Microbial control of lactic acidosis in grain-fed sheep

A Thesis

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by

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List of abbreviations

A	absorbance
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BHI	brain heart infusion
CoA	coenzyme-A
e.g.	for example
EDTA	ethylenediamine tetra acetic acid
et al.	et alia (and others)
g	gram
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
l	litre(s)
M	molar
m	metre(s)
MFS	methylgreen-formaldehyde-saline
mg	milligram
µg	microgram(s)
µl	microlitre(s)
mM	millimolar
NADH	nicotinamide-adenine dinucleotide (reduced)
NADPH	nicotinamide-adenine dinucleotide phosphate (reduced)
^{o}C	degrees centigrade (Celsius)
Pi	inorganic phosphate
rpm	rotation per minute
sp.	species
TCA	trichloroacetic acid
TPP	thiamin pyrophosphate
VFA	volatile fatty acid

w/v	weight:volume
%	percent
<	less than
>	more than

Summary

An increasing human population and improving living standards in developing countries has led to increased consumption of animal protein. However, a shortage of arable land in developing countries restricts the extent of animal agriculture. There is a rapidly growing need therefore for developing nations to optimise the efficiency of animal production on limited resources.

Livestock production can be increased in two ways: one is by increasing the output of the individual animal per unit of feed input, and the other is by increasing the number of animals per unit of land. Concentrate supplementation of forage or fibre is the most popular method used to achieve increased animal production, because concentrates contain high levels of readily fermentable carbohydrate and are the cheapest feed per unit of energy output. Under drought conditions the use of concentrate meals becomes important since green feed availability is minimal. Supplementation with a high carbohydrate diet can increase dry matter intake, enhance apparent digestibilities of dry matter and nitrogen (de Faria and Huber, 1984), improve average daily gains and elevate feed efficiency of young animals (Glimp et al., 1989; Drought Feeding and Management of Cattle, 1982). However, in grazing ruminants an abrupt changeover from roughage to a diet containing high fermentable carbohydrates can cause significant digestive disturbances, in particular, lactic acidosis. In contrast, when ruminants are slowly introduced to a predominantly carbohydrates diet (up to 71% concentrate meal and mollases with 18% maize stover), the condition of acidosis does not arise (Mackie et al, 1978). This is because the rumen microbial population has had time to adapt to the increasing amounts of lactic acid by establishing compensatory populations of bacteria that can readily utilise the lactic acid as it is produced.

Although it is possible to prevent acidosis by slow adaptation of the animal, there is an associated loss of production due to slow growth during this period. If it were possible to speed up the process of adaptation, production costs would be reduced and production efficiency increased. There are some possible ways that can be applied to speed up the process of adaptation without upsetting the rumen ecosystem. These are the addition of buffers or antibiotics to the feeds or inoculation with crude rumen fluid obtained from an animal already adapted to a concentrate diet. The buffer and antibiotic treatments have been reported to be effective only for a short time period, and crude rumen fluid inoculation has been considered impractical. Therefore, a more powerful treatment, that is practical and long lasting, needs to be investigated. The research project described here was aimed at investigating the use of microbial inoculants to prevent the onset of acidosis in acutely grain fed animals, and to find out the most effective combination of virginiamycin and lactic acid utilising bacteria (*Selenomonas ruminantium* subsp. *lactilytica* and *Megasphaera elsdenii*) in controlling lactic acid accumulations *in vitro*.

Populations of lactic acid utilising bacteria in the sheep rumen were studied during the process of adaptation to a predominantly grain diet. *Selenomonas ruminantium* subsp. *lactilytica* was found to be the major lactic acid utiliser and its population increased 70 fold in 20 days from 10^6 cfu to 7×10^7 cfu/ml of rumen fluid. *Megasphaera elsdenii* was detectable at most stages during adaptation but its numbers remained relatively constant at about 2×10^6 cfu/ml rumen fluid. Ruminal lactate was maintained at low levels (less than 5 mM) and the ruminal pH was higher than 5.3. The concentration of total volatile fatty acids increased gradually as the grain proportion was increased in the rations. The concentration and proportion of propionate and butyrate increased at the expense of acetate.

Pre-inoculation of the rumen with *in vitro* grown cultures of *Selenomonas ruminantium* subsp. *lactilytica* maintained ruminal lactate levels below 5 mM for 24 hours. This treatment also maintained higher ($P<0.05$) ruminal pH and total volatile fatty acid

concentrations compared to the controls. However, this treatment failed to prevent acidosis for longer than 24 hours. This may be the result of catabolite repression where *S. ruminantium* subsp. *lactilytica* preferentially fermented glucose generated from starch rather than utilising the lactic acid produced by other bacteria. Alternatively the *Selenomonas ruminantium* subsp. *lactilytica* population may have declined after 24 hours when all the lactic acid had been utilised. Therefore, in the long term, increased levels of ruminal lactic acid could not be prevented by pre-inoculation with *Selenomonas ruminantium* subsp. *lactilytica*. This problem was eliminated when a combination of *Selenomonas ruminantium* subsp. *lactilytica* and *Megasphaera elsdenii* was used as inoculum. This combination was found to be effective in preventing lactic acidosis for up to 4 days by significantly ($P<0.05$) maintaining higher ruminal pH and lower lactic acid concentrations compared to the controls. However, consistent *in vivo* results were difficult to obtain due to individual animal-animal variation and varying histories of the experimental animals. Therefore, continuous culture studies *in vitro* were carried out.

A model system was established where *Streptococcus bovis* strain 2B was used as the starch fermenter and generator of lactic acid. *Selenomonas ruminantium* subsp. *lactilytica* and *Megasphaera elsdenii* were used as lactic acid utilisers. Combinations of bacteria and the antibiotic virginiamycin were used to investigate the most effective treatment for preventing lactic acid accumulation in starch-induced acidosis over a short time period (24 hours). A combination of virginiamycin and *Megasphaera elsdenii* significantly ($P<0.05$) reduced lactic acid concentration compared to controls and other treatments. This treatment also significantly ($P<0.05$) increased total volatile fatty acid concentrations and depressed the *S. bovis* population compared to controls. This treatment was then used to control the lactic acid accumulation in rumen fluid cultures over 3 days. It was found that a single inoculation of this combination was only effective for up to 24 hours, where lactic acid concentrations were reduced by 75% compared to the controls. After that time lactic acid production resumed at a rate similar to the controls. A more effective treatment was achieved by double inoculations (at 12 and 36 hours) with a combination of

Selenomonas ruminantium and *Megasphaera elsdenii* and virginiamycin. This treatment significantly ($P<0.05$) decreased lactic acid production in the cultures compared with control and other treatment groups. This treatment also significantly ($P<0.05$) increased total bacteria and total volatile fatty acids in the fermenter. In addition, the inoculation increased the proportion of propionate and butyrate at the expense of acetate. This is a very promising result and the use of this inoculation regime *in vivo* may be useful, not only in preventing the development of lactic acidosis, but also in increasing the protein and energy supply for the host.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being available for photocopying and loan.

Komang G. Wiryawan

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Chapter 1 Introduction and Review of Literature

1.1 Introduction

The fermentation of feed by rumen microorganisms has been studied extensively over the last 3 decades. In recent years, renewed interest in rumen microorganisms has been stimulated by the possibility of enhancing rumen fermentation using genetically engineered rumen microorganisms. However in developing countries (e.g. Indonesia) that have limited funds and facilities, the use of ecological approaches to overcome nutritional problems is still a priority. There are a number of areas in which ecological approach to modifications of the rumen fermentations can be attempted such as, feed additives that enhance or retard certain microbes (Harrison and McAllan, 1980; Owens and Goetsch, 1986; Demeyer and Van Nevel, 1986), protection of high quality protein from microbial degradation (Van Nevel and Demeyer, 1988), and probiotics (live inoculants) (Theodorou et al., 1990). This project was aimed at studying the use of probiotics to manipulate rumen fermentation so as to prevent lactic acidosis in grain fed sheep. In the sections that follow I will consider, briefly, the rumen itself, ecology and effects of lactic acidosis, and approaches taken to alleviate this problem.

1.2 Review of Literature.

1.2.1 The rumen and its microbes.

Ruminant animals (e.g. sheep, cattle) are distinguishable from monogastric animals in that they possess a complex fermentation chamber called the reticulo-rumen. The reticulo-rumen contains a variety of species of anaerobic bacteria, protozoa and fungi, which carry out a complex series of reactions and interactions with feed consumed by the animals. Optimum conditions within the rumen environment include a temperature range from 38^{o} to 41^{o} C, a pH that is maintained between 5.7 and 7.3 by the buffering effect

of saliva, and a mean redox potential of -350 mV, kept low by the rapid utilisation of oxygen and the production of H_2S to help maintain anaerobiosis (Wolin, 1981; Hoover and Miller, 1992).

The regular mix of digesta in the rumen, regurgitation and remastication of plant materials allows the proliferation of microorganism to high densities. Total bacterial numbers can reach levels of 10^9 - 10^{10} cells per gram of rumen contents.

Church (1988) classified ruminal bacteria into 8 distinct groups based on their utilisation of different dietary components (cellulose, hemicellulose, starch, sugars, intermediate acids, protein, lipid) or on methane production. In this review only three main important groups will be discussed (cellulolytic, proteolytic and amylolytic bacteria). The first group is cellulolytic bacteria. Based on numbers and their ability to degrade cellulose, the major cellulolytic bacteria are *Fibrobacter succinogenes, Ruminicoccus flavefaciens, Ruminococcus albus* and *Butyrivibrio fibrisolvens*. Cellulose can be broken down by these bacterial species, releasing soluble sugars. Most of the released sugars are eventually converted to volatile fatty acids by the non-cellulolytic bacteria (Hobson and Wallace, 1982a,b). The second group of important ruminal bacteria are the proteolytic bacteria. Rumen bacteria included in this group are *Ruminobacter amylophilus*, *Prevotella ruminicola*, some strains of *Butyrivibrio fibrisolvens* and *Streptococcus bovis* (Russell et al., 1981). These bacteria rapidly break down dietary protein in the rumen into polypeptides, amino acids and ammonia, which are used for bacterial protein synthesis. The third group is the amylolytic bacteria. This group is particularly important for animals fed a diet rich in readily fermentable carbohydrates. The major starch-degrading bacteria in the rumen are: *Ruminobacter amylophilus*, *Streptococcus bovis, Succinimonas amylolytica* and *Prevotella ruminicola* (Baldwin and Allison, 1983). These bacteria hydrolyse starch to produce maltose and glucose. This will be discussed in more detail in section 1.2.5.

Another major group of microorganisms in the rumen is the protozoa. Protozoal numbers can be as high as 10^5 to 10^6 cells per gram of rumen content (Hoover and Miller, 1992), and the majority are ciliate. Protozoa can comprise about 2% of the weight of rumen contents, 40% of total microbial nitrogen and produce 60% of microbial fermentation products in the rumen (Church, 1988). All protozoa are strict anaerobes. Ciliated protozoa are divided into 2 families: 1) *Isotrichidae*, consists of genus *Isotricha* and *Dasytricha* and 2) *Ophryoscolechidae*, consists of genus *Entodinium*, *Diplodinium*, *Epidinium*, and *Ophryoscolex* (Ogimoto and Imai, 1981). The ciliated protozoa can attack and ingest wide varieties of plant constituents including cellulose, hemicellulose, pectin, starch, soluble sugars and lipid. *Isotricha* is able to utilise starch and many sugars, but not maltose, while *Dasytricha* can utilise many sugars including maltose, but not starch. The *Entodinium* species is present in significant numbers in the presence of a high-starch diet.

As well as digesting plant polymers (cellulose, starch) protozoa also actively ingest bacteria as a protein source. They do not show any preference for particular rumen bacterial species. The ingestion and digestive capacities of protozoa have been widely discussed (Coleman, 1975; 1979; 1980; 1989). A single protozoan is estimated to engulf 10^2 to 10^4 bacteria per hour. If this is extrapolated to the real rumen environment (10^5 to 10^6 protozoa/ml), as many as 10^7 to 10^{10} bacteria are engulfed per hour (Coleman and Sandford, 1979). This represents from 0.1% to almost 100% of the total bacterial biomass. The predation of bacteria by protozoa decreased the protein flow into duodenum (Rowe et al., 1985; Kayouli et al., 1986). The opposite result was reported by Demeyer and Van Nevel (1979) that microbial nitrogen in the rumen was greater in faunated than defaunated sheep, as the protozoal biomass was usually large; 20-70% of rumen microbial nitrogen consists of protozoa (Coleman, 1979). The beneficial and detrimental effect of protozoa on animal performance is still questioned (Veira, 1986; Hobson and Jouany, 1988). It seems that the main role of protozoa in the rumen is as a fermentation stabilising factor. By ingesting feed particles and storing reserve

polysaccharides, the protozoa control the level of substrate availability and by ingesting bacteria they may serve to hold the fermentation in check, especially when high-grain diets are fed.

Anaerobic fungi are also found in the rumen. Orpin (1975) identified one species for the first time when he reported that certain flagellated cells thought to be protozoa were, in fact, zoospores of fungi. Since then, several genera have been discovered by Barr et al. (1989), Breton et al. (1989) and Phillips (1989). The rumen fungi may contribute up to 8% of the microbial mass in animals fed high-roughage diets (Orpin, 1981). Anaerobic fungi isolated from the rumen can be grouped into the three morphological types: *Neocallimastic* sp. with a polyflagellated spore and a highly branched rhizoid (Orpin, 1975), *Piromonas* sp. with a monoflagellated spore and a branched rhizoid (Orpin, 1977a), and *Sphaeromonas* sp. with a monoflagellated zoospore and a bulbous rhizoid (Orpin, 1976). Anaerobic fungi use all plant polysaccharides, with the exception of pectin and polygalacturonic acid. Most *Neocallimastix* grow on microcrystalline and amorphous cellulose, xylan, pullulan, pustulan, inulin, and starch (Bauchop and Mountfort, 1981; Orpin and Letcer, 1979; Lowe et al., 1987; Phillips and Gordon, 1988; Williams and Orpin, 1987a,b; Breton et al., 1989). All *Piromonas* sp. can use xylan (Phillips and Gordon, 1988). Cellulolytic activity is usually found in this genus (Fonty et al., 1988b). *Sphaeromonas* genus seems to utilise a more restricted range of polysaccharides than *Neocallimastix* and *Piromonas*. All *Sphaeromonas* isolates are able to use xylan, but only some utilise cellulose (Orpin, 1981; Fonty et al., 1989; Gordon and Phillips, 1989). In relation to their ability to degrade plant cell walls, rumen fungi produce a wide variety of extracellular polysaccharidases and glycosidases (Mountfort and Asher, 1985, 1989; Pearce and Bauchop, 1985; Williams and Orpin, 1987a,b).

Rumen fungi have been shown to interact with bacteria and protozoa. Rumen fungi can form stable cocultures with methanogenic bacteria (Bauchop and Mountfort, 1981) because rumen fungi produce high concentrations of hydrogen. The association with

methanogens increases the rate and extent of cellulose hydrolysis from filter paper (Bauchop and Mountfort, 1981; Fonty et al., 1988b). Some inhibition effects have also been observed. The cellulolytic activity of *N. frontalis* was partially inhibited in coculture with *R. flavefaciens* and *R. albus* (Richardson et al., 1986), but in coculture with *Selenomonas ruminantiun* (a noncellulolytic rumen bacteria), cellulose degradation by *N. frontalis* is completely inhibited (Bernalier et al., 1988). In contrast, *Sphaeromonas communis* showed better cellulolytic activity in the presence of *Selenomonas ruminantium* than in monoculture. In coculture with the lactate-metabolising bacteria *Megasphaera elsdenii*, the cellulolytic activity of *N. frontalis* and *P. communis* is inhibited (Bernalier and Fonty from Fonty and Joblin, 1991), while Richardson et al (1986) reported that *M. elsdenii* enhanced the fungal cellulolytic activity of *N. frontalis*. The interaction between protozoa and fungi has not been extensively studied. Because ciliate protozoa can ingest large bacteria (Coleman, 1989) of a size similar to that of zoospores, it is probable that protozoal predation of fungal zoospores occurs. This is supported by defaunation studies that show removal of protozoa from the rumen causing an increased population density of fungal zoospores (Orpin, 1977b).

1.2.2 Rumen fermentation.

The most important aspect of rumen fermentation is the degradation of structural carbohydrates including cellulose, hemicellulose and xylan. These compounds are almost undegradable in monogastric animals. Even in the rumen, they can not be efficiently digested. The digestibility of these structural carbohydrates varies from 30% to 90% depending on energy levels in the diet (Czerkawski, 1979). Soluble sugars and starch are almost completely fermented in the rumen. Overall, about 70% of carbohydrates can be fermented (Czerkawski, 1979). Increased digestibility of structural carbohydrates can result in significant increases in ruminant productivity.

Microbial fermentation of plant materials produces a numbers of end products. The main products are volatile fatty acids (VFA's), mainly acetate, propionate, and butyrate. Although different diets can influence the bacterial population in the rumen, the ruminal VFA's are very stable with the molar ratio of acetate:propionate:butyrate being near 65:25:10 with roughage and 50:40:10 for concentrate diets (Church, 1988) The efficiency of food energy utilisation depends upon the relative proportion of the major VFA's produced by fermentation. The efficiency (%) capture of hexose energy in the major VFA's are 62.5, 100.0 and 78.0 for acetate, propionate and butyrate respectively (Hungate, 1966). The propionate to acetate ratio is higher in concentrate than roughage diets. VFA's are absorbed through the rumen wall into the blood stream of the animal and are utilized by the animal as carbon and energy sources (Church,1988). It is estimated that VFA's constitute 50-70% of the energy requirement of the ruminant animal (Vermorel, 1988).

Ruminal VFA concentrations represent the balance between rates of production and rates of removal for each VFA as well as their interconversions (Owens and Goetsch, 1988). The rate of VFA absorption is influenced by concentration, osmolality, chain length of the individual acids and ruminal pH. Reduced ruminal pH increases the VFA absorption (Black and Kennedy, 1984). Increasing chain length also results in increased absorption rates with relative rates of absorption of undissociated acids as follows: butyric>propionic>acetic (Arnold, 1970). The rate of VFA metabolism by cells of the rumen wall are in order of butyrate>propionate>acetate. Acetate enters the blood in the greatest quantity because it is present in the greatest concentration within the rumen and it is metabolised at the slowest rate by the epithelial cell of the rumen walls. Of the absorbed VFA's, propionate is used for the synthesis of glucose; acetate and butyrate mainly contribute to fat synthesis, particularly in milk, and are oxidised as energy sources (Czerkawski, 1986).

The main nitrogen source for the ruminant animal is derived from the breakdown of bacterial cells as well as some undigested plant protein in the abomasum (Hungate, 1966; Jarrige, 1988). It is estimated that up to 80% of the protein nitrogen supply to the ruminant animal is derived from bacterial protein (Buttery et al., 1985). Another feature of ruminants is that they can survive on a diet low in protein because of nitrogen recycling via saliva and activity of urea cycle (McDougall, 1948). The amount of urea nitrogen transferred to the rumen is determined by the rate of salivary secretion, the plasma urea concentration, and the urea flux across the rumen wall.

Rumen fermentation pattern is greatly influenced by feed supplied to animals. The microbial processing of cereal grains differs profoundly from that of forage materials. The feeding of cereal grains sets in motion an ecological succession that gives rise to a fermentation in which the production of acids and other products may exceed the absorptive and digestive capacity of the animal, and may predispose the ruminant to digestive disturbance such as acidosis (McAllister et al., 1990).

1.2.3 Lactic acidosis.

1.2.3.1 Lactic acidosis and its consequences to the rumen.

Lactic acidosis is defined as a condition of pathologically high acidity of the blood. The term acidosis in ruminants is expanded to include acidic conditions in the rumen (rumen acidosis) Huntington (1988). Lactic acidosis is also referred to as acid indigestion, acute impaction, rumen overload and feed (grain) engorgement (Dunlop, 1972). There are two kinds of lactic acidosis ; a) acute acidosis, a disturbance in animals that are definitely sick (ruminal stasis, unable to stand, rumen damage and possibly death), b) chronic or sub-acute acidosis, perhaps the more economically important problem (Dunlop and Hammond from Slyter, 1976). Animals with chronic acidosis may not appear sick, but their feed intake and performance are reduced.

The primary cause of the disease appears to be the ingestion of excessive amounts of readily fermentable carbohydrate that is rapidly metabolised to lactic acid in the rumen. Materials such as starch (Krogh, 1961), lactose (Krogh, 1960), sucrose (Krogh, 1960), glucose (Krogh, 1959), apples (Merrill, 1952), lush pasture (Annison et al., 1959a,b), mangolds (Scarisbrick, 1954), brewers grains (Owens, 1959), fodder beet (Williams and Coup, 1952), and grapes (Portway, 1957) have all been found to induce lactic acidosis in ruminants. Terry et al. (1973) reported that grains such as wheat, barley and corn also induced lactic acidosis because they provide less buffering power to the rumen than do natural forages. Wheat appears to be more of a problem than corn (Oltjen et al., 1966) or sorghum (Morris, 1971) possibly because of the differing rates of release of the fermentable carbohydrate

The amount of feed required to cause death from acidosis in ruminants varies and is influenced by a number of factors including the species of animal, the type of diet, the amount of feed the animal is accustomed to eating, and the pre-existing intestinal microbial populations (Slyter, 1976). On a body weight basis, sheep can consume more feed without inducing acidosis than can cattle. Well-nourished merino sheep developed digestive problems when 75 to 80 g/kg body weight of crushed wheat was supplied. Meanwhile, sheep in poor condition succumb after a dose of 50 g/kg body weight. Acidosis symptoms (reduced feed intake) have developed when cattle were fed 13 to 23 gram of wheat/kilogram body weight (Oltjen et al., 1966). Dunlop (1970) reported that larger animals were more vulnerable than smaller ones when dosage was based on body weight. Therefore it was suggested that metabolic size (kg body weight$^{0.75}$) rather than body weight be used in calculating the dosage for grain engorgement.

Lactic acidosis was first indicated by the lowering of pH in the rumen, together with an increase in the concentration of lactic acid from less than 1 mM to about 150 mM (Briggs et al., 1957; Ghorban et al., 1966). Rumen pH also dropped to less than 5 destroying

the protozoa and other bacterial populations in the rumen (Hungate et al., 1952). *Streptococcus bovis* and *Lactobacilli* are markedly increased as the pH drops to 5.0 and they may also increase the level of lactic acid in the rumen because of the increase in substrate availability. Lactic acid then crosses the rumen wall into the blood stream and dissociates to lactate and the H^+ ion. The latter combines with bicarbonate to form carbonic acid which then dissociates into carbon dioxide and water. If high enough, the blood carbon dioxide will increase the carbon dioxide tension, depress the respiratory centre, decrease the blood pressure and reduce the blood pH (Huber, 1976). Low blood pH causes an imbalance of electrolytes due to the loss in the rumen and the gut and to high acid concentrations. In severe cases, acidosis interferes with renal functions on oxygen transport. The rapid increase in blood lactic acid was also attributed to a failure in aerobic metabolism and falling systemic blood pressure. This decreased perfusion pressure and oxygen supply to peripheral tissues.

The influence of low rumen pH on rumen function has also been investigated. Bruce and Huber (1973) found that the drop in rumen pH inhibited the amplitude and frequency of ruminal contractions. Kay et al. (1969) suggested that the pathological changes in the rumen wall may be due to low rumen pH, characteristically found in animals fed barley, and may begin with a thickening of the epithelium and lamina propria. When the ruminal epithelium is exposed to low pH for longer periods, hyperkeratosis, papillary clumping and rumenitis develop (Kay et al., 1969; McMannus et al., 1977)

Lactic acidosis also reduces ruminal motility, therefore decreasing rumen turnover rate. This digestive disturbance is likely to result in reduced microbial biosynthetic activity per unit of energy utilized, and the protein availability to the host animal for productive purposes will therefore be reduced (Slyter, 1976).

Krogh (1961) found that cellulose digestion in the rumen decreased at reduced pH. It was probably caused by the reduction in numbers of cellulolytic bacteria at pH less than

5. Meanwhile, VFA absorption into the animal increased as the acidity of rumen ingesta increased.

1.2.3.2 The influence of lactic acidosis on the animal.

Although losses by death from lactic acidosis in the feedlot is low (only 0.009%), the economic losses due to decreased cattle performance from chronic acidosis are considered to be substantial (Braddy, 1976). Clinical signs of acidotic animals depends upon the degree of ruminal hyperacidity. In mild cases, the animals show a transient reduction or loss of appetite (off feed). In more severely affected animals, an obvious indigestion with marked intoxication within 12-24 hour after overfeeding occurs (Dirksen, 1970). Another symptom of acidosis in animals is lameness with hot and swollen extremities. Other general symptoms may include anorexia, dullness, staggering gait, labored respiration and variable changes in heart rate and temperature. Diarrhoea was also found in acidotic animals (Huber, 1971). This happens because body water (shared by plasma, interstitial, and intracellular fluid) enters the rumen when ruminal ingesta become hypertonic to plasma.

Acidosis has also been reported to cause other feedlot ailments such as laminitis, polioencephalomalacia, and liver abscesses. There were some reports of the relationship between laminitis and acidosis. Dirksen (1970) observed hoof sloughing in the succeeding months after lactic acidosis; Maclean (1969) reported 1 to 9 percent laminitis incidence in 'barley beef' units in Great Britain. Histamine is considered to be responsible for laminitis because histamine is found in the rumen fluid of lactic acidotic animals (Dain et al., 1955) and because the histopathology of laminitis resembles the circulatory effects of histamine (increased capillary permeability and arteriolar dilitation), and the use of antihistamine may prevent laminitis (Jubb and Kennedy, 1970). In contrast, Brent (1976) reported that laminitis is unlikely to be caused by ruminal

histamine because histamine is poorly absorbed and is rapidly metabolised. High levels of orally administered histamine have not produced laminitis (Brent, 1976).

Polioencephalomalacia (PEM) is also called cerebrocortical necrosis (CCN). The signs of the polioencephalomalacia include dullness and sometimes blindness, progressing to muscular tremors (especially of the head), and opisthotonos (Jubb and Kennedy, 1970). Jensen and Mackey (1971) reported that PEM was attributed to a wide variety of causes, but it was finally shown (Davies et al., 1965) to respond to large, intravenous doses of thiamin. PEM often occurs on a high concentrate diet, where exogenous thiamin should be high (Brent, 1976). The signs must be caused by a reversible biochemical defect, since recovery is rapid when thiamin is given soon enough. Brent also reported that cells might be thiamin deficient; or a thiamin antimetabolite might be blocking thiamin dependent reactions. Edwin et al. (1968a,b) reported an enzyme, thiaminase, may be responsible for PEM. Since then, thiaminase I-producing *Clostridium sporogenes* has been isolated from PEM animals by Shreeve and Edwin (1974). Lactic acidosis is related to PEM because thiaminase I producing anaerobic bacteria such as *Clostridium sporogenes* prefer to grow at low rumen pH.

Rumenitis-liver abscesses also occur in acidotic animals. Liver abscesses were reported in about 10.8 % of cattle slaughtered in the USA in 1973. Rumenitis-liver abscesses are initiated by the development of microvacuoles in the rumen epithelial tissue. As soon as the mucosa loses its protective epithelium, bacteria (*Fusobacterium necrophorusm Actinomyces pyogenes* and others) or fungi may invade (Dunlop, 1967). Deep ulcers may be produced by these microbes, as well as metastatic infection in other organs, particularly in the liver. There is some evidence that liver abscesses are related to infections by rumen microorganisms: a) most liver abscesses contain *Spherophorus (Fusiformis) necrophorus* (Newsom, 1938; Madin, 1949), b) *Spherophorus necrophorus* is in the rumen and feces of most healthy and diseased cattle (Robinson et al., 1951), and c) *Spherophorus necrophorus* injected into the portal vein produces liver

abscesses (Jensen et al., 1954). The development of rumenitis-liver abscesses in acidotic animals may be caused by high ruminal lactic acid, low ruminal pH, high ruminal osmotic pressure, bacterial endotoxin or the combination of these factors rendering the epithelium susceptible to mechanical injury.

1.2.4 Microbiology of lactic acidosis.

1.2.4.1 Lactic acid producing bacteria.

Since it is recognized that rumen microorganisms are responsible for generating lactic acidosis, many experimental programs have been established to study these microorganisms. Lactic acid producing bacteria in the rumen include : *Lactobacilli, Streptococcus bovis, Ruminococcus sp, Succinivibrio dextrinosolvens, Lachnospira multiparus*, and *Eubacterium sp* (Bryant, 1959, 1963; Hungate, 1966). Lactic acid is also produced by *Butyrivibrio sp* (Bryant and Small, 1956), *Bacteroides amylophylus* (Hamlin and Hungate, 1956), *Succinimonas amylolytica* (Bryant et al., 1958), and some strains of *Selenomonas ruminantium* (Bryant, 1956). Among these microorganisms *Streptococci* and *Lactobacilli* are found to be the major lactate producers in ruminants (Krogh, 1961). Chaplin and Jones (1973) found that the numbers of *Streptococci* increased from $9x10^5$ cfu at 0 hour to a peak of $1x10^9$ cfu at 18 hour. *Lactobaccilli* which were undetectable at the start, peaked at $9x10^8$ cfu after 48 hours.

The rapid proliferation of *Streptococcus bovis* is a result of its very short doubling time i.e., 9 minutes under suitable conditions (Bruggeman and Giesecke, 1965). It can increase its numbers 2000-fold in 6 hour (Hungate et al., 1952). Thus if the amount of grain added to the diet exceeds the capacity of protozoa to remove a large proportion of it, *S. bovis* will be able to utilise this feed source and multiply much more rapidly than the protozoa in response to the excess substrate. Fermentation by *S. bovis* will then proceed at a rapid rate leading to a high rate of production of lactic acid.

According to Hungate (1968) the reason for the outgrowth of *Streptococcus bovis* is as follows: "As long as carbohydrate is limiting, the fermentation efficiency in ATP production from a given quantity of carbohydrate has competitive value and bacteria obtaining more than 2 ATP per sugar can compete successfully with *Streptococcus bovis*. In acute indigestion the great excess of starch or sugar makes carbohydrate no longer limiting. *Streptococcus bovis* can metabolize the carbohydrate faster than the competing types that use such molecules more efficiently. The ATP yield of *Streptococcus bovis* per molecule of sugar is low, but when carbohydrate is in excess, the yield of ATP per unit of time is considerably greater than in competing species. When the carbohydrates have been utilised, the rumen acidity returns to normal and more efficient producers of ATP compete succesfully".

1.2.4.2 Lactic acid utilising bacteria.

It would seem likely that animals adapted to a high grain diet should carry a microbial population that use lactic acid efficiently.

Some experiments have been carried out to isolate lactate utilising bacteria from the rumen. Johns (1948) was first to isolate a lactic acid utilizing bacterium from sheep. The bacteria was called *Veillonella gazogenes* = *Micrococcus lactilyticus* (Foubert and Douglas, 1948), and now it is named *Veillonella alcalescens*. The cells were spherical, 0.3 - 0.6 μ in diameter, occuring singly, in pairs, and in large irregular groups, gram-positive in young cultures but gram-negative after 12 - 18 hours growth. Strictly anaerobic conditions were essential for growth, with temperature and pH optima of 37°C and 6.0 - 8.0 respectively. This bacteria fermented lactate to propionate, acetate, hydrogen, and carbon dioxide. Johns (1951b) also isolated another lactate utilizer, *Propionibacterium acnes* . This is an anaerobic, gram-positive bacterium. The cells are pleiomorphic, usually arranged in single, pairs or V and Y configuration. This bacteria can not ferment glucose because of a lack of hexokinase (Rogosa et al., 1965).

Elsden and Lewis (1953) discovered LC (large coccus), later named *Peptostreptococcus elsdenii* and now called *Megasphaera elsdenii* , for the first time in sheep. The cells are large cocci, occuring in pairs or singly, sometimes in chains. These gram negative cocci need strictly anaerobic conditions for growth. This filamentous bacterium ferments lactate to acetic, propionic, butyric, valeric, and caproic acids, carbon dioxide, and a trace of hydrogen.

Bryant (1956) found *Selenomonas ruminantium* sub-species *lactilytica* which can ferment lactate to propionic and acetic acids. This bacterium was described as a rather large, curved, crescent-shaped rods, usually 0.8 - 1.0 μ in width and 2.0 - 7.0 μ long with blunt ends. They were usually arranged as single cells with occasional diplo-rods and short chains. The cells generally had a single tuft of a few to 12 or more flagella and they appeared to originate on the concave side of the cell at a central position or near the end.

Mackie et al. (1978) found *Anaerovibrio* as a major lactate-utilising bacteria in sheep adapted to high grain diets. *Selenomonas ruminantium, Megasphaera elsdenii, Propionibacterium*, and *Veillonella alcalescens* were also found to be present. Huber et al. (1976) found that *Selenomonas ruminantium* was the major lactate utilising bacterium in a steer, followed by *Megasphaera elsdenii,* and *Peptococcus asaccharolyticus*.

1.2.4.3 The role of protozoa in lactic acidosis.

Protozoa may play an important role in preventing lactic acidosis, especially the genus of *Entodinium*, since this microorganism rapidly takes up starch and, unlike holotrich protozoa, produces very little lactic acid (Abou Akkada et al., 1960). Entodinia also ferment starch at a slower rate than bacteria. In addition, ciliate protozoa ingest many bacteria (Coleman, 1975) and may, as a result, reduce bacterial numbers which further slows down the lactate production in the rumen.

Because of the long division time of Entodinia, 5.5 h in sheep fed hay (Warner, 1962) and 7.3 h in sheep fed lucerne pellets (Hungate et al., 1971), the amount of grain that can be introduced into the ration is limited. Under conditions of lactic acidosis where the diet is abruptly changed from roughage to a high grain diet, Entodinia can not compete with lactic acid-producing bacteria. The protozoa are destroyed as the rumen pH falls below 5 (Purser and Moir, 1959). Consequently, soluble carbohydrate would remain free in the rumen fluid and allow further proliferation of lactic acid-producing bacteria.

