfidproduktionen från rajgräs som torkats i 48, 24, 12, 3 eller 0 timmar mättes. I försöket ingick även att studera vilken effekt olika behandlingar av ensilage har på vätesulfidbildning. I detta experiment ingick ensilage utan tillsatsmedel och ensilage som behandlats med myrsyra. Dessutom analyserades ensilage som behandlats med två olika kulturer av mjölksyrabakterier (Power Start ^{TM} och Pioneer). I det fjärde experimentet studerades om olika tanninninnehåll i kärringtand ledde till skillnader i mängd bildad vätesulfid..

I alla experimenten inkuberades växtmaterialet tillsammans med vomvätska i medicinflaskor under 12 timmar. Mätningar av bildad mängd H_2S gjordes 2, 4, 6, 8 och 12 timmar efter att inkubationen påbörjats. Mängden bildad vätesulfid jämfördes även med råproteininnehållet hos de analyserade växterna.

I studien framkom att det var signifikant mindre vätesulfidproduktion från baljväxten kärringtand än från gräs. Det framkom i studien att varianten av kärringtand med högt innehåll av tanniner producerade mer vätesulfid än de andra exemplaren.

Vid jämförelser mellan gräs som torkats under olika lång tid, påvisades inga stora skillnader i den totala bildningen av H_2S men när mängden sattes i relation till råproteininnehållet påvisades signifikant lägre andel vätesulfid i de flaskor med gräs som torkats under 48 timmar.

När effekten av tillsatsmedel i ensilage undersöktes, visades att mindre mängd vätesulfid bildades i flaskorna med ensilage utan tillsatsmedel än för de där tillsatsmedel använts.

Gas *in vitro*-metoden som användes i detta arbete var lätt att utföra och gav i några fall liknande svar som i litteraturen, men även olikheter påvisades. I de experiment som undersökte effekten av tillsatsmedel i ensilage och tanniner i kärringtand, skilde sig resultaten tydligt från litteraturen. Möjligen är inte analys av sulfider en bra måttstock på proteinnedbrytning hos dessa växtmaterial. Dessa resultat understryker vikten av behovet av mer forskning inom området och utveckling av att förbättra noggrannheten hos utrustningen.

2. Introduction

Large areas of land in livestock systems exist as permanent or improved pastures and are sown with forage crops such as grasses and legumes. Grassland-based systems of ruminant production are characterised by the biological inefficiency of converting plant biomass, which is relatively low in protein, into animal products that are relatively high in protein. Ruminant farming generates large quantities of ammonia through an imbalance of release of products from protein degradation in plant biomass, and the availability of energy to rumen micro-organisms. This means that if the supply of energy is limited while there is an excess of peptides and amino acids of plant origin, rumen microbes use amino acids for energy and liberate ammonia through deamination. Ammonia cannot be used by the animal for growth; in fact it is toxic and has to be converted to urea and eliminated in the urine. To overcome the problem with inefficiency of N use, supplementation of protein is common on farms which can exacerbate the problems of pollution of land, water and air (Kingston-Smith and Theodorou, 2000).

Feed protein is extensively degraded by microbial (and plant) enzymes in the rumen, and this process, proteolysis, is very rapid and extensive during the first few hours after feeding. Microbial degradation involves breaking down proteins into peptides and amino acids. Some amino acids are further degraded into organic acids, ammonia (NH_3) and carbon dioxide (CO_2) (Figure 1). Rumen microbes use ammonia, small peptides and free amino acids to synthesise proteins for their own demands (producing 'microbial protein') (Mc Donald *et al.,* 1995).

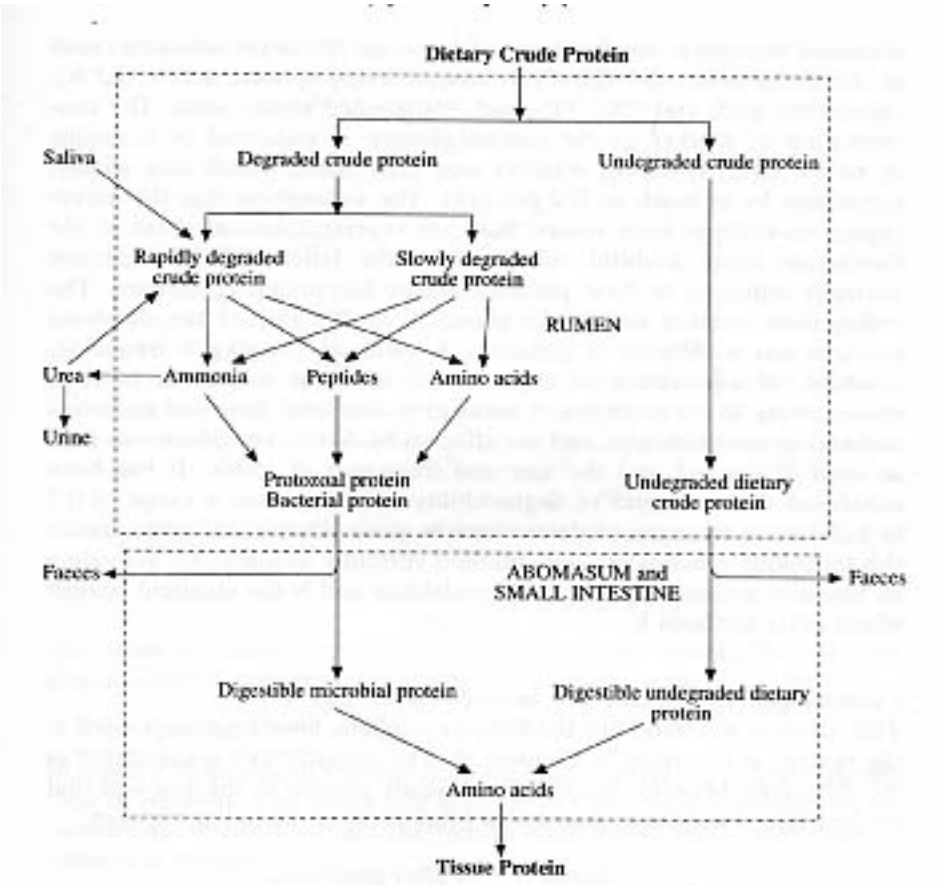

Figure 1. Fate of dietary crude protein in the ruminant animal (from Mc Donald *et al.,* 1995).

N-use efficiency could be improved by increasing the stability of plant protein over the first 2 hours of incubation in the rumen (Nugent and Mangan, 1981). Analysis of proteolysis in the rumen is of interest in order to allow comparison of different feeds and, hopefully, improvements in protein efficiency.

Over the years, there have been a number of *in vitro* and *in vivo* experiments set up to investigate the process of rumen protein degradation (proteolysis) (Beever and Siddons, 1986). The value of these techniques may be limited because they are difficult to accomplish and because of their inability to distinguish effects on a short time period (these are of interest when studying proteolysis). Some of the techniques have a further limitation- being invasive and requiring surgically-modified animals, like fistulated cows (Dewhurst *et al.,* 1995).

To overcome the above mentioned dilemmas, and find a technique that is both easy to use, gives reliable results and is less invasive to animals, it would be interesting to analyse the gas produced in the rumen or breath. Analysis of breath or the rumen headspace gas (the layer of gas placed above the rumen fluid) are relatively easy to perform and less invasive to animals (Bray and Till, 1975).

The objective of this project was to provide missing information to underpin the development of a gas production technique for the study of rumen proteolysis.

This new approach to study protein degradation in fresh herbage is based on the fact that sulphides are important products of rumen proteolysis (Mc Donald *et al.,* 1995).

Sulphur amino acids contain over 90 % of the sulphur in the plant material (Havlin *et al.,* 1999) and by utilising this information, measurements of the level of sulphides will be made to study the dynamics of protein degradation.

3. Literature review

3. 1. Rumen sulphide production

Sulphur is an essential nutrient for ruminants and their microbes. Most of the sulphur in the animal body occurs in proteins containing the amino acids cysteine and methionine. These proteins are involved in e.g. milk production, muscle growth and wool production. Wool is rich in cysteine and contains about 4 % sulphur. Insulin, biotin, thiamine and the important metabolite coenzyme A, also contain sulphur. Sulphur-containing compounds are also important in the respiratory process from haemoglobin through to cytochromes (Mc Donald *et al.,* 1995).

In the process of protein degradation in the rumen methionine and cysteine are catabolised by rumen bacteria to yield ammonia and hydrogen sulphide (Bird and Hume, 1971). Hydrogen sulphide might also originate from inorganic sulphate (Bird and Moir, 1971) but only a small amount of sulphur is present in the body in inorganic form (Mc Donald *et al.,* 1995). Figure 2 illustrates the fate of sulphur in the animal and Figure 3 shows the metabolic pathways in the rumen.

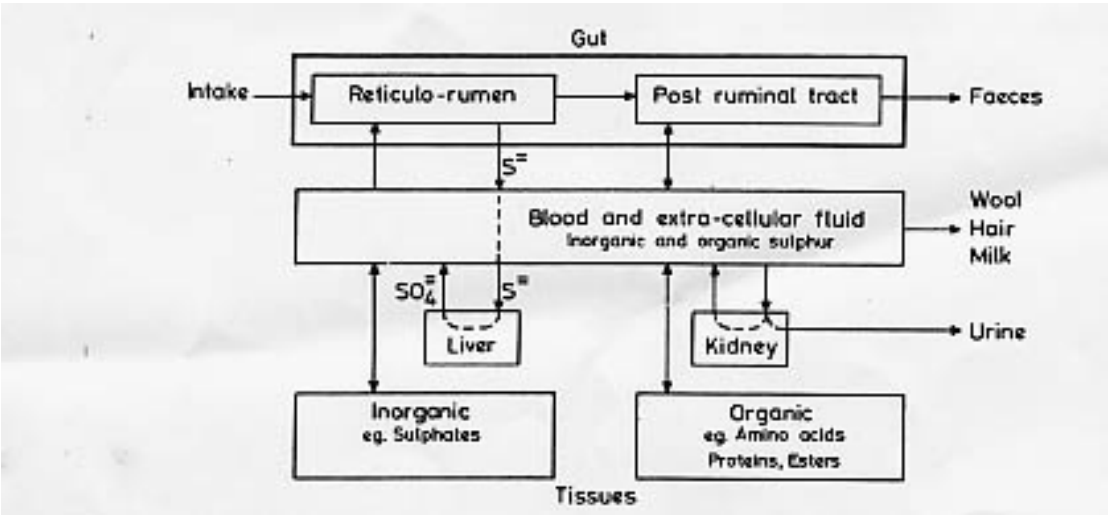

Figure 2. The fate of sulphur in ruminants. Boxes and arrows represent pools and flows of sulphur in the animal (from Bray and Till, 1975).

