THE CONTROL AND MANIPULATION OF SILAGE FERMENTATION

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SUMMARY

A series of experiments was conducted to investigate the mechanisms of the control and manipulation of silage fermentation.

Preliminary experiments, using silage additives conventionally described as fermentation inhibitors and fermentation stimulants, demonstrated the extent to which the chemical composition of silage may be manipulated (Chapter 3). In Experiment 1, high levels of addition of formic acid or a mixture of ammonium hexaformate, ammonium hexapropionate and caprylic acid (more than 6 l/t) inhibited fermentation and preserved 78-81% of the water soluble carbohydrate content of the forage ensiled. These additives at lower levels of addition and mixed ammonium tri-hydrogen tetraformate and ammonium tri-hydrogen tetrapropionate encouraged ethanol accumulation, presumably by yeasts, in the later stages of ensilage. In Experiment 2, an inoculant of *Lactobacillus plantarum* encouraged a rapid homolactic fermentation in the early stages of ensilage and appeared to deter undesirable micro-organisms but adding sucrose (40 kg/t grass) with the inoculant resulted in the accumulation of 61 g ethanol/kg dry matter (DM) silage, suggesting that yeasts assumed a more prominent role in the fermentation. Addition of sodium bicarbonate with the inoculant and sucrose sustained the homolactic fermentation (maximum 194 g lactic acid/kg DM after 120d) by encouraging a relatively high pH, and this vigorous lactic fermentation seemed to be effective against yeast activity (maximum 13 g ethanol/kg DM). In Experiment 3, the effects of some of these manipulations on microbial numbers were examined. Microbial numbers were not a reliable indication of the chemical composition of the silage.

It was recognised that an assessment of the metabolic status, as well as numbers, of micro-organisms on silage was required. Therefore the experiments described in Chapter 4 were conducted to develop techniques for the *in vitro* measurement of metabolic activity of silage micro-organisms. In Experiments 1 and 2, conditions were optimised for the *in vitro* assay of glucose uptake by an inoculum of silage micro-organisms. Further experiments (Experiments 3 and 4) to validate the assay demonstrated the sensitivity of an inoculum to perturbations to environmental conditions *in vitro*; e.g. glucose uptake by an inoculum prepared from 2-d-old silage was restricted by almost 30% per mmol undissociated lactic acid present. In addition, the assay proved useful for the assessment of metabolic activity of an inoculum prepared at timed intervals during ensilage. In preparation for a series of experiments to investigate the action of some silage additives, Experiments 5 and 6 evaluated techniques for the preparation of laboratory-scale silos. Ensiling chopped grass in polythene bags within an anaerobic cabinet was adopted as a standard method. Freezing and thawing

did not adversely affect the ensilability of grass, although there were problems related to the period over which the grass was left to thaw, so grass from a single harvest was frozen and stored at -20°C before use in series of experiments.

In Chapter 5, the techniques developed in Chapter 4 were used to study the microbial changes underlying the effects on chemical composition of the silage produced by fermentation inhibitors (Experiment 1) and fermentation stimulants (Experiment 2). The silage micro-organisms exhibited maximum activity soon after ensilage, reflected by high rates of *in vitro* glucose utilisation. This was associated with maximum rates of lactic acid accumulation in the silage, and led to maximum population sizes within 2-5 d. When compared with the extent of fermentation, as judged by the area under the curve of microbial activity during the first 20 d of ensilage, in the control silage all additives except sodium bicarbonate alone restricted fermentation; sodium bicarbonate encouraged a more extensive fermentation.

Changes in chemical composition indicated that acid additives, apart from high levels of addition of formic acid, restricted fermentation by undesirable micro-organisms only when combined with an active lactic fermentation. Surprisingly, numbers of lactic acid bacteria were stimulated by addition of lactic acid and sodium lactate (0.07 mol/l each). The reasons for this were not clarified, but rates of glucose utilisation *in vitro* and amounts of lactic acid produced in these silages were not greater than in the control. Furthermore, during the later stages of ensilage, after exhaustion of water soluble carbohydrates, lactic acid was degraded, possibly by lactic acid bacteria or saccharolytic clostridia encouraged by the provision before ensilage of these alternative substrates.

The early establishment of a very low pH (less than 3.50), with 6 l/t sulphuric acid or as a result of the rapid fermentation elicited by the inoculant with additional sucrose, appeared to cause the fermentation of substrates along alternative pathways. The inclusion of sodium bicarbonate with the inoculant and sucrose increased peak metabolic activity and maintained a homolactic fermentation which guaranteed deterrence of undesirable epiphytic micro-organisms, but led to utilisation of most of the water soluble carbohydrates in the silo by day 10. The incorporation of sodium bicarbonate alone maintained pH above 7.00 which seemed to contribute to the survival of coliform bacteria and saccharolytic clostridia as well as encouraging a rapid lactic fermentation. The control of these unwanted bacteria, therefore, appears to be reliant on a low pH as well as a vigorous lactic fermentation.

By using an anaerobic cabinet to maintain strict anaerobic conditions it was possible to provide additional carbohydrates during ensilage (Experiment 3), simulating to some extent the conditions resulting from the hydrolysis of plant fibre. Whilst continued fermentation by lactic acid bacteria apparently deterred yeasts in Experiments 1 and 2, the abundance of extra

hexoses in the silage when added 30 d after the grass was originally ensiled, allowed commensal growth and development of both groups of micro-organisms; after a further 30 d, an additional 27 and 21 g lactic acid/kg DM silage and 27 and 33 g ethanol/kg DM silage had accumulated from added glucose or fructose, respectively (18 g/kg forage ensiled). Yeasts apparently began to ferment the substrate immediately while lactic acid bacteria required a period of adaptation (approximately 10 d). Additional xylose supplied in the same way was not fermented.

In conclusion, although conservation of water soluble carbohydrates was difficult without high levels of addition of organic acids, lactic acid concentration and pH were shown to have important regulatory roles. Undissociated lactic acid was apparently a particularly potent inhibitor of microbial activity. But these factors were weaker individually. The results indicate that the rapid fermentation by homofermentative lactic acid bacteria is important to ensure a stable environment in the early hours after ensilage and thus to avoid deterioration during storage.

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1. INTRODUCTION

The principles of the conservation of fresh herbage as silage are well understood (there is evidence that the procedure was in use in Egypt as long ago as 1000-1500 BC (see Schukking, 1976)), and silage of a reasonable quality may be produced without additives by adherence to a few principles. However, increasing economic pressure to produce silage that is consistently both well preserved and of a high nutritive value, and evidence of improvements in performance in animals given additive-treated silages, make it attractive to use additives to manipulate processes in the silo to ensure the desired fermentation. This approach is not new; Watson and Nash (1960), summarising the literature, cited instances of additive use dating from as early as 1886. However, more information concerning the nature of fermentative processes during silage production, and the factors regulating them, is required before adequate control can be achieved.

The Ensilage Process

There is a wide choice in the detail of the procedures that may be used for silage making but the success and efficiency of the conservation process, and the feeding value of the silage produced, depend primarily on a limited number of factors. Chief amongst these are the chemical composition of the crop, the post-mowing, pre-ensiling changes in crop composition, and the fermentation in the silo.

The Composition of the Crop

For leafy materials typical of those used for silage making, water usually accounts for 780-850 g/kg of the total weight of the crop. The remainder consists of carbohydrates, nitrogenous compounds, lipids and minerals.

The plant carbohydrates may be classified into two main groups: the water-soluble or non-structural carbohydrates that are found in the plant cells, and the structural carbohydrates that are components of plant cell walls. The water-soluble carbohydrates (WSC) include simple sugars (mainly glucose, fructose and sucrose) and organic acid intermediates of sugar metabolism (malate, citrate, oxaloacetate etc.), along with complex insoluble polysaccharides that the plant synthesises as an energy store (Table 1.1). In temperate grasses the energy store is in the form of fructosans, while in legumes the predominant storage polysaccharide is starch. The cell wall is often referred to as the plant "fibre", and is an association of a number of components.

Table 1.1 The concentration of sugars, storage polysaccharides and cell wall components in samples of perennial ryegrass (g/kg dry matter (DM)) (after Thomas and Morrison, 1982). Values are for leafy material at an immature stage of growth.

Glucose + fructose	46
Sucrose	32
Fructosan	60
Cellulose	213
Hemicellulose	158
Pectin	24
Lignin	27

Of these, cellulose consists of chains of linear molecules of β,1-4 linked glucose residues aligned and cross-linked by hydrogen bonds to form a highly ordered molecular complex. In addition, the cell wall contains a range of other carbohydrate and non-carbohydrate components, and current views are that these materials are also linked together in a complex by covalent bonds. The main carbohydrate in this complex is hemicellulose; few hemicelluloses actually contain a single sugar and the main hemicelluloses in grasses are arabinoxylans, whilst in legumes arabinoxylans and glucomannans occur. Even though lactic acid bacteria can only use soluble sugars as substrate, hemicellulose may be the source for a portion of the lactic and acetic acids produced during ensilage. Before it can be fermented, however, hemicellulose must be hydrolysed to 5- or 6-carbon sugars. This hydrolysis is accomplished by chemical hydrolysis or by plant enzymes in the forage (Dewar *et al.*, 1963). Pectin, composed of α,1-4 linked methyl-substituted galacturonic acid residues, is also present in the cell wall structure of legumes and, to a lesser extent, grasses. The major non-carbohydrate component in the cell wall complex is the aromatic polymer, lignin, but there are small amounts of phenolic acids and acetic acid that are esterified to the other fibre components.

The concentration of crude protein (CP) in grass is typically 120-160 g/kg dry matter (DM) (Thomas and Morrison, 1982). The forage nitrogenous components include proteins and non-protein materials such as nitrates, ammonium salts, peptides, free amino acids, and the purine and pyrimidine bases of nucleic acids. Generally, 750-850 g/kg of the total nitrogen is accounted for as plant protein, the amino acid composition of which appears to vary relatively little with plant source (Lyttleton, 1973).

Plant lipids (typically 10-50 g/kg DM in grasses) are a mixture of triglycerides, glycolipids, phospholipids, waxes and sterols. The first three of these components provide a significant dietary source of long-chain fatty acids for the animal and, characteristically, the mixture of fatty acids is rich in unsaturated acids.

Factors Influencing Crop Composition The chemical composition of forage crops is influenced by many factors including the type, species and strain of plant, growing conditions (e.g. temperature, light intensity and water availability), fertilizer treatment, stage of growth, and cutting or grazing management. Even for a single strain of grass or clover it is impossible to predict the chemical composition under a particular set of field conditions with precision (see Agricultural Research Council (A.R.C.), 1976). However, some important and consistent trends in composition occur as plants mature. Maturation is associated with a reduction in the proportion of leaf, and alterations in leaf and stem composition. Characteristically, the

contents of WSC, protein and lipid are reduced and the amount of cellulose, hemicellulose and lignin is increased. Lignin is not digested by the ruminant, and its bonding to hemicellulose and the "encrustation" of the cellulose with the lignin-hemicellulose complex also limits the extent to which these carbohydrates are digested. Therefore, with advancing maturity and increased lignification, the digestibility of the cell wall components is reduced and there are accompanying effects on the digestibility of organic matter, gross energy and protein.

Low contents of WSC are also found in samples of regrowth grass and in these circumstances they are accompanied by higher protein contents (Waite, 1965). In addition, the WSC content is reduced, in primary and regrowth cuts, by the application of nitrogenous fertilisers; when the fertiliser is applied 1-2 weeks before cutting, protein content may be elevated but, when the nitrogen is applied, as is more typical, soon after each cut, the protein content of the next cut may be unchanged or reduced (see A.R.C., 1976).

Post-harvest changes in crop composition

The crop when it is placed in the silo is alive and therefore respiring actively. Although plants can and do respire proteins and lipids, carbohydrates are the major respiratory source and immediately after harvesting both sucrose and fructosans are rapidly hydrolysed to glucose and fructose. The liberated energy which accompanies the process will appear as heat, which accumulates and consequently increases the rate of respiration. When all of the oxygen has been consumed, carbohydrates are transformed anaerobically, but the changes are incomplete and a large number of intermediate compounds and organic acids are also formed.

There are indications (McDonald *et al.*, 1991) that extensive proteolysis occurs in the crop prior to, and during, the early stages of ensilage. The involvement of plant enzymes is implied since aseptically-grown and sterilised grass sustain an increase in non-protein nitrogen. After harvesting, rapid proteolysis may reduce the protein content of the material by 50 to 60 % to yield a wide variety of products including ammonia, γ-aminobutyric acid and histamine. These metabolites are detectable even in well preserved silage (Kemble, 1956). The amino acid composition of the fermented, non-protein fraction is not the same as that of the parent protein; most amino acids are present in lowered concentrations while the concentration of some, e.g. proline, glutamine and asparagine, is increased (Kemble and Macpherson, 1954). Chamberlain *et al.* (1982) and McGinn *et al.* (1990) found that some silage additives containing formaldehyde and/or formic acid reduced proteolysis, and Wetherall *et al.* (1990) used biological inhibitors to specifically restrict some proteolytic activities. However, proteolysis remains a poorly understood, and unfortunate, consequence

of cutting and ensiling the crop.

Microbial Fermentation

The presence of bacteria on the aerial parts of plants has long been recognised (Allen *et al.* 1937). Moon and Henk (1980), using a scanning electron microscope, observed the total number of bacteria on fresh grass to vary between 10^6 and 10^9/g DM. The bacteria were located primarily on the outer surfaces of the leaves. The great majority of these bacteria are strict aerobes which contribute little or nothing to silage preservation (Gibson *et al.*, 1958), and since anaerobiosis is achieved rapidly after the silo is sealed, their growth is soon inhibited. The coliforms (members of the Enterobacteriaceae family) are the most abundant of those organisms capable of anaerobic growth. In addition, *Bacillus* spp., *Clostridium* spp. and fungi are found in small numbers on fresh grass material (Gibson *et al.*, 1958). Clostridia, being obligate anaerobes, occur only in endospore form and probably result from soil contamination or slurry application. Lactic acid bacteria, which are the most important species during ensiling, are usually present on grass in numbers 1000 times lower than their main competitors, the fungi and enterobacteria.

Accumulation of acid end-products increases the antimicrobial activity in fermented products (Baird-Parker, 1980). The production of acid and the accompanying pH decrease extend the lag phase of sensitive organisms (Smulders *et al.*, 1986). Ingram *et al.* (1956) proposed that the following three factors were important for the preservative action of acidic substances: (i) the pH effect, (ii) the extent of the dissociation of the acid and (iii) specific effects of the molecule itself. Lipophilic acids such as acetic and lactic acid in their undissociated form can penetrate the microbial cell, interfere with essential metabolic functions and reduce the intracellular pH (Baird-Parker, 1980; Smulders *et al.*, 1986). The minimal inhibitory concentration of an undissociated acid for a specific spoilage organism is usually lower at a given pH than the inhibitory concentration of the total acid (Lindgren and Dobrogosz, 1990). Other fermentation end-products, such as formic acid, acetoin, 2,3-butanediol (Lindgren and Dobrogosz, 1990), diacetyl (Jay, 1982), hydrogen peroxide (Gilland and Speck, 1977), carbon dioxide (Clark and Takacs, 1980; Blickstad *et al.*, 1981) and antimicrobial proteins (bacteriocins) (Mattick and Hirsch, 1947), are known to be active in the preservation by lactic acid bacteria of other food and fermented feeds, although their role in the silage fermentation remains obscure.

The changes in bacterial population during the first few days are critical to the success or failure of the subsequent fermentation. If conditions are suitable, the lactic acid bacteria will quickly acidify the environment to such an extent that competing organisms will not be

able to survive. If the pH is not lowered quickly enough the undesirable micro-organisms will be able to compete for nutrients and, in so doing, further reduce the chances of obtaining a stable silage since many of their products do not aid preservation, and will reduce the nutritive value of the silage.

The Lactic Acid Bacteria Six genera (*Lactobacillus, Pediococcus, Enterococcus, Lactococcus, Streptococcus and Leuconostoc*), noted for their ability to produce lactic acid, are collectively characterised as lactic acid bacteria. Although lactic acid bacteria seem to co-exist with plants, their role on the plant surface is still unclear. It has been suggested that they may offer protection from pathogenic micro-organisms by producing antagonistic compounds such as acids, bacteriocins and anti-fungal agents (Visser *et al.*, 1986). Table 1.2 lists some of the genera found associated with forage. The predominant organism in silage is not constant, but, of all the lactic acid bacteria, *Lactobacillus plantarum* can most easily and successfully colonise freshly ensiled forage as this species can ferment a wide variety of substrates, is highly competitive and produces large amounts of acid quickly.

A distinctive feature of the lactic acid bacteria is their high acid tolerance. The pH range for growth is 4.0 to 6.8, although some species, such as *Pediococcus cerevisiae*, will grow at pH 3.5 (Kandler and Weiss, 1986). The minimum pH for growth and fermentation is strain dependent and the accumulation of acidic end-products of fermentation eventually lowers the internal pH below a critical level. Lactic acid bacteria are most often described as facultative anaerobes, since they can grow under aerobic or anaerobic conditions, but unlike other organisms which change from fermentative to respiratory pathways in the presence of oxygen, their metabolism remains fermentative (Gill *et al.*, 1986).

The pathways through which substrates are fermented differ according to the species of lactic acid bacteria present and may alter according to the nature of the substrate. The fermentation of hexoses is either homolactic or heterolactic; in the former case one mole of glucose (or fructose) is fermented to 2 moles of lactate, with no loss of carbon (Equation 1.1), whereas in the latter case acetic acid and ethanol are also formed (Equation 1.2).

Equation 1.1 Fermentation of glucose and fructose by homofermentative lactic acid bacteria.

$$\text{Glucose (or fructose)} + 2\text{ADP} + 2\text{Pi} \longrightarrow 2\ \text{Lactate} + 2\text{ATP} + 2\text{H}_2\text{O}$$

Equation 1.2 Fermentation of glucose and fructose by heterofermentative lactic acid bacteria.

a) Glucose + ADP + Pi ------→ Lactate + Ethanol + CO_2 + 2ATP + H_2O

b) 3 Fructose + 2ADP + 2Pi ---→ Lactate + Acetate + 2 Mannitol + CO_2 + 2 ATP + H_2O

c) Glucose + 2 Fructose + 2ADP + 2Pi ------→ Lactate + Acetate + 2Mannitol + CO_2 + 2ATP + H_2O

Thus, heterolactic fermentation is less efficient in terms of acid production, especially if fructose is more abundant than glucose, as is the case with grass. Homo- and heterofermentative lactic acid bacteria share a common pathway for the fermentation of pentoses.

Equation 1.3 Fermentation of pentoses by homofermentative and heterofermentative lactic acid bacteria:-

Xylose (or Arabinose) + 2ADP + 2 Pi ------→ Lactate + Acetate + 2ATP + $2H_2O$

The disappearance of organic acids during ensilage is probably due entirely to their fermentation by lactic acid bacteria and can be demonstrated under conditions of low glucose availability. These reactions are summarised in Equation 1.4a-1.4h.

Equation 1.4 Fermentation of citrate and malate by homofermentative and heterofermentative lactic acid bacteria:-

a) Citrate + ADP + Pi ------→ 2 Acetate + Formate + CO_2 + ATP

b) 2 Citrate + 2H ------→ 2 Acetate + 2,3-Butanediol + $4CO_2$

c) 2 Citrate + ADP + Pi ------→ 3 Acetate + Lactate + $3CO_2$ + ATP

d) Citrate + 4H ------→ Acetate + Ethanol + Formate + CO_2

e) Malate ------→ Lactate + CO_2

f) 2 Malate ------→ 2,3-Butanediol + $4CO_2$ + 2H

g) Malate + ADP + Pi ------→ Acetate + Formate + CO_2 + 2H + ATP

h) Malate + 2H ------→ Ethanol + Formate + CO_2

Table 1.2 Some lactic acid bacteria of importance during ensilage (after McDonald *et al.*, 1991).

Genus	Glucose fermentation	Morphology	Lactate	Species
Lactobacillus	Homofermentative	Rod	DL	*L. acidophilus*
			L(+)	*L. casei*
			D(L)*	*L. coryniformis*
			DL	*L. curvatus*
			DL	*L. plantarum*
			L(+)	*L. salivarius*
	Heterofermentative	Rod	DL	*L. brevis*
			DL	*L. buchneri*
			DL	*L. fermentum*
			DL	*L. viridescens*
Pediococcus	Homofermentative	Coccus	DL	*P. acidilactici*
			DL	*P. damnosus (cerevisiae)*
			DL	*P. pentosaceus*
Enterococcus	Homofermentative	Coccus	L(+)	*E. faecalis*
			L(+)	*E. faecium*
Lactococcus	Homofermentative	Coccus	L(+)	*L. lactis*
Streptococcus	Homofermentative	Coccus	L(+)	*S. bovis*
Leuconostoc	Heterofermentative	Coccus	D(-)	*L. mesenteroides*

* About 15-20 % of the total lactic acid is the L(+) isomer.

While many bacteria can attack lactate in the presence of oxygen, very few can do this anaerobically in the absence of another energy source or electron acceptor. However, the work of Henderson *et al.* (1987b) and Lindgren *et al.* (1990) confirmed that lactate could be dissimilated by lactic acid bacteria when all other fermentable substrate in the silage had become exhausted.

Lactic acid bacteria are virtually non-proteolytic and, since they have limited powers of amino acid synthesis, an external supply of amino acids is necessary for their growth. Their ability to ferment amino acids also appears to be restricted and it is thought that only two, serine and arginine, are extensively attacked by some of these organisms (McDonald *et al.*, 1991).

Coliform bacteria The principal fermentation product of coliforms is acetate, although they also produce lactate, ethanol and 2,3-butanediol and may, in part, be responsible for the appearance of these compounds in silage. In addition, coliforms are able to deaminate and decarboxylate some amino acids (Beck, 1978), but possess only weak proteolytic properties.

The conditions inhibitory to enterobacteria in the silo are not clear, but may be related to a combination of pH and lactic acid production, rather than to pH alone (Chamberlain and Quig, 1982).

Clostridia Clostridial growth is stimulated by high storage temperature, low DM content and delayed sealing of the silo. Saccharolytic clostridia act against preservation by utilising lactate and sugars, principally hexoses, to form butyrate and smaller amounts of formate, acetate, propionate, ethanol and butanol, as shown in Equation 1.5.

Equation 1.5 Fermentation of glucose and lactate by clostridia.

a) $Glucose + 3ADP + 3Pi \longrightarrow Butyrate + 2CO_2 + 2H_2 + 3ATP + 3H_2O$

b) $2\ Lactate + ADP + Pi \longrightarrow Butyrate + 2CO_2 + 2H_2 + ATP + H_2O$

Proteolytic clostridia reduce the nutritional value of the product by degrading amino acids. The fermentative actions are of three basic types: deaminative, decarboxylative and the Strickland type, which involves a coupled oxidation-reduction of pairs of amino acids (Barker, 1961). Some clostridia can reduce nitrate and nitrite to ammonia (Smith and Hobbs, 1974; Bousset-Fatianoff *et al.*, 1971); purines and pyrimidines are also degraded by some clostridia (Vogels and van der Drift, 1976).

The pH and water activity are the two main factors of importance in surpressing the growth of clostridia during ensilage. Clostridia cannot tolerate acid conditions (the optimum

pH for their growth is in the range 7.0 to 7.4) (Pelczar and Reid, 1972) and the pH limiting for growth is dependent on the actual concentration of acids, particularly the undissociated acids, in the silage (Woolford, 1975a; Jonsson, 1989). In addition, clostridial growth can be restricted if crops are wilted so that DM levels are above 300 g/kg (Gibson, 1965).

The Fungi Fungi are eukaryotic, heterotrophic organisms which grow either as single cells, the yeasts, or as multicellular filamentous colonies, the moulds. They obtain nutrients by secreting extracellular enzymes to break down complex organic molecules into simple monomers, which can then be absorbed through their cell membranes. The majority of fungi are strict aerobes although some, including a number of yeasts and a few filamentous fungi, can grow under anaerobic conditions.

Yeasts Under anaerobic conditions yeasts must obtain energy from the fermentation of sugars, as depicted in Equation 1.6.

Equation 1.6 Fermentation of glucose by yeasts.

$$\text{Glucose} + 2\text{ADP} + 2\text{Pi} \longrightarrow 2\ \text{Ethanol} + 2\text{CO}_2 + 2\text{H}_2\text{O}$$

Yeasts are not inhibited by the pH levels reached during ensilage, and most yeasts will grow within the pH range 3 to 8 (McDonald *et al.*, 1991). Inhibition of yeasts by short-chain organic acids, such as lactic and acetic acids, is achieved by undissociated acid molecules entering the cells by passive diffusion. Their subsequent dissociation releases H^+ ions which reduce the intracellular pH to a level which will rapidly kill the cell unless the ions are expelled again by active transport. This inducible proton-lactate symport system requires energy (Cassio *et al.*, 1987), so the presence of adequate sugar is essential for the survival of yeasts in acidic, anaerobic environments. Under aerobic conditions, however, yeasts are able to withstand organic acids better than most micro-organisms, and may utilise a variety of substrates, including lactate, acetate, citrate, malate, succinate, propionate, and ethanol (Kreger-van-Rij, 1984) and lactate-assimilating yeasts have been associated with aerobic deterioration (Daniel *et al.*, 1970; Jonnson and Pahlow, 1984).

Moulds The conditions in the silo associated with well-preserved silage are unfavourable for the growth of moulds and they are generally only associated with those areas of silage, such as on the sides and surface, that are exposed to air. Their presence is undesirable since they break down not only sugars and lactic acid via normal respiratory pathways but they are also able to hydrolyse and metabolise cellulose and other cell wall components. Furthermore, some moulds produce mycotoxins such as aflatoxin, patulin and zearaleone which are harmful

to animals and humans. There has been some interest in the use of inocula containing propionic acid bacteria to stimulate the production in the silo of propionic acid which, in its undissociated form, prevents the growth of moulds at low pH levels.

Other micro-organisms have been identified, in lower numbers, in silage. Bacilli are spore-forming bacteria, scarce on fresh plant material and deterred by a rapid multiplication of lactic acid bacteria (Gibson *et al.*, 1961) but some species have been implicated with the aerobic deterioration of silage (Woolford and Cook, 1978; Woolford, 1984; Woolford and Wilkie, 1984). Listeria are facultatively anaerobic bacteria found in relatively low numbers in silage, and are not thought to survive at low pH (Vetter and von Glan, 1978). However, where oxygen filters slowly into the silage, such as at the tie-end of big-bale silage bags, the low O_2 concentrations are favourable for listeria. The organism is known to be responsible for meningitis, encephalitis and septicaemia and to cause abortion. The increase in incidence of listeriosis coincides with the introduction of big-bale silage, and also with an increase in the use of untreated human sewage and animal slurry as a fertiliser (Watson, 1985); slurries are thought to be the most important source of contamination. Acetic acid bacteria have been associated with the aerobic deterioration of whole-crop maize silage (Spoelstra *et al.*, 1988) but it is not known whether they are important in grass silages.

Chemical aspects

Acid hydrolysis During the prolonged storage period, direct acid hydrolysis of cell wall polysaccharides may contribute to an increase in WSC. Dewar *et al.* (1963) demonstrated considerable breakdown of hemicelluloses at pH 4 by incubating hemicellulose preparations at various pH levels (4 to 6) for 90 days (Table 1.3). Morrison (1979, 1988), also noted increased hemicellulose breakdown when silages were made with certain acid additives, notably sulphuric and formic acids. It is not known whether natural acid production in silages causes any hydrolysis.

Buffering capacity The buffering capacity of plants, i.e. their ability to resist pH change, is an important factor in ensilage. Measurements of the buffering capacity are made by determining the amount of acid (either mineral, lactic or other organic acids) which must be added to a fresh crop to obtain the desired pH (McDonald and Henderson, 1962). Most of the buffering properties of herbage can be attributed to the anions present (organic acid salts, orthophosphates, sulphates, nitrates and chlorides) with only about 10 to 20 % resulting from the action of plant proteins (Playne and McDonald, 1966).

Table 1.3 Effect of pH on the hydrolysis of ryegrass hemicellulose over a 90-day period (after Dewar *et al.*,1963). Results expressed as xylose (g/kg hemicellulose).

pH	Temperature (^{0}C)		
	22	30	37
4.0	71	76	84
5.0	46	48	48
6.0	38	44	44

The implication for conservation is that up to 70 % of the lactic acid may be neutralised by plant constituents, so that only a small portion of the lactic acid may be present in the silage as free acid. O' Meara *et al.* (1987) suggested that it is not the amount of lactic acid present in silage which is the critical factor in determining stability, rather the amount neutralised by plant constituents.

Silage Additives

Extensive experimental evidence and much practical experience clearly indicate the considerable benefits that additives can confer. They can control, and thus improve, the fermentation in the silo, reduce waste and make a more acceptable product for livestock. Silage additives can be classified into five main categories (Table 1.4).

Fermentation Stimulants

Inoculants In some silages the development of lactic acid bacteria is very slow and this can lead to the growth of undesirable micro-organisms in the early stages of fermentation. Lindgren *et al.* (1983) found mostly heterofermentative species on fresh crops, numbers being especially low at the beginning and end of the growing season (Lindgren *et al.*, 1985). There is a strong case for the use of specific cultures of homofermentative lactic acid bacteria, applied at the rate of at least 10^6 colony forming units (CFU)/g fresh crop, if silage preserved by natural fermentation is the desired aim. It is important that strains selected for such use are genetically stable, able to carry out a rapid homofermentation of hexoses at ambient temperatures, and exhibit no proteolytic activity (Seale, 1986).

Although *L. plantarum* satisfies most of these criteria, some strains are slow to produce lactic acid until the pH falls below 5.0 (Woolford, 1972); the ideal inoculum should, therefore, also include lactic acid bacteria which are active within the pH range 5.0 to 6.5, such as streptococci and pediococci.

The commercial exploitation of cultures of lactic acid bacteria as additives for silage came with the development of freeze-drying and encapsulation techniques. Unlike acids, the products are safe to handle and do not corrode farm machinery, and are more attractive to the farmer.

Carbohydrate sources A combination of inoculum with sugar may be more efficient in influencing the fermentation than an inoculum used alone to augment the accumulation of lactic acid (Heron, 1985).

Table 1.4 Classification of silage additives (after McDonald *et al.*,1991).

Fermentation stimulants		Fermentation inhibitors		Aerobic deterioration inhibitors	Nutrients	Absorbents
Bacterial cultures	**Carbohydrate sources**	**Acids**	**Others**			
Lactic acid bacteria	Glucose	Mineral acids	Formaldehyde	Lactic acid bacteria	Urea	Barley
	Sucrose	Formic acid	Paraformaldehyde	Propionic acid	Ammonia	Straw
	Molasses	Acetic acid	Glutaraldehyde	Caproic acid	Biuret	Sugar beet pulp
	Cereals	Lactic acid	Sodium nitrite	Sorbic acid	Minerals	Polymers
	Whey	Benzoic acid	Sulphur dioxide	Pimarcin		Bentonite
	Beet pulp	Acrylic acid	Sodium metabisulphite	Ammonia		
	Citrus pulp	Glycollic acid	Ammonium bisulphite			
	Potatoes	Sulphamic acid	Sodium chloride			
	Cell wall degrading enzymes	Citric acid	Antibiotics			
		Sorbic acid	Carbon dioxide			
			Carbon bisulphide			
			Hexamethylenetetramine			
			Bronopol			
			Sodium hydroxide			

Addition of either glucose or sucrose to the crop at the time of ensiling ought to improve the fermentation; of the two sugars, glucose is preferable as some of the fructose component of sucrose may be converted by indigenous heterofermentative lactic acid bacteria to the neutral product mannitol. However, Chamberlain (1988) found that, rather than augmenting the accumulation of lactic acid, 80% of added glucose was fermented to ethanol, presumably by yeasts. Added xylose was not readily metabolised and approximately 30 to 50 % remained in the silage after 100 d, but this may have encouraged lactate-assimilating yeasts as there was a secondary fermentation of lactate to acetate.

In practice, sucrose, glucose and fructose are not normally used because of their high cost; molasses, a cheaper by-product, is preferred.

Cell Wall Degrading Enzymes The use of cellulolytic and hemicellulolytic enzymes as silage additives has the potential to increase the availability of fermentable sugars and to improve the digestibility of the organic matter.

Availability of fermentable carbohydrates Most commercial enzyme preparations contain cellulases and hemicellulases, and the combined actions of these enzyme systems on plant polysaccharides will result in a mixture of hexose and pentose sugars. The application of hemicellulase may increase the rate of the natural hydrolysis of hemicellulose, but has little effect on the extent of the hydrolysis, suggesting that most of the additional sugar is likely to come from the cellulose fraction (Henderson *et al.*, 1990). Honig and Pahlow (1990) warned that despite a measurable enzyme effect, the fermentation was not improved, with released pentoses fermented to acetic acid and ethanol. They concluded that improvements could only be expected if the enzyme additive is able to release a minimum of approximately 10 g hexoses/kg fresh weight during the critical first hours of fermentation.

Several workers have recognised the potential advantages of high levels of enzyme addition. Spoelstra (1990) used application rates up to 30 times higher than recommended by manufacturers; increasing amounts of enzyme led to additional neutral detergent fibre (NDF) degradation. Most cell wall was degraded in lower DM silages, incubated at 40^{0}C, with increasingly lower breakdown at higher DM contents, and increased maturity limited cell wall degradation. Prolonged incubation up to 150 days gave additional reductions in NDF, although most cell wall degradation occurred within 50 days. Spoelstra (1990) demonstrated very little effect of pH on enzyme action, but there were indications that plant proteolytic enzymes may have inactivated the hydrolytic enzymes during the initial stages of ensilage.

Many commercial enzyme preparations now incorporate an inoculum of homofermentative lactic acid bacteria to optimise the utilisation of released substrates, and Merry and Braithwaite (1987) and Henderson *et al.* (1987b) noted a higher lactic acid content and a lower pH in the early stages of fermentation than in corresponding untreated control or inoculated silages.

Digestibility of Organic Matter Although cellulases may increase the soluble carbohydrate content of herbage, experiments to demonstrate possible increases in digestibility have found them to be less effective (Whittemore and Henderson, 1977; van Vuuren *et al.*, 1979). Bacteria, fungi and protozoa in the rumen colonise practically all ingested plant material (Bauchop, 1980), and, since these micro-organisms have the enzymic potential to degrade cellulose and hemicellulose (Akin, 1986), enzymic pre-treatment of the forage in the silo may not necessarily increase overall digestibility. Indeed, a tendency for reduced digestibility in response to enzyme treatment has been reported (Jacobs and McAllan, 1987) and this may reflect a loss of soluble products of fibre degradation in silage effluent. Nevertheless, the rate of digestion in the rumen may be increased and fermentable carbohydrates may be released more rapidly, thus improving the efficiency of rumen microbial protein synthesis (Choung and Chamberlain, 1992). More work is obviously needed to clarify the action and control of fibrolytic enzymes in the silo, and the consequences for animal production.

Fermentation Inhibitors

Mineral Acids Virtanen (1933) developed a method of preserving crops using mineral acids. The main objective of the so-called AIV process was to lower the pH of forage to a level at which plant and microbial enzymes would be inhibited completely. Ideally, the pH of the crop should be reduced to below 3.0, but at this level silage is unpalatable to animals. There has been renewed interest in sulphuric acid (42-45% w/v) as a reasonably cheap silage additive, and the current objective is to supplement the natural fermentation process by the addition of moderate amounts of acid (Chamberlain and Quig, 1987), rather than to suppress the fermentation.

Organic acids Although formic acid is the strongest acid in the fatty acid series, it is considerably weaker than mineral acids, and no attempt is made, when using it commercially, to reduce the pH of the crop to below 4.0.

The antibacterial effect of formic acid, and of other fatty acids, is due partly to the hydrogen ion concentration effect and partly to the selective bactericidal action of the undissociated acid (McDonald and Henderson, 1974; Beck, 1978). Within the fatty acid homologous series, the hydrogen ion concentration effect decreases with increasing molecular

weight while the antimicrobial effect increases up to the C12 acid (Galbraith *et al.*, 1971; Woolford, 1975a). However, because of their low solubility in water, acids with chain lengths greater than C10 or C11 are not particularly efficient in this respect. Woolford (1975a) examined the first 12 acids in the fatty acid series for antimicrobial activity against micro-organisms associated with silage (Table 1.5). In general, the minimum inhibitory concentration decreases with increasing chain length.

Yeasts have been found to be particularly tolerant of formic acid, and high counts of these organisms, leading to high ethanol contents, have been observed in silages treated with this additive (Henderson *et al.*, 1972). In a study by Chamberlain and Quig (1987), silage was well-preserved with the application of 2 and 6 litres formic acid per tonne grass, but with 4 l/t the fermentation was poor. The low level of formic acid aided the natural fermentation and the high level severely restricted the fermentation, but the intermediate level inhibited the lactic acid bacteria to a greater extent than the enterobacteria; although the numbers of enterobacteria were reduced progressively by increasing levels of acid addition, formic acid did not prevent a rise in population as ensilage proceded. By day 90 the highest numbers were present in the silages prepared with formic acid at 4 and 6 l/t. As enterobacteria are known to generate formic acid, this acid probably has less effect on reducing their growth.

High levels of formic acid have also been shown to reduce rates of proteolysis and deamination, (Waldo *et al.*, 1971; Wilson and Wilkins, 1973; McDonald and Edwards, 1976), have an inhibitory effect on plant respiration (Castle and Watson, 1970; Henderson *et al.*, 1972; Kennedy, 1987), and to hydrolyse polysaccharides during the storage period (Morrison, 1979; 1988).

Salts of formic acid To overcome the corrosion and handling problems associated with the use of formic acid, a complex salt, ammonium tetraformate, was developed for commercial use. It has a lower acid content than formic acid, which is rectified by a higher rate of application. Several studies (Chamberlain *et al.*, 1990b; McGinn, 1990) demonstrated effective preservation of sugars with a high level of Maxgrass (B.P. Chemicals Ltd) (a mixture containing 870 g/kg tetra-ammonium formate, 100 g/kg propionic acid and 30 g/kg caprylic acid).

Formaldehyde Although formalin-acid mixtures are very effective on grass, the potential health risks of exposure to formaldehyde are now recognised (Selikoff and Hammond, 1981) and their use has been banned in some countries (McDonald *et al.*, 1991).

Table 1.5 Minimum inhibitory concentrations (mmol/l) for a series of short chain fatty acids against various groups of micro-organisms at pH 4 and 3 (after Woolford, 1975a).
(* denotes antimicrobial effect due to pH)

	Group of micro-organisms					
	pH 4				pH 3	
	Lactic acid bacteria					
	Homo.	Hetero.	Yeasts	Moulds	Yeasts	Moulds
Formic	13	*	75	100	25	50
Acetic	94	94	94	94	94	< 12
Propionic	63	31	63	63	31	16
Butyric	63	39	16	< 8	16	< 8
Valeric	*	12	12	6	12	6
Caproic	31	4	6	3	4	3
Enanthic	16	16	6	2	3	2
Caprylic	16	4	6	4	4	3
Pelargonic	4	4	4	2	2	< 0.5

The efficiency of conservation of nutrients during ensilage

Losses in silage making occur in the field and in the silo during preservation and storage of the crop. Watson and Nash (1960), summarising the literature, concluded that DM losses averaged 12-19% but could be as high as 40%. Corresponding figures for crude protein and metabolisable energy loss were 11-20% and 20-30%, respectively. Subsequent studies (Papendick, 1974; Waldo, 1977; Lingvall, 1978) have shown a similar range of DM loss but they indicate that with good ensilage techniques losses may be restricted to values approaching 10 %.

Field losses

Field losses arise through plant respiration, weather damage and inefficiencies in the harvesting of the crop. Respiration losses will account, under many conditions, for approximately 1 to 2 % of plant DM/day, although the loss of nutritive value is somewhat greater since it is the soluble, highly digestible carbohydrate that is affected. Where weather conditions are poor, field losses rise sharply, particularly if wilting is prolonged. Rain leaching of nutrients is exacerbated if there has been bruising or laceration of the herbage to release the cell sap.

In-silo losses

Losses in the silo are due to plant and microbial aerobic metabolism, avoidable and unavoidable fermentation, and effluent. Prolonged aerobiosis following the initial ensilage of the crop results in the oxidation of plant sugars and the development of undesirable microbial populations. Where air is satisfactorily excluded from the silo an anaerobic fermentation proceeds, but the efficiency of the process depends on the type of fermentation and the time taken to achieve the pH needed for preservation. Homolactic fermentations are the most efficient since they yield products of the maximum acidity with little loss of DM or energy. Heterolactic fermentations are less efficient, giving acidic and neutral products, and a higher loss of DM, although energy loss differs little from that with homolactic fermentation, because some of the fermentation products have a high energy content.

Effluent is a particular problem with silages of low DM content and may account for a substantial loss of soluble nutrients (up to 6 % of the crop DM); this loss reduces to zero at a forage DM content of between 25-30%.

In some circumstances, particularly with crops of a high DM and a high residual soluble carbohydrate content, there may be additional losses through aerobic deterioration by bacteria, yeasts and fungi after the silo has been opened (Beck, 1978; Woolford, 1978).

Silage as a feed

Nutritional limitations

The pre-fermented nature of silage imposes important limitations on its nutritional value. Silage nitrogen is rapidly broken down in the rumen, resulting in high concentrations of ruminal ammonia (McDonald and Edwards, 1976).

There is clear evidence of a pronounced net loss of N between mouth and duodenum due to ammonia absorption from the rumen (Thomas and Chamberlain, 1982a; Chamberlain *et al.*, 1982; Chamberlain *et al.*, 1986) arising from inefficient capture of NH_3-N by rumen bacteria. The cause of this intra-ruminal recycling of nitrogen and the low rates of microbial protein synthesis with silage diets can partly be explained by low yields of ATP generated from digestion of silage fermentation products in the rumen (Thomas, 1982). The short-chain fatty acids in silage (mainly lactic acid and acetic acid plus some propionic acid) may be absorbed across the rumen wall and utilised by the host animal but are of little or no calorific value to the rumen microbiota, since, in the rumen:

1 mole hexose----------→ 4 ATP

but if the hexose is fermented in the silo, then at best (homolactic fermentation):

1 mole hexose----------→ 2 lactic acid

with a net yield of 1 ATP in the rumen; the loss is greater if pentoses are fermented:

1 mole pentose----------→ 1 lactic acid + 1 acetic acid

with a net yield of 0.5 mole ATP in the rumen (Chamberlain, 1987).

Thus, the presence of the end-products of fermentation in the silo reduces the yield of ATP per unit of organic matter fermented in the rumen. It has been estimated that feeding silage could deprive the micro-organisms in the rumen of approximately one third of the energy supply potentially available in the original herbage (Tamminga, 1982). Chamberlain (1987) calculated that ATP yield in the rumen with a well-preserved naturally fermented silage (containing 150 and 40 g/kg DM, respectively, of lactic and acetic acid) was 15 to 20 % less than with silage of restricted fermentation (60 and 20 g/kg DM lactic and acetic acid). Consequently, a reduction in ATP yield and in microbial growth in the rumen leads to a reduction in the supply of amino acids to the host animal, since the ruminant's major source of protein is derived from microbial cells leaving the rumen and under-going digestion in the small intestine. Furthermore, in nutritional terms, the effect is magnified since microbial proteins, relative to most dietary proteins, are rich in essential amino acids such as methionine and lysine, the supplies of which can limit animal production (Thomas and Chamberlain,

1982).

Although some of the nutritional limitations associated with silage feeding may be alleviated by supplementation with extra fermentable carbohydrate (Chamberlain *et al.*, 1985; Rooke *et al.*, 1987) and with protein concentrates (Castle and Watson, 1976; Castle, Gill and Watson, 1983; Rae *et al.*, 1986), it would appear sensible to consider methods of manipulating fermentation in the silo that not only inhibit undesirable groups of micro-organisms, but also produce a silage that is utilised more efficiently by the ruminant. In this respect the objective should be to improve the preservation of the original protein in the forage and to produce a silage that contains a supply of readily-fermentable substrates.

Relation of Silage Composition to Nutritional Value

Silages of similar chemical composition can differ widely in nutritional value for reasons that are not altogether understood. Gordon (1987), using 2.2 l/t formic acid, 3.2 l/t Ecosyl and an untreated control, found only minor differences in fermentation within the silo, yet the inoculant-treated silage was associated with higher DM intakes and milk yield. Other workers (Hooper and Armstrong, 1987; Steen *et al.*, 1989; Chamberlain *et al.*, 1990a) demonstrated similar benefits associated with inoculant additives. By contrast, Appleton and Done (1987) found no differences in silage fermentation using 4 l/t Add-F or an inoculant, but formic acid-treated silage resulted in higher intakes and improved animal performance. There have been clear nutritional benefits attributable to the preservation of fermentable carbohydrates and a consequent increased efficiency of silage N utilisation for rumen microbial protein synthesis (Chamberlain *et al.*, 1990a,b; Henderson *et al.*, 1990). On other occasions, although additives have improved fermentation in the silo, these improvements have not been reflected in animal performance. Chamberlain *et al.* (1987), comparing an inoculant of *L. plantarum*, a cellulase/hemicellulase preparation and 2.3 l/t formic acid against an untreated control, and Kennedy (1987), using a similar set of treatments, saw no significant differences in any of the intake or animal performance parameters despite readily-measurable differences in silage composition related to additive type. The data would suggest that the utilisation of silage and the mechanisms by which silages of quite different chemical composition promote equally good animal production are more complicated and not determined simply by the extent of fermentation.

Rumen fermentation patterns can differ between silages, there being a positive correlation between the concentration of lactic acid in the silage and the molar proportion of propionic acid in the rumen (Gordon, 1989a, b; Mayne, 1990; Martin *et al.*, 1990). The higher (acetate plus butyrate): propionate ratios in the rumen for formic acid-treated silages

were associated with higher milk fat concentrations, presumably reflecting differences in the ratio of lipogenic to glucogenic precursors in the products of digestion and, possibly, homonally-mediated effects on nutrient utilisation. Lower concentrations of milk fat from animals fed inoculant-treated silages, compared with those of animals fed restricted fermentation-silage diets (Chamberlain *et al.*, 1987; Gordon, 1989 a,b; Mayne, 1990), may relate to these differences in the pattern of rumen fermentation. The theoretical arguments for restricting fermentation may, therefore, at times be over-ridden by factors such as the need for milk of a particular quality.

AIMS AND OBJECTIVES

Until the action of silage additives is properly understood and until the effect on the chemical composition of the silage can be predicted, progress to identify factors regulating animal performance from silage-based diets will be limited. An underlying problem is our lack of knowledge of factors controlling the extent and pattern of fermentation in the silo.There is a real need to investigate more closely the action of some silage additives on the fermentation of grass. The experiments reported in this thesis were designed to differentiate the regulating factors involved in the silage fermentation and to clarify the mode of action of some silage additives.

2. MATERIALS AND METHODS

Silage production

S.23 perennial ryegrass (*Lolium perenne*) was cut with a Deutch-Fahr disc-mower, harvested using a precision-chop Taarup forage harvester (chop length approximately 20 mm) and ensiled within 2 h of harvest, restricting wilting to a minimum.

The field was totally committed to silage after the application of a compound fertiliser 10-12 weeks before harvesting and Nitram (ICI) at least 8 weeks before harvesting. The first cut was taken May-June, and a second in July, and a third August-September.

Excess grass was chilled at 4°C for 24 h, mixing thoroughly to avoid heating of the mass, and subsequently stored frozen at -20°C in 25 kg-capacity plastic bags.

Triplicate sub-samples of grass from each harvest were stored frozen at -20°C or dried and milled for analysis.

1 tonne (cube) silos These were constructed from cubic fibre-glass water tanks (1.82 m^3); 1 tonne of grass was loaded and consolidated as the cube was filled, and sealed with plastic silage sheet (125 μm thick, Plasti-covers, Irvine Industrial Estate, Irvine). A layer of sand-filled plastic fertiliser sacks was placed over the sheet to ensure compaction and to restrict infiltration by air. Finally a plywood cover was fastened over the top as protection against the elements. Effluent was allowed to drain from the bottom where the cubes were standing on wooden pallets. When additives were required, 1 tonne grass was loaded into a feeder wagon (Roco-Mengele mixer wagon), mixed with the appropriate additive, unloaded via the feeder chute and subsequently ensiled. Sub-samples were taken from each silo using a petrol-driven corer.

8 kg (tube) silos These were constructed from plastic drain pipes (17 l capacity) with a perforated disc at the bottom allowing free escape of effluent. 8 kg grass was loaded and consolidated; each silo was sealed with sheeting and weighted with 2 kg lead-shot which was contained in a plastic bag to evenly distribute the weight over the surface of the grass and restrict infiltration of air. Wet additives were applied as a fine mist over the grass using a hand-held applicator (plant feeder); dry additives were sprinkled evenly over the grass and mixed thoroughly.

Measuring cylinder silos Grass was ensiled in 250 ml polypropylene measuring cylinders (approximately 300 g/laboratory silo) which were securely sealed with plastic fermentation traps. Additives were applied as for the 8 kg silos.

Polythene bag silos Sterile 31 cm x 18 cm polythene bags (standard polythene) (Seward

Medical, Great Suffolk Street, London) were filled loosely with approximately 150 g grass (following appropriate additive application) within an anaerobic cabinet (Don Whitley Scientific Limited, Shipley, West Yorkshire) in which the gas atmosphere was CO_2 (97%) and H_2 (3%). The open ends were folded over and secured to restrict evaporation from the fermenting grass. The bags were left in the cabinet for the duration of the experiment to protect them from oxygen exposure.

Chemical analysis of the silage

Analyses were performed on forage samples that had been minced with a household mincer and stored at -20°C.

Dean and Stark DM This was determined by distillation of a minced silage sample with toluene, following the procedure of Dewar and McDonald (1961).

Minced silage (25 g) was placed in a round-bottomed 1 l flask and immediately covered with 300 ml of redistilled toluene. The refluxing was continued until the level of water in the receiver did not change over a period of 15 min. The water receiver was disconnected and left for 1 h and the volume of water recorded. After discarding the toluene, 10 ml water was pipetted into a 25 ml volumetric flask and diluted to volume. The acidity of the water was measured; 10 ml of the diluted liquid with 40 ml neutral ethanol was titrated with NaOH (0.1 mol/l) using phenolphthalein as indicator.

Calculation

Volume correction = 2.5 x V/10 x T x 0.0055 ml = 0.001375VT ml

At 20°C weight of water = 0.998V(1-0.001375T)

Weight of silage DM = W-0.998(1-0.001375T)

% of DM = {100[W-0.998V(1-0.001375T)]}/W

where W = weight of fresh silage (g)

V = observed volume of water (ml)

T = titre of NaOH (0.1 mol/l) (ml)

pH of silage A representative sample of 20 g silage was mixed with 20 ml distilled water and the pH recorded using a Corning 120 pH meter.

Total nitrogen (TN) This was measured using an automated version of the Kjeldahl method, the Kjeltec 1030 apparatus (Tecator Ltd., Bristol). Silage (1 g) was digested at 420°C with N_2-free H_2SO_4 (980 g/l) and catalyst tablets (3.5 g K_2SO_4 and 0.4 g $CuSO_4.5H_2O$). The digested sample was distilled with NaOH (460-480 g/l) and nitrogen content determined by titration of the distillate with HCl (0.02 mol/l) using bromocresol green/methylene red mixed indicator solution.

Crude protein (CP) was calculated by multiplying the TN content of the sample by 6.25.

True protein (TP) and non-protein nitrogen (NPN) in silage The TP content of silage was determined by Kjeldahl analysis of the material precipitated by tannic acid (Van Roth, 1939). NPN content was calculated by subtracting the TP content from the CP content.

Reagent

Tannic acid solution was prepared by dissolving 4.45 g tannic acid and 0.1 ml concentrated H_2SO_4 in 100 ml distilled water, allowing to stand for 24 h, before filtering through Whatman N°42 filter paper.

The sample (1 g) was weighed into a centrifuge tube and incubated at 100°C for 15 min with 20 ml boiling tannic acid solution, cooled for 15 min and centrifuged at 1500 x *g* for 10 min. The supernatant was discarded and the residue resuspended in 25 ml distilled water and re-centrifuged as before. The washing and centrifuging was repeated twice more and the residue placed in a Kjeldahl digestion tube for nitrogen determination as described above.

Ammonia nitrogen in silage This was determined on a water extract of the sample. The extract was prepared by incubating 20 g silage and 200 ml distilled water at 40°C for 30 min, stirring intermittently. The extract was filtered through muslin and centrifuged at 1500 x *g* for 20 min. The supernatant was retained for analysis.

Silage extract (10 ml) was distilled with NaOH (460-480 g/l) and the distillate titrated with HCl (0.02 mol/l) using bromocresol green and methylene red mixed indicator solution using a Kjeltdec 1030 auto-analyser.

Lactic acid in silage This was determined by the method of Elsden and Gibson (1954). Lactic acid is oxidised to acetaldehyde which combines with sodium metabisulphite and is determined iodometrically. Sugars which may give rise to carbonyl compounds, and nitrogenous compounds such as proteins are removed with copper sulphate and calcium hydroxide.

Silage extract (9.5 ml) was mixed with 0.5 ml $CuSO_4$ (200g/l) and 1 g calcium hydroxide, left for 30 min, and centrifuged until clear. An aliquot (1 ml) of the clear supernatant with 4 ml H_2O and 0.5 ml H_2SO_4 (5 mol/l) was distilled with 5 ml ceric sulphate solution (ceric sulphate (20 g) in H_2SO_4 (1 l) (0.5 mol/l)). The distillate (15 ml) was collected in 2 ml fresh sodium metabisulphite (5 g/l), cooled, and mixed with 1 ml starch solution (20 g/l). Iodine solution (0.05 mol/l containing 20 g/l potassium iodide and 13 g/l iodine) was added to give a permanent blue colour, which was decolourised with sodium

thiosulphate (0.1 mol/l); iodine (5 mmol/l) was added until a pale blue colour persisted and this was decolourised with 1 g sodium hydrogen carbonate. Finally, the solution was titrated with iodine (5 mmol/l) until a pale blue colour persisted for not less than 1 min.

Water soluble carbohydrates (WSC) in silage These were determined by a method similar to that of Somogyi (1945).

Reagents

Reagent A contained (g/l), sodium carbonate, 25; Rochelle salt (potassium sodium tartrate, 25; sodium sulphate, 20).

Reagent B was a solution of $CuSO_4$ (150 g/l), with 1 or 2 drops of concentrated sulphuric acid per 100 ml.

Reagent C contained 25 parts reagent A with 1 part reagent B.

Arsenomolybdate reagent:- 25 g ammonium molybdate and 21 ml Analar concentrated sulphuric acid were dissolved in 450 ml water, and 25 ml disodium hydrogen arsenate (30 g/l) added; the mixture was incubated at 55°C for 25 min, with continuous stirring, and transferred to a brown bottle.

A sample (5 ml) of silage extract was hydrolysed at 100°C for 30 min with 0.1 ml H_2SO_4 (1 mol/l), and neutralised with 0.1 ml NaOH (1 mol/l) after cooling. The hydrolysate (2 ml) was deproteinised with 4 ml $ZnSO_4$ (50 g/l) and 4 ml NaOH (0.3 mol/l). The suspension was mixed and centrifuged at 1500 x *g* for 10 min and 2 ml supernatant or standard was incubated at 100°C for 10 min with reagent C. Arsenomolybdate reagent (2 ml) was added to the cooled mixture and the solution transferred to a 50 ml volumetric flask and made up to volume with distilled water. The absorbance was read on a Lambda 5 spectrophotometer (Perkin Elmer, Beaconsfield, Buckinghamshire) at 500 nm against a blank of distilled water. The total soluble sugars in samples were calculated by reference to a calibration graph derived with standard solutions containing 50 to 250 mg/l 50:50 glucose and xylose.

Ethanol in silage Ethanol was determined by gas chromatography by the method of Huida (1982) using methanol as an internal standard. Dry methanol (30 μl) was added to 5 ml silage extract and 1 μl injected on to the column of an 8310 gas chromatograph (Perkin-Elmer, Buckinghamshire) fitted with a flame ionisation detector. The columns were 2 m long, 2 mm internal diameter and packed with Chromosorb 101. The oven setting was 100°C and the carrier gas (N_2) flow was 60 ml/min.

Total and individual volatile fatty acids (VFA) The VFA in silage extract was determined by gas chromatography by the procedure of Cottyn and Boucque (1968).

Reagents

The preservative mixture contained 30 ml metaphosphoric acid (250 g/l) and 20 ml distilled water.

The internal standard contained 2 g/l hexanoic acid.

The VFA standard solution contained 4 ml acetic acid (6 g/100 ml), 2 ml propionic acid (7.2 g/100 ml), 2 ml butyric acid (8.4 g/100 ml), 2 ml isobutyric acid (0.8 g/100 ml), 2 ml valeric acid (0.96 g/100 ml) and 2 ml isovaleric acid (0.96 g/100 ml) in 100 ml distilled water.

Silage extract (5 ml), 1 ml preservative and 1 ml hexanoic acid were mixed well, allowed to stand for 20 min, and centrifuged at 1500 x *g* for 20 min. The supernatant was analysed using a Shimadzu GC-8A gas chromatograph (Dyson Instruments, Ltd., Hetton, Tyne and Wear). The sample (1-3 μl) was injected onto a glass column packed with 5% Carbowax 20M/TPA on Chromosorb G 80/100 mesh. The oven temperature was 135°C and carrier gas (N_2) flow rate was 60 ml/min.

Neutral detergent fibre (NDF) and acid detergent fibre (ADF) The NDF and ADF contents in silage were determined by the method of Goering and Van Soest (1970) on samples that had been dried at 100°C to constant weight and milled.

Reagents

Neutral detergent (ND) solution contained (g or ml/l): sodium lauryl sulphate , 30; disodium ethylene diaminetetraacetic acid dihydrate, 18.6; sodium borate decahydrate, 6.81; anhydrous di-sodium hydrogen phosphate, 4.56; 2-ethoxyethanol, 10.

