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Optimisation of cell growth and shelf life stability of *Megasphaera elsdenii* NCIMB 41125

by

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Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae (Microbiology)

In the

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I, the undersigned, declare that the thesis has not been submitted to any university for a degree.

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Date: _____

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Supervisor: Dr M.S. Thantsha
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ABSTRACT

Economic demands for milk and meat products force farmers to increase the carbohydrate content of grains fed to animals. One of the consequences of this intervention is the lactic acidosis condition in animals fed the high-concentrate diets, which is the accumulation of lactic acid in the rumen. Symptoms of the condition include lameness, bloatedness, epistaxis and dilated pupils. Methods such as ionophore antibiotics, gradual transition from high to low-concentrate diets and yeast cultures have in the past been used to avert this condition but all had their disadvantages. Microorganisms may develop resistance to the ionophore antibiotics; gradual transition time may be too long for production and yeast cultures have been reported to be ineffective when used alone. *Megasphaera elsdenii*, a major lactate utiliser of the rumen of animals, has been used as a direct-fed microbial in the management of lactic acid levels. Studies on *M. elsdenii* NCIMB 41125 have shown that the bacterium is an effective lactic acid utiliser.

Megasphaera elsdenii NCIMB 41125 was cultured in a Biostat Braun B fermenter where growth yields were attempted to be optimised by using a pulse-and-shift method. A semi-defined lactate (SDL) and corn steep liquor (CSL) media, which contained reducing agents, to ensure anaerobiosis, were used in the optimisation

and shelf-life studies. Culture stability studies were performed on samples from a fermenter, and subsequently in stainless-steel kegs. Samples for analysis were then taken from the kegs. Preservation of *M. elsdenii* NCIMB 41125 and prevention of cell settlement methods were also evaluated using a combination of sodium lactate / glycerol and pure xanthan gum / gelatin, respectively. The cultures were harvested using either continuous or fed-batch fermentations.

Shelf-life was better for cultures grown on SDL medium with a lower concentration of lactic acid, a finding which related to the substrate affinity of *M. elsdenii* NCIMB 41125. Higher growth yields were obtained from secondary cultures which had been continuously harvested into stainless-steel kegs. Shelf-life results obtained from the use of corn steep liquor (CSL) medium were almost similar to those obtained when SDL medium was used, however, the problem with CSL data was the variability between batches. None of the preservation or prevention of cell settlement methods resulted in positive responses, although pure xanthan gum preserved cultures for the six days evaluated.

In order to avert a sudden reduction of viable cells when high concentrations of lactic acid are used, it could be necessary to harvest cells during the secondary growth phase.

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SDL: Semi-defined lactate

CSL: Corn steep liquor

DFM: Direct-fed microbials

VFA: Volatile fatty acids

LUB: Lactate utilising bacteria

LPB: Lactate producing bacteria

μ_m: Maximum specific growth rate

K_s: Saturation constant

S_r: Substrate concentration in medium

x: Biomass

Y: Growth yield factor

℃: Degrees Celsius

mℓ: Millilitres

ℓ: Litres

I.D: Internal diameter

E.D: External diameter

OD: Optical density

RO: Reverse osmosis

n.d: Not determined

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INTRODUCTION

Ruminant animals are naturally distributed, often in large herds, over a wide diversity of habitats and climatic zones, ranging from Arctic regions to tropical rain forests and, from deserts characterised by large diurnal and seasonal temperature variations, to swamps. This wide distribution is related to their ability to adapt to the utilisation of a broad variety of plant materials and to efficiently digest the most abundant organic material on earth, cellulose, as present in plant cell walls. Ruminants owe this remarkable nutritional adaptability to the presence of a broad spectrum of microbial species, largely obligate anaerobes, in their rumen. As a complex community these microbial species degrade organic material presented in a macerated form, by the host animal, to a limited range of low molecular weight compounds.

These important compounds include straight-chain and branched-chain volatile fatty acids and ammonia. They are actively absorbed by the rumen wall and serve as energy sources or as building blocks for biosynthesis of body tissue. The undigested residues of feedstuff degradation, plus microbial cells generated in the rumen, are transported to downstream compartments of the digestive tract where they are subjected to the action of enzymes, secreted by the host and by the microbial populations resident in those organs. In this way, further nutrients, e.g. B-vitamins, are released and become available for metabolism by the host.

As in other nutrient-rich microbial habitats there is, on the one hand, intense competition of species capable of metabolizing the readily available energy sources. Those species which are best adapted to fill a particular niche gain dominance. On the other hand, there is also synergistic interaction between species at the end of a food chain which degrade highly polymeric feed components like cellulose and starch, and other species utilising low molecular compounds released in this process. Gradual transitions in the diet of ruminants produce shifts in the composition of the microbial population of the rumen without significantly affecting the nature and proportions of fermentation products made available to the host. However, abrupt and substantial changes in the composition of feedstuffs tend to cause severe digestive and metabolic disturbances, sometimes with fatal consequences.

Under non-intensive farming conditions this may occasionally happen, for instance when domestic animals accidentally gain access to large quantities of grain. However, in intensive feedlot farming practices, economic pressures demand that cattle which were raised on pasture, should be fattened in the shortest possible time by rapidly increasing the energy and protein content of their diet. A similar situation holds for high producing dairy cattle immediately after calving. In both cases, human intervention places demands on the adaptive capacities of domestic ruminants for which they were not equipped by nature.

Lactic acidosis is among the negative consequences of such interventions. Lactic acidosis is a disorder that is characterised by an accumulation of organic acids, especially lactic acid, in the rumen as result of the intake of high-concentrate diets (Dawson and Allison, 1988). Two forms of lactic acidosis are, acute (sudden) and sub-acute (chronic) acidosis. Acute acidosis is a ruminal condition characterised by pH values below 5.0, approaching 4.5 or lower (Stock and Britton, 1993; Owens *et al*., 1998; Krause and Oetzel, 2006). Sub-acute acidosis is a ruminal condition characterised by pH values between 5.0 and 5.6 (Stock and Britton, 1993).

The combined symptoms of these conditions include decreased milk production, reduced fat test, poor body condition despite adequate energy intake, high cull rates, epistaxis, diarrhoea, bloating, lameness, dilated pupils and elevated heart rate.

A number of curative methods have in the past been developed but almost all have not been without limitations. Gradual adaptation from low to high-concentrate diet limits the ability of the farmer to produce beef or milk in line with market demands. Antibiotic usage presents resistance and environmental risks. The use of probiotics, or microorganims naturally occurring in the rumen, provides the benefit of utilising lactate as their carbon source. However, the challenge for microbiologists is to isolate lactate utilisers, commonly known as lactate utilising bacteria (LUB), for enrichment in laboratory growth experiments.

The main LUB in the rumen are *Veillonella parvula, Veillonella alcalescens, Propionibacterium acnes, Butyribacterium, Corynebacterium enzymicum, Selenomonas ruminantium* and *M. elsdenii* (Slyter, 1976). *Selenomonas ruminantium* and *M. elsdenii* have proven to be more effective as lactate utilisers, than other LUB (Slyter, 1976). However, *S. ruminantium* has the tendency to be lost in enrichment studies.

In feedlot and dairy pens, *M. elsdenii* could be used as a direct-fed microbial (DFM). DFM's are microbial cultures that consist of naturally occurring culture preparations fed to animals for beneficial post-ruminal effects, for example, improved establishment of the normal gut microflora. The concept is similar to probiotics, which are microbial cultures that are commonly used in humans, for example, yoghurt cultures. Ghorbani (2002) reported that supplementing cattle with DFM's resulted in a decreased risk of lactic acidosis and had no effect on ruminal or blood pH.

Cultivation of *M. elsdenii* has been conducted in batch and continuous culture systems. The latter method provides the advantage of steady state growth. However, both these methods would be applicable in cases where the culture is harvested into harvest vessels for inoculation trials involving cattle.

Kemira Phosphates (2006) recently isolated a Gram-negative, fast-growing, acid and ionophore resistant lactate utiliser, *M. elsdenii* NCIMB 41125. Animal trials indicated that *M. elsdenii* NCIMB 41125 is effective in the prevention of lactic acidosis. This organism will be used throughout this study.

Logistics of handling large volumes of culture demand that the culture be stored in vessels for certain periods before delivery to inoculation sites at feedlots. This might affect the viability of the cells after dosing into the cattle. Sodium lactate (carbon source) and glycerol (preservative) will be used and tested in this study as post-harvest additives. The degree of viability of the cells in the culture, before dosing the animals, will be evaluated.

Settlement of the culture at the bottom of kegs occurs as a result of storage and hence access to nutrients, would be limited and consequently a decrease in the shelf-life of the culture in the vessels. The use of thickening agents

(stabilising agents) would be applicable in keeping cells in suspension throughout storage periods. Gum xanthan and gelatin are two additives that will also be evaluated for this function.

The basis of this study was formulated upon the optimisation of a selective medium that supports growth of *M. elsdeni.* The shelf-life of *M. elsdenii* NCIMB 41125 was determined so that inoculation of cattle with predetermined cell numbers from the vessel, resulted in the ability of the inoculum to multiply in the rumen. For better shelf-life of the culture, conditions were optimised to at least match the anaerobic, rumen environment.

To our knowledge, this thesis is the first to report on shelf-life of anaerobic microorganisms and therefore, no similar results are available for comparison.

CHAPTER 1

LITERATURE REVIEW

1.1 Microflora of the rumen

A broad array of animals consumes and survives on plant materials. Terrestrial mammals have different types of gut and are hence designated as monogastric or ruminants. Ruminant animals have four different gut compartments. Apart from a large variety of wild animals, ruminants include many different domesticated species which play an important role in the economic and cultural lives of human communities in diverse climatic zones of the earth, e.g. cattle, sheep, goats, water buffalo, camels, reindeer, lama, alpaca and yak. The animals have different metabolisms and microbial diversity due to different gut shapes. As far as nutrition is concerned, ruminants are remarkably adapted and their digestive system is primarily geared to the utilisation of the most abundant, but slowly digestible, carbohydrate in nature, *viz*. cellulose. Where farming practices require higher rates of production of meat and milk, cattle are usually fed more readily digestible carbohydrates, especially grain and other sources of starch in addition to roughage. Provided moderate amounts of such readily fermentable carbohydrates are fed and the transition from a high-roughage diet is gradual, the animals normally adapt successfully to the dietary change. However, in modern intensive farming practices, there is an increasing tendency to make the transition from roughage diets to diets with a very high energy content in the shortest possible time for economic reasons. Under these circumstances, the problem of lactic acidosis arises.

The prevalence and economical importance of lactic acidosis in feedlot systems necessitates in-depth research to control and/or prevent this metabolic disorder.

1.2 Fermentation by rumen microflora

The microbial ecosystem is normally relatively stable but can be dynamic when acted upon by external factors. These factors may be feed, water or air. The microbial population is adapted to survive and to change when the rumen is subjected to new feed ingredients (Kamra, 2005). The changes in microbial

populations, as a result of feed intake, may hence be associated with changes in fermentation patterns of nutritional factors by the microorganisms.

Kamra (2005) listed a broad range of substrates including cellulose, starch, pectin, urea and proteins that have specific affinities with members of bacteria, bacteriophages, fungi, protozoa and archaeae. Bacteria form a larger pool of the microbial population and hence are important in fermentation of substrates. Fermentation patterns of ruminants on forage-based feed show a balance in the roles of microoganisms as feeds are ingested until the point of excretion (Mackie and Gilchrist, 1979; Brown, 2006). However, increased availability of fermentable substrates such as starch and sugars, results in an increase in microbial growth rates and fermentation activities (Nagaraja and Lechtenberg, 2007). This situation leads to increased production of fermentation products, more especially volatile fatty acids (VFAs) and lactic acid. While VFAs provide energy to the animal, the lactic acid may cause a fatal disorder known as lactic acidosis.

1.3 Lactic acidosis in ruminant animals

By definition, lactic acidosis is a digestive disorder in ruminants that may occur when there is a sudden excessive intake of readily fermentable carbohydrates, particularly when ruminants are switched from a diet of roughage to energy-rich concentrate diet containing high levels of starch. The disorder is characterised by accumulation of organic acids, especially lactic acid, in the rumen (Dawson and Allison, 1988). Ruminal acidity and osmolality increase markedly as the organic acids accumulate. This may lead to damage of the intestinal wall, increased blood pressure and dehydration that may prove fatal (Owens *et al*., 1998). Destruction of the papillae (finger-like projections lining the rumen wall that aid in the absorption of nutrients in the rumen) and damage to the linings of the intestines, may result in poor absorption of nutrients and consequently to low gains and poor feed efficiencies.

The total volatile fatty acid (VFA) concentration generally increases from the onset of acidosis, but with progression of acidosis, their VFA concentrations decrease dramatically because of the destruction of the bacterial flora and ruminal dilution from influx of fluids to compensate for increased osmolality (Huber, 1976).

Russell and Rychlik (2001) showed that ruminal acidosis can also be of some major concern to humans when high grain rations can stimulate *E. coli* such as 0157:H7, due to low intestinal pH. Two forms of lactic acidosis have been described, namely, acute and sub-acute acidosis.

1.3.1 Acute acidosis

Acute acidosis is a ruminal condition characterised by pH values below 5.0, approaching 4.5 or lower (Stock and Britton, 1993; Owens *et al*., 1998; Krause and Oetzel, 2006). Effects of acute acidosis include a large increase in lactic acid concentration, an increase in VFA concentration and a large decrease in protozoa (Nagel and Broderick, 1992). The affected animal is depressed and usually ataxic, off-feed, with dilated pupils and an elevated heart rate. The affected animal suffers from diarrhoea and may become recumbent and die within 2 to 5 days after the insult (Nordlund, 1995).

During acute acidosis, blood flow to the gastrointestinal tract is decreased, thereby reducing the absorption of organic acids from the rumen (Huber, 1971). Acute acidosis presents specific signs and symptoms which, if recognised in time, could be treated (Nocek, 1997). Mohamed-Nour (2006), for example, reported a case of acute acidosis in a goat which ingested large quantities of sorghum flour kept for human consumption.

1.3.2 Sub-acute acidosis

Signs of sub-acute rumen acidosis are different from those of acute acidosis and are difficult to diagnose (Stock and Britton, 1993). Sub-acute acidosis is a consequence of maximising energy intake, which requires provision of

approximate levels of physical and chemical dietary components (Nocek, 1997). A ruminal pH range between 5.0 and 5.6 is regarded as sub-acute or chronic acidosis (Stock and Britton, 1993). The major sign of sub-acute acidosis is reduced or inconsistent feed intake. Other symptoms include decreased milk production, reduced fat test, poor body condition despite adequate energy intake, high cull rates, diarrhoea and laminitis (Nocek, 1997).

The management of acidosis is thus clearly of utmost importance. Although the costs associated with sub-acute ruminal acidosis are difficult to pinpoint, the potential costs to the dairy industry are huge (Donovan, 1997; Hutjens, 1999). Sub-acute acidosis occurs more in dairy cattle than in feedlot cattle because the duration of feeding dairy cattle is longer than for feedlot cattle (Nagaraja and Chengappa, 1998).

1.3.3 Ruminal microbiological changes

Early microbiological studies of the aetiology of lactic acidosis in cattle and sheep on high-concentrate diets provided much information on the most important bacterial species involved in excessive production of lactic acid on the one hand and in the utilisation of this acid on the other hand. Their fermentative activities, nutritional requirements, growth physiological characteristics and enzyme pathways were studied in considerable detail. However, since these studies require the isolation of pure cultures, followed by extensive phenotypical characterisation, it was not possible to follow changes in the microbial population at short intervals after a change of diet of the host animal, e.g. from a high-roughage to a high-concentrate diet. This situation has changed drastically with more recent advances in molecular genetics which made it possible for the development of genetic probes of varying specificity for genera species and even strains of a microorganism. Such probes allow the rapid and sensitive detection of organisms of interest present in complex microbial populations, such as rumen contents.

Dietary engorgements result in changes in rumen microbial populations (Fig. 1.1). The proportion of lactate-producing bacteria (LPB) in the rumen can be

as high 90 to 95% of total culturable bacteria in grain-fed animals (Leedle and Hespell, 1980).

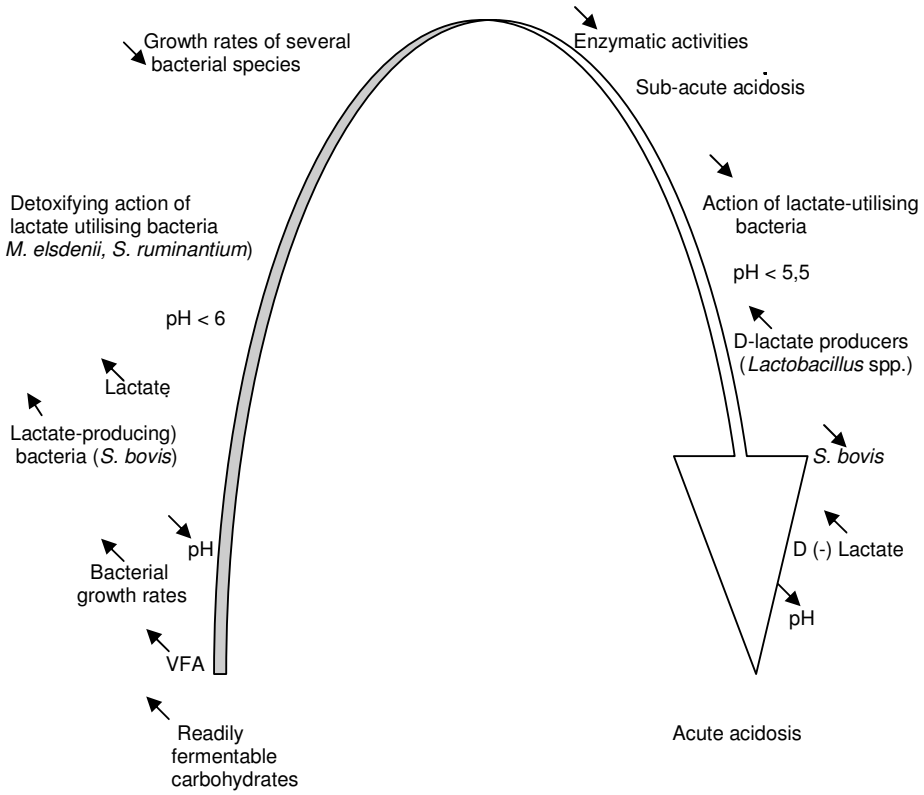

Fig: 1.1: Ruminal changes leading to lactic acidosis. (↘) decrease, (↖) increase. Adapted from: Fonty and Chaucheyras-Durand (2006).

The predominant LPB and maltose-utilising bacteria in the rumen include species of *Bifidobacterium*, *Butyrivibrio*, Eubacteria, *Lactobacillus*, *Mitsukella*, *Prevotella*, Ruminobacteria, Selemonads, *Streptococcus*, *Succinimona* and *Succivibrio* (Kotarski *et al*., 1992; Chesson and Forsberg, 1997; Steward *et al*., 1997). Mackie and Gilchrist (1979) reported that *Butyrivibrio*, Eubacteria and lactobacilli were the major genera in sheep adapted to high grain diets. Examples of ruminal bacteria that have fast growth rates and which rapidly ferment starch or soluble sugars, and contribute to rapid accumulation of DL-lactic acid and VFA, include *S. ruminantium*, *S. bovis* and anaerobic lactobacilli.

Selenomonas ruminantium, a Gram-negative curved rod, is a predominant species in the rumen and consistently increases in concentration in grain-fed

animals. It may be the most dominant organism in animals adapted to high-concentrate diets (Caldwell and Bryant, 1966, Latham *et al*., 1971). Ruminal selemonads are classified into two subspecies, i.e. *ruminantium* and *lactilytica*, based mainly on their ability to utilise lactate and glycerol (Ricke *et al*., 1996). Strains that utilise lactate and glycerol are placed under the subspecies *lactilytica,* and all other strains are grouped under the subspecies *ruminantium*. Therefore, *S. ruminantium* can contribute to both lactic acid production and utilisation.

The explosive growth of *S. bovis*, in response to availability of fermentable carbohydrates, is only observed in situations where the animal is unadapted to a grain diet. Wells *et al*. (1993) found a 10 000-fold decrease in the numbers of *S. bovis* that was similar to results found in forage-fed cattle; this decline is entirely related to ruminal pH. The role of *S. bovis* is to initiate the chain of events that will eventually lead to acute ruminal acidosis (Nagaraja and Miller, 1989; Gill *et al*., 2000). *S. bovis* cannot ferment lactate but is capable of fermenting carbon sources such as glucose and maltose.

Ruminal lactobacilli are more resistant to low pH than *S. bovis*, which explains why they become dominant in the acidic rumen (pH < 5.6). A significant increase in the population of lactobacilli is a common feature of both acute and sub-acute acidosis (Slyter, 1976; Nagaraja and Miller, 1989; Goad *et al*., 1998).

Two predominant species of lactobacilli that have been identified and characterised, particularly in grain-adapted animals, were *L. ruminis* and *L. vitulinus* (Sharpe e*t al*., 1973; Al Jassim and Rowe, 1999). The former species produces primarily L (+) lactic acid and the latter produces only the D-isomer.

Mackie and Gilchrist (1979) reported an increase in protozoa in proportion with the amount of readily fermentable carbohydrates (RFC) fed during a stepwise adaptation from low to high grain diets. After 7 days, the number of LPB outnumbered lactate-utilising bacteria (LUB). Protozoal populations, which decreased markedly towards day 7, started to increase in order to

regain control of fermentation. The levels of cellulolytic bacteria remained the same, even after 54 days of feeding, despite the reported acid sensitivity of *Bacteriodes* spp.

M. elsdenii and *Selenomonas ruminantium* convert acetate to propionate if the dietary shift is gradual (Owens *et al*., 1998). pH-sensitive ruminal bacteria e.g. cellulolytic bacteria are inhibited if the ruminal pH is less than 6.0 (Russell and Wilson, 1996). pH-resistant ruminal bacteria (*Streptococcus bovis, Prevotella ruminicola*, *Clostridium aminophilum* and *S. ruminantium*) allow their pH to decline which protects the organisms from the influx and accumulation of fermentable acid anions (Russell, 1991).

In steers, adapted to a concentrate diet, *S. ruminantium* subsp. *lactilytica* was reported to be the major LUB (Huber *et al*., 1976), followed by *M. elsdenii* and *Peptococcus asaccharolyticum*. *Anaerovibrio* species were reported to be the major LUB in concentrate-adapted sheep (Mackie *et al*., 1978). The fermentative capacities of the main rumen bacteria are listed in Table 1.1. Although a number of lactate-utilising bacterial species have been isolated from rumen contents, including *Veillonella parvula, Veillonella alcalescens*, *Anaerovibrio lipolytica, Propionibacterium acnes, Butyribacterium*, *Corynebacterium enzymicum,* the two species which appear to play the major role in lactate turnover in the rumen are *M. elsdenii* and *Selenomonas ruminantium* subsp. *lactilytica*.

1.4 Consequences of lactic acidosis to the animal industry

The prevalence of lactic acidosis has negative consequences in animal welfare and for cattle farms. Efforts to obtain data on current estimates of the economical impact for South Africa or other parts in the world were unsuccessful; however, the reviews of Donovan (1997), Enemark (2009) and Plaizier *et al*. (2009) are important in this regard.

Table 1.1: Fermentative capacities of the main ruminal bacterial species

	Cellulose	Hemi-celluloses	Starch	Pectines	Proteins	Triglycerides	Cellobiose	Maltose	Glucose	Glycerol	Lactate	Succinate	Fumarate	Peptides	Amino acids	Hydrogen
Fibrobacter succinogenes	+	+	-	+	-	-	+	+	+	-				-		
Ruminococcus albus	+	+	-	+	-		-	-	-					-		
Ruminococcus flavefaciens	+	+	-	+	-		+	-	-	-				-		
Butyrivibrio fibrisolvens	-	+	+	+	+		+	+	+	+				+		
Prevotella ruminicola	-	+	+	+	+		+	+	+	+	-	-		+		
Selenomonas ruminantium	-	-	+	+	+			+	+	+	+	+	-	+	+	
Streptococcus bovis	-	-	+	+	+		+	+	+	-	-			+	+	
Ruminobacter amylophilus	-	-	+	-	+		-	+	-	-				-	-	
Eubacterium ruminantium	-	-	-			-	+	+								
Megasphaera elsdenii	-	-	-	-	-	-		+	+	+	+		-	+	+	
Anaerovibrio lipolytica	-	-	-	-	-	+	-	-	-	+				-	-	
Methanobacterium ruminantium	-	-	-		-		-	-	-		-			-	-	+
Wolinella succinogenes	-	-	-		-		-			-			+	-	-	+

(+) positive, (-) negative. Adapted from: Fonty and Chaucheyras-Durand (2006).

Stone (1999) reported that the impact of sub-acute ruminal acidosis on cows on a large dairy farm in Wisconsin, in New York State, resulted in reduced milk yields of 2.7 kg/d, milk fat production by 0.35% and milk protein production by 0.12%. The percentage reduction of milk fat and milk protein for an entire lactation period of the cows was estimated at $400 per cow per lactation. Gröhn and Bruss (1990) reported a 0.3% incidence of acute ruminal

acidosis in Ayrshire cows, which was the highest during the first month post-calving and relatively non-existent after three months. Donovan (1997) estimated the cost for the incidence of sub-acute ruminal acidosis between $500 million and $1 billion a year.

The problem of lactic acidosis can also be a huge concern for humans. Russell and Rychlick (2001) reported that low ruminal acidity may result in the shedding of the enterohemorrhagic *E. coli* strain 0157:H7.

1.5 Management and prevention of lactic acidosis

Measures which have been and are currently being used to prevent or manage lactic acidosis in ruminants, fed high-energy diets, can be classified as follows:

- Measures based on the manipulation of diets and feed regimens to counteract the effects of overgrowth of lactate-producing bacteria in the rumen.
- Inclusion of ionophore antibiotics in animal feeds to change the pattern of carbohydrate fermentation away from lactate accumulation.
- Measures aimed at restoring the balance of the microbial population of the rumen, after perturbation by excessive intake of readily fermentable carbohydrates, by the administration of (live) microbial cultures.

1.5.1 Gradual adaptation of animals to concentrate diets

Gradual adaptation from roughage to concentrate diets results in reduction of the proportion of lactate producers, but also a decrease in production efficiencies (Moir and Williams, 1950; Gilchrist and Kistner, 1962; Warner, 1962; Mackie and Heath, 1979; Counotte and Prins, 1981 and Therion *et al.,* 1982). Mackie and Heath (1979) reported that the numbers of lactate-utilising bacteria (LUB) increased gradually through the 60% concentrate diet, followed by a further 16-fold increase when the 71% concentrate-diet was fed.

Molecular-based and culture-based enumeration showed a reduction in *Butyrivibrio* as more grain was fed, but molecular techniques indicated a more prominent role by *Selenomonas* and *Megasphaera* genera (Klieve *et al*., 2003). Based on molecular-based techniques, *S. ruminantium* increased dramatically during 3 days of feeding more than 45% concentrate, whereas *M. elsdenii* became numerous by day 5 of feeding more than 60% concentrate and increased to 10^8 cells.mℓ$^{-1}$ within approximately an additional 7 days of feeding a 75% concentrate diet. The increase of LUB at a later stage than LPB, may relate to a relatively slower growth rate of lactate utilisers. Specific growth rates of lactate utilisers, *Propionibacterium (*0.2 to 0.35 h^{-1}), *S, ruminantium* (0.5 to 1.0 h^{-1}), *M. elsdenii* (0.4 to 0.6 h^{-1}) and *Anaerovibrio* (0.2 h^{-1}) at optimum pH were lower than LPB, *Butyrivibrio* (0.7 h^{-1}) or streptococci (2.35 h^{-1}) (Counotte and Prins, 1981; Therion *et al.,* 1982).

Adaptation of feedlot cattle with increases in dietary concentration from approximately 55 to 90% of diet dry matter in less than approximately 14 days, and allowing *ad libitum* access to feed, generally results in reduced performance during adaptation or over the entire feeding period (Brown *et al*., 2006). Although adaptation could be controlled in feedlot and dairy industries, complications by animal to animal variation in adaptation rates could limit its potential (Wiryawan and Booker, 1995).

