The Development of Novel Silage Inoculants using Strain Selection and Genetic Manipulation Techniques.

Thesis presented for the degree of Doctor of Philosophy by

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July, 1993.

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Aidan Fitzsimons Date: 17.9.93

Acknowledgements

I wish to express my gratitude to my supervisor, Dr. Michael O' Connell, for his guidance, enthusiasm and belief, to Dr. Padraig O' Kiely for being so generous with much appreciated advice and technical assistance, to Prof. Jean Delcour for hosting me in his laboratory and for ensuring that my stay was enjoyable and productive and to Dr. Thecla Ryan for introducing me to all this and for her encouragement over the years.

To the many members of The Good Time Club, and in particular my fellow crewmates in the good ship John Barry; Dan, Sharon, Margareth, Hugh, Geraldine, John, Fiona, Maura, Brian and Sue-Ann- a big 'yo!' for all the fond memories and don't forget to keep in touch. To my Belgian colleagues; Bernard, Brigitte, Cathy, Dominique, Jacques, Jean-Yves, Nathalie, Pascal, René, Sabine and Thierry I wish to express my gratitude for the wonderful hospitality and friendship extended to me by them and their families and friends. Thanks also to Suzanne, Maria, Carol and all the gang at Thornton Park Equestrian Centre for much fun, a few aches and pains and some welcome inspiration.

Last, and most certainly not least, a tip of the floppy hat to 3 very special people; Phillip, Marie and most especially Susan, for friendship, support and for always being there.

To my parents, Brian and Annie,

For their support, financial and otherwise,

through my many years of eduction:

I promise I'll get a proper job now.

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Abstract

Eighteen strains of *Pediococcus acidilactici* were screened in the laboratory for their potential as silage inoculants. The grass silage isolate, *P. acidilactici* G24, was found to be the most suitable, primarily on the basis of its short lag phase. Inoculation of grass of water soluble carbohydrate content greater than 100g/kg DM with *P. acidilactici* G24 at a rate of 10^6/g forage consistently aided silage preservation, as indicated by a more rapid rate of lactic acid production and pH decrease, and silage with a lower pH, a lower ammonia nitrogen content and a higher level of crude protein than uninoculated controls. Furthermore, when compared to inoculants consisting of *Lactobacillus plantarum* or mixtures of *L. plantarum* and *P. acidilactici*, *P. acidilactici* G24 initiated a more rapid rate of pH decrease and maintained the same silage pH up to 60 days after ensilage, implying that *P. acidilactici* G24 is superior to these more traditional inoculants.

When tested on grass of water soluble carbohydrate content less than 60g/kg DM, *P. acidilactici* G24 failed to aid preservation. A second inoculant was therefore developed to cope with low water soluble carbohydrate conditions where starch is available as an alternative source of water soluble carbohydrate. This situation is frequently encountered in the ensilage of alfalfa. While no amylolytic strain of lactic acid bacteria suitable as a silage inoculant could be isolated, the amylase enzyme of *Lactobacillus amylovorus* was found to be well adapted to typical ensiling conditions. A region of DNA coding for the *L. amylovorus* α-amylase enzyme was therefore cloned and integrated into the Cbh gene of the competitive inoculant strain, *L. plantarum* LP80. A *Lactobacillus* host strain was chosen in order to comply with regulations governing the release of genetically engineered organisms into the environment. The amylolytic derivative, *L. plantarum* LPGAFΔ5, secreted 49% of the amylase secreted by *L. amylovorus*, stably maintained amylase activity for 50 generations under non-selective conditions and displayed no reduction in growth rates on glucose based media compared to the parent strain, *L. plantarum* LP80.

1. Introduction.

1.1. FOREWORD.

1.1.1. What is silage?

Silage is defined as the product formed when material of sufficiently high moisture content, liable to spoilage by aerobic micro-organisms, is stored anaerobically (Woolford 1984). While silage is frequently made from fish and poultry offal, potatoes, and agricultural by-products such as tomato, grape, and beet pulp, the most commonly ensiled crops are grasses, corn and legumes such as alfalfa. The objective of silage making is the efficient preservation of the crop so that losses in nutritional value are minimised. This is accomplished via an acid fermentation under anaerobic conditions in which lactic acid bacteria present on the forage convert crop water soluble carbohydrates to lactic, acetic and butyric acids. The resulting fall in pH, combined with the toxicity of the undissociated acids, inhibits the growth of spoilage organisms and ensures crop preservation. Silage has a number of advantages over the more traditional hay making as a means of crop preservation; it is less dependent on weather conditions, it allows a number of harvests to be made in a single growing season and it generally gives rise to a more nutritious product. It has therefore assumed increasing importance as a means of crop preservation and by 1978 had replaced hay as the major method of forage preservation in northern Europe (Wilkinson 1980).

1.1.2. The silage making process: a brief overview.

Grass and alfalfa for silage making are best harvested reasonably early in the growth cycle when the crops have a relatively high fermentable carbohydrate and low fibre content. This corresponds to a high leaf to stem ratio. Corn, on the other hand, is harvested when the grain is of a doughy consistency at which stage the leaves are beginning to turn brown. Two methods of harvesting are practised and the resulting silages are referred to as direct cut or pre-wilted. As the name suggests, pre-wilted silage is made by cutting the crop and leaving it in the field to wilt for up to 48 hours. Turning or tedding may be employed to accelerate the wilting process. This method is only employed in the making of grass and alfalfa silage, as corn has a naturally low moisture content at harvesting. After wilting, or immediately after cutting in the case of direct cut silage, the crop is chopped into short lengths by a forage harvester and conveyed to the silo, which is normally of the bunker or tower type. The former usually consists of a concrete rectangular structure having only three walls, which may slope towards the base to facilitate packing. Tower silos are constructed from concrete, steel or fibreglass and have a domed roof. Once the silo is filled and the crop packed, the contents are sealed from the atmosphere. Respiratory activity by plant enzymes and aerobic micro-organisms results in a rapid decrease in oxygen levels (Langston et al 1958, Mc Donald 1981). Once anaerobic conditions are established, lactic acid bacteria present on the crop prior to harvesting, rapidly multiply and ferment crop water soluble carbohydrates to lactic and acetic acids. The low pH and the toxicity of the undissociated acids restrict further microbial activity, and facilitate preservation of the remaining crop nutrients (Seale 1986). If a low pH is not rapidly established, a secondary fermentation can occur in which saccharolytic clostridia ferment lactic acid and crop water soluble carbohydrates to butyric acid and carbon dioxide. In this process two moles of lactic acid are converted to one mole of the weaker butyric acid and an increase in pH takes place. Conditions then become favourable for the growth of proteolytic clostridia which convert crop proteins and

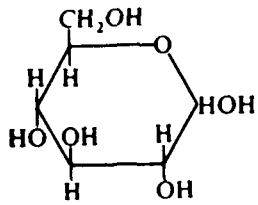
amino acids to ammoniacal compounds, thus further increasing the pH and degrading crop nutrients in the process (Woolford 1984). Crop preservation is therefore dependent on the rapid onset of a lactic acid fermentation, bringing about a sharp decrease in pH.

1.2. THE CHEMICAL COMPOSITION OF THE RAW MATERIAL.

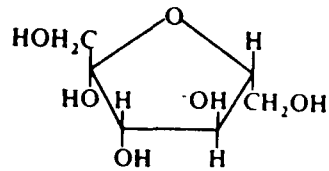
The principal crop variables influencing the outcome of the silage fermentation are water soluble carbohydrate levels, dry matter content and buffering capacity. Good preservation is most easily attained from crops such as maize with a high water soluble carbohydrate level and dry matter content and a low buffering capacity. By contrast alfalfa is difficult to ensile, having a very high buffering capacity and a low level of water soluble carbohydrate and dry matter. The aforementioned characteristics vary considerably in grass but the crop is usually considered intermediate between maize and alfalfa in its ensilibility.

1.2.1. Crop Carbohydrates.

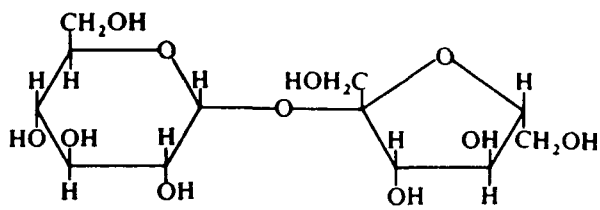
Crop carbohydrates are crucial to the silage fermentation as they constitute the substrate for conversion to lactic acid which effects preservation. Since micro-organisms require an aqueous environment for their growth, it is the water soluble component of the crop carbohydrate which is of primary interest in the silage fermentation. Figures vary depending on the dry matter content and buffering capacity of the crop but it is generally agreed that 60-80g/kg dry matter (DM) water soluble carbohydrate are required to facilitate preservation. (Wierenga 1962, Wierenga 1969, Smith 1973). The principal water soluble carbohydrates in grass are fructans, glucose, fructose and sucrose , the structures of which are shown in figure 1.1. Fructans are the most abundant form of water soluble carbohydrate in temperate grasses (Laidlaw and Reid 1952). They represent the major storage carbohydrate in grass and are concentrated in the stem of the plant where levels may exceed 150g/kg DM (Mackenzie and Wylam 1957). Leaves seldom contain more than 40g/kg DM (Mackenzie and Wylam 1957). Sucrose levels in grasses range from 20 to 80g/kg DM while fructose and glucose accounts for 10-30g/kg DM (McDonald et al 1960). Fructose tends to predominate over glucose with the ratio ranging from 1.1:1 to 3.9:1 (McDonald et al 1960, MacKenzie and Wylam 1957).



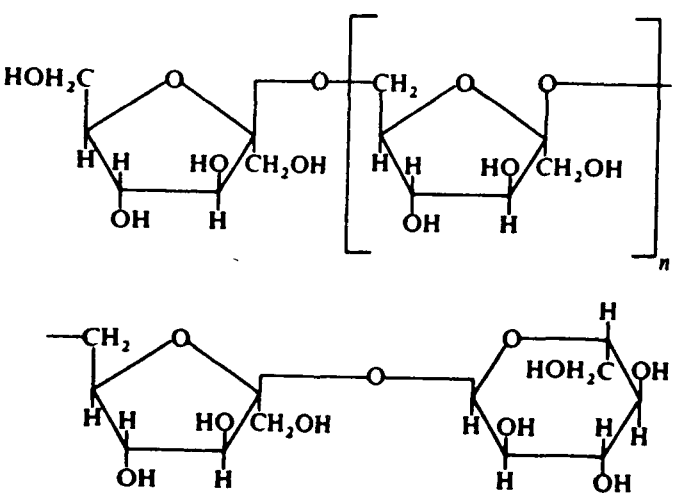
Glucose



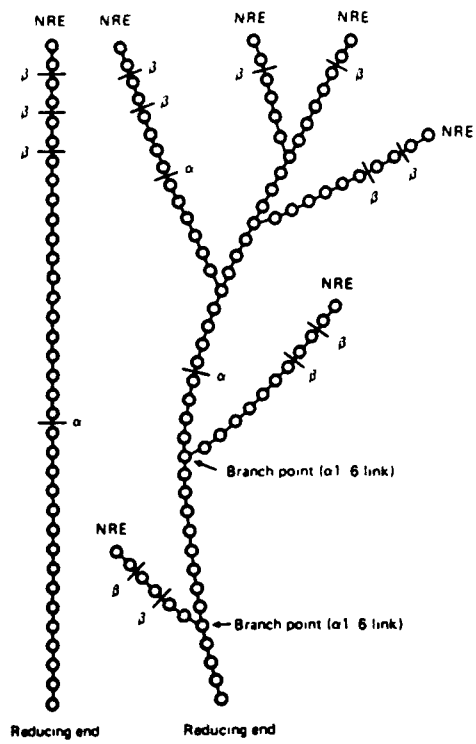
Fructose



Sucrose



Fructans



Amylose Amylopectin

Figure 1.1: Structure of the principal water soluble carbohydrates occuring in forage crops.
NRE: Non reducing end.
α and β represent typical cleavage points in amylose and amylopectin for α and β-amylases.

The water soluble carbohydrate content of grasses varies considerably depending on a number of factors, the most important of which are the species, the stage of maturity, the time of day, weather conditions and fertiliser levels (Smith 1973). Typical water soluble carbohydrate levels for a number of grass species commonly grown for silage are presented in table 1.1.

Species	Mean water soluble carbohydrate (g/kg DM)
Italian Ryegrass	181
Perennial Ryegrass	170
Timothy	110
Meadow Fescue	96
Cocksfoot	79

Table 1.1: Mean water soluble carbohydrate levels in 5 grass species. (Henderson 1973)

Clearly water soluble carbohydrate levels tend to be highest in the ryegrasses and lowest in cocksfoot. Different cultivars of the same species may also vary in their water soluble carbohydrate levels. The tetraploid cultivars, Reveille and Petra for example, have been shown to have consistently higher water soluble carbohydrate levels than diploid cultivars of the same species (Henderson 1973, Dent and Aldrich 1963). Water soluble carbohydrate levels vary considerably throughout the growing season and are generally highest in late May/ early June and in late July and August. (Henderson 1973, Waite and Boyd 1953). Since carbohydrate levels in the plant are dependent on photosynthesis, sunshine invariably has a major influence on water soluble carbohydrate levels. Stirling (1954) showed that the sugar content of a crop harvested after several days of dull weather was lower than

in material harvested from the same plot during sunny weather. This effect is mediated by light intensity and not temperature (Deinum 1966). Indeed high temperatures have an adverse effect on water soluble carbohydrate levels which are highest under conditions of high light intensity and low temperature (Bathurst and Mitchell 1958, Archibald et al 1960, Deinum 1966). Grass water soluble carbohydrate levels also vary throughout the day, rising to a peak in mid afternoon and then falling until daylight the following day (Smith 1973). This variation occurs mainly in the sucrose component of the water soluble carbohydrate (Waite and Boyd 1953). The application of nitrogenous fertilisers reduces the level of water soluble carbohydrate in the plant (Waite 1958, Wierenga 1962, Smith 1973). This decrease occurs in the fructose component of the water soluble carbohydrate and is caused by the acceleration in herbage growth promoted by the fertiliser (Smith 1973).

The carbohydrate content of maize and alfalfa differs from that of grass in that starch is the major storage carbohydrate. The starch component of plant cells exists in two forms. Amylose, the unbranched form, consists of glucose residues linked by α 1-4 bonds. In the branched form, amylopectin, the α 1-4 linked glucose chain is interrupted about once in every 30 glucose residues by an α 1-6 bond, thereby creating a branched structure (see fig. 1.1). Unlike fructans both forms of starch are insoluble in cold water. Glucose, fructose and sucrose are the principal water soluble carbohydrates in maize and alfalfa (Raguse and Smith 1966, McAllan and Phipps 1977) and are more plentiful in the former. Water soluble carbohydrate levels in maize and alfalfa peak at about 300g/kg DM and 110g/kg DM respectively early in the growing season but fall to 80g/kg DM and 60g/kg DM at maturity. As the crop develops the starch content of maize rises from 70 to 280g/kg DM while that of alfalfa has been shown to fall from 87g/kg DM to 45g/kg DM (Wilkinson and Phipps 1979, Raguse and Smith 1966).

1.2.2. Dry Matter (DM).

High crop dry matter contents benefit the silage fermentation by virtue of the greater tolerance of lactic acid bacteria to low moisture compared to that of the vegetative forms of clostridia. Increases in the dry matter content of the crop restrict microbial activity by increasing the osmotic pressure (Wierenga 1958a) and result in anaerobic stability being achieved at a higher pH (Gibson and Stirling 1959, Wierenga 1969, Woolford et al 1979). Dry matter contents within the range of 20%-25% are optimal for the silage fermentation and at levels above 33% no undesirable microbial activity will occur (Lanigan 1963). The dry matter content of forage crops increases with maturity. Typical dry matter contents for grasses at harvest range from 12 to 25% (Brady 1960, Rooke et al 1988, Haigh 1987, Haigh 1988, Mayne 1990, Nesbakken and Broch-Due 1991) but may be increased to 30-40% by wilting (Henderson et al 1972, O' Kiely et al 1988). Maize tends to have a higher dry matter than grasses, ranging from 23 to 33% as the season progresses (Wilkinson and Phipps 1979). The dry matter content of alfalfa ranges from 19-39% (Carpintero et al 1969, Dellaglio and Torriani 1985, Ely et al 1981, 1982) but may be increased to as much as 60% by wilting (Kung et al 1984).

1.2.3. Buffering Capacity.

The buffering capacity of plants, or their ability to resist changes in pH, is crucial to the silage fermentation as it determines the amount of acid required to achieve anaerobic stability. Since forage crops have a pH of approximately 6 after maceration which drops to about 4 in well preserved silages, it is the buffering capacity in this range that is of prime importance. Buffering capacity is normally determined by first titrating crop extracts to pH 3 with 0.1M HCl to release bicarbonate as CO_2 and then titrating to pH 6 with 0.1M NaOH. The result is expressed as the milliequivalents (mE) of alkali required to change the pH from 4 to 6 /kg DM (Playne and McDonald 1966). Typical buffering capacities of a number of forage crops are shown in table 1.2.

Crop	Buffering Capacity (mE NaOH/kg DM)
Forage corn	200
Orchardgrass	300
Perennial ryegrass	350
Italian ryegrass	430
Alfalfa	480

Table 1.2: Buffering capacities of a range of forage crops.(Wilkinson 1978).

The buffering capacity of grasses is normally intermediate between that of corn and alfalfa. Organic acids, eg. malic, citric and quinic acids, are responsible for 68-80% of plant buffering capacities, with plant proteins accounting for another 10-20% (Playne and McDonald 1966). The protein content and therefore the buffering capacity of forage crops decreases with maturity (Waite and Gorrod 1959, Wilkinson and Phipps 1979) but is increased by the application of nitrogenous fertiliser (Lyttleton 1973). The high buffering capacity of alfalfa has been attributed to its high organic acid content, which generally ranges from 60-80g/kg DM (Fauconneau and Jarrige 1954), but may be as high as 100g/kg DM (Fauconneau 1958). Alfalfa also tends to contain greater amounts of protein than other forage crops comprising 15-20% of the dry matter (Ely et al 1981,1982), further contributing to the high buffering capacity.

1.3. THE MICROBIOLOGY OF THE RAW MATERIAL.

Successful crop preservation by ensilage depends on a complex microbial fermentation in which lactic acid bacteria dominate, suppressing undesirable micro-organisms such as coliforms, clostridia and fungi. The relative numbers of each type of organism and their biochemical activities therefore play a crucial role in determining the outcome of the silage fermentation.

1.3.1. The Lactic Acid Bacteria.

Lactic acid bacteria are micro-aerophilic , gram positive, non sporeforming organisms which ferment sugars to lactic acid (Orla Jensen 1919). Categorisation of the group is based primarily on the type of sugar fermentation; homofermentative in which hexoses are fermented primarily to lactic acid and heterofermentative in which other products, principally ethanol and CO_2 are formed. Further division is based on cell morphology. Using this classification, the lactic acid bacteria commonly associated with silage and their sugar fermentation profiles are listed in table 1.3.

Bacterium	Fermentation of:			Main form of lactic acid produced
	Glucose	Fructose	Sucrose	
HOMOFERMENTATIVE				
Rods:				
Lactobacillus casei	+	+	sd	D(-)
Lactobacillus coryneformis	+	+	+	L(+)
Lactobacillus curvatus	+	+	-	DL(-)
Lactobacillus plantarum	+	+	+	DL(-)
Cocci:				
Pediococcus acidilactici	+	+	w	DL(-)
Pediococcus damnosus	+	+	-	L(+)
Pediococcus pentosaceus	+	+	w	DL(-)
Enterococcus faecalis	+	+	+	?
Enterococcus faecium	+	+	s	?
HETEROFERMENTATIVE				
Rods:				
Lactobacillus brevis	+	+	s	DL(-)
Lactobacillus buchneri	+	+	s	DL(-)
Lactobacillus fermentum	+	+	+	DL(-)
Lactobacillus viridescens	+	+	s	DL(-)
Cocci:				
Leuconostoc mesenteroides	+	+	+	D(-)

+: Positive reaction by >90% of strains
-: Negative reaction by >90% of strains
w: Weak reaction
s: Some strains positive, others negative
d: Delayed reaction

Table 1.3: Classification of lactic acid bacteria important in silage.
Compiled from Buchanan and Gibbons (1974), McDonald (1981), Sharpe (1981), Teuber and Geis (1981).

Throughout the course of the silage fermentation, the temperature and pH may vary considerably, and the ability of lactic acid bacteria to withstand these changes is therefore of great significance. While most lactic acid bacteria are mesophilic organisms, strains vary in their ability to withstand high temperatures. Leuconostocs and lactobacilli cannot grow at temperatures greater than 30°C and 45°C respectively while enterococci and some pediococci can tolerate temperatures as high as 50°C. In terms of ability to grow at various values of pH, pediococci and lactobacilli are the most versatile of the lactic acid bacteria, having growth optima of 5.5-6 and tolerating pH values as low as 3.5 and 4 respectively. By contrast enterococci and leuconostocs exhibit little growth below pH 4.5 (Bryan-Jones 1969, Buchanan and Gibbons 1974).

1.3.1.1. The biochemistry of Lactic Acid Bacteria.

Three biochemical activities of the lactic acid bacteria are of major significance in the ensilage process; the fermentation of carbohydrates, the dissimilation of organic acids including lactic acid, and the breakdown of nitrogenous compounds.

(a) The fermentation of carbohydrates.

The major products of carbohydrate metabolism by homofermentative and heterofermentative lactic acid bacteria are outlined in table 1.4.

Homofermentative:

Aerobic:

1 glucose (or 1 fructose) ⟶ 1 lactic acid + 1 pyruvic acid + 1 H_2O.

Anaerobic:

1 glucose (or 1 fructose) ⟶ 2 lactic acid.

1 pentose ⟶ 1 lactic acid + 1 acetic acid.

Heterofermentative:

Aerobic:

1 glucose (or 1 fructose) ⟶ 1 lactic acid + 1 acetic acid+ 1 CO_2 + 2 H_2O.

Anaerobic:

1 glucose ⟶ 1 lactic acid + 1 ethanol + 1 CO_2.

3 fructose ⟶ 1 lactic acid + 1 acetic acid + 2 mannitol + 1 CO_2.

2 fructose + 1 glucose ⟶ 1 lactic acid + 1 acetic acid + 1 CO_2 + 2 mannitol.

1 pentose ⟶ 1 lactic acid + 1 acetic acid.

Table 1.4: Major products of sugar metabolism by lactic acid bacteria.

Under the anaerobic conditions which prevail in the silage fermentation, homofermentative lactic acid bacteria ferment the major crop carbohydrates, glucose and fructose to 2 moles of lactic acid. After prolonged incubation traces of acetate, acetoin, diacetyl, 2-3 butanediol and ethanol may also be produced (El-Bendy et al 1982, Murphy and Condon 1984).

By contrast, heterofermentative lactic acid bacteria ferment one mole of glucose to one mole each of lactate, ethanol and CO_2, and three moles of fructose to one mole each of lactate, acetate, and CO_2 and 2 moles of mannitol. Alternatively members of the *L. brevis L. buchneri* complex which lack the enzyme acetaldehyde dehydrogenase, required for the normal heterofermentative fermentation of glucose use fructose as an external electron acceptor. Two moles of fructose and one of glucose are therefore required to form a single mole of lactate. The heterofermentative fermentation is thus less efficient in terms of lactic acid produced per mole of hexose fermented. This is especially significant in crops such as grass where fructose predominates over glucose since the heterofermentative pathway for the fermentation of fructose is considerably more wasteful than that for glucose.

(b) The dissimilation of organic acids.

The major organic acids found in forage crops; citrate and malate, are rapidly hydrolysed during ensilage (Playne et al 1967, Hirst and Ramstad 1957), mainly due to their fermentation by lactic acid bacteria (Woolford 1984). The fermentation of malate has been reported in *Enterococcus faecalis, Enterococcus faecium, Leuconostoc mesenteroides, L. plantarum* and some *Pediococcus* spp. (McDonald 1981). Only *E. faecalis* and *E. faecium* are capable of fermenting citrate alone while *L. brevis* and some *L. mesenteroides* strains can do so in the presence of an additional carbohydrate source (McDonald 1981, Sneath et al 1986). The products formed from organic acid degradation by lactic acid bacteria include lactate, formate, acetate, ethanol, 2-3 butane diol and acetoin (Woolford 1984), and the end products may vary with pH. As the pH increases a greater proportion of formate and acetate are formed until at pH 7 no lactate is produced (Gunsalus and Campbell 1944).

In addition to fermenting crop organic acids, lactic acid bacteria may also break down some of the lactic acid formed during the initial fermentation period. Many lactic acid bacteria oxidise lactic acid to acetic acid and CO_2 under aerobic conditions, but since the silage fermentation is predominantly an anaerobic process this activity is only of importance during feedout. However in prolonged incubations the anaerobic degradation of lactic acid to acetic acid by *Lactobacillus brevis, Lactobacillus buchneri*, some pediococci and *Lactobacillus plantarum* has been reported (Peterson and Fred 1920, Beck 1978, Lindgren et al 1990). *Lactobacillus brevis* is capable of degrading lactic acid to acetic acid and CO_2 in cultures where lactic acid is the sole carbon source (Peterson and Fred 1920). By contrast Lindgren et al (1990) observed that *L. plantarum* ferments lactic acid to acetic and formic acid only in the presence of citric acid. In cultures in which glucose and citrate were the sole carbon sources the authors observed the degradation of as much as 30-40% of the lactate initially formed from glucose over a 60 day incubation period. They proposed that a pyruvate formate lyase was responsible for this activity and suggested that it might account for the degradation of lactic acid and simultaneous increase in acetic acid noted in some silages inoculated with *L. plantarum* and stored for long periods.

(c) The fermentation of nitrogenous compounds.

The fermentation of nitrogenous compounds in silage (eg. crop proteins and amino acids) is undesirable as it results in the formation of ammoniacal compounds which tend to increase the pH. Furthermore it is preferable that dietary protein per se enters the duodenum of the animal and that proteolysis is limited as free amino acids are of nutritional value only to the rumen microflora (Woolford 1984). Lactic acid bacteria are responsible for little significant breakdown of nitrogenous compounds in silage. They are generally regarded as non-proteolytic although weak proteolytic activity has been reported in *L. plantarum, Lactobacillus casei* and *Lactobacillus mesenteroides* subs *cremoris* (Orla Jensen et al 1947, Khalid and Marth 1990, Broome and Hickey 1991). The only amino

acids extensively attacked by lactic acid bacteria are serine and arginine, which are deaminated by *L. plantarum* and some *Pediococcus* spp. to ammonia, CO_2 and acetoin and ornithine respectively (Buchanan and Gibbons 1974, McDonald and Whittenbury 1973, Brady 1966). Some deamination of amino acids by lactic acid bacteria tends to occur in even the best preserved silages. The decarboxylation of amino acids to amines has been reported in several strains of *E. faecalis* and a number of *Lactobacillus* strains, though not those commonly found in silage (Gale 1940, Rodwell 1953, Recsei and Snell 1972). Some lactic acid bacteria, including *L. plantarum* but not *L. brevis*, *E. faecalis* or *Pediococcus* spp. are capable of reducing nitrate, first to nitrite and then to ammonia (Oshima and McDonald 1978). However since a large part of the nitrate in herbage is reduced in the early stages of ensilage by aerobic bacteria (McDonald 1981) this action of lactic acid bacteria is of little significance.

1.3.1.2. Lactic acid bacteria numbers.

Lactic acid bacteria numbers on the standing crop are low; often less than 1 X 10^2/g fresh matter (Woolford 1984). Lactic acid bacteria seem to be particularly scarce on alfalfa with Kroulik and Wiseman (1955) and Muck (1989) failing to detect any isolates from the standing crop. Numbers on maize are somewhat higher; in a survey of 533 fields of maize throughout the U.S.A. Speckman et al (1981) found that 42% of samples had less than 1 X 10^2 lactic acid bacteria/g fresh matter and 77% had less than 2 X 10^4. Results from grass vary, ranging from no isolates (Stirling and Whittenbury 1963, Fenton 1987) to 1.2 X 10^4/g fresh matter (Ostling and Lindgren 1991). However numbers are normally in the region of 10^2/g fresh matter (Stirling 1951, Kroulik et al 1955, McDonald et al 1962, Stirling and Whittenbury 1963). Two studies on the identity of lactic acid bacteria occuring on the standing crop are available. Stirling and Whittenbury (1963) reported that of 400 isolates from various plants leuconostocs accounted for 80% and lactobacilli and pediococci for 10% each. Leuconostocs occured on a wide number of plants while pediococci were found mainly on grasses and cereals. Lactobacilli were

limited to grass, especially sheath material and decaying leaves at the base of the crop where nutrients are more accessible. By contrast Fenton (1987) isolated no leuconostocs from standing grass and found that *Pediococcus acidilactici* and *Enterococcus* species each accounted for 40% and lactobacilli for 6% of isolates. Given the conflicting data from the only 2 studies available it is difficult to make any firm conclusions on the dominant types of lactic acid bacteria on standing crops.

1.3.2. The clostridia.

The major challenge to the preservative effect of lactic acid bacteria is presented by the clostridia. Clostridia are gram positive, spore forming rods which grow under anaerobic conditions and ferment sugars, organic acids or proteins (Buchanan and Gibson 1974). They are divided into 2 main groups: the saccharolytic clostridia which ferment mainly carbohydrates and organic acids and possess only limited proteolytic activity and the proteolytic clostridia which ferment mainly proteins and have limited carbohydrate degrading ability. However some strains eg. *Clostridium perfringens* exhibit both saccharolytic and proteolytic activity. Gibson (1965) identified 7 clostridial species which are of note in the silage fermentation and classified them as lactate or amino acid fermentors as shown in table 1.5.

Lactate fermenters	Amino Acid fermenters	Others
C. butyricum	*C. bifermentans*	*C perfringens*
C. paraputrificum	*C. sporogenes*	*C. sphenoides*
C. tyrobutyricum		

Table 1.5: Classification of clostridia of importance to the silage fermentation.

Saccharolytic clostridia ferment 1 mole of glucose or fructose or 2 moles of lactic acid to 1 mole of butyric acid and 2 moles each of carbon dioxide and hydrogen (McDonald 1981). Proteolytic species ferment amino acids by one of three pathways as shown in table 1.6. (Whittenbury 1968):

1. Stickland reaction
1 alanine + 2 glycine → 3 acetic acid + $3NH_3$ + CO_2
2. Deamination
3 alanine → 2 propionc acid + acetic acid + $3NH_3$+ CO_2
1 valine → isobutyric acid + NH_3 + CO_2
1 leucine → isovaleric acid + NH_3 + CO_2
3. Decarboxylation
Histidine → Histamine
Lysine → Cadaverine
Arginine → Ornithine → Putrescine

Table 1.6: Amino acid fermentation pathways of the proteolytic clostridia.

Crucial to the control of clostridia in the silage fermentation is their inability to withstand low values of pH and water activity. The optimum pH for clostridial growth is 7-7.4 (Pelczar and Reid 1972) and the critical pH below which growth ceases is determined by the water activity. This was aptly demonstrated by Wieringa (1958) who showed that *Clostridium tyrobutyricum* could grow in broth adjusted with HCl to pH 4.1 but when the water activity was decreased by adding 35g NaCl/kg pH 5.4 was the lower limit for growth. Muck (1988) subsequently developed a relationship between crop dry matter contents and the minimum pH for clostridial growth (see figure 1.2).

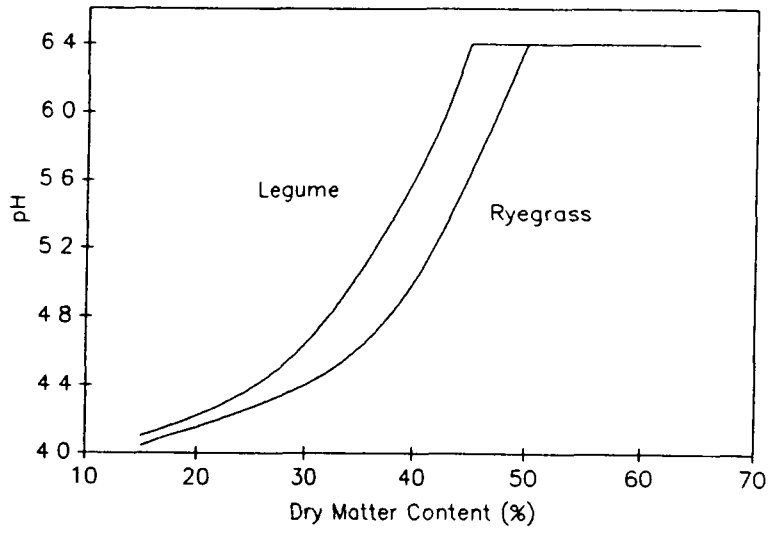


Fig. 1.2: The effect of crop dry matter on the pH at which growth of *C. tyrobutyricum* ceases (Muck 1988).

Clostridia occur as spores on the standing crop and are usually present in very low numbers, less than 100/g forage (Gibson et al 1958, Gibson et al 1961, Allen and Harrison 1937, Martos 1941). They are considered not to be members of the normal crop microflora but contaminants from the soil introduced during harvesting. The application of farmyard manure can lead to a short term rise in the numbers of clostridial spores on the standing crop but this tends to be nullified 6-7 weeks after application due to clostridial spores being washed off the crop by rainfall (Ostling and Lindgren 1991).

1.3.3. The coliform bacteria.

The coliform bacteria span a wide range of species, with the most important of the silage isolates being of the family *Enterobacteriaceae*. Members of this group are gram negative, non spore forming and facultatively anaerobic and are generally non pathogenic rod shaped bacteria. They ferment carbohydrates, primarily to acetic acid, and are capable of the deamination and decarboxylation of amino acids (Beck 1978). The optimal pH for growth is 7.0 (McDonald 1981).

Enterobacteria numbers on the standing crop tend to be high compared to those of lactic acid bacteria, ranging from 10^1 to 10^6/g (Ostling and Lindgren 1991, Kroulik et al 1955), and are largely unaffected by slurry application earlier in the growing season (Rooke 1990, Ostling and Lindgren 1991). Ostling and Lindgren (1991) reported that more than 90% of the enterobacteria on standing grass crops were of the genus *Enterobacter* with 72% of these being *Enterobacter agglomerans*. Similarly Kroulik et al (1955) found that the majority of the coliform bacteria isolated from various standing crops were of the genus *Aerobacter*, now known as *Enterobacter* (Buchanan and Gibbons 1974). Other members of the *Enterobacter* family isolated from forage and silage include *Erwinia herbicola, Escherichia coli, Hafnia alvei* and *Klebsiella* spp. (Gibson et al 1958,, Spoelstra 1987).

1.3.4. Fungi.

Fungi are eukaryotic micro-organisms which grow either as single cells, the yeasts, or as multicellular filamentous colonies, the moulds. They obtain nutrients for growth by secreting extracellular enzymes such as proteases, amylases and cellulases which break down complex organic molecules which can then be absorbed into the cell. The majority are strict aerobes though a number have been shown to be capable of growth under anaerobic conditions (Deacon 1980). Beck (1978) divided the yeasts of importance in silage into 2 groups; the bottom growing or sedimentary yeasts, eg *Torulopsis*, which ferment sugars to ethanol but have little or no activity on lactic acid and the top growing or pellicle yeasts which have a weak fermentation capacity but a high respiration capacity for lactic acid. Species of the genera *Hansenula, Pichia, Candida* and *Saccharomyces* are included in this group. Both activities are obviously undesirable in silage. Most yeasts are acid tolerant and are capable of growth at pH values as low as 3.5 to 3.8 (Pelczar and Reid 1972).

The moulds are the most diverse and least understood micro-organisms of relevance to the silage fermentation. Most are unable to grow under conditions of low pH and anaerobiosis. Their presence in silage is undesirable since they break down not only sugars and lactic acid via normal respiratory pathways, but also hydrolyse and metabolise cellulose and other cell wall components. Furthermore some moulds produce mycotoxins which are harmful to animals (McDonald 1981).

Yeast and mould numbers tend to increase as the season progresses. Henderson et al (1972) reported less than 10 yeasts and moulds/g crop on standing grass in early June. Kroulik et al (1955) reported a yeast and mould count on grass of 10^3-10^4/g in late June which increased to 10^6/g by mid July. Yeast and mould numbers on alfalfa were similar ranging from 10^2/g in May to 10^4/g in June and 10^6/g in mid July. Numbers on maize followed the same pattern but were slightly higher and peaked at 10^7/g in August. Middelhoven and van Baalen (1988) observed a yeast count of up to 10^5/g on maize in late September and identified *Candida ingeniosa, Cryptococcus laurentii, Sporobolomyces roseus* and *Sporidiobolus salmonicolor* among the isolates.

1.4. CHEMICAL AND MICROBIOLOGICAL CHANGES DURING ENSILAGE.

1.4.1. Wilting.

The beneficial effects of wilting on the silage fermentation were aptly demonstrated by Haigh (1987), who in a survey of 1713 farm silages found a strong positive correlation between high dry matter contents and good preservation with 83% of silages made from material of greater than 26% DM being well fermented. The benefits of wilting on the fermentation are mediated by an increase in dry matter content resulting in a concentration of soluble substances in the water phase and an increase in osmotic pressure (Wierenga 1958). The greater tolerance of lactic acid bacteria compared to clostridia of such conditions gives the former a competitive advantage. This may obviate the need for a preservative. Other benefits of wilting include a reduction in the weight of material which must be transported from the field and decreased levels of highly polluting effluent production.

Wilting is only necessary in the ensilage of high moisture crops such as grass and alfalfa which are generally wilted to dry matter contents of 25-30% and 35-40% respectively (O' Kiely 1989, Carpintero et al 1969). While this is the primary aim of wilting significant changes in microbial numbers, water soluble carbohydrate and protein levels may also occur.

A number of studies have shown increases in lactic acid bacteria and enterobacteria numbers of widely varying degrees during wilting. Ostling and Lindgren (1991) reported that after harvesting, crops wilted overnight contained 5 times the number of lactic acid bacteria and enterobacteria found in unwilted crops. Similarly Kroulik et al (1955) showed a 7 to 17 fold increase in total bacterial numbers during a 2 to 5 hour wilt while Henderson et al (1972) observed an increase in lactic acid bacteria numbers from 100/g on the standing crop to 1.7×10^4/g after a 25 hour wilt and 1.1×10^5 after 31 hours. A large increase in total counts, to which enterobacteria presumably contributed, was also observed. A comprehensive study of bacterial numbers prior to ensiling by Muck (1989) concurred with these results. He reported that lactic acid bacteria numbers increased from undetectable levels on the

standing crop to an average of 52/g after cutting, due to inoculation from the mower, to 5 X 10^4/g after a 24 hour wilt. The rate of increase of lactic acid bacteria numbers then fell away, presumably due to decreases in moisture and water soluble carbohydrate levels. The growth of lactic acid bacteria during wilting increased with increasing temperatures above 15°C but was very slight below this temperature. Lactic acid bacteria numbers were found to be lower at the top of the swath. The author suggested that this was due to the sterilising effect of UV radiation though lower levels of moisture are another possibility. It therefore seems probable that since no increases in clostridia occur during wilting due to the prevailing aerobic conditions, the increase in lactic acid bacteria numbers combines with their increased competitiveness compared to clostridia to make a successful fermentation more likely. However prolonged wilting, beyond the period of maximum lactic acid bacteria growth, should be avoided since the increase in aerobic to facultative anaerobic bacterial and fungi (Kroulik et al 1955) numbers may lead to stability problems during feedout (Woolford 1984).

Changes in the water soluble carbohydrate content of crops during wilting and the early stages of ensiling are brought about primarily by respiration by plant enzymes (Melvin 1963, Festenstein 1966). Respiration may be summarised as follows:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 2870kj.$$

In the living plant the energy released is captured in the form of ATP. However after, harvesting, most biosynthetic reactions cease and this energy is instead dissipated as heat. Respiration continues in the harvested plant as long as substrate and oxygen are available and the pH, which varies little during wilting (Henderson et al 1972, Carpintero et al 1979), remains suitable. Losses of water soluble carbohydrate due to respiration would therefor be expected during wilting. Decreases in water soluble carbohydrate levels from 10.5% DM to 8.6% DM and from 17.7% DM to 16.8% DM have been observed in alfalfa after a 25 hour wilt and in ryegrass after a 31 hour wilt respectively (Carpintero et al 1969, Henderson et al 1972). Clark (1974) noted losses of as much as 50% of the water soluble

carbohydrate over a 12 hour wilt of Italian ryegrass though the level fluctuated somewhat afterwards, perhaps due to release of sugars from plant polysaccharides. By contrast Carpintero et al (1979) noted negligible loss of water soluble carbohydrate in ryegrass over a 48 hour wilt. While this suggests that water soluble carbohydrate losses during wilting might be controlled they are normally accepted as an inevitable part of a process that is of overall benefit to the fermentation.

Plant enzymes are also responsible for substantial losses of crop protein during wilting. Kemble and McPherson (1954) reported a 20% loss of ryegrass protein over a 3 day wilt and a 50% loss was reported by Ohyama (1970) after a 5 day wilt of cocksfoot. Brady (1960) examined the nitrogenous fraction of wilted ryegrass and found that the majority of protein nitrogen was converted to amino nitrogen, with only small increases in volatile nitrogen and amide nitrogen. The extent of proteolysis is dependent on the rate of drying and if a rapid wilt is achieved little protein nitrogen is lost (Woolford 1984).