Acid detergent (AD) solution consisted of 20 g cetyl-trimethyl ammonium bromide per l H_2SO_4 (0.5 Mol/l).

Approximately 1 g of sample was mixed in a 500 ml round-bottomed flask with 100 ml ND solution, 2 ml Dekalin and 0.5 g sodium sulphite, for NDF determination, or with 100 ml AD solution and 2 ml Dekalin, for ADF determination. The flask was refluxed for 60 min after the onset of boiling, and the contents transferred to a pre-weighed sintered glass crucible (porosity 1) which was set on a filter manifold. The flask and the inside of the crucible were washed twice with boiling water and twice with acetone. The crucible and its contents were dried overnight in an oven at 100°C and reweighed after cooling in a desiccator. The sample was ashed at 580°C for 3 h and the ash-free concentrations of NDF and ADF recorded as the subsequent loss in weight.

Microbial enumeration

The methods were based on the techniques described by Seale *et al.* (1986). 10 g of each minced silage sub-sample was placed in a sterile, polythene (standard polythene) stomacher

bag (31 x 18 cm) (Seward Medical) with 90 ml anaerobic diluent, and homogenised for 2 min in a stomacher, Lab Blender 400 (A. J. Seward, Blackfriars Road, London). The homogenate was strained through muslin into a clean, CO_2-filled 100 ml Erlenmeyer flask and ten-fold serial dilutions prepared in anaerobic diluent-filled Hungate tubes.

Aliquots (1 ml) of the appropriate dilution was used for triplicate, double-layered pour-plates of the following media:

a) Yeast Extract agar for total viable colony forming units (CFU),

b) Tween Acetate agar for lactic acid bacteria,

c) Violet Red Bile agar for coliform bacter ia, and

d) Malt Extract agar for yeasts and moulds.

Oxoid media constituents were obtained from Unipath Limited, Wade Road, Basingstoke.

Each medium was poured at 45-47°C and, when set, the petri dishes were inverted and incubated at 35°C; coliforms were enumerated after 2 d and other microbial groups after 5 d incubation.

Anaerobic diluent (Bryant and Burkey, 1953) contained (g or ml/l): K_2HPO_4, 0.45; KH_2PO_4, 0.45; $(NH_4)_2SO_4$, 0.90; $MgSO_4.7H_2O$, 0.09; $CaCl_2.2H_2O$, 0.14; cysteine hydrochloride, 0.05; Na_2CO_3, 3; rezazurin (1 mg/ml), 1. Oxygen was removed by boiling and gassing until cool with CO_2; the pH was adjusted to 6.6. The diluent was sterilised by autoclaving at 121°C for 15 min.

Yeast Extract agar This was based on the medium of Woolford and Cook (1978) and contained (g/l) yeast extract (Oxoid L.21), 5; lab lemco (Oxoid, L.29), 5; neutralised bacteriological peptone (Oxoid L.34), 5; fructose, 5; $MgSO_4.7H2O$, 0.5; technical grade agar (Oxoid L.13), 15. The pH was adjusted to 6.8-7.0, and the constituents boiled and dispensed before autoclaving at 121°C for 15 min.

Tween Acetate agar This was based on the medium of Keddie (1951) and contained (g or ml/l) yeast extract (Oxoid L.21), 5; lab lemco (Oxoid L.29), 8; neutralised bacteriological peptone (Oxoid, L.34), 10; glucose, 10; Tween 80, 0.5; triammonium citrate, 2; $MgSO_4.7H_2O$, 0.4; $MnSO_4.4H_2O$, 0.1; technical grade agar (Oxoid, L.13), 20. The pH was adjusted to 5.4 and the constituents were boiled and dispensed before autoclaving at 121°C for 15 min.

Before pouring, sterile sodium acetate trihydrate (17 g/l) / glacial acetic acid (233.2 g/l) buffer (2 mol/l), pH 5.4, was thoroughly mixed with the medium to give a final concentration of buffer in the medium of 0.2 mol/l.

Violet Red Bile agar This was prepared according to the manufacturer's recommendations (Oxoid CM 107), and dispensed before autoclaving at 121°C for 15 min.

Malt Extract agar This was prepared according to the manufacturer's recommendations (Oxoid CM 59) and, after boiling to dissolve the constituents, was autoclaved at 121°C for 15 min. Lactic acid (1 ml/l) was added to the molten media, after it had cooled to 55°C, prior to pouring.

Petri dishes Sterile, triple-vent 90 mm diameter, plastic petri dishes (Bibby Sterilin Limited, Tilling Drive, Stone) were used for all cultures.

Enumeration of micro-organisms Whenever possible, plates containing 30-300 CFU were counted. An average result for the triplicate plates was calculated and corrected for dilution. The number of micro-organisms present was expressed per g FW or DM, as indicated in the text.

Metabolic assay

The assay estimated the fermentative activity of an inoculum prepared from silage by measurement of glucose utilisation in an *in vitro* system.

Preparation of inoculum Minced silage (10 g) was homogenised for 2 min in 90 ml anaerobic diluent (pH 6.6) using a Stomacher 400 Lab Blender. The extract was strained through muslin into CO_2-filled 100 ml Erlenmeyer flasks, and 2 x 35 ml samples were centrifuged at 27000 x *g* for 15 min at room temperature in CO_2-filled gas-tight polycarbonate centrifuge tubes. Each cell pellet was resuspended in 3.5 ml anaerobic diluent and combined. The final concentration of microbes in the inoculum (7 ml) was similar to that found on the original silage.

Incubation procedure Hungate tubes containing 7 ml reduced buffered glucose solution (20 μmol/ml glucose, 0.2 mg/ml dithiothreitol in phosphate buffer (0.1 mol/l), pH 6.5, CO_2-gassed), prepared anaerobically, were inoculated with 3 ml cell suspension and incubated at 37°C for 3 h. Sub-samples (1 ml) were withdrawn using sterile syringes every 30 min during the 3 h incubation, centrifuged at 11600 x *g* for 10 min and the supernatant stored at -20°C. Appropriately diluted sub-samples were later analysed for glucose concentration and the rate of glucose utilisation was taken as a measure of the fermentative activity of the silage inoculum.

Analysis of the glucose concentration in the supernatant The concentration of residual glucose in the supernatant of sub-samples taken during the *in vitro* assay was determined enzymically using glucose oxidase with a chromogen; the glucose was oxidised by glucose oxidase (GOD) and the hydrogen peroxide formed, in the presence of peroxidase (POD),

oxidised the chromogen, either o-dianisidine or Perid (2,2 azino-di[3 ethyl-benzthiazoline sulphonate]).

$$\beta\text{-D-Glucose} + O_2 + H_2O \xrightarrow{GOD} \text{D-Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{O-Dianisidine (reduced)} \xrightarrow{POD} \text{O-Dianisidine (oxidised)} + H_2O$$

Reagents

10 ml sodium phosphate buffer, 0.5 mol/l, pH 7.00

1 mg peroxidase (40 units)

2 mg glucose oxidase in 30 μl stock solution (30 units)

50 μl O-dianisidine (10 g/l in H_2O)

Appropriately diluted supernatant (5 μl) was incubated with 250 μl reagent at 37°C for 1 h. Blank determinations were performed on distilled water. The absorbance at 450 nm was read on a Titertek spectrophotometer aand glucose concentration was determined by reference to a standard curve.

Lactic acid concentration in the supernatant D- and L-lactic acid concentrations were determined enzymically using D- and L-lactate dehydrogenase (D- and L-LDH) (U.V. method, Boehringer Mannheim). In the presence of D-LDH, D-lactic acid is oxidised by nicotinamide adenine dinucleotide (NAD) to pyruvate. The oxidation of L-lactic acid is performed by L-LDH.

$$\text{D- or L-lactic acid} + NAD^+ \xrightarrow{LDH} \text{pyruvate} + NADH + H^+$$

Glutamate-pyruvate transaminase (GPT), in the presence of L-glutamate, catalyses conversion of pyruvate, displacing the equilibrium of the reaction in favour of pyruvate and NADH.

$$\text{Pyruvate} + \text{L-glutamate} \xrightarrow{GPT} \text{L-alanine} + \text{2-oxoglutarate}$$

Reagents

A: 440 mg L-glutamic acid dissolved in 30 ml glycylglycine buffer (0.5 mol/l), pH 10;

B: Freeze dried NAD (35 mg/ml);

C: GPT suspension (1571 units/ml);

D: D-LDH solution (5429 units/ml);

E: L-LDH solution (5429 units/ml).

Supernatant (0.1 ml) or water blank was incubated at 20-25°C for 5 min with 1 ml reagent A, 0.20 ml reagent B, 0.02 ml reagent C and 1 ml distilled water, and the absorbance at 340 nm was recorded. Following incubation for 20 min with 0.02 ml reagent D absorbance at 340 nm was recorded again. The difference in absorbance was proportional to the concentration of D-lactic acid. The reaction mixture was incubated with 0.02 ml reagent E for a further 20

min, and the difference in absorbance at 340 nm from that of the original was proportional to the concentration of L-lactic acid.

Total carbohydrate assay The method of Dubois *et al.* (1956) was used for estimation of residual carbohydrate concentrations *in vitro*, and also, on occasion, for total carbohydrate analysis of the silage. Phenol (0.5 ml) was mixed with 0.5 ml diluted sample and 2.5 ml conc. H_2SO_4 was added. The sample was mixed thoroughly and on cooling the absorbance at 490 nm was recorded. Carbohydrate concentration was determined by reference to a standard curve prepared from serial dilutions of a 1 mg/ml solution of the appropriate carbohydrate.

3. MANIPULATION OF FERMENTATION USING SILAGE ADDITIVES

Information on the extent to which fermentation could be manipulated using various additive treatments was needed before detailed investigations into the control and manipulation of fermentation were initiated. In Experiment 1 additives that tend to restrict fermentation, formic acid, ammonium salts of formic and propionic acid and caprylic acid, were used and in Experiment 2 additives designed to stimulate fermentation, an inoculant of lactic acid bacteria, sucrose and sodium bicarbonate, were added before ensiling. Cube silos (1 tonne capacity) were used rather than smaller, laboratory-scale silos in order to represent more closely practical conditions. Experiment 3 addressed the question of whether changes in chemical composition can be explained in terms of changes in the numbers of the various types of micro-organisms present. For this experiment drain-pipe silos (8 kg capacity) were chosen to enable greater replication of treatments thus increasing the precision of the experiment, to improve the accuracy with which additives could be applied and to avoid the errors associated with core-sampling of silos. Late-season grass was chosen to provide a control that would be especially sensitive to the various manipulations; there is a tendency for high CP and low WSC in autumn-cut grass, which makes the crop difficult to ensile without additives (Wilkinson, 1988).

Experiment 1 The effect of formic acid, a mixture of ammonium salts of formic and propionic acids and a mixture of ammonium salts of formic and propionic acids with caprylic acid on the ensilage of late-season perennial ryegrass

Introduction Organic acids and their salts are widely used to induce a rapid initial reduction in the pH of the crop to around 4.5 and to deter undesirable micro-organisms and thus encourage the proliferation of lactic acid bacteria. These additives have given widely differing results, related to their effectiveness on a particular crop, variations in crop characteristics such as species and stage of growth which have marked effects on the buffering capacity of the crop, and application rate of the acid. This experiment looked at the effect of different levels of formic acid, the ammonium salts of formic and propionic acids and the ammonium salts of formic and propionic acids with caprylic acid.

Materials and methods Grass (*L. perenne*) was harvested in September (1989) and 1 tonne thoroughly mixed with the following treatments: 1) no additive (control); 2) formic acid

(850 g/l) at 2, 4, 6 and 8 l/t (acid F); 3) a mixture of ammonium tri-hydrogen tetraformate (550 g/l) and ammonium tri-hydrogen tetrapropionate (150 g/l) at 4, 6 and 8 l/t (acid S); 4) a mixture of ammonium hexaformate (680 g/l), ammonium hexapropionate (110 g/l) and caprylic acid (20 g/l) at 2, 4, 6 and 8 l/t (acid X). The grass was ensiled in 1 tonne-capacity silos, as described in Chapter 2, and duplicate sub-samples taken by coring on day 7, 21, 49 and 120 were bulked and analysed for changes in chemical composition.

The lowest level of acid S was chosen to contain an amount of formic acid comparable to that in the lowest levels of acid F and acid X.

Results The composition of the grass is shown in Table 3.1 and the composition of the silages on day 7, 21, 49 and 120 is shown in Tables 3.2a, b, c and d, respectively. The control silage was well preserved with reasonably low levels of ammonia, pH 4.39 and negligible amounts of butyric acid. The fermentation of material with 2 l/t mixed acids (acid X) followed a similar pattern to the control, with higher concentrations of lactic acid after 49d and concurrently lower acetic acid; low levels of formic acid (2 l/t acid F) and the ammonium salt (4 l/t acid S) did little to improve the fermentation,with lower concentrations of lactic acid and higher concentrations of acetic acid and ethanol throughout the time course. Similarly, although 4 l/t acid X and acid S (4-8 l/t) apparently restricted the lactic fermentation, residual WSC were fermented to ethanol. High levels of acid F (4, 6, 8 l/t) and acid X (6, 8 l/t) restricted fermentation and preserved WSC (74-78% and 44-81% of that present in the grass as ensiled, respectively). The higher pH associated with 6 and 8 l/t acid X suggests that the restriction was an antimicrobial effect of the organic acids rather than an effect of pH.

The proportion of major products of fermentation (lactic acid, acetic acid and ethanol) in each silage is shown in Table 3.3; the concentrations of metabolites in sub-samples from day 21-120 were meaned and this value used as an estimate of the final concentration because, on close examination, the observed variations in chemical composition in the last 100 d could not, with confidence, be considered real and could be due to errors associated with core-sampling. The theoretical proportion of metabolites expected from homolactic and heterolactic fermentations were calculated assuming a 50/50 mixture of glucose and fructose in the hexose fraction and using the acetic acid concentration in the control silage to calculate the maximum contribution of pentoses, ignoring other possible sources of acetate such as heterofermentative breakdown of fructose or catabolism of lactate, i.e. assuming a substrate mixture of 0.24 glucose:0.24 fructose:0.52 pentose.

Table 3.1 Chemical composition of the grass ensiled in 1 tonne-capacity silos in Experiment 1 (g/kg DM, unless stated otherwise).

pH	6.18
Dry matter (g/kg)	141
Crude protein	230
True protein (g/kg TN)	799
Ammonia (g/kg TN)	37
WSC	102
NDF	547
ADF	245
(NDF-ADF)	302

Table 3.2a The effect of organic acids on the chemical composition of silages after 7 d (g/kg DM, unless stated otherwise).

	Control	Acid F (l/t)				Acid X (l/t)				Acid S (l/t)		
		2	4	6	8	2	4	6	8	4	6	8
pH	4.41	4.66	4.28	3.87	3.76	4.38	4.59	4.39	4.15	4.84	4.68	4.56
Dry matter (g/kg)	154	169	180	186	184	162	168	185	186	168	168	176
Crude protein	221	241	222	223	223	233	245	237	236	254	247	256
True protein (g/kg TN)	459	630	684	617	634	485	537	574	619	565	587	528
Ammonia (g/kg TN)	79	34	37	31	31	80	58	62	76	72	86	93
Lactic acid	80	8	6	2	3	63	5	4	6	15	9	7
WSC	28	32	104	101	92	30	80	100	118	44	54	88
Ethanol	4	11	3	3	3	1	5	3	3	1	1	1
Acetic acid	2	3	0	1	0	7	3	3	1	4	2	1
Propionic acid	0	0	0	2	0	3	4	5	6	4	5	8
NDF	442	504	500	459	462	475	459	463	484	488	486	481
ADF	258	264	243	234	230	250	247	236	229	255	251	240
NDF-ADF	184	240	257	225	232	226	211	228	255	233	235	240

Table 3.2b The effect of organic acids on the chemical composition of silages after 21 d (g/kg DM, unless stated otherwise).

	Control	Acid F (l/t)				Acid X (l/t)				Acid S (l/t)		
		2	4	6	8	2	4	6	8	4	6	8
pH	4.33	4.79	4.42	4.00	3.84	4.37	4.65	4.55	4.31	5.04	4.69	4.63
Dry matter (g/kg)	146	165	170	184	178	161	168	172	187	164	166	171
Crude protein	243	245	256	238	236	258	260	252	248	274	268	271
True protein (g/kg TN)	381	541	558	538	552	445	479	527	554	482	490	520
Ammonia (g/kg TN)	100	57	39	34	39	104	67	71	77	105	96	96
Lactic acid	95	22	3	2	2	76	12	4	3	31	15	3
WSC	18	18	97	117	116	17	39	93	116	16	23	53
Ethanol	5	18	6	4	4	6	12	6	4	19	16	10
Acetic acid	12	8	3	0	0	14	0	4	3	10	5	2
Propionic acid	1	0	0	0	0	3	4	3	4	5	7	9
NDF	459	525	506	475	495	480	505	500	477	538	517	531
ADF	277	315	285	241	258	249	281	268	241	286	266	273
NDF-ADF	182	210	221	234	238	231	221	233	236	252	251	258

Table 3.2c The effect of organic acids on the chemical composition of silages after 49 d (g/kg DM, unless stated otherwise).

	Control	Acid F (l/t)				Acid X (l/t)				Acid S (l/t)		
		2	4	6	8	2	4	6	8	4	6	8
pH	4.42	4.92	4.23	4.10	3.84	4.37	5.23	4.43	4.28	5.10	4.86	4.65
Dry matter (g/kg)	154	164	178	185	188	173	161	176	201	168	176	180
Crude protein	228	260	256	232	232	244	261	253	241	272	266	232
True protein (g/kg TN)	363	438	443	519	538	370	460	496	589	413	474	559
Ammonia (g/kg TN)	130	112	40	43	40	121	128	72	76	163	123	111
Lactic acid	100	25	3	6	3	117	18	4	4	38	29	6
WSC	7	10	79	87	88	6	8	61	75	8	9	27
Ethanol	7	27	11	6	3	8	28	12	6	23	15	19
Acetic acid	20	17	5	3	3	14	28	3	0	29	12	3
Propionic acid	0	4	0	2	0	3	0	4	4	4	7	7
NDF	457	465	485	468	476	498	487	497	534	480	504	536
ADF	265	278	263	244	245	287	302	259	285	277	280	281
NDF-ADF	192	187	222	224	231	211	185	238	250	203	224	254

Table 3.2d The effect of organic acids on the chemical composition of silages after 120 d (g/kg DM, unless stated otherwise).

	Control	Acid F (l/t)				Acid X (l/t)				Acid S (l/t)		
		2	4	6	8	2	4	6	8	4	6	8
pH	4.39	5.44	4.19	3.99	3.87	4.26	4.78	4.70	4.21	5.31	4.84	4.45
Dry matter (g/kg)	167	139	203	202	218	180	194	199	205	168	179	189
Crude protein	214	253	235	234	229	229	232	236	244	260	261	251
True protein (g/kg TN)	356	416	510	530	564	437	457	575	522	456	479	523
Ammonia (g/kg TN)	125	165	40	43	40	120	118	69	76	184	139	101
Lactic acid	78	15	3	2	3	87	25	6	5	19	23	11
WSC	6	5	76	81	78	5	8	45	83	3	7	29
Ethanol	5	29	9	5	3	6	24	13	5	27	28	22
Acetic acid	25	45	4	6	4	18	19	8	7	45	20	6
Propionic acid	0	0	0	0	0	1	2	3	3	5	5	5
NDF	467	472	466	519	492	482	457	572	464	483	541	513
ADF	296	288	292	290	282	291	285	324	269	284	303	309
NDF-ADF	171	184	174	229	210	190	172	248	195	199	238	204

Table 3.3 Proportion of major fermentation products formed during ensilage (Experiment 1).

		Lactic acid	Acetic acid	Ethanol	Total metabolites (g/kg DM)
Control		0.78	0.16	0.05	116
Acid F (l/t)	2	0.30	0.33	0.36	69
	4	0.19	0.25	0.56	16
	6	0.27	0.27	0.45	11
	8	0.38	0.25	0.38	8
Acid X (l/t)	2	0.81	0.13	0.06	115
	4	0.33	0.29	0.38	55
	6	0.25	0.25	0.50	20
	8	0.33	0.25	0.42	12
Acid S (l/t)	4	0.36	0.35	0.29	80
	6	0.39	0.21	0.40	57
	8	0.25	0.14	0.61	28

Theoretical proportions of metabolites:-

Homolactic fermentation: 0.74 lactic acid : 0.27 acetic acid

Heterolactic fermentation: 0.50 lactic acid : 0.36 acetic acid : 0.14 ethanol

Although the observed data have little meaning when the overall extent of fermentation is very low, a comparison between the actual and expected ratios reveals a dramatic shift from a predominantly homolactic fermentation with the control silage and with 2 l/t acid X to a fermentation yielding a higher proportion of ethanol, suggesting a more vigorous yeast involvement, with 2 l/t acid F, 4 l/t acid X and 4, 6 and 8 l/t acid S.

Discussion The results indicate that increasing levels of organic acids inhibit the fermentation, preserving large amounts of the original WSC. The lower levels of acid addition restricted the activities of micro-organisms on the silage to different degrees; formic acid at 2 l/t and acid X at 4 l/t restricted the lactic fermentation and appeared to stimulate ethanol production from the sugars ultimately leading to an unstable silage, as judged from its high pH. However, 2 l/t acid X improved the efficiency of preservation by a stimulation of lactic acid production, to the detriment of the ethanolic fermentation. Chamberlain and Quig (1987) found an adverse effect on the fermentation using 4 l/t formic acid, while 2 l/t stimulated a lactic fermentation. This suggests that the grass used in this experiment needed less than 2 l/t formic acid to restrict activities of undesirable micro-organisms (e.g. epiphytic heterolactic and coliform bacteria) in the early stages of fermentation, perhaps owing to a lower buffering capacity of the crop, although this was not quantified. Stimulation of the lactic fermentation with 2 l/t acid X does imply that this is a less potent agent on a volume for volume basis for the restriction of fermentation. Similarly the inability of even high levels of acid S to preserve WSC suggests that formic acid is a more effective inhibitor of microbial activity than the ammonium salts.

Experiment 2 The effect of an inoculant, sucrose and sodium bicarbonate on the ensilage of late-season perennial ryegrass

Introduction In Experiment 2 the aim was to examine to what extent fermentation could be stimulated or extended by the use of an inoculum of lactic acid bacteria, the provision of extra fermentable carbohydrate as sucrose and the addition of sodium bicarbonate to counteract the restrictive effect of reduction of pH on the lactic fermentation.

Materials and Methods Grass (*L.perenne*) was harvested in October (1989) and 1 tonne mixed with the following additives: 1) no additive, control (C); 2) an inoculant (Ecosyl, ICI plc., Billingham) providing L. plantarum at 10^6 CFU/g fresh weight (I); 3) sucrose, 30kg/t

(S); 4) the inoculant plus sucrose, 30kg/t (30S); 5) the inoculant plus sucrose, 30kg/t, and sodium bicarbonate, 20kg/t (30SB); 6) the inoculant plus sucrose, 40kg/t (40S); 7) the inoculant plus sucrose, 40kg/t, and sodium bicarbonate, 20kg/t (40SB); 8) the inoculant plus sucrose, 30kg/t, and sodium bicarbonate, 30kg/t (60SB); 9) the inoculant plus sucrose, 40kg/t, and sodium bicarbonate, 30kg/t (70SB).

Treated grass was ensiled in 1 tonne-capacity cube silos as described in Chapter 2, and sub-samples were obtained by coring on day 3, 7, 21, 49 and 120 and analysed for changes in chemical composition.

Results The composition of the grass is shown in Table 3.4 and that of the silages after 3, 7, 21, 49 and 120 d is shown in Tables 3.5a, b, c, d and e, respectively. The proportion of the major products of fermentation (lactic acid, acetic acid and ethanol) in each silage is shown in Table 3.6. As in Experiment 1, a number of assumptions were made. The concentrations in sub-samples from day 21-120 were meaned and used as an estimate of the final values; although the changes in chemical composition during the last 100 days were apparently consistent, the fermentation "end-point" was difficult to define. The expected proportion of fermentation products was calculated from the amount of WSC utilised, assuming a 50/50 mixture of glucose and fructose and assuming the acetic acid was derived entirely from pentose fermentation, i.e. assuming a substrate mixture of 0.40 glucose : 0.40 fructose : 0.20 pentose. The control silage was well preserved with a low pH, a low concentration of ammonia and negligible concentrations of butyric acid. After 120 d ensilage there were low levels of residual WSC, with the concentrations of lactic acid, acetic acid and ethanol showing little evidence of heterolactic fermentation or yeast activity. The effects of sodium bicarbonate on the changes in pH and concentrations of WSC, lactic acid, acetic acid, ethanol and ammonia are shown in Figure 3.1a, b, c, d, e and f, respectively, using data from treatments 30S, 60SB and 40S, 70SB.

The forage ensiled with the inoculant had high concentrations of lactic acid after 3 d, reflecting a faster rate of fermentation in the early stages (Figure 3.1c). The addition of extra substrate with or without inoculant increased the accumulation of lactic acid, but ultimately resulted in the production of ethanol (Figure 3.1e). The bicarbonate treatments maintained a higher pH (Figure 3.1a) and resulted in a slower rate of lactic acid appearance but an increased rate of acetic acid production in the early stages of ensilage (Figure 3.1d), although by day 7 lactic acid was the predominant metabolite, and with the 70SB treatment reached a peak of 220 g/kg after 21 d.

Table 3.4 Chemical composition of the forage ensiled in 1 tonne-capacity silos in Experiment 2 (g/kg DM, unless stated otherwise).

pH	6.22
Dry matter (g/kg)	141
Crude protein	218
True protein (g/kg TN)	793
Ammonia (g/kg TN)	52
WSC	146
NDF	523
ADF	230
NDF-ADF	293

Table 3.5a The effect of an inoculant (I), sucrose (S) or an inoculant with sucrose (30S, 40S) or combinations of inoculant, sucrose and sodium bicarbonate (30SB, 40SB, 60SB, 70SB) on the chemical composition of the silages after 3 d (g/kg DM, unless stated otherwise).

	Control	I	S	30S	40S	30SB	60SB	40SB	70SB
pH	5.33	4.98	4.68	4.88	4.73	9.64	9.92	9.46	9.95
Dry matter (g/kg)	156	147	185	178	189	211	211	205	221
Crude protein	232	244	195	195	192	186	183	172	171
True protein (g/kg TN)	570	558	590	568	521	758	706	724	719
Ammonia (g/kg TN)	53	52	48	55	50	34	36	35	34
Lactic acid	30	40	40	34	38	5	8	40	20
WSC	115	144	249	262	247	214	201	181	207
Ethanol	1	0	1	0	1	1	0	1	1
Acetic acid	7	3	9	6	1	6	7	16	15
Propionic acid	0	0	0	0	0	0	0	0	0
NDF	511	504	468	417	400	461	425	422	426
ADF	252	265	231	217	221	225	211	190	189
NDF-ADF	259	238	238	200	179	236	215	232	238

Table 3.5b The effect of an inoculant (I), sucrose (S) or an inoculant with sucrose (30S, 40S) or combinations of inoculant, sucrose and sodium bicarbonate (30SB, 40SB, 60SB, 70SB) on the chemical composition of the silages after 7 d (g/kg DM, unless stated otherwise).

	Control	I	S	30S	40S	30SB	60SB	40SB	70SB
pH	4.40	4.22	4.23	4.23	4.14	5.04	8.42	4.94	6.51
Dry matter (g/kg)	153	153	176	178	189	194	203	203	194
Crude protein	237	223	198	193	177	184	174	170	176
True protein (g/kg TN)	443	451	490	425	447	590	579	537	551
Ammonia (g/kg TN)	70	65	64	65	70	66	68	76	81
Lactic acid	80	95	82	82	95	138	114	131	145
WSC	53	48	182	202	200	37	16	76	38
Ethanol	3	2	3	3	3	3	5	4	3
Acetic acid	7	6	3	3	4	20	38	17	25
Propionic acid	0	0	0	0	0	0	0	0	0
NDF	422	432	384	366	362	376	360	360	370
ADF	250	250	223	217	196	214	195	191	184
NDF-ADF	173	182	161	150	166	162	165	169	186

Table 3.5c The effect of an inoculant (I), sucrose (S) or an inoculant with sucrose (30S, 40S) or combinations of inoculant, sucrose and sodium bicarbonate (30SB, 40SB, 60SB, 70SB) on the chemical composition of the silages after 21 d (g/kg DM, unless stated otherwise).

	Control	I	S	30S	40S	30SB	60SB	40SB	70SB
pH	4.20	4.06	4.08	4.01	4.01	4.43	4.90	4.48	4.65
Dry matter (g/kg)	160	160	167	165	159	192	193	191	194
Crude protein	216	218	220	216	213	193	174	187	184
True protein (g/kg TN)	422	423	402	403	368	399	441	498	474
Ammonia (g/kg TN)	96	101	86	96	93	106	155	94	122
Lactic acid	106	120	120	129	170	188	215	176	226
WSC	50	17	96	59	85	18	9	26	14
Ethanol	4	5	15	32	40	6	9	7	6
Acetic acid	8	11	9	13	15	20	25	21	24
Propionic acid	0	0	0	0	0	0	0	0	0
NDF	422	432	410	384	401	348	321	348	345
ADF	230	259	234	232	231	197	192	205	198
NDF-ADF	162	173	176	151	169	151	129	143	146

Table 3.5d The effect of an inoculant (I), sucrose (S) or an inoculant with sucrose (30S, 40S) or combinations of an inoculant, sucrose and sodium bicarbonate (30SB, 40SB, 60SB, 70SB) on the chemical composition of the silage after 49 d (g/kg DM, unless stated otherwise).

	Control	I	S	30S	40S	30SB	60SB	40SB	70SB
pH	4.21	4.14	4.13	4.07	3.99	4.85	5.04	4.40	4.56
Dry matter (g/kg)	179	163	181	189	182	212	201	207	204
Crude protein	204	216	217	205	201	185	183	177	175
True protein (g/kg TN)	401	448	435	453	392	451	450	463	457
Ammonia (g/kg TN)	97	115	100	100	99	124	177	103	138
Lactic acid	91	117	108	123	148	169	226	192	205
WSC	40	9	13	14	18	8	5	12	8
Ethanol	4	7	32	25	48	10	12	13	11
Acetic acid	8	12	12	10	11	21	31	17	23
Propionic acid	0	0	0	0	0	0	0	0	0
NDF	446	475	458	432	420	362	351	355	339
ADF	299	292	296	264	255	222	209	215	205
NDF-ADF	147	182	162	168	165	140	142	140	135

Table 3.5e The effect of an inoculant (I), sucrose (S) or an inoculant with sucrose (30S, 40S) or combinations of inoculant, sucrose and sodium bicarbonate (30SB, 40SB, 60SB, 70SB) on the chemical composition of the silages after 120 d (g/kg DM, unless stated otherwise).

	Control	I	S	30S	40S	30SB	60SB	40SB	70SB
pH	4.26	4.22	4.12	4.02	4.02	4.56	4.88	4.46	4.66
Dry matter (g/kg)	186	185	189	181	179	199	209	199	218
Crude protein	207	195	205	210	206	183	181	181	174
True protein (g/kg TN)	420	461	470	407	383	430	442	471	469
Ammonia (g/kg TN)	97	106	94	90	96	132	153	107	129
Lactic acid	92	80	113	126	120	189	185	178	194
WSC	19	6	9	13	14	6	3	8	5
Ethanol	10	7	47	54	61	9	10	13	8
Acetic acid	12	16	14	16	12	25	27	24	24
Propionic acid	0	0	0	0	0	0	0	0	0
NDF	468	483	520	497	439	418	434	404	421
ADF	315	307	324	309	284	258	266	236	250
NDF-ADF	154	177	196	187	155	159	169	168	171

Table 3.6 Proportion of major fermentation products formed during ensilage in 1 tonne-capacity silos (Experiment 2).

	Lactic acid	Acetic acid	Ethanol	Total metabolites (g/kg DM)
Control	0.86	0.08	0.05	111
I	0.85	0.10	0.05	125
S	0.73	0.08	0.20	157
30S	0.72	0.07	0.21	176
40S	0.71	0.06	0.24	207
30SB	0.86	0.10	0.04	211
60SB	0.85	0.09	0.05	212
40SB	0.85	0.11	0.04	247
70SB	0.86	0.10	0.03	240

Theoretical proportions of metabolites:-

Homolactic fermentation 0.89 lactic acid : 0.11 acetic acid

Heterolactic fermentation 0.50 lactic acid : 0.26 acetic acid : 0.24 ethanol

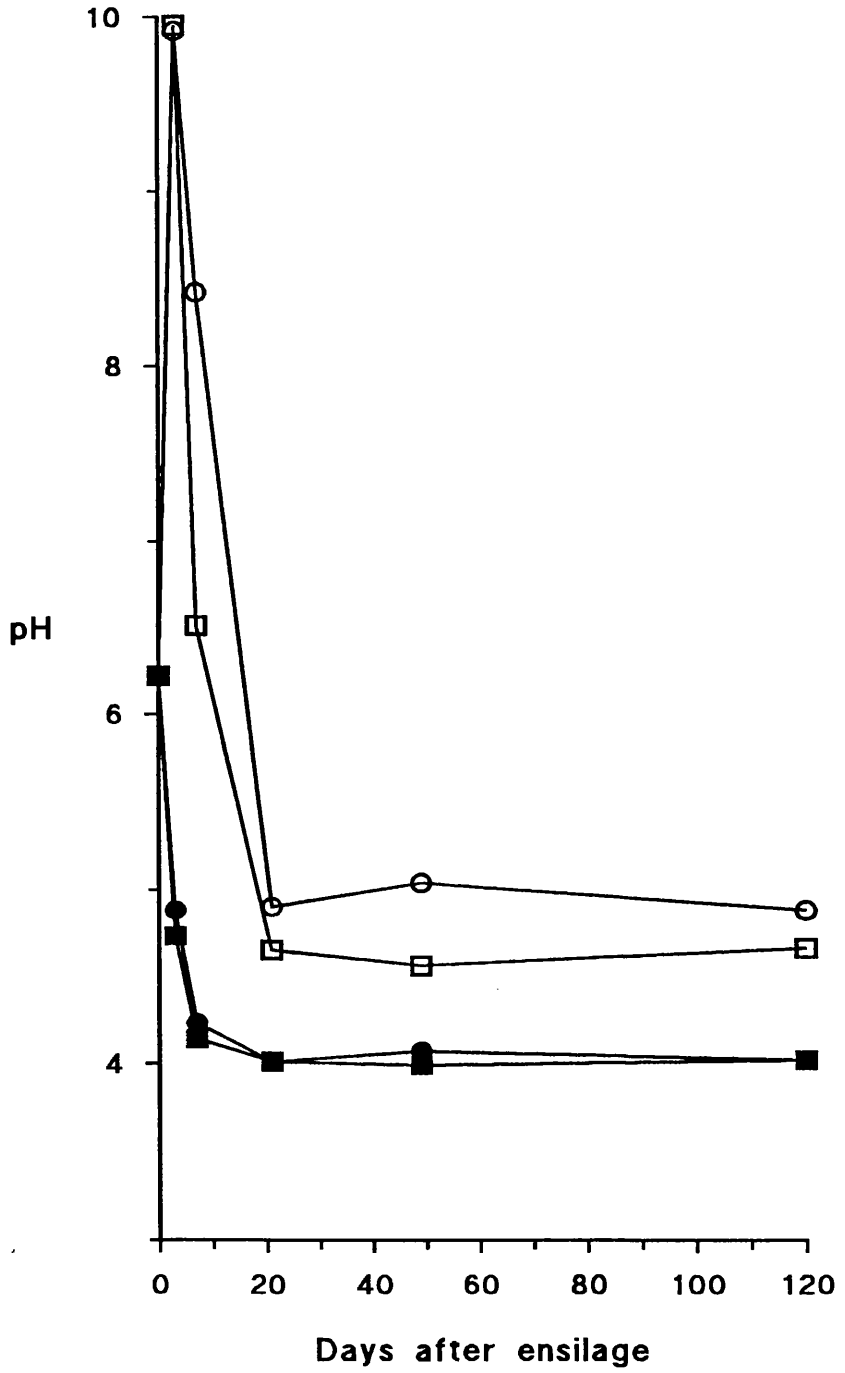


Figure 3.1a Changes in pH during ensilage of perennial ryegrass, in 1-tonne cube silos, with an inoculant plus 30 kg/t sucrose (●), an inoculant plus 30 kg/t sucrose and 30 kg/t sodium bicarbonate (○), an inoculant plus 40 kg/t sucrose (■), and an inoculant plus 40 kg/t sucrose and 30 kg/t sodium bicarbonate (□).

Values are derived from bulked triplicate sub-samples.

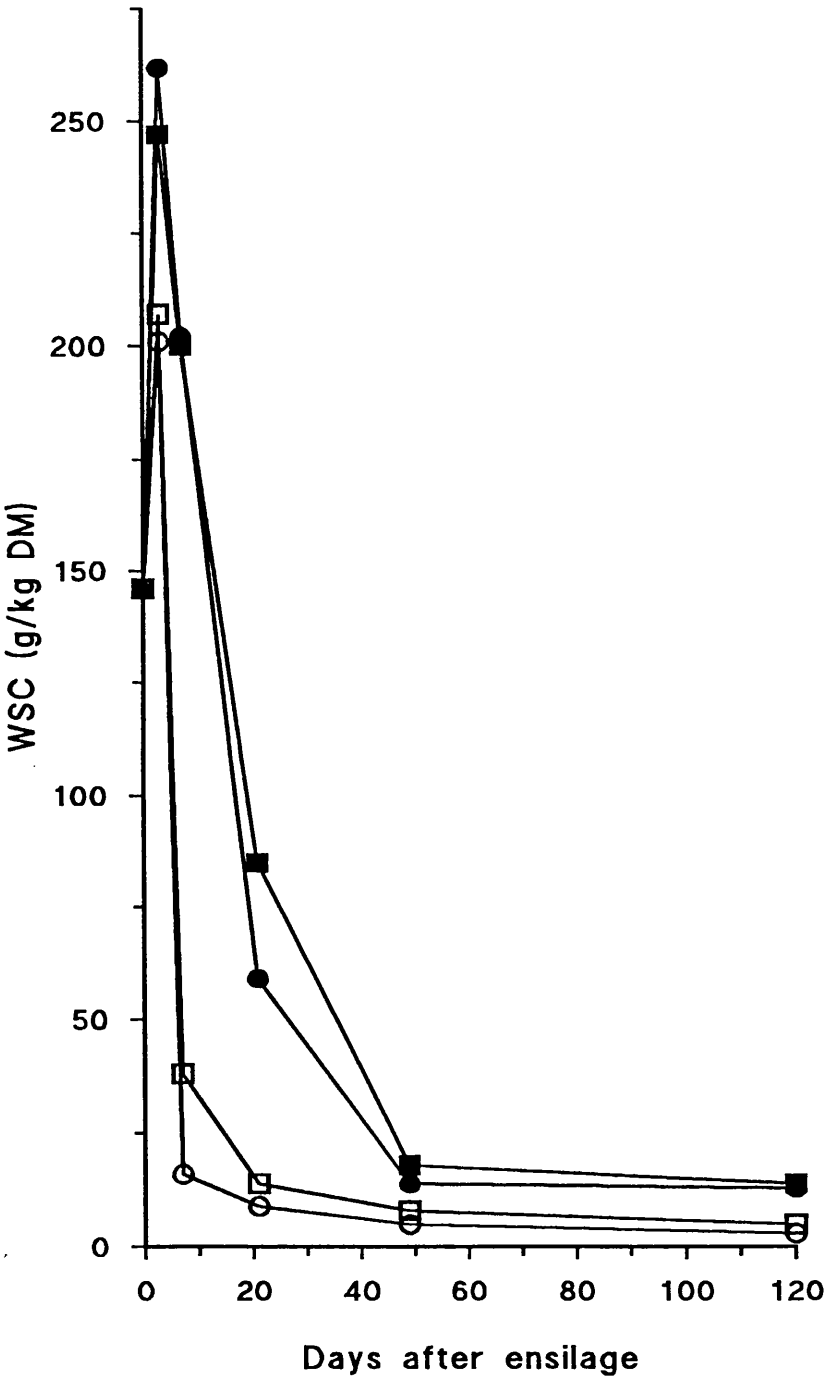


Figure 3.1b Changes in the concentrations of WSC (g/kg DM) during ensilage of perennial ryegrass, in 1-tonne cube silos, with an inoculant plus 30 kg/t sucrose (●), an inoculant plus 30 kg/t sucrose and 30 kg/t sodium bicarbonate (○), an inoculant plus 40 kg/t sucrose (■), and an inoculant plus 40 kg/t sucrose and 30 kg/t sodium bicarbonate (□).
Values are derived from bulked triplicate sub-samples.

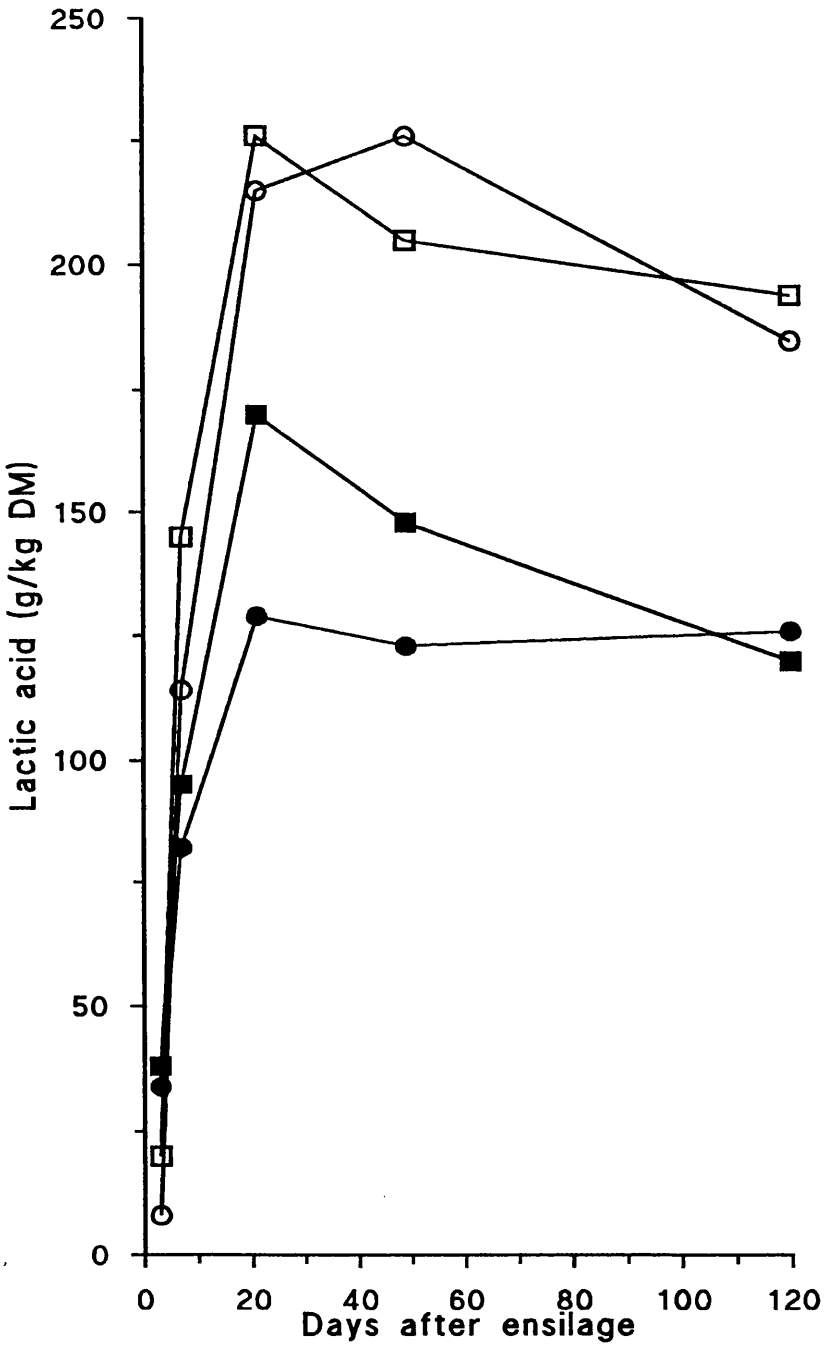


Figure 3.1c Changes in the concentrations of lactic acid (g/kg DM) during ensilage of perennial ryegrass, in 1-tonne cube silos, with an inoculant plus 30 kg/t sucrose (●), an inoculant plus 30 kg/t sucrose and 30 kg/t sodium bicarbonate (○), an inoculant plus 40 kg/t sucrose (■), and an inoculant plus 40 kg/t sucrose and 30 kg/t sodium bicarbonate (□). Values are derived from bulked triplicate sub-samples.

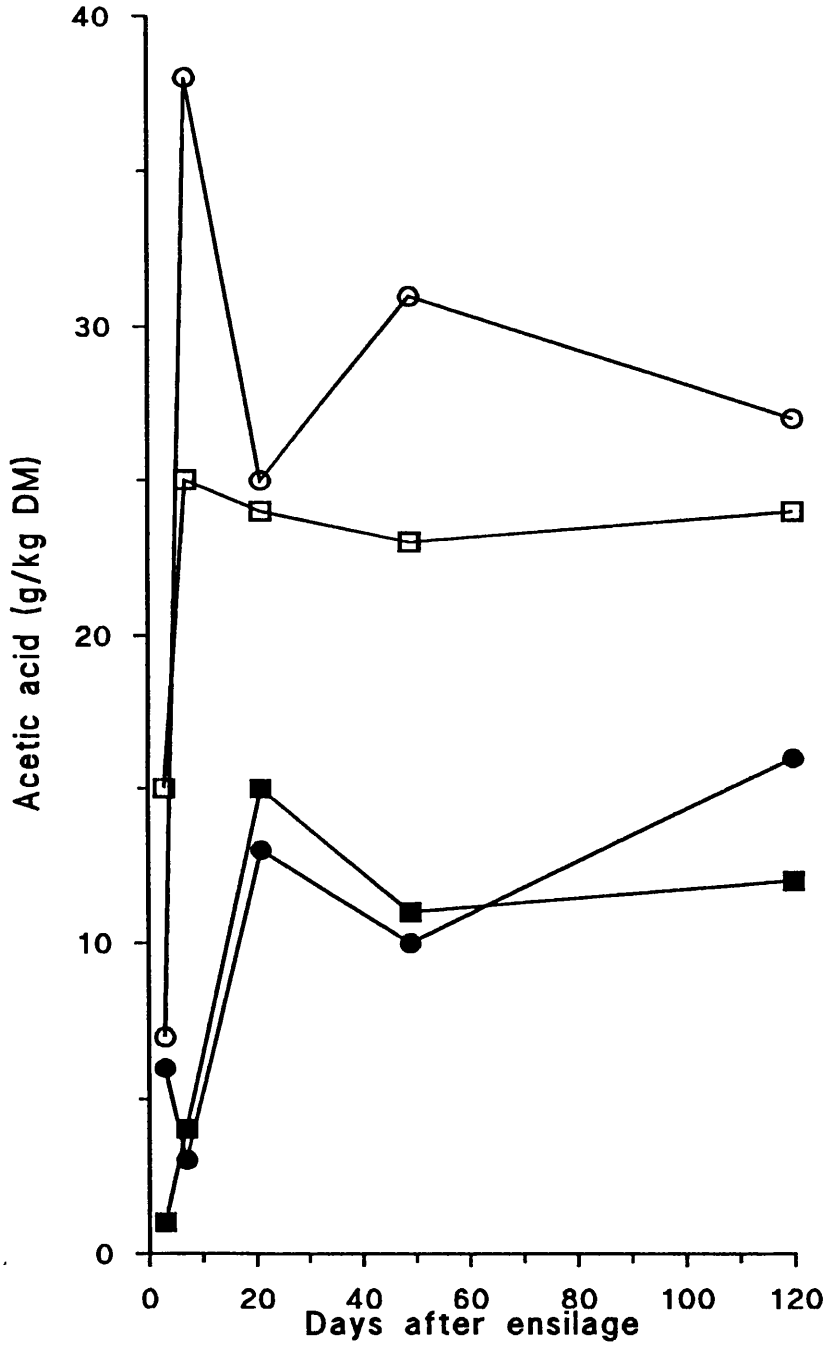


Figure 3.1d Changes in the concentrations of acetic acid (g/kg DM) during ensilage of perennial ryegrass, in 1-tonne cube silos, with an inoculant plus 30 kg/t sucrose (●), an inoculant plus 30 kg/t sucrose and 30 kg/t sodium bicarbonate (○), an inoculant plus 40 kg/t sucrose (■), and an inoculant plus 40 kg/t sucrose and 30 kg/t sodium bicarbonate (□). Values are derived from bulked triplicate sub-samples.

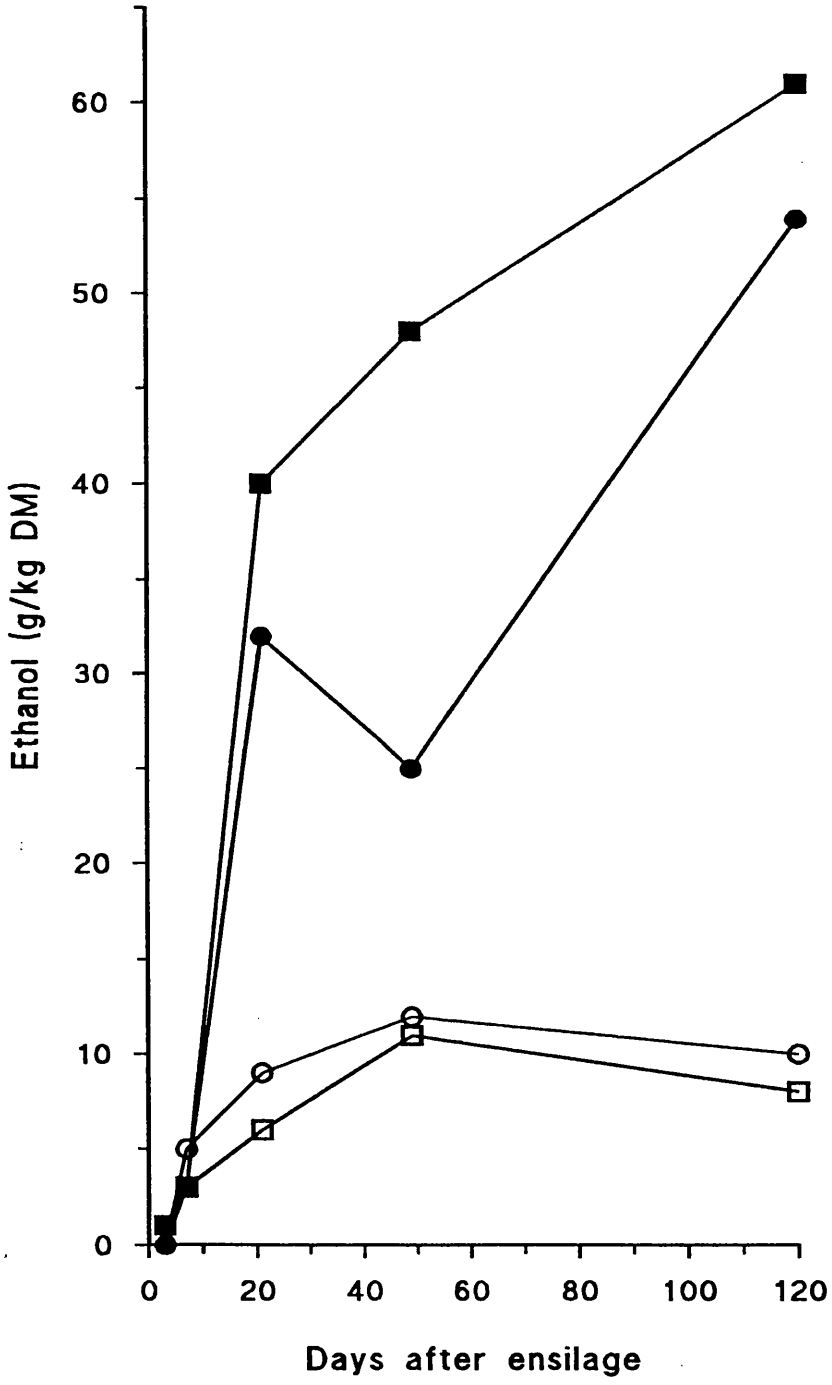


Figure 3.1e Changes in the concentrations of ethanol (g/kg DM) during ensilage of perennial ryegrass, in 1-tonne cube silos, with an inoculant plus 30 kg/t sucrose (●), an inoculant plus 30 kg/t sucrose and 30 kg/t sodium bicarbonate (○), an inoculant plus 40 kg/t sucrose (■), and an inoculant plus 40 kg/t sucrose and 30 kg/t sodium bicarbonate (□).
Values are derived from bulked triplicate sub-samples.

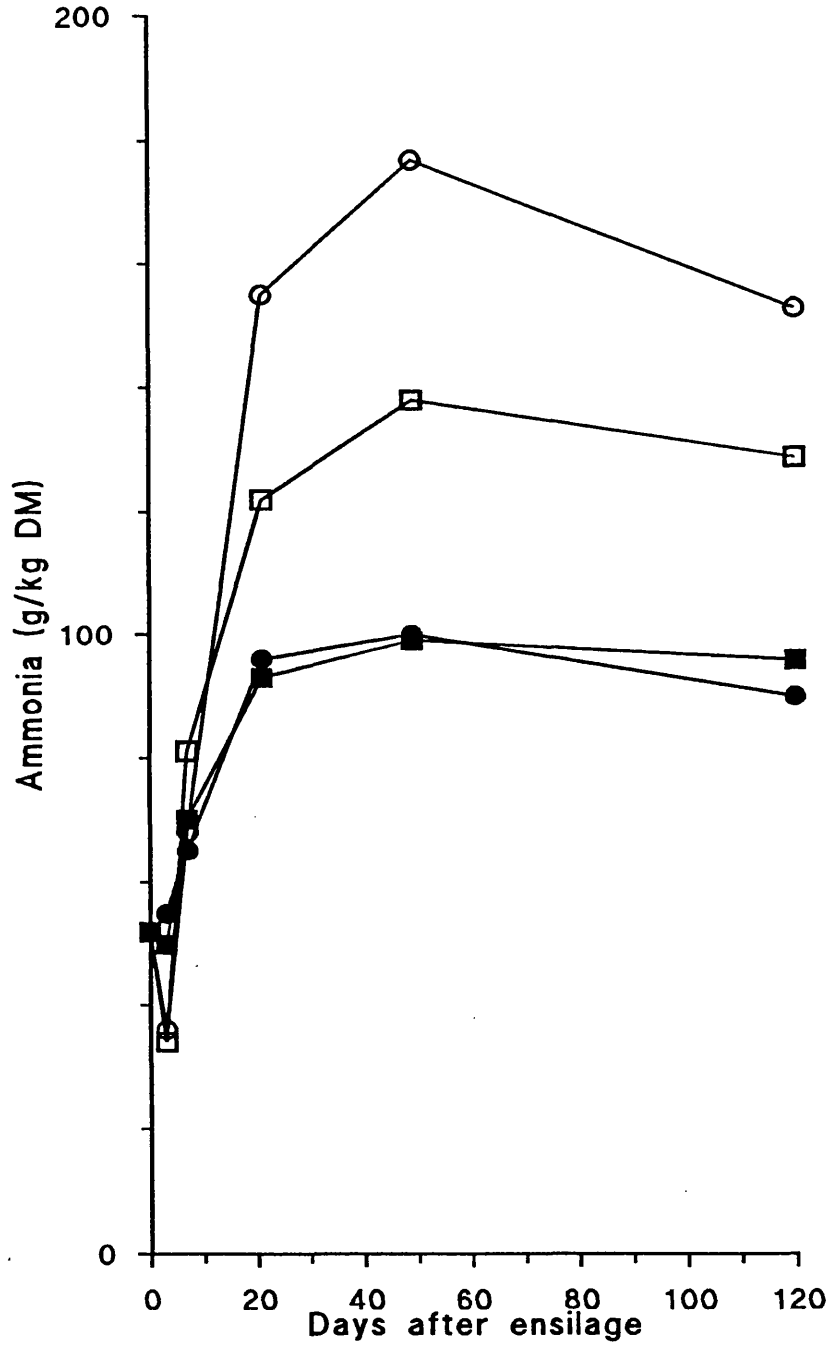


Figure 3.1f Changes in the concentrations of ammonia (g/kg DM) during ensilage of perennial ryegrass, in 1-tonne cube silos, with an inoculant plus 30 kg/t sucrose (●), an inoculant plus 30 kg/t sucrose and 30 kg/t sodium bicarbonate (○), an inoculant plus 40 kg/t sucrose (■), and an inoculant plus 40 kg/t sucrose and 30 kg/t sodium bicarbonate (□). Values are derived from bulked triplicate sub-samples.

High concentrations of ammonia were another characteristic of the sodium bicarbonate treated silage (Figure 3.1f).

A comparison of observed and theoretical ratios of metabolites reveals the predominantly homolactic nature of the fermentation of control and of inoculant- and bicarbonate-treated silages. Additional substrate provided with the inoculant was apparently fermented to ethanol (S, 30S and 40S), presumably the result of yeast activity since ethanol production continued after lactic acid production apparently abated (Figures 3.1c and 3.1e). The concentration of total metabolites shows that sodium bicarbonate increased the extent of the homolactic fermentation to double that of the inoculant-treated silage.

Discussion The provision of the inoculant increased the rate of lactic fermentation, deterring the activities of undesirable micro-organisms in the early stages. Although there are indications that the lactic acid bacteria in the inoculant treatment may have been suffering a nutrient deficit which was satisfied with the addition of extra sucrose, there is a suggestion from the higher ethanol concentrations with higher levels of substrate provision that the lactic acid bacteria did not ferment excess WSC but that these were utilised by yeasts in the later stages of ensilage.