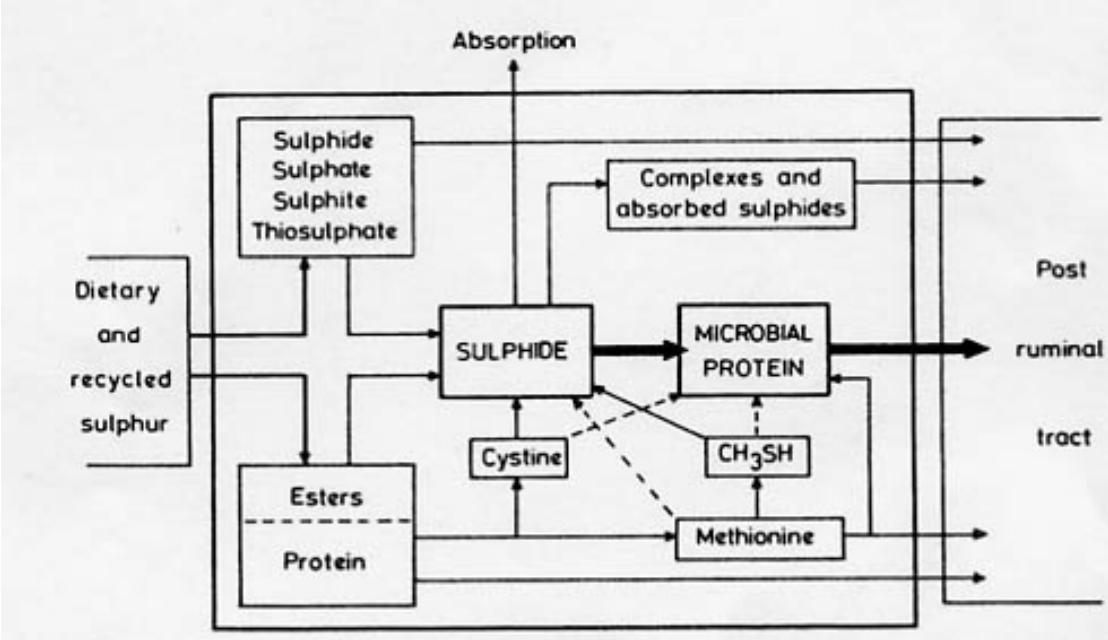

Figure 3. The major pathways of sulphur metabolism in the rumen (from Bray and Till, 1975).

Apart from hydrogen sulphide (up to 200 mg/kg) it has been showed that methanethiol (up to16 mg/kg) and dimethyl sulphide (up to 11 mg/kg) are present in rumen gas (Dewhurst *et al.,* 2001). As reported by Zikakis and Salsbury (1969) methanethiol is produced by degradation of methionine. The origin of dimethyl sulphide is unclear but it has been suggested by Bird (1972) that methanethiol may be oxidised to dimethyl sulphide when it has left the highly reduced environment of the rumen.

Ruminants have a potential for efficient absorption of ingested sulphate due to the micro-organisms in the rumen. Sulphide can be absorbed from the rumen after it has been reduced from inorganic sulphate by the rumen micro-organisms. The absorption rate is dependent on the rumen pH (Bird and Moir, 1971).

The sulphate-reducing bacteria are classified as assimilatory or dissimilatory. Sulphate reducers that are assimilatory generate limited amount of sulphide for immediate metabolic requirements. This sulphate reduction is inhibited by cysteine and other organic sulphur compounds. Dissimilatory sulphate-reducing bacteria are strict anaerobes that use sulphate as a terminal electron acceptor to derive energy for growth and metabolism. Unlike assimilatory sulphate reducers these produce more sulphide than necessary for cell growth and dissimilatory sulphur reduction may be inhibited by the toxic effect of excess sulphide. With higher sulphate rations the capacity of the rumen microbes to reduce sulphate to sulphide increases (Cummings *et al.,* 1995).

In plants, normal concentrations of sulphur range between 0.1-0.5% of dry matter and the main part of the sulphur (approximately 90%) is present in amino acid form (Havlin *et al.,* 1999). Plant protein has an average N:S ratio about 15:1 which is similar to that in tissue protein and milk. For cattle, the desirable N:S ratio in feeds is about 13.5 to 15:1 and about 10 to 13.5:1 for sheep (Bray and Till,1975).

In the breath of ruminants sulphides are present at high levels. The highest value of sulphide in rumen gas tolerated by cattle is 0.1% of the total rumen gas volume. Ruminal sulphide concentration is diet dependent. It is recommended to feed ruminants with a diet with a total sulphur concentration of 0.2- 0.3% (Cummings *et al.,* 1995).

Sulphides are interesting to analyse to address questions about proteolysis. As reported by Bray and Till (1975), the half-life of sulphides in the rumen is short (10-15 minutes) and changes in sulphide concentrations are both rapid and large. These traits provide the sensitivity needed to get important information about the process of protein degradation. A 10-fold increase in the concentration of sulphides in rumen head-space gas within 1 hour of cows consuming grass silage has been demonstrated (Dewhurst *et al.,* 2001).

3.2. The *in vitro* gas production technique

The *in vitro* gas production technique was developed from earlier *in vitro* methods measuring forage digestion. One, frequently used method is the rumen liquor, acid pepsin *in vitro* technique of Tilley and Terry (1963). It involves two main stages. In the first, rumen liquor and a buffer solution is added to dried forage and incubated for 48 hours. In the second step, a pepsin solution is added to the residues and incubated for another 48 hours. Pepsin is added to digest protein that has not been digested after the first stage. This technique measures the dry matter (DM) losses of the feed and it has the ability to monitor the time course of fermentation.

Another common method is the polyester bag technique that can be used to estimate both the rate and extent of disappearance of feed constituents. When using this technique, fistulated animals are used for rumen incubation of polyester bags filled with forage samples. The bags are removed at different times after insertion and the residues can be used for further analysis. This technique is used to provide estimates of rates of disappearance of feed components, such as protein (Mehrez and Orskov, 1977.)

A method to measure the gaseous products, carbon dioxide and methane of microbial metabolism by using computerized pressure sensors, was described by Pell and Schofield (1993). This technique was developed to facilitate the study of the kinetics of forage digestion *in vitro* by measuring gas production. Whilst other, earlier mentioned, *in vitro* methods follow the disappearance of one component of a substrate, gas measurements focuses on the appearance of fermentation products. One advantage with the gas method is that the fermentation products that are monitored are products from both soluble and insoluble substrates. Other methods do not consider the soluble substrates- since these wash out of nylon bags whether or not they are fermentable. It was also reported that the small sample size makes this technique useful with tissue culture samples or synthetic compounds. The method can also be useful to determine the importance of some different feed fractions (monosaccharides, polysaccharides, pectins, starch, cellulose and hemicellulose) in providing energy to the microbes and to determine whether compounds inhibit microbial activity.

The method used in this work was developed by Theodorou *et al.,* (1994) to measure the production of fermentation gas from ruminant feeds inoculated in rumen fluid. The most important equipment in this technique is the gas-tight bottles which are used to hold the rumen *in vitro* fermentations. Gases that are produced from the fermentation of feed samples accumulate in the head-space (the space in the bottle above the rumen fluid), as the fermentation proceeds. By the use of a pressure transducer, the volume and the pressure of the accumulated gas in the bottles can be measured. These data can provide researchers with important information on the kinetics of the analysed feed.

Sulphur is a component of the amino acids cysteine and methionine, and appearance of H_2S is related to the breakdown of proteins that contain these amino acids (Dewhurst, 2002). The level of H_2S was measured in this work as a tool to show the extent of protein degradation in the forages being studied.

3. 3. Effects of plant composition/ characteristics and treatment on proteolysis

3.3.1. Effects of species and varieties

There is a relationship between the chemical and physical characteristics of a plant and its feeding value. To provide a chemical characterisation of forages, the dry matter content of a crop is divided into cell contents and cell walls. Cell walls contain peptic substances, lignin and the structural polysaccharides, hemicellulose and cellulose. The cell contents are comprised of the cell nucleus and cytoplasm. Therefore it accounts for the major part of proteins, peptides, nucleic acids, lipids, sugars and starches in the plant.

Changes in the chemical composition of a crop occur during its stages of growth. As a grass plant matures, the proportion of cell walls increases and the cell content fraction decreases. In contrast, compared with grasses, legumes show less of an increase in cell walls when they mature.

Other factors which influence the chemical composition of herbage include forage species, variety within a species, environment and management of the sward (Holmes, 1989).

Due to the chemical composition, the protein content differs between species and varieties of crops. Timothy, Meadow Fescue and Cocksfoot are characterised by higher cell wall contents than ryegrasses and hence they have a lower level of protein (Holmes, 1989). As reported by Phillips *et al.,* (1999) the crude protein content in Perennial Ryegrass is higher than in Cocksfoot and Timothy. It was showed that the value of crude protein in Perennial Ryegrass was 24.3 % of DM (dry matter), Timothy (19.6 %) and Cocksfoot (19.1%). The crude protein content of *Lotus* is approximately 19.6 % of DM (Spörndly, 1999).

The description of chemical composition of forages doesn't give all the information about their availability to the animal. The rate of protein degradation in the rumen and

the digestibility in the whole digestive tract are of interest to evaluate the feeding value of a crop.

There are differences in digestibility and the rate of protein degradation between species and varieties of grasses and legumes. The grass Cocksfoot is characterised by a lower value of digestibility of its total dry matter content than other grass species due to its high cell wall content (Holmes, 1989). Legumes break down more easily into shorter particles than grasses due to weaker breaking points in their tissue structure (Wilson *et al.,* 1996). The legume *Lotus* contains chemical compounds as tannins and cyanogenic glycosides as defence against herbivores (Goldstein and Spencer, 1985).

