The effects of specific *Saccharomyces cerevisiae* strains and monensin supplementation on rumen fermentation *in vitro*

by

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Declaration

I, Vaughn Barry Holder, declare that this dissertation for the degree M.Sc. (Agric) at the University of Pretoria has not been submitted by me for a degree at any other university.

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Summary

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In recent times there has been much concern among animal product consumers about the safety and use of antimicrobial substances in the production of food for human consumption. This has been driven by the ban of the use of antibiotics at subtherapeutic levels for food animal production in Europe. For this reason, producers are always looking at 'natural' alternatives to antibiotics to improve production from their animals. One such alternative is the use of yeast cultures of *Saccharomyces cerevisiae* in ruminant diets to manipulate rumen fermentation. Yeast culture fed to ruminants has increased production from beef and dairy cattle and sheep as well as stabilizing rumen fermentation under conditions such as rumen acidosis. Yeast culture has been shown to increase the microbial protein supply from the rumen by stimulating growth of bacteria in the rumen. Yeast culture may be used to alleviate the negative effects of rumen antimicrobials such as monensin on rumen microbial populations and fibre digestion. Four separate sets of experiments were undertaken. In the first set of experiments, the effects of 10 specific yeast cultures on the growth of 3 selected rumen bacteria was evaluated. The rumen bacteria evaluated were *Ruminococcus albus*, *Selenomonas ruminantium* and

Ruminobacter amylophilus. It was found that only two of the ten strains of yeast tested were able to consistently decrease the lag time of the selected rumen bacteria. In the second set of experiments, the effects of yeast culture addition on a rumen fluid based batch culture fermentation was analysed by measuring the gas pressure produced by the fermentation. The results obtained were too variable to draw any conclusions from the data. In the third set of experiments, the effects of yeast, monensin and their combination were evaluated in rumen simulating continuous cultures. It was found that monensin increased the efficiency of the fermentation but decreased the total anaerobic bacteria. Yeast culture increased the total anaerobic bacteria. UNI yeast alleviated the reduction in anaerobic bacteria when combined with monensin. The last set of experiments were an attempt to develop an assay to measure the potential of certain yeast strains to stimulate rumen fermentation. The potential assays were based on the ability of yeasts to stimulate a growing culture of *Ruminococcus albus*. None of the assays attempted showed obvious potential as a future assay. From the study it seems that yeasts stimulate the growth of certain species of ruminal bacteria but not all yeast strains are able to do so. Yeast supplementation may be fed in combination with monensin in order to reduce the impact of monensin on the microbial populations of the rumen.

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Chapter 1

Introduction and motivation

There is a large body of literature that indicates that yeast culture supplementation, specifically with strains of *Saccharomyces cerevisiae*, may have an impact on the productive performance of ruminant animals by manipulation of the rumen fermentation. *Saccharomyces cerevisiae* yeast strains have been tested in the majority of experiments although there are examples of the use of other strains of *Saccharomyces* spp. (*Saccharomyces boularrdii*) (Oeztuerk *et al.*, 2005) as well as other yeast species (*Trichosporon sericeum*) (Mwenya *et al.*, 2005) as feed additives. Yeast cultures in this review, unless otherwise specified, refer to yeast cells (either live or not specified) together with remnants of the media on which it was grown.

There is emerging interest in using supplements like yeast cultures to alter rumen fermentation. Yeast culture supplements are natural products that have been used as feed additives for more than 100 years. Yeasts have been used in human nutrition for years and are perceived as natural and safe products. They are also rich in vitamins, enzymes and nutrients (Dawson *et al.*, 1995) and may be economically produced from inexpensive raw materials. Yeast supplementation may serve as a substitute for antibiotics for manipulating the microbial populations of the rumen. The use of antibiotics in animal production is a growing concern (Newbold *et al.*, 1995), and the use of sub-therapeutic levels of antibiotics in animal production has been banned in the EU.

There are a wide range of potential effects of yeast culture supplements on ruminant animal production, including increased rate of body weight gain (Dawson *et al.*, 1990), increased milk production (Dawson *et al.*, 1990, Williams *et al.*, 1991), increased feed intake (Dawson *et al.*, 1990, Erasmus *et al.*, 1992) and decreased occurrence of rumen instability related to depression in rumen pH. There is, however, much variability in the recorded responses to yeast supplementation. Some researchers have reported little or no

effect (Smith *et al.*, 1993, Soder *et al.*, 1999) or even negative effects on the previously mentioned production parameters. More research is needed concerning the effects of yeast supplementation on rumen fermentation in order to clarify the mode of action by which animal production is improved. This should help to clarify the conditions under which yeast culture supplementation might be beneficial. It has also been suggested that yeast and the ionophore antibiotic monensin may be used complimentarily to benefit animal production as these additives differ in their mode of action in the rumen. The combination of yeast and monensin has not yet been extensively tested in continuous culture.