1.2.5 Biochemistry of lactic acidosis.

1.2.5.1 Anabolism of lactic acid.

Grain degradation is started when it is taken into the mouth and ground up briefly. The grain is lubricated along the gullet into the rumen by the saliva from the mouth. Rumen bacteria will metabolize the carbohydrate, converting part of it into sugar then fatty acids. Some of the coarse grain will be regurgitated and ruminated more thoroughly before the bacteria metabolize it. Some, of course, will flow into the omasum and abomasum. Waldo (1973) reported that 94% $\pm$ 2.4% of barley starch was digested prior to the abomasum or duodenum. Much more corn than barley starch escaped ruminal digestion. Corn starch digestion was 78% $\pm$ 12.5% prior to the abomasum or duodenum. The rate of digestion of cereal starch is slower than that of sugars and is dependent on several factors such as the grain type (particularly if whole grain is used, Toland, 1976; Rowe et al., 1989), and the extent (Ørskov and Fraser, 1975; Lee et al., 1987) and method of processing (Mould et al., 1983).

It is estimated that grain consists of about 80% starch. Starch is a plant polymer of glucose and serves as a nutritional reservoir in plants. There are two forms of starch; amylose and amylopectin. Amylose is comprised of an unbranched polymer of glucose.

Amylopectin, consists of a branched chain polymer of glucose (Albanese, 1963). Amylose or amylopectin can be hydrolysed by α and β amylases to maltose and finally to glucose. This glucose can be converted by lactic acid-producing bacteria to acetic and lactic acids.

The amount of lactic acid produced from glucose varies, according to the pathway used for fermentation (Gottschalk, 1979) and pH (Russell, 1985). The homofermentative pathway yields 2 mol of lactate per mol of glucose (Figure 1.1), whereas the heterofermentative pathway yields 1 mol each of lactate, ethanol, and carbon dioxide per mol of glucose (Figure 1.2). The bifidum pathway yields acetate and lactate in a ratio of 3 to 2 (Figure 1.3). Bacteria that use the homofermentative pathway include all *Streptococci* and some species of Lactobacillus. The ATP produced from this pathway is 2 mol per mol of glucose. The heterofermentative pathway is mainly used by Lactobacillus (*L. brevis* and *L. fermentum*). Bacteria that use this pathway produce 1 mol ATP per mol glucose utilised or half of the ATP produced in homofermentative pathway. The bifidum pathway is used by *Bifidobacterium bifidum*. Bacteria that use this pathway produce the highest ATP per mol glucose utilised (2.5 mol ATP per mol glucose) compared to the other two pathways.

1.2.5.2 Catabolism of lactic acid.

Some experiments have been conducted to study the metabolism of lactic acid in rumen microorganisms. Jayasuriya and Hungate (1959) found acetate to be the major end product of lactate catabolism. In addition, butyrate, methane, and carbon dioxide were also formed. Baldwin et al. (1962) showed that the ratio of propionate to acetate increased with increasing dietary carbohydrate. Johns (1951a,b) found that *Veillonella gazogenes* produced propionate from lactate via fixation of carbon dioxide with the formation of succinate.

Ladd (1959) studied the metabolic pathway of 2-^{14}C-lactic acid by using *Megasphaera elsdenii*. Carboxyl-labelled acetate and methylene-labelled propionate were the main products of lactate catabolism. Carbon dioxide, hydrogen, butyrate, valerate, and caproate were also formed. There is general agreement that lactic acid catabolism occurs via two routes. First, the dicarboxylic acid pathway will be discussed (Figure 1.4). Bacteria that use this pathway include: *Selenomonas ruminantium*, *Propionibacterium acnes*, and *Veillonella gazogenes*. ATP produced by the bacteria using this pathway is 1 mol per mol of lactate utilised. The second pathway is the direct reductive or non-randomizing pathway (Figure 1.5) (Elsden et al., 1956 and Ladd, 1959). Bacteria using this pathway include *Megasphaera elsdenii*, and *Clostridium propionicum*. The bacteria using this pathway produce 1 mol ATP per 3 moles of lactate. It was reported by Whanger and Matrone (1964) that the balance between the two pathways may depend upon the diet and the balance of species in the ruminal microbial population. The acrylate pathway (non-randomising pathway) predominated in ovine ruminal microorganisms on sulfur-adequate diets. In contrast, the succinate pathway was preferred on sulfur-free diets.

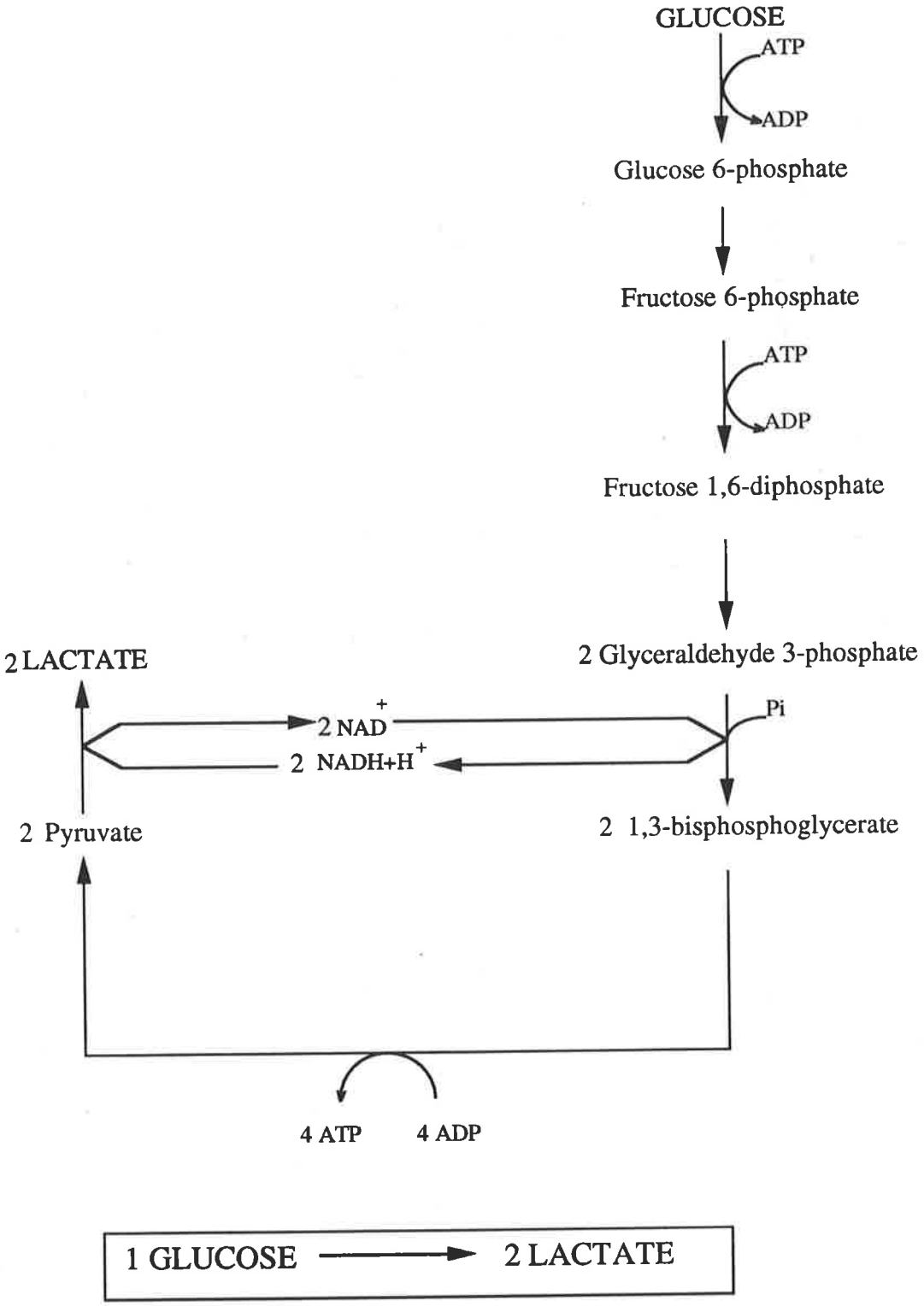

Figure 1.1 The Homofermentative Pathway

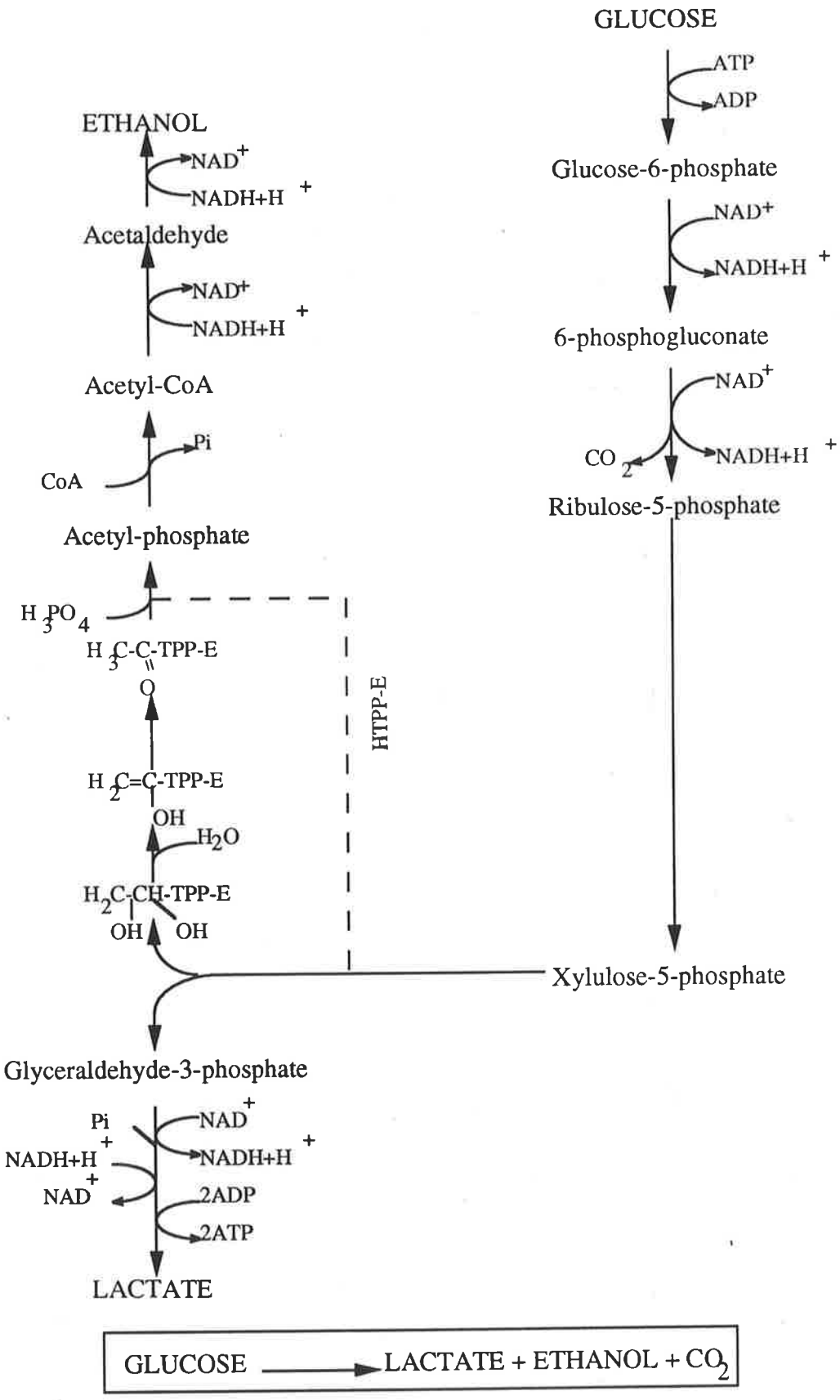

Figure 1.2 The Heterofermentative Pathway.

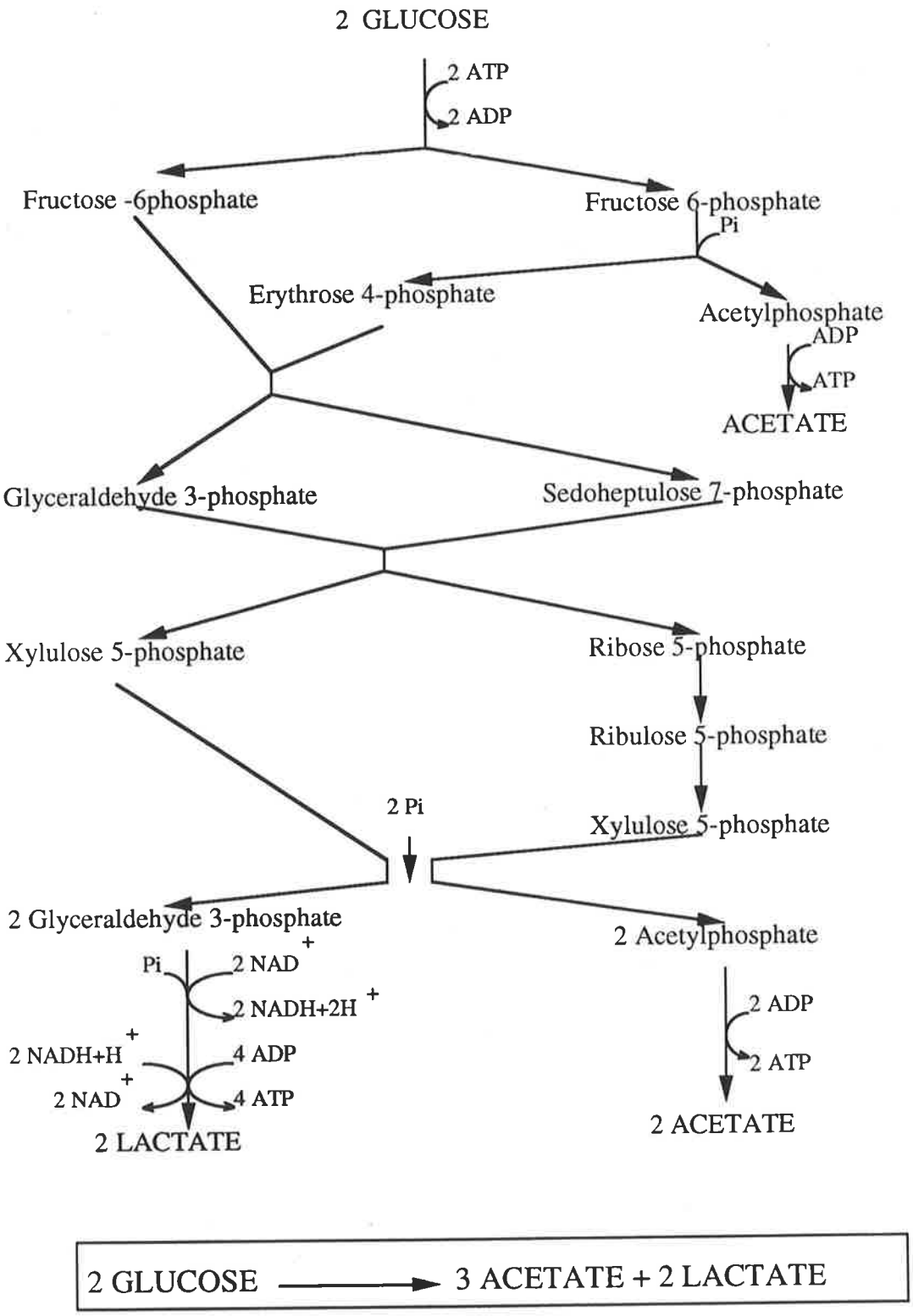

Figure 1.3 The Bifidum Pathway

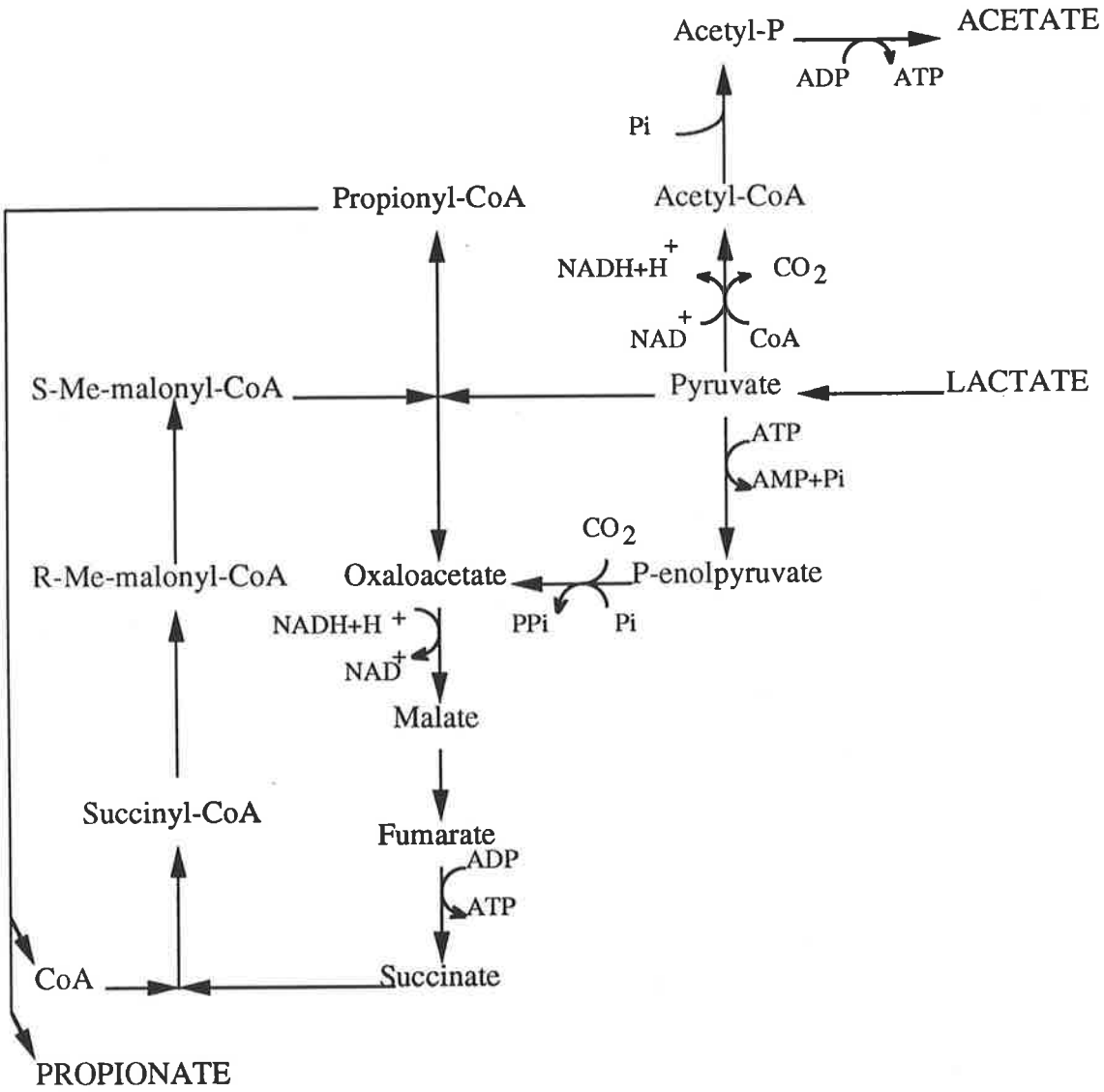

Figure 1.4 The Succinate-propionate Pathway.

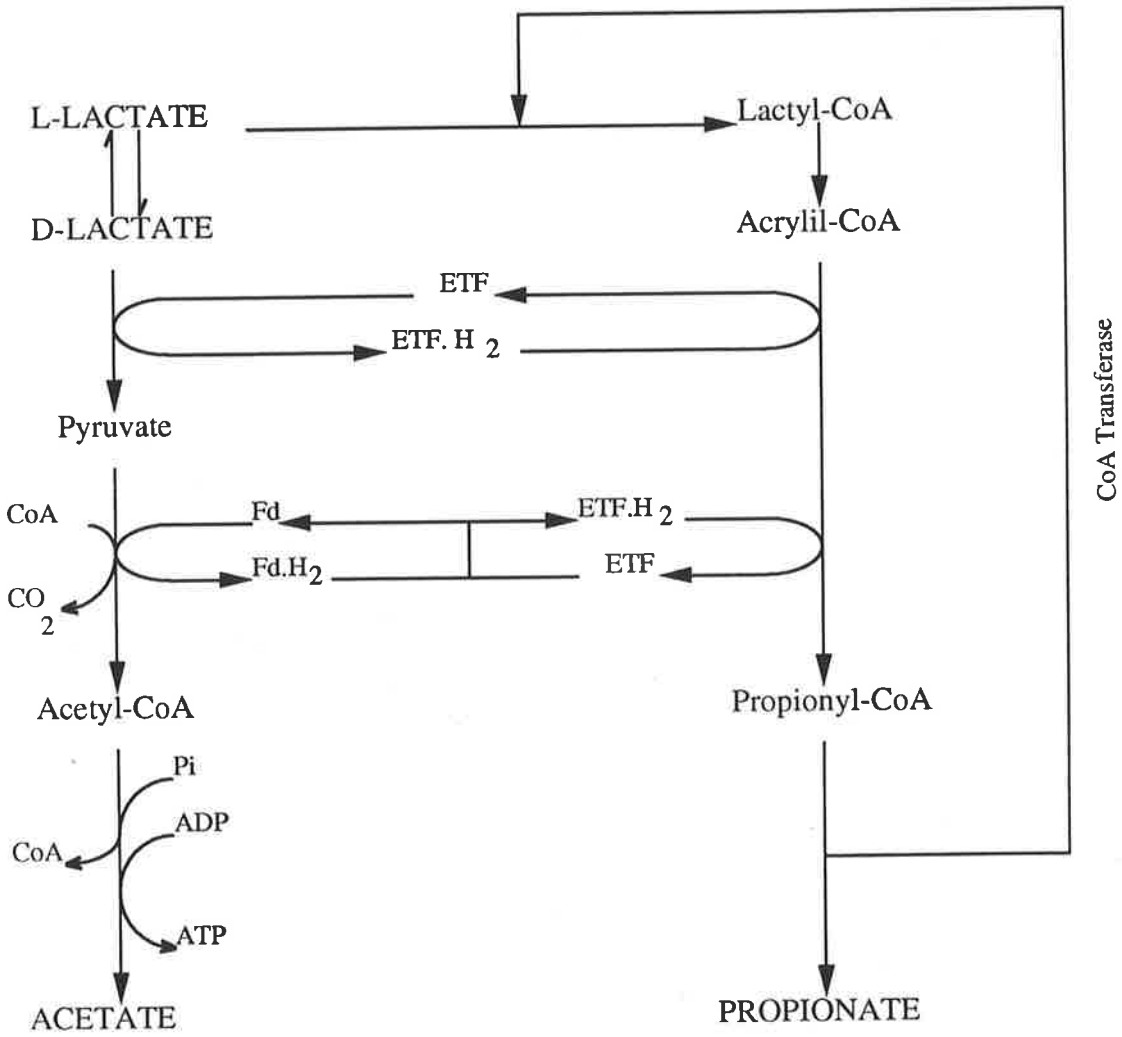

Figure 1.5 The Acrylate Pathway

1.2.6 Possible strategies for alleviation of lactic acidosis.

1.2.6.1 Slow adaptation.

Many investigations have been conducted in an attempt to prevent lactic acidosis problems from occurring in ruminants. One of them is a slow adaptation process. The animal is adapted to a high grain diet by increasing the amount of grain in rations for several days or weeks. The aim of the approach is to control lactic acid production and to allow lactic acid utilising-bacteria to proliferate and utilise the lactic acid produced by other bacteria and protozoa. This idea is based on the reality that lactic acid utilising bacteria have a doubling time much longer than lactic acid producing-bacteria.

Mackie et al. (1978) studied the changes in lactate producing and lactate utilising bacteria in sheep during stepwise adaptation to a high-concentrate diet. During this experiment it was found that the increase in lactic acid-producing bacteria was followed by an increase in lactic acid utilising-bacteria, which resulted in low lactic acid accumulation in the rumen. Amongst the lactate utilisers, *Veillonella* and *Selenomonas* predominated in the early stages, but were superseded with time by *Anaerovibrio* and *Propionibacterium*. *Megasphaera elsdenii* appeared intermittently throughout the experiment. *Anaerovibrio* was the major lactic acid-utilising bacteria found in the experiment. Among the lactic acid-producing bacteria, *Bacteroides* was superseded by more acid tolerant *Lactobacillus* and *Eubacterium*.

A similar experiment (Huber et al. 1976), found that in a steer adapted to a high grain ration, *Selenomonas ruminantium* was the major lactate utiliser . *Megasphaera elsdenii* and *Peptococcus asaccharolyticus* were also found to be present. There were no lactic acidosis problems found in either experiment. The concentration of total lactic acid in the rumen ranged from 0.7 - 4.26 mM with the D-isomer constituting 32 - 81% of the total. Total VFA's ranged from 92 - 156 mM, and the rumen pH ranged from 5.78-6.71.

The adaptation process seems to be very useful and the easiest way to prevent lactic acidosis in ruminants. However, a slow adaptation process is considered to be time consuming because it takes several weeks to complete. Problems of reduced weight gain during adaptation, labour costs for feed formulation and variation in adaptation rate among the animals are also significant. Therefore, a slow adaptation process is considered financially unbeneficial.

1.2.6.2 Rumen buffering.

The first effect of lactic acidosis in the rumen is a drop in the rumen pH. At the rumen pH less than 5, most bacteria and protozoa are killed. Therefore, some researchers have tried to prevent the change in rumen acidity by mixing dietary buffers into the ration.

Horn et al. (1979) studied the effects of the addition of 2% sodium bentonite, 1 % sodium bentonite plus 1 % dolomitic limestone (dolomite), or 1 % sodium bentonite plus 1 % potassium bicarbonate in the ration. The results of these experiments showed that lactic acid concentrations in steers fed the control, bentonite, bentonite plus dolomite and bentonite plus bicarbonate diets increased by 58.7, 50.1, 28.7, and 43.6 mM, respectively, by 2 h post feeding. Total VFA concentrations, and molar percentages of individual VFA's were not influenced by the treatments. The ruminal pH values were similar for all rations with the exception that the pH values of steers fed the rations containing bentonite plus bicarbonate were consistently higher than those of steers fed the other diet. Rowe et al. (1989) studied the effect of bentonite clay on the production of lactic acid in sheep. The addition of bentonite clay to the rations could not reduce lactic acid production in the rumen. This result is similar to that reported by Aitchison et al. (1987) for bicarbonate together with avoparcin.

The addition of dietary buffers into the rations may partially prevent lactic acid accumulation in the rumen. However its efficacy is restricted to a short time period,

since the buffers contribute relatively little before an adverse effect on palatability constrains their level of inclusion.

1.2.6.3 Antibiotic treatments.

Lactic acidosis is initiated by rapid proliferation of amylolytic bacteria such as *Lactobacilli* and *Streptococci* that can ferment grain very rapidly and produce elevated levels of lactic acid in the rumen. Therefore another approach to controlling acidosis is to prevent the proliferation of these bacteria with selective antibiotics such as tetracycline hydrochloride and penicillin G (Nakumara et al., 1971), capreomycin disulfate (Beede and Farlin, 1977), oxamycin and thiopeptin (Muir and Barreto, 1979; Kezar and Church, 1980). According to Church (1988) antibiotics are classified into two groups: 1, antibiotics that have systemic effect (e.g. chlortetracycline, tylosin, erythromycin and zinc bacitracin. These antibiotics play an important role in reducing the incidence of abscessed livers and 2, antibiotics that have ruminal effects include the ionophores monensin and lasalocid. These antibiotics alter feed intake and/or ruminal fermentation patterns in ways that facilitate microbial adaptation to increased concentrate intake.

Some antibiotics have been used to control lactic acid-producing bacteria in the rumen. Nagaraja et al. (1981; 1982) used lasalocid, monensin and thiopeptin to prevent lactic acidosis in cattle by administering the antibiotics for 7 days before inducing acidosis with corn (27.5 gram/kg body weight). These antibiotics could reduce the production of lactic acid and maintain the rumen pH when acidosis was induced by intraruminal administration of glucose, but these antibiotics could neither reduce lactic acid production nor maintain rumen pH when acidosis was induced by intraruminal administration of ground corn.

Muir et al. (1980) used thiopeptin and related antibiotics to prevent lactic acidosis in sheep. Thiopeptin could prevent lactic acidosis by reducing rumen lactate by 80 to 90 %.

At least 0.18 mg/kg body weight was required to control acute acidosis. Penicillin, however, inhibited ruminal VFA production as well as lactate synthesis. In addition, the effective period for penicillin in the rumen was only 8 to 16 h, after which lactate fermentation was re-established.

Rowe et al. (1989) used virginiamycin to control the proliferation of lactic acid-producing bacteria in the rumen using an *in vitro* technique. The antibiotic virginiamycin could control lactic acid production even at concentration of 0.5 μg/ml rumen fluid. Rowe et al. (1989) concluded that the antibiotic feed additives has a far greater potential than bentonite or buffers in controlling acidosis during the introduction of ruminants to a high grain diet. This result is similar to that reported by Aitchison et al. (1987) for bicarbonate relative to avoparcin.

The use of antibiotics does not completely solve the problem of lactic acidosis because antibiotics cause depression of rumen fermentation as evidenced by the decrease in VFA production (Mitchel et al., 1969; Muir et al. (1980). The depression of VFA production may be caused by antibiotics killing not only lactic acid producing bacteria, but also killing other rumen bacteria. In addition, many bacteria develop resistance to them (Hungate, 1966; Wiseman et al., 1960). The use of antibiotics may also increase the cost of production and the residues may be found in animal products especially when high dosages (> 200 ppm) of antibiotics were given to animals. The latter problem may be prevented by withdrawing the antibiotics several days before slaughter (Maynard and Loosli, 1956).

1.2.6.4 Microbial inoculations.

The use of probiotics (live inoculants) to maintain microbial interactions in a way that maintains the stability of the ecosystem and provides optimal health for the host has been

studied extensively in humans and animals (Fuller, 1992). In ruminants the use of bacterial and fungal probiotics has been used to enhance the development and maintenance of stable rumen fermentations. Theodorou et al. (1990) reported that a probiotic based on an anaerobic rumen fungus (*Neocallimastix* sp.) increased intake and live weight gain in calves following weaning. Products based on yeast or aerobic fungi such as *Saccharomyces cerevisiae* and *Aspergillus oryzae* fermentation extract have been reported to increase feed intake and live weight gain after weaning in both calves (Hughes, 1988) and lambs (Jordan and Johnston, 1990). These findings provide some hope that probiotics, based on rumen microorganisms may be applied in the prevention of lactic acidosis in grain-fed animals.

1.2.6.4.1 Crude rumen fluid.

Slow adaptation to a ration containing large quantities of readily fermentable carbohydrate can usually be accomplished with no ill effects to the animal. Changes in number or relative population of ruminal microorganisms have been proven to be important factors in adaptation. It was demonstrated by Mackie et al. (1978) that the increase in lactic acid producing bacteria in the rumen during the adaptation process had always been followed by an increase in lactic acid utilising bacteria. Based on this fact, it should be possible to accelerate the adaptation process by inoculating ruminal material from an animal already adapted to a high grain ration.

Huber (1974) studied the effects of intraruminal inoculation on adaptation of lambs and heifers to a high-energy ration. Intraruminal inoculation with either 200 or 400 ml of ruminal fluid from animal already adapted to a high grain diet prevented lactic acidosis from occurring in lambs. Inoculation did not increase average daily body weight gain but, increased feed efficiency when compared with those of lambs that were adapted to the ration over a 13-day period. Intraruminal inoculation of heifers with 1 litre or 2 litres

ruminal fluid increased feed intake by 31% and 35% per day compared with uninoculated heifers.

Allison et al. (1964) also studied the effects of intraruminal inoculation of rumen fluid on adaptation of lambs to a ration containing wheat. Lambs inoculated with 600 grams of ruminal fluid did not become acidotic, whereas three of four lambs in the uninoculated group became sick.

Intraruminal inoculation with ruminal fluid can clearly prevent lactic acidosis problems in sheep and cattle, but its application in industry seems to be a problem because considerable amounts of rumen fluid would be needed for inoculation. Also there is a problem of variable quality of rumen fluid from different animals.

1.2.6.4.2 Purified bacteria.

The evidence that bacteria play an important role in preventing lactic acidosis becomes clearer from the studies of the changes of the types of ruminal microorganisms present during slow adaptation and intraruminal inoculations with ruminal fluid. Based on this evidence, Cook et al. (1975) evaluated the performance of cattle inoculated with unidentified monocultures of ruminal lactic acid-utilizing bacteria. There were three pure cultures of lactic acid-utilizing bacteria used; 2C, R3, and LC. One liter of each culture was administered into the rumen of heifers fed an 85% concentrate ration. The heifers which were inoculated with 1 litre of R3 bacteria had an average daily body weight gain 0.21 kg higher than that of uninoculated heifers, and less feed was needed to produce 1 kg of body weight gain.

This work has provided a new insight into solving the lactic acidosis problem in ruminants, but it needs to be extended especially in the isolation and characterization of bacteria which predominate in the rumen of animals adapted to a high grain diet.

Potential isolated bacteria may then be commercially prepared in the form of capsules or lyophilysed bacteria for inoculation purposes. This will provide practical applications in the animal production industry.

1.2.7 Aims of project.

Because of the weaknesses of the strategies mentioned before, the immediate aims of this project are :

1. To study the changes in population of lactic acid utilising bacteria (e.g. *Selenomonas* etc.) during the period of rumen adaptation to grain supplementation.

2. To investigate the effect of introducing concentrated cultures of isolated bacteria into sheep at varying times during the feeding regimes.