3.3.2. Duration of wilting

Cell death in plants is an important mechanism for the survival of the whole organism. Only after cell death has occurred, can proteolysis (protein degradation) start. Availability of active oxygen is a requirement for cell death to occur. Hence when plants are damaged by grazing herbivores or being harvested, there will be oxygen reaching the plant tissue and providing the signal for cell death to begin- which further enables proteolysis (Kingston-Smith and Theodorou, 2000). Hydrolysis of proteins (proteolysis) to peptides and amino acids starts soon after the cutting of the crop. Then amino acids will be degraded to amines, amides and ammonia by microbial deamination and decarboxylation (Charmley *et al.,* 1990). In fresh herbage, 75-90 % of the total nitrogen is present as protein but after harvesting and a few days wilting on the field, the true protein content may be reduced by 50 %. The extent of protein degradation varies with plant species, DM content and temperature (Mc Donald *et al.,* 1995).

Proteolysis associated with plant enzymes (proteases) commences soon after cutting of the fresh grass. Protease activity is required by plants for a wide range of important functions. One of the functions is to dissolve storage protein during seed germination. The proteases are also responsible for the process of protein turnover, the degradation of damaged proteins and for the process of remobilising valuable N during senescence (degenerative process in the life circle of plants) (Kingston- Smith and Theodorou, 2000).

In prior work of Zhu *et al.* (1999) it was hypothesised that proteolysis in grazing ruminants eating fresh forage may be mediated by the proteases within the plant. It was showed that grass incubated anaerobically (39°C over night) with rumen microbes available, had the same reduction in protein content as grass incubated without rumen microbes. These data implies that the plant enzymes were responsible for the major portion of proteolysis.

Hristov and Sandev (1998) reported that wilting of forages would increase the levels of degraded protein (non-protein-N)- through the action of plant proteases.

3.3.3. Effects of silage inoculants

Silage is produced as a result of the fermentation of water- soluble-carbohydrates (WSC) in herbage, particularly by lactic acid bacteria. This fermentation produces mainly lactic acid but also other products (Davies *et al.,* 1998) and a reduced pH value will be obtained by the production of lactic acid (Winters et al., 2000).

A rapid protein breakdown occurs during the process of ensilage (Mc Donald *et al.,* 1995*)* and the amount of degradation can distinctly influence the nutritive value of silage (Winters et al., 2002). There are reports of how the value of non-protein-nitrogen (NPN) compounds such as free amino acids and ammonia, which are products from protein degradation, increases during ensiling. These compounds do not have the same value to the animal as protein that by-pass the rumen digestive process. This protein which is not degraded in the rumen is a potential source of amino acids for absorption in the intestine (Salawu et al., 1999a).

It is generally known that a rapid rate of pH decline limits protein breakdown during ensilage by inhibiting plant proteolytic activity and growth of clostridia. In previous studies, it has been demonstrated that inoculation with *Lactobacillus plantarum* gave a significantly lower percentage free amino acids of total amino nitrogen, compared to uninoculated silage. These data indicate that silage inoculated with lactic acid producing bacteria has reduced protein breakdown. It has also been shown that inoculated silage is closer in amino acid composition to fresh herbage compared with other treatments. This relates to a minimal loss of essential amino acids in inoculated silage compared to untreated silage (Winters *et al.,* 2000).

Formic acid can be used to improve the fermentation in silage due to its ability to reduce the pH value and therefore inhibit protein degradation and growth of undesirable microbes. There are risks involved by using formic acid because it is toxic and may cause skin, eye and respiratory irritations and may release toxic fumes (Salawu et al., 1999a).

The handling of lactic acid bacteria is not as hazardous as with formic acid but both treatments have a similar effect on silage preparation. Both treatments can achieve a rapid decline of the pH value and therefore accomplish high-quality silage. Compared to inoculation with lactic acid bacteria, it has been shown that silage treated with formic acid, has a higher value of water soluble carbohydrates (WSC) but lower level of lactic acid produced (Merry *et al.,* 1995).

3. 3. 4. Effects of tannins

Condensed tannins (CT) also called proanthocyanidins, are polyphenolic compounds normally occurring in plant vacuoles. CT binds easily and strongly through hydrogen bonding to protein. There is a hypothesis that some plants evolved production of CT as a chemical defence against invasion by pathogenic microorganisms as well as being eaten by insects and grazing herbivores. It is reported that the protein-CT complex will result in a reduction of the proteolysis of forage protein in the rumen (Barry and Mc Nabb, 1999).

Jones and Mangan, 1977) showed the bonding between CT and forage protein is pH dependent. The protein-CT complex is stable at pH 3.5-7.5, which matches up to the rumen environment, but dissociates below pH 3, 5 enabling digestion and absorption

of amino acids to occur in the intestine. The action of CT binding to protein is to slow the rates of both making the protein soluble and degradation of forage proteins by rumen micro organisms (Barry and Mc Nabb, 1999).

A higher amount of protein reaching the abomasum will lead to an increased level of amino acids absorbed. The degradation of S containing amino acids to inorganic sulphide in the rumen will also decreased by CT bonding to protein. This will result in increased intestinal absorption of the amino acid cysteine, which contains S, and enhance its important involvement in essential reactions in the body (e.g. milk production and live- weight gain) (Mc Nabb *et al.,* 1993).

The components of the feeding value are voluntary feed intake (VFI), the digestion process and how efficiently the digested nutrients are utilized (Ulyatt 1973). It has been reported that high CT concentration (63-106 g/kg DM) decrease the feed intake in sheep, while medium concentrations of CT (45 g/kg DM) did not affect the intake of feed. These data accord well with plant CT production being a defence against being eaten by herbivores (Barry and Mc Nabb, 1999).

There are question marks about how much of the increased rumen by- pass protein that results from the action of CT will actually be absorbed in the intestine and hence be valuable for the consumer. It is likely that the amount of protein being released in the intestine is influenced by several factors, such as the molecular structure of the tannins, the pH in the intestinal tract and the nature of the proteins. It has been shown that leaves from the tannin rich legume tree *Calliandra* gave poor utilisation of by-pass protein in the intestine (Salawu et al., 1999b).

The chemical structures of condensed tannins may differ between species, which in turn affect the biological effect of the CT. For example *Lotus corniculatus* has got a lower molecular mass of CT than *Lotus pedunculatus* and they have got different types of subunits. The tannins of Lotus corniculatus bind more fully to protein than those in Lotus pedunculatus (Barry and Mc Nabb, 1999).

Tannins may also have toxic effects on ruminants. One negative effect is that CT may damage the mucosa of the gastrointestinal tract, decreasing the absorption of nutrients. Condensed tannins may also reduce the absorption of essential amino acids, especially methionine and lysine. Decreased methionine availability could increase the toxicity of cyanogenic glycosides, because methionine is involved in the detoxification of cyanide via methylation to thiocyanate (Cornell University homepage, 2004-01-21).

4. Materials and Methods

All experiments in this work were set up at IGER, Aberystwyth, U.K in June-July, 2003.

4.1 Rumen donor animals

The rumen fluid that was used as incubation medium in the experiments was collected from fistulated non-lactating Holstein Friesian cows grazing grass. The cattle were from Trawsgoed research farm, IGER, Aberystwyth, U.K.

4.2 Forage preparation

Fresh herbage was crushed using a wooden roller on a wooden board. Crushed herbage was cut into 5 mm sections and samples (5 g) transferred into the medicine bottles used for the in vitro gas production method. Thereafter 100 ml of rumen fluid was added to each bottle. Bottles only filled with rumen fluid were used as controls. The bottles were then sealed with butyl rubber stoppers and incubated in a controlled environment room with a temperature of 39°C (which is similar to the rumen temperature). In the experiment with freeze-dried silage, 1g of the sample was used - otherwise the treatments were the same as described above.

4.3 Effects of species and varieties on *in vitro* hydrogen sulphide production

Triplicates of Cocksfoot, *Lotus corniculatus var.* Leo S41 (5% tannins), Ryegrass, Tall Fescue, Timothy and duplicates of Meadow Fescue were set up. Three replicates of rumen fluid with no forage additions were used as blanks. The date of harvest and incubations of plants was the 31st of July 2003. The grass used was grown on demonstration plots, and the *Lotus* was grown in a green house, at IGER, Aberystwyth, UK.

4.4 The effect of wilting on *in vitro* hydrogen sulphide production

Three replicates of fresh grass (cut just before the start of incubations) and grasses that were wilted 3, 12, 24 and 48 hours before incubations started were used. The grasses were wilted in unsealed plastic bags at room temperature in the laboratory. Three replicates of rumen fluid without forage were set up as blanks. The grass material used in this experiment was a mixture of three ryegrasses AberComo, AberDart and Aber Linnet. The last time this sward had been cut before the harvesting in this experiment was on the 23rd of June 2003 and was fertilised on the 25th of June 2003. The amounts used for the fertilising were 250 kg NPK (27-5-5) per hectare. The date of incubations was on the 17th of July 2003.

4.5 The effect of inoculants in silage on *in vitro* hydrogen sulphide production

Three replicates of silages prepared with different additives: Pioneer inoculant, Power Start inoculant or Formic acid were used in the experiment. Power Start is a freshly cultured silage inoculant that contains two strains of *Lactobacillus Plantarum* and one strain of *Lactobacillus lactis.* It is applied to the forage before ensiling to give an inoculation rate of 10^6 Colony forming units per gram of fresh matter. Power Start is supplied by Genus Ltd Nantwich, Cheshire, UK.

Pioneer is a freeze-dried silage inoculant that contains one strain of *Lactobacillus Plantarum* and one strain *of Lactobacillus buchneri*. It is applied to the forage before ensiling to give an inoculation rate of 10^5 Colony forming units per gram of fresh matter. Pioneer is supplied (specially to IGER) by Pioneer Hi-Bred, Apenser Str 198, 21 614 Buxtehude, Germany.

There were also three replicates of silage prepared with no additive and three replicates of rumen fluid without forage as blanks. The silage that was used in the experiment was sampled from an experiment with high-sugar grasses, conducted at IGER during 2001. The date of incubations was on the 22nd of July 2003.