The objectives of this study were to:

- Examine the effects of supplementation with specific strains of *Saccharomyces cerevisiae* on the growth of representative pure culture rumen bacteria
- Examine the effects of yeast culture supplementation on rumen fluid based mixed culture fermentations
- Examine the potential for the combined use of yeast culture supplements and monensin for manipulation of rumen fermentation.
- Develop a rapid quality control assay for testing the ability of yeast culture to stimulate rumen bacteria

Chapter 2

Literature Review - The effect of yeast culture and monensin supplementation on rumen fermentation and animal production

1. The effects of yeast culture supplementation on *in vitro* and *in vivo* rumen fermentations

1.1 The effects of yeast culture supplementation on microbial fermentations *in vitro*

1.1.1 The effects of yeast culture supplementation on *in vitro* pure culture bacterial fermentations

In experiments to study the effects of yeast culture supplementation on rumen bacteria, the growth of *Selenomonas ruminantium* strain H18 and *Megasphaera elsdenii* strain B159 (major lactate utilizing species) were stimulated by increment levels of yeast culture supplementation (Callaway and Martin, 1997). In a similar fashion, the growth of major cellulose digesters *Fibrobacter succinogenes* strain S85 (Callaway and Martin, 1997) and *Ruminococcus albus* strain B199 (Callaway and Martin, 1997) were stimulated by the supplementation of yeast culture. Researchers have reported increases in the rate of cellulose digestion by the major cellulose digesting species: *Fibrobacter succinogenes* and *Ruminococcus flavifaciens* in response to yeast culture supplementation (Callaway and Martin, 1997, Sullivan and Martin, 1999). There are reports that yeast culture supplementation increased the total volatile fatty acids produced by *Selenomonas ruminantium* strains HD4 and H18 (Callaway and Martin, 1997). In addition, yeast

culture supplementation has led to alteration in the proportion of fatty acids produced, with increased relative proportion of acetate produced by *S. ruminantium* strain HD4 and increased relative proportion of propionate produced by *S. ruminantium* strain H18 (Callaway and Martin, 1997).

1.1.2 The effects of yeast culture supplementation on rumen fluid based mixed culture batch fermentations

In an attempt to simplify experiments, or to avoid the time, expense and complexity of rumen simulating continuous culture experiments and *in vivo* trials, single batch fermentations of feed or feed ingredients in dilute rumen fluid can give relative values for digestibility and of relative proportions of fermentation end products (Stern *et al.*, 1997). Yeast supplementation of rumen fluid based mixed culture fermentations has led to:

- Altered concentrations and ratios of major fermentation products including:

Yeast culture supplementation has increased total VFA production from a fermentation of Bermuda grass (Sullivan and Martin, 1999). Yeast culture supplementation has led to increases in propionate production and a decrease in the acetate to propionate ratio from a fermentation of Lucerne hay (Sullivan and Martin, 1999, Lynch and Martin, 2002). Yeast culture supplementation has increased the molar proportion of acetate produced and decreased the butyrate production from a diet composed of hay plus concentrate (60:40) (Lila *et al.*, 2004). Lila *et al.* (2004) also reported an increase in total VFA production as well as a linear decrease in lactate accumulation from all of the analysed feedstuffs, and decreased methane production and hydrogen accumulation in hay plus concentrate (60:40) diets. Lynch and Martin (2002) reported an increase in final pH of batch cultures supplemented with yeast culture.

- Altered nutrient digestion:

Supplementation with yeast culture has led to increased *in vitro* dry matter digestibility of a hay pus concentrate (60:40) diet (Lila *et al.*, 2004). Lynch and Martin (2002) reported a decrease in *in vitro* dry matter disappearance.

1.1.3 The effects of yeast culture supplementation on rumen simulating continuous culture fermentations

Rumen simulating continuous culture systems were developed as an alternative to labour intensive, complicated and expensive feeding trials with sheep and cattle. Rumen simulators provide a cheaper, more rapid method for determining the effects of various diets and feed ingredients on rumen fermentation. The strict control of operating variables in laboratory conditions allows researchers to detect smaller effects of additives on the rumen fermentation than would be possible in practical *in vivo* trials (Garret and Yoon, 1997). However, there are some areas in which the true rumen and rumen simulators differ. Products of fermentation such as volatile fatty acids are absorbed in the true rumen and are only diluted out by overflow in rumen simulators. This leads to higher concentrations of end products in artificial rumens. A consequence of end product accumulation is that bacterial populations will be lower, rumen protozoa may be absent, and digestibility may be lower (Bergen, 1977). However, comparisons of *in vivo* data and data produced by rumen simulator trials suggest that rumen simulators may be used to compare feed ingredients and additives, as they are ranked in the same order as those determined by *in vivo* measures (Bergen, 1977, Garret and Yoon, 1997).