3. To study the effect of the combinations of concentrated cultures plus antibiotics (e.g. virginiamycin) *in vitro* (fermenter studies).

Chapter 2 General Materials and Methods.

2.1 Materials.

2.1.1 A list of media used during this experiment.

2.1.1.1 Minimal medium for *Selenomonas ruminantium* subspecies *lactilytica*. Modification of M medium (Tiwari et al., 1969): g/100 ml.

Trypticase	0.10
Yeast extract	0.01
L-cysteine	0.08
n-valeric acid	0.035 ml
Mineral solution I	4.0 ml
Mineral solution II	4.0 ml
Vitamin solution	0.035 ml
Trace element solution	0.035 ml
Resazurin	0.035 ml
Sodium carbonate	0.30
Fumaric acid	0.10
Lactic acid	1.00

The pH was adjusted to 7.5 before autoclaving.

2.1.1.2 Mineral solution I (Bryant and Burkey, 1953): g/100 ml.

K_2HPO_4	0.60
Distilled water	100 ml

2.1.1.3 Mineral solution II (Bryant and Burkey, 1953): g/100 ml.

KH_2PO_4	0.60
NaCl	1.20
$(NH_4)_2SO_4$	1.20
$CaCl_2.2H_20$	0.12
$MgS0_4.7H_20$	0.25
Distilled water	100 ml

2.1.1.4 Vitamin solution (Schaefer et al., 1980): mg/100 ml.

Thiamine HCl	20.00
Ca-D-pantothenate	20.00
Nicotinamide	20.00
Riboflavin	20.00
Pyridoxine HCl	20.00
PABA	1.00
Biotin	0.25
Folic acid	0.25
Cyanocobalamine	0.20

The solution was made up to 1000 ml in 5 mM HEPES. The pH was adjusted to 7.3 and autoclaved for 5 minutes.

2.1.1.5 Trace element solution (Biebl and Pfennig, 1981): g/l

HCl (1 M)	3 ml
$ZnCl_2$	0.07
$MnCl_2.4H_20$	0.10
H_3BO_4	0.06
$CoCl_2.6H_2O$	0.20

$CuCl_2.2H_20$	0.02
$NiCl_2.6H_20$	0.02
$Na_2Mo0_4.2H_20$	0.04
Distilled water	1000 ml

This was prepared by first adding the HCl to the distilled water, then dissolving the seven salts in an aliquot of this before combining. The solution was then autoclaved and stored at 4°C.

2.1.1.6 Media for total culturable bacteria (Mackie and Heath, 1979): g/100 ml.

Glucose	0.05
Maltose	0.05
Cellobiose	0.05
Soluble starch	0.05
Xylan	0.05
Trypticase	0.20
Yeast extract	0.05
VFA solution	1.0 ml
Trace element solution	1.0 ml
Hemin solution (0.1%)	1.0 ml
Mineral solution I	1.0 ml
Mineral solution II	1.0 ml
Cysteine	0.025
Sodium carbonate	0.30

2.1.1.7 Volatile fatty acids solution (Caldwell and Bryant, 1966).

Acetic acid	17 ml
Propionic acid	6 ml
Butyric acid	4 ml
Iso-butyric acid	1 ml
n-valeric acid	1 ml
Iso-butyric acid	1 ml
DL-a-methylbutyric	1 ml

2.1.1.8 Hemin solution (Caldwell and Bryant, 1966): g/100 ml.

Hemin	0.01
Ethanol (95%, vol/vol)	25 ml
NaOH	0.02

Hemin is dissolved in 50ml of 0.02% NaOH and 50 ml of 50% ethanol. Stored at 4°C. Use at 10 ml/litre.

2.1.1.9 Brain Heart Infusion (BHI) Medium: g/100 ml.

BHI powder (Oxoid)	3.70
Cysteine	0.05
Hemin solution	0.10 ml

2.1.1.10 Artificial saliva (McDougall, 1948): g/l.

$NaHCO_3$	9.80
$Na_2HPO_4.10\ H_20$	9.30
NaCl	0.47
KCl	0.57

$MgCl_2.6H_20$	0.09
$CaCl_2$	0.05

The pH of the solution was adjusted to 7.0, filter sterilized and CO_2 equilibrated.

2.1.2 Solutions used for VFA analysis (Erwin et al., 1961).

2.1.2.1 Analytical standard.

Stock VFA mixture	1.5 ml
Distilled water	1.0 ml
Protein precipitant	0.5 ml
Internal standard	0.5 ml

2.1.2.2 Stock VFA mixture.

Acetic acid	10.00 ml
Propionic acid	2.50 ml
Iso-butyric acid	0.25 ml
n-butyric acid	2.50 ml
Iso-valeric acid	5.00 ml
n-valeric acid	5.00 ml
Distilled water	100 ml

2.1.2.3 Protein precipitant.

Metaphosphoric acid	375 g
Formic acid	500 ml
Distilled water	2000 ml

2.1.2.4 Internal standard.

Caproic acid	10.5 ml
Distilled water	2000 ml

2.1.2.5 Stock VFA solution.

Acetic acid	1.0 M
Propionic acid	1.0 M
Iso-butyric acid	1.0 M
n-butyric acid	1.0 M
Iso-valeric acid	0.1 M
n-valeric acid	0.1 M

2.1.3 Solutions used for starch analysis (Trace Scientific N.S.W., Australia).

2.1.3.1 0.2 M Acetate buffer: g/litre.

$CH_3COONa.3H_20$	27.2
Distilled water	1000 ml

2.1.3.2 Glucose oxidase reagent:

Active ingredients	Concentrations
Glucose oxidase	20.000 Units/L
Peroxidase	250 units/L
4-aminoantipyrine	0.5 mM
4-hydroxybenzoic acid	10 mM

Phosphate buffer	110 mM

Also contains non-reactive fillers and stabilizers. pH 7.0 ± 0.2 (25° C).

2.1.4 Lactic acid assay (Sigma Diagnostic).

2.1.4.1 Protein precipitant: g/l.

Metaphosphoric acid	250
Distilled water	1000 ml

2.1.4.2 Trichloroacetic acid (TCA) solution: g/100ml.

Trichloroacetic acid	10
Distilled water	100 ml

2.1.4.3 Hydrazine buffer: g/100 ml.

Glycine	3.0
Hydrazine	6.0 ml
EDTA	0.1

2.1.4.4 Enzymes (Sigma).

L-Lactate dehydrogenase. LD (bovine heart) suspension in ammonium sulphate. Approximately 1000 U/ml when prepared. 1 Unit represents the amount of enzyme that converts 1 µmole of lactate to pyruvate in one minute.

D-Lactate dehydrogenase. The enzyme was extracted from *Lactobacillus leichmannii*. The enzyme was suspended in ammonium sulphate. Approximately 2000 U/ml when prepared.

2.1.5 Solutions for microbiological assay.

2.1.5.1 Dilution solution (Bryant and Burkey, 1953) : g/100 ml.

Mineral solution I	7.5 ml
Mineral solution II	7.5 ml
Cysteine	0.05
Sodium carbonate	0.3
Resazurin, 0.1%	0.1 ml
Distilled water	100 ml

2.1.5.2 Methylgreen-Formaldehyde-Saline (MFS) solution (Ogimoto and Imai, 1981): g/litre

35% formaldehyde solution	100 ml
methylgreen	0.6
Sodium chloride	8.0
Distilled water	900 ml

2.1.6 Gram stain solutions (Harrigan and McCance, 1966).

2.1.6.1 Ammonium oxalate crystal violet (Hucker):

Solution A :

Crystal violet (90% dye content)	2 g
Ethyl alcohol	20 ml

Solution B :

Ammonium oxalate	0.8 g
Distilled water	80 ml

The two solutions were mixed in the above quantities.

2.1.6.2 Safranin solution:

Safranin (2.5% solution in 95% alcohol)	10 ml
Distilled water	100 ml

2.1.6.3 Lugol's iodine solution.

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 ml

2.2 Methods.

2.2.1 Preparation of anaerobic media.

Anaerobic media were prepared by boiling the solutions and bubbling with 100% O_2 free CO_2 (passed over copper turnings at 300°C). The required volumes were dispensed under 100% CO_2 into Hungate tubes, or into 100 ml crimp-top bottles before autoclaving. After autoclaving, the media were stored in a Coy anaerobic hood. The hood was filled with 95% CO_2 and 5% H_2. These gas conditions were used in all experiment in which the coy anaerobic hood was used.

2.2.2 Preparation of anaerobic plates.

Anaerobic plates were prepared by adding 1.5% agar to the media before autoclaving. The media were autoclaved for 15 minutes at 121°C and 15 psi. Plates were poured in a laminar floor hood when the temperature of the media reached 45°C and left with the lids off until the agar had set. Plates were placed in a coy anaerobic hood to allow the removal of O_2 from the media by diffusion.

2.2.3 VFA Analysis (Modification of that described by Erwin et al, 1961).

Sample preparation. Rumen fluid was collected either through a rumen fistula or a stomach tube. It was then filtered through two layers of gauze. 5 ml of filtered rumen fluid was mixed with 1 ml of protein precipitant (section 2.1.2.3) and 1 ml internal standard (section 2.1.2.4). It was then centrifuged at 8000 x g for 10 minutes. 0.2 µL of the supernatant was injected into the gas chromatograph. The gas chromatography used was a Shimadzu Gas Chromatograph (GC-14A) combined with Delta Data System (SGE analytical products, part no. 054474). The column used was: phase BP 21; 0.5 µm film with 25 m x .53 mm ID filled with polyethylene glycol. Initial temperature is 100°C. Temperature is increased at the rate of 9°C per minute, until maximum temperature is 150°C achieved. Injector temperature is 240°C and detector temperature is 280°C. Sample injection size is 0.2 µl. The same procedure was done for measuring VFA concentration from the fermenter. The concentration of component acids was estimated by comparing the ratio of acid peak area to internal standard peak area with the corresponding ratios measured on standard VFA mixtures.

2.2.4 Starch analysis (Trace Scientific N.S.W.).

The general reaction for starch analysis is as follows. The enzyme amyloglucosidase (EC 3.2.1.3) hydrolyses both α-1,4 and α-1,6 bonds of starch and the glucose released can be quantified by the following coupled enzyme procedure.

Glucose + O_2 + H_2O ----------> Gluconic acid + H_2O_2

H_2O_2 + HBA + 4-aminoantipyrine ----------> Quinoneimine dye + H_2O

Preparation of the amyloglucosidase. The enzyme provided was prepared from *Aspergillus niger* and when purchased was supplied in a glucose solution to stabilize it. The glucose was separated from the enzyme using a gel filtration column (Sephadex column-Pharmacia PD-10). The sephadex column was uncapped and filled with 0.05 M acetate buffer pH 4.5. The buffer was allowed to run into the column. This step was repeated twice. 2.5 ml of amyloglucosidase (10 units/ml) were added. When this has run in, a test tube was placed under the outlet and 3.5 ml of the acetate buffer were added to the column to elute the enzyme.

Sample preparation. One ml of sample taken from the fermenter was hydrolysed with 1 ml amyloglycosidase at 55°C for 15 minutes. The sample was then boiled for 2 minutes and cooled to room temperature. 0.1 ml of this starch hydrolysate was mixed with 2.5 ml of the glucose oxidase reagent and incubated at 37° C for 10 minutes. The concentration of the starch was calculated from the calibration curve.

Calibration curve. Data for the calibration curve were obtained from 5 dilutions of 5 mM glucose solution. 0.1 ml of each of the glucose solutions was mixed with 2.5 ml of glucose oxidase reagent (Trace Scientific, N.S.W. Australia). The blank consisted of 0.1 ml water and 2.5 ml glucose oxidase reagent. All test tubes were incubated at 37°C for 10 minutes, then the absorbance was read at 520 nm using a spectrophotometer.

2.2.5 Lactic acid assay (Sigma Diagnostic).

Sample preparation. 5 ml of filtered rumen fluid was deprotenized with 1 ml of 25% metaphosphoric acid (section 2.4.2.1). The mixture was centrifuged at 5000 x g for 10 minutes. The supernatant was removed to fresh tubes and stored at -20°C until analysed. Blood samples were treated similar to that of the rumen fluid except the blood samples were deprotenized with 10% trichloroacetic acid [TCA (section 2.1.4.2)] in the ratio of 2 ml blood to 4 ml TCA.

Sample analysis. 10 mg Nicotinamide Adenine Dinucleotide (NAD) was mixed with 2 ml glycine buffer (section) plus 4 ml distilled water. 0.1 ml of L- or D- lactate dehydrogenase was added. 0.9 ml of the mixture was pipetted into BLANK and TEST cuvettes. To blank cuvette, 0.1 ml of water was added meanwhile, 0.1 ml of sample was added to test cuvette. Both cuvettes were then incubated for 25 minutes at 37°C. The absorbance of test cuvette was read using a NOVASPEC spectrophotometer at 340 nm with the blank as reference.

Calculation of lactic acid:

$$\text{Rumen fluid (mM)} = \frac{A340}{6.22} \times \frac{6}{5} \times \frac{1}{0.1}$$

$$\text{Blood (mM)} = \frac{A340}{6.22} \times \frac{6}{2} \times \frac{1}{0.1}$$

A_{340} = final maximum absorbance at 340 nm.

6.22 = millimolar absorptivity of NADH at 340 nm

$\frac{6}{5}$ = dilution for rumen fluid.

$\frac{6}{}$ = dilution for blood.

2

1 = reaction volume (ml).

0.1 = volume (ml) of sample in cuvette.

2.2.6 Gram stain procedure (Harrigan and McCance, 1966).

One drop of distilled water was placed on a cooled microscope slide. Bacterial cells from a single colony were picked and resuspended in the drop of water. This sample was mixed and spread thinly on the slide and allowed to air dry. The sample was then fixed by passing it through a burner flame two or three times. The heat-fixed smear was stained for 1 minute with ammonium oxalate crystal violet (section 2.1.6.1). The slide was then washed gently with tap water. The smear was flooded with Lugol's iodine solution (section 2.1.6.3) and allow to stand 1 minute. The slide was then washed in tap water and blotted dry. The smear was decolorized 30 seconds by gentle agitation in 95% alcohol, and blotted dry. It was then counterstained 10 to 30 seconds in safranin (section 2.1.6.2) and washed in tap water and blot dried. A drop of immersion oil was put on the smear and examined under the microscope with 1000 times magnification.

2.2.7 Bacterial colony counts.

0.1 ml rumen fluid or fermenter cultures were added to 0.9 ml of dilution solution (section 2.1.5.1). From this mixture, 0.1 ml was taken out and mixed with another 0.9 ml dilution solution. This was done until a 10^{-6} or 10^{-7} dilution was obtained. 0.1 ml of each of the last three dilutions were plated out on to agar plates.The plates were incubated at 39° C in the anaerobic hood for 5 days.

2.2.8 Protozoal counts.

One ml of rumen fluid was mixed with 5 ml methylgreen-formaldehyde-saline (MFS) (section 2.1.5.2) solution and shaken gently. A drop of this mixture was placed on to a haemocytometer and a cover slip placed on top. The protozoa were counted in five-1 mm^2 fields.

Chapter 3 Changes in the sheep rumen environment during stepwise adaptation to a high grain diet.

3.1 Introduction.

As discussed previously, lactic acidosis will usually not arise if the ruminant is slowly adapted to a high carbohydrate diet by gradually increasing the amount of grain in the feed ration. This is probably due to a gradual change in the ruminal bacterial population and in the proportion of lactic acid utilising bacteria. However, the precise species and proportions of lactic acid utilising bacteria have not been established. Mackie and Gilchrist (1979) and Huber et al. (1976) reported changes in the chemical and microbiological contents of the rumen of sheep and a steer. Different animals contained different populations of rumen bacteria. Mackie et al. reported that *Veillonella* and *Selenomonas* were the predominant lactic acid utilisers found in the early stages of the adaptation process in sheep, but were superseded with time by *Anaerovibrio* and *Propionibacterium.* Meanwhile, Huber et al found that *Selenomonas ruminantium* was the major lactic acid utiliser in a steer adapted from hay feeding to a diet high in fermentable carbohydrates. In experiments described here, I have examined changes in species and population densities of lactic acid utilising bacteria in the sheep rumen during the process of adaptation to a predominantly grain diet. The physical environment (pH, volatile fatty acids, lactate) was also studied during the adaptation. I also report the isolation and characterisation of the predominant lactic acid utilising bacteria obtained from an experimental animal.

3.2 Materials and Methods.

3.2.1 Animal and diet.

A one year old fistulated merino ewe (40 kg) was used in this experiment. It was fed with 800 g of lucerne chaff for six months before the experiment was begun. After six months, every four days, a proportion of lucerne chaff was replaced with an equal proportion of whole wheat grain until the final diet contained 80% grain and 20% lucerne (Table 3.1). The animal was fed once daily at 9.00 AM.

Table 3.1. Proportion (%) of lucerne chaff to grain during adaptation process.

Day	Lucerne (% of dry matter)	Grain (% of dry matter)
0	100	0
1-4	90	10
5-8	80	20
9-12	60	40
13-16	40	60
17-20	20	80

3.2.2 Sample collection.

Samples for chemical and microbiological examination were taken three hours after feeding at the first and last day of each ration combination. Ruminal samples were taken by vacuum with a 15 mm internal diameter tube through the rumen fistula and samples were strained through two layers of gauze.

3.2.3 Chemical analysis.

The strained samples were analysed for lactic acid and volatile fatty acid content as described in Chapter 2. Rumen pH was measured directly from whole rumen fluid using a pH meter (HANNA Instruments B 417). Blood samples were collected via the jugular

vein and stored in heparin-containing tubes until analysed for lactic acid concentration (see Chapter 2).

3.2.4 Isolation of lactic acid utilising bacteria.

Lactic acid utilising bacteria were isolated by streaking serially diluted rumen fluid on to lactate agar plates (see Chapter 2). The plates were then incubated at 39°C in the anaerobic hood for five days. Bacterial identification was based on : Gram stain, cell morphology, lactate and glucose utilisation, and end product metabolism.

3.2.5 Lactic acid utilisation tests.

The ability of isolates of bacteria (*Selenomonas ruminantium* subsp. *lactilytica* and *Megasphaera elsdenii*) to utilise lactic acid was investigated. 250 µl of overnight cultures were grown in 10 ml M medium (section 2.1.1.1) containing 1% lactic acid in Hungate tubes. The density of the cells was measured using a NOVASPEC spectrophotometer at 600 nm for three days for *Selenomonas ruminantium* and for two days for *Megasphaera elsdenii* against M medium as a blank. The effect of intermediate additions (8 mM aspartate, 8 mM fumarate) on lactic acid utilisation was also observed. The bacteria were also grown on glucose as a comparison with growth on lactic acid.

3.2.6 Bacterial volatile fatty acid production tests.

The volatile fatty acid production was measured after the cells had been grown for 24 hours in 1% lactate or glucose (BHI)-containing liquid media in Hungate tubes, in duplicate. The volatile fatty acid analysis was by gas chromatography combined with Delta Data system (section 2.1).

3.3 Results.

3.3.1 Identification of the predominant types of lactic acid utilising bacteria.

Strains identified morphologically as *S. ruminantium* subsp. *lactilytica* had the following characteristics: mainly single, motile, Gram negative (Figure 3.2a) rods (banana shape), with occassional doublets and long chains occurring depending on the growth stage (Figure 3.1a). In glucose containing (BHI) medium or lactate containing medium (see Chapter 2) the bacteria produced mainly acetate and propionate (Figure 3.3b,c). *Megasphaera elsdenii* consisted of large, Gram negative (Figure 3.2b), non motile cocci . They occurred mainly in doublets and short chains (Figure 3.1b). They produced acetate, propionate, butyrate, and valerate from lactic acid (Figure 3.3d) but only butyrate, isovalerate and caproate were produced from glucose (Figure 3.3e).

3.3.2 Changes of lactic acid utilizing bacteria during adaptation.

Changes in the predominant lactic acid utilising bacteria were followed during the adaptation to lactic acid production in the rumen. Lactic acid utilising bacteria were identified by picking at least 20 colonies per plate and morphologically examining them by light microscopy. During the adaptation period, *S. ruminantium* subspecies *lactilytica* increased overall at least 70 fold from 10^6 cfu/ml to 7 x 10^7 cfu/ml of rumen fluid, with a ten fold increase occuring over the first 12 days of adaptation. *Megasphaera elsdenii* was detectable at most stages during adaptation but the numbers remained relatively constant at about 2 x 10^6/ml over the whole adaptation period and were always at least 10 fold less than *S. ruminantium* subsp. *lactilytica* (Figure 3.4). There was about 5% non-lactilytic bacteria detected on the plates, but the cololies were very small and the bacteria could not grow in liquid medium that contained lactic acid as a sole carbon source.

Figure 3.1a Light micrograph of unstained *S. ruminantium* subspecies *lactilytica*.
The light micrograph (40 times magnification) shows the general shape and size of unstained *S. ruminantium* subspecies *lactilytica*.

Figure 3.1b Light micrograph of unstained *M. elsdenii*.
The light micrograph (40 times magnification) shows the general shape and size of unstained *M. elsdenii*.

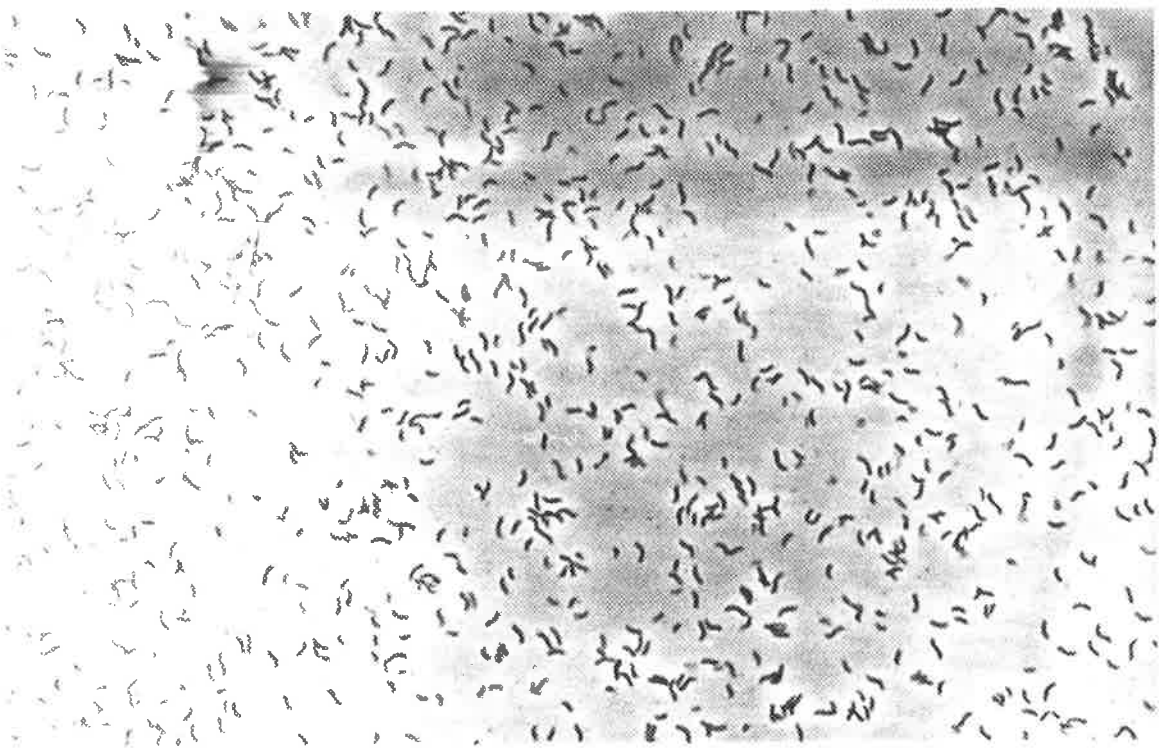

Figure 3.1a Light micrograph of unstained *S. ruminantium* subspecies *lactilytica*.

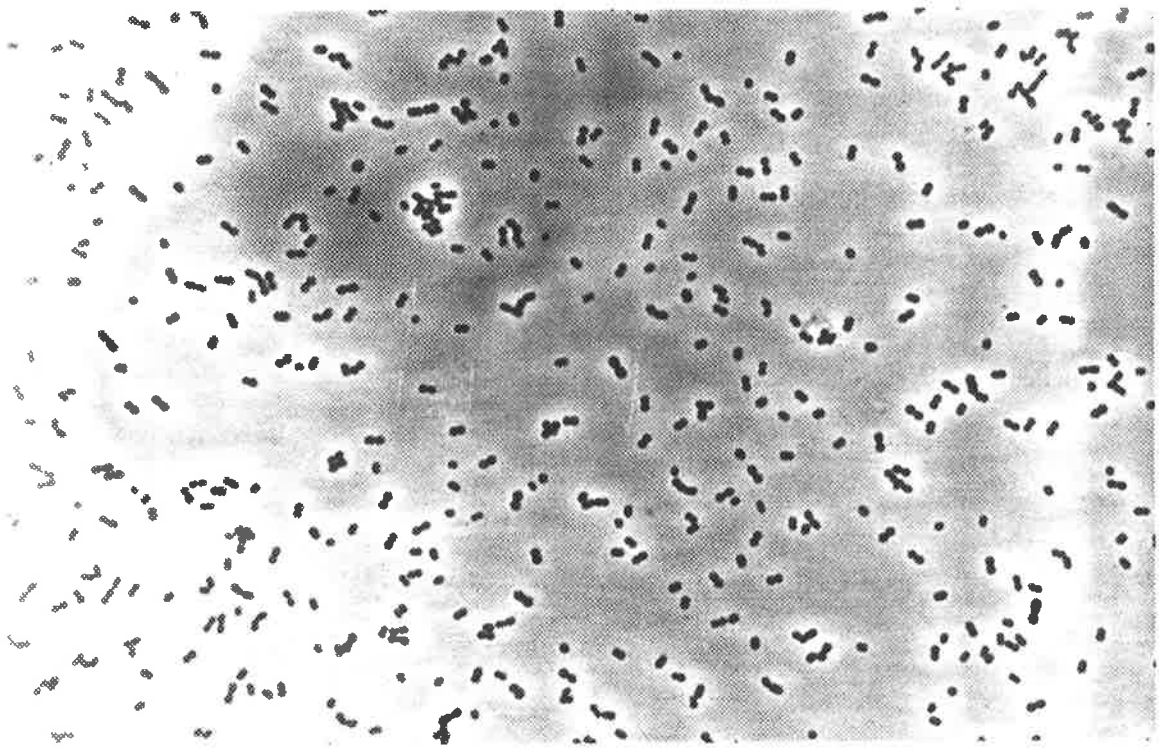

Figure 3.1b Light micrograph of unstained *M. elsdenii*.

Figure 3.2a Light micrograph of a Gram-stained preparation of *S. ruminantium* subspecies *lactilytica*. The light micrograph (100 times magnification) shows that the cells are Gram negative.

Figure 3.2b Light micrograph of a Gram-stained preparation of *M. elsdenii*. The light micrograph (100 times magnification) shows that the cells are Gram negative.

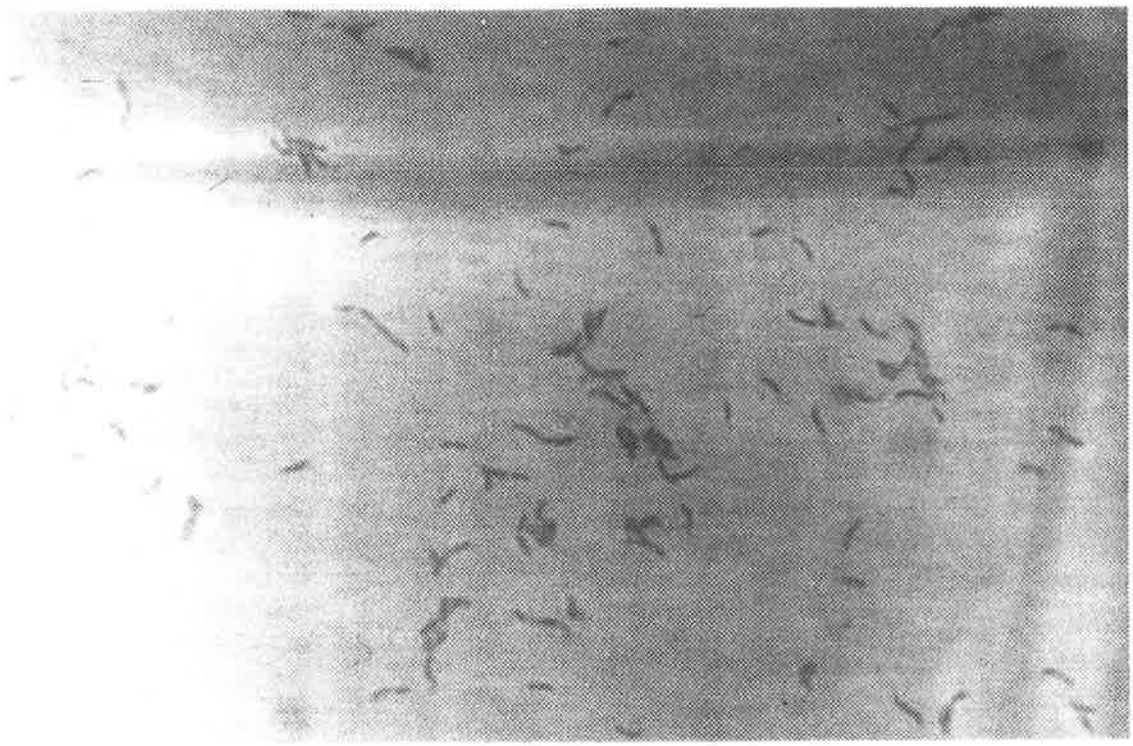

Figure 3.2a Light micrograph of Gram-stained *S. ruminantium* subsp. *lactilytica*.

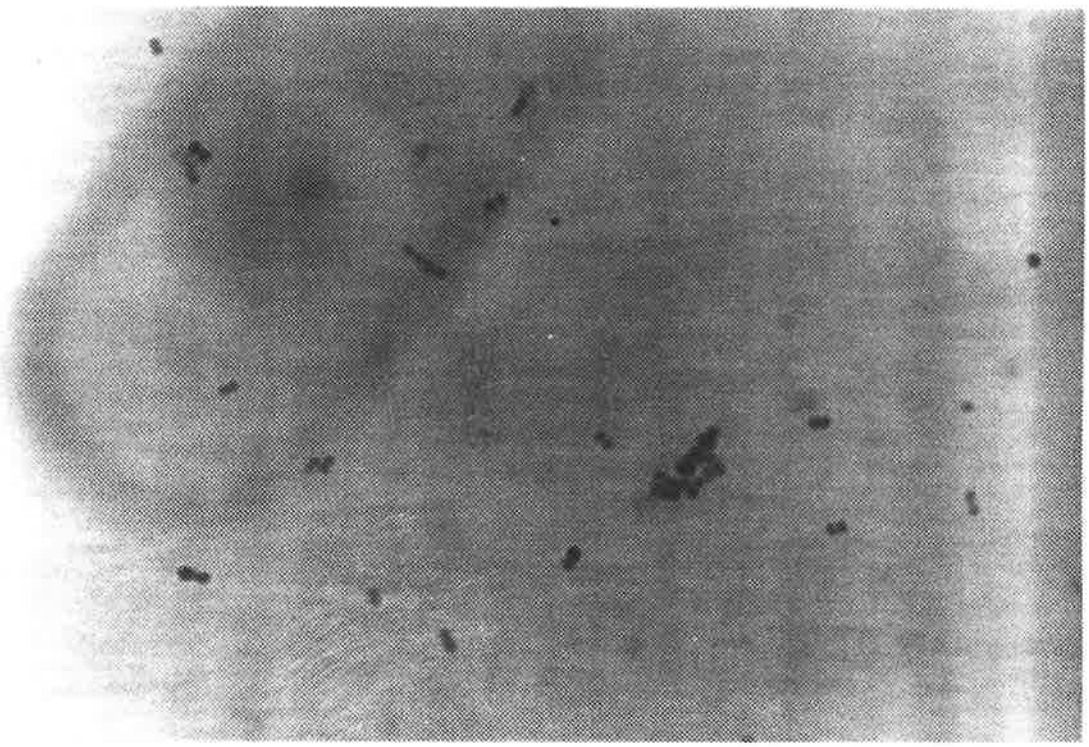

Figure 3.2b Light micrograph of Gram-stained *M. elsdenii*.

Figure 3.3a Volatile fatty acid standards. Peak separation was achieved using a Shimadzu Gas Chromatograph combined with Delta Data System.