4.6 The effect of tannins on *in vitro* hydrogen sulphide production

Triplicate samples of 5 different *Lotus* were used in this experiment. The date of harvest of plants and incubations was on the 29th of July 2003. The plants used were grown in pots in a green house at Plas Gogerddan, IGER, Aberystwyth, UK. There were two types of Lotus with a low content of tannins: *Lotus corniculatus*, variant USDA (typically 0.5% tannins) and *Lotus corniculates* variant Leo S50 (typically 1% tannins). Two types of *Lotus corniculates* with a higher amount of tannins were also used, and that was variant Leo S41 (typically 5% tannins) and Maitland (typically 6% tannins). These are all different varieties with same tannin structure. One *Lotus*, of a different species and a with a different tannins structure, called *Lotus pedunculatus* (typically 9% tannins), was used as well.

4.7 Gas production method

Recordings of gas produced and the concentration of hydrogen sulphide in gas were made at 2, 4, 6, 8 and 12 hours after the start of incubations. To measure gas production in the head- space of the bottles a 3-way syringe valve connected to a pressure transducer and a LED digital read-out voltmeter was used (Figure 4).

Figure 4. The pressure transducer connected to a digital display measuring gas pressure and volume of fermentation gas.

At each reading the syringe needle was inserted through the butyl rubber stopper into the head-space of the bottle above the culture medium. Thereafter the pressure could be recorded on the digital display. As gas was withdrawn through the syringe by pulling on the syringe plunger, the digital display showed the decrease in gas pressure. The pulling continued until the display read zero. Then the volume of gas in the syringe was recorded before the needle was removed from the bottle and the gas was transferred to a plastic bottle which contained 800 ml of CO_2.

4.8 Hydrogen sulphide measurement

The amount of H_2S produced (ml) was calculated by measuring the H_2S concentration (ppm) in the gas sample using a H_2S meter (Triple Plus+) (Figure 5).

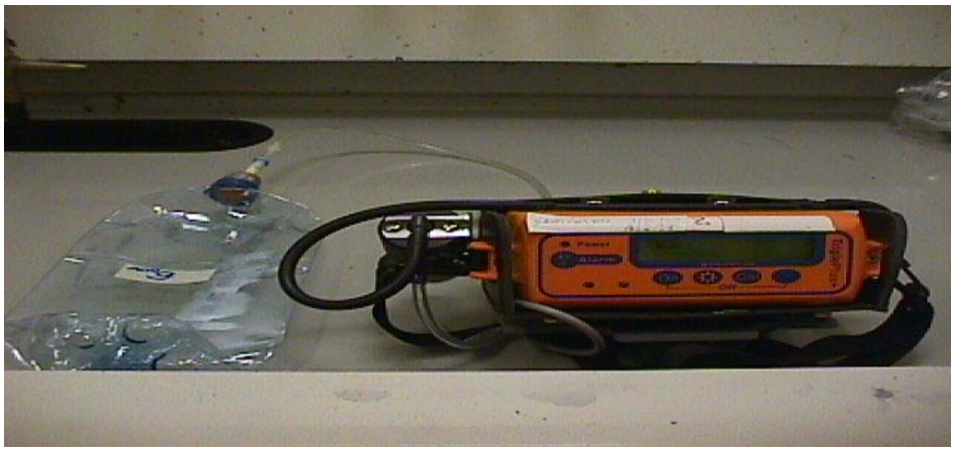

Figure 5. A H_2S meter connected to a plastic bottle filled with fermentation gas and 800 ml CO_2.

4.9 Statistics

All statistical analysis was done using the SAS system for Windows(SAS 6.12, TS level 020, SAS Institute, Cary, NC, USA). All data were analysed by the mixed model procedure (Littell et al., 1996). The following model was used:

$Y=ų + a + t + a*t + e$

Where Y= dependent variable; ų = overall mean; a= fixed effect of treatment (additive, wilting etc.); t= fixed effect of time; a*t = two-way interaction; e = random residual error. The random statement was batch number within treatment.

5. Results and discussion

5.1 Effects of species and varieties on *in vitro* hydrogen sulphide production

Production of H_2S

Table 1a and Figure 6 show that *Lotus* had significant lower production of H_2S than all the other samples at most incubation times. Ryegrass (PRG) was found to have the highest level of H_2S production. After 2 hours of incubation there was no significant difference in H_2S production between the samples (Table 1b).

Table 1a. The effect of species and varieties on mean values of H_2S produced (ml) from different herbage after different length of incubation (hours; h). The standard error (SE) value is shown in parentheses under each mean value. Uncorrected mean values (ml) for control.

Sample	0-2 h	2-4 h	4-6 h	6-8 h	8-12 h
Cocksfoot	0,011 (0,005)	0,047 (0,005)	0,067 (0,005)	0,065 (0,005)	0,101 (0,005)
Lotus	0,016 (0,005)	0,034 (0,005)	0,04 (0,005)	0,042 (0,005)	0,066 (0,005)
Meadow Fescue	0,012 (0,007)	0,054 (0,007)	0,074 (0,007)	0,069 (0,007)	0,099 (0,007)
PRG	0,019 (0,005)	0,074 (0,005)	0,087 (0,005)	0,079 (0,005)	0,12 (0,005)
Tall Fescue	0,025 (0,005)	0,066 (0,005)	0,082 (0,005)	0,076 (0,005)	0,099 (0,005)
Timothy	0,014 (0,005)	0,047 (0,005)	0,063 (0,005)	0,059 (0,005)	0,085 (0,005)
Control	0,012	0,005	0,008	0,009	0,027

Table 1b. The effect of forage species and varieties. Significance levels (P-values) for pairwise comparisons of H_2S production from different herbage in table 1a. All comparisons are for incubations of the same duration (hours).

Sample	*Lotus*	Tall Fescue	PRG	Cocksfoot	Meadow Fescue
Timothy					
2	0,7010	0,1577	0,4706	0,7327	0,8785
4	0,1260	0,0216	0,0020	1,0000	0,4048
6	0,0072	0,0265	0,0055	0,5945	0,2169
8	0,0519	0,0309	0,0180	0,3746	0,1429
12	0,0258	0,0009	0,0002	0,0401	0,1249
Lotus					
2		0,2927	0,7327	0,4706	0,6203
4		0,0006	0,0001	0,1260	0,0344
6		0,0001	0,0001	0,0022	0,0009
8		0,0001	0,0001	0,0078	0,0032
12		0,0001	0,0001	0,0002	0,0014
Tall Fescue					
2	0,2927		0,4706	0,0852	0,1578
4	0,0006		0,2927	0,0216	0,1796
6	0,0001		0,4724	0,0773	0,3902
8	0,0001		0,7977	0,1796	0,5808
12	0,0001		0,4967	0,0999	0,0708
PRG					
2	0,7327	0,4706		0,2927	0,8785
4	0,0001	0,2927		0,0020	0,4048
6	0,0001	0,4724		0,0178	0,4371
8	0,0001	0,7977		0,1080	0,4823
12	0,0001	0,4967		0,0258	0,7169
Cocksfoot					
2	0,4706	0,0852	0,2927		0,8785
4	0,1260	0,0216	0,0020		0,4048
6	0,0022	0,0773	0,0178		0,4371
8	0,0078	0,1796	0,1080		0,4823
12	0,0002	0,0999	0,0258		0,7169
Meadow Fescue					
2	0,6203	0,1578	0,4261	0,8785	
4	0,0344	0,1796	0,0294	0,4048	
6	0,0009	0,3902	0,1416	0,4371	
8	0,0032	0,5808	0,4370	0,4823	
12	0,0014	0,0708	0,0205	0,7169	

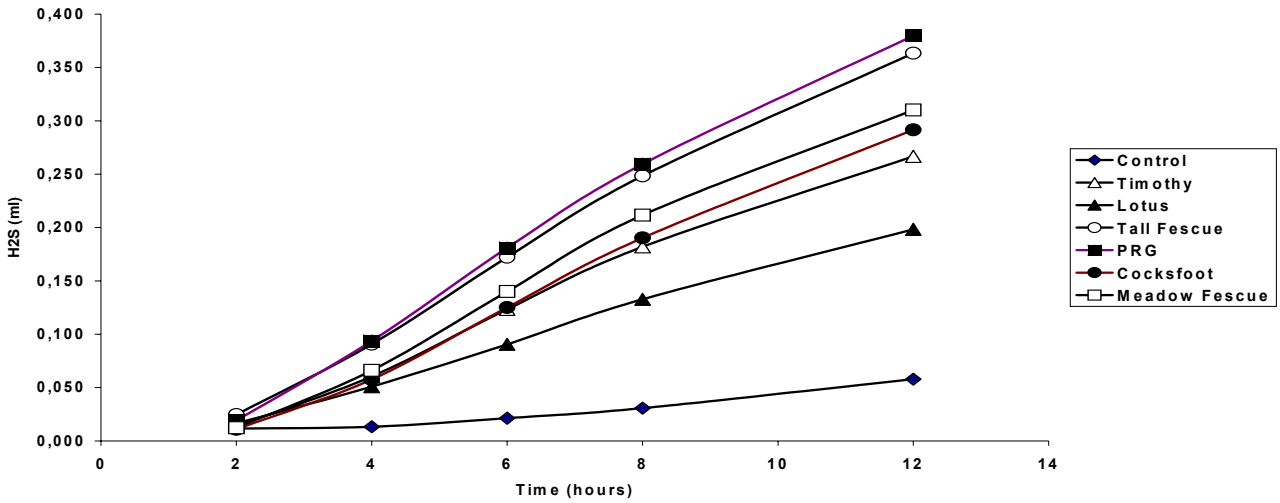

Figure 6. The effect of species and varieties on cumulative H_2S production (ml).

Production of H_2S on a crude protein (CP) content basis

When results were expressed on a crude protein basis, it was shown that *Lotus* produced less H_2S than the grasses (Table 2a and Figure 7). More of the differences reached statistical significance when the H_2S production was calculated on a crude protein basis (Table 2b).

Table 2a. The effect of species and varieties on mean values of H_2S production (ml per g of crude protein) from different herbage at different length of incubation in hours (h). The standard error (SE) value is shown in parentheses under each mean value.