1.5.2 Rumen buffering

A buffer is an aqueous solution that resists a change in pH when a strong acid or base is added to it (Erdman, 1988). Dietary buffers are normally used when cows are fed high-energy diets and this often results in low ruminal acidity, which in turn affects parameters such as dry matter intake, milk yield and milk composition (Erdman, 1988; Tucker *et al*., 1992; Kennely *et al*., 1999). They are also used when there is a shortfall of saliva and natural feed buffering constituents. They are useful in prevention of post-prandial increases in ruminal fluid hydrogen ion concentration (H^+) and are most beneficial when diets contain corn silage (Erdman, 1988).

A number of chemicals have been used as dietary buffers. Hutjens (1991) reportedly used magnesium oxide, sodium bentonite, calcium carbonate and sodium bicarbonate as dietary buffers. Although the chemical properties of these compounds are not the same, their role is to stabilise the ruminal pH. Kennely *et al*. (1999) found a decrease in ruminal pH (*P = 0.01*) when cows were fed diets containing buffers.

It is evident from the literature that dietary buffers are not used primarily as a cure for lactic acidosis but for the stabilisation of the rumen acid/base balance in order to increase parameters such as dry matter, milk yield and milk composition

1.5.3 Use of rumen contents

Allison *et al*. (1964) conducted an experiment in which eight lambs were fed cracked wheat through ruminal fistula. Four of these lambs were inoculated intra-ruminally with ruminal contents from sheep adapted to a diet containing wheat. Three of the four lambs which were not inoculated with ruminal contents from an adapted animal, became sick on the fourth day of the experiment, while none of the four lambs that were inoculated, became sick. In the animal not adapted to a concentrate diet, the ingestion of relatively high amounts of starch or sugar tends to produce conditions that are conducive to rapid growth of the LPB, *S. bovis*, followed by lactobacilli spp. (Hungate *et al*., 1952; Krogh, 1961). The growth of these organisms appears to be controlled when an adapted animal is similarly fed.

In heifers, intra-ruminal inoculation with crude rumen fluid from concentrate fed cattle, increased daily feed intake by 35% (Cook *et al*., 1975). However, direct application of this approach is inefficient due to the amount of rumen fluid needed for inoculating large numbers of animals and the extreme variability of the procedure (Wiryawan and Brooker, 1995).

1.5.4 Vaccine immunisation

Systemic vaccination of ruminants has previously been shown to induce strong mucosal antibody responses (Sheldrake *et al*., 1985). This strategy has been reported to induce the production of mucosal (IgA and IgGi) antibody responses in sheep against rumen-dwelling commensally ciliates (Gnanasampanthan, 1993). Gill *et al*. (2000) used this method by introducing live or killed *Streptococcus bovis* Sb-5 vaccine with or without adjuvant, via intramuscular injections in sheep which were subsequently fed grain. After the challenge with grain, the sheep maintained high levels of feed intake, high pH, low diarrhoea after 24 h and lower L-lactate concentrations than in the control group. Higher amounts of *S. bovis*-specific antibodies were found in samples of saliva, rumen fluid and serum from sheep immunised with live vaccine than killed vaccine.

The authors suggested the mechanism in the reduction of lactic acid involved binding of the vaccine to the lactate-producing organisms, thereby interfering with their biological antibodies functions. One of the reasons attributed to the less impact of killed vaccine was formalin, which may have reduced the antigenicity of the vaccine. The authors also suggested that resistance to the accumulation of lactic acid could be due to a generalized immunopotentiation rather than an antigen-specific effect. However, specifically generated immune responses could have constituted the major source of the antigen anti-*S. bovis* antibodies in the rumen, which acted against lactic acid-producing bacteria, reducing the severity of lactic acidosis. The authors acknowledged the practical impossibilities of applying this immunisation practice in feedlot cattle.

1.5.5 Use of antibiotics

Ionophores are antimicrobial compounds that are commonly fed to ruminant animals to improve feed conversion efficiency (Callaway *et al*., 2003), but were originally used to control intestinal parasites in poultry (Bergen and Bates, 1984). These compounds transport ions across cell membranes of

susceptible bacteria, dissipating ion-gradients and uncoupling energy expenditures killing these bacteria. Examples of ionophores include monensin, tetranosin, salinomycin, virginiamycin, lysocellin, lasalocid and narasin.

1.5.5.1 Monensin and tetranosin

Monensin has been the mostly used and successful ionophore and plays a role in the decrease of lactic acid (Dennis *et al*., 1981). Monensin treatment increases production of the most reduced VFA, propionate (Dinius *et al*., 1976; Richardson *et al*., 1976; Van Nevel and Demeyer, 1977). Propionate-producing ruminal bacteria such as *S. ruminantium* and *M. elsdenii* are not inhibited by monensin (Callaway *et al*., 1999). Monensin reduces mortality, especially among feedlot animals, by reducing the incidence of acute and sub-acute acidosis, ruminal acidosis, bloat and bovine emphysema (Galyean and Owens, 1988). Monensin reduces acidosis by directly inhibiting LPB (Dennis *et al.,* 1981). A disadvantage of monensin is protein deamination (Russell and Strobel, 1989). Protein deamination by rumen microorganisms is a wasteful process that often produces more ammonia than can be used in microbial growth (Annison, 1956).

Newbold and Wallace (1988) studied the effect of ionophores, monensin and tetranosin, on simulated development of lactic acid *in vitro* by using a co-culture of *S. ruminantium, S. bovis, M. elsdenii* and lactobacilli. Immediately after introduction of glucose, all the bacterial species were stimulated. However, the faster growth rate of S. *bovis* under conditions of excess substrate (Russell and Baldwin, 1978) meant that this bacterium quickly became dominant. The high levels of lactate production associated with *S. bovis* at high growth rates (Russell and Hino, 1985) caused the pH to drop, inhibiting the growth of *S. ruminantium* and *M. elsdenii*. The results with monensin agree with both the *in vitro* results of (Dennis *et al*., 1981), in which lactate production from various carbohydrates was reduced, and *in vivo* results in which monensin prevented the onset of acidosis in artificially stressed animals (Nagaraja *et al*., 1982; Nagaraja and Bartley, 1983).

Furthermore, the results suggested that tetranosin may have a curative and preservative role during the sub-acute stages of lactic acidosis. Tetranosin inhibited the growth of lactobacilli and *S. bovis* after introduction to the co-culture and could have been more effective in the recovery phase, where overgrowth of lactobacilli had already occurred. Monensin was unable to stop the growth of lactobacilli. However, both ionophores would be expected to provide protection against lactic acidosis *in vivo* due to their effective inhibition of *S. bovis* at concentrations that are likely to occur in the rumen (Newbold *et al*., 1998).

1.5.5.2 Salinomycin

Salinomycin is an antibiotic produced by *Streptococcus albus* and is highly effective against Gram-positive bacteria, including *Mycobacterium* species, but it is ineffective against Gram-negative bacteria and fungi (Liu, 1982; Miyazaki, 1974). Olumeyan *et al*. (1986) reported that salinomycin had no effect on cellulolytic and LUB, but the proportion of LPB was higher in salinomycin-fed steers than in control steers. Based on this observation, salinomycin cannot be considered as an effective ionophore in the prevention of lactic acidosis.

1.5.5.3 Virginiamycin

Wiryawan and Brooker (1995) reported that *S. bovis* treated with 0.75 $\mu g.m\ell^{-1}$ of virginiamycin resulted in the prevention of lactic acidosis for up to 12 h, but by 24 h, lactate levels had increased to the levels of untreated controls. However, the combination of virginiamycin and *M. elsdenii* was evaluated for its ability to control lactic acid accumulation for up to 3 days in strained crude rumen fluid cultures incubated in the presence of soluble starch. Starch was added 12 h before the antibiotic-bacteria combination. After 24 h (12 h after introduction of virginiamycin and bacteria), lactic acid in treated cultures was approximately 20% of that in the culture. However, after 36 h, lactate had increased by 3-fold and by 72 h, was 30 mM compared with 50 mM in the controls.

When the combination of virginiamycin and *M. elsdenii* was introduced at 12 h and again at 36 h, lactate levels remained at 15 mM for the duration of the experiment. A combination of *S. ruminantium* subsp. *lactilytica, M. elsdenii* and virginiamycin administered at 12 and 36 h was the most effective treatment and the concentration of lactate in the fermenter did not exceed 5 mM. This is possibly because *S. ruminantium* subsp. *lactilytica* preferentially ferments glucose and produces lactate that sustains *M. elsdenii*.

1.5.5.4 Lysocellin

Kung *et al*. (1992) studied the effect of lysocellin on *in vitro* ruminal fermentation and found that the LPB are sensitive to the ionophore, a result similar to the findings of Dennis *et al*. (1981). However, lysocellin did not reduce the number of LPB.

1.5.5.5 Lasalocid and narasin

The potential use of lasalocid and narasin in the prevention of lactic acidosis was investigated by Nagaraja *et al*. (1987). These authors used ruminal fluid from cattle, fed high alfalfa diet, which was incubated in a buffered medium in the presence of these antimicrobial agents. Results showed that the use of narasin was more effective at low doses while lasalocid was effective at higher doses. The usage of both narasin and lasalocid as ionophores has probably decreased in recent years since little or no information could be found in the literature in this regard. This may be due to new introduced legislation in countries such as the United States of America, which prohibit the use of antimicrobial agents in the animal industry due to the toxicities that accompany these compounds.

Although ionophores could provide solutions for variables such as weight gain, milk increase and reduction of ruminal lactic acidosis, disadvantages may arise. Some of the disadvantages of ionophores are depressed ruminal fermentation, development of resistance, increased production costs and possible presence of antibiotic residues (Wiryawan and Brooker, 1995).

1.5.6 Use of defined biological agents

Compared with the use of rumen contents from 'adapted' animals, the application of defined biological agents has the advantage that a particular strategy for preventing or ameliorating lactic acidosis, may be designed by the choice of biological agent or simple mixture of agents with promising characteristics. As in the case of industrially produced pharmaceuticals versus herbal remedies, the concentration or activity of the preparation can be checked before use and a more reproducible response can be expected than from the use of rumen contents.

1.5.6.1 Yeast cultures

The effect of laboratory-grown cultures of yeast cells in the prevention of lactic acidosis has been extensively studied (Nisbet and Martin, 1991; Rossi *et al*., 1995; Chaucheyras-Durand *et al*., 1996; Newbold *et al*., 1998; Rossi *et al*., 2004). Font and Chaucheyras-Durand (2006) showed that viable yeast cells were capable of exhibiting their metabolic effects when used in combination with other bacteria. Brossard *et al*. (2004) showed that a strain of *Saccharomyces cerevisiae* stimulated ciliate Entodiniomorphid protozoa, which are known to engulf starch granules very rapidly (Coleman, 1974) and compete effectively with LPB for their substrate (Owens, 1998). These studies showed that yeast cells could be effective in the prevention of lactic acidosis through their interaction with other ruminal microbial species.

The ability of yeast cells to scavenge oxygen has been reported by Newbold, (1995). As much as 60 ℓ oxygen can enter the rumen via feed intake, water intake, rumination or salivation. This finding is also supported by Chaucheyras-Durand and Fonty (2002) who introduced live yeast cells in lambs and observed a lower redox potential of the rumen fluid.

1.5.7 Direct-fed microbials

The concept of direct-fed microorganisms (DFM) has been employed recently. DFM are microbial cultures that consist of naturally occurring culture preparation and was primarily based on the potential beneficial post-ruminal effect, including improved establishment of beneficial gut microflora (Fuller, 1999). The concept is similar to probiotics, which are microbial cultures that are commonly used in humans and examples include yoghurt. DFM have also been used to describe viable microbial cultures, culture extracts or enzyme preparations (Yoon and Stern, 1995). Ghorbani *et al*., (2002) reported that supplementing cattle with DFM resulted in a decreased risk of lactic acidosis and had no effect on ruminal or blood pH.

Escherichia coli O157:H7 and *Salmonella* play a significant role in food-borne infections. In humans *E. coli* O157:H7 causes acute haemorrhagic colitis with a small percentage resulting in haemolytic uremic syndrome (Potter *et al*., 2004), whereas *Salmonella* causes salmonellosis. The effect of DFM on fecal shedding of *E. coli* O157:H7 and *Salmonella* in naturally infected feedlot cattle were also evaluated by these authors in a clinical trial involving 138 feedlot steers. A significant reduction (32%) in fecal shedding of *E. coli* O157:H7, but not *Salmonella,* was observed among the treatment steers compared with a control group during finishing. This study suggested that DFM were capable of significantly reducing fecal shedding of *E. coli* O157:H7 in naturally infected cattle, but not *Salmonella*.

A study to investigate the effects of oral supplementation of the lactic acid-producing bacterium *Enterococcus faecium* EF212 alone, or in combination with *Saccharomyces cerevisiae* on mediators of the acute phase response in feedlot steers, showed that supplementation of feed had no effect on concentrations of SSA, lipopolysaccharide binding protein, haptoglobin, or α_1-AGB in plasma compared with those of the control groups. Feeding of *E. faecalis* and yeast increased plasma concentrations of serum amyloid A (SAA), lipopolysaccharide binding protein and haptoglobin but had no effect on α_1-acid glycoprotein (AGB).

The combination of yeast and *Enterococcus faecalis* has also been reported in a study by Nocek and Kautz (2006). The study entailed the use of DFM on minimal digestion, health and performance of pre- and post-partum of dairy cattle. Cows supplemented with DFM had higher estimated ruminal available dry matter (DM) for both corn silage and haylage, than in control cows. Supplemented cows consumed more DM during both the pre- and post-partum periods. In addition, those supplemented with DM produced 2.3 kg more milk per cow per day than did non-supplemented cows. There was no difference in 3.5% fat-corrected milk. Milk fat percentage was lower, but not depressed for cows receiving DFM. There were no differences in milk fat yield or milk protein percentage and yield. Cows consuming DFM had higher blood glucose post-partum, as well as lower β-hydroxybutyrate levels both pre-partum and on day 1 post-partum. Plasma non-esterified fatty acid concentration was not statistically affected by DFM, but was numerically lower pre-partum and higher post-partum for supplemented cows.

The use of DFM has not always provided required results. A study by Raeth-Knight *et al*. (2007) showed that supplementing dairy cows in mid-lactation with DFM products containing *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* for 84 days did not affect DMI or milk production. There was also no effect of DFM supplementation on diet digestibility or rumen fermentation.

Nocek *et al*. (2000) reported a reduced risk of acidosis in dairy cows fed a combination of lactate producing bacteria, lactobacilli and enterococci, presumably because the presence of these bacteria caused the rumen microflora to adapt to their presence within the rumen. Greening *et al*. (1991) found that inoculation with *M. elsdenii* significantly reduced ruminal pH and lactate concentrations in acidosis-induced cattle. Inoculation of an *in vitro* fermentation with *M. elsdenii* also prevented accumulation of lactic acid (Kung and Hession, 1995).

1.6 Taxonomy and physiology of *M. elsdenii*

1.6.1 Taxonomic characteristics

Identification and classification of microorganisms is a cornerstone in Microbiology. Failure to classify these diverse populations may hinder progress as some of these microorganisms could, for example, be harmful to the environment. *M. elsdenii* was discovered more than five decades ago but it was only in the 1970's that this bacterium was correctly assigned its name. Recent studies, especially molecular techniques, have provided more answers to the taxonomy of *M. elsdenii.*

Megasphaera belongs to the Sporumusa sub-branch of the phylum of Gram-negative bacteria, *Firmicutes* (Stackerbrandt *et al*., 1985; Schleifer *et al*., 1990; Doyle *et al*., 1995; Carlier *et al*., 2002). Within the *Firmicutes*, it belongs to the family *Veillonellaceae* (Rogosa 1971; Both *et al*., 1992; Willems and Collins, 1995; Carlier *et al*., 2002).

Rogosa (1971) describes *Megasphaera* as Gram-negative cocci, 2.0 µm or more in diameter that occurs as single cells, pairs and occasionally in chains. *M. elsdenii* is the major lactate-utilising organism in the rumen of adapted cattle, fed high grain diets. This bacterium, (formerly *Peptostreptococcus elsdenii),* is an obligately anaerobic bacterium known as a normal inhabitant of the rumen of cattle and sheep, can use both carbohydrates and organic acids, and is considered to be one of the organisms involved in lactic acid metabolism and amino acid deamination (Hungate, 1966). High numbers of *M. elsdenii* can also be cultured from intestinal contents of human and pigs (Giesecke *et al.,* 1970; Werner, 1973; Vervaeke and Van Assche, 1975). *M elsdenii* has an outer membrane, but 16S rRNA sequencing indicated that it is more closely related to Gram-positive bacteria than to Gram-negative species (Stackerbrandt *et al*., 1985).

1.6.2 Genotypic characteristics of *M. elsdenii*

The advent of molecular techniques has placed the microbiology sphere in a better position to elucidate the analysis of the relationships among ruminal organisms. The other important benefit entails the comparison of bacterial species in a broad range of ruminants. Píknová *et al.* (2006) studied the genetic variability in sheep, lamb and calf. The authors showed that the 16S rDNA was similar among *M. elsdenii* strains. The phylogenetic analysis of *M. elsdenii* T5 was identical (different in less than 5 nucleotides) to sequences of *M. elsdenii* which originates from different habitats. These isolates originated from swine colon and cow rumen in the USA (strain Y-J4), Australia (strain LA 03) and South Africa (ATCC 25940). The authors speculated that the low genetic variability reflected a nutritional strategy of *M. elsdenii* in that its role in the rumen is largely the utilisation of lactate. The other explanation for the low genetic variability could be the invariable spectrum of substrates, which imply that *M. elsdenii* may not be significantly affected by the dietary changes which may force this organism to quickly adapt to the different energy sources. The authors also speculated that *M. elsdenii* could have evolved from an ancestral strain, which contained epigenetic factors such as mobile gene elements (plasmids, transposons or bacteriophages), gene arrangements and mutations that may promote the transfer of genes between phylogenetically homologous populations contributing to the genetic variability.

1.6.3 Metabolism of lactate by *M. elsdenii:* the acrylate pathway

In the rumen, lactic acid is produced from the fermentation of glucose, fructose, maltose, lactose and cellobiose but not from pentoses, except ribose (Slyter, 1976). Usually, one group of organisms produces lactic acid and the second group ferments it to VFA, although the first stage occurs at a more rapid rate than the second. The VFA (acetate, propionate and butyrate) are the main products of lactate metabolism (Marounek and Bartos, 1987; Marounek *et al.*, 1989; Hino *et al.*, 1994).

Butyrate and propionate are the major VFA of lactate utilisation by *M. elsdenii.* The production of propionate normally occurs via a randomizing pathway. However, Ladd (1959) confirmed in one of his studies that the production of propionate from lactate occurred via an acrylate pathway. *M. elsdenii* was found to be the only organism to use the acrylate pathway in the production of propionate from lactate. More studies by Baldwin *et al*. (1962) and Counotte and Prins (1979, 1981), showed similar results to the findings by Ladd. Their results indicated that *M. elsdenii* could ferment between 60 and 80% of the DL-lactate in the rumen to either propionate or butyrate.

The production of VFA, especially propionate and butyrate, can stimulate the rumen environment (McLeod and Baldwin, 2000). Weigand *et al*. (1975) reported that approximately 0.90% butyrate can be absorbed by the rumen, hence providing energy for rumen wall thickening, papillae and capillary development.

Volatile fatty acids form part of the end-products of starch metabolism but are readily absorbed from the rumen (Bergman, 1990). The absorption rates of the VFA are butyrate>propionate>acetate. The quantities of these VFA in the effluent are in reverse order due to the preferential metabolism of butyrate and propionate. It follows that VFA are utilised through intermediate metabolites following formation of the respective CoA metabolites. The proportions of the VFA are not the same in specific tissue - for example, a large proportion of butyrate and propionate in the bloodstream is metabolised in the liver whereas as much as 90% acetate is metabolised in the arterial blood.

Butyrate plays a specific role in supporting the normal function of the large intestine, for example, the promotion of the growth of epithelial cells (Young and Gibson, 1995; Sakata, 1997; Tsukahara *et al*., 2003), the stimulation of mucous release (Shimotoyodome *et al*., 2000), the absorption of minerals and water (Roediger and Moore, 1981; Holtug *et al*., 1992) and the prevention of the colorectal cancer (Kameue *et. al*., 2004).

Butyrate production plays also a role in the gut of pigs due its role in the proliferation of epithelial cells. Tsukahara *et al*. (2006) reported on the use of *M. elsdenii* in combination with *Lactobacillus acidophilus* for the production of butyrate. The rationale for using the two bacteria emanated from the fact that sodium gluconate, a bifidogenic saccaharide, often escapes from the small intestine. By using the two bacteria, when pigs are fed sodium gluconate, absorption in the large is enhanced. It was also found that butyrate was produced in higher concentrations when *M. elsdenii* and *L acidophilus* were used in combination than when used individually.

In another study, butyrate was produced in high amounts during continuous growth of *M. elsdenii* cultured on a medium containing a yeast additive (Soto-Cruz *et al*., 2001). The presence of a yeast additive resulted in more butyrate than propionate and acetate. The authors suggested that soluble compounds in the yeast additive filtrate enhanced the anabolism of *M. elsdenii* and modified the carbon fluxes through its pathway, by increasing butyrate and decreasing valerate production in the culture.

While *M. elsdenii* is known to utilise lactate via the acrylate pathway (Asanuma and Hino, 2002), *S. ruminantium* is only capable of utilising D-lactate to pyruvate, via the NAD-independent D-lactate dehydrogenase, because this organism lacks both the lactate racemase and the L-lactate dehydrogenase. *S. ruminantium,* however, is capable of metabolising lactate, if lactate racemase is introduced into the cells. It follows that in order to increase lactate utilisation, *S. ruminantium* is augmented with both lactate racemase and ID-LDH (Asanuma and Hino, 2005).

1.6.3.1 Lactate uptake

Under anaerobic conditions, pyruvate is converted to lactate to regenerate the NAD used in glycolysis. Ruminal and silage microbes produce two forms of lactate, the D+ and L-form. The L-form is identical to that produced from glucose by exercising muscle and can be readily metabolised by the liver and heart tissue. In contrast, D+ lactate, typically 30 to 38% of the total lactate

found in the rumen, is not produced by mammalian tissues (Owens *et al*., 1998). A study by Waldrip and Martin (1993) showed that lactate uptake is not inhibited in the presence of sugars such as maltose, glucose and sucrose. Furthermore, pH values of between 4 and 5 increased the lactate uptake. However, lactate (L-lactate) was inhibited by protonophores. These results suggest that protons are involved in the uptake of lactate.

1.6.3.2 Substrate preferences

Research has shown that catabolite regulatory mechanisms, where bacteria exhibit preferential and sequential use of one or more substrates to the exclusion of others, play a role in substrate utilisation, that influence lactate in the rumen (Russell and Baldwin, 1978). In the case of *S. ruminantium,* lactate utilisation is suppressed in the presence of glucose, sucrose and xylose. Hishinunuma *et al.* (1968) reported inhibition of lactate utilisation by glucose, when this bacterium was used. However, *M. elsdenii* can utilise glucose, maltose and lactate simultaneously (Russell and Baldwin, 1978).

S. ruminantium undergoes catabolite repression (Russell and Baldwin, 1978) and would compete with LPB such as *S. bovis* for substrates. Although substrate preference is important, other factors such as substrate affinity, maintenance energy expenditure, growth yields and maximum growth rates on multiple substrates are also implicated in substrate utilisation in the complex rumen environment (Russell and Baldwin, 1979; Russell *et al*., 1979). The sporadic appearance of *S. bovis* in an adaptation experiment using a high concentrate diet containing 71% maize and molasses (Mackie *et al*., 1978; Mackie and Gilchrist, 1979) can be explained by its low affinity to glucose. If L-lactate is the predominant isomer, then *S. bovis* would be implicated as the cause of acute acidosis, since it only produces L-lactic acid (Slyter, 1976; Russell and Baldwin, 1978).

The utilisation of lactate by lactate-utilising bacteria such as *M. elsdenii*, *Selenomonas ruminantium* and *Veillonella parvula* may present problems associated with methanogenesis (Asanuma and Hino, 2005). Methanogenesis

occurs as a result of *M. elsdenii* building due to the available hydrogen, which is one of the end-products of lactate metabolism. *S. ruminantium* metabolises lactate to mainly propionate and succinate, thereby producing a lower amount of hydrogen than *M. elsdenii* (Asanuma and Hino, 2004). Some strains of *S. ruminantium* reduce nitrate and nitrite to ammonia (Iwamoto *et al*., 2001; Yoshii *et al*., 2003). The reduction of nitrate and nitrite in the rumen is important because it can decrease methane production (Hino and Asanuma, 2003). However, nitrate can be toxic to the rumen environment if supplied in high concentrations. To avert this situation, lactate is fed at adequate amounts as it can stimulate the reduction of nitrite.

In the rumen, *M. elsdenii* and *S. ruminantium* have been reported to produce cobalt, which helps in the synthesis of vitamin B_{12} (Tiffany *et al*., 2006). Vitamin B_{12} is a growth factor of ruminal bacteria such as *Prevotella ruminicola* and *Methanomicrobium mobile* (Tanner and Wolfe, 1988).

Malate, which has been reported to stimulate lactate utilisation by *Selenomonas ruminantium* (Callaway and Martin, 1996), exhibited no effect on *M. elsdenii* (Khampa and Wanapat, 2006). This was after the authors supplemented malate and urea in concentrate cassava chip, which was then offered to four lactating cows.

M. elsdenii has been implicated in the production of conjugated linoleic acid (CLA) (Kim *et al*., 2002). CLA is a fatty acid with two conjugated unsaturated double bonds at different positions, which is mainly an intermediate during the biohydrogenation of linoleic acid in the rumen (Kepler *et al*., 1966). CLA has various health benefits such as anti-carcinogenic, anti-diabetic, anti-atherogeneic and anti-dipogenic (Nagpal *et al.,* 2007). Puniya *et al.* (2008) conducted an *in vitro* trial to examine the potential of *Lactobacillus* isolates, namely, *Lactobacillus viridescens, Lactobacillus lactis* and *Lactobacillus brevis* for the production of linoleic acid. Results showed that *Lactobacillus brevis* may be used as a direct-fed microbial because of the higher production of CLA when lower concentrations sunflower oil were used. Similarly, a human intestine isolate of *M. elsdenii* (Alonso, 2003), may be tested in *in vitro*

studies similar to those reported by Puniya *et al*. (2008) , and subsequently tested in *in vivo* trials.

1.7 Selective isolation of lactic acid utilising bacteria

Around 1992, the Agricultural Research Council, with financial backing by Kynoch Feeds, which was later taken over by Kemira Phosphates, decided to embark on a fresh approach to the isolation and screening of cultures of lactic acid-utilising bacteria from rumen contents of high-producing dairy cows and feedlot animals, adapted to energy-rich diets. The aim was to obtain highly active cultures which would serve as a starting point for the development of a commercially viable preparation for prevention and treatment of lactic acidosis in feedlot and dairy cattle.

The ideal lactate utiliser for such therapeutic treatment should possess a combination of attributes: It should be able to multiply rapidly under the conditions prevalent in the rumen of animals on high-concentrate diets. This would include the presence of relatively high concentrations of lactic acid and pH values below 5.5. The organisms should be able to utilise both optical isomers of lactic acid at a rapid rate, even in the presence of soluble carbohydrates such as maltose and glucose. The products of lactate metabolism should be beneficial to the host animal. Also, the growth of the introduced cultures should not be inhibited by the ionophore antibiotics commonly used in feedlot operations to counteract lactic acidosis (Kemira Phosphates, 2006).

A two-pronged strategy was followed. On the one hand, suitable dilutions of rumen fluid were plated directly on habitat-simulating agar media containing sodium lactate as the only added energy source. To enhance the selectivity of the medium, a pH indicator was included to show up those colonies in whose vicinity a rise in pH occurred as a result of conversion of lactic acid to weaker acids. A large number of such colonies were isolated and passed through a series of screening tests for other desired attributes. The most

promising strains were subjected to more detailed biochemical and growth-physiological tests, and all of these large cocci, resembled *Megasphaera*.

The second strategy was aimed at enrichment, from the already pre-selected microbial population present in rumen contents of animals on high-concentrate diets, those strains which could thrive under sustained, simultaneous pressure of a combination of selective factors. The application of continuous culture systems was considered to be highly suitable for this purpose (Veldkamp, 1970; Krieg, 1981). However, by opting for the pH-auxostat (Martin and Hempfling, 1976) version of continuous culture and keeping the pH differential between control point and the incoming poorly-buffered medium small, a further selection pressure was applied. This was selection, within the heterogeneous population, for the fastest-growing individuals under the given, constant environmental conditions. These individuals determined the rate at which lactic acid was converted to weaker acids, and thus the rate of deviation from pH set point and the rate at which fresh medium was added. *i.e.* the dilution rate of the system. In time, slower growing species and strains in the population were washed out of the fermenter, which made isolation of pure cultures relatively easy. Of the seven cultures thus obtained, six were morphologically typical of the genus *Megasphaera*, while the seventh was a spore-former. Selenomonads were observed microscopically in the very early stages of some of the enrichments but they were soon ousted by *Megasphaera* strains.