1.4.2. Harvesting.

While lactic acid bacteria numers on the standing crop are low, they increase during harvesting to 10^3-10^7/g herbage at the point of ensilage (Henderson et al 1972, Ely et al 1981, Moon et al 1981, Pahlow and Dintner 1987, Rooke 1990, Jonsonn 1991, Kung et al 1991, Nesbakken and Broch Due 1991). This increase has been attributed to a combination of growth by indigenous bacteria and inoculation from harvesting machinery (Stirling and Whittenbury 1963, Woolford 1984). Fenton (1987) maintained that, during operation, the forage harvester and mower are rapidly coated with a layer of sap within which lactic acid bacteria multiply. After two days of operation lactic acid bacteria numbers on the harvesting machinery ranged from 5 X 10^7 to 1 X $10^{10}/m^2$ with enterococci, leuconostocs and lactobacilli predominating. Muck (1989) agreed that in swaths with a low level of lactic acid bacteria (<100/g) the forage harvester was a source of inoculation, at a rate of approximately 10^3 lactic acid bacteria/g forage. The inoculation rate was observed to increase with increasing

air temperature and decreased during cool days. However in swaths with lactic acid bacteria counts of greater than 10^4/g the growth of these bacteria during the harvesting process could account for lactic acid bacteria numbers at the point of ensiling. By contrast, Pahlow and Ruser (1989) maintained that increases in lactic acid bacteria numbers during harvesting could not be accounted for by microbial growth and that harvesting machinery could not harbour sufficient lactic acid bacteria to inoculate the large amounts of forage cut. Instead they suggested that substances such as manganese released from plant cells during harvesting allow lactic acid bacteria, which would otherwise not be detected, to survive isolation. This would imply that increases in lactic acid bacteria numbers during harvesting are only apparent and that the microbial population of the fresh crop has been consistently underestimated by a factor of 10^2-10^7.

1.4.3. Chemical and Microbial changes in the silo.

In the initial stages of the silage fermentation the major changes in the water soluble carbohydrate and protein content of the crop are brought about by the activity of plant enzymes (Whittenbury 1968, Woolford 1984). Sucrose and fructans are rapidly hydrolysed to their component monomers, glucose and fructose, which thus become the major water soluble carbohydrates available to bacteria (Woolford 1984). A combination of plant enzyme activity and subsequent acid hydrolysis may result in as much as 55% of crop hemicellulose being broken down to arabinose and xylose (Dewar et al 1963, McDonald et al 1962, 1968). Since oxygen is present in the early stages of ensilage, respiration by plant enzymes continues, resulting in a loss of water soluble carbohydrate and a rise in temperature. In well sealed silos this temperature rise is of the order of 3-4°C (McDonald et al 1966), but in poorly sealed silos, temperatures as high as 55-60°C have been recorded (Kempton and San Clemente 1959). Extensive proteolysis also continues during this period. Kemble (1956) showed that within 12-24 hours of ensilage the non protein nitrogen content of the crop can increase from 200g/kg of total nitrogen to 400g/kg of total nitrogen and demonstrated

using sterile ryegrass that plant enzymes were responsible for this activity.

Once plant respiration has exhausted oxygen supplies, which in well sealed silos may take as little as 5 hours (Woolford 1984), a series of microbial successions, as described in figure 1.3, begins which leads to the eventual stabilisation of the crop.

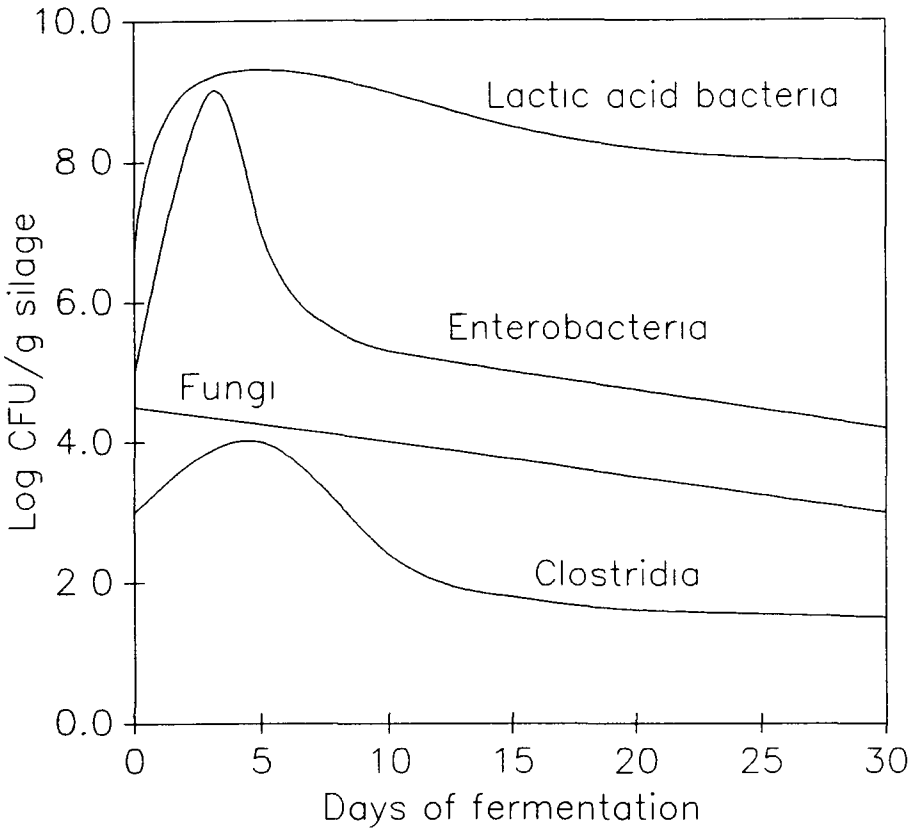


Figure 1.3: Typical changes in major microbial groups during the ensilage of forage crops (Muck 1988).

In the initial period of ensiling coliforms, which are present at levels of 10^3-10^8/g fresh material, rapidly multiply and peak at around 10^9/g silage after 1-2 days (Kung et al 1991, Nesbakken and Broch Due 1991). During the early stages of anaerobiosis some clostridial growth is also observed; Henderson et al (1972) observed an increase in clostridial numbers from 10^3/g on the harvested crop to 10^5/g 3 days after ensilage. This accounts for the presence of some butyric acid in well preserved silage. The lactic acid cocci (pediococci, enterococci and leuconostocs) have a higher pH optimum and a greater tolerance of aerobic conditions than lactobacilli. They are therefore the dominant lactic acid bacteria during the first 2 days of ensilage and initiate the lactic acid fermentation (Ely et al 1981, Moon et al 1981, Lindgren et al 1983). The extensive growth of the heterofermentative leuconostocs and the enterobacteria, which both ferment sugars to acetate, in the initial stages of ensilage leads to acetic acid being temporarily produced in relatively high quantities compared to lactic acid, even in silages which ultimately preserve well.

The pH reduction initiated by the lactic acid cocci brings about a major shift in the microbial population. The enterobacteria and clostridia have pH optima of 7-7.4 and the fall in pH brings about a rapid reduction in their numbers (Woolford 1984). The pediococci and enterococci therefore play a vital role in suppressing enterobacteria and clostridia in the initial stages of ensilage and thereby preventing an acetic acid/ butyric acid fermentation and crop spoilage (Langston et al 1962, Lindgren et al 1983). Yeasts and mould are relatively unaffected by acid conditions and their numbers remain constant or decline slowly during ensilage (Nilsson 1956, Dellaglio and Torriani 1985, Chamberlain and Quig 1987). Lactic acid bacteria numbers peak at 10^8-10^9/g after 2-4 days and then decline in a slow steady manner to 10^7-10^8/g (Henderson et al 1972, Seale 1986, Nesbakken and Broch Due 1991, Kung et al 1991). The fall in pH also causes a shift within the lactic acid bacteria population with the enterococci and leuconostocs being superceeded by the more acid tolerant lactobacilli and pediococci and 7 days after

ensilage pediococci and homofermentative lactobacilli can comprise as much as 75% of the bacteria present in the silo (Langston et al 1962). These species are highly efficient in lactic acid production and ensure a rapid fall in pH to about 4. This low pH not only inhibits the growth of undesirable micro-organisms but also suppresses the activity of plant proteases. Plant proteases have a pH optimum of 5-6 (Tracey 1948) and while Carpintero et al (1979) have shown that some proteolysis may occur at pH values as low as 3.5-3.8, most if not all proteolysis ceases once a pH of 4.3 is reached (MacPherson 1952). The rate of acidification therefore determines the extent of proteolysis during ensilage.

During the latter stages of ensilage a large increase in heterofermentative lactobacilli occurs. By day 62 Langston et al (1962) observed that 45% of isolates were of this type, 40% were homofermentative lactobacilli and 15% pediococci. A similar shift from homofermentative to heterofermentative lactobacilli was noted by Beck (1972). He observed that acidification was initiated by homofermentative species, with *L. plantarum* and *L. curvatus* dominating by day 4. However by day 142, 75-97% of the lactobacilli were heterofermentative, with *L. brevis* and *L. buchneri* predominating. This shift in fermentation type was attributed to the greater tolerance of the heterofermentative lactobacilli to the low pH prevailing in the latter stages of the silage fermentation and to the presence of acetic acid, a major product of their fermentation (Beck 1978). In some cases the acid tolerant homofermentative pediococci dominate the microflora in the latter stages of the fermentation along with the heterofermentative lactobacilli (Whittenbury 1968, McDonald and Whittenbury 1973). This may explain why lactic acid concentrations in the silo continue to increase relative to those of acetic acid during this period, in spite of the high numbers of heterofermentative lactic acid bacteria present. It has also been suggested that heterofermentative lactic acid bacteria may switch to the homofermentative production of lactic acid under low pH conditions as was observed for *S. bovis* by Russell and Hino (1985).

It is generally agreed that a lactic: acetic acid ratio of 4-5:1 and a final lactic acid concentration of 100g/kg DM are required to attain a pH of 4 and ensure good preservation (Henderson and McDonald 1984, Kung et al 1984, Woolford 1984, Kung et al 1991, Nesbakken and Broch Due 1991, Tengerdy et al 1991). This represents 2-3 times the amount of lactic acid predicted on the basis of the buffering capacity of the original herbage (McDonald and Henderson 1962). The discrepancy is due to an increase in buffering capacity during ensilage caused by the undesirable action of lactic acid bacteria on organic acids, mainly citrate and malate. While these constitute the major buffering components of the fresh crop the end products of their fermentation by lactic acid bacteria are either neutral (acetoin, 2,3-butane diol and ethanol), salts of organic acids (lactate, acetate, carbonates and formate) or alkaline (cations released on decarboxylation) and have an even greater buffering effect; the lactate and acetate formed have a greater buffering capacity than their compounds of origin while cations released in decarboxylation directly neutralise fermentation acids formed from crop sugars (Whittenbury 1968). The CO_2 produced also represents a loss in dry matter and the overall effect is a 2 to 4 fold increase in crop buffering capacity (McDonald and Henderson 1962, Playne and McDonald 1966, Greenhill 1964 (a)). However this activity is unavoidable unless fermentation is suppressed completely.

The major challenge to the preservative effect of the lactic acid bacteria is presented by the clostridia. While some clostridial growth often occurs in the early stages of ensilage, it is only in the latter stages that extensive clostridial activity is observed and their metabolic end products occur only in more mature silages (Woolford 1984). Clostridial growth is favoured by a low ratio of crop water soluble carbohydrate to buffering capacity, low crop dry matter contents, low initial levels of lactic acid bacteria, high initial pH, delayed sealing of the silo and storage at high temperatures (Gibson 1965, Woolford 1984, Leibensperger and Pitt 1987). Clostridial development is initiated by the more acid tolerant saccharolytic species (Woolford 1984). These ferment 2 moles of lactate to one of

butyrate, thereby raising the pH towards neutrality. Such a pH rise was observed in wheat silage by Moon et al (1981) from 5.0 after 3 days fermentation to 5.25-5.4 after 25 days and was accompanied by a rise in butyric acid levels and the near disappearance of lactic acid. This rise in pH creates a more favourable environment for the growth of proteolytic clostridia which ferment amino acids by deamination, oxidation/reduction or decarboxylation, leading to the formation of ammonia, fatty acids, CO_2 and amines. The production of ammonia leads to a further rise in pH and since it is produced in relatively small quantities by other silage microorganisms (McDonald 1981) it is frequently used as an indicator of proteolytic clostridial activity. Ammonia nitrogen levels as high as 23% of the total nitrogen have been reported in poorly preserved silages (O' Kiely 1985). As well as a loss in nutritive value due to amino acid degradation, the proteolytic activity of clostridia also poses potential health risks to the animal due to amine formation. The amines cadaverine, putrescine, histamine, γ-amino butyric acid, β-alanine, tyramine and tryptamine have all been detected in silages (Whittenbury 1968). Clostridial silages are characterised by a high level of butyric acid and ammonia and a high pH. This leads to poorly preserved forage, low dry matter intake and poor utilisation of silage nitrogen by the animal.

In preventing clostridial silages, it is the rate of pH fall at the beginning of the fermentation as opposed to the final pH achieved which is crucial (Macpherson and Violante 1966, Whittenbury 1968). A sharp fall in pH during the initial stages of ensilage depends on rapid sealing of the silo and maintainance of anaerobic conditions. Delayed sealing of the silo has been shown to result in a lactic acid fermentation being replaced by a butyric one (Miller et al 1961, Henderson and McDonald 1975). Wilson and Flynn (1979) observed that a 24 hour delay in sealing the silo resulted in a final silage with a pH of 5.41 compared to 4.19 in controls, a four fold increase in butyric acid levels and a similar decrease in lactic acid content. Two factors, both of which result in a diminished lactic acid fermentation, contribute to this shift in fermentation

pattern. Greenhill (1964) has shown that autolysis of plant cells is necessary for the growth of lactic acid bacteria and that this release of plant juice is delayed by up to 3 days by the presence of air. Furthermore in the presence of oxygen, aerobic micro-organisms utilise water soluble carbohydrate that would otherwise be fermented to lactic acid. This theory is supported by the greater susceptibility of low water soluble carbohydrate crops to the deleterious effects of air ingress (Henderson and McDonald 1975). Presumably high water soluble carbohydrate crops can withstand greater losses of sugars through non-fermentable activity without seriously affecting the fermentation.

1.4.4. Feedout.

The presence of air in the silo also encourages the proliferation of yeasts (Ruxton and McDonald 1974) and gram negative bacteria (Langston et al 1962, Ohyama et al 1975). This is of great significance during feedout when the anaerobic environment is changed to an aerobic one. Micro-organisms which were previously dormant due to lack of oxygen now multiply, resulting in deterioration of the silage. The timescale for deterioration varies from hours to weeks and the factors determining aerobic stability are not fully understood. Aerobic deterioration results in an increase in temperature and pH, high nutritional losses, restricted intake (Moon et al 1980) and in extreme cases death due to toxin ingestion (Beck 1978). Moon and Ely (1979) have reported temperature increases of as much as 12°C during the first 48 hours of aerobic deterioration and a pH increase of 3.5 units after 96 hours. Investigations of the factors predisposing crops to aerobic deterioration have only been partially successful. Air ingress into the silo, delayed sealing and excessive wilting are all known to lead to a proliferation of aerobic micro-organisms which become active once the silo is opened, resulting in crop deterioration. (Weise 1968,1971, Takahashi 1970, Koch et al 1973, Honig 1975, Weise and Honig 1975, Woolford et al 1979). Attempts to predict the aerobic stability of a silage from its chemical constituents have been largely unsuccessful. A number of studies have found no

relationship between aerobic stability and levels of residual sugar, lactic acid, volatile fatty acids, ammonia, free amino acids or pH (Ohyama et al 1975, Henderson et al 1979, Ohyama and McDonald 1975, Ohyama et al 1977). Daniel et al (1970) reported that silage with a high dry matter content is susceptible to aerobic deterioration though Ohyama et al (1975) and Henderson et al (1979) found this not to be the case. Carbohydrate rich silages such as those made from maize and stemmed potatoes (Gross and Beck 1970) and those in which fermentation has been restricted (Barry and Fennessy 1972) have also been observed to be prone to aerobic deterioration. While there is some debate about factors predisposing silage to aerobic deterioration it is well established that the products of a butyric acid fermentation; butyric acid, isobutyric acid and isovaleric acid, confer aerobic stability on silage (McDonald 1981, Woolford 1984, Henderson et al 1979) through the inhibitory action of higher volatile fatty acids on microbial growth (Woolford 1975).

At a microbial level yeasts, moulds and bacteria all play a role in the aerobic deterioration of silage. Yeasts have long been considered the most significant micro-organisms in this process and crops with a yeast count of greater than 10^5/g have been regarded as particularly prone to aerobic deterioration (Daniel et al 1970). However a number of studies have shown crops with lower numbers of yeasts to deteriorate with equal rapidity (Ohyama and McDonald 1975, Henderson et al 1979, Woolford et al 1979). The yeasts involved comprise the acid utilising species Candida, Endomycopsis, Hansenula and Pichia and to a lesser extent the sugar utilising Torulopsis (Ohyama and Hara 1975, Moon and Ely 1979). These yeasts assimilate lactic acid resulting in a rise in temperature and pH and extensive nutrient losses. Moulds also play a significant role in aerobic deterioration, particularly in grass silages (Ohyama et al 1977). Moulds break down sugars, lactic acid, cellulose and other cell wall components and may produce harmful mycotoxins (McDonald 1981). A wide range of moulds have been isolated from silage, the most common of which belong to the genera *Monascus, Geotrichum, Byssochlamys, Mucor, Aspergillus, Penicillium* and *Fusarium.* (McDonald

1981). The bacteria involved in aerobic deterioration are mainly *Bacillus sp.* possessing both saccharolytic and proteolytic activity, though lactic acid bacteria may also be involved (McDonald 1981, Woolford 1984).

Attempts to relate the aerobic deterioration of silage made from different crops to the action of either yeasts, moulds or fungi have proved inconclusive and one would tend to conclude that all three types of organism have a role to play in the aerobic deterioration of grass, corn and alfalfa silage (Woolford et al 1978 and 1979, Barry et al 1980, Crawshaw et al 1980, Moon et al 1980, Woolford and Wilkie 1984, Lindgren et al 1985, Spoelstra et al 1988). Whatever their identity, the organisms responsible for aerobic deterioration seem to be indigenous to silage as silage exposed to filtered air has been shown to deteriorate at an equal rate as the same silage exposed to unfiltered air (Woolford et al 1978). However this does not exclude the possibility of contamination during unloading operations on the farm. It is unlikely that aerobic deterioration can be countered by chemical additives and the problem is best controlled by good ensiling technique, ie. ensuring rapid and complete sealing of the silo and minimising exposure time of the silo face to air during feedout. This is best accomplished using narrow silos.

1.5. SILAGE ADDITIVES.

From the preceeding description of the silage fermentation it is evident that a wide range of factors, some of which (eg. lactic acid bacteria numbers on the crop), are very difficult to control, influence the outcome of the ensiling process. In order to ensure consistent preservation from such a variable raw material the use of additives has been proposed. A wide variety of chemicals have been suggested as potential silage additives, the most successful of which has been formic acid. Applied at a rate of 2-4l/t fresh crop (Woolford 1984) it directly reduces the crop pH and inhibits microbial growth and plant enzymes. However formic acid, like many chemical additives, is dangerous to handle and corrosive to farm machinery. Interest has therefore grown in biological additives which are safe to handle and act by aiding the natural fermentation. This is accomplished by increasing the water soluble carbohydrate available for fermentation either directly, or indirectly, by the addition of cellulolytic or amylolytic enzymes. Alternatively the population of desirable lactic acid bacteria may be boosted.

1.5.1. Sugar additives.

The benefits of adding sugar sources to crops at ensilage, thereby increasing the substrate available for fermentation to lactic acid, has been demonstrated by a number of workers using pure sugar solutions. Weise (1967) added 10g/kg sucrose to grass of 150g/kg DM and 100g WSC/kg DM and observed an accelerated growth of lactic acid bacteria, a more rapid pH fall and the early elimination of coliform bacteria. The addition of 20g/kg glucose to Italian Ryegrass with a WSC content of 41g/kg DM by Ohyama et al (1971) resulted in a pH drop to 3.69 compared to 5.71 in controls. Glucose addition to alfalfa has also been shown to reduce proteolysis and improve the stability of amino acids (McDonald 1981).

While pure sugars are undoubtedly successful in aiding preservation they are too expensive for use on commercial farms. Molasses, a relatively cheap by-product of sugar refining, has been used instead. Molasses has a dry matter content of 70-75% and a WSC content of 650g/kg DM, of which

sucrose is the main component (McDonald et al 1981). Carpintero et al (1969) added 40g/kg of molasses to lucerne, raising the WSC from 70 to 190 g/kg DM. The final silage had a pH of 4.1, a lactic acid content of 93.3 g/kg DM and an ammonia nitrogen level of 78 g/kg of total nitrogen, compared to values of 4.8, 51.2 and 203 for the control. Similar improvements in fermentation patterns on addition of molasses at ensiling have been reported by a number of authors (Salsbury et al 1949, Andrews and Stob 1958, Becker et al 1970, Umana et al 1991). Crucial to the success of molasses in aiding fermentation is the application rate which must be sufficient to compensate for the buffering capacity of the crop, and for losses due to plant respiration and heterolactic fermentation. Estimates of the proportion of added sugar lost as CO_2 range from 2% (Hartfiel and Marquering 1968) to 42% (Jung 1972) and a treatment rate of 20-40 g/kg is generally recommended (Woolford 1984). Application at such a level at the forage harvester is impossible due to the high viscosity of molasses and it is therefore poured onto each layer of forage of approximately 30 cm as the silo is filled. O' Kiely (1988) showed that when applied to the top of a filled silo molasses only penetrates the top layer of silage and the major part of the crop remains untreated. This difficulty in applying molasses has led to a decline in its popularity as a silage additive.

1.5.2. Enzyme additives.

Forage crops contain large reserves of carbohydrate in the form of complex polysaccharides, such as cellulose, hemicellulose, pectin and starch, which cannot be fermented by most lactic acid bacteria. It has therefore been proposed to add cellulolytic and amylolytic enzymes to forage crops at ensiling which would degrade these polysaccharides to simple sugars, thereby increasing the substrate available for fermentation to lactic acid. Furthermore increased rates of digestion in the rumen have been related to maximum feed intake (Mertens and Ely 1979) which in turn is negatively correlated with forage cell wall content (Osbourn 1976). Therefore any breakdown of cell wall components by cellulase would be expected to

increase silage digestibility.

Commercial cellulolytic silage additives usually contain cellulase enzymes from *Aspergillus* and *Trichoderma* spp., which are active in the silage pH range 4-6. In view of the complex nature of the forage carbohydrate a mixture of cellulases, hemicellulases and pectinases is frequently employed. Tengerdy et al (1991) ensiled fresh and wilted alfalfa with 2 enzyme mixtures; one comprising 0.025% *T. reesei* cellulase DM/g forage and 0.005% alkaline cellulase and the other 0.025% each of *T. reesei* cellulase and viscozyme, a hemicellulase and pectinase preparation from an *Aspergillus* spp. . Both enzyme treatments induced a more rapid pH drop and an increase in lactic acid bacteria numbers and lactic acid production compared to controls. Enzyme effects were more pronounced in fresh than wilted alfalfa, suggesting that the lower dry matter content of fresh alfalfa provided more favourable conditions for enzyme hydrolysis. The mixture of cellulase, hemicellulase and pectinase was more effective than that of acid and alkaline cellulases in making glucose and pentoses available for fermentation and in stimulating a homolactic fermentation, indicating that these enzymes may have a synergistic role in the breakdown of plant polysasccharides. Improved fermentation patterns arising from cellulase addition have also been reported by Henderson and MacDonald 1977, van Vuuren et al 1989 and Jacobs and McAllan 1991. However literature data reveals no consistent increase in crop digestibility associated with cellulase addition. Stokes (1992) added a mixture of cellulase, xylanase, cellobiase and glucose oxidase to grass legume forage and observed increases in digestibility in the resulting silage. When treated silage was fed to cows increases in dry matter intake, milk production and milk protein were noted. Similar results were reported by Chamberlain and Robertson (1989). However Huhtanen et al (1985) and Kung et al (1990) observed no increases in digestibility of cellulase treated corn silage. Indeed decreases in digestibility of corn silage associated with cellulase treatment were reported by Jacobs and McAllan (1991) and may indicate that cellulase degraded the more digestable fraction of the structural polysaccharide,

leaving a less digestible residue.

While cellulose represents the major storage carbohydrate in grass, corn and lucerne also contain large quantities of starch. Like cellulose, starch is unavailable to most lactic acid bacteria but may be released by the action of amylase enzymes. Barancic and Sevcic (1966) applied *A. oryzae* amylase at a rate of 20 kg/t to chopped alfalfa and observed an increase in lactic acid and a reduction in acetic acid, butyric acid and ammonia compared to control silages. Leahy et al (1990) applied an α 1-4 amylase to chopped corn at a rate of 0.05% on a wet crop basis and observed a 7% increase in liveweight gains in animals fed the resulting silage. When these animals were returned to a normal diet they still exhibited higher liveweight gains than control animals fed the same ration. Companion studies (Leahy 1988) revealed no effect of amylase treatment on silage water soluble carbohydrate or lactic acid levels but an increase in ruminal microbial activity in animals fed the treated silage. This suggests that the liveweight gain increases reported were linked to an improved microbial flora in the rumen. By contrast Froetschel et al (1991) observed increases in lactic acid levels in wheat silages treated with α amylase in crops of 41.6% DM but not in crops of 23% DM and no improvements in animal performance.

In an assessment of amylolytic and cellulolytic additives for silage, Pitt (1990) considered that the crucial parameter in determining their success was whether enzyme activity was sufficiently high to achieve substantial substrate hydrolysis within the 2 week fermentation period. Silo conditions were considered unfavourable for enzyme activity with the complex structure of the substrate, diffusion limitations of enzymes and products, low water activities and the presence of plant proteases combining to limit enzyme activity in silage to .01 to .00001 of those reported in laboratory studies. Application rates of 5000g/t and 100g/t of cellulase and amylase respectively were therefore recommended. Woolford (1984) concluded that such application rates were uneconomical and that the use of enzyme additives depended on the isolation of enzymes with high specific activities in crude extracts.

1.5.3. Lactic acid bacteria additives.

Lactic acid bacteria numbers on the crop at ensiling range from 10^3 to 10^7/g which one might suppose would be sufficient to ensure a lactic acid fermentation. However desirable homofermentative acid tolerant lactic acid bacteria constitute a small proportion of this population (Fenton 1987) and inoculation with such bacteria has been proposed as a means of aiding preservation. The criteria required of any such inoculant were outlined by Whittenbury (1961) as follows:

(i) It must have a high growth rate and be capable of dominating other bacteria likely to occur in silage.

(ii) It must be homofermentative.

(iii) It must be acid tolerant and rapidly reduce the pH to 4.

(iv) It must be capable of fermenting glucose, fructose, sucrose and preferably fructosans and pentosans.

(v) It must not produce dextran or mannitol from sucrose.

(vi) It should have no action on organic acids.

(vii) It should have a broad temperature range, extending up to 50°C.

Other researchers have added other criteria including lack of proteolytic activity (Wieringa and Beck 1964), activity under aerobic and anaerobic conditions and over a wide dry matter range, cellulase activity (McDonald 1983), growth at low temperature and utilisation of starch (Lindgren 1984). These criteria exclude leuconostocs and heterofermentative lactobacilli on metabolic grounds and enterococci on grounds of acid tolerance. Homofermentative lactobacilli and pediococci would therefore appear to have the greatest potential as silage inoculants.

Owing to the difficulty emphasised by Wierenga and Beck (1964) and Woolford and Sawczyc (1984) of isolating a single strain fulfilling all the aforementioned characteristics a mixed culture would appear to have greatest potential as a silage inoculant. *Lactobacillus plantarum* has been singled out as the most suitable inoculant based on the criteria of Wieringa (McDonald 1981). However *L. plantarum* is slow to produce acid until

the pH falls below 5 (McDonald 1981), while grass at ensiling has a pH of approximately 6. Many inoculants therefore contain, in addition to *L. plantarum*, a strain to initiate the fermentation and rapidly lower the pH to 5, at which point *L. plantarum* becomes optimally active. Common choices include *P. acidilactici* and *Enterococcus* spp. both of which have high pH optima compared to *L. plantarum*. *Enterococcus* spp. have the added advantage of a high growth rate under the aerobic conditions which prevail in the initial stages of ensilage.

Numerous reports cite improvements in fermentation patterns (Ely et al 1981, Henderson and McDonald 1984, Dellaglio and Torriani 1985, Weinberg et al 1988, Nesbakken and Broch-Due 1991, Kung et al 1991) and animal performance (Rooke et al 1988, Gordon 1989, Mayne 1990, Petit and Flipot 1990, Martinsson 1991, Wohlt 1989) associated with the use of bacterial inoculants. Typical of these reports is that of Gordon (1989). He ensiled grass of 24g/kg WSC and 15.3% using an *L. plantarum* inoculant at a rate of 10^6 CFU/g grass. Inoculation resulted in a more rapid rate of lactic acid production and pH decrease; 3 days after ensiling the pH and lactic acid content of the inoculated silage were 3.77 and 84g/kg compared to 3.95 and 73g/kg in controls. By feedout time there were no significant differences in dry matter, butyric acid levels or the pH of inoculated and uninoculated silages. However the inoculated silage had higher crude protein, fibre and soluble carbohydrate levels and lower ammonia nitrogen and acetic acid contents than controls. Animals fed this silage had significantly higher dry matter intakes and a 7% higher milk yield than those fed control silage. At a microbial level inoculants have been shown to increase the lactic acid bacteria population and decrease numbers of yeasts, moulds and coliform bacteria (Kung et al 1991). The animal performance studies previously referenced indicate that lactic acid bacteria inoculants bring about improvements in silage nutritional value by ensuring a more rapid decrease in pH and not by reducing the final pH achieved. A rapid pH fall was previously described as being crucial in minimisig losses due to proteolysis by plant enzymes and by clostridial action. The strong positive correlation in the literature

between increased rates of pH decrease associated with bacterial inoculants, lower ammonia nitrogen and acetate levels in the resulting silage and improvements in animal performance would suggest that this is the mechanism by which bacterial inoculants exert their benefit.

While the data discussed above would suggest a positive influence by bacterial inoculants on the silage fermentation numerous reports cite no benefits associated with inoculation (Burghardi et al 1980, Thonney et al 1980, Ely et al 1982, Kung et al 1984, Haigh et al 1987, Shockey et al 1988, Cleale et al 1990). However, when taken as a whole, literature data suggests that bacterial inoculants aid silage preservation as long as the following points are taken into consideration:

(i) Strains for use as silage inoculants must be carefully selected. Poor strain selection is a likely cause for the failures of mixtures of *Bacillus subtilis, Aspergillus oryzae* and *Leuconostoc mesenteroides* (Thonney et al 1980) and of a *Candida* spp. and *Lactobacillus acidophilus* (Moon et al 1981) to aid preservation. *Lactobacillus acidophilus* for example is a natural inhabitant of the digestive tract of animals, with a temperature optimum of 30-35°C and fastidious nutrient requirements. Its failure to successfully compete under silo conditions is therefore hardly surprising. However care in strain selection is no guarantee of success. Woolford and Sawczyc (1984) subjected 21 lactic acid bacteria strains, including some isolated from silage, to a comprehensive series of laboratory tests based on the criteria previously outlined. The 3 strains exhibiting greatest potential were tested in laboratory silos but failed to improve preservation. While these results are somewhat discouraging some of the most successful inoculant trials reported in the literature have involved lactic acid bacteria strains isolated from silage (Gordon 1989, Mayne 1990, Kung et al 1991, Nesbakken and Broch-Due 1991). One can therefore conclude that such strains are, as one might expect, best adapted to compete under silo conditions, and are therefore most likely to aid preservation.

(ii) For inoculation to be successful, enough water soluble carbohydrate (generally 60-80 g/kg DM) must be available to

provide sufficient substrate for fermentation to lactic acid. This was well ilustrated by Henderson et al (1984) who ensiled lucerne of 49 g/kg DM water soluble carbohydrate using no additive, an inoculant, a sugar additive and the two treatments combined. A decrease in silage pH values and ammonia nitrogen levels was only observed using both the inoculant and the sugar additive.

(iii) Domination of the epiphytic microflora requires that an inoculant be applied at a rate of 10^6/g grass, preferably in a liquid form. The mathematical model of the silage fermentation developed by Leibensperger and Pitt (1987) predicts that an inoculation rate of 10^6/g is required to ensure a successful fermentation, a conclusion borne out by the review of Woolford (1984) who noted that such an inoculation rate produced well preserved silages from a variety of materials. Lower inoculation rates may be responsible for the failure of a number of inoculant trials (Henderson and McDonald 1984, Haigh et al 1987, Shockey et al 1988, Cleale et al 1990). Henderson and McDonald (1984) and Haigh et al (1987) attributed the failure of a number of commercial inoculants to two factors; application at a low rate and in a powdered form. Application in liquid form at the forage harvester as opposed to a dry form at the pit ensures better distribution throughout the crop and a shorter lag phase. Ideally inoculants should be cultured overnight prior to application at a rate of 10^6/g as practised by Nesbakken and Broch-Due (1991) in a series of very successful inoculant trials. This ensures that sufficient numbers of bacteria in a highly active metabolic state are well distributed throughout the crop, thereby maximising the potential for a rapid fermentation and domination of the epiphytic microflora.

(iv) Inoculants are most successful under good ensiling conditions, where uninoculated controls preserve reasonably well anyway. They therefore tend to make good silages better and are of little benefit in circumstances where unsuitable crop composition or poor ensiling technique would lead to poor preservation.

1.5.4. Future developments in silage additives.

1.5.4.1. Strain development strategies.

In view of their ease of application, their success in a number of well publicised silo trials, their low cost compared to enzymes, and their potential for future development, bacterial inoculants now dominate other types of biological additives both in terms of sales and research efforts. While the circumstances and manner of inoculant application maximising the chances of a successful fermentation are well understood, strain development remains a major challenge. Natural selection and genetic engineering represent the two main strategies for the development of inoculant strains. Natural selection may involve the isolation of strains which are better adapted to the silage fermentation than those in existing inoculants. Alternatively strains bearing novel desirable characteristics may be added to existing inoculants as any strain bearing such a characteristic is unlikely to be competitive enough under silage conditions to control the fermentation on its own. However the additional strain must be sufficiently competitive to express the desired activity in the silo and must not possess any activities detrimental to preservation. The choice of such strains is therefore virtually limited to the lactic acid bacteria.

Genetic engineering facilitates the introduction of DNA coding for single desirable characteristics from strains otherwise unsuitable as inoculants, into competitive inoculant strains. Since the strain initially possessing the characteristic of interest is not actually used in the inoculant the choice of strain is not limited by its suitability for use in the silage fermentation. However the choice is constrained by guidelines governing the use of genetically engineered strains in the environment which dictate that strains bearing only DNA from within the same genus are acceptable for release. Since most inoculant strains are lactobacilli or pediococci any novel characteristics to be introduced into inoculant strains would therefore have to originate from within these genera. Genetic manipulation may also involve the elimination or disruption of DNA coding for undesirable characteristics in inoculant strains, in which case such problems do not

arise.

1.5.4.2. Potential improvements in inoculant strains.

The most obvious improvement in silage inoculant strains would involve an increase in their general competitiveness and adaptation to silo conditions. Such an improvement would involve a number of characteristics and would therefore be best accomplished by natural selection. Other more defined developments in which genetic engineering might play a role include inactivating genes coding for the decarboxylation and deamination of amino acids, altering the ratio of lactic acid production in favour of the L(+) isomer, thereby reducing the potential for acidosis associated with high intake of the D(-) form, increasing competitiveness of inoculants by incorporating the ability to produce substances antagonistic towards competing micro-organisms, and conferring amylolytic or cellulolytic activity on inoculant strains thereby increasing the substrate available for fermentation to lactic acid. The last option is particularly attractive for a number of reasons. A shortage of crop water soluble carbohydrate is the rationale behind using sugar sources or enzymes as silage additives and is a well established cause of inoculant failure. Therefore any inoculant strain capable of making further reserves of fermentable carbohydrate available for itself would combine the advantages of all three types of biological additives. This is borne out by models of the silage fermentation and farm scale silo experiments which show amylases, cellulases and inoculants to have a synergistic effect on the silage fermentation. (Pitt 1990, Tengerdy et al 1991). The high cost of the enzyme component of such an additive would be overcome if the enzyme was produced by the inoculant itself during fermentation. This could be achieved by including a strain producing amylase or cellulase in an inoculant mixture or by cloning a suitable amylase or cellulase gene into a competitive inoculant strain. The natural selection strategy avoids any regulatory difficulties and would therefore be preferable. However it depends on the isolation of a lactic acid bacterium secreting a cellulase or amylase enzyme active under silage conditions, and which

is itself reasonably competitive under such conditions. Unfortunately no lactic acid bacterium secretes cellulase (Buchanan and Gibbons 1974) and the development of a cellulolytic inoculant is therefore unlikely in the short to medium term. However amylolytic activity has been reported in lactic acid bacteria of the genera *Enterococcus*, *Leuconostoc* and *Lactobacillus* but not *Pediococcus*. Assuming a suitable strain could be isolated, the development of an amylolytic inoculant for use on starch containing crops is therefore possible. Since forage at ensiling has a pH of about 6 and the final silage pH is approximately 4 the primary criterium for selection of an amylolytic strain for use in the silage fermentation is the degree of amylase activity in this pH range. Amylolytic strains of *E. bovis, E. lactis, E. faecalis, E. equinas, E. liquefaciens* and a number of *Leuconostoc* spp. have been isolated (Seeley and Dain 1960, Langston and Bouma 1960a, Walker 1965, Boyer and Hartman 1971, Wirahadikusumah et al 1972, Lindgren and Refai 1984), though the corresponding amylase enzymes remain largely uncharacterised. Of those studied, the amylases produced by *E. equinis* and some *Leuconostoc* strains show little activity below pH 5 (Boyer and Hartman 1971, Lindgren and Refai 1984), and this, combined with the inability of enterococci and leuconostocs to grow below pH 4.5 suggests that these genera are unlikely to be a source of an amylase enzyme with a pH profile suitable for use in the silage fermentation.

Strains of the lactobacilli *L. casei, L. cellobiosus, L. amylovorus, L. amylophilus and L. plantarum* along with strains resembling *L. acidophilus* and *L. vitelinus* have all been reported to be capable of degrading starch (Langston and Bouma 1960b, Nakamura and Crowell 1979, Nakamura 1981, Champ et al 1983, Sen and Chakrabarty 1986, Giraud et al 1991). Of the few corresponding amylases which have been characterised, that of *L. cellobiosus* has an unsuitable pH range (Sen and Chakrabarty 1986) but those of *L. amylovorus* and *L. plantarum* show some potential for use in silage. The *L. amylovorus* amylase has a broad pH range with an optimum of 5.5 (Nakamura 1981). In common with *L. amylophilus, L. amylovorus* was isolated from cattle waste corn

fermentations, but has a greater amylolytic activity, producing 3 times the level of lactic acid produced by *L. amylophilus* in a 20 hour fermentation on MRS containing soluble starch. It is a homofermentative organism, capable of fermenting glucose and fructose, the major forage crop carbohydrates to lactic acid (Nakamura and Crowell 1979, Nakamura 1981). Its growth is inhibited by glucose concentrations in the region of 100g/l (Zhang and Cheryan 1991) but such high levels of glucose are unlikely to be encountered in silage. The optimum temperature and pH for lactic acid production from starch is 40-45°C and 5-6 respectively. A sharp decrease in lactic acid production is observed below 30°C (Cheng et al 1991) and this, combined with the limited ability of the organism to grow below 20°C (Nakamura 1981) would seem to make the strain unsuitable for inclusion in a silage inoculant. However Petit and Flipot (1990) reported the inoculation of alfalfa timothy silage with a commercial inoculant mixture consisting of *L.plantarum* (40%), *L. casei* (20%), *L. amylovorus* (20%), *E. faecium* (15%) and *Cellulomonas flavigena* (5%). While inoculation resulted in increased intake compared to control silage, the breakdown of starch during fermentation was not monitored and it is therefore impossible to assess whether the amylase activity of *L. amylovorus* exerted any effect.

Lactobacillus plantarum was previously described as the organism best suited for use as a silage inoculant and the amylolytic *L. plantarum* strains isolated from grass silage by Langston and Bouma (1960b) would therefore appear to have considerable potential in the development of an amylolytic inoculant. Unfortunately no further data was reported on these highly interesting strains. However Giraud et al (1991) have recently characterised an amylolytic strain of *L. plantarum*, designated A6, isolated from cassava roots during retting. The corresponding amylase enzyme seems well suited to the silage fermentation with a pH optimum of 5 and 70% activity at pH values of 4 and 6. While the enzyme has a high temperature optimum of 55°C it still exhibits 30% activity at 20°C. Furthermore the strain seems highly competitive, exhibiting growth rates and lactic acid yields on glucose and starch close to

those observed for a type strain of *L. plantarum* on glucose. While amylase is not produced at glucose levels greater than 6.7g/l this is unlikely to cause problems in the silage fermentation as at higher glucose levels the amylylolytic activity would not be needed for a successful fermentation. Both *L. amylovorus* and *L. plantarum* therefore seem to have some potential for inclusion in an inoculant mixture for use on crops containing starch. Should this prove impractical due to a lack of competitiveness under silo conditions the possibility of formulating an amylolytic inoculant based on these strains still exists. This would be accomplished by cloning the amylase gene from either strain into a competitive *L. plantarum* inoculant strain.