The most consistently reported effect of yeast culture supplementation is an increase in the number of total anaerobic and cellulolytic bacteria. Newbold *et al.* (1995) reported a more than 35% increase in total anaerobic and cellulolytic bacteria in yeast supplemented rumen simulating cultures. Dawson *et al.* (1990) reported 5-40 fold increases in the concentration of cellulolytic bacteria in rumen simulating cultures supplemented with yeast culture. There are reports of changes in the concentrations of fermentation products. Miller-Webster *et al.* (2002) reported an increase total VFA concentration in rumen simulating cultures supplemented with yeast culture. Miller-Webster *et al.* (2002) also reported increase in molar proportion of propionate and decrease in the proportion of acetate in yeast supplemented rumen simulating cultures. Dawson *et al.* (1990) reported no significant change in the relative concentrations of VFA in rumen simulating cultures supplemented with yeast culture. Newbold *et al.* (1995) reported no

changes in daily output of fermentation end products in response to yeast culture supplementation. Miller-Webster *et al.* (2002) reported an increase in the ammonia concentration in yeast supplemented rumen simulating cultures while Dawson *et al.* (1990) reported no significant change in the concentration of ammonia in yeast supplemented rumen simulators. Dawson *et al.* (1990) and Newbold *et al.* (1995) reported no significant change in the pH of yeast supplemented rumen simulators. There are reports of altered nutrient digestibility in yeast supplemented continuous cultures. Miller-Webster *et al.* (2002) reported an increase in dry matter digestibility in rumen simulating cultures supplemented with yeast. Newbold *et al.* (1995) reported no difference in dry matter digestibility in yeast supplemented rumen simulating cultures. Miller-Webster *et al.* (2002) reported an increase in the protein digestibility in yeast supplemented rumen simulating cultures.

1.2 The effects of yeast culture supplementation on rumen fermentation and animal production *in vivo*

Dairy cattle production is generally expressed in terms of daily milk production, energy corrected milk production or feed efficiency. Production of meat producing animals is measured by measuring growth parameters such as average daily gain and feed conversion ratio. Yeast culture supplementation has resulted in increases in production of both beef and dairy cattle (Williams *et al.*, 1991, Wohlt *et al.*, 1991, Kung *et al.*, 1997, Wohlt *et al.*, 1998). However, there are reports of negligible effect of yeast culture supplementation on both milk production (Arambel *et al.*, 1990, Higginbotham *et al.*, 1994, Kung *et al.*, 1997, Schingoethe *et al.*, 2004) and growth parameters (Mir and Mir, 1994). Dawson (2000), in a review of 22 trials with more than 9039 dairy cattle and 22 trials with beef cattle, reported an average increase of 7.3% (ranging between 2% and 30%) in milk production from dairy cattle and 8.7% (ranging between 0% and 20%) mean increase in average daily gain of beef cattle. In addition to milk production, there are reports of altered milk composition. These include increases in milk fat and milk protein percentages (Besong *et al.*, 1996). Others have reported that yeast

supplementation had no effect on milk composition (Arambel *et al*., 1990, Higginbotham *et al.*, 1994, Kung *et al.*, 1997, Schingoethe *et al*., 2004).

Yeast culture supplementation may alter the digestibility of feed in the rumen. Increases in the rate or extent of digestibility of feed, especially roughage (Wohlt *et al.*, 1991, Newbold *et al.*, 1995, Wohlt *et al.*, 1998) or protein (Wohlt *et al.*, 1991, Wohlt *et al.*, 1998) have been noted. However, there are reports of negligible effect of yeast culture supplementation on nutrient digestibility (Putnam *et al*., 1997, Doreau and Jouany, 1998).

Yeast culture supplementation in ruminant diets has led to increased dry matter intake (DMI) by animals (Williams *et al.*, 1991, Wohlt *et al.*, 1991, Erasmus *et al.*, 1992, Wohlt *et al.*, 1998). Other researchers reported either no effects on DMI (Erdman *et al*., 1989, Kung *et al.*, 1997, Schingoethe *et al*., 2004) or a depression in DMI at high levels of supplementation (Besong *et al.*, 1996).

There have been reports of yeast supplementation increasing the efficiency of milk production per unit feed under conditions of heat stress (Schingoethe *et al*., 2004). There is also mention in the literature of trials where supplementation with yeast cultures has led to decreased rectal temperatures of cattle in hot weather (Huber *et al*., 1994).

There are reports that yeast culture supplementation has led to an alteration in the proportions of VFA's produced by the rumen fermentation with most researchers reporting a reduction in the acetate to propionate ratio (Williams *et al.*, 1991, Besong *et al.*, 1996). Yeast culture supplementation has also led to decreased concentrations of ammonia in the rumen (Erasmus *et al.*, 1992).

There is evidence in the literature that yeast culture supplementation may lead to increased rumen pH (Williams *et al.*, 1991) and reduced lactic acid concentrations (Williams *et al.*, 1991, Erasmus *et al.*, 1992) in the rumen. However, others have reported no effect of yeast culture supplementation on rumen pH (Erasmus *et al.*, 1992, Besong *et al.*, 1996, Doreau and Jouany, 1998).