The isolates with the most favourable set of characteristics from the two selection procedures were then compared with regard to maximum specific growth rate, effect of pH on growth rate, biomass output rates in continuous culture, and end products of lactate metabolism. Two strains were eventually introduced into the rumens of sheep on a high-concentrate diet in a controlled experiment and both were capable of preventing acidosis by preventing the accumulation of lactic acid and consequent drop in pH. Isolate CH4 was selected for further study as it met all the requirements intended for. The isolate was patented and transferred to a culture collection and hence given a new name, *M. elsdenii* NCIMB 41125.

1.8 Physiological properties of *M. elsdenii* NCIMB 41125

M. elsdenii NCIMB 41125 is capable of proliferating at relatively low pH values i.e. below pH 5.0 and as low as 4.5, characterised as acute acidosis. It is resistant to antibiotics commonly added to feedlots diets, especially monensin and lasalocid. It is also capable of utilising lactate as a carbon source in the presence of soluble carbohydrates such as maltose and glucose. *M. elsdenii* NCIMB 41125 has a relatively high growth rate, i.e. more than 0.94 h^{-1} (Kemira Phosphates, 2006) and is capable of fermenting both D (+) and L (-) lactic acid with the formation of acetic, propionic, butyric and valeric acids (Wiederhold, 1994).

Figure 1.2 shows a phase-contrast microscopic image of *M. elsdenii* NCIMB 411254, taken at the Agricultural Research Council (South Africa). Even though the sample from which the picture was taken contained less dense populated cells, the appearance and morphology of the cells show single, pairs and a chains of four cocci.

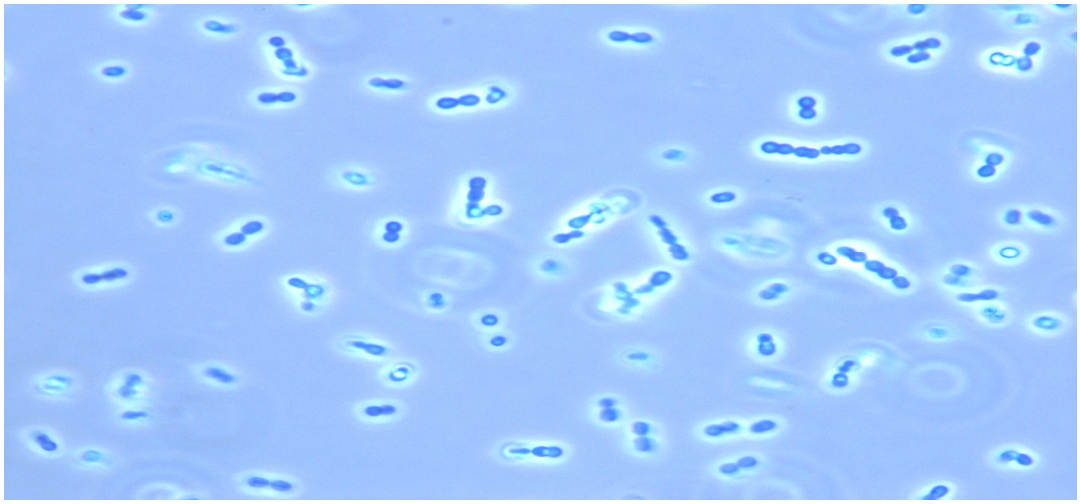

Fig. 1.2: Phase-contrast microscopic image of *M. elsdenii* NCIMB 41125

The appearance of *M. elsdenii* NCIMB 41125, shown in Fig. 1.2, is consistent according to Rogosa (1971), who described cells of *M. elsdenii* as appearing in single, pairs and occasionally in chains.

1.9 Phylogenetic relationship of *M. elsdenii* NCIMB 41125 and other bacteria

M. elsdenii type strain (ATCC 25940), *M. elsdenii (*NCIMB 41125 and CH7) and representatives of some major rumen bacteria, were used for the construction of a phylogenetic tree. *M. elsdenii* CH7 is the isolate obtained during evaluation of several isolates as potential candidates for the prevention of lactic acidosis (Kemira Phosphates, 2006). The sequences for *M. elsdenii* NCIMB 41125 and CH7 were obtained from the 16S-rDNA gene.

The phylogenetic analysis (Fig. 1.3) was constructed using the sequence information in the 16S rRNA molecule, currently the most widely used for inferring phylogenetic relationships among microorganisms (Stackebrandt and Rainey, 1995; Woese, 1987; Vandamme *et al.,* 1996). A phylogenetic tree was subsequently constructed using the Treetool program and the "FastDNAml" algorithm of Olsen *et al.* (1994), which is based on the maximum likelihood algorithm of Felsenstein (1981). All strains of *Megasphaera* clustered together, with the type strain showing close relatedness to the strain of interest, *M. elsdenii* NCIMB 41125. *S. ruminantium*, another LUB, appears within the same main root of the tree, while *S. bovis*, an LPB, does not show proximity to *M. elsdenii* NCIMB 41125. Methanogens and *E. coli* are distanced from the LUB and LPB, suggesting that they play no role in lactic acidosis.

1.10 Molecular technology studies on ruminal microbial populations

Traditional methods such the most probable number (MPN) and roll-tube methods have been extensively used in the past for the estimation of rumen microbial populations (Hungate, 1969; Dehority *et al*., 1989). New modern technologies that are now used offer more information on microbial dynamics.

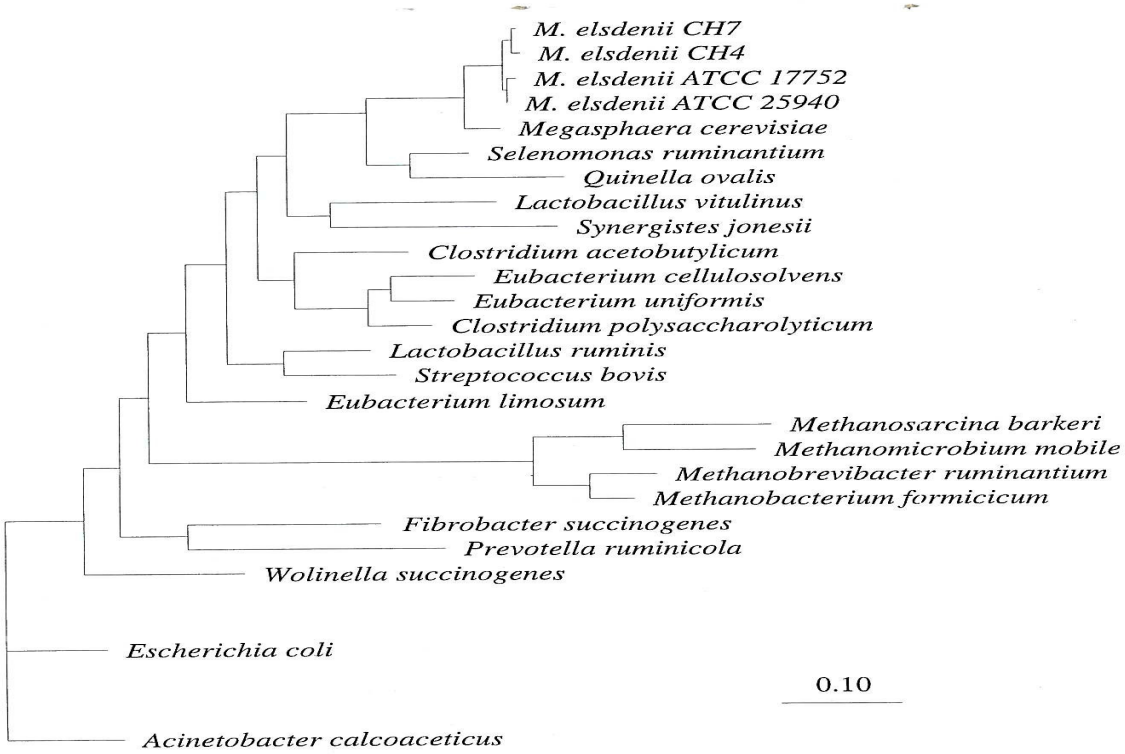

Fig. 1.3: Phylogenetic tree, based on 16 rDNA sequences, showing the evolutionary relationships between members of the genus *Megasphaera* and other representatives of bacterial and archaeal taxa occurring in the rumen. Adapted from: Greyling (1996).

Failure to cultivate certain organisms, which were perhaps difficult to cultivate using conventional laboratory methods, could be avoided. Deng *et al*. (2008) outlined a detailed background on a number of techniques for the quantification of microbial populations, which included Blot hybridization, fluorescence *in situ* hybridization (FISH), competitive PCR (cPCR) and real-time PCR. These authors reported that real-time PCR provided better

advantage than other methods as it overcame the disadvantages of hybridization. The real-time PCR entails the amplification of nucleic acid and sequencing to acquire data on the 16S/18S rDNA/rRNA sequences.

Stevenson and Weimer (2007) obtained samples from lactating cows, for which *Prevotella* species accounted 42 - 60% of the bacterial rRNA gene copies while *M. elsdenii* populations comprised <0.003% of the bacterial rRNA gene copies. By using the quantitative real-time PCR technique, populations of *M. elsdenii* were monitored, although they represented a lower percentage population compared to species of *Prevotella*.

The real-time PCR may be used together with other conventional microbiological methods to ensure the most accurate results for the desired objectives, especially the quantification of microbial populations.

Conclusion

There are several methods that are currently used for alleviation of lactic acidosis in animals. Each of these methods has its own disadvantages and/or limitations. Of the methods mentioned in this literature review, use of microbial inoculations for the prevention of lactic acidosis, is the method of interest for our research group, specifically for feedlot cattle. *M. elsdenii* NCIMB 41125 isolated by the Agricultural Research Council (ARC-Animal Production Institute, South Africa), is one of the lactate-utilising bacteria that can be used to control lactic acidosis. However, there are still problems with regards to growth of this bacterium and its subsequent survival during storage. This is an indication that much work still has to be done to ensure optimum growth and survival of this promising lactate utiliser. The main aims of this study were therefore to optimise growth conditions for *M. elsdenii* NCIMB 41125 and to establish methods for preservation of the culture such that its numbers do not diminish during storage.

CHAPTER 2

MATERIALS AND METHODS

2.1 Anaerobic microbiological procedures

All culture manipulations (inoculation and incubation) were performed in an Anaerobic cabinet, Model 1024, with a controlled atmosphere composition at specific concentrations of carbon dioxide (30%), hydrogen (5%) and balance nitrogen.

Fig. 2.1: Anaerobic cabinet used in this study

2.2 Preparation of stock solutions and growth media

2.2.1 Composition of growth media

The compositions of the different stock solutions used in the preparation of the growth media for *M. elsdenii* NCIMB 41125 are shown in Table 2.1. The compositions of semi-defined lactate (SDL) and corn steep liquor (CSL) media are shown in Table 2.2.

Table 2.1: Composition of stock solutions

Stock solution	Components of stock solution	Concentration ($g.\ell^{-1}$)
Mineral solution 1	K_2HPO_4	11.84
Mineral solution 2	$(NH_4)_2SO_4$	7.971
	KH_2PO_4	7.077
	NaCl	1.777
	$MgSO_4.7H_2O$	3.747
	$CaCl_2.2H_2O$	2.411
Mineral solution 3	KH_2PO_4	40
	$(NH_4)_2SO_4$	120
	$MgSO_4.7H_2O$	8
	$CaCl_2.2H_2O$	2.4
Mineral solution 5	KCl	6.4089
	$MgSO_4.7H_2O$	4.7162
	$CaCl_2.2H_2O$	0.2223
	$FeSO_4.7H_2O$	0.1485
	Citric acid	13.4
Trace mineral solution	$MnSO_4.H_2O$	0.308
	$ZnSO_4.7H_2O$	0.44
	$CuSO_4.5H_2O$	0.079
	$CoCl_2.6H_2O$	0.081
	$Na_2MoO_4.2H_2O$	0.05
	KI	0.013
Vitamin solution 2	Ca-D- Pantothenate	0.2
	Pyridoxal hydrochloride	0.2
	Biotin	0.1

Table 2.2: Composition of SDL and CSL media

Components	Medium	
	SDL	CSL
	ℓ^{-1}	
Peptone	5 g	5 g
Yeast extract	5 g	5 g
L - Sodium lactate 60% (w/w)	33.2 g	25 g
Mineral solution 3	25 mℓ	-
Indigocarmine 0.5 % (w/v)	1 mℓ	1 mℓ
Vitamin solution 2	2 mℓ	2 mℓ
Trace mineral solution	0.5 mℓ	0.5 mℓ
L-cysteine.HCl	2 mℓ	2 mℓ
Sodium sulphide solution	2 mℓ	2 mℓ
Mineral solution 5	-	5 mℓ
Corn steep liquor	-	34 mℓ

2.2.2 Reducing agent solutions

L-cysteine.HCl (12.5% m/v) and sodium sulphide (12.5% m/v) were weighed out and separately dissolved in previously boiled distilled water and each transferred to a 500 mℓ serum bottle (volumes as indicated in Table 2.2). The bottles were sealed with 20 × 20 mm rubber stoppers and crimped with metal screw caps. The bottles were autoclaved at 121ºC for 25 min, cooled to room temperature and gassed with O_2-free N_2 for 10 min. During gassing, a sterile needle was inserted into the septum of the rubber stopper for venting. The bottles were then stored at room temperature and the reducing agent solutions were ready to use until a maximum period of six months.

2.2.3 Preparation of serum bottles

Serum bottles (30 mℓ), rubber stoppers and caps were stored in the anaerobic cabinet overnight before use. After storage, the rubber stoppers and caps were fitted to the bottles and the caps were crimped. The bottles were taken

out of the cabinet, autoclaved for 25 min and transferred back to the anaerobic cabinet.

2.2.4 SDL medium for working cultures

Liquid medium (Table 2.2), (without the reducing agents), was prepared in 1 ℓ screw-necked Schott bottles. The bottles were provided with polypropylene (PP) lids which sealed against the inner surface of the bottle neck with two O-rings and which were fitted with three threaded female Luer-lok-to-3 mm tubing ports (Fig 2.2). When the contents of the bottle were to be purged with O_2-free gas, under aseptic conditions, the inlet and vent ports were fitted with sawn-off 3 mℓ PP syringe barrels fitted with cotton wool (Fig 2.2). The lower ends of the gas inlet and medium outlets were extended to the bottom of the bottle with 3 mm I.D. Norprene tubing (Fig. 3.2). During autoclaving, the gas vent port retained its cotton wool filter, while other two ports were sealed with PP Luer-lok caps. The medium was autoclaved for 20 min and cooled in a waterbath at 55ºC, while gassing with O_2-free N_2 by removing one of the two male plastic Luer-lok plugs connected to the ports. The reducing agents, L-cysteine.HCl, followed by sodium sulphide, were added to the medium through the venting port, using sterile needles connected to the 3 mℓ syringes. When the medium was reduced (yellow colour), the gas line was disconnected and the male plastic Luer-lok plug replaced to its port.

The bottle was then transferred to the anaerobic cabinet for dispensing into the sterile 30 mℓ serum bottles. SDL medium (10 mℓ) was withdrawn from the Schott bottle by fitting a sterile syringe on an opened port and gently pulling the plunger. A sterile needle was fitted on the syringe and the medium transferred, by gently pushing down the plunger, and through the septum of the rubber stopper, into the glass bottle. The serum bottles were then taken out of the anaerobic cabinet and allowed to cool at room temperature before use. For the preparation of SDL agar medium, bacteriological agar (15 $g.\ell^{-1}$) was added in the SDL medium After adding the reducing agents, the SDL agar was transferred to the anaerobic cabinet and immediately plated-out onto Petri-dishes containing dispensed serial dilutions.

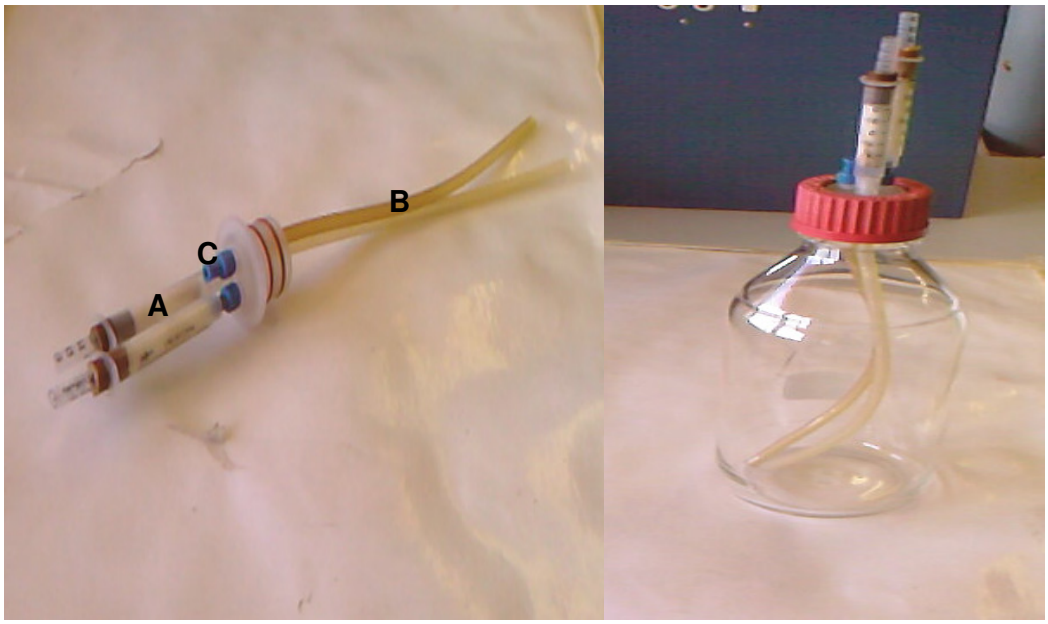

Fig. 2.2: Components of screw-necked Schott bottle: (A) Syringe barrel, (B) Norprene tubing, (C) Luer-lok cap

2.2.5 Diluents

The method for the preparation of diluents was adapted from Caldwell and Bryant (1966). Distilled water (1365 mℓ) was transferred to a 2 ℓ Schott bottle. Indigocarmine (1.5 mℓ) and mineral solutions 1 and 2 (37.5 mℓ each) were added to the bottle. The bottle was fitted to a three-port lid as described in section 2.2.4. Sixty millilitres of $NaHCO_3$ stock solution (39.6 $g.\ell^{-1}$) were pippeted into a 250 mℓ Schott bottle and fitted with a three-port lid which consisted of the tubes with the same length as the bottle. The two bottles were autoclaved at 121ºC for 25 min. The solutions were allowed to cool to room temperature whilst gassing with oxygen-free N_2. The $NaHCO_3$ solution, which had not been gassed, was withdrawn from the bottle with a sterile 60 mℓ syringe and added to the 2 ℓ Schott bottle through the venting port. Similarly, the reducing agent solutions, starting with L-cysteine.HCl, followed by sodium sulphide, were also added to the bottle. The bottle with the reduced solution was gassed for 15 min and transferred to the anaerobic cabinet. The lid of the bottle was replaced with a dispenser top, set at 9 mℓ for injection into sterile long tubes.

2.2.6 Media for fed-batch and continuous cultures

Distilled water was measured into stainless-steel kegs of 20 ℓ to 50 ℓ capacity. (Fig. 2.3), to a volume as determined by the aim of the experiment. All components of the medium, except reducing agent solutions, (Table 2.2) were added to the kegs, shaken to dissolve the solid materials, and the kegs then tightly sealed. The reducing agent solutions, L-cysteine.HCl, followed by sodium sulphide, were withdrawn from the glass bottles and added to the sealed kegs using sterile syringes as shown in Fig. 3.4. Boiled, distilled water was similarly added to the kegs after each reducing agent to rinse excess concentrate solutions from the inner walls of lancets. In-between additions of reducing agent solutions and water, as described above, the female steel Luer was kept closed with a syringe used to add the respective liquid. The tops of the sealed kegs (lancet heads) were covered with aluminium foil and the kegs were autoclaved at 121ºC for 60 min.

Fig. 2.3: Stainless-steel keg: (A) Lancet, (B) Stainless-steel keg

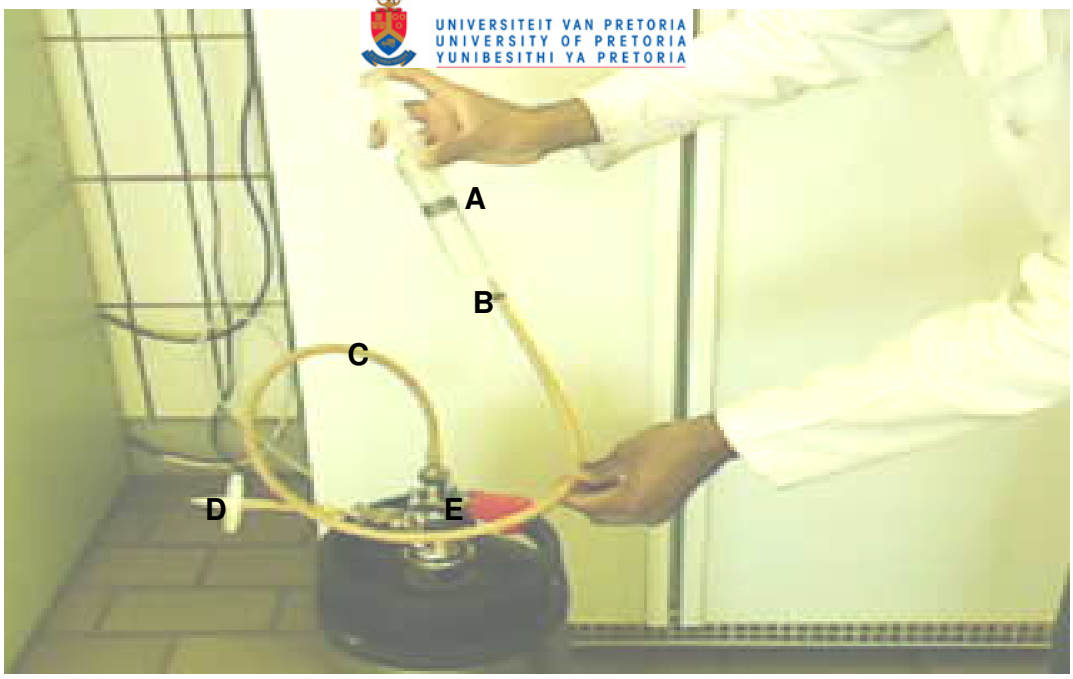

Fig. 2.4: Transfer of reducing agents to kegs: (A) Syringe, (B) Female steel Luer fitted to syringe, (C) Norprene tube, (D) plastic filter, (E) Connector fitted to the sealed lancet top

2.3 Bacterial cultures

Semi-defined lactate agar slant cultures of *M. elsdenii* NCIMB 41125 were obtained from Kemira Phosphates Pty (Ltd) and stored at -196ºC in liquid nitrogen.

2.3.1 Cultivation of *M. elsdenii* NCIMB 41125

M. elsdenii NCIMB 41125 was cultivated in either 30 mℓ sealed, pre-gassed, serum bottles or a 5 ℓ fermenter.

2.3.1.1 Preparation of mother culture and working culture

A mother culture was prepared by inoculating a loopful of the slant culture (SDL) into 30 mℓ serum bottles containing 10 mℓ SDL broth. Working cultures were prepared by transferring 1 mℓ of mother culture into 30 mℓ serum bottles containing 10 mℓ SDL broth. All the cultures were incubated at 39ºC overnight.

2.3.1.2 Cultivation in stirred fermenters

A clean 5 ℓ B-Braun fermenter vessel (model Biostat-B) with the fitted calibrated pH electrode (pH 4 and 7), was autoclaved at 121ºC for 30 min. After autoclaving, the exhaust and water connections of the sterile fermenter were connected to the control unit. The fermenter was gassed with O_2-free N_2 at a rate of 0.2 ℓ.min^{-1} for 1h. The SDL or CSL medium (3 ℓ) was transferred, with gassing of the glass break, to the fermenter using Watson-Marlow 505*S* pumps (Fig. 2.5). The reducing agent solutions, 6 mℓ each, were separately added to the fermenter until the redox potential reached -250 mV. The redox potential of the sample was measured with a redox meter in an anaerobic cabinet. Fifty-milliliters of inoculum containing approximately 10^8 cfu.mℓ$^{-1}$ of *M. elsdenii* NCIMB 41125 was injected through the inoculation port, into the fermenter containing 5 ℓ medium. The fermentation parameters were set as follows: temperature 39ºC, stirrer speed 200 rpm, pH control at 5.5 ± 0.1 by automatic addition of either H_3PO_4 or KOH solutions; foam suppression by the addition of silicone antifoam on detection of foam by a sensor.

2.3.1.2.1 Batch culture

M. elsdenii NCIMB 41125 was cultivated in the fermenter as described in section 2.3.1.2. In subsequent experiments, batch cultures were started by removing, with the harvest pump, all but approximately 0.5 ℓ of culture from a previous run which had been left to stand at room temperature for a maximum of 10 days. The culture was brought to normal working volume by addition of fresh medium with the medium pump, while reinstating temperature, pH and foam control and stirring speed.

2.3.1.2.2 Fed-batch culture

Growth conditions of fed-batch cultures were similar to those of batch culture. However, after 17 h of incubation, 2.5 ℓ of the fermenter constituents was pumped into an empty harvest keg for discarding. Fresh medium was added

to the fermenter to a total volume of 5 ℓ. After a further 17 h incubation, 4.5 ℓ was harvested into a sterile 20 ℓ keg (keg 1) for experimental purposes.

Fig. 2.5: B-Braun fermenter (model Biostat-B) containing *M. elsdenii* NCIMB 41125 culture: (A) Printer, (B) Culture vessel, (C) Control Unit, (D1) Acid reservoir, (D2) Base reservoir, (E) Gas line, (F1) Harvest keg, (F2) Medium keg, (G1) Watson-Marlow 505*S* pump for harvesting, (G2) Watson-Marlow 505*S* pump for medium

The remaining 0.5 ℓ culture in the fermenter was diluted to 3 ℓ with fresh medium and incubated for 7 h, after which 2.5 ℓ was removed and discarded. Fresh medium (4.5 ℓ) was then added to the fermenter and the fed-batch was again incubated for 17 h and 4.5 ℓ then harvested into a sterile keg (keg 2). This procedure was repeated for keg 3, after which the fermenter was emptied, cleaned and re-autoclaved. All the kegs with the harvest were stored at room temperature for shelf-life studies. Shelf-life was determined by

sampling the kegs daily (except for weekends and holidays), and performing viability counts for up to 21 days.

This experiment was repeated but only a single batch of harvest was collected in the second experiment

2.3.1.2.3 Continuous culture

Growth conditions for continuous culture were similar to those for batch growth. After growth of the first batch, 2.5 ℓ of the culture was pumped into an empty harvest keg. An amount of 4.5 ℓ of the fresh medium was added to the fermenter to a total of 5 ℓ working volume. After 17 h incubation, continuous growth was started by activating the medium addition and harvest removal pumps. The *M. elsdenii* NCIMB 41125 culture was continuously grown with combinations of lactic acid concentrations and dilution rates as shown in Table 2.3. The culture was continuously harvested into sterile stainless-steel kegs and stored at room temperature.

Table 2.3: Lactic acid concentrations and dilution rates for continuous cultures

Medium	Lactic acid ($g.\ell^{-1}$)	Dilution rates (h^{-1})
SDL	4.8	0.29
SDL	8.0	0.34
SDL	9.6	0.21
CSL	9.6	0.21
SDL	16	0.34
SDL*	8.0	0.40

* Pure xanthan gum included in the medium

Setting of dilution rates of continuous cultures

The medium dosage peristaltic pumps used (Watson-Marlow 505 S) have electronically controlled speeds adjustable between 0.25 and 55 rpm, in steps of 0.25 rpm. To determine the relationship between rotational speed of the pump impellor and the displacement rate of a particular type and size of tubing, the pump speed was set at an intermediate value and the effluent collected in a measuring cylinder over a timed period. From this a calibration factor 'mℓ per r.p.m' was calculated. As the physical characteristics of the tubing tend to change with repeated sterilisations and extended use, the calibration was repeated from time to time. The volumetric displacement rate of the harvest removal pump was always set to a slightly higher rate than that of the medium addition pump. A certain amount of gas from the head space of the fermenter was thus removed together with the harvest.