Sodium bicarbonate addition maintained a higher pH throughout the course of ensilage resulting in a more extensive fermentation. A subsidiary effect was the accumulation of ammonia. The higher pH in the early stages of ensilage was above the optimum for lactic acid bacteria (Kandler and Weiss, 1986) and may have encouraged the proliferation of organisms not normally encountered in silage making, as well as stimulating coliform bacteria which are associated with deaminative activity (Henderson, 1984) and the formation of acetic acid and other end-products such as succinate, acetoin, butanediol, formate, H_2 and CO_2 (Wood, 1961). This may partly explain the rapid drop in WSC concentration, not accounted for by acetic acid production. Nevertheless, the subsequent maintenance of a homolactic fermentation to the exhaustion of WSC discouraged the activities of yeasts.

Experiment 3 The effect of manipulating the fermentation using a mixture of organic acids, sucrose and an inoculant

Introduction In the previous experiments it was shown that the fermentation can be influenced very markedly by various additive treatments applied at ensiling. This experiment addressed the question of whether changes in chemical composition can be explained in terms

of changes in the numbers of the various types of micro-organisms present. The treatments selected were a) a high level of application of mixed organic acids to inhibit fermentation, b) addition of sucrose to augment the supply of fermentable sugars and c) an inoculant to increase the numbers of lactic acid bacteria in the very early stages of ensilage.

Materials and Methods Grass (*L. perenne*) was harvested in November (1989) and the following treatments were applied as a fine mist over the grass using a hand-held applicator: no additive, control; a mixture of ammonium hexaformate (680 g/l), ammonium hexapropionate (110 g/l) and caprylic acid (20 g/l), 6 l/t; sucrose (500 g/l), 20 kg/t; an inoculant (*L. plantarum*), to provide 10^6 CFU/g fresh weight. The grass was ensiled in 36 x 8 kg-capacity drain-pipe silos as described in Chapter 2.

Triplicate silos from each treatment were opened after 5 d, 12 d and 70 d and the silages analysed using standard chemical and microbiological techniques (Chapter 2). **Statistical analysis** was by one-way analysis of variance using GENSTAT.

Results The chemical composition of the grass and the microbial populations present are shown in Tables 3.7a and b. The composition of the crop (low sugar/high protein) was typical of autumn-cut grass, nevertheless the control silage was well preserved, with a low pH (4.14) and negligible butyric acid concentration.

The chemical composition of the silages at the three opening times is given in Table 3.8. The lactic acid concentration of the control silage reached 152 g/kg, presumably owing to release of fermentable sugars from hemicellulose (suggested by an apparent reduction in (NDF-ADF) concentration) while the organic acids mixture severely restricted fermentation, associated with low concentrations of metabolites (Table 3.8, Figure 3.2) but also high numbers of lactic acid bacteria (more than 10^8 CFU/g FW) (Figure 3.3). The inoculant ensured a more rapid fermentation than the control in the early stages (5 d opening time), reflected by higher numbers of lactic acid bacteria (238×10^7 CFU/g FW) (Figure 3.3), but after 12 d, the numbers on the control, sucrose- and acids-treated silage were very similar; the lactic acid bacteria accounted for more than 95% of total viable organisms in all treatments (Figure 3.3).

The addition of sucrose, while not stimulating a more rapid fermentation than the control, encouraged a higher concentration of lactic acid by day 70, and the lower pH was associated with higher yeast counts (19×10^3 CFU/g FW). The inoculant too encouraged higher yeast counts (10×10^3 CFU/g FW by day 70).

Table 3.7a Chemical composition of the grass ensiled in Experiment 3 (g/kg DM, unless stated otherwise).

pH	5.80
Dry matter (g/kg)	137
Crude protein	242
True protein (g/kg TN)	503
Ammonia (g/kg TN)	106
WSC	77
NDF	501
ADF	250
NDF-ADF	251

Table 3.7b Microbial populations on the grass ensiled in Experiment 3 (CFU/g fresh weight).

Total viable organisms	$30x10^6$
Lactic acid bacteria	$< 10^3$
Coliform bacteria	$6x10^6$
Yeasts and moulds	$< 10^2$

Table 3.8 Effect of addition of a mixture of organic acids, an inoculant and sucrose on the chemical composition of the silage (g/kg DM, unless stated otherwise).

	Day 5				Day 12				Day 70				SED
	Control	Mixed acids	Inoculant	Sucrose	Control	Mixed acids	Inoculant	Sucrose	Control	Mixed acids	Inoculant	Sucrose	(n=36)
pH	4.47	4.34	4.22	4.33	4.15	4.39	4.13	4.02	4.14	4.19	4.19	4.01	0.03
Dry matter (g/kg)	145	153	138	152	147	153	142	156	147	152	145	152	4.2
Crude protein	234	224	240	217	213	226	236	202	212	217	210	198	6.4
True protein (g/kg TN)	335	385	371	344	325	373	305	350	284	285	268	255	15.2
Ammonia (g/kg TN)	134	132	127	128	152	136	137	145	172	157	191	170	4.4
Lactic acid	85	19	112	75	104	9	106	101	152	37	151	172	4.7
WSC	43	114	27	107	13	119	17	53	7	128	5	9	4.3
Ethanol	1	0	1	1	2	1	2	2	4	5	4	7	0.6
Acetic acid	17	3	13	19	19	4	14	20	31	5	32	27	2.3
Propionic acid	0	4	0	0	0	4	0	0	1	5	2	2	1.1
NDF	453	474	469	460	419	444	458	408	403	408	418	400	7.0
ADF	254	250	252	260	248	234	259	233	242	245	248	233	4.1
NDF-ADF	199	225	217	199	171	209	199	175	161	162	170	167	6.1

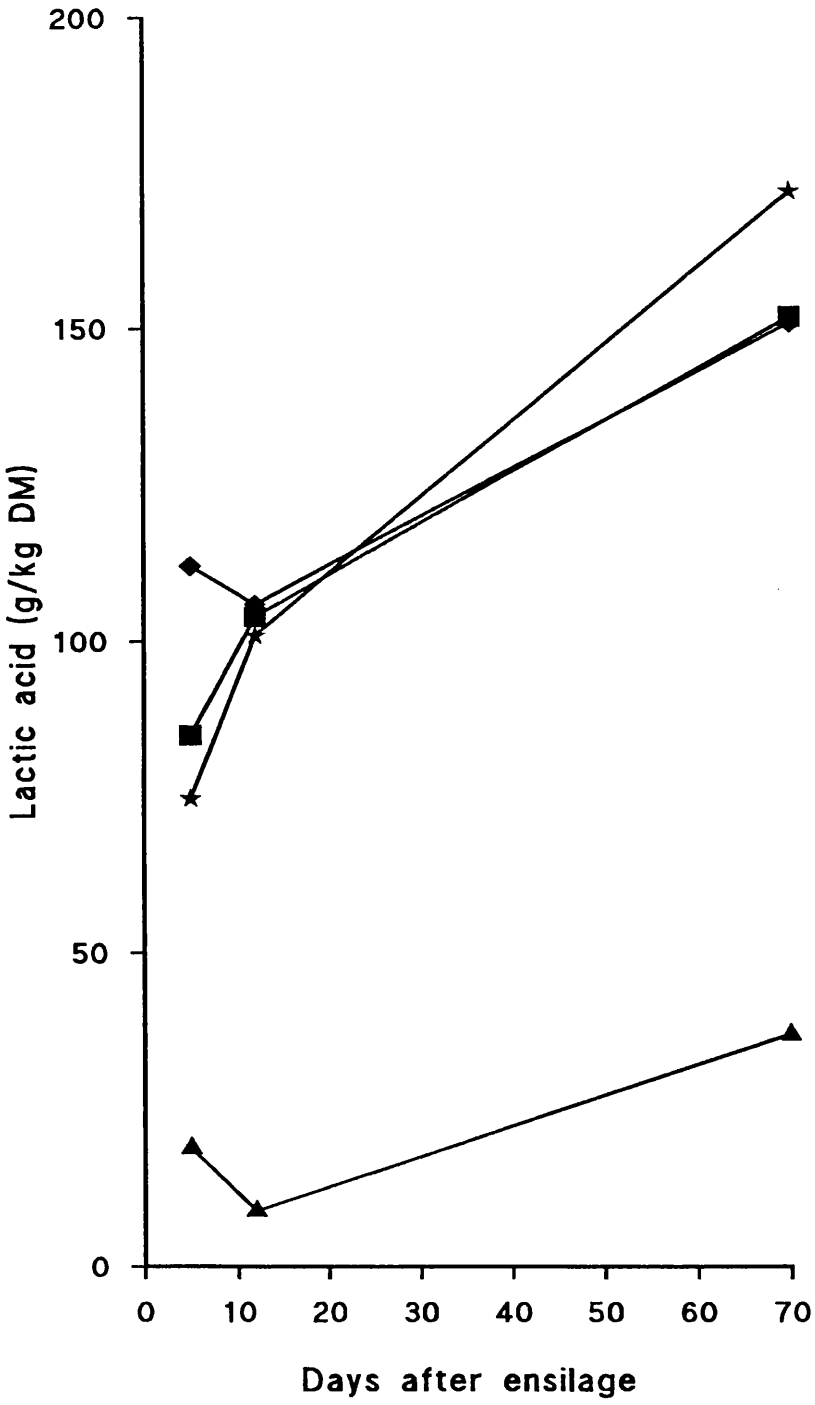


Figure 3.2 Changes in the concentrations of lactic acid during ensilage of perennial ryegrass, in polypropylene measuring cylinder silos, with a mixture of organic acids (▲), an inoculant (◆) or sucrose (★), or without additive (■).

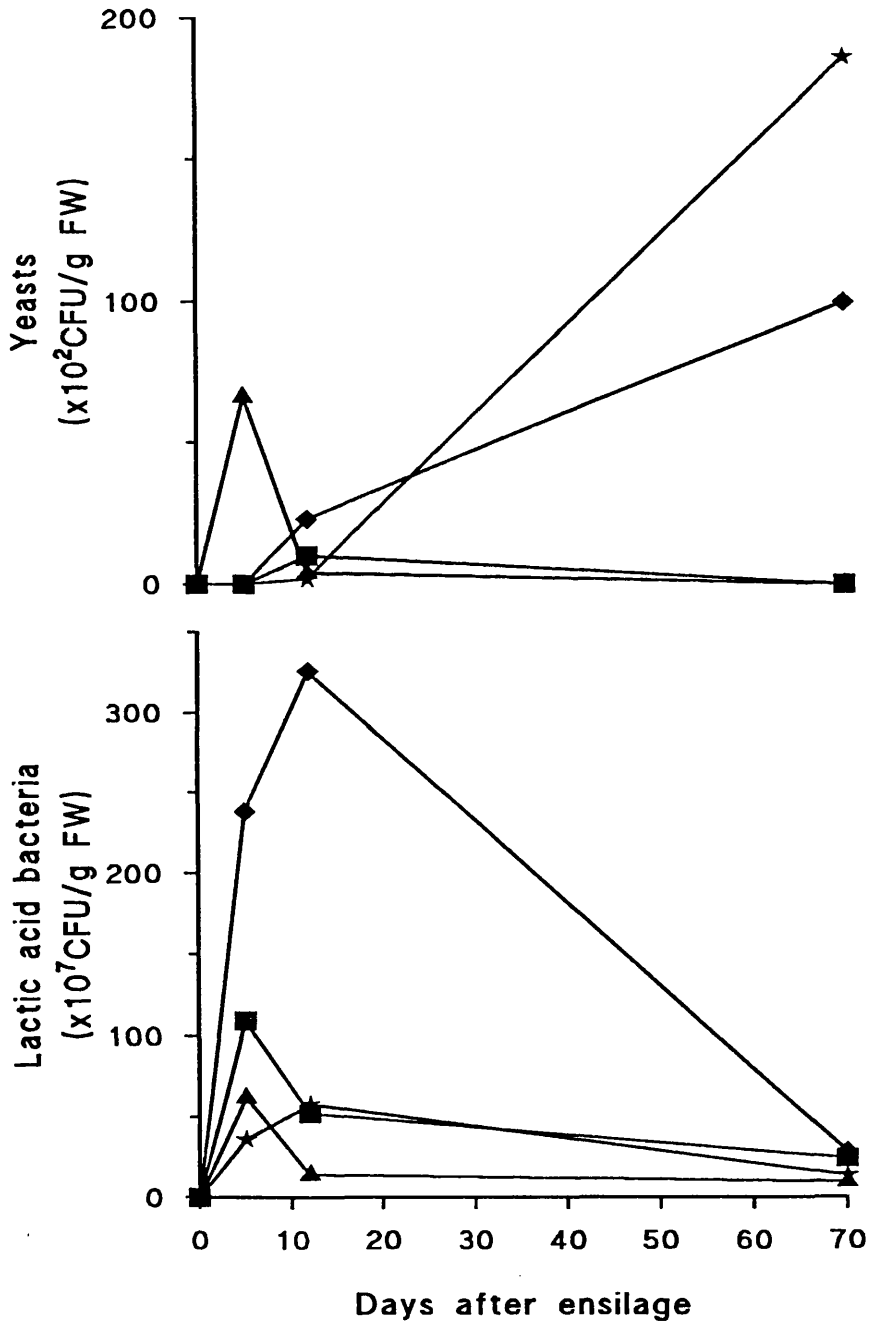


Figure 3.3 Changes in the numbers of yeasts ($x10^2$) and lactic acid bacteria ($x10^7$) (CFU/g FW) during ensilage of perennial ryegrass, in polypropylene measuring cylinder silos, with a mixture of organic acids (▲), an inoculant (◆) or sucrose (★), or without additive (■).

Discussion The enumeration of micro-organisms belonging to the major groups found on the silage is a useful technique, and the data can be used to explain some of the changes in chemical composition. The vigorous growth rate of the lactic acid bacteria in the inoculant-treated silage corresponded with the fastest rates of lactic acid production in the first 5 d. However, although numbers remained high throughout the 70 d, the rate of lactic acid production was slower, and although the organic acids mixture restricted fermentation, bacterial numbers were still high; similarly higher yeast counts with some of the treatments were not reflected by changes in chemical composition.

The CFU count represents the number of micro-organisms with potential for growth given optimum conditions, whereas conditions in the silo eventually restrict their activity. The implication is that microbial numbers give no reliable indication of microbial fermentative activity.

Conclusions It was somewhat surprising that the control silages in all three experiments were well preserved without additive treatment. Despite the grass being cut late in the season this did not deter a predominantly homolactic fermentation, which suggests that the efficient preservation of the crop was due to the presence of a large proportion of homofermentative lactic acid bacteria. This illustrates that it can be misleading to form general ideas regarding the ensilability of a crop without knowledge of the type or activity of micro-organisms present. The chemical composition of the grass used in Experiment 1 and 2 was not high nitrogen/low WSC as would have been expected, which compounds the problems associated with predicting the need of additives to assist the fermentation.

The use of 1 tonne-capacity cubes represented more closely a practical situation, as there was a larger mass of silage to ferment than in the 8 kg-capacity tubes used in Experiment 3, however there was consequently a more variable composition resulting from the heterogenous nature of the silage. The use of smaller silos in Experiment 3 helped to standardise the procedure and ensured a more homogenous material, while rapid filling and more rigorous compaction encouraged the early establishment of anaerobic conditions. Triplicate silos were emptied on each opening day and a representative sample taken from each silo, avoiding the sources of error inherent with core-samples which were bulked to provide one sample per silo. Triplicate samples allowed statistical analysis of data and differentiation of treatment effects from natural variation.

The grass used in Experiment 3 contained the lowest concentration of WSC (76 g/kg DM), but fermentation resulted in the highest lactic acid concentration (152 g/kg DM); the

stability achieved in the control silage in Experiment 1 from 78 g/kg DM lactic acid may reflect a lower buffering capacity of the grass, with less lactic acid neutralised by plant constituents. Presumably the breakdown of hemicellulose and release of fermentable carbohydrates contributed to the extended fermentation of the control silage in Experiment 3. Although there is apparently a large loss of hemicellulose suggested by the NDF-ADF data from Experiments 1 and 2, this is difficult to reconcile with the relatively small contribution of fermentable pentose sugars, calculated from acetic acid concentrations in the silage. Released pentoses may be lost in effluent or, alternatively, the apparent breakdown of hemicellulose may not necessarily result in a direct release of fermentable carbohydrates. Although Henderson and McDonald (1984), Seale *et al.*(1986) and Chamberlain (1987) found an approximate 30% increase in the supply of fermentable substrate, during the early stages of ensilage, as the pH drops, plant enzymes, thought to be partly responsible for degradation of hemicellulose bonds, are inhibited (Dewar *et al.*, 1963) and the oligosaccharides may not be cleaved further, leaving fragments of hemicellulose which may be neither fermented nor identified by conventional chemical analysis.

In conclusion, fermentation was inhibited by high levels of acid addition and extended by the partial removal of the inhibitory effect of pH. However, the differential restriction of specific groups of micro-organisms, in order to control the fermentation, was more difficult to achieve. In Experiment 1 low levels of organic acid addition restricted the activities of undesirable bacteria, but the extent to which the homofermentative lactic acid bacteria were tolerant or succeeded by yeasts was unpredictable. There may be a critical level of application which excludes the activities of undesirable bacteria yet allows lactic acid bacteria to prosper and discourage yeasts. Sodium bicarbonate extended the lactic fermentation, to the exclusion of yeasts, by maintenance of a higher pH. This suggests that pH, or the combined effects of pH and lactic acid, may be important in regulating senescence of lactic acid bacteria, although there was not a clear pattern to the extent of accumulation of undissociated lactic acid (Table 3.9).

In Experiment 3 the enumeration of micro-organisms demonstrated the changes in microbial numbers, and provided some useful data relating to the early hours of fermentation. However, CFU enumeration can be misleading when trying to relate numbers to changes in chemical composition in the later stages of ensilage since conditions in the silo restrict the fermentative activities of most micro-organisms. The fermentation profiles (Table 3.3 and 3.6) assisted the interpretation of the data and illustrated the shift in fermentation pathways elicited by the various additive treatments. To understand further the roles played

by specific micro-organisms during ensilage, there is a need to assess the fermentative activity of the micro-organisms *in situ*.

Chapter 4 describes the development of an *in vitro* assay to measure the rate of glucose utilisation by an inoculum prepared from the silage, and the application of this technique to demonstrate the effect of various perturbations on the fermentative activity of silage micro-organisms.

Table 3.9 The effect of sodium bicarbonate on the concentration of total and undissociated lactic acid after 21 d (Experiment 2).
Values shown are the mean of days 21-120.

	pH	Total lactic acid (moles/kg DM)	Undissociated lactic acid (mmoles/l)
30S	4.03	126	121
60SB	4.94	209	45
40S	4.01	146	139
70SB	4.62	208	89

Proportion of undissociated lactic acid was calculated from the Henderson:Hasselbach equation:-

proportion of the total concentration which is undissociated = 1/[1+antilog(pH-pKa)]

4. THE METABOLIC ACTIVITY OF SILAGE MICRO-ORGANISMS MEASURED USING AN *IN VITRO* ASSAY OF GLUCOSE UTILISATION

The currently accepted methods for monitoring the course of silage fermentation involve the selective enumeration of viable micro-organisms and chemical analyses. However, whilst microbial numbers may increase rapidly after ensilage and remain high, their continued presence is not always reflected in further changes in chemical composition of the silage. The implication is that microbial numbers give no reliable indication of microbial metabolic activity.

A series of experiments was carried out with the following objectives:
1) to optimise conditions to assay fermentative activity, measuring the rate of glucose utilisation *in vitro* by an inoculum of silage micro-organisms, 2) to assess the effect of environmental perturbations on fermentation and microbial activity and 3) to study changes in activity over a short time course. As related topics, attention was given also to developing ensilage techniques using an anaerobic cabinet and to examining the suitability of freezing as a means of storage of grass for use in later experiments.

Experiment 1 Preliminary experiments to measure the utilisation of glucose *in vitro* by an inoculum prepared from silage

Introduction As the initial step towards standardising *in vitro* conditions, in Experiment 1a the pH of the incubation medium was controlled using various zwitterionic buffers, phosphate buffer or anaerobic diluent, at pH 6.5. The stability of the pH was challenged by gassing with CO_2, by addition of solid or homogenised silage or by addition of an extract of the silage prepared in water. The buffers were compared to determine which best maintained a stable pH.

An inoculum with a reasonable level of fermentative activity was required to optimise conditions for the measurement of glucose utilisation. In Experiment 1b, the age of the silage, within the first 15 d of ensilage, that sustained micro-organisms with the highest rate of glucose utilisation was identified using inocula prepared at timed intervals from material ensiled in laboratory-scale silos. The inoculum was taken from a strained, 10 x diluted homogenate prepared in anaerobic diluent.

The restoration of the microbial population density in the inoculum to that present on the original forage, by centrifugation and resuspension of the homogenate, was examined in

Experiment 1c as a possible means of increasing the rate of glucose utilisation.

Materials and Methods Experiment 1a The zwitterionic buffers, 2-(N-morpholino) ethanesulphonic acid (MES), morpholinopropane sulphonic acid (MOPS) and N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid (HEPES) (0.1 mol/l), phosphate buffer (0.1 mol/l KH_2PO_4 - 0.1 mol/l K_2HPO_4) and anaerobic diluent (described in Chapter 2), were used at pH 6.5 in trials to determine which would best maintain a stable pH while a) gassing with CO_2 (flow rate approximately 250 ml/min) for 1 h, b) adding silage extract (prepared as described in Chapter 2) (10ml), pH 3.88, c) adding 0.2g silage, pH 3.88, and d) adding a 10 x diluted homogenate of silage prepared in anaerobic diluent, pH 6.6-7.3 (10 ml).

Experiment 1b Grass (*L.perenne*) was ensiled in 18 laboratory-scale silos (250 ml polypropylene measuring cylinders). On days 0, 1, 2, 5, 7 and 15 after ensilage, triplicate silos were opened and the contents bulked and minced. Minced silage (10g) was homogenised with anaerobic diluent (90ml) for 2 min in a Stomacher 400 Lab Blender (Seward Medical) and the homogenate was strained through double-layered muslin into a CO_2-filled 100 ml Erlenmeyer flask. This 10 x diluted inoculum was incubated in a buffered glucose solution (5 mmol/l glucose in CO_2-gassed 0.1 mol/l phosphate buffer, pH 6.5), under a CO_2 head-space atmosphere, in duplicate, anaerobic glass vials (20 ml per incubation). The incubation was performed at 30°C and sub-samples (1 ml) were withdrawn every hour for 8 h and prepared for glucose analysis; the sub-samples were centrifuged at 11,600 x *g* for 10 min and the supernatant was frozen and stored at -20°C before measurement of glucose concentration, as described in Chapter 2.

Experiment 1c Grass (*L.perenne*) was ensiled in triplicate laboratory-scale silos and after 2 d the contents were bulked and minced and prepared for microbial enumeration; minced silage (10 g) was homogenised with 90 ml anaerobic diluent for 2 min and the homogenate was strained through double-layered muslin into a CO_2-filled 100 ml Erlenmeyer flask. This 10 x diluted homogenate was used in serial dilutions for enumeration of viable micro-organisms on Yeast Extract agar, while 2 and 3 ml (containing a number of micro-organisms equivalent to that on 0.2 and 0.3 g FW silage) were incubated in a buffered, CO_2-gassed glucose solution (20 mmol/l glucose in 0.1 mol/l phosphate buffer, pH 6.5) in duplicate, CO_2-filled Hungate tubes (10 ml per incubation).

A second inoculum was prepared by centrifuging an aliquot of the strained homogenate (70 ml) at 27,000 x *g* for 15 min at room temperature in CO_2-filled, gas-tight plastic centrifuge tubes and resuspending the cell pellet in anaerobic diluent (7 ml),

maintaining strict anaerobic precautions. Serial dilutions were used for enumeration of viable micro-organisms on Yeast Extract agar, and 2 and 3 ml of this concentrated inoculum (containing a number of micro-organisms equivalent to that on 2 and 3 g fresh material) were incubated in a buffered, CO_2-gassed glucose solution (20 mmol/l glucose in 0.1 mmol/l phosphate buffer, pH 6.5) in duplicate, CO_2-filled Hungate tubes (10 ml per incubation).

The incubations were performed at 30°C. Sub-samples (1 ml) were withdrawn every 30 min for 3 h and prepared for glucose analysis, as described previously. Comparison was made between the two preparations of the rate of *in vitro* glucose utilisation and of the reproducibility of the measurements with the two preparations.

Results Experiment 1a The data in Table 4.1 show the change in pH of the different buffers under test conditions; phosphate buffer appeared to be most resistant to change.

Experiment 1b *In vitro* glucose utilisation over 8 h by an inoculum prepared from freshly minced 2-d-old silage is shown in Figure 4.1, displaying a sigmoidal pattern of uptake, which suggests adaptation by the micro-organisms to *in vitro* conditions during prolonged incubation.

Figure 4.2 shows the dynamic characteristics of the fermentative activity of silage micro-organisms during the early stages of ensilage. The low level of *in vitro* glucose uptake at day 0 (less than 1 μmol/h/g FW) increased to 60.9 μmol/h/g FW after 2 d, followed by a relatively gradual decline in activity to 10.3 μmol/h/g FW by day 15.

Refrigeration of the minced material obtained on day 2, and subsequent preparation and incubation of an inoculum, had an adverse effect on the activity and resulted in a reduced rate of glucose utilisation (17.7 μmol/h/g FW), although there was no effect of this treatment on total viable numbers.

Experiment 1c Suspension and incubation for 3 h in 0.1 mol/l phosphate buffer did not adversely affect numbers of viable micro-organisms (Table 4.2) and centrifugation of the inoculum did not affect the viability of silage micro-organisms; $80x10^8$CFU/g fresh weight were recovered from both the diluted homogenate and the reconstituted inoculum. Concentration of the inoculum resulted in larger changes in glucose concentration during the *in vitro* incubation, which were proportional to inoculum size (Table 4.3); the concentrated inoculum (either 2 or 3 ml) utilised the equivalent of 10 μmol glucose/h/g fresh material (correlation coefficient between glucose concentration and time, $r = 0.9321$ and 0.9783, respectively) whereas the rates of glucose utilisation by 2 and 3 ml of diluted inoculum differed and were, respectively, 18 and 24 μmol/h/g fresh material.

Table 4.1 Change in pH of 18 ml buffer solution under test conditions.

	Gassed with CO_2 for 1 h	Added 10 ml silage extract, pH 3.88	Added 10 ml silage homogenate, pH 6.6-7.3	Added 0.2 g silage, pH 3.88
Anaerobic diluent	+0.10	-0.31	-0.17	+0.20
Phosphate buffer	-0.40	-0.33	-0.14	+0.03
MES	-0.43	-0.40	-0.20	+0.23
MOPS	-0.52	-0.42	-0.11	+0.26
HEPES	-0.54	-0.44	-0.21	+0.28

Table 4.2 Effect of sample preparation and incubation on microbial populations on forage (CFU/g FW).

	Total viable organisms	Lactic acid bacteria	Coliform bacteria
Silage	$21x10^8$	$81x10^6$	$25x10^5$
Inoculum (pre-incubation)	$37x10^8$	$42x10^6$	$32x10^5$
Inoculum (after 3 h incubation)	$23x10^8$	$69x10^6$	$59x10^5$

Table 4.3 Effect of inoculum preparation on glucose uptake.

Size of inoculum (ml)	Preparation: homogenised (H), or homogenised and centrifuged (C)	Rate of glucose utilisation (μmol/h/g FW)
2.0	H	18
2.0	C	10
3.0	H	24
3.0	C	10

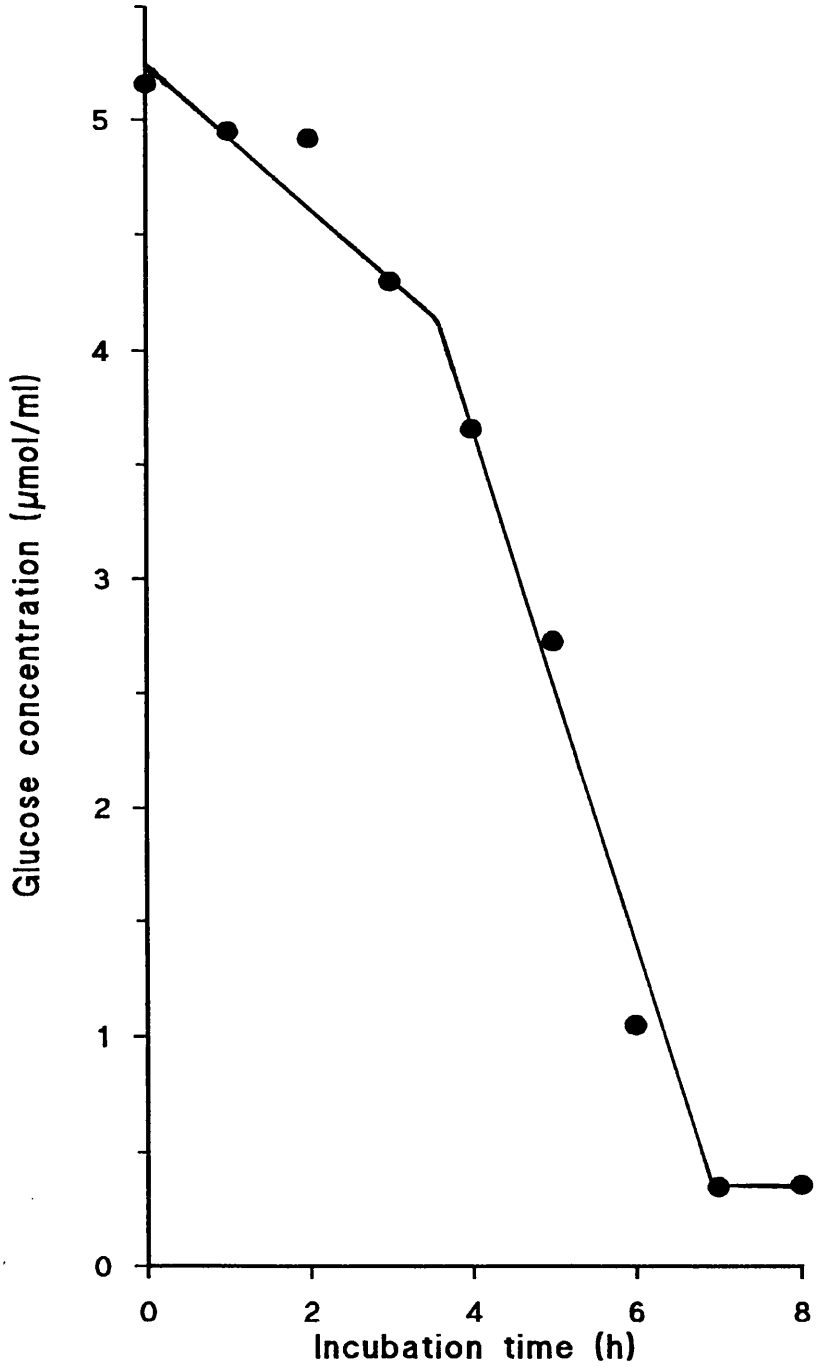


Figure 4.1 The effect of length of incubation (up to 8 h) on glucose concentration *in vitro* (µmol/ml) during incubation with a dilute inoculum prepared from 2-d-old silage.

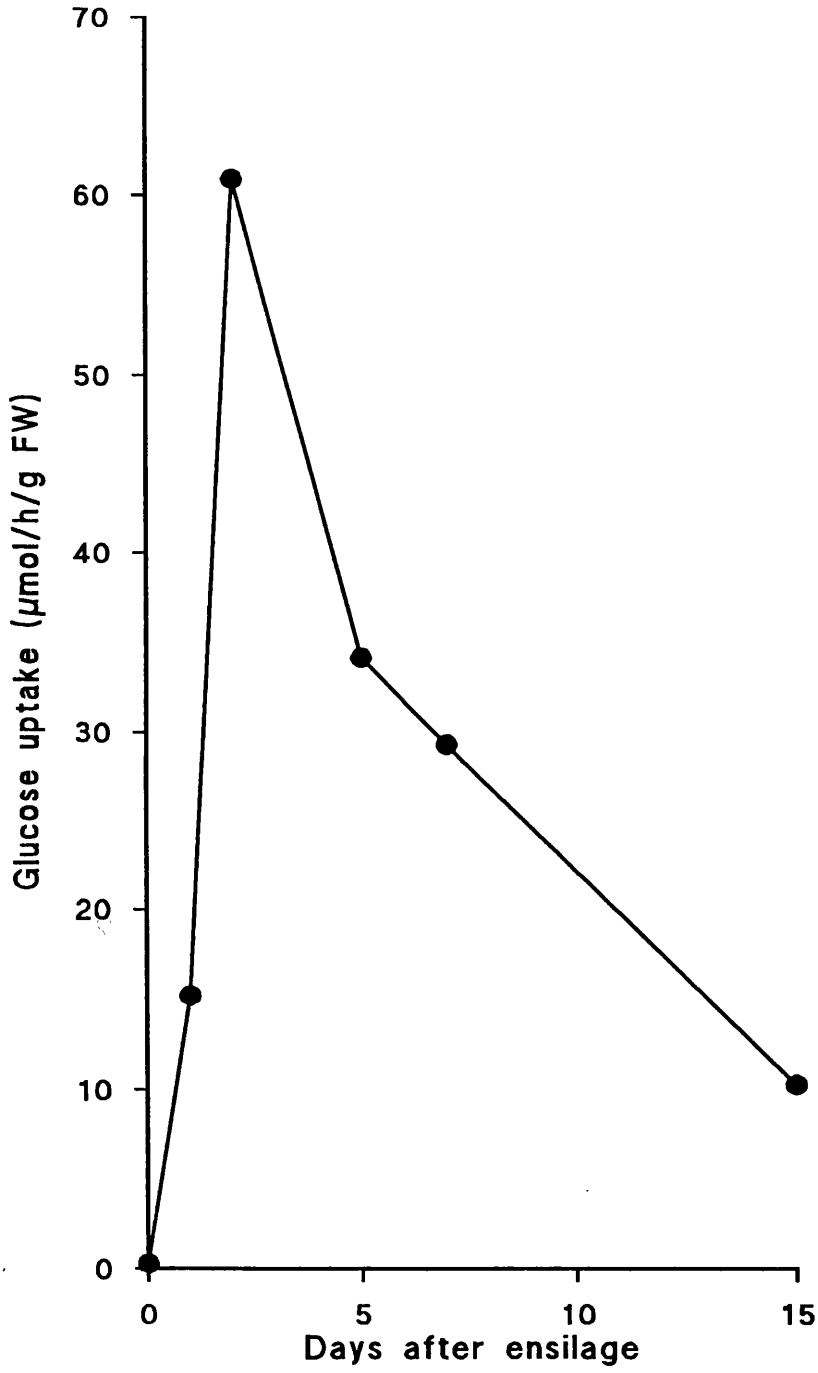


Figure 4.2 Glucose uptake over 3 h incubation (μmol/h/g FW) by an inoculum of silage micro-organisms prepared at timed intervals over a 15 d time-course.

These rates were calculated from relatively small changes in glucose concentration, which were less well correlated with time (r=0.5307 and 0.7263).

Discussion Assay conditions which are selected in advance of a full validation of the method may be far from ideal. As the buffer used for suspending the bacterial cells can be critical for avoiding cell damage and for sustaining a viable culture, identification of a suitable buffer was chosen as the starting point for the development of the assay. There were no great differences in the ability of the chosen buffers to maintain a stable pH in these trials, but phosphate buffer, the major constituent of the anaerobic diluent, was used for future assays to maintain, as far as possible, continuity between media. Phosphate buffers are used most often for the cultivation of lactic acid bacteria for *in vitro* studies, although the effect of phosphate on enzyme activity is not fully understood (Garvie, 1980). The pH of the buffer is also important, and the effect of different pH values was studied in a later series of experiments in which conditions for the *in vitro* measurement of glucose utilisation were optimised.

Measurement of glucose utilisation over 8 h, albeit utilisation by a dilute inoculum, revealed adaptation of the micro-organisms to *in vitro* conditions after 3 h, characterised by multiplication and an exponential pattern of utilisation; to avoid adaptation of the inoculum to *in vitro* conditions, the change in glucose concentration over 3 h was used as the measure of metabolic activity of the inoculum.

Although the conditions for the *in vitro* measurement of glucose utilisation were still crude, it was clear that a large rise in metabolic activity occurred during the early stages of ensilage (Experiment 1b). The period of greatest activity was identified as 48-72 h after ensilage and an inoculum prepared from 2-d-old silage was used during the subsequent development of the assay. Because refrigeration of the forage prior to preparation of an inoculum appeared to adversely affect the subsequent activity, for all future assays the inoculum was prepared from freshly sampled material.

Data from microbial enumeration in Experiment 1c suggest that micro-organisms were not adversely affected by the preparation of a concentrated inoculum, although it is recognised that incubation for 5 d in ideal conditions before enumeration allows the opportunity for repair and regeneration. The activity of the concentrated inoculum was not as high as anticipated; glucose utilisation, per g FW, by the concentrated inoculum was approximately 0.5 times the rate of glucose utilisation determined using the 10 x diluted homogenate. This suggests that the preparation may have impaired the activity of the inoculum or, alternatively, that the dilution led to inaccuracies, associated with the measurement of small changes in

glucose concentration, in the determination of glucose uptake. The second explanation seems the more likely given the higher correlation coefficient for the change in glucose concentration over 3 h during incubation with the concentrated inoculum (mean 0.9552), reflecting a more accurate assessment of glucose utilisation and fermentative activity.

Experiment 2 Validation of the standardised *in vitro* assay

Introduction Using the techniques and conditions identified in Experiment 1 as those necessary to maximise the rate of change of glucose concentration, a series of trials was performed to optimise conditions for the *in vitro* measurement of the metabolic activity of silage micro-organisms. An inoculum prepared from 2 d-old silage was incubated in the buffered glucose solution using a range of pH values and different temperatures. Glucose uptake was measured under aerobic and anaerobic conditions, and with reducing agents to secure an anoxic environment. The concentration of glucose supplied was varied, within the range necessary to maintain precision of analysis, to determine whether the availability of substrate affected the rate of utilisation. Once the optimum level of each factor was identified, this was incorporated in the subsequent assays.

Materials and Methods A small volume of homogeneous silage was required for these trials, so laboratory-scale silos were used to prepare the material; grass (*L. perenne*) was ensiled in triplicate 250 ml polypropylene measuring cylinders and after 2 d the contents were bulked to provide homogeneous material from which a concentrated inoculum was prepared (as described in Chapter 2).

The effects of concentration of glucose (4-40 mmol/l), length of incubation (up to 10 h) and volume of inoculum (0.5-3 ml) were examined in duplicate incubations. Glucose uptake was measured under aerobic and anaerobic conditions (using N_2- or CO_2-gassed buffer and head-space) and with addition of reducing agents (0.2 mg/ml dithiothreitol or 0.1 mg/ml neutralised cysteine-hydrochloride), and using a range of pH (4-8) and temperature (30-50^0). Residual glucose concentration and lactic acid accumulation were analysed as described in Chapter 2.

Results Phosphate buffer (0.1 mol/l, pH 6.5) maintained a stable pH; the terminal pH differred from the initial pH by less than 0.2, after 3 h incubation. The rate of glucose utilisation was independent of glucose concentration in the range 4-40 mmol/l.

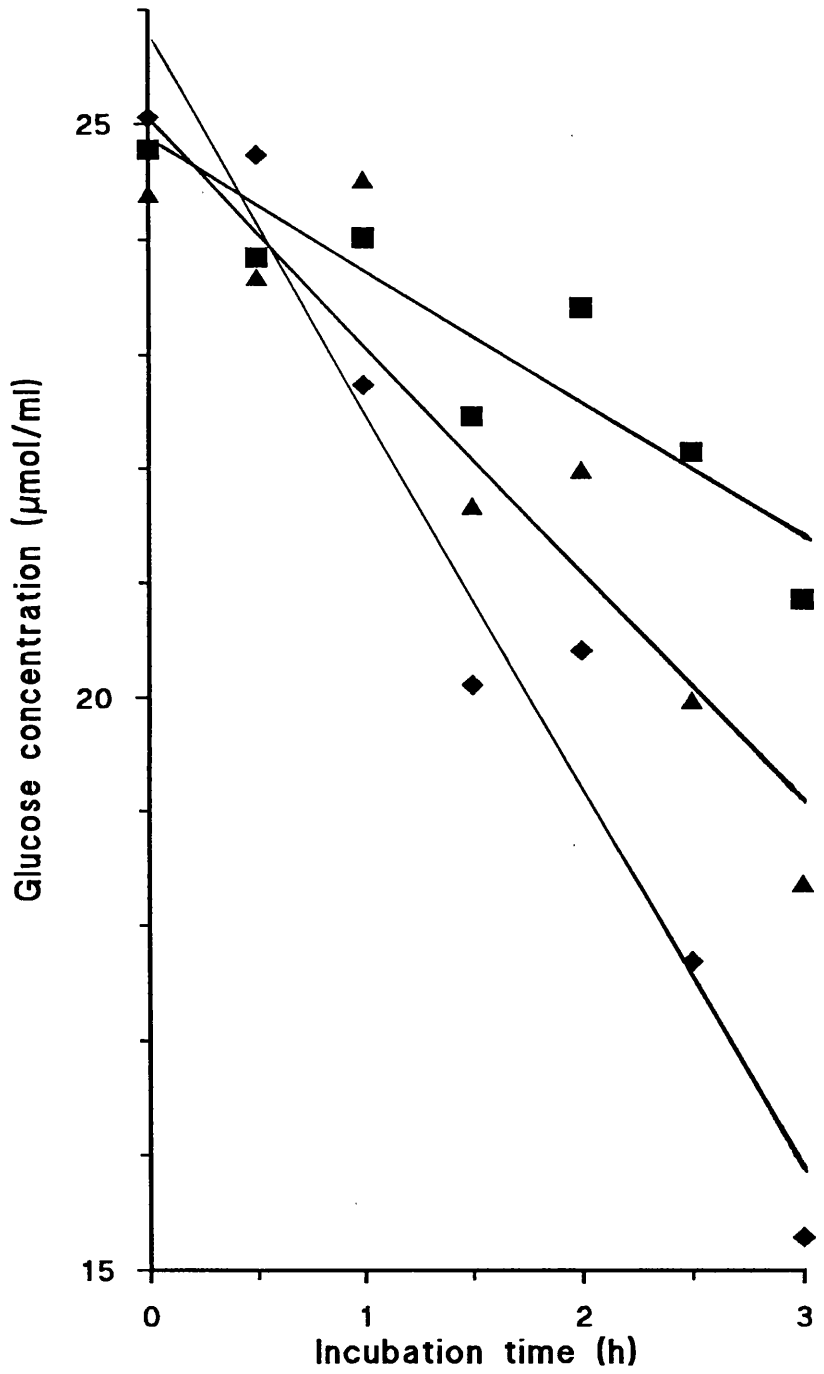


Figure 4.3 The effect of inoculum size (ml) on glucose concentration *in vitro* (µmol/ml) over 3 h incubation:- 1.5 ml (■), regression equation y=24.77-1.13x, r=0.9122; 2.0 ml (▲), regression equation y=25.07-2.00x, r=0.9321; 3.0 ml (◆), regression equation y=25.77-3.27x, r=0.9783. Values are means of duplicate determinations.

Table 4.4 Effect of reducing agents (dithiothreitol, 200 mg/ml, and neutralised cysteine hydrochloride, 100 mg/ml) on glucose uptake and L-lactic acid production over 3 h incubation.

	Glucose uptake (μmol/h/g FW)	L-lactic acid production (μmol/h/g FW)	Molar ratio of lactate production/glucose utilisation
No reducing agent	58.8	101.8	1.7
Dithiothreitol	48.8	103.2	2.1
Cysteine hydrochloride	41.2	88.0	2.1

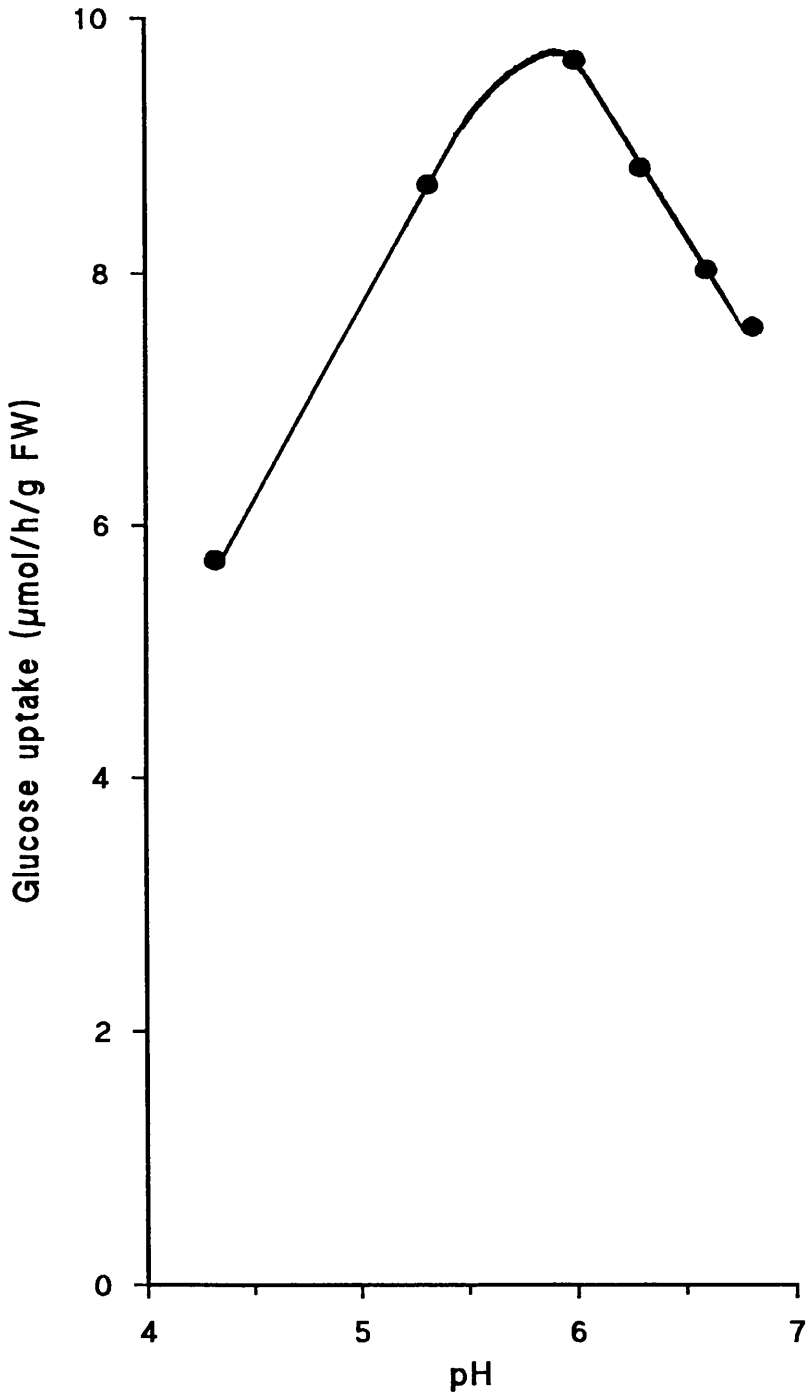


Figure 4.4 The effect of pH on glucose uptake over 3 h incubation (µmol/h/g FW) by an inoculum prepared from 2-d-old silage. Values are means of duplicate determinations.

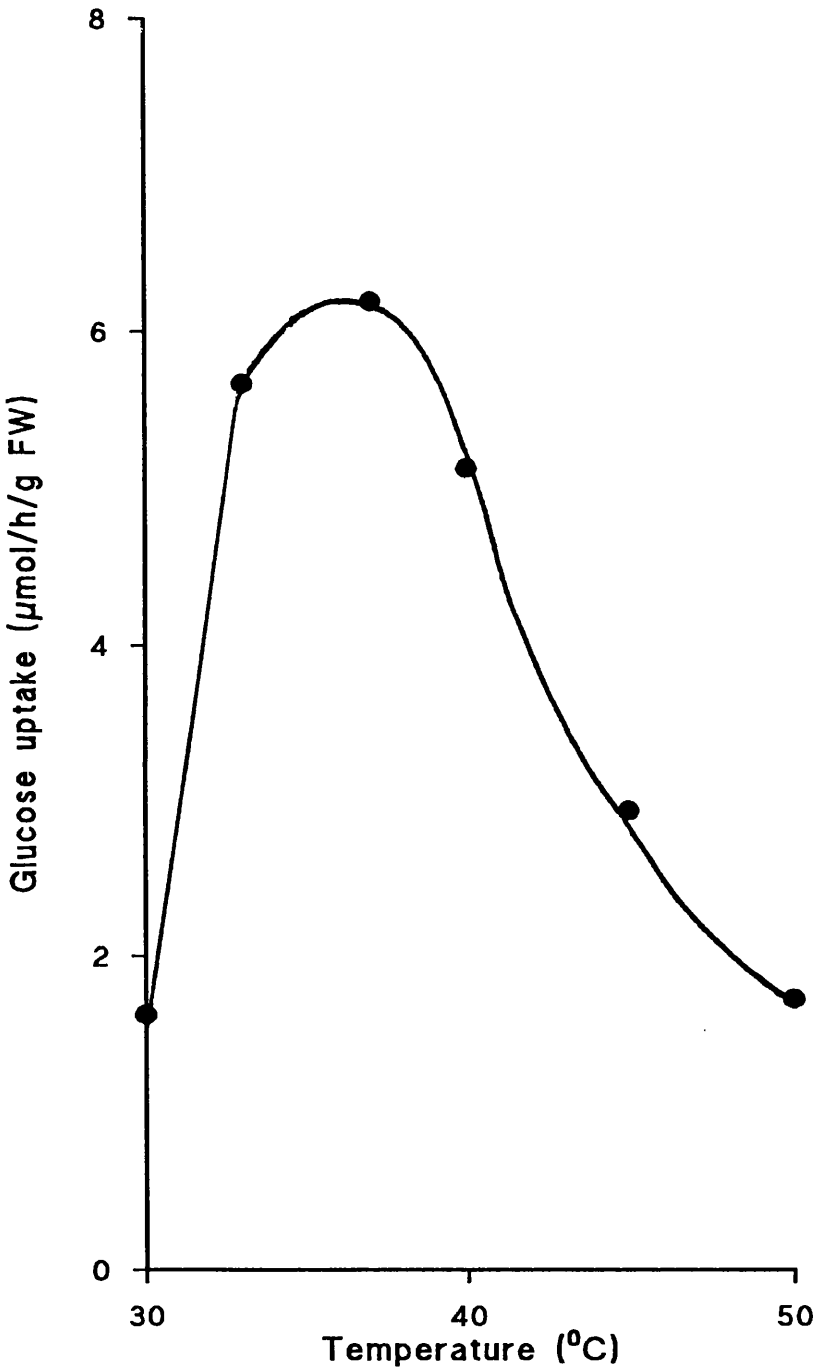


Figure 4.5 The effect of temperature (°C) on glucose uptake over 3 h incubation (μmol/h/g FW) by an inoculum prepared from 2-d-old silage. Values are means of duplicate determinations.

The rates of glucose uptake were proportional to inoculum size (2.0-3.0 ml) and glucose utilisation was linear throughout the course of a 3 h incubation period (Figure 4.3). Bacterial numbers did not increase and adaptation occurred only when the incubation period was extended; after 10 h incubation total viable micro-organisms had increased in number from $79x10^8$ to $692x10^8$ CFU/g fresh weight.

Glucose uptake was pH- and temperature-dependent, for example the rate at pH 5 and 7 was respectively 21% and 29% lower than at pH 6.0, (Figure 4.4) and, as compared with the rate at 37°C, there was a 60% and 80% reduction at 30°C and 50°C respectively (Figure 4.5). Glucose uptake was similar under aerobic and anaerobic conditions (although reducing agents restricted glucose uptake slightly) and the reduced medium enhanced the accumulation of lactic acid from glucose metabolism (Table 4.4).

Discussion Glucose uptake *in vitro* was linear and proportional to inoculum size using 2 ml and 3 ml inoculum; less than 2 ml compromised the proportionality of glucose uptake with respect to inoculum size, possibly an effect of the lower amount of utilisation and inaccuracies associated with its measurement. To optimise the rate of change of glucose concentration 3 ml was used and, to ensure the accuracy of the measurement of glucose concentration in the supernatant, 20 mmol/l glucose was included in the incubation medium; although 4 mmol/l glucose was not found to limit utilisation, it was decided to use a higher glucose concentration in view of reports of altered end-product distribution and of the *in vitro* reconversion of lactate to pyruvate where nutrients were limiting (Garvie, 1980).

The assay was shown to be sensitive to incubation conditions; those adopted were chosen to resemble as closely as possible the silage environment, yet to optimise glucose utilisation by the inoculum. Thus, a compromise was made with the choice of pH, since the rate of change of glucose concentration over 3 h at a pH normally occurring in the silo (less than pH 5.0) was too small to measure accurately. Glucose uptake *in vitro* was maximal at pH 5.5-6.5, and a pH of 6.5 was chosen as the standard incubation pH since this was the pH of the medium (anaerobic diluent) into which the micro-organisms were originally extracted. Although pH has been found to affect enzyme activities (Montville *et al.*, 1987; Tseng and Montville, 1990), with consequent changes in end-product distribution, the fermentation in the assay at pH 6.5 was predominantly homofermentative. Lactic acid production was not adversely affected by aerobic or anaerobic conditions; Hickey *et al.* (1983), however, demonstrated the evolution of acetate, acetoin and lactate in the ratio 6:2:1 when pure cultures of lactic acid bacteria were incubated in an aerobic medium for 2 h. The provision

of pyruvate in this earlier *in vitro* study probably allowed the fermentation of substrates to products other than lactate since there was no accumulation of NADH from glycolysis and hence no necessity for regeneration of NAD via reduction of pyruvate to lactic acid. Since glucose utilisation was not impeded, the assay was conducted taking rigorous anaerobic precautions to mimic conditions in the silo, and dithiothreitol was included to ensure a reduced environment.

The basic assay accommodated the measurement of the rate of glucose utilisation and metabolite formation by an inoculum, under standard optimized conditions, and provided a means whereby the effect of environmental conditions on the metabolic activity of the epiphytic micro-flora associated with silage could be investigated. The length of incubation was restricted to 3 h to avoid the adaptation of the inoculum to *in vitro* assay conditions, and thus to provide an indication of the fermentative activity of silage micro-organisms as found on the silage.

Experiment 3 Factors affecting the fermentative activity of silage micro-organisms

Introduction The objective of these trials was to expose an inoculum prepared from silage to perturbations *in vitro* to validate the sensitivity of the assay and to identify factors that influence fermentative activity of micro-organisms associated with silage.

The ability of an inoculum of epiphytic silage micro-flora to metabolise various carbohydrates was studied. The carbohydrates used were glucose, fructose and sucrose (the major soluble carbohydrates in forages), xylose and arabinose (products of hemicellulose breakdown), and starch (the predominant storage polysaccharide in legumes). Since some species of lactic acid bacteria are important in fermentation of lactose to lactic acid during the souring of milk, the rate of utilisation of lactose *in vitro* by silage micro-organisms was also studied.

The conditions resulting in the cessation of a natural fermentation are not clearly defined, although a combination of pH and the concentration of lactic acid is thought to play an important role. The pH/lactic acid concentrations were manipulated in the *in vitro* system in an attempt to clarify these mechanisms. Low concentrations of volatile fatty acids (formic, acetic, propionic, butyric and valeric acid) accumulate during ensilage and have been used at higher concentrations to inhibit the fermentation (McDonald and Henderson, 1974; Woolford, 1975a). Ethanol, too, is produced during fermentation, mainly by yeasts, but also during heterofermentation of glucose, and is potentially inhibitory. The effect of organic

acids, sulphuric acid and ethanol on microbial activity *in vitro* was studied.

Common salt is a well established food preservative and was used by Goett *et al.* (1970) and Goering and Gordon (1973) as a silage additive, and antibiotics have restricted fermentation in experimental silages (Woolford, 1975b; Woolford and Wilkins, 1975; Merry and Haskins, 1989). The effect of sodium chloride, penicillin, streptomycin and chloramphenicol on the fermentative activity of silage micro-organisms was, therefore, examined *in vitro*. Ammonia has been used as a silage additive (Kung *et al.*, 1984; Deschard *et al.*, 1987) and as an inhibitor of aerobic deterioration (Honig, 1975), and, more recently, as a means of delignifying forage (Morrison and Brice, 1983/84; Morrison, 1988). To examine the effects of ammonia on an inoculum of silage micro-organisms, ammonium nitrate was included in the incubation medium; although used as food preservatives, nitrates are not recognised as having antibiotic properties but dissociation was expected to release ammonia into solution.

Materials and Methods For each trial, grass (approximately 300 g) was ensiled in triplicate 250 ml polypropylene measuring cylinders and, after 2 d, the contents were bulked and an inoculum prepared for duplicate *in vitro* incubations.

Assay conditions were varied in a series of experiments and the following additions were included in the incubation (mmol/l): glucose, fructose, sucrose, xylose, arabinose, lactose, 25; sodium lactate, 70-400; sodium acetate, 20-100; sulphuric acid, 50; individual volatile fatty acids (formate, acetate, propionate, butyrate and valerate), 50; ethanol, 100-400; sodium chloride, 200-1000; ammonium nitrate, 100-500; and, (mg/l), penicillin, 600; streptomycin, 100; chloramphenicol, 100. Starch was also added (4.5 mg/ml).

The techniques for total carbohydrate analysis (Dubois *et al.*, 1956), described in Chapter 2, were used to assay residual fructose, sucrose, xylose, arabinose, lactose and starch.

Results The silage inoculum readily fermented glucose, sucrose and fructose, but not xylose, arabinose, lactose or starch (Table 4.5). Glucose utilisation was stimulated (50%) by 60 mmol/l sodium acetate and by 33% by 50 mmol/l acetic acid (Table 4.7) (Fig.4.6). In contrast, antibiotics (Table 4.6), sodium lactate (Figure 4.7), sulphuric acid and volatile fatty acids other than acetic acid (Table 4.7), ethanol (Figure 4.8), sodium choride (Figure 4.9) and ammonium nitrate (Figure 4.10) were all inhibitory.