Time 10:12:55 DELTA CHROMATOGRAPHY DATA SYSTEM - AREA PERCENT REPORT Date 25/01/94

METHOD :VFA-ANAL
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DILUTION : 1.000

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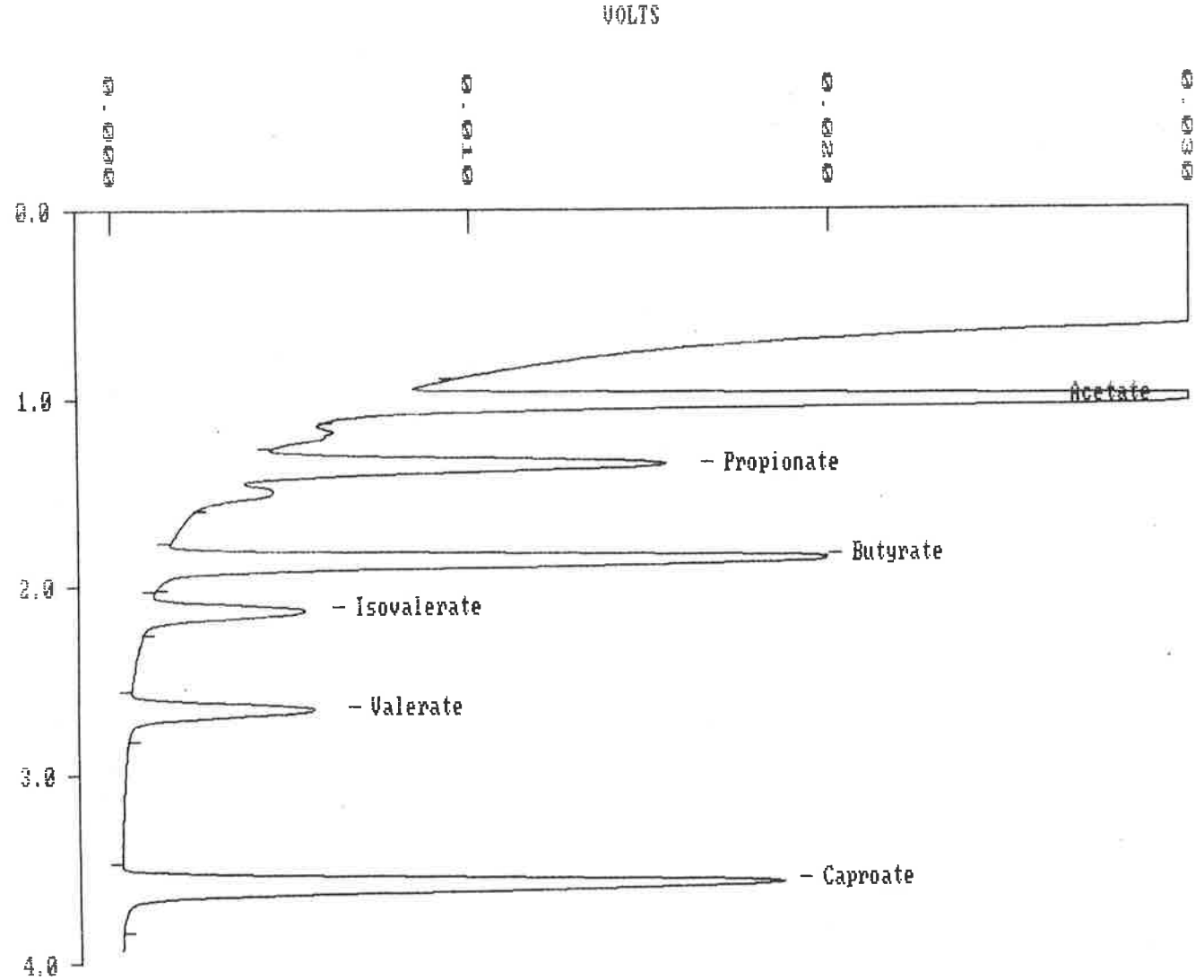
CHROMATOGRAM SOURCE :ACQUIRE

quired by Method :VFA-ANAL on 25/01/94



EAK #	COMPT #	COMPOUND	RETENTION TIME (MIN)	WIDTH (sec)	HEIGHT (mV)	AREA (mV.Sec)	AREA PERCEI
1	1	Acetate	1.0	4.3	27.73	118.94	29
2	2	Propionate	1.3	4.8	11.49	63.56	15
3	3	Butyrate	1.8	4.6	18.48	89.31	21
4	4	Isovalerate	2.1	4.7	4.36	21.09	5
5	5	Valerate	2.6	4.7	5.20	25.35	6
6	6	Caproate	3.6	4.8	18.51	92.42	22
					85.77	410.66	100

Figure 3.3b Volatile fatty acid production of *Selenomonas ruminantium* subsp. *lactilytica* grown for 24 hours on BHI medium.

Time 10:39:24 — Date 25/01/94

DELTA CHROMATOGRAPHY DATA SYSTEM - AREA PERCENT REPORT

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DILUTION : 1.000

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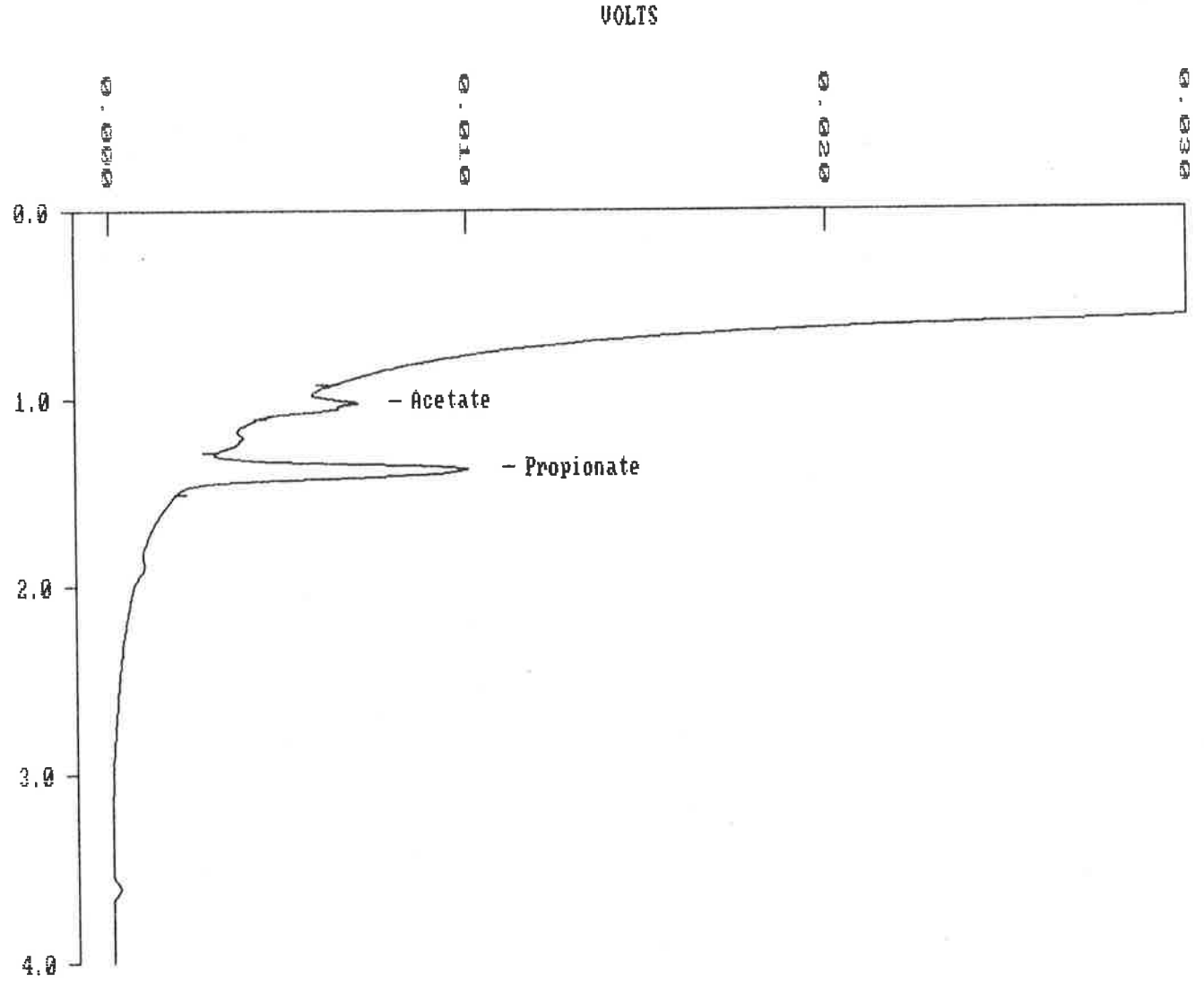
quired by Method :VFA-ANAL on 25/01/94



EAK #	COMPT #	COMPOUND	RETENTION TIME (MIN)	WIDTH (sec)	HEIGHT (mV)	AREA (mV.Sec)	AREA PERCEN
1	1	Acetate	1.0	4.1	1.89	7.39	17.
2	2	Propionate	1.4	4.6	7.47	36.10	83.
					9.36	43.49	100.

Figure 3.3c Volatile fatty acid production of *Selenomonas ruminantium* subsp. *lactilytica* grown for 24 hours on lactate containing medium.

****

Time 10:47:59 DELTA CHROMATOGRAPHY DATA SYSTEM - AREA PERCENT REPORT Date 25/01/94

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SAMP :SEL-LF
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DILUTION : 1.000

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CHROMATOGRAM SOURCE :ACQUIRE

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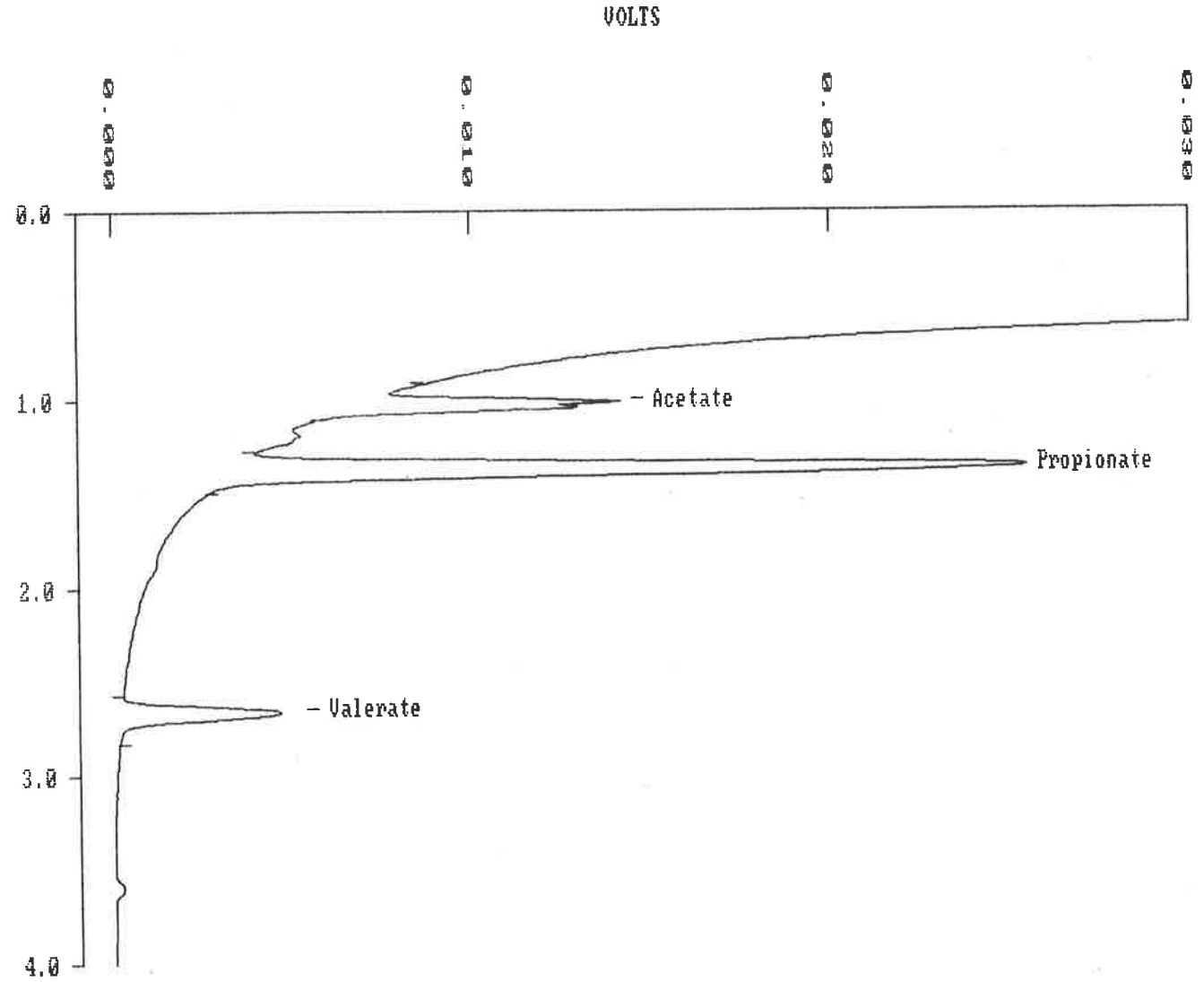
quired by Method :VFA-ANAL on 25/01/94



AK #	COMPT #	COMPOUND	RETENTION TIME (MIN)	WIDTH (sec)	HEIGHT (mV)	AREA (mV.Sec)	AREA PERCEI
1	1	Acetate	1.0	4.1	7.06	27.89	18
2	2	Propionate	1.4	4.6	21.96	104.34	67
3	5	Valerate	2.6	4.7	4.46	21.50	14
					33.48	153.74	100

Figure 3.3d Volatile fatty acid production of *Megasphaera elsdenii* grown for 24 hours on BHI medium.

Time Date
10:22:55 DELTA CHROMATOGRAPHY DATA SYSTEM - AREA PERCENT REPORT 25/01/94

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SAMP :ME-BHI
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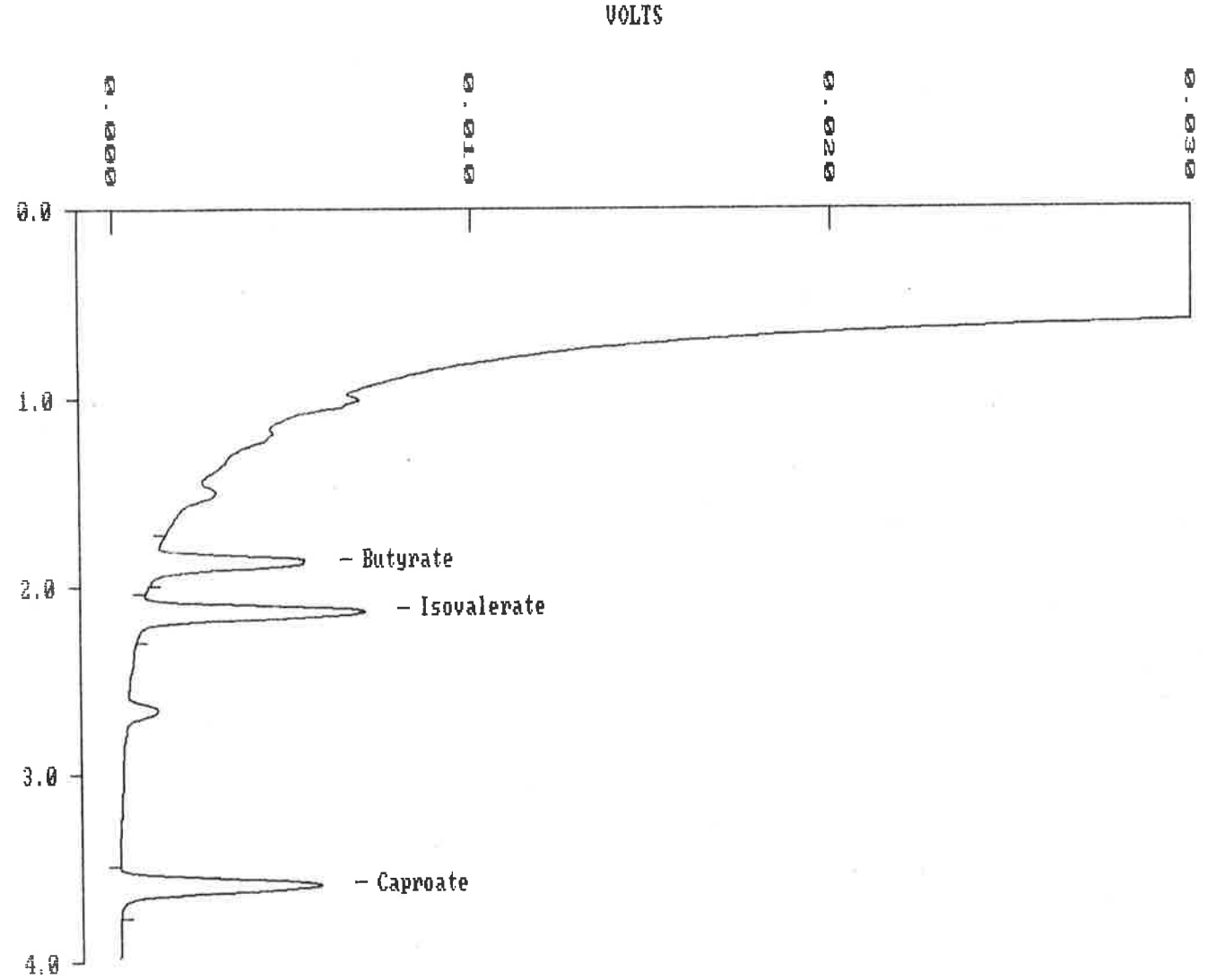

EAK #	COMPT #	COMPOUND	RETENTION TIME (MIN)	WIDTH (sec)	HEIGHT (mV)	AREA (mV.Sec)	AREA PERCEI
1	3	Butyrate	1.8	4.6	4.15	19.73	25
2	4	Isovalerate	2.1	4.6	6.25	29.91	38
3	6	Caproate	3.6	4.8	5.64	28.06	36
					16.05	77.70	100

Figure 3.3e Volatile fatty acid production of *Megasphaera elsdenii* grown for 24 hours on lactate containing medium.

Time 10:30:57 DELTA CHROMATOGRAPHY DATA SYSTEM - AREA PERCENT REPORT Date 25/01/94

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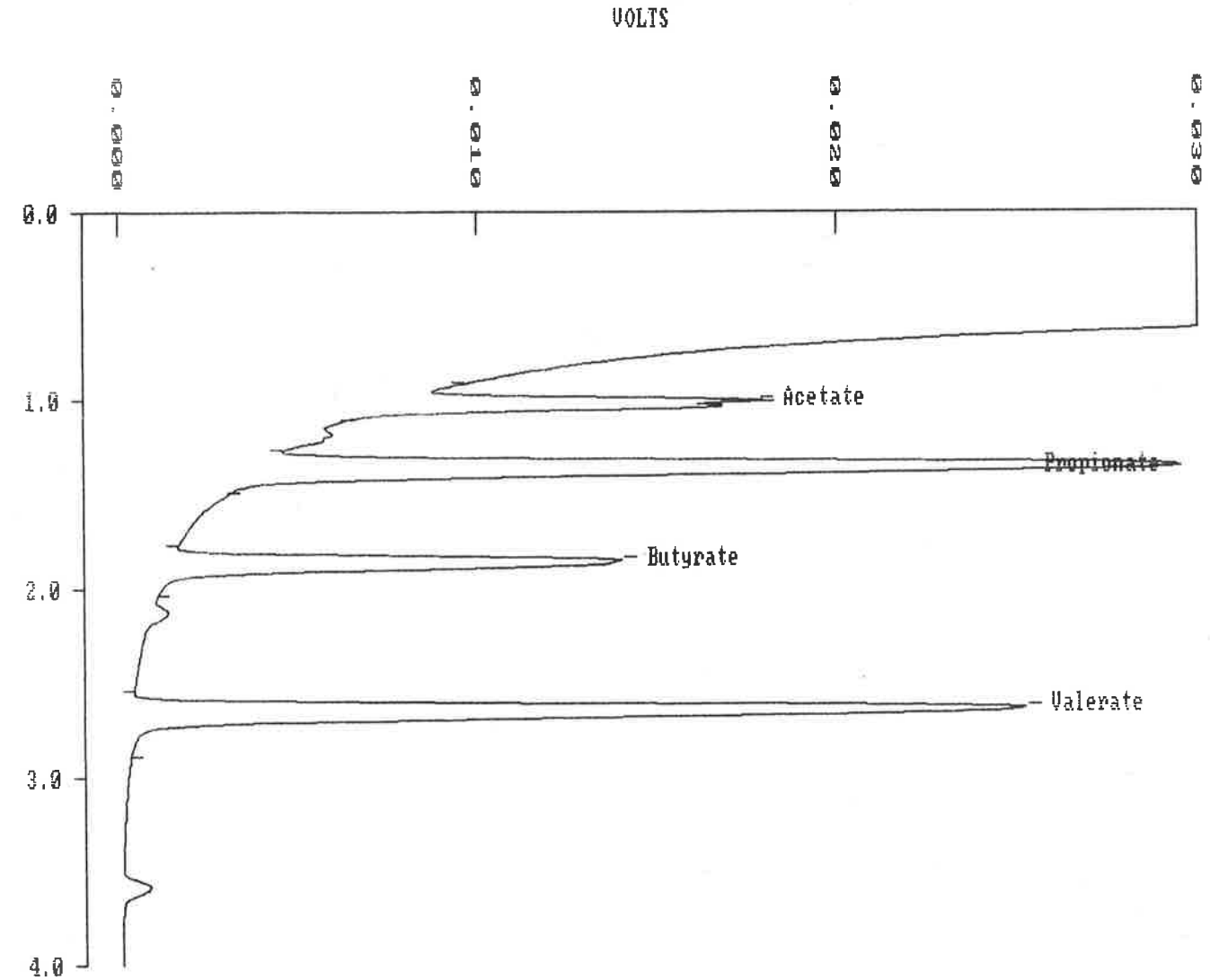
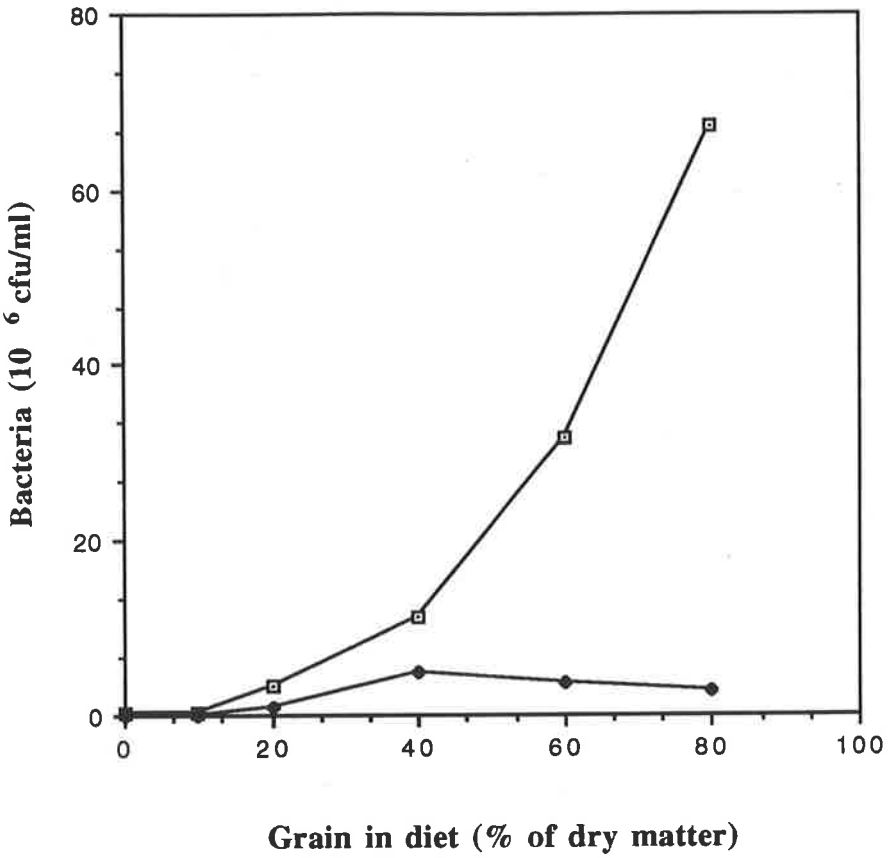


EAK #	COMPT #	COMPOUND	RETENTION TIME (MIN)	WIDTH (sec)	HEIGHT (mV)	AREA (mV.Sec)	AREA PERCEI
1	1	Acetate	1.0	4.2	10.24	41.85	12
2	2	Propionate	1.4	4.7	25.50	124.31	35
3	3	Butyrate	1.8	4.6	12.57	59.75	17
4	5	Valerate	2.6	4.7	24.86	120.89	34
					73.17	346.80	100

Figure 3.4 Lactilytic bacterial populations during the adaptation period. Colony counts of lactic acid utilising bacteria were taken on the fourth day of each ration combination. For colony counts, bacteria were grown on M medium with lactic acid as the sole carbon source. (n = 1?)

Selenomonas ruminantium subsp. lactilytica
Megasphaera elsdenii

Figure 3.4 Lactilytic bacterial populations during adaptation process.



3.3.3 Chemical changes in the rumen and blood during adaptation.

Ruminal parameters of pH, lactate, and volatile fatty acids are good indicators for determining bacterial population changes in the rumen. Therefore changes in these parameters were investigated during the 20 day adaptation period. Table 3.2 shows that rumen pH values were above 5.3 over the 20 day period of the experiment. The lowest value was obtained on the first day on which the 20% grain+80% lucerne diet was introduced. Total VFA concentration increased as the grain proportion was increased. The VFA concentration on the first day of the diet showed a little fluctuation during the experiment, however the total VFA values at the fourth day on the diets showed a progressive increase as the grain proportion was increased. The peak values were found on a 60% grain+40%lucerne diet and were slightly reduced on the 80%grain+20% lucerne diet. Lactic acid concentration in the rumen was found to be very low. Both L(+) and D(-) lactic acid were detected, with D(-) lactic acid constituting between 0 and 74% of the total lactic acid, but it disappeared after 40% grain+60% lucerne diet was introduced to the animal. Only L(+) lactic acid was found in the blood. Its concentration was gradually reduced as the proportion of grain was increased in the diet.

The proportion of acetic acid gradually decreased from 71.9% on 100% lucerne diet to 59.6% on an 80%grain+20%lucerne diet (Table 3.3). In contrast, the proportion of propionic acid increased from 14.3% on an all lucerne diet to 19.4% on an 80% grain+20% lucerne diet. Butyric acid also increased from 10.2% to 15.7% as the grain proportion was increased in the ration.

Table 3.2 Rumen pH, total VFA, rumen and blood lactic acid levels during stepwise adaptation to a high grain diet.

Grain (%)	Day	Rumen pH	Total VFA (mM)	Lactic acid (mM)			
				rumen		blood	
				L(+)	D(-)	L(+)	D(-)
0	0	5.7	99.0	1.3	3.4	3.2	-
10	1	5.8	94.5	1.1	3.1	3.0	-
	4	5.6	103.4	2.1	3.3	2.6	-
20	1	5.3	146.0	0.9	1.9	2.8	-
	4	5.7	104.3	-	-	2.7	-
40	1	6.0	96.5	-	-	2.0	-
	4	5.6	104.4	0.6	-	3.2	-
60	1	6.0	108.4	2.9	-	2.9	-
	4	5.5	121.2	1.9	-	1.9	-
80	1	5.6	136.9	0.5	-	2.1	-
	4	5.6	117.7	-	-	2.1	-

Table 3.3 Individual VFA's in the rumen during adaptation to a high grain diet.

VFA		Grain in ration (% of dry matter)					
		0	10	20	40	60	80
Acetate	mM	71.1	68.1	69.0	62.7	73.6	70.1
	%	71.9	65.8	66.1	60.0	60.7	59.6
Propionate	mM	14.1	20.4	18.9	23.5	23.0	22.8
	%	14.3	19.7	18.1	22.5	19.0	19.4
Butyrate	mM	10.1	11.9	12.4	13.9	18.1	18.4
	%	10.2	10.8	11.9	13.3	14.9	15.7

3.3.4 Lactic acid utilisation *in vitro* by isolated bacteria.

To test the ability of the bacterial isolates to utilise lactic acid and to determine whether there is a common response of lactilytic species to intermediate metabolites of the randomising succinate-propionate pathway, the growth rates of *S.ruminantium* subsp. *lactilytica* and *M. elsdenii* were determined in media containing either DL-lactate, glucose, aspartate, fumarate, or a combination of DL-lactate+aspartate or DL-lactate+fumarate. With *S. ruminantium* subsp. *lactilytica*, maximum optical density (OD at 600 nm) was obtained over a 24 hour period with glucose as the carbon source. When lactate alone was used as carbon source the optical density was only 30% of that of glucose in 24 hours. However when lactate was supplemented with 8 mM aspartate or fumarate, an OD of 70% of that with glucose was obtained. Fumarate or aspartate alone did not support growth (Figure 3.5a). For *M. elsdenii*, there was no difference in growth rate using either glucose, lactate, or lactate plus aspartate as carbon sources (Figure 3.5b).

The rate of utilisation of lactate by the two lactilytic species described above was determined *in vitro*, by culture in the presence of 51.3 mM (D)- and 51.3 mM (L)- lactate plus 8 mM fumarate. One ml samples were taken every 24 hours over 2 days of culture and lactate levels were measured. Viable bacterial counts were also made at each time point. The results (Figure 3.6a,b) are expressed as µmoles of lactate remaining in the media per 10^7 viable bacteria. Both (D)-and (L)-lactate were metabolized at 20.89 µmoles/hour/10^7 cfu in *S. ruminantium* and 71.27 µmoles/hour/10^7 cfu in *M. elsdenii*. By 2 days, residual lactate concentration in both cultures was less than 10mM.

Figure 3.5a Growth curves of *Selenomonas ruminantium* subsp. *lactilytica* on M medium with:

- Blank (without carbon source),
- Lactate,
- Aspartate,
- Fumarate,
- Lactate and aspartate,
- Lactate and fumarate,
- Glucose as carbon source.

The cells were incubated at 39°C, and cell densities were measured every 24 hours using a NOVASPEC spectrophotometer at 600 nm against a M medium blank. All measurements were done in duplicate, and the results are expressed as the mean.

Figure 3.5a Growth curves of *S. ruminantium*.

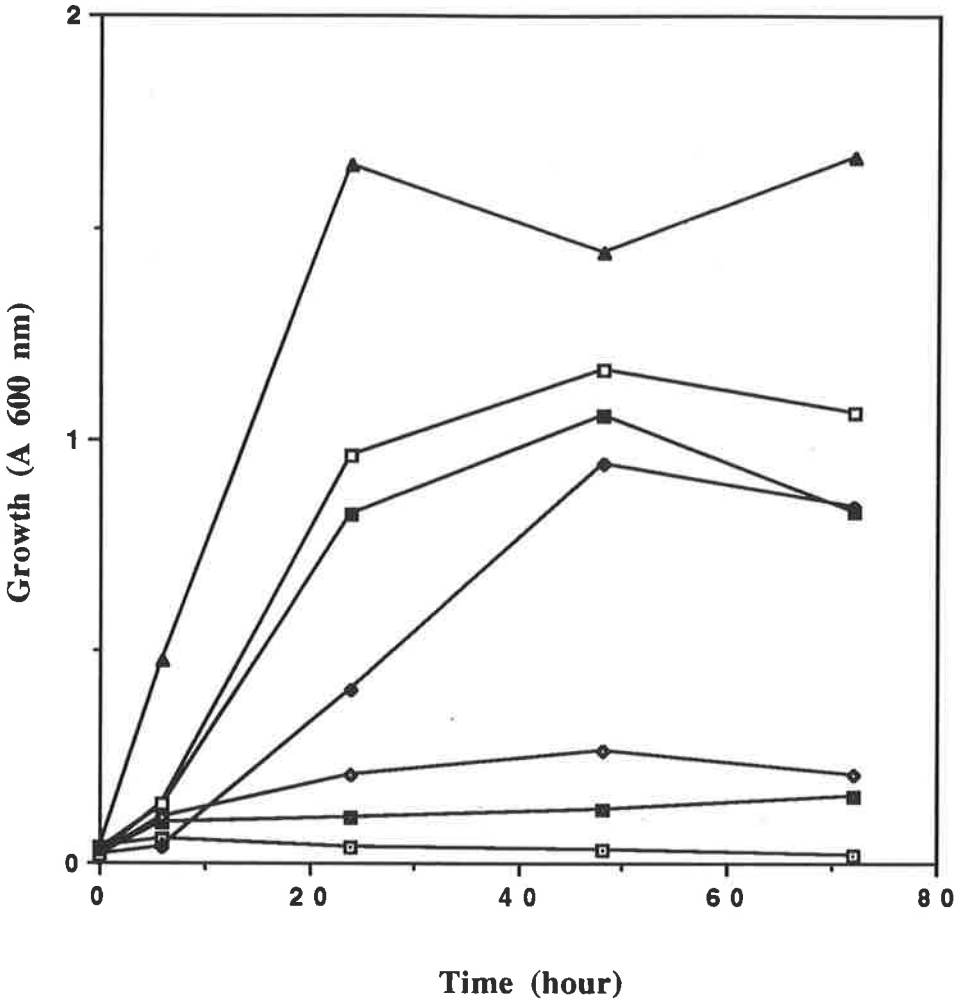
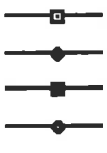


Figure 3.5b Growth curves of *Megasphaera elsdenii* on M media with:



blank (without carbon source),
lactate,
lactate and aspartate,
glucose as carbon source.

The cells were incubated at 39°C, and cell densities were measured every 24 hours using a NOVASPEC spectrophotometer at 600 nm against a M medium blank. All measurements were done in duplicate, and the results are expressed as the mean.

Figure 3.5b Growth curves of *M.elsdenii* on different carbon sources.