The working volume of the fermenter at a given impellor speed was set by adjusting the position of the harvest suction tube until, the liquid/gas interphase stabilised at a pre-selected volume mark on the fermenter wall. The above method of setting the dilution rate was adequate for most purposes. However, in section 2.9 and 2.10, precise setting of the dilution rate to one-half of maximum specific growth rate of this strain was required. In this case the medium keg was placed on a platform scale (capacity 100 kg, discrimination 10 g) and the medium removal rate was monitored over approximately 6 h periods in terms of rate of mass change of the keg contents. The slope of the regression line, calculated from the data, represented the medium flow rate in $kg.h^{-1}$.

Likewise, the working contents of the fermenter were adjusted in terms of mass. The dilution rate was then calculated as $kg.h^{-1}/kg$, which reduces to the normal unit h^{-1}.

2.4 Sampling of *M. elsdenii* NCIMB 41125 cultures

M. elsdenii was aseptically sampled from fermenters, sealed bottles and stainless steel kegs to perform microscopic examinations and shelf-life studies. Sampling methods and volumes for different sources are described below.

2.4.1 Sampling from sealed serum bottles

Twenty-millilitres of the culture was withdrawn from the serum bottles by fitting a sterile needle to a sterile 60 mℓ syringe and piercing the rubber stopper of the crimped serum bottle, in the anaerobic cabinet. The culture was transferred to a long sterile tube.

2.4.2 Sampling from fermenters

Forty-millilitres of the culture was aseptically withdrawn from the sampling port of the fermenter using sterile 60 mℓ syringes and transferred to 100 mℓ serum bottles. The serum bottles were then transferred to the anaerobic cabinet.

2.4.3. Sampling from stainless-steel kegs

The liquid outlet of a keg connector was fitted with a length of Norprene tubing ending in a male Luer fitting, and a plastic gas filter (0.22 µm) was fitted on the gas inlet of the keg head. This assembly was autoclaved and used to replace the connection between fermenter and keg immediately after harvesting. It remained on the keg for the duration of the experiment. Before sampling, the kegs were shaken vigorously to ensure homogeneous distribution of the cells. A supply of O_2-free N_2 was then connected to the gas inlet filter, the Norprene tubing was inserted into a Watson-Marlow 505*S* peristaltic pump (Fig. 2.6), the tubing clamp upstream of the Luer fitting released, and the pump started. The clamp closest to the lancet was then released and the first 100 mℓ of culture outflow collected in a beaker and

discarded. This was performed to ensure that culture remaining in the lancet and pump tubing from a previous sampling procedure was not included in the sample for viability counts. The next 100 mℓ were then pumped, via a 19-gauge hypodermic needle, into a sterile, sealed sampling bottle previously gassed with O_2-free N_2. The serum bottles were transferred to the anaerobic cabinet.

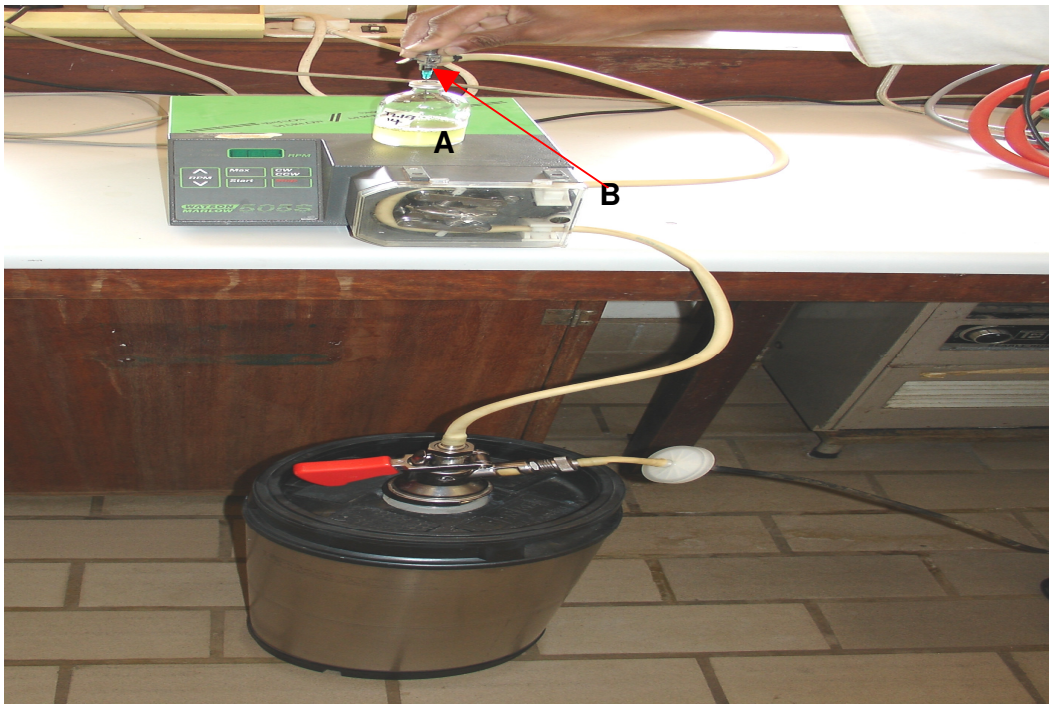

Fig. 2.6: Sampling of the culture from stainless-steel kegs: (A) Sampling bottle, (B) Female Luer connected to a needle

2.5 Viability counts

All viability counts of *M. elsdenii* NCIMB 41125 were performed by using the pour plate method.

2.5.1 Plate counts

All requisites were stored in the anaerobic cabinet overnight for the preparation of dilution series. After storage, a serum bottle containing *M.*

elsdenii was transferred to the anaerobic cabinet where 20 mℓ of the culture was withdrawn with a sterile syringe and transferred to a long sterile tube. Ten-fold dilution series were prepared in triplicate. One millimetre volume of the appropriate dilution was pour-plated in SDL agar plates. The plates were incubated in the anaerobic incubator for 48 or 72 h at 39ºC. For shelf-life studies, viable cell counts were performed for the required number of days. The counts were expressed as colony forming units per mℓ of the sample.

2.5.2 Microscopic analysis

Average chain lengths were microscopically estimated from samples used for dilution series and plating, by viewing several fields under the 600× magnification of a phase contrast microscope. The colony counts were multiplied by this factor to obtain a more realistic estimate of the number of viable cells per millilitre of sample.

2.6 Effect of post-harvest additives on shelf-life

Additives used were glycerol (99.99% w/v); L - sodium lactate (60% w/w); pure xanthan gum (0.2% w/v) and gelatin (4% w/v). Serum bottles (100 mℓ) containing measured volumes of the additives (Table 2.4), rubber stoppers and caps were transferred to and left in the anaerobic cabinet overnight. After storage, the rubber stoppers and caps were fitted to the bottles and the caps were crimped. The bottles were autoclaved at 121ºC for 25 min and stored at room temperature.

Different volumes of 17 h *M. elsdenii* NCIMB 41125 culture were injected into 100 mℓ serum bottles containing different volumes of additives. The inoculated serum bottles were stored at room temperature (on the bench) for 6 d. The short-term effect of post-harvest additives on shelf-life of *M. elsdenii* was determined by performing viable cell counts on day 0 and 6.

Sampling from the fermenter was performed from the sampling port using a sterile 60 mℓ syringe, whereas sampling from the serum bottles was performed using a sterile 60 mℓ syringe with a needle, the latter in the

anaerobic cabinet. Fifty-millilitres of culture was collected from the fermenter, from which appropriate volumes of the culture and additives were dispensed into sterile serum bottles, on the bench. Viability counts were performed using the same method as in section 2.5. A count for the control sample (*M. elsdenii* NCIMB 41125 culture) was performed on day 0.

Table 2.4: Volume proportions of mixtures of 1) gelatin and pure xanthan gum used for the prevention of cell settlement and; 2) glycerol and L- sodium lactate used for the preservation of *M. elsdenii* NCIMB 41125.

Additive and *M. elsdenii* NCIMB 41125	**Volumes (mℓ)**			
Gelatin	5	10	20	40
M. elsdeni NCIMB 41125	45	40	30	10
Glycerol	5	10	20	40
M. elsdeni NCIMB 41125	45	40	30	10
L - Sodium lactate	15	20	30	40
M. elsdeni NCIMB 41125	35	30	20	10
Pure xanthan gum	15	20	30	40
M. elsdeni NCIMB 41125	35	30	20	10

2.7 Effect of xanthan gum / carboxymethylcellulose mixture and pure xanthan gum on cell settlement

These procedures were performed using the same conditions as previously described for batch and continuous growth. Continuous growth for keg 0 was initially carried out for 6 h at the dilution rate of 0.28 h^{-1}. The dilution rate of 0.4

h^{-1} was used for 18 h. The 0.4 h^{-1} dilution rate was also used for the shelf-life kegs, (kegs 1 and 2).

Keg 0 was shaken immediately after removal from the harvest line to ensure homogeneity of the culture. It was then left undisturbed for 14 days. A sampling device was assembled consisting of a disk of polypropylene (PP), 64 mm diameter x 6 mm thick, with a central 4.8 mm hole through which was inserted a 575 mm length of 4.8 mm external diameter (E.D) stainless steel tubing (tight fit). Reference marks were made on the tubing to coincide with the top of the PP disk when this was pressed against the rim of the keg opening with the tubing slid to:

(a) bottom of the keg 'bottom',

(b) 180 mm above this (approximate level of 7.5 ℓ medium in the keg) 'middle' and

(c) 370 mm above the bottom of the keg (just below the level of 15 ℓ) 'top'

A female Luer-to-tubing fitting was connected to the top of the sampling tube which was then placed in a 6 mm internal diameter (I.D) aluminium tube for sterilisation at 121℃ for 1 h. On day 14 the seal of the keg was removed and the sampling device carefully lowered through the PP disk to the 'top' sampling position. A sterile 60 mℓ syringe was attached and a 50 mℓ sample withdrawn and injected into a sterile, pre-gassed sampling bottle. The same procedure was followed for the 'middle' and 'bottom' sampling sites, except that initial 10 mℓ samples were drawn and discarded to get rid of culture from intermediate strata which had been forced into the sampling tube while lowering it to the next site. By pressing the PP disk against the sealing rim of the keg throughout the sampling procedure, the sampling tube was steadied in the centre line of the keg for minimal disturbance of the strata within the culture. The remainder of the culture in the keg was discarded after withdrawal of samples.

The sampling procedures for kegs 1 and 2 were the same as in earlier shelf-life studies, i.e. the kegs were thoroughly mixed by shaking before successive samplings.

2.8 Pulse-and-shift technique for nutritional studies

The culture was grown continuously until the steady-state was maintained. Then different nutrients (Table 2.5) were pulsed into the fermenter using a sterile syringe. Samples were taken from fermenter pre- and post-pulsing for optical density measurements, biomass yield determination and quantification of residual lactic acid.

Table 2.5: Details of the design of pulse-and-shift experiment

Nutrients pulsed into fermenter		Dilution rate (h^{-1})	Working volume of fermenter (ℓ)	Nominal concentration of lactic acid in the incoming medium ($g.ℓ^{-1}$)
Type	**Quantities**			
Lactic acid*	8.0 g	0.4	1.5	8.0
	38.6 g	0.43	1	24.0
Vitamin solution 1	2 mℓ**	0.43	1	24.0
Vitamin solution 2	2 mℓ**	0.43	1	24.0
Mineral solution 3	25 mℓ**	0.4	1.5	24.0
Trace mineral solution	1 mℓ**	0.4	1.5	8.0
Yeast extract	5.0 g**	0.43	1	24.0
Peptone	5.0 g**	0.43	1	24.0

* Added as the equivalent quantity of 60% w/w L - sodium lactate concentrate

**Quantities normally present in 1 ℓ medium

With the low yield expected from anaerobic lactate fermentation, fairly large cultures were required for gravimetric determinations of acceptable precision, e.g. 40 mℓ in quadruplicate. Withdrawal of such a volume from a fermenter of 1 ℓ working volume would have disturbed the steady-state. To obviate this, a sampling device consisting of a sterile Schott bottle (capacity 500 mℓ when dry mass was determined, otherwise 50 mℓ) with a three-port closure was temporarily inserted in the harvest line and the required volume of culture collected over a period of time determined by the overflow rate. The Schott bottle was chilled in an ice bath to arrest further utilisation of lactate and an associated increase in biomass during the collection period.

2.8.1 Optical density measurements

The bacterial growth in liquid media was followed by measurement of OD at 580 nm, pathlegth 10 mm using a DMS 90 UV Visible spectrophotometer. A 1 mℓ sample of culture was diluted 10-fold with distilled water and briefly vortexed before taking OD measurements.

2.8.2 Dry mass determination

Quadruple 40 mℓ of cultures were each transferred into pre-weighed, dry stainless-steel tubes. The tubes were centrifuged at 17 000 rpm for 12 min and the supernatant discarded. The pellets were washed with 10 mℓ portions of distilled water and the tubes re-centrifuged using the same conditions. The rinse water was removed without disturbing the pellets. The tubes were dried overnight in an oven at 105ºC, cooled in a desiccator over silica gel and weighed on an analytical balance.

2.8.3 Residual lactic acid quantification

Twenty millilitres (20 mℓ) of sample was removed from the fermenter and added into a 30 mℓ serum bottle. The metabolic activity was immediately

stopped by adding 200 µℓ phosphoric acid (85%). In the later phases of the investigation, the samples were preserved by mixing 9 mℓ with 1 mℓ 10% (w/v) NaOH. The analysis results were corrected by an appropriate dilution factor.

L-lactate was determined with an YSI 2700 SELECT analyzer fitted with an YSI 2329 L-lactate membrane. The analysis makes use of the following reactions:

1) L-lactate + O_2 $\xrightarrow{\text{L-lactate oxidase (immobilized)}}$ H_2O_2 + pyruvate

2) H_2O_2 $\xrightarrow{\text{Pt anode}}$ $2H^+ + O_2 + 2\ e^-$

The electron flow, which is detected by the instrument, is linearly proportional to the steady-state H_2O_2 concentration and, therefore, to the concentration of lactate.

2.9 Determination of saturation constant for growth yield

2.9.1 Medium preparation

Two 40 ℓ batches of SDL medium, containing 20 $g.ℓ^{-1}$ of a 60% (w/w) L-sodium lactate solution, were prepared in 50 ℓ stainless-steel kegs.

The medium preparation procedure differed from that used in section 2.2.6, in two ways: Peptone and yeast extract were prepared separately in 5 ℓ beakers. The pH values of both solutions were adjusted to pH 5.6 before adding to the contents of the keg. L-cysteine.HCl and sodium sulphide stock solutions were added to the kegs as the last medium components after the volume of the keg contents had been adjusted with distilled water to 39.84 ℓ (indirectly, by weighing). After this, the kegs were immediately sealed with lancets and autoclaved at 121℃ for 60 min.

2.9.2 Growth of cultures

Batch and continuous cultivation were performed as described in section 2.3.1.2.1 and 2.3.1.2.2, respectively. The working volume of the fermenter was adjusted to 1.0 ℓ and the dilution rate was set at 0.47 h^{-1}. In later stages of the experiments the medium keg was placed on a platform scale and the

medium removal rate was monitored over approximately 8 h periods in terms of rate of mass change of the keg contents.

2.9.3 Measurement of optical density, residual lactic acid and dry mass

Dry mass, residual lactic acid and optical density were determined as described in section 2.8. Measurements of residual lactic acid and optical density were performed at increasing dilution rates of 0.1, 0.2, 0.3, 0.45 and 0.6 h^{-1} for Lineweaver-Burke plots. Measurements at a dilution rate of 0.45 h^{-1} were performed in triplicate for 3 consecutive days for the estimation of saturation constant (K_s) from the steady-state lactic acid concentration at half-maximum specific growth rate of *M. elsdenii* NCIMB 41125.

2.10 Effect of storing harvest at 25°C

Continuous growth was started on an incoming SDL medium, containing 9.6 $g.\ell^{-1}$ lactic acid, at a dilution rate of 0.46 h^{-1}. After 24 h, an SDL medium with 24 $g.\ell^{-1}$ lactic acid was used as an incoming medium, at the same dilution rate. The culture was continuously harvested for 56 h and stored at 25°C for 69 h.

The above procedure was repeated but the following changes were introduced: 1) Continuous growth was started from an overnight fed-batch culture. 2) The culture was harvested continuously for 25 h and stored at 25°C for 24 h.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Shelf-life stability of *M. elsdenii* NCIMB 41125

3.1.1 Fed-batch growth to determine changing patterns in cell populations

The fed-batch growth method that was used to determine changing patterns in cell population is described in section 2.3.1.2.2.

The experiment was carried out in kegs and was divided into two trials from which comparisons in viable cell counts were made. There was a general increase in viable cell counts from day 0 to day 1 (Fig. 3.1). The reason could be that lactic acid was not depleted at the time of the first sampling, after 17 h of growth (Fig. 3.1). Viable cell counts in trial 1, keg 1 (T1K1) were higher than those in trial 2, keg 1 (T2K1) from day 0 to 4. Trial 2 was performed 14 days after trial 1 (Fig. 3.1). The counts between the trials overlapped on day 5. There was a sudden decrease in viable cell counts from day 5 to 6 and day 4 to 5 for T1K2 and T1K3 respectively (Fig. 3.1). For T1K1, this decrease was observed from day 7 to 8. After these sharp decreases, the viable cell counts levelled-out until day 10, although the decrease was steeper for T1K1 (Fig. 3.1). The rate in loss of viability was low from day 10 to day 14 for all cultures in trial 1 (Fig. 3.1).

Although the viable cell counts during the first five days in trial 2 were less than those in trial 1, the decreases were relatively small. The counts in trial 2 started to level-out from day 8 but those in T2K1 showed a steeper decrease.

A comparison of viable cell counts between the two trials show similarities in the corresponding keg numbers. The counts in T1K1 and T2K1 did not differ much from each other. This trend was also found for the counts in T1K2 & T2K2 and T1K3 & T2K3. The improvement of shelf-life, as shown by viable cell counts, in ascending order of the kegs between the two trials over a 14-day period, may be explained by the abundance of nutrients and less competition in the remaining culture. These results suggest that higher initial viable cell counts are desirable for better survival of *M. elsdenii* NCIMB 41125

under fed-batch conditions using the SDL medium containing 9.6 g lactic acid per litre as stated in 2.3.1.2.2.

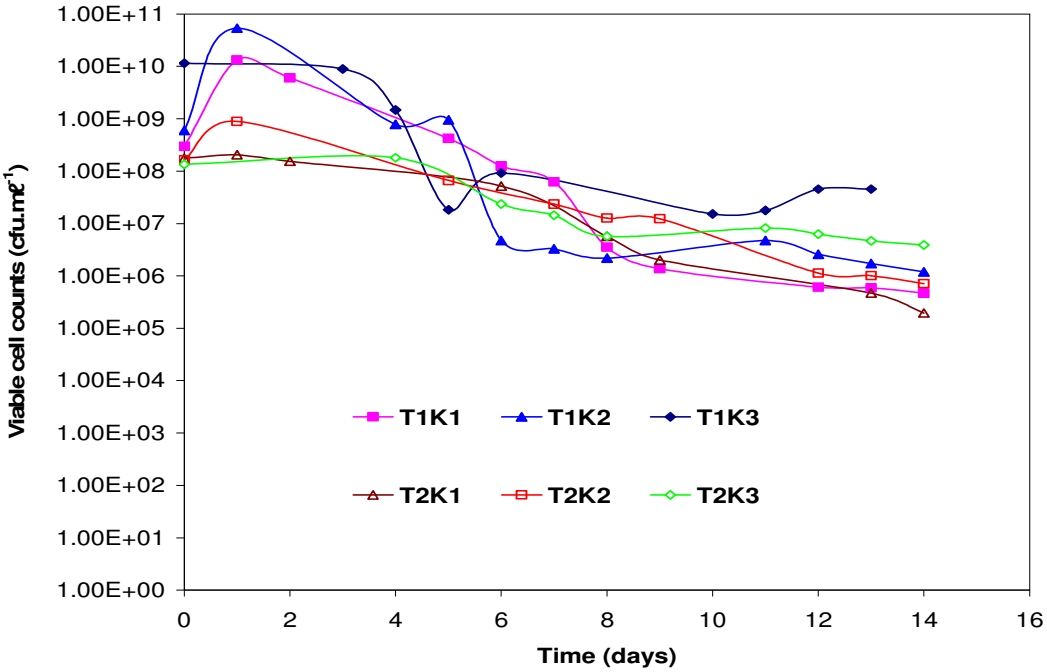

Fig 3.1: Changes in viable cell counts of *M. elsdenii* NCIMB 41125 populations, produced in fed-batch culture. (S_r lactic acid, 9.6 g.ℓ^{-1})

In this section, viable cell counts were corrected for chain length of 4. Chain lengths of 2 were also observed, especially from day 10 to 14.

3.1.2 Continuous growth to determine changing patterns in cell populations at different lactic acid concentrations

In this section, 4.8, 8.0, 9.6 and 16 g lactic acid per litre in SDL medium and 9.6 g lactic acid per litre in CSL medium, were used for continuous growth of *M. elsdenii* cells and after harvesting in kegs, stored for different periods at room temperature.

Fig 4.2 shows the results of the change in viable cell counts after 8 days when using 4.8 g.ℓ^{-1} lactic acid at a dilution rate of 0.29 h^{-1}. There was a slight increase in cell numbers from day 0 (6.0×10^7 cfu.mℓ^{-1}) to day 1 (1.1×10^8 cfu.mℓ^{-1}) whereafter the numbers stayed approximately the same to day 7

when the numbers declined to 3.6 × 10^7 cfu.mℓ$^{-1}$. This indicated a 1.7-fold decline in viable cell numbers, which is a small factor.

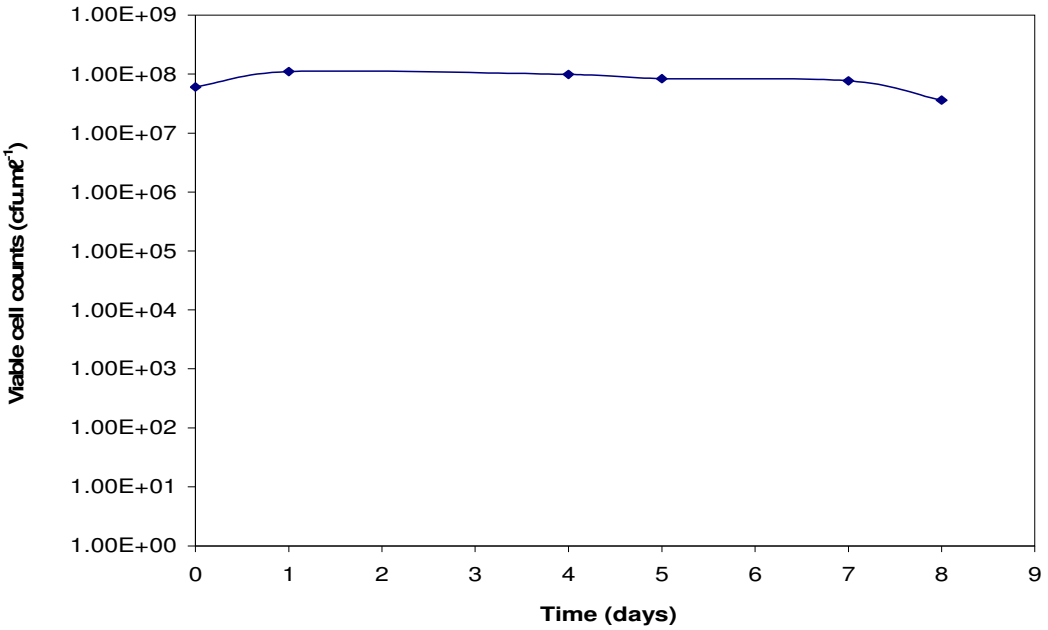

Fig. 3.2: Changes in viable cell counts of *M. elsdenii* NCIMB 41125 populations, produced in continuous cultures. (S_r lactic acid, 4.8 g.ℓ$^{-1}$; D, 0.29 h^{-1})

When the lactic acid concentration was increased to 8.0 g.ℓ$^{-1}$ at a dilution rate of 0.34 h^{-1}, there was a relatively faster decrease in viable cell numbers after 8 days (Fig. 3.3), compared to results when using 4.8 g.ℓ$^{-1}$ (Fig. 3.2). Doubling the concentration of lactic acid in the medium did not, as expected, increase the viable cell counts on day 0 (A sample for day 0 was taken during steady-state conditions in the fermenter, before harvesting into the keg). Viable cell counts of 1.5 × 10^9 and 1.22 × 10^9 cfu.mℓ$^{-1}$ from keg 1 and 2 respectively, on day 1, were higher than 1.1 × 10^8 cfu.mℓ$^{-1}$ when using 4.8 g.ℓ$^{-1}$. There was an average of 2.3-fold decrease in viable cell counts in keg 1 and 2 (Fig. 3.3) compared to 1.1-fold decrease in keg 1 (Fig. 3.2) from day 1 to 2.

An increase in both S_r lactic acid and dilution rate resulted in a quicker decrease in viable counts from day 0 to day 8, when compared to 4.8 g.ℓ$^{-1}$ lactic acid at the dilution rate of 0.29 h^{-1}. It seems at this stage that the increase in S_r lactic acid resulted in a rapid decrease in viable cell counts upon storage of the culture at room temperature.

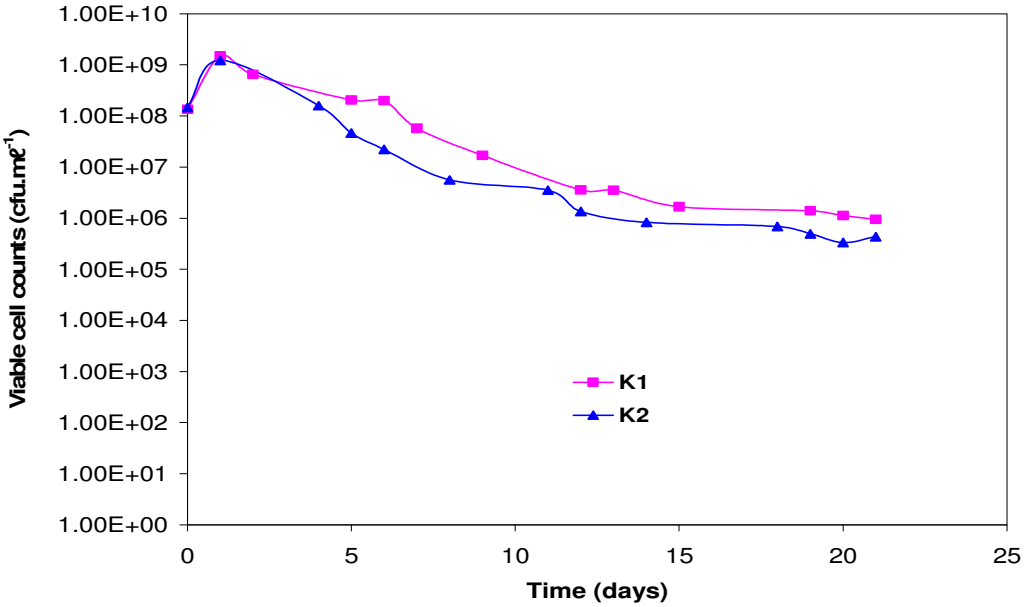

Fig. 3.3: Changes in viable cell counts of *M. elsdenii* NCIMB 41125 populations, produced in continuous cultures. (S_r lactic, 8.0 g.ℓ$^{-1}$; D, 0.34 h^{-1})

When the concentration of lactic acid was further increased to 16 g.ℓ$^{-1}$ at a dilution rate of 0.34 h^{-1}, there was an even faster decrease in viable cell counts over the first 8 days and after 21 days (Fig. 3.4). The counts decreased from on day 0, to cfu.mℓ$^{-1}$ and cfu.mℓ$^{-1}$ on day 8 and 21 respectively.

Up to this point, increasing the lactic acid concentration resulted in an increase in viable cell counts over the first few days but not an improvement of shelf-life. In order to improve the shelf-life, 9.6 g.ℓ$^{-1}$ lactic acid was used. Fig. 3.5 shows the results of a shelf-life experiment using SDL medium with a lactic concentration of 9.6 g.ℓ$^{-1}$ at a dilution rate of 0.21 h^{-1}. The cultures were harvested during three separate continuous runs, each harvested for 24 h Viable cell counts from cultures in keg 1 and keg 2, from day 1 to day 4, were almost the same. A slight decrease of counts from keg 1 on day 5 was.