Sample	0-2 h	2-4 h	4-6 h	6-8 h	8-12 h
Cocksfoot	0,739 (0,345)	3,164 (0,345)	4,551 (0,345)	4,437 (0,345)	6,876 (0,345)
Lotus	0,573 (0,345)	1,196 (0,345)	1,382 (0,345)	1,473 (0,345)	2,285 (0,345)
Meadow Fescue	0,831 (0,422)	3,640 (0,422)	5,014 (0,422)	4,859 (0,422)	6,680 (0,422)
PRG	1,235 (0,345)	4,801 (0,345)	5,624 (0,345)	5,077 (0,345)	7,769 (0,345)
Tall Fescue	1,914 (0,345)	5,120 (0,345)	6,332 (0,345)	5,919 (0,345)	8,930 (0,345)
Timothy	0,797 (0,345)	2,727 (0,345)	3,676 (0,345)	3,419 (0,345)	4,955 (0,345)

Table 2b. The effect of forage species and varieties. Significance levels (P-values) for pairwise comparisons of H_2S production (on a crude protein content basis) from different herbage in Table 2a. All comparisons are for incubations of the same duration.

Sample	*Lotus*	Tall Fescue	PRG	Cocksfoot	Meadow Fescue
Timothy					
2	0,6521	0,0350	0,3819	0,9072	0,9508
4	0,0060	0,0001	0,0005	0,3823	0,1119
6	0,0002	0,0001	0,0009	0,0906	0,0252
8	0,0009	0,0001	0,0034	0,0521	0,0171
12	0,0001	0,0001	0,0001	0,0011	0,0056
Lotus					
2		0,0137	0,1927	0,7376	0,6422
4		0,0001	0,0001	0,0009	0,0003
6		0,0001	0,0001	0,0001	0,0001
8		0,0001	0,0001	0,0001	0,0001
12		0,0001	0,0001	0,0001	0,0001
Tall Fescue					
2	0,0137		0,1816	0,0276	0,0634
4	0,0001		0,5207	0,0009	0,0147
6	0,0001		0,1643	0,0020	0,0271
8	0,0001		0,1023	0,0074	0,0685
12	0,0001		0,0293	0,0006	0,0007
PRG					
2	0,1927	0,1816		0,3239	0,4692
4	0,0001	0,5207		0,0037	0,0482
6	0,0001	0,1643		0,0418	0,2788
8	0,0001	0,1023		0,0001	0,6935
12	0,0001	0,0293		0,0843	0,0619
Cocksfoot					
2	0,7376	0,0276	0,3239		0,8683
4	0,0009	0,0009	0,0037		0,3941
6	0,0001	0,0020	0,0418		0,4072
8	0,0001	0,0074	0,0001		0,4497
12	0,0001	0,0006	0,0843		0,7241
Meadow Fescue					
2	0,6422	0,0634	0,4692	0,8683	
4	0,0003	0,0147	0,0482	0,3941	
6	0,0001	0,0271	0,2788	0,4072	
8	0,0001	0,0685	0,6935	0,4497	
12	0,0001	0,0007	0,0619	0,7241	

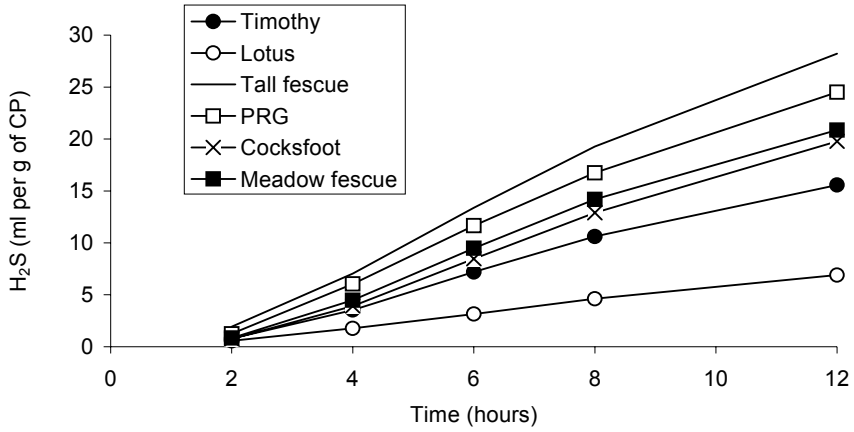

Figure 7. The effect of species and varieties on cumulative H_2S production (ml per g of crude protein).

Lotus had a lower level of H_2S production than the grasses. This might be due to the fact that *Lotus* are cyanogenic (they produce HCN as a plant defence mechanism when cells are damaged) and HCN reacts with sulphur to form thiocyanate (Bazin *et al.,* 2002). The effect of tannins decreasing the level of proteolysis might also have contributed to the lower levels of H_2S produced in the samples with *Lotus* (Barry and Mc Nabb, 1999). However, the confounding effect of HCN production means that the technique is not suitable for this type of forage. This conclusion also applies to white clover, which is also cyanogenic.

The analysis of N showed that the crude protein content in *Lotus* is higher than in the other forages, which would lead to higher values of H_2S production. Due to the fact that *Lotus* are cyanogenic (Bazin *et al.,* 2002) and contain tannins the formation of H_2S might be limited.

5.2 The effect of wilting duration on *in vitro* hydrogen sulphide production

Production of H_2S

The levels of H_2S produced in all bottles increased over the time, but between the readings after 6 and 8 hours of incubation a decline in the rate of H_2S production was observed (Table 3a). Grass wilted for 48 hours had a significantly lower level of H_2S production than the other samples at the reading after 4 hours of incubation (Table 3b and Figure 8.)

Table 3a. The effect of wilting duration (h) on mean values of H_2S produced (ml) from herbage at different length of incubation in hours (h). The standard error (SE) value is shown in parentheses under each mean value. Uncorrected mean values (ml) for control.

Duration of wilting	**0-2 h**	**2-4 h**	**4-6 h**	**6-8 h**	**8-12 h**
48 h	0,024 (0,0058)	0,050 (0,0050)	0,085 (0,0050)	0,064 (0,0050)	0,147 (0,0050)
24 h	0,026 (0,0050)	0,071 (0,0050)	0,090 (0,0050)	0,076 (0,0050)	0,149 (0,0050)
12 h	0,026 (0,0050)	0,072 (0,0050)	0,090 (0,0050)	0,074 (0,0050)	0,147 (0,0050)
3 h	0,029 (0,0050)	0,085 (0,0050)	0,095 (0,0050)	0,074 (0,0050)	0,148 (0,0050)
0 h	0,020 (0,0058)	0,076 (0,0050)	0,085 (0,0050)	0,076 (0,0050)	0,141 (0,0050)
Control	0,001	0,002	0,003	0,005	0,014

Table 3b. The effect of wilting duration (h). Significance levels (P-values) for pairwise comparisons of H_2S production from different herbage in table 3a. All comparisons are for incubations of the same duration.

Duration of wilting	*3 h wilting*	*12 h wilting*	*24 h wilting*	*48 h wilting*
0 h wilting				
2	0,2379	0,4019	0,4019	0,5183
4	0,2252	0,5340	0,4800	0,0005
6	0,1396	0,4295	0,4247	0,9283
8	0,7546	0,7724	0,9794	0,1230
12	0,3292	0,4008	0,2162	0,3798
3 h wilting				
2		0,7058	0,7058	0,6206
4		0,0707	0,0592	0,0001
6		0,4815	0,4867	0,1642
8		0,9813	0,7351	0,2142
12		0,8903	0,7890	0,9210
12 h wilting				
2	0,7058		1,0000	0,8829
4	0,0707		0,9321	0,0029
6	0,4815		0,9933	0,4832
8	0,9813		0,7527	0,2060
12	0,8903		06853	0,9691
24 h wilting				
2	0,7058	1,0000		0,8829
4	0,0592	0,9321		0,0037
6	0,4867	0,9933		0,4780
8	0,7351	0,7527		0,1171
12	0,7890	06853		0,7139
48 h wilting				
2	0,6206	0,8829	0,8829	
4	0,0001	0,0029	0,0037	
6	0,1642	0,4832	0,4780	
8	0,2142	0,2060	0,1171	
12	0,9210	0,9691	0,7139	

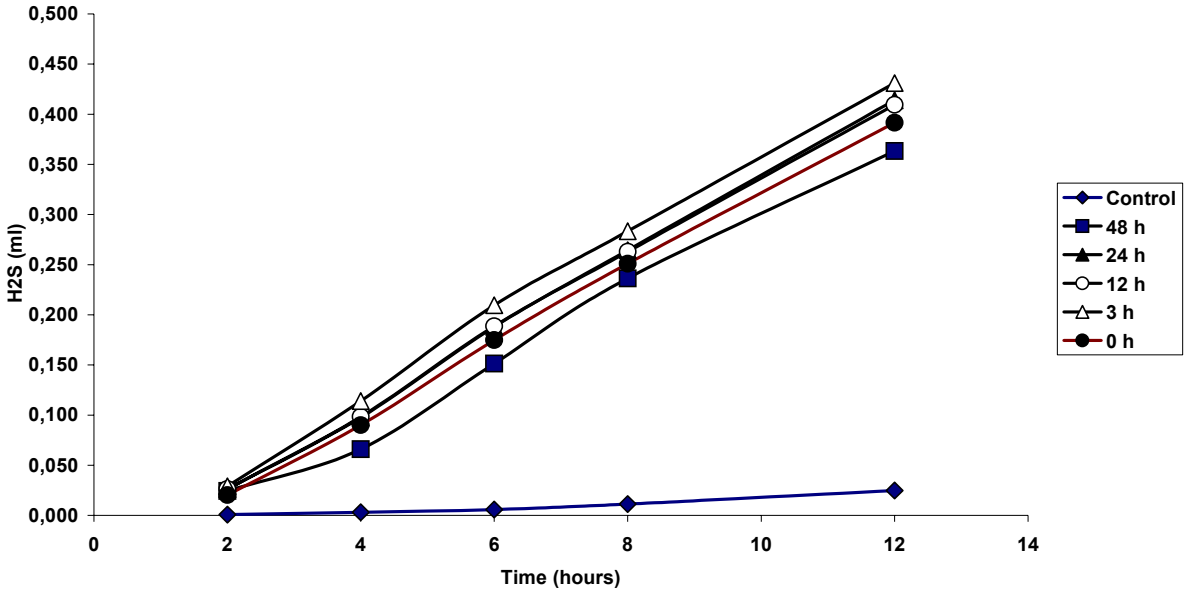

Figure 8. The effect of wilting duration (h) on cumulative H_2S production (ml).