There are fairly consistent reports that yeast culture supplementation increases the total number of anaerobic and cellulolytic bacteria in the rumen of cattle and sheep (Dawson *et*

al., 1990, Newbold *et al.*, 1995, Kumar *et al.*, 1997). Increase in microbial protein production by the rumen in response to yeast supplementation has also been recorded (Newbold *et al.*, 1995). This is consistent with the reports of increased bacterial numbers in the rumen. Microbial protein is a valuable source of high quality protein to ruminant animals. Others have reported no effect of yeast culture supplementation on microbial protein production (Doreau and Jouany, 1998).

Some workers indicate that yeast culture supplementation may play a role in development of the functional rumen in calves (Quigley *et al.*, 1992, Chaucheyras-Durand and Fonty, 2001, Lesmeister *et al*., 2004).

2. Possible modes of action by which yeast culture supplementation may improve animal production

2.1 Mechanisms by which yeast culture may improve animal production *via* effects on rumen fermentation

- **Oxygen scavenging**

The majority of bacterial species responsible for the fermentation of feed in the rumen are strictly anaerobic. Oxygen is regularly introduced in to the rumen with ingested feed and water. This oxygen increases the redox potential of the rumen which inhibits strict anaerobes (Williams and Newbold, 1990). Some researchers have suggested that the stimulatory effect of yeast culture on rumen fermentation may be due to the fact that yeasts rapidly metabolise oxygen introduced into the rumen. This will lower the redox potential of the rumen and create a more favourable growth medium for the anaerobic bacterial species (Wallace, 1994, Miller-Webster *et al.*, 2002). This would lead to stimulation of the strict anaerobic bacterial species responsible for fermentation of feed in the rumen.

- **Modulation of rumen pH**

Modern ruminant diets usually contain rapidly degradable carbohydrates in the form of starch from grains such as maize. These feed ingredients are fermented rapidly in the rumen and can lead to accumulation of fermentation intermediates such as lactate. Accumulation of lactate in the rumen can cause the pH of the rumen to decrease as lactic acid concentration is a major determinant of rumen pH. Depression in rumen pH inhibits the growth of many beneficial bacterial species in the rumen and is also strongly inhibitory to those organisms responsible for ruminal fibre digestion.

There are reports of decreases in total and peak lactate production in response to yeast culture supplementation (Williams *et al.*, 1991, Erasmus *et al.*, 1992, Mir and Mir, 1994, Lila *et al.*, 2004). Yeast culture supplementation has also shown to stimulate the growth of major lactate utilizing bacteria *in vitro* (Callaway and Martin, 1997). It follows that there are numerous reports of yeast supplementation increasing the pH of the rumen (Williams *et al.*, 1991, Beauchemin *et al.*, 2003, Sauvant *et al.*, 2004). Decreased rumen lactic acid concentration, and therefore increased pH would lead to stimulation of the acid sensitive bacterial species responsible for fermentation of important feed ingredients in the rumen.

- **Increased production of VFA**

There are reports of increases in the total VFA produced by *in vitro* rumen fermentation of substrate supplemented with yeast culture (Miller-Webster *et al.*, 2002, Lila *et al.*, 2004). Volatile fatty acids (VFA) are the principle source of energy for ruminants and therefore increases in total VFA produced may improve animal production. This has been demonstrated in *in vitro* trials with pure culture bacteria as well as with rumen simulating continuous culture fermenters (Miller-Webster *et al.*, 2002, Lila *et al.*, 2004).

- **Altered proportions of VFA**

Researchers have found that supplementation with yeast culture has led to depression in the acetate to propionate ratio. This usually occurs when propionate is produced at the expense of acetate. This effect has been recorded both *in vitro* (Dawson *et al.*, 1990, Williams *et al.*, 1991, Miller-Webster *et al.*, 2002, Lila *et al.*, 2004) and *in vivo* (Williams *et al.*, 1991). Depression in the acetate to propionate ratio may stimulate animal production due to the fact that propionate has a higher enthalpy and thus provides more energy to the animal than acetate.

2.2 The effect of yeast culture on animal production *via* changes in diet digestibility

- **Improved Rumen digestion**

Workers have reported increases in dry matter and fibre digestibility in response to yeast supplementation (Lila *et al.*, 2004). Increases in digestion of feed will lead to more nutrients being available to the animal and thus better animal production. However, other researchers reported no change in diet digestibility (Putnam *et al.*, 1997).

- **Rate of digestion**

There have been reports that yeast supplementation in ruminants may increase the rate of fibre digestion (Williams *et al.*, 1991, Newbold *et al.*, 1995, Lila *et al.*, 2004). There is a general agreement in the literature that the effect of yeast on digestion, particularly fibre digestion, is on the rate rather than the extent of digestion. Increases in the rate of feed digestion may lead to a greater quantity of nutrients available for production. In addition, it will increase the rate of emptying of the rumen. This may lead, in turn, to an increase in DMI.