Table 4.5 Effect of substrate supply (25mmol/l) on utilisation by silage micro-organisms (μmol/h/g FW, unless stated otherwise).

Substrate supply	Substrate utilisation
Glucose	4.3
Arabinose	0.8
Sucrose	4.0
Fructose	5.5
Xylose	0.3
Lactose	0
Starch	72 μg/h/g FW

Table 4.6 Effect of penicillin (600 mg/l), streptomycin (100 mg/l) and chloramphenicol (100 mg/l) on glucose uptake (μmol/h/g FW) by silage micro-organisms.

	Glucose utilisation
Control	45
Penicillin plus streptomycin	22
Chloramphenicol	11
Penicillin plus streptomycin plus chloramphenicol	9

Table 4.7 Effect of organic and mineral acid (50 mmol/l) on *in vitro* glucose uptake (μmol/h/g FW) by silage micro-organisms and pH of incubation medium.

	Glucose uptake	**Terminal pH of buffer**
No acid	10.3	6.23
Formic acid	8.0	5.51
Acetic acid	13.7	5.50
Propionic acid	7.8	5.68
Butyric acid	7.0	5.76
Sulphuric acid	0.4	2.48

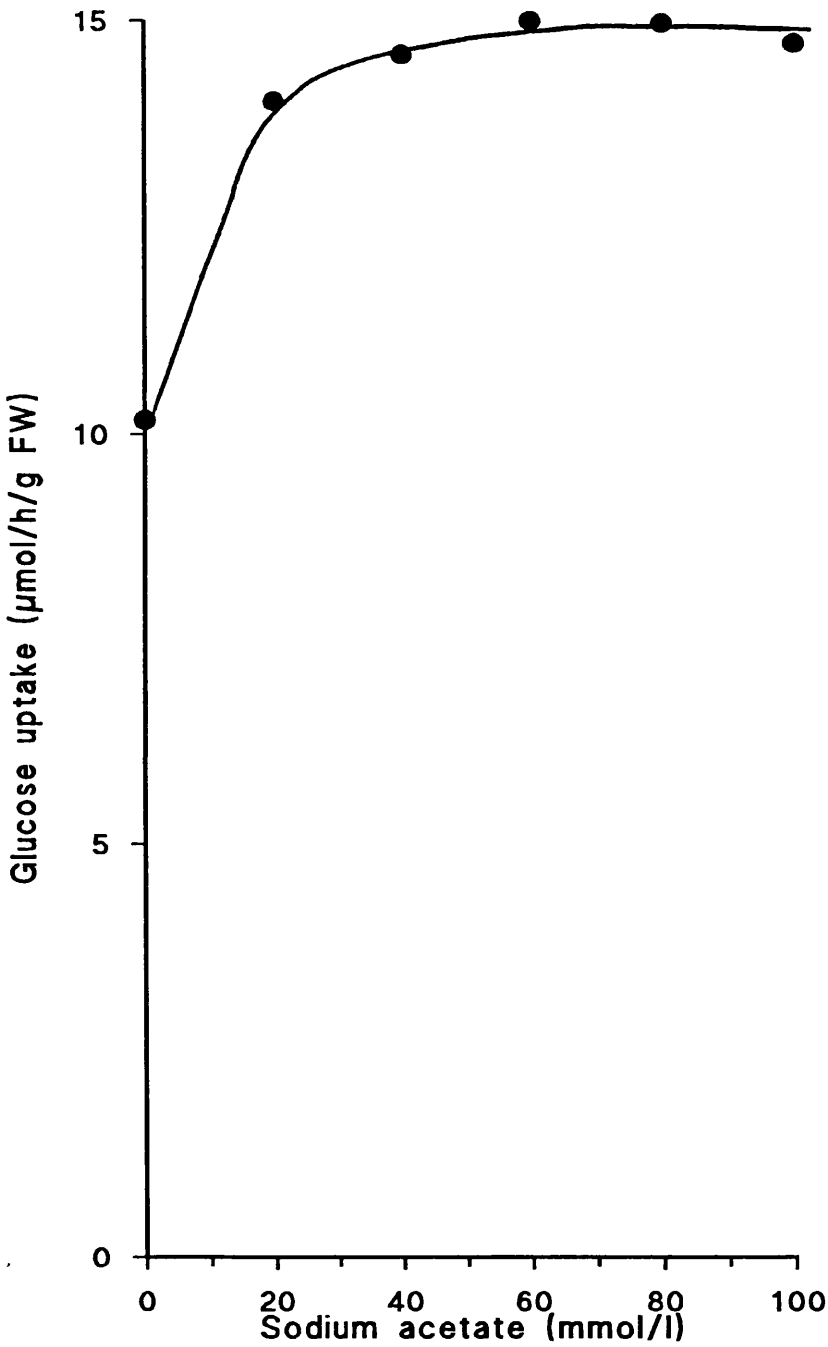


Figure 4.6 The effect of sodium acetate (mmol/l) on glucose uptake during 3 h incubation *in vitro* of an inoculum prepared from 2-d-old silage (µmol/h/g FW).

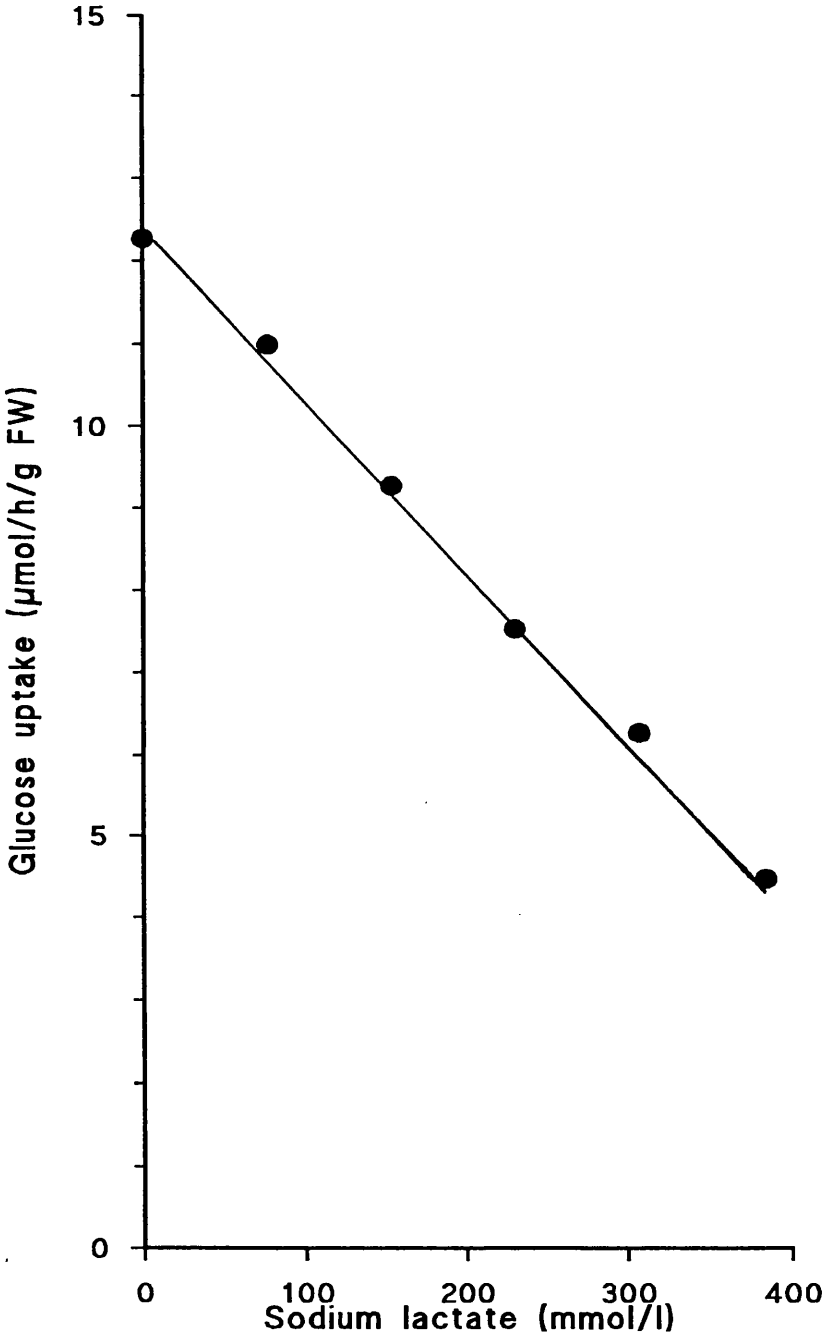


Figure 4.7 The effect of sodium lactate (mmol/l) on glucose uptake during 3 h incubation *in vitro* of an inoculum prepared from 2-d-old silage (µmol/h/g FW). Regression equation, y=12.39-0.02x; r=0.9988.

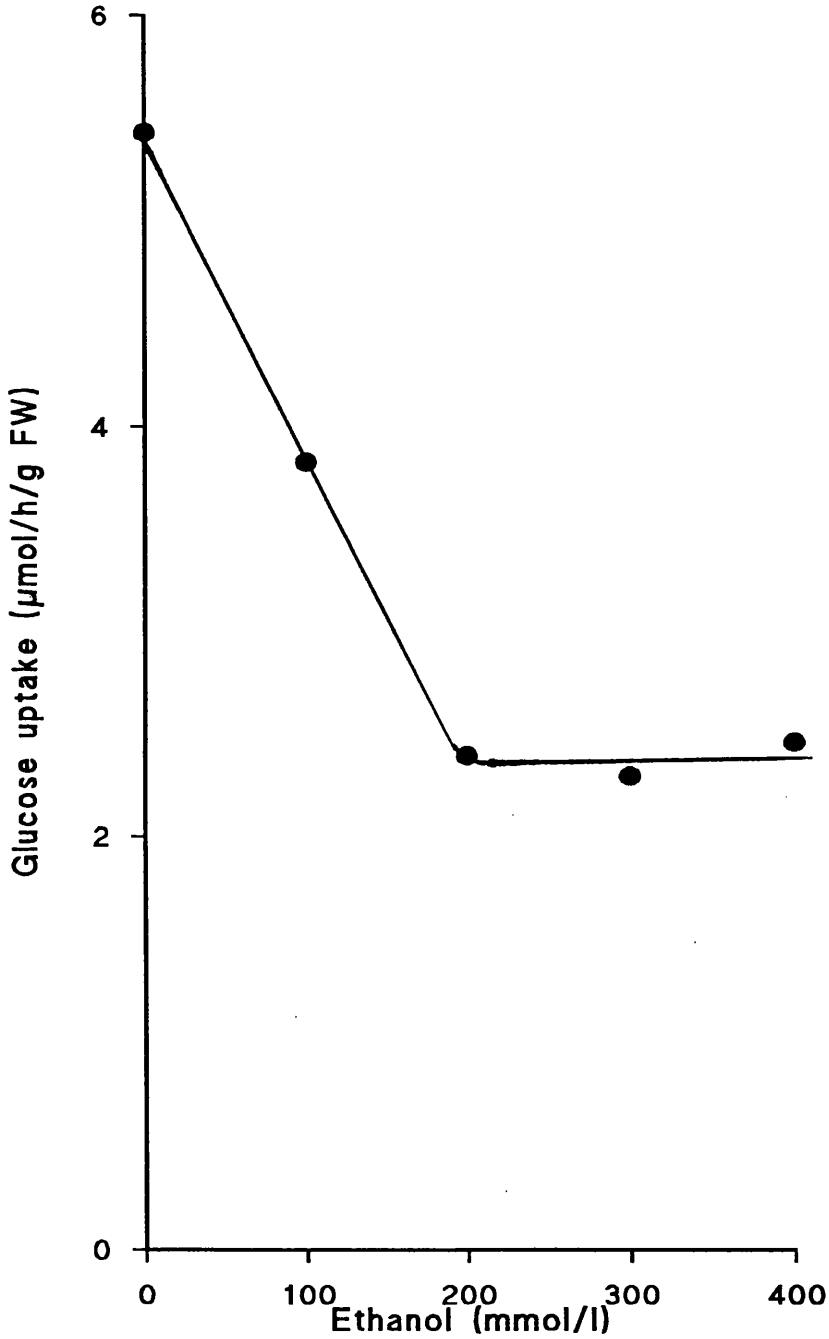


Figure 4.8 The effect of ethanol (mmol/l) on glucose uptake during 3 h incubation *in vitro* of an inoculum prepared from 2-d-old silage (µmol/h/g FW).

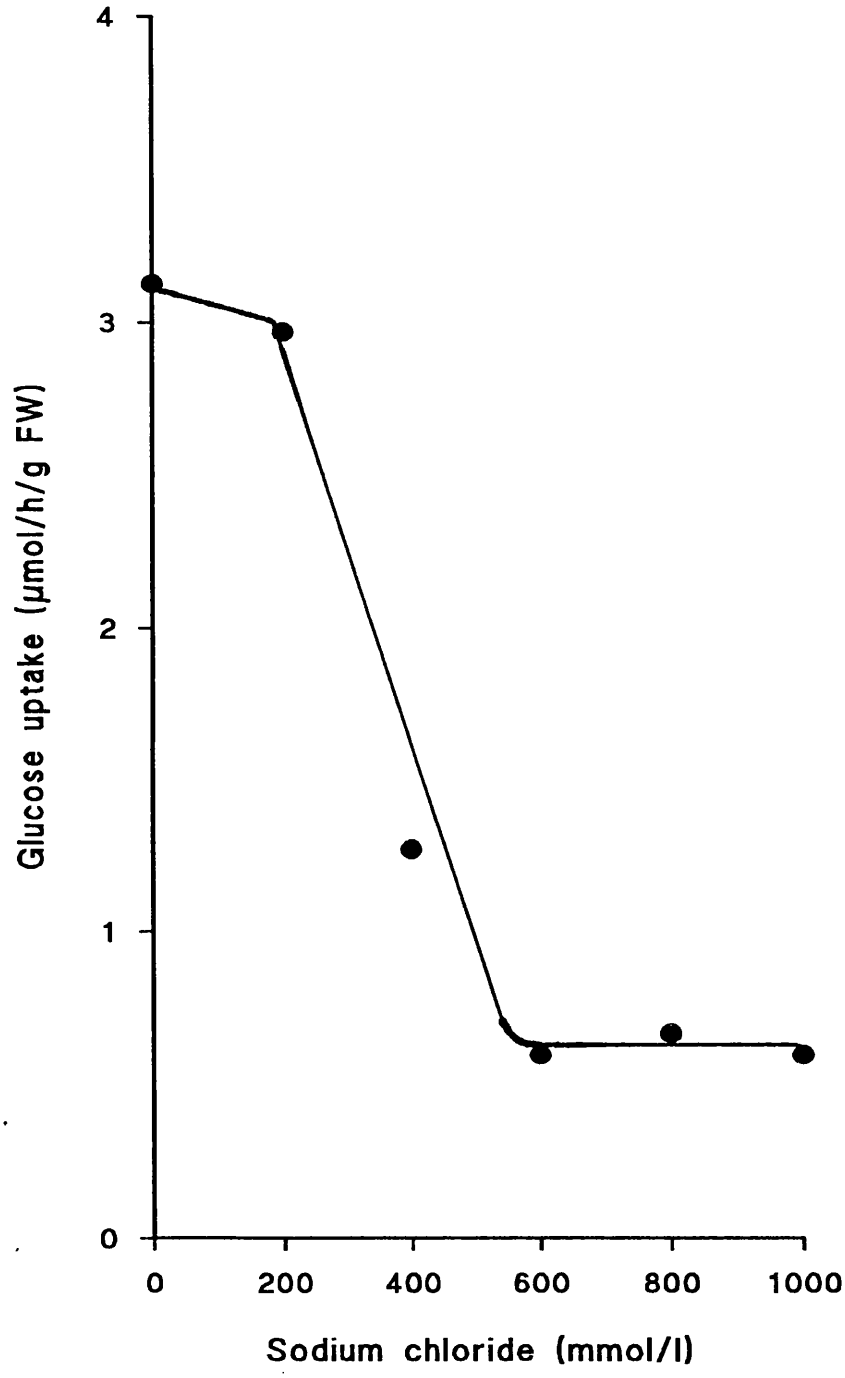


Figure 4.9 The effect of sodium chloride (mmol/l) on glucose uptake during 3 h incubation *in vitro* of an inoculum prepared from 2-d-old silage (µmol/h/g FW).

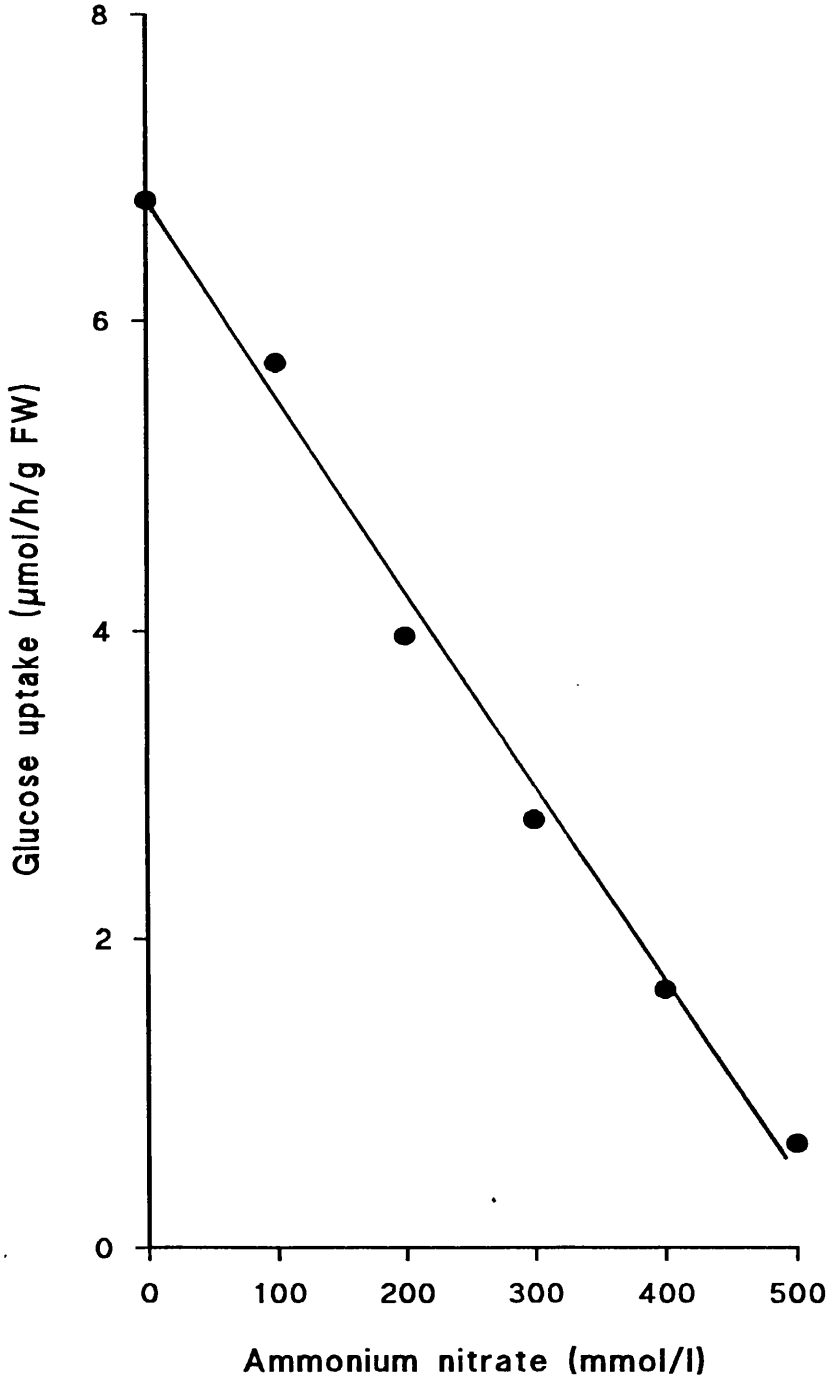


Figure 4.10 The effect of ammonium nitrate (mmol/l) on glucose uptake during 3 h incubation *in vitro* of an inoculum prepared from 2-d-old silage (µmol/h/g FW). Regression equation, y=6.75-0.013x; r=0.9961.

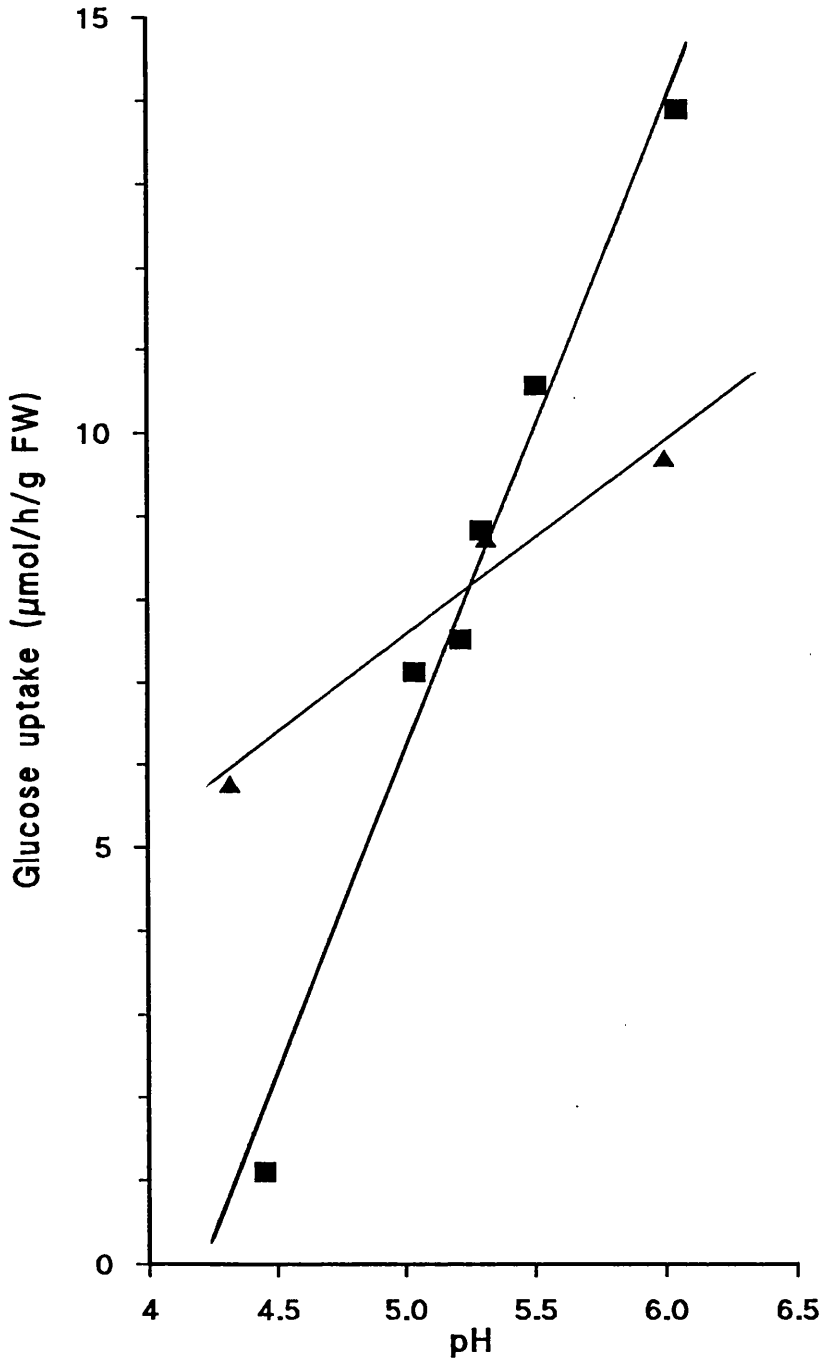


Figure 4.11 The effect of pH ▲ and 0.1 M sodium lactate ■ on glucose uptake during 3 h incubation *in vitro* of an inoculum prepared from 2-d-old silage (µmol/h/g FW) :- regression equation for glucose uptake without sodium lactate inclusion (▲), y=2.39x-4.44, r=0.9846; regression equation for glucose uptake with sodium lactate inclusion (■), y=7.99x-33.84, r=0.9912.

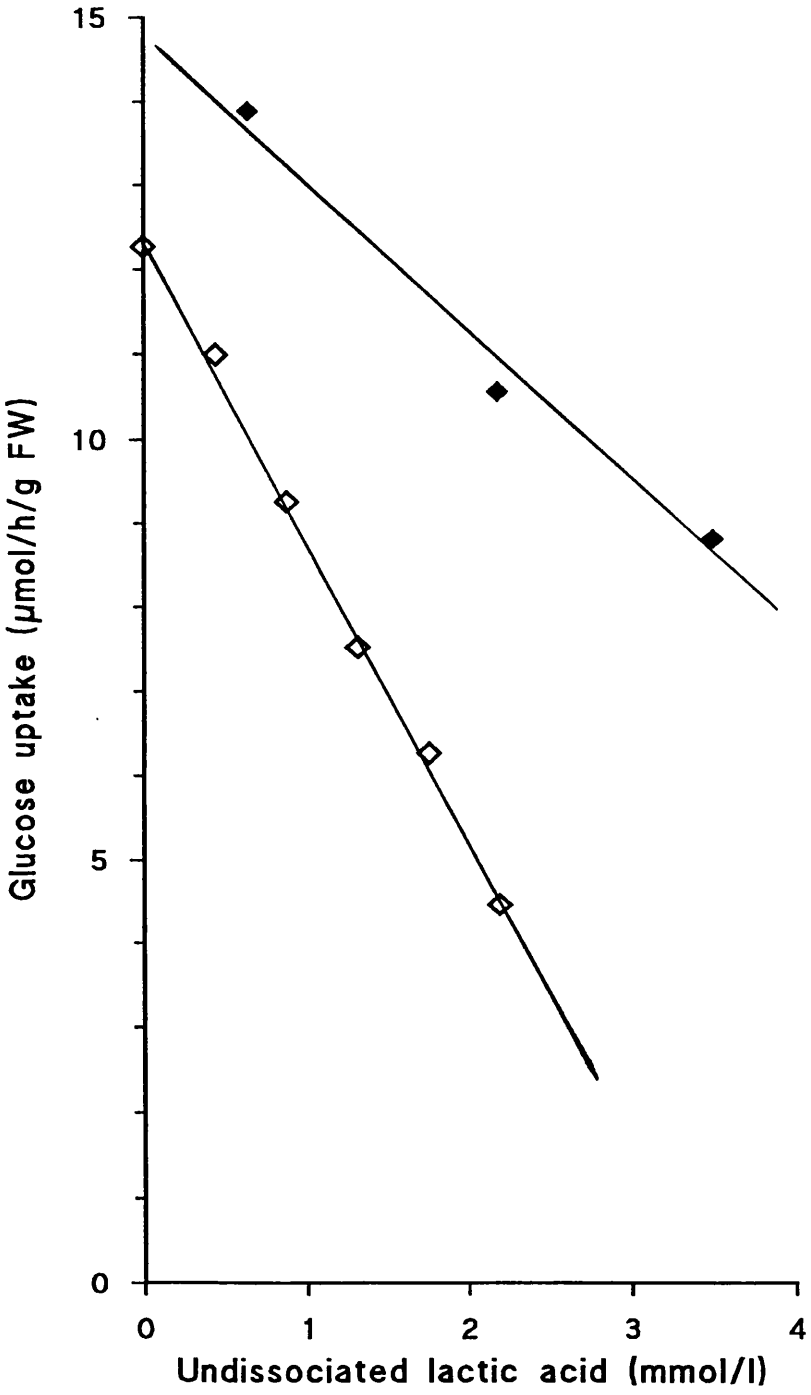


Figure 4.12 The effect of undissociated lactic acid (mmol/l) on glucose uptake during 3 h incubation *in vitro* of an inoculum prepared from 2 d-old silage (µmol/h/g FW) :- data from Figure 4.7 (◇), regression equation y=12.39-3.57x, r=0.9988; data from Figure 4.11 (◆), regression equation y=14.87-1.78x, r=0.9914.

Declining pH (6.81-4.32) at a constant lactate concentration (0.1 mol/l) and increasing concentrations of lactate (0.07-0.35 mol/l) at a constant pH (6.10) inhibited glucose utilisation (Figure 4.11 and 4.7, respectively). The effect of undissociated lactic acid concentration, calculated from these data, is illustrated in Figure 4.12, showing a similar pattern of inhibition (12 and 29 %, respectively, per mmol lactic acid present) whether increases in undissociated lactic acid were achieved by reducing the pH or by adding lactate.

Discussion The *in vitro* studies showed that the rate of glucose utilisation was sensitive to perturbations to incubation conditions, and the inhibition by antibiotics (penicillin, streptomycin and chloramphenicol) confirmed that glucose uptake *in vitro* was bacterial.

Hexose sugars were readily fermented, fructose more so than glucose and sucrose, perhaps reflecting selection pressures bearing on the epiphytic micro-flora, fructose being the most abundant hexose in grasses. Pentose sugars, however, were not readily fermented. This agrees with the observations of Rogosa (1974) who showed that some silage lactic acid bacteria do not readily ferment xylose and Chamberlain (1988) who found that approximately 30-50% of xylose added to the silo remained unused after 100 d. In addition, lactose, traditionally regarded as a natural substrate for lactic acid bacteria, and starch were not utilised by silage micro-organisms. The micro-organisms present possibly did not possess the necessary complement of enzymes (which may be induced after a period of adaptation), or alternatively it may require the presence of different strains of lactic acid bacteria to ferment these substrates. Further investigation is necessary to clarify the potential of the silage lactic acid bacteria to utilise different substrates, particularly in relation to pentose sugars (mainly xylose and arabinose) released during hemicellulose hydrolysis.

Sodium acetate and acetic acid (60 and 50 mmol/l, respectively) stimulated the rate of *in vitro* glucose utilisation. McFeeters and Chen (1986) found that acetate increased the rate of mannitol fermentation *in vitro* by *L. plantarum* and they suggested that acetate acted as an electron acceptor; the provision of an exogenous electron acceptor may facilitate a more exhaustive oxidation of the available substrate. Alternatively, the reaction of sodium acetate with lactic acid (a stronger acid) may displace undissociated lactic acid and increase the concentration of acetic acid, which is less inhibitory to bacterial activity; Woolford (1975a) found that 375 mmol/l acetic acid was needed to inhibit lactic acid bacteria at pH 6.0. More work is needed to clarify the potentially buffering impact of anions in the silage. Formic, propionic and butyric acids inhibited glucose uptake; the pH of the buffer was 5.5-5.7, which is higher than would be considered inhibitory, confirming that it was a property of the organic

acids which restricted the fermentation. In the case of sulphuric acid it is likely to be the impact of pH which restricts the fermentation.

Sodium lactate inclusion enhanced the effect of declining pH on glucose uptake (Figure 4.11), suggesting potent inhibition by the undissociated lactic acid molecule. The key role of undissociated acid is further supported by the much weaker inhibitory effect of added sodium lactate at constant pH (Figure 4.7). The range of concentrations of lactic acid used contributed up to approximately 70 mmol undissociated lactic acid/kg DM (assuming 60 g DM/l *in vitro*), and were responsible for similar degrees of inhibition in different trials, supporting the theory that the undissociated molecule may be an important regulator of the silage fermentation. The different degrees of restriction in the two experiments (Figure 4.12) may relate to the metabolic status of the two inocula, thus a reflection of conditions in the silo. Similarly, differences in the rate of glucose utilisation in Figures 4.6-4.10 at zero concentration of inclusions (mean 7.6 $\pm$ 1.47 μmol/h/g DM), presumably relate to the metabolic activity of the inoculum in response to conditions in the silo.

Ethanol, the neutral product of heterolactic fermentation by lactic acid bacteria and of ethanolic fermentation by yeasts, is a well recognised preservative, although its role in the silage fermentation is not clear. In the *in vitro* studies, the concentration which inhibited fermentative activity (200 mmol/l) corresponded to 153 g/kg DM, assuming a dry matter content *in vitro* of 60 g/l. The physiological inhibition of micro-organisms by ethanol is unlikely to be of significance in the practical situation since it would require 300 g/kg DM hexose to be fermented exclusively to ethanol to attain such a concentration.

Ammonia has been used as a silage additive, though with variable results. Kung *et al.* (1984) found that ammonia treatment (17-29 g/kg DM) stimulated lactic acid production in lucerne silages, but when Deschard *et al.* (1987) ensiled whole-crop winter wheat with higher levels (35 g/kg DM) the lactic acid content was reduced and the silage was poorly preserved. In the experiment reported here, the lowest level of ammonium nitrate (100 mmol/l) contributed approximately 21 g/kg DM ammonia, assuming full dissociation of the salt in solution and an equivalent DM content of 60 g/l *in vitro*. The nitrate anion may, however, have been at least partly responsible for the inhibition of the fermentative activity and, in retrospect, to differentiate the effects of ammonia, the experiment should be repeated using other ammonium salts, for example ammonium chloride or ammonium sulphate.

Common salt has long been used for preserving foods for human consumption and Gouet *et al.* (1970) used 10 g salt/kg lucerne but found it to be ineffective as a silage additive. Inhibition of glucose uptake *in vitro* was achieved by 0.4-0.6 mol/l (77-116 g/kgFW)

sodium chloride, which may reflect the effects of osmolarity on the micro-organisms utilising the glucose, but is unlikely to be of much use in practice and might even be toxic if fed to livestock.

The imposition of perturbations revealed, to some extent, the fermentation characteristics of silage micro-organisms and the response of these micro-flora to adverse conditions *in vitro*, and demonstrated the sensitivity of the assay to variations in metabolic activity. Experiment 4 investigated the reaction of epiphytic micro-organisms to ensilage and the change in fermentative activity of these micro-organisms *in situ* during the natural fermentation of grass.

Experiment 4 The effect of ensilage on the fermentative activity of epiphytic micro-organisms

Introduction Following the development of an assay to measure microbial glucose utilisation, and the use of this assay to study the effect of a range of perturbations on the *in vitro* activity of silage micro-organisms, the assay was used to study changes in microbial activity during ensilage.

Materials and methods Late-season perennial ryegrass, harvested in September (1991), was ensiled in 36x250 ml polypropylene measuring cylinders (as described in Chapter 2) and triplicate silos were opened on days 0, 1, 2, 3, 4, 7, 10, 15, 20, 30, 60 and 90, and an inoculum prepared from the fresh material was assayed for microbial populations and metabolic activity, including, on days 2, 7 and 30, measurement of D- and L-lactic acid production. Silage samples were stored frozen at -20^0 C for chemical analyses, as described in Chapter 2.

Results The grass ensiled was a low sugar/high nitrogen autumn crop (Table 4.8) and produced a poorly preserved silage with a high pH and low concentrations of lactic acid, although the concentrations of ammonia and butyric acid also remained low. The changes in chemical composition during the ensilage period are shown in Table 4.9 and Figure 4.13a. The WSC content (originally 54 g/kg DM) was reduced to 8 g/kg DM after 7 d, associated with a rise in lactic acid concentration to 54 g/kg DM and a fall in pH to 4.33. After 30 d ensilage, lactic acid concentration declined (16 g/kg DM, day 90), with an associated increase in the concentration of acetic acid (47 g/kg DM, day 90). The pH was 5.10 after 90 d.

Table 4.8 Chemical composition of the grass ensiled (g/kg DM, unless stated otherwise).

Dry matter (g/kg)	199
Crude protein	217
Water soluble carbohydrates	54

Table 4.9 Chemical composition of the silage after 90 d (g/kg DM unless stated otherwise).

pH	5.10
Dry matter (g/kg)	175
Crude protein	223
Ammonia nitrogen (g/kg TN)	176
Lactic acid	16
Water soluble carbohydrates	4
Ethanol	6
Acetic acid	47
Propionic acid	9

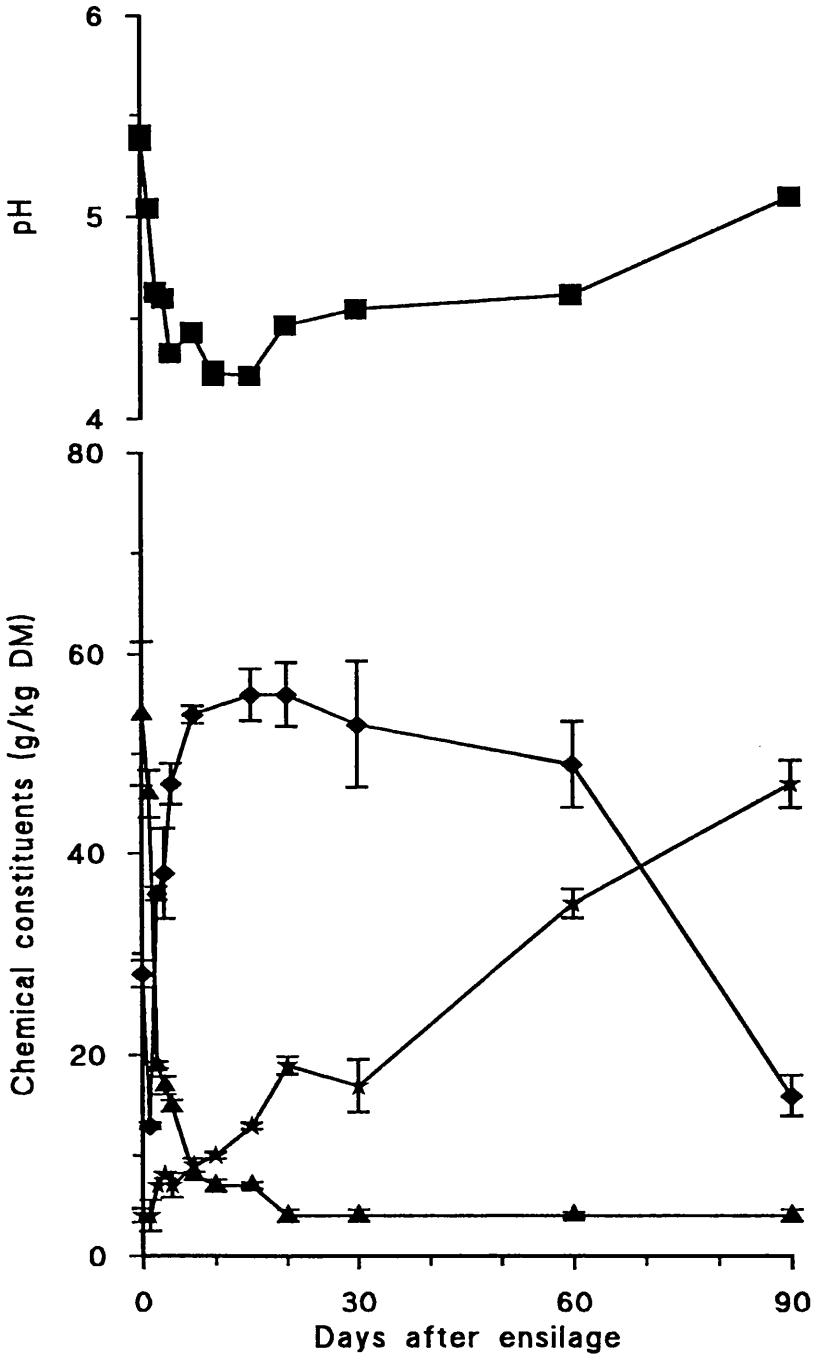


Figure 4.13a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), and acetic acid (★) (g/kg DM) during ensilage of perennial ryegrass in polypropylene measuring cylinders (Experiment 4). Error bars represent SE of triplicate silos.

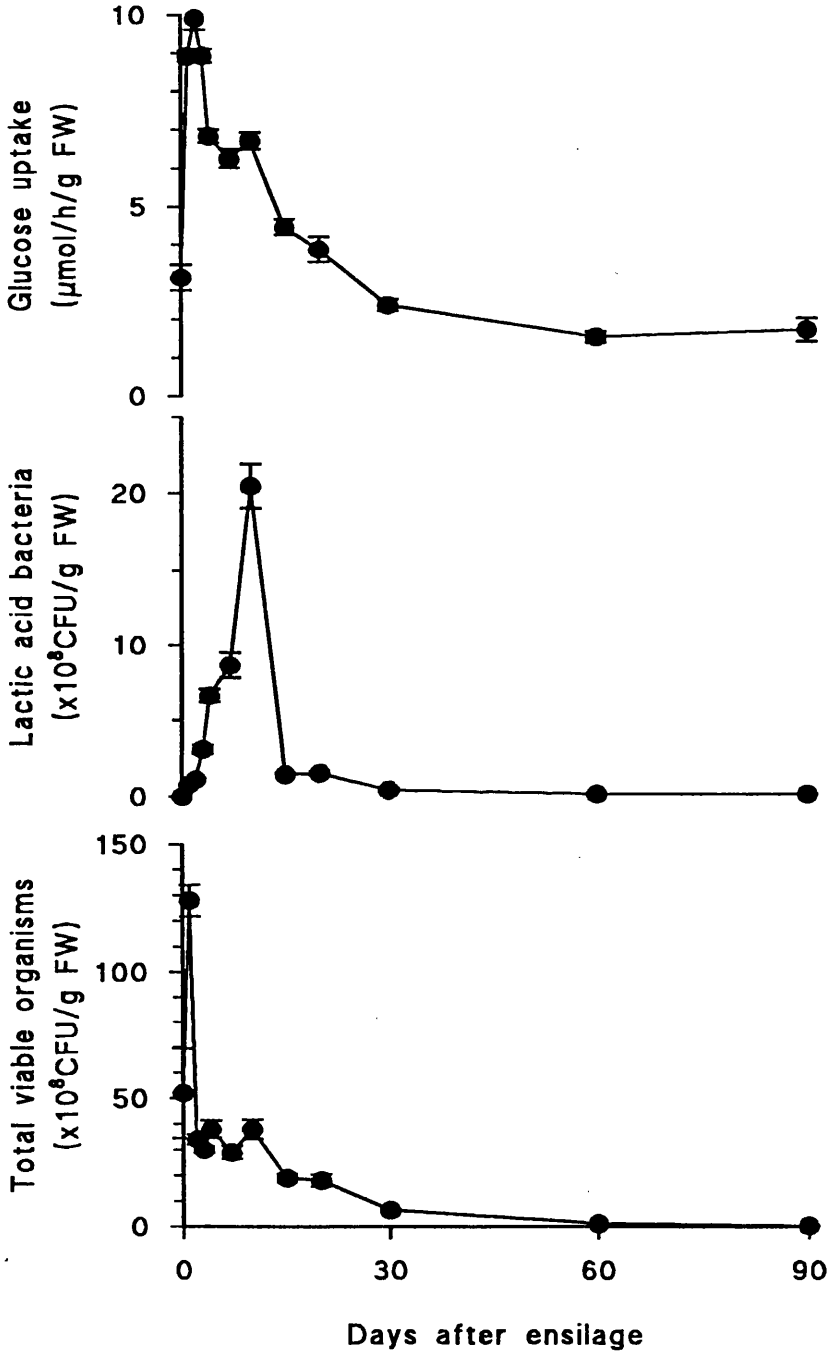


Figure 4.13b Changes in the numbers of total viable organisms and lactic acid bacteria (CFU/g FW) and the rate of glucose uptake *in vitro* (μmol/h/g FW) during ensilage of perennial ryegrass in polypropylene measuring cylinders (Experiment 4). Error bars represent the SE of triplicate silos.

Table 4.10 Glucose uptake and D- and L-lactic acid production (μmol/h/g FW) by an inoculum prepared from silage on days 2, 7 and 30 (Experiment 4).

	Glucose uptake	L-lactic acid production	D-lactic acid production
Day 2	9.9	13.2	2.6
Day 7	6.7	3.2	4.9
Day 30	2.4	2.8	0

The changes in microbial populations and the rate of *in vitro* glucose utilisation are shown in Figure 4.13b. Total viable and lactic acid bacterial populations ($52x10^8$ and $39x10^8$ CFU/g FW, respectively, on day 0) increased to maximum numbers ($13x10^9$ and $66x10^7$ CFU/g FW) by days 2-5; the rate of glucose uptake (metabolic activity) likewise increased rapidly to a maximum at day 2 (9.9 μmol/h/g FW), but although the viable count remained relatively unchanged the measured activity declined. After 10 d the rate of glucose utilisation had decreased 32 % to 6.73 μmol/h/g FW. The metabolic activity continued to decline to day 30 (2.4 μmol glucose consumed/h/g FW) and thereafter remained close to this value. Closer examination of the data showed that glucose utilisation per 10^8 total viable organisms followed a similar pattern during the period of active fermentation, rising to 298 nmol/h/10^8 CFU at day 3 and subsequently falling 41% to 177 nmol/h/10^8 CFU by day 10. However, after 30 d, microbial glucose utilisation per 10^8 CFU increased to 400 nmol/h/10^8 CFU and remained high during the remainder of the time-course. The rates of D- and L-lactic acid production (μmol/h/g FW) were, respectively, 2.6 and 13.2 on day 2, 4.9 and 3.2 on day 7 and 0 and 2.8 on day 30 (Table 4.10).

The coliform population decreased rapidly from $38x10^7$ CFU/g FW initially to less than 10^2 /g FW after 7 d, rising again to $51x10^3$ in the later stages of fermentation.

Discussion The first 48 h were the most dynamic in the course of the silage fermentation; the lactic acid bacteria population increased in number and metabolic activity to a peak, corresponding with a rapid fermentation of most of the WSC to lactic acid. The early decline of coliform bacteria may have been due to the drop in pH, the increase in lactic acid production or the increase in numbers of lactic acid bacteria, or a combination of these factors. In the later stages of the fermentation the decrease in lactic acid concentration and rise in pH encouraged an increase in the number of coliform bacteria, although no deleterious effect on the silage composition attributable to these bacteria was apparent. There was no increase in the number of yeasts and no significant rise in ethanol concentration; presumably the lactic acid bacteria fermented most of the WSC, leaving insufficient readily available substrate to support fermentation by yeasts or coliform bacteria.

Small amounts of acetic acid produced during the early stages of ensilage may have been the consequence of the heterolactic fermentation of fructose or the release and subsequent metabolism of pentose sugars, or a result of coliform metabolism. But between days 30 and 90, after exhaustion of WSC, there was a greater accumulation of acetic acid, associated with the degradation of lactic acid. Clostridia are known to metabolise lactate to

acetate and butyrate in the silo (Gibson, 1965), and the rise in pH would have encouraged clostridial activity, although the chemical composition of the silage is not consistent with this explanation. Lindgren *et al.* (1990) described the anaerobic degradation of L-lactate by *L. plantarum*, using oxaloacetate as an electron acceptor, and it may be that a lack of soluble carbohydrates led to a similar pattern of metabolism here. Nicotinamide adenine dinucleotide (NAD)-independent lactate dehydrogenases (i-LDHs) are found in some anaerobic bacteria, and enable them to use lactate as a source of carbon (Garvie, 1980). The activities of the i-LDHs in lactic acid bacteria are usually low when a cell is actively forming lactate (Garvie, 1980) but, when nutrients are in short supply, the i-LDHs may scavenge some of the lactate and reconvert it to pyruvate, which the cell can then use. The accumulation of acetate associated with lactate disappearence suggests a fermentation of pyruvate to acetate.

The proportions in which D- and L-lactic acid were produced changed from predominantly L-lactic acid production on day 2 to a more even balance of D- and L-lactate production on day 7. After 30 d, lactate production was exclusively of the L-isomer. Environmental conditions have been found to affect the proportion of D- and L-lactic acid production by lactic acid bacteria (Garvie, 1967; Stetter and Kandler, 1973; Gordon and Doelle, 1975) and the NAD-linked D- and L-lactate dehydrogenases (n-DLDH and n-LLDH), responsible for the production of D- and L-lactic acid respectively, are known to be different in different genera and species and even in different strains of the same species (Garvie, 1980). For example, all *Leuconostoc* spp. form D-lactate from glucose as they have a single n-DLDH and no n-LLDH (Garvie, 1969). Some lactic acid bacteria form DL-lactate, but the ratio of the isomers can change in cultures growing without pH control (Garvie, 1967; Stetter and Kandler, 1973; Gordon and Doelle, 1975), although for strains of *L. plantarum* the ratio of L- to D-lactate formed does not change much (Garvie, 1967; Gasser *et al.*, 1970). The data of this experiment suggest that either different lactic acid bacteria dominate the silo at different times or that the conditions in the silo may modify the fermentation pathways of the existing micro-flora. More work is needed to clarify the situation.

This experiment illustrates the sensitivity of the assay to detect changes in metabolic activity of the microbes in response to changing conditions in the silo during ensilage. The *in vitro* assay measures the activity of those micro-organisms present to ferment available substrate under optimised conditions, and allows the analysis of the fermentation patterns of an inoculum prepared from the silage. An incubation period of 3 h limits adaptation to the assay conditions whereas the CFU enumeration technique allows the inoculum to grow in ideal conditions for 5 d. In the later stages of ensilage the numbers of total viable organisms

were frequently lower than the numbers of lactic acid bacteria; this may be an adverse effect of competition for nutrients by organisms growing on Yeast Extract agar, or a consequence of the disruption of aggregates of bacteria by Tween 80 in the Tween Acetate agar. More work is needed to explain these discrepancies, but unusual observations such as these further compromise the validity of microbial enumeration data. Microbial glucose utilisation *in vitro*, per 10^8CFU, rose from 177 nmol/h/10^8 CFU at day 10 to 400 nmol/h/10^8 CFU by day 30, but during this period the chemical composition of the silage was relatively stable, demonstrating the poor relationship between microbial population size and microbial activity and, further, the importance of a measure of activity independent of CFU enumeration.

It was planned to use the assay, in conjunction with CFU enumeration, to study the fermentation of additive-treated grass, and the effect of addition, to a pre-fermented silage, of extra sugars. This addition was intended to simulate the action of fibrolytic enzymes. Prior to this, Experiments 5 and 6 were instigated to investigate the feasibility of ensiling 100-150g grass (fresh or frozen) in sterile polythene bags within the anaerobic cabinet as a method of producing small quantities of uniform silage.

Experiment 5 A preliminary experiment to compare the ensilage of grass in laboratory silos and inside an anaerobic cabinet

Introduction In order to investigate the effect of changing conditions within the silo and manipulating the fermentation after ensilage, a technique was required whereby the forage may be ensiled and subsequently treated without the incursion of air. In Experiment 5, a series of preliminary trials was performed, a) to clarify the requirements of a laboratory silo and the implications of pretreatment of grass (chopping, bruising and mincing) for subsequent ensilage in laboratory-scale silos, and b) to test the importance of anaerobic conditions and the effectiveness of a range of techniques for achieving and maintaining anaerobiosis. Thick plastic or glass is considered less permeable to gases than thin plastic, and one concern was that the latter may allow gaseous exchange and perhaps loss of volatiles.

Materials and methods Experiment 5a Chopped grass (*L.perenne*) was compacted into duplicated 250 ml polypropylene measuring cylinder laboratory silos (as described in Chapter 2) and 100-150 g was loosely filled into duplicate sterile polythene bags, inside and outside an anaerobic cabinet, with the open end of the bag folded over and secured with adhesive tape. Duplicate wide-necked plastic sample bottles (100 ml-capacity) were used to ensile grass

inside the anaerobic cabinet after a) chopping the grass, b) chopping the grass and bruising 50 g portions for 5 min in a stomacher (Lab Blender 400) and c) chopping the grass and mincing with a domestic food mincer. The grass was loosely filled or compacted into the vessels, and the neck of the bottle was securely sealed with i) a fermentation trap, which allowed the release of gases, ii) a loosely fitted screw-cap, which allowed free exchange of gases, or iii) a tightly fitted rubber O-ring screw-cap, which did not permit exchange of gases. In addition, chopped, minced grass was compacted into duplicate 20 ml-capacity glass vials closed with a butyl rubber seal and aluminium cap, and duplicate 20 ml-capacity McCartney bottles closed with an air-tight rubber-sealed screw-cap. The pH of the material on days 0, 2, 7 and 14 was recorded.

Experiment 5b Grass was ensiled in duplicate 500 ml-capacity thick-walled screw-capped plastic reagent bottles, inside the anaerobic cabinet: a) 50 g loosely ensiled, b) 100 g loosely ensiled and c) 450 g compacted. In addition, grass was ensiled loosely in duplicate glass Kilner jars inside the anaerobic cabinet, isolated from the atmosphere of the cabinet by means of gas-tight rubber seals, and 100-150 g was loosely packed in duplicate sterile polythene bags. Duplicate measuring cylinder silos were filled outside the anaerobic cabinet as a positive control.

Results Experiment 5a The pH of the grass used in Experiment 5a was 6.33. Table 4.11 shows the change of pH of the material prepared in measuring cylinders and polythene bags. Where the ingress of air had been prevented, all silages fermented well, reflected by low pH values after 2 d. Grass in the anaerobic cabinet fermented more quickly than that ensiled outside the anaerobic cabinet, with lower pH values by day 7. The pH of the material ensiled outside the anaerobic cabinet in sterile polythene bags was unstable.

Table 4.12 shows the change in pH of the chopped, bruised and minced material ensiled in 100 ml-capacity plastic sample bottles and Table 4.13 shows the change in pH of the material prepared in glass vials and McCartney bottles. There were no consistent differences in pH values attributable to type of silo and chopping, bruising and mincing the grass before ensilage all produced well preserved, low pH silage after 14 d.

Experiment 5b All silos produced acceptable silages after 12 d (Table 4.14a). WSC (54g/kgDM) were fermented almost exclusively to lactic acid with low concentrations of acetic acid and ethanol produced.

Table 4.11 Change in pH of grass ensiled in duplicate measuring cylinder silos and polythene bag silos, inside and outside an anaerobic cabinet.

	pH		
	Day 2	**Day 7**	**Day 14**
Measuring cylinder silos (outside anaerobic cabinet)	4.92	4.26	4.32
Polythene bag silos (outside anaerobic cabinet)	5.34	7.52	6.83
Measuring cylinder silos (inside anaerobic cabinet)	4.60	4.06	4.41
Polythene bag silos (inside anaerobic cabinet)	4.56	4.07	4.41

Table 4.12 Effect of pre-treatment of forage and different degrees of enclosure on rate of change of pH of grass ensiled in 100 ml-capacity plastic sample bottles, within an anaerobic cabinet.

		Chopped grass			Chopped and bruised grass			Chopped and minced grass		
		Day 2	**Day 7**	**Day 14**	**Day 2**	**Day 7**	**Day 14**	**Day 2**	**Day 7**	**Day 14**
Fermentation trap	**Compacted**	4.44	4.13	4.58	4.46	4.14	4.56	4.38	4.04	4.33
	Loosely filled	4.47	4.07	4.34	4.44	4.06	4.51	4.30	4.05	4.09
Screw-cap	**Compacted**	4.46	4.10	4.56	4.39	4.22	4.56	4.38	4.03	4.42
	Loosely filled	4.48	4.09	4.46	4.48	4.06	4.21	4.30	4.05	4.15
O-ring seal	**Compacted**	4.42	4.08	4.23	4.42	4.08	4.45	4.37	4.08	4.06
	Loosely filled	4.43	4.10	4.40	4.37	4.14	4.55	4.34	4.10	4.14

Table 4.13 Change in pH of chopped, minced grass ensiled in 20 ml-capacity glass containers within an anaerobic cabinet.

	pH		
	Day 2	**Day 7**	**Day 14**
McCartney bottles	4.31	4.10	4.01
Glass vials	4.31	4.07	4.02

Table 4.14a Chemical composition of the silages after 12 d in different vessels within an anaerobic cabinet (g/kg DM, unless stated otherwise).

	Type of silo					
	Plastic bottles			Polythene bag	Kilner jar	Measuring cylinder
	50 g	100 g	Compacted			
pH	3.86	3.88	3.88	3.89	3.89	3.91
Dry matter (g/kg)	177	166	175	190	170	187
Crude protein	205	231	205	192	231	224
Ammonia (g/kg TN)	99	97	117	97	94	94
Lactic acid	60	63	58	53	62	56
WSC	11	10	5	9	10	8
Ethanol	3	4	3	3	4	2
Acetic acid	1	2	2	1	1	2
Recovery (%) of material ensiled.	100	99	100	99	100	88

Table 4.14b Microbial populations on grass and silage after 12 d (CFU/g FW).

	Grass	Type of silo					
		Plastic bottle			Polythene bag	Kilner jar	Measuring cylinder
		50 g	100 g	Compacted			
Total viable organisms	$48x10^8$	$56x10^7$	$105x10^7$	$57x10^7$	$89x10^7$	$97x10^7$	$105x10^7$
Lactic acid bacteria	$< 10^2$	$13x10^6$	$60x10^6$	$70x10^6$	$40x10^6$	$70x10^6$	$147x10^6$
Coliform bacteria	$17x10^6$	$81x10^2$	$46x10^2$	$< 10^2$	$24x10^2$	$37x10^2$	$130x10^2$
Yeasts	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$

There was a tendency for higher numbers of coliform bacteria in the control silage, but, in general, the microbial populations were not affected by the type of silo (Table 4.14b). There was only minimal weight loss from the experimental silos (Table 4.14a) but some discoloration of liquid in the fermentation lock on the measuring cylinder silos indicated that effluent leakage into the lock may have occurred.

Discussion The results from the preliminary studies in Experiment 5 emphasise the importance of an anaerobic environment to ensure successful preservation. The method by which anoxic conditions were maintained did not appear to be relevant and inclusion or exclusion of the atmosphere provided inside the anaerobic cabinet did not affect the rate of change of pH. The release of gases did not appear to be necessary either, but, where closed vessels were used (inside the anaerobic cabinet) the pH fell quicker, probably because of accumulation of CO_2 and volatiles. Mincing the grass before ensilage encouraged a more rapid fermentation, probably due to faster access of the epiphytic micro-flora to the plant cell constituents, although by day 14 all the silages had a similar pH. The chopping of the grass appeared to be sufficient pretreatment to ensure release of WSC and adequate preservation. Compaction, aided by chopping, reduces oxygen penetration, thereby reducing deterioration (Hoxey and Billington, 1987), but where the maintenance of anaerobic conditions was controlled by the anaerobic cabinet, compaction did not confer an advantage on the fermentation and, in the control silage of Experiment 5b, may have contributed to cellular disruption and a loss of material in effluent via the fermentation lock. The material from which the silos were constructed affected neither microbial changes nor chemical changes during ensilage; the chemical composition of the silage revealed a predominantly homolactic fermentation in all treatments.