1.5.4.3. The genetic manipulation of inoculant strains.

Until recently, the genetic manipulation of lactic acid bacteria has been restricted by the lack of (a) suitable cloning vectors and (b) an efficient means of transformation. However a wide range of cloning vectors for lactic acid bacteria are now available including pTV1, pAMβ1 and pGK12 (Youngman et al 1983, LeBlanc and Lee 1984, Kok et al 1984). These vectors typically contain a gram positive origin of replication, a suitable antibiotic resistance marker and a number of unique restriction sites facilitating the introduction of heterologous DNA. Some also contain gram negative origins of replication, allowing them to be manipulated in easy to handle *Escherichia coli* strains. Furthermore vector DNA can now be efficiently transformed into most lactic acid bacteria by electroporation. This involves exposing cells to a brief high voltage electrical discharge, rendering them permeable to DNA, and is the method of choice for introducing DNA into lactobacilli , pediococci, enterococci and leuconostocs (Luchansky et al 1988, David et al 1989, Bringel and Hubert 1990, Kim et al 1992). Transformation efficiencies vary considerably with strain and range from 1-60/υg DNA to 5 X 10^6/υg DNA (Kim et al 1992, Bringel and Hubert 1990).

The simplest method of applying this technology to construct amylolytic inoculant strains involves cloning an

amylase gene, and the sequences required for its expression and secretion, into a suitable cloning vector and introducing the resulting construct into the desired strain by electroporation. Such a strategy was used by Jones and Warner (1990), Scheirlinck et al (1989) and Cocconcelli et al (1991) to introduce amylase genes from *Bacillus stearothermophilus* and *Bacillus amyloliquefaciens* into *L. plantarum* and, in the case of Cocconcelli et al (1990), into *L. reuteri, L. acidophilus* and *L. helveticus.* The vectors used by the three groups are described in figure 1.4.

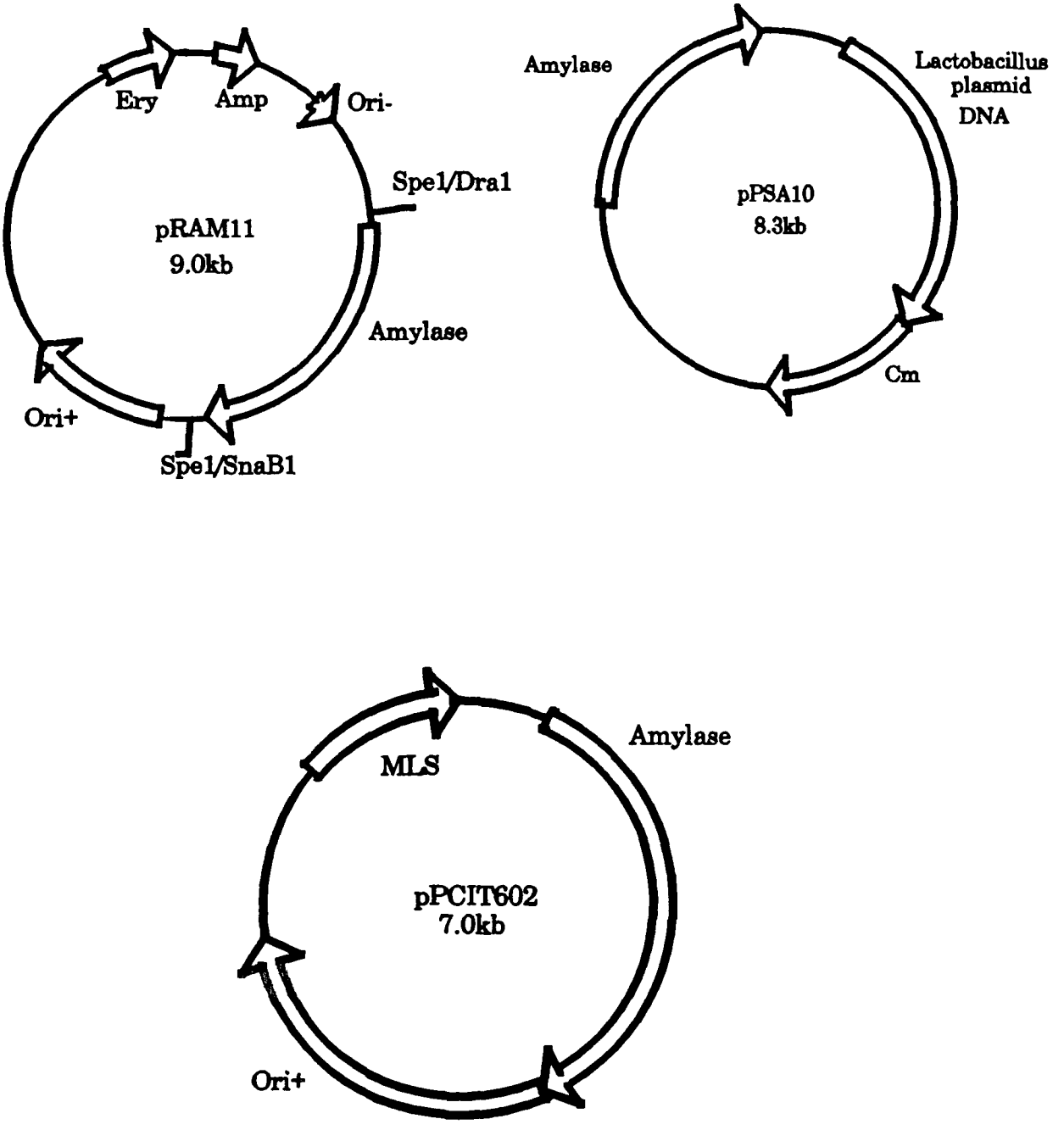


Fig. 1.4: Plasmids pRAM11 (Scheirlinck et al 1989), pPSA10 (Cocconcelli et al 1991) and pPCIT602 (Jones and Warner 1990)

In all three cases, transformants were stable under selective pressure and secreted active amylase, albeit at lower levels than those observed in the natural host. This may be a result of incompatibility between the expression and secretion signals of *Bacillus spp.* and those of *L. plantarum*. Under nonselective conditions the plasmid pRAM11 was unstable and after 26 generations only 10% of the *L. plantarum* cells contained plasmid (Scheirlinck et al 1989). By contrast Cocconcelli et al (1991) observed that 95% of *L. reuteri* cells transformed with pPSA10 retained the plasmid after 600 generations growth under nonselective conditions. Silage inoculants only undergo about 10 generations of growth in a typical fermentation (Bates et al 1989) and while stability studies were not carried out in *L. plantarum*, one would expect an *L. plantarum* strain carrying pPSA10 to be completely stable under silo conditions. This plasmid would therefore seem to be an ideal basis for constructing an amylolytic *L. plantarum* inoculant strain as it is stable, is maintained at a high copy number of about 60 per cell and confers on the host strain significant levels of amylolytic activity. In order to comply with regulatory requirements the *B. stearothermophilus* amylase gene would have to be replaced by one of *Lactobacillus* origin and the non *Lactobacillus* sequences coding for antibiotic resistances would have to be removed. However this would not cause a problem as these sequences are not involved in plasmid maintainance or heterologous gene expression and transformants could be selected by their amylase positive phenotype. The contrast in stability observed between pRAM11 and pPSA10 is difficult to explain as both plasmids contain a *B. stearothermophilus* amylase gene, sequences coding for antibiotic resistance and an origin of replication of *Lactobacillus* origin. The suggestion that plasmids replicating via single stranded DNA are unstable on insertion of foreign DNA (Jones and Warner 1990) does not apply as this was shown to be the mechanism of replication of pPSA10. Possible explanations include DNA of gram negative origin in pRAM11 contributing to instability or a hitherto unknown stabilising effect of the *L. plantarum*

origin of replication in pPSA10. Both of these possibilities could be investigated using suitable constructs.

Another means of ensuring the stable maintainance of heterologous genes under nonselective conditions is by integration of the gene into the host chromosome. This occurs via homologous recombination in which a cross over event between homologous sequences on a plasmid and on the host chromosome results in integration of the entire plasmid into the chromosome. Such integrants are normally very stable; Sharp et al (1992) reported a 100% survival rate for a chromosomally integrated plasmid in an *L. plantarum* inoculant after a 30 day silage fermentation. However the foreign gene is only present in a single copy per cell and levels of heterologous gene expression are therefore lower than for autoreplicative plasmids which may be present in hundreds of copies per cell. In view of the instability of pRAM11 Scheirlinck et al (1989) developed an integration vector, pH421 (see figure 1.5) for the inoculant strain *L. plantarum* 80, based on a 3.6kb chromosomal DNA fragment.

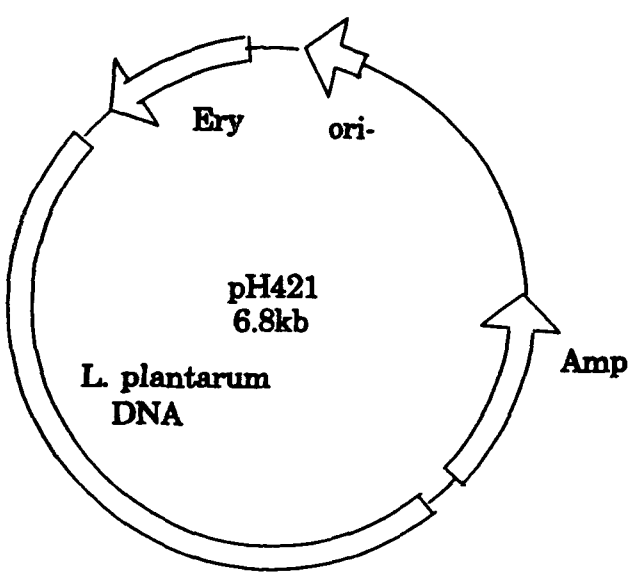


Figure 1.5: Plasmid pH421 (Scheirlinck et al 1989).

The vector contained an erythromycin marker and a gram negative but not a gram positive origin of replication and was therefore incapable of replicating in *L. plantarum*. On transformation of *L. plantarum* 80 with pH421 twenty erythromycin resistant colonies were isolated each of which was shown to contain plasmid pH421 integrated into the host chromosome at a different location. Only three of these integrants had altered growth characteristics compared to the parent strain, presumably due to integration disrupting a nonessential gene. The others exhibited no alterations in growth characteristics and stably maintained erythromycin resistance in the absence of selection pressure. The vector, pH421, therefore seemed to be a suitable tool for introducing foreign genes into *L. plantarum* 80 and a cassette containing the *Bacillus stearothermophilus* α-amylase and the *Clostridium thermocellum* endoglucanase genes along with sequences directing their expression and secretion was cloned into pH421. Following electroporation, fourteen transformants/υg DNA were isolated compared to 3 X 10^5/υg for autoreplicative plasmid DNA. This difference in transformation efficiency reflects the low frequency at which homologous recombination takes place. The genome structure at the point of integration of a typical transformant is shown in figure 1.6.

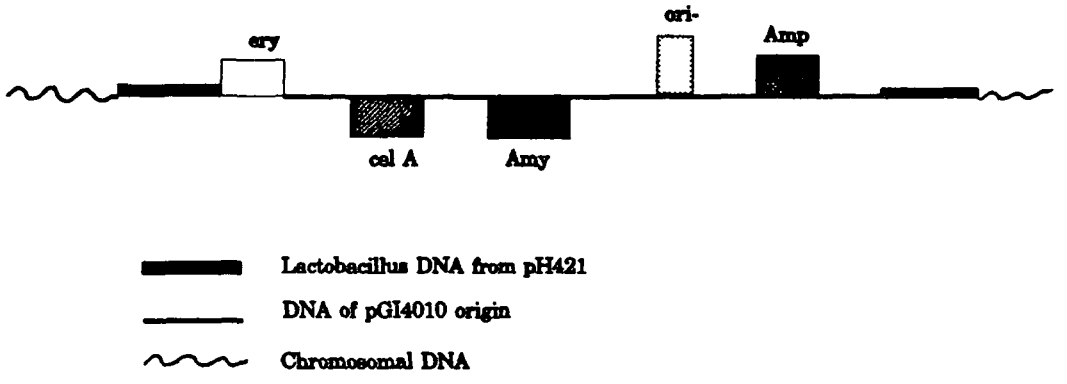


Figure 1.6: Structure of *L. plantarum* LP80 chromosome at site of integration of cellulase and amylase genes (Scheirlinck et al 1989).

Most of the transformants stably maintained erythromycin resistance and secreted endoglucanase at up to 40% of the levels observed using autoreplicative plasmids. However none secreted significant levels of amylase. The authors suggested that this was due to the amylase gene being present only in a single copy as the *Bacillus stearothermophilus* regulatory sequences had successfully directed amylase secretion when present on the multicopy plasmid, pRAM11. However it is possible that the high copy number of the gene when present on an autoreplicative plasmid compensated to some extent for the effect of incompatibility between the regulatory sequences of *Bacillus stearothermophilus* and the expression and secretion mechanism of *L. plantarum*. A single copy of the *Bacillus stearothermophilus* amylase gene under the control of *L. plantarum* regulatory sequences integrated into the chromosome might therefore direct secretion of significant quantities of amylase enzyme. While this problem could be rectified, figure 1.5 reveals a more fundamental problem associated with integrating foreign genes into the host chromosome via a single homologous recombination; even if the inserted gene originates from within the same genus as the host, the recombinant strain still contains DNA of gram negative origin from the integrative plasmid. The presence of such sequences is likely to prohibit the strain from being accepted for environmental release and thereby render it useless commercially. It is not practical to eliminate these sequences from the integrating plasmid since the resulting construct would have no origin of replication. The ligation mix would therefore have to be transformed directly into the inoculant strain and in view of the aforementioned low frequency of homologous recombination it is unlikely that sufficient DNA of the desired structure would enter the cell in order to allow the selection of integrants. To overcome this problem, Hols et al (1992) have developed a 2 step integration procedure in which only the gene of interest is ultimately integrated into the host chromosome. This strategy is outlined in figure 1.7.

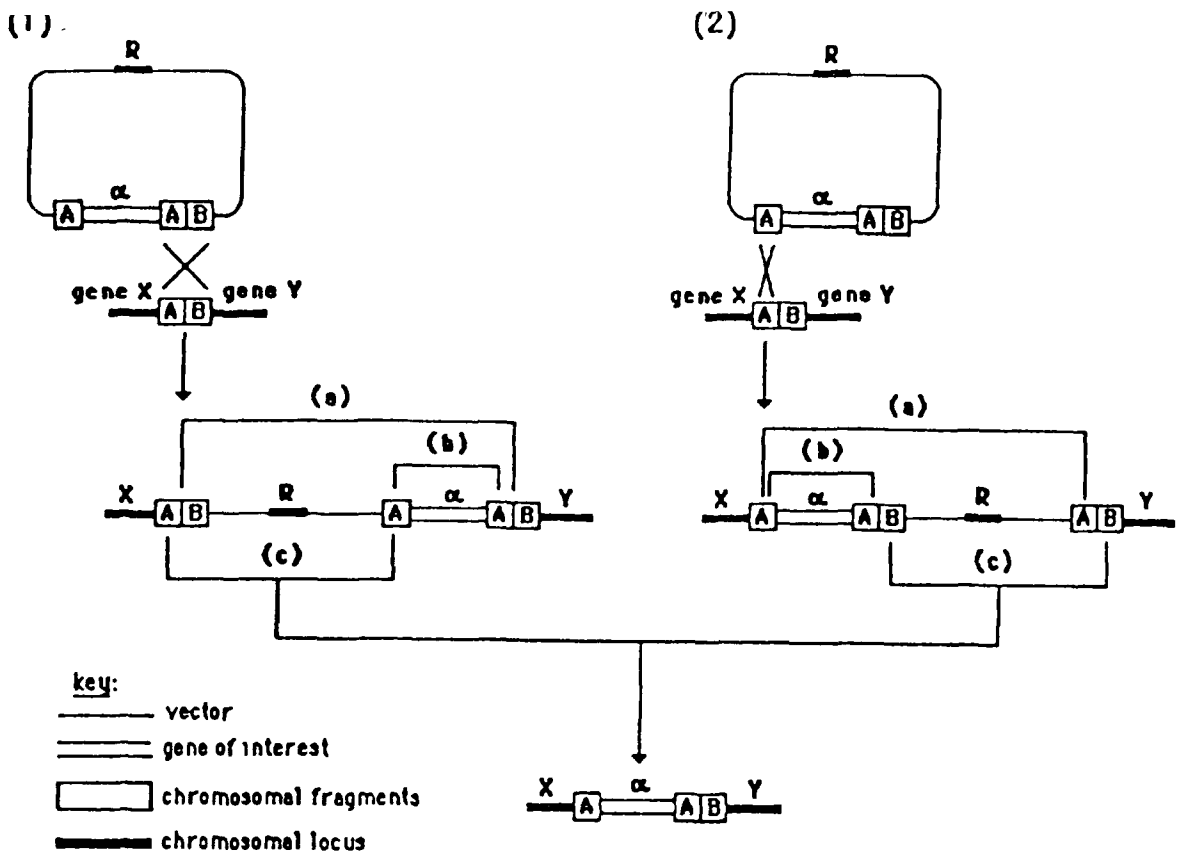


Figure 1.7: Two step integration procedure for integration of heterologous genes into host chromosome (Hols et al 1992).

The vector, which may be of the unstable autoreplicative or nonreplicative type, carries a resistance marker **R** and an integration-excision module composed of the gene of interest (α) sandwiched between a chromosomal DNA fragment (**AB**) on the right and the 5′ moiety of it (**A**) on the left. Integration of the whole plasmid takes place by recombination between the homologous chromosomal locus and the right plasmid fragment **AB** (case 1) or the left plasmid fragment **A** (case 2). These integrants can easily be selected since they have acquired the vector resistance marker **R** together with the gene of interest. Subsequent intrachromosomal recombination between direct repeats can occur in different ways; either the whole plasmid (a), the gene of interest alone (b) or the vector alone (c) can be excised. These recombinants can be selected phenotypically; negative for the resistance marker and the gene of interest (a), positive for the marker but negative for the gene (b) or negative for the marker but positive for the gene (c). Type c recombinants are chosen. To be functionally nondisruptive the gene X must end in **A** upstream and the gene Y must end in **A** or **B** downstream.

This strategy was applied to integrate the amylase gene of *Bacillus licheniformis* into the chromosome of the lactic acid bacterium *Enterococcus faecalis* (Hols et al 1992). An expression cassette comprising transcription, translation and secretion signals from *E. faecalis* was used as the **AB** fragment. This ensured that the integrated gene would be preceeded in the chromosome by the sequences directing gene expression and protein secretion in the ultimate host. The integration excision module was initially ligated to a nonreplicative vector but in spite of a transformation efficiency of $10^5/\upsilon g$ control DNA no transformants were detected due to the low frequency of integration. The module was therefore introduced into *E. faecalis* using an unstable autoreplicative vector as suggested by Rixon et al (1990). This allows the complete plasmid to be maintained in the host by applying selective pressure for sufficient generations to allow integration to take place. Following successful transformation the 2 step selection procedure was applied and 1 colony bearing the final desired phenotype were shown to contain a single copy of the amylase gene which had integrated in an **AB** type event. The final construct was stable with the amylase positive phenotype being lost at a frequency of only 3×10^{-4} per generation, which is similar to that reported by Cocconcelli et al (1991) for the autoreplicative plasmid pPSA10. While amylase production in the recombinant strain was not quantified and therefore cannot be compared to the 1850 units/l reported for *L. plantarum* transformed with pPSA10 a single copy of a foreign amylase gene integrated into the host chromosome was observed to be capable of directing significant levels of amylase secretion. This contrasts with the results of Scheirlinck et al (1989) and suggests that the regulation of foreign gene expression and secretion by sequences compatible with host mechanisms is crucial to maximising secretion of the protein product of single copy heterologous genes. The strategy of Hols et al (1992), if applied to *L. plantrum,* using the amylase gene of *L. amylovorus* or *L. plantarum* A6, therefore offers an alternative to the strategy of Cocconcelli et al (1991) for the construction of an amylolytic inoculant strain which would satisfy commercial and regulatory requirements.

1.6. FOREWORD TO EXPERIMENTAL WORK.

It was proposed to develop a lactic acid bacteria silage inoculant based on the theory that it is the rate of pH decrease at the beginning of ensilage, and not the final pH achieved, which is crucial in ensuring preservation (MacPherson 1952, Gibson et al 1958, MacPherson and Violante 1966, Whittenbury 1968). Such an inoculant would therefore have to be highly competitive, capable of rapidly dominating the fermentation. For this reason attention was focussed on the lactic acid cocci; the enterococci, the leuconostocs and the pediococci, which initiate the silage fermentation (Ely et al 1981, Moon et al 1981, Lindgren et al 1983). Of these genera the pediococci appeared the most suitable as, unlike the others, they are homofermentative and, since they are capable of tolerating low pH, are active throughout the entire course of the fermentation (Woolford 1984). *Pediococcus acidilactici* was selected as having the greatest potential, primarily on the basis of its ability to ferment the major crop carbohydrates, glucose and fructose, to lactic acid, and its tolerance of temperatures as high as 50°C (Teuber and Geis 1981, Woolford 1981). Literature data suggested that a suitable strain would most likely originate from silage (Gordon 1989, Mayne 1990, Kung et al 1991, Nesbakken and Broch-Due 1991) though strains from other sources would also be assessed for the purpose of comparison.

While a highly competitive inoculant strain would be expected to induce a rapid decrease in pH and ensure good preservation when sufficient water soluble carbohydrate is available for fermentation to lactic acid, such a strain would be incapable of producing enough lactic acid to ensure preservation under conditions of low water soluble carbohydrate. This problem is occasionally encountered when ensiling all forage crops, primarily due to adverse weather conditions in the period immediately prior to harvesting, but is common in the ensilage of alfalfa, which by nature has a low water soluble carbohydrate content and a high buffering capacity (Raguse and Smith 1966, Wilkinson 1978). Low levels of crop water soluble carbohydrate could be overcome by adding a sugar source along with an inoculant,

but this would represent a somewhat cumbersome and expensive approach. A more economical solution would involve designing an inoculant capable of fermenting forage carbohydrates which are normally unavailable to lactic acid bacteria (eg starch, cellulose) to lactic acid. In view of the criteria for inoculant selection (Whittenbury 1961, Wierenga and Beck 1964) and regulatory restrictions, silage inoculants will continue to be based almost exclusively on lactic acid bacteria. Since no lactic acid bacteria secrete cellulase, the range of substrates available to inoculants could only be extended to starch, using an amylolytic lactic acid bacterium. It was therefore proposed to develop such a strain, specifically designed to cope with low water soluble carbohydrate conditions where starch is available as an alternative carbohydrate source. As indicated by section 1.5.4.2., the lactobacilli are the only lactic acid bacteria secreting amylase enzymes which are potentially useful in silage. If a sufficiently competitive amylolytic strain of *Lactobacillus* could be isolated then it could be used directly as an inoculant. Otherwise the possibility of transferring a gene coding for a suitable amylase enzyme to a competitive inoculant strain remains. Regulations governing the release of genetically manipulated bacteria into the environment dictate that the inoculant strain would have to originate from the same genus as the amylase gene and that the gene would have to be stably integrated into the host chromosome.

2. Materials and Methods.

2.1. BACTERIAL STRAINS AND PLASMIDS.

Strain	Phenotype/Genotype	Source/Reference
Lactobacillus		
L. amylophilus NCIB11546	Amylase +	Nakamura and Crowell (1979)
L. amylovorus NRRLB4540	Amylase +	Nakamura (1981)
L. plantarum DCU-101	Silage isolate	DCU stocks
L. plantarum B2	Silage isolate	F. Duffner, DCU.
L. plantarum LP80	Silage isolate	P. Hols, Université Catholique de Louvain, Belgium.
L. plantarum NCIB8826		P. Hols.

L. plantarum NCYYZ2110	*L. plantarum* NCIB8826 transformed with pYYZ2110 Amy+, Em^r, Cm^r.	This study
L. plantarum NCYYZ2111	*L. plantarum* NCIB8826 transformed with pYYZ2111 Amy+, Em^r, Cm^r.	This study
L. plantarum NCYYZ2112	*L. plantarum* NCIB8826 transformed with pYYZ2112 Amy+, Em^r, Cm^r.	This study
L. plantarum NCYYZ2113	*L. plantarum* NCIB8826 transformed with pYYZ2113 Amy+, Em^r, Cm^r.	This study
L. plantarum NCYYZ2114	*L. plantarum* NCIB8826 transformed with pYYZ2114 Amy+, Em^r, Cm^r.	This study
L. plantarum NCYYZ2115	*L. plantarum* NCIB8826 transformed with pYYZ2115 Amy+, Em^r, Cm^r.	This study
L. plantarum NCYYZ2116	*L. plantarum* NCIB8826 transformed with pYYZ2116 Amy+, Em^r, Cm^r.	This study
L. plantarum NCYYZ2117	*L. plantarum* NCIB8826 transformed with pYYZ2117 Amy+, Em^r, Cm^r.	This study

L. plantarum NCGAFΔ1*	*L. plantarum* NCIB8826 with amylase gene integrated at CBH locus. Amy +, Em^s	This study
L. plantarum NCGAFΔ2	*L. plantarum* NCIB8826 with amylase gene integrated at CBH locus. Amy +, Em^s	This study
L. plantarum NCGAFΔ3	*L. plantarum* NCIB8826 with amylase gene integrated at CBH locus. Amy +, Em^s	This study
L. plantarum NCGAFΔ4	*L. plantarum* NCIB8826 with amylase gene integrated at CBH locus. Amy +, Em^s	This study
L. plantarum NCGAFΔ5	*L. plantarum* NCIB8826 with amylase gene integrated at CBH locus. Amy +, Em^s	This study
L. plantarum LPYYZ2112	*L. plantarum* LP80 transformed with pYYZ2112	This study
L. plantarum LPGAFΔ2	*L.plantarum* LP80 with amylase gene integrated at CBH locus. Amy +, Em^s	This study

L. plantarum LPGAFΔ3	*L. plantarum* LP80 with amylase gene integrated at CBH locus. Amy +, Em^s	This study
L. plantarum LPGAFΔ4	*L. plantarum* LP80 with amylase gene integrated at CBH locus. Amy +, Em^s	This study
L. plantarum LPGAFΔ5	*L. plantarum* LP80 with amylase gene integrated at CBH locus. Amy +, Em^s	This study

Pediococcus

P. acidilactici	PLL01	V. Laffitte,
"	PLL02	Lacto-Labo,
"	PLL03	Dange-St.-Romain,
"	PLL04	France.
"	PLL05	"
"	PLL06	"
"	PLL07	"
"	PLL08	"
"	PLL09	"

P. acidilactici	M408		F. Dellaglio,
"	P17		Piacenza, Italy.
"	E92		"
"	E112		"
"	E165		"
P. acidilactici	A12	Silage isolates	This study.
"	B14	" "	" "
"	C20	" "	" "
"	G24	" "	" "

Miscellaneous

Bacillus cereus	Protease +	DCU stocks
Escherichia coli TG1	supE hsdΔ5 thi Δ(lac-proAB) F'(traD 36 proAB$^+$ lacI^{q}lacZΔM15)	Gibson (1984)

* *Lactobacillus plantarum* NCGAFΔ1 to NCGAFΔ5 and LPGAFΔ2 to LPGAFΔ5 represent independent integrants, all of which were constructed using the plasmid pGAF002.

Table 2.1: Bacterial strains used in this study.

Plasmid	Phenotype	Source/Reference
pGK13	Cm ^{r}, Em^{r}	Kok et al(1984)
pYYZ2110*	pGK13 containing fragment of *L. amylovorus* α-amylase gene. Amy +, Em^{r}, Cm^{r}	This study
pYYZ2111	pGK13 containing fragment of *L. amylovorus* α-amylase gene. Amy +, Em^{r}, Cm^{r}	This study
pYYZ2112	pGK13 containing fragment of *L. amylovorus* α-amylase gene. Amy +, Em^{r}, Cm^{r}	This study
pYYZ2113	pGK13 containing fragment of *L. amylovorus* α-amylase gene. Amy +, Em^{r}, Cm^{r}	This study
pYYZ2114	pGK13 containing fragment of *L. amylovorus* α-amylase gene. Amy +, Em^{r}, Cm^{r}	This study
pYYZ2115	pGK13 containing fragment of *L. amylovorus* α-amylase gene. Amy +, Em^{r}, Cm^{r}	This study
pYYZ2116	pGK13 containing fragment of *L. amylovorus* α-amylase gene. Amy +, Em^{r}, Cm^{r}	This study

pYYZ2117	pGK13 containing fragment of *L. amylovorus* α-amylase gene. Amy +, Em^r, Cm^r	This study
pGIPCBH	CBH +, Em^r	P. Hols.
pGAF001	pGIPCBH with Sma1 adaptor in Xba1 site. Em^r	This study
pGAF002	pGAF001 with fragment of *L. amylovorus* α-amylase gene in Sma1 site. Amy +, Em^r	This study

*Plasmids pYYZ2110 to pYYZ2117 originate from cloning of *L. amylovorus* α-amylase gene and contain fragments of this gene of various length.

Table 2.2: Plasmids used in this study.

2.2. MICROBIOLOGICAL MEDIA.

Luria Bertani (LB) medium was routinely used for the culturing of *E. coli* strains and was prepared as follows:

Tryptone	10g
Yeast extract	5g
NaCl	5g
H_2O	To 1l

MRS (Oxoid) was used for the routine culture of lactic acid bacteria. On occasions where MRS containing an alternative sugar source to the usual 20g/l glucose was required, MRS was prepared from its constituents as follows:

Peptone	10g
Tryptone	8g
Yeast Extract	4g
Tween 80	1ml
di-Potassium Hydrogen Phosphate	2g
Sodium acetate.$3H_2O$	5g
tri-Ammonium Citrate	2g
Magnesium sulphate $7H_2O$	0.2g
Manganese sulphate $4H_2O$	0.05g
Carbohydrate source	As required
H_2O	to 1l
pH	6.2

Solid agar was prepared by adding 1.2% Oxoid No. 3 technical agar. All media were autoclaved at 15lb/in^2 for 20 minutes unless otherwise stated.

LP agar:

Peptone	10g
Tryptone	8g
Yeast Extract	5g
Sugar Source	20g
Tween 80	1ml
di-Potassium Hydrogen Phosphate	6g
Sodium acetate.$3H_2O$	2.5g
tri-Ammonium Citrate	2g
Magnesium sulphate $7H_2O$	0.2g
Manganese sulphate $4H_2O$	0.1g

The above constituents were dissolved in 900ml distilled water and 20ml of indicator solution containing 0.1g bromocresol green in 30ml 0.01N NaOH was added. The pH was brought to 5.5 using glacial acetic acid and agar was added to 1.2%. The total volume was brought to 1l with distilled water and the medium was autoclaved at 110^{o}C for 20 minutes.

SOC:

Tryptone	5g
Yeast extract	5g
NaCl	0.5g

The above were dissolved in 950ml distilled water, 10ml of 250mM KCl was added, and the pH was adjusted to 7 with 5M NaOH. The final volume was brought to 1l with distilled water. After autoclaving at 15 lb/ sq. inch, 5ml of 2M $MgCl_2$, sterilised by autoclaving, and 20ml of a 1M glucose solution, sterilised by filtration through a 0.22υm filter, were added.

Antibiotics, when required, were added after autoclaving, when the medium had cooled to 55^{o}C.

2.3. BUFFERS AND SOLUTIONS.

TE:

pH 7.6:

10mM Tris.Cl (pH 7.6)

1mM EDTA (pH 8.0)

DENHARDT'S SOLUTION (50X):

Ficoll	5g
Polyvinylpyrrolidone	5g
Bovine serum albumin	5g
H_2O	to 500ml

The solution was filtered through a 0.22υm filter, dispensed into 25ml aliquots, and stored at -20°C.

PRONASE:

Pronase was dissolved in dH_2O at a concentration of 20 mg/ml, incubated at 37°C for 1 hour, and stored at -20°C.

RNase FREE OF DNase:

RNase A was dissolved in 10mM Tris.Cl (pH 7.5), 15mM NaCl at a concentration of 10 mg/ml, heated to 100°C for 15 minutes, aliquoted, and stored at -20°C.

TRIS ACETATE BUFFER (TAE) 50X:

Tris base	242g
Glacial acetic acid	57.1ml
0.5M EDTA (pH 8.0)	100ml
H_2O	To 1l

TRIS BORATE (TBE) 5X:

Tris base	54g
Boric acid	27.5g
0.5M EDTA	20ml

GEL LOADING BUFFER (6X):

0.25% bromophenol blue

0.25% xylene cyanol

40% (w/v) sucrose in H_2O

AMPICILLIN:

A 25 mg/ml stock solution of the sodium salt was prepared in sterile H_2O and stored in aliquots at -20°C.

CHLORAMPHENICOL

A 25 mg/ml stock solution was prepared in 100% ethanol and stored at -20°C.

ERYTHROMYCIN

A 25 mg/ml stock solution was prepared in 98% ethanol and stored at -20°C.

PHENOL:

Solid phenol was melted at 68°C and 8-hydroxyquinoline added to a concentration of 0.1%. The melted phenol was extracted once with 1.0M Tris (pH 8.0) and a number of times with 0.1M Tris (pH 8.0), 0.2M β-mercaptoethanol until the pH of the aqueous phase was greater than 7.6. Phenol was stored at 4°C, in the dark under 0.1M Tris (pH 8.0), 0.2M β-mercaptoethanol.

SSC (20X):

Sodium citrate (88.2g) and NaCl (175.3g) were dissolved in 800ml dH_2O. The pH was adjusted to 7 with a few drops of 10M NaOH and the solution was made up to 1l.

SODIUM IODIDE:

NaI	90.8g
H_2O to	100ml

The solution was stirred until as much NaI as possible was dissolved. After filtering through Whatman No. 1 filter paper 1.5g of Na_2SO_3 was added and the solution was stored at 4^0C in a light proof bottle.

NEW WASH:

Ethanol	50%
Tris-HCl, pH 7.5	10mM
EDTA	1mM

The solution was stored at -20^0C.

STET BUFFER:

8% w/v Sucrose

5% w/v Triton X-100

50mM EDTA

50mM Tris.HCl, pH 8.0

SOLUTION 1:

50mM glucose

25mM Tris.Cl pH 8.0

10mM EDTA

SOLUTION 2:

0.2M NaOH

1% SDS

SOLUTION 3:

60 ml 5M potassium acetate

11.5 ml glacial acetic acid

28.5 ml H_2O

TRITON MIX:

5ml 20% Triton X-100

12.5ml 0.25M EDTA pH 8.0

2.5ml 1.0M Tris.Hcl pH 8.0

H_2O to 50ml.

TEN:

0.1M Tris.HCl

0.1M EDTA

0.15M NaCl

BLOCKING SOLUTION:

Supplied in DIG labelling kit (Boehringer Mannheim)

HYBRIDISATION SOLUTION:

5X SSC

0.1% N lauroylsarcosine Na salt

1% blocking solution

0.02% SDS

BUFFER 1:

0.1M maleic acid

0.15M NaCl

pH 7.5

BUFFER 2:

1% blocking solution in buffer 1

BUFFER 3:

0.1M Tris.Cl

0.1M NaCl

0.05M $MgCl_2$

pH 9.5

ANTIBODY CONJUGATE SOLUTION:

0.02% antibody in buffer 2

COLOUR SOLUTION:

135ul NBT solution

115ul X phosphate solution

40ml buffer 3

POLYACRYLAMIDE GEL ELECTROPHORESIS:

Separating Gel:

2.5ml 1.5M Tris. pH 8.8, 0.4% SDS

2.5ml 40% acrylamide 0.8% N, N'-methylene-bis-acrylamide

1ml 0.2% starch

150υl 50% glycerol

3.85ml H_2O

3υl TEMED

35υl 10% ammonium persulphate

Stacking Gel:

2.0ml 0.5M Tris. pH 6.8, 0.4% SDS

0.8ml 40% acrylamide 0.8% N, N'-methylene-bis-acrylamide

5.2ml H_2O

32υl TEMED

96υl 10% ammonium persulphate

3X Sample Buffer:

6ml glycerol

3ml β-mercaptoethanol

1.8g SDS

7.8ml 0.5M Tris pH 6.8, 0.4% SDS

0.1% bromophenol blue

H_2O to 20ml

Running Buffer:

30g Tris.HCl

14.4g glycine

0.1% SDS

H_2O to 20ml

2.4. LABORATORY METHODS FOR THE ASSESSMENT OF POTENTIAL SILAGE INOCULANTS.

2.4.1. Isolation of *P. acidilactici* strains from silage:

Pediococcus acidilactici strains A12, B14, C20 and G24 were isolated from different grass silages on the basis of their ability to grow at 50°C and to ferment D-arabinose to acid (Buchanan and Gibbons, 1974). The isolation procedure involved the addition of a 10g silage sample to 90ml of sterile Ringers solution followed by homogenisation. The silage extract was diluted in tenfold steps to 10^{-4} and 0.1ml of each dilution spread on MRS (Oxoid) medium containing 2υg/ml amphotericin B (Gibco). Following overnight incubation at 50°C, colonies were transferred onto MRS plates containing 20g/l D-arabinose as the sole carbon source and 20ml/l of a 30g/l solution of bromocresol green in 0.01N NaOH. Colonies capable of fermenting D-arabinose appeared yellow after overnight incubation at 37°C and were subjected to microscopic examination. All gram positive cocci were tested for their ability to ferment glucose, fructose, galactose, arabinose, xylose and maltose to acid, to confirm their identity. Glucose fermentation was assayed in MRS broth containing 0.0016% bromocresol green as an indicator. Other fermentation tests required the replacement of glucose with the appropriate sugar. Sugar utilisation was indicated by a colour change in the medium from green to yellow due to acid production.

2.4.2. Growth conditions:

Pediococci and lactobacilli were cultured on MRS (oxoid) at 37°C except where otherwise stated.

2.4.3. Growth characteristics:

The design of methods for determining growth characteristics was based on the characteristics required of a successful silage inoculant. All growth characterisations (growth curves, kinetic studies, sugar to lactate conversions etc.) were carried out using MRS as the growth medium.

Optimal growth temperatures were determined by inoculation

of culture media, preincubated at selected temperatures from 15°C to 45°C, followed by incubation for 24 h at the desired temperature. The effect of pH values between 4 and 8 on growth was assessed by inoculation of culture media, which had been adjusted to the desired pH using conc. HCl/H_2SO_4, and subsequent incubation at 37°C for 12 h. A 0.1% (vol/vol) inoculum from a static culture was used in all cases and culture turbidity at 600nm was used to monitor cell growth. Optical densities were plotted against pH and temperature and the optimum values of pH and incubation temperature for growth determined graphically. The percentage of maximum growth attained after 12 hours at 20°C was determined by simultaneously inoculating MRS broth preheated to both 20°C and the temperature optimum for the strain involved. Following incubation for 12 hours at these temperatures optical densities were compared.

Growth curves were determined using 100ml of MRS containing glucose or fructose as the sole carbon source, which was inoculated from a static culture at a rate of 10^6 cells/ml. Cell numbers were monitored by sampling and dilution in Ringers solution followed by plating on MRS and overnight incubation at 37°C. Growth curves were carried out at 30°C as opposed to 37°C in order to approximate more closely to silo conditions.

Exponential growth rates were calculated from the bacterial concentrations X_o and X_t at the times t_o and t according to the equation $\upsilon = (\lg X_t - \lg X_o) / \lg_e (t - t_o)$ where $\lg_e = 0.43429$ (Schlegel, 1985).

The lag phase was defined as the time interval between inoculation and the establishment of the maximum growth rate (Schlegel, 1985) and was determined graphically using a plot of log cell number vs time.

The conversion efficiencies of glucose and starch to lactate were calculated on the basis that 10g starch yields 11.11g glucose (Nakamura and Crowell 1981) and that the the fermentation of 1 mole of glucose to 2 moles of lactate represents 100% conversion.

2.4.4. Inhibition of *L. plantarum* growth:

Potential inoculants were screened for inhibiton of *L. plantarum* growth by dotting single clonies onto MRS agar using sterile toothpicks and incubating overnight at 37°C. Colonies were lysed by exposing to chloroform for 30 minutes and overlaid with soft MRS agar seeded with 10^6 *L. plantarum* cells/ml. Once solid, plates were incubated overnight at 37°C and screened for zones of inhibition.

2.4.5. Proteolytic activity:

Cells were streaked on MRS medium containing 5% gelatin as the sole carbon source. After 48 h incubation at 30°C plates were flooded with a solution of 15g mercuric chloride in 20ml conc. HCl and 100ml distilled water. *Bacillus cereus* was used as a positive control and proteolysis was detected as a clear zone surrounding areas of bacterial growth against a hazy white background.

2.4.6. Lactic acid determination:

Lactic acid levels in culture broths and silage extracts were determined enzymatically using beef heart L-(+)-lactic dehydrogenase and *Lactobacillus leichmanii* D-(-)-lactic dehydrogenase (Boehringer Mannheim Corp.).