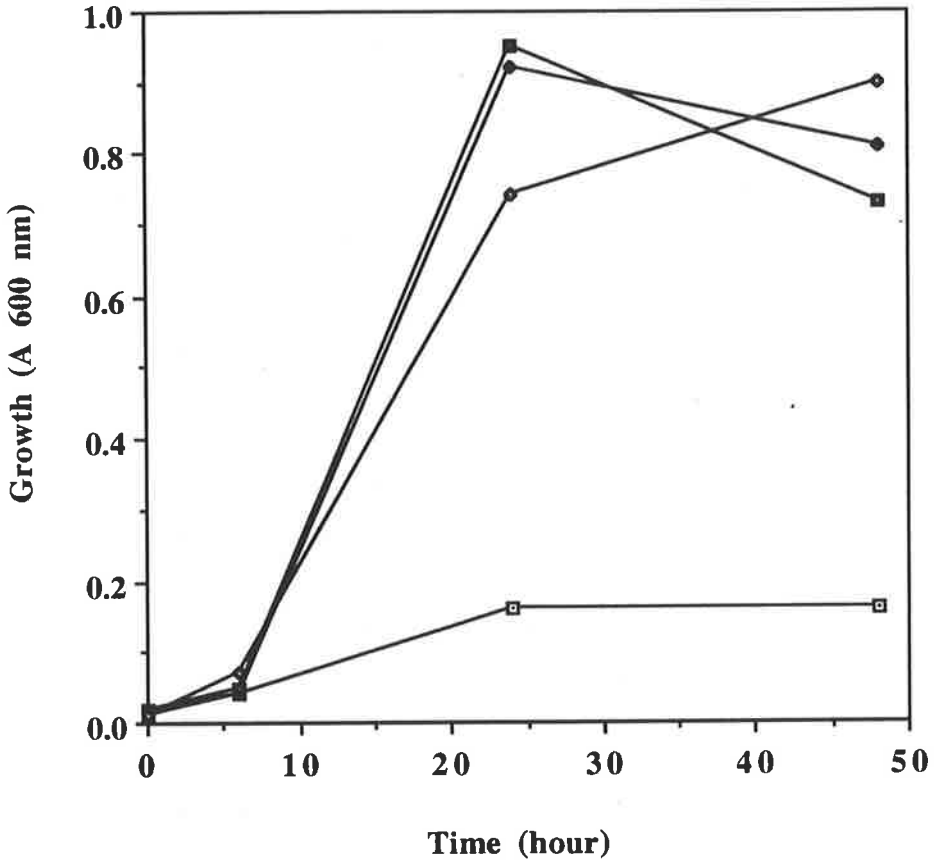


Figure 3.6a Lactic acid utilisation by *S. ruminantium* subsp. *lactilytica.*

—□— L-lactate,

—◆— D-lactate.

The bacteria were grown on M medium with 51.32 mM (D)-and 51.32 mM (L)-lactate plus 8 mM fumarate and incubated at 39°C. The lactic acid concentrations were measured every 24 hours. The results were expressed as µmole quantities of lactate remaining in the media per 10^7 viable bacteria.

Figure 3.6a Lactic acid utilisation by *S. ruminantium*.

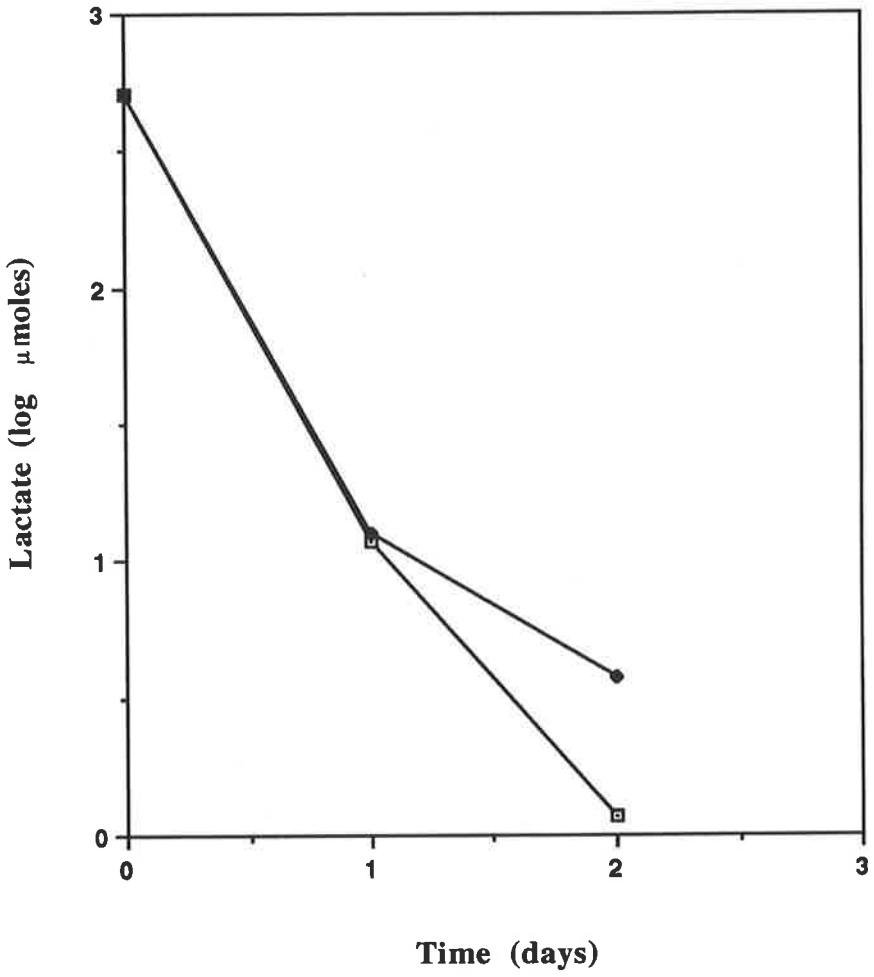


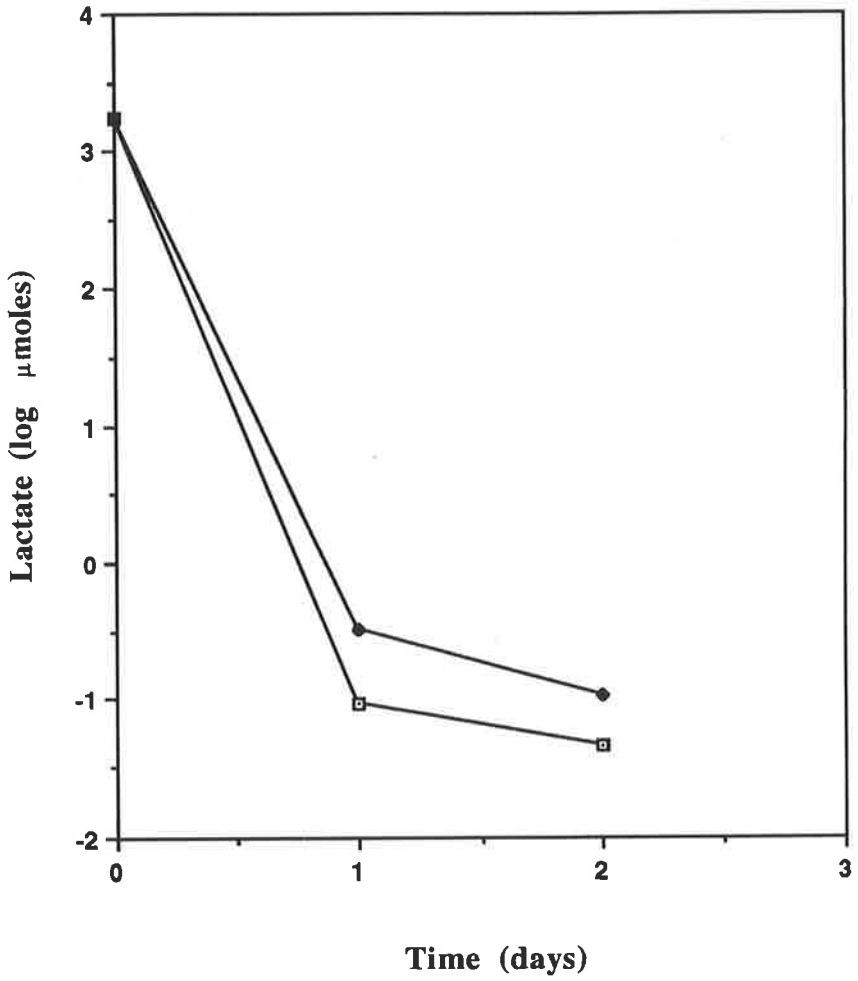
Figure 3.6b Lactic acid utilisation by *M. elsdenii.*

—□— L-lactate,

—●— D-lactate.

The bacteria were grown on M medium with 51.32 mM (D)-and 51.32 mM (L)-lactate plus 8 mM fumarate and incubated at 39°C. The lactic acid concentrations were measured every 24 hours. The results were expressed as µmoles quantities of lactate remaining in the media per 10^7 viable bacteria.

Figure 3.6b Lactic acid utilisation by *M. elsdenii*.



3.4 Discussion.

There are two main advantages of grain supplementation. Firstly, it increases the total volatile fatty acid concentration in the rumen probably as a result of the rapid fermentation of grain compared with the more fibrous lucerne chaff. Secondly, the change in microbial populations towards lactic acid utilisers results in an increased ruminal proportion of propionate versus acetate. This is very important in animals because efficient degradation of dietary carbohydrates by rumen microorganisms leads to a glucose deficiency in the host (Church, 1988). Therefore, propionate plays a crucial role as a precursor for gluconeogenesis when it is an energy source or converted to glucose in the liver.

The result of this experiment supports previous experiments by Mackie and Gilchrist (1979) and Huber et al. (1976), that gradual adaptation to a high grain diet over a 20 day period of time can prevent the development of lactic acidosis in sheep. This is demonstrated by the stability of ruminal pH, which never dropped below 5.3 during the experiment. In addition, the ruminal concentration of lactic acid was maintained at a low level (less than 5 mM).

During stepwise adaptation to a high grain diet, the predominant lactilytic bacterial specie identified was *S. ruminantium* subspecies *lactilytica*. In contrast to the data of Mackie and Gilchrist (1979), *Anaerovibrio sp.* were not observed during morphological examinations of rumen samples. A possible explanation for this is that the maintenance diet prior to the experiment reported here was lucerne containing a high level of protein nitrogen (18-19% of dry matter). In contrast, the sheep used in the experiment of Mackie and Gilchrist were maintained on a relatively low-protein containing corn stover diet (8-12% of dry matter). Since *S. ruminantium* is proteolytic, there may have been a positive selection for Selenomonads to become predominant members of the flora. In addition, the grain supplied in the work of Mackie and Gilchrist was maize, containing rumen

insoluble zein. This may have restricted bacterial access to the grain and so influenced the bacterial populations present.

The numbers of lactic acid utilisers found in this experiment were much lower than that investigated by Mackie and Gilchrist (1979). This may be due to the differences in the bacterial isolation media used. In their work, 2% trypticase and 0.2% yeast extract were used. This high concentration of trypticase and yeast extract allowed not only lactic acid utilizers but also proteolytic bacteria to grow. In the experiment described here, 0.1% (w/v) trypticase and 0.01% (w/v) yeast extract were used. This low concentration of yeast extract and trypticase may inhibit the growth of bacteria which do not use lactic acid as a carbon and energy source. Higher lactic acid utilising bacterial populations in Mackie's work may also be influenced by the use of more complex and balanced diets used in the experiment.

The increase in lactic acid utilizing bacteria during the experiment is not merely a reflection of the increased lactic acid concentration in the rumen, but is also due to the increased availability of glucose from wheat grain. Both *S. ruminantium* subspecies *lactilytica* and *M. elsdenii* are able to use glucose as a carbon source.

Previous work by Nisbet and Martin(1990) has shown that the growth of *S. ruminantium* subspecies *lactilytica* was stimulated by the presence of 10 mM malate. This is probably due to the involvement of the randomising succinate-propionate pathway for lactate metabolism in this species (Linehan et al., 1978) and may be important in maintaining effective long term levels of *S. ruminantium* subspecies *lactilytica* in the rumen following inoculation. The results of the experiment examining the effect of fumarate and aspartate on growth of *S. ruminantium* subspecies *lactilytica* suggests that dietary supplementation with either of these compounds, perhaps also including lactic acid, may help maintain the introduced lactilytic population of *Selenomonas* following inoculation. This result is in agreement with the work of Nisbet and Martin (1990) in which they demonstrated

enhanced growth of *S. ruminantium* on a lactate medium supplemented with aspartate, malate or fumarate and enhanced lactate uptake, particularly with malate. The effect of these compounds was not tested on other lactilytic species. The results reported here show that *M. elsdenii* growth was not stimulated by either fumarate or aspartate. The differences in response of *S. ruminantium* subspecies *lactilytica* and *M. elsdenii* is probably related to the different metabolic pathways involved in lactate utilization of the two species; *S. ruminantium* subspecies *lactilytica* uses the succinate-propionate pathway in which malate and fumarate are intermediates (Ladd, 1959) and aspartate can be metabolized to fumarate. Linehan et al. (1978) has suggested that the growth enhancing effect of dicarboxylic acids is due to the sparing effect on oxaloacetate that is otherwise used for gluconeogenesis. *M. elsdenii* utilizes the acrylate pathway in which neither fumarate nor malate are intermediates. Whether a pathway intermediate such as pyruvate would stimulate *M. elsdenii* growth is not known.

3.5 Conclusion.

The result of this experiment confirms previous findings that gradual adaptation to a high grain diet over a 20 day period of time can prevent lactic acidosis in sheep. In addition, grain feeding increases total volatile fatty acid concentration, producing an important energy source for the animal. Grain feeding also increases the proportion of ruminal propionate at the expense of acetate.

In contrast to the data of Mackie and Gilchrist (1979), *Selenomonas ruminantium* subspecies *lactilytica* was found to be a predominant lactic acid utilizer in this experiment.

Chapter 4 Control of lactic acidosis in grain-fed sheep by bacterial inoculations.

4.1 Introduction.

Some investigators have reported that the accumulation of lactic acid in the rumen of sheep abruptly fed with a high grain diet is caused by a rapid proliferation of lactate producing bacteria (e.g. *S. bovis* and *Lactobacillus*). In contrast, lactic acid utilising bacteria multiply at a very slow rate.

The previous chapter showed that gradual adaptation to a high grain diet can prevent lactic acidosis in sheep and it was found that the lactilytic bacterial population built up as the amount of grain was increased in the ration. Therefore, the logical approach to controlling the accumulation of lactic acid in the rumen when the animals are fed with a high grain diet is by inoculation with lactate utilizing bacteria . There are some problems related to this: a) the number of bacteria required for an effective inoculum, b) the time of inoculation, and c) which bacterial species is likely to be the most effective in reducing lactate concentrations. In this experiment *Selenomonas ruminantium* subsp. *lactilytica* and *Megasphaera elsdenii* were used as inoculants because they were the predominant lactic acid utilisers found when the animal was adapted to a high grain diet (Chapter 3). The use of dialysis tubing as a vessel for delivery of the inoculum in the second experiment was aimed to separate the introduced bacteria from the endogenous bacteria and to prevent rapid bacterial wash out from the rumen. At the same time this would allow the bacteria to adapt to the rumen.

The purpose of the experiments described here were to determine which bacteria may be used as useful inoculants and are as follows:

1. To study the short term effect of *S. ruminantium* subsp. *lactilytica* inoculation on the accumulation of lactic acid in the sheep rumen.

2. To study the effect of the inoculation of combined *S. ruminantium* subsp. *lactilytica* and *M. elsdenii* on lactic acid levels in the rumen.

4.2 Materials and Methods.

4.2.1 Feeds and animals

In *S. ruminantium* subsp. *lactilytica* inoculation experiment, eight merino ewes (average body weight 40 kg) were used. Four were control animals (uninoculated) and the other four were each inoculated with a pure culture of *S. ruminantium* subsp. *lactilytica* isolated in the previous experiment. All sheep were fed with 800 g of lucerne per day for six weeks before the experiment started. The sheep were starved for 48 hours before being drenched with grain. Acidosis was induced with 1200 g (80%) of cracked wheat diluted into 1200 ml of warm water (39° C) and administered through a stomach tube. 300 g of lucerne chaff (20% of total diet) were also made available every 24 hours to the animals as feed during the experiment.

In the other experiment (inoculation with a mixture of *S. ruminantium* subsp. *lactilytica* and *Megasphaera elsdenii*), five fistulated merino ewes (average body weight 40 kg) were fed with 800 g of lucerne chaff for six weeks before the experiment was begun. Prior to the experiment, all sheep were starved for 24 hours. Three of the sheep were inoculated with bacteria (see section 4.2.2) whilst the other two sheep were used as controls. Wheat slurry [640 g cracked wheat and 640 ml warm water (39°C)] was introduced intraruminally as a bolus via a rumen fistulae.

4.2.2 Bacterial inoculum preparation.

For *S. ruminantium* inoculation, one ml of *S. ruminantium* subsp. *lactilytica* was inoculated into 1 litre of lactate-containing medium (see section 2.1.1.1). For the experiment involving a mixture of *S. ruminantium* and *M. elsdenii*, one ml cultures of *S.ruminantium* and *M. elsdenii* were separately used to inoculate 500 ml of lactate containing medium. The bacteria were incubated for 16 hours at 39°C. Cells were then pelleted at 6000 rpm (6400 *g*) for 10 minutes. The pellet was resuspended in 100 ml of the lactate medium. In the *S. ruminantium* experiment, the sheep to be treated were inoculated orally with 100 ml of bacteria plus 100 ml medium, and control sheep were inoculated with 200 ml medium one hour after grain administration. In the mixed *S. ruminantium* plus *M. elsdenii* experiment, treated sheep were intraruminally inoculated with 100 ml of a mixture of *S. ruminantium* and *M. elsdenii* one hour after grain administration. Two sheep were directly-inoculated (bacteria were mixed with rumen contents) and the third sheep was indirectly-inoculated (bacteria were contained in dialysis-tubing, pore size 10000 Dalton, which was suspended in the rumen). Control sheep were inoculated with 200 ml medium.

4.2.3 Sampling and analysis.

In the *S. ruminantium* experiment, samples were taken using a stomach tube at 0 time (before feeding) and every 8 hours for 24 hours. In the mixed *S. ruminantium* plus *M. elsdenii* experiment, the first sample was collected 3 hours after the animals were fed with 800 g of lucerne chaff (before the animals were starved). The following samples were collected every 24 hours after feeding with grain (prior to the next feeding) for 4 days. The samples from control sheep were collected only up to 24 hours as the animals had become very sick and their treatment was discontinued. Sample treatments were the same as in the chapter 3 (see section 3.2.3). Parameters measured included: rumen pH, blood

and ruminal lactic acid, rumen volatile fatty acids (VFA,s), and numbers of total and lactic acid utilising bacteria.

4.2.4 Statistical analysis.

Statistical analysis was done using analysis of variance with Genstat 5. The degree of significance between controls and treatments were compared using the F test.

4.3 Results.

4.3.1 Inoculation of sheep with *Selenomonas ruminantium* subsp. *lactilytica*.

It has been well understood that the prevention of lactic acidosis in animals adapted to a high grain diet is caused by the proliferation of lactic acid utilising bacteria (Mackie and Gilchrist, 1978; Huber et al., 1976; this thesis). The previous chapter showed that during the adaptation process, *S. ruminantium* subsp. *lactilytica* was the predominant lactic acid utiliser found in the rumen. In this experiment, the ability of *S.ruminantium* subsp. *lactilytica* to prevent lactic acidosis was tested when the animals were abruptly fed with high-wheat rations.

4.3.1.1 Ruminal pH.

The first sign of acidotic animals is the decrease in rumen pH caused by the accumulation of lactic acid in the rumen. To investigate whether inoculation with *S. ruminantium* was able to prevent lactic acidosis, ruminal pH values of control and inoculated sheep were monitored. One of the four inoculated sheep developed acidosis following the introduction of high grain diet, and the data of this animal was not included in the statistical analysis. Table 4.1 shows changes in the ruminal pH of control and inoculated

sheep following grain engorgement. Initial pH values (time 0) were approximately 7.7 and VFA levels were depressed to 50 mM in all sheep. This may be as a consequence of saliva contamination plus the period of starvation prior to commencement of the experiment. pH values significantly ($P<0.001$) declined to 4.7 in uninoculated sheep following the administration of the cracked wheat drench and remained in this level for the duration of the experiment. In contrast, the pH in sheep inoculated with *S. ruminantium* subsp. *lactilytica* was stable at between 6.0 to 7.0, except for sheep 4, which did not conform.

4.3.1.2 Ruminal volatile fatty acids.

The effect of the *S. ruminantium* inoculation on total and individual volatile fatty acid concentrations was investigated during the experiment In inoculated animals, total ruminal VFA's increased significantly ($P<0.001$) from 50 to 92 mM, but then returned to 65 mM. In control animals, total ruminal VFA values fell to less than 20 mM in 8 hours and to less than 10 mM by 16 hours. These changes in inoculated sheep were the result of a significant ($P<0.01$) 2 fold increase in propionate (from 8.7 mM to 17 mM), and a 4 fold increase in butyrate (from 3.5 mM to 14.5 mM), compared with a 2.6 fold decrease in propionate and 6.6 fold decrease in butyrate in control sheep (Table 4.2). Acetate significantly ($P<0.01$) increased by 1.5 fold in inoculated sheep but then returned to original levels, after 24 hours. In control sheep, acetate fell to a level that was 16% of the initial value but then increased to reach 35% of the initial value after 24 hours. In all sheep, other VFA's such as isobutyrate, valerate and isovalerate were detectable but their levels were each less than 3% of total VFA's and are not shown in Table 4.2. The acetate:propionate ratio of control sheep was relatively constant at 3.2 - 3.5 whereas in inoculated sheep, the ratio fell from 3.9 to 1.9. This is largely a reflection of the elevated concentration of propionate.

4.3.1.3 Ruminal lactate.

Ruminal lactic acid concentration is the most important criterion in studying the effect of the *S. ruminantium* inoculation on the development of acidosis in animals. In most cases ruminal L-lactate levels are less than 1 mM. Following grain engorgement, ruminal L-lactate increased significantly to 108 mM within 8 hours in control sheep, but increased to only 2 mM in inoculated animals (Table 4.1). D-lactate was 6-10 fold lower than L-lactate in control animals and no D-lactate was obtained in inoculated animals. Blood lactate levels were similar for both groups of animals and varied between 1.7 and 2.6 mM. Three of the four sheep inoculated with *S. ruminantium* subsp. *lactilytica* appeared to suffer no effect from the grain diet whereas the control sheep exhibited symptoms of physiological distress; diarrhoea, listlessness and lack of appetite. At the completion of the 24 hour experiment, inoculated sheep immediately resumed normal feeding on the original lucerne diet, whereas control sheep took another 3 days before they resumed feeding. When inoculated animals were continued on a grain diet for longer than 24 hours, signs of acidosis occurred (distress, lack of appetite) and the experiment was stopped due to concern for welfare of the animals.

4.3.1.4 Rumen bacterial population.

The effect of bacterial inoculation on ruminal lactic acid utilising bacteria was observed before and after inoculation. This was aimed at monitoring the introduced bacteria following the inoculation. Table 4.3 shows the population of lactic acid utilising bacteria of control and inoculated sheep. In control sheep at zero time, *S. ruminantium* subsp. *lactilytica* was 3.2×10^6 cfu/ml. Following grain feeding, neither *S. ruminantium* subsp. *lactilytica* nor *M. elsdenii* were detected at 10^{-3} dilution. In inoculated sheep, the introduced population of *S. ruminantium* remained between 2.2 to 3.7×10^7 cfu/ml. *M. elsdenii* was detectable around 16 hours at 4×10^5 cfu/ml and had doubled by 24 hours. The total numbers of *M. elsdenii* were always less than 1% of *S. ruminantium* subsp. *lactilytica* .

Table 4.1 Values of ruminal pH, lactate, total VFA and blood lactate of wheat-fed sheep inoculated with *S. ruminantium*.

Parameters	Sampling time (hour)	Control[a]	Inoculated[b]
Rumen pH	0	7.73 ± 0.04	7.59 ± 0.10
	8	4.69 ± 0.06	6.27 ± 0.28***
	16	4.91 ± 0.20	6.44 ± 0.23**
	24	4.92 ± 0.10	6.58 ± 0.29**
Rumen lactate	0	0	0
(mM)	8	120.10 ± 16.18	0
	16	97.43 ± 8.45	0
	24	109.33 ± 12.86	0
Blood lactate	0	2.25 ± 0.26	1.69 ± 0.20
(mM)	8	1.67 ± 0.22	2.15 ± 0.46
	16	1.84 ± 0.24	2.33 ± 0.33
	24	1.93 ± 0.16	2.60 ± 0.63
Total VFA	0	52.09 ± 7.08	50.87 ± 4.03
(mM)	8	15.03 ± 1.06	91.85 ± 10.41***
	16	6.16 ± 1.26	79.80 ± 10.44***
	24	11.38 ± 2.50	64.75 ± 7.88***

a) Mean ± standard error mean of four replications.
b) Mean ± standard error mean of three replications
**) very significant (P<0.01) difference
***) highly significant (P<0.001) difference

Table 4.2 Effect of *S. ruminantium* inoculation on individual VFA's of wheat-fed sheep.

Parameters	Sampling time (hour)	Control[a]	Inoculated[b]
Acetate mM	0	31.0 ± 3.65	35.88 ± 3.21^{ns}
	8	10.74 ± 1.13	53.80 ± 8.06**
	16	5.05 ± 0.96	42.61 ± 8.39**
	24	10.65 ± 2.50	31.67 ± 20.0*
Acetate %	0	63.94 ± 2.50	70.46 ± 1.18^{ns}
	8	70.94 ± 2.92	58.16 ± 2.71*
	16	83.50 ± 4.25	52.78 ± 4.72*
	24	92.10 ± 2.71	47.76 ± 5.28***
Propionate mM	0	8.84 ± 0.92	9.16 ± 1.03^{ns}
	8	3.38 ± 0.38	21.37 ± 2.39***
	16	0.73 ± 0.42	21.13 ± 2.53***
	24	0	17.0 ± 0.90
Propionate %	0	19.13 ± 2.66	18.03 ± 1.65^{ns}
	8	22.96 ± 3.25	23.27 ± 0.10^{ns}
	16	9.45 ± 5.48	26.79 ± 2.81*
	24	0	27.20 ± 4.28
Butyrate mM	0	3.21 ± 0.50	3.43 ± 0.24^{ns}
	8	0.48 ± 0.03	13.64 ± 1.95***
	16	0.28 ± 0.05	13.65 ± 1.13***
	24	0.47 ± 0.04	14.51 ± 1.36***
Butyrate %	0	6.55 ± 0.67	6.76 ± 0.06^{ns}
	8	3.22 ± 0.14	15.15 ± 2.72**
	16	5.14 ± 1.12	17.53 ± 2.15**
	24	5.12 ± 1.70	22.60 ± 0.94***

a) Mean ± standard error mean of four replications
b) Mean ± standard error mean of three replications
*) significant ($P<0.05$) difference
**) very significant ($P<0.01$) difference
***) highly significant ($P<0.001$) difference
ns) not significant
%) percent of total VFA's

Table 4.3 Lactic acid utilising bacterial populations of wheat-fed sheep inoculated with *S. ruminantium.*

Sampling time (hour)	*Selenomonas ruminantium*		*Megasphaera elsdenii*	
	Control[a]	Inoculated[b]	Control[a]	Inoculated[b]
	---------- x 10^{-5}/ml rumen fluid ----------			
0	32.3 ± 5.9	35.8 ± 6.6	< 10^3	< 10^3
8	< 10^3	222.7 ± 55.3	< 10^3	<10^3
16	< 10^3	374.7 ± 22.5	<10^3	4
24	< 10^3	315.7 ± 37.5	<10^3	9

a) Mean ± standard error mean of four replications
b) Mean ± standard error mean of three replications
<10^3 = the population is less than 1000

4.3.2 Inoculation with a mixture of *Selenomonas ruminantium* subsp. *lactilytica* and *Megasphaera elsdenii*.

Since *S. ruminantium* subsp. *lactilytica* and *M. elsdenii* were found to be the predominant lactic acid utilisers in the rumen of the sheep adapted to grain diet (Chapter 3), the ability of these bacteria in preventing lactic acidosis in wheat-fed sheep was investigated. In order to monitor the introduced bacteria, and the effect of inoculation on endogenous bacteria, the inoculum was contained in a dialysis tubing (pore size 10000 Dalton) in one of the three treated sheep.

4.3.2.1 Ruminal pH, lactate, and volatile fatty acids.

Animals suddenly fed with a diet containing high fermentable carbohydrate will usually develop acidosis. Ruminal parameters such as pH, lactate, and volatile fatty acids were drastically changed in acidotic animals. To investigate the effectiveness of inoculation with a combination of *S. ruminantium* and *M. elsdenii* in preventing lactic acidosis in grain fed sheep, the parameters above were measured. Following intraruminal administration of wheat slurry, control sheep given no bacterial inoculum showed a dramatic decrease in ruminal pH from 5.9 to 4.6 (Figure 4.1). All inoculated sheep, directly or indirectly, had a higher ruminal pH ($P<0.05$) than the control sheep and the values remained higher than 5.5 throughout the experiment.

Grain administration significantly ($P<0.05$) elevated the lactic acid concentration of control sheep from 0 mM to 99.0 mM in 24 hours. Meanwhile, the L-lactic acid concentration of inoculated sheep remained at less than 1 mM for three days (Figure 4.2).

All animals had total VFA values of more than 100 mM before grain administration (Figure 4.3). One day later, total VFA's for inoculated animals slightly decreased to 65 mM in directly inoculated sheep and to 73 mM in indirectly inoculated sheep. However, in control animals, total VFA values fell significantly ($P<0.05$) to 21 mM.

There was no significant difference ($P>0.05$) in rumen acetate, propionate and butyrate concentrations between control and treated animals. The proportion of acetate in control sheep decreased from 71 to 60 percent of total VFA (Table 4.4). On the other hand, the proportion of propionate and butyrate slightly increased from 17 to 25 percent and from 12 to 15 percent, respectively. In directly inoculated sheep, the proportion of acetate of total volatile fatty acids fell to 40 percent one day after inoculation,but then gradually increased to 50 percent in the following days. The proportion of propionate did not change throughout the experiment. However, the proportion of butyrate went up from 12 percent before inoculation to 40 percent one day after inoculation, but it gradually went down during the following days. The same pattern was found in indirectly inoculated sheep. The proportion of acetate decreased from 69 percent to 17 percent one day after inoculation, but then it increased again during the following days. The proportion of butyrate increased more than five fold one day after the inoculation, but then decreased during the next three days.

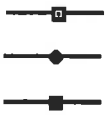
4.3.2.2 Rumen bacterial population.

Grain engorgement that leads to lactic acidosis also changes the rumen microbial population due to alterations in the rumen environment. The changes of total and lactic acid utilising bacteria following grain feeding were monitored in controls and inoculated sheep. Figure 4.4 shows the number of lactate utilising bacteria in the rumen fluid. In control animals, counts of lactate utilising bacteria did not change one day after grain administration. On the other hand, counts of lactate utilising bacteria in directly and indirectly inoculated sheep increased significantly ($P<0.05$) to more than 6×10^7 cfu/ml of rumen fluid in four days.

Total bacterial numbers of control and inoculated sheep are shown in Figure 4.5. The bacterial count from control sheep did not change during the investigation. In contrast, the total number of bacteria from inoculated sheep, both directly and indirectly inoculated,

increased significantly ($P<0.05$) to more than 9×10^{10} cfu/ml of rumen fluid. There were at least 4 or 5 different bacterial species observed from the inoculated sheep compared to only 2 species in controls. About 50% of total bacteria in inoculated animals was *Selenomonas*-like bacteria. Meanwhile, control animals were dominated by small cocci bacteria, possibly *Streptococcus bovis*.

Figure 4.1 Ruminal pH of wheat-fed sheep inoculated with a mixture of *S ruminantium* and *M. elsdenii*.



control (without inoculation),
direct inoculation,
indirect inoculation.

Rumen pH were measured directly after sampling. The data were average of two replications for control and direct inoculations but only one measurement for indirect inoculation. Bars = standard error means. *= significant (P<0.05) difference.

Figure 4.1 Ruminal pH of wheat-fed sheep inoculated with a mixture of S. ruminantium and M. elsdenii.

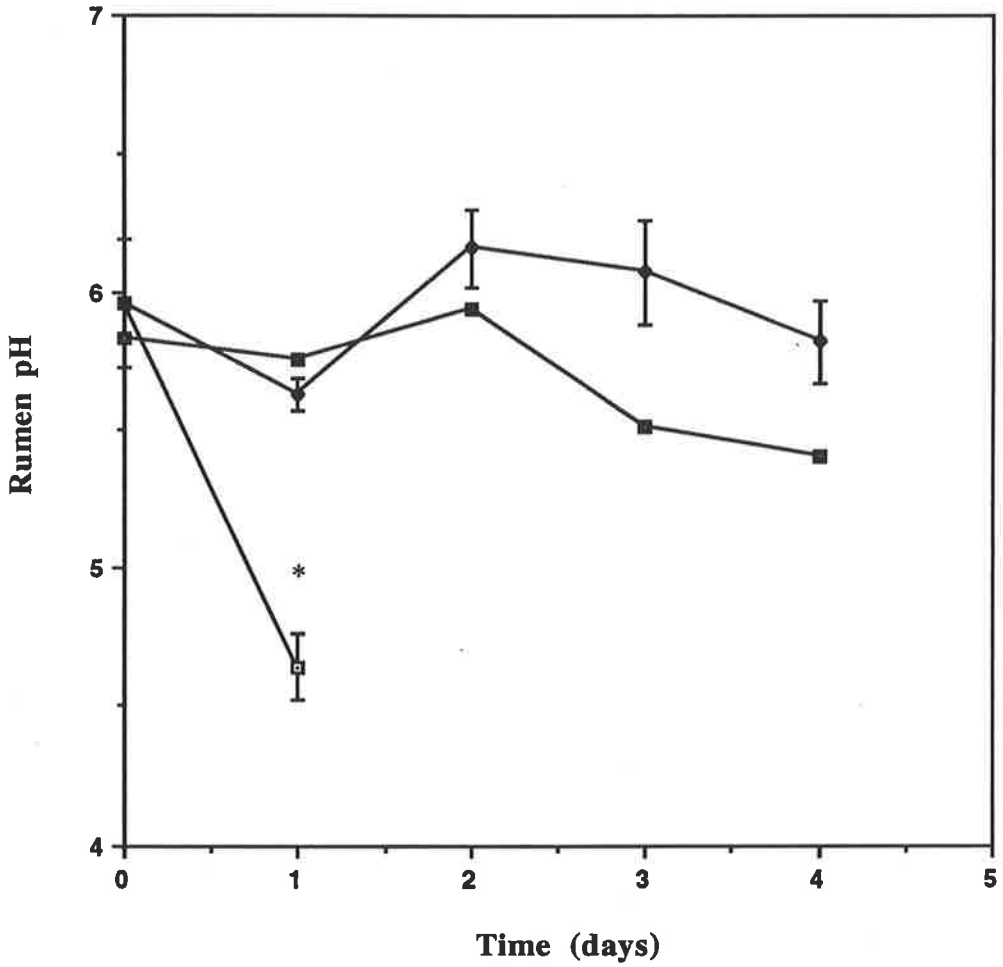
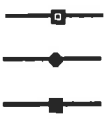


Figure 4.2 Ruminal lactic acid concentrations of wheat-fed sheep inoculated with a mixture of *S. ruminantium* and *M. elsdenii*.



control (without inoculation),
direct inoculation,
indirect inoculation.