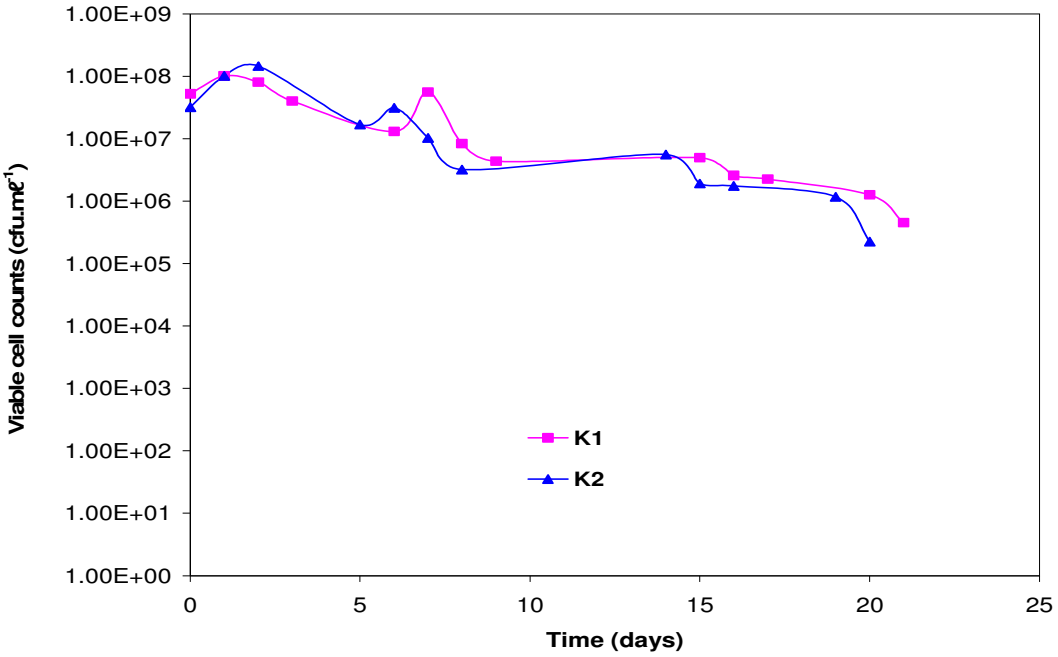

Fig. 3.4: Changes in viable cell counts of *M. elsdenii* NCIMB 41125 populations, produced in continuous cultures. (S_r lactic acid, 16.6 g.ℓ$^{-1}$; D, 0.34 h^{-1})

followed by a steady decrease until day 13. There were relatively high viable cell counts in keg 3 during the first two days, however, on day 5, the counts were similar to those from keg 1 and keg 2. Thereafter, a steady decrease in cell numbers was observed up to day 14 when the counts in all the kegs were almost the same.

In the next experiment, the same lactic acid concentration and dilution rate were used, except that the culture was harvested continuously into three different kegs. It means that the three kegs (Fig. 3.6) contained sequential lots of a harvest from a single chemostat run. These cultures were harvested in order of succession, from, keg 1, keg 2 and keg 3, and each harvested for 24 h. The culture in keg 1 contained 8.5 × 10^9 cfu.mℓ$^{-1}$ on day 2 which decreased almost the same as in keg 1. The counts in keg 3 decreased much slower than in both kegs 1 and 2, from 7.1 × 10^7 cfu.mℓ$^{-1}$ on day 1, to approximately 10^6 cfu.mℓ$^{-1}$ on day 14. No viable cell counts were available from keg 3 on day 2 while counts on day 6 were 5.9 × 10^6 cfu.mℓ$^{-1}$, which were higher than those from keg 1 and lower that those from keg 2 on the same day (day 6).

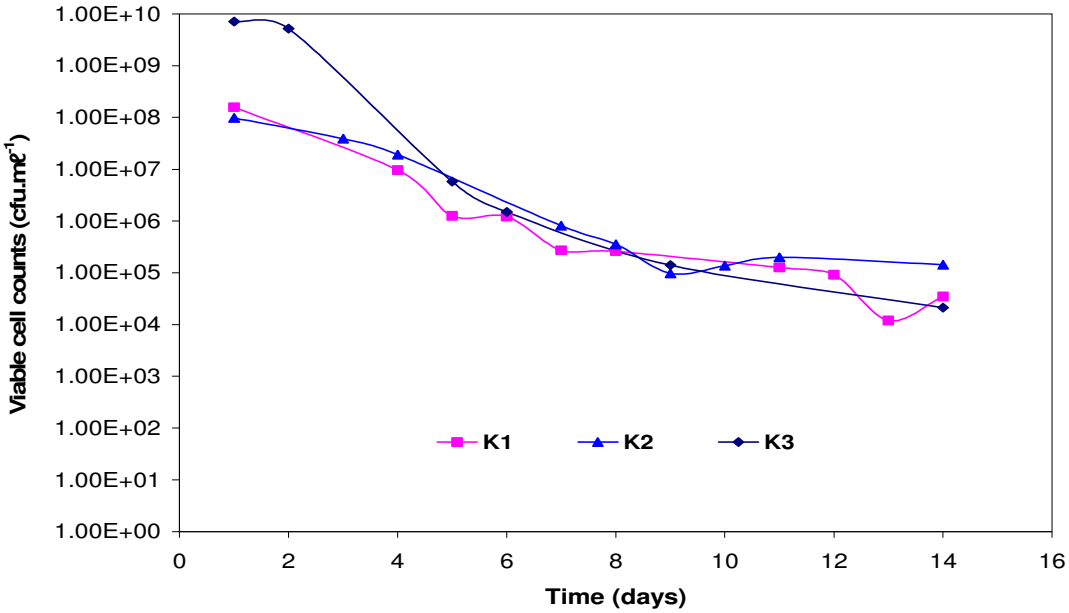

Fig. 3.5: Changes in viable cell counts of *M. elsdenii* NCIMB 41125 populations, produced in continuous cultures using SDL medium. (S_r lactic acid, 9.6 g.ℓ^{-1}; D, 0.21 h^{-1})

There seems to be a faster reduction of counts when the initial counts were high. This tends to affect the shelf-life for SDL medium at a S_r lactic acid of 9.6 g.ℓ^{-1} because the keg 1 culture resulted in poorer stability in 14 days. One of the reasons may be that a high number of cells metabolise the available nutrients much quicker than low number of cells, hence result in a more rapid decrease in cells. Although the dilution rates and the medium concentrations were the same, the results showed that there was a better shelf-life in keg 3, which had lower initial counts compared to the other kegs. It shows that better shelf-lives may be obtained as more culture is harvested in a single chemostat run.

In Fig. 3.7 the shelf-life of *M. elsdenii* NCIMB 41125 was determined when corn steep liquor (CSL) was used in the culturing medium. CSL is considered to be a good source of nutrients such as B-vitamins, amino acids and some lactic acid (Liggett and Koffler, 1948). The decrease of viable cell counts in keg 1 culture from day 1 to day 5 was followed by an increase on day 6 and a decrease from day 7 to day 16. No explanation for the increase on day 6

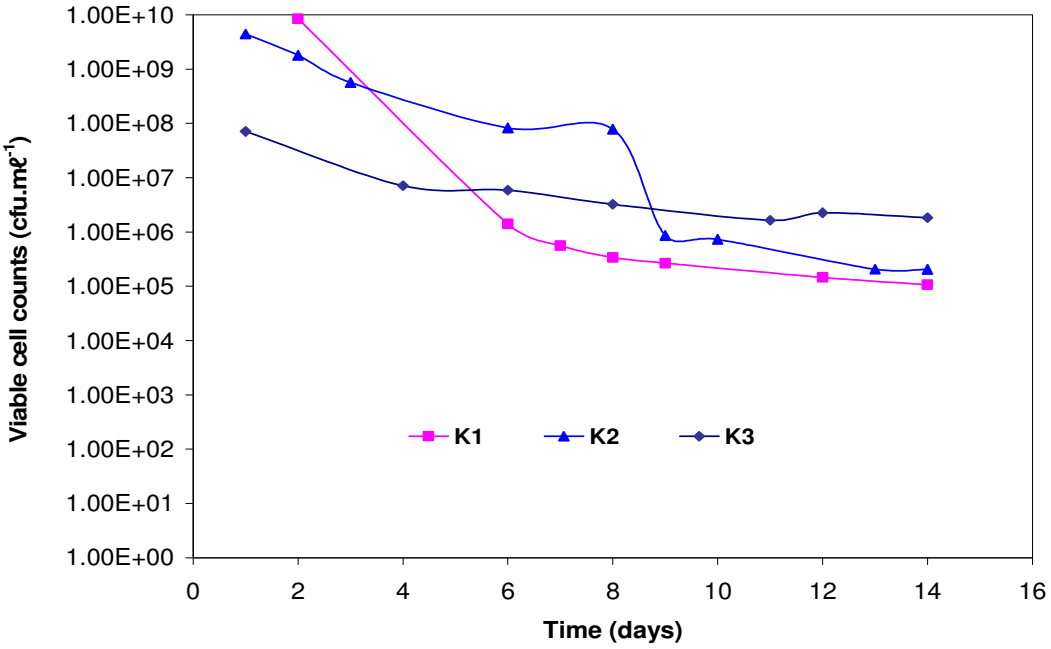

Fig. 3.6: Changes in viable cell counts of *M. elsdenii* NCIMB 41125 populations, produced in continuous cultures using SDL medium. (S_r lactic acid, 9.6 g.ℓ$^{-1}$; D, 0.29 h^{-1})

With the exception of the results shown in Fig. 3.4 (keg 2), viable cell counts from continuous cultures did not generally increase from day 1 to day 2. The reason provided for the increase in viable cell counts from day 0 to day 1 may be that lactic acid was not depleted on day 0, hence this substrate was still utilised until day 1 (Figs. 4.1 and 4.2).

Better shelf-life was found in keg 2 counts compared to keg 1, from day 1 to day 12 (Fig. 3.7). There was a pronounced difference of viable cell counts on day 12; 1.3 × 10^5 cfu.mℓ$^{-1}$ and 7.7 × 10^7 cfu.mℓ$^{-1}$ for keg 1 and keg 2, respectively. This pronounced difference of viable cell counts between keg 1 and keg 2 could not be explained. However, comparison of counts (1.3 × 10^5 cfu.mℓ$^{-1}$ in keg 1) on day 12 (Fig. 3.7), to those obtained in Figs. 4.3 and 4.4, showed almost the same counts. In all these instances, the same dilution rate of 0.21 h^{-1} and lactic acid concentration of 9.6 g.ℓ$^{-1}$, were used. The higher counts of 7.7 × 10^7 cfu.mℓ$^{-1}$ in keg 2 (Fig. 3.7) could be due to more favourable growth conditions. On that basis, the CSL medium looks promising as growth medium that will result in better survival of *M. elsdenii* during storage.

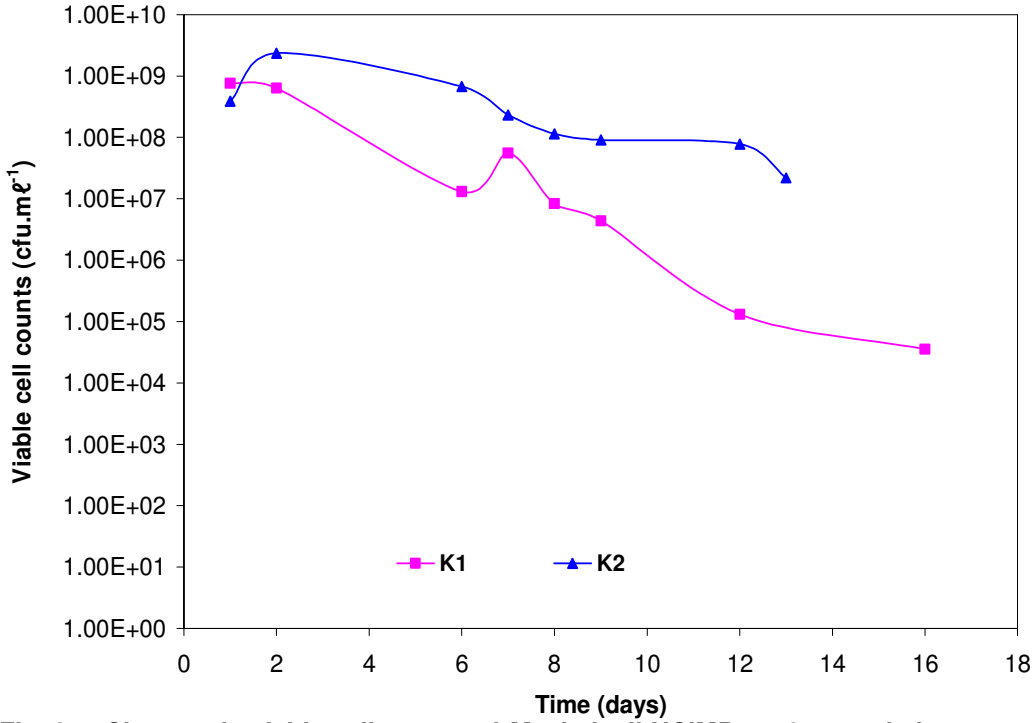

Fig. 3.7: Changes in viable cell counts of *M. elsdenii* NCIMB 41125 populations, produced in continuous culture using CSL medium. (S_r lactic acid, 9.6 g.ℓ$^{-1}$; D, 0.21 h^{-1})

In all the continuous growth experiments, the viable cell counts were not corrected for chain length as compared to fed-batch experiments.

3.1.3 Summary of shelf-life studies

To our knowledge, this thesis is the first to report on shelf-life of obligate anaerobic microorganisms and therefore, no similar results are available for comparison. However, results were compared to shelf-life studies of other anaerobic bacteria though not of rumen origin.

In one study, viable probiotic counts were determined from 15 commercial feed supplements. Of the 15 samples tested, only three commercial products contained no lactobacilli at all (Gilliland, 1981), although the supplements were supposed to contain *L. acidophilus*. This shows variability of certain strains for certain supplements. However, the strain (*M. elsdenii* NCIMB 41125) used in this study obviates any difficulties of determining the shelf-life

due to its specificity for lactic acid and that there are no contaminants as observed with the commercial feed supplements.

The presence of oxygen plays a major role in the survival of probiotic bacteria (Brunner *et al.*, 1993). This was also observed in our shelf-life laboratory experiments using *M. elsdenii* NCIMB 41125 in which redox potential values of cultures which had been exposed to oxygen were high (Agricultural Research Council, unpublished data). Prolonged storage of such cultures resulted in a faster reduction of viable cell counts.

Improved shelf-life obtained from the use of lower concentrations of lactic acid, points to the substrate inhibition concept (Pirt, 1975). The concept states that increasing the substrate concentration results in cell biomass increase. However, if the substrate reaches a critical concentration, the biomass decreases. These patterns were also observed in the case of phenols and alcohols. It is mentioned in section 3.5 that the biomass resulting from an SDL medium, containing a lactic concentration of 24 $g.\ell^{-1}$, is almost half than that obtained when a lactic concentration of 9.6 $g.\ell^{-1}$, at 0.45 h^{-1}, was used. This shows an inhibitory effect of high lactic acid concentrations on the growth of *M. elsdenii* NCIMB 41125 and therefore relates to the substrate inhibition concept.

Viable cell counts of 10^5 $cfu.m\ell^{-1}$ for 14 days or more were targeted and interpreted as sufficient for inoculating feedlots (Agricultural Research Council, unpublished data). This quantity, 10^5 $cfu.m\ell^{-1}$, was achieved at all lactate concentrations used in this study. In comparison, the shelf-life data from fed-batch and continuous cultures, as used in this study, did not show any significant differences.

3.2 Effect of post-harvest additives

3.2.1 Effect of glycerol and L-sodium lactate on the preservation of *M. elsdenii* NCIMB 41125

Four volume proportions of sodium lactate between 30 and 80% as well as glycerol, with volume proportions of 10 to 80%, were used to establish the effects on the preservation of cells (Table 3.1).

For the 30% volume proportion of sodium lactate, the viable cell counts decreased from 5.7×10^8 to 1.7×10^6 cfu.mℓ$^{-1}$ on day 0 and 6 respectively. The difference in viable cell counts between the control and the 30% volume proportion was approximately 29%. For the 40, 60 and 80% volume proportions, there was a significant decrease, from 10^8 to less than 10^4 cfu.mℓ$^{-1}$, indicating a significant loss of viability. This indicated a pronounced reduction rate and therefore shows that when sodium lactate was used at volume proportions of 30% or higher, a phenomenon described in section 3.1.3, i.e. substrate inhibition, could have occurred. Lactate could have a repressive effect on the growth of *M. elsdenii* NCIMB 41125 if used at high concentrations approaching critical levels.

The use of glycerol has been shown to preserve cells from losing viability (Bryukhanov and Netrusov, 2006), although cultures were stored at -70°C for three years. It was envisaged in this study, that glycerol, as a cryoprotective agent, may preserve cells over a 6-day period at room temperature. The same trend was found with glycerol using 10 to 80% volume proportions, where the cell numbers decreased from 10^8 to less than 10^4 cfu.mℓ$^{-1}$ over 6 days (Table 3.1).

The count on 'day 0' was typical of counts obtained on fed-batch cultures on SDL medium after 17 h (Table 3.1). The loss of viability of the control culture after 6 days storage at room temperature (± 25°ºC) was somewhat higher than normal.

The use of glycerol resulted in a rather repressive effect. One of the possible reasons could be the room temperature effect (± 25ºC) as opposed to much lower temperatures for which glycerol was previously used (Bryukhanov and Netrusov, 2006). These results showed that sodium lactate and glycerol were not effective in preserving cultures of *M. elsdenii* NCIMB 41125.

Table 3.1: Viable cell counts of cultures using different concentrations of L-sodium lactate and glycerol.

Additive	Volume proportions (%) of additive in mixture	Viable cell counts (cfu.mℓ$^{-1}$), corrected for average chain length of 4	
		Day 0	Day 6
None	0	8.2×10^8	1.5×10^7
L-sodium lactate (60% w/v)	30	5.7×10^8	1.7×10^6
	40	4.9×10^8	$<1 \times 10^4$
	60	3.3×10^8	$<1 \times 10^4$
	80	1.6×10^8	$<1 \times 10^4$
Glycerol (10% v/v)	10	7.4×10^8	$<1 \times 10^4$
	20	6.5×10^8	$<1 \times 10^4$
	40	4.9×10^8	$<1 \times 10^4$
	80	1.6×10^8	$<1 \times 10^4$

3.3 Prevention of cell settlement and extension of shelf-life

3.3.1 Effect of xanthan gum / carboxymethylcellulose mixture addition in kegs

As the name implies, the genus *Megasphaera* is characterised by large spherical cells. Moreover, these occur largely in pairs and in chains of variable length (Rogosa, 1971). It was therefore tacitly assumed that the cells would have a tendency to settle down during prolonged storage of cultures and this would lead to a degree of inhomogeneity of the product supplied to feedlots.

Carboxymethylcellulose (CMC) is a polymeric compound, produced in different grades consisting of molecular weights ranging from 80 000 to 750 000 g.mol^{-1}. Some of the properties of CMC in aqueous solutions are: thickening agent, stabiliser, suspension of materials and water retention (Kelco, 2005). Xanthan gum is an extracellular polysaccharide produced by an aerobic bacterium, *Xanthomonas campestris*. In addition to its high molecular

weight (approximately 2 million $g.mol^{-1}$), xanthan gum is also pseudoplastic (Rosalam and England, 2006). This property implies that the viscosity of stirred cultures in the fermenter should be lower than that of the unstirred harvest in the kegs.

A mixture of CMC and xanthan gum was envisaged to provide pseudoplastic properties and viscosity to the culture. It was also anticipated that this mixture would stabilise the culture while at the same time acting as barrier against potential metabolites which could negatively affect cells during storage of cultures at room temperatures. This mixture was aimed at the prevention of cell settlement, which would render cells easy access to nutrients.

Three kegs: keg 0, 1 and 2, were used. Samples in keg 1 and keg 2 were sampled daily from day 0 to day 14 and analysed for viable cell counts. After harvesting in keg 0, the keg was sealed and left at room temperature for 14 days. On day 14, the keg contents were sampled at the top, middle and bottom levels and analysed for viable cell counts. The results in Fig. 3.8 showed that the counts remained in the region of 10^9 $cfu.m\ell^{-1}$ for the first 2 days. Thereafter, the counts decreased steadily over the next 12 days, ending up at 2.5 x 10^6 $cfu.m\ell^{-1}$ for keg 1 and 6.3 x 10^6 $cfu.m\ell^{-1}$ for keg 2 on day 14. This does not represent a significant improvement over the results of earlier shelf-life studies of *M. elsdenii* NCIMB 41125, stored for a similar period in the absence of additives (Section 3.1). Viable cell counts from different depths of keg 0 showed that the xanthan gum / CMC mixture was not effective in keeping cells in suspension. The top, middle and bottom depths had counts of $<10^3$, $<10^5$ and $<10^7$ $cfu.m\ell^{-1}$, respectively. These counts varied significantly between the top and bottom samples. The viable cell count of 5.2 × 10^6 $cfu.m\ell^{-1}$, for the bottom level, is representative of those obtained from shelf-life studies, in which no stabilising agent was used (Section 3.1). It is assumed that at least part of the stabilising agent mixture, possibly the carboxymethylcellulose (CMC) component, settled out during storage, thus aggravating the tendency of culture towards sedimentation.

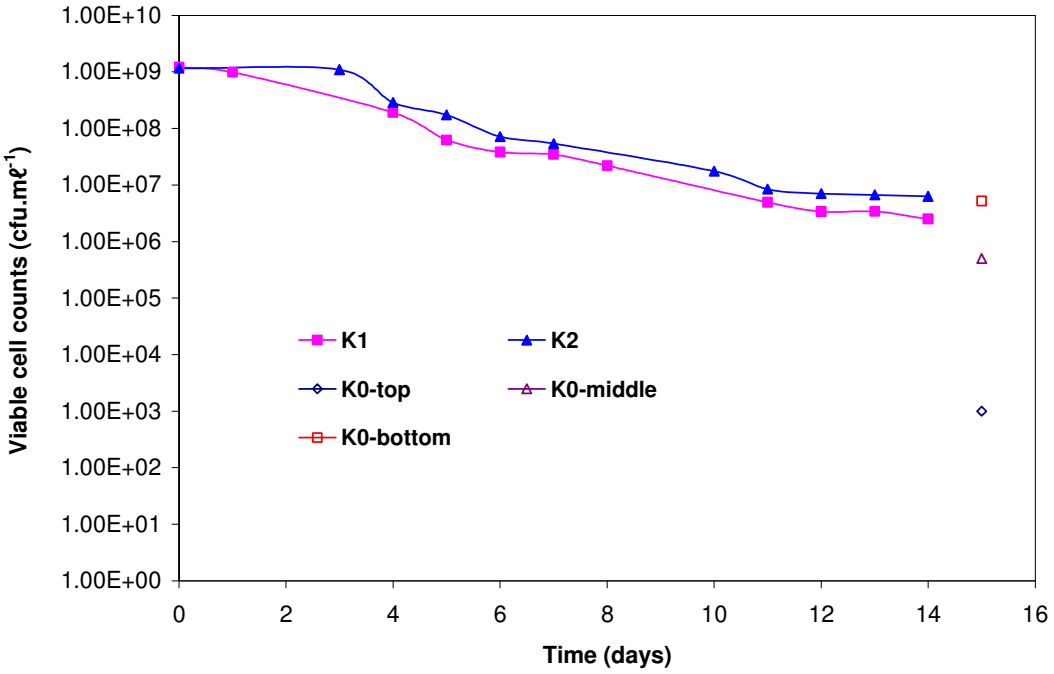

Fig. 3.8: Changes in viable cell counts of *M. elsdenii* NCIMB 41125 populations, produced in continuous culture using SDL medium. (S_r lactic acid, 9.6 g.ℓ$^{-1}$, D, 0.4 h^{-1}). Medium contained 0.2% xanthan gum/CMC mixture.

3.3.2 Effect of pure xanthan gum addition to kegs

The advantage of using pure xanthan gum as a single compound as opposed to the CMC / xanthan gum mixture is the reduced pseudoplasticity and hence viscosity.

The same procedure was followed as in Section 3.3.1 except that only xanthan gum was used and that after sampling the top, middle and bottom levels, the keg was shaken and thereafter a sample taken to perform viable cell counts.

Different viable cell counts from 'keg 0' suggested that pure xanthan gum was not evenly suspended in the kegs after storage for 15 days at room temperature (Fig. 3.9). Viable cell counts at the 'bottom' were higher than the sample taken after the keg has been shaken. This shows that pure xanthan gum was not effective for keeping cells in suspension in the kegs. However, all counts except the 'bottom' sample, which had approximately 10^8 cfu.mℓ$^{-1}$,

fell within the same log unit i.e. 10^6 cfu.mℓ$^{-1}$. No viable cell counts were determined for 'keg 0' on day 0.

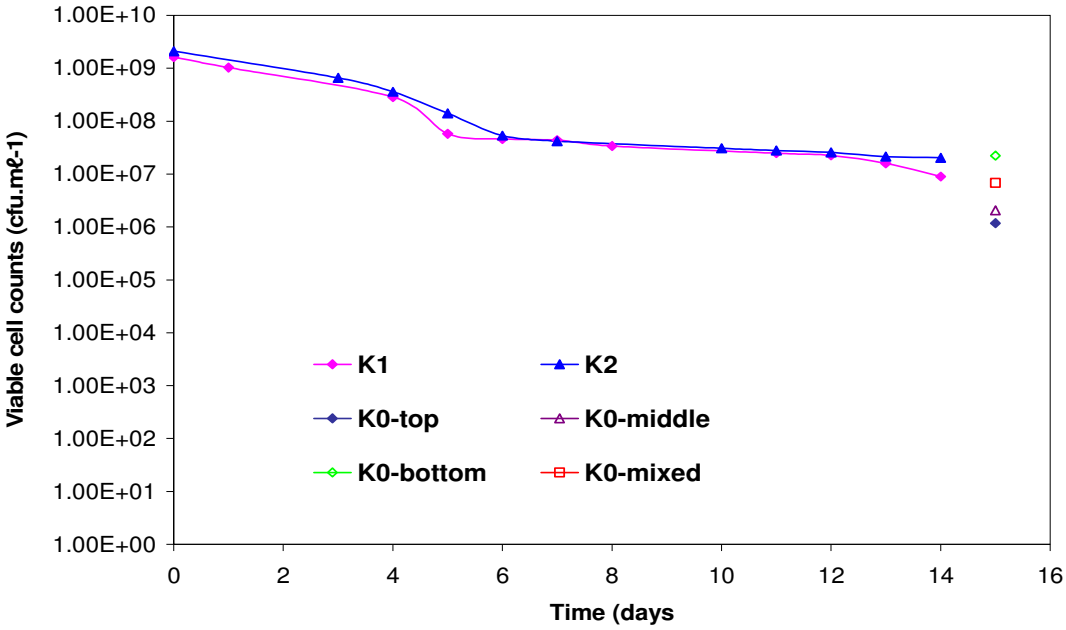

Fig. 3.9: Changes in viable cell counts of *M. elsdenii* NCIMB 41125 populations, produced in continuous culture using SDL medium. (S_r lactic acid, 9.6 g.ℓ$^{-1;}$ D, 0.4 h^{-1}). Medium contained 0.2% pure xanthan gum.

There was a rapid decrease in viable cell counts from day 0 to 5 (Fig. 3.9), but as also observed in section 3.1.1 and 3.1.2, a levelling-out trend appeared from day 6 to 14. There was a general improvement in viable cell counts, with counts of 8.88 × 10^6 and 2.02 × 10^7 cfu.mℓ$^{-1}$ for keg 1 and keg 2, respectively, on day 14. These results were significant since definite higher counts were obtained for the first time in stainless-steel keg studies.

Thus, the results obtained from the use of pure gum xanthan gum suggested that this polysaccharide acts as a protective layer to the settled/sedimented cells, thereby improving the shelf-life of *M. elsdenii* NCIMB 41125.

3.3.3 Effect of gelatin and pure xanthan gum in serum bottles

Four volume proportions of gelatin between 10 and 80% as well as for pure xanthan gum, with volume proportions of 30 to 80%, were used to test their effects on cell settlement and extension of shelf-life (Table 3.2).