2.4.7. Determination of amylase activity:

The presence of α-amylase activity in culture supernatants was detected using a Ceralpha kit available from Biocon Biochemicals Ltd., kindly donated by Helen Sheehan. This assay uses as a substrate *p*-nitrophenyl maltoheptaoside, chemically end blocked at the non-reducing end, which is resistant to attack by β-amylase, glucoamylase and α-glucosidase (McCleary and Sheehan, 1987). The nitrophenyl-maltooligosaccharides released by α-amylases are cleaved by glucoamylase and α-glucosidase in the kit to glucose plus free *p*-nitrophenol, which is measured spectrophotometricaly. Cultures for amylase determinations were grown for 24 hours in MRS containing 0.5% glucose and 0.5% starch as the fermentable carbohydrate source and buffered with 1% $CaCO_3$. A 0.2ml aliquot of culture supernatant was added to 0.2ml of the substrate mixture and incubated at 40°C for 10 minutes. The reaction was

terminated by addition of 3ml 1% Tris.HCl and the absorbance measured at 410nm. The α-amylase activity in Units/ml was calculated as follows:

(A_{410nm} /incubation time) X (total vol. / Vol. assayed) X (dilution / EmM) where EmM = 17.8.

Since this assay depended on a second enzyme system, it was not suitable for determining the temperature or pH optimum of the amylase enzyme. The Phadebas method was therefore used instead. A suitable volume of sample was diluted to 4ml in 0.2M Na_2HPO_4/NaH_2PO_4 which had been previously adjusted to the desired pH. A single Phadebas tablet was added, the tube vortexed for 10 seconds, and incubated at the desired temperature for 1 hour. The sample was then centrifuged at 5,000g for 10 minutes and the absorbance of the supernatant at 620nm measured. The level of amylase activity in the sample was determined from the standard curve supplied with the kit and the result expressed in Units where 1 unit of amylase activity was defined as the amount of enzyme catalysing the hydrolysis of 1υmol glucosidic linkage per minute.

The molecular weight of amylolytic proteins was determined using polyacrylamide gels containing starch as described in section 2.3. Samples for electrophoresis were mixed with sample buffer, boiled for 10 minutes and loaded onto the gel along with Rainbow molecular weight markers purchased from Amersham. The gel was run at 45 volts until the magenta marker (molecular weight 14,300) had reached the bottom of the gel. The gel was then washed for three 30 minute periods at room temperature with agitation in 100ml 10mM Tris.HCl, 0.2% starch, pH 5.5 and was incubated for 2 hours at 55°C in fresh washing solution and stained in 0.1% KI, 0.01% I_2.

2.4.8. Cell extracts.

Cultures for the preparation of cell extracts were diluted and plated to determine the initial number of cells. Cells were harvested by centrifugation at 5000g for 10 minutes at 4°C, the pellets were washed twice in half the original volume of 0.2M sodium phosphate buffer, pH 5.5, and resuspended in 1/10 the original volume of sodium phosphate buffer. The cell suspension was placed on ice and subjected to sonication for 10 cycles of 30 seconds at 65 watts with 30 second intervals using a Labsonic 2000 ultrasonic homogenizer. The sonicated cell suspension was diluted and plated in order to determine the number of cells remaining intact. The lysed cells were centrifuged at 5000g for 10 minutes at 4°C to remove cell debris and the supernatant stored at -20°C. Amylase activity was determined using the Phadebas system and activities expresses in Units per cell lysed.

2.5. SMALL SCALE SILO TRIALS.

2.5.1. Grass silage trials:

All grass silage trials were carried out using plastic pipe silos of 6kg capacity as described in figure 2.1. Inocula were grown to stationary phase in MRS at 37°C. Plate counts on MRS from cultures grown previously under identical conditions of initial cell concentration, MRS batch and incubation temperature and time were used to estimate inoculant cell density. The culture volume required to treat 7kg of grass at the desired rate of bacteria/g grass (2-4ml) was diluted to 10ml in sterile Ringers solution. Seven kilograms of perennial ryegrass (*Lolium perenne*) chopped by a precision-chop harvester was spread on a sterile plastic sheet and half the inoculum added dropwise using a syringe. The grass was hand mixed and the remaining inoculum added. It was assumed that small amounts of MRS which had supported bacterial growth to stationary phase would have no effect on the silage fermentation and uninoculated controls were therefore treated with 10ml of sterile Ringers solution. Following inoculation, 6kg of treated grass was packed into a test silo which was immediately sealed. For each treatment sufficient silos were set up to allow 3 silos to be opened on each day on which sampling was required. Silos were stored under cover at ambient temperature. Silos were sampled by emptying and thoroughly mixing the entire contents of the silo and then removing a sample of approximately 100g.

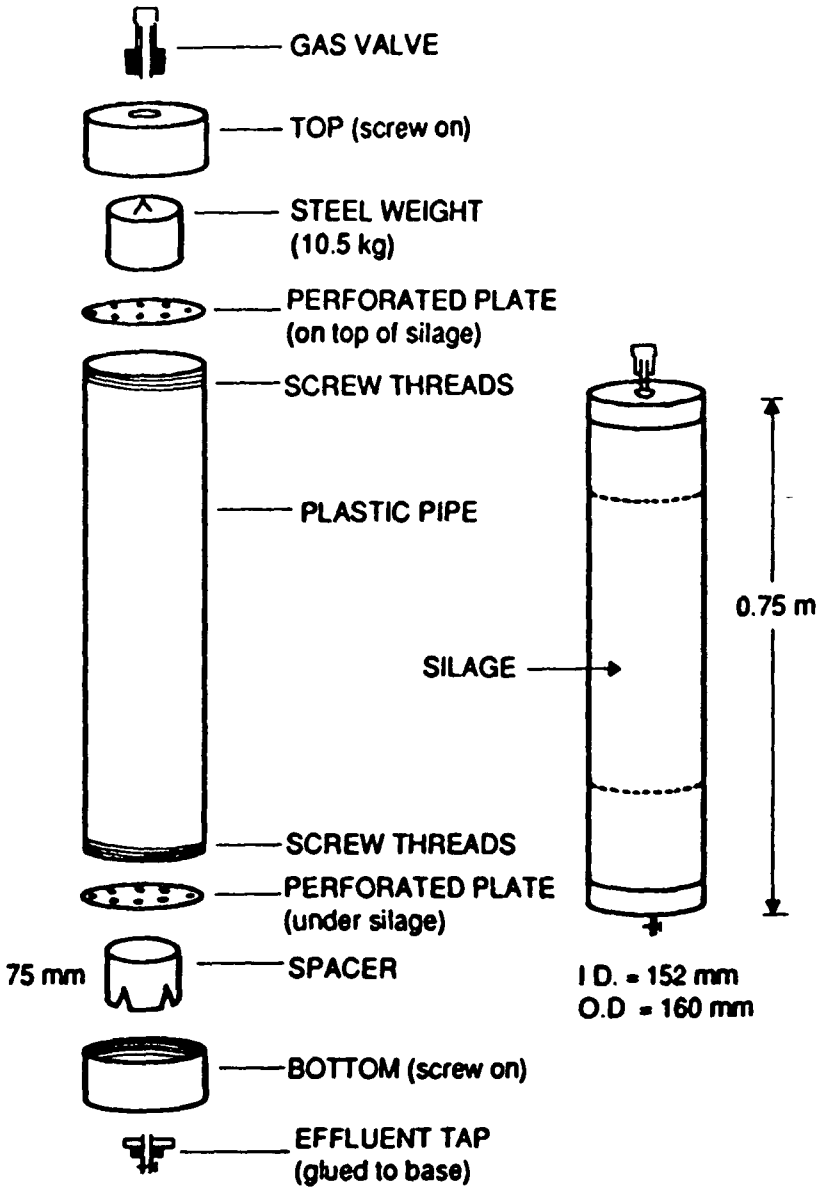


Figure 2.1: Plastic pipe silos used for grass silage trials (O' Kiely and Wilson 1991)

2.5.2. Cereal silage trials:

Only a small quantity of raw material was available for cereal silage trials and the relatively large plastic pipe silos were therefore deemed impracticle for this purpose. All cereal trials were instead performed using 100ml glass test tubes, fitted with a rubber stopper, through which was inserted a water filled gas release valve. Inoculants were prepared as described for grass silage. The substrate for ensiling consisted of a mixture of ground barley and chopped barley straw in a 1:1 ratio by dry weight. The substrate required for each treatment was spread on a plastic sheet and rehydrated while mixing to 30% dry matter with ringers solution containing the required inoculant using a mist applicator of the type commonly available in garden centres. This ensured an even distribution of inoculant. A 100g sample of treated substrate was packed into each test tube, the silo sealed, and stored at 15°C. As before each treatment was performed in triplicate for each day on which sampling was required and silos were sampled as described for grass silage.

2.5.3. Microbiological analysis of silage:

A 10g sample of silage was added to 90ml of sterile Ringers solution and homogenised. An extract was diluted in tenfold steps in sterile Ringers solution and the appropriate dilution plated.

Lactobacillus plantarum numbers were estimated by plating on LP agar, a selective medium based on the growth characteristics of lactobacilli as listed by Sharpe (1981) and on the sugar fermentation profile of *L. plantarum* (Buchanan and Gibbons, 1974). Plates were incubated for 48 hours at 30°C. *Lactobacillus plantarum* colonies appeared large and yellow against a background of small green/blue colonies. The low pH, high concentration of acetate ions and the presence of the growth stimulant Tween 80 in LP medium facilitates selection of lactobacilli (Sharpe, 1981). The use of sorbitol as the sole sugar source ensures that of the lactobacilli only *L. plantarum, L. salivarius, L. casei* and some strains of *L. coryneformis* produce acid (Buchanan and Gibbons, 1974) and form large yellow

colonies. Of these 4 strains only *L. plantarum* ferments both ribose and sorbitol (Buchanan and Gibbons, 1974) and 500 yellow colonies isolated in this manner from each treatment were replica plated on LP medium containing each of these sugars as the sole carbon source. The percentage of yellow colonies positively identified as *L. plantarum* varied from 97-98% in samples from silos which had not been inoculated with *L. plantarum* to 99-100% in samples from treated silos. The number of yellow colonies on LP medium was therefore used as an estimate of *L. plantarum* numbers.
No selective medium based on sugar fermentation profiles could be developed for *P. acidilactici*. However *P. acidilactici* is one of the few silage micro-organisms capable of growth at temperatures as high as 50°C (Buchanan and Gibbons, 1974). Samples were therefore plated on MRS containing 2υg/ml amphotericin B to inhibit fungal growth and incubated overnight at 50°C. *Pediococcus acidilactici* ferments glucose, fructose, galactose, arabinose and xylose but not maltose to acid and 500 colonies isolated in this manner from each treatment were tested for their ability to ferment these sugars. More than 95% of colonies tested were *P. acidilactici* and MRS counts at 50°C were therefore used to estimate *P. acidilactici* numbers.
Control silos revealed that throughout the ensiling period less than 20 amylolytic colonies/g silage were detected using MRS containing 1% soluble starch as the sole fermentable sugar source. This medium was therefore used for the enumeration of *L. amylovorus* in silage samples. Starch degradation was directly visible as a clear halo surrounding colonies producing amylase.

2.5.4. Biochemical analysis of silage:
Dry matter contents of silages and crops for ensiling respectively were determined by drying 50g of sample at 40°C for 48 h or at 98°C for 24 h.
Water soluble carbohydrate and pH determinations were carried out on aqueous extracts from grass, rehydrated cereals, or silage.
The measurement of pH was performed directly on aqueous

extracts using a pH meter.

Water soluble carbohydrates were measured colorimetrically using the method of Wilson (1978). A 10ml sample of aqueous extract was heated to boiling point over a bunsen burner, cooled in tap water and filtered overnight at 4°C through Whatman No. 5 filter paper. A 0.2ml aliquot of filtered sample was transferred to a fresh test tube and 1ml of 5% phenol and 5ml of conc. H_2SO_4 added. The absorbance of the resulting solution was measured at 490nm and the water soluble carbohydrate concentration of the sample calculated using a standard curve prepared using samples of known glucose concentration.

Ammonia nitrogen levels were estimated using an adaptation of the phenol hypochlorite method of O' Keeffe and Sherrington (1983). A 50ml aliquot of distilled water was added to 2g sample of chopped forage and the sample mixed. The sample was placed in a water bath at 50°C for 20 mins and after mixing the sample was filtered through Whatman No. 1 filter paper. To 0.1ml of sample filtrate, 5ml of a 10g/l phenol, 50.7mg/l sodium nitroprusside solution and 5ml of a solution containing 5g/l sodium hydroxide and 0.4% (v/v) sodium hypochlorite were added. The sample was mixed, incubated at 37°C for 15 minutes and the absorbance at 550nm measured. The ammonia nitrogen concentration was calculated from a standard curve prepared using solutions of known ammonium sulphate concentration.

Crude protein measurements were carried out on dried milled forage samples using a Tecator Kjeltec AUTO 1030 analyser.

Statistical analysis on results from laboratory silos was carried out using t-tests as described by O' Kiely and Wilson (1991).

2.6. GENETIC TECHNIQUES.

2.6.1. Preparation of total DNA from *Lactobacillus* strains: A 10ml aliquot of an overnight culture was centrifuged for 10 minutes at 3000g. The pellet was washed in 5ml of 6.7% sucrose in TE and resuspended in 2ml of the sucrose solution. A 0.2ml aliquot of 20mg/ml lysozyme in 25mm Tris pH 8 was added . After 20 minutes incubation at 37°C, with occasional agitation, 1ml of 0.25M EDTA (pH 8.0) and 1ml of 10% SDS was added. The sample was incubated for a further 10 minutes at 37°C and extracted twice with phenol and once with chloroform isoamylalcohol 24:1. A 1ml aliquot of 3M sodium acetate pH 5.2 followed by an equal volume of isopropanol at -20°C was then added. The sample was inverted a couple of times and the total DNA, which appeared as a fluffy white thread, was removed to a sterile tube using a pasteur pipette. The DNA was washed twice in 5ml of 70% ethanol, dried briefly under vacuum and 1ml of sterile water added. The pellet was resuspended by incubating at 65°C for 20 minutes and on cooling, 1υl of a 10mg/ml solution of RNase was added. The DNA was left overnight at room temperature and then stored at 4°C.

2.6.2. Small scale isolation of plasmid DNA from *E. coli*: 1. Lysis by boiling: Cells were grown overnight on LB agar containing the appropiate antibiotic. A patch of growth was transferred to an eppendorph containing 300υl of STET buffer using a sterile cocktail stick. The cells were completely resuspended and 20υl of a 10mg/ml lysozyme solution added. After 10 minutes incubation at room temperature the samples were placed in a boiling water bath for 60 seconds and were immediately centrifuged at 12.000g for 10 minutes. The resulting viscous pellet was removed using a sterile cocktail stick and 300υl of isopropanol added. The samples were incubated at -20°C for 30 minutes and centrifuged at 12,000g for 10 minutes. The resulting pellet was washed with 70% ethanol, dried under vacuum, resuspended in 30υl TE (pH 8.0), and 1υl RNase was added. The DNA was stored at -20°C.

2. Lysis by alkali: A single colony was inoculated into 10ml LB containing the appropiate antibiotic and incubated overnight at 37°C with vigorous agitation. A 1.5 ml culture aliquot was transferred to an eppendorph and centrifuged at 12,000g for 5 mins. The supernatant was removed by aspiration, the pellet was resuspended in 100 υl of ice cold solution 1, and 200 υl of fresh solution 2 added. Following a 5 minute incubation period on ice, 150 υl of soltion 3 was added and the sample was immediately vortexed for 10 seconds. After a further 5 minute incubation on ice, the sample was centrifuged at 12,000g for 5 minutes and the supernatant extracted with phenol and chloroform:isoamyl alcohol 24:1. Two volumes of ethanol were added, the sample was vortexed briefly and incubated at room temperature for 2 minutes. The plasmid DNA was recovered by centrifugation at 12,000g for 5 minutes, the supernatant was removed by aspiration and the pellet was washed with 1 ml of 70% ethanol. The pellet was dried under vacuum, resuspended in 30 υl TE (pH 8.0), and 1υl RNase added. The DNA was stored at -20°C. When larger quantities of plasmid DNA were required without the time and expense involved in performing a maxi prep, this procedure was scaled up using 8ml of culture, 200υl solution 1, 400υl solution 2, 300υl solution 3, 600υl isopropanol and resuspending the DNA in a final volume of 100υl.

2.6.3. Large scale preparation of plasmid DNA from *E. coli*: A 1l flask containing 250ml LB and the appropriate antibiotic was inoculated with 1ml of an overnight culture of *E. coli* and incubated with vigorous agitation at 37°C for 24 hours. The cells were harvested by centrifuging at 4000g for 10 minutes and resuspended to 2ml in ice cold 25% sucrose in 50mM Tris pH 8.0. The cell suspension was transferred to a 15 ml Corex tube and a 0.4ml aliquot of lysozyme (20mg/ml in 0.25M Tris) was added. After 10 minutes incubation on ice, 0.8ml of ice cold 0.25M EDTA pH 8 was added. The cells were incubated for a further 15 minutes on ice and 3.2ml of Triton mix added. The suspension was mixed and left on ice for a further 20 minutes. The sample was then centrifuged at 15,000g for 60 minutes at 4°C. The cleared lysate was removed to a 10ml

sterile tube and 6.9g of cesium chloride added and dissolved by gentle inversion. The solution was transferred to a Beckman quickseal polyallomer tube and 180ul of 10mg/ml ethidium bromide was added. The tube contents were brought to 14.1g using 10mM EDTA pH 8. The tube was filled using mineral oil, heat sealed and centrifuged at 50,000 RPM for 24 hours at 18°C. The tubes were viewed under ultra violet light and the lower plasmid band extracted using a sterile 18 guage needle and a 1ml syringe. For applications such as sequencing where extremely clean DNA was required, a second gradient was performed. A quickseal tube was filled to within 2cm of the top with a solution containing 116.8 g/100ml cesium chloride and 0.8 ml/100ml 0.5M EDTA, pH 8.0 and the DNA from the first gradient added. Following addition of 180ul 10mg/ml ethidium bromide, the tube was filled with mineral oil. The tube was sealed, centrifuged and the plasmid DNA removed as before. Ethidium bromide was removed from the plasmid DNA by extracting 3 times with isopropanol saturated with 20X SSC and the DNA was dialysed overnight against several changes of distilled water. The DNA was precipitated by adding 2 volumes of ice cold ethanol, incubating at -20°C for 1 hour and centrifuging at 14,000g for 20 minutes. The DNA pellet was dried under vacuum and resuspended to the desired concentration.

2.6.4. Preparation of electrocompetent *E. coli* cells:

A 1l flask, containing 250 ml LB warmed to 37°C, was inoculated with 2.5 ml of an overnight culture of *E. coli*, and incubated at 37°C with vigorous agitation until an OD_{600} of 0.6 to 0.8 was reached. The cells were chilled on ice for 30 minutes and harvested by centrifugation at 4000g for 15 minutes at 4°C using a prechilled rotor. The cells were resuspended by gentle swirling in 250ml of ice cold distilled water and centrifuged as above. The cells were then resuspended in 125ml of sterile water and centrifuged as above. A further resuspension in 5ml of ice cold 10% glycerol followed by centrifugation as above was performed. The cells were finally resuspended to a volume of 0.5 to 0.75ml in ice cold 10% glycerol, dispensed into 100ul aliquots and stored at -70°C.

2.6.5. Electroporation of *E. coli* cells:

A 100υl aliquot of competent cells was removed from the freezer and thawed gently at room temperature. The cells were transferred to ice and plasmid DNA dissolved in 2-5υl of TE (pH 8.0) added. The contents of the eppendorph were mixed well by flicking and incubated on ice for 1 minute. The Bio-Rad Gene Pulser unit was set to 25υF, 2.5KV and 200 ohms. The mixture of DNA and cells was transferred to an ice cold 0.2cm electroporation cuvette and the contents of the cuvette were brought to the bottom by a sharp tap on the bench. The cuvette was dried briefly with a tissue, rapidly inserted into the Gene Pulser unit and a single pulse applied. A 1ml aliquot of SOC medium at room temperature was immediately added to the cuvette and the cells were transferred to an eppendorph. The cells were incubated for 1 hour at 37°C with vigorous agitation and plated onto selective medium.

2.6.6. Preparation of electrocompetent *L. plantarum* cells:

A 2l flask, containing 500ml of MRS broth, preheated to 37°C, was inoculated with 10ml of an overnight culture of *L. plantarum* and incubated at 37°C, with vigourous agitiation until an $O.D._{600nm}$ of 0.6 was reached. The cells were harvested by centrifugation at 6000g at room temperature, washed twice in 500ml TEN and 3 times in 200ml dH_2O. The cells were resuspended in 500υl filter sterilised 30% PEG 1000, aliquoted in 100υl lots and stored at -80°C.

2.6.7. Electroporation of *L. plantarum* cells:

A 100υl aliquot of cells was removed from the freezer and allowed to thaw on ice. A 10υl aliquot of ice-cold plasmid DNA was added and the cell suspension was mixed and transferred to a cold 2mm electroporation cuvette. The eletroporator was set to 25υFD, 1.7KV and 400 ohms and a single pulse applied. The cuvette was transferred immediately to ice and after a 30 minute incubation, 1ml of MRS was added and the sample incubated at 30°C for 2 hours. The cells were then plated in 200υl aliquots on MRS containing the appropiate antibiotic.

2.6.8. Isopropanol precipitation:

DNA was precipitated by adding 1/10 sample volume 5M sodium perchlorate, 1/10 volume 5mg/ml tRNA and an equal volume of isopropanol. Samples were mixed well and centrifuged at 14,000g for 20 minutes. The supernatant was removed and the pellet washed with 70% ethanol. The DNA pellet was dried under vacuum and resuspended in TE (pH 8.0).

2.6.9. Column cleaning:

The bottom of a 0.75ml microfuge tube was pierced with a sterile 18 gauge needle and 10υl of glass beads and 500υl of a 70% solution of sepharose 4-CL in ethanol added. The tube was placed in a 1.5ml eppendorph to collect the eluate and centrifuged at 1,500 RPM for 2 minutes. A volume of TE (pH 8.0) between 20 and 100υl, of equal volume to the sample, was added and centrifuged as above. This process was repeated 5 times. The 1.5ml eppendorph was then replaced by a fresh sterile eppendorph, the sample was added to the glass beads, and the tubes were centrifuged as above.

2.6.10. DNA Restrictions:

Restriction enzymes and buffers were purchased from BRL and were used according to the manufacturer's instructions.

2.6.11. Alkaline phosphatase treatment:

A 1υg aliquot of plasmid DNA was restricted in a total volume of 10υl. After digestion was complete 1υl of a 1 Unit/υl solution of bacterial alkaline phosphatase was added. Following incubation at 37°C for 20 minutes, 10υl STE, 10υl 10mmEDTA, 5υl 10% SDS, 60υl sterile water and 100υl TE were added. The sample was heated to 70°C for 10 minutes and extracted twice with phenol and once with ether. The DNA was incubated at 55°C until no ether odour remained. An equal volume of ethanol was added and the sample incubated at -20°C for 1 hour. The sample was then centrifuged at 13,000g for 30 minutes and the pellet resuspended in 10υl TE.

2.6.12. Ligations:

Ligations were performed using T_4 ligase and 5X ligation buffer purchased from BRL. Ligations were carried out in a total volume of 10υl using 1υl T_4 ligase and a 10 fold molar excess of insert over plasmid DNA. Samples were incubated for 3 hours at room temperature or overnight at 14°C. Prior to transformation, protein was removed from ligations by column cleaning or by extraction with phenol and chloroform isoamylalcohol 24:1 followed by isopropanol precipitation.

2.6.13. Agarose gel electrophoresis:

Agarose was dissolved to a concentration of 0.7% in TAE by boiling and the gel poured. Gel loading buffer (6X) was added to the sample before loading. Electrophoresis was carried out at 100V for 1 hour on a horizon 58 minigel apparatus or at 50V overnight on a Pharmacia GNA-200 maxigel apparatus. Gels were stained for 20 minutes in 5υg/ml ethidium bromide, rinsed in water and viewed on a UV transilluminator.

Gels were photographed using an Olympus OM-20 camera, Kodak T max 100 35mm film, and an A003 Red filter (Cokin, France).

2.6.14. Excision of DNA from agarose gels by Geneclean:

Gels were viewed on a UV transilluminator and the band of interest cut out and weighed. A 1ml aliquot of sodium iodode was added per 0.5g gel and the gel slice was dissolved at 55°C. Following addition of 1υl of Silica 325 glass beads obtained from Stratech Scientific Ltd., the sample was vortexed briefly and incubated on ice for 5 minutes. The sample was centrifuged for 10 seconds at 12,000g and the pellet washed as above, three times in 50υl ice cold New Wash. The pellet was then resuspended in 10υl TE and incubated at 55°C for 10 minutes to elute the DNA. The glass beads were removed by centrifugation at 12,000g for 5 minutes and the supernatant containing the DNA of interest was transferred to a fresh tube.

2.6.15. Estimation of DNA concentration:

1. Gel electrophoresis: DNA samples were serially diluted and electrophoresed alongside serial dilutions of molecular weight marker DNA of known concentration. The gel was stained in ethidium bromide, photographed, and the concentration of the sample estimated by comparing band intensities to those of the molecular weight markers.

2. Spectrophotometric analysis: A 1υl aliquot of sample DNA was added to 1ml H_2O and the absorbance of the solution recorded at 260 and 280nm. If the ratio of the readings at 260nm and 280nm equalled 1.8 (+/- 0.1), indicating that the DNA was free of contaminants, the concentration was estimated by assuming that an $O.D._{260}$ of 1.0 corresponds to 50υg/ml double stranded DNA, 40υg/ml single stranded DNA or 20υg/ml of single stranded oligonucleotides (Sambrook et al 1989).

2.6.16. Partial digestion of total DNA:

Five eppendorphs were prepared on ice; the first of which contained 18υl of total DNA and 2υl of the appropiate restriction buffer. Half these amounts was added to each of the remaining eppendorphs. A 1υl aliquot of restriction enzyme was introduced into the first eppendorph, mixed well, and 10υl was then transferred to the 2nd tube. The contents of the 2nd tube were mixed and 10υl transferred to the next eppendorph. This process was repeated until the last eppendorph was reached. The samples were incubated at 37°C for 15 minutes and the enzyme inactivated at 68°C for 15 minutes. The samples were electrophoresed on a 0.4% agarose gel at 60 volts along with molecular weight markers, stained in ethidium bromide, and photographed. The enzyme dilution giving the most DNA of the required size range was selected and a large scale digest carried out using this dilution.

2.6.17. Fractionation of partially digested total DNA:
Partially digested total DNA was loaded into a single large well, created by joining a number of teeth on the gel comb together using masking tape, in a 0.4% agarose gel. Molecular weight markers were loaded into the single wells flanking the large central well. The gel was run at 60V and stained briefly in ethidium bromide. Only the lanes containing the molecular weight markers were viewed under UV and the area of the gel corresponding to partially digested DNA of the required size was located and cut out. The remainder of the gel was then photographed. DNA was recovered from the agarose using a Type NrE51 Elektroelution Apparatus supplied by Biometra, Gottingen. The apparatus was filled with 10mM Tris (pH 8.0) and thin slices of the gel were placed in the sample cups. A 50υl aliquot of 20% sodium acetate, 0.25% bromophenol blue was added to each of the V-shaped channels and a voltage of 100V was applied for 1 hour. The Tris buffer was slowly removed using a 50ml syringe connected to a piece of rubber tubing which was inserted down the hollow barrel of the valve handle. The salt solution containing the eluted DNA was then carefully removed from the V-shaped channels using a pipette, extracted with phenol and chloroform isoamylalcohol 24:1 and 2.5 volumes of ethanol was added. The sample was incubated at -20°C for 10 minutes and the DNA recoverred by centrifugation at 12,000g for 30 minutes at 4°C. The supernatant was removed by aspiration, the pellet was dissolved in 100υl H_2O and 10υl of 5M sodium perchlorate and 100υl of isopropanol was added. The DNA was recovered by centrifugation at 14,000g for 20 minutes and the pellet was washed twice in 70% ethanol. The DNA pellet was dried under vacuum and resuspended in 50υl TE (pH 8.0).

2.6.18. Southern blotting of agarose gels:
The gel was stained in ethidium bromide, photographed, and denatured in 3 volumes of 1.5M NaCl, 0.5M NaOH for 1 hour at room temperature, with constant shaking. The gel was then neutralised in 1.5M NaCl, 1M Tris.Cl (pH 8.0) for 1 hour, at room temperature, with constant shaking. Meanwhile a piece of 3MM Whatman paper of equal width but 10cm longer than the gel was placed on a sheet of glass, slightly

larger than the gel in both dimensions. The glass was placed on top of a baking dish, filled with 10X SSC, with both ends of the Whatman paper extending into the buffer. The 10X SSC was allowed to soak into the Whatman paper by capillary action and any air bubbles were smoothed out with a glass rod. While wearing gloves, a piece of nitrocellulose paper, 1-2mm longer than the gel in both dimensions was cut out and floated in 2X SSC until it had wetted completely. The nitrocellulose was then immersed in the 2X SSC for 2-3 minutes. A window, 1mm smaller than the gel in both dimensions, was defined in the damp Whatman paper, using strips of parafilm. The neutralised gel was inverted and places over this window, taking care not to trap air bubbles between the gel and the Whatman paper. The wet nitrocellulose paper was placed on top of the gel and a window was defined in the nitrocellulopse paper as described above. This ensured that the only pathway for movement of the buffer was through the gel into the nitrocellulose paper. Two pieces of Whatman 3MM paper, the same size as the gel, were soaked in 2X SSC and placed on top of the nitrocellulose paper. A 10cm stack of paper towels, slightly smaller than the 3MM paper, was placed on top of the 3MM paper, followed by a glass plate and a 1kg weight. Transfer of DNA was allowed to proceed overnight. The towels and the 3MM paper above the nitrocellulose paper were then removed and the dehydrated gel and the nitrocellulose paper were lifted off together and placed, gel side up, on a dry sheet of 3MM paper. The position of the wells were marked on the nitrocellulose filter, the gel was peeled off, and the filter was soaked in 6X SSC for 5 minutes at room temperature. The filter was air dried on a sheet of 3MM Whatman paper, placed between 2 sheets of Whatman paper, and baked for 2 hours at 80°C. The filter was stored at room temperature.

2.6.19. Hybridisation with non-radioactive DNA probes:

Non radioactive probes were labelled by random priming with digoxigenin dUTP, using the DIG labelling kit supplied by Boehringer Mannheim. Probes consisted of linear double stranded DNA, obtained directly from restriction or recovered from agarose gels by Geneclean. The probe was dissolved in 10υl TE (pH 8.0), boiled for 10 minutes, cooled on ice, and 5υl H_2O, 2υl dNTP, 2υl hexanucleotide and 1υl of klenow enzyme added. The probe was incubated at 37°C for between 1 and 24 hours, and 2υl 0.2M EDTA, 2.5υl LiCl and 75υl of ethanol were added. Following incubation for between 1 and 3 hours at -70°C, the labelled probe was recovered by centrifugation at 12,000g at room temperature for 1 hour. The probe was washed with 100υl 70% ethanol at 4°C, recovered by centrifuging as above for 30 minutes, dried under vacuum and resuspended in 50υl TE (pH 8.0).

The nitrocellulose filter, approximately 400cm^2, was placed in a heat sealer bag and 80ml of hybridisation solution added. The bag was sealed and the filter prehybridised at 64°C for 2 hours. A 20υl aliquot of probe was boiled for 10 minutes and added to 10ml of fresh hybridisation solution, heated to 64°C. The bag containing the filter was cut at one corner, the hybridisation solution removed, and the fresh hybridisation solution, containing the probe, added. The bag was resealed and hybridised at 64°C overnight. The filter was removed from the bag, and washed with shaking, twice at room temperature for 5 minutes in 200ml 2X SSC, 0.1% SDS and twice for 15 minutes at 68°C in 200ml 0.1X SSC, 0.1% SDS. The filter was then washed at room temperature, with shaking, for 1 minute in 100ml buffer 1, for 30 minutes in 400ml buffer 2, for 30 minutes in 80ml of antibody conjugate solution, for 2 periods of 15 minutes in 400ml buffer 1 and for 2 minutes in 80ml buffer 3. The filter was then immersed in 40ml colour solution and incubated in the dark without agitation for up to 24 hours, depending on the time required for bands to form at a suitable intensity. The colour solution was then removed, 100ml buffer 4 was added, and the filter was photographed. The filter was air dried and stored at room temperature. For subsequent viewing the filter was rewetted in buffer 4.

2.6.20. Hybridisation with radioactive probes:

Radioactive probes were labelled using a Random Priming Labelling kit from BRL. Probes consisted of linear double stranded DNA and were denatured by boiling for 5 minutes in a volume of 8υl TE. The probe was rapidly cooled on ice and 1υl dTTP, 1υl dCTP, 1υl dGTP, 7.5υl random primer buffer mixture, 0.5υl ^{35}S ATP (1000 Ci/mmol) and 5υl H_2O added. The contents of the tube were mixed, 0.5 units of klenow enzyme were added and labelling was allowed to proceed at 25°C for 3 hours. The reaction was terminated by adding 2.5υl stop solution.

The nitrocellulose filter was rehydrated in 6X SSC, placed between 2 sheets of 3MM Whatman paper and inserted into a heat sealable bag. The sheets of Whatman paper were removed and 10ml 50% formamide, 7X SSC, 5X Denhardt's solution were added. Any air bubbles in the bag were removed, the bag was sealed and incubated at 42°C for 2-4 hours in a shaking water bath. The buffer solution was then removed, replaced with 3ml fresh buffer and the labelled probe added. The bag was resealed and hybridisation was allowed to proceed overnight at 42°C in a shaking water bath. The hybridisation solution was removed and the blot washed successively for 15 minutes at 37°C once in 30% formamide, 5X SSC, twice in 2X SSC, twice in 0.5X SSC and twice in 0.2X SSC. The blot was dried for 1 hour at 80°C and exposed, in the dark, to Fuji x-ray film for 24-48 hours. The X-ray film was placed in a bath of Kodak X-Omat developer for 5 minutes, rinsed briefly in water and fixed for 5-10 minutes in Kodak X-Omat fixer. At this stage the film could be exposed to light and was rinsed thoroughly in water and allowed to dry at 37°C.

2.6.21. DNA sequencing:

Double stranded plasmid DNA for sequencing was prepared using 2 successive cesium chloride gradients as described in section 2.6.3. After precipitation, the DNA pellet was resuspended in 100υl TE (pH 8.0), and the concentration was estimated spectrophotometrically and adjusted to 1 υg/υl.

Oligonucleotide primers of 20 base pairs in length, complementary to DNA sequences 50-100 base pairs from the region where sequencing was required to begin, were obtained from Eurogentec, Belgium. The concentration of primers was estimated spectrophotometrically and was adjusted to 70 υg/υl, equivalent to 10 ρmoles/υl.

Sequencing was carried out using the T7 sequencing kit supplied by Pharmacia. A 2υl aliquot of primer and 1.5υl 1M NaOH was added to 2υg of double stranded plasmid DNA in 8υl TE. The sample was vortexed and centrifuged briefly, and incubated at 65°C for 5 minutes. The denatured DNA was transferred to a 37°C water bath and 1.5υl 1M HCl and 2υl annealing buffer added. The sample was then incubated for 10 minutes at 37°C and for 5 minutes at room temperature.

Meanwhile a 2.5υl aliquot of the A, C, G, and T Mix-short reagents was added to 4 colour coded eppendorphs and the T7 DNA polymerase was diluted 1/5 in ice cold enzyme dilution buffer.

The tube containing the freshly denatured and annealed primer/template was centrifuged briefly and 3υl of labelling mix, 1υl of ^{35}S dATP αS (>1000Ci/mmmol) and 2υl diluted T7 DNA polymerase were added. The contents of the tube were mixed by gentle pipetting, centrifuged briefly, and incubated at room temperature for 5 minutes. Meanwhile the 4 eppendorphs containing the Mix-short reagents were warmed to 37°C.

After incubation of the labelling reaction for 5 minutes, 4.5υl was transferred into each of the pre-warmed colour coded eppendorphs containing the Mix-short reagents. The contents of each tube were mixed by gentle pipetting, incubated at 37°C for 5 minutes, and 5υl of stop solution added. The samples were stored at -20°C until needed.

Electrophoresis of sequencing reactions was carried out using a model S2 sequencing rig, purchased from BRL. The glass plates were washed succesively in detergent, tap

water, and distilled H_2O and were then rinsed 3 times in ethanol and allowed to air dry. The smaller of the 2 plates was placed in a fume hood and 5ml of dimethyldichlorosilane solution was poured on and rubbed onto the plate. The plate was left to dry for 5 minutes, polished and rinsed with distilled H_2O. The large plate was laid flat on the bench, the 2 spacers positioned along the sides and the smaller plate was placed on top. The two plates were taped together, taking particular care to form a watertight seal at the bottom corners. The gel solution was prepared by adding 42g urea to 10ml 10X TBE and 15ml 40% acrylamide, 0.8% bis-acrylamide and adjusting the volume to 75ml with H_2O. The urea was dissolved by heating to 55°C and H_2O added to a volume of 100ml. The gel solution was allowed to cool, filtered through a 0.45μm Millipore filter, and 360μl TEMED and 60μl 10% ammonium persulphate added. The gel was poured and the flat side of a shark's tooth comb inserted to a depth of 0.5cm. The plates were clamped together on all four sides using bulldog clips and the gel was left for 1 hour to set. If the gel was not to be used immediately the comb was surrounded with tissue soaked in 1X TBE, the tissue was wrapped in Saran wrap and the gel was stored at 4°C for up to 16 hours.

Prior to running the gel, the tape and bulldog clips were removed and the gel placed in position in the sequencing rig. The top and bottom reservoirs were filled with 1X TBE and the comb was carefully removed. The flat surface of the gel was washed with 1X TBE and the comb reinserted, with the teeth just sticking into the surface of the gel. The gel was prerun at 55 W for 45 minutes and the samples were heated to 80°C for 2 minutes. The wells were washed with 1X TBE and 2.5μl of the 4 samples corresponding to each template/primer loaded. The gel was run at 55W until the upper of the 2 blue dyes was 7-8cm from the bottom of the gel, at which time a second loading was performed as described above. After the third and final loading the gel was allowed to run until the lower of the 2 blue dyes reached the end of the gel.

The power was then switched off and the gel removed from the sequencing rig and left for 15 minutes to cool, during which time the radioactive running buffer was disposed of. The smaller of the two plates was then carefully removed from the gel along with the spacers and combs. A piece of 3MM Whatman paper, cut to the same size as the gel, was placed on the gel and firmly smoothed onto the gel surface by rubbing with a glass rod. The Whatmann paper, with the gel attached, was then carefully removed, the gel surface covered with Saran wrap, and the gel was dried at 80°C under vacuum for 2 hours. The Saran wrap was then removed, the Whatman paper was placed gel side up in a sequencing cassette and in the dark, a sheet of Fuji X-ray film was placed on top, and the gel left to expose for 24-48 hours. Again in the dark, the X-ray film was placed in a bath of Kodak X-Omat developer for 5 minutes, rinsed briefly in water and fixed for 5-10 minutes in Kodak X-Omat fixer. At this stage the film could be exposed to light and was rinsed thoroughly in water and allowed to dry at 37°C.

2.6.22: Sequence analysis.

Analysis of DNA sequence information was carried out using the PCGene programme, supplied by Intelligenetics, Belgium. Homology searches were conducted using the BLAST facility at EMBL, Heidelberg.

3. RESULTS

3.1. THE DEVELOPMENT OF A *P. acidilactici* INOCULANT FOR GRASS SILAGE.

3.1.1. Strain isolation.

As indicated in section 1.6., *P. acidilactici* was chosen as the lactic acid bacterium most likely to initiate a rapid decrease in silage pH and benefit the overall fermentation under normal ensiling conditions. A total of 18 strains of *P. acidilactici* from various sources were collected for assessment of their potential as silage inoculants. *Pediococcus acidilactici* PLL01, PLL02, PLL03, PLL04, PLL05, PLL06, PLL07, PLL08, PLL09 and M408, P17, E92, E116, E165 were kindly donated by Texel and Prof. Franco Dellaglio from their respective culture collections. *Pediococcus acidilactici* A12, B14, C20 and G24 were isolated from 4 grass silage samples from various locations in Ireland as described in materials and methods. *Pediococcus acidilactici* ferments glucose, fructose, galactose, arabinose and xylose but not maltose to acid (Buchanan and Gibbons 1974, Teuber and Geis 1981) and a single isolate from each sample with such a sugar fermentation profile was selected for further study. Strains A12, B14, and C20 were isolated from well preserved silages sampled more than 1 month after ensilage while G24 was isolated after 2 days ensilage.