The data were average of two replications for control and direct inoculations but only one measurement for indirect inoculation. Bars = standard error means.
*= significant ($P<0.05$) difference.

Figure 4.2 Ruminal lactate concentrations of wheat-fed sheep inoculated with a mixture of S. ruminantium and M. elsdenii

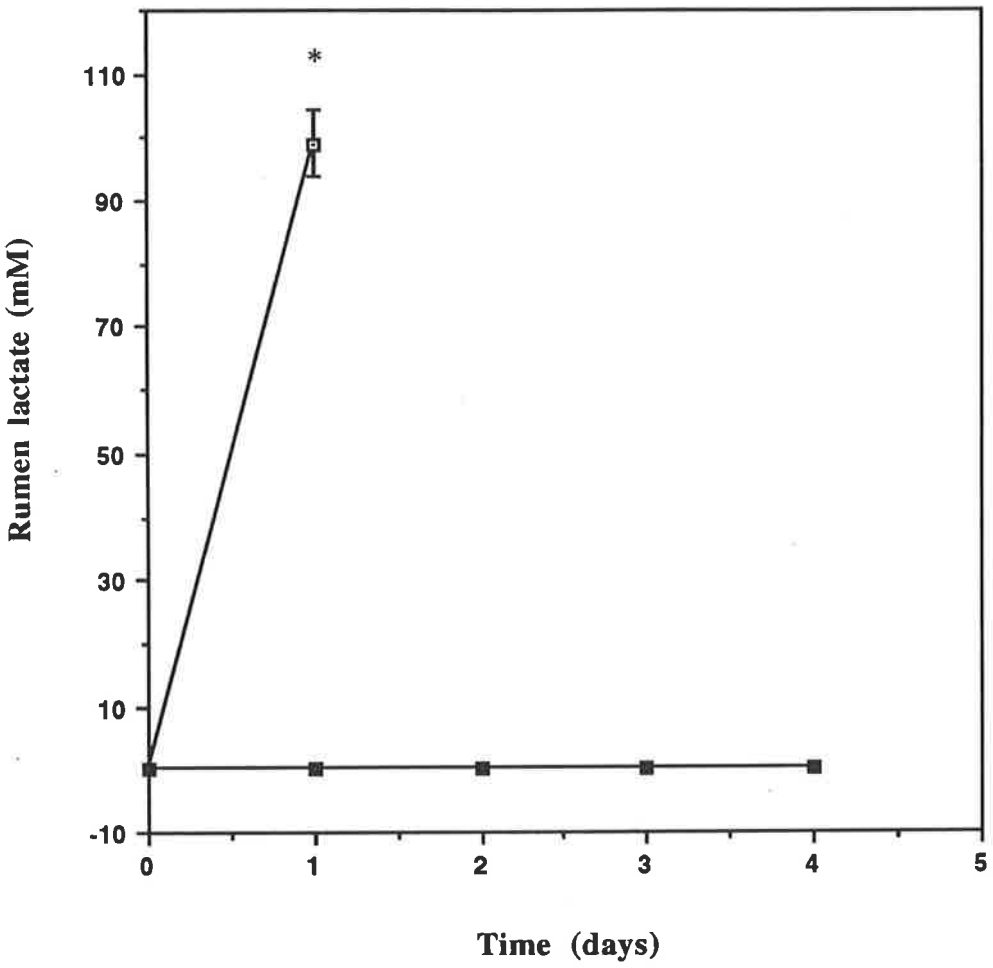


Figure 4.3 Ruminal total VFA's of wheat-fed sheep inoculated with a mixture of *S. ruminantium* and *M. elsdenii*.

control (without inoculation),
direct inoculation,
indirect inoculation.

The data were average of two replications for control and direct inoculation but only one measurement for indirect inoculation. Bars = standard error means.
*= significant (P<0.05) difference.

Figure 4.3 Ruminal total VFA concentrations of wheat-fed sheep inoculated with a mixture of S. ruminantium and M. elsdenii.

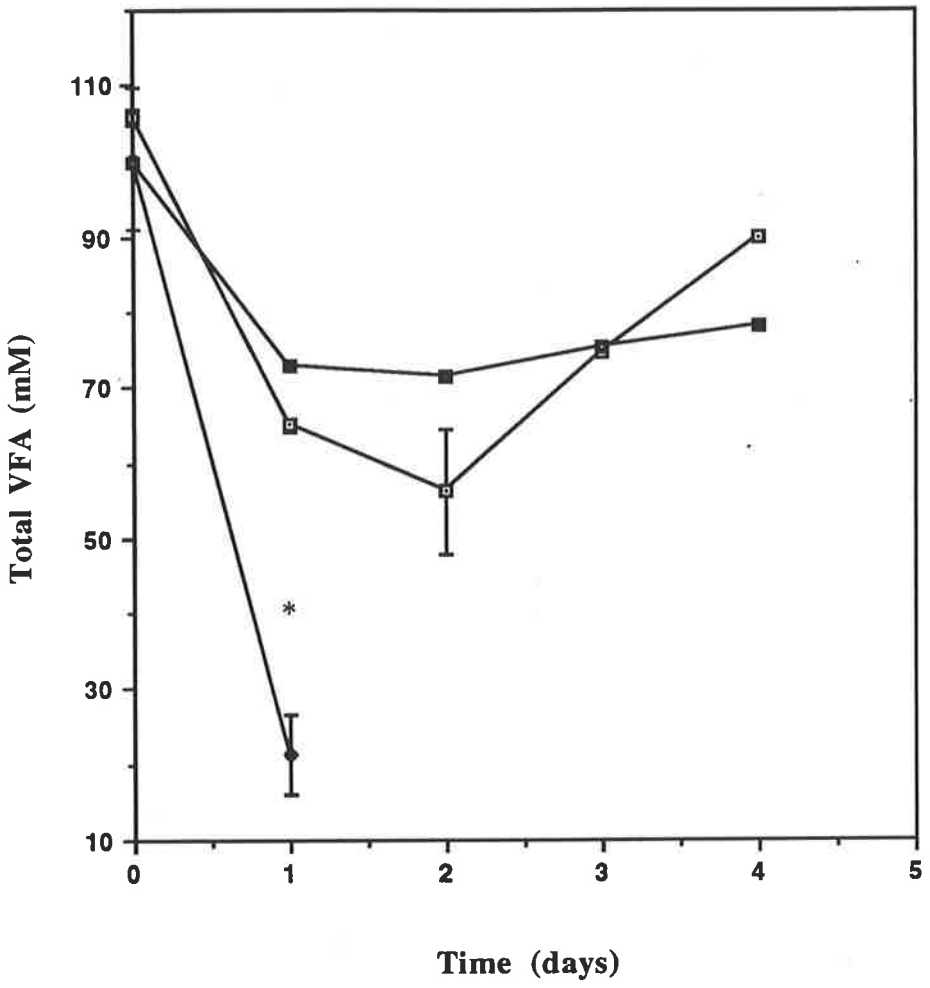
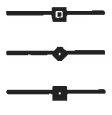


Figure 4.4 Ruminal lactic acid utilising bacterial populations of wheat-fed sheep inoculated with a mixture of *S. ruminantium* and *M. elsdenii*.



control (without inoculation),
direct inoculation,
indirect inoculation.

100 µl serially diluted rumen samples (10^{-4} to 10^{-6}) were plated out onto lactate-containing medium and incubated at 39° C for five days. At least twenty colonies were picked and morphologically examined under light microscopy. The data were average of two replications for control and direct inoculations but only one measurement for indirect inoculation. Bars = standard error means. *= significant ($P<0.05$) difference.

Figure 4.4 Lactic acid utilising bacterial populations of wheat-fed sheep inoculated with S. ruminantium and M. elsdenii.

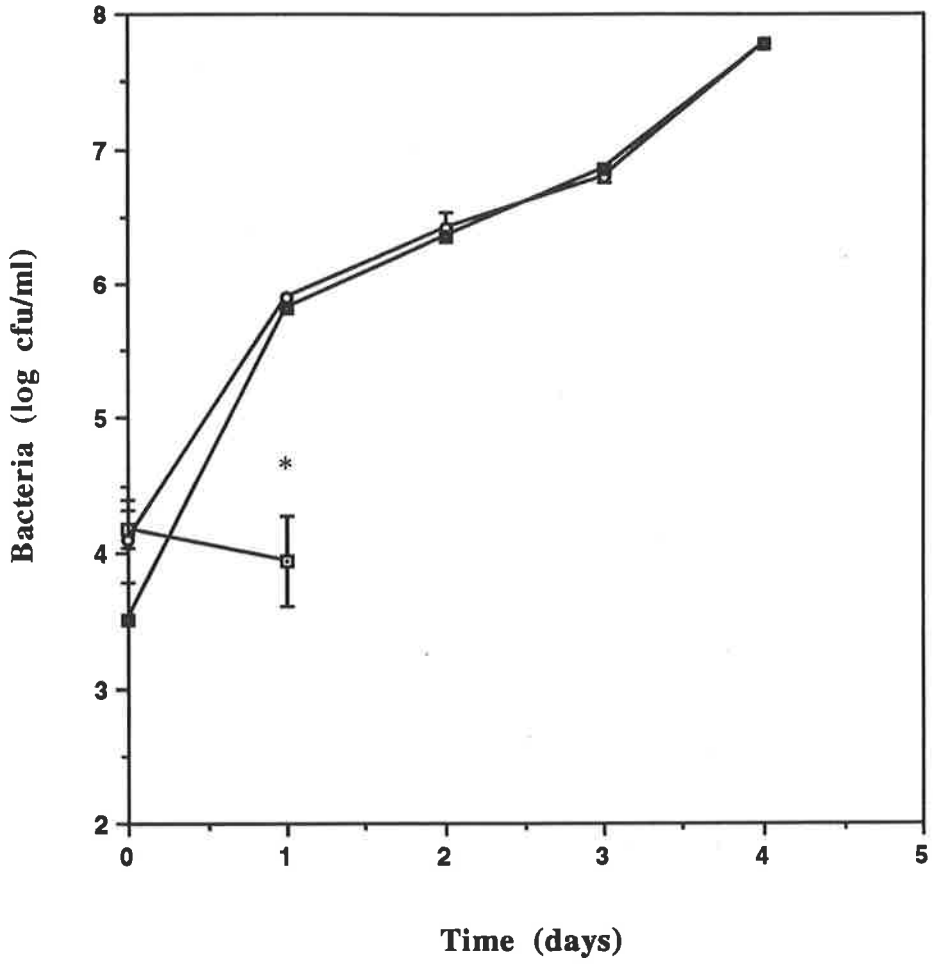


Figure 4.5 Ruminal total bacterial populations of wheat-fed sheep inoculated with a mixture of *S. ruminantium* and *M. elsdenii*.

—□— control (without inoculation),
—●— direct inoculation,
—■— indirect inoculation.

100 µl serially diluted rumen samples (10^{-6} to 10^{-7}) were plated out onto complex medium and incubated at 39° C for five days. The colonies were counted from plates containing at least twenty colonies. The data were average of two replications for control and direct inoculations but only one measurement for indirect inoculation.
Bars = standard error means. *= significant ($P<0.05$) difference.

Figure 4.5 Total bacterial populations of wheat-fed sheep inoculated with S. ruminantium and M. elsdenii.

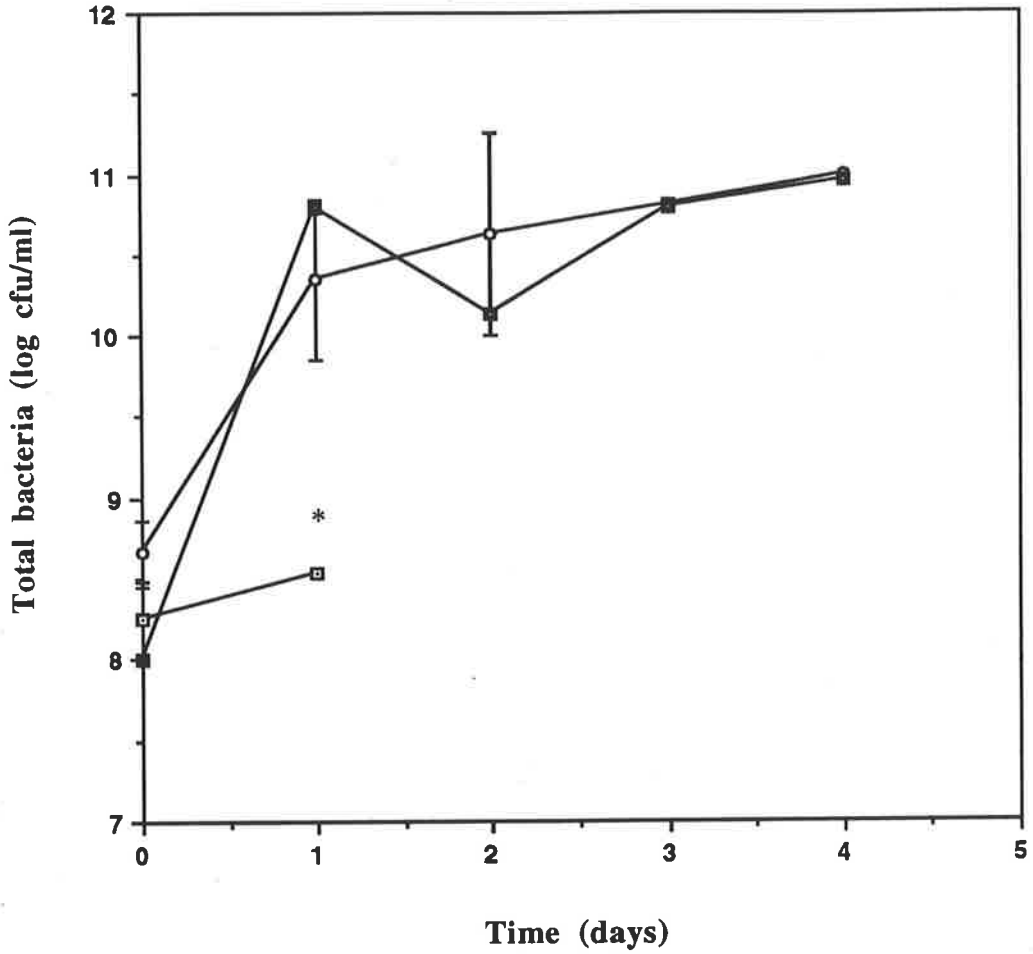


Table 4.4 Effect of inoculation with a mixture of *S. ruminantium* and *M. elsdenii* on individual VFA's of wheat-fed sheep.

Parameters	Sampling time (day)	Control[a]	Direct[a] Inoculation	Indirect[b] Inoculation
Acetate mM	0	70.19 ± 7.61	72.12 ± 0.70	66.67
	1	12.51 ± 2.44	24.41 ± 7.07	10.44
	2	na	26.11 ± 4.41	30.66
	3	na	34.13 ± 2.13	38.74
	4	na	42.57 ± 2.90	40.35
Acetate %	0	71.38 ± 1.26	68.98 ± 1.30	67.44
	1	59.64 ± 3.35	40.32 ± 10.90	17.37
	2	na	50.32 ± 2.18	44.64
	3	na	48.32 ± 1.92	55.08
	4	na	49.72 ± 4.34	54.90
Propionate mM	0	16.53 ± 1.14	19.91 ± 2.84	20.72
	1	5.26 ± 1.07	12.28 ± 8.07	3.26
	2	na	10.40 ± 1.04	8.61
	3	na	13.97 ± 1.21	10.87
	4	na	15.17 ± 2.40	16.22
Propionate %	0	16.88 ± 0.37	19.02 ± 2.55	20.96
	1	25.02 ± 1.18	20.13 ± 12.99	5.42
	2	na	20.22 ± 0.55	12.54
	3	na	19.84 ± 2.17	15.45
	4	na	17.75 ± 3.14	22.07
Butyrate mM	0	11.46 ± 0.18	12.54 ± 1.19	11.47
	1	3.51 ± 1.78	23.50 ± 13.88	46.42
	2	na	15.10 ± 1.07	29.41
	3	na	22.47 ± 0.69	20.73
	4	na	28.03 ± 6.94	16.93
Butyrate %	0	11.75 ± 0.89	12.01 ± 1.25	11.60
	1	15.35 ± 4.53	39.55 ± 23.88	77.21
	2	na	29.47 ± 1.63	42.82
	3	na	31.84 ± 0.26	29.47
	4	na	32.54 ± 7.46	23.03

a) Mean ± standard error means of two replications
b) data from one sheep
na) not available
mM) millimolar
%) percent of total VFA's

4.4 Discussion.

Imbalance between production and utilisation of lactic acid in the rumen of animals fed with a high concentrate diet leads to lactic acidosis. This is due to a slow proliferation of lactic acid utilising bacteria. Therefore, inoculation with high numbers of lactic acid utilising bacteria may be necessary to prevent lactic acid accumulation in the rumen. From these experiments it was shown that *S.ruminantium* subsp. *lactilytica* can be used as an inoculum for controlling lactic acidosis in grain fed sheep for a short period of time. The data showed that inoculated sheep had no symptoms of acidosis whereas control sheep demonstrated the typical metabolic disturbance associated with acute acidosis; low ruminal pH, depressed total VFA's, an almost complete absence of propionate or butyrate and high ruminal lactate as well as symptoms of physiological distress; diarrhoea, listlessness and lack of appetite. Resumption of feeding on the original lucerne diet was also delayed in control versus inoculated animals. This may be attributed to several possible causes such as: (1) profound modification of the rumen flora; (2) damage to the ruminal epithelium; (3) to the systemic effects of toxic or pyrogenic substances produced in the rumen (Phillipson, 1969)

The result of these experiments can be contrasted with values obtained at the end of the 20 day stepwise adaptation period (Chapter 3). Ruminal (D)- and (L)- lactate levels in adapted and inoculated sheep were negligible. Ruminal pH was similar in both groups of animals and total VFA's were in excess of 60 mM in both groups. The final concentrations of propionate and butyrate were similar for both groups of sheep and were at least 2 fold higher than initial values.

One of the four sheep inoculated with *S. ruminantium* in the 24 hour experiment developed acidosis. This may have been due to individual differences in tolerance to a grain diet, especially the differences in individual animal physiology or metabolism. Similar results were reported by Allison et al. (1964), when one of the four control lambs

that were induced with wheat did not develop acidosis.

Blood lactic acid did not increase in all sheep in the trial. This is supported by the report by Muir et al. (1980) that plasma lactate did not increase during lactic acidosis because elevation of plasma lactate appears to lag at least 10 hour behind an elevation in rumen lactate. This may be the time required to cause low pH induced damage to the rumen epithelial tissue.

Abnormally high initial ruminal pH (7.7) in *S. ruminantium* inoculation experiment was perhaps due to the saliva contamination as samples were taken using a stomach tube through the mouth. This suggests that the use of fistulated animals was necessary in studying rumen environments in order to avoid the saliva contamination problem as saliva contamination may lead to the misinterpretation of the data obtained. High initial ruminal pH may also be caused by low concentration of volatile fatty acids in the rumen. This could have been the result of 48 hours starvation prior to the experiment so as to minimise the effect of the previous feeding to the results obtained.

In both experiments, ruminal pH of control animals was very low compared with inoculated animals. This is a result of the accumulation of lactic acid in the rumen, as lactic acid absorption across the rumen wall is very slow. It is estimated that the rate of lactate absorption from the rumen is only 10% of that of VFA's (Argenzio and Whipp, 1980). Low rumen pH may also be due to a reduction in saliva production as the animals were fed with grain. Church (1988) estimated that saliva production was reduced by 20% when grain was introduced to animals compared to animals on a long hay diet. The reduction in the saliva production is due to failure in stimulation of chewing and rumination processes. The rapid drop of the rumen pH could also be the result of a decrease in ammonia concentration in the rumen since ammonia can act to prevent decreases in rumen pH, particularly when the concentration of ammonia is 20 mg/dl or greater (Hoover and Wolin, 1992).

The relatively low concentration of ruminal VFA's in control sheep in both experiments may be due to an increase in their absorption under low pH conditions (Annison and Armstrong, 1970). It may also be the result of reduced production in the rumen during acidosis, since bacterial diversity in the rumen is reduced in acidotic animals, and most lactic acid producing bacteria produce only small amount of VFA's. Low VFA concentration can also be influenced by the increase in rumen fluidity, since rumen hypertonicity causes body fluid to enter the rumen (Huber, 1971).

The increase in the proportion of butyrate of total VFA's in inoculated sheep especially in the experiment with a mixture of bacteria as inoculants (see Table 4.4) may be due to the increase in the *M. elsdenii* population in the rumen as this species produces high butyric acid from lactate (see Figure 3.4e). Some researchers have reported that the increase in butyrate proportion in the rumen may be contributed by the increase in protozoal population as protozoa produce mainly butyric acid from carbohydrate fermentation (Hungate, 1966) and large populations in the rumen have been associated with high butyrate fermentation patterns (Eadie et al., 1970). In contrast, Eadie et al. (1970) also reported that in defaunated (protozoa-free) animals, the proportion of butyrate in the rumen increases. This means that the role of protozoa to the increase of butyrate proportion in the rumen is questioned.

From these experiments it was noted that bacterial inoculation can be used to manipulate the proportion of individual VFA's in the rumen. Depending on the purpose of the experiment, certain individual VFA's may be increased by inoculating a specific bacterium into the rumen. In feedlot conditions, inoculation with propionate-producing bacteria such as *S. ruminantium* may be very beneficial as propionate can be used as a precursor of gluconeogenesis.

Although modifications such as the addition of intermediate substances (e.g. fumarate)

and multiple bacterial inoculations have been examined, the prevention of lactic acidosis by inoculation with *S. ruminantium* for longer than 24 hours was not successful. This may be because *S. ruminantium* prefers to use maltose or glucose generated from starch to produce lactic acid rather than to utilise the lactic acid produced by other bacteria (see Figure 5.5). It is also possible that rapid lactic acid utilisation in the first 24 hours reduced the selective advantages for *S. ruminantium* and therefore resulted in a reduced population that could not keep up with lactic acid producing bacteria in the following days. Therefore, lactic acidosis developed, after 24 hours.

It is not clear whether the increase in lactic acid utilising bacteria in inoculated sheep is due to the proliferation of the introduced bacteria or the proliferation of the original lactic acid utilising rumen bacteria. This is because there is no marker carried by the introduced bacteria. It is possible that both groups of bacteria utilise lactate, glucose and other nutrients in the rumen and inhibit the outgrowth of amylolytic bacteria by restricting the availability of other growth factors (e.g. ammonia and minerals). It could also be possible that the introduced bacteria help to utilise lactic acid at the early stage of the experiment and then allow the original lactic acid utilising rumen bacteria to adapt and proliferate at the later stages. This is supported by evidence of an increase in the *M. elsdenii* population after 16 hours of grain feeding in the *S. ruminantium* inoculated sheep (see Table 4.3).

The use of dialysis tubing in the sheep treated with a mixture of *S. ruminantium* and *M. elsdenii* was aimed at separating the introduced bacteria from endogenous bacteria and preventing rapid wash out of introduced bacteria from the rumen, but still allow low molecular weight substances (e.g. lactate, VFA's) to pass through the tubing. Introducing the bacteria in the dialysis tubing also allowed time for the bacteria to adapt to the conditions in the rumen. Although only one animal was used in this treatment, the data demonstrate the importance of bacterial inoculation in building up the endogenous lactic acid utilising bacteria in the rumen. The number of lactic acid utilising bacteria

inside the tube decreased from 10^{10} cfu to 10^{7} cfu after two days. This may be due to an increase in the endogenous lactic acid utilising bacterial population in the rumen, a limited flow of lactic acid into the tubing or because the dialysis tubing floated on the surface of the rumen digesta and thus restricted nutrient supply to the bacteria.

The use of dialysis tubing may be very useful in studying the ecology of the rumen bacteria especially in relation to the survival of introduced bacteria into the rumen, the interaction between introduced and endogenous rumen bacteria and the co-existence of two or more different species of bacteria when they are introduced into the rumen.

The effectiveness of the mixture of *S. ruminantium* and *M. elsdenii* in preventing lactic acidosis may be due to a rapid utilisation of lactic acid especially by *M. elsdenii*. Low lactic acid concentrations inhibited the domination of lactic acid producing bacteria in the rumen. Low ruminal lactic acid may therefore cause a decline in the population of the *M. elsdenii*. However, in the presence of *S. ruminantium*, this decline may be prevented, possibly because *S. ruminantium* could produce lactic acid from other metabolic products of starch. The ability of the mixture of these bacteria to prevent lactic acidosis may also be due to production of intermediate(s) that can stimulate growth of other bacteria, therefore inhibiting the domination of *S. bovis* in the rumen. This is evidenced by the increase in total bacteria (Figure 4.5) one day after grain feeding. The increase in total bacteria may also be due to the increase in readily available carbohydrate in the rumen compared with time zero when the animals were fed with lucerne chaff.

4.5 Conclusion.

From these experiments it was concluded that the use of *S. ruminantium* subspecies *lactilytica* as a probiotic (live-bacterial inoculant) can prevent lactic acidosis at least for a 24 hour period, but failed to do so for longer periods of time. A single inoculation with a mixture of *S. ruminantium* subspecies *lactilytica* and *M. elsdenii* prevented lactic acid accumulation for four days. It seems likely that *M. elsdenii* plays an important role in lactic acid utilisation as demonstrated by the increase in butyric acid especially in indirectly inoculated sheep. However, because of animal-animal variations further studies of the effect of individual and combinations of the bacterial inoculations will be carried out *in vitro*.

Chapter 5 Control of lactic acid accumulation by antibiotic treatment and bacterial inoculations *in vitro*.

5.1 Introduction.

Continuous culture studies have been carried out by many researchers who are involved in ruminant nutrition (Russell and Baldwin, 1978; Russell and Dombrowski, 1980; Russell et al., 1981; Isaacson et al., 1974). These continuous culture techniques have allowed investigators to study rumen microbial metabolism under conditions which more closely approximate *in vivo* fermentation than batch culture conditions. The advantages of using a continuous culture fermenter are that the conditions are reproducible and it is convenient to study particular aspects by modifying specific conditions without the confounding factors of physiological differences between animals. Of course, there are some weaknesses in the continuous culture system, such as disappearance of protozoa from the cultures. The length of the experiment is therefore limited.

The development of RUSITEC (rumen simulation technique) has helped to minimise these problems (Hoover et al., 1976; Czerkawski and Breckenridge, 1979). This technique was designed to simulate differential flows of liquids and solids that occur in the rumen. In addition, this technique can maintain protozoa longer than a commercial fermenter. In this study, the use of RUSITEC was not considered necessary as the lactic acid production was induced with soluble starch.

The studies described here were carried out because of the variability that one sees with *in vivo* experiments, due to animal-animal variations. *S. bovis* was chosen in this study to act as a marker for acidosis because this is the predominant lactic acid producing bacterium found in animals fed a high concentrate diet (Krogh, 1961; Mackie et al., 1978). They are also easy to grow *in vitro* and produce high concentrations of lactic acid.

The antibiotic, virginiamycin, was used due to the very low concentration (0.75 μg/ml) required to control lactic acid production in the rumen (Rowe et al., 1989) compared to other commonly used antibiotics in ruminants [Thiopeptin (1.5 μg/ml); lasalocid and monensin (15 μg/ml) (Nagaraja et al., 1982)]. Virginiamycin also is specifically active against Gram-positive bacteria (Gale et al., 1981).

The aims of the fermenter trials described here were to investigate the most effective treatment in controlling lactic acid accumulation in continuous cultures: 1) in pure cultures using *Streptococcus bovis* 2B as the lactic acid producing bacteria, 2) in whole rumen fluid media (to better simulate actual rumen conditions).

5.2 Materials and Methods.

5.2.1 Organisms and Antibiotic.

The organisms used were *Streptococcus bovis* 2B, *Selenomonas ruminantium* subsp. *lactilytica* and *Megasphaera elsdenii* (isolated from sheep adapted to a high wheat diet as described in Chapter 3). The antibiotic used was virginiamycin (Stafac 500) provided by Dr. J.B.Rowe (Western Australian Department of Agriculture).

5.2.2 Fermenter conditions.

The fermenter used in this experiment was a Bioflow IIC Microprocessor Controlled Laboratory-Scale fermenter (New Brunswick Scientific Co., Inc.). During the experiment, the fermenter had 600 ml working volume and the pH was maintained at 6.0 by the automatic pumping of acid or base (either 1 M HCl or 1 M KOH). The fermenter was sparged at a rate of 20 ml/minute with CO_2 and N_2 gases in a ratio of 3:1. The temperature was set at 39° C. The cultures were continuously mixed by a built in stirrer at 100 rotations per minute in the pure culture studies and at 50 rotations per minute in the

rumen fluid studies.

5.2.3 Medium and growth.

S. bovis 2B was grown in the fermenter in M medium (see section 2.1.1.1), except lactate was replaced with 2 g/l starch. The culture was continuously infused with the same medium at a rate of 30 ml/hour (5% of working volume) until steady state was achieved or the absorbance (OD at 600 nm) stabilised. Normally, steady state was achieved in 30 hours when the OD reached 0.4 - 0.5. Acidosis was then induced with 36 g of soluble starch (Difco) at 0 hour and again at 12 hours. At the same time as starch addition, medium infusion was replaced with artificial saliva (McDougall, 1948) also containing 1 g/l of trypticase and 0.5 g/l yeast extract. Artificial saliva was infused at the rate of 10% of total volume (60 ml/hour).

5.2.4 Rumen fluid preparation.

One litre of rumen fluid was taken from a fistulated merino sheep 24 hours after the animal was fed 800 g lucerne chaff on each of five collection days. This is to minimise the effect of previous feeding on the results obtained. Rumen fluid was then filtered through four layers of gauze and immediately brought to the laboratory and used as inoculum for the fermenter.

5.2.5 Bacterial and antibiotic treatments.

In the pure culture study, inoculations with lactic acid utilising bacteria were carried out at zero hour (the same time as acidosis was induced with starch). The addition of virginiamycin was carried out at 2 hours. In the rumen fluid study, virginiamycin and bacterial inoculations were carried out at 12 hours for single inoculations, and at 12 hours and 36 hours for multiple inoculations. Virginiamycin was added to a final concentration

of 0.75 μg/ml. Bacterial inoculations were with 100 ml (10^9 cfu) of an overnight (16 hours) culture grown in BHI medium.

5.2.6 Sampling and analysis.

In the pure culture studies, 30 ml samples were collected via the sampling port every 4 hours for 24 hours. Meanwhile, in the rumen fluid studies, samples were collected every 12 hours for three days. Parameters measured included: concentrations of lactic acid, starch, VFA's, numbers of *S. bovis*, *Selenomonas ruminantium*, *Megasphaera elsdenii*, protozoa, and total bacteria. Sample analysis and colony counts were done as described previously (see Chapter 2).

5.2.7 Statistical analysis.

The data were analysed using Analysis of Variance (ANOVA) by GENSTAT 5. The degree of significance between treatments was done using the students T-test.

5.3 Results.

5.3.1 Study of starch-induced lactic acidosis in pure cultures.

Streptococcus bovis has been reported to proliferate rapidly when animals were fed with a diet high in readily fermentable carbohydrate and this bacterium has been associated with a high lactic acid concentrations in the rumen. In this continuous culture experiment, *S. bovis* was used to simulate the lactic acid production in the rumen. The effectiveness of virginiamycin and lactic acid utilising bacterial inoculations (*S. ruminantium* and *M. elsdenii*) in controlling lactic acid accumulation was also investigated.

5.3.1.1 Lactic acid, starch, and volatile fatty acids.

To study the effect of virginiamycin and bacterial inoculations in preventing high lactic acid levels in continuous culture, lactic acid and volatile fatty acid concentrations were investigated. There were no significant ($P>0.05$) differences in the lactic acid concentration between control and treated cultures at 0 hour (Figure 5.1). Following starch addition, the lactic acid concentration increased rapidly from 13 mM to 35 mM in uninoculated cultures over 4 hours. Lactic acid increased further to around 48 mM by 8 hours and remained at that level until the end of the experiment. Cultures inoculated with *S. ruminantium* (although statistically not significant, $P>0.05$) had higher lactic acid levels than the controls throughout the experiment, indicating that *S. ruminantium* prefers to use other carbon sources (e.g. glucose) rather than lactate. In addition, *S. ruminantium* produced lactic acid from glucose (Figure 5.5). In cultures treated with virginiamycin, the rapid increase in lactic acid production was prevented for up to 12 hours and it was significantly ($P<0.05$) lower than controls and *S. ruminantium* treated cultures. After 12 hours, lactic acid production was almost the same as controls. In cultures treated with *S. ruminantium* plus virginiamycin or *M. elsdenii* alone, lactic acid concentrations were reduced by 50% and 65% respectively during the investigation. The best result was shown by *M. elsdenii* plus virginiamycin. Lactic acid concentration was maintained at a low level (less than 5 mM), and it was significantly ($P<0.05$) lower than either controls or other treatments.

Overall, there was no significant ($P>0.05$) difference between starch concentrations in control and treated cultures or amongst treated cultures. However it was noted that at 8 hours, the starch concentration of virginiamycin treated cultures was significantly ($P<0.05$) higher than the controls and *S. ruminantium* treated cultures (Figure 5.2). This is probably due to a reduction in *S. bovis* population in virginiamycin treated cultures (Figure 5.4).