Production of H_2S on a crude protein (CP) content basis

There was some evidence (at a few time points) of significantly lower levels of H_2S produced compared to the crude protein content in the bottle with grass wilted for 48 hours than for the other samples (Table 4a, Table 4b and Figure 9). A decline in the rate of H_2S production was observed between the readings at 6 and 8 hours (Table 7).

Table 4a. The effect of wilting duration (h) on mean values of H_2S production (ml per g of crude protein) of herbage at different length of incubation in hours (h). The standard error (SE) value is shown in parentheses under each mean value.

Duration of wilting	0-2 h	2-4 h	4-6 h	6-8 h	8-12 h
48h	0,949 (0,289)	1,914 (0,256)	3,283 (0,256)	2,496 (0,256)	5,631 (0,256)
24h	1,254 (0,256)	3,399 (0,256)	4,305 (0,256)	3,635 (0,256)	7,123 (0,256)
12h	1,290 (0,256)	3,536 (0,256)	4,431 (0,2569)	3,635 (0,256)	7,195 (0,2556)
3h	1,309 (0,256)	3,838 (0,256)	4,311 (0,256)	3,335 (0,256)	6,643 (0,256)
0h	0,878 (0,289)	3,540 (0,256)	3,908 (0,256)	3,509 (0,256)	6,457 (0,256)

Table 4b. The effect of wilting duration (h). Significance levels (P-values) for pairwise comparisons of H_2S production (on crude protein content basis) from different herbage in table 4a. All comparisons are for incubations of the same duration.

Duration of wilting	*3 h wilting*	*12 h wilting*	*24 h wilting*	*48 h wilting*
0 h wilting				
2	0,2742	0,2958	0,3400	0,8632
4	0,4204	0,9909	0,7004	0,0002
6	0,2784	0,1635	0,2852	0,0989
8	0,6371	0,7298	0,7305	0,0105
12	0,6118	0,0537	0,0797	0,0327
3 h wilting				
2		0,9581	0,8788	0,3594
4		0,4140	0,2388	0,0001
6		07445	0,9874	0,0096
8		0,4165	0,4170	0,0303
12		0,1421	0,1998	0,0106
12 h wilting				
2	0,9581		0,9204	0,3853
4	0,4140		0,7089	0,0002
6	07445		0,7326	0,0045
8	0,4165		0,9993	0,0047
12	0,1421		0,8428	0,0003
24 h wilting				
2	0,8788	0,9204		0,4377
4	0,2388	0,7089		0,0005
6	0,9874	0,7326		0,0100
8	0,4170	0,9993		0,0047
12	0,1998	0,8428		0,0004
48 h wilting				
2	0,3594	0,3853	0,4377	
4	0,0001	0,0002	0,0005	
6	0,0096	0,0045	0,0100	
8	0,0303	0,0047	0,0047	
12	0,0106	0,0003	0,0004	

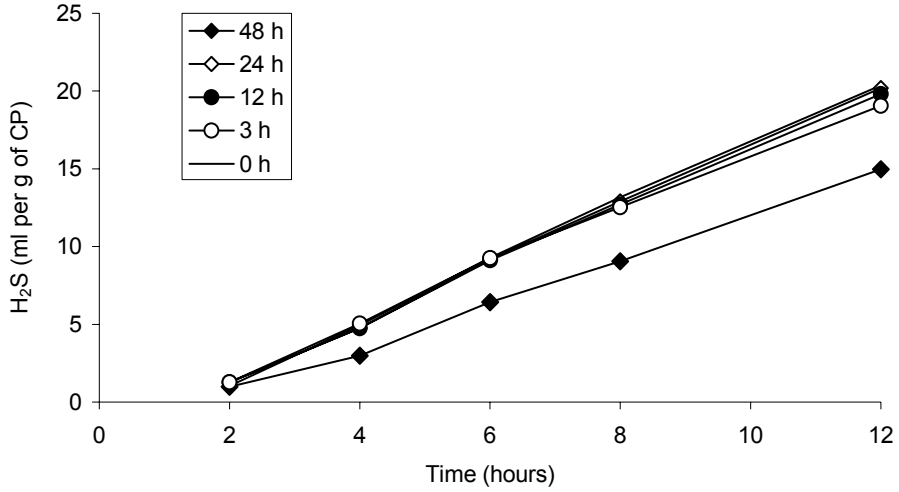

Figure 9. The effect of wilting duration (hours) on cumulative H_2S production (ml per g crude protein).

The results from this experiment did not show big differences in the amount of H_2S produced from grass wilted for different lengths of time. That might due to the fact that all the grass were crushed just before incubation started and not at the time they were cut. In practice the grass could had been crushed by the mower at time for harvesting- particularly if a mower-conditioner is used. The crushing mechanism of the mower will enlarge the opportunity for plant proteases to degrade the protein (Richard Dewhurst, personal communication, 2003). There might be larger differences between the different treated grasses if they had been crushed at the time the harvesting took place. In future work it would be to consider crushing the grass when cutting it.

Another possible cause of the result might be that all the grass was wilted in plastic bags in the laboratory and didn't get much access of oxygen. As reported by Kingston- Smith *et al.,* (2000), the contact with oxygen stimulates the cell death to appear, which further encourage the start of proteolysis by plant proteases. In the plastic bags it might were small amounts of oxygen available which perhaps prevented the action of proteolysis. In future work it maybe would be better to not wilt the grass in plastic bags.

When the levels of H_2S produced were compared to the crude protein content of the grass used in the experiment other interesting data was observed. Grass wilted for 48 hours had significantly lower values of H_2S production than the other samples after 4 to 12 hours of incubation.

The lower values of H_2S produced in the sample with grass wilted for 48 hours may be due to the activity of plant proteases. As reported by Charmley *et al.,* (1990) the plant proteases degrade the protein into peptides and amino acids in the period between cutting and start of incubation. Because of that sulphur is a part of the amino acids cysteine and methionine there will be less sulphur at the start of the incubations.

That might explain why the production of H_2S per unit of the crude protein content is lower when grass is wilted for longer time.

In the bottles with unwilted grass the proteolysis has just started when the incubation begins which might contribute to a higher content of sulphur and explain the high values of H_2S produced compared to the crude protein content of these grass samples. It might also be that fresh grass was easier to crush than wilted grass and due to that crushing stimulates the action of plant proteases, this might explain why the production of H_2S is lower for wilted grass (Dewhurst, personal com., 2003).

5.3 The effect of inoculants in silage on *in vitro* hydrogen sulphide production

Production of H_2S

All samples had increasing values of H_2S production until 6 hours of incubation. Silage treated with formic acid, Pioneer, or Power Start had reduced levels of H_2S production after 8 hours (Table 5a). After 12 hours of incubation the levels of H_2S produced had peaked for all samples. The untreated silage had significantly lower levels of H_2S production at all readings in comparison with silage treated with formic acid (Table 5b and Figure 10).

Table 5a. The effect of inoculants on mean values of H_2S produced (ml) from silage at different length of incubation in hours (h). The standard error (SE) value is shown in parentheses under each mean value. Uncorrected mean values (ml) for control.

Treatment	0-2 h	2-4 h	4-6 h	6-8 h	8-12 h
No inoculants	0,029 (0,035)	0,040 (0,029)	0,044 (0,029)	0,043 (0,029)	0,063 (0,029)
Pioneer	0,031 (0,029)	0,055 (0,029)	0,057 (0,029)	0,054 (0,029)	0,079 (0,029)
Power start	0,035 (0,035)	0,049 (0,029)	0,056 (0,029)	0,049 (0,029)	0,076 (0,029)
Formic acid	0,039 (0,029)	0,055 (0,029)	0,060 (0,029)	0,052 (0,029)	0,082 (0,029)
Control	0,002	0,005	0,008	0,008	0,024

Table 5b. The effect of inoculants in silage. Significance levels (P-values) for pairwise comparisons of H_2S production from different herbage in table 5a. All comparisons are for incubations of the same duration.

Treatment	*No inoculants*	*Pioneer*	*Power start*
Formic acid			
2	0,0316	0,0688	0,4290
4	0,0007	0,9357	0,1513
6	0,0003	0,5728	0,3354
8	0,0580	0,5195	0,5195
12	0,0001	0,5195	0,1748
No inoculants			
2		0,5975	0,1981
4		0,0006	0,0339
6		0,0018	0,0056
8		0,0129	0,2010
12		0,0003	0,0023
Pioneer			
2	0,5975		0,3787
4	0,0006		0,1304
6	0,0018		0,6868
8	0,0129		0,2010
12	0,0003		0,4689
Power start			
2	0,1981	0,3787	
4	0,0339	0,1304	
6	0,0056	0,6868	
8	0,2010	0,2010	
12	0,0023	0,4689	

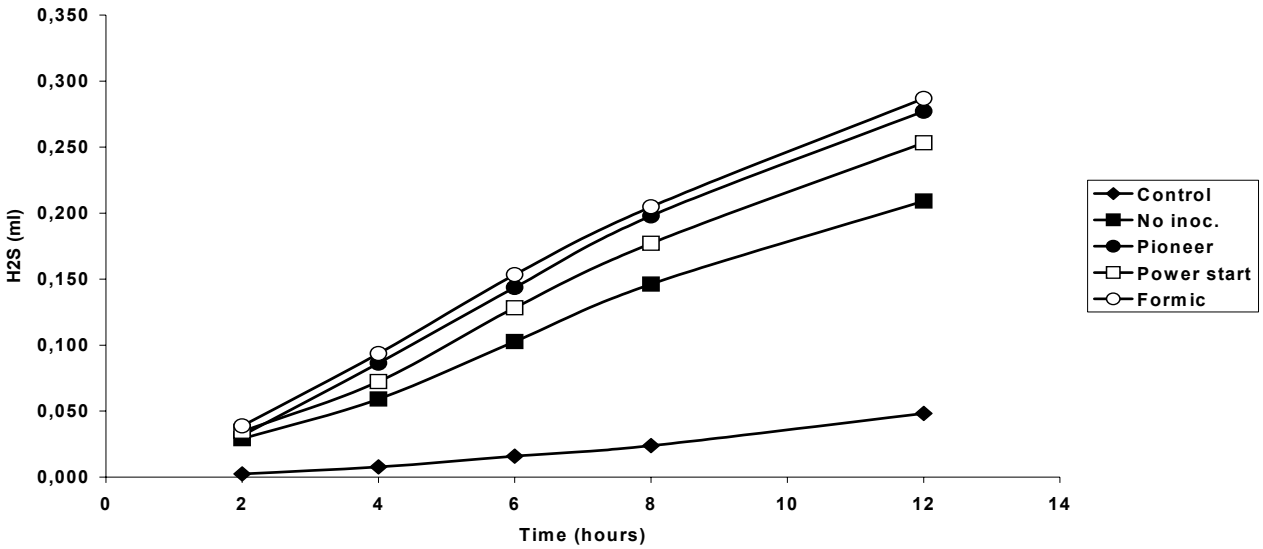

Figure 10. The effect of inoculants in silage on cumulative H_2S production (ml).