Table 3.2: Viable cell counts of cultures using different concentrations of gelatin and pure xanthan gum

Additive	Volume proportions (%) of additive in mixture	Viable cell counts (cfu.mℓ$^{-1}$), corrected for average chain length of 4	
		Day 0	Day 6
None	0	6.3×10^8	1.8×10^7
Gelatin (4% w/v)	10	5.7×10^8	2.1×10^8
	20	5.0×10^8	9.5×10^7
	40	3.8×10^8	6.9×10^7
	80	1.3×10^8	2.9×10^7
Pure xanthan gum (0.2% w/v)	30	5.7×10^8	1.5×10^8
	40	5.0×10^8	1.0×10^8
	60	3.8×10^8	7.5×10^7
	80	1.3×10^8	5.9×10^7

After 24 h, the visual appearance of the cultures in the serum bottles was identical. Visual observations of the cultures showed that there was cell sedimentation after 5 days. In all 9 cultures, the viable cell counts decreased from day 0 to day 6 (Table 3.2). The reduction in viable cell counts was less for all 8 cultures containing the stabilising agents, than for the control (35-fold for the control, as against between 2.2 and 5.5 fold for the culture with added stabilising agents). The viable cell counts were higher for cultures with pure xanthan gum after 6 days, compared to the same levels of gelatin. It thus seems that pure xanthan gum has better preservation properties than gelatin. Increasing the percentage/concentration of both additives had a positive effect on shelf-life, indicated by lower reductions in viable cell counts on day 6, compared to initial counts. As with the use of pure xanthan gum in kegs (Section 3.3.2), the use of gelatin and pure xanthan gum in serum bottles, improved shelf-life but resulted in sedimentation of cells.

Increasing the concentration of gelatin and pure xanthan gum resulted in better survival of the culture in relatively high numbers, indicated by a lesser reduction in viable cell counts after 6 days of storage. Comparing highest and lowest concentration of additives used, it was observed that when 10% volume proportion of gelatin was used, there was a loss of 3.6×10^8 cfu.mℓ$^{-1}$ after 6 days, while, when 80% volume proportion was used, there was a loss of 1.1×10^8 cfu.mℓ$^{-1}$. A similar observation was made for pure xanthan gum where 30% resulted in a loss of 5.5×10^8 cfu.mℓ$^{-1}$, while 80% resulted in a loss of 7.1×10^7 cfu.mℓ$^{-1}$.

3.4 Optimisation of biomass yield

3.4.1 Pulse-and-shift technique for nutritional studies

The pulse-and-shift technique consists of identifying growth-limiting nutrients by observing changes in optical density and/or cell dry mass in a continuous culture system, in response to injection of suspected growth-limiting nutrients into the growth vessel (Mateles and Battat, 1974). Whenever a limiting nutrient or group of nutrients is identified, its concentration in the medium reservoir is increased to the point that the limitation is eliminated. The search for the next growth-limiting nutrient is then continued. These authors reported that this procedure provides an efficient means for optimising culture media in terms of increasing cell concentration the media are capable of supporting. In this study, different constituents of the growth medium (SDL) were pulsed and their effect on biomass yield was investigated.

3.4.1.1 Pulsing with lactic acid

Lactate is the growth-limiting nutrient of *M. elsdenii* (Giesecke *et al*., 1970). According to Mateles and Battat (1974), pulsing of the growth-limiting substrate into a steady-state culture should result in a biomass increase 1 or 2 h after pulsing. In this study, the duration of the response in terms of cell growth measured in terms of OD, was determined at 5, 10 or 15 minutes intervals.

When 8 g lactic acid was pulsed into a culture volume of 1.5 ℓ containing 8 g lactic acid per litre medium, varying results were observed for different batches. In one batch, there was a decrease in OD 15 min after pulsing followed by a slight increase after 25 min of pulsing (Fig. 3.10). The highest cell biomass was observed after 1 h, and then decreased steadily during the next 100 min. After 160 min, the OD was approximately the same as before pulsing with 8 g lactic acid. The same tendency was found in a similar experiment (Fig. 3.11), except that a slight increase in OD occurred after 150 min. Even after 250 min after pulsing, the cell biomass decreased steadily within the next 100 min.

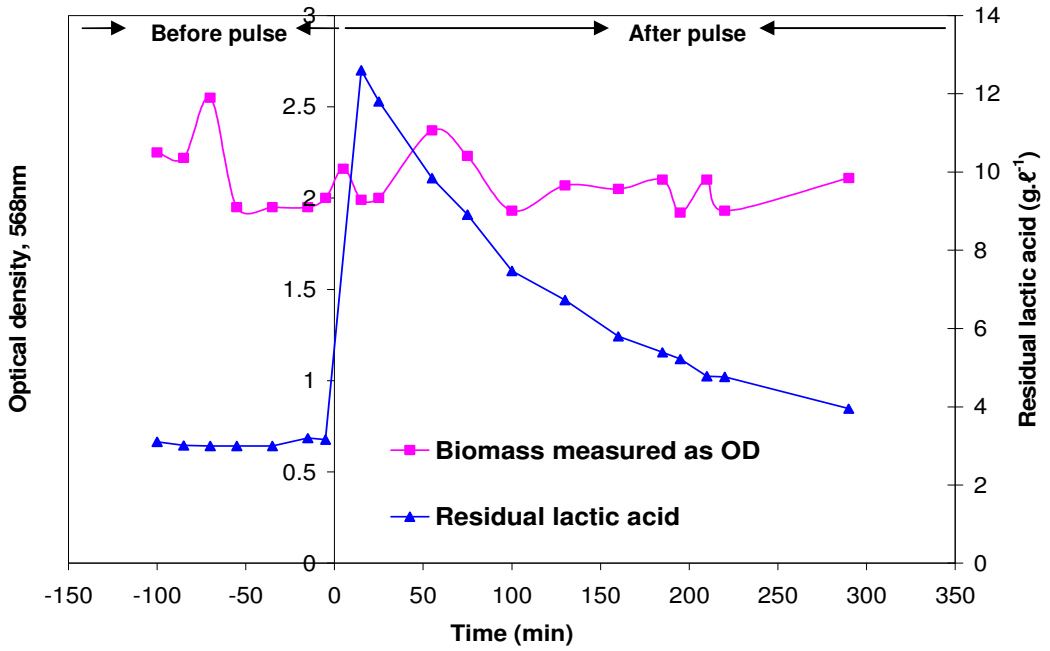

Fig. 3.10: Optical density and residual lactic acid of a culture of *M. elsdenii* NCIMB 41125 at a dilution rate of 0.4 h^{-1} before and after pulsing with lactic acid at time 0. S_r lactic acid in the SDL medium, 8.0 $g.\ell^{-1}$.

In Fig. 3.10, the residual lactic acid before pulsing was approximately 2.5 $g.\ell^{-1}$ Fifteen minutes after pulsing, the value increased to 12.5 $g.\ell^{-1}$. After 160 min of pulsing, the lactic acid was about 6 $g.\ell^{-1}$, indicating utilisation of 7 $g.\ell^{-1}$. In the other experiment, the lactic acid was about 5 $g.\ell^{-1}$ after 160 min of pulsing, indicating utilisation of 6 $g.\ell^{-1}$ lactic acid (Fig. 3.11). A lactic concentration of 4 $g.\ell^{-1}$ was obtained after 250 min.

The subsequent rate of decline in lactic acid concentration after pulsing in both batches was steeper than could be accounted for by the given dilution rate, indicating a higher rate of uptake than before pulsing - the culture may eventually reach steady-state values of 2.5 g.ℓ^{-1}, which were obtained before pulsing.

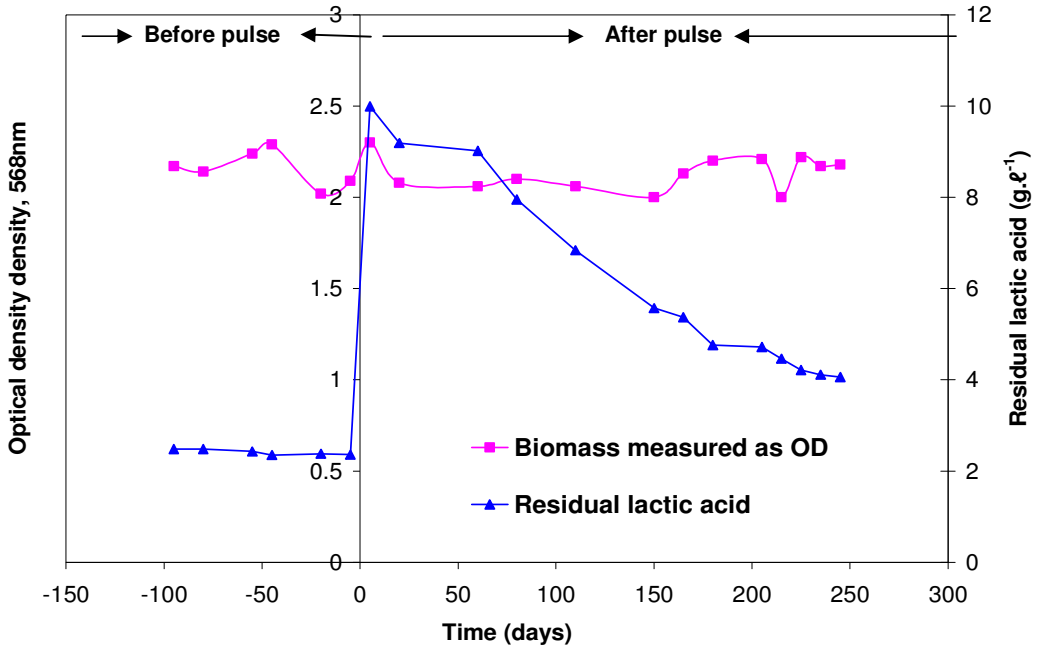

Fig. 3.11: Optical density and residual lactic acid of a culture of *M. elsdenii* NCIMB 41125 at a dilution rate of 0.4 h^{-1} before and after pulsing with lactic acid at time 0. S_r lactic acid in the SDL medium, 8.0 g.ℓ^{-1}.

Increasing the pulse to 38.6 g lactic acid in a medium containing 24 g lactic acid per litre produced a distinctly negative effect on culture density, resulting in a gradual wash-out 1 h after pulsing (Fig. 3.12). This suggests that concentrations of lactic acid, in the order of between 24 and 45 g.ℓ^{-1}, have a growth rate-depressing effect on *M. elsdenii* NCIMB 41125. Although the culture did not wash-out during steady-state, the pulse might be too high and hence, beyond the critical concentration of *M. elsdenii* NCIMB 41125 at the dilution rate of 0.43 h^{-1}. Pirt (1975) showed that high substrate concentrations could have inhibitory effects on the culture, thereby resulting in a decrease in biomass. The author also reported that phenols and alcohols show the same trend.

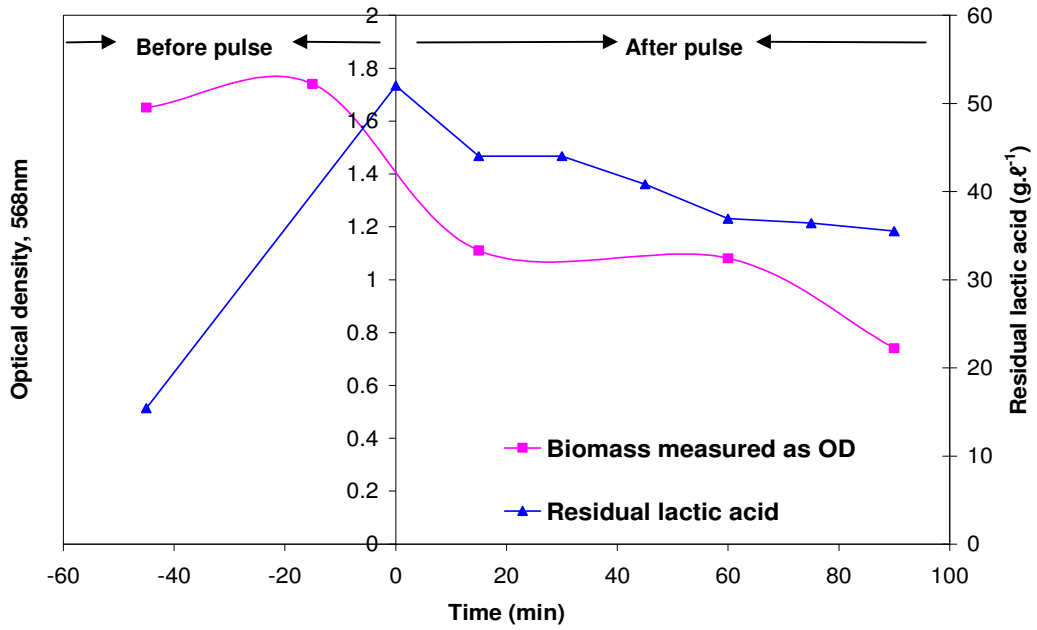

Fig. 3.12: Optical density and residual lactic acid of *M. elsdenii* NCIMB 41125 at a dilution rate of 0.43 h^{-1} before and after pulsing with 38.6 g of lactic acid, S_r lactic acid in the SDL medium, 24 $g.\ell^{-1}$.

Higher concentrations of lactic acid, as shown in section 3.1.2, had a negative effect on the shelf-life of *M. elsdenii* NCIMB 41125. This observation could also be used to explain the gradual wash-out of the culture as shown in Fig 3.12. Pulsing with lower lactic acid concentrations may result in biomass increase although positive responses could happen until lactic acid concentrations reach growth-depressing levels at a given dilution rate. The wash-out of the cells occurred only after pulsing with 38.6 $g.\ell^{-1}$ lactic acid and this implies inhibition of cells by lactic acid.

4.4.1.2 Pulsing with vitamin solutions

The residual lactic acid increased before pulsing and this was accompanied by a decrease in OD (Fig. 3.13). This tendency continued after pulsing but visual observations of the fermenter contents and prolonged incubation of the culture did not indicate wash-out of the cells. Similarly, the results in Fig. 3.14 suggest that steady-state conditions did not exist at the time of pulsing, but this should not affect the interpretation. Mateles and Battat (1974) stated that

pulse additions of potential limiting nutrients could be made even before the continuous culture system had attained a steady state after the previous

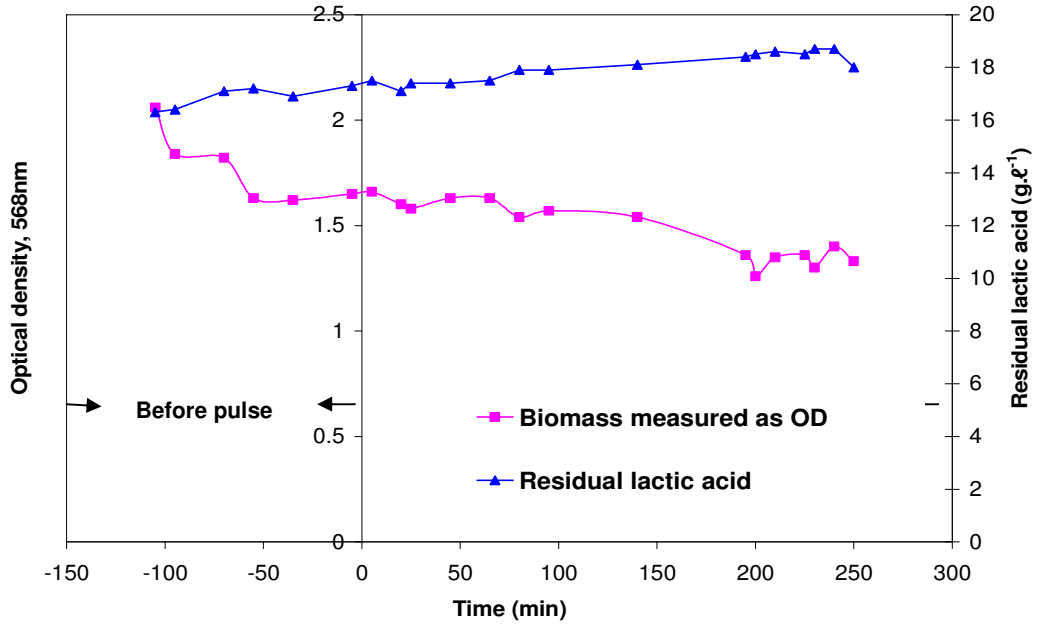

Fig. 3.13: Optical density and residual lactic acid of a culture of *M. elsdenii* NCIMB 41125 at a dilution rate of 0.43 h^{-1} before and after pulsing with vitamin solution 1. S_r lactic acid in the SDL medium, 24 g.ℓ^{-1}.

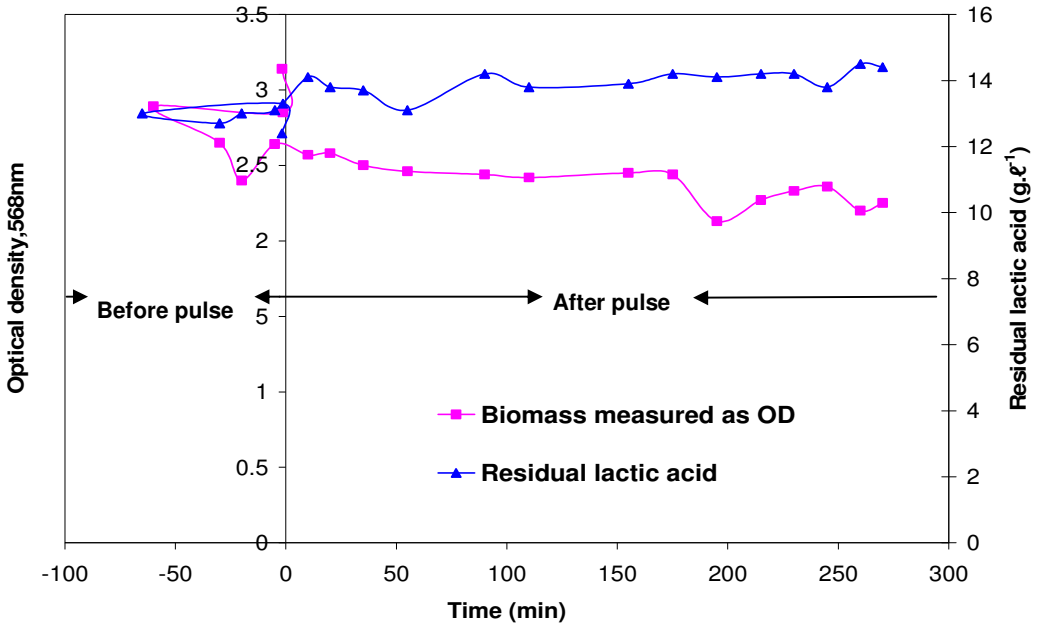

Fig. 3.14: Optical density and residual lactic acid of a culture of *M. elsdenii* NCIMB 41125 at a dilution rate of 0.43 h^{-1} before and after pulsing with vitamin solution 2. S_r lactic acid in the SDL medium, 24 g.ℓ^{-1}.

intervention. Pirt (1975) reported that growth yields from vitamins are often higher when the vitamin is growth-limiting than when it is in excess. However, the absence of any significant responses in this study suggested that there could be other nutrients which are growth-limiting for *M. elsdenii* NCIMB 41125. It appears that the effect of pulsing with vitamin solutions resulted in a decrease in the utilisation of lactic acid by the *M. elsdenii* NCIMB 41125 culture, consequently, with no effect on biomass yield (Fig. 3.13 and 3.14).

3.4.1.3 Pulsing with mineral solutions

Pulsing with mineral solution 3, did not result in a significant response, though the interpretation is complicated by fluctuations in the optical density readings (Fig. 3.14). However, it was clear that there was a gradual decrease in the levels of residual lactic acid. These results indicates the possibility of minerals being growth limiting for *M. elsdenii* NCIMB 41125 though a conclusion could not be drawn due to observed fluctuations in OD measurements.

After pulse-adding the trace mineral solution to the culture, a slight, delayed increase in optical density and a pronounced decrease in the steady-state concentration of lactic acid were observed after 40 min (Fig. 3.16). The levels of lactic acid reduction obtained after addition of trace mineral solution were

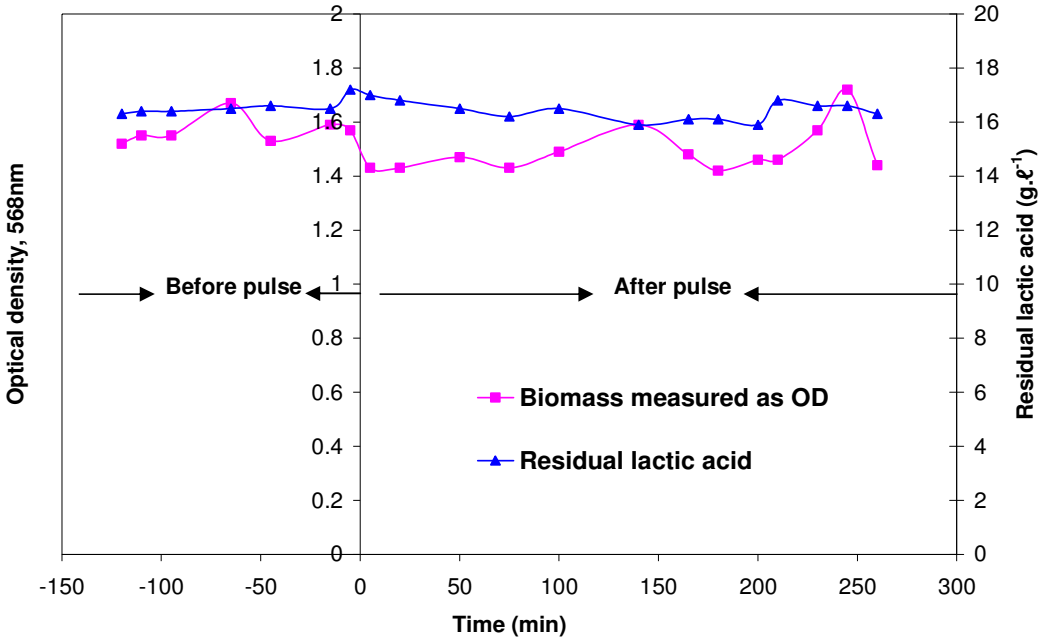

Fig. 3.15: Optical density and residual lactic acid of a culture of *M. elsdenii* NCIMB 41125 at a dilution rate of 0.43 h^{-1} before and after pulsing with mineral solution 3. Lactic acid concentration in the SDL medium, 24 g.ℓ^{-1}.

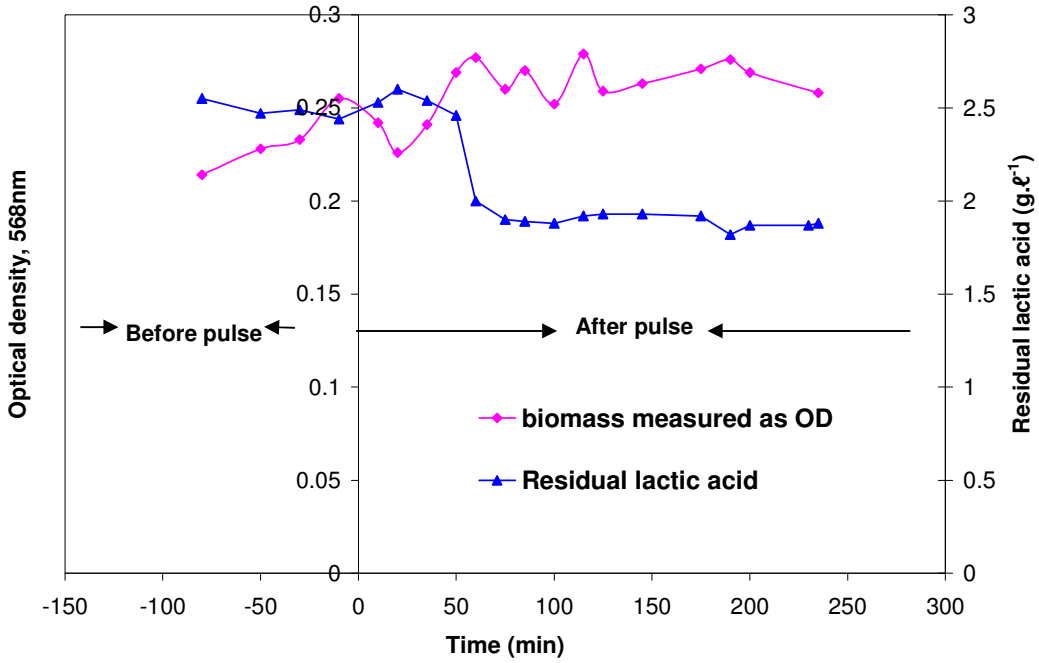

Fig. 3.16: Optical density and residual lactic acid of a culture of *M. elsdenii* NCIMB 41125 at a dilution rate of 0.4 h^{-1} before and after pulsing with trace mineral solution. S_r lactic in the SDL medium, 8.0 $g.\ell^{-1}$.

higher than reductions obtained with any of the medium constituents. This might have been due to a possible transition from growth limitation by a trace mineral to limitation by the energy source, lactate. Under these conditions, pulsing with a nutrient in short supply in the basal medium could be expected to produce a minimal increase in culture density before growth rate would have become lactate-limited.

This result indicates that the trace mineral solution used at this concentration would contribute to the optimisation of biomass yield.

4.4.1.4 Pulsing with yeast extract and peptone

Addition of yeast extract to a culture with a residual lactic acid concentration of 16 $g.\ell^{-1}$ resulted in an increase in optical density but no clear increase in lactic acid utilisation (Fig. 3.17). Assuming that yeast extract supplies to *M. elsdenii* NCIMB 41125 a limiting nutrient in very low concentrations, a significant growth response to doubling of the yeast extract content of the medium was expected since the concentration of the yeast extract was doubled. Yeast

extract is known to provide vitamins and co-factors which stimulate the growth of bacteria (Leclerc *et al.*, 1998). The co-factors are involved in metabolic pathways such as reduction of acetate to glucose, which then serves as an energy substrate. It was also envisaged that the pulse of yeast extract would exhibit a growth response by virtue of its nutrients, in addition to vitamins which are supplied by the vitamin solution 2, which is one of the components of the SDL medium.

There were fluctuations in residual lactic acid responses after pulsing with peptone for about 75 min (Fig. 3.18). During the same period, no clear trend was observed for the OD measurements taken. However, an increase in the level of lactic acid utilisation and a decrease in cell biomass were observed after 170 min of pulsing. After 210 min, the OD increased and residual lactic acid did not change. The residual lactic acid and OD results after 320 min of pulsing with peptone did, however, not suggest positive responses for the two variables.

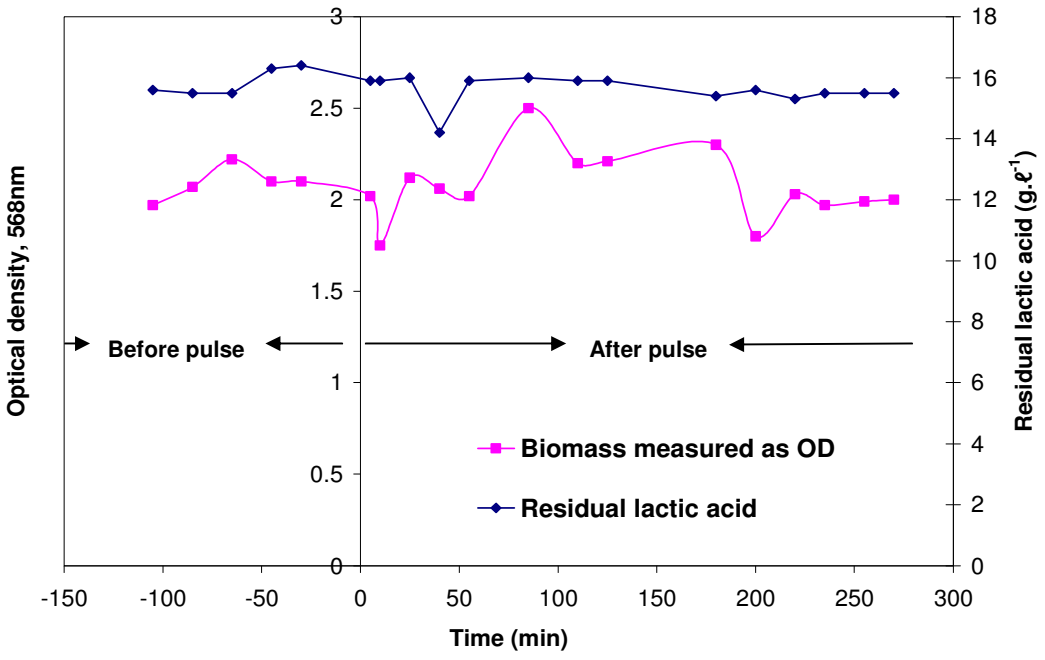

Fig. 3.17: Optical density and residual lactic acid of a culture of *M. elsdenii* NCIMB 41125 at a dilution rate of 0.43 h^{-1} before and after pulsing with yeast extract. S_r lactic acid in the SDL medium, 24 $g.\ell^{-1}$.