- **Dry Matter Intake (DMI)**

Although DMI is not an estimate of digestibility, it is included with this section as it impacts digestibility directly and because it is thought that an increase in the rate of digestion may in turn increase the emptying rate of the rumen and therefore increase animal DMI (Wallace, 1994). Many workers have reported an increase in DMI of ruminants fed yeast culture (Williams *et al.*, 1991, Erasmus *et al.*, 1992, Putnam *et al.*, 1997). Increased feed intake will lead to increased productive output of animals by providing more nutrients to the animal. Other researchers reported no effects of yeast culture supplementation on DMI (Erdman *et al.*, 1989, Kung *et al.*, 1997).

2.3 The effect of yeast culture supplementation on animal production *via* effect on the rumen microbial population

- **Increase in total anaerobic and cellulolytic bacteria**

Many workers have shown an increase in total culturable anaerobic and/or cellulolytic bacteria in response to yeast culture supplementation (Dawson *et al.*, 1990, Newbold *et al.*, 1995, Lila *et al.*, 2004, Sauvant *et al.*, 2004). This appears to be the most consistently reported effect of yeast culture supplementation on ruminants. Anaerobic and cellulolytic bacteria are predominantly responsible for fermentation in the rumen. It therefore follows that higher concentrations of bacteria could improve digestion or rate of digestion, thus making more nutrients available to the ruminant animal. In addition, higher numbers of bacteria in the rumen could lead to increased flow of microbial protein from the rumen to the small intestine (Erasmus *et al.*, 1992).

- **Increased Microbial protein production**

An increase in the concentration of bacteria in the rumen may lead to an increase in the amount of microbial protein available for digestion in the small intestine (Newbold *et al.*, 1995, Beauchemin *et al.*, 2003). There are also indications that yeast supplementation may alter the proportions of amino acids entering the small intestine (Erasmus *et al.*,

1992). Increased amino acid supply may be partially responsible for the positive effects of yeast culture supplementation on ruminant production.

- **Improved Microbial efficiency**

Microbial efficiency is reported as grams of microbial protein produced per kilogram of organic matter fermented. Some workers have reported an increase in the efficiency of production of microbial protein (Olsen *et al*., 1994). Increases in microbial efficiency would result in more protein being available for production per unit of feed intake. Others have found no effect on microbial efficiency (Miller-Webster *et al.*, 2002).

2.4 Global representation of the possible mode of action whereby yeast culture stimulates ruminant animal production

It appears that stimulation of rumen bacteria is central to the mode of action whereby yeast culture stimulates rumen fermentation. The exact mechanism by which the yeast culture stimulates rumen bacteria remains unclear (Dawson, 2000). It may be due to the removal of oxygen from the rumen environment or due to some unidentified growth factor provided by the metabolically active yeast cells (Dawson *et al.*, 1995). Selective stimulation of lactate utilizing bacteria (Callaway and Martin, 1997) may result in stabilization of rumen pH. The stabilized pH may further stimulate the growth of the pH sensitive bacterial populations of the rumen and provide a suitable environment for fibre digestion.

Bacteria are predominantly responsible for digestion in the rumen and it follows that higher bacterial numbers increase the animal's digestion rate and therefore it digestive capacity. Increased rate of digestion of feed results in additional nutrients being available to the animal as well as making more space in the rumen resulting in increased DMI. Higher bacterial numbers also result in additional microbial protein being available to the animal for digestion in the small intestine. The final result is that more nutrients are available for the ruminant for productive purposes.

Figure 2.1 is a schematic representation of an attempt to bring together the possible modes of action whereby yeast supplementation stimulates ruminant animal production (adapted from Wallace, R. J., 1994 and Dawson *et al.*, 1995).