Experiment 6 The ensilage of grass, either fresh or when thawed after deep freezing, in sterile polythene bags

Introduction A technique was required whereby the fermentation could be manipulated during the ensilage process, without the incursion of air. Experiment 6a was undertaken to examine the fermentation of loosely packed grass (approximately 150 g) in sterile polythene bags within an anaerobic cabinet; comparison was made between the fermentation characteristics of these and the more conventional laboratory silos (250 ml measuring cylinders) over a 30 d time course.

In addition, a system in which the chemical composition and the micro-flora could be controlled or standardised would be useful for the evaluation of silage additives and for studying the importance of different micro-organisms during ensilage in series of experiments. Controlled-environment chambers could be used to produce crops which are harvested at a standard growth stage and chemical composition and a standard micro-flora could be obtained by sterilising the crop at harvest by gamma irradiation and inoculating with the required micro-flora (Gouett *et al.*,1972). The use of a cellular matrix impregnated with a nutrient solution and inoculated with silage micro-organisms was reported by Woolford and Wilkins (1975). Alternatively, and more simply, a large quantity of crop could be harvested on one occasion and then frozen until required for ensiling on a laboratory scale. Experiment 6b was designed to determine the effects of freezing and thawing on the suitability of a sample of grass for ensilage.

The microbiological data are expressed on a DM basis to allow comparison between silages of different DM content.

Materials and methods **Experiment 6a** Grass (*L. perenne*) was harvested in late June (1991) and ensiled in 250 ml polypropylene measuring cylinders and in sterile polythene bags (18x31cm) (Seward Medical) inside an anaerobic cabinet under an atmosphere of 95% CO_2/5% H_2. Triplicate silos were opened on days 0, 1, 2, 3, 4, 5, 7, 10, 15, 20 and 30 and microbial numbers and activity assayed immediately; the forage was subsequently frozen and stored at -20°C before chemical analysis. Excess grass from the harvest was frozen and stored at -20°C, as described in Chapter 2, for use in Experiment 6b.

Experiment 6b Grass (*L. perenne*), harvested in late June (1991) and stored at -20°C for 9 months, was thawed and ensiled in sterile polythene bags within an anaerobic cabinet. Triplicate bags were removed on days 0, 1, 2, 3, 4, 5, 7, 10, 15, 20 and 30 and the microbial numbers and activity assayed immediately; the forage was stored at -20°C for subsequent chemical analysis.

Statistical analysis was by one-way analysis of variance (MINITAB).

Results **Experiment 6a** The grass was from a high sugar/low CP summer crop (Table 4.15a, b) and was well preserved in measuring cylinders and polythene bags, with low concentrations of ammonia and butyric acid after 30 d. There were significant, though small, differences in chemical composition between the two silages (Table 4.16).

Table 4.15a Chemical composition of the grass ensiled in polypropylene measuring cylinders and in polythene bags within an anaerobic cabinet (g/kg DM, unless stated otherwise) (Experiment 6a).

pH	5.73
Dry matter (g/kg)	216
Crude protein	110
Ammonia-nitrogen	1
Water soluble carbohydrates	163

Table 4.15b Microbial populations on the grass ensiled in polypropylene measuring cylinders and in polythene bags within an anaerobic cabinet (CFU/g DM) (Experiment 6a).

Total viable organisms	$12x10^6$
Lactic acid bacteria	$< 10^2$
Coliform bacteria	$62x10^4$
Yeasts	$< 10^2$

Table 4.16 Chemical composition (g/kg DM, unless stated otherwise) of 30-d-old silage prepared from fresh grass ensiled in 250 ml polypropylene measuring cylinders and in sterile polythene bags within an anaerobic cabinet (Experiment 6a) and from thawed grass ensiled in sterile polythene bags within an anaerobic cabinet (Experiment 6b).

	Fresh grass ensiled in polypropylene measuring cylinders (control)	Fresh grass ensiled in polythene bags within an anaerobic cabinet (Experiment 6a)	SED(1)[1] (n=62)		Thawed grass ensiled in polythene bags within an anaerobic cabinet (Experiment 6b)	SED(2)[1] (n=53)	
pH	3.56	3.59	0.03		3.65	0.03	
Dry matter (g/kg)	169	191	7.2	**	207	8.9	
Ammonia-N	3	2	0.1	***	1	0.2	***
Lactic acid	152	139	7.2		93	12.0	***
WSC	68	101	7.7	***	51	8.4	***
Ethanol	15	5	1.0	***	6	0.8	
Acetic acid	12	7	1.9	*	12	2.5	
Propionic acid	1	2	0.7		1	0.7	

[1] Comparison between the control and polythene bag silos in Experiment 6a were made using SED(1) and comparisons between the polythene bags in Experiments 6a and 6b were made using SED(2). SED(2) is the higher of two values derived from unbalanced sets of data. Asterisks accompanying SED values denote statistical differences between treatments :- *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

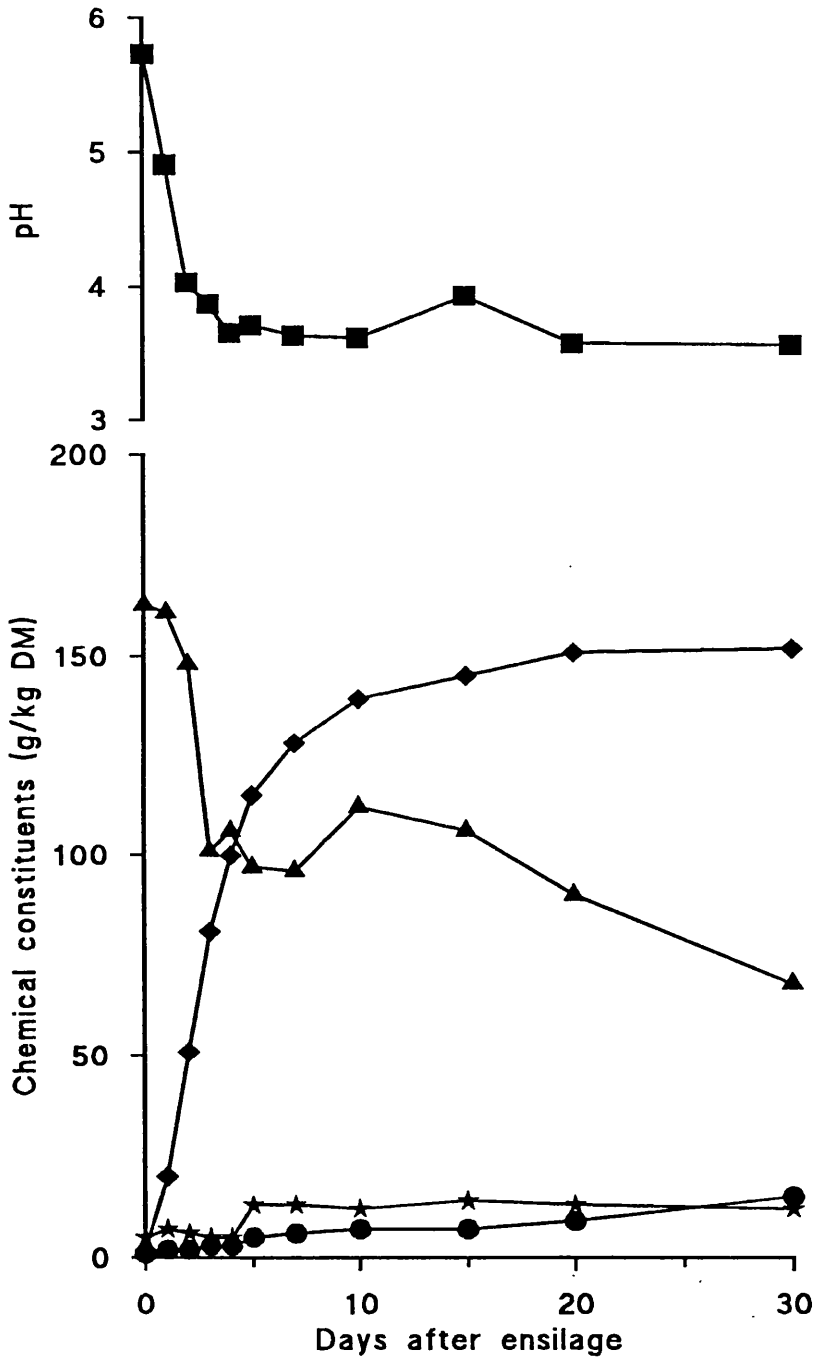


Figure 4.14a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of "fresh" perennial ryegrass in polypropylene measuring cylinders (Experiment 6a).

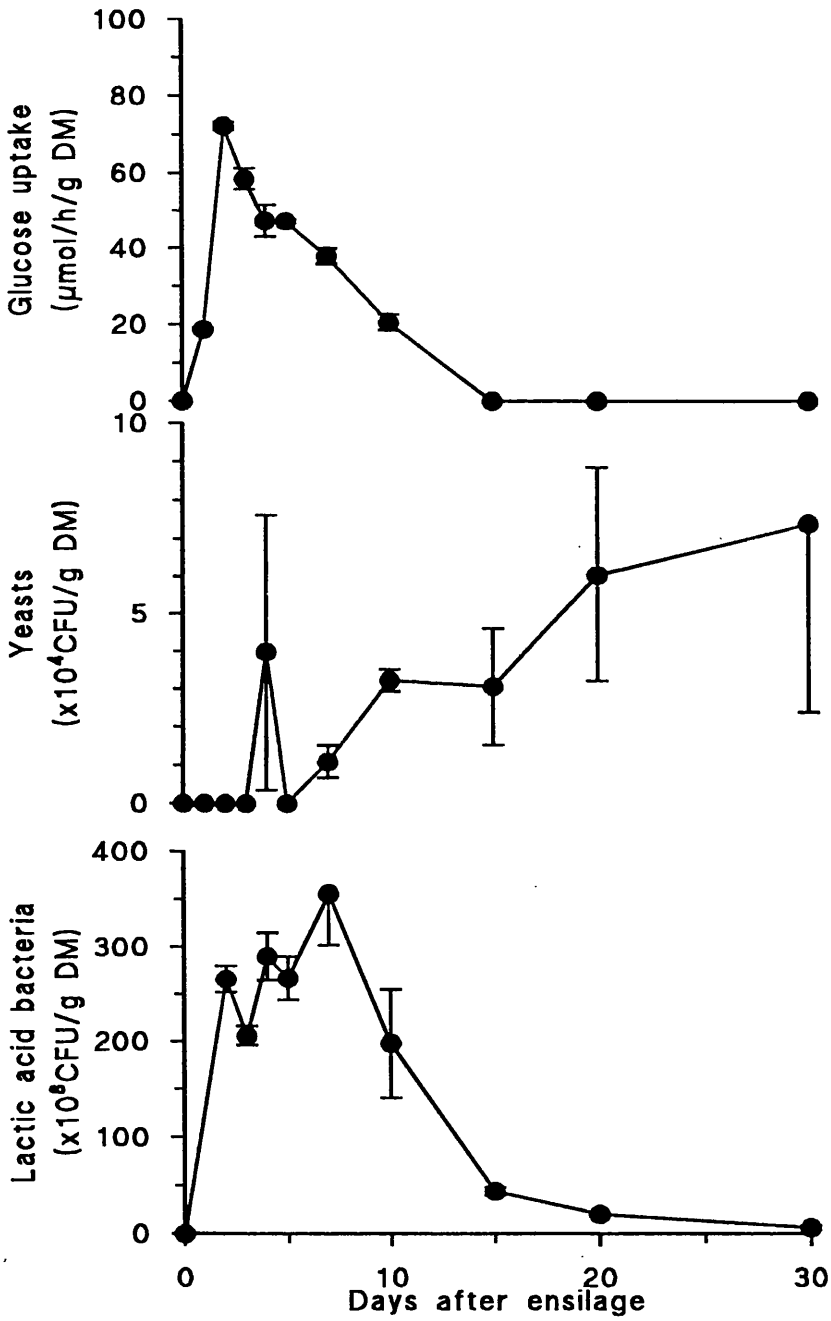


Figure 4.14b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (μmol/h/g DM) during ensilage of "fresh" perennial ryegrass in polypropylene measuring cylinders (Experiment 6a).

Error bars represent SE of triplicate silages.

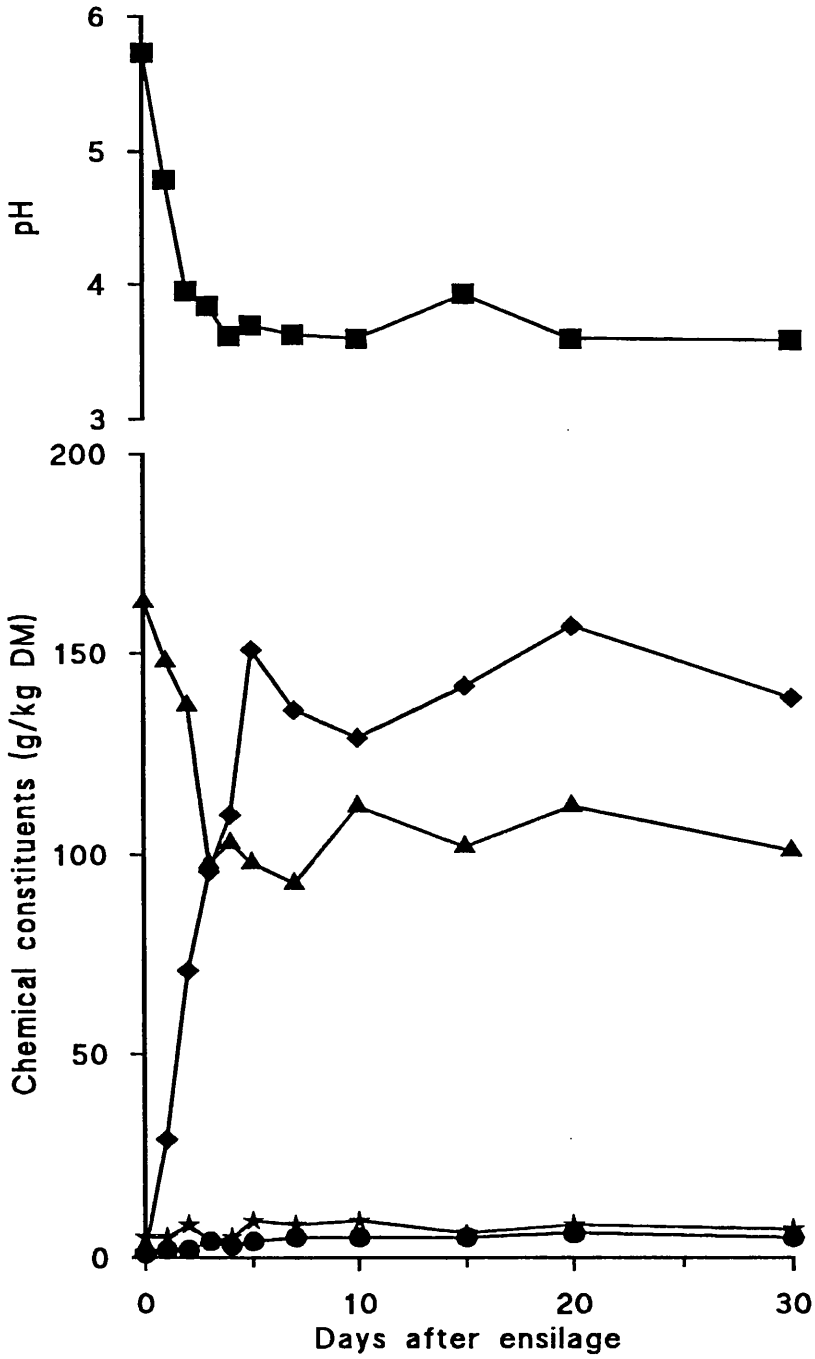


Figure 4.15a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of "fresh" perennial ryegrass in polythene bags inside the anaerobic cabinet (Experiment 6a).

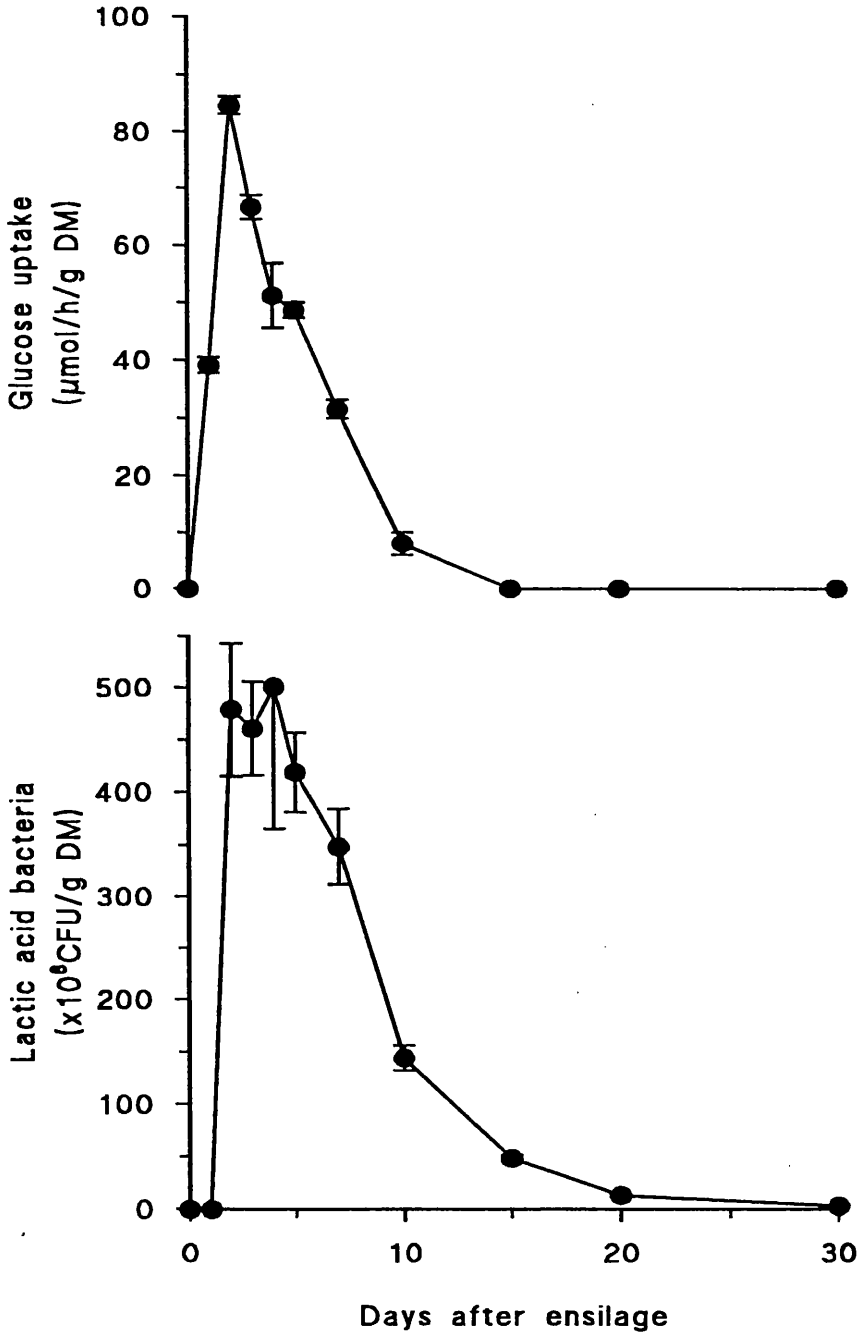


Figure 4.15b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during ensilage of "fresh" perennial ryegrass in polythene bags within an anaerobic cabinet (Experiment 6a).
Error bars represent SE of triplicate silages.

Table 4.17a Chemical composition of the fresh and thawed grass (g/kg DM, unless stated otherwise) (Experiment 6b).

	Fresh	**Thawed**
pH	5.73	5.59
Dry matter (g/kg)	216	192
Crude protein	110	127
Ammonia	1	1
Water soluble carbohydrates	163	179

Table 4.17b Microbial populations on the fresh and thawed grass (CFU/g DM) (Experiment 6b).

	Fresh	**Thawed**
Total viable organisms	$12x10^6$	$20x10^6$
Lactic acid bacteria	$< 10^2$	$67x10^3$
Coliform bacteria	$62x10^4$	$60x10^3$
Yeasts	$< 10^2$	$< 10^2$

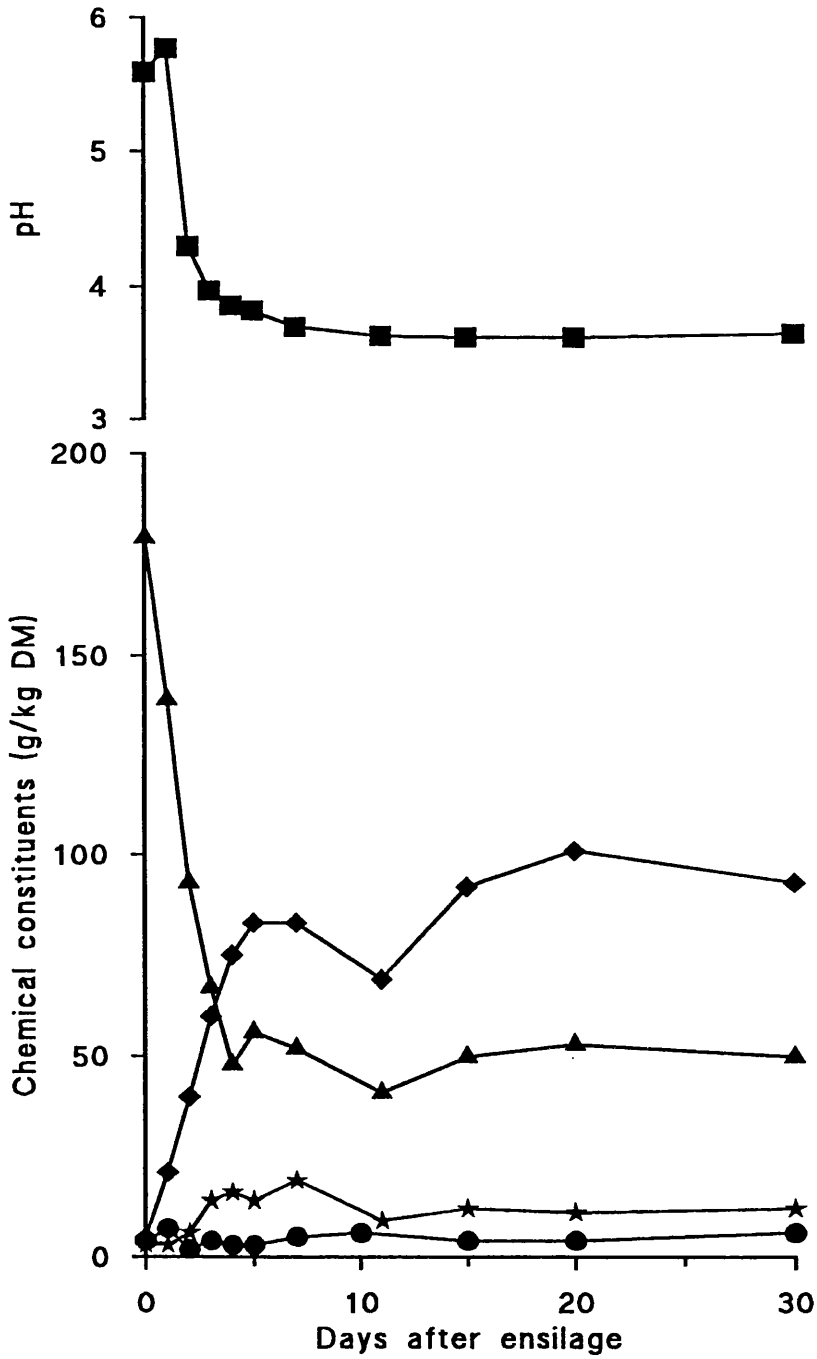


Figure 4.16a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass in polythene bags within an anaerobic cabinet (Experiment 6b).

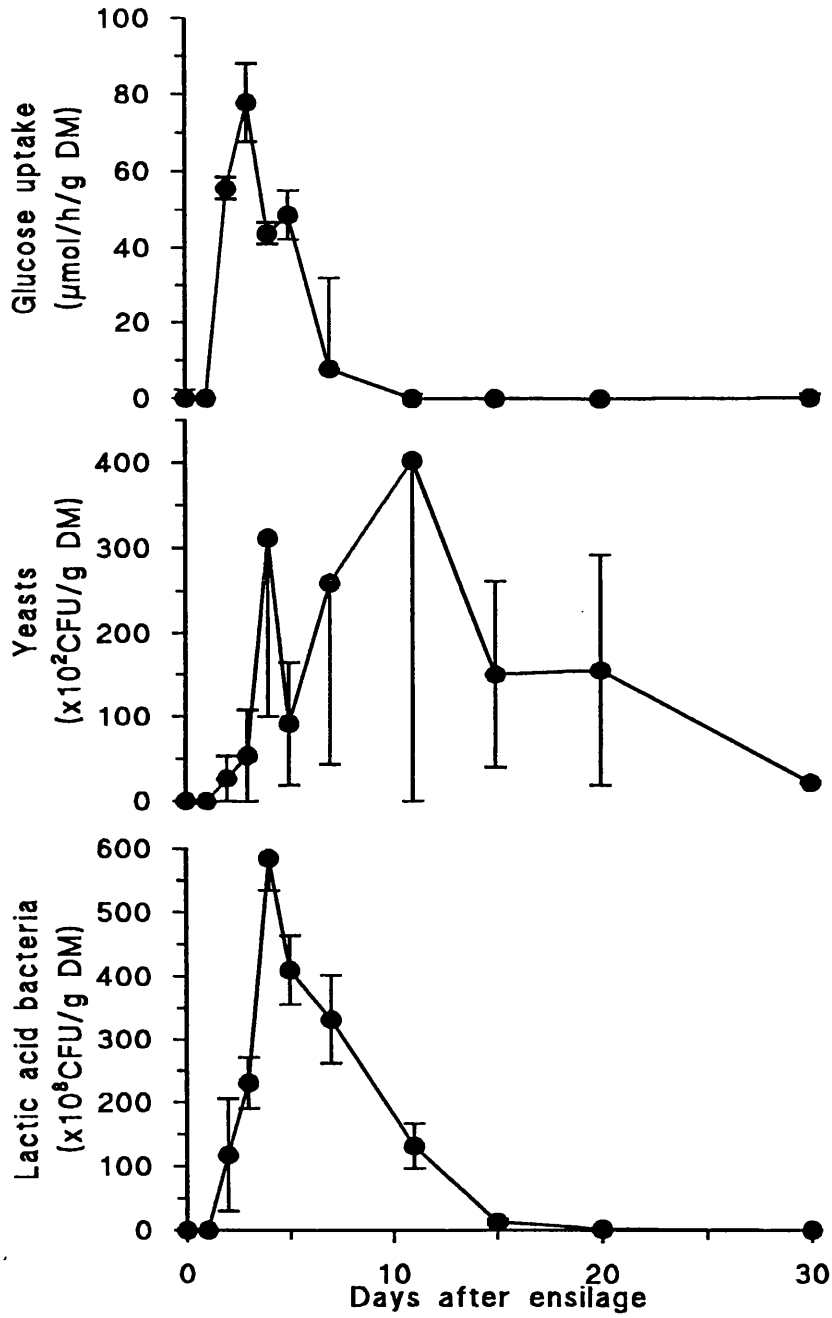


Figure 4.16b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during ensilage of thawed perennial ryegrass in polythene bags within an anaerobic cabinet (Experiment 6b).
Error bars represent SE of triplicate silages.

The grass ensiled in polythene bag silos underwent a more rapid fermentation (Figures 4.14a and 4.15a) reflected by an early proliferation of lactic acid bacteria and higher microbial activity (84.7 and 72.3 μmol glucose utilisation/h/g DM on day 2 for polythene bags and measuring cylinders respectively) (Figures 4.14b and 4.15b). This was associated with lower concentrations of acetic acid, ammonia and ethanol and higher residual WSC after 30 d.

Irrespective of the type of silo, within 48 h lactic acid bacteria predominated and coliform bacteria were inhibited by day 4, despite high numbers on the grass (Table 4.15b) and rapid multiplication during the first 24 h. Moulds were detected on day 7 ($44\pm21.3x10^2$ CFU/g DM) and day 10 ($90\pm14.6x10^2$CFU/g DM) in measuring cylinder silos. Yeast (undetected in the polythene bag silos) were probably responsible for the accumulation of ethanol in the later stages of ensilage in the measuring cylinders.

Experiment 6b Freezing and thawing did not greatly affect the chemical composition of the grass or the epiphytic microbial populations on the crop (Table 4.17a, b), and, despite small differences between the two silages (Table 4.16), the patterns of fermentation were similar (Figures 4.15a, b and 4.16a, b). The fresh grass underwent a more rapid fermentation, with greater lactic acid production and a faster drop in pH during the first 5 d reflected by higher numbers of lactic acid bacteria ($48x10^9$ and $12x10^9$ CFU/g DM, after 2 d, fresh and thawed material respectively) (Figure 4.16b), and higher levels of glucose uptake *in vitro* (84.7 and 55.7 μmol/h/g DM after 2 d, fresh and thawed material respectively); the pH of the thawed material did not fall until 2 d after ensilage and this was associated with a greater accumulation of acetic acid and an overall "lag" in the fermentation process.

Higher numbers of coliform bacteria on the fermenting fresh grass ($31x10^8$ CFU/g DM at day 1) may have contributed to the slightly higher concentrations of ammonia, but the presence of yeasts on the fermenting thawed material was not associated with greater ethanol production.

Discussion In Experiment 6a, the earlier and more comprehensive exclusion of air and establishment of anaerobic conditions in the polythene bag silos within the anaerobic cabinet deterred the activities of undesirable epiphytic micro-organisms and ensured the proliferation of homofermentative lactic acid bacteria, resulting in a faster drop in pH and a more efficient fermentation and preservation of a higher concentration of WSC. There were higher numbers of yeasts and higher ethanol concentrations in the silage prepared in the measuring cylinder silos, perhaps a consequence of the less stringent maintenance of anaerobiosis. Similarly, the growth of moulds in the control silage on days 7 and 10 may result from the incursion of

air. In Experiment 6b, freezing and thawing did not adversely affect the chemical composition of the grass or the epiphytic microflora on the grass and consequently the fermentation pattern was not different from that of the fresh forage. The "lag" preceding the initiation of fermentation of the thawed grass may have allowed the proliferation of undesirable micro-organisms, such as species of heterofermentative lactic acid bacteria, and resulted in the early accumulation of acetic acid.

Grass (150g) loosely filled into sterile polythene bags within the anaerobic cabinet proved to be a satisfactory technique for producing small volumes of experimental silage, and grass stored at -20°C may be confidently used for a series of experiments to study the action of a range of manipulative treatments.

Conclusions It is clear from the time-course experiments described in this chapter that the fermentation is most intense during the first days of ensilage, with virtually all the WSC expended and the pH lowered within 4 d. The proliferation of lactic acid bacteria ensured a rapid drop in pH which deterred competition and maintained the stability of the silage. Control during the first hours of ensilage is crucial to ensure a homolactic fermentation and the efficient conversion of sugars to lactic acid. Although fresh and thawed grass underwent similar patterns of fermentation, there were small differences in the chemical composition after 30 d, probably a result of the early proliferation of heterofermentative lactic acid bacteria during the apparent "lag"; perhaps the epiphytic homolactic micro-flora require more opportunity for recovery after a period of storage at -20°C. This deficiency may be overcome by inoculation with a suitable concentration of homofermentative lactic acid bacteria.

The fermentation of forages prepared in measuring cylinder silos, in Experiments 4 and 6, were associated with higher concentrations of acetic acid than the forages ensiled in sterile polythene bags within the anaerobic cabinet. The significant differences in fermentation characteristics, small though they were, may be due to the ingress of small volumes of air, possibly encouraging different species of lactic acid bacteria, although there are no data to support this. This raises the question whether the fermentation, and the effect of silage additives, inside the anaerobic cabinet bears any relationship at all to real silage making conditions, where complete anaerobiosis is rarely achieved. However, the anaerobic cabinet affords a set of controlled conditions under which specific effects of silage additives on the micro-flora and chemical composition can be examined.

Enumeration of micro-organisms provides some useful data during the early stages of ensilage, when the rapid fermentation is reflected by the high numbers of microbes.

However, in the later stages, when the chemical composition is relatively stable, it is not sufficient to rely on microbial numbers as a measure of fermentative activity within the silo. The measurement of glucose utilisation *in vitro* by an inoculum prepared from the silage gives a clearer indication of the fermentative activity *in situ*. Other metabolic parameters could be measured to assess microbial activity. For instance, fructose is fermented more rapidly than glucose *in vitro* (Experiment 3) and would probably be well suited as a substrate. In Experiment 4, where the later stages of ensilage were characterised by a catabolism of lactic acid, the activity of the silage micro-organisms may be better reflected by the utilisation of lactic acid *in vitro*. Alternatively, the accumulation of end-products (ethanol, carbon dioxide as well as lactic acid) may be assayed. Measurement of residual glucose concentration in the supernatant after incubation is quick and relatively cheap and, in most cases, the utilisation of glucose reflects the metabolic status of silage micro-organisms.

The assay of glucose uptake *in vitro* identified higher residual microbial activity in the poorly preserved, high pH-silage in Experiment 4, where lactic acid was extensively metabolised to acetic acid in the later stages of ensilage, than in the silage in Experiment 6, where there was no secondary fermentation. Despite a predominantly homolactic fermentation, insufficiency of WSC in Experiment 4 led to a poorly preserved silage, while the grass ensiled in Experiment 6 had higher concentrations of WSC which ensured high concentrations of lactic acid and a low pH.

Coliform bacteria were deterred within the early stages of ensilage of all the experiments reported here, and so it is difficult to differentiate inhibition by lactic acid bacteria, concentration of lactic acid or pH. However, the high numbers in the later stages of ensilage in Experiment 4 may be a consequence of the lactate degradation and rise in pH. A proliferation of yeasts in conditions of high substrate availability was anticipated, but, although there was reasonably high preservation of WSC in the silages in Experiment 6, there were only low numbers of yeasts throughout. More work is needed to clarify the conditions conducive to maintenance of stability of the silage or selection for, and survival of, undesirable epiphytic micro-organisms and spoilage of apparently well preserved silage.

In Chapter 5 the ensilage of thawed grass from a single harvest was manipulated using a range of silage additives including fermentation stimulants, fermentation inhibitors and extra substrates added during ensilage to simulate sugar release by fibrolytic enzymes. The fermentations were followed over 60 d and the rate of change of chemical composition and microbial populations and activities were measured and compared.

5. MANIPULATION OF THE FERMENTATION BY SILAGE ADDITIVES

In the experiments in this chapter the *in vitro* assay of glucose utilisation was used, in conjunction with microbial enumeration and analysis of chemical composition, to clarify the mechanisms underlying the effects of a range of silage additives. The data is expressed on a DM-basis to allow interpretation between silages of different DM content.

Thawed grass from a single harvest was ensiled in sterile polythene bags after additive treatment, in Experiment 1, with organic acids (formic and lactic acid), a mineral acid (sulphuric acid) and the sodium salt of lactic acid and, in Experiment 2, with an inoculant containing *L. plantarum* (Ecosyl, ICI plc) with added sucrose and sodium bicarbonate, and a high level of addition of a commercial cell wall-degrading preparation (Clampzyme). In Experiment 3, glucose, fructose and xylose were added to pre-fermented material (30-d-old silage), within an anaerobic cabinet, to simulate conditions resulting from the release of fermentable carbohydrates from the cell walls by the action of fibrolytic enzymes.

Experiment 1 The effect of formic acid, sulphuric acid, lactic acid and sodium lactate on the ensilage process

Introduction Experiment 1 was performed to clarify the effect of a low and a high rate of addition of formic acid, a high rate of addition of sulphuric acid and equimolar amounts of lactic acid and sodium lactate on silage micro-organisms.

The inhibition of fermentation by high levels of organic acids was shown in Chapter 3, and previously by others (Chamberlain and Quig, 1987). Satisfactory and reproducible control is, however, more difficult to achieve with lower levels of addition (Chamberlain and Quig, 1987). The buffering capacity of the crop is thought to play an important role in dictating the level of application of the acids necessary to control the fermentation, but this relationship, as well as the effect of acids on silage micro-organisms, is poorly defined.

Whereas formic acid is thought to affect the silage fermentation with its antimicrobial properties, sulphuric acid is used to reduce the pH to inhibit the microbial fermentation. The rate of application of sulphuric acid provided the same normality as the high level of addition of formic acid. Since the mechanisms whereby lactic acid, dissociated or undissociated, controls fermentation are still poorly understood, the effects of lactic acid and sodium lactate were examined in Experiment 1 in an attempt to clarify the preservative role of lactic acid. In a preliminary trial, titrating a homogeneous preparation of grass with lactic acid, it was

found that 0.07 mmol lactic acid/g FW reduced the pH from approximately 6.0 to 4.2. This level of addition was thought to be sufficient to reduce the pH but not to inhibit activity, simulating a partly fermented silage, and the rates of application of sodium lactate and lactic acid for ensilage were each equivalent to 0.07 mmol lactate/g grass.

Materials and methods Thawed grass (*L.perenne*), harvested and frozen for up to 9 months, was ensiled in 33 sterile polythene bags (150 g/bag) inside the anaerobic cabinet following treatment of the grass with a) 2.3 l/t formic acid (850 g/l), b) 6 l/t formic acid (850 g/l), c) 6 l/t sulphuric acid (450 g/l), d) 6.31 kg/t lactic acid and e) 7.85 kg/t sodium lactate. A control (untreated grass) was ensiled similarly. Triplicate silos were removed from the cabinet on days 0, 1, 2, 3, 4, 5, 7, 10, 15, 20, 40 and 60 after ensilage and prepared for microbial enumeration and assay of fermentative activity, as described in Chapter 2. The remaining sub-sampled silage was frozen and stored at -20^{0}C before chemical analysis.

As in Chapter 3, the expected proportions of metabolites were calculated, assuming that the acetic acid in the control silage (mean 20-60 d) was derived exclusively from fermentation of pentose sugars released by cell-wall degradation, and that the WSC content of the grass comprised 0.50 glucose and 0.50 fructose, i.e. assuming a substrate mixture of 0.41 glucose : 0.41 fructose : 0.18 pentose. Theoretical and actual proportions of metabolites formed during ensilage were compared, except where a secondary fermentation was identified, to derive more information regarding the fermentation pathways; since too much emphasis cannot be placed on the data for single timed-sample analyses, to derive a more representative estimate of the effect of the additives on the composition of the silages the concentration of the chemical constituents of the last three sub-samples (20-60 d) were meaned.

Statistical analysis was by one-way analysis of variance, using MINITAB.

Results The grass ensiled for the control was a high sugar/low CP crop, harvested in June and stored at -20^{0}C, as described in Chapter 4 (Table 4.17).

The chemical composition and the microbial constitution of the grasses ensiled after treatment is shown in Table 5.1 and the changes in the chemical composition and the microbial populations during ensilage are shown in Figures 5.1-5.6 and Table 5.2.

Control The control silage was well preserved with a low pH (lower than 4.00 after 7d), low concentrations of ammonia and negligible butyric acid, and contained 0.23 of the WSC content of the original forage (mean 20-60 d). Lactic acid accumulated rapidly (more than 94

g/kg DM after 4 d) (Figure 5.1a), and, despite some acetic acid production (16 g/kg DM after 2 d), the proportion of metabolites produced throughout ensilage reveals a predominantly homolactic fermentation (Table 5.3).

In vitro glucose uptake was maximal after 2 d (66.7 μmol/h/g DM) (Figure 5.1b), falling 82% to 11.9 μmol/h/g DM after 20 d ensilage, and after 60 d low levels of fermentative activity remained (31.4 μmol/h/g DM). Lactic acid bacteria increased to $32x10^9$ CFU/g DM by day 3 (Figure 5.1b) and dominated the subsequent fermentation ($22x10^7$ after 60 d) (Table 5.2). Coliform bacteria were deterred from the silage after 3 d, following multiplication from $157x10^2$ to $276x10^3$ CFU/g DM within the first 48 h. Yeasts were identified after 20 d ensilage (Figure 5.1b).

Formic acid (2.3 l/t) Treatment of the grass with 2.3 l/t formic acid effected a well preserved silage after a more extended fermentation than the control silage (Figure 5.2a). Addition of the acid reduced the pH initially to 4.90 and lactic acid production (26 g/kg DM after 4 d ensilage) reduced the pH to less than 4 by 15 d. WSC were fermented throughout the period of storage, associated with more acetic acid production in the later stages of ensilage. Comparison of the proportions of end-products of metabolism with the theoretical proportions (Table 5.3) reveals a more heterolactic fermentation than the control silage.

Lactic acid bacteria multiplied slowly to $103x10^8$ CFU/g DM after 10 d (Figure 5.2b), and subsequently decreased to $209x10^6$ CFU/g DM after 60 d, and glucose uptake *in vitro* was restricted throughout the period of ensilage (maximal after 4 d, 44.8 μmol glucose utilised/h/g DM).

Coliform bacteria ($178x10^3$ CFU/g DM at day 0) declined steadily and were deterred by 4d. Although yeasts were identified between day 2 ($120x10^2$ CFU/g DM) and day 10 ($21x10^5$ CFU/g DM), these did not apparently contribute to the fermentation.

Formic acid (6 l/t) Addition of 6 l/t formic acid preserved 0.81 of the WSC present in the original forage. Lactic acid concentrations were low (8 g/kg DM, mean 20-60d) and negligible amounts of acetic acid and ammonia accumulated. The concentration of WSC fluctuated quite markedly (Figure 5.3a), but this was not reflected in other changes in the chemical composition of the silage and no explanation was apparent. The inhibition of fermentation was reflected by the low rates of glucose uptake *in vitro* (maximum 24.7 μmol/h/g DM on day 4, Figure 5.3b) and low numbers of lactic acid bacteria (maximum $14x10^8$ CFU/g DM on day 10, Figure 5.3b). Coliform bacteria were deterred after 2 d, and yeasts were not present throughout the period of storage.

Table 5.1a Chemical composition of the grasses after treatment (g/kg DM, unless stated otherwise)(Experiment 1).

	pH	Dry matter (g/kg)	Ammonia	Water soluble carbohydrate	Lactic acid
Control	6.17	213	1	191	7
2.3 l/t formic acid	4.90	192	2	84	8
6 l/t formic acid	3.91	214	1	192	5
6 l/t sulphuric acid	3.47	235	1	152	0
Lactic acid	4.97	178	2	105	29
Sodium lactate	5.93	175	2	110	44

Table 5.1b Microbial populations on the forage ensiled (CFU/g DM) (Experiment 1).

	Total viable organisms	Lactic acid bacteria	Coliform bacteria	Yeasts
Control	$64x10^6$	$15x10^4$	$157x10^2$	$<10^2$
2.3 l/t formic acid	$255x10^6$	$11x10^4$	$178x10^3$	$<10^2$
6 l/t formic acid	$48x10^6$	$19x10^4$	$129x10^2$	$<10^2$
6 l/t sulphuric acid	$11x10^6$	$64x10^2$	$13x10^2$	$<10^2$
Lactic acid	$14x10^6$	$14x10^4$	$<10^2$	$<10^2$
Sodium lactate	$23x10^6$	$24x10^4$	$<10^2$	$<10^2$

Table 5.2a Chemical composition of the silages after 60 d (g/kg DM, unless stated otherwise) (Experiment 1).

	pH	Dry matter (g/kg)	Ammonia	Lactic acid	Water soluble carbohydrate	Ethanol	Acetic acid	Butyric acid
Control	3.65	232	1	88	37	2	15	tr.[1]
2.3 l/t formic acid	3.78	181	3	87	5	2	15	tr.
6 l/t formic acid	3.73	234	1	9	169	1	2	1
6 l/t sulphuric acid	3.31	212	1	55	26	3	5	tr.
Lactic acid	4.16	159	5	16	3	7	40	2
Sodium lactate	5.28	163	8	17	0	7	46	29
SED	0.08	5.7	0.4	10.1	12.7	2.9	4.1	2.7
(n=182)	***	***	***	***	***	*	***	***

The SED value quoted is the higher of two derived from unbalanced sets of data. Statistically significant differences are:- *, $P<0.05$ and ***, $P<0.001$.
[1] tr. denotes trace amounts detected.

Table 5.2b Microbial populations on the silage after 60 d (CFU/g DM) (Experiment 1).

	Total viable organisms	Lactic acid bacteria	Coliform bacteria	Yeasts
Control	$49x10^6$	$224x10^6$	$<10^2$	$64x10^4$
2.3 l/t formic acid	$21x10^6$	$209x10^4$	$<10^2$	$<10^2$
6 l/t formic acid	$2x10^6$	$1x10^6$	$<10^2$	$<10^2$
6 l/t sulphuric acid	$14x10^4$	$510x10^6$	$<10^2$	$67x10^2$
Lactic acid	$177x10^6$	$236x10^8$	$<10^2$	$<10^2$
Sodium lactate	$918x10^6$	$121x10^8$	$<10^2$	$<10^2$

Table 5.3 Proportion of major fermentation products formed during ensilage (Experiment 1).

	Lactic acid	Acetic acid	Ethanol	Total metabolites (g/kg DM)
Control	0.84	0.14	0.02	115
2.3 l/t Formic acid	0.77	0.21	0.02	82
6 l/t Formic acid	0.80	0.10	0.10	10
6 l/t Sulphuric acid	0.91	0.05	0.04	76
Lactic acid	0.33	0.56	0.12	52
Sodium lactate	0.21	0.38	0.06	82

Theoretical ratio of metabolites:-

Heterolactic fermentation 0.50 lactic acid : 0.25 acetic acid : 0.25 ethanol

Homolactic fermentation 0.91 lactic acid : 0.09 acetic acid

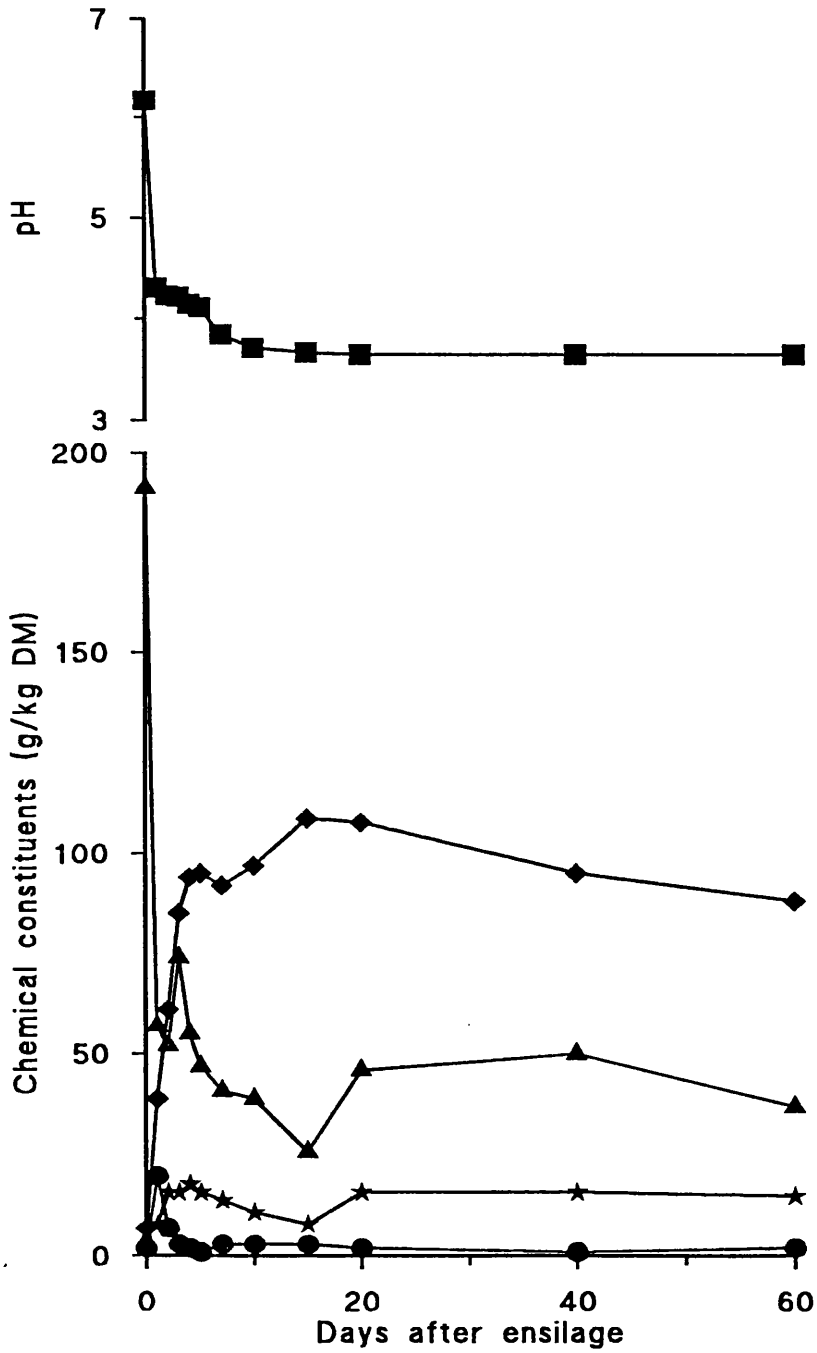


Figure 5.1a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, without additive, in polythene bags within an anaerobic cabinet.

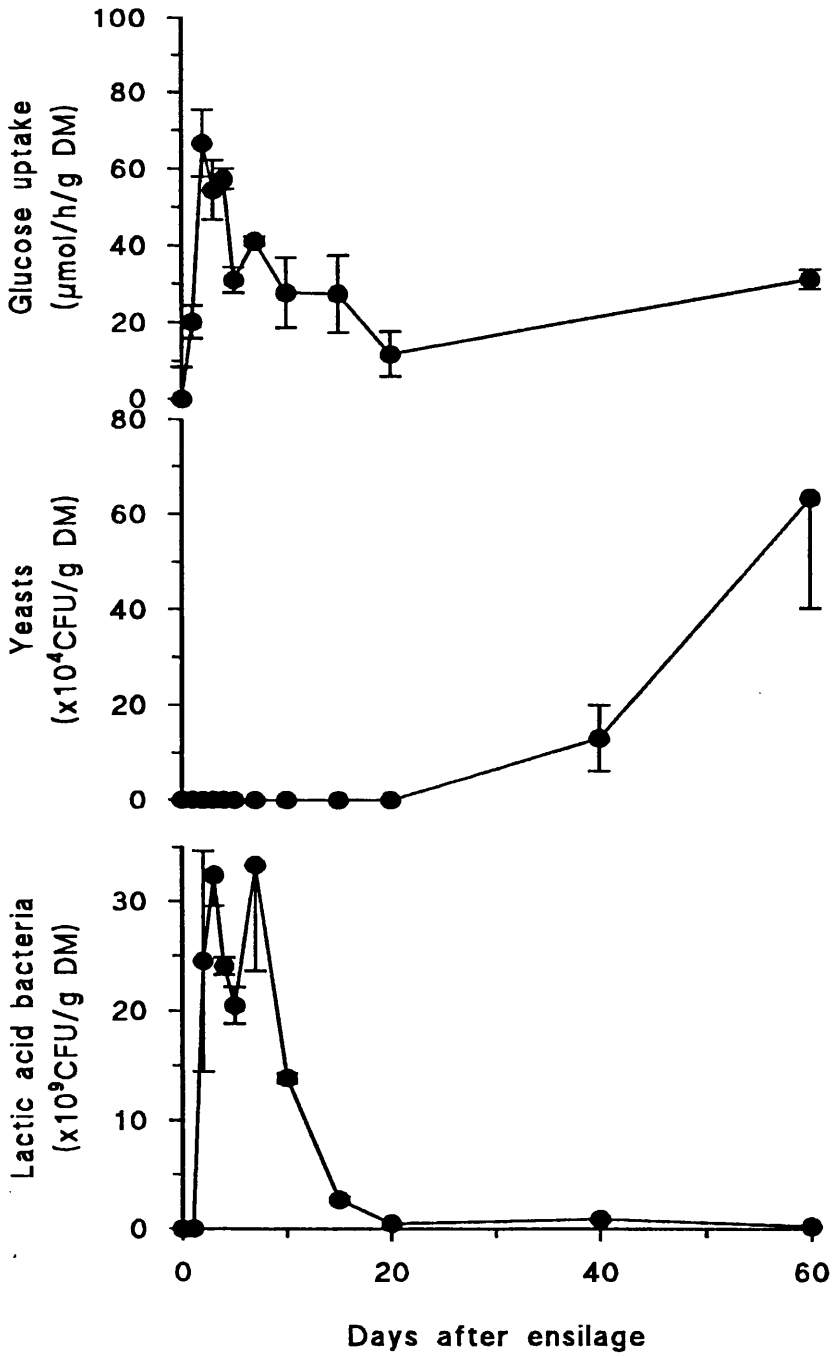


Figure 5.1b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (μmol/h/g DM) during ensilage of thawed perennial ryegrass, without additive, in polythene bags within an anaerobic cabinet.

Error bars represent SE of triplicate silages.

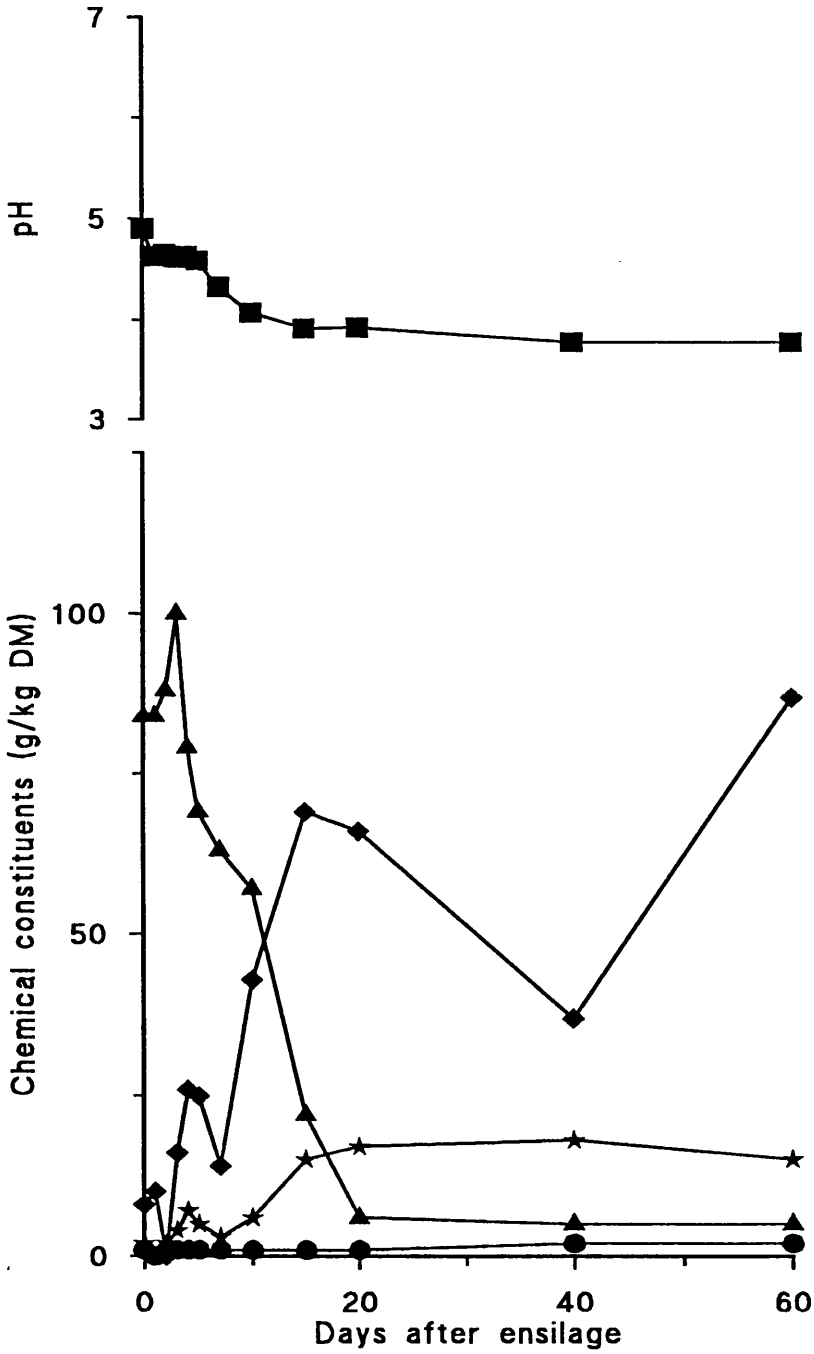


Figure 5.2a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, with 2.3 l/t formic acid, in polythene bags within an anaerobic cabinet.