There were no volatile fatty acids detected in control and antibiotic treated cultures. This is because starch is mostly converted to lactic acid and most lactic acid producing bacteria (including *S. bovis* 2B) produce very little volatile fatty acids. The highest volatile fatty acid concentration was produced by cultures containing *M. elsdenii* or combinations of *M. elsdenii* and virginiamycin (Figure 5.3).

5.3.1.2 Bacterial population.

The level of lactic acid accumulation in the cultures was influenced by the balance of lactic acid producing and utilising bacteria present. Therefore, monitoring the fluctuation of these bacteria following treatments were essential. All cultures started with the same levels ($P>0.05$) of *S. bovis* (Figure 5.4). The cultures treated with virginiamycin either alone or in combination with bacteria had a significantly ($P<0.05$) lower population of *S. bovis* in the first 12 hours. The *S. bovis* population in cultures treated with virginiamycin alone rapidly increased after 12 hours and was significantly ($P<0.05$) higher than controls at 20 and 24 hours. This is due to the short term effect of the antibiotic as many other researchers have reported (e.g. Muir et al., 1980). Meanwhile, the *S. bovis* population in the cultures treated with a combination of *S. ruminantium* plus virginiamycin and *M. elsdenii* plus virginiamycin remained significantly ($P<0.05$) lower than controls throughout the experiment.

There were no lactic acid utilising bacteria detected in control or antibiotic treated cultures (Figure 5.6). The initial numbers of *S. ruminantium* and *M. elsdenii* were the same ($\pm$ 4 x 10^6 cfu/ml) in bacterial treated cultures. The numbers of lactic acid utilising bacteria in cultures treated with a combination of virginiamycin and bacteria increased faster than cultures treated with bacteria alone. This may be the result of an increase in growth factors available in the cultures as *S. bovis* growth was depressed by the virginiamycin.

Figure 5.1 Lactic acid concentrations in pure cultures treated with:

■ Control (without treatment),
▨ Virginiamycin,
▩ *S. ruminantium*,
▨ *M. elsdenii*,
□ *S. ruminantium* and virginiamycin,
■ *M. elsdenii* and virginiamycin,
▤ *S. ruminantium* and *M. elsdenii*.

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. Bacterial inoculations (10^9 cfu) were carried out at 0 hour and the virginiamycin (0.75 µg/ml) was added at 2 hours. The data represent the average of two replications.
Bars = standard error means.

Figure 5.2 Starch concentrations in pure cultures treated with:

■ Control (without treatment),
▨ Virginiamycin,
▩ *S. ruminantium*,
▨ *M. elsdenii*,
□ *S. ruminantium* and virginiamycin,
■ *M. elsdenii* and virginiamycin,
▤ *S. ruminantium* and *M. elsdenii*.

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. Bacterial inoculations (10^9 cfu) were carried out at 0 hour and the virginiamycin (0.75 µg/ml) was added at 2 hours. The data represent the average of two replications.
Bars = standard error means.

Figure 5.1 Lactic acid concentrations in pure cultures.

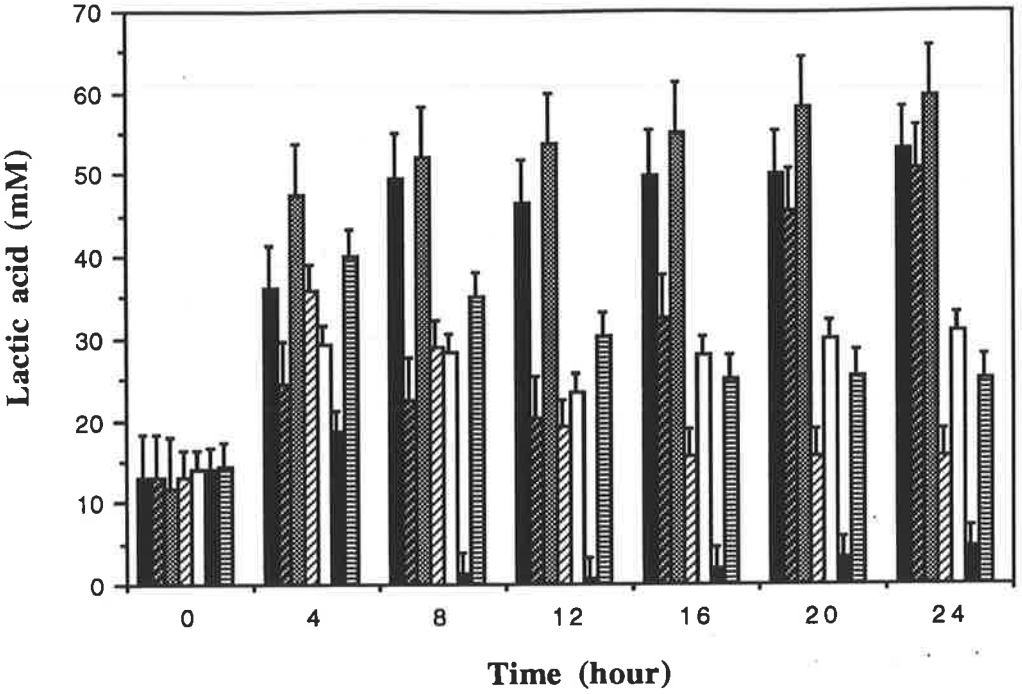


Figure 5.2 Starch concentrations in pure cultures.

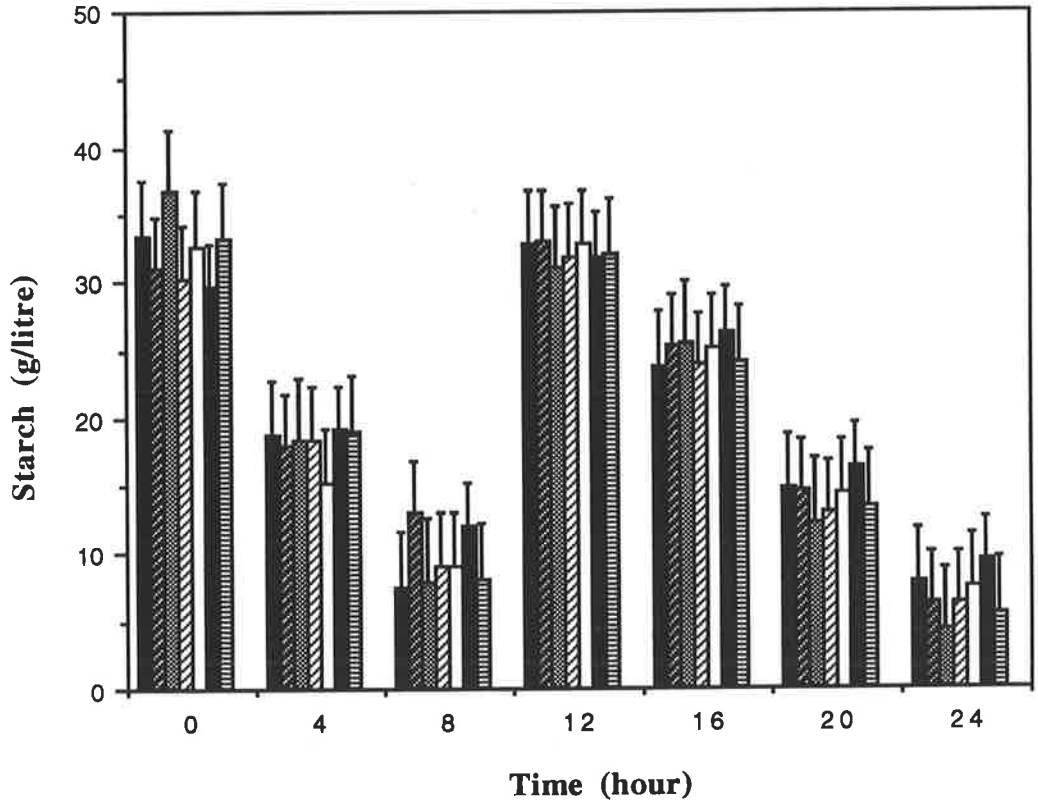


Figure 5.3 Total volatile fatty acid concentrations in *S. bovis* cultures treated with:

- *S. ruminantium.*
- *M. elsdenii,*
- *S. ruminantium* and virginiamycin,
- *M. elsdenii* and virginiamycin,
- *S. ruminantium and M. elsdenii*

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. Bacterial inoculations (10^9 cfu) were carried out at 0 hour and the virginiamycin (0.75 µg/ml) was added at 2 hours. The data represent the average of two replications.
Bars = standard error means.

Figure 5.4 *Streptococcus bovis* populations in pure cultures treated with:

- Control (without treatment),
- Virginiamycin,
- *S. ruminantium,*
- *M. elsdenii,*
- *S. ruminantium* and virginiamycin,
- *M. elsdenii* and virginiamycin,
- *S. ruminantium* and *M. elsdenii.*

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. The bacterial inoculations (10^9 cfu) were carried out at 0 hour and the virginiamycin (0.75 µg/ml) was added at 2 hours. The data represent the average of two replications.
Bars = standard error means.

Figure 5.3 Total VFA concentrations in pure cultures.

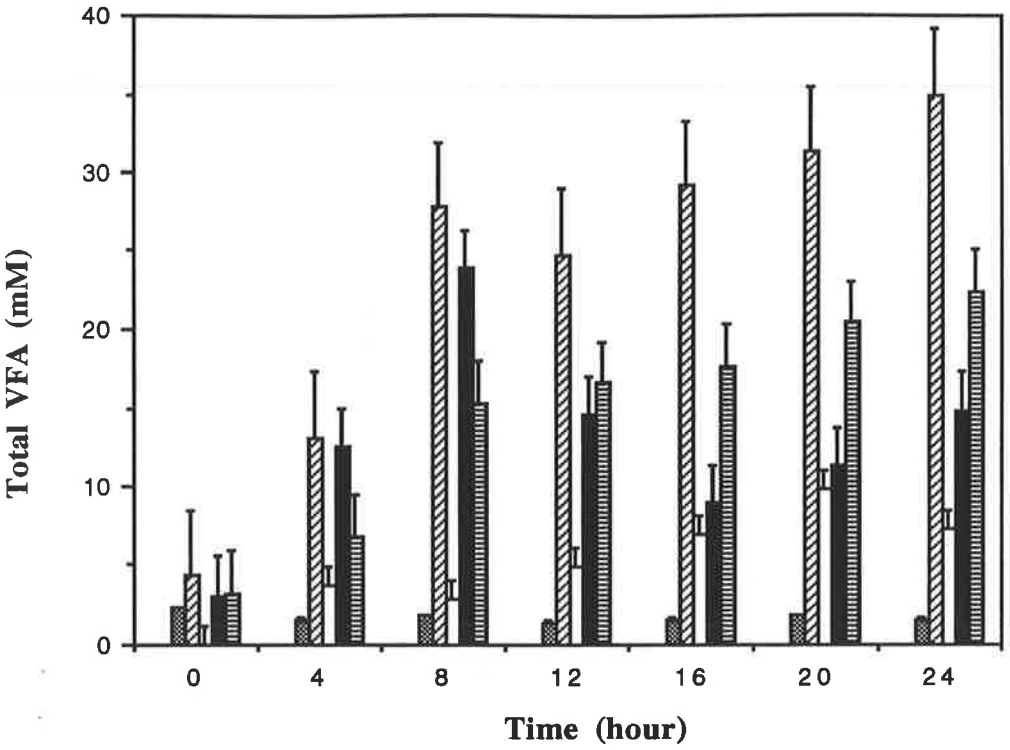


Figure 5.4 S. bovis populations in pure cultures.

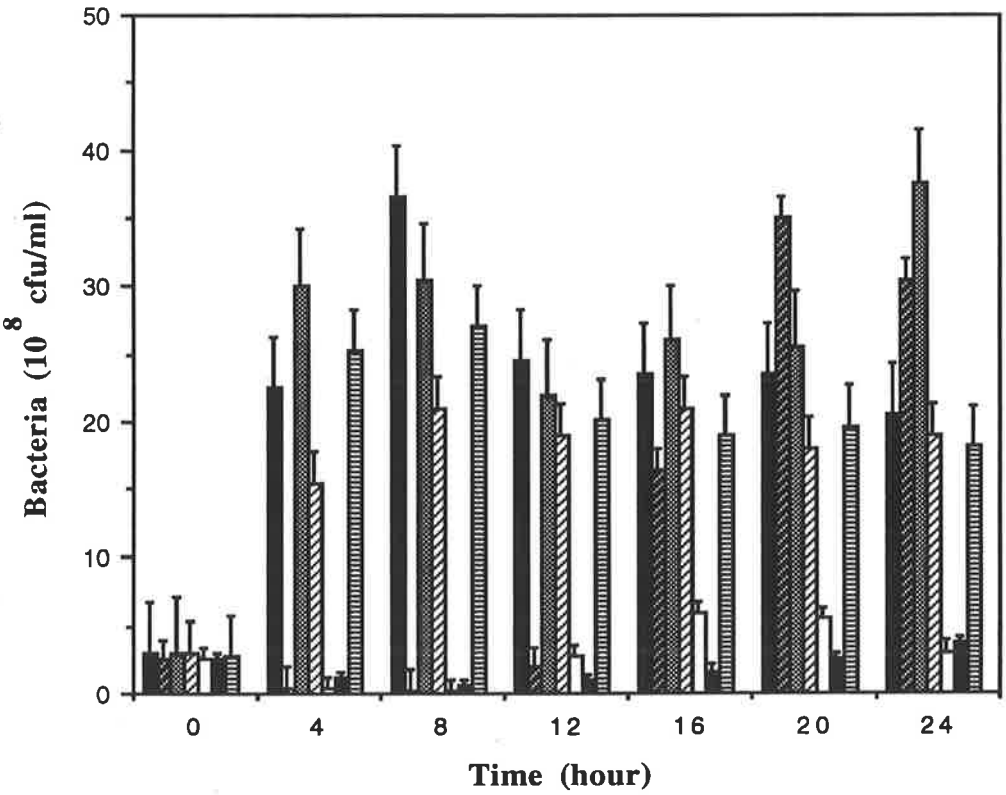
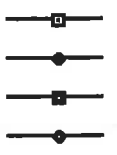


Figure 5.5 Lactic acid utilisation by:



S. ruminantium in M medium containing 0.5% lactate,
S. ruminantium in M medium containing 0.5% lactate and 0.5% glucose,
M. elsdenii in M medium containing 0.5% lactate,
M. elsdenii in M medium containing 0.5% lactate and 0.5% glucose

Cells were grown in 10 ml M medium in Hungate tubes and incubated at 39° C. The data represent the average of two replications.

Figure 5.6 Lactic acid utilising bacterial populations in *S. bovis* cultures treated with:

S. ruminantium.
M. elsdenii,
S. ruminantium and virginiamycin,
M. elsdenii and virginiamycin,
S. ruminantium and M. elsdenii

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. Bacterial inoculations (10^9 cfu) were carried out at 0 hour and the virginiamycin (0.75 μg/ml) was added at 2 hours. The data represent the average of two replications.
Bars = standard error means.

Figure 5.5 Lactic acid utilisation by S. ruminantium and M. elsdenii in the presence and absence of glucose.

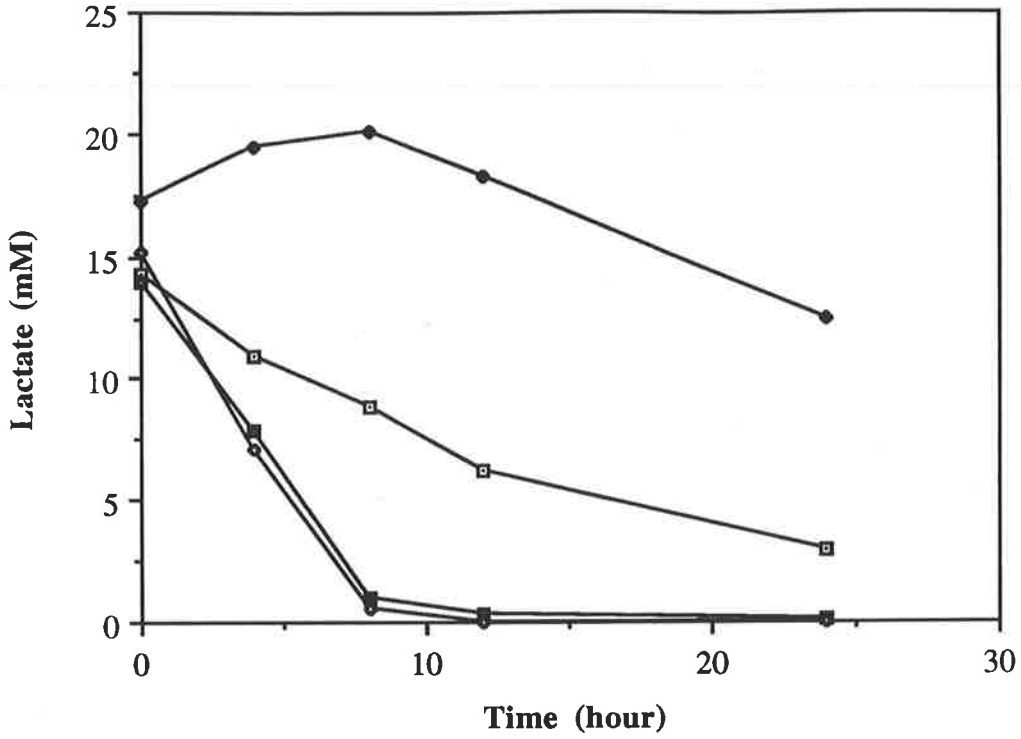
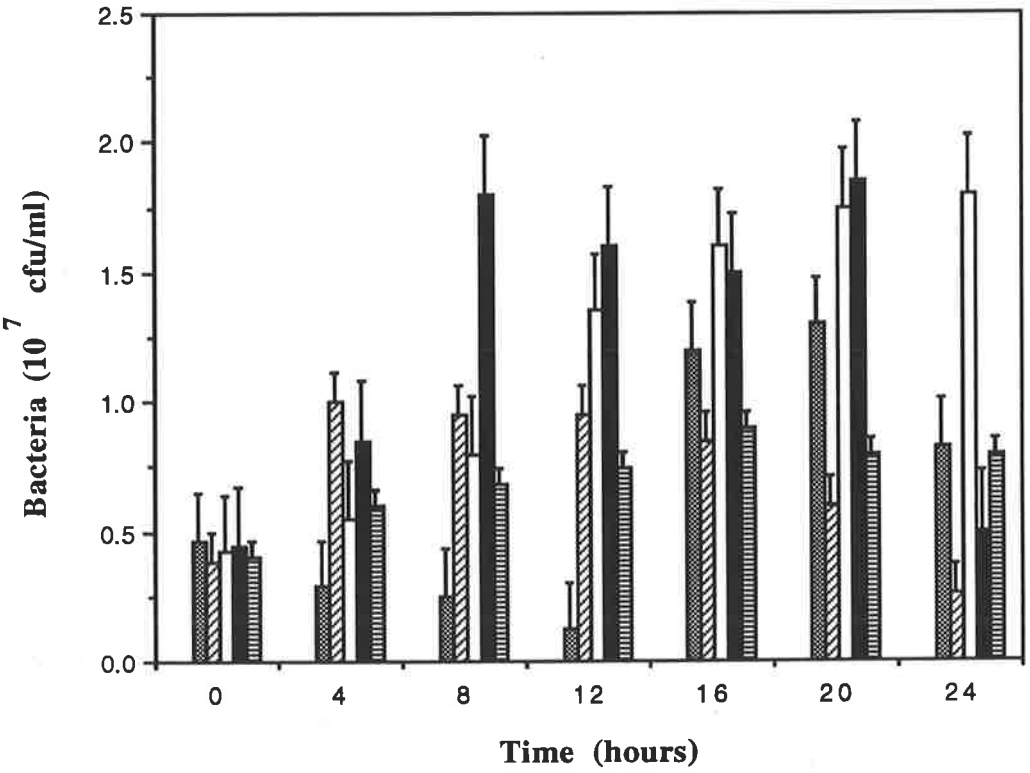


Figure 5.6 Lactic acid utilising bacterial populations in pure cultures.



5.3.2 Study of starch-induced lactic acidosis *in vitro*.

From pure culture studies with *S. bovis* as a lactic acid producing bacteria, it was found that the combination of virginiamycin and *M. elsdenii* was the most effective treatment in preventing the lactic acid accumulation for 24 hours. In the experiment described here, this combination was tested for the ability to control lactic acid accumulation for a longer period of time (3 days) in strained rumen fluid cultures. The effectiveness of other treatments was also investigated. Rumen fluid was used in this experiment in order to better simulate *in vivo* conditions.

5.3.2.1 Lactic acid, starch, and volatile fatty acids.

Lactic acid and volatile fatty acids are generated from starch. The concentration of lactic acid and volatile fatty acids are affected by the bacterial population present in the cultures. Therefore, the influence of virginiamycin and bacterial inoculations on the production of lactic acid and volatile fatty acids from starch were investigated. Lactic acid concentrations in all cultures were not significantly ($P>0.05$) different at time zero, and the concentrations were less than 5 mM (Figure 5.7). 12 hours after starch addition, lactic acid levels in all cultures increased to 14 - 19 mM. At 24 hours (12 hours after the addition of virginiamycin and bacteria), lactic acid concentrations of treated cultures were significantly ($P<0.05$) lower than controls. However, lactic acid concentrations of cultures treated with a single inoculation of *M. elsdenii* plus virginiamycin rapidly increased 36 hours after treatment and were significantly ($P<0.05$) higher than multiply treated cultures at 48 hours. All cultures treated with multiple inoculations had significantly ($P<0.05$) lower lactic acid concentrations compared to the control and single inoculated cultures. Amongst the multiply inoculated cultures, the combination of *M. elsdenii* plus *S. ruminantium* plus virginiamycin had significantly ($P<0.05$) lower lactic acid concentrations than *S. ruminantium* plus virginiamycin and *M. elsdenii* plus virginiamycin treated cultures with the lactic acid concentration maintained at less than

1mM until the end of the experiment.

Starch utilisation was not significantly (P>0.05) different between control and treated cultures and amongst the treated cultures (Figure 5.8). This could be the result of starch utilisation by species of rumen bacteria that may be less sensitive to virginiamycin, or because samples were taken each time after the addition of starch.

Figure 5.9 shows total VFA concentrations in the cultures. At time zero, total VFA concentrations were not significantly (P>0.05) different in all cultures. Total VFA concentration of controls went up 12 hours after starch addition and then decreased gradually and stabilised after 48 hours. In general, the cultures treated with *M. elsdenii* plus virginiamycin and *M. elsdenii* plus *S. ruminantium* plus virginiamycin had significantly (P<0.05) higher total VFA's than other treatments after 48 hours.

5.3.2.2 Bacterial and protozoal populations.

The effect of virginiamycin and bacterial inoculations on total and lactic acid utilising bacterial and protozoal populations was also studied. The total number of bacteria in the control cultures at the start of the experiment was significantly (P<0.05) higher than treated cultures. This may be due to the normal daily fluctuations in the rumen bacterial population. Total bacteria of all cultures increased gradually and stabilised after 36 hours. The total bacterial population of cultures treated with a combination of virginiamycin plus bacteria was significantly (P<0.05) higher than controls, especially after 48 hours. This means that the inoculation was not only reducing the lactic acid levels in the cultures but may also be supporting the growth of other rumen bacteria (Figure 5.10).

Lactic acid utilising bacteria in control cultures were significantly (P<0.05) higher than treated cultures at time zero, but the population was decreasing as acidosis developed (Figure 5.11). After 12 hours the cultures treated with *M. elsdenii* plus *S. ruminantium*

plus virginiamycin and *M. elsdenii* plus virginiamycin had significantly (P<0.05) higher lactic acid utilising bacteria (mainly *S. ruminantium* and *M. elsdenii* with occasional low numbers of *Anaerovibrio*) than controls and other treatments. This could be related to the utilisation of lactic acid as evidenced by low lactic acid concentrations in the cultures (Figure 5.7).

There was little variation in the protozoal population among the cultures, and generally protozoa were washed out from the cultures by 48 hours (Figure 5.12). This was probably due to the lack of solid particles for attachment of protozoa. The pattern of protozoa disappearance was similar in all cultures and there was no effect of treatments on the protozoal populations.

5.3.2.3 Individual volatile fatty acids.

Changes in the concentrations and proportions of individual volatile fatty acids is one important aspect in rumen microbial manipulation. In this experiment, these changes were investigated following the virginiamycin and lactic acid utilising bacterial inoculations. Table 5.1 shows the concentration of the individual volatile fatty acids (acetate, propionate, and butyrate) in the cultures. In general, the acetic acid concentration was increased after starch addition, but the increase in acetic acid in controls was significantly (P<0.05) higher than treated cultures after 36 hours. In contrast, the concentration of butyrate was significantly (P<0.05) higher in treated cultures than controls.

The proportion of acetate did not change in control cultures during the investigation (Table 5.2), but the proportion of acetate in treated cultures was significantly (P<0.05) decreased. The cultures treated with the combination of *M. elsdenii* and virginiamycin had higher proportions of propionate than controls and *S. ruminantium* plus virginiamycin treatments. All treated cultures, except the cultures treated with single

inoculation of *M. elsdenii* plus virginiamycin, had significantly ($P<0.05$) higher butyrate proportions than controls. The increase of propionate and butyrate proportions in treated cultures may be the result of lactic acid utilisation by *S. ruminantium* and *M. elsdenii.*

Figure 5.7 Lactic acid concentrations in rumen fluid cultures treated with:

- Control (without treatment),
- *S. ruminantium* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *S. ruminantium* and *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 hours.

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. The data represent the average of two replications. Bars = standard error means.

Figure 5.8 Starch concentrations in rumen fluid cultures treated with:

- Control (without treatment),
- *S. ruminantium* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *S. ruminantium* and *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 hours.

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. The data represent the average of two replications. Bars = standard error means.

Figure 5.7 Lactic acid concentrations in rumen fluid cultures.

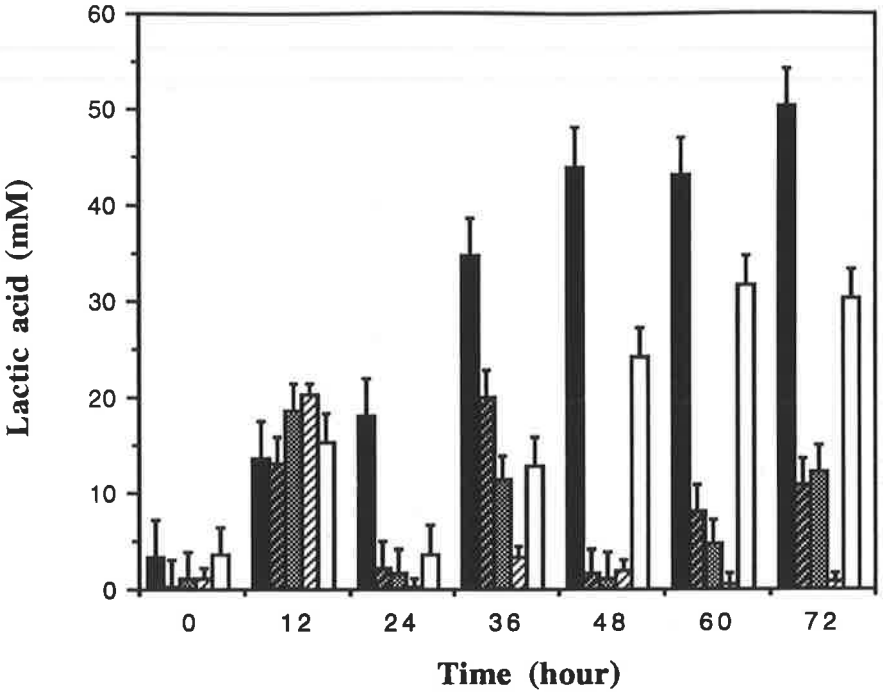


Figure 5.8 Starch concentrations in rumen fluid cultures.

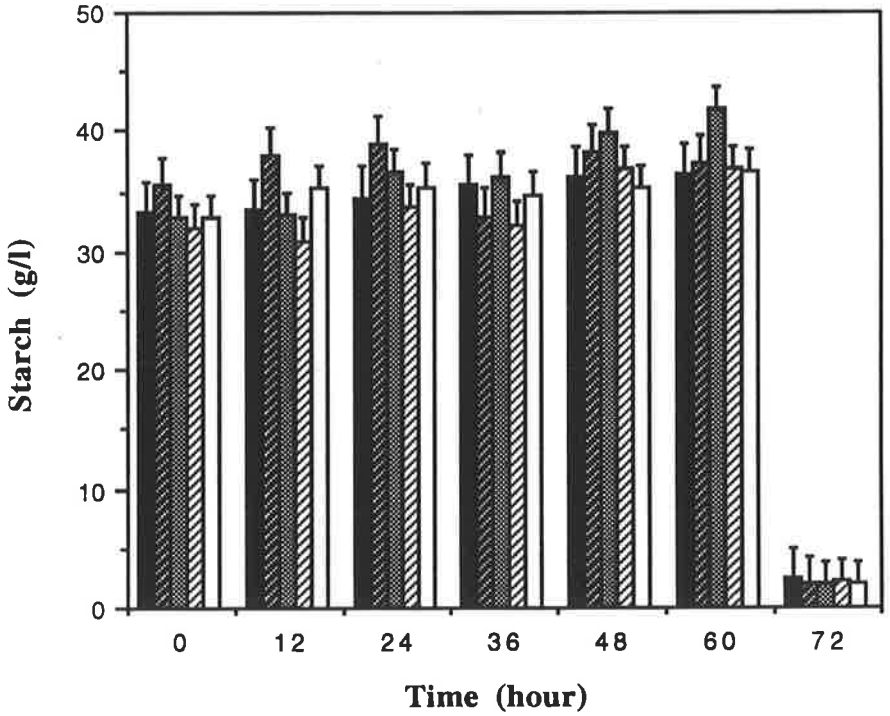


Figure 5.9 Total volatile fatty acid concentrations in rumen fluid cultures treated with:

- Control (without treatment),
- *S. ruminantium* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *S. ruminantium* and *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 hours.

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. The data represent the average of two replications. Bars = standard error means.

Figure 5.10 Total bacterial populations in rumen fluid cultures treated with:

- Control (without treatment),
- *S. ruminantium* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *S. ruminantium* and *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 hours.

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. The data represent the average of two replications. Bars = standard error means.

Figure 5.9 Total VFA concentrations in rumen fluid cultures.

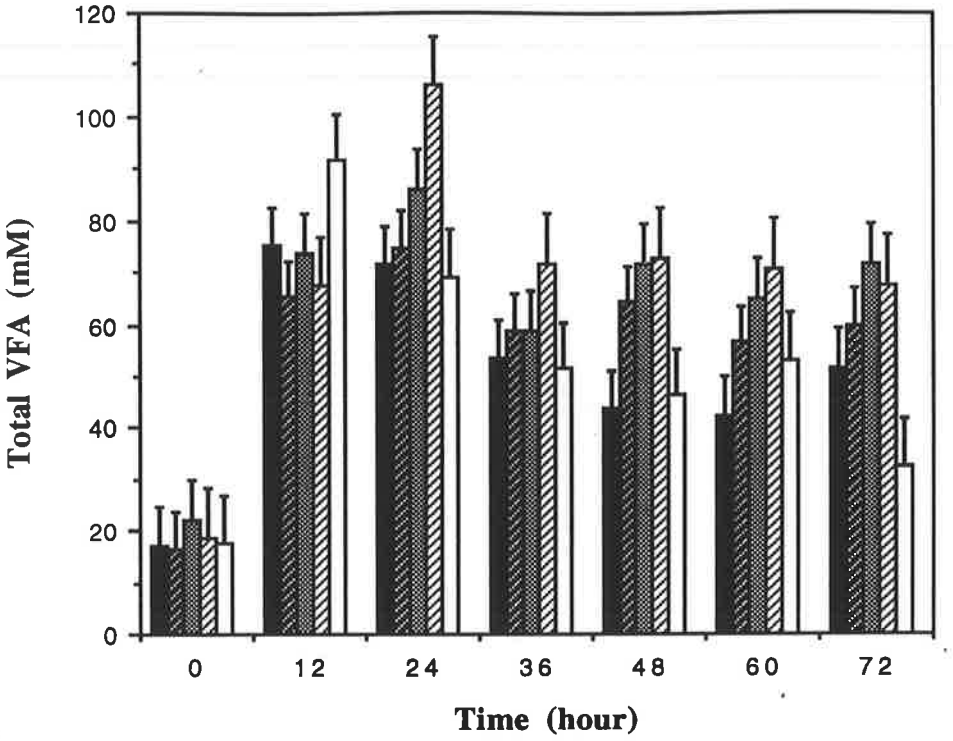


Figure 5.10 Total bacterial populations in rumen fluid cultures.