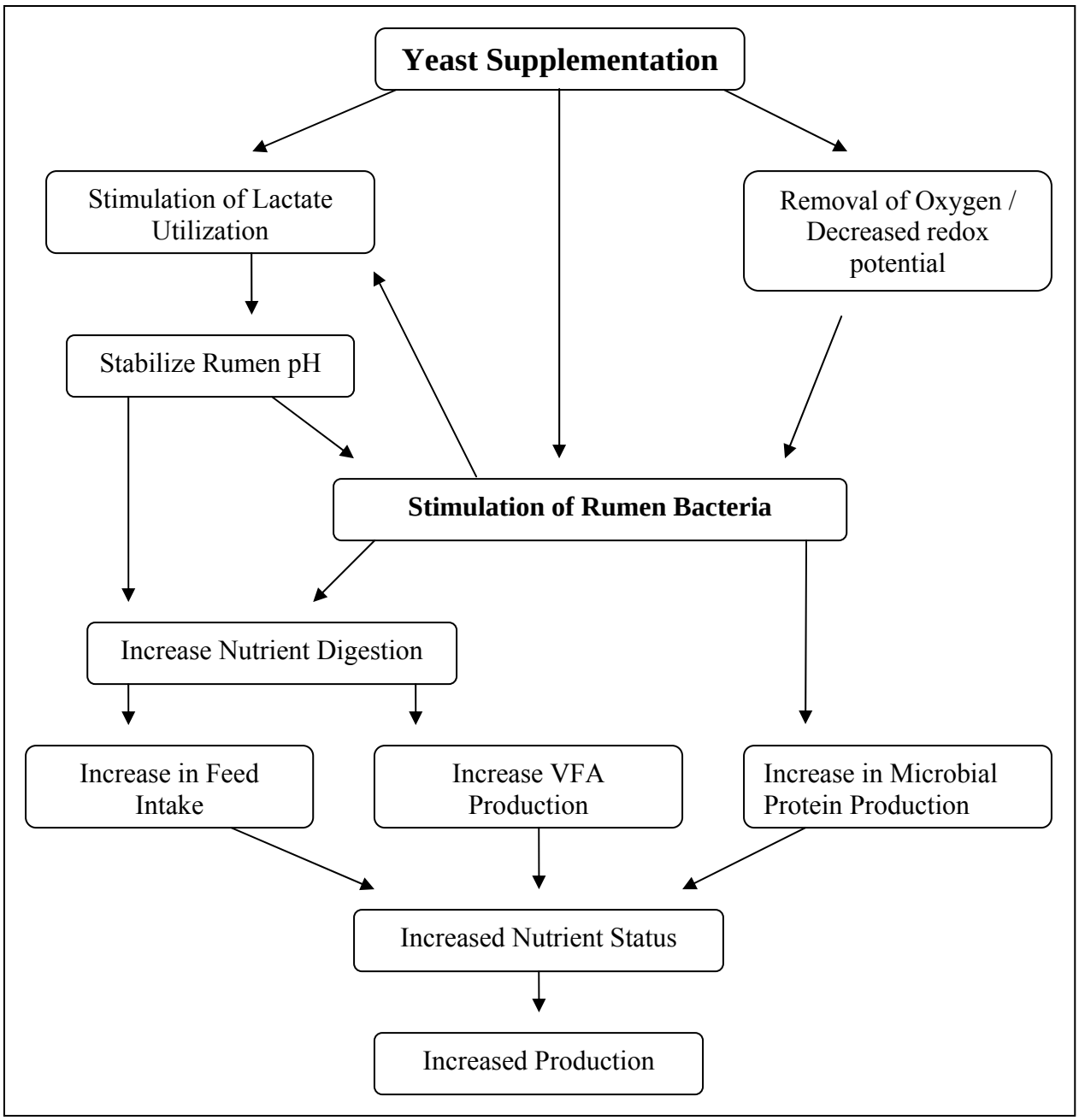

Figure 2.1 Schematic representation of the mode of action whereby yeast stimulates production in ruminants

3. Factors causing variability in response to yeast supplementation

The response of ruminant production to yeast supplementation is variable (Newbold *et al.*, 1995, Miller-Webster *et al.*, 2002). In some cases, many of the commonly reported effects are observed and in others, little or no effect is observed. Dawson (2000) summarized 22 trials with dairy cattle and 22 with beef cattle. He found that the range of response in milk production expressed as a percentage increase was between +2% to +30% with a mean increase across experiments of +7.3%. Similarly, in beef cattle, he found that response of average daily gain to yeast supplementation ranged from 0% to +20% with a mean increase across experiments of +8.7%. There may be a number of reasons for this variability and these will now be discussed.

3.1 The effect of diet composition on animal response to yeast supplementation

Researchers suggest that the response to yeast supplementation may depend on the composition of the diet fed (Williams *et al.*, 1991, Dawson *et al.*, 1995, Lila *et al.*, 2004). There is however, no clear understanding of what diet composition would result in the best response to yeast supplementation (Dawson *et al.*, 1995). The effects of yeast supplementation on DM intake and digestibility are more common in high concentrate diets and may be related to the maintenance of fibre digestion by mediation of rumen pH despite the presence of high quantities of concentrates (Carro *et al*., 1992).

3.2 The effect of yeast strain on animal response to yeast supplementation

There are more than 1000 strains of *Saccharomyces cerevisiae* that have been identified and characterized. Of these, only a handful have shown to have beneficial effects on micro-organisms *in vitro* and on the productive performance of animals (Dawson *et al.*, 1995). There is much data that indicates that not all yeast strains have the ability to

stimulate rumen fermentation (Newbold *et al.*, 1995). In fact, few of the yeast strains tested in the literature have any effect on rumen fermentation. There is also evidence that specific yeast strains may be more effective under certain dietary and environmental circumstances (Dawson *et al.,* 1995). There is clear evidence for the difference in stimulatory activity between yeast strains. This may account for some of the variation in the responses observed in the literature.