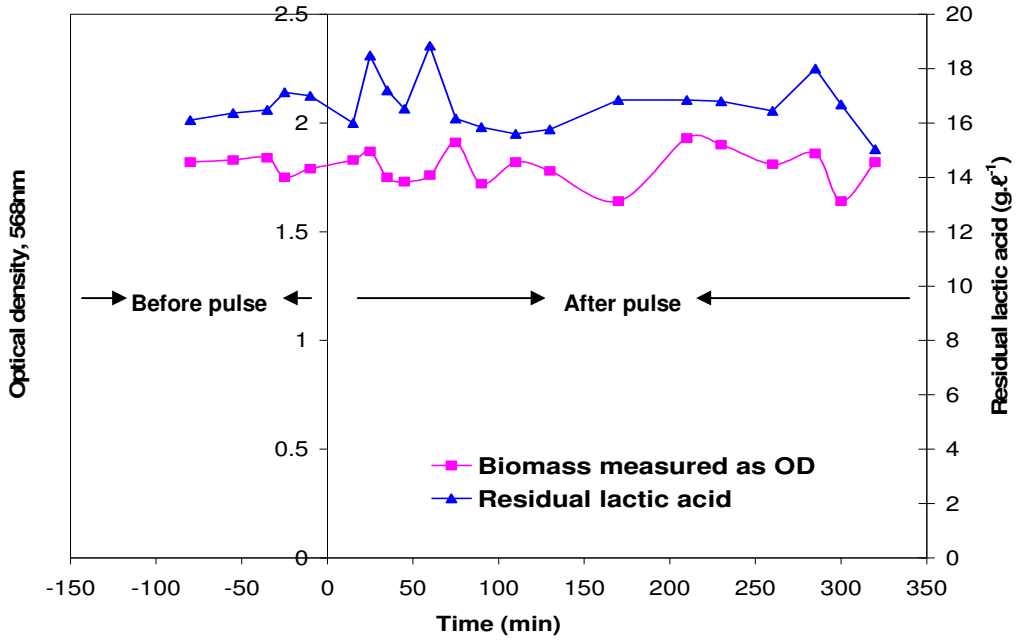

Fig. 3.18: Optical density and residual lactic acid of a culture of *M. elsdenii* NCIMB 41125 at a dilution rate of 0.43 h^{-1} before and after pulsing with peptone. S_r lactic acid in the SDL medium, 24 $g.\ell^{-1}$.

3.4.2 Summary of pulses

The results showed that pulsing with lactic acid at 8 g and 38.6 g did not result in positive effects on cell biomass under the experimental conditions. It should, however, be kept in mind that the medium which was continuously fed, contained 8 g lactic acid (Figs. 3.10 and 3.11) and 24 $g.\ell^{-1}$ lactic acid (Fig. 3.13). The lactic acid concentration of the SDL medium, 24 $g.\ell^{-1}$, could be considered too high for continuous cultures at the dilution rates of 0.4 and 0.43 h^{-1}. If that is the case, the positive responses obtained after pulsing with the trace mineral solution, which occurred when the lactic acid concentration in the medium was 8.0 $g.\ell^{-1}$, could indicate that at this concentration (8.0 $g.\ell^{-1}$), lactic acid is still growth-limiting.

The absence of positive responses after pulsing with components of the SDL medium as shown above may indicate unique properties of *M. elsdenii* NCIMB 41125 for it's specificity for lactate. It is shown in section 3.5 that at a dilution rate of 0.45 h^{-1}, the dry mass of *M. elsdenii* NCIMB 41125 obtained, using lactic acid concentration in the SDL medium of 24 $g.\ell^{-1}$, was 0.17 $g.\ell^{-1}$, as

opposed to the dry mass of 0.6 g.ℓ^{-1}, which was obtained at the same dilution rate but SDL containing 9.6 g.ℓ^{-1} lactic acid. This explains the repressive effect of lactic acid towards the culture. Therefore, the negative responses after pulsing with essential nutrients of *M. elsdenii* NCIMB 41125 is explained by the substrate inhibition concept (Pirt, 1975). Kuhn *et al.* (1979) showed that methionine and biotin yielded positive responses in a pulse-and-shift technique on *Bacillus caldotenax* but no repressive effects of the substrates were reported.

3.4.3 Determination of saturation constant for growth yield

3.4.3.1 Determination of saturation constant using Lineweaver-Burke plots

The Lineweaver-Burke plot of reciprocals of dilution rate and residual lactic acid at six dilution rates showed such a scatter of points that construction of a regression line was deemed worthless. The analytical data were collected while the culture was not in true steady state. The only useful information from this study is the residual lactic acid concentration at µm/ 2 (0.45 h^{-1}) which was of the same order as the results of residual lactic acid shown in Table 3.3.

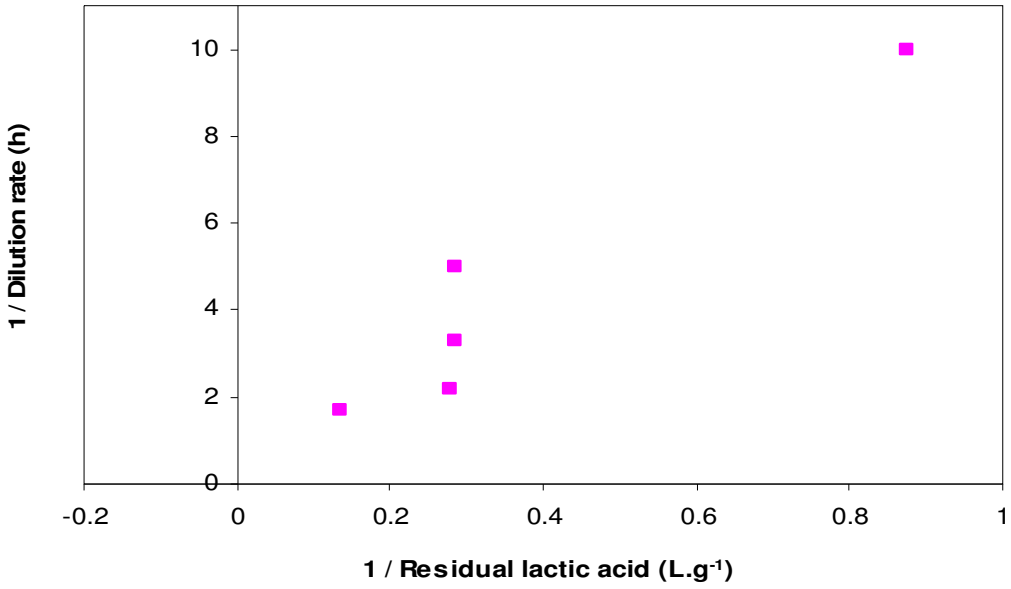

Fig. 3.19: Lineweaver-Burke plot of residual lactic acid vs. dilution rate

3.4.3.2 Measurements of saturation constant at steady-state concentration of residual lactic acid at μ_m/ 2 (0.45 h^{-1})

The results of measurements on three consecutive days are shown in Table 3.3. The mean residual lactic acid concentration of six determinations which represent the K_s value is 1.68 g.ℓ^{-1} or 19 mM. (It increased to 1.96 g.ℓ^{-1} or 22 mM when the value from the Lineweaver-Burke plot is included in the mean). Compared to the value of 0.37 mM reported by Russell and Baldwin (1978) for strain B159, it is very high, indicating a low affinity of strain NCIMB 41125 for lactic acid.

The assumption that $S_r >> K_s$, which is normally made in the derivation of the equation describing the growth of micro-organisms in continuous culture is, thus, not valid for *M. elsdenii* NCIMB 41125 growing on SDL medium. It follows that the critical dilution rate, D_c, at which results in complete washout of the culture, does not correspond to the maximum specific growth rate (μ_m). This is illustrated by Fig. 3.20 where the steady-state concentrations of biomass, residual lactic acid and rate of output of biomass have been plotted for dilution rates rising from 0 to 0.8 h^{-1}. These values were calculated by inserting experimentally determined values for concentration of lactic acid in the medium reservoir (S_r), saturation constant (K_s) and yield factor (Y) for lactic acid in the theoretical equations predicting growth in chemostat culture. The results predict a critical dilution rate of 0.75 h^{-1}, that is well below the typical value of 0.91 h^{-1} for maximum specific growth rate. It should be noted that the expected initial lactic acid concentration of SDL medium, 9.69 g.ℓ^{-1}, is six-fold more than the K_s value. The residual lactic acid concentrations in Table 3.3 showed a decreasing trend over a three day period. There was an increase in biomass concentration, from day 1 to day 2, but from day 2 to day 3 there was a decrease, which could not be explained.

Table 3.3: Measurements of the saturation constant at steady-state concentration of residual lactic acid at μ_m/ 2 (0.45 h^{-1})

Time (days)	Dry mass (g.ℓ^{-1})	Residual lactic* acid (g.ℓ^{-1})	Optical density** at 568nm
1	n.d.	2.21	0.304
	0.58	n.d.	0.308
	n.d.	2.01	0.297
2	n.d.	1.68	0.299
	0.65	n.d.	0.308
	n.d.	1.57	0.323
3	n.d.	1.51	0.322
	0.60	n.d.	0.334
	n.d.	1.16	0.333

* Samples obtained from the fermenter at 7 h intervals

** Samples obtained from the fermenter at 1 h intervals

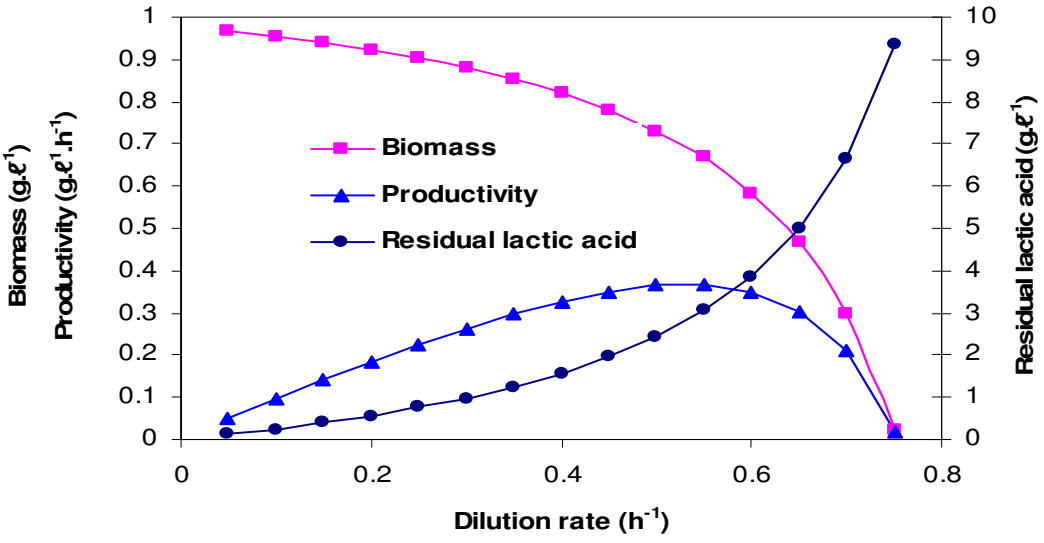

Fig. 3.20: Effect of dilution rates on concentrations of *M. elsdenii* NCIMB 41125 and residual lactic acid at steady-state. Medium, SDL; S_r lactic acid, 9.6 g.ℓ^{-1}, K_s = 2.0 g.ℓ^{-1}, μ_m = 0.91 h^{-1}, Y = 0.102

3.4.3.3 Dilution rates determined as rate of mass loss of medium from kegs

As mentioned in section 2.3.1.2.3, measurement of the rate of medium loss from kegs provided a convenient method of determining dilution rates. Representative graphs for experiments performed in sections 2.8 and 2.9 are shown in Figs. 3.20 and 3.21, respectively.

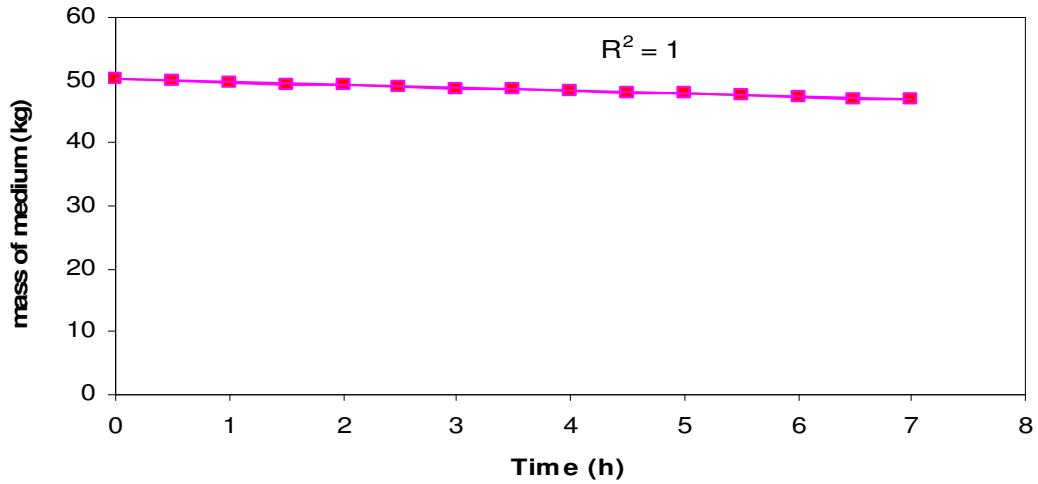

Fig. 3.21: Rate of medium flow to the fermenter measured as mass loss of medium from reservoir against time. Slope (dilution rate) , 0.456 h^{-1}

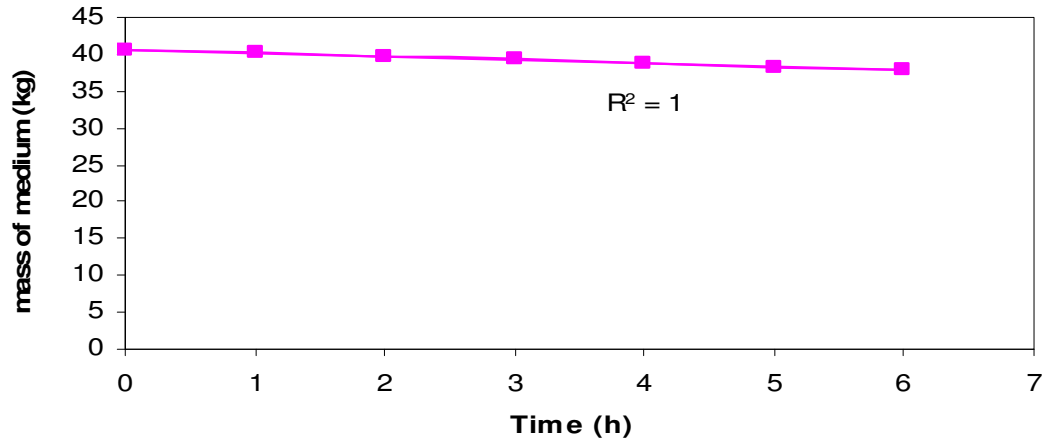

Fig. 3.22: Rate of medium flow to the fermenter measured as mass loss of medium from reservoir against time. Slope (dilution rate), 0.462 h^{-1}

The correlation coefficient of 1 shows the reliability and consistency of medium delivery rate for the expected dilution rate of 0.45 h^{-1}; hence the actual dilution rates of 0.456 and 0.462, respectively.

3.5 Viability of harvested cultures after storage at 25ºC

The culture was continuously collected from the fermenter into a keg, and thereafter stored at 25°C and then sampled for analyses (See Table 3.4 and 3.5 for the periods of collecting the culture and storing the culture).

The data shown in Tables 3.4 and 3.5 confirm the observations made during change in populations of *M. elsdenii* NCIMB 41125 harvested cultures. It indicates that further utilisation of lactic acid and growth occurred during storage of the harvest at room temperatures in the region of 25°C. After 24 h storage at 25°C this process was still ongoing (Table 3.5), whereas after 69 h the dry mass concentration had increased 5.6-fold and the residual lactic acid had fallen 30-fold, in comparison with the steady-state values pertaining to day 2 (Table 3.4).

Table 3.4: Optical density, dry mass and residual lactic acid determinations on *M. elsdenii* NCIMB 41125 samples collected from the fermenter and from the harvest vessel (collected over 56 h)

Measurements	Fermenter		Harvest vessel after 69 h storage
	Day 1	Day 2	
Optical density*	0.12	0.13	0.33
Dry mass ($g.\ell^{-1}$)	0.19	0.14	0.79
Residual lactic acid ($g.\ell^{-1}$)	17.6	17.6	0.58

* Measured at 568 nm, path length 10 mm, on a 10^{-1} dilution of the culture in R.O. water

Table 3.5: Optical density, dry mass and residual lactic acid determinations on *M. elsdenii* NCIMB 41125 samples collected from the fermenter and from the harvest vessel (collected over 25 h)

Measurements	Fermenter after 24 h	Harvest vessel after 24 h storage
Optical density*	0.13	0.198
Dry mass ($g.\ell^{-1}$)	n.d.	0.517
Residual lactic acid ($g.\ell^{-1}$)	11.1	2.73

* Measured at 568 nm, path length 10 mm, on a 10^{-1} dilution of the culture in R.O. water; n.d.; not determined

However, the yield factors for the conversion of lactate to biomass were low, being 0.035, 0.026 and 0.035 for days 1, 2 and the overall process, respectively. Earlier work indicated yield factors of 0.06 and higher (Unpublished data).

Results of this section suggest that it should be profitable to find optimal conditions for exploiting the 'maturation' stage which is inherent to the procedure where continuous cultivation is followed by batch processing of the harvest.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

- Better shelf-life results for fed-batch and continuous growth experiments were obtained after several cultures were harvested i.e. in one growth trial, cultures from third kegs had better shelf-lives than those from second and first kegs. Both the fed-batch and the continuous growth methods showed comparable results during a 14-day period.

- Higher concentrations of lactic acid showed negative effects on the shelf-life of *M. elsdenii* NCIMB 41125 at room temperature. Although there was no substantial difference between SDL and CSL media, the corn steep liquor medium contributed towards a better shelf-life and growth yields. However, the shelf-life data was variable, suggesting variation in the composition of corn steep liquor batches, due to manufacturing processes.

- Higher concentrations of lactic acid in the medium during continuous growth were partially inhibitory, as shown by S_r lactic acid of 24 $g.\ell^{-1}$ at the dilution rate of 0.45 h^{-1}.

- The dilution rate of 0.45 h^{-1} and lactic acid concentration of 16 $g.\ell^{-1}$ in SDL medium, may be useful for continuous growth of *M. elsdenii* NCIMB 41125, compared to lower dilution rates as the dry mass obtained at 0.45 h^{-1} was appreciable. At this dilution rate, the lactic acid concentration of 16 $g.\ell^{-1}$ did not inhibit the culture as compared to 24 $g.\ell^{-1}$ lactic acid.

- During storage at room temperature, *M. elsdenii* NCIMB 41125 settled at the bottom of stainless-steel kegs. The use of pure xanthan gum was not very effective at keeping cells in suspension, however, the shelf-life was improved.

- Pulsing the SDL medium, containing 8.0 $g.\ell^{-1}$ lactic acid, with trace mineral solution provided a positive response in growth yields - it was shown that this concentration was growth-limiting. Based on lower

growth yields obtained with S_r lactic acid of 24 g.ℓ^{-1}, pulsing with trace mineral solution at this relatively high S_r lactic acid concentration, may probably not have improved growth yields.

- The SDL, rather than CSL medium, may be preferable for the optimisation of growth yields and shelf-life stability of *M. elsdenii* NCIMB 41125.

- The saturation constant helped in identifying the range of K_s values in the order of 1.6 - 2.0 g.ℓ^{-1}, where *M. elsdenii* NCIMB 41125 was growth-limiting.

- The increase in dry mass when optimising growth yields during secondary growth in the kegs, i.e. after harvest of the culture from continuous growth, showed the efficiency of the secondary growth method. However, this method suffers from a lack of pH and temperature control in the kegs.

- Gassing the stainless-kegs with O_2-free N_2 before harvesting the culture, may improve anaerobic conditions.

- An alternative method that may be investigated in future for the optimisation of growth yields and shelf-life of *M. elsdenii* NCIMB 41125 is the freeze-drying method. Large volumes of liquid (culture) are not required at the farming site during dosage/inoculation of ruminants. However, large quantities of the culture will be required for filtration purposes as large amounts will be lost as effluent. The benefit will far outweigh carrying large quantities of the culture to the farms. The shelf-life is expected to be improved as there will be no need for *M. elsdenii* NCIMB 41125 to access nutrients in the liquid (culture).

- Shelf-life and growth yield studies on *M. elsdenii* NCIMB 41125 is of utmost importance in the feedlot and dairy industry as it allows the farmer to maintain the culture for the desired time. The challenge for

microbiologists is to identify appropriate conditions for prolonged survival and obtaining high growth yields.

- The role of *M. elsdenii* NCIMB 41125 as a lactic acid utiliser can be further manipulated if microbiologists work closely with farmers.

REFERENCES

Agricultural Research Council. Progress Reports 1991/92 - Animal Production Centre, p 67. Animal Production Institute. Private Bag X2, Irene, 0162, South Africa.

Al Jassim R.A.M. and **Rowe J.B.** 1999. Better understanding of acidosis and its control. Rec. Adv. Anim. Nutr. Aust. **12:** 91-97.

Allison M.J., Bucklin J.A. and **Dougherty R.W.** 1964. Ruminal changes after overfeeding with wheat and the effect of intraruminal inoculation on adaptation to a ration containing wheat. J. Anim. Sci. **23:** 1164-1171.

Alonso L., Cuesta E.P. and **Gilliland S.E.** 2003. Production of free conjugated linoleic acid *by Lactobacillus acidophilus* and *Lactobacillus casei* of human intestinal origin. J. Dairy Sci. **86:** 1941-1946.

Annison E.F. 1956. Nitrogen metabolism in the sheep. Biochem. J. **64:** 705-714.

Asanuma N. and **Hino T**. 2002. Understanding metabolic regulation in the ruminal bacteria: *Streptococcus bovis, Selonomonas ruminantium* and *Megasphaera elsdenii*. In: Martin SA (ed.), Gastrointestinal Microbiology in Animals. 61-87. Research Signpost, Kerala.

Asanuma N. and **Hino T**. 2004. Prevention of rumen acidosis and suppression of ruminal methanogenesis by augmentation of lactate utilization. Nihon Chikusan Gakkaiho. **75:** 543-550.

Asanuma N. and **Hino T**. 2005. Ability to utilize lactate and related enzymes of a ruminal bacterium. *Selonomonas ruminantium*. Anim. Sci. J. **76:** 345-352.

Baldwin R.L., Wood W.A. and **Emery R.S.** 1962. Conversion of lactate-C14 to propionate by the rumen microflora. J. Bacteriol. **83:** 907-913.

Bergen W.G. and **Bates D.B.** 1984. Ionophores: Their effect on production efficiency and mode of action. J. Anim. Sci. **58:** 1465-1483.

Bergman E.N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. **70:** 567-590.

Both B., Buckel W., Kroppenstedt R. and **Stackebrandt E.** 1992. Phylogenetic and chemotaxonomic characterization of *Acidaminococcus fermentans.* FEMS Microbiol. Lett. **76:** 7-11.

Brossard L., Martin C., Chaucheyras-Durand F. and **Mlichalet-Doreau B.** 2004. Effect of the microbial additive *Saccharomyces cerevisiae* CNCMI-1077 on protein and peptide degrading activities of rumen bacteria grown in vitro. Reprod. Nutr. Dev. **44 (3):** 195-206.

Brown M.S., Ponce C.H. and **Pulikanti R.** 2006. Adaptation of beef cattle to high-concentrate diets: Performance and ruminal metabolism. J. Anim. Sci. **81 (E. Suppl.):** E25-E33.

Bryukhanov L. and **Netrusov A.I.** 2006**.** Long-term storage of obligate anaerobic microorganisms in glycerol. Appl. Biochem. Microbiol. **42 (2):** 177-180.

Callaway T.R. and **Martin S.A.** 1996. Effects of organic acid and monensin treatment on in vitro mixed ruminal microorganism fermentation of cracked corn. J. Anim. Sci. **74:** 1982-1989.

Callaway T.R., Adams K.A. and **Russell J.B.** 1999. The ability of 'low G+C gram-positive' ruminal bacteria to resist monensin and counteract potassium depletion. Curr. Microbiol. **39:** 226-230.

Callaway T.R., Edrington T.S., Rychlik J.L., Genovese K.J., Poole T.L. Jung Y.S., Bischoff K.M., Anderson C. and **Nisbet D.J.** 2003. Ionophores:

Their use as ruminant growth promotants and impact on food safety. Curr. Issues Intest. Microbiol. **4:** 43-51.

Caldwell D.R. and **Bryant M.P.** 1966. Medium without rumen fluid for non-selective enumeration and isolation of rumen bacteria. Appl. Microbiol. **14:** 794-801.

Carlier J.P., Marchandin H., Jumas-Bilak E., Lorin V., Henry C., Carriere C. and **Jean-Pierre H.** 2002. *Anaeroglobus geminatus* gen. nov., sp. nov., a novel member of the family *Veillonellaceae*. Int. J. Syst. Evol. Microbiol. **52:** 983-986.

Chaucheyras-Durand F., Fonty G., Bertin G., Salmon J.M. and **Gouet P.** 1996. Effects of a strain of *Saccharomyces cerevisiae* (Levucell SC1), a microbial additive for ruminants, on lactate metabolism in vitro. Can. J. Microbiol. **42:** 927-933.

Chaucheyras-Durand and **Fonty G.** 2002. Influence of a probiotic yeast (*Saccharomyces cerevisiae* CNCM I-1077) on microbial colonization and fermentations in the rumen of newborn lambs. Microbial Ecol. Health Dis. **14:** 30-36.

Chesson A. and **Forsberg C.W.** 1997. Polysaccharide degradation by rumen microorganisms. The Rumen Microbial Ecosystem. Hobson P. N. and Stewart C. S. ed. Blackie Academic and Professional, New York, NY. 329-381.

Coleman G.S. 1974. Digestion and Metabolism in the Ruminant, In: Mcdonald I.W. and Werner A.C.I. (eds). University of New England Publishing, Armidale. 149-164.

Counotte G.H. and **Prins R.A.** 1979. Regulation of rumen lactate metabolism and the role of lactic acid in nutritional disorders of ruminants. Vet. Sci. Comm. **2:** 277-303.

Counotte G.H. and **Prins R.A.** 1981. Regulation of lactate metabolism in the rumen. Vet. Res. Commun. **5:** 101-115.

Cook M.K., Cooley J.H. and **Huber T.L.** 1975. Performance of cattle inoculated with rumen bacteria. J. Anim. Sci. **41:** 396-400.

Dawson K.A. and **Allison M.J.** 1988. Digestive disorders and nutritional toxicity. *In* The rumen microbial ecosystem, Edited by P.N. Hobson. London: Elsevier Appl. Sci. 445-459.

Dehority B.A., Tirabasso P.A. and **Grifo A.P.** 1989. Most-probable-number procedures for enumerating ruminal bacteria, including the simultaneous estimation of total and cellulolytic numbers in one medium. Appl. Environ. Microbiol. **55:** 2789-2792.

Deng W.D., Xi D., Mao H.M. and **Wanapat M.** 2008. The use of molecular techniques based on ribosomal RNA for rumen microbial ecosystem studies: A review. Mol. Biol. Rep. **35:** 265-274.

Dennis S.M., Nagaraja T.G. and **Bartley E.E.** 1981. Effects of lasalocid or monensin on lactate-producing or lactate-using rumen bacteria. J. Anim. Sci. **52:** 418-426.

Dinius D.A., Simpson M.E. and **Marsh P.B.** 1976. Effect of monensin fed with forage on digestion and the ruminal ecosystem of steers. J. Anim. Sci. **42:** 229-234.