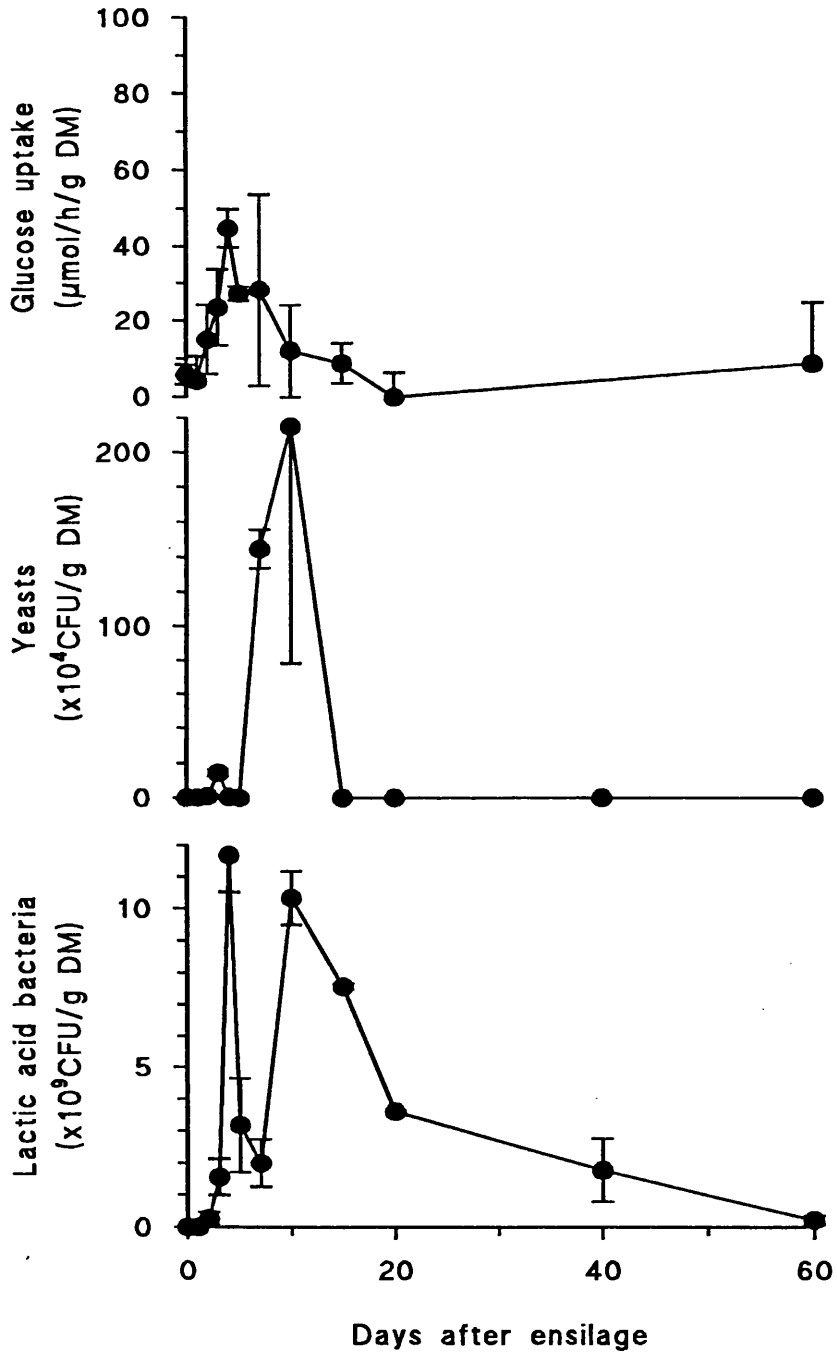


Figure 5.2b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during ensilage of thawed perennial ryegrass, with 2.3 l/t formic acid, in polythene bags within an anaerobic cabinet.
Error bars represent SE of triplicate silages.

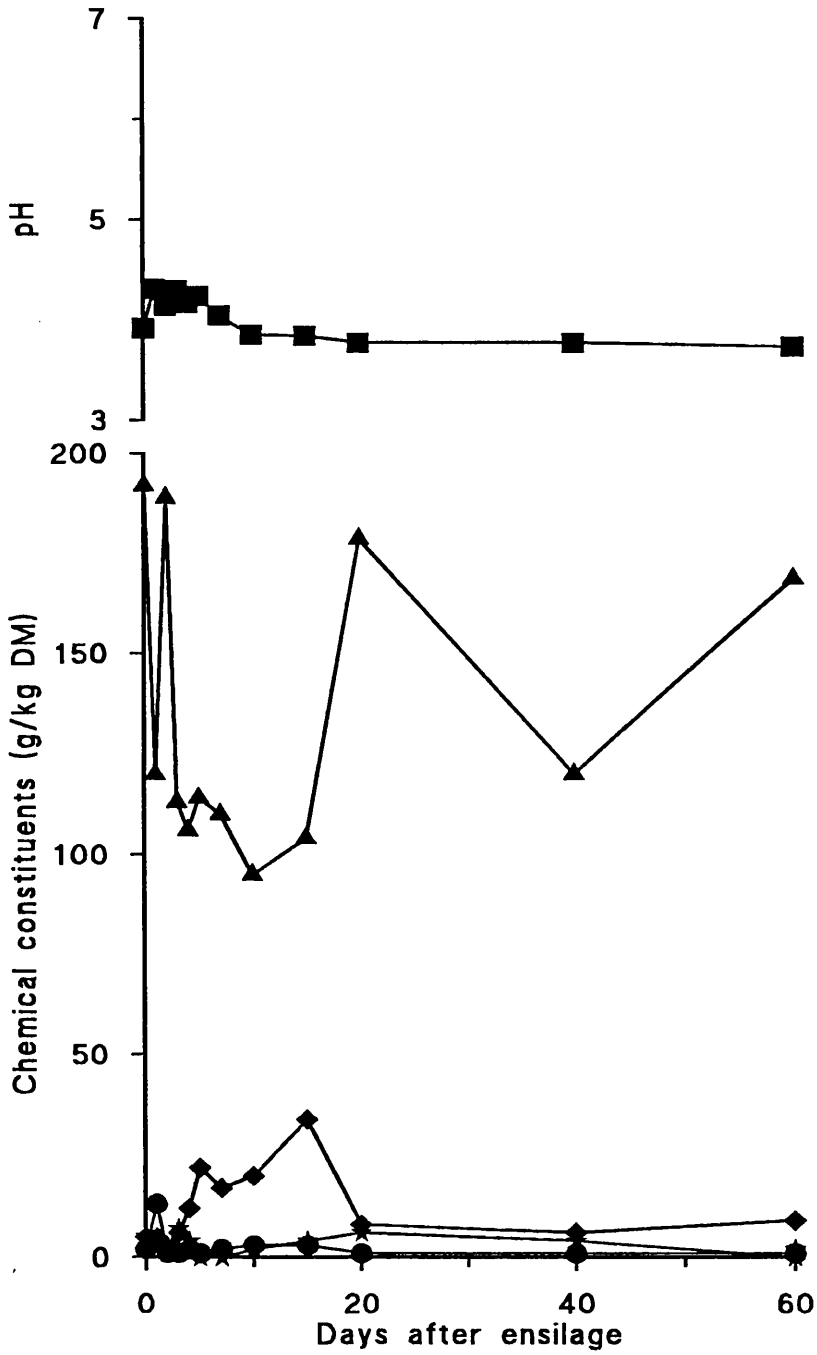


Figure 5.3a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, with 6 l/t formic acid, in polythene bags within an anaerobic cabinet.

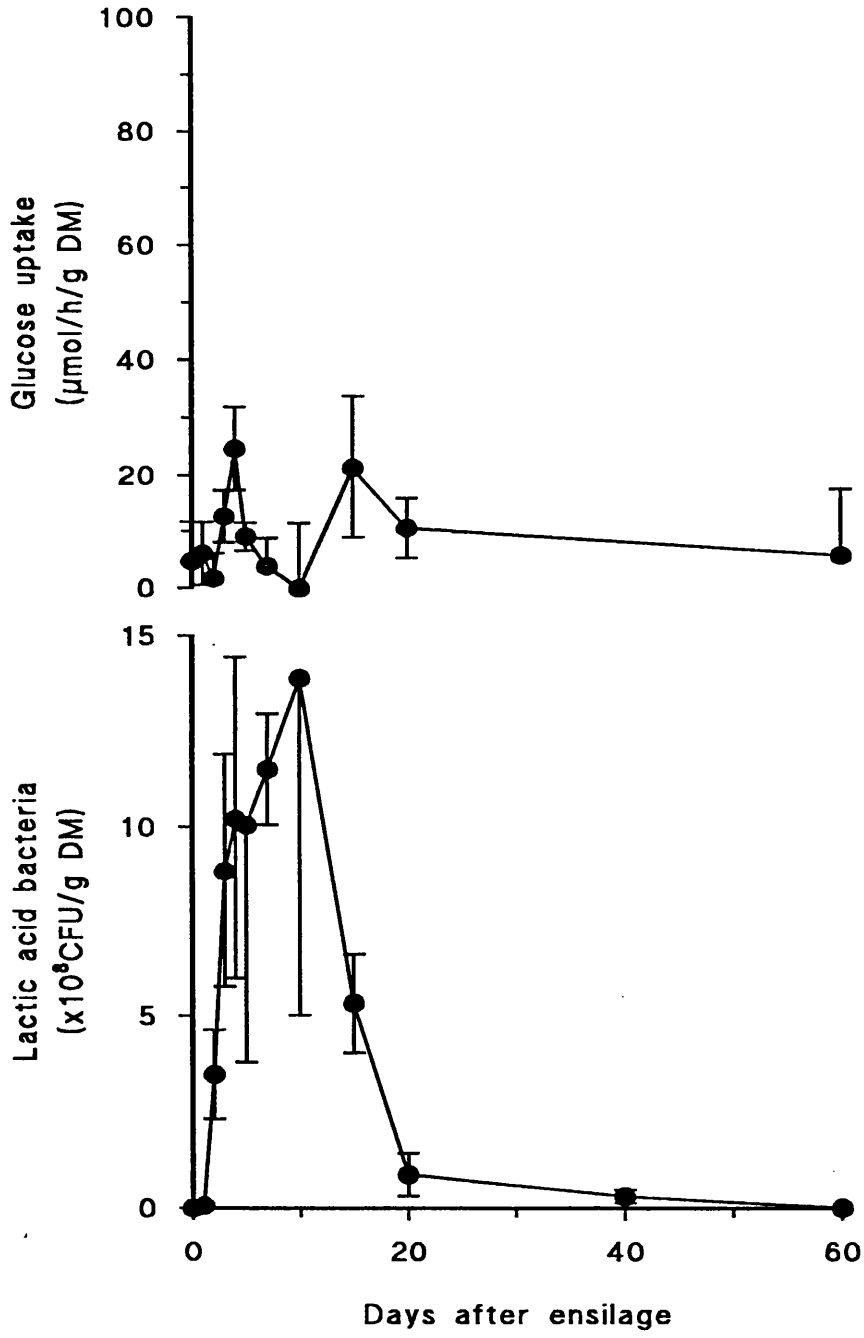


Figure 5.3b Changes in the numbers of lactic acid bacteria (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during ensilage of thawed perennial ryegrass, with 6 l/t formic acid, in polythene bags within an anaerobic cabinet.

Error bars represent SE of triplicate silages.

Sulphuric acid (6 l/t) A high level of sulphuric acid reduced the pH of the grass to 3.47 and restricted the fermentation in the early stages of ensilage; WSC were fermented slowly to lactic acid (57 g/kg DM after 10 d, and 69 g/kg DM mean 20-60 d), low concentrations of acetic acid and negligible ethanol. Although the proportions of metabolites suggest an exclusively homolactic fermentation (Table 5.3), there was loss of WSC in the later stages of fermentation not accounted for by measured end-products (Table 5.2a).

Maximum glucose uptake *in vitro* was higher than the control (70.4 μmol/h/g DM, Figure 5.4b), although not until 5 d after ensilage, when lactic acid bacteria numbers were high ($203x10^8$ CFU/g DM). Although yeasts were identified in high numbers ($24x10^6$ CFU/g DM on day 15) their presence was not reflected by increased concentrations of ethanol. Coliform bacteria did not multiply and disappeared after 3 d.

Lactic acid (6.31 kg/t) Treatment of the forage with lactic acid (equivalent to 0.07 mol/kg fresh weight) resulted in a poorly preserved, high pH silage after 60 d (Table 5.2a). The addition of lactic acid reduced the pH of the grass to 4.97 and after 5 d the fermentation of WSC and further accumulation of lactic acid had reduced the pH to 3.96. However, during the final 20 d of the storage period the lactic acid concentration fell from 105 g/kg DM to 16 g/kg DM, accompanied by the accumulation of acetic acid and ethanol and an increase in pH (Figure 5.5a)

High numbers of lactic acid bacteria (maximum $257x10^9$ CFU/g DM after 7 d) were maintained following the period of peak fermentative activity, between day 2 and day 5, when the rate of glucose utilisation *in vitro* was greater than 40 μmol/h/g DM (Figure 5.5b), and more than 10^9 remained after 60 d (Table 5.2b). Coliform bacteria were inhibited by treatment of the grass with lactic acid. Yeasts multiplied to $10x10^6$ CFU/g DM after 10d but were not sustained.

Sodium lactate (7.85 kg/t) Treatment of the grass with sodium lactate similarly resulted in poorly preserved silage with high pH and high concentrations of ammonia, acetic acid and butyric acid after 60 d (Table 5.2). Lactic acid was produced rapidly (121 g/kg DM after 5 d) (Figure 5.6a), but the pH remained above 4.00 and, after 20 d, the concentration of lactic acid fell and ammonia, acetic acid and butyric acid accumulated.

Lactic acid bacteria multiplied to $234x10^9$ CFU/g DM after 5 d, corresponding with the period of maximum *in vitro* glucose utilisation (greater than 50 μmol/h/g DM between days 2-5), and high numbers of lactic acid bacteria were sustained throughout ensilage ($121x10^8$ CFU/g DM after 60d) (Figure 5.6b).

Coliform bacteria and yeasts were deterred from the silage.

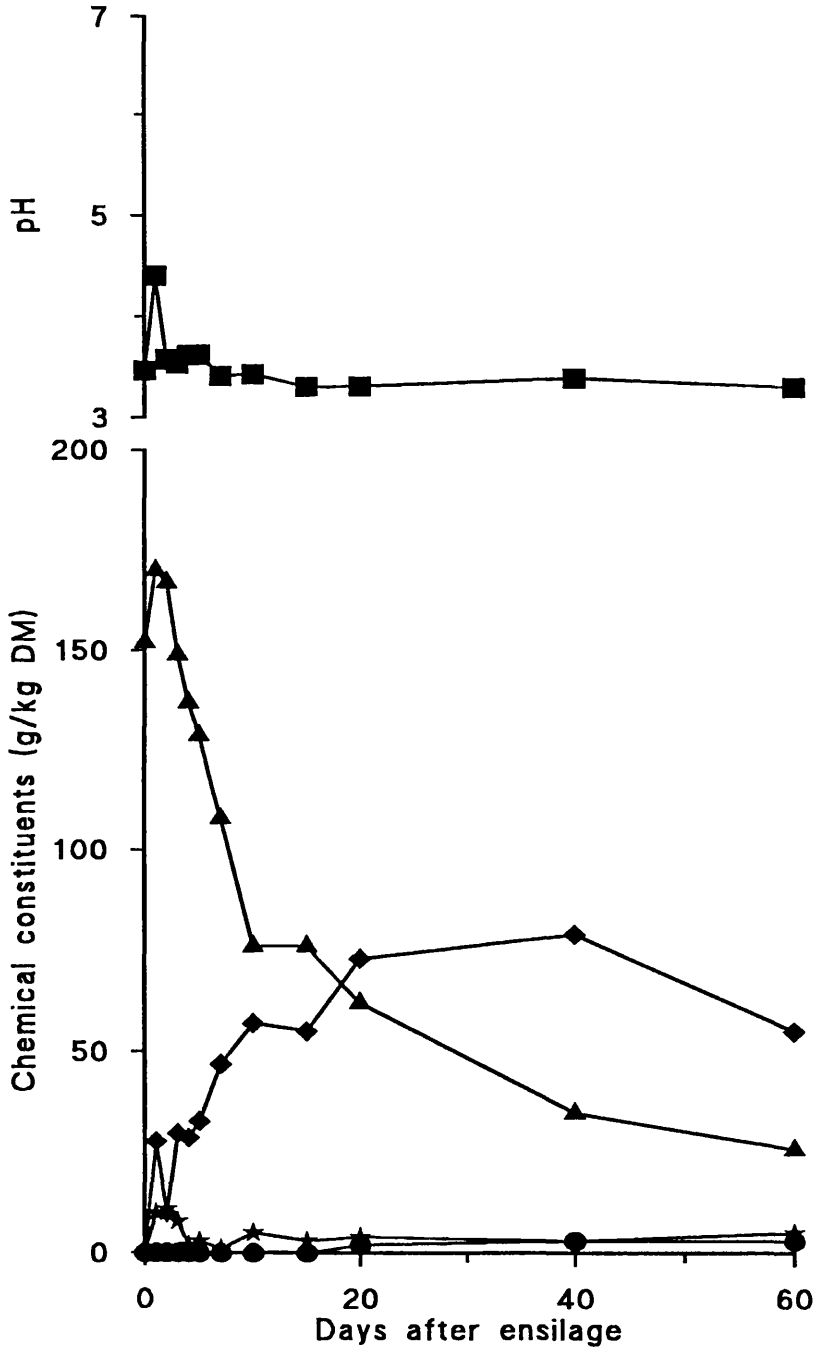


Figure 5.4a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, with 6 l/t sulphuric acid, in polythene bags within an anaerobic cabinet.

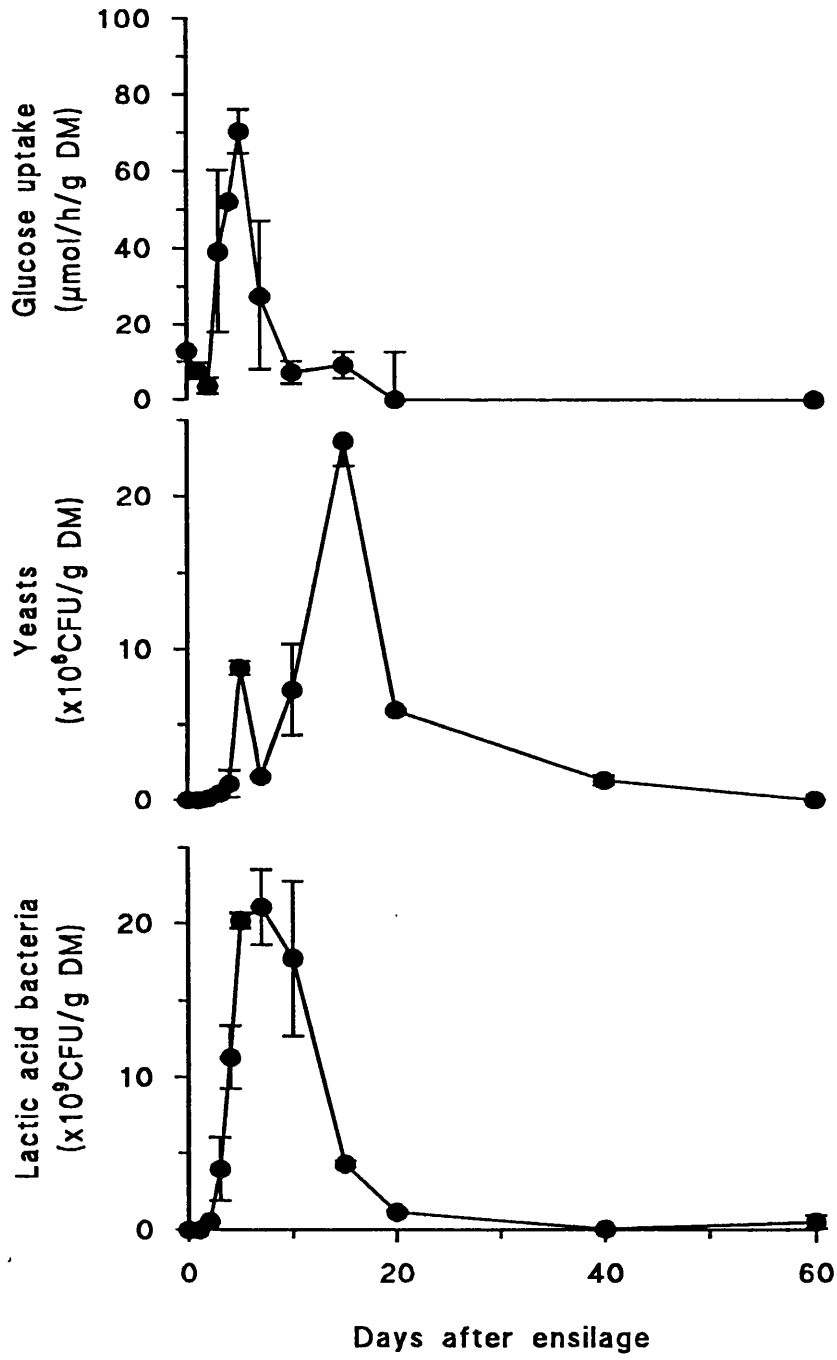


Figure 5.4b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during ensilage of thawed perennial ryegrass, with 6 l/t sulphuric acid, in polythene bags within an anaerobic cabinet.
Error bars represent SE of triplicate silages.

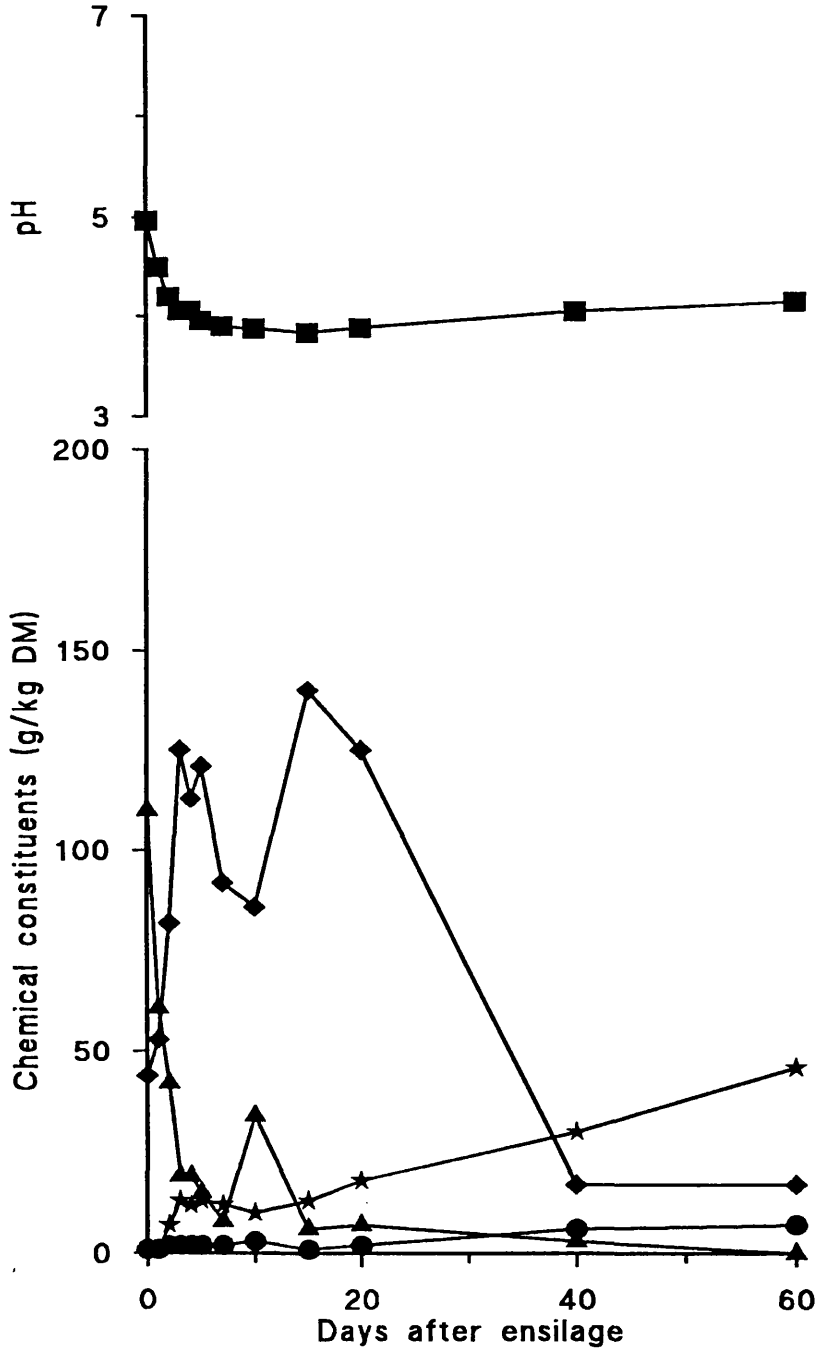


Figure 5.5a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, with 6.31 kg/t lactic acid, in polythene bags within an anaerobic cabinet.

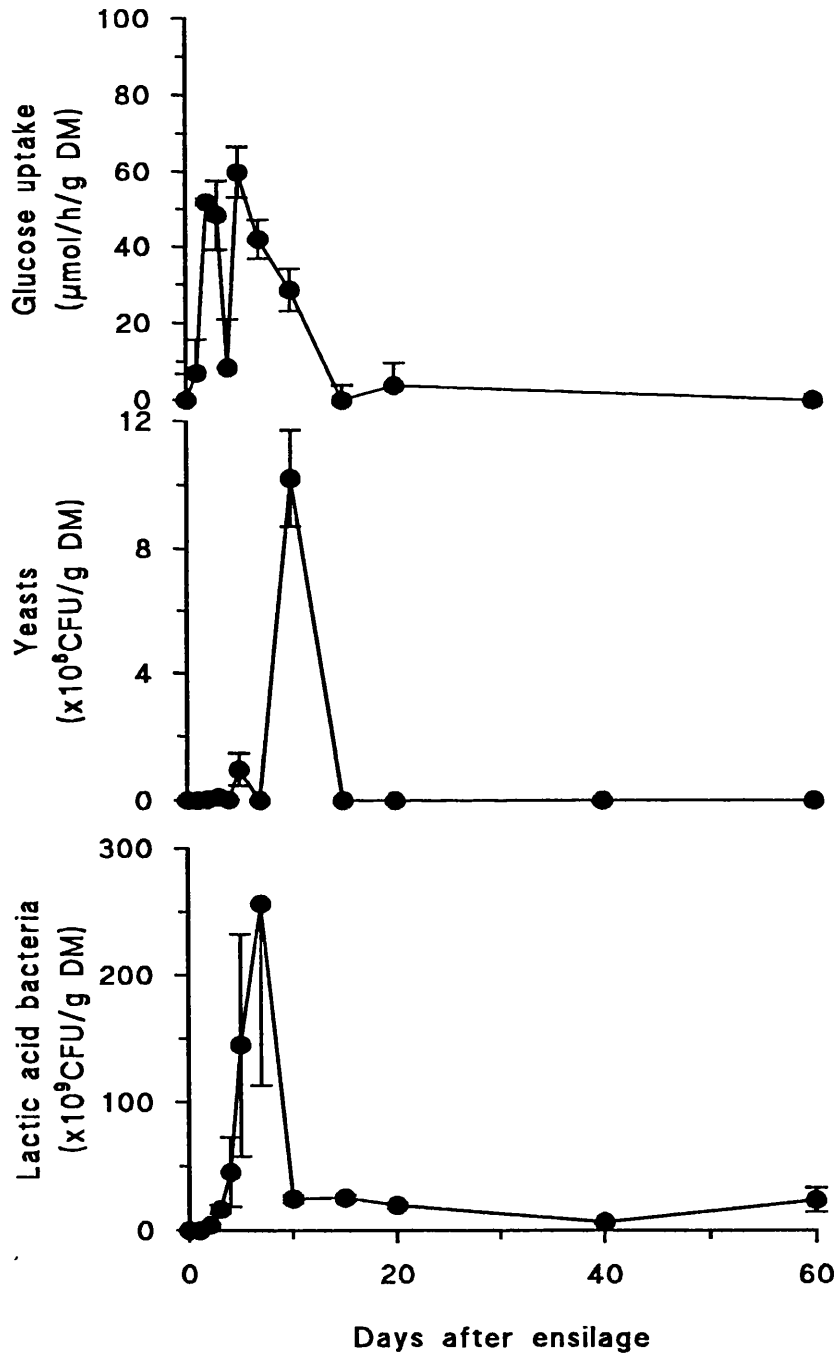


Figure 5.5b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (μmol/h/g DM) during ensilage of thawed perennial ryegrass, with 6.31 kg/t lactic acid, in polythene bags within an anaerobic cabinet.
Error bars represent SE of triplicate silages.

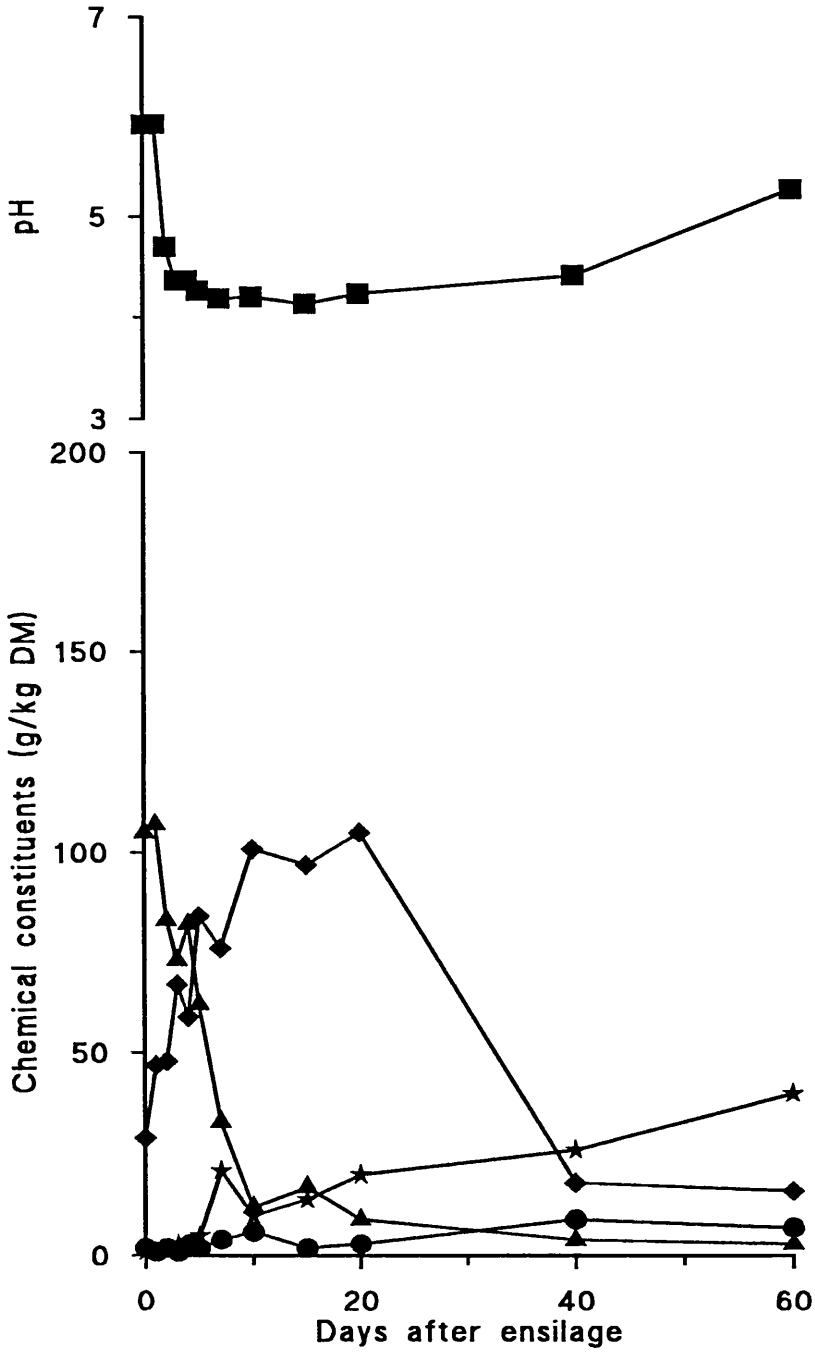


Figure 5.6a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, with 7.85 kg/t sodium lactate, in polythene bags within an anaerobic cabinet.

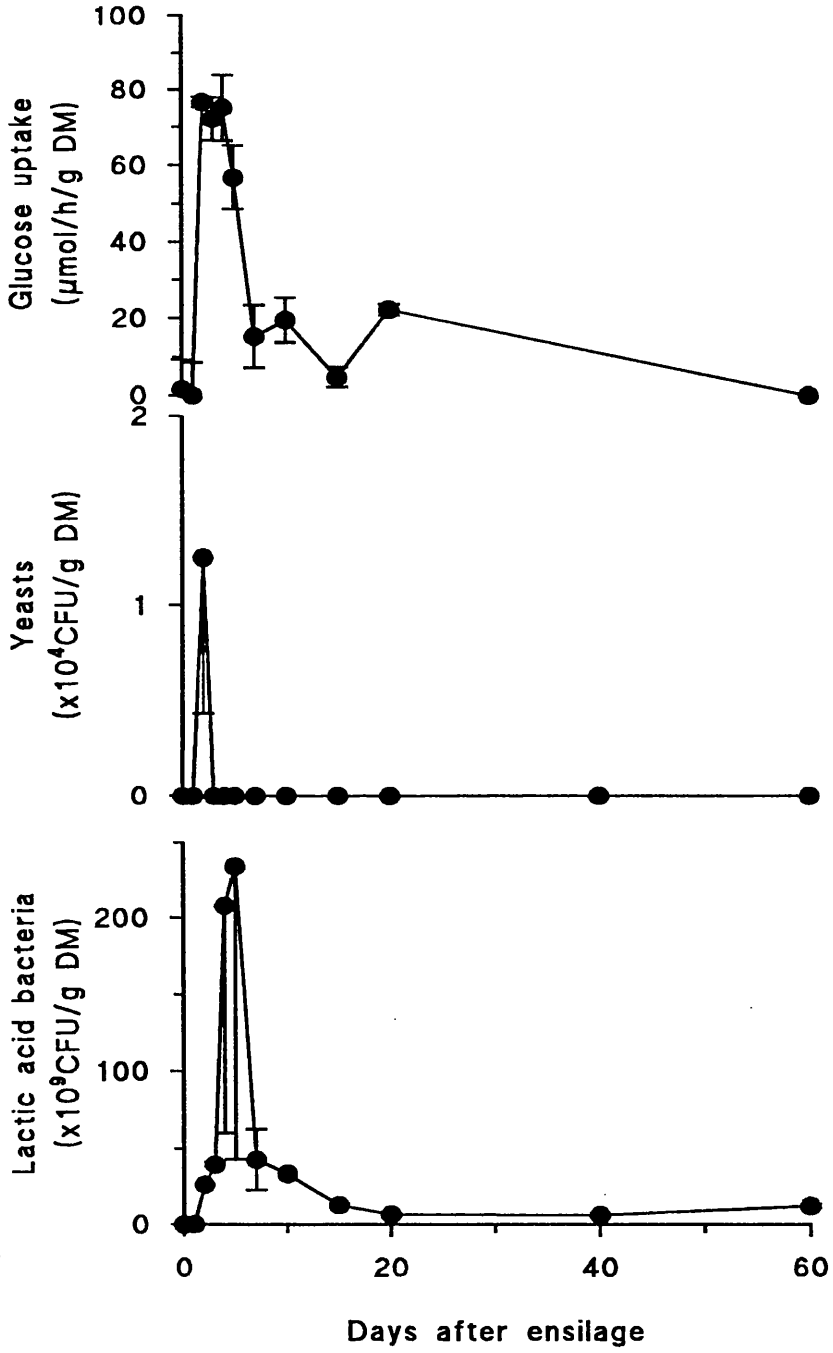


Figure 5.6b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during ensilage of thawed perennial ryegrass, with 7.85 kg/t sodium lactate, in polythene bags within an anaerobic cabinet.
Error bars represent SE of triplicate silages.

Discussion It was not possible to initiate all of the experiments simultaneously in the anaerobic cabinet, so the time-course experiments were staggered. Two bags of frozen grass, stored at -20°C until 36 h before ensilage, were used at each starting day, one for each treatment. Analysis of triplicate sub-samples of the forage taken on day 0, after application of the additives, revealed differences in the chemical composition of the crop ensiled. Whilst it may sometimes be misleading to regard single timed-sample data with too much reverence, the concentrations of WSC in the ensiled forage, after treatment with 2.3 l/t formic acid, sodium lactate and lactic acid, do suggest that there was some loss of WSC. To check that the differences in WSC content were not the result of analytical errors, an alternative method for analysis of total soluble carbohydrates (Dubois *et al.*, 1956) was used, and the data followed a similar pattern. Respiration of WSC may account for some of the sugar losses, since there were no by-products of fermentation at day 0, although the addition of formic acid ought to have restricted the rate of respiration. Because of the apparently lower concentrations of WSC in some of the ensiled forage, the interpretation of the fermentation characteristics should be treated with caution.

The grass used in this experiment was well preserved after 60 d without additive treatment, but the acetic acid and ammonia production in the early stages of ensilage, perhaps a consequence of competition by coliform bacteria or a combination of heterolactic and coliform bacteria, was prevented by the addition of acid additives. The residual levels of metabolic activity in the later stages of ensilage of the control were not reflected in changes in the chemical composition and may have been overestimated as the actual amounts of glucose utilised *in vitro* were very small.

The higher level of addition of formic acid inhibited fermentation of WSC, restricted the numbers of lactic acid bacteria and deterred yeasts and coliform bacteria. The inhibition of a normal lactic fermentation avoided the accumulation of lactic acid, hence a higher pH was maintained. Treatment of the grass with 2.3 l/t formic acid deterred the production of acetic acid initially, but once the vigorous lactic fermentation stopped, the fermentation of WSC continued with the accumulation of acetic acid. This suggests that at this level of addition formic acid was effective only in combination with the inhibition afforded by the lactic fermentation. The low pH achieved by addition of 6 l/t sulphuric acid (pH 3.47) restricted fermentation in the early hours of ensilage. Maximum *in vitro* glucose utilisation was not reached until 5 d after ensiling, but was nevertheless comparable to the maximum seen in the control, consistent with the lack of an antimicrobial effect of sulphuric acid. The pH effect of sulphuric acid may subsequently have been more effective in restricting an

undesirable fermentation when complemented by endogenous production of lactic acid. However, after the lactic fermentation abated, there was evidence of a continued fermentation, possibly elicited by acid-tolerant species of lactic acid bacteria and associated with the production of alternative end-products (formic acid, 2,3-butanediol, acetoin or mannitol). This revival of, and continued fermentation by, lactic acid bacteria may have deterred the activities of acid tolerant yeasts.

The initial pH of the lactic acid- and sodium lactate-treated forages was higher than was expected from the titration data described in the introduction, and may have been the result of a higher concentration of buffering constituents in the forage used in this experiment, but the fermentation of substrates in the lactic acid-treated forage ensured a low pH. However, following exhaustion of WSC by the peculiarly large population of lactic acid bacteria, despite the virtual cessation of fermentative activity, as measured by *in vitro* glucose utilisation, most of the exogenous and endogenous lactic acid was degraded, with an associated increase in the concentration of acetic acid and ethanol. Whilst the supply of WSC may have been limiting, the pH before secondary fermentation began (pH 3.88) ought to have maintained stability. It therefore seems more likely that the provision of lactic acid to the grass at the point of ensilage encouraged the survival of epiphytic micro-organisms which either had the potential to metabolise lactic acid in times of nutrient shortage or had adapted to preferentially ferment lactic acid. In the sodium lactate-treated silage, the fermentation was similarly restricted but the rapid exhaustion of WSC by an augmented number of lactic acid bacteria led to the degradation of lactic acid in the later stages of the storage period. The accumulation of ammonia and butyric acid suggest proliferation of saccharolytic clostridia, their survival perhaps encouraged by the provision of lactate as a substrate and their growth stimulated by the failure of lactic acid bacteria to reduce the pH sufficiently. These results illustrate the need for more work to clarify the conditions leading to the spoilage of apparently well preserved silages; the provision of lactate as a substrate for the *in vitro* assay of microbial activity, or in the cultivation media for the enumeration of micro-organisms, may be useful, and detailed analysis of the supernatant after incubation *in vitro* may reveal the nature of the fermentation pathways and the organisms responsible.

Although lactic acid and sodium lactate stimulated larger populations of lactic acid bacteria and ensured inhibition of yeasts and coliform bacteria, after the lactic fermentation had slowed, degradation of lactic acid and spoilage of the silage began. Similarly, the impotence of 2.3 l/t formic acid and 6 l/t sulphuric acid against contaminants, after the lactic fermentation apparently finished, suggests that the fermentative activity of lactic acid bacteria,

and the rapid accumulation of lactic acid, may be a more competent inhibitor than acid additives of the activity of undesirable silage micro-organisms.

Experiment 2 The effect of an inoculant containing *Lactobacillus plantarum* plus sucrose, with and without added sodium bicarbonate, of bicarbonate alone, and of an enzyme preparation (Clampzyme) added to the grass before ensilage

Introduction The activity of silage micro-organisms can be restricted by the addition of acids, but some capacity for substrate utilisation may be retained. From the data of Experiment 1, the rapid production of lactic acid was apparently a more potent deterrent to fermentative activity than other organic or mineral acids. In order to study in more detail the role of lactic acid in the fermentation, the rate and extent of accumulation was manipulated by adding an inoculum, by increasing the concentration of substrate and by incorporating sodium bicarbonate. Also, since fibrolytic enzymes have been shown to degrade plant cell walls and to release fermentable carbohydrates during ensilage (Chamberlain *et al.*, 1987; Rauramaa *et al.*, 1987), a high rate of addition of a commercial hemicellulase/cellulase preparation (Clampzyme, Forum Chemicals Limited, Redhill, U.K.) was included among the treatments.

Materials and methods The grass used in Experiment 2 was from the same harvest as that used in Experiment 1 and the design of Experiment 2 was similar to that in Experiment 1. Thawed grass (*L.perenne*), harvested and frozen for up to 9 months, was ensiled in 33 sterile polythene bags (150 g/bag) inside the anaerobic cabinet after treatment of the grass with a) 0.5 l/t Clampzyme, b) an inoculant of *L. plantarum*, providing 10^6 CFU/g FW (Ecosyl, 3.0 l/t), with 40 kg/t FW sucrose, c) the inoculant with 40 kg/t sucrose plus 30 kg/t FW sodium bicarbonate and d) 30 kg/t sodium bicarbonate alone. The Clampzyme and Ecosyl additives were applied as a fine mist over the grass using a hand-held applicator and the sucrose and sodium bicarbonate were sprinkled evenly over the grass and mixed well before ensiling. Untreated grass was ensiled as a control as in Experiment 1. Triplicate silos were removed from the cabinet on days 0, 1, 2, 3, 4, 5, 7, 10, 15, 20, 40 and 60 after ensilage and prepared for microbial enumeration and assay of fermentative activity, as described in Chapter 2. The remaining sub-sampled silage was frozen and stored at -20°C before chemical analysis.

Theoretical proportions of metabolites were calculated, assuming, as in Experiment 1, a substrate mixture composed of 0.41 glucose : 0.41 fructose : 0.18 pentose. These and the actual proportions of metabolites formed during ensilage were compared, except where a secondary fermentation was identified, to derive more information regarding the fermentation pathways; the concentrations of metabolites in the last three sub-samples (day 20-day 60) were meaned to provide an estimate of the concentrations at a theoretical end-point of fermentation.

Statistical analysis was by one-way analysis of variance, using MINITAB.

Results The grass ensiled for the control was a high sugar/low CP summer crop, as described in Chapter 4 (Table 4.17). The chemical composition and the microbial populations of the starting material is shown in Table 5.4 and the changes in chemical composition and microbial populations are shown in Figures 5.7-5.11 and Table 5.5.

Control The control silage was well preserved with low pH, low ammonia and negligible butyric acid after 60 d (Table 5.5). WSC were rapidly fermented (41 g/kg DM remaining after 7 d) to lactic acid (92 g/kg DM after 7 d) and acetic acid (14 g/kg DM after 7 d) (Figure 5.7a). Table 5.6 illustrates the predominantly homofermentative nature of the process.

The early period of dynamic fermentative activity was reflected by the rate of glucose uptake *in vitro* which peaked at day 2 (66.7 μmol glucose utilised/h/g DM) before falling 82% by day 20 (11.9 μmol/h/g DM) (Figure 5.7b). Lactic acid bacteria multiplied to $32x10^9$ CFU/g DM by day 3, and coliform bacteria were soon deterred (less than 10^2 CFU/g DM after 3 d). Yeasts were identified in low numbers after 40 d.

Clampzyme Treatment with Clampzyme effected a fermentation similar to that of the control silage, with low pH, low ammonia, low acetic acid and negligible butyric acid after 60 d (Table 5.5a). Residual WSC (0.19 of the WSC present on the original forage) remained after 20 d (mean days 20-60), following a predominantly homolactic fermentation (Table 5.6). The continued accumulation of lactic acid during the later stages of ensilage resulted in higher concentrations after 60 d than in the control silage, suggesting the fermentation of substrates released by hydrolysis of the cell walls. Corresponding with a delay before peak microbial activity (maximum *in vitro* glucose uptake 65.3 μmol/h/g DM after 4 d), lactic acid bacteria were slow to reach maximum numbers ($48x10^9$ CFU/g DM on day 10) (Figure 5.8b). Coliform bacteria were nevertheless deterred by 4 d, while yeasts, maximum $306x10^4$ CFU/g DM on day 4, were present throughout ensilage.

Table 5.4a Chemical composition of the grasses after treatment (g/kg DM unless stated otherwise) (Experiment 2).

	pH	Dry matter (g/kg)	Ammonia	Water soluble carbohydrate
Control	6.17	213	1	191
Clampzyme	5.00	221	1	140
Ecosyl plus sucrose	6.07	225	2	246
Ecosyl plus sucrose plus sodium bicarbonate	9.65	231	1	218
Sodium bicarbonate	9.63	214	1	77

Table 5.4b Microbial populations on the forage ensiled (CFU/g DM) (Experiment 2).

	Total viable organisms	Lactic acid bacteria	Coliform bacteria	Yeasts
Control	$64x10^6$	$15x10^4$	$157x10^2$	$<10^2$
Clampzyme	$15x10^6$	$79x10^3$	$<10^2$	$<10^2$
Ecosyl plus sucrose	$42x10^6$	$44x10^5$	$23x10^2$	$<10^2$
Ecosyl plus sucrose plus sodium bicarbonate	$104x10^6$	$53x10^5$	$45x10^2$	$<10^2$
Sodium bicarbonate	$82x10^6$	$37x10^3$	$<10^2$	$<10^2$

Table 5.5a Chemical composition of the silages after 60 d (g/kg DM, unless stated otherwise) (Experiment 2).

	pH	Dry matter (g/kg)	Ammonia	Lactic acid	Water soluble carbohydrates	Ethanol	Acetic acid	Propionic acid	Butyric acid
Control	3.65	232	1	88	37	2	15	tr.	tr.
Clampzyme	3.66	215	1	133	28	4	12	1	1
Ecosyl plus sucrose	3.58	180	3	131	15	9	9	2	1
Ecosyl plus sucrose plus sodium bicarbonate	4.49	228	4	215	7	5	19	2	1
Sodium bicarbonate	7.35	144	19	23	0	9	115	15	76
SED	0.12	10.84	0.7	14.4	16.2	1.5	4.0	0.5	4.1
(n=132)	***	***	***	***	*	***	***	***	***

SED value quoted is the higher of two determinations derived from unbalanced sets of sample data. Statistically significant differences are:- *, $P<0.05$ and ***, $P<0.001$.

Table 5.5b Microbial populations on the silage after 60 d (CFU/g DM) (Experiment 2).

	Total viable organisms	Lactic acid bacteria	Coliform bacteria	Yeasts
Control	$49x10^6$	$224x10^6$	$<10^2$	$64x10^4$
Clampzyme	$3x10^6$	$112x10^6$	$<10^2$	$12x10^3$
Ecosyl plus sucrose	$6x10^6$	$696x10^6$	$<10^2$	$<10^2$
Ecosyl plus sucrose plus sodium bicarbonate	$204x10^6$	$2192x10^6$	$<10^2$	$<10^2$
Sodium bicarbonate	$31x10^6$	$344x10^6$	$<10^2$	$<10^2$

Table 5.6 Proportion of major metabolites formed during ensilage (Experiment 2).

	Lactic acid	Acetic acid	Ethanol	Total metabolites (g/kg DM)
Control	0.84	0.14	0.02	115
Clampzyme	0.88	0.09	0.02	128
Ecosyl plus sucrose	0.91	0.05	0.04	140
Ecosyl plus sucrose plus sodium bicarbonate	0.90	0.08	0.02	216
Sodium bicarbonate	0.20	0.75	0.05	114

Theoretical ratio of metabolites:-

Heterolactic fermentation — 0.50 lactic acid : 0.25 acetic acid : 0.25 ethanol

Homolactic fermentation — 0.91 lactic acid : 0.09 acetic acid

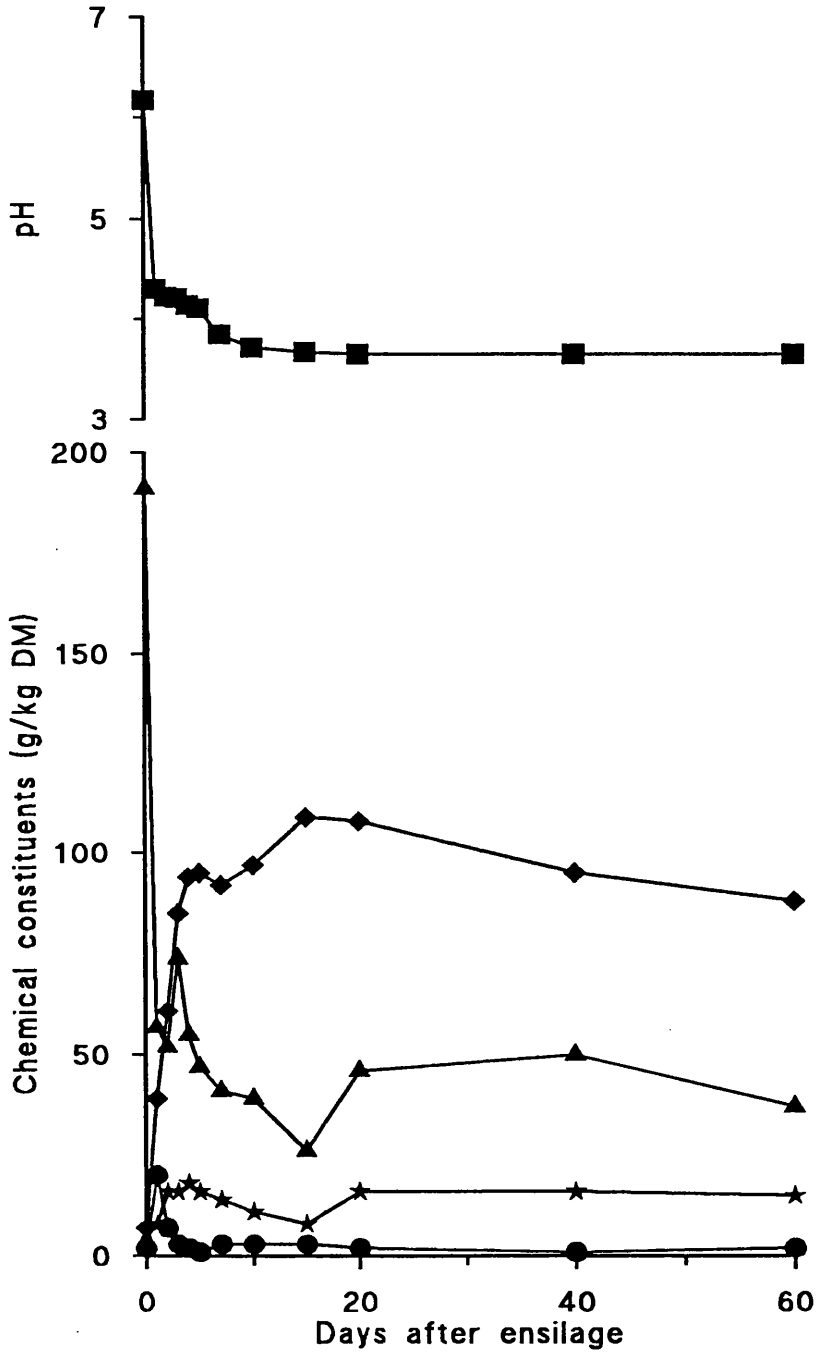


Figure 5.7a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, with no additive, in polythene bags within an anaerobic cabinet.

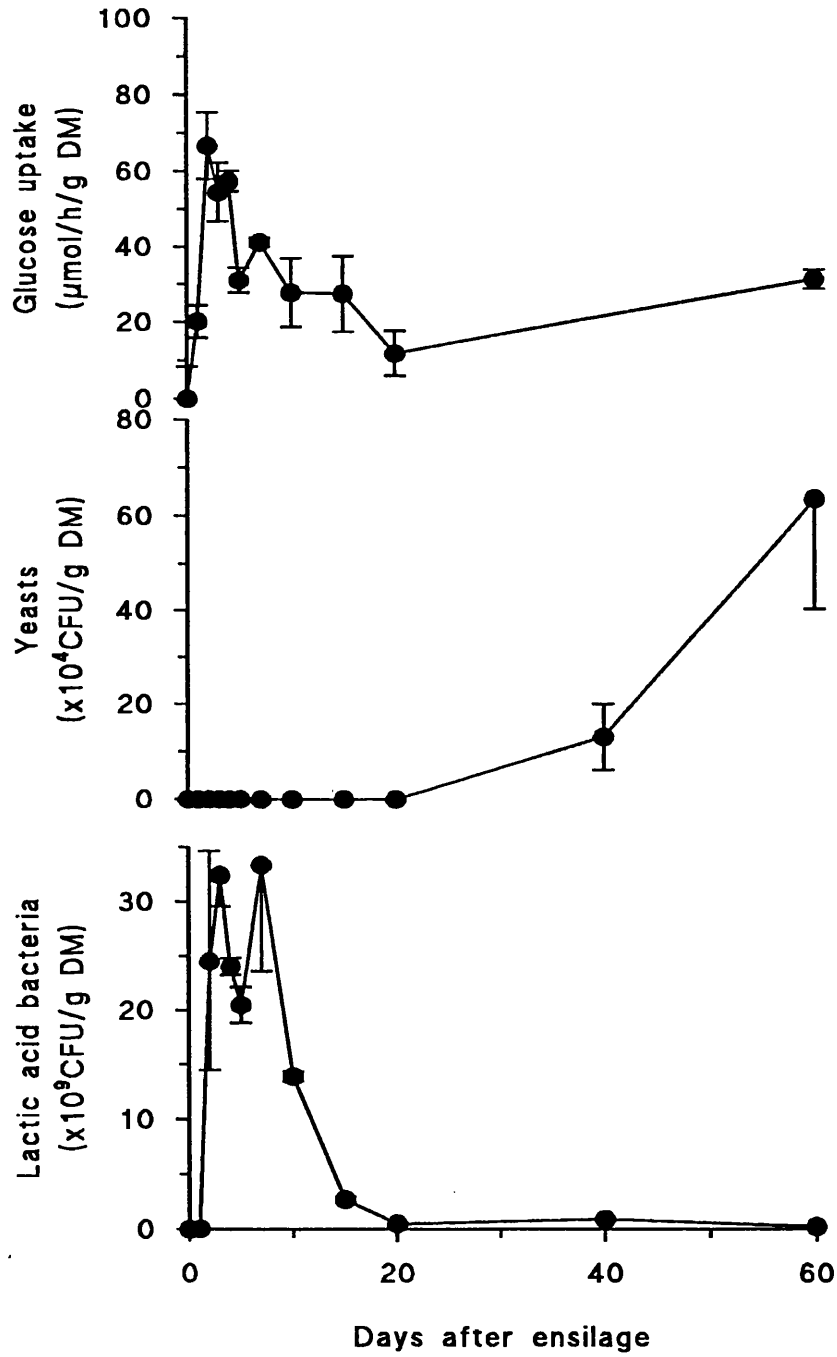


Figure 5.7b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (μmol/h/g DM) during ensilage of thawed perennial ryegrass, without additive, in polythene bags within an anaerobic cabinet.

Error bars represent SE of triplicate silages.

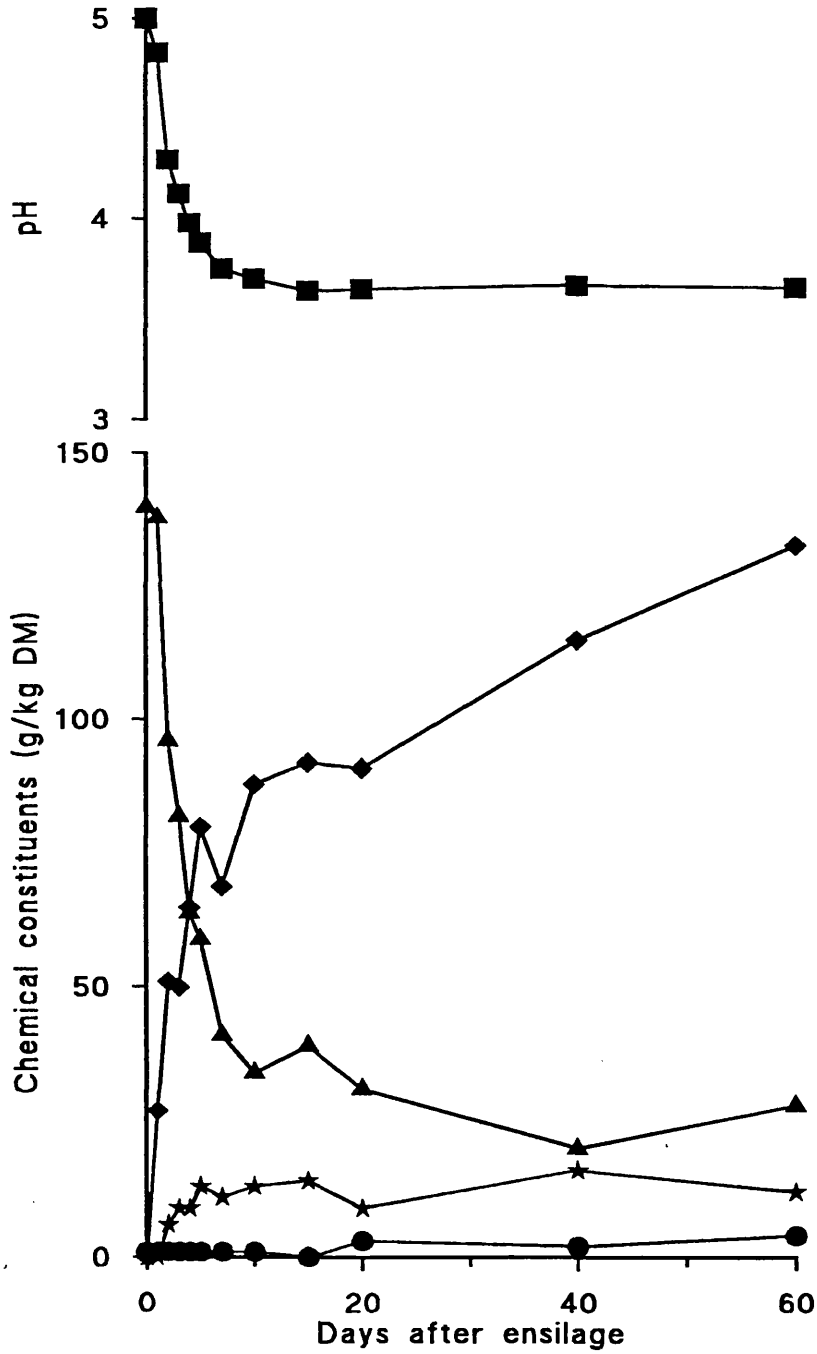


Figure 5.8a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, with 0.5 l/t Clampzyme, in polythene bags within an anaerobic cabinet.