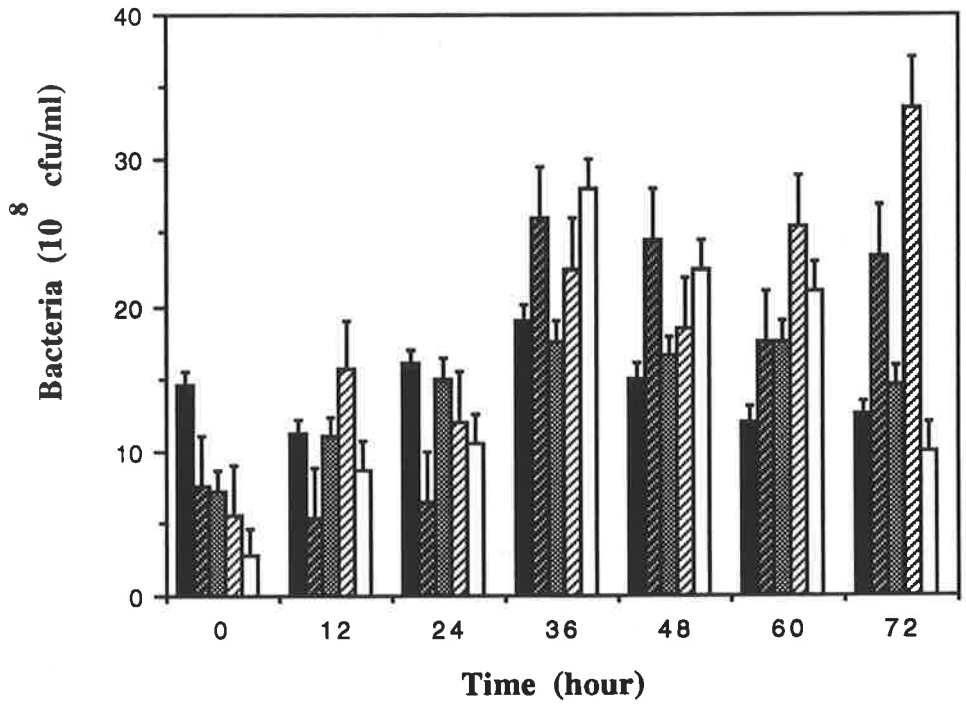


Figure 5.11 Lactic acid utilising bacterial populations in rumen fluid cultures treated with:

- Control (without treatment),
- *S. ruminantium* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *S. ruminantium* and *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 hours.

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. The data represent the average of two replications. Bars = standard error means.

Figure 5.12 Protozoal populations in rumen fluid cultures treated with:

- Control (without treatment),
- *S. ruminantium* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *S. ruminantium* and *M. elsdenii* and virginiamycin at 12 and 36 hours,
- *M. elsdenii* and virginiamycin at 12 hours.

The fermenter temperature was maintained at 39° C and the pH was maintained at 6.0. The data represent the average of two replications. Bars = standard error means.

Figure 5.11 Lactic acid utilising bacterial populations in rumen fluid cultures.

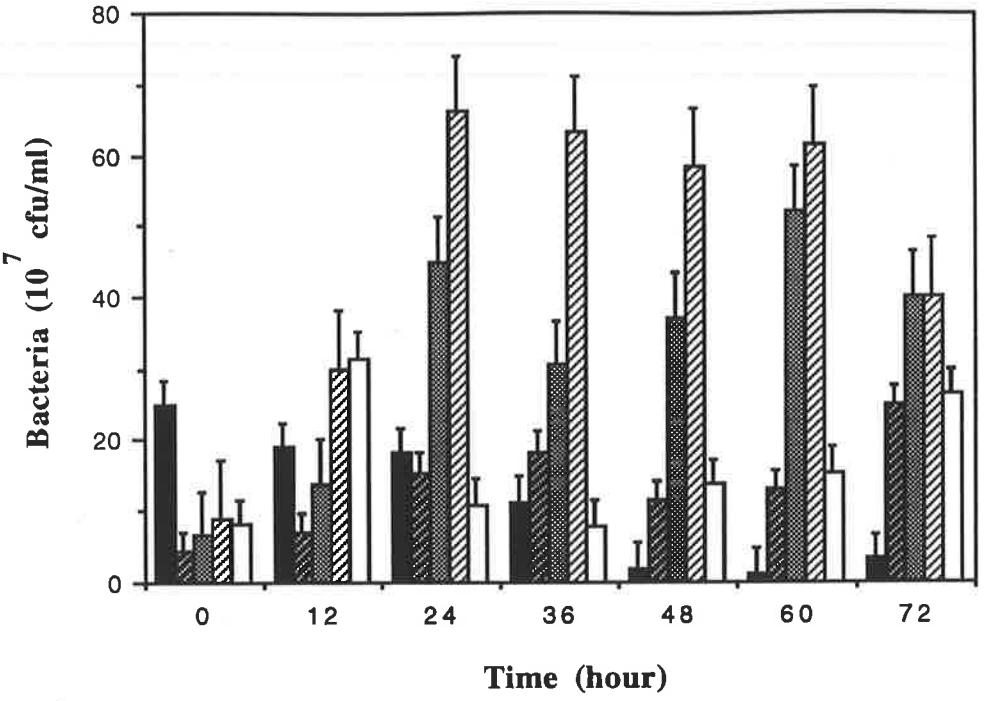


Figure 5.12 Protozoal populations in rumen fluid cultures.

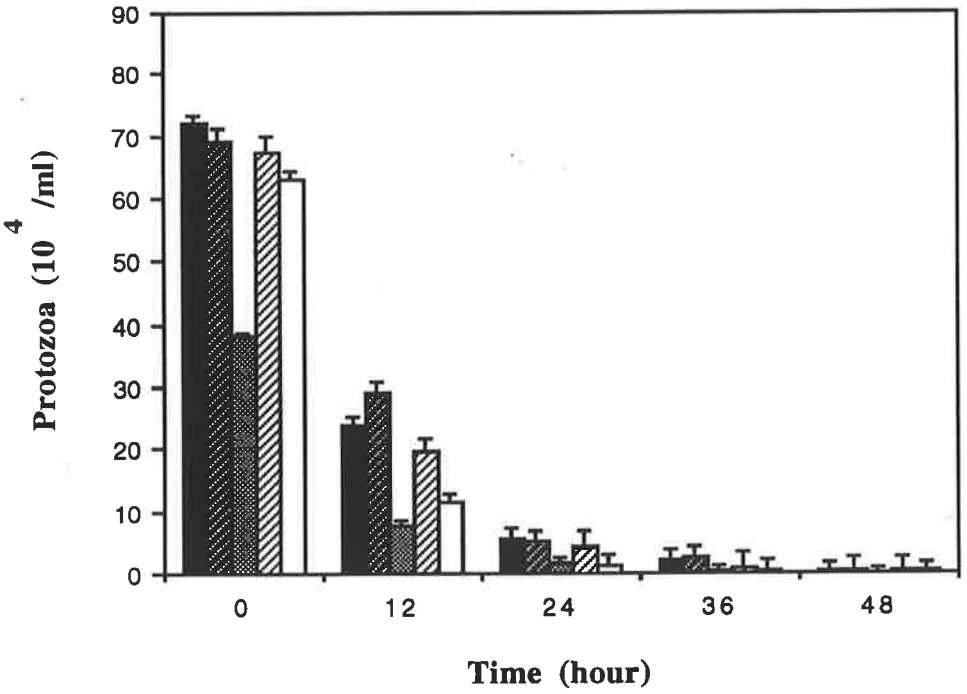


Table 5.1 Concentration (mM) of individual volatile fatty acids in rumen fluid cultures.

Time (hour)	Treatments[a]					SEM
	Control	Sel.+A	Meg.+A	Sel.+Meg.+A	Meg.+A1	
	Acetate					
0	11.80	9.48	13.30	11.08	11.23	1.81
12	39.60	34.69	43.73	32.81	50.68	2.19
24	36.85	28.98	30.98	35.62	27.92	1.68
36	29.29	16.44	17.99	18.27	16.61	1.44
48	26.22	17.51	18.81	16.65	16.65	1.37
60	26.70	16.90	17.70	17.10	22.10	4.17
72	32.10	18.80	21.70	16.10	15.50	3.42
	Propionate					
0	4.24	4.92	5.63	5.74	4.14	1.13
12	30.10	22.40	22.0	29.60	28.40	2.55
24	27.50	18.80	25.30	46.50	18.70	3.74
36	18.70	12.70	12.80	21.30	13.50	3.03
48	12.50	14.20	21.0	23.30	14.10	2.33
60	10.32	10.51	13.70	16.97	16.74	2.16
72	11.27	11.26	16.40	13.59	9.99	2.07
	Butyrate					
0	1.81	2.12	3.06	1.84	2.37	0.58
12	5.19	8.13	5.83	4.86	8.68	1.38
24	5.90	20.0	20.20	13.90	16.70	2.46
36	4.90	20.36	18.44	16.85	15.47	1.22
48	4.90	21.50	19.46	15.02	11.68	1.26
60	5.46	20.03	19.70	17.43	11.06	2.04
72	7.69	21.31	20.87	20.20	5.36	1.00

Sel.= *Selenomonas ruminantium* subsp. *lactilytica*
Meg.= *Megasphaera elsdenii*
A= Antibiotic (Virginiamycin)
a= average of two replications
SEM= standard error of means

Table 5.2 Proportion (%) of individual volatile fatty acids in rumen fluid cultures.

Time (hour)	Treatments[a]					SEM
	Control	Sel.+A	Meg.+A	Sel.+Meg.+A	Meg.+A1	
	Acetate					
0	65.50	57.70	60.50	60.10	62.50	4.96
12	52.92	53.43	60.88	48.86	57.85	1.96
24	52.46	42.92	40.43	37.09	44.14	1.87
36	55.32	33.22	36.60	32.40	36.63	0.67
48	60.15	32.75	31.48	30.25	39.83	1.86
60	62.80	35.60	34.60	33.10	43.0	2.31
72	62.80	36.50	36.60	31.90	49.10	2.89
	Propionate					
0	24.20	29.90	25.60	29.90	24.20	3.83
12	40.17	34.26	30.48	43.97	32.40	1.58
24	39.20	27.60	33.0	48.40	28.60	4.33
36	35.60	25.60	25.80	37.70	28.40	4.50
48	28.80	26.50	35.30	42.50	32.20	3.73
60	24.40	22.10	26.80	32.60	35.30	3.11
72	22.0	20.40	27.60	26.80	34.10	3.91
	Butyrate					
0	10.10	12.60	13.90	10.0	13.30	2.30
12	6.93	12.30	8.13	7.16	9.76	1.24
24	8.30	29.40	26.20	14.50	27.30	4.28
36	9.10	41.20	37.60	29.90	35.0	3.89
48	11.07	40.23	32.69	27.27	28.0	1.97
60	12.85	42.26	38.56	34.35	21.69	1.57
72	15.20	41.50	35.80	41.30	16.80	3.58

Sel.= *Selenomonas ruminantium* subsp. *lactilytica*
Meg.= *Megasphaera elsdenii*
A= Antibiotic (Virginiamycin)
a= average of two replications
SEM= standard error of means

5.4 Discussion.

A single bacterial species was used in the fermenter trials as this reduces the complexity of the rumen ecosystem and reduces the variations in the bacterial populations. These experiments were based on the concept that if a rapidly growing ruminal lactic acid producing bacteria can be controlled, it will be easier to control other lactic acid producing bacteria in the rumen. There are a number of reasons why *Streptococcus bovis* was used as the lactic acid producing bacteria in pure culture experiment. Many researchers have reported that this bacterium rapidly increased when animals were fed with a diet containing highly fermentable carbohydrate (Krogh, 1960; Mackie et al., 1978). *S. bovis* is also easy to grow and has a very short doubling time (± 9 minutes under certain conditions; Bruggemann and Giesecke, 1965). *S. bovis* uses the homofermentative pathway (see Figure 1.1) which produces more lactic acid per mol of glucose than the other pathways.

The results of the pure culture experiment showed that lactic acid production of *S. bovis* can be as high as 35 mM in four hours (Figure 5.1), but the addition of *S. ruminantium* subspecies *lactilytica* to the cultures increased the lactic acid concentration to 47 mM. This could be the result of lactic acid production by *S. ruminantium* in the presence of other carbon sources generated from starch (see Figure 5.5) or because *S. ruminantium* produced intermediate(s) that can increase the growth of *S. bovis*, thus increasing the lactic acid concentration in the cultures. The latter was supported by the evidence that *S. bovis* population in *S. ruminantium* treated cultures was higher than controls (Figure 5.4) and this led to an increase in starch utilisation at 24 hours (Figure 5.2).

In virginiamycin treated cultures, the *S. bovis* population rapidly increased after 12 hours. This suggests that the efficacy of the virginiamycin is limited to 12 hours. In contrast, *S. bovis* populations in the cultures treated with combination of *S. ruminantium* and virginiamycin or *M. elsdenii* and virginiamycin were maintained at a level 1/3-1/5 that

of the controls. This may be the result of a combined effect of virginiamycin and lactic acid utilising bacteria. Virginiamycin acted to reduce the *S. bovis* populations in the early stage, therefore increased carbon source and other growth factors were available in the cultures. This led to an increase of the lactic acid utilising bacterial population since these bacteria were also more resistant to virginiamycin. High populations of lactic acid utilisers may further inhibit the rapid growth of *S. bovis* at the later stage. It seems unlikely that volatile fatty acids support the growth of *S. bovis*, because total volatile fatty acid concentrations in *S. ruminantium* treated cultures were low compared with *M. elsdenii* alone or a combination of *M. elsdenii* and virginiamycin or *M. elsdenii* and *S. ruminantium* treated cultures.

The accumulation of lactic acid was much slower in the rumen fluid than in the pure culture experiments. This is understandable because in the rumen, there are other bacteria that grow and produce lactic acid more slowly than *S. bovis* or that do not produce lactic acid from starch. There may also be competition for starch utilisation between protozoa and bacteria, where protozoa help slow down lactic acid production by taking up starch faster than bacteria and metabolising it at a slower rate. There may also be a predation of bacteria by protozoa that may further slow down lactic acid production. Slower lactic acid accumulation in the rumen fluid studies compared to *in vivo* studies (Chapter 4) may be caused by the maintenance of the pH at 6.0 that may restrict the domination of lactic acid producing bacteria since all other bacteria can grow and take part in normal fermentation. The pH of the cultures was maintained at 6.0 because it was found from the *in vivo* experiment (Chapter 3 and 4) that rumen pH was around 6 when the animals were fed with high grain diets. This was also to avoid possible fluctuations in the pH caused by gas flow into the fermenter since CO_2 lowered the pH. Enrichment of lactic acid producing bacteria might happen in controls as lactic acid production increased rapidly after 24 hours, and stabilised at about 45 mM after 48 hours. The cultures treated with a combination of *S. ruminantium* and *M. elsdenii* and virginiamycin had the lowest lactic acid concentration compared with controls or other treatments. This is a reflection

of the high lactic acid utilising bacterial population in these cultures (Figure 5.11).

In general, total bacterial populations increased after 24 hours (12 hours after treatments) in the rumen fluid study. Similar results were found in pure culture experiments, where total bacterial numbers in bacterial treated cultures were greater than controls. This supported the conclusion that *S. ruminantium* or *M. elsdenii* might produce intermediate(s) that support the growth of other bacteria. Another explanation may be that more energy may be released from glucose when lactic acid is not produced since growth of lactic acid producing bacteria was depressed by virginiamycin. This means a more efficient use of carbon source and greater cell growth. The increase in total bacteria may also be caused by the disappearance of protozoa from the cultures. The protozoal disappearance may be due to the lack of solid particles in the cultures for attachment.

The increase in total bacteria in turn increased total volatile fatty acid concentrations. This experiment also showed that there is no relationship between the increase in propionate and butyrate proportions and the size of the protozoal population. Therefore, the increase in propionate and butyrate proportions may be mainly caused by the utilisation of lactic acid by *S. ruminantium* and *M. elsdenii*. High concentrations of acetic acid in controls may be due to the domination of lactic acid producing bacteria that also produce acetate.

Virginiamycin is selectively active against Gram-positive bacteria without depressing the population of other bacteria. This is demonstrated by the higher population of total bacteria and lower lactic acid concentrations in treated cultures compared with controls. In addition, virginiamycin did not decrease total volatile fatty acid concentrations in the fermenter. This means that high volatile fatty acid producers such as *S. ruminantium* and *M. elsdenii* were not affected by virginiamycin. The amount of antibiotic used in this experiment was not designed to kill all Gram positive bacteria, but to slightly reduce or slow down the lactic acid production thus allowing lactic acid utilising bacteria to proliferate and remain at a high level during the experiment. Higher concentrations of

virginiamycin used in these experiments than that of Rowe's was due to the dilution effect of the continuous culture. Rowe et al. (1989) reported that 0.5 μg virginiamycin per ml rumen fluid can reduce lactic acid production in batch cultures by up to 60% . In this experiment 0.75 μg virginiamycin per ml of culture can reduce the lactic acid produced by *S. bovis* by 50%. This is understandable because *S. bovis* produced more lactic acid than other ruminal lactic acid producing bacteria.

The combination of *S. ruminantium* and *M. elsdenii* and virginiamycin was found to be the most effective treatment in controlling lactic acid accumulation in the fermenter. This could be the result of the ability of the *S. ruminantium* to maintain a high population of *M. elsdenii* by producing lactic acid from maltose or glucose generated from starch, as the growth of other lactic acid producing bacteria was inhibited by virginiamycin. This is evidenced by the increase of the lactic acid concentration when *S. ruminantium* was cocultured with *S. bovis*. The effectiveness of *S. ruminantium* and *M. elsdenii* and virginiamycin may also be caused by the production of intermediate(s) that supported the growth of other bacteria that inhibited the *S. bovis* domination in the cultures.

5.5 Conclusion.

It is concluded that the combination of *M. elsdenii* and virginiamycin is the most effective treatment for controlling lactic acid accumulation in starch-induced acidosis in pure cultures. *S. ruminantium* treatment increased lactic acid levels and this is due the production of lactic acid by fermentation of maltose or glucose.

The combination of *M. elsdenii* and virginiamycin in a single treatment step can prevent lactic acid accumulation for only 24 hours. Multiple inoculations of this combination can maintain low levels of lactic acid for at least three days. The combination of *S. ruminantium* and *M. elsdenii* and virginiamycin was the most effective treatment in controlling lactic acid accumulation in the fermenter. Further studies *in vivo* are necessary to test this treatment regime.

Chapter 6 General discussions and Future studies.

6.1 General discussions.

Grain supplementation is very important, especially in drought and feedlot conditions because 1) green feed is less available in dry seasons and 2) grain can increase feed intake, body weight gain and feed efficiency. However, an abrupt changeover from roughage to a grain diet can cause problems. One of these is lactic acidosis. Lactic acidosis is defined as a condition of pathologically high acidity of the blood. The term acidosis in ruminants is expanded to include acidic conditions in the rumen (rumen acidosis) Huntington (1988). Lactic acidosis occurs because lactic acid producing bacteria such as *Streptococcus bovis* and *Lactobacilli* multiply rapidly and metabolise starch, producing large amounts of lactic acid. In contrast, lactic acid utilising bacteria grow more slowly. The resulting high concentration of ruminal lactic acid reduces the ruminal pH to as low as 4.0, leading to the death of protozoa and many bacteria. *S. bovis* and the *Lactobacilli* are more resistant to low pH, and therefore continue to produce lactic acid after protozoa and other bacteria are destroyed (Slyter et al., 1970). The effect of low pH on the rumen is itself, very damaging. Effects range from inhibition of the amplitude and frequency of rumen contractions to damage of the rumen epithelium. This can lead to bacterial infection reaching the bloodstream, and liver abscesses. Rumen dysfunction causes various problems, ranging from a reduction or loss of appetite to death of the animal.

Lactic acidosis development can be prevented in several ways. A gradual adaptation process is reported to be the safest way to control lactic acid accumulation. This is carried out by gradually increasing the amount of grain in the ration over 3 to 4 weeks, allowing the lactic acid utilising bacteria to build up slowly and utilise lactic acid as it is produced in the rumen. In the work described here, *Selenomonas ruminantium* subspecies *lactilytica*

was the predominant lactic acid utilising bacteria in the rumen of sheep adapted to a high wheat diet. Although the isolates were able to grow on selective lactate-containing medium, the numbers in the rumen were not merely a reflection of lactic acid availability in the rumen as *S. ruminantium* can utilise and grow on other carbon sources such as maltose and glucose.

The domination of *S. ruminantium* in the experiment described here may be influenced by the use of a high protein lucerne chaff diet during pre-experiment feeding since *S. ruminantium* is also proteolytic (Hungate, 1966; Wallace and Brammall, 1985). This can be contrasted to the finding of Mackie and Gilchrist (1979) where they reported that *Anaerovibrio sp* was the predominant lactic acid utilising bacteria found in sheep adapted to a high corn diet. The differences may be due to the diet given before and during the experiments. Mackie and Gilchrist fed the animals with a corn-containing diet which was generally low in protein, but what protein was present (zein) was insoluble in the rumen. Based on these two experiments, it is concluded that it is likely to be necessary to isolate local bacteria for future inoculation purposes as geographical and feed differences will determine different predominant bacteria in the rumen.

Although *Megasphaera elsdenii* was found to utilise lactic acid three times faster than *S. ruminantium*, its numbers were always less than *S. ruminantium* during the adaptation process. The lower *M. elsdenii* population may be due to the higher sensitivity of this bacterium to low rumen pH compared to *S. ruminantium*. Another reason may be that *S. ruminantium* can use a greater variety of carbon sources than *M. elsdenii* and *M. elsdenii* may not be able to compete with other rumen microorganisms for nutrients as well as *S. ruminantium*.

The hypothesis of the experiment described in Chapter 4 was that the development of lactic acidosis is due to an imbalance in production and utilisation of lactic acid in the rumen. It was reported that this is a result of slow proliferation of lactic acid utilising

bacteria. Therefore a logical approach to the control of lactic acid accumulation is to inoculate animals with lactic acid utilising bacteria at a high enough population density so as to have an effect.

S. ruminantium subsp. lactilytica and *M. elsdenii* strains described in Chapter 3 were used as inocula when animals were fed with a high wheat ration. In the *S. ruminantium* only inoculation, the results showed that *S. ruminantium* inoculations can prevent lactic acid accumulation for up to 24 hours but failed to do so for longer periods of time. This may be the result of an initial rapid utilisation of the lactic acid produced in the rumen, which in turn may have led to a decrease in *S. ruminantium* population when the carbon source was eliminated. It may also be that other carbon sources were utilised by *S. ruminantium* in preference to lactic acid, leading to a further increase in lactic acid as was evidenced by the *in vitro* studies (see Figure 5.5). When the other carbon sources had been completely utilised, *S. ruminantium* may have been unable to compete successfully with lactate-producing bacteria. The *S. ruminantium* population may have dropped to such a level that it was not capable of responding to the further accumulation of lactic acid in the rumen. It may also be that the inoculum of *S. ruminantium* was too small and that this strain did not establish itself quickly enough. Therefore the numbers of bacteria remaining after 24 hours may have been too low and not able to prevent lactic acid accumulation. It may be useful to inoculate animals with different numbers of bacteria in order to find out the most effective concentration to use in preventing the development of lactic acidosis.

To determine the most effective concentration of bacteria to use for inoculation is not straight forward because it is influenced by the rate of lactic acid production in the rumen. This is in turn affected by the number and types of lactic acid producing bacteria present in the rumen and types of feed and feed processing. There have been many bacterial trials in ruminants, with conflicting results. Some show that introduced populations can be established and others have shown the opposite. The crucial factors seem to be the timing

of inoculation, number of cells, and that recent isolates may be better than laboratory strains. No one has yet tried a range of different strains at the same time. This becomes important since Zhang et al. (1991) and Flint and Bisset (1990) isolated 20 and 28 different strains of *S. ruminantium* respectively from crude rumen fluid. This raises the question as to whether a number of strains are required for inoculation such that many niches can be filled with competitive bacterial populations. Flint and Bisset (1990) also showed that in different strains of *S. ruminantium,* lactate dehydrogenase has different stereospecificities. This may be important in conferring a competitive advantage in different niches. It would be interesting to repeat the acidosis experiment with a range of recently isolated strains of *S. ruminantium.*

The combination of *S. ruminantium* and *M. elsdenii* was able to control lactic acid accumulation in the rumen over four days. It seems likely that *M. elsdenii* plays an important role in controlling the high level of lactic acid in the rumen as demonstrated by the increased proportions of butyrate in the rumen. The effectiveness of this combination may be due to *S. ruminantium* helping to maintain a high population of *M. elsdenii* by itself producing lactic acid from maltose or glucose. The depression of lactic acid production by other bacteria may be the result of short supply of nutrients in the rumen in the presence of high numbers of *S. ruminantium* and *M. elsdenii.*

Monitoring changes in lactic acid utilising bacteria in the rumen was made easier by using dialysis tubing. The use of dialysis tubing was aimed at separating introduced bacteria from endogenous bacteria. This tubing allows low molecular weight substances to pass through but inhibits the exchange of bacteria to and from the rumen. Although the bacterial population in the tubing decreased from 10^{10} to 10^{7} cfu in two days, the lactic acid levels were kept low. This means that the inoculants helped to utilise lactic acid at the early stages and allowed endogenous bacteria to build up. The increase in endogenous lactic acid utilising bacteria may then have reduced the concentration of lactic acid in the rumen. This in turn would reduce the number of lactic acid utilising bacteria in

the tubing.

The use of dialysis tubing has broad applications in studies of rumen ecology as this technique provides a tool for studying interactions between introduced bacteria and endogenous rumen microorganisms. This tubing may also be used practically as a capsule for bacterial inoculum since the tubing is made from cellulose and can be degraded slowly in the rumen. Freeze dried concentrated cultures will probably have to be used to ensure a small capsule size. The dialysis tubing experiments also showed that bacterial inoculation of the rumen by this method may allow the inoculum to establish without predation by protozoa. This application may prove useful in the short term control of certain rumen conditions (e.g. lactic acidosis), but has yet to be tested for long term effects.

Another interesting finding of the *S. ruminantium* and *M. elsdenii* inoculation experiment was that the proportion of propionate and butyrate was increased. This means that bacterial inoculation has promising future applications as the inoculation can be targeted to produce certain VFA's depending upon the needs required for animal production. In feedlot conditions, inoculation with propionate producing bacteria may be useful since propionate can be used as a precursor of gluconeogenesis. Meanwhile in dairy industries, inoculation with acetate producing bacteria may be more useful as acetate can increase the fat content of milk.

The use of antibiotics in feedlot conditions is a common practice to reduce the incidence of acidosis. Some antibiotics (oxamycin, thiopeptin, lasalocid, monensin and capreomycin disulfate) have been reported to be effective in preventing rapid proliferation of lactic acid producing bacteria (Beede and Farlin, 1977; Muir and Barreto, 1979; Kezar and Church, 1980; Nagaraja et al., 1981, 1982). The use of 1.30 mg/kg body weight of thiopeptin, lasalocid, and monensin on acidosis-induced cattle reduced lactic acid levels in the rumen by 20% and maintained higher ruminal pH compared to controls. However,

these antibiotics were only effective for 12 hours. This may be due to washout of the antibiotics from the rumen. Because of the short efficacy of antibiotics in the rumen, supplementation with them has to be continued for an extended time period. This creates new problems because many bacteria develop resistance to the antibiotic (Hungate, 1966; Wiseman et al., 1960), the production costs increase, and the antibiotic residues may be found in animal products especially when high dosages of antibiotics are used.

In this experiment (Chapter 5), virginiamycin was used because it is effective at a very low concentration (0.75 μg/ml) compared to commonly used antibiotics in ruminants (1.5 μg/ml of thiopeptin; 15 μg/ml of lasalocid and monensin [Nagaraja et al., 1982]) and only affects Gram-positive bacteria (Gale et al., 1981). In addition, virginiamycin supplementation was needed for only two days when added together with the bacterial inoculation.

The role of virginiamycin in these experiments was to slow down the proliferation of lactic acid producing bacteria in the early stages of the experiments, thereby allowing *S. ruminantium* and *M. elsdenii* to establish themselves. The prevention of lactic acid accumulation by *S. ruminantium* and *M. elsdenii* provided an opportunity for other microorganisms to grow and compete for nutrients. The diversity of microorganisms in the cultures will inhibit domination of certain bacteria, thereby reducing the likelihood that lactic acidosis will develop.

As described in Chapter 5, lactic acid studies were carried out *in vitro* using pure cultures of *S. bovis* or strained rumen fluid. These experiments were conducted because of the variation in results obtained *in vivo* due to variation between individual animals. The advantages of using a continuous culture fermenter are that the conditions are reproducible and it is convenient to study particular aspects by modifying specific conditions without the confounding factors of physiological differences between animals. In pure culture studies, it was found that inoculation with a combination of *M. elsdenii*

and virginiamycin was the most effective treatment for controlling lactic acid levels, compared with virginiamycin alone, bacteria alone, or a combination of *S. ruminantium* and *M. elsdenii*. This may be the result of the activity of virginiamycin in the early stages to slow down lactic acid production by *S. bovis*, therefore enabling *M. elsdenii* to grow and maintain a high population in 24 hours. Meanwhile, inoculation with *S. ruminantium* alone increased lactic acid concentrations above that of the controls. This may be due to the production of intermediate(s) by *S. ruminantium* that supported the growth of *S. bovis*, and production of lactic acid by *S. ruminantium* itself.

In the second experiment, the combination of *M. elsdenii* and virginiamycin was tested for 3 days in strained-rumen fluid cultures in the fermenter. The effectiveness of other combinations of virginiamycin plus lactic acid utilising bacteria were also tested. The results showed that a single inoculation with the combination of *M. elsdenii* and virginiamycin can only prevent lactic acidosis for 24 hours, after which the lactic acid concentration increased. The most effective treatment was obtained when multiple inoculations with a combination of *S. ruminantium* and *M. elsdenii* and virginiamycin were used as inoculum. Multiple inoculations (12 and 36 hours) with this combination maintained low lactic acid levels and simultaneously led to an increase in the total number of bacteria and in total volatile fatty acid production compared to controls. Increased total bacteria may be the result of intermediate(s) produced by *S. ruminantium* and *M. elsdenii* that stimulate the growth of other bacteria. It may also be caused by the more efficient use of carbon sources when lactic acid is not produced. The increase in the concentration of total VFA's and the proportion of propionate and butyrate in the fermenter may be contributed to by *M. elsdenii* and *S. ruminantium* as these bacteria produce a high level of volatile fatty acids and are more resistant to virginiamycin compared to lactic acid producing bacteria (e.g. *S. bovis*). These experiments indicate that multiple inoculations may be necessary for manipulating rumen fermentation, as this may allow the establishment of a population in the rumen which does not otherwise occur with a single inoculation.

The increase in total numbers of bacteria may mean an increase in the amount of protein available to the animal, and an increase in total VFA's means an increase in energy supply for animals. Therefore, bacterial inoculation coupled with antibiotic use is beneficial, not only because it can prevent the development of lactic acidosis but also because it supplies the animal with higher levels of protein and energy.

6.2 Future studies.

During the adaptation experiment described in Chapter 3, the concentration of lactic acid in the rumen was very low. This was probably caused by the increase in lactic acid utilising bacterial population in the rumen. However, it is not impossible that it may be caused by increases in other bacteria that can use starch or maltose or glucose but not produce lactic acid. Therefore it may be useful to examine such bacteria during adaptation and use these as inocula when the animals are fed a high concentrate diet.

The use of strictly anaerobic lactic acid utilising bacteria as an inoculant may also be problematic in the application, especially in remote areas unless the bacteria are supplied in tablets or capsules that provide insulation against oxygen. Another alternative may be the use of facultative anaerobic lactic acid utilising bacteria as an inoculant. This approach will enable us to mix dried bacteria with the feed ration, and will ensure the presence of lactic acid utilising bacteria every time the animals eat.

Much rumen bacterial research over the past decade has concentrated on developing techniques to genetically alter rumen bacteria and thereby modify rumen fermentation. However, although there has been a lot of effort put into the field of genetic manipulation, there are still only a few species of rumen bacteria that have been succesfully manipulated (*Prevotella ruminicola*, Thomson et al., 1992; *S. bovis*, Brooker et al., 1993; *Butyrivibrio fibrisolvens*, Whitehead, 1992), even though plasmids from quite a few species have been isolated and characterised (e.g. Zhang et al., 1991). This puts a

limitation on the bacteria which may be genetically manipulated (e.g. genes for lactate utilising pathways added to species that do not carry them, up-regulation of lactate utilisation in species that already utilise lactate), with reference to control of lactic acidosis, and indeed, none of them may be suitable candidates. However, it is possible to speculate on the genetic characteristic one wishes to alter.

In concentrate fed animals, *S. bovis* and *Lactobacilli* are responsible for the accumulation of lactic acid in the rumen. Genetically, *S. bovis* (Brooker et al., 1993) has been extensively studied compared with *Lactobacilli*. Therefore modification of *S. bovis* may be possible by transferring gene(s) responsible for lactic acid catabolism from *S. ruminantium* or *M. elsdenii* to *S. bovis*. Therefore *S. bovis* can produce and utilise lactic acid at the same time. This will eliminate the growth rate differences between lactic acid producing and utilising bacteria.

From the experiment described in Chapter 3, *S. ruminantium* was found to be the predominant lactic acid utiliser in the rumen during adaptation to a high-wheat diet. Hungate (1966) reported that this bacterium is present under most dietary conditions. This bacterium can achieve numbers up to 51% of the total bacterial count in the rumen (Caldwell and Bryant, 1966). Therefore, this bacterium is a perfect target for modification in the rumen. Genetic studies of this bacterium have been conducted in our laboratory (Zhang et al., 1991; Zhang and Brooker, 1993; Attwood and Brooker, 1992) and other laboratories (Orpin et al., 1986; Hazelwood and Teather, 1988). Two of the *S. ruminantium* subsp. *lactilytica* (strains MA2 and MA11) isolated from grain-fed sheep in our laboratory contain plasmids. These extra-chromosomal genetic elements may form the basis of a gene transfer system for this bacterial species. In future, it may be possible to transfer gene(s) that regulate lactic acid utilisation from *M. elsdenii* to *S. ruminantium* thereby increasing the utilisation of lactic acid by *S. ruminantium*.

None of the aforementioned genetically engineered strains have so far been used in *in vivo* trials, so their potential for establishing in the rumen has not been tested. Again a lot of strains being used are old and may not be able to compete. It took 30 years of research to achieve the current understanding of *E. coli* genetics, and this may be the case with rumen bacteria. Therefore, it is important that ecological studies (as described in this thesis) be carried out to determine whether non-genetically altered bacteria can be used for rumen manipulation. These studies have provided valuable information and confirmed that probiotics can be used to prevent lactic acidosis.

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