3.1.2 Laboratory assessment of potential silage inoculants.

Since the time and expense involved in testing 18 strains in small scale silos was deemed prohibitive, the *P. acidilactici* isolates were initially screened for their potential as silage inoculants using a series of laboratory tests based on the criteria outlined by Whittenbury (1961). As might be expected, the 18 strains had many characteristics in common. All were incapable of hydrolysing gelatin, exhibited no detectable lactate breakdown and had an optimum growth temperature of 37°C. They grew well at temperatures between 25°C and 45°C and rapidly initiated growth at pH values between 4.5 and 8.0. Growth tapered off rapidly at lower values of temperature and initial medium pH. The influence of temperature and

initial pH on the growth of 2 strains; *P. acidilactici* G24 and PLL07 is depicted in figure 3.1. Exponential growth rates varied only slightly, ranging from $1.5h^{-1}$ to $2.4h^{-1}$ on glucose and from $1.3h^{-1}$ to $1.9h^{-1}$ on fructose. However, as can be seen from table 3.1, significant variation was observed among the strains in their lag phases (5 to 11 h), in the culture pH after 26 h growth on glucose and fructose (3.62 to 4.18 and 3.69 to 4.46 respectively) , in their glucose to lactate conversion efficiencies after 12 h growth (43 to 78%), in their pH optima for growth (6.0 to 7.3) and in the percentage of maximum growth exhibited at 20°C (12 to 80%). A good correlation was observed between growth and acidification, with those strains exhibiting the shortest lag phases bringing about the most rapid decrease in pH. Three strains; *P. acidilactici* PLL03, PLL04 and PLL07, were found to secrete metabolites other than lactic acid, presumed to be pediocins, which inhibited *L. plantarum* growth. On the basis of these results *P. acidilactici* G24 was deemed to have the greatest potential as a silage inoculant and was selected for testing in small scale silos. This choice was made on the basis of its short lag phase of 5 h, its ability to rapidly reduce the culture pH to a value of 3.62 after 26 h growth, its compatibility with *L. plantarum*, its glucose to lactate conversion efficiency of 76% after 12 h and its ability to produce 80% of the cell mass attained at 37°C at incubation temperatures as low as 20°C. By contrast, *P. acidilactici* PLL07 exhibited a long lag phase of 8.3 h, an inability to substantially reduce the culture pH and inhibited *L. plantarum* growth and was therefore selected as a negative control for small scale silo experiments. Growth curves for these two strains in MRS using glucose as the sole sugar source are presented in figure 3.2. A similar pattern was observed when fructose was used as the sole sugar source (data not shown).

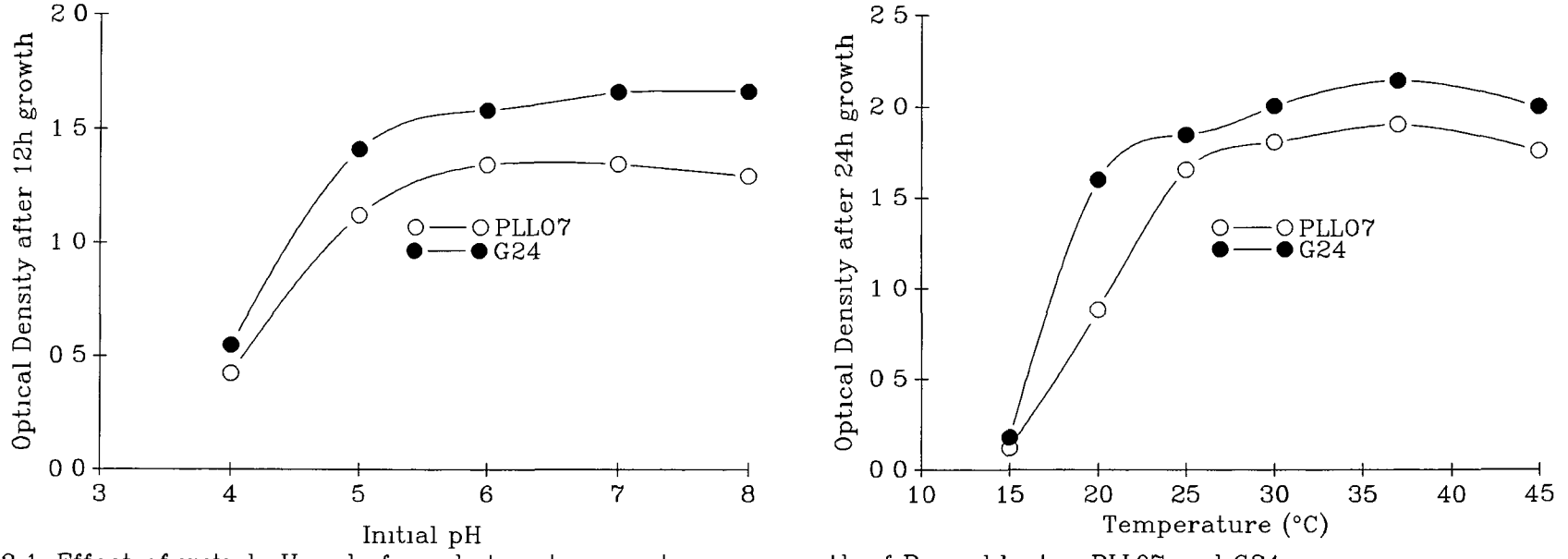


Fig 3 1 Effect of initial pH and of incubation temperature on growth of P acidilactici PLL07 and G24

	pH AFTER 26 hrs GROWTH		SUGAR TO LACTATE CONVERSION RATE		pH* OPTIMUM	% MAX GROWTH AT 20°C	Length of lag phase (h)
	GLUCOSE	FRUCTOSE	GLUCOSE	FRUCTOSE			
PLL01	3.88(.03)	4.23(.05)	67(3.5)	72(4.6)	6.6(.2)	44(3.2)	9.0
PLL02	3.94(.05)	4.30(.04)	63(2.8)	57(3.7)	6.6(.1)	46(3.7)	10.0
PLL03	3.96(.02)	4.29(.03)	59(3.6)	58(6.2)	6.0(.3)	12(1.8)	10.2
PLL04	4.01(.06)	4.26(.06)	69(3.9)	64(5.1)	6.2(.3)	30(3.2)	11.0
PLL05	3.94(.01)	4.17(.01)	65(4.2)	68(3.8)	6.5(.2)	53(3.5)	7.9
PLL06	3.93(.08)	4.25(.07)	78(1.7)	68(4.1)	6.6(.4)	28(2.6)	10.0
PLL07	4.18(.06)	4.46(.04)	43(5.6)	57(4.0)	6.5(.1)	50(4.3)	8.3
PLL08	4.14(.04)	4.38(.04)	60(4.8)	58(4.6)	6.9(.1)	41(5.1)	8.7
PLL09	4.05(.05)	4.21(.03)	56(3.2)	58(3.8)	6.9(.5)	54(6.2)	7.3
M408	3.73(.02)	3.97(.04)	62(4.3)	57(1.6)	7.2(.4)	47(4.8)	8.5
P17	3.91(.06)	4.17(.03)	58(4.1)	65(2.8)	6.8(.2)	57(5.3)	8.0
E92	3.99(.08)	4.12(.07)	45(3.6)	56(1.7)	7.0(.3)	63(6.4)	7.5
E112	3.95(.06)	4.10(.08)	66(3.9)	67(1.6)	7.3(.4)	55(4.7)	6.9
E165	3.72(.05)	3.82(.01)	55(3.2)	56(3.9)	6.9(.2)	60(6.8)	8.5
A12	3.78(.07)	3.97(.04)	76(4.7)	68(4.1)	7.2(.3)	67(6.9)	6.6
B14	3.82(.08)	4.03(.04)	56(5.6)	67(3.8)	7.1(.6)	59(6.3)	7.1
C20	3.78(.10)	3.95(.05)	69(6.8)	63(3.7)	6.9(.1)	69(6.9)	6.1
G24	3.62(.06)	3.79(.05)	74(2.6)	68(3.6)	7.2(.1)	80(6.3)	5.0

Table 3.1:Growth characteristics of *P. acidilactici* strains.
(Assays were performed in duplicate and standard deviations are in parenthesis)
*: Indicates optimum pH for growth initiation.

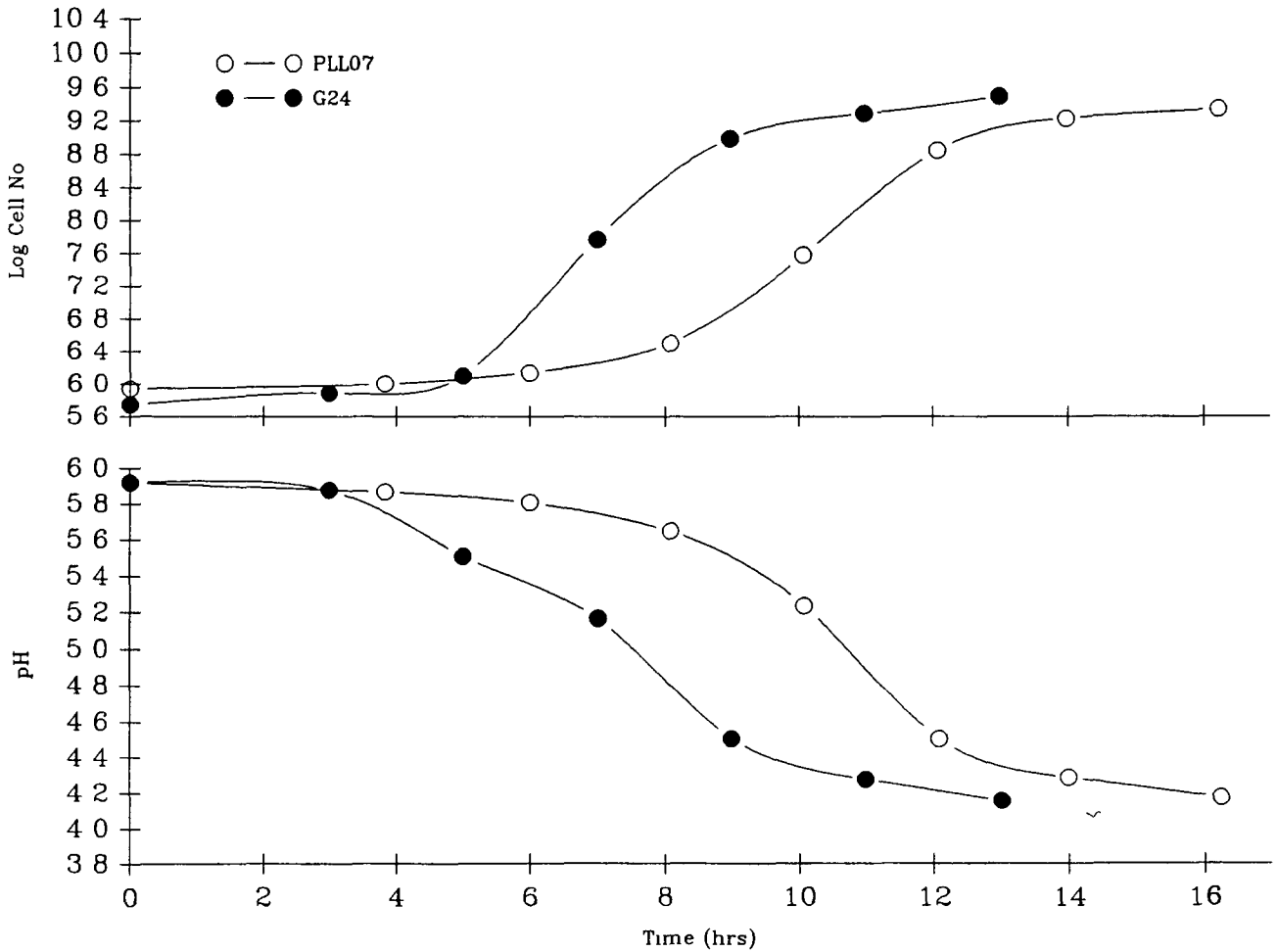


Fig 3 2 Growth curves of P acidilactici PLL07 and G24 on glucose

3.1.3. Small scale silo trials of *P. acidilactici*.

The ability of *P. acidilactici* G24 to effect preservation of grass silage was assessed in small scale silos as described in materials and methods. The first silo trial was designed to assay for any effect of inoculation with *P. acidilactici* on preservation, to compare the effectiveness of *P. acidilactici* G24 and PLL07 as silage inoculants and to investigate any stimulation of *L. plantarum* growth resulting from inoculation with *P. acidilactici* which might lead to improvements in preservation. This was prompted by literature reports of mixtures of *P. acidilactici* and *L. plantarum* being particularly effective as inoculants (Heron and Henderson 1981, Lindgren et al 1983, Pettersson et al 1983, Henderson and McDonald 1984, Henderson et al 1984). The grass isolate *L. plantarum* B2, which had been shown to be highly competitive and to aid preservation of grass silage (F. Duffner, personal communication) was chosen for use in all grass silage trials. The treatments used are outlined in table 3.2. Grass at ensiling had a dry matter content of 15.4%, a water soluble carbohydrate content of 132g/kg DM and a crude protein content of 166g/kg DM. The changes in the silage microflora, lactic acid levels and pH over the ensiling period and the silage composition after 6 days fermentation are presented in tables 3.3 to 3.7 and figure 3.3.

Treatment	Inoculation rate/g grass
1	0
2	10^4 *L. plantarum* B2
3	10^6 *P. acidilactici* G24
4	10^6 *P. acidilactici* G24 + 10^4 *L. plantarum* B2
5	10^6 *P. acidilactici* PLL07 + 10^4 *L. plantarum* B2

Table 3.2: Silo Trial 1: Treatments used.

TREATMENT	DAY 0	DAY 1	DAY 2	DAY 3	DAY 6
1	6.03	5.95^a	4.53^a	4.19^a	3.90^a
2	6.03	5.93^a	4.57^a	4.19^a	3.87^a
3	6.03	5.85^b	4.32^b	4.04^b	3.81^b
4	6.03	5.85^b	4.35^b	4.04^b	3.81^b
5	6.03	5.91^a	4.55^a	4.16^a	3.88^a

Table 3.3: Changes in pH over ensiling period.

Treatments as described in Table 3.2.
Values within columns differing significantly from one another followed by different superscripts.

Treatment	Day 1	Day 2	Day 3	Day 6
1	0.55[a]	0.98[a]	1.34[a]	1.74[a]
2	0.58[a]	1.02[a]	1.38[a]	1.84[a]
3	0.54[a]	1.20[b]	1.56[b]	2.06[b]
4	0.52[a]	1.26[b]	1.52[b]	1.92[ab]
5	0.46[a]	1.00[a]	1.48[ab]	1.84[a]

Table 3.4: Silo Trial 1: Changes in lactic acid concentration (%) over ensiling period.

Treatments as described in Table 3.2.
Values within columns differing significantly from one another followed by different superscripts.

TREATMENT	DAY 0	DAY 1	DAY 2	DAY 3	DAY 6
1	2.06[a]	4.69[a]	5.03[a]	5.17[a]	5.33[a]
2	2.06[a]	4.32[a]	5.26[a]	5.03[a]	5.28[a]
3	6.04[b]	6.54[b]	8.59[b]	9.67[b]	8.92[b]
4	6.04[b]	6.78[b]	8.02[b]	9.65[b]	8.60[b]
5	6.07[b]	5.10[c]	5.65[c]	6.67[c]	6.56[c]

Table 3.5: Silo Trial 1: Changes in Log *P. acidilactici* numbers over ensiling period.

Treatments as described in Table 3.2.
Values within columns differing significantly from one another followed by different superscripts.

TREATMENT	DAY 0	DAY 1	DAY 2	DAY 3	DAY 6
1	0.40^{a}	1.01^{a}	1.04^{a}	2.53^{a}	3.91^{a}
2	4.01^{b}	4.57^{b}	5.54^{b}	5.95^{b}	7.10^{b}
3	0.40^{a}	1.12^{a}	2.06^{c}	3.54^{c}	4.56^{c}
4	4.01^{b}	4.51^{b}	5.14^{d}	5.63^{d}	6.78^{d}
5	4.01^{b}	4.03^{c}	5.37^{bd}	6.04^{b}	7.35^{b}

Table 3.6: Silo Trial 1: Changes in Log *L. plantarum* numbers over ensiling period.

Treatments as described in Table 3.2.
Values within columns differing significantly from one another followed by different superscripts.

Treatment	WSC (g/kg aqueous extract)	Crude Protein (g/kg DM)	Ammonia N (% Total N)
1	1.0^{a}	161^{a}	5.7^{a}
2	0.9^{a}	175^{ab}	5.1^{a}
3	1.0^{a}	179^{b}	4.6^{b}
4	0.9^{a}	177^{b}	4.6^{b}
5	0.8^{a}	170^{a}	5.4^{a}

Table 3.7: Silo Trial 1: Composition of silages after 6 days of fermentation.
Treatments as described in Table 3.2.
Values within columns differing significantly from one another followed by different superscripts.

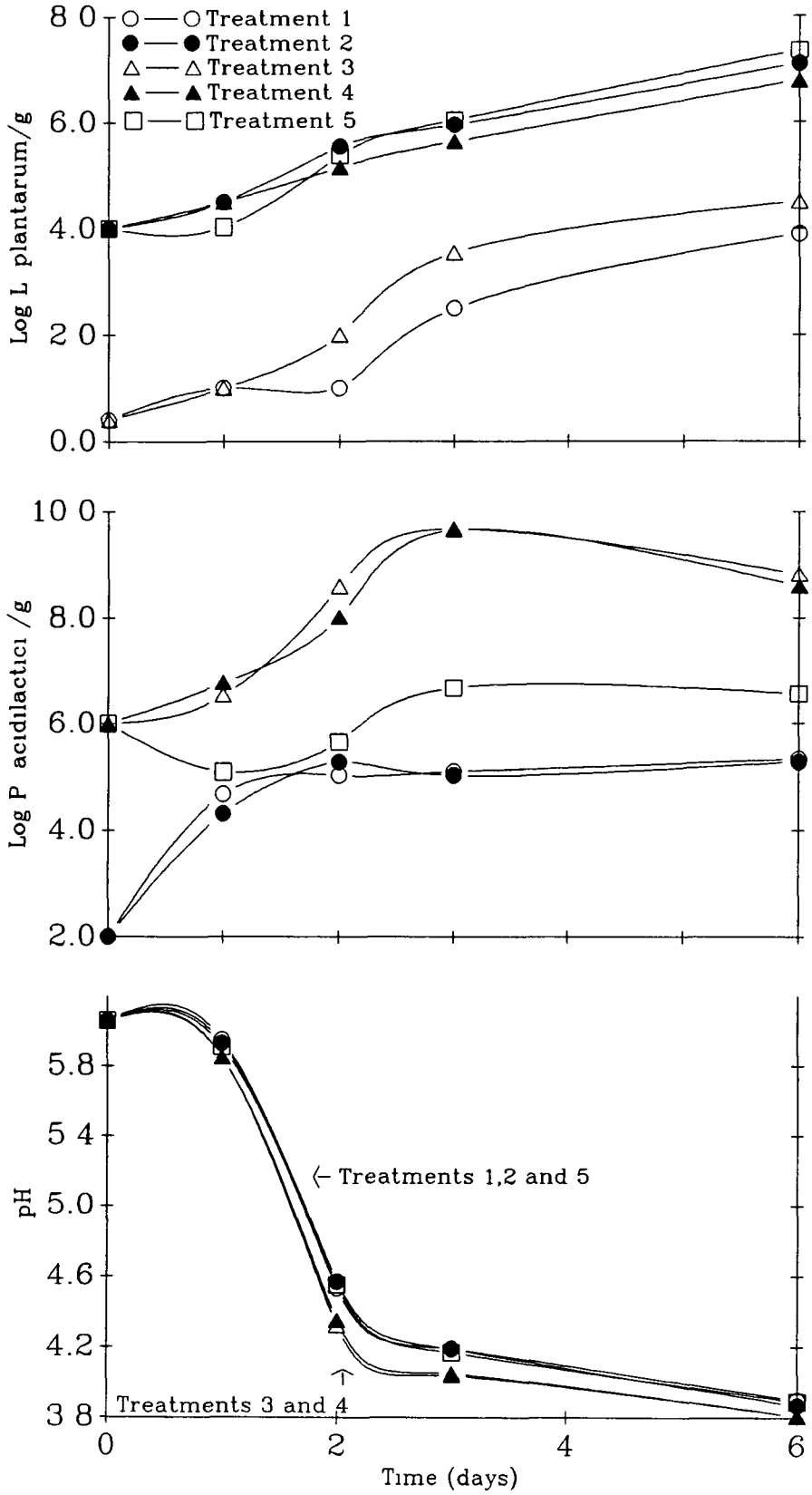


Fig 3 3 Silo trial 1, Changes in microbial flora and pH
Treatments as described in table 3 2

Inoculation with 10^6 *P. acidilactici* G24 alone/g grass (treatment 3) led to a rapid proliferation of *P. acidilactici*, a tenfold increase in L. *plantarum* numbers and a significantly greater rate of lactic acid production and pH decrease compared to uninoculated controls at all sampling points after day 1. A decrease in the ammonia nitrogen level of the final silage was also observed. The addition of 10^4 *L. plantarum* cells/g grass to the G24 inoculum (treatment 4) gave rise to no additional effect on pH, lactic acid production, crude protein or ammonia nitrogen levels. *Pediococcus acidilactici* numbers were unchanged while *L. plantarum* numbers were significantly lower after 3 days ensilage than when *L. plantarum* alone was used as an inoculum. The lower pH and higher lactic acid levels associated with inoculation with *P. acidilactici* were most significant in the early stages of fermentation, 2 to 3 days after ensiling. This correlated with a peak in the *P. acidilactici* population at this time of approximately 10^9/g. Over the next 3 days a tenfold reduction in *P. acidilactici* numbers was observed. By contrast, *L. plantarum* numbers were still rising 6 days after ensilage. Inoculation with 10^4 *L. plantarum* cells/g grass (treatment 2) had no effect on *P. acidilactici* numbers, on the rate of lactic acid production and pH decrease or on the ammonia nitrogen level of the silage after 6 days fermentation. Inoculation with *P. acidilactici* PLL07 in conjunction with *L. plantarum* (treatment 5) gave rise to no beneficial effect compared to controls or inoculation with *L. plantarum* alone. *Pediococcus acidilactici* numbers were significantly lower than when G24 was used and *L. plantarum* numbers were unaffected by the presence of strain PLL07. The *P. acidilactici* counts suggest that *P. acidilactici* PLL07 failed to make any impact on preservation due to a lack of competitiveness, as was expected from the laboratory results. This preliminary silo trial revealed a statistically significant beneficial effect on preservation associated with inoculation with *P. acidilactici* G24 at a rate of 10^6/g grass. However since *P. acidilactici* and *L. plantarum* inoculation rates were different no comparison could be made between their

abilities to aid preservation. A second trial was therefore carried out to compare the ensiling abilities of the 2 strains and to test for any synergy between them. The treatments used are outlined in table 3.8. Grass at ensiling had a dry matter content of 16.98% and a water soluble carbohydrate content of 119.2g/kg DM. The silos were sampled after 1, 3 and 7 days of fermentation and the results of the microbiological and biochemical analyses performed are presented in tables 3.9 to 3.11 and figure 3.4.

Treatment	Inoculation rate/g grass		G24:B2
	G24	B2	
1	-	-	-
2	9.14×10^5	-	-
3	-	1.06×10^6	-
4	4.56×10^5	5.30×10^5	1 : 1.16
5	7.62×10^5	1.76×10^5	4.33 : 1
6	1.52×10^5	8.80×10^5	1 : 5.79

Table 3.8: Silo Trial 2: Treatments used.

Treatment	Log *L. plantarum* numbers			
	Day 0	Day 1	Day 3	Day 7
1	1.67^{a}	3.61^{a}	5.21^{a}	5.99^{a}
2	1.77^{a}	3.58^{a}	5.12^{a}	6.38^{a}
3	6.03^{b}	6.35^{b}	8.08^{b}	8.52^{b}
4	5.73^{c}	6.23^{b}	7.57^{c}	8.29^{b}
5	5.25^{d}	5.90^{c}	6.91^{d}	7.74^{c}
6	5.95^{e}	6.33^{b}	7.30^{e}	8.45^{b}

Table 3.9: Silo Trial 2: Log L. plantarum numbers over ensiling period.

Treatments as described in Table 3.8.
Values within columns differing significantly from one another followed by different superscripts.

Treatment	Log *P. acidilactici* numbers			
	Day 0	Day 1	Day 3	Day 7
1	2.08^{a}	3.36^{a}	6.61^{a}	6.85^{a}
2	5.96^{b}	6.27^{b}	9.04^{b}	8.52^{b}
3	2.03^{a}	3.20^{a}	6.56^{a}	6.74^{a}
4	5.66^{c}	6.01^{c}	8.33^{c}	8.53^{b}
5	5.88^{d}	6.22^{bc}	8.49^{c}	8.60^{b}
6	5.18^{e}	5.75^{d}	7.61^{c}	7.85^{c}

Table 3.10: Silo Trial 2: Log *P. acidilactici* numbers over ensiling period.

Treatments as described in Table 3.8.
Values within columns differing significantly from one another followed by different superscripts.

Treatment	pH			
	Day 0	Day 1	Day 3	Day 7
1	6.20^{a}	5.60^{a}	4.51^{a}	4.20^{a}
2	6.20^{a}	5.28^{b}	4.38^{b}	4.14^{b}
3	6.20^{a}	5.60^{a}	4.45^{c}	4.13^{b}
4	6.20^{a}	5.47^{ac}	4.43^{c}	4.11^{c}
5	6.20^{a}	5.39^{c}	4.46^{c}	4.15^{b}
6	6.20^{a}	5.46^{ac}	4.47^{c}	4.13^{bc}

Table 3.11:Silo Trial 2: Variation in pH over ensiling period.

Treatments as described in Table 3.8.
Values within columns differing significantly from one another followed by different superscripts.

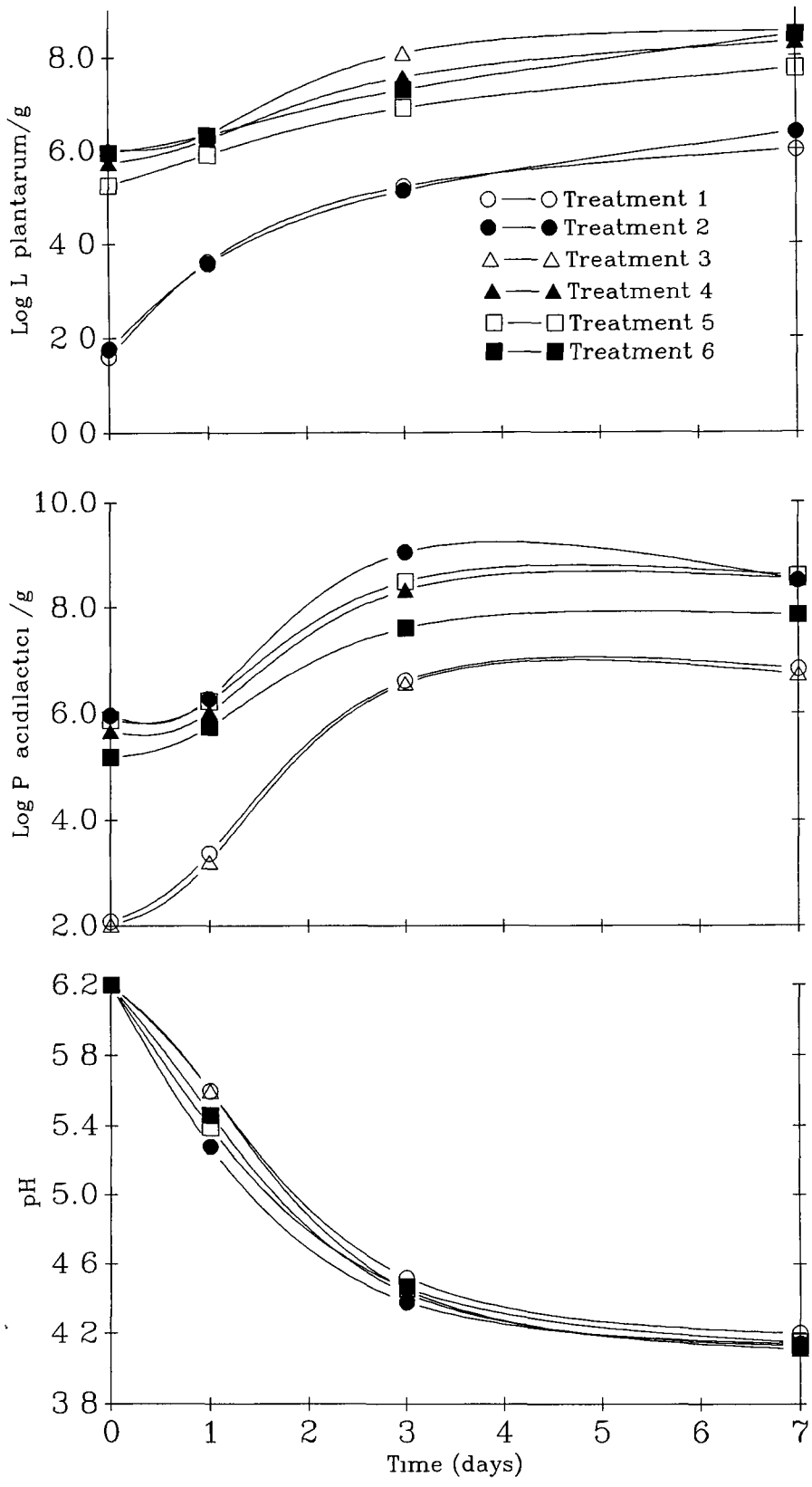


Fig 3 4 Silo trial 2, Changes in pH and microbial flora
Treatments as described in Table 3 8

As indicated by table 3.11 and figure 3.4, each of the 5 treatments of 10^6 lactic acid bacteria/g grass led to a lower silage pH after 7 days fermentation compared to controls. The final pH observed in silos inoculated with either *P. acidilactici* G24 or *L. plantarum* B2 alone or in a ratio of 5:1 or 1:5 was not significantly different. While the pH after 7 days fermentation was slightly but significantly lower in silos inoculated with a 1:1 ratio of *P. acidilactici* G24 to *L. plantarum* B2, the most desirable fermentation pattern, i.e. a rapid decrease in pH, was observed in silos inoculated with *P. acidilactici* G24 alone. The major impact of inoculation with *P. acidilactici* at a rate of 10^6/g grass on the pH in the early stages of fermentation observed here was in agreement with the observations from the previous silo trial. Furthermore, in both experiments the lower pH compared to other treatments observed 3 days after ensilage coincided with a peaking of *P. acidilactici* numbers at approximately 10^9/g silage. By contrast, in other treatments where a less rapid decrease in pH was observed, *P. acidilactici* numbers were less than 4 X 10^8/g silage after 3 days fermentation and did not peak until later in the fermentation. The results therefore indicate a link between a rapid proliferation of *P. acidilactici* in the early stages of ensilage and a rapid decrease in pH. This is best accomplished by inoculation with high numbers of *P. acidilactici* alone. However the slightly lower pH in silos inoculated with an equal mixture of *P. acidilactici* and *L. plantarum* compared to those inoculated with *P. acidilactici* alone after 7 days fermentation, which correlates with a 100 fold excess of *L. plantarum* numbers in the former silos at this time, suggested that inoculation with *L. plantarum* in conjunction with *P. acidilactici* might be necessary in order to maintain the low pH attained during the initial fermentation. A long term silo trial was therefore performed to test this hypothesis. Grass with a dry matter content of 16.5% and a water soluble carbohydrate level of 116.9g/kg DM was inoculated with either 1.16 X 10^6 *P. acidilactici* G24/g grass, 1.12 X 10^6 *L. plantarum* B2/g grass or a mixture of 5.8 X 10^5 *P. acidilactici* G24 and 5.6

X 10^5 *L. plantarum* B2 /g grass. The silos were sampled after 20 and 60 days' fermentation and the results of the biochemical and microbial analyses performed on the silage samples are presented in table 3.12.

TREATMENT	pH		Log *L. plantarum*		Log *P. acidilactici*	
	Day 20	Day 60	Day 20	Day 60	Day 20	Day 60
Control	4.36^{a*}	4.54^{a*}	5.64^{a}	5.34^{a}	6.22^{a}	6.12^{a}
L. plantarum	3.94^{b}	3.97^{b}	8.23^{b*}	7.65^{b*}	6.35^{a}	6.37^{a}
P. acidilactici	3.97^{b}	3.96^{b}	5.85^{a}	5.47^{a}	7.98^{b*}	7.34^{b*}
L. plantarum + *P. acidilactici*	3.95^{b}	3.97^{b}	8.16^{b*}	7.68^{b*}	6.88^{c*}	6.34^{c*}

Table 3.12: Silo Trial 3: Biochemical and microbiological changes during the latter stages of fermentation.
Values within columns differing significantly from one another followed by different superscripts.
*: Difference between day 20 and day 60 value significant.

No significant difference in silage pH was noted between silos treated with *P. acidilactici* alone, *L. plantarum* alone or a mixture of *P. acidilactici* and *L. plantarum* after 20 days fermentation. Furthermore, no significant increase in pH was observed in any of these silos between 20 and 60 days after ensilage. It was therefore concluded that inoculation with *P. acidilactici* alone was sufficient to rapidly establish and subsequently maintain a low silage pH and that no detectable additional benefit could be obtained by adding *L. plantarum* to the inoculant.

All silo trials to date were performed using grass with a water soluble carbohydrate content of greater than 100g/kg DM. In order to investigate the hypothesis suggesting low levels of crop water soluble carbohydrate as a cause of inoculant failure (Woolford 1984, Henderson et al 1984), grass with a water soluble carbohydrate content of only 51g/kg DM and a dry matter content of 13.5% was inoculated with *P. acidilactici* G24 at a rate of 10^6/g grass. As indicated by figure 3.5 and table 3.13, *P. acidilactici* numbers increased slowly and there was no significant improvement in the rate of lactic acid production and pH decrease or in the final crude protein level compared to the uninoculated control silages, in marked contrast to previous trials. While a 14% decrease in the final ammonia nitrogen level compared to controls was noted, the results of silo trial 4 suggest that when *P. acidilactici* growth in the early stages of ensilage is limited by a shortage of water soluble carbohydrate, it exerts little or no effect on the outcome of the fermentation.

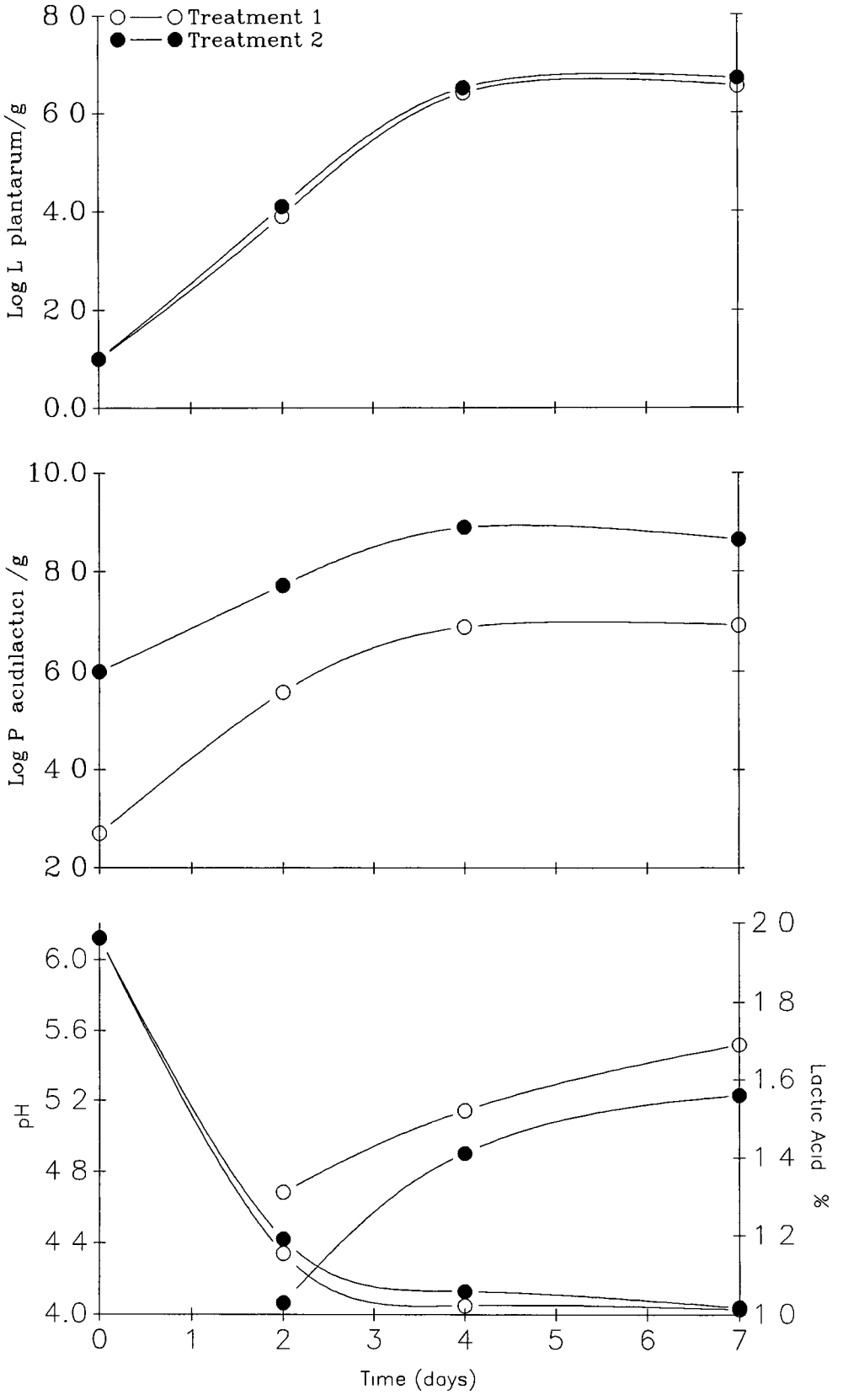


Fig 3 5 Silo trial 4, Changes in pH, lactic acid levels, and microbial flora

Treatment	WSC (g/kg aqueous extract)	Crude Protein (g/kg DM)	Ammonia N (% Total N)
1(control)	0.4^{a}	215^{a}	4.6^{a}
2(G24)	0.2^{b}	221^{a}	3.9^{b}

Table 3.13: Silo Trial 4: Composition of silages after 7 days ensilage.
Values within columnns differing significantly from one another followed by different superscripts.

3.2. THE DEVELOPMENT OF AN AMYLOLYTIC INOCULANT STRAIN.

The results presented in section 3.1 indicated that while *P. acidilactici* G24 was effective as an inoculant when sufficient water soluble carbohydrate was available for fermentation, it was of little benefit when water soluble carbohydrate levels were low. This is frequently the case in the ensiling of alfalfa which, on account of its low water soluble carbohydrate content and high buffering capacity, is considered one of the most difficult crops to ensile (Raguse and Smith 1966, Wilkinson 1978). However crops such as alfalfa, maize and cereals contain significant levels of starch (Woolford 1984) which are unavailable for fermentation by most currently employed inoculants. The problem of insufficient water soluble carbohydrates for a successful fermentation might therefore be overcome by employing an amylolytic inoculant strain, which would be capable of fermenting starch to lactic acid. An amylolytic inoculant was therefore developed according to the strategy outlined in section 1.6.

3.2.1. Strain selection.

The first step in the development of an amylolytic inoculant was the selection of a suitable amylolytic lactic acid bacterium strain. As indicated by section 3.1, such a strain would ideally originate from silage, and extracts from a number of grass silage samples were therefore plated onto MRS containing starch as the sole fermentable carbohydrate source, incubated at 30°C for 48 hours and screened for the presence of starch degrading colonies. Despite repeated attempts, no amylolytic lactic acid bacteria were isolated from grass silage. The amylolytic strains, *Lactobacillus amylovorus* and *Lactobacillus amylophilus* were therefore obtained from culture collections and assessed in the laboratory for their potential as silage inoculants.

3.2.2. Laboratory assessment of *L. amylovorus* and *L. amylophilus* as potential silage inoculants.

The potential of *L. amylovorus* and *L. amylophilus* as silage inoculants was assessed using similar criteria to those employed previously (section 3.1.2.). Both strains were found to have the basic metabolic features required of a suitable silage inoculant; the ability to ferment the major crop water soluble carbohydrates, glucose, fructose and starch , to acid in a homofermentative manner, and an absence of any detectable proteolytic or lactic acid degrading activity. Only *L. amylovorus* fermented sucrose to acid.