Production of H_2S on a crude protein (CP) basis

When the production of H_2S was related to the crude protein content of the silage the results showed a similar pattern to the analysis of the total amount of H_2S produced (Figure 11.) It was shown that untreated silage had lower levels of H_2S produced compared to its crude protein content at several of the readings (Tables 6a and 6b).

Table 6a. The effects of inoculants on mean values of H_2S production (ml per g of crude protein) from silage at different length of incubation in hours (h). The standard error (SE) value is shown in parentheses under each mean value.

Treatment	0-2 h	2-4 h	4-6 h	6-8 h	8-12 h
No inoculants	1,648 (0,217)	2,257 (0,180)	2,475 (0,180)	2,463 (0,180)	3,567 (0,180)
Pioneer	1,724 (0,180)	3,007 (0,180)	3,140 (0,180)	2,974 (0,180)	4,342 (0,180)
Power start	2,040 (0,217)	2,868 (0,180)	3,251 (0,180)	2,842 (0,180)	4,433 (0,180)
Formic acid	2,211 (0,180)	3,119 (0,180)	3,398 (0,180)	2,934 (0,180)	4,673 (0,180)

Table 6b. The effect of inoculants in silage. Significance levels (P-values) for pairwise comparisons of H_2S production (on crude protein basis) from different herbage in table 6a. All comparisons are for incubations of the same duration.

Treatment	*No inoculants*	*Pioneer*	*Power start*
Formic acid			
2	0,0541	0,0647	0,5494
4	0,0018	0,6628	0,3314
6	0,0009	0,3187	0,5683
8	0,0728	0,8768	0,7182
12	0,0001	0,2023	0,3540
No inoculants			
2		0,7899	0,2108
4		0,0058	0,0220
6		0,0133	0,0045
8		0,0527	0,1461
12		0,0045	0,0017
Pioneer			
2	0,7899		0,2708
4	0,0058		0,5690
6	0,0133		0,6657
8	0,0527		0,6064
12	0,0045		0,7207
Power start			
2	0,2108	0,2708	
4	0,0220	0,5690	
6	0,0045	0,6657	
8	0,1461	0,6064	
12	0,0017	0,7207	

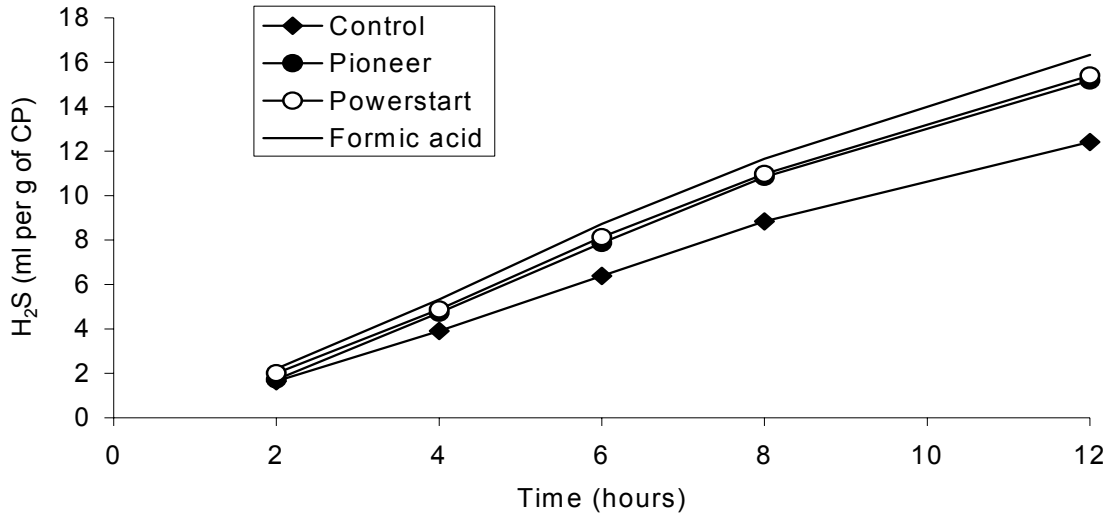

Figure 11. The effect of inoculants in silage on cumulative H_2S production (ml per g of crude protein).

It is interesting that the untreated silage has lower values of H_2S than the silage treated with inoculants. The effect of lactic acid producing bacteria were expected to decrease the proteolysis in the inoculated samples and hence due to a lower level of H_2S produced. In a prior study (Winters *et al.,* 2000) it was showed that silage inoculated with *Lactobacillus plantarum* reduced the level of free amino acids which indicated that a decline in protein degradation had occurred compared to untreated silage.

5.4 The effect of tannins on *in vitro* hydrogen sulphide production

Production of H_2S

Lotus corniculatus var. USDA, which contained least tannins had the lowest value of H_2S production (Table 7a and Figure 12). It was showed that *Lotus pedunculatus,* which has the highest content of tannins, had significantly higher levels of hydrogen sulphide (Table 7b). The three other samples with *Lotus* had similar and intermediate values of H_2S production.

Table 7a. The effect of tannins on mean values of H_2S produced (ml) from *Lotus* at different length of incubation in hours (h). The standard error (SE) value is shown in parentheses under each mean value. Uncorrected mean values (ml) for control.

Sample	0-2 h	2-4 h	4-6 h	6-8 h	8-2 h
USDA (0,5%)	0,017 (0,003)	0,026 (0,003)	0,029 (0,003)	0,037 (0,003)	0,040 (0,003)
Leo S50 (1%)	0,018 (0,003)	0,032 (0,003)	0,040 (0,003)	0,041 (0,003)	0,075 (0,003)
Leo S41 (5%)	0,016 (0,003)	0,030 (0,003)	0,041 (0,003)	0,044 (0,003)	0,077 (0,003)
Maitland (6%)	0,015 (0,003)	0,032 (0,003)	0,038 (0,003)	0,041 (0,003)	0,083 (0,003)
Pedunculatus (9%)	0,016 (0,003)	0,032 (0,003)	0,041 (0,003)	0,055 (0,003)	0,107 (0,003)
Control	0,005	0,008	0,008	0,014	0,032

Table 7b. The effect of tannins in *Lotus*. Significance levels (P-values) for pairwise comparisons of H_2S production from different herbage in table 7b. All comparisons are for incubations of the same duration.

Treatment	*Leo S 50*	*Maitland*	*Pedunc.*	*USDA*
Leo S 41				
2	0,6512	0,7174	0,4763	1,0000
4	0,7174	0,7174	0,7174	0,2102
6	0,7174	0,7174	0,7174	0,0062
8	0,4705	0,4705	0,0062	0,0769
12	0,4705	0,1302	0,0001	0,0001
Leo S 50				
2		0,4173	0,2608	0,6512
4		1,0000	1,0000	0,1098
6		0,4705	1,0000	0,0024
8		1,0000	0,0009	0,2813
12		0,0290	0,0001	0,0001
Maitland				
2	0,4173		0,7047	0,7174
4	1,0000		1,0000	0,1098
6	0,4705		0,4705	0,0153
8	1,0000		0,0009	0,2813
12	0,0290		0,0001	0,0001
Pedunc.				
2	0,2608	0,7047		0,4763
4	1,0000	1,0000		0,1098
6	1,0000	0,4705		0,0024
8	0,0009	0,0009		0,0001
12	0,0001	0,0001		0,0001
USDA				
2	0,6512	0,7174	0,4763	
4	0,1098	0,1098	0,1098	
6	0,0024	0,0153	0,0024	
8	0,2813	0,2813	0,0001	
12	0,0001	0,0001	0,0001	

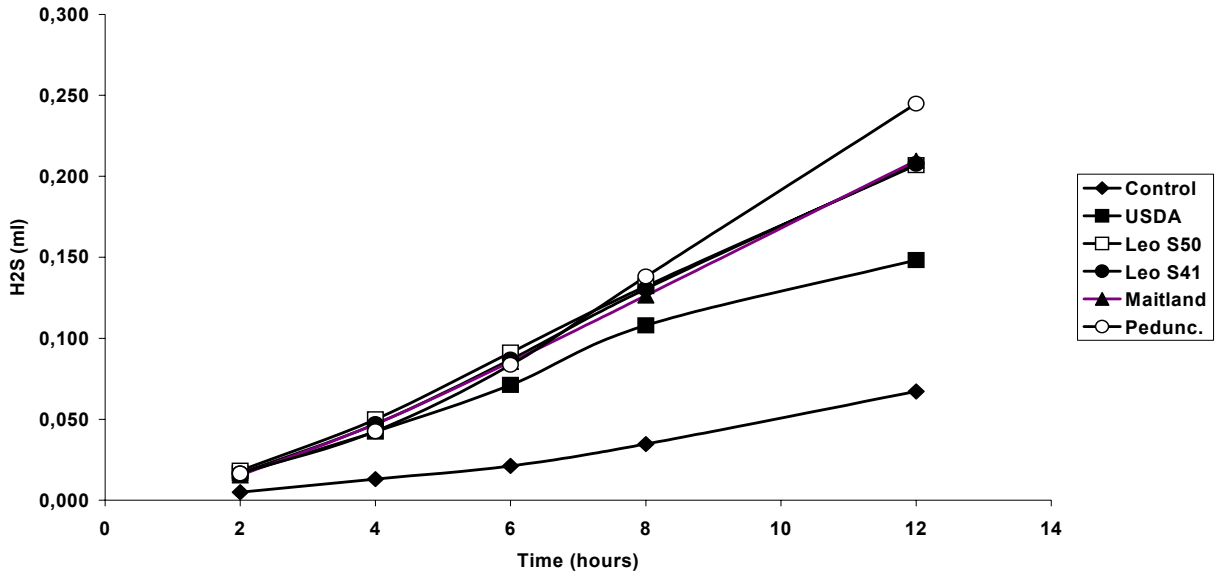

Figure 12. The effect of tannins in *Lotus* on cumulative H_2S production (ml).