Donovan J. 1997. Subacute acidosis is costing us millions. Hoards Dairyman, 25 September, 666.

Doyle L.M., McInerney J.O., Mooney J., Powell R., Haikara A. and **Moran A.P.** 1995. Sequence of the gene encoding the 16S rRNA of the beer spoilage organism *Megasphaera cerevisiae.* J. Ind. Microbiol. **15:** 67-70.

Enemark J.M.D. 2009. The monitoring, prevention and treatment of sub-acute ruminal acidosis (SARA): A review. Vet. Journal. **176:** 32-43.

Erdman R.A. 1988. Dietary buffering requirements of the lactating dairy cow: A review. J. Dairy Sci. **71 (12):** 3246-3266.

Felsenstein J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. **17:** 368-376.

Fuller R. 1999. Probiotics for farm animals. In: G. W. Tannock (ed.) Probiotics - A Critical Review. p 15. Horizon Scientific Press, Wymondham, England.

Fonty G. and **Chaucheyras-Durand F.** 2006. Effects and modes of action of live yeasts in the rumen. Cel. Mol. Biol. **61 (6):** 741-750.

Galyean M.L. and **Owens F.N.** 1988. Effects of monensin on growth, reproduction, and lactation in ruminants. In: ISI Atlas of Science: Anim. Plant Sci. ed. ISI Press, Philadelphia, PA. 71-75.

Ghorbani G.R., Morgavi D.P., Beauchemin K.A. and **Leedle J.A.Z.** 2002. Effects of bacterial direct-fed microbials on ruminal fermentation, blood variables, and the microbial populations of feedlot cattle. J. Anim. Sci. **80:** 1977-1986.

Giesecke D., Wiesmayr S. and **Ledinek M.** 1970. *Peptostreptococcus elsdenii* from the caecum of pigs. J. Gen. Microbiol. **64:** 123-126.

Gilchrist F.M.C. and **Kistner A.** 1962. Bacteria of the ovine rumen. 1. The composition of the population on a diet of poor teff hay. J. Agric. Sci. **59:** 77-83.

Gill H., Shu S.Q. and **Leng R.A.** 2000. Immunization with *Streptococcus bovis* protects against lactic acidosis in sheep. Vaccin. **18:** 2541-2548.

Gnanasampanthan G. 1993. Immune responses of sheep to rumen ciliates and the survival and activity of antibodies in the rumen fluid. Ph.D. thesis, University of Adelaide, Adelaide.

Goad D.W., Goad C.L. and **Nagaraja T.G.** 1998. Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. J. Anim. Sci. **76:** 234-241.

Greening R.C., Smolenski W.J., Bell R.L., Barsuhn M.M. and **Robinson J.A.** 1991. Effects of inoculation of *Megasphaera elsdenii* strain 407A (UC-12497) on ruminal pH and organic acids in beef cattle. J. Anim. Sci. **69 (Suppl. 1):** 518.

Greyling B.J. 1996. The development and evaluation of a specific probe for *Megasphaera elsdenii*. MSc. Thesis. Rand Afrikaans University.

Gröhn Y.T. and **Bruss M.L.** 1990. Effect of disease, production and season on traumatic reticuloperitonitis and natural acidosis in dairy cattle. J. Dairy Sci. **73:** 2355-2363.

Hino T., Shimoda K. and **Maruyama T.** 1994. Substrate preference in a strain of *Megasphaera elsdenii*, a ruminal bacterium, and its implications in propionate production and growth competition. Appl. Environ. Microbiol. **60:** 1827-1831.

Hino T. and **Asanuma N.** 2003. Suppression of ruminal methanogenesis by decreasing the substrates available to methanogenic bacteria. Nutrition abstracts and Reviews, Series B: Livestock Feeds and Feeding. **73:** 1-8.

Hishinunuma F., Kanegasak S. and **Takahaski H.** 1968. Ruminal fermentation and sugar concentrations. Agric. Biol. Chem. **32:** 1325-1330.

Holtug K., Rasmussen H.S. and **Mortensen P.B.** 1992. An in vitro study of short-chain fatty acid concentrations, production and absorption in pig colon. Com. Biochem. Physiol. **103:** 189-197.

Huber T.L. 1971. Effect of acute indigestion of compartmental water volumes and osmolarity in sheep. Am. J. Vet. Res. **32:** 887-890.

Huber T.L. 1976. Physiological effects of acidosis on feedlot cattle. J. Anim. Sci. **43:** 902-909.

Hungate T.L., Dougherty R.W., Bryant M.P. and **Cello R.M.** 1952. Microbiological and physiological changes associated with acute indigestion in sheep. Cornell Vet. **42 (4):** 423-449.

Hungate R.E. 1966. The rumen and its microbes. New York: Academic Press.

Hungate R.E. 1969. A roll tube method for cultivation of strict anaerobes. In: Norris JR, Ribbons DW (eds). Met. Microbiol. Vol 3B. Academic Press, London and New York, 117-132.

Hutjens M.F. 1991. Feed additives. Veterinary clinics of North America. Food Animal Practice 7, 525-540.

Hutjens M.F. 1999. How and when feed additives may or may not pay. Hoards Dairyman. 25 September.

Iwamoto M., Asanuma N. and **Hino T**. 2001. Effects of energy substrates on nitrate reduction and nitrate reductase activity in a ruminal bacterium, *Selenomonas ruminantium*. Anaerobe. **7:** 315-321.

Kamra D.N. 2005. Rumen microbial ecosystem. Curr. Sci. **89 (1):** 124-135.

Kameue C., Tsukahara T., Yamada K., Koyama H., Iwasaki Y., Nakayama K. and **Ushida K.** 2004. Dietary sodium gluconate protects rats from large bowel cancer by stimulating butyrate production. J. Nutr. **134:** 940-944.

Kelco-Company. 2005. Carboxymethlycellulose book. USA. Inc. 1-26.

Kemira Phosphates. 2006. *Megasphaera elsdenii* strain and its uses. US Patent Application Serial Number 10/521,847.

Kennelly J.J., Robinson B. and **Khorasani G.R.** 1999. Influence of carbohydrate source and buffer on rumen fermentation characteristics, milk yield and milk composition in early lactation Holstein cows. J. Dairy Sci. **82:** 2486-2496.

Kepler C.R., Hirons K.P., McNeill J.J. and **Tove S.B.** 1966. Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. J. Biol. Chem. **6:** 1350-1354.

Khampa S. and **Wanapat M.** 2006. Supplementation of urea level and malate in concentrate containing high cassava chip on rumen ecology and milk production in lactating cows. Pak. J. Nutr. **5 (6):** 530-535.

Kim Y.J., Liu R.H., Rychlik J.R. and **Russell J.B.** 2002. The enrichment of a ruminal bacterium *Megasphaera elsdenii* (YJ-4) that produces the *trans-10, cis-12* isomer of conjugated linoleic acid. J. Appl. Microbiol. **92:** 976-982.

Klieve A.V., Hennessy D., Ouwerkerk D., Foster R.J., Mackie R.I. and **Attwood G.T.** 2003. Establishing populations of *Megasphaera elsdenii* YE 34 and *Butyrivibrio fibrisolvens* YE 44 in the rumen of cattle fed high grain diets. J. Appl. Microbiol. **95:** 621-630.

Kotarski S.F., Waniska R.D. and **Thurn K.K.** 1992. Starch hydrolysis by the ruminal microflora. J. Nutr. **122:** 178-190.

Krause K.M. and **Oetzel G.R.** 2006. Understanding and preventing subacute ruminal acidosis in dairy herds: A review. Anim. Feed Sci. Technol. **126:** 215-236.

Krieg R. 1981. Enrichment and isolation. In: Manual of Methods for General Bacteriology. Gerhardt, P. (Ed.), American Society for Microbiology, Washington, 112-114.

Krogh. N. 1961. Studies on alterations in the rumen fluid of sheep, especially concerning the microbial composition when readily available carbohydrates are added to the food. Acta. Vet. Scandinavica. **2:** 103-107.

Kuhn H., Friederich U. and **Fiechter A.** 1979. Defined minimal medium for a thermophilic *Bacillus* sp. developed by a chemostat pulse and shift technique. Europ. J. Appl. Microbiol. Biotechnol. **6:** 341-349.

Kung L., Tung R.S. and **Slyter L.L.** 1992**.** In vitro effects of the ionophore lysocellin on ruminal fermentation and microbial populations. J. Anim. Sci. **70:** 281-288.

Kung L. and **Hession A.O.** 1995. Preventing in vitro lactate accumulation in ruminal fermentation by inoculation with *Megasphaera elsdenii*. J. Anim. Sci. **73:** 250-256.

Ladd J.N. 1959. The fermentation of lactic acid by a gram-negative coccus. Biochem. **71:** 16-22.

Latham M.J., Sharpe M.E. and **Sutton J.D.** 1971. The microbial flora of the rumen of cows fed hay and high cereal rations and its relationship to the rumen fermentation. J. Appl. Bacteriol. **34:** 425-434.

Leclerc M., Elfoul-Bensaid L. and **Bernalier A.** 1998. Effect of yeast extract on growth and metabolism of H_2-utilizing acetogenic bacteria from the human colon. Curr. Microbiol. **37 (3):** 166-171.

Leedle J.A.Z. and **Hespell R.B.** 1980. Diurnal variations in bacterial numbers and fluid parameters in ruminal contents of animals fed low or high-forage diets. Appl. Environ. Microbiol. **44:** 402-412.

Liggett R.W. and **Koffler H.** 1948. Corn steep liquor in Microbiology. Bacteriol. Rev. **12 (4):** 297-311.

Liu C.M. 1982. Microbial aspects of polyether antibiotics: activity, production and biosynthesis, *In* J.W. Westley (ed.), Polyether antibiotics: Vol. Biology. Marcel Dekker Inc., New York. 43-102.

Mackie R.I., Gilchrist F.M.C., Robberts A.M., Hannah P.E. and **Schwartz H.M.** 1978. Microbiological and chemical changes in the rumen during stepwise adaptation of sheep to high concentrate diets. J. Agric. Sci, Camb. **90:** 241-254.

Mackie R.I. and **Gilchrist F.M.C.** 1979. Changes in lactate-producing and lactate-utilizing bacteria in relation to pH in the rumen of sheep during stepwise adaptation to a high concentrate diet. Appl. Environ. Microbiol. **38 (3):** 422-430.

Mackie R.I. and **Heath S.** 1979. Enumeration and isolation of lactate-utilizing bacteria from the rumen of sheep. Appl. Environ. Microbiol. **38 (3):** 416-421.

Marounek M. and **Bartos S.** 1987. Interactions between rumen amylolytic and lactate-utilizing bacteria in growth on starch. J. Appl. Bacteriol. **63:** 233-238.

Marounek M., Fliegrova K. and **Bartos S.** 1989. Metabolism and some characteristics of ruminals strains of *Megasphaera elsdenii*. Appl. Environ. Microbiol. **55:** 1570-1573.

Martin G.A. and **Hempfling W.P.** 1976. Method for the regulation of microbial population density during continuous culture at high growth rates. Arch. Microbiol. **107:** 41-47.

Mateles R.I. and **Battat E.** 1974. Continuous culture used for media optimization. Appl. Microbiol. **28 (6):** 901-905.

McLeod K.R. and **Baldwin R.L.** 2000. Effects of diet forage: concentrate ratio and metabolizable energy intake on visceral organ growth and in vitro oxidative capacity of gut tissues in sheep. J. Anim. Sci. **78:** 760-770.

Miyazaki Y., Shibuya M., Sugawara H., Kawaguchi D., Hirose C., Nagatsu J. and **Esumi S.** 1974. Salinomycin: A new polyether antibiotic. J. Antibiot. **27:** 814-821.

Mohamed-Nour M.S. 2006. Surgical treatment of experimentally induced lactic acidosis in Nubian goats. J. Anim. Vet. Adv. **5 (1):** 49-52.

Moir R.J. and **Wiliams V.J.** 1950. Ruminant flora studies in the sheep. 2. The effect of the level of nitrogen intake upon the total number of free microorganisms in the rumen. Austr. J. Sci. Res. **83:** 381-392.

Nagaraja T.G., Avery T.B., Bartley E.E., Roof S.K. and **Dayton A.D.** 1982. Effect of lasalocid monensin or thiopeptin on lactic acidosis in cattle. J. Anim. Sci. **54:** 649-658.

Nagaraja T.G. and **Bartley E.E**. 1983. Prevention of lactic acidosis with lasalocid or monensin. Agri-Practice **4 (3):** 5-12.

Nagaraja T.G., Taylor M.B., Harmon D.L. and **Boyer J.E.** 1987. In vitro lactic acid inhibition and alterations in volatile fatty acid production by antimicrobial feed additives. J. Anim. Sci. **65:** 1064-1076.

Nagaraja T.G. and **Miller G.W.** 1989. Rumen microbial changes in ionophore antibiotic-treated steers with experimentally induced acidosis. Australas. J. Anim. Sci. **2:** 465-468.

Nagaraja T.G. and **Chengappa M.M.** 1998. Liver abscess in feedlot cattle: A review. J. Anim. Sci. **76:** 287-298.

Nagararaja T.G. and **Lechtenberg K.F.** 2007. Acidosis in feedlot cattle. Vet. Cli. Food Anim. **23:** 333-350.

Nagel S. and **Broderick G.A.** 1992. Effect of formic acid or formaldehyde treatment of alfalfa silage on nutrient utilization by dairy cows. J. Dairy Sci. **5:** 140-154.

Nagpal R., Yadav H., Puniya A.K., Singh K., Jain S. and **Marotta F.** 2007. Conjugated linoleic acid: sources, synthesis and potential health benefits - an overview. Curr. Top Nutraceutical Res. **5 (2/3):** 55-66.

Newbold C.J. and **Wallace R.J.** 1988. Effects of the ionophores monensin and tetronasin on simulated development of ruminal lactic acidosis in vitro. Appl. Environ. Microbiol. **54:** 2981-2985.

Newbold C.J. 1995. Microbial feed additives for ruminants. In: Wallace R.J. and Chesson A, (eds) Biotechnology in Animal Feeds and Animal Feeding, VCH, Weinheim.

Newbold C.J., McIntosh F.M. and **Wallace R.J.** 1998. Changes in the microbial population of a rumen-simulating fermenter in response to yeast culture. Can. J. Anim. Sci. **78:** 241-244.

Nisbet D.J. and **Martin S.A.** 1991. Effect of *a Saccharomyces cerevisiae* culture on lactate utilization by the ruminal bacterium *Selenomonas ruminantium* J. Anim. Sci. **69:** 4628-4633.

Nocek J.E. 1997. Bovine acidosis: implications on laminitis. J. Dairy Sci. **80:** 1005-1028.

Nocek J.E., Kautz W.P., Leedle J.A.Z. and **Allman J.G.** 2000. Altering diurnal pH and in situ digestion in dairy cows with ruminal supplementation of direct-fed microbials (DFM) and yeast. J. Dairy Sci. **83 (Suppl. 1):** 1242 (Absr.).

Nocek J.E. and **Kautz W.P.** 2006. Direct-fed microbial supplementation on ruminal digestion, health and performance on pre- and postpartum dairy cattle. J. Dairy Sci. **89:** 260-266.

Nordlund K.V. 1995. Questions and answers regarding rumenocentesis and the diagnosis of herd based subacute rumen acidosis. Proc. 4-State Appl. Nutr. Conf., University of Wisconsin Extension Service, Madison, WI.

Olsen G.J., Matsuda H., Hagstrom R. and **Overbeek R.** 1994. FastDNAml - a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. CABIOS **10:** 41-48.

Olumeyan D.B., Nagaraja T.G., Miller G.W., Frey R.A. and **Boyer J.E.** 1986. Rumen microbial changes in cattle fed diets with or without salinomycin. Appl. Environ. Microbiol. **51:** 340-345.

Owens F.N., Secrist D.S., Hill W.J. and **Gill D.R.** 1998. Acidosis in cattle: A review. J. Anim. Sci. **76:** 275-286.

Píknová M., Bireš O., Javorsky P. and **Pristaš P.** 2006. Limited genetic variability in *Megasphaera elsdenii* strains. Folia. Microbiol. **51 (4):** 299-302.

Pirt S.J. 1975. Principles of microbe and cell cultivation, Oxford: Blackwell Scientific Publications. 29-41.

Plaizier J.C., Krause D.O., Gozho G.N. and **McBride B.W.** 2009. Sub-acute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. **176**: 21-31.

Potter A.A., Klashinski S., Li Y., Frey E., Townsend H., Rogan D., Erickson G., Hinkley S., Klopfenstein T., Moxley R.A., Smith D.R. and **Finlay B.B.** 2004. Decrease shedding of *Escherichia coli* O157:H7 by cattle following vaccination with type iii secreted proteins. Vaccine. **22:** 362-369.

Puniya A.K., Chaitanya S., Tyagi A.K. and **Kishan Singh S.De.** 2008. Conjugated linoleic acid producing potential of lactobacilli isolated from the rumen of cattle. J. Ind. Microbiol. Biotechnol. **35:** 1223-1228.

Raeth-Knight M.L., Linn G.L. and **Jung H.G.** 2007. Effect of direct-fed microbials on performance, diet digestibility and rumen characteristics of Holstein dairy cows. J. Dairy Sci. **90:** 1802-1809.

Richardson L.F., Raun A.P., Potter E.L., Cooley C.O. and **Rathmacher R.P.** 1976. Effect of monensin on rumen fermentation *in vitro* and *in vivo*. J. Anim. Sci. **43:** 657-664.

Ricke S.C., Martin S.A. and **Nisbet D.J.** 1996. Ecology, metabolism and genetics of ruminal selenomonads. Crit. Rev. Microbiol. **22:** 27-65.

Roediger W.E.W. and **Moore A**. 1981. The effect of short-chain fatty acid on sodium absorption in the isolated human colon perfused through vascular bed. Digestive Disease and Sciences. **26:** 100-106.

Rogosa M. 1971. Family: Veillonaceae. In Bergeys Man. Syst. Bacteriol. 685**.**

Rogosa M. 1971. Transfer of *Peptostreptococcus elsdenii* Gutierrez *et al.,* to a new genus, *Megasphaera* [*M. elsdenii* (Gutierrez *et al.,*) comb. Nov.] Int. J. Systemic Bacteriol. **21:** 187-189.

Rosalam S. and **England R.** 2006. Review of xanthan gum production from unmodified starches by *Xanthomonas campestris* sp. Enzyme Microbiol. Technol. **39 (2):** 197-207.

Rossi F., Concconcelli P.S. and **Masoero F.** 1995. Effect of a *Saccharomyces cerevisiae* culture on growth and lactate utilization by the ruminal bacterium *Megasphaera elsdenii.* Ann. Zootech. **44:** 403-409.

Rossi F., Di Luccia A., Vincenti D. and **Concconcelli P.S.** 2004. Effects of peptidic fractions from *Saccharomyces cerevisiae* culture on growth and metabolism of the ruminal bacteria *Megasphaera elsdenii.* Anim. Res. **53:** 177-186.

Russell J.B. and **Baldwin R.L.** 1978. Substrate preference in rumen bacteria: Evidence of catabolite regulatory mechanisms. Appl. Environ. Microbiol. **36:** 319-329.

Russell J.B. and **Baldwin R.L.** 1979. Comparison of substrate affinities among several rumen bacteria: a possible determinant of rumen bacterial competition. Appl Environ. Microbiol. **37(3):** 531-536.

Russell J.B., Delfino F.J. and **Baldwin R.L.** 1979. Effects of combinations of substrates on maximum growth rates of several rumen bacteria. Appl. Environ. Microbiol. **37:** 544-549.

Russell J.B. and **Hino T.** 1985. Regulation of lactate production in *Streptococus bovis*: a spiraling effect that contributes to rumen acidosis. J. Dairy Sci. **68:** 1712-1721.

Russell J.B. and **Strobel H.J.** 1989. Effect of ionophores on ruminal fermentation. Appl. Environ. Microbiol. **55:** 1-6.

Russell J.B. 1991. Intracellular pH of acid-tolerant ruminal bacteria. Appl. Environ. Microbiol**. 57:** 3383-3384.

Russell J.B. and **Wilson D.B.** 1996. Why are some ruminal cellulolytic bacteria unable to digest cellulose at low pH? J. Dairy Sci**. 79:** 1503-1509.

Russell J.B. and **Rychlik J.L.** 2001. Factors that alter rumen microbial ecology. Sci. **292:** 119-122.

Schleifer K.H., Leuteritz M., Weiss N., Ludwig W., Kirchof G. and **Seidel-Rüfer H.** 1990. Taxonomic study of anaerobic, Gram-negative, rod-shaped bacteria from breweries: Emended description of *Pectinatus cerevisiiphilus* and description of *Pectinatus frisingensis* sp. nov., *Selenomonas lacticifex* sp. nov., *Zymophilus raffinosivorans* gen. nov., sp. nov., and *Zymophilus paucivorans* sp. nov. Int. J. Syst. Bacteriol. **40:** 19-27.

Sakata T. 1997. Influence of short-chain fatty acids on intestinal growth and functions. In: Krotchevsky D, Bonfield C (eds), *Dietary fibre in health and disease*, 191-199. Plenum Press , New York.

Sharpe M.E., Latham M.J., Garvie E.I., Zirngibi J. and **Kandler O.** 1973. Two new species of *Lactobacillus* isolated from the rumen, *Lactobacillus ruminis* sp. nov. and *Lactobacillus vitulinus* sp. nov. J. Gen. Microbiol. **77:** 37-49.

Sheldrake R.F., Scicchitano R. and **Husband A.J.** 1985. The effect of lactation on the transport serum-derived immunoglobulin A into bile of sheep. Immunol. **54:** 471-478.

Shimotoyodome A., Megura S. Hase T., Tokimitsu I. and **Sakata T.** 2000. Short- chain fatty acids but not lactate or succinate stimulate mucous release in the rat colon. Comparative Biochemistry and Physiology, **125:** 525-531.

Slyter L.L. 1976. Influence of acidosis on rumen function. J. Anim. Sci. **43:** 910-929.

Soto-Cruz., Chavez-Rivera R. and **Saucedo-Castaneda G.** 2001. Stimulation of *Megasphaera elsdenii's* butyrate production in continuous culture by a yeast additive. Braz. Arch. Biol. Tech. **44: (2)** 179-184.

Stackebrandt E., Pohla H., Kroppenstedt R., Hippe H. and **Woese C.R.** 1985. 16S rRNA analysis of Sporomusa, *Selenomonas*, and *Megasphaera* on the phylogenetic origin of Gram-positive. Eubacteria Arch. Microbiol. **143**: 270-276.

Stackebrandt E. and **Rainey F.A.** 1995. Partial and complete 16S rDNA sequences, their use in generation of 16S rDNA phylogenetic trees and their implications in molecular ecological studies. Kluwer Academic Publishers, Netherlands. Mol. Microb. Ecol. Manual. **3.1.1**:1-17.

Stevenson D.M. and **Weimer P.J.** 2007. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantitative real-time PCR. Appl. Microbiol. Biotechnol. **75:** 165-174.

Stewart C.S., Flynt H.J. and **Bryant M.P.** 1997. The rumen bacteria. The Rumen Microbial Ecosystem. Hobson P. N. and Stewart C. S. ed. Blackie Academic and Professional, New York, NY. 10-72.

Stock R.A. and **Britton R.** 1993. Acidosis in feedlot cattle. *In*: Scientific Update on Rumensin/Tylan for the Professional Feedlot Consultant. Elanco Animal Health, Indianapolis, IN. A1-A13.

Stone W.C. 1999. The effect of sub-clinical acidosis on milk components. In: Cornell Nutrition Conference for Feed Manufacturers. Cornell Univ., Ithaca, NY, 40-46.

Tanner R.S. and **Wolfe R.S.** 1988. Nutritional requirements of *Methanomicrobium mobile.* Appl. Environ. Microbiol. **54:** 625-628.

Therion J.J., Kistner A. and **Kornelius J.H.** 1982. Effect of pH on growth rates of rumen amylolytic and lactilytic bacteria. Appl. Environ. Microbiol. **44 (2):** 428-434.

Tiffany M.E., Fellner V. and **Spears J.W.** 2006. Influence of cobalt concentrations on vitamin B_{12} production and fermentation of mixed microorganisms grown in continuous culture flow-through fermentors. J. Anim. Sci. **84:** 635-640.

Tsukahara T., Iwasaki Y., Nakayama K. and **Ushida K**. 2003. Stimulation of butyrate production in the large intestine of weaning piglets by dietary fructooligosaccharides and its influence on the histological variables of the large intestinal mucosa. J. Nutr. Sci. Vitaminol. **49:** 414-421.

Tsukahara T., Hashizume K., Koyama H. and **Ushida K**. 2006. Stimulation of butyrate production through the metabolic interaction among lactic acid bacteria, *Lactobacillus acidophilus*, and lactic acid-utilizing bacteria, *Megasphaera elsdenii*, in porcine cecal digesta. Anim. Sci. J. **77:** 454-461.

Tucker W.B., Aslam M., Lema M., Shin I.S., Le Ruyet P., Hogue J.F., Buchanan D.S., Miller T.P. and **Adams G.D.** 1992. Sodium bicarbonate or multielement buffer via diet or rumen: Effects on performance and acid-base status of lactating cows. J. Dairy Sci. **75:** 2409-2420.

Vandamme P., Pot B., Gillis M., De Vos P., Kersters K.O. and **Swings J.** 1996. Polyphasic taxonomy,a consensus approach to bacterial systematics. Microbiol. Rev. **60:** 407-438.

Van Nevel C.J. and **Demeyer D.I.** 1977. Effect of monensin on rumen metabolism in vitro. Appl. Environ. Microbiol. **34:** 251-257.

Veldkamp H. 1970. Enrichment cultures of prokaryotic organisms. In: Methods in Microbiology, Vol. 3A, Norris, J.R. and Ribbons, D.W. (Eds), Academic Press, London, 305-361.

Vervaeke I.J. and **Van Assche P.F.** 1975. Occurrence of *Megasphaera elsdenii* in faecal samples of young pigs. Zentralbl. Bakteriol. Hyg. 1 Abt. Orig. A. **231**: 145-152.

Warner A.C. 1962. Enumeration of microorganisms. J. Gen. Microbiol. **28:** 119-128.

Waldrip H.M. and **Martin S.A.** 1993. Effects of an *Aspergillus oryzae* fermentation extract and other factors on lactate utilization by the ruminal bacterium *Megasphaera elsdenii*. J. Anim. Sci. **71:** 2770-2776.

Weigand E., Young J.W. and **McGilliard D**. 1975. Volatile fatty acid metabolism by rumen mucosa from cattle fed hay or grain. J. Dairy Sci. **58:** 1294-1300.

Wells S.J., Trent A.M., Marsh W.E., McGovern P.G. and **Robinson R.A**. 1993. Individual cow risk factors for clinical lameness in lactating dairy cows. Prev. Vet. Med. **17:** 95-109.

Werner H. 1973. *Megasphaera elsdenii* - a normal inhabitant of the human intestine? Zentralbl. Bakteriol. Hyg. 1 Abt. Orig. **A. 223:** 343-347.

Wiederhold A.H. 1994. Isolation, selection and cultivation of lactic acid-utilising rumen bacteria for the treatment of chronic and acute acidosis. MSc. thesis. University of Natal, Pietermaritzburg.

Willems A. and **Collins M.D.** 1995. Phylogenetic placement of *Dialister pneumosintes* (formerly *Bacteroides pneumosintes*) within the *Sporomusa* sub-branch of the Clostridium subphylum of the Gram-positive bacteria Int. J. Syst. Bacteriol. **45:** 403-405.

Wiryawan K.G. and **Brooker J.D.** 1995. Probiotic control of lactate accumulation in acutely grain-fed sheep. Aust. J. Agric Res. **46:** 1555-1568.

Woese C.R. 1987. Bacterial evolution. Microbiol. Rev. **51:** 221-271.

Yoon I.K. and **Stern M.D.** 1995. Influence of direct-fed microbials onruminal fermentation and performance of ruminants: A review. Australas. J. Anim. Sci. **8:** 533-555.

Yoshii T., Asanuma N. and **Hino T.** 2003. Number of nitrate- and nitrite-reducing *Selenomonas ruminantium* in the rumen, and possible factors affecting its growth. Anim. Sci. J. **74:** 483-491.

Young G.P. and **Gibson P.R.** 1995. Butyrate and the human cancer cell. In: Cummings JH, Sakata T (eds), Physiological and clinical aspects of short-chain fatty acids, 19-33. Cambridge University Press, Cambridge.