The ability of *L. amylovorus* and *L. amylophilus* to successfully compete under ensiling conditions was assessed by comparing them to *L. plantarum* DCU101, a silage isolate which had previously been shown to be effective in aiding preservation of grass silage (see table 3.11). The effect of temperature and initial medium pH on the growth of the 3 strains is depicted in figure 3.6. *Lactobacillus amylovorus* had a similar temperature and pH profile for growth to *L. plantarum*, with an initial pH of 6 and a temperature of 35-40°C being optimal for growth. Significant growth inhibition was observed below an initial pH of 4 and 25°C and above 45°C. By contrast growth of *L. amylophilus* was not greatly reduced until a temperature below 20°C was reached but was inhibited at pH values less than 5.0. Optimum growth of *L. amylophilus* was observed at pH 7 and 20-25°C. The fermentation profiles of the 3 strains at 30°C on MRS containing glucose as the sole carbon source and of *L. amylovorus* and *L. amylophilus* on MRS containg starch as the sole carbon source are presented in figures 3.7 and 3.8. The growth curves of both *L. amylovorus* and *L. amylophilus* were largely unaffected by changing the carbon source. The shortest lag phase of 12h on both glucose and starch was exhibited by *L. amylophilus*, compared to 14h and 16h for *L. plantarum* and *L. amylovorus* respectively. However once a pH of 4.7-4.8 was reached, growth of *L. amylophilus* slowed and after 28 and 32h respectively,

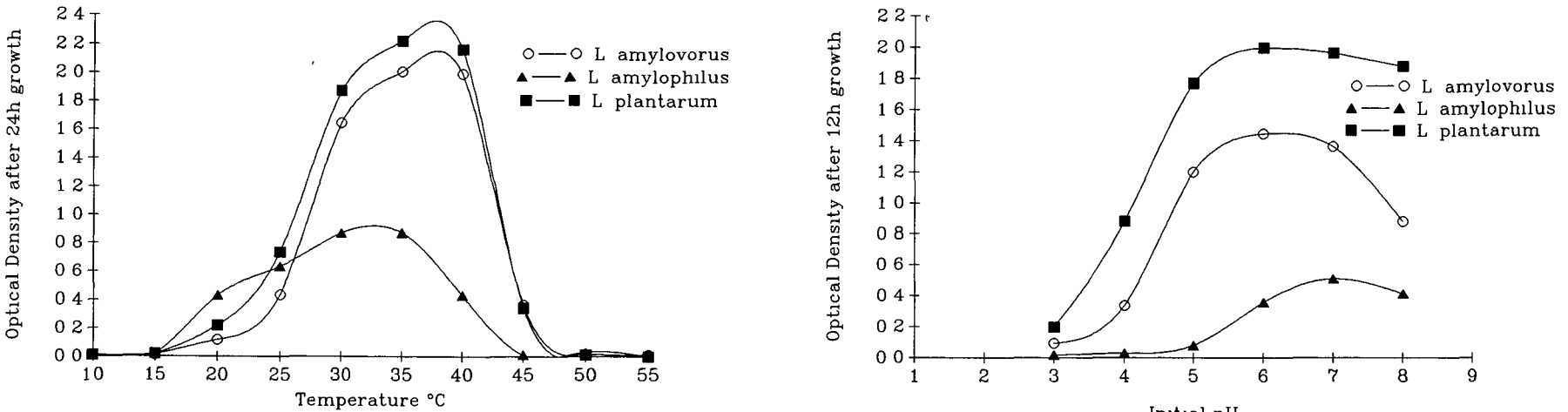


Fig 3 6 Effect of incubation temperature and initial pH on growth of L amylovorus, L amylophilus and L plantarum

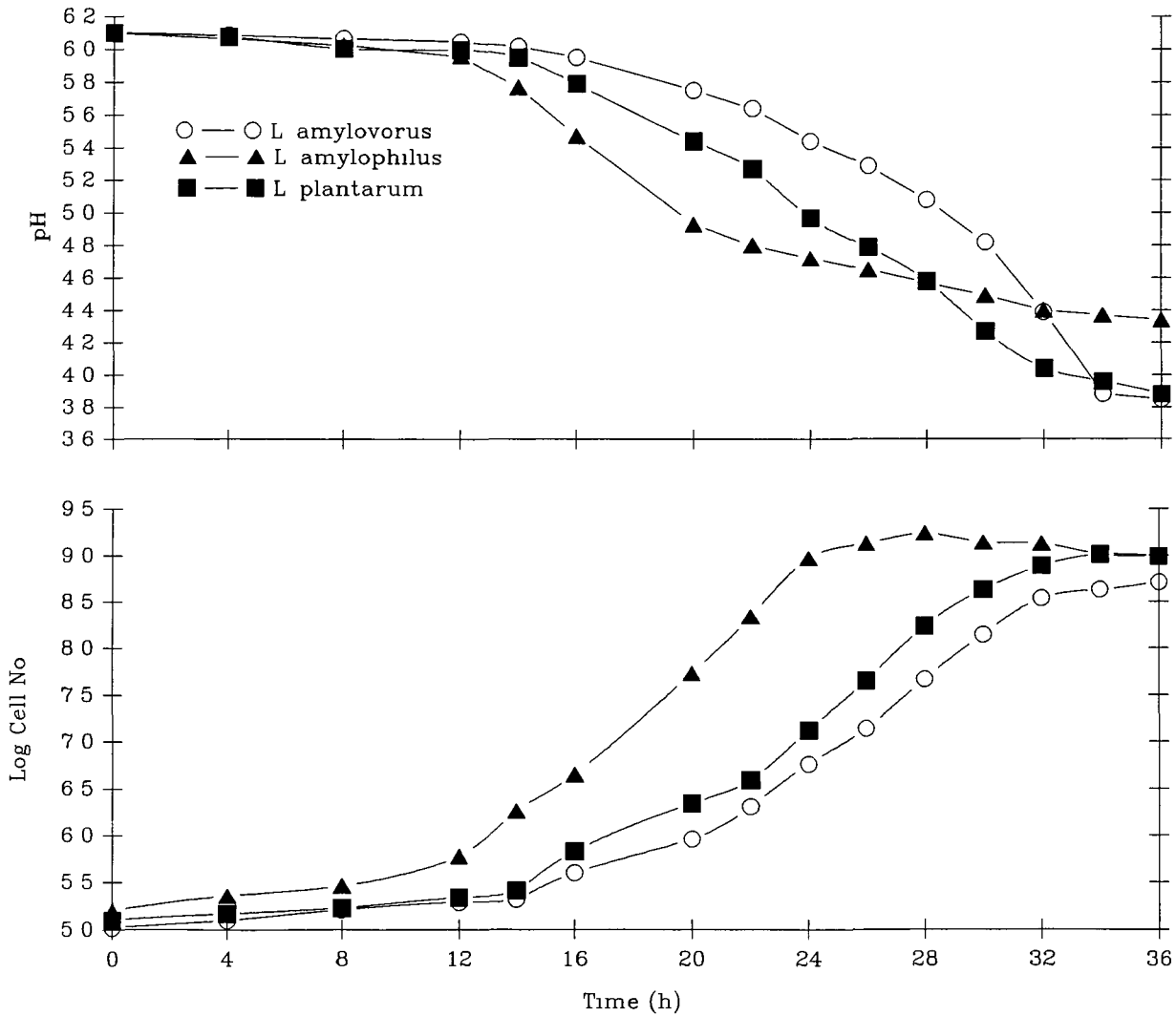


Fig 3 7 Growth curves of L amylovorus, L amylophilus and L plantarum on glucose at 30 degrees C

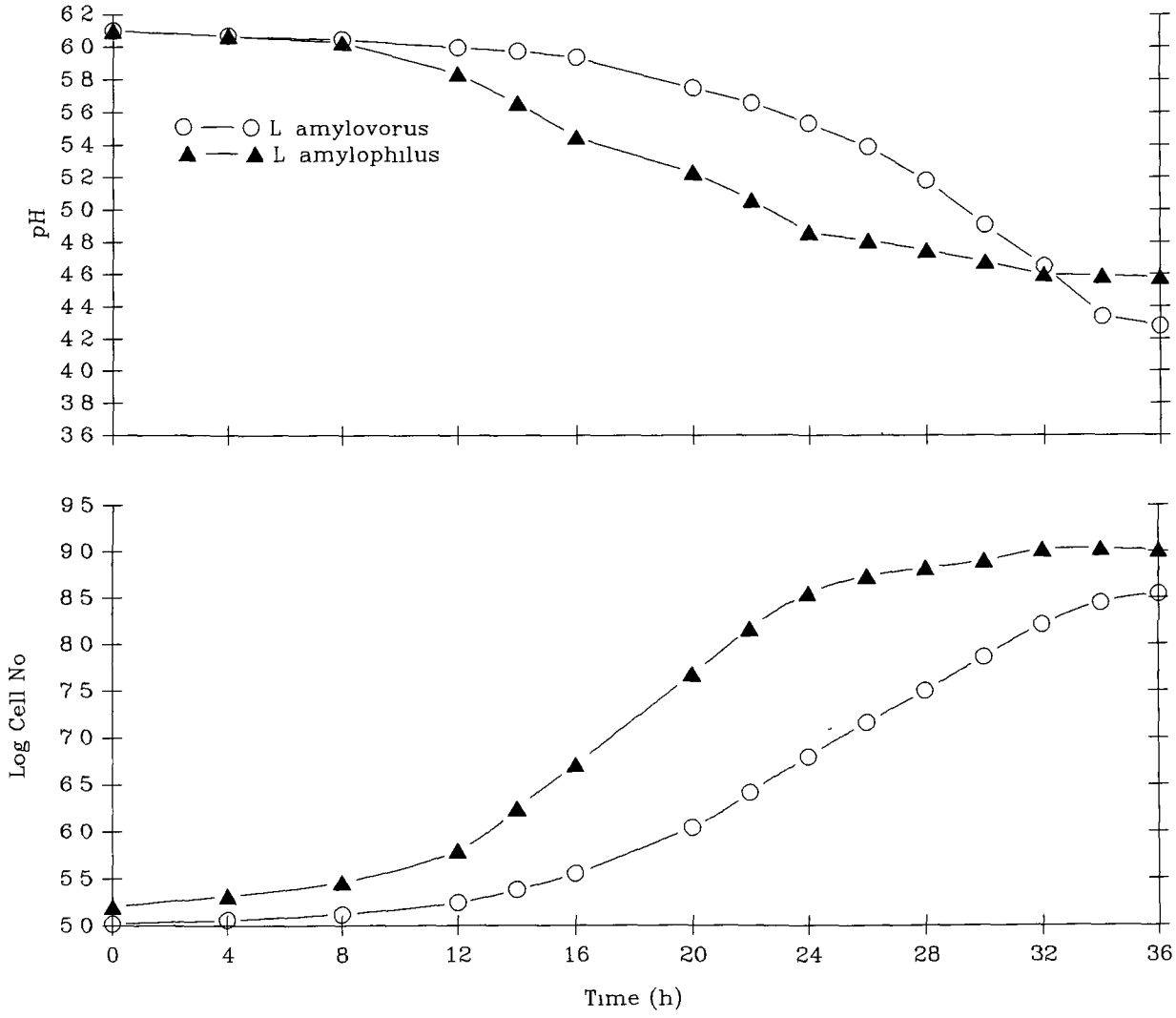


Fig 3 8 Growth curves of L amylovorus and L amylophilus on starch at 30 degrees C

cultures of *L. plantarum* and *L. amylovorus* exhibited a lower pH than those of *L. amylophilus*. Following 36 hours of growth in MRS at 30°C the pH of *L. amylovorus*, *L. amylophilus* and *L. plantarum* cultures was 3.82, 4.34 and 3.84 respectively.

Biochemical analysis revealed that both *L. amylovorus* and *L. amylophilus* secreted an α-amylase into the culture medium. *Lactobacillus amylovorus* was the more amylolytic of the 2 strains, secreting 1.06 X 10^{-10} U/cell amylase after 48 hours growth at 30°C compared to the 3 X 10^{-11} U/cell secreted by *L. amylophilus*. The effect of pH and temperature on the amylase enzymes produced by the 2 strains is presented in figures 3.9 and 3.10 respectively. The *L. amylovorus* amylase had a lower pH optimum of 5.5 compared to that of 7.5 for *L. amylophilus* and over the pH range 4-6 exhibited 3 to 5 times the amylolytic activity of *L. amylophilus*. Both enzymes had a temperature optimum of 50-55°C but the *L. amylovorus* amylase had a greater temperature stability, withstanding incubation temperatures as high as 65°C. At temperatures less than 35°C culture supernatants from both strains exhibited similar levels of amylolytic activity.

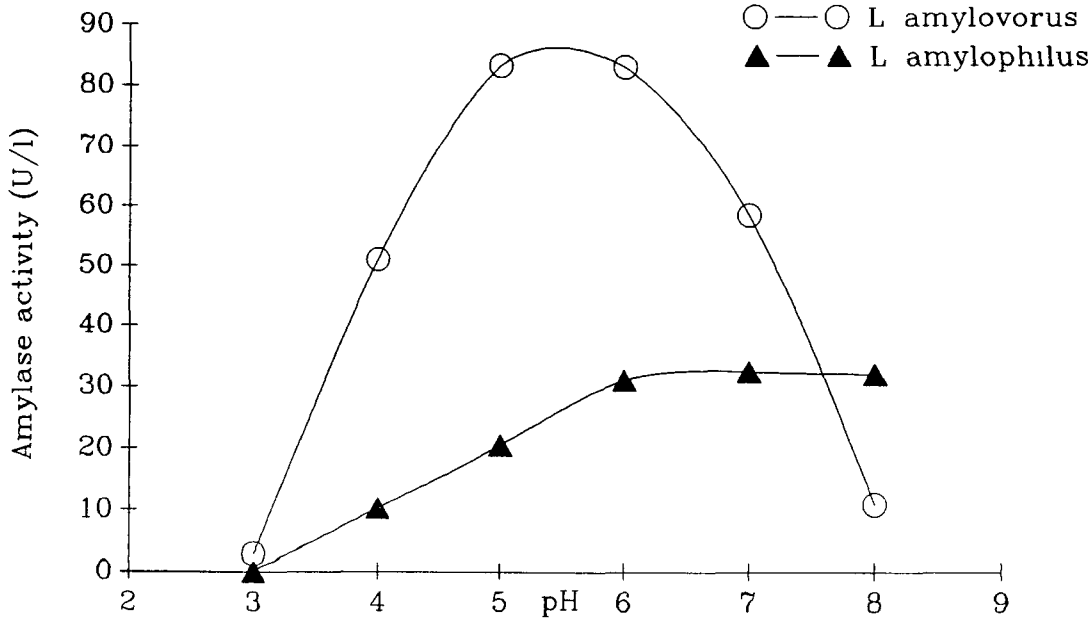


Fig 3 9 Effect of pH on amylase activity of L amylovorus and L amylophilus

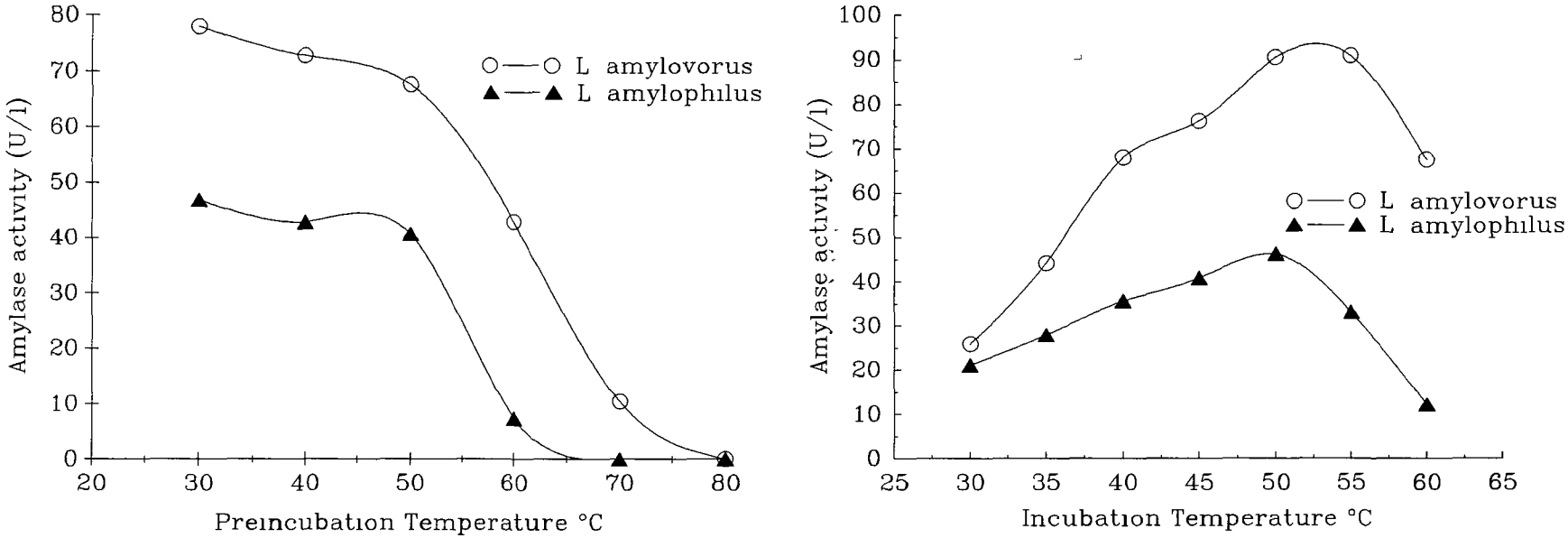


Fig 3 10 Effect of preincubation and incubation temperature on amylase activity of L amylovorus and L amylophilus

The low level of amylase activity exhibited by the *L. amylophilus* amylase enzyme over the silage pH range of 4-6 implied that this strain would be unsuitable as a silage inoculant for crops containing starch. By contrast, the *L. amylovorus* amylase enzyme was well suited to environmental conditions experienced in the silo and the strain exhibited a similar pH and temperature profile and only a marginally longer lag phase compared to an inoculant strain of *L. plantarum*. The ability of *L. amylovorus* to enhance the preservation of cereal silage was therefore tested.

3.2.3. Cereal silo trials of *L. amylovorus*.

The ensiling potential of *L. amylovorus* was assessed using a mixture of milled straw and ground barley in a 1:1 ratio by dry weight, which was rehydrated to a dry weight of 34.1%. Three treatments were employed; a control, 1.03 X 10^6 CFU *L. amylovorus*/g forage and 1.07 X 10^6 CFU *L. plantarum* LP80/g forage. *Lactobacillus plantarum* LP80 was included in this trial instead of *L. plantarum* B2 or DCU101 as, unlike the latter two strains, *L. plantarum* LP80 could be successfully transformed with plasmid DNA. Data on its competitiveness under silo conditions would therefore prove useful should genetic engineering techniques be required to construct an amylolytic inoculant strain. The inoculant numbers and silage pH 1, 2, and 4 days after ensilage are presented in table 3.14.

Treatment	pH			Log Inoculant No.*		
	DAY 1	Day 2	Day 4	Day 1	Day 2	Day 4
Control	6.16[a]	4.59[a]	4.01[a]	2.85[a]	5.06[a]	6.71[a]
L. amylovorus	6.22[a]	4.56[a]	4.08[a]	6.62[b]	6.79[b]	6.94[a]
L. plantarum	5.16[b]	3.77[b]	3.61[b]	6.91[c]	7.13[c]	7.47[b]

Table 3.14. Cereal silo trial 1: Changes in pH and bacterial numbers over the ensiling period. Values within columns differing significantly from one another followed by different superscripts.

* Inoculant numbers for control refer to *L. plantarum*.

Inoculation with *L. amylovorus* failed to reduce the silage pH compared to controls, while *L. plantarum* LP80 initiated a rapid reduction in silage pH and was clearly effective as an inoculant. Microbiological analyses suggested that the contrasting effectiveness of the 2 strains was related to their competitiveness; with *L. plantarum* LP80 numbers increasing much more rapidly than those of *L. amylovorus*. However laboratory results indicated that the amylase enzyme of *L. amylovorus* was well adapted to the silage microenvironment and it was therefore decided to transfer the amylase gene of *L. amylovorus* to the more competitive *L. plantarum* LP80.

3.2.4. Cloning of the *L. amylovorus* amylase gene:

Chromosomal DNA was isolated from *L. amylovorus* NRRLB4540 and partially digested with *MboI*. DNA fragments between 2 and 10 kb were isolated by electroelution and ligated to *BamHI* digested, alkaline phosphatase treated pGK13 vector DNA. Plasmid pGK13 is a shuttle vector containing both gram positive and gram negative origins of replication and encodes resistance to both erythromycin and chloramphenicol (see figure 3.11).

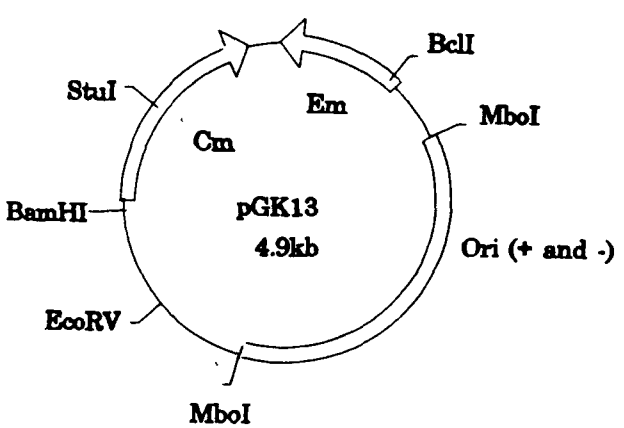


Figure 3.11: Plasmid pGK13 (Kok et al 1984).

Escherichia coli TG1 cells were transformed with the ligation mix, plated onto LB agar containing 20ug/ml chloramphenicol and 0.2% soluble starch, and incubated at 37°C for 48 hours. The plates were stained with iodine vapour, and of the 10,000 transformants, 8 amylase positive clones, designated pYYZ2110 to pYYZ2117, were identified. The erythromycin resistance and ampicillin sensitivity of each clone was confirmed, eliminating the possibility that the clones were any of the many ampicillin resistant, amylolytic strains commonly used in the laboratory. Restriction analysis of plasmid DNA revealed that all clones contained insert DNA of 2 to 5kb. The restriction profile of each clone was different, but all 8 clones shared a 1.3kb fragment of DNA on digestion with *Hpa*II. This 1.3kb fragment was isolated from a *Hpa*II digest of plasmid DNA from a single clone using Geneclean, labelled with digoxigenin dUTP, and used to probe a Southern blot of the gel described in plate 3.1. The *L. amylovorus* chromosomal DNA used for the gel was from a preparation independent to that used for the cloning of the amylase gene. As indicated by plate 3.1, the 1.3kb *Hpa*II fragment hybridised to undigested chromosomal but not to plasmid DNA from *L. amylovorus* and to 1.3kb and 11kb fragments from *Hpa*II and *HindII* digests respectively of *L. amylovorus* total DNA. No hybridisation to a *Hin*dIII digest of *L. plantarum* total DNA was observed. These results confirmed the *L. amylovorus* chromosome as the origin of the 1.3kb *Hpa*II fragment. The fragment also hybridised to a 1.3kb *Hpa*II fragment from each of the 8 clones, indicating that all 8 clones shared this fragment of insert DNA. Other faint bands of hybridisation to *Hpa*II digests of plasmid DNA from the 8 clones were presumably caused by the presence of small quantities of partially digested DNA containing the 1.3kb fragment.

Plate 3.1: Hybridisation of cloned 1.3kb *Hpa*II fragment to *Hpa*II digest of chromosomal DNA from *L. amylovorus*.
Lanes: (1) λ ladder, (2) *L. amylovorus Hpa*II, (3) *L. amylovorus Hin*dIII, (4) *L. amylovorus* uncut, (5) *L. plantarum Hin*dIII, (6-14) pYYZ2110-2118 *Hpa*II.

3.2.5. Preliminary assessment of amylase production in *L. plantarum*.

In order to determine which, if any, of the clones contained the sequences required for the efficient expression and secretion of α-amylase in *L. plantarum*, plasmid DNA from each of the original clones was introduced into *L. plantarum* NCIB8826 by electroporation. The corresponding transformants were designated *L. plantarum* NCYYZ2110 to NCYYZ2117 and all 8 recombinant *L. plantarum* strains were amylase positive. The laboratory strain, *L. plantarum* NCIB8826, was used for the preliminary assessment of the ability of constructs to direct amylase expression in *Lactobacillus* on account of its high transformation efficiency of 10^6/μg DNA.

The molecular weight of the amylases secreted by *L. amylovorus* and 5 of the recombinant *L. plantarum* strains was determined by polyacrylamide gel electrophoresis, as described in section 2.4.7, and is presented in plate 3.2 and table 3.15. The similarities between the multiple amylolytic proteins indicate that the smaller proteins may derive from proteolytic cleavage of the largest amylolytic protein at specific sites. The molecular weights of the largest amylolytic proteins secreted by the recombinant *L. plantarum* strains were of 71-105kd, smaller than the 155kd amylase secreted by *L. amylovorus*. This suggests that fragments of the *L. amylovorus* α-amylase gene, of various length, and not the complete gene may have been cloned.

Plate 3.2: Amylolytic proteins of *L. amylovorus* and recombinant *L. plantarum* strains.

Lane 1: *L. plantarum* NCYYZ2112
" 2: *L. plantarum* NCYYZ2113
" 3: *L. plantarum* NCYYZ2115
" 4: *L. plantarum* NCYYZ2116
" 5: *L. plantarum* NCYYZ2117
" 6: *L. amylovorus*

L. amylovorus	*L. plantarum* NCYYZ:				
	2112	2113	2115	2116	2117
155					
135					
126					
105				105	
94		94		94	
88		88		88	
78	78	78	78	78	
71	71	71	71	71	71
65	65	65	65	65	65
61	61	61	61	61	61
58	58	58	58	58	58

Table 3.15: Molecular weight (kda) of amylolytic proteins produced by *L. amylovorus* and recombinant strains of *L. plantarum*.

As indicated by table 3.16 culture supernatants from recombinant strains of *L. plantarum* secreting amylolytic proteins of greater molecular weight tended to contain greater quantities of amylase, though differences in amylase production per cell were not significant.

L. plantarum recombinant strain	Mol. Wt. of largest amylolytic protein produced	Amylase produced Units/cell
NCYYZ2112	78	1.55×10^{-10}
NCYYZ2113	94	2.52×10^{-10}
NCYYZ2115	78	8.40×10^{-11}
NCYYZ2116	105	2.78×10^{-10}
NCYYZ2117	71	1.62×10^{-10}

Table 3.16: Amylase production by recombinant strains of *L. plantarum*.

3.2.6. Sequencing of the α-amylase gene.

The nucleotide sequence of the N terminal end of the *L. amylovorus* amylase gene was determined using plasmid pYYZ2112, and is presented in figure 3.12, along with the derived amino acid sequence of the encoded protein. A large open reading frame beginning with an GTG codon was identified. A potential ribosome binding site, 16bp upstream of the start codon, a putative promoter, containing typical RNA polymerase binding and recognition sites, and a potential signal sequence comprising the first 37 amino acids of the protein were all identified, and their positions are indicated on the figure.

AGTTACTAAAACTAATACTGACTATAAAACAGAAGCAAAAAAATTTTCGATTTTTATGA

-35 -10
AAACGGTTGCAAAGAAGTTAGCAAAAATATATAATTTCTTTTGAAATTGTTCACTTGGC

CAAGCTGCAGTTTCAATATTTTAATAAAGGGGGCAGTAAAAAGTGAAAAAAAAGAAAAG
SD
V K K K K S

TTTCTGGCTTGTTTCTTTTTTAGTTATAGTAGCTAGTGTTTTCTTTATATCTTTTGGAT
F W L V S F L V I V A S V F F I S F G

TTAGCAATCATTCTAAACAAGTTGCTCAAGCGGCTAGTGATACGACATCAACTGATCAC

F S N H S K Q V A Q A A S D T T S T D H
↑
Cleavage site

TCAAGCAATGATACAGCTGATTCTGTTAGCGACGGTGTTATTTTGCATGCATGGTGCTG

S S N D T A D S V S D G V I L H A W C

GTCGTTCAACACGATTAAAAACAACTTGAAACAGATTCATGACGCCGGCTACACAGCGG

W S F N T I K N N L K Q I H D A G Y T A

TTCAACTTCACCTGTTAATGAAGTTAAAGTTGGAAATAGCGGGTCTAAGTCATTAAATA

V Q T S P V N E V K V G N S G S K S L N

ACTGGTATTGGCTATATCAGCCAACTAAATATAGTATTGGTAACTATTATTTAGGAACG

N W Y W L Y Q P T K Y S I G N Y Y L G T

GAAGCTGAATTTAAGTCAATGTGCGCTGCTGCTAAAGAATATAATATCAGGATCATTGT

E A E F K S M C A A A K E Y N I R I I

CGATGCAACTCTGAATGATACAACAAGTGATTATAGTGCAATTTCGGATGAAATTAAAA

V D A T L N D T T S D Y S A I S D E I K

GTATTCCAAATTGGACACATGGTAACAAACAAATTTCGAATTGGAGTGATCGTGAAGAT

S I P N W T H G N K Q I S N W S D R E D

GTTACTCAAAATTCGTTGTTAGGTTATATGATTGGAATACTCAAANNNNAATATACTAA

V T Q N S L L G Y M I G I L K X X Y T

CCTTATGAGTTTTAAGAGTTCAAGTCTGCATAAACTTCTTAATAAACCTTGCGAACTAA

N L M S F K S S S L H K L L N K P C E L

AGACTGCCTCGAAGTCCGAAAGCAATACTACGTCGANCAAGATTTTTGAAAATGAACGT

K T A S K S E S N T T S X K I F E N E R

GGCAGCAAAGGGGTTGTTTTTGCAAACGCTTCCGACAGTTCATATAGTTTGAATGTTAA

G S K G V V F A N A S D S S Y S L N V

AACTAGTTTAGCTGATGGGACTTATGAAAACAAGGCTGGTTCAGATGAATTTACCGTTA

K T S L A D G T Y E N K A G S D E F T V

AAAATGGTTATTTAACCGGTACAATTCAAGGACGTGAAGTTGTTGTTCTTTACGGGGAT

K N G Y L T G T I Q G R E V V V L Y G D

CCAACAAGCAGCAGCAGTACAACAACAGAAACTAAAAAGGTTTATTTTGAAAAGCCTTC

P T S S S S T T T E T K K V Y F E K P

AAGTTGGGGTAGTAGAGTTTATGCCTATGTTTATAATAAAAATACGAATAAAGCTATAA

S S W G S R V Y A Y V Y N K N T N K A I

CTTCAGCTTGGCCTGGCAAAAAAATGACCGCTTTAGGTAACGACAAATATGAATTGGAT

T S A W P G K K M T A L G N D K Y E L D

CTCGACACTGATGAAGATGACTCTGATTTAGCTGTTATCTTTACCGATGGGACAAAGCA

L D T D E D D S D L A V I F T D G T K

AACACCAGCAGCTAATGAGGCTGGTTTTACCTTTACGCTGATGCCACTTATGATCCAGG

Q T P A A N E A G F T F T L M P L M I Q

Fig. 3.12: Nucleotide sequence and derived amino acid sequence of the N terminal end of the *L. amylovorus* α-amylase gene. Putative -35 and -10 sequences in the promoter region and the proposed ribosome binding site (Shine Dalgarno sequence) are overlined and underlined respectively. The cleavage site between the signal sequence and the mature α-amylase is indicated by a vertical arrow. Amino acid symbols used are indicated in appendix A.

The derived amino acid sequence of the N terminal region of the *L. amylovorus* α-amylase gene was found to display significant homology with α-amylases from *Bacillus subtilis* and *Clostridium acetobutylicum* with 70% of amino acids over a 159 residue stretch of the *B. subtilis* amylase and 42% of amino acids over a 67 residue stretch of the *C. acetobutylicum* amylase being identical to the *L. amylovorus* α-amylase amino acid sequence. The comparison of the *L. amylovorus* α-amylase amino acid sequence with those of *B. subtilis* and *C. acetobutylicum* is presented in figure 3.13. Slight homology was also observed with rat and mouse pancreatic α-amylases and mouse salivary α-amylase.

```
                      *  *     *   **  ** *   * ***** ***** *
B. subtilis  : 25 LAGPAAASAETANKSNELTAPSIKSGTILHAWNWSFNTLKH
L. amylovorus: 33 VAQAASDTTSTDHSSND-TADSVSDGVILHAWCWSFNTIKN

                 * ******** **** * ** ** * **  ********* * *
B. subtilis  : MKDIHDAGYTAIQTSPINQVKEGNQGDKSMSNWYWLYQPTSYQI
L. amylovorus: LKQIHDAGYTAVQTSPVNEVKVGNSGSKSLNNWYWLYQPTKYSI

                 ** ***** *** ***** ** *  ****  * ***** *** *
B. subtilis  : GNRYLGTEQEFKEMCAAAEEYGIKVIVDAVINHTTSDYAAISNE
L. amylovorus: GNYYLGTEAEFKSMCAAAKEYNIRIIVDATLNDTTSDYSAISDE

                  ********** ** ***** *********
B. subtilis  : VKSIPNWTHGNTQIKNWSDRWDVTQNSLLG 184
L. amylovorus: IKSIPNWTHGNKQISNWSDREDVTQNSLLG 191

Predicted No.: 6.04 X 10^-40


                        ****** *  *  * *  **  * * ** * *
C. acetobutylicum: 112 ERGSKGAVIVNEGD-SFNLNTPTNLEDGNYDNHGSA
L. amylovorus    : 241 ERGSKGVVFANASDSSYSLNVKTSLADGTYENK-AG

                       *  **  *  ***       * *       *
C. acetobutylicum: TDSLTVSQGRMTGTVPANSIIVIYNKNSNPGS 178
L. amylovorus    : SDEFTVKNGYLTGTIQGREVVVLYGDPTSSSS 307

Predicted No.: 2.44 X 10^-3
```

* indicates identical amino acids.

The predicted number is the number of results expected by chance to have a score greater than or equal to the score of the result presented.

Fig. 3.13: Homology between derived amino acid sequence of N terminal region of *L. amylovorus* α-amylase gene and α-amylases from *B. subtilis* and *C. acetobutylicum*.

3.2.7. Construction of amylolytic inoculant strains of *L. plantarum*.

An amylolytic derivative of the inoculant strain *L. plantarum* LP80 was constructed by transforming LP80 with the plasmid pYYZ2112, to give *L. plantarum* LPYYZ2112. However as discussed previously (see section 1.6), any potential inoculant strain must satisfy the regulations governing the release of recombinant organisms into the environment which dictate that strains carrying heterologous genes stably integrated into the host chromosome are more acceptable than their counterparts carrying autoreplicative plasmids. A strategy for the integration of the *L. amylovorus* amylase gene into the *L. plantarum* LP80 chromosome was therefore devised using the plasmid pGIPCbh. This plasmid is described in figure 3.14 and contains both a gram negative and gram positive origin of replication, originating from pJDC9 and pE194 respectively, a gene coding for erythromycin resistance, and the conjugated bilary acid hydrolase (Cbh) gene from *L. plantarum*, within which is situated a unique *Xba*I site.

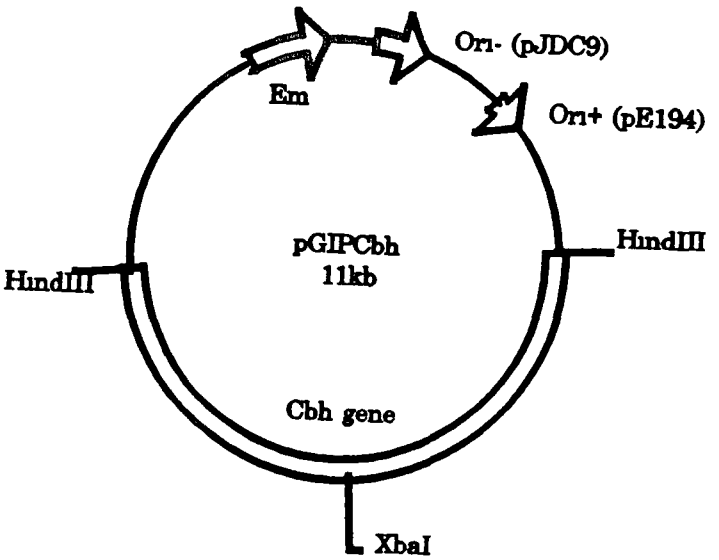


Figure 3.14: Plasmid pGIPCbh.

Conjugated bilary acid hydrolase is involved in cell metabolism in the intestinal environment only (Christiaens et al 1992) and disruption of this gene would not be expected to affect *L. plantarum* growth rates in silage. Leer et al (1993) demonstrated that the chromosomal Cbh gene in *L. plantarum* LP80 was a suitable site for targetting integration of heterologous genes by substituting an interrupted Cbh gene containing a chloramphenicol resistance gene for the chromosomal Cbh gene by homologous double cross over recombination. The resulting strain had a Cbh^-, Cm^r phenotype which was stably maintained for more than 100 generations under non selective conditions. It was proposed to insert the *L. amylovorus* α-amylase gene fragment from pYYZ2112 into the Cbh gene of pGIPCbh, and introduce the resulting plasmid into *L. plantarum* LP80 by electroporation. The entire plasmid would be expected to integrate into the *L. plantarum* LP80 chromosome and a subsequent excision event would eliminate all DNA of pGIPCbh origin. This two step integration procedure represents a similar strategy to that adopted by Hols et al (1992), (see figure 1.7 and section 1.5.4.3) and is described in figure 3.15.

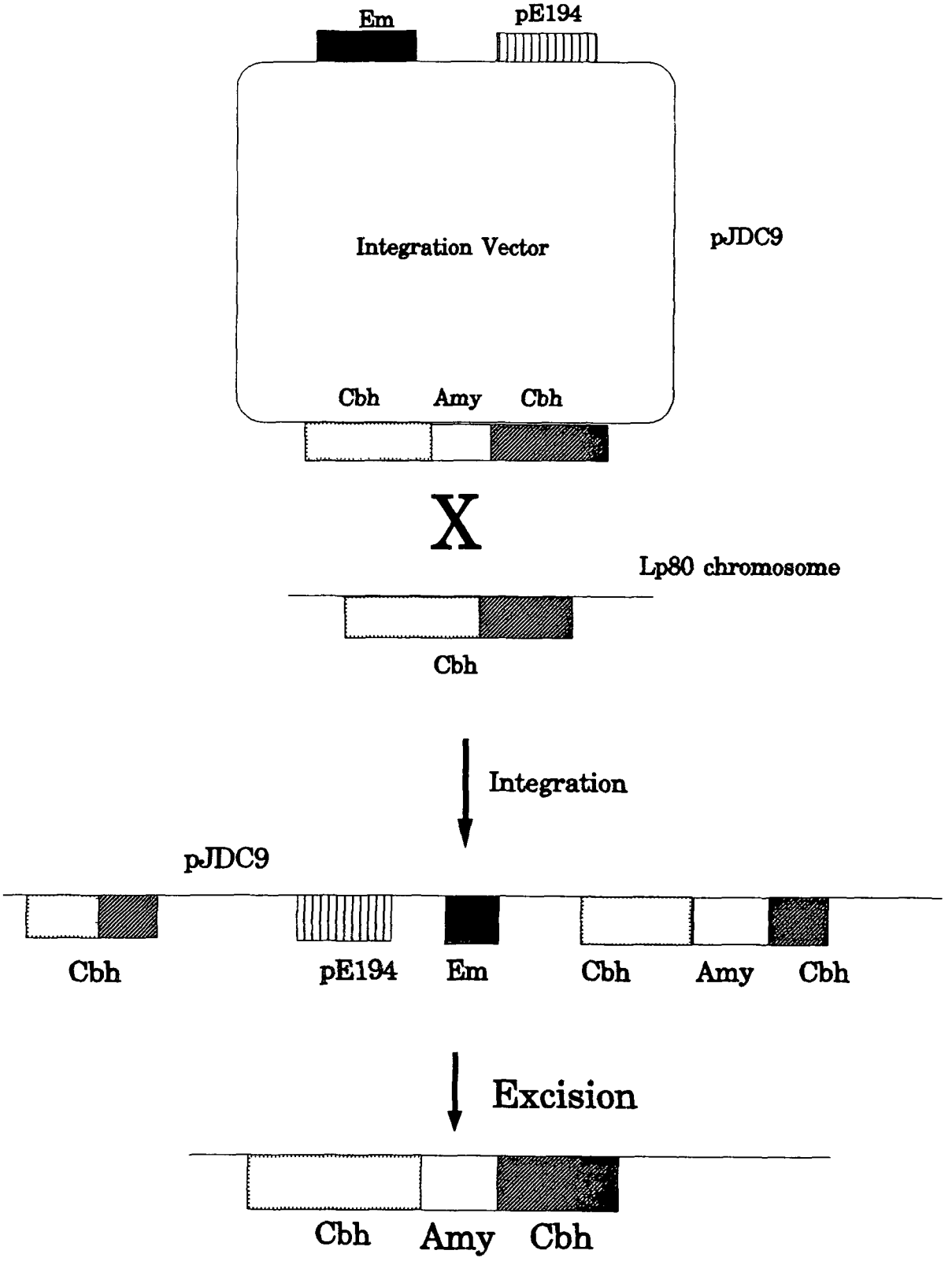


Figure 3.15: Strategy for the integration of a fragment of the *L. amylovorus* α-amylase gene into the chromosome of *L. plantarum* LP80.

The unique *Xba*I site in pGIPCbh, within the Cbh gene, was the obvious choice for introducing a fragment of the*L. amylovorus* α-amylase gene into pGIPCbh. However restriction analysis of pYYZ2112 revealed that it contained no site for *Xba*I and that the most suitable fragment containing the amylase gene for subcloning into pGIPCbh was a blunt ended *Eco*RV/*StuI* fragment of 3.2kb, comprising a 2.1kb DNA fragment of *L. amylovorus* origin and 1.1kb of flanking DNA from pGK13. The integration of DNA of pGK13 origin would render the resulting *L. plantarum* LP80 derivative unsuitable for release into the environment but would facilitate assessment of the competitiveness and amylolytic activity of such a strain. In order to facilitate subcloning of a blunt ended fragment into the Cbh gene in pGIPCbh the unique *Xba*I site within pGIPCbh was modified to generate a *Sma*I site (CCCGGG) which would accomadate a blunt ended fragment using a short oligonucleotide with the sequence CTAGCCCGGG, as described in figure 3.16.