3.3 The effect of the amount of yeast fed on animal response to yeast supplementation

There is little uniformity in the amount of yeast supplemented to ruminants. The usual measure of content of viable cells in a product is its content of colony forming units (CFU). But there is variation here too with some researchers reporting the CFU content of the supplement and not quantity of product fed to the animals and others not reporting CFU content at all (Quigley *et al.*, 1992, Putnam *et al.*, 1997, Miller-Webster *et al.*, 2002, Giger-Reverdin *et al.*, 2004, Lesmeister *et al.*, 2004). It seems logical that the amount of yeast should be related to the amount of feed an animal eats to get an estimate of the relative concentration of yeast in the digesta. However, some researchers do not report feed intake even though they may indicate how much yeast was fed (Higginbotham *et al.*, 1994, Oeztuerk *et al.*, 1998, Doreau and Jouany, 1998). For this reason the quantity of yeast fed will be referred to by CFU per kilogram feed in this review in an attempt to find a standard of comparison between experiments indicating the relative concentration of viable yeast in the digesta. Dawson *et al.* (1990) reported in both *in vitro* and *in vivo* trials the yeast supplementation range of 1.4 – 4.2 x 10^9 CFU/kg feed and reported increased total and cellulolytic bacteria in both continuous culture and in the rumens of steers. In trials with feedlot cattle, Beauchemin *et al.* (2003) used a yeast supplement of approximately 8 x 10^8 CFU/kg feed and reported little effect on rumen fermentation in steers. Erasmus *et al.* (1992) used a yeast supplement on dairy cows with approximately 2 x 10^9 CFU/kg feed and found that yeast culture increased DMI, decreased peak lactic acid concentration in the rumen and increased the flow of microbial protein to the small intestine. Williams *et al.* (1991) used a yeast supplement on dairy cows with

approximately 3 x 10^9 CFU/kg feed and found that yeast supplementation increased DMI, increased peak pH, increased the ratio of acetate to propionate and increased the rate of fibre digestion. In a series of trials on beef cattle, Mir and Mir (1994) used a yeast supplement of approximately 5 x 10^9 CFU/kg feed and reported negligible effects on rumen fermentation or animal production. In an *in vitro* trial and in an *in vivo* trial using sheep, Newbold *et al.* (1995) directly compared several yeast strains ranging in viable cell count between 8.6 x 10^4 and 8.7 x 10^8 CFU/kg feed. Wohlt *et al.* (1998) observed a numerical increase in response to increment levels of yeast supplementation providing evidence that the level of supplementation will affect the response. It appears that there is a wide range in concentration of viable yeast fed to ruminants and this may indeed have lead to variability in response to supplementation. However, there have been cases where fairly high concentrations of yeast have been fed but with little effect, suggesting that it is not the only factor affecting animal response to yeast supplementation (Kung *et al.*, 1997).

3.4 The effect of yeast viability in supplements on the animal response to yeast supplementation

There is evidence in the literature that the presence of metabolically active yeast cells is a requirement for yeast to be an effective form of supplementation. This is demonstrated by the fact that live yeast supplementation increased total anaerobic and cellulolytic bacteria and increased the proportion of propionate in rumen simulating continuous cultures whereas autoclaved yeast cells had no effect (Dawson *et al.*, 1990, El Hassan *et al.*, 1993). However, yeast cells sterilized by irradiation have shown to retain their ability to stimulate rumen bacteria (El Hassan *et al.*, 1993). Yeast cells sterilized by irradiation may still be metabolically active although they cannot reproduce. This suggests that live or metabolically active yeast cells are required for yeast supplementation to be effective or that there is a stimulatory substance in yeast that is heat labile and is destroyed in the autoclaving process.

3.5 The effect of the method of preparation of yeast culture on the animals' response to yeast supplementation

It has been found that the method of preparation of the yeast product may affect the response to yeast supplementation. Live or metabolically active yeast has shown to have a greater effect in stimulating rumen fermentation than yeasts that are killed in the production process (Dawson *et al.*, 1990). There is also considerable variation in the amount of live yeast cells that are present in the yeast culture product (Erasmus *et al.*, 1992).

3.6 Individual circumstances that may affect animals' response to yeast supplementation

Response to yeast supplementation may vary according to the conditions of the rumen and environment. For example, if the average rumen pH of a dairy herd is low, then yeast supplementation may have an effect in increasing the rumen pH and stabilizing the rumen fermentation. However, if the rumen pH of the herd is already at an optimum, it is unlikely that yeast supplementation will have any effect on rumen pH (Williams *et al.*, 1991, Newbold *et al.*, 1995). Dawson *et al.* (1995) suggested that activities of the microbial population are increased by yeast supplementation and may improve nutrient status, particularly that of protein. However, he points out that supplementation will have negligible effect in this regard if protein or the nutrient in question is not limiting in the first place. There may be variation in the response due to other parameters such as stage of lactation (Wohlt *et al.*, 1991, Kung *et al.* 1997) or the way the yeast is presented (e.g. In an adlib ration or once daily top dressed on the feed). Therefore the response to supplementation with yeast culture may be more likely when animals are fed a poor quality diet or when rumen fermentation is disturbed (Dawson *et al.*, 1995).