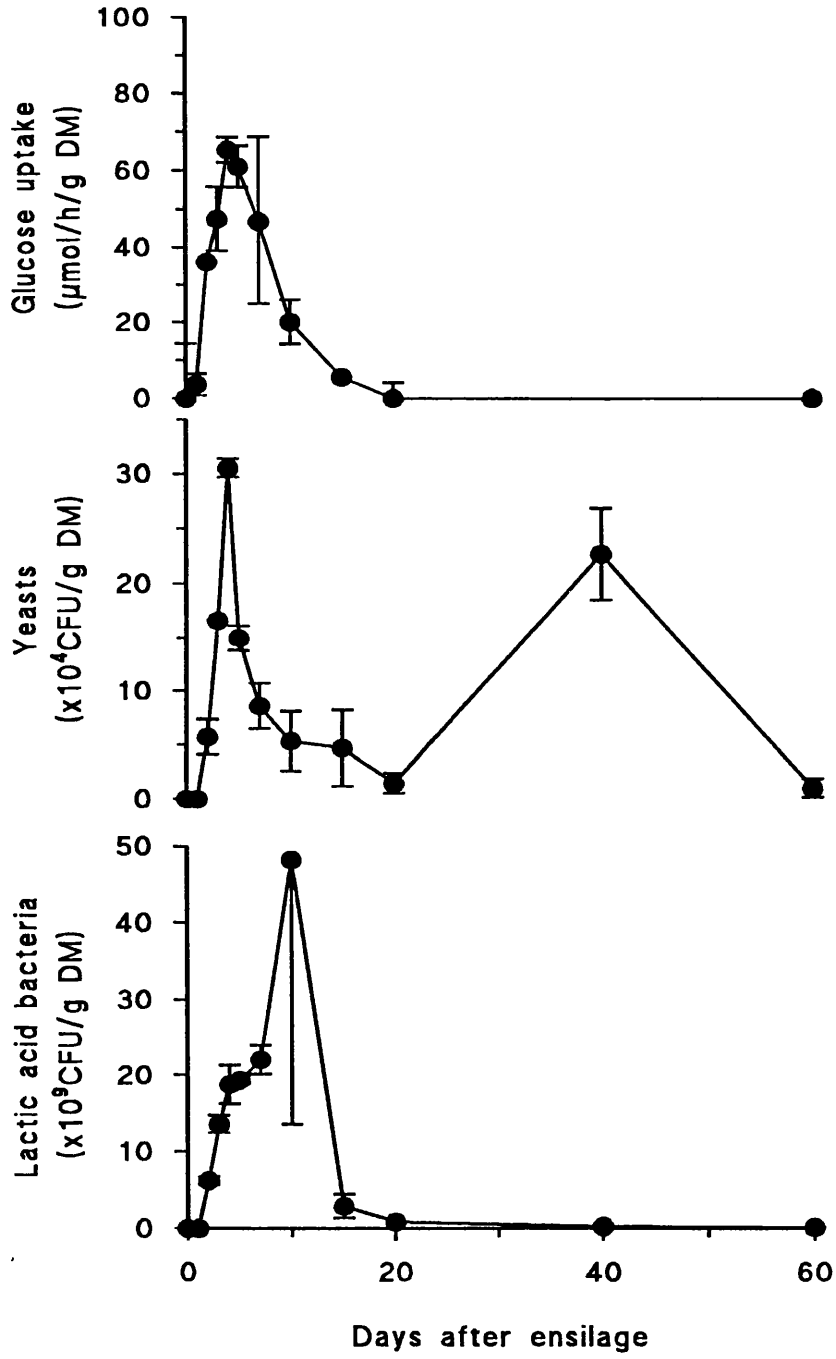
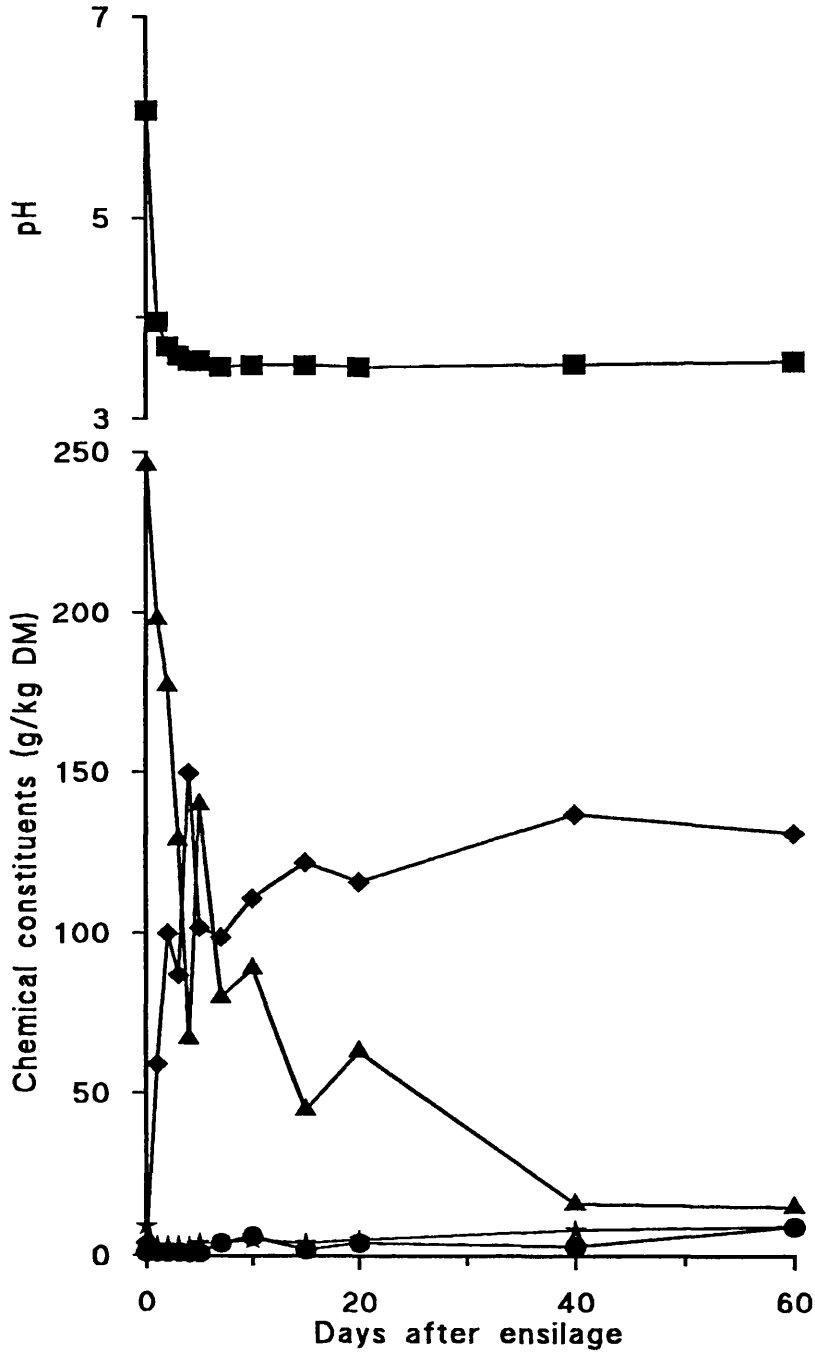


Figure 5.8b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during ensilage of thawed perennial ryegrass, with 0.5 l/t Clampzyme, in polythene bags within an anaerobic cabinet.
Error bars represent SE of the mean of triplicate silages.



5.9a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, with 3 l/t Ecosyl and 40 kg/t sucrose, in polythene bags within an anaerobic cabinet.

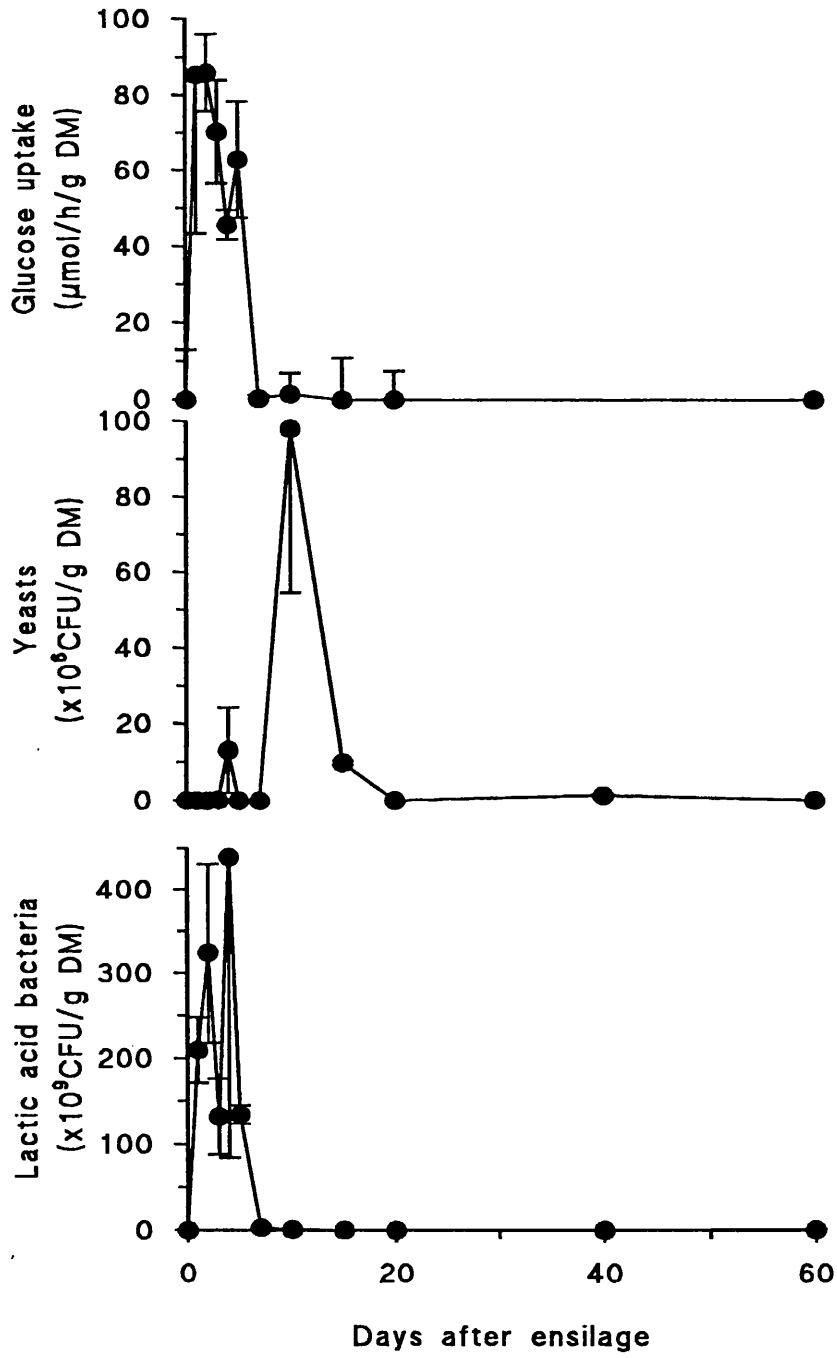


Figure 5.9b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during ensilage of thawed perennial ryegrass, with 3 l/t Ecosyl and 40 kg/t sucrose, in polythene bags within an anaerobic cabinet. Error bars represent SE of the mean of triplicate silages.

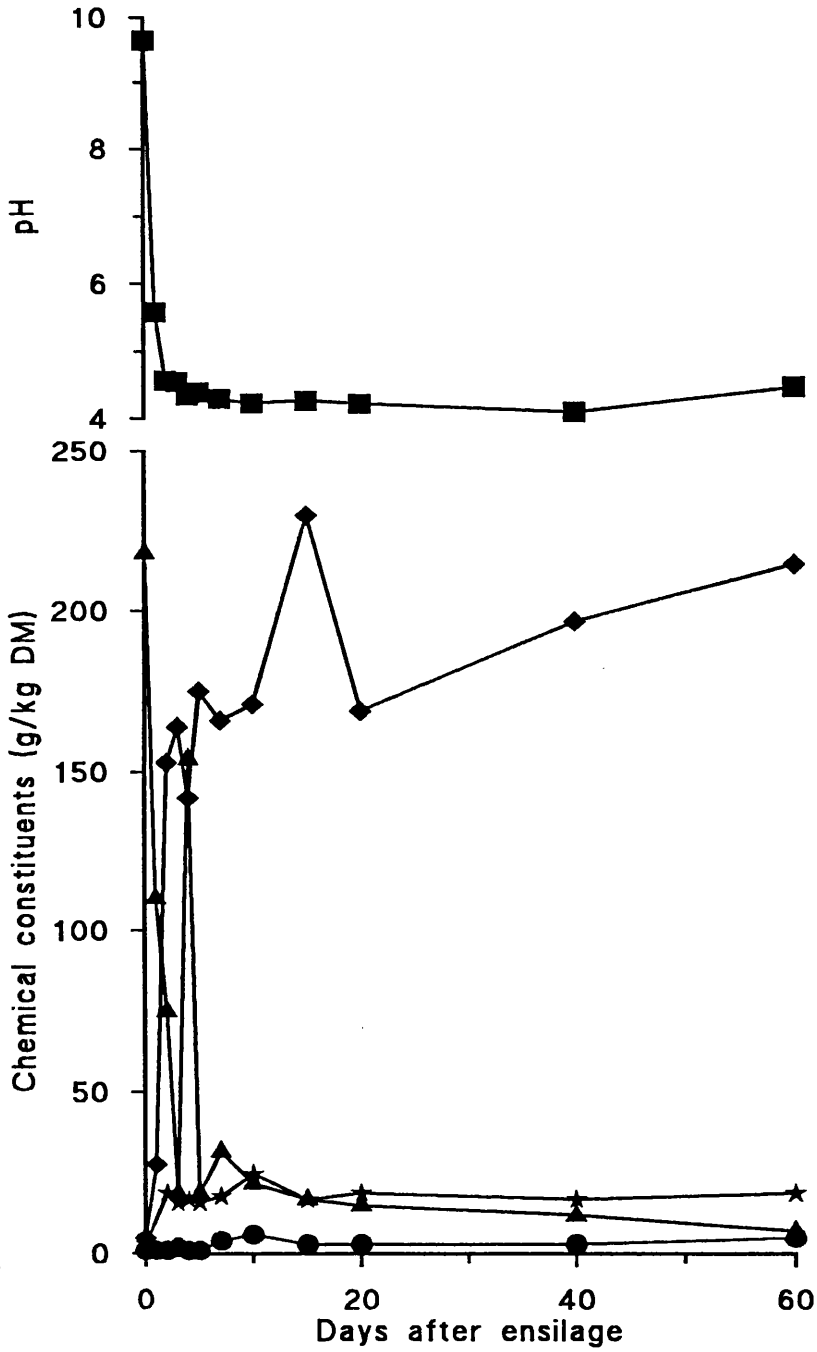


Figure 5.10a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, with 3 l/t Ecosyl and 40 kg/t sucrose and 30 kg/t sodium bicarbonate, in polythene bags within an anaerobic cabinet.

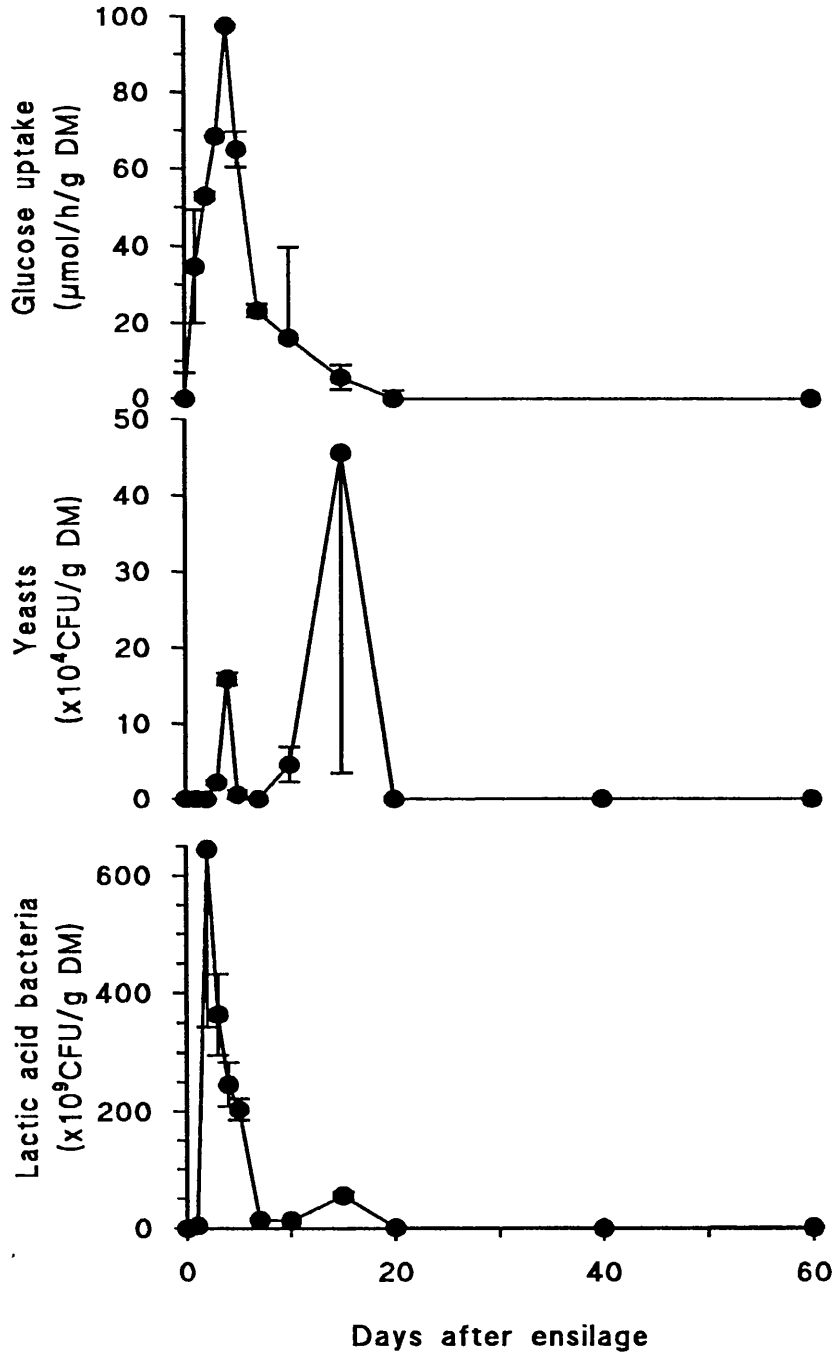


Figure 5.10b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during ensilage of thawed perennial ryegrass, with 3 l/t Ecosyl and 40 kg/t sucrose and 30 kg/t sodium bicarbonate, in polythene bags within an anaerobic cabinet.

Error bars represent SE of the mean of triplicate silages.

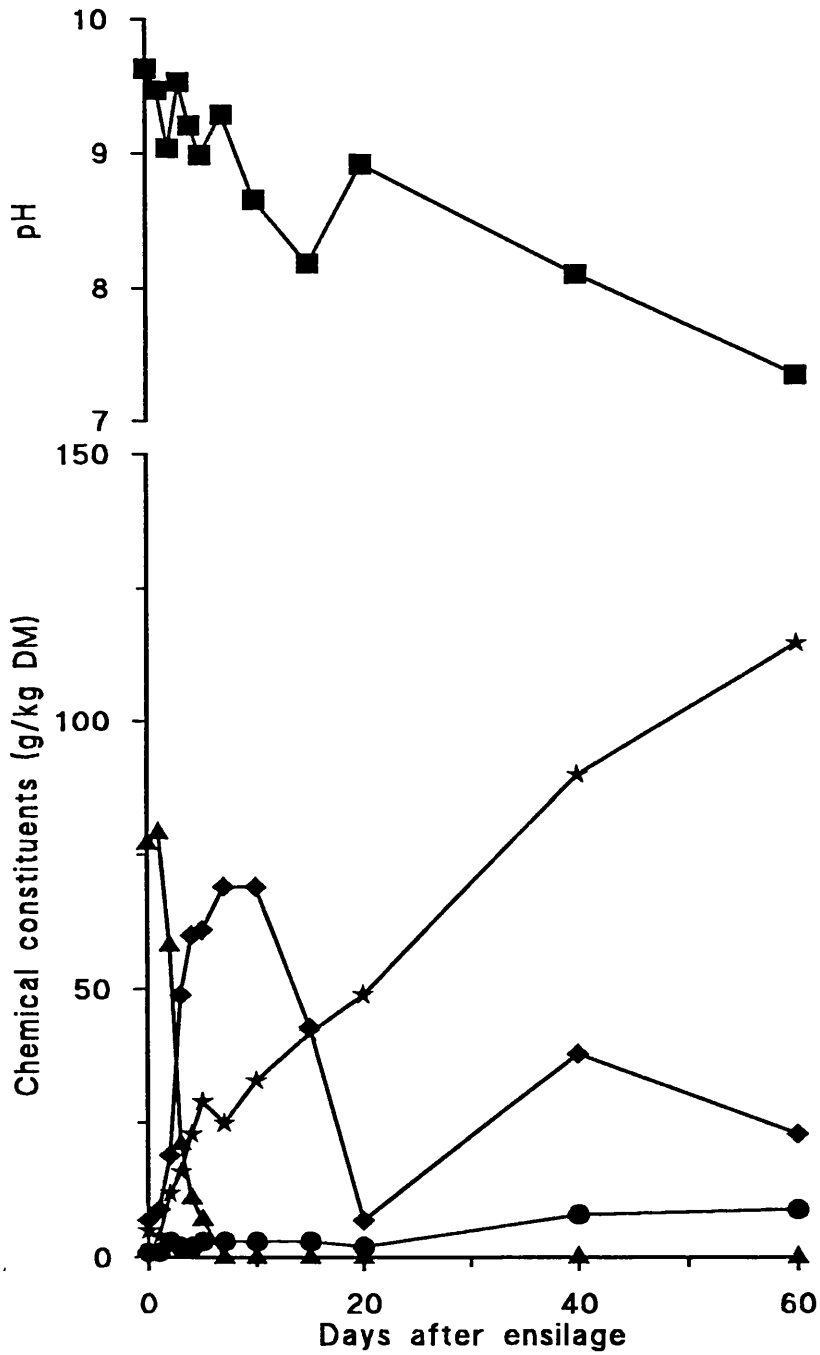


Figure 5.11a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during ensilage of thawed perennial ryegrass, with 30 kg/t sodium bicarbonate, in polythene bags within an anaerobic cabinet.

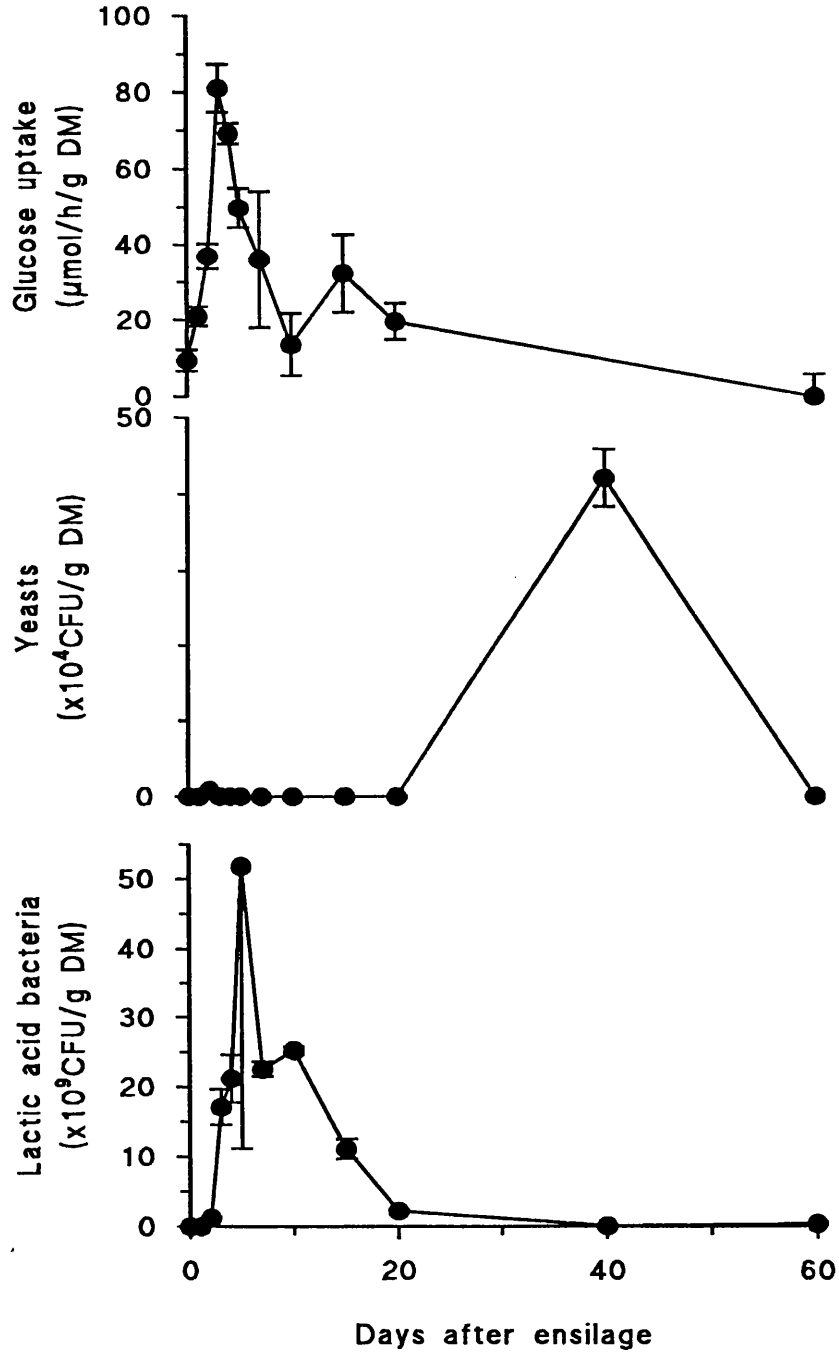


Figure 5.11b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during ensilage of thawed perennial ryegrass, with 30 kg/t sodium bicarbonate, in polythene bags within an anaerobic cabinet. Error bars represent SE of the mean of triplicate silages.

Ecosyl plus sucrose Addition of ecosyl and sucrose stimulated a concentrated period of intense fermentative activity. The pH fell from 6.07 to 3.96 in the first 24 h (Figure 5.9a), and during the first 7 d of ensilage 166 g/kg DM WSC were fermented, with the accumulation of 100 g/kg DM lactic acid. The remaining WSC was fermented at a slower rate with the continued accumulation of lactic acid, and production of ethanol and acetic acid (9 g/kg DM of each) in the later stages of fermentation (Figure 5.9a). There may also have been an additional fermentation, with unmeasured metabolites produced, since more WSC disappeared than can be accounted for. Nevertheless, Table 5.6 confirms the predominantly homolactic nature of the fermentation.

The vigorous production of lactic acid is reflected in the microbial constitution of the silage; lactic acid bacteria ($5x10^6$ at day 0) increased to $44x10^{10}$ CFU/g DM after 4 d. The rate of glucose uptake *in vitro* similarly increased rapidly to a maximal level after 2 d (85.9 μmol/h/g DM) and subsequently fell to negligible activity after 7 d. Coliforms were not detected beyond 24 h after ensilage, whereas numbers of yeasts were relatively high ($98x10^6$ CFU/g DM) by day 10, and it is surprising more ethanol was not produced.

Ecosyl plus sucrose plus sodium bicarbonate The inclusion of sodium bicarbonate with Ecosyl and sucrose encouraged the fermentation of 196 g/kg DM WSC in the first 10 d, with the accumulation of 171 g/kg DM lactic acid and 18 g/kg DM acetic acid, during a predominantly homolactic fermentation (Table 5.6). Nevertheless, the pH remained above 4.00 throughout, associated with higher ammonia production in the early stages after ensilage.

Lactic acid bacteria numbers ($65x10^{10}$ CFU/g DM on day 2) and *in vitro* glucose uptake (maximum 98 μmol/h/g DM on day 4) were both higher than the control silage. Coliform bacteria multiplied to $974x10^2$ CFU/g DM and persisted until 5 d after ensilage, while yeasts were present in low numbers and did not participate in the fermentation.

Bicarbonate Addition of sodium bicarbonate alone to the grass had an adverse effect on the preservation. A high pH was maintained throughout the period of storage, WSC (77 g/kg DM at day 0) were exhausted by day 7, and, after 10 d, lactic acid concentration (69 g/kg DM) declined, associated with a rise in concentration of butyric acid to 33 g/kg DM after 20 d and 76 g/kg DM after 60 d, acetic acid (115 g/kg DM after 60 d), ammonia (19 g/kg DM after 60 d) and propionic acid (15 g/kg DM after 60 d).

Glucose uptake *in vitro* was maximal after 3 d (81.1 μmol/h/g DM), falling 55% to 36.2 μmol/h/g DM on day 7, and was negligible after 20 d. Although lactic acid bacteria did not proliferate to such an extent as in the Ecosyl-sucrose-bicarbonate treated-forage, high numbers were maintained throughout the period of storage (maximum $52x10^9$ CFU/g DM).

In addition, coliform bacteria multiplied to $23x10^6$ by day 5 and persisted until 40d after ensilage. Generally, low numbers of yeasts (less than 10^2) were present throughout (Figure 5.11b).

Discussion The control silage was well preserved and the rapid, predominantly homolactic, fermentation contributed to preservation of 0.23 of the WSC content of the original forage. In this respect, from a practical standpoint, there would be little benefit from the use of silage additives. However, the provision of extra substrate with the inoculum and the inclusion of sodium bicarbonate were responsible for some interesting changes to the course of the fermentation.

Just as in Experiment 1, the time-course experiments were staggered, with two different treatments initiated at once. Triplicate sub-samples of the forage on day 0, after application of the silage additives, revealed differences between the WSC concentrations, and that of the forage treated with sodium bicarbonate alone was particularly low, even after re-analysis of total soluble carbohydrate contents using the method of Dubois *et al.* (1956). Similarly, addition of 40 g sucrose per kg forage ought to have increased the WSC content to at least 250 g WSC/kg DM. Respiration of WSC before ensilage may be an explanation. Although more sugars may have been released in the later stages of ensilage with sodium bicarbonate treatment (presumably a consequence of plant enzyme-mediated polysaccharide hydrolysis), which would account for the high concentrations of acetic acid, interpretation of the data and assessment of the effect of sodium bicarbonate in particular must be treated with caution.

On the basis of previous results it would be expected that the low pH and high WSC, and absence of a lactic fermentation, in the later stages of ensilage following treatment with Ecosyl and sucrose would be conducive to ethanolic fermentation by yeasts. There was a continued loss of WSC but the absence of ethanolic fermentation and the low residual WSC content suggest that other products were formed (formate, diacetyl, 2,3-butanediol or mannitol) by competing acid-tolerant species of lactic acid bacteria, although it is difficult to reconcile continued fermentation with conditions in the silo (pH 3.54, lactic acid concentration 122 g/kg DM after 15 d) or with the low rates of glucose utilisation *in vitro*. Similarly, despite relatively high numbers of yeasts on Clampzyme-treated forage, the continued competition by lactic acid bacteria apparently inhibited accumulation of ethanol. The competition for substrates and the rapid production of lactic acid may suppress the expression of yeast activity or, alternatively, the production of other, unidentified, antibiotic

agents by actively fermenting lactic acid bacteria may have an adverse effect on yeast metabolism. Although bacteriocins (proteins secreted by bacteria which inhibit the growth of other closely related bacteria) (Hardy, 1975) have not been identified in the silage process, Hurst (1981) described the production of nisin by *Lactococcus lactis* subsp. *lactis* and West and Warner (1988) identified the production of plantacin B from *L. plantarum* NCDO 1193, while Axelsson *et al.* (1989) first reported the production of reuterin by *Lactobacillus reuteri*. Reuterin is a potent broad spectrum antimicrobial substance effective against Gram-negative and Gram-positive bacteria, yeasts, fungi and protozoa.

The high pH of sodium bicarbonate alone-treated forage had little control over the mixed population of viable epiphytic micro-organisms, possibly including saccharolytic clostridia, which are thought to be tolerant of a higher pH (optimum pH 7-7.4, Pelczar and Reid, 1972), and resulted in a mixed fermentation and a poorly preserved silage. Although lactic acid bacteria eventually dominated, coliform bacteria remained until 40 d after ensilage and probably contributed to the accumulation of acetic acid. The commensal development of lactic acid bacteria and coliform bacteria suggests that the inhibition of coliforms may not be achieved simply by encouraging the accumulation of lactic acid. The pH was not lowered enough to suppress clostridia, and these may have metabolised lactic acid with the associated production of butyric acid and acetic acid. The accumulation of ethanol may be a result of yeast activity, heterolactic fermentation by lactic acid bacteria, or mixed acid fermentation and butanediol fermentation by coliform bacteria (McDonald *et al.*, 1991); such an unusually high pH in the silo may be expected to elicit unusual patterns of fermentation.

Clampzyme-treatment apparently retarded initiation of fermentation, as measured by *in vitro* glucose utilisation, but this is more likely to be due to inadequate thawing before ensilage, as described in Chapter 4, Experiment 6, and not necessarily a direct effect of enzyme treatment. Examination of the ratio of metabolites (days 20-60 after ensilage) formed with enzyme-treated silage reveals a more extended homolactic fermentation of WSC to lactic acid than in the control silage, reflected by higher numbers of lactic acid bacteria in the later stages of ensilage, perhaps encouraged by the release of WSC during fibre degradation; it is not possible to deduce whether the carbohydrates were fermented immediately after release or whether a period of adaptation was necessary before fermentation continued. The sample was too small to allow analysis of the concentration of NDF and ADF in the treated forage and without these data it is difficult to describe the effects of the enzyme in more detail, but the absence of a higher concentration of acetic acid, and no increase in the concentration of residual WSC, does suggest that pentose sugars were not released. It would be interesting

to investigate the effects of an increase in the concentration of substrates during the ensilage process on the survival and growth of the micro-organisms present at the time. Chamberlain (1988) found that extra xylose applied before ensilage encouraged the proliferation of lactate-utilising species of yeasts, with the metabolism of lactic acid to acetic acid a consequence. Thus, the provision of extra fermentable substrates should be carefully controlled to ensure maximum efficiency of utilisation and may have implications for the preservation and stability of the silage.

Experiment 3 The effect of the addition, during ensilage of *L. perenne*, of glucose, fructose and xylose.

Introduction Commercial enzymes are applied to grass to release additional sugars to act as substrate for lactic acid bacteria during ensilage and, by improving the digestibility of the organic matter, to improve the efficiency of utilisation of conserved forage. The release of sugars from plant fibres by the action of fibrolytic enzymes has been demonstrated by Chamberlain *et al.* (1987) and Rauramaa *et al.* (1987), and Gonzalez-Yanez *et al.* (1990) concluded that with the application of commercial enzymes at the recommended rates of application most of the additional substrate will be likely to come from the activity of the cellulases.

Released carbohydrates may accumulate, should there be negligible microbial activity in the later stages of ensilage, and the increase in WSC may improve the nutritive value of the silage, although additional sugars may be vulnerable to losses in effluent. Conversely, additional substrates may be fermented, and the risk of yeasts capitalising on released carbohydrates in the later stages of ensilage, after the lactic fermentation has abated, is an important consideration and may have implications for the aerobic stability of the silage (McDonald, 1991).

Glucose, fructose and xylose were added to prefermented material to examine the effect on the micro-flora of an increased availability of substrate. Because of the design of the experimental silos, no effluent loss was permitted, which would help to illustrate the fate of extra substrates during storage.

Materials and methods Thawed grass (*L. perenne*) from the same harvest as that used in Experiments 1 and 2 was used in this experiment. Untreated grass was ensiled in 36 polythene bags within the anaerobic cabinet. After 30 d all the bags were opened, emptied onto the

cleaned base of the anaerobic cabinet, mixed and combined in three equal portions before the addition of one sugar to each. Sugars were added in equimolar amounts (kg/t fresh weight): glucose, 18; fructose, 18; xylose, 15. The forage was re-ensiled in the polythene bags, maintaining strict anaerobic precautions throughout. Triplicate bags were removed from the cabinet on days 0, 1, 2, 3, 4, 5, 7, 10, 20 and 30 after addition of the substrates. All fresh samples were prepared for microbial enumeration and inocula for *in vitro* assay of metabolic activity were prepared from the sub-samples taken on days 0, 2, 4, 10, 20 and 30. The forage was frozen and stored at -20°C before chemical analysis.

Statistical analysis The data were analysed by one-way analysis of variance using MINITAB.

Results The material re-ensiled after 30 d was a well preserved forage of low pH (3.65), low ammonia (1.21 g/kg DM) and acetic acid (12 g/kg DM) with negligible butyric acid concentration and moderately low (47 g/kg DM) residual WSC following a predominantly homolactic fermentation (93 g/kg DM lactic acid) (Table 5.9a). The microbial populations present are shown in Table 5.9b. There was negligible fermentative activity of the silage micro-organisms.

The changes in chemical composition following treatment of the forage are shown in Figures 5.12- 5.14 and Table 5.10. All the treated silages remained well preserved with a low pH and negligible butyric acid and only slight increases in acetic acid concentration. Extra glucose and fructose was readily fermented to 33 and 39 g/kg DM ethanol and 40 and 20 g/kg DM lactic acid (glucose- and fructose-treated material, respectively) (Figures 5.12a and 5.13a). Xylose was only gradually fermented, with an increase of 11 g/kg DM lactic acid and 5 g/kg DM ethanol (Figure 5.14a). The data in Figure 5.11a suggest that there may have been some degradation of ethanol in the glucose-treated forage, although this does not correlate well with the relatively stable concentration of acetic acid and is more likely to be due to sampling errors.

Although the rates of *in vitro* glucose uptake were generally low, there was a suggestion of revived activity, up to day 4, for all treatments; fructose treatment elicited the greatest rate of utilisation (maximum 21.1 μmol/h/g DM on day 4). An unexpected feature of the data is the rate of *in vitro* glucose utilisation by micro-organisms from the xylose-treated silo (maximum 19 μmol/h/g DM on day 4), which was not reflected in the relatively stable chemical composition.

Table 5.7a Chemical composition of the forage re-ensiled (g/kg DM, unless stated otherwise) (Experiment 3).

pH	3.65
Dry matter (g/kg)	207
Ammonia	1
Lactic acid	93
Water soluble carbohydrates	47
Ethanol	6
Acetic acid	12
Propionic acid	1
Butyric acid	tr.

Table 5.7b Microbial populations and activity on the forage re-ensiled (CFU/g DM) (Experiment 3).

Total viable organisms	$18x10^6$
Lactic acid bacteria	$19x10^6$
Coliform bacteria	$<10^2$
Yeasts	$22x10^2$
***In vitro* glucose utilisation (μmol/h/g DM)**	0

Table 5.8a Chemical composition of the silages after addition of glucose, fructose and xylose and storage for a further 30 d (g/kg DM, unless stated otherwise) (Experiment 3).

	pH	Dry matter (g/kg)	Ammonia	Lactic acid	Water soluble carbohydrates	Ethanol	Acetic acid	Propionic acid	Butyric acid
Glucose	3.60	208	1	120	17	33	15	tr.	0
Fructose	3.62	199	1	114	19	39	15	0	0
Xylose	3.62	207	1	103	109	10	9	tr.	tr.
SED (n=60)	0.02	7.5	0.1	9.5	11.1 ***	2.9 ***	2.3 *	0.6	0.6

Statistically significant differences are:- *, $P<0.05$ and ***, $P<0.001$.

Table 5.8b Microbial populations on the silage after addition of glucose, fructose and xylose and after storage for a further 30 d (CFU/g DM) (Experiment 3).

	Total viable organisms	Lactic acid bacteria	Coliform bacteria	Yeasts
Glucose	$70x10^5$	$22x10^8$	$<10^2$	$<10^2$
Fructose	$10x10^5$	$22x10^8$	$<10^2$	$340x10^3$
Xylose	$60x10^4$	$33x10^6$	$<10^2$	$81x10^3$

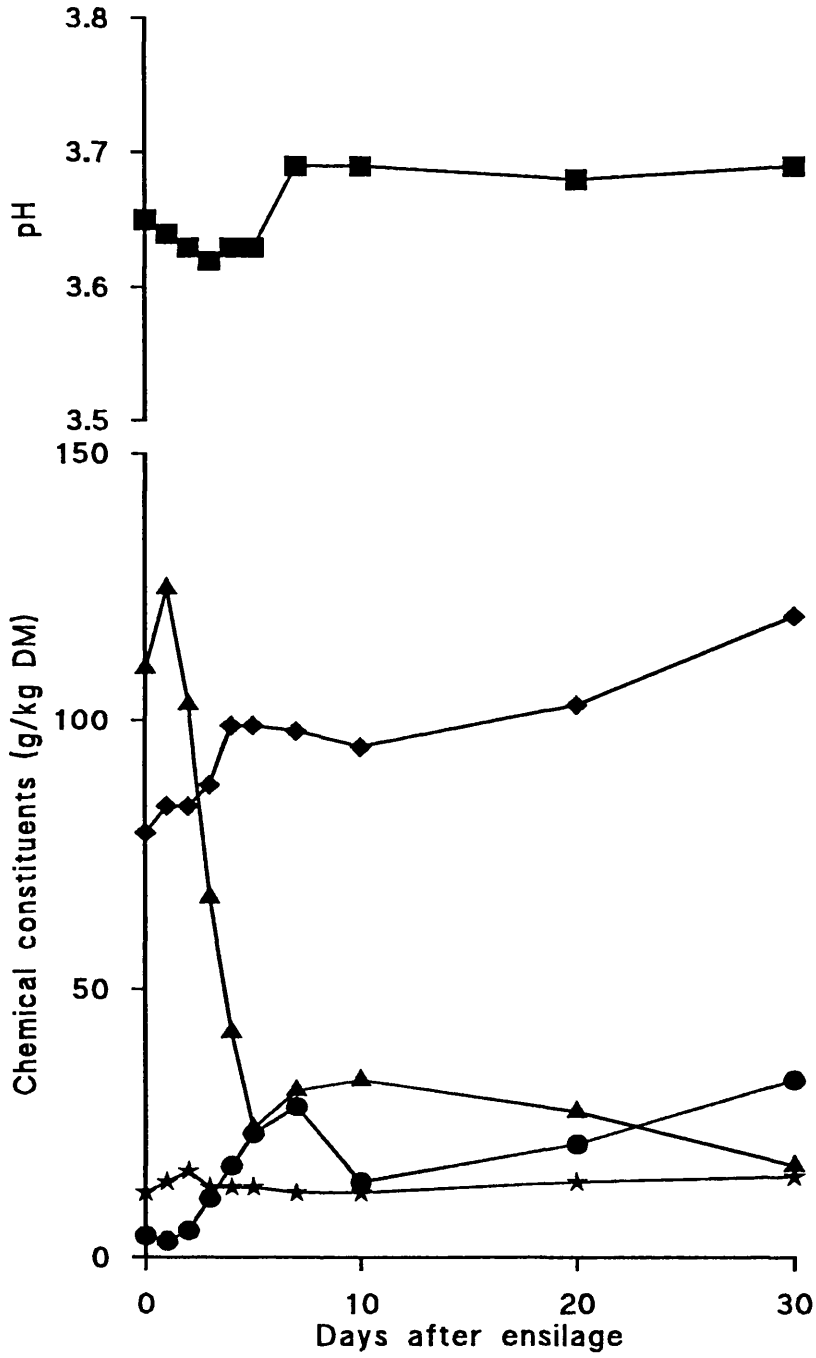


Figure 5.12a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during re-ensilage of thawed perennial ryegrass, with 18 kg/t glucose, in polythene bags within an anaerobic cabinet.

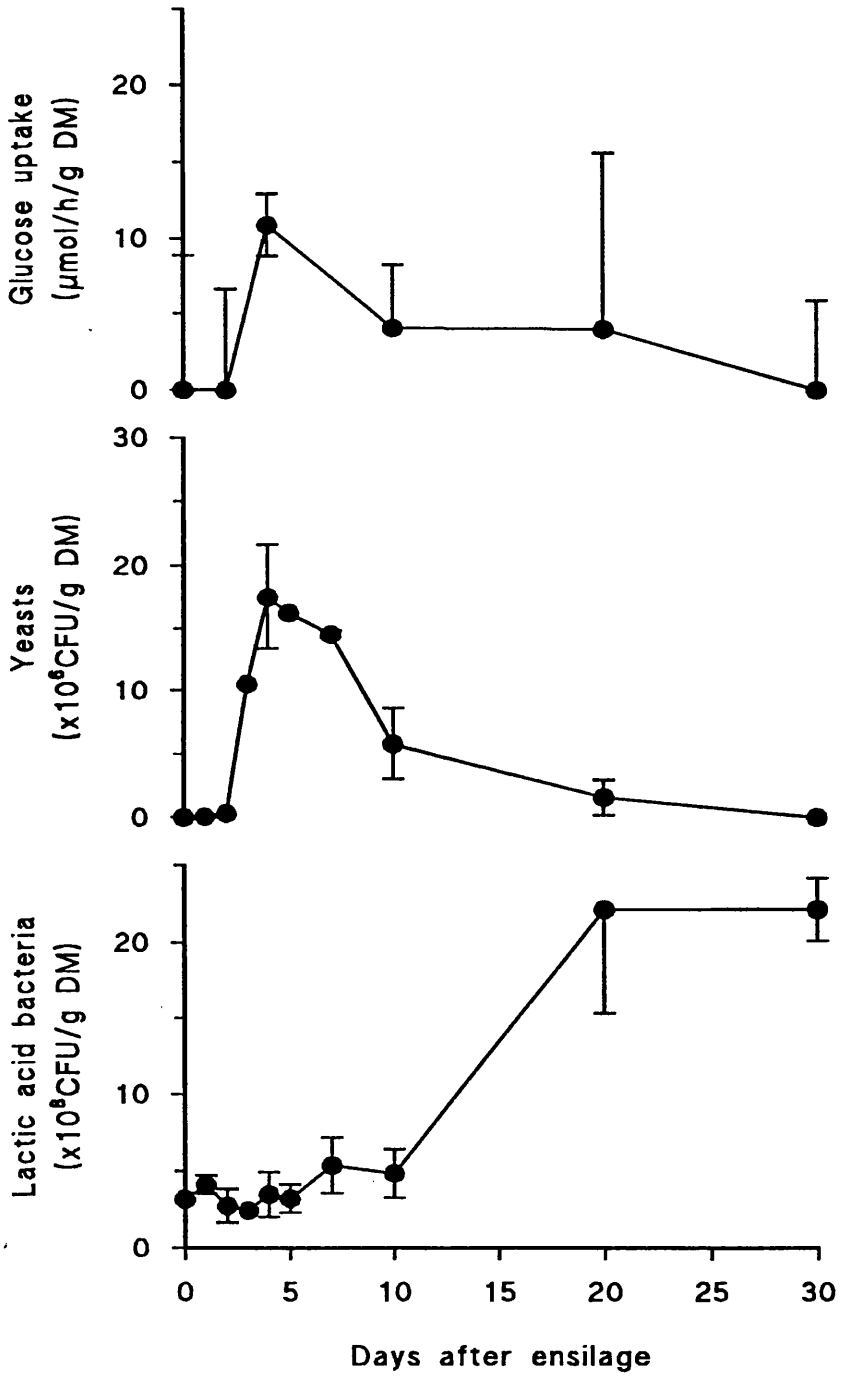


Figure 5.12b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during re-ensilage of thawed perennial ryegrass, with 18 kg/t glucose, in polythene bags within an anaerobic cabinet.
Error bars represent SE of the mean of triplicate silages.

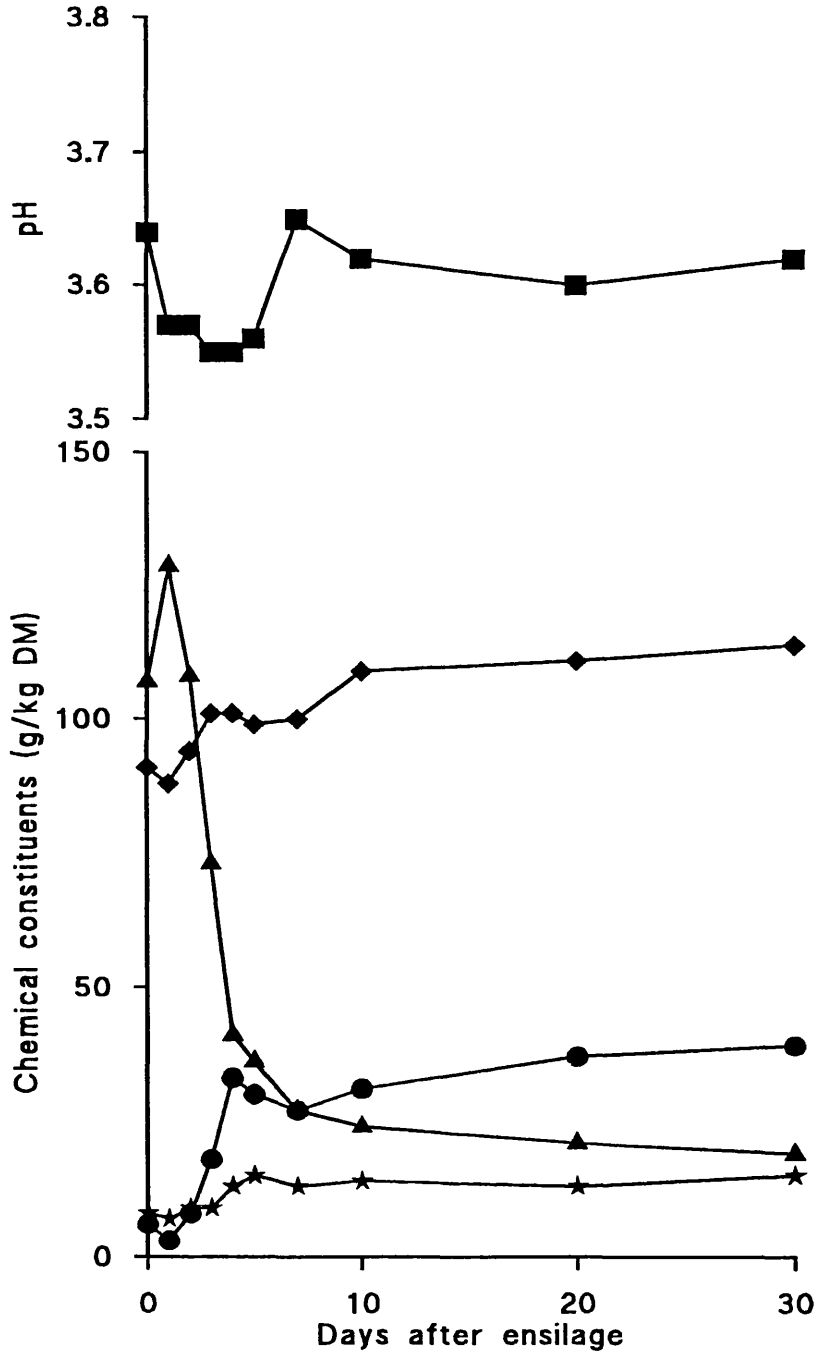


Figure 5.13a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during re-ensilage of thawed perennial ryegrass, with 18 kg/t fructose, in polythene bags within an anaerobic cabinet.

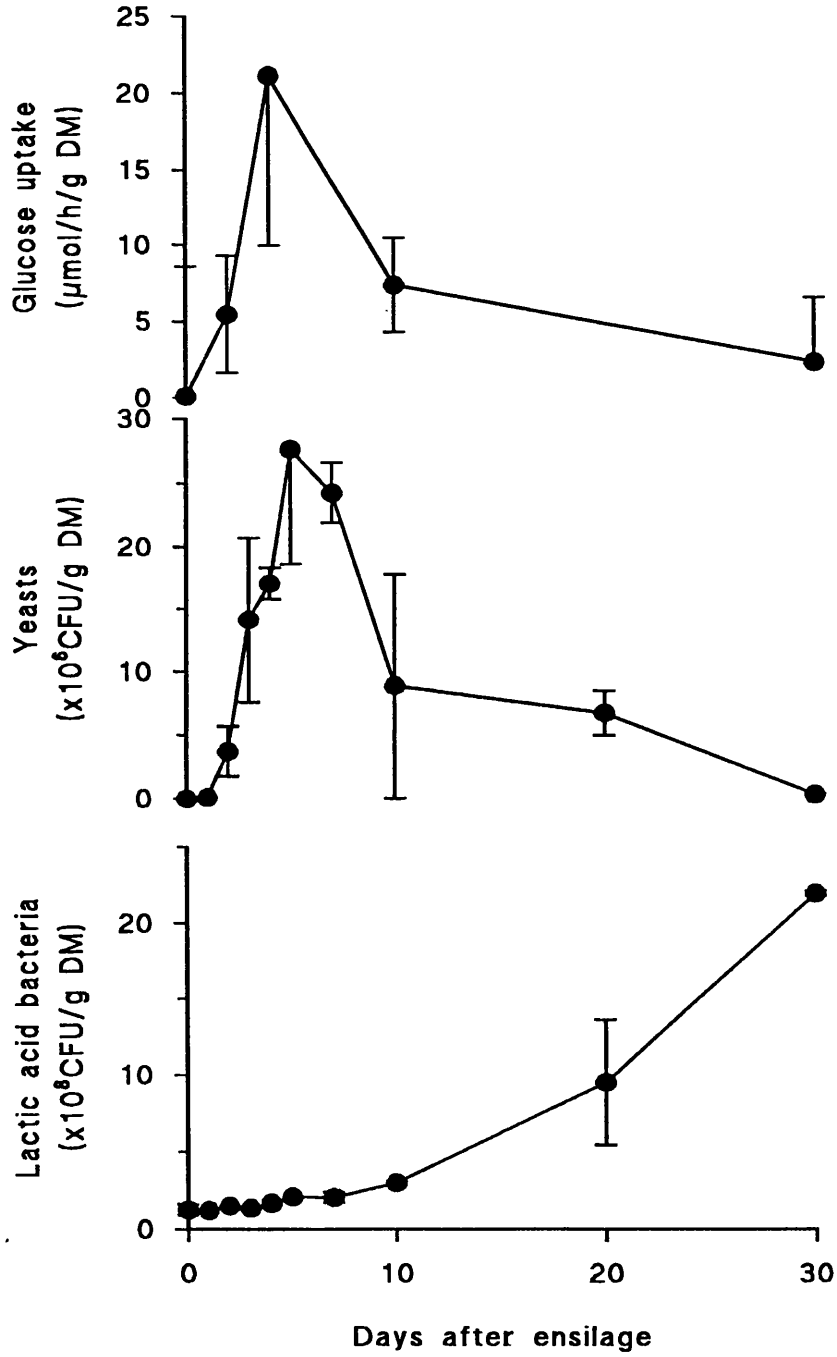


Figure 5.13b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g FW) and the rate of glucose uptake *in vitro* (µmol/h/g FW) during re-ensilage of thawed perennial ryegrass, with 18 kg/t fructose, in polythene bags within an anaerobic cabinet.
Error bars represent SE of the mean of triplicate silages.