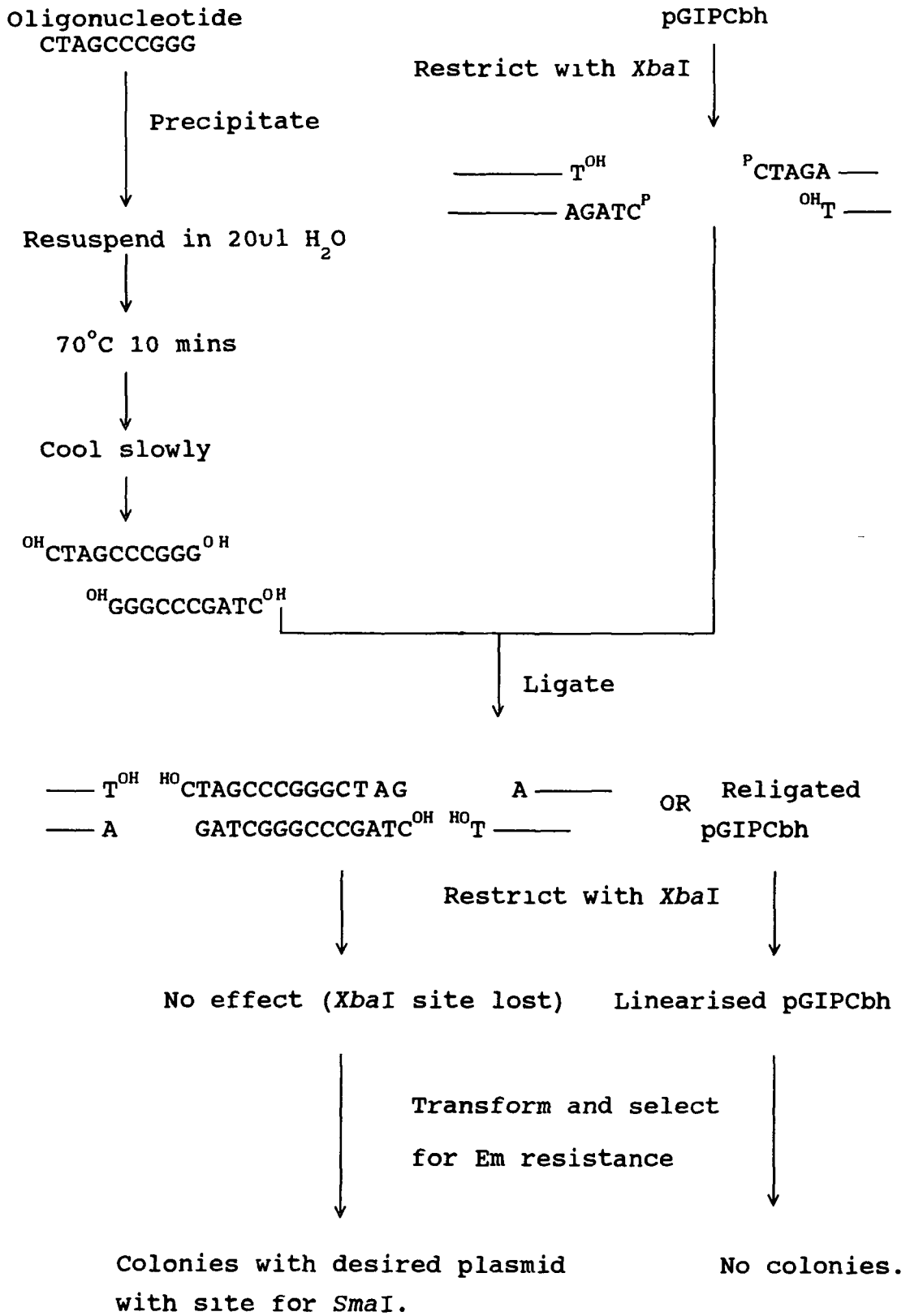


Fig. 3.16: Construction of pGAF001.

The concentration of oligonucleotide was adjusted to approximately 1 nmole/ul and 50ul of this DNA was precipitated to remove any impurities by adding 12ul 5M sodium acetate and 125ul cold ethanol and incubating at -70°C for 1 hour. The DNA was recovered by centrifugation at 12,000g for 20 minutes, washed in 1ml 70% ethanol, dried under vacuum and resuspended in 20ul H_2O. The oligonucleotide was heated to 70°C for 10 minutes and allowed to cool to room temperature over a period of 2 hours to permit annealing to take place. The annealed oligonucleotide was then ligated for 3 hours at room temperature to 10 nmoles pGIPCbh which had been restricted with *Xba*I. Since the oligonucleotide was not phosphatased it could not anneal to itself, and the only possible constructs to emerge from the ligation were pGIPCbh containing a single insert of the annealed oligonucleotide (with a single nick in each strand as shown in figure 3.16) and religated pGIPCbh. The *Xba*I site in the desired construct had been replaced by a *Sma*I site and the ligation mix was therefore restricted with *Xba*I to eliminate any circular pGIPCbh arising from religation without any insertion. The restricted ligation mix was then used to transform electrocompetent *E. coli* TG1 cells and 90% of the resulting erythromycin colonies contained the desired construct, pGAF001, possessing a site for *Sma*I but not *Xba*I.

A 3.2kb *Eco*RV/*Stu*1 fragment from pYYZ2112, containing the *L. amylovorus* amylase gene, was ligated to *Sma*I restricted pGAF001 DNA, and the ligation mix was used to transform electrocompetent *E. coli* TG1 cells. However none of the erythromycin resistant transformants were amylolytic. It was suspected that insertion of the *L. amylovorus* amylase gene within the Cbh gene of pGAF001 resulted in amylase expression levels that were sufficiently high to be toxic in *E. coli*. The high transformation efficiency of *L. plantarum* NCIB8826 (10^6/ug pGK13 DNA) made it a useful host into which ligation mixes could be transformed under such circumstances and the ligation was therefore repeated and used to transform electrocompetent *L. plantarum* NCIB8826 cells. Approximately 5% of the erythromycin resistant transformants expressed amylase and contained the desired

plasmid.

The resulting construct, pGAF002, was used to transform electrocompetent *L. plantarum* LP80 cells, yielding erythromycin resistant amylase positive transformants. Six amylase positive, erythromycin resistant transformants of *L. plantarum* LP80 were cultured for 30 generations in MRS broth containing 5ug/ml erythromycin, to allow the initial integration event to take place. The cells were then spread on MRS plates supplemented with 0.2% starch and 5ug/ml erythromycin, and 2 amylase positive colonies from each of the original 6 cultures were grown for 10 generations in MRS broth without selection. The cells were spread on MRS plates supplemented with 0.2% starch and 8 of the 12 cultures were found to stably maintain amylase activity. It was presumed that the desired integration event had occured in these 8 cultures, which were then maintained for 30 generations in MRS broth without selection, to allow excision of the erythromycin resistance gene to occur. Cells with the desired phenotype (amylase positive, erythromycin sensitive) were then identified by spreading on MRS plates containing 0.2% starch and replica plating amylase positive colonies on MRS plates with and without erythromycin. Approximately 60% of the cells were amylase positive, of which 15% were erythromycin sensitive. Four independent amylase positive erythromycin sensitive clones, designated *L. plantarum* LPGAFΔ2 to *L. plantarum* LPGAFΔ5 were retained for further study.

Integration of the *L. amylovorus* α-amylase gene at the desired location in the *L. plantarum* LP80 chromosome was confirmed by Southern blotting. A *Hin*dIII digest of total DNA from *L. plantarum* LP80 and LPGAFΔ5, one of the putative integrants, was probed with ^{35}S labelled pGIPCbh plasmid DNA, which contains the Cbh gene. As indicated by plate 3.3, the chromosomal *Hin*dIII fagment containing the Cbh gene was observed to have increased in size from 4.3kb in *L. plantarum* LP80 to 7.5kb in LPGAFΔ5, confirming the insertion of a 3.2kb fragment within the Cbh gene.

The above integration procedure was repeated using *L. plantarum* NCIB8826, giving rise to integrants *L. plantarum* NCGAFΔ1 to NCGAFΔ5. This suggests that integration within the Cbh gene could be directed by pGAF002 in a number of *L.*

plantarum strains. All integrants gave rise to substantial zones of starch degradation when grown on MRS containing starch as the sole fermentable carbohydrate and subsequently stained with iodine vapour (see plate 3.4)

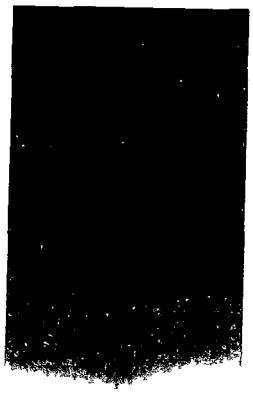


Plate 3.3: Hybridisation of Cbh gene to *Hin*dIII digest of total DNA from *L. plantarum* LP80 and *L. plantarum* LPGAFΔ5.
Lane 1: λ marker
" 2: *L. plantarum* LP80 *Hin*dlII
" 3: *L. plantarum* LPGAFΔ5 *Hin*dIII
" 4: pGAF001 *Sma*I

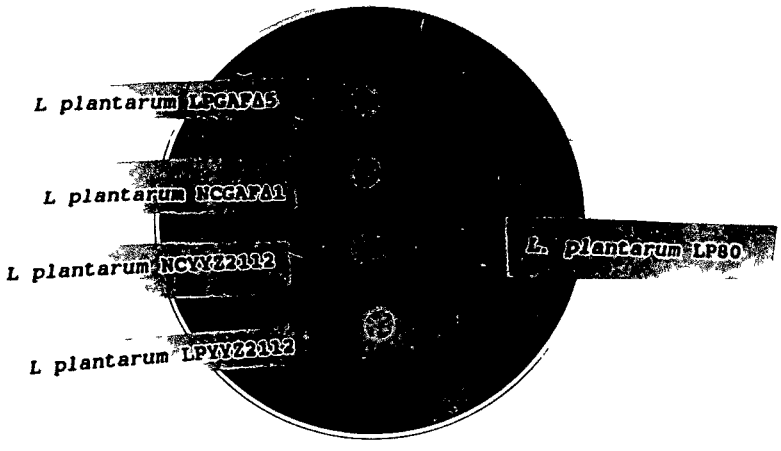


Plate 3.4: Amylolytic activity of recombinant *L. plantarum* strains.

3.2.8. Characterisation of *L. plantarum* LPGAFΔ5.

The stability of *L. plantarum* LPGAFΔ5 was compared to that of *L. plantarum* LPYYZ2112, in which the α-amylase gene of *L. amylovorus* was maintained on an autoreplicative plasmid. Only 1% of *L. plantarum* LPYYZ2112 cells but 100% of *L. plantarum* LPGAFΔ5 cells maintained amylase activity after 50 generations of growth under non-selective conditions.

The quantity and percentage of total amylase secreted into the extracellular medium by *L. amylovorus* and by *L. plantarum* LP80 and NCIB8826 carrying the *L. amylovorus* amylase gene on an autoreplicative plasmid or integrated into the host chromosome after 24 hours' growth in MRS containing 0.5% starch and 0.5% glucose as the fermentable carbohydrate source are presented in table 3.17.

Strain	Amylase secreted		% of total amylase secreted
	U/l	U/cell	
L amylovorus	47 2	$9\ 44 \times 10^{-11}$	88
L plantarum NCYYZ2112	69 2	1.39×10^{-10}	82
L plantarum NCGAFΔ1	25.8	5.14×10^{-11}	85
L plantarum LPYYZ2112	35.6	$6\ 45 \times 10^{-11}$	86
L. plantarum LPGAFΔ5	23.8	4.63×10^{-11}	82

Table 3.17: Amylase secretion levels in *L. amylovorus* and recombinant *L. plantarum* strains.

The recombinant strains of *L. plantarum* LP80 and *L. plantarum* NCIB8826 carrying the *L. amylovorus* α-amylase gene integrated into the chromosome secreted 72% and 37% respectively of the amylase secreted by their autoreplicative counterparts, on a per cell basis. *Lactobacillus plantarum* LP80 seemed to be a less suitable host for amylase production than *L. plantarum* NCIB8826, secreting 46% (autoreplicative) to 91% (integrant) of the amylase secreted by corresponding strains of *L. plantarum* NCIB8826. Only *L. plantarum* NCYYZ2112 secreted more amylase per cell than *L. amylovorus*, while *L. plantarum* NCGAFΔ1 and *L. plantarum* LPGAFΔ5 secreted 54% and 49% respectively of the amylase secreted by the wild type strain. All strains secreted 82-88% of the total amylase produced.

The growth characteristics and pattern of amylase production of *L. amylovorus*,*L. plantarum* LP80 and *L. plantarum* LPGAFΔ5 grown in MRS with 0.5% glucose, 0.5% starch or 0.5% starch and 0.5% glucose as the fermentable carbohydrate source are presented in figures 3.17 to 3.19. The amylolytic integrant *L. plantarum* LPGAFΔ5 displayed similar growth characteristics to the wild type LP80 strain when grown on glucose as the major carbohydrate source. However,the recombinant strain exhibited a marked competitive advantage over the wild type strain when starch alone or a mixture of starch and glucose were employed as fermentable substrates. On starch alone *L. plantarum* LP80 failed to reduce the culture pH below 5.92 or to grow beyond a cell density of 5.54 X 10^6/ml. By contrast *L. plantarum* LPGAFΔ5 cultures displayed a final pH of 4.63 and a cell density of 7.41 X 10^8/ml. The recombinant strain also displayed a competitive advantage when a mixture of glucose and starch was employed as a fermentable substrate, though this only became apparent after 8 hours' growth, at which point fermentable carbohydrate levels presumably became limiting for growth of *L. plantarum* LP80. After 14 hours' growth *L. plantarum* LPGAFΔ5 cultures displayed a pH of 4.06 and a cell density of 3.16 X 10^9/ml compared to 4.81 and 1.13 X 10^9/ml for cultures of the wild type strain.

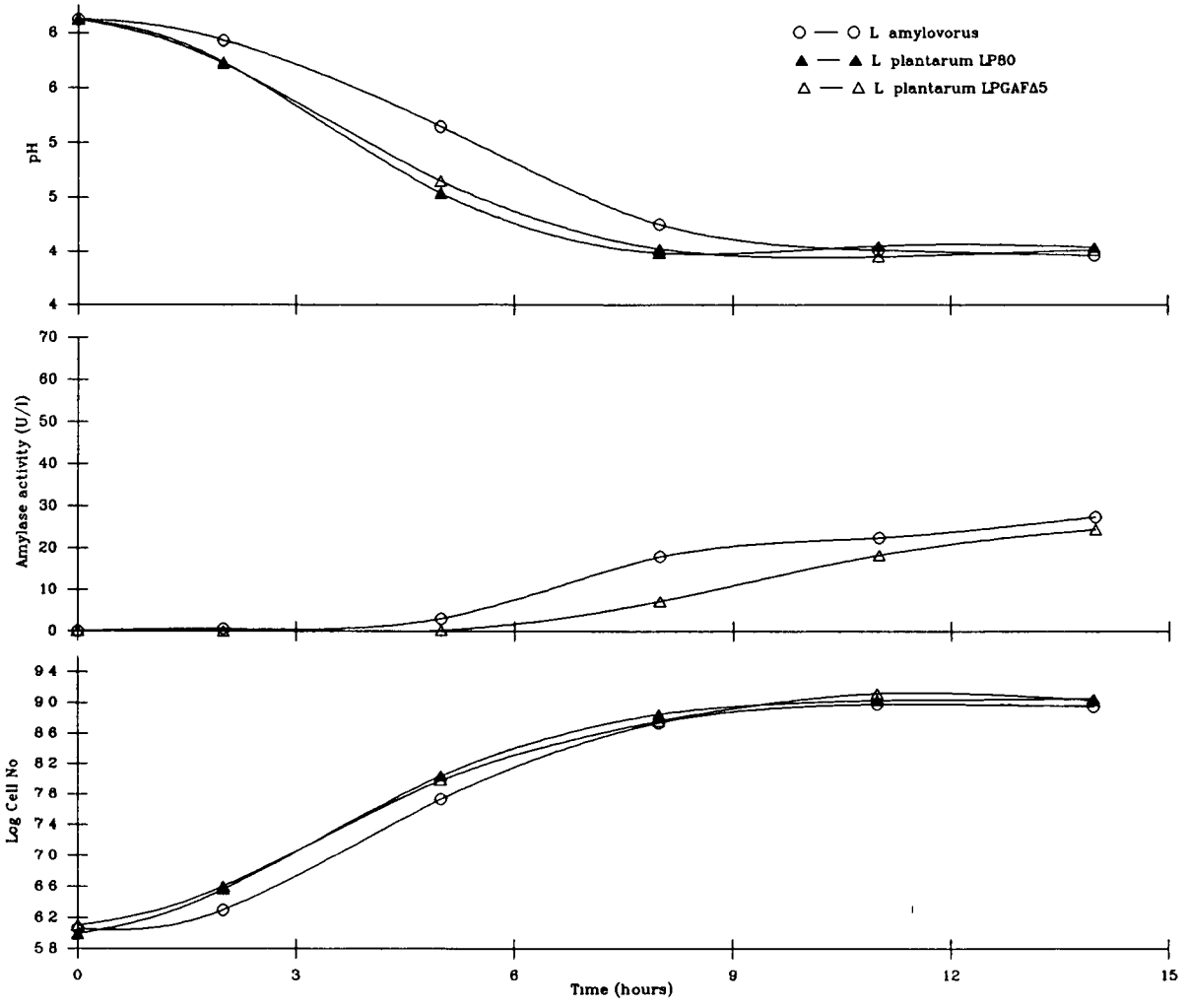


Fig 3 17 Growth curves of L amylovorus L plantarum LP80 and L plantarum LPGAFΔ5 on glucose

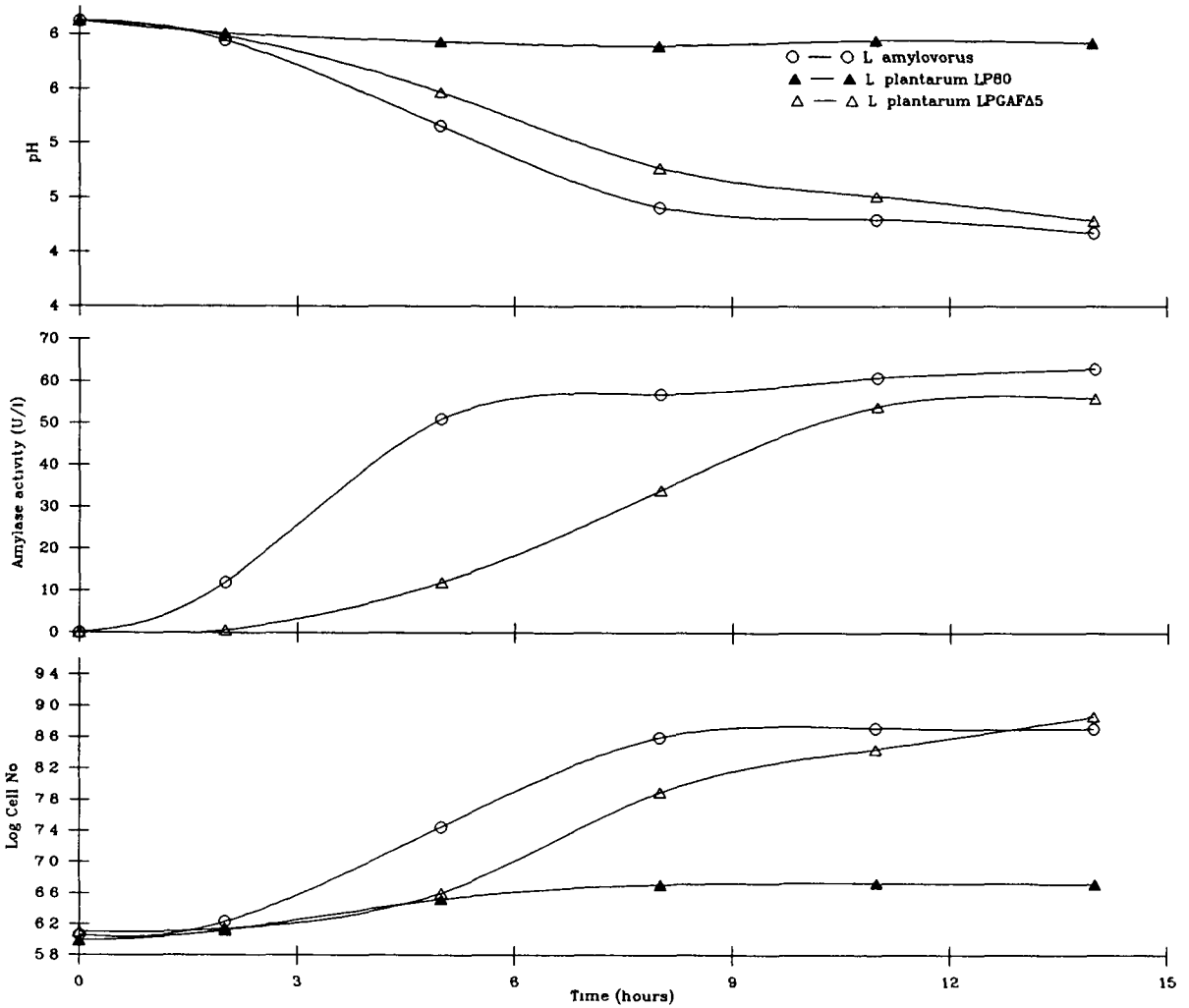


Fig 3 18 Growth curves of L amylovorus L plantarum LP80 and L plantarum LPGAFΔ5 on starch

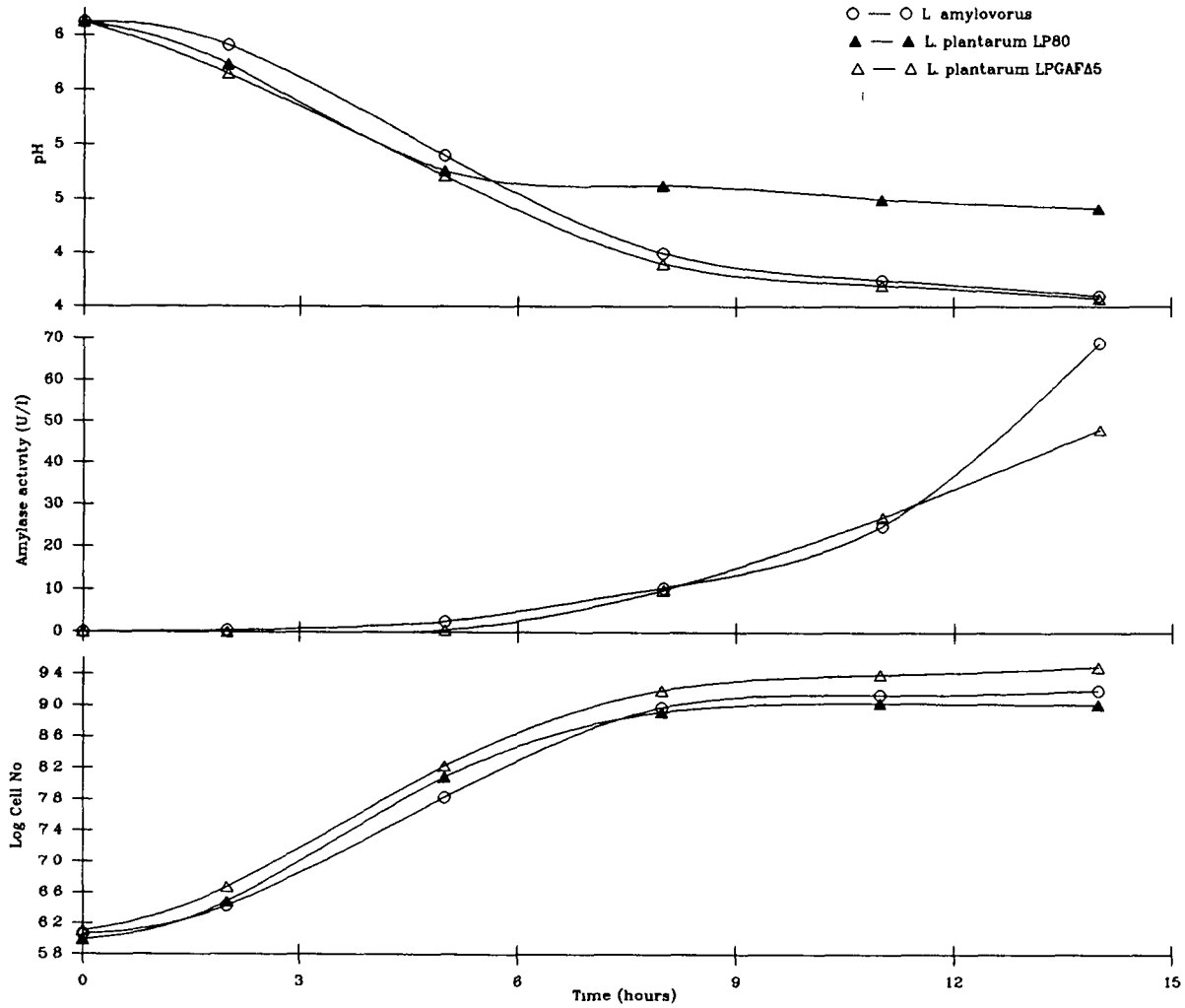


Fig 3 19 Growth curves of L amylovorus L plantarum LP80 and L plantarum LPGAFΔ5 on glucose and starch

When grown on glucose or a mixture of glucose and starch *L. plantarum* LPGAFΔ5 exhibited a similar final culture pH compared to *L. amylovorus*, but initiated a more rapid reduction in pH. By contrast, when starch was employed as the sole fermentable substrate, *L. amylovorus* displayed a competitive advantage over *L plantarum* LPGAFΔ5, with a shorter lag phase and a slightly lower final culture pH. The comparatively long lag phase of *L. plantarum* LPGAFΔ5 when grown on starch alone correlated with a low level of amylase production during the early stages of fermentation. Amylase production was observed to be controlled by catabolite repression in both *L. amylovorus* and *L. plantarum* LPGAFΔ5 in that amylase production was inhibited by the presence of glucose in the culture medium. When amylase levels were considered on a per cell basis, no stimulatory effect of starch on amylase production was observed in either strain.

4. Discussion.

4.1. The development of an inoculant for grass silage.

The approach adopted to the design of a suitable inoculant for grass silage was based on the premise that a rapid fall in pH during the first few days of ensilage is crucial to minimising nutrient losses due to the action of plant enzymes and Gram negative bacteria during this period, and of clostridia later on in the fermentation. Attention was therefore focussed on the lactic acid cocci which dominate the initial stages of the silage fermentation (Ely et al 1981, Moon et al 1981, Lindgren et al 1983). Of the lactic acid cocci, *P. acidilactici* was selected as the most suitable on the basis of its homofermentative metabolism and ability to tolerate high temperatures. Furthermore, compared to *L. plantarum*, *P. acidilactici* has a greater tolerance of low pH (Bryan Jones 1969), and while *L. plantarum* is well known to be superceded by heterofermentative lactobacilli during the latter stages of fermentation (Langston et al 1962, Beck 1972), there is some evidence that *Pediococcus* spp. dominate the silage microflora, along with the heterofermentative lactobacilli, at this time (Whittenbury 1968, McDonald and Whittenbury 1973). This suggested that an inoculum consisting solely of a *P. acidilactici* strain would ensure a rapid fall in silage pH, and might also be capable of subsequently dominating the fermentation over a long period of time. This hypothesis was borne out by small scale silo trials which indicated that *P. acidilactici* G24, which was selected from 18 *P. acidilactici* strains screened in the laboratory as having the greatest potential as an inoculant, when applied at a rate of 10^6/g forage, consistently aided the preservation of grass silage of water soluble carbohydrate content greater than 100g/kg DM, as indicated by an accelerated rate of lactic acid production and pH decrease, and a final silage with a lower pH, a lower ammonia nitogen content and a higher crude protein content compared to controls. Furthermore *P. acidilactici* G24 proved superior as an inoculant to *L. plantarum* B2 or various mixtures of *P. acidilactici* G24 and *L. plantarum*: While all inoculated silages had the same pH after 7, 20 or 60 days' fermentation, the rate of pH

decrease was greatest in those silos inoculated with *P. acidilactici* G24 alone. Ammonia N is widely accepted as an indicator of clostridial activity (McDonald 1981) and the correlation observed between inoculation with *P. acidilactici*, a rapid rate of pH decrease, and a lower level of ammonia nitrogen in the resulting silages is in agreement with the hypothesis that a rapid rate of pH decrease restricts subsequent protein degradation and clostridial activity (Gibson et al 1958, MacPherson 1962, MacPherson and Violante 1966, Whittenbury 1968).

The rapid rate of pH decrease initiated by inoculation with *P. acidilactici* G24 alone distinguished this inoculant as superior to *L. plantarum* based inoculants tested. Microbiological analysis revealed a strong positive correlation between this sharp decrease in pH and a rapid proliferation of *P. acidilactici* to a peak of 10^9/g forage 3 days after ensilage. Any action limiting this initial proliferation, eg. a change in inoculant composition or a reduction in the substrate available for fermentation, resulted in a slower rate of pH decrease. The inability of *L. plantarum* based inoculants, when used at a rate of 10^6 CFU/g forage to initiate such a rapid rate of pH decrease was linked to their inability to provide such a high number of beneficial lactic acid bacteria (*L. plantarum* and *P. acidilactici*) by day 3 of the fermentation; the maximum being a total of 3 X 10^8/g forage compared to 1.1 X 10^9/g when *P. acidilactici* alone was used. After 3 days of fermentation *P. acidilactici* numbers began a slow decline which continued until 60 days after ensilage. However when silos inoculated with either *P. acidilactici* or *L. plantarum* alone were compared, the numbers of the inoculant strain were not found to be significantly lower in those silos inculated with *P. acidilactici* alone at any time point right up to 60 days after ensilage. This indicates that, as predicted from literature data, *P. acidilactici* is just as capable as *L. plantarum* of dominating the fermentation over a prolonged period of time.

While the results of the silo trials presented here are in agreement with literature data on the biochemical characteristics of *Pediococcus* spp. and their role in the

silage fermentation, they are in marked contrast to reviews of silage inoculants which suggest a mixture of *P. acidilactici* and *L. plantarum* as an ideal inoculant (Woolford 1984, Seale 1986). This hypothesis stems from the inability of *L. plantarum* to produce large quantities of lactic acid until a pH of less than 5 is reached (McDonald 1981) and proposes that *P. acidilactici* would initiate the fermentation and rapidly reduce the pH to 5, at which point the *L. plantarum* would take over, completing the fermentation. This theory is supported by a number of silo trials in which inoculation with mixtures of *L. plantarum* and *Pediococcus* spp. resulted in beneficial effects compared to uninoculated controls (Henderson and McDonald 1984, Kung et al 1984, Weinberg et al 1988, Froetschel et al 1991, Martinsson 1991, Tengerdy et al 1991, Stokes 1992), as it should be emphasised, was observed in the present study. However the literature reports failed to investigate whether a similar or greater beneficial effect could be obtained by inoculation with a *Pediococcus* spp. alone. Instead it seems to have become accepted that *L. plantarum* is a necessary component of any silage inoculant in order to complete the fermentation in spite of the aforementioned background data on *P. acidilactici* and *L. plantarum* suggesting the former to have at least equal preserving potential. However the data presented here indicating the potential of *P. acidilactici* G24 as the sole component of a silage inoculant is in agreement with results of Torriani et al (1987) and Lesins and Schulz (1968) respectively who found that inoculation of maize silage with *P. acidilactici* and of sedge, but not alfalfa silage with an unidentified *Pediococcus* spp. resulted in improved preservation compared to controls. The pattern of *Pediococcus* growth in both studies was similar to that reported here with pediococci numbers peaking at approximately 10^9/g forage 2-4 days after ensilage. Furthermore Lesins and Schulz (1968) noted that, as reported here, the difference in pH between control silos and those inoculated with a *Pediococcus* spp. alone was greatest in the early stages of fermentation and that at 30°C, inoculation with pediococci alone resulted in a

greater pH drop compared to controls than inoculation with a mixture of pediococci and *L. plantarum*. The results of the 3 studies discussed above therefore indicate the definite potential of *P. acidilactici* as the sole component of a silage inoculant and support the heretofore ignored conclusion of Lesins and Schulz (1968) that "the generally held view that *Lactobacillus spp.* should be looked upon as the only suitable basis for inoculum needs revision".

While the results discussed above point to a definite beneficial effect from inoculation with *P. acidilactici* certain limitations also emerge:

(i) *P. acidilactici* strains for use as silage inoculants must be carefully selected and cannot be chosen from a culture collection on an *ad hoc* basis. The contrasting abilities of *P. acidilactici* G24 and *P. acidilactici* PLL07 to aid preservation under identical ensiling conditions points to the variability of *P. acidilactici* strains in their ensiling potential. The need for care in selecting strains for use as silage inoculants was further emphasised by the heterogeneity of *P. acidilactici* strains, evident from the laboratory results. While the 18 strains of *P. acidilactici* tested shared many features characteristic of the species (Buchanan and Gibbons 1974, Teuber and Geise 1981) including the ability to ferment glucose and fructose, a temperature optimum of 37°C, and the ability to grow rapidly between 25°C and 45°C and between pH values of 4.5 and 8.0, they differed in the length of their lag phases, the rate at which they reduced the culture pH, their ability to produce pediocins and their tolerance of low growth temperatures, factors crucial in determining their suitability as silage inoculants. The degree of strain heterogeneity suggests that individual strains, while retaining the basic characteristics of *P. acidilactici*, had adapted to different environments. The greater tolerance of low growth temperatures (20°C and less) displayed by isolates from Irish grass silage compared to their counterparts originating from the warmer climates of France and Italy was a particularly striking

example of adaptation to a particular environment.
Another important consequence of natural selection is that strains required to compete effectively in a particular environment are best isolated from that same environment. While this might appear obvious, literature reports of silo trials using organisms selected from culture collections (Thonney et al 1980) or from habitats such as the digestive tract (Moon et al 1981) indicate that this simple guideline has been frequently ignored. The contrasting abilities of *P. acidilactici* G24 and *P. acidilactici* PLL07 to aid preservation, as evidenced by laboratory results and small scale silo trials, are undoubtedly linked to the former strain being of grass silage origin. Furthermore, the distinguishing feature of the latter strain compared to the other grass silage isolates screened, its extremely short lag phase, is also consistent with its original habitat: *P. acidilactici* G24 was isolated from the early stages of a silage fermentation where a short lag phase would be essential for a strain to establish itself. By contrast the other silage isolates originated from silages which had undergone more than 30 days' fermentation, where a short lag phase would not be necessary to achieve dominance. The laboratory results therefore point to the potency of natural evolutionary processes in selecting strains adapted to a particular environment, particularly when ill-defined multi-component characteristics such as competitiveness are involved.
The correlation observed here between laboratory selection procedures and small scale silo trials, using *P. acidilactici* G24 and PLL07, establishes the effectiveness of such screening procedures in selecting potential inoculants. The laboratory screening procedure was designed to identify an isolate with a suitable metabolism for use as an inoculant and which would be capable of rapidly dominating the fermentation. The emergence of a strain from the screen originating from the initial stages of a silage fermentation and which was subsequently shown to be effective as an inoculant demonstrated the effectiveness of the screening procedure. However this could also be interpretated as suggesting that such a screen is

superfluous and that common sense would dictate that a strain of such origin would be most likely to succeed in silo trials. This would represent a high risk strategy; effective if one was fortunate enough to select the isolate with the best ensiling potential but expensive and frustrating otherwise. Consider, for example, the outcome if the strain selected failed to aid preservation; would one continue to test the remaining strains with no logical basis bar original habitat for choosing the next strain to test? There is also the possibility that one might cease screening once a strain giving some beneficial effect had been found, possibly discarding a more effective inoculant. By contrast a preliminary laboratory screen ensures that only the strain with the greatest potential is tested in small scale silos and if it is ineffective one can be reasonably certain that the other strains would be even less suitable. Furthermore the comparison of results from the laboratory and from small scale trials facilitates better understanding of the characteristics required of an effective silage inoculant, eg. in this study a short lag phase emerges as being crucial in determining a strain's potential. The resulting improved understanding of silage inoculants is essential if future inoculants are to be developed in a scientific manner.

The success reported here in determining ensiling potential using laboratory screening procedures is in contrast to the experience of Woolford and Sawczyc (1984) who tested 21 strains of lactic acid bacteria, including a number of silage isolates, using a similar but more thorough laboratory screen to the one employed here. None of the isolates satisfied all of the criteria used and the three strains with the greatest ensiling potential failed to aid preservation of grass silage. It is possible that the authors were unfortunate in that the original collection of 21 strains did not contain a suitable silage inoculant and given the thoroughness of their screening protocol it is likely that, of the 21 strains tested, the three selected had the greatest ensiling potential. This study does not therefore serve to invalidate laboratory screening procedures in the selection of silage inoculants, but

establishes the need for including a strain of known ensiling potential in any screen to act as a standard against which the test strains can be assessed.

(ii) While natural selection implies that silage isolates will have the greatest potential as silage inoculants, it also suggests that isolates from one type of silage may not be suitable for the ensiling of another crop. It is striking that in the work presented here and in the data presented by Lesins and Schulz (1968) and Torriani et al (1987) *Pediococcus* strains isolated from grass, sedge and maize silage respectively were successfully used to aid preservation of silage of the same crop type from which the strains had originally been isolated. Furthermore Lesins and Schulz (1968) found that their sedge silage isolate was ineffective in aiding preservation of alfalfa silage. The crop specific nature of successful inoculants suggested by these results requires further investigation and would be of great significance in areas of the world where a number of different crops are routinely ensiled.

(iii) Inoculation with *P. acidilactici* G24 was successful in aiding preservation of grass of water soluble carbohydrate content greater than 100g/kg DM but not of 51g/kg DM. The shortage of water soluble carbohydrate was seen to limit the proliferation of *P. acidilactici*, thus eliminating the preservative effect associated with the strain. This observation was in keeping with literature data which suggests that a water soluble carbohydrate content of 60-80g/kg DM is required to ensure a successful fermentation (Wierenga 1962, 1969, Smith 1973) and emphasises the importance of assessing the sugar content of a crop prior to harvesting. Crops deficient in fermentable sugar could be treated with an additive combining a sugar source and an inoculant or ensiled using an alternative additive such as formic acid.

4.2. The development of an amylolytic inoculant strain.

In view of the successful isolation of a competitive *P. acidilactici* strain from grass silage, a similar approach was adopted to the isolation of a starch degrading amylolytic inoculant strain. However no starch degrading lactobacilli were obtained from grass silage samples. This was in marked contrast to the experience of Langston and Bouma (1960b) who found that 93% of *L. plantarum* isolates from grass silage were capable of fermenting starch to acid. Given the absence of starch from grass, the failure to isolate starch degrading lactobacilli reported here is hardly surprising. It was suspected that amylolytic lactobacilli might be more easily isolated from silage made from crops containing starch but samples from silage of this type were not available. Two starch degrading lactobacilli; *L. amylovorus* and *L. amylophilus* (Nakamua 1981, Nakamura and Crowell 1979), isolated from waste corn fermentations, were therefore obtained from culture collections and tested for their ensiling potential in the laboratory. *Lactobacillus amylovorus* was found to have the greater potential for the ensilage of starch containing crops as its amylase enzyme showed greater activity over the silage pH range of 6-4. Furthermore when compared with an inoculant strain of *L. plantarum*, *L. amylovorus* had a similar pH and temperature profile for growth and only a slightly longer lag phase. In spite of this encouraging laboratory data, *L. amylovorus* was found to be incapable of aiding the preservation of cereal silage under conditions where *L. plantarum* LP80 proved effective as an inoculant. Microbiological analysis revealed that the poor performance of *L. amylovorus* in aiding preservation was caused by a lack of competitiveness under silo conditions. This suggests that the small difference in lag phase observed between *L. amylovorus* and *L. plantarum* in laboratory growth media was greatly exaggerated under silo conditions. The ineffectiveness of *L. amylovorus* as an inoculant for cereal silage observed here is consistent with the results of Petit and Flipot (1990) who observed no effect of inoculation with a mixture of lactic acid bacteria including 5 X 10^9 *L. amylovorus* /g forage on the pH or

ammonia nitrogen content of cereal silage.

As an alternative to including *L. amylovorus* directly in a silage inoculant, it was proposed to transfer the amylolytic activity from *L. amylovorus* to *L. plantarum* LP80, which had proved competitive in cereal silage (see section 3.2.3). Two factors suggested that the amylase from *L. amylovorus* would be particularly suitable for this purpose; the enzyme was well adapted to silo conditions of temperature and pH, and since *L. amylovorus* and *L. plantarum* belong to the same genus, it was suspected that the expression and secretion signals of the *L. amylovorus* α-amylase gene would be more compatible than those originating from other species with the expression and secretion mechanisms of *L. plantarum*. A recombinant *L. plantarum* inoculant strain carrying the *L. amylovorus* α-amylase gene would thus be expected to produce greater quantities of amylase than strains carrying genes from *Bacillus* spp. as constructed by Jones and Warner (1990), Scheirlinck et al (1989), and Conconcelli et al (1991). The *L. amylovorus* α-amylase gene was therefore cloned in *E. coli* using the shuttle vector pGK13. The 8 clones were analysed by expressing them in *L. plantarum* NCIB8826, an easily manipulated laboratory strain, and by nucleotide sequencing. This revealed that while the clones contained the expression and secretion signals associated with the *L. amylovorus* α-amylase gene and successfully induced amylase secretion in *L. plantarum*, none contained the complete gene, as indicated by the absence of a stop codon in the amylase open reading frame and the lower molecular weights of the recombinant α-amylase compared to the wild type.