Production of H_2S on a crude protein (CP) content basis

After 12 hours of incubation the sample with *Lotus Corniculatus* var. USDA, which contained least tannins had a significantly lower level of H_2S produced compared to its crude protein content than the other samples (Table 8a and 8b and figure 12).

Table 8a. The effect of tannins on mean values of H_2S production (ml per g of crude protein) from Lotus at different length of incubation in hours (h). The standard error (SE) value is shown in parentheses under each mean value.

Sample	0-2 h	2-4 h	4-6 h	6-8 h	8-12 h
USDA (0,5%)	0,658 (0,118)	1,017 (0,118)	1,142 (0,118)	1,463 (0,118)	1,588 (0,118)
Leo S50 (1%)	0,843 (0,118)	1,490 (0,118)	1,944 (0,118)	1,936 (0,118)	3,521 (0,118)
Leo S41 (5%)	0,674 (0,118)	1,232 (0,118)	1,627 (0,118)	1,781 (0,118)	3,149 (0,118)
Maitland (6%)	0,743 (0,118)	1,560 (0,118)	1,885 (0,118)	2,007 (0,118)	4,073 (0,118)
Pedunculatus (9%)	0,440 (0,136)	1,032 (0,118)	1,342 (0,118)	1,780 (0,118)	3,485 (0,118)

Table 8b. The effect of tannins in *Lotus*. Significance levels (P-values) for pairwise comparisons of H_2S production (on crude protein basis) from different herbage in table 8a. All comparisons are for incubations of the same duration.

Treatment	*Leo S 50*	*Maitland*	*Pedunc.*	*USDA*
Leo S 41				
2	0,3217	0,6844	0,2028	0,9211
4	0,1334	0,0591	0,2405	0,2080
6	0,0675	0,1328	0,0988	0,0071
8	0,3587	0,1866	0,9988	0,0675
12	0,0339	0,0001	0,0537	0,0001
Leo S 50				
2		0,5545	0,0324	0,2770
4		0,6759	0,0105	0,0085
6		0,7258	0,0012	0,0001
8		0,6771	0,3579	0,0085
12		0,0026	0,8301	0,0001
Maitland				
2	0,5545		0,1025	0,6136
4	0,6759		0,0037	0,0030
6	0,7258		0,0030	0,0001
8	0,6771		0,1861	0,0030
12	0,0026		0,0015	0,0001
Pedunc.				
2	0,0324	0,1025		0,2361
4	0,0105	0,0037		0,9293
6	0,0012	0,0030		0,2409
8	0,3579	0,1861		0,0677
12	0,8301	0,0015		0,0001
USDA				
2	0,2770	0,6136	0,2361	
4	0,0085	0,0030	0,9293	
6	0,0001	0,0001	0,2409	
8	0,0085	0,0030	0,0677	
12	0,0001	0,0001	0,0001	

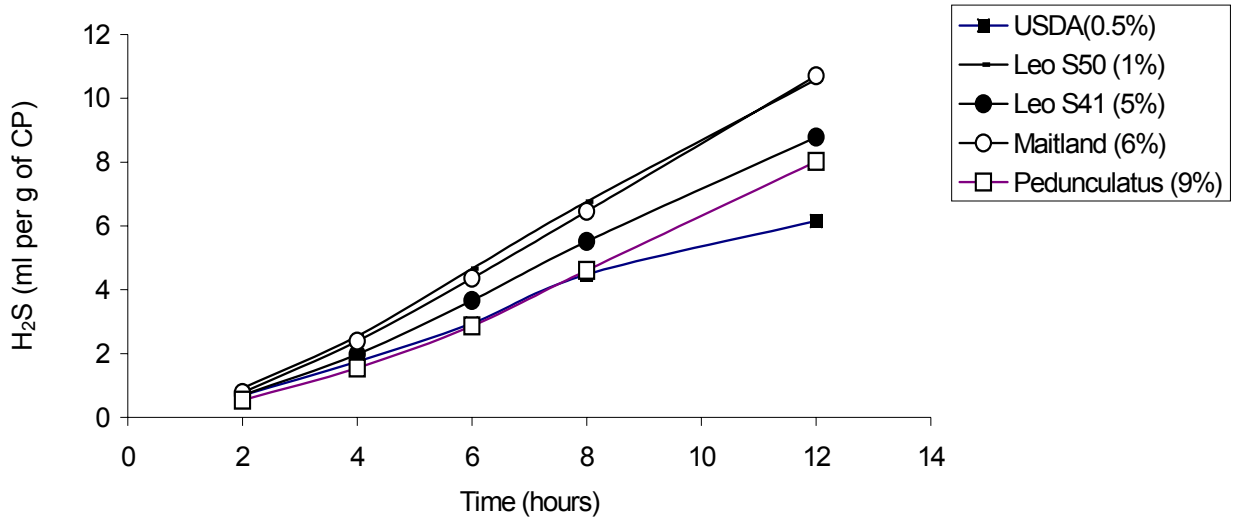

Figure 13. The effect of tannins in *Lotus* on cumulative H_2S production (ml per g of crude protein).

The amount of H_2S produced was larger from *Lotus pedunculatus* which has the highest level of tannins while *Lotus corniculatus* var. USDA, that contains least tannins, had the lowest value of H_2S production. These results are the reverse of what was expected because in previous work (Mc Nabbb *et al*., 1993; Barry and Mc Nabb, 1999; Salawu *et al*., 1999 a) increased levels of tannins protect the protein from being degraded in the rumen.

An explanation as to why there was less H_2S produced from *Lotus* containing low levels of tannins could be that the activity of cyanogenic compounds was high in these samples. *Lotus* contains cyanogenic glycosides that form toxic HCN as a defence mechanism. HCN react with sulphur to form thiocyanate which will lead to decreased levels of H_2S production (Bazin *et al.,* 2002). Goldstein and Spencer (1985) showed that tannins inhibit the action of cyanogenic glycosides and therefore the formation of HCN. Increased levels of tannins could lead to reduce production of HCN, which will enable more H_2S to be produced. However, it is not possible to distinguish effects of tannins and cyanogenesis in this work.

The cyanogenic nature of *Lotus* means that measuring H_2S production will not be a reliable tool to evaluate protein degradation.

6. General discussion

The overall objective of the work to which this small project relates is to evaluate differences in rumen protein degradation of forages in order to provide information that will enable improvements of protein efficiency. The purpose of this study was to measure the production of hydrogen sulphide in rumen fermentation gas to monitor the level of protein degradation.

The results from the experiment where different species and varieties were compared showed that *Lotus* had significantly lower levels of hydrogen sulphide production than grass samples. Explanations as to why the value of H_2S produced in the bottles with *Lotus* was lower than with the grass samples, could due to the fact that *Lotus* are cyanogenic. This means that *Lotus* contains toxic cyanogenic glycosides (HCN), and to make these substrates harmless sulphur reacts with HCN to form thiocyanate (Bazin *et al.,* 2002).

Earlier work showed that condensed tannins can contribute to a reduction in the level of protein degradation (Barry and Mc Nabb, 1999) so it was expected that increased levels of tannins would lead to reduced levels hydrogen sulphide. The data from the experiment where different varieties of *Lotus* containing various levels of tannins were compared, showed the reverse result from this hypothesis. Tannins inhibit the action of cyanogenic glycosides (Goldstein and Spencer, 1985), which will enable more H_2S to be produced from *Lotus* with high levels of tannins. As a result of the cyanogenic nature of *Lotus* (Bazin *et al.,* 2002)- measuring H_2S production will not be a reliable tool to evaluate the extent of protein degradation of this species.

It was noticed that all the samples with *Lotus* stayed green in their colour during the incubations. These results differed from the experiment with wilted ryegrass, where it was observed that the grass was yellow after incubations. The green pigment of all plants, chlorophyll, is located in the chloroplasts, where proteins and lipids also are sited. Due to their vicinity of each other it seems like the metabolism of these compounds is closely co-ordinated (Thomas *et al.,* 1985). The samples of *Lotus* produced less H_2S than the grass samples so it may be that the fermentation process and colour of the plants are correlated.

Data from the experiment where different silage were analysed showed that untreated silage has got lower values of H_2S than the silage treated with inoculants. This is contrary to the results of a prior study by Winters *et al.,* (2000) where it was showed that silage inoculated with *Lactobacillus plantarum* reduced the level of free amino acids which indicated that a decline in protein degradation had occurred compared to untreated silage.

There has been no analysis made on the distribution of amino acids in the feeds used in these studies. The content of amino acids containing sulphur (cysteine and methionine) may differ between the material used in the experiments, which might affect the levels of H_2S production. In future work it would be useful to analyse the distribution of amino acids to enable more accurate predictions of the process of proteolysis.

As described by Bird *et al.* (1971), inorganic sulphate can be reduced to hydrogen sulphide by rumen micro-organisms. The levels of inorganic sulphate in the herbage used in the experiments is not known and that might affect the results- though it is likely that any effect would be small.

In earlier experiments using this technique the samples were incubated in rumen fluid diluted with a buffer solution to keep the pH value stable. This was done in the first experiment (which is not included in this report) but it was found to be a bad idea

because it meant that the microbial population was diluted below normal rumen levels during the early phase of incubation. As a result, instead of measuring the proteolysis the amount of energy available to the microbes will be a limiting factor for their growth. For example, plant material with a higher sugar content would produce more H_2S due to its ability to provide the rumen microbes with more energy enabling them to degrade the protein, than plants poorer with sugar (Dewhurst, 2003, personal communication). To obtain a similar energy supply for all samples, whole rumen fluid was used as inoculum in all experiments. Measurements of the pH values were made before the start of incubations and after 12 hours to see if it is possible to use whole rumen liquor. It was found that the pH values were in a range of 5.5 to 6.9 during the incubation time which was found to be rather stable. If the incubations would last for longer time it might be consider to use a buffer solution.

The gas production technique has the potential to be developed to study all aspects of rumen fermentation and protein degradation through the study of gas composition. Further work is needed to develop the approach and measure fermentation products in addition to H_2S. The equipment used to measure H_2S production was quite simple and consideration should be given to use of more expensive gas analysis equipment- particularly equipment that would measure other gases as well.

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