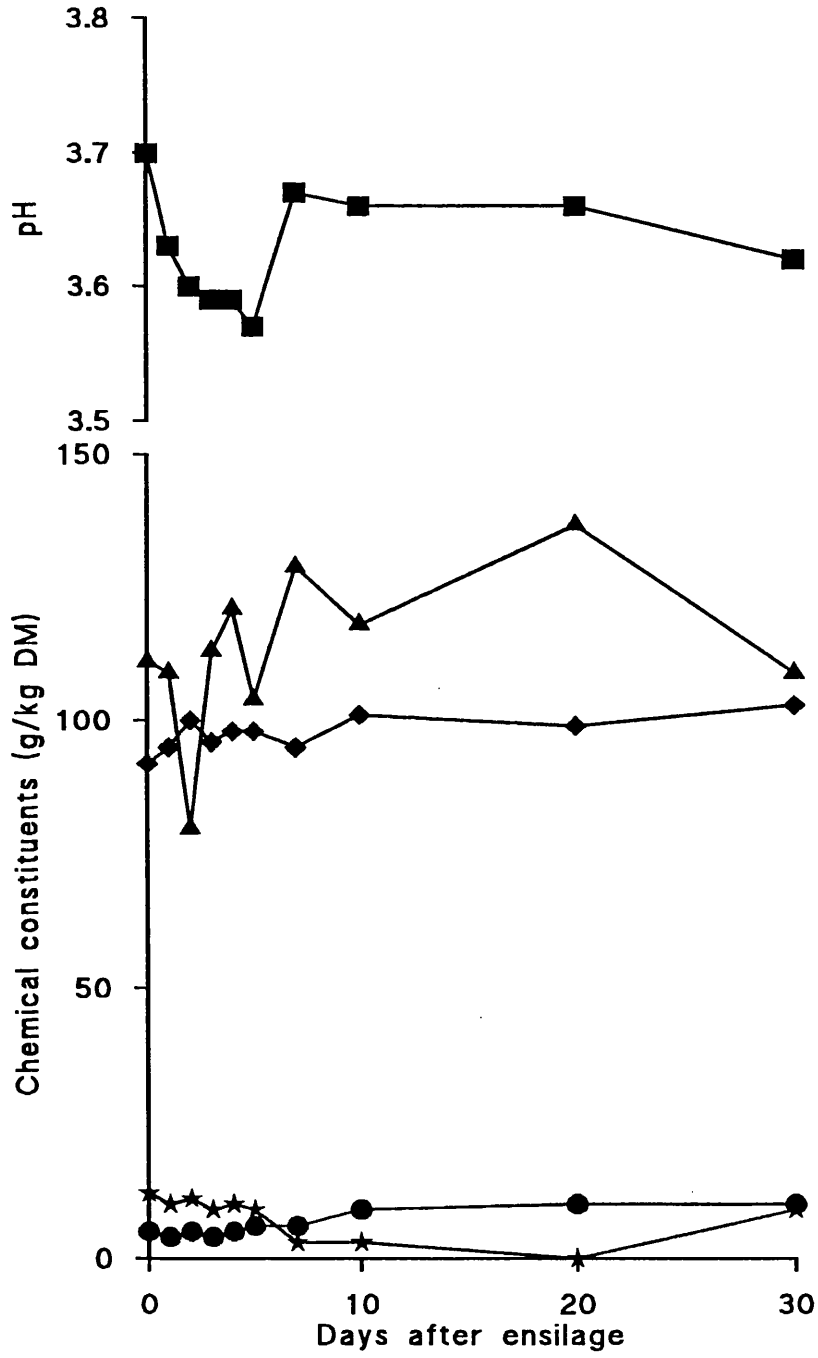


Figure 5.14a Changes in pH and the concentrations of WSC (▲), lactic acid (◆), acetic acid (★) and ethanol (●) (g/kg DM) during re-ensilage of thawed perennial ryegrass, with 15 kg/t xylose, in polythene bags within an anaerobic cabinet.

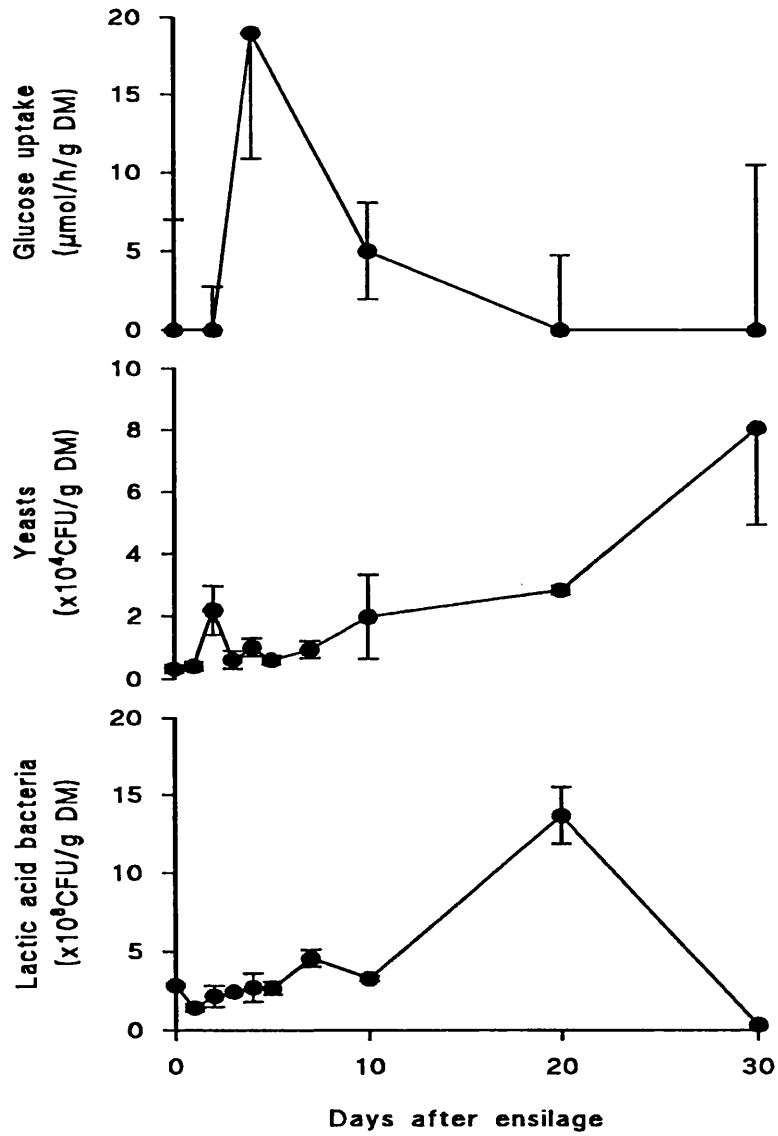


Figure 5.14b Changes in the numbers of lactic acid bacteria and yeasts (CFU/g DM) and the rate of glucose uptake *in vitro* (µmol/h/g DM) during re-ensilage of thawed perennial ryegrass, with 15 kg/t xylose, in polythene bags within an anaerobic cabinet.
Error bars represent SE of the mean of triplicate silages.

Lactic acid bacteria in the hexose-treated forages multiplied after 10 d ensilage, and, over the 30 d period, there was a 7-fold increase in numbers with glucose treatment and a 17-fold increase with fructose treatment (Figures 5.12b and 5.13b). Yeasts multiplied rapidly during the first 5 d of re-ensilage with hexose sugars (maximum $28x10^6$ CFU/g DM on day 5 and $18x10^6$ CFU/g DM on day 4 for fructose and glucose treatments respectively), corresponding with the accumulation of ethanol.

Discussion The provision of hexose sugars stimulated the continued fermentation by lactic acid bacteria and yeasts; the rapid multiplication of lactic acid bacteria after approximately 10 d suggests that a period of adjustment or adaptation preceded growth and multiplication. However, these higher numbers were not responsible for an augmented rate of lactic acid production and were probably limited by the supply of substrates, with much of the additional sugar already fermented to ethanol. The fermentation of a sample of grass from the same harvest, ensiled for 60 d in Experiments 1 and 2 (control), slowed after 20 d (Figures 5.1a and 5.7a) and, despite multiplication of yeasts in the later stages (Figures 5.1b and 5.7b), there was little change in chemical composition, with more than 40 g/kg DM WSC remaining (mean 20-60 d). The provision of extra hexose (more than 100 g/kg DM) during storage may have prompted yeast activity and encouraged adaptation of the existing lactic acid bacteria to the otherwise prohibitive conditions in the silo, and resulted in a revival of fermentation and accumulation of first ethanol and then lactic acid, with low levels of residual WSC after 30 d. *In vitro* glucose utilisation was low and, judging by the consistent pattern of increasing activity for all of the treatments, may reflect the increasing populations of micro-organisms on the silage, stimulated by the provision of extra carbohydrates; presumably, the low pH of the re-ensiled forage restricted the level of substrate utilisation in the silo. The apparent stimulation of microbial glucose utilisation by xylose addition may not have been specifically related to the chemistry of the substrate, but due indirectly to the increased concentration of carbohydrate in the silo, even though the pentose sugar was virtually unfermentable. We know little about the rates of uptake and utilisation of different substrates, and more work is needed to clarify the potential of different sugars to revive the silage micro-organisms.

In the study by Chamberlain (1988), lactate-tolerant yeasts apparently survived with xylose added before ensilage and may have been responsible for the degradation of lactic acid in the later stages of ensilage. In the experiment reported here, the predominantly homolactic fermentation and the competition for substrate by lactic acid bacteria probably deterred the activities of the majority of undesirable epiphytic micro-organisms before the application of

xylose; only 2% WSC was fermented after re-ensilage with xylose. Pentose sugars are released by hydrolysis of hemicelluose and may accumulate in the early stages of ensilage, possibly supporting populations of undesirable epiphytic micro-organisms. Release of hexose sugars, too, should be controlled to resist colonisation and proliferation by yeasts, since the apparent delay before lactic acid bacteria adaptated to the conditions of augmented substrate availability encouraged the activities of yeasts.

Conclusions The use of frozen grass derived from the same harvest for the three experiments in Chapter 5 ought to have allowed comparison between all of the treatments, as if they were imposed within a single experiment. The grass was from an early harvest and would be considered, by conventional standards, easily ensilable without silage additives. There was, however, some variation in the chemical composition of the samples of grass ensiled with the additives; the most disturbing differences were in the WSC concentrations. The interpretation of the fermentation patterns and the effect of a wide range of different additive types on the same grass is not, therefore, as simple as anticipated and the results are discussed with this precaution borne heavily in mind.

The effect of the silage additives on the fermentation characteristics is summarised in Tables 5.9, 5.10 and 5.11. Table 5.9 illustrates the effects of the range of silage additives on the chemical composition of the forage. In the control silage, the acetic acid was produced during the first 3 d of ensilage, suggesting a mixed fermentation initially. This was corroborated by the early accumulation of >0.90 of the total ammonia concentration produced during storage. Competition by the coliform bacteria or a combination of heterofermentative lactic acid bacteria and coliform bacteria may have been responsible for the mixed fermentation in the early hours of ensilage.

Only the high level of addition of formic acid inhibited fermentation and preserved WSC. The effect of 2.3 l/t formic acid may have combined with the inhibition resulting from the lactic fermentation to deter the activities of undesirable micro-organisms. Information concerning the inhibitory activity of acid combinations is not readily available, although Lindgren and Dobrogosz (1990) proposed a synergistic relationship between lactic acid and acetic acid for the inhibition of yeasts, due perhaps to an increase in concentration of undissociated acetic acid as a consequence of the strong acidic effect of lactic acid. Formic acid and lactic acid have similar dissociation constants (dissociation constant (pKa) is 3.86 for lactic acid and 3.75 for formic acid) and may act in combination to deter undesirable micro-organisms. High levels of addition of sulphuric acid inhibited fermentation in the early stages

of ensilage by virtue of the low pH, although it is not clear whether, in the later stages of fermentation, the sulphuric acid may only have been so effective against the high numbers of yeasts because of the combined effects of the production of endogenous lactic acid and competition for substrate by acid-tolerant lactic acid bacteria.

The provision of lactic acid to the grass at the point of ensilage did not restrict fermentation, rather it encouraged the survival of epiphytic micro-organisms which later contributed to the deterioration of the silage by metabolising lactic acid to acetic acid. The identity of these micro-organisms was not clarified, but what was demonstrated was the importance of an active lactic fermentation, rather than the total concentration of lactic acid in the silage, for the control of the microflora. The addition of sodium lactate and lactic acid each encouraged an augmented number of lactic acid bacteria (Table 5.10), but while lactic acid-treated forage underwent an accelerated fermentation and a rapid fall in pH, the pH of the sodium lactate-treated forage remained relatively high and did not deter the activities of contaminants such as clostridia. More work is needed to explain the apparent stimulation of lactic acid bacteria by exogenous lactic acid and sodium lactate.

In Experiment 2, although high numbers of yeasts were detected on the Ecosyl plus sucrose treatment, lactic acid bacteria fermented most of the WSC. In addition, there was apparently an extended fermentation of WSC by acid-tolerant species which obstructed the yeasts in the later stages of ensilage. It is perhaps interesting to note that at the low pH of sulphuric acid-treated silage WSC were apparently fermented but no end-products detected. The establishment of a low pH (less than approximately 3.50) may not, therefore, inhibit utilisation of substrates, but simply alter the pathways of metabolism. A detailed analysis of the supernatant after *in vitro* incubation may have revealed the nature of these fermentations. The addition of sodium bicarbonate alone maintained a much higher pH throughout the period of storage (higher than pH 7.00), and, as well as the proliferation of coliform bacteria, this may have encouraged saccharolytic bacteria and the degradation of the accumulated lactic acid. The regulation of pH may, therefore, play a principal role in the control of the changing microbial populations in the silo.

The results of Experiment 3 show that hexose sugars added to pre-fermented material are readily fermented by yeasts but that lactic acid bacteria may require a period of adaptation. The failure of yeasts to exploit the provision of extra substrates via enzyme-treatment in Experiment 2 may be due to competition by lactic acid bacteria, since the gradual release of substrates by the action of cellulolytic enzymes may avoid the requirement by lactic acid bacteria for a period of adaptation. The single addition of substrate in Experiment 3

more clearly demonstrated the reaction of silage micro-organisms confronted with an augmented pool of fermentable substrate after a period of nutrient shortage.

In the early stages of ensilage, the rapid increase in the rate of glucose utilisation *in vitro* reflects the period of greatest lactic acid production in the silo, preceding maximal microbial populations. The effect of the additive treatments on *in vitro* glucose utilisation is summarised in Table 5.11. Despite an apparent "lag" before fermentation of thawed material in Chapter 4, Experiment 6, the control silage of the experiments in Chapter 5 was not affected; there was, however, with some of the treatments an apparent delay. Whether this was a result of additive treatment or physical pretreatment of the forage was not clarified, but, for this reason, details regarding the effect of silage additives on *in vitro* glucose uptake during the first hours of ensilage should be treated with caution. The "area under the curve" was calculated from the data of the first 20 d of ensilage for each treatment; this measurement may be considered an index of the overall extent of fermentative activity, being a function of both the duration and level of activity. These data correspond well with the utilisation of WSC in the silo within the first 10 d. Except for sodium bicarbonate alone, the various treatments restricted the extent of the fermentative activity. Treatment of the forage with sodium bicarbonate apparently encouraged a more extensive fermentation than the control silage, leading to an abundance of acetic acid, butyric acid and ammonia in the silage after 60 d. With some of the treatments, in the later stages of ensilage, there was continued fermentation in the silo (for example sodium bicarbonate-, sodium lactate- and lactic acid-treated silage all underwent secondary fermentations) which was not reflected by the *in vitro* assay. More work is needed to improve the sensitivity of the measurement of metabolic activity in the later stages of ensilage, especially where the measurement of glucose utilisation *in vitro* may not be representative of fermentation pathways in the silo.

The manipulation of the silage fermentation with silage additives, and the predictability of the effects of some additive treatments, have helped to illustrate some of the mechanisms thought to regulate the ensilage process. These are discussed in the next, and final, chapter in relation to the control of the fermentation process.

Table 5.9a Summary of changes in chemical composition of the silages in Experiments 1 and 2 (g/kg DM, unless stated otherwise).

	pH				WSC content of the forage	Proportion of WSC utilised				Residual WSC (mean 20-60 d)	Lactic acid accumulation			
	2 d	5 d	10 d	Mean 20-60 d		2 d	5 d	10 d	Mean 20-60 d		2 d	5 d	10 d	Mean 20-60 d
Control	4.23	4.12	3.72	3.65	191	0.73	0.75	0.80	0.77	44	61	95	97	97
2.3 l/t formic acid	4.65	4.59	4.08	3.83	84	0	0.18	0.32	0.94	5	12	25	43	63
6 l/t formic acid	4.13	4.23	3.85	3.76	192	0.02	0.41	0.51	0.19	156	2	22	20	8
6 l/t sulphuric acid	3.58	3.63	3.44	3.34	152	0	0.15	0.50	0.73	41	10	33	57	69
lactic acid	4.20	3.96	3.88	4.04	105	0.21	0.41	0.89	0.95	5	48	84	101	46
sodium lactate	4.70	4.27	4.21	4.65	110	0.62	0.86	0.69	0.97	3	82	121	86	53
clampzyme	4.30	3.88	3.70	3.66	140	0.31	0.58	0.76	0.81	27	51	80	88	113
ecosyl plus sucrose	3.72	3.58	3.54	3.55	246	0.28	0.43	0.64	0.87	32	100	102	111	128
ecosyl plus sucrose plus sodium bicarbonate	4.57	4.40	4.24	4.28	218	0.66	0.91	0.90	0.95	11	153	175	171	194
sodium bicarbonate	9.04	8.99	8.66	8.13	77	0.25	0.91	1.00	1.00	0	19	61	69	23

Table 5.9b Summary of the changes in chemical composition of the silages used in Experiments 1 and 2 (g/kg DM, unless stated otherwise).

	Acetic acid accumulation				Ethanol accumulation				Butyric acid accumulation			
	2 d	5 d	10 d	Mean 20-60 d	2 d	5 d	10 d	Mean 20-60 d	2 d	5 d	10 d	60 d
Control	16	16	11	16	7	1	3	2	0	0	0	tr.
2.3 l/t Formic acid	2	5	6	17	1	1	1	2	0	0	0	tr.
6 l/t Formic acid	3	2	6	1	1	1	3	1	0	0	0	1
Sulphuric acid	11	3	5	4	0	0	tr.	3	0	0	0	tr.
Lactic acid	1	5	10	29	2	2	6	6	1	tr.	0	2
Sodium lactate	7	13	10	31	2	2	3	5	tr.	1	tr.	29
Clampzyme	6	13	13	12	1	1	1	3	0	0	0	1
Ecosyl plus sucrose	3	4	5	7	1	1	6	5	0	0	0	1
Ecosyl plus sucrose plus sodium bicarbonate	19	16	25	18	1	1	6	4	0	0	0	1
Sodium bicarbonate	12	29	33	85	3	3	3	6	0	0	0	76

Table 5.10 Summary of the changes in microbial populations on the silages in Experiments 1 and 2.

	Population size of lactic acid bacteria (CFU/g DM)				Period of survival of coliform bacteria	Maximum population of yeasts
	After 2 d	After 5 d	After 10 d	Mean 20-60 d		
Control	$246x10^8$	$205x10^8$	$139x10^8$	$264x10^6$	3 d	$64x10^4$ at 60 d
2.3 l/t Formic acid	$265x10^6$	$318x10^7$	$103x10^8$	$186x10^7$	4 d	$215x10^4$ at 10 d
6 l/t Formic acid	$347x10^6$	$101x10^7$	$139x10^7$	$39x10^6$	2 d	Not detected
6 l/t Sulphuric acid	$549x10^6$	$202x10^8$	$177x10^8$	$565x10^6$	3 d	$236x10^5$ at 15 d
Lactic acid	$420x10^7$	$145x10^9$	$244x10^8$	$165x10^8$	2 d	$10x10^6$ at 10 d
Sodium lactate	$264x10^8$	$234x10^9$	$335x10^8$	$822x10^7$	1 d	$125x10^2$ at 2 d
Clampzyme	$620x10^7$	$194x10^8$	$482x10^8$	$389x10^6$	4 d	$306x10^3$ at 4 d
Ecosyl plus sucrose	$324x10^9$	$134x10^9$	$89x10^6$	$239x10^6$	1 d	$98x10^6$ at 10 d
Ecosyl plus sucrose plus sodium bicabonate	$645x10^9$	$204x10^9$	$130x10^8$	$149x10^7$	5 d	$46x10^4$ at 15 d
Sodium bicarbonate	$120x10^7$	$518x10^8$	$252x10^8$	$862x10^6$	20 d	$421x10^2$ at 40 d

Table 5.11 Summary measures of the changes in rate of glucose utilisation *in vitro* of an inoculum prepared from the forages used in Experiments 1 and 2.

	Maximum rate of glucose uptake (μmol/h/g DM)	Period during which glucose uptake >10 μmol/h/g DM	Mean rate of glucose uptake (μmol/h/g DM)	"Area under the curve" for first 20 d
Control	66.7 at 2 d	1-60 d	33.6	627
2.3 l/t Formic acid	44.8 at 4 d	2-10 d	16.3	295
6 l/t Formic acid	24.7 at 4 d	3-4 d	9.2	205
6 l/t Sulphuric acid	70.4 at 5 d	3-7 d	20.9	358
Lactic acid	76.6 at 2 d	2-10 d	31.3	506
Sodium lactate	59.9 at 5 d	2-10 d	22.7	435
Clampzyme	65.3 at 4 d	2-10 d	26.0	469
Ecosyl plus sucrose	85.9 at 2 d	1-5 d	32.0	388
Ecosyl plus sucrose plus sodium bicarbonate	97.5 at 4 d	1-10 d	33.1	502
Sodium bicarbonate	81.1 at 3 d	1-20 d	33.6	644

6. GENERAL DISCUSSION

As discussed in Chapter 1, factors affecting the ensilage process include the composition of the forage, the thermal and physical conditions in the silo and the fermentation by different micro-organisms on the forage. The interaction of these factors results in a high degree of variability in silage quality, a certain unpredictability in the success of the ensilage process in conserving the forage, and an uncertainty about the optimum management practices for a particular crop and silo. The use of silage additives may offer the potential for more control over the process. Experiments reported here, in which a variety of different additive treatments were applied, serve to illustrate the very considerable range in the type and extent of fermentation that can occur when grass is ensiled. For example, in Chapter 5, Experiment 1, preservation of 156 g/kg DM WSC (0.81 of that present in the original forage) by 6 l/t formic acid contrasts with the stimulation of the fermentation of WSC, by inoculation and treatment with sodium bicarbonate, to 194 g/kg DM lactic acid and 18 g/kg DM acetic acid. There is a strong theoretical argument in favour of restricting fermentation in the silo to the minimum compatible with good preservation (Chamberlain, 1987) since the fermentation of WSC in the silo markedly reduces the supply of readily fermentable substrate for micro-organisms in the rumen and can, consequently, reduce the ATP available for microbial protein synthesis, with adverse effects on the supply of amino acids to the host animal. There are, however, other complicating factors to consider, relating to the influence of the extent of fermentation and subsequent rumen fermentation patterns on nutrient utilisation, before reaching definite conclusions about the ideal qualitative composition of silage. But, whatever the ideal silage composition, from a nutritional standpoint, may be, it is clear that a greater degree of control over the fermentation is required if we are ever to produce consistently silages of the desired type.

To be able to achieve a desired extent of fermentation requires a knowledge of the factors which determine microbial activity. Pitt *et al.* (1985) produced a comprehensive discussion concerning the factors controlling the lactic fermentation but were unable to show which factors may regulate the demise and decline of silage microbial populations. Specifically, what remains unclear is the regulation of the senescence of the lactic acid bacteria; we know little more than that their activity is stopped by an ill-defined combination of pH and lactic acid concentration, and that there are considerable differences between species and strains in their susceptibility to inhibition.

Clearly pH plays an important role in the deterrence of undesirable contaminating

bacteria; *Clostridia* spp. and coliform bacteria are not tolerant of low pH conditions, whereas a high acid tolerance is a distinctive feature of the lactic acid bacteria, though of course yeasts are not inhibited by the pH levels reached during ensilage. Addition of sulphuric acid (6 l/t) lowered the pH of the forage and restricted fermentation in the silo, although the high levels of glucose uptake *in vitro* (nearly 3 times higher than that for 6 l/t formic acid treatment) showed that this was not a specific antimicrobial effect of the acid molecule itself. Inhibition of fermentation may be achieved by high levels of application of formic acid, as shown in Chapter 3 and Chapter 5, Experiment 1, to such an extent that only low levels of lactic acid accumulate, and hence no drastic drop in pH ensues (Chamberlain *et al.*, 1982). In this case, the antibiotic effect of the organic acids may over-ride the effect of pH.

If the inhibition of fermentation were attributable to a critical level of lactic acid, or other fermentation products, it would be reasonable to expect to find a certain maximum limit to the production of acids. The stimulation of fermentation of available substrates to a range of lactic acid concentrations in Chapter 3, Experiment 2 and Chapter 5, Experiment 2 (97-194 g/kg DM), and the successful preservation at all levels, suggests that total lactic acid concentration does not determine the end-point of fermentation. The addition of extra lactic acid in Chapter 5, Experiment 1 restricted metabolic activity (glucose uptake *in vitro*) but not to a sufficient extent to lead to a premature end to the fermentation process, and, in the later stages of fermentation, had adverse effects on the stability of the silage, presumably due to the survival of lactate-assimilating micro-organisms.

The concentration of undissociated lactic acid may play an important role in regulating the extent of the lactic fermentation and the survival of lactic acid bacteria. At the low pH levels found in silage, much of the organic acid content (lactic acid and acetic acid) is present in the undissociated form and thus able to penetrate the microbial cell, reduce the intracellular pH and interfere with essential metabolic functions (Baird-Parker, 1980; Smulders *et al.*, 1986); in the *in vitro* studies, glucose uptake was reduced by almost 30% per mmol undissociated lactic acid present. Lindgren and Dobrogosz (1990) explained that acetic acid was more inhibitory against yeasts than lactic acid since acetic acid has 2 to 4 times more of the acid in the undissociated state than lactic acid at pH 4.0-4.6. This may have useful implications for the aerobic stability of some silages in which especially high concentrations of acetic acid have been detected (Henderson *et al.*, 1987a, b; Honig and Pahlow, 1990; Selmer-Olsen, 1990; Davies, 1990).

The accumulation of undissociated lactic acid during the ensilage of the untreated forages used in Chapters 3, 4 and 5 is discussed below (Table 6.1); because the action of

silage additives may distort the inhibition of the fermentation, only the untreated silages were considered. The conditions prevalent at the end of the initial burst of activity were examined, while the silage micro-flora comprised predominantly lactic acid bacteria and before any lactic acid turnover or secondary fermentation complicated the calculations. The initial period of fermentation was identified as that which lasted until the pH reached a relatively stable level and, because of the difficulties and possible errors associated with single timed-sample data, approximate concentrations were calculated using values for the 10 days around this asymptote.

If the hypothesis is correct, the final concentration of undissociated lactic acid must be that which restricts further fermentation in the silo, at least by lactic acid bacteria, if not yeasts too. Although the control silages were all well preserved, there was no clear relation between the different measurements of the extent of fermentation. Multi-variate analysis (Principal Component Analysis, Minitab) did not reveal any relationship between the theoretical end-point of fermentation and silage composition either. Thus, it is unreasonable to suggest that any one of the parameters, including undissociated lactic acid, is constant at a theoretical end-point of fermentation. Nevertheless, it was clear that conditions which limited the expression of fermentative activity of lactic acid bacteria soon developed in the silo; microbial activity and lactic acid production peaked at 2-3 d after ensilage, followed by maximal microbial population sizes.

As ryegrass matures, the content of organic acids decreases (Waite *et al.*, 1964) and so the buffering capacity of the crop may be expected to fall. However, late-season regrowths are likely to contain low concentrations of WSC and have a high protein content yet, being physiologically immature, may also have a high buffering capacity. The grass used in Chapter 3 and Chapter 4 (Experiment 4) was from a late-season regrowth (September-October), while that used for Chapter 4 (Experiment 5 and Experiment 6) and Chapter 5 was from an earlier harvest (end of June). With the exception of the silage in Chapter 4, Experiment 4, lactic acid production with the late-season grasses was not limited by substrate supply and was sufficient to maintain stability. Persistence of relatively high pH, and consequently low concentrations of undissociated lactic acid, was consistent with a higher buffering capacity of the regrowth swards.

Table 6.1 Composition of forages after the initial burst of fermentation (g/kg DM, unless stated otherwise).

	pH	Dry matter (g/kg)	Water soluble carbohydrates	Lactic acid		Acetic acid	Ethanol	Undissociated lactic acid (mmoles/l)
				g/kg DM	mmoles/l			
Chapter 3, Experiment 1 (10-20 d)	4.38	150	21	91	178	7	4	41
Chapter 3, Experiment 2 (10-20 d)	4.24	158	50	99	206	7	3	58
Chapter 3, Experiment 3 (10-20 d)	4.18	147	15	105	201	19	-	65
Chapter 4, Experiment 4 (5-15 d)	4.30	190	8	55	143	11	-	39
Chapter 4, Experiment 5a (5-15 d)	3.64	184	97	130	326	14	6	203
Chapter 4, Experiment 5b (5-15 d)	3.64	203	100	147	416	8	9	258
Chapter 4, Experiment 6 (5-15 d)	3.72	206	53	88	254	14	4	147
Chapter 5 (10-20 d)	3.69	221	43	103	325	14	3	194

A sufficiency of fermentable carbohydrate is important to allow adequate fermentation by lactic acid bacteria, accumulation of lactic acid and fall in pH to ensure a successful fermentation and to maintain preservation. There was a suggestion in Chapter 4 (Experiment 4) and in Chapter 5 (Experiment 1) that, after exhaustion of WSC, lactic acid bacteria had in the later stages of ensilage metabolised lactic acid to acetic acid to derive energy for growth. This suggestion is supported by the similar findings of Henderson *et al.* (1987a, b). Thus, silages containing high concentrations of acetic acid may either be a consequence of slow fermentation in the crucial, early stages of ensilage leading to high pH and the prevention by contaminating micro-organisms of the efficient conversion of WSC to lactic acid, or the result of a rapid initial fermentation leading to a shortage of available substrates which, in turn, has led to a secondary fermentation and a rise in pH.

However, there are potential drawbacks to having an efficient lactic acid fermentation, or to inhibiting the fermentation, where this leaves excess fermentable WSC available since yeasts apparently thrive in these silages (Chapter 3, Experiments 1, 2 and 3) and readily metabolise residual WSC leading to substantial losses in nutritive value and possibly also increasing the likelihood of aerobic deterioration on opening the silo. In the light of advances in the production of viable inoculants of homolactic fermenters, the concentration of WSC in the crop required to ensure successful preservation may be substantially less than currently perceived. In this context there is also a need to refine the use of enzyme additives for the provision of extra substrate during ensilage via the hydrolysis of polysaccharides so making the success of the fermentation less dependent on the WSC content of the crop. From the stand-point of making a significant contribution to the silage fermentation, the timing of the release of extra substrate is all important and for maximum benefit should be encouraged in the crucial early stages of ensilage; after the lactic fermentation has stopped, yeasts may more readily utilise additional WSC before lactic acid bacteria (Chapter 5, Experiment 3). Attention should be directed towards preventing the needless fermentation of the products of cell wall breakdown or, alternatively, selecting the enzyme complexes to produce degradation products that are non-fermentable in the silo, but available for ruminal metabolism. For example, while 0.3-0.5 added xylose remained largely unfermented in the silo in the study by Chamberlain (1987), and more so in Experiment 3, Chapter 5, Chamberlain *et al.* (1985) suggested that xylose might be more efficiently used for ruminal microbial protein synthesis than would hexose sugars. However, cell wall carbohydrates may be hydrolysed in the rumen anyway, and a premature release of carbohydrates in the silo may lead to unnecessary energy losses if not via fermentation then via losses in effluent. A weakening of the fibre matrices and an

increase in the rate of digestion of cellulose in the rumen, rather than increased overall organic matter digestibility, may be a more attractive objective, and may ultimately improve the efficiency of utilisation of preserved forage by rebalancing to some extent the poorly synchronised release of nitrogen and energy in the rumen. Choung and Chamberlain (1992) recently demonstrated an improved amino acid supply to the small intestine with enzyme-treated silage, which may reflect a more efficient digestion of dietary forage and better ruminal nutrition.

This series of experiments has encompassed a wide range of additive types, and has produced a diverse range of silages of widely different chemical composition. The results have shown that pH may play an important role in the deterrence of undesirable micro-organisms, that competition for substrate, or limited availability of substrate, undoubtedly suppresses the fermentative activity of the microbiota as a whole, and that undissociated lactic acid contributes to the antibiosis of the silage environment. It is possible to predict to some extent the conditions predisposing to the success or failure of fermentation, and to the same degree to suggest, given the chemical composition and the microbial constitution of the crop, suitable additives to assist the preservation process. But, because of the dynamic, multi-variate nature of silage, there is no independence and one component cannot be separated and manipulated without affecting another. In common with most ecosystems, there is the potential for all of the microbial communities in the silo to survive and grow, given the right opportunities. The key to the control and manipulation of the process may be to selectively encourage one member of the community, to dominate the environment, to the exclusion of all others.

Although, because of the complexity of the silage ecosystem, it was difficult to demonstrate more clearly the regulatory mechanisms, the techniques developed offer genuine promise for future studies. Development of a simple, fast method of ensilage on a laboratory scale, to provide small amounts of easily accessible, homogeneous duplicate and triplicate samples at timed intervals throughout the fermentation (Chapter 4), allowed detailed study of the silage fermentation. An important advantage of loosely packing material in the sterile polythene bags inside the anaerobic cabinet was the ease and efficiency with which additions could be made, thus allowing anaerobic manipulations of the fermentation. Anaerobic cabinets are expensive and not readily available in many laboratories, but cheaper glove bags, available from several commercial suppliers (e.g. Sigma Chemical Company) may represent an equally effective alternative. There were small, significant differences in fermentation characteristics between forage ensiled in measuring cylinder silos and that ensiled in polythene

bags within the anaerobic cabinet, possibly reflecting ingress of small volumes of air to the measuring cylinder silos. While it might therefore be argued that the anerobic cabinet may not recreate exactly practical silage making conditions, where complete anaerobiosis is rarely achieved, it does allow the specific effects of silage additives on the micro-flora and chemical composition to be demonstrated.

Freezing did not adversely affect the ensilability of the grass. However, in retrospect, the period of thawing before ensilage should be carefully controlled to avoid excessive respiration losses, and ensilage with microbial inoculants may overcome the initial "lag" period problems experienced in some of the experiments.

There are valid conclusions to be drawn from enumeration of micro-organisms, especially during the early stages of ensilage, when the higher numbers of microbes reflect the rapid fermentation. However, in the later stages of ensilage the interpretation of microbial enumeration is complicated, since the relatively stable chemical composition of the silage does not reflect the presence of high numbers of microbes. The assay of fermentative activity (glucose uptake), *in vitro*, defines the status of the bacterial population more accurately than determinations of the actual number of micro-organisms. The assay was sensitive enough to identify the effects of different additives on the initial rate of fermentation (Table 5.16), and also aided interpretation of the unusual fermentations in Chapter 4 (Experiment 4), when lactic acid was metabolised due to a shortage of fermentable substrate, and in Chapter 5 (Experiment 2), where treatment of the forage with sodium bicarbonate maintained an enhanced level of metabolic activity among the silage micro-flora. In the later stages of ensilage, however, chemical changes may not always be reflected by changes to *in vitro* glucose utilisation. Treatment with lactic acid and sodium lactate resulted in poorly preserved material, and in these instances the measurement of lactic acid degradation *in vitro* may provide a more meaningful index of fermentative activity in the silo. Similarly, the rate of production of lactic acid or ethanol or other metabolites *in vitro* may be measured and used to define the involvement of specific groups of micro-organisms. The *in vitro* fermentation may be manipulated by inclusion of lactic acid or antibiotics to control different groups of microbes or, alternatively, specific strains may be incubated to investigate the effect of silage additives or other perturbations on metabolic activity. These approaches will provide a more rapid and more reliable assessment of the microbial processes underlying the chemical changes occurring in the silo than conventional enumeration methodology.

REFERENCES

Agricultural Research Council (ARC), 1976. *The Nutrient Requirements of Farm Livestock, Number 4; Composition of British feeding stuffs*, Agricultural Research Council, London.

Akin, D.E. 1986. In *Control of Digestion and Metabolism in Ruminants*, pp. 139-157, Eds. Milligan, L.P., Grovum, W.L. and Dobson, A.; Prentice-Hall, Englewood Cliffs, New Jersey.

Allen, L.A., Harrison, J., Watson, S.J. and Ferguson, W.S. 1937. *Journal of Agricultural Science, Cambridge* **26**, 271-293.

Appleton, M and Done, D. 1987. In *Proceedings of the Eighth Silage Conference*, pp 15-16, Institute of grassland and animal production, Hurley.

Axelsson, L.T., Chung, T.C., Dobrogosz, W.J. and Lindgren, S.E. 1989. *Microbial Ecology of Health and Disease* **2**, 131-136.

Baird-Parker, A.C. 1980. In *Microbial Ecology of Foods*, pp. 126-135, Ed. Silliker, J.H.; Academic Press, New York.

Barker, H.A. 1961. In *The Bacteria, Volume 2*, pp. 151-207, Eds. Gunsalus, I.C. and Stanier, R.Y.; Academic Press, New York and London.

Bauchop, T. 1980. In *Contemporary Microbial Ecology*, pp. 305-326, Eds. Ellwood, D.C., Hedger, J.N., Latham, J.N., Lynch, J.M. and Slater, J.H.; Academic Press, London.

Beck, T. 1978. In *Fermentation of silage - a review*, pp 61-115. Ed. McCullough, M.E.; National Feed Ingredients Association, Iowa.

Blickstad, E., Enfors, S.-O. and Molin, G. 1981. *Journal of Applied Bacteriology* **50**, 493-504.

Bousset-Fatianoff, N., Gouet, P., Bousset, J. and Contrepois, M. 1971. *Annals de Biologie Animale, Biochemie et Biophysique* **11**, 715-723.

Bryant, M.P. and Burkey, L.A. 1953. *Journal of Dairy Science* **36**, 205-217.

Cassio, F. Leao, C. and van Uden, N. 1987. *Applied and Environmental Microbiology* **53**, 509-513.

Castle, M.E., Gill, M.S. and Watson, J.N. 1983. *Grass and Forage Science* **38**, 135-140.

Castle, M.E. and Watson, J.N. 1970. *Journal of the Brittish Grassland Society* **25**, 65-70.

Castle, M.E. and Watson, J.N. 1976. *Journal of the British Grassland Society* **31**, 191-195.

Chamberlain, D.G. 1987. *Process Biochemistry*. April 1987. 60-63.

Chamberlain, D.G. 1988. *Journal of the Science of Food and Agriculture* **46**, 129-138.

Chamberlain, D.G., Martin, P.A. and Robertson, S. 1990a. In *Proceedings of the Ninth Silage Conference*, pp. 122-123, University of Newcastle Upon Tyne, Newcastle.

Chamberlain, D.G., Martin, P.A., Robertson, S. and Hunter, E.A. 1992. *Grass and Forage Science* **47**, 391-399.

Chamberlain, D.G., Robertson, S., Martin, P.A. and Jackson, D.A. 1990b.In *Proceedings of the Ninth Silage Conference*, pp. 120-121, University of Newcastle Upon Tyne, Newcastle.

Chamberlain, D.G. and Robertson, S. 1992. *Animal Feed Science and Technology* **37**, 257-264.

Chamberlain, D.G., Thomas, P.C. and Anderson, F.J. 1983. *Journal of Agricultural Science, Cambridge* **101**, 47-58.

Chamberlain, D.G., Thomas, P.C. and Robertson, S. 1987. In *Proceedings of the Eighth Silage Conference*, pp.31-32, Institiute of Grassland and Animal Production, Hurley.

Chamberlain,D.G., Thomas, P.C., Wilson, W., Newbold, C.J. and MacDonald, J.C. 1985. *Journal of Agricultural Science, Cambridge* **104**, 331-340.

Chamberlain, D.G., Thomas, P.C. and Robertson, S. 1987. In *Proceedings of the Eighth Silage Conference*, pp. 31-32, Institute of Grassland and Animal Production, Hurley.

Chamberlain, D.G., Thomas, P.C. and Quig, J., 1986. *Grass and Forage Science* **41**, 31-48.

Chamberlain, D.G., Thomas, P.C. and Wait, M.K. 1982. *Grass and Forage Science* **37**, 159-164.

Chamberlain, D.G. and Quig, J. 1987. *Journal of the Science of Food and Agriculture* **38**, 217-228.

Choung, J.J. and Chamberlain, D.G. 1992. *Journal of the Science of Food and Agriculture* **60**, 525-527.

Clark, D.S. and Takacs, J. 1980. In *Microbial Ecology of Foods*, pp. 170-180, Ed. Silliker, J.H.; Academic Press, London.

Cottyn, B.G. and Boucque, G.N. 1968. *Journal of Agricultural and Food Chemistry* **16** 105-107.

Daniel, P., Honig, H., Weise, F. and Zimmer, E. 1970. *Das Wirtschaftseigene Futter* **16**, 239-256.

Davies, O.D. 1990. In *Proceedings og the Ninth Silage Conference*, pp. 74-75, University of Newcastle Upon Tyne, Newcastle.

Deschard, G., Tetlow, R.M. and Mason, V.C. 1987. *Animal Feed Science and Technology* **18**, 283-293.

Dewar, W.A., McDonald, P. 1961. *Journal of the Science of Food and Agriculture* **12**, 790-795.

Dewar, W.A., McDonald, P. and Whittenbury, R. 1963. *Journal of the Science of Food and Agriculture* **14**, 411-417.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. 1956. *Analytical Chemistry* **28**, 350-356.

Elsden, S.R. and Gibson, Q.H. 1954. *Biochemical Journal* **58**, 154-158.

Galbraith, H., Miller, T.B. Paton, A.M. and Thompson, J.K. 1971. *Journal of Applied Bacteriology* **34**, 803-813.

Garvie, E.I. 1967. *Journal of Dairy Research* **34**, 31-38.

Garvie, E.I. 1969. *Journal of General Microbiology* **58**, 85-94.

Garvie, E.I. 1980. *Microbiological Reviews* **44**, 106-139.

Gasser, F. 1970. *Journal of General Microbiology* **62**, 223-239.

Gibson, T. 1965. *Journal of Applied Bacteriology* **28**, 56-62.

Gibson, T., Stirling, A.C., Keddie, R.M. and Rosenberger, R.F. 1958. *Jounal of General Microbiology* **19**, 112-129.

Gibson, T., Stirling, A.C., Keddie, R.M. and Rosenberger, R.F. 1961. *Journal of Applied Bacteriology* **24**, 60-70.

Gill, M., Siddons, R.C., Beever, D.E. and Rowe, J.B. 1986. *British Journal of Nutrition* **55**, 399-407.

Gilland, S.E. and Speck, M.L. 1977. *Journal of Food Protection* **40**, 820-823.

Goering, H.K. and Gordon, C.H. 1973. *Journal of Dairy Science* **56**, 1347-1351.

Goering, H.K. and Van Soest, P.J. 1970. Forage Fibre Analysis in *Agricultural Handbook N°379.*

Gonzalez-Yanez, M., Henderson, A.R., McGinn, R. and Kerr, W.D. 1990. In *Proceedings of the Ninth Silage Conference*, pp. 16-17, University of Newcastle Upon Tyne, Newcastle.

Gordon, F.J. 1987. In *Proceedings of the Eighth Silage Conference*, pp. 19-20. Institute of Grassland and Animal Production, Hurley.

Gordon, F.J. 1989a. *Grass and Forage Science* **44**, 169-179.

Gordon, F.J. 1989b. *Grass and Forage Science* **44**, 353-357.

Gordon, G.L. and Doelle, H.W. 1975. *Journal of Bacteriology* **121**, 600-607.

Gouett, P.H., Contrepois, M., Bousset, J. and Bousset-Fatianoff, N. 1972. *Annals of Biology and Biochemistry and Biophysics* **12**, 159.

Gouett, P.H., Fatianoff, N. and Sauvant, D. 1970. *Agricultura* **18**, 61-70.

Hardy, K.G. 1975. *Bacteriological Reviews* **39**, 464-515.

Henderson, A.R. 1984. In *Edinburgh School of Agriculture Annual Report*, pp. 102-104.

Henderson, A.R., Anderson, D.H., Neilson, D.R. and Hunter, E.A. 1987a. In *Proceedings of the Eighth Silage Conference*, pp. 13-14, Institute of Grassland and Animal Production, Hurley.

Henderson, A.R., Anderson, D.H., Scott, N.A. and Hunter, E.A. 1990. In *Proceedings of the Ninth Silage Conference*, pp. 70-71, University of Newcastle Upon Tyne, Newcastle.

Henderson, A.R. and McDonald, P. 1984. *Research and Development in Agriculture* **1**, 171.

Henderson, A.R., McDonald, P. and Woolford, M.K. 1972. *Journal of the Science of Food and Agriculture* **23**, 1079-1087.

Henderson, A.R., McGinn, R. and Kerr, W.D. 1987b. In *Proceedings of the Eighth Silage Conference*, pp. 29-30, Institute of Grassland and Animal Production, Hurley.

Heron, S.J.E. 1985. PhD Thesis, University of Edinburgh.

Hickey, M.W., Hillier, A.J. and Jago, G.R. 1983. *Australian Journal of Biological Science* **36**, 487-496.

Honig, H. 1975. *Das Wirtschaftseigene Futter* **21**, 25-32.

Honig, H. and Pahlow, G. 1990. In *Proceedings of the Ninth Silage Conference*, pp. 18-19. University of Newcastle Upon Tyne, Newcastle.

Hooper, P.G. and Armstrong, D.G. 1987. In *Proceedings of the Eighth Silage Conference*, pp. 17-18, Institute of Grassland and Animal Production, Hurley.

Hoxey, R.P. and Billington, R.S. 1987. In *Proceedings of the Eighth Silage Conference*, pp. 131-132, Institute of Grassland and Animal Production, Hurley.

Huida, L. 1982. *Journal of the Agricultural Society in Finland* **54**, 137-143.

Hurst, A. 1981. *Advances in Applied Microbiology* **27**, 85-123.

Ingram, M., Ottoway, F.J.H. and Coppock, J.B.M. 1956. *Chemistry and Industry* **42**, 1154-1165.

Jacobs, J.L. and McAllan, A.B. 1987. In *Proceedings of the Eighth Silage Conference*, pp. 107-108, Institute of Grassland and Animal Production, Hurley.

Jay, J.M. 1982. *Applied and Environmental Microbiology* **44**, 525-532.

Jonsson, A. 1989. *Swedish University of Agricultural Sciences, Report N°42*, Uppsala.

Jonsson, A. and Pahlow, G. 1984. *Animal Research and Development* **20**, 7-22.

Jouany, J.P. and Ushida, K. 1989. In *The Rumen Ecosystem*, pp. 139-150, Eds. Hoshino,S., Onodera, R., Minato, H. and Itabashi, H.; Japan Scientific Press, Tokyo.

Kandler, O. and Weise, N. 1986. In *Bergeys Manual of Systematic Bacteriology, Volume 2*, pp. 1209-1234, Eds. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G.; Williams and Wilkins, Baltimore.

Keddie, R.M. 1951. *Proceedings of the Society of Applied Bacteriolgy* **14**, 157-160.

Kemble, A.R. 1956. *Journal of the Science of Food and Agriculture* 7, 125-130.

Kemble, A.R. and Macpherson, H.T. 1954. *Biochemical Journal* **58**, 46-49.

Kennedy, S.J. 1987. In *Proceedings of the Eighth Silage Conference*, pp. 25-26, Institute of Grassland and Animal Production, Hurley.

Kreger-van-Rij, N.J.W. 1984. In *The Yeasts-a taxonomic survey, 3rd edition*; Elsevier Science Publishers, B.V., Amsterdam.

Kung, L., Grieve, D.B., Thomas, J.W. and Huber, J. 1984. *Journal of Dairy Science* **67**, 299-306.

Lindgren, S.E. 1990. In *Proceedings of the Ninth Silage Conference*, pp. 52-53, University of Newcastle Upon Tyne, Newcastle.

Lindgren, S.E., Axelsson, L.T. and McFeeters, R.F. 1990. *Federation of European Microbiological Societies (FEMS) Letters* **66**, 209-214.

Lindgren, S.E. and Dobrogosz, W.J. 1990. *FEMS Microbiology Reviews* **87**, 149-164.

Lindgren, S.E., Lingvall, P., Kaspersson, A., de Kartzow, A. and Rydberg, E. 1983. *Swedish Journal of Agricultural Research* **13**, 91-100.

Lindgren, S.E., Pettersson, K., Jonsson, A., Lingvall, P. and Kaspersson, A. 1985. *Swedish Journal of Agricultural Research* **15**, 9-18.

Lingvall, P. 1978. In *Proceedings of the Fifth Silage Conference*, pp. 50-51, Ed. Harkess, R.D.; Hannah Research Institute, Ayr.

Lyttleton, J.W. 1973. Proteins and Nucleic Acids. In *Chemistry and Biochemistry of Herbage, Volume 1*, pp. 63-103, Eds. Butler, G.W. and Bailey, R.W.; Acedemic Press, London.

Mattick, A.T.R. and Hirsch, A. 1947. *Lancet* (ii), 5-7.

McDonald, P. and Edwards, R.A. 1976. *Proceedings of the Nutrition Society* **35**, 201-211.

McDonald, P., Henderson, A.R. and Heron, S.J.E. 1991. Eds. *The Biochemistry of Silage, 2nd edition*; Chalcombe Publications.

McDonald, P. and Henderson, A.R. 1962. *Journal of the Science of Food and Agriculture* **13**, 395-400.

McDonald, P. and Henderson, A.R. 1974. *Journal of the Science of Food and Agriculture* **25**, 791-795.

McFeeters, R.F. and Chen, K.H. 1986. *Food Microbiology* **3**, 73-81.

McGinn, R., Kerr, W.D. and Hinks, S. 1990. In *Proceedings of the Ninth Silage Conference*, pp. 88-89, University of Newcastle Upon Tyne, Newcastle.

Martin, P.A., Chamberlain, D.G. and Robertson, S. 1990. In *Proceedings of the Ninth Silage Conference*, pp. 46-47, University of Newcastle Upon Tyne, Newcastle.

Mayne, C.S. 1990. *Animal Production* **51**, 1-14.

Merry, R.J. and Braithwaite, 1987. In *Proceedings of the Eighth Silage Conference*, pp. 27-28, Institute of Grassland and Animal Production, Hurley.

Merry, R.J. and Haskins, A.K. 1990 In *Proceedings of the Ninth Silage Conference*, pp. 102-103, University of Newcastle Upon Tyne, Newcastle.

Moon, N.J. and Henk, W.G. 1980. *Applied and Environmental Microbiology* **40**, 1122-1129.

Montville, T.J., Hsu, A. H.-M. and Meyer, M.E. 1987. *Applied and Environmental Microbiology* **53**, 1798-1802.

Morrison, I.M. 1979. *Journal of Agricultural Science, Cambridge* **93**, 581-586.

Morrison, I.M. 1988. *Journal of Agricultural Science, Cambridge* **111**, 35-39.

Morrison, I. and Brice, R.E. 1983/84 *Animal Feed Science and Technology* **10**, 229-238.

O'Meara, T. Gilheany, D.G. and Wilson, R.K. 1987. In *Proceedings of the Eighth Silage Conference*, pp. 7-8, Institute of Grassland and Animal Production, Hurley.

Papendick, K. 1974. *Proceedings of the Fifth General Meeting of the European Grassland Federation*, 1973, Uppsala. *Vaxtodling* **28**, 81-89.

Pelczar, M.J. and Reid, R.D. 1972. *Microbiology*; McGraw-Hill Book Company, New York.

Pitt, R.E., Muck, R.E. and Leibensperger, R.Y. 1985. *Grass and Forage Science* **40**, 279-303.

Playne, M.J. and McDonald, P. 1966. *Journal of the Science of Food and Agriculture* **17**, 264-268.

Rae, R.C., Golightly, A.J., Marshall, D.R. and Thomas, C. 1986. *Animal Production* **42**, 435 (abstract).

Rauramaa, A., Setala, J., Moisio, T., Heikkila, T. and Lampila, M. 1987. *Journal of Agricultural Science in Finland* **59**, 361-370.

Rogosa, M. 1974. In *Bergey's manual of Determinative Bacteriology*, 8thedition, pp. 576-593, Eds. Buchanan, R.E. and Gibbons, N.E.; The Williams and Wilkins Book Company, Baltimore.

Rooke, J.A., Lee, N.H. and Armstrong, D.G. 1987. *British Journal of Nutrition* **57**, 89-98.

Schukking, S. 1976. *Stikstof* **19**, 2-11.

Seale, D.R. 1986. *Journal of Applied Bacteriology Symposium Supplement*, pp.9s-26s.

Seale, D.R., Henderson, A.R., Petterson, K.O. and Lowe, J.F. 1986. *Grass and Forage Science* **41**, 61-67.

Selikoff, I.J. and Hammond, E.C. 1981. *Carcinogenicity of Formaldehyde*: a final report to the American Cancer Society by the Environmental Cancer Information Unit, Environmental Science Laboratory, Mount Sinai School of Medicine, New York.

Selmer-Olsen, I, 1990. In *Proceedings of the Ninth Silage Conference*, pp. 54-55, University of Newcastle Upon Tyne, Newcastle.

Sharp, R., Hooper, P.G. and Armstrong, D.G. 1990. In *Proceedings of the Ninth Silage Conference*, pp. 48-49, University of Newcastle Upon Tyne, Newcastle.

Smith, L.D.S. and Hobbs, G. 1974. In *Bergey's manual of Determinative Bacteriology, 8th edition*, pp. 551-572, Eds. Buchanan, R.E. and Gibbons, N.E.; The Williams and Wilkins Book Company, Baltimore.

Smulders, F.J.M., Barensden, P., van Logtestijn, J.G., Mossel, D.A.A. and van der Marel, G.M. 1986. *Journal of Food Technology* **21**, 419-436.

Somogyi, M. 1945. *Journal of Biological Chemistry* **160**, 69-73.

Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. 1986. Eds. *Bergeys Manual of Systematic Bacteriology, Volume 2*; Williams and Wilkins, Baltimore.

Spoelstra, S.F. 1990. In *Proceedings of the Ninth Silage Conference*, pp. 27-28, University of Newcastle Upon Tyne, Newcastle.

Spoelstra, S.F., Courtin, M.G. and van Beers, J.A.C. 1988. *Journal of Agricultural Science, Cambridge* **111**, 127-132.

Steen, R.W.J., Unsworth, E.F. Gracey, H.I., Kennedy, S.J. and Anderson, R. 1989. *Grass and Forage Science* **44**, 381-390.

Stetter, K.O. and Kandler, O. 1973. *Archives of Microbiology* **94**, 221-247.

Tamminga, S. 1982. In *Forage Protein Conservation and Utilisation*, pp. 83-97, Eds. Griffiths, T.W. and Maguire, M.F.; Commissions of the European Communities, Brussels.

Tseng, C. and Montville, T.J. 1990. *Applied and Environmental Microbiology* **56**, 2761-2763.

Thomas, P.C. 1982. In *Forage Protein in Ruminant Animal Production*, pp. 67-68, Eds. Thomson, D.J., Beever, D.E. and Gunn, R.G.; British Society of Animal Production Occasional Publication N°6.

Thomas, P.C. and Chamberlain, D.G. 1982a. In *Forage Protein Conservation and Utilisation*, pp. 121-146, Eds. Griffiths, T.W. and Maguire, M.F.; Commissions of the European Communities, Brussels.

Thomas, P.C. and Chamberlain, D.G. 1982b. In *Silage for Milk Production, Technical Bulletin N°2*, pp. 63-101, Eds. Rook, J.A.F. and Thomas, P.C.; National Institute for Research in Dairying, Reading.

Thomas, P.C. and Morrison, I.M. 1982. In *Silage for Milk Production, Technical Bulletin N°2*, pp. 13-37, Eds. Rook, J.A.F. and Thomas, P.C.; National Institute for Research in Dairying, Reading.

Van Vuuren, A.M., Mergsma, K., Frol-Kramer, F. and van Beers, J.A.C. 1989. *Grass and Forage Science* **44**, 223-230.

Vetter, R.L. and von Glan, K.N. 1978. In *Fermentation of Silage-a review*, pp. 281-332, Ed. McCullough, M.E.; National Feed Ingredients Association, Iowa.

Van Roth, C. 1939. *Vorratspflege und Lebensmittelforschung* **2**, 22.

Virtanen, A.I. 1933. *The Empire Journal of Experimental Agriculture* **1**, 143-155.

Visser, R., Holzapfel, W.H., Bezuidenhout, J.J. and Kotze, J.M. 1986. *Applied and Environmental Microbiology* **52**, 552-555.

Vogels, G.D. and van der Drift, C. 1976. *Bacteriological Reviews* **40**, 403-468.

Waite, R. 1965. *Proceedings of the Nutrition Society* **24**, 38-46.

Waite, R., Johnston, M.J. and Armstrong, D.G. 1964. *Journal of Agricultural Science, Cambridge* **62**, 391-398.

Waldo, D.R. 1977. *Journal of Dairy Science* **60**, 306-326.

Waldo, D.R., Keys, J.E., Smith, L.W. and Gordon, C.H. 1971. *Journal of Dairy Science* **54**, 77-83.

Watson, D.C. 1985. *Journal of Applied Bacteriology* **59**, 955-1035.

Watson, S.J. and Nash, M.J. 1960. *The Conservation of Grass and Forage Crops*. Oliver and Boyd, Edinburgh.

West, C.A. and Warner, P.J. 1988. *FEMS Microbiology Letters* **49**, 163-165.

Wetherall, J.A., Armstrong, A.G. and Rooke, J.A. 1990. In *Proceedings of the Ninth Silage Conference*, pp. 13-14, University of Newcastle Upon Tyne, Newcastle.

Wilkinson, J.M. 1988. In *Silage UK, 5th edition*; Chalcombe publications, Marlow Bottom.

Wilson, R.F. and Wilkins, R.J. 1973. *Journal of Agricultural Science, Cambridge* **80**, 225-231.

Whittemore, C.T. and Henderson, A.R. 1977. *Journal of the Science of Food and Agriculture* **28**, 506-510.

Wood, W.A. 1961. In *The Bacteria, Volume 2*, pp. 58-149, Eds. Gunsalus I.C. and Stanier, R.Y.; Acedemic Press, New York.

Woolford, M.K. 1972. *Herbage Abstracts* **42**, 105-111.

Woolford, M.K. 1975a. *Journal of the Science of Food and Agriculture* **26**, 219-228.

Woolford, M.K. 1975b. *Journal of the Science of Food and Agriculture* **26**, 229-237.

Woolford, M.K. 1978. *ARC Research Report* **4**, 8-12.

Woolford, M.K. 1984. *The Silage Fermentation*. Marcel Dekker, New York.

Woolford, M.K. and Cook, J.E. 1978. *Animal Feed Science and Technology* **3**, 89-94.

Woolford, M.K. and Wilkie, A.L. 1984. *Journal of Agricultural Science, Cambridge* **102**, 97-104.

Woolford, M.K. and Wilkins, R.J. 1975. *Journal of the Science of Food and Agriculture* **26**, 869-878.