Zymograms of culture supernatants from *L. amylovorus* and the recombinant strains of *L. plantarum* revealed two interesting features; the large molecular weight of 155 kda of the *L. amylovorus* α-amylase enzyme and the presence of multiple amylolytic proteins. While most α-amylases have molecular weights of 40-60 kda, amylases of 160 and 200 kda are secreted by *Bacillus polymyxa* (Kawazu et al 1987) and an unidentified gram positive spore former (Burgess-Cassler

et al 1991) respectively. The large molecular weight reported here is also in keeping with previous reports indicating that the *L. amylovorus* α-amylase is of molecular weight 140-150 kda (Imam et al 1991, Burgess-Cassler and Imam 1991). The molecular weights of the multiple amylolytic proteins observed in culture supernatants of *L. amylovorus* and *L. plantarum* expressing truncated fragments of the *L. amylovorus* α-amylase gene of various lengths were identical, apart from the absence of a number of the larger proteins from the recombinant *L. plantarum* strains. This suggests that the multiple amylolytic proteins derive from the largest protein present by a process of proteolytic cleavage at specific sites. Multiple amylolytic proteins have also been observed in the culture supernatants of *Bacillus stearothermophilus* (Thudt et al 1985), *Streptomyces griseus* (Garcia-Gonzalez et al), *Bacillus circulans* (Siggens 1987) and an unidentified gram positive spore former (Burgess-Cassler et al 1991) and have been proven to arise from proteolytic cleavage of precursors in *Bacillus polymyxa* (Kawazu et al 1987) and *Bacillus* sp. H-167 (Shirokizawa et al 1989). The correlation between the secretion of an amylase of unusually high molecular weight and the presence of multiple amylolytic proteins in culture supernatants observed in *B. polymyxa* (Kawazu et al 1987), a gram positive spore former (Burgess-Cassler et al 1991) and the present study suggests this may be a general phenomenon. However Imam et al (1991) have reported the existence of only a single amylolytic protein of molecular weight 150 kda in supernatants of *L. amylovorus*. The discrepancy between this result and those reported here may relate to the carbohydrate source in the culture medium; Imam et al (1991) used glucose which is known to inhibit protease activity while starch was used in this study. The ability of truncated portions of the *L. amylovorus* α-amylase gene to direct expression of active amylase in *L. plantarum*, and the amylolytic activity of proteins as small as 57 kda which are presumed to derive from a 150 kda protein leads one to examine the reasons for the evolution of such large amylase enzymes. The correlation observed between increases in the molecular weight of the largest

amylolytic protein secreted in *L. plantarum* and in the total amylolytic activity in culture supernatants, though not significant, suggests that while a protein of only 57 kda is essential for amylase activity, the remaining 100 kda of the amylase enzyme does contribute towards overall amylolytic activity. This may involve enhancing the stability of the active amylase or assisting in substrate binding.

Nucleotide sequence data confirmed that while a truncated form of the *L. amylovorus* α-amylase gene had been cloned, the cloned portion contained all the sequences necessary for efficient gene expression and protein secretion (ie. the promoter, ribosome binding site and signal sequence). As discussed previously the presence of these regions within the cloned region was essential to the strategy pursued to obtain high levels of amylase expression and secretion in recombinant inoculant strains of *L. plantarum*. Signal sequences of α-amylases are typically 29-41 amino acids in length (Vihinen and Mantsala 1989) and like all signal peptides display little amino acid sequence homology but a high degree of structural homology, consisting of 3 physico-chemically distinct regions; a positively charged amino terminal n-region, a central hydrophobic h-region, rich in apolar residues, and a more polar carboxy terminal c-region conforming to the (-3,-1) rule (Von Heijne 1988). As indicated by figure 4.1 the signal peptide of the *L. amylovorus* α-amylase gene, 37 amino acids in length, conforms to this pattern.

Figure 4.1:Signal peptide of the *L. amylovorus* α-amylase.

The n-region is 6 amino acids long, with a positive charge of 5. This is typical of gram positive signal peptides which tend to have a higher net positive charge than their gram negative counterparts (Von Heijne 1988). The h-region

consists of 21 apolar amino acids and the border between the h- and c- regions is signalled by a serine, another feature typical of signal peptides (Von Heijne 1988). The c-region conforms to the (-3,-1) rule (Perlman and Halvorson 1983), with the residues 3 and 1 amino acids upstream from the cleavage site being small and uncharged, in this case alanine, and separated by a large residue (glutamine).

The derived amino acid sequence of the N terminal region of the *L. amylovorus* α-amylase displayed 70% amino acid identity with a 159 residue stretch of the *Bacillus subtilis* α-amylase (Yang et al 1983). As indicated by figure 4.2, this region includes one of the 4 regions of conserved amino acid sequence common to all α-amylases. α-Amylases are metalloenzymes, binding one mole of calcium per mole of enzyme and this region has been postulated as the calcium binding site (Rogers 1985).

Lactobacillus amylovorus	VDATLND
Bacillus subtilis	VDAVINH
Barley	ADIVINH
Pig	VDAVINH
Mouse	VDAVINH
Human	VDAVINH

Figure 4.2: Conserved α-amylase amino acid sequences among various species. (Source- EMBL databank, Heidelberg)

The region of homology between the *L. amylovorus* and *B. subtilis* amylases extends far beyond the conserved region, spanning 159 residues, 70% of which are identical. By comparison α-amylases from two species as closely related as *Bacillus stearothermophilus* and *Bacillus licheniformis* share only 62% amino acid homology (Gray et al 1986). This suggests an evolutionary link between the α-amylase of these two organisms, a supposition further strengthened by the fact that most *B. subtilis* α-amylases share with the *L. amylovorus* α-amylase pH and temperature optima of 5-6 and 55°C-60°C respectively (Vihinen and Mantsala 1989). The *L.*

amylovorus α-amylase also displayed 42% amino acid identity with a 65 residue stretch from the α-amylase of *Clostridium acetobutylicum,* which has a pH optimum of 5.5 but a temperature optimum of only 37°C (Verhasselt et al 1989), suggesting a more distant evolutionary link between amylases from these organisms.

In order to construct an amylolytic strain of *L. plantarum* which would be effective as an inoculant and which would stably maintain amylase activity under non-selective conditions, the cloned fragment of the *L. amylovorus* α-amylase gene was integrated into the chromosome of the competitive inoculant strain, *L. plantarum* LP80, within the inessential conjugated bile hydrolase (Cbh) gene. For purposes of comparison, the *L. amylovorus* α-amylase gene was also integrated into the chromosome of the laboratory strain, *L. plantarum* NCIB8826, and was introduced into both strains on an autoreplicative vector. Characterisation of the amylolytic integrant, *L. plantarum* LPGAFΔ5, led to the following conclusions concerning its ensiling potential:

(i) When glucose was employed as the sole fermentable carbohydrate source, *L. plantarum* LPGAFΔ5 was as competitive as the wild type strain, *L. plantarum* LP80. The competitiveness of the recombinant strain in the presence of glucose may be linked to a reduction of amylase production compared to when glucose was absent, thereby ensuring that energy was not wasted in the large scale production of amylase when the enzyme was not needed. A similar phenomenon was observed with *L. amylovorus*, suggesting that sequences regulating amylase production with regard to the carbohydrate environment were cloned and integrated into *L. plantarum* along with the *L. amylovorus* α-amylase gene. The competitiveness of *L. plantarum* LPGAFΔ5, compared to the wild type strain, also implies that integration within the Cbh gene has no adverse effects on cell growth, confirming the Cbh gene as a suitable locus for chromosomal integration. Interestingly a naturally occuring amylolytic *L. plantarum* strain isolated by Giraud et al (1991) displayed a growth rate 76% of that displayed by a typical *L. plantarum* strain and given the importance

of inoculant competitiveness, it is likely that *L. plantarum* LPGAFΔ5 would outperform this natural isolate in effecting preservation.

(ii) The integrant, *L. plantarum* LPGAFΔ5, displayed a huge competitive advantage over *L. plantarum* LP80, but was considerably slower than *L. amylovorus* in initiating a reduction in pH, when grown on starch as the sole fermentable carbohydrate source. This correlated with a slower rate of amylase production compared to *L. amylovorus*, during the early stages of growth. However, since all forage crops contain some glucose or fructose, the fermentation characteristics of *L. plantarum* LPGAFΔ5 on a mixture of starch and glucose are of more relevance to inoculation of silage. Under these conditions, both *L. plantarum* LP80 and the integrant strain induced a more rapid initial decrease in medium pH compared to *L. amylovorus*. However growth of *L. plantarum* LP80 soon slowed, presumably due to glucose limitation, and the culture pH did not fall below 4.8. By contrast both *L. amylovorus* and *L. plantarum* LPGAFΔ5 made a smooth transition to utilising starch as a carbohydrate source, without any perturbation in the rate of cell growth or pH decrease, and produced a final culture pH of just above 4. The small quantities of amylase produced during growth on glucose were presumably responsible for the absence of any lag phase after glucose supplies were exhausted. The use of MRS containing 0.5% glucose and 0.5% starch is representative of the situation frequently encountered in ensiling alfalfa, where available carbohydrates are insufficient to allow *L. plantarum* inoculants to produce enough lactic acid to ensure preservation while a reserve of starch remains unfermented. The laboratory data suggests that *L. plantarum* LPGAFΔ5 would be capable of effecting preservation under such circumstances by utilising starch to maintain its initial high rate of growth and lactic acid production.

The competitive advantage of *L. plantarum* LPGAFΔ5 over the wild type *L. plantarum* LP80 on starch based media was due to a high level of amylase production by the integrant

strain; after 24 hours growth on MRS containing 0.5% starch and 0.5% glucose culture supernatants of *L. plantarum* LPGAFΔ5 contained 23.8 U/l of amylase. This represents 49% of the amylase produced by *L. amylovorus* under similar conditions. By contrast in the only comparable study, Scheirlinck et al (1989) observed barely detectable levels of amylase activity when they integrated the α-amylase gene of *B. stearothermophilus* into the chromosome of *L. plantarum* LP80. Two factors may contribute towards the superior levels of amylase production observed in this study. The promoter, ribosome binding site and signal sequence of the integrated α-amylase gene, though not originating from *L. plantarum*, still originate from within the *Lactobacillus* genus, and would therefore be expected to be more compatible with the expression and secretion mechanisms of *L. plantarum* than sequences originating from *Bacillus* spp.. Furthermore if the *L. amylovorus* α-amylase gene was integrated into the chromosomal Cbh gene of *L. plantarum* LP80 in the same orientation as the Cbh gene, then the Cbh promoter may stimulate expression of the α-amylase gene along with the gene's own promoter.

While amylase expression levels in *L. plantarum* LPGAFΔ5 and NCGAFΔ1 are high compared to corresponding literature values they represent only 72% and 37% respectively of the amylase secreted by the corresponding strains carrying autoreplicative plasmids. The difference may be due to a gene dosage effect; the integrant strains presumably only contain a single copy of the amylase gene in the chromosome while numerous copies of an autoreplicative plasmid may exist within a single cell. *Lactobacillus plantarum* LP80 derivatives consistently produced less amylase than similar constructs in *L. plantarum* NCIB8826, with levels ranging from 46% (autoreplicative) to 91% (integrant). This suggests a role for genetic background in determining amylase production levels and that it would be worthwhile testing amylase production levels in a range of competitive *L. plantarum* strains.

In conclusion, an active fragment of the *L. amylovorus* α-amylase gene, free of any antibiotic resistance markers, was integrated into the Cbh gene of the *L. plantarum* LP80

chromosome. The integrant stably maintained amylase activity over 50 generations under non selective conditions, displayed no loss of competitiveness compared to the parent strain, secreted 49% of the amylase secreted by *L. amylovorus* and exhibited a significant competitive advantage over *L. plantarum* LP80 when grown in starch based media. These encouraging results would appear to justify conducting silo trials with *L. plantarum* LPGAFΔ5, preferably using a difficult to ensile crop with low levels of water soluble carbohydrate. Should such trials demonstrate an economic advantage of using amylolytic *L. plantarum* strains as silage inoculants, the integration strategy employed here could be applied to the development of such a strain completely devoid of any non *Lactobacillus* DNA, which would be acceptable for release into the environment.

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Appendix A

Amino acid symbols

Amino Acid	Symbol
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanıne	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	Z

Appendix B

Publications

Part of the data presented here has been previously published, as indicated below.

Full papers:

Fitzsimons., A., F. Duffner, D. Curtin, G. Brophy, P. O' Kiely and M. O' Connell. 1992.
Assessment of *Pediococcus acidilactici* as a potential silage inoculant.
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Abstracts:

Fitzsimons, A. and M. O' Connell. 1990.
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Fitzsimons, A., A. Berns, G. Brophy and M. O' Connell. 1990.
Amylase production in two *Lactobacillus* strains.
FEMS Microbiology Reviews. **87:** D22.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept 1992, p 3047–3052 Vol 58, No 9
0099-2240/92/093047-06$02 00/0
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Assessment of *Pediococcus acidilactici* as a Potential Silage Inoculant

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Received 2 December 1991/Accepted 4 July 1992

Eighteen *Pediococcus* strains were screened for their potential as silage inoculants. *Pediococcus acidilactici* G24 was found to be the most suitable, exhibiting a short lag phase on both glucose and fructose, a rapid rate of acid production, a high sugar-to-lactate conversion efficiency, no detectable breakdown of proteins or lactic acid, and the ability to grow within a broad range of pH and temperature. When tested in laboratory silos using grass with a water-soluble carbohydrate content of 24 g/kg of aqueous extract, *P acidilactici* G24 stimulated the natural *Lactobacillus plantarum* population and accelerated the rates of lactic acid production and pH decrease. After 6 days of fermentation, the inoculated silage exhibited a 12% decrease in ammonia nitrogen and an 11% increase in crude protein levels compared with uninoculated controls. The use of an *L. plantarum* inoculant at a rate of 10^4 bacteria per g of grass in conjunction with *P acidilactici* G24 produced no additional beneficial effect. Inoculation of grass with a water-soluble carbohydrate level of 8 g/kg of aqueous extract with *P acidilactici* G24 led to no acceleration in the rate of *L plantarum* growth or pH decrease. However, after 7 days of fermentation the inoculated silage had a 14% lower ammonia nitrogen protein content than did uninoculated controls. The results suggest that *P acidilactici* G24 may be useful as a silage inoculant for crops with a sufficiently high water-soluble carbohydrate level.

Silage is defined as the product formed when grass or other material of sufficiently high moisture content, liable to spoilage by aerobic microorganisms, is stored anaerobically (25) The objective of silage making is efficient preservation of the crop, so that losses in nutritional value are minimized After cutting, the crop is placed in a silo and sealed from the atmosphere Respiratory activity by plant enzymes and aerobic microorganisms results in a rapid decrease in oxygen levels (6) Once anaerobic conditions are established, lactic acid bacteria present on the crop prior to harvesting rapidly multiply and ferment crop water-soluble carbohydrates to lactic and acetic acids The low pH and the toxicity of the undissociated acids restrict further microbial activity and facilitate preservation of the remaining crop nutrients (17) If a low pH is not rapidly established, a secondary fermentation can occur in which clostridia ferment lactic acid and crop water-soluble carbohydrates to butyric acid and carbon dioxide In this process, 2 mol of lactic acid is converted into 1 mol of the weaker butyric acid and an increase in pH takes place Conditions then become favorable for the growth of proteolytic clostridia, which convert crop proteins and ammo acids to ammoniacal compounds, thus further increasing the pH and degrading crop nutrients in the process (25) Crop preservation is therefore dependent on the rapid onset of lactic acid fermentation, bringing about a sharp decrease in pH While numbers of lactic acid bacteria on fresh grass vary from 10^3 to 10^7/g of herbage (11, 14, 15), homofermentative acid-tolerant lactic acid bacteria, which are highly efficient in lactate production, constitute a small fraction of the total population (4) Inoculation of silage with large quantities of homofermentative lactic acid bacteria has therefore been proposed as a means of aiding preservation For this strategy to be successful, the inoculant must satisfy certain criteria, as outlined by Whittenbury (22) These include rapid growth and lactate production, leading to a sharp decrease in pH, the ability to ferment the major crop carbohydrates, glucose and fructose, to lactate in a homofermentative fashion, tolerance of environmental conditions of temperature and pH in the silo, and absence of proteolytic activity Of the lactic acid bacteria, only the pediococci and the homofermentative lactobacilli satisfy these criteria (2) Owing to the difficulty of isolating a single strain that meets all of these criteria, a mixed culture appears to have greater potential as an effective inoculant The use of *Lactobacillus plantarum* strains only m silage inoculants is uncommon, since grass at ensiling has a pH of approximately 6 while *L plantarum* is slow to produce lactic acid until a pH of less than 5 is reached (10) A number of researchers have found that pediococci dominate the early stages of ensilage (7, 9, 23) and have proposed that a mixed culture of pediococci, which would initiate fermentation and rapidly decrease the pH to 5, and *L plantarum*, which would then further decrease the pH, might prove effective as a silage inoculant Experimental studies support this theory, with mixed inocula of *L plantarum* and *Pediococcus acidilactici* proving particularly effective (3, 5, 21) These findings are reflected in the composition of the 16 inoculants currently available on the Irish market, of which 13 contain both *L plantarum* and *Pediococcus* strains, 2 contain only *L plantarum*, and one contains a single *Pediococcus* strain (20) While considerable data on the evaluation of *L plantarum* strains as silage inoculants are available relatively few publications have focussed on the selection of *Pediococcus* strains, with most *Pediococcus*-related publications dealing with preformulated mixed-inoculum performance This is unfortunate considering the well-documented role of pediococci in silage fermentation The object of the experiments reported here was evaluation of a number of *P acidilactici* strains as potential silage inoculants

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MATERIALS AND METHODS

Bacterial strains. *Bacillus cereus* was obtained from laboratory stocks *P acidilactici* PLL01, PLL02, PLL03, PLL04, PLL05, PLL06, PLL07, PLL08, and PLL09 and *L plantarum* L115 were obtained from V Laffitte, Lacto Labo, Dange-Saint-Romain, France *P acidilactici* M408, P17, E92, E112, and E165 were supplied by F Dellaglio, Universita Cattolica del Sacro Cuore, Piacenza, Italy *P acidilactici* A12, B14, C20, and G24 were isolated from different grass silages on the basis of the ability to grow at 50°C and to ferment D-arabinose to acid (2) The isolation procedure involved addition of a 10-g silage sample to 90 ml of sterile Ringer's solution and homogenization The silage extract was diluted in 10-fold steps to 10^{-4}, and 0 1 ml of each dilution was spread on MRS (Oxoid) medium containing 2 μg of amphotericin B (GIBCO) per ml Following overnight incubation at 50°C, colonies were transferred onto MRS plates containing 20 g of D-arabinose per liter as the sole carbon source and 20 ml of a 30-g/liter solution of bromocresol green per liter in 0 01 N NaOH Colonies capable of fermenting D-arabinose appeared yellow after overnight incubation at 37°C and were subjected to microscopic examination All gram-positive cocci were tested for the ability to ferment glucose, fructose, galactose, arabinose, xylose, and maltose to acid to confirm their identity Glucose fermentation was assayed in MRS broth containing 0 0016% bromocresol green as an indicator Other fermentation tests required replacement of glucose with the appropriate sugar Sugar utilization was indicated by a change in the medium from green to yellow due to acid production

Growth conditions. Pediococci and lactobacilh were cultured on MRS (Oxoid) at 37°C, except as otherwise stated

Growth characteristics. The design of methods for determining growth characteristics was based on the characteristics required of a successful silage inoculant All growth characterizations (growth curves, kinetic studies, sugar-to-lactate conversions, etc) were done with MRS as the growth medium

Optimal growth temperatures were determined by inoculation of culture medium preincubated at selected temperatures from 15 to 45°C, followed by incubation for 24 h at the desired temperature The effect of pH values between 4 and 8 on growth was assessed by inoculation of culture medium which had been adjusted to the desired pH by using concentrated HCl-H_2SO_4 and subsequent incubation at 37°C for 12 h A 0 1% (vol/vol) inoculum from a static culture was used in all cases, and culture turbidity at 600 nm was used to monitor cell growth Optical densities were plotted against pH and temperature, and the optimum values of pH and incubation temperature for growth were determined graphically The percentage of maximum growth attained after 12 h at 20°C was determined by simultaneously inoculating MRS broth preheated to both 20 and 37°C (the temperature optimum for *P acidilactici*) Following incubation for 12 h at these temperatures, optical densities were compared

Growth curves were determined by using 100 ml of MRS containing glucose or fructose as the sole carbon source which was inoculated from a static culture at a rate of 10^6 cells per ml Cell numbers were monitored by sampling and dilution in Ringer's solution, followed by plating on MRS and overnight incubation at 37°C Growth curves were determined at 30°C, as opposed to the optimum growth temperature of 37°C, to approximate silo conditions more closely Exponential growth rates were calculated from the bacterial concentrations X_0 and X_t at times t_0 and t with the equation $\mu = (\log X_t - \log X_0)/\log e\ (t - t_0)$, where $\log e = 0\ 43429$ (16)

The lag phase was defined as the time between inoculation and establishment of the maximum growth rate (16) and was determined graphically by using a plot of log cell number versus time

Cultures used for growth curves were sampled after 12 h for determination of sugar-to-lactate conversion efficiencies and were also used to measure culture pH after 26 h of growth Glucose-to-lactate conversion efficiencies were calculated on the basis of the fact that fermentation of 1 mol of glucose to 2 mol of lactate represents 100% conversion Sampling at such an early stage in the growth cycle led to conversion efficiencies lower than those that might be expected for a homofermentative organism such as *P acidilactici* However, this was deemed necessary in view of the importance of the early stages of silage fermentation in determining the success of preservation

Inhibition of *L plantarum* Test strains were dotted onto MRS agar by using sterile toothpicks and incubated overnight at 37°C Colonies were lysed by exposure to chloroform for 30 mm and overlaid with soft MRS agar seeded with 10^6 *L plantarum* cells per ml Once solid, plates were incubated overnight at 37°C and screened for zones of inhibition

Proteolytic activity. Cells were streaked on MRS medium containing 5% gelatin as the sole carbon source After 48 h of incubation at 30°C, plates were flooded with a solution of 15 g of mercuric chloride in 20 ml of concentrated HCl and 100 ml of distilled water *B cereus* was used as a positive control, and proteolysis was detected as a clear zone surrounding areas of bacterial growth against a hazy white background

Biochemical assays. Reducing sugar concentrations were measured by using 5-dinitrosalicylic acid and the method of Bernfeld (1) Lactic acid levels in culture broths and silage extracts were determined enzymatically by using beef heart L-(+)-lactic dehydrogenase and *L leichmannii* D-(−)-lactic dehydrogenase (Boehringer Mannheim Corp)

Laboratory scale silos. Cylindrical silos with a 6-kg capacity, as described by O'Kiely and Wilson (13), were used in all experiments Inocula were grown to the stationary phase in MRS at 37°C Plate counts on MRS from cultures grown previously with identical initial cell concentrations MRS batches, and incubation temperatures and times were used to estimate inoculant cell density The culture volume required to treat 7 kg of grass at the desired rate of bacteria per g of grass (2 to 4 ml) was diluted to 10 ml in sterile Ringer's solution Seven kilograms of perennial ryegrass (*Lolium perenne*) chopped by a precision chop harvester was spread on a sterile plastic sheet, and half of the inoculum was added dropwise by using a syringe The grass was hand mixed, and the remaining inoculum was added It was assumed that small amounts of MRS which had supported bacterial growth to the stationary phase would have no effect on the silage fermentation, and uninoculated controls were therefore treated with 10 ml of sterile Ringer's solution Following inoculation, 6 kg of treated grass was packed into a test silo which was immediately sealed For each treatment, sufficient silos were set up to allow three silos to be opened on each day on which sampling was required Silos were sampled by emptying and thoroughly mixing the entire contents of each silo and then removing a sample of approximately 100 g

Microbiological analysis of silage. A 10-g sample of silage was added to 90 ml of sterile Ringer's solution and homog-

enized An extract was diluted in 10-fold steps in sterile Ringer's solution, and the appropriate dilutions were plated *L plantarum* numbers were estimated by plating on LP agar, a selective medium based on the growth characteristics of lactobacilli as listed by Sharpe (19) and described below and the sugar fermentation profile of *L plantarum* The medium was prepared by dissolving 10 g of peptone, 5 g of tryptone, 5 g of yeast extract, 6 g of KH_2PO_4, 2 5 g of sodium acetate, 2 g of diammonium citrate, 1 ml of Tween 80, 0 2 g of $MgSO_4$ $7H_2O$, 0 1 g of $MnSO_4$ $4H_2O$, and 20 g of sorbitol in 900 ml of distilled water Following addition of 20 ml of indicator solution containing 0 1 g of bromocresol green in 30 ml of 0 01 N NaOH, the pH was brought to 5 5 by using glacial acetic acid Agar was added to 1 2%, and the total volume was brought to 1 liter with distilled water The medium was autoclaved at 110°C for 20 min, and on cooling to 55°C, 2 μg of amphotericin B per ml was added to prevent fungal growth Plates were incubated for 48 h at 30°C *L plantarum* colonies appeared large and yellow against a background of small green-blue colonies The low pH, the high concentration of acetate ions, and the presence of the growth stimulant Tween 80 in LP medium facilitate selection of lactobacilli (19) Use of sorbitol as the sole sugar source ensures that of the lactobacilli only *L plantarum*, *L salivarius*, *L casei*, and some strains of *L coryneformis* produce acid (2) and form large yellow colonies Of these four species, only *L plantarum* ferments both ribose and raffinose (2), and 500 yellow colonies isolated in this manner from each treatment were replica plated on LP medium containing each of these sugars as the sole carbon source The percentage of yellow colonies positively identified as *L plantarum* varied from 97 to 98% in samples from silos which had not been inoculated with *L plantarum* to 99 to 100% in samples from treated silos In each treatment, the number of yellow colonies was multiplied by the relevant conversion rate to determine the actual number of *L plantarum* colonies

No such selective medium based on sugar fermentation profiles could be developed for *P acidilactici* However, *P acidilactici* is one of the few silage microorganisms capable of growth at temperatures as high as 50°C (2) Samples were therefore plated on MRS containing 2μg of amphotericin B per ml to inhibit fungal growth and incubated overnight at 50°C *P acidilactici* ferments glucose, fructose, galactose, arabinose, and xylose but not maltose to acid, and 500 colonies isolated in this manner from each treatment were tested for the ability to ferment these sugars Conversion factors were somewhat lower than for LP medium, ranging from 80 to 85% in umnoculated silos to 98 to 100% in treated silos Raw data were multiplied by the relevant conversion factor as described above

Chemical analysis of silages. Dry-matter contents of silages and grasses, respectively, were obtained by drying 50 g samples at 40°C for 48 h or at 98°C for 24 h Water-soluble carbohydrate and pH determinations were carried out on aqueous extracts from grass or silage

Water-soluble carbohydrates were measured colorimetrically by using the method of Wilson (24) Ammonia nitrogen levels were estimated by using an adaptation of the phenol hypochlorite method of O'Keefe and Sherrington (12)

Crude protein measurements were done on dried, milled forage samples by using a Tecator Kjeltec AUTO 1030 analyzer Statistical analysis of results from laboratory silos was done by the method of O'Kiely and Wilson (13) Least-squares differences were used to determine significance

RESULTS

Isolation of *P. acidilactici* strains from silages. *P acidilactici* isolates were obtained from four grass silage samples from various locations in Ireland as described in Materials and Methods *P acidilactici* ferments glucose, fructose, galactose, arabinose, and xylose but not maltose to acid (2), and a single isolate from each sample with such a sugar fermentation profile was selected for further study Strains A12, B14, and C20 were isolated from well-preserved silages, while G24 was isolated after 2 days of ensilage

Growth characteristics. The 18 strains of *P acidilactici* were assessed in the laboratory for their potential as silage inoculants by using a variety of relevant biochemical parameters All strains were incapable of hydrolyzing gelatin, exhibited no detectable lactate breakdown, and had an optimum growth temperature of 37°C The 18 strains grew well at temperatures ranging from 25 to 45°C and at pH values between 4 5 and 8 Growth tapered off rapidly at lower values of temperature and pH Exponential growth rates varied only slightly, ranging from 1 5 to 2 4 h^{-1} on glucose and from 1 3 to 1 9 h^{-1} on fructose However, significant variation was observed among the strains in their lag phases (5 to 11 h), in the culture pHs after 26 h of growth on glucose and fructose (3 62 to 4 18 and 3 69 to 4 46, respectively), in the glucose-to-lactate conversion efficiencies after 12 h of growth (43 to 78%), in the pH optima for growth (6 0 to 7 3), and in the percentages of maximum growth exhibited at 20°C (12 to 80%) Three strains, *P acidilactici* PLL03, PLL04, and PLL07, were found to inhibit *L plantarum* growth On the basis of these results *P acidilactici* G24 was selected as having the greatest potential as a silage inoculant by virtue of its short lag phase of 5 h, its ability to produce 80% of the cell mass attained at 37°C at incubation temperatures as low as 20°C, its compatibility with *L plantarum*, its glucose-to-lactate conversion efficiency of 76% after 12 h, and its ability to reduce the culture pH to 3 62 after 26 h of growth *P acidilactici* PLL07 was also tested in laboratory scale silos as a negative control in view of its long lag phase of 8 3 h, its inhibition of *L plantarum* growth, and its poor ability to reduce the culture pH Growth curves for these two strains and PLL06 in MRS

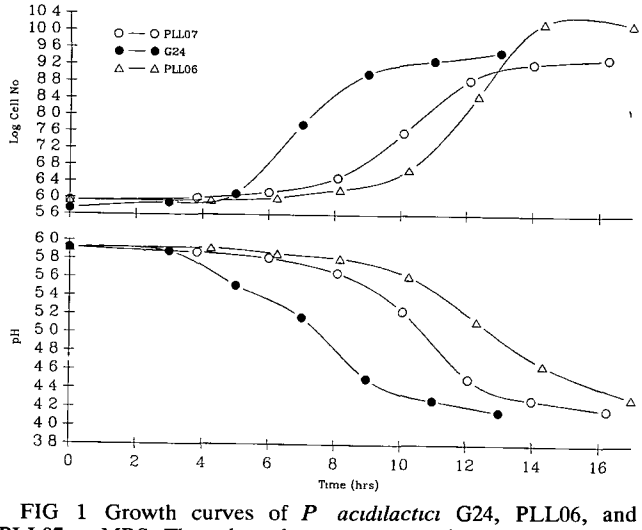

FIG 1 Growth curves of *P acidilactici* G24, PLL06, and PLL07 on MRS The sole carbon source was glucose

TABLE 1 Treatments used in test silos

Treatment	No of bacteria added/g of grass	
	Expt 1	Expt 2
1	0	0
2	10^4 *L plantarum*	10^6 G24
3	10^6 G24	
4	10^6 G24 + 10^4 *L plantarum*	
5	10^6 PLL07 + 10^4 *L plantarum*	

with glucose as the sole sugar source are presented in Fig 1 A similar pattern was observed when fructose was used as the sole sugar source A good correlation between growth and acidification was observed, with G24, the most active of the strains, rapidly entering the log phase and bringing about the most rapid drop in pH

Performance in test silos. Two experiments were carried out with the treatments used and the grass compositions outlined in Tables 1 and 2 The changes in the silage microflora and pH over the ensiling period and the silage composition after 6 days are presented in Fig 2 and 3 and Table 3 Under the good ensiling conditions of experiment 1, inoculation with 10^4 *L plantarum* cells per g of grass (treatment 2) had no effect on *Pediococcus* numbers, on the rate of lactic acid production and pH decrease, or on the ammonia nitrogen level of the silage after 6 days of fermentation Inoculation with *P acidilactici* G24 alone (treatment 3) led to rapid proliferation of pediococci, a 10-fold increase in *L plantarum* numbers, a significantly greater rate of lactic acid production and pH decrease, and a lower level of ammonia nitrogen than in the uninoculated control Addition of 10^4 *L plantarum* cells per g of grass to the G24 inoculum (treatment 4) gave rise to no additional effect on pH, lactic acid production, crude protein, or ammonia nitrogen levels *P acidilactici* numbers were unchanged, while *L plantarum* numbers were significantly lower after 3 days of ensilage than when *L plantarum* alone was used as an inoculum Inoculation with *P acidilactici* PLL07 in conjunction with *L plantarum* (treatment 5) gave rise to no beneficial effect compared with inoculation with *L plantarum* alone *P acidilactici* numbers were significantly lower than when G24 was used, and *L plantarum* numbers were unaffected by the presence of strain PLL07

In experiment 2, only *P acidilactici* G24, the most successful inoculant from experiment 1, was tested under more challenging ensiling conditions (Table 2 contains a description of the conditions) Figure 3 indicates that inoculation led to a less rapid rise in *Pediococcus* numbers than in experiment 1 and no significant improvement in the rates of lactic acid production and pH decrease or in the final crude protein level compared with the uninoculated control silages was observed However, the ammonia nitrogen content of the silage after 7 days of fermentation, at 3 9% of total N, was

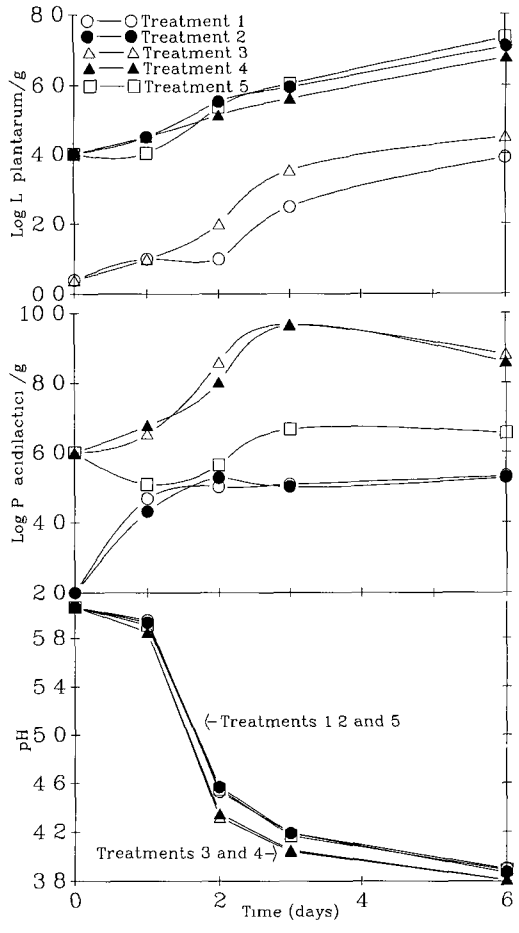

FIG 2 Changes in silage pH and microbial flora in experiment 1

significantly lower than the uninoculated control value of 4 6%

DISCUSSION

Of the 18 *P acidilactici* strains studied, strains isolated from grass silage tended to show common characteristics, distinguishing them from the remaining isolates Silage isolates A12, B14, C20, and G24 were well adapted to growth at low temperatures, exhibited a short lag phase on glucose and fructose, had a pH optimum for growth of close to 7, and had good acidification efficiency Since the rapid establishment of a low pH and the domination of the epiphytic microflora are criteria which, above all others, should decide which organisms are used as inoculants (17), *P acidilactici* G24, which exhibited the shortest lag phase, the most rapid acidification rate, and the fastest growth at 20°C, was se-

TABLE 2 Composition of grasses ensiled

Expt	Mean amt of dry matter (g/kg) (SD)	Mean amt of WSC[a] (g/kg of aqueous extract) (SD)	Mean amt of crude protein (g/kg of dry matter) (SD)	Mean pH (SD)	Mean no of *L plantarum* /g	Mean no of *P acidilactici* /g
1	154 (2 3)	24 (2 6)	166 (3 6)	6 04 (0 01)	<10	<10
2	135 (2 0)	8 (0 4)	213 (15 6)	6 12 (0 02)	<10	30 (2 5)

[a] WSC, water soluble carbohydyrate

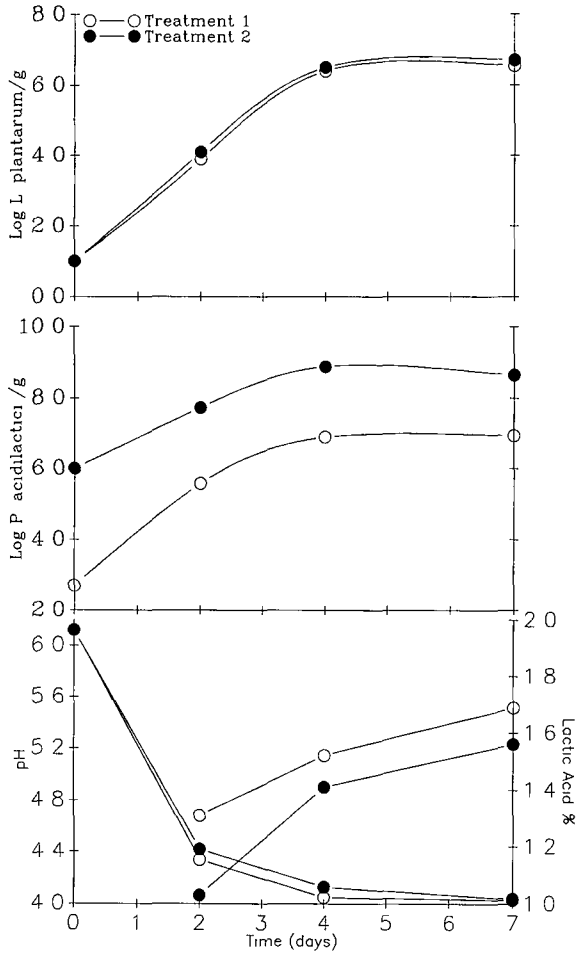

FIG 3 Changes in silage pH, lactic acid, and microbial flora in experiment 2

lected for testing in laboratory silos This strain also fulfilled the remaining criteria for a useful silage inoculant described previously

The laboratory silo trials were designed in such a manner as to demonstrate whether addition of *P acidilactici* would affect the silage fermentation, whether any difference could be observed in the performance of *P acidilactici* strains that differ in their growth characteristics on laboratory growth medium, and to show clearly any synergistic effect between *L plantarum* and *P acidilactici* The inoculation rates of *L plantarum* were therefore 100-fold less than those of *P acidilactici* so that any stimulation of *L plantarum* growth could be easily seen

In experiment 1, only treatments 3 and 4, consisting, respectively, of *P acidilactici* G24 alone and in conjunction with *L plantarum*, were successful, as evidenced by an increased rate of lactic acid production and pH decrease and a final silage with a lower ammonia nitrogen content than the uninoculated control Addition of 10^4 *L plantarum* cells per g of grass to the *P acidilactici* G24 inoculum led to no additional effect on the criteria outlined above The success of *P acidilactici* G24 alone as an inoculant is in keeping with the data of Lesins and Schulz (8) and Langston et al (7), who noted a correlation between high numbers of pediococci in the early stages of ensilage and good preservation This suggests that a *Pediococcus* sp capable of dominating the early stages of ensilage would make an effective inoculant Of the 18 *P acidilactici* strains studied, G24 is uniquely suited to this task, having been isolated from the early stages of a silage fermentation

A stimulatory effect of *P acidilactici* on *L plantarum* numbers was seen only with treatment 3, in which *P acidilactici* G24 was used on its own By day 2, a 10-fold increase in indigenous *L plantarum* numbers was evident compared with uninoculated controls Inoculation with a cocktail containing a 10^6 10^4 ratio of *P acidilactici* and *L plantarum* per g of grass led to a halving of *L plantarum* numbers compared with treatment 2, in which *L plantarum* was used on its own This suggests that either *P acidilactici* G24 stimulates the growth of indigenous *L plantarum* only or that any synergistic effect is dependent on the initial ratio of the two strains in the silo, i e , when *P acidilactici* and *L plantarum* are present in an initial ratio of 10^6 10/g of grass, *P acidilactici* stimulates *L plantarum* growth However, at higher *L plantarum* levels the two strains compete for water-soluble carbohydrate, with *P acidilactici* becoming dominant in the trials described here The microbiological data also indicate that it is in the early stages of silage fermentation that pediococci exert the most influence, with *P acidilactici* numbers peaking after 3 days of ensilage and *L plantarum* numbers still rising after 6 days This is in agreement with previous studies on succession (7, 9, 23)

We therefore propose that a bacterial inoculant containing only an active *P acidilactici* strain could prove useful as a silage inoculant, with the *Pediococcus* sp dominating the early stages of ensilage and promoting a rapid pH decrease while stimulating the natural *L plantarum* population, ensuring that it will dominate in the latter stages However, care must be taken in the selection of *P acidilactici* strains for use as silage inoculants, as indicated by the failure of *P acidilactici* PLL07 to aid preservation in laboratory silos The contrasting effects of *P acidilactici* G24 and PLL07 on preservation appear to validate the laboratory selection methods employed here, in particular, the use of growth curves and acidification rates on MRS Consideration must

TABLE 3 Composition of silages in experiment 1 after 6 days of ensilage[a]

Treatment	pH	% Lactic acid	Log no of *P acidilactici*	Log no of *L plantarum*	WSC (g/kg of aqueous extract)	Crude protein (g/kg of dry matter)	Ammonia N (% total N)
1	3 90*	1 74*	5 33*	3 91*	1 0*	161*	5 7*
2	3 87*	1 84*	5 28*	7 10†	0 9*	175†‡	5 1†
3	3 81†	2 06†	8 92†	4 56‡	1 0*	179†	4 6‡
4	3 81†	1 92*†	8 60†	6 78§	0 9*	177†	4 6‡
5	3 88*	1 84*	6 56‡	7 35†	0 8†	170‡	5 4*†

[a] Values within rows that differ significantly from one another are followed by different superscripts The values shown represent means from three silos
[b] WSC, water soluble carbohydrate

also be given to crop characteristics before selecting an additive, as under the more difficult ensiling conditions of experiment 2, *P acidilactici* G24 failed to produce any beneficial effect This may have been due to the low level of crop water-soluble carbohydrate, which has frequently been cited as the reason for inoculant failure (17, 18, 25) This might be overcome by addition of a carbohydrate source, e g , molasses, to the crop

ACKNOWLEDGMENTS

This work was supported in part by the Commission of the European Communities, ECLAIR programme, AGRE 0041

We thank Tom Keating, John Marron, Jim Hammill, Stephen O'Connell, and Noel Tonge for assistance

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