4. Effects of monensin on animal production

4.1 Mode of action of monensin

The primary mode of action of monensin on the rumen is bacteriostatic inhibition of certain bacterial populations. A secondary mode of action is the control of the protozoa that cause coccidiosis. Inhibition is achieved by disruption of ion transport in the microbial cells leading to excessive uptake of sodium ions and loss of potassium ions from the cells (Russell and Strobel, 1989, Ipharraguerre and Clark, 2003). Gram negative bacteria are generally unaffected by monensin as they have a complex outer membrane that is impermeable to larger molecules. Gram positive bacteria generally lack the complex outer membrane structure and so are susceptible to the inhibitory effects of monensin (Bagg, 1997, Ipharraguerre and Clark, 2003).

Monensin inhibits organisms that produce hydrogen, a precursor for methane synthesis. Methanogenic bacteria are not affected by monensin directly (Russell and Strobel, 1989). Monensin inhibits lactate producing organisms (Russell and Strobel, 1989).

It is the general belief that monensin exerts many of its effects by modifying the composition of microbial populations in the rumen (Dawson, 2005). However, monensin has also been shown to control microbial activity in the small intestine (Parker *et al*., 1987). Intestinal tissue is highly metabolically active and has a high cell turnover rate. Supplementation with monensin reduces the gut turnover by controlling microbial activity and thus decreases the requirement of gut tissues for essential nutrients and makes more nutrients available for productive purposes. This may be another mode of action whereby monensin increases the feed efficiency of ruminant animals.

4.2 Effects of monensin on energy metabolism

Monensin has led to decreased methane production in *in vitro* fermentations with rumen fluid (Russell and Strobel, 1988, Sullivan and Martin, 1999) as well as *in vivo* with dairy cows (Odongo *et al*., 2007). As much as 12% of the animals feed energy may be lost to methane production (Russell and Strobel, 1989). Monensin supplementation has led to an increased in the ratio of propionate to acetate (Daenicke *et al.*, 1982, Russell and Strobel, 1988, Sullivan and Martin, 1999, McGuffey *et al.* 2001, Mutsvangwa *et al*., 2002, Broderick, 2004, Shinzato *et al.*, 2006). Propionate has a higher enthalpy than acetate and can therefore provide more energy to the animal for productive purposes (Russell and Strobel, 1989). Increase in propionate enhances glucose status (Ipharraguerre and Clark, 2003, Broderick, 2004) which is essential for production of lactate, the precursor of lactose which is essential for milk synthesis (McGuffey *et al.* 2001).

4.3 Effects of monensin on protein metabolism

Supplementation with monensin has led to decreased degradation of peptides and decreased de-amination of amino acids in *in vitro* (McGuffey *et al.* 2001) and *in vivo* (Russell and Strobel, 1988) studies. Proteolysis and de-amination of proteins and amino acids in the rumen often leads to excessive production of ammonia. If this ammonia exceeds the capacity of rumen microbes to utilize it for protein synthesis, ammonia will accumulate in the rumen. This ammonia crosses the rumen wall into the blood and will be converted to urea by the liver. Urea synthesis has an energy cost to the animal. Some of this urea is recycled to the rumen but much is lost in the urine (Russell and Strobel, 1989). Monensin reduces proteolytic activity in the rumen and may improve nitrogen status in animals that are fed readily degradable protein sources by increasing flow of protein and amino acids of dietary origin to the small intestine (McGuffey *et al.* 2001).

4.4 Effects of monensin on digestive disorders

Some forms of ruminant production such as intensive beef production require that animals are fed a diet high in concentrates with a large proportion of readily fermentable carbohydrates. Rapid fermentation of these carbohydrates can result in accumulation of lactate in the rumen which often results in a decrease pH and digestive disturbance (Nocek, 1997). Monensin leads to decreased production of lactate, and may alleviate severe depressions in pH of ruminants receiving high concentrate diets (Russell and Strobel, 1989, Sullivan and Martin, 1999, McGuffey *et al.* 2001).

High producing ruminants, such as dairy cows, often suffer from digestive disorders related to a lack of glucogenic precursors (Ipharraguerre and Clark, 2003). This happens due to the rapid change in energy required by the animal between late gestation and early lactation. Monensin enhances propionate production which is a major substrate for glucose production in the liver (Ipharraguerre and Clark, 2003). Supplementation with monensin, therefore, has led to a decrease in the occurrence of nutrition related diseases such as ketosis and displaced abomasum (Duffield *et al*., 2002).

4.5 Effects of monensin on ruminant production

Increased production of both beef (Daenicke *et al.*, 1982) and dairy cattle (Beckett *et al.*, 1998, Mutsvangwa *et al*., 2002) supplemented with monensin are consistently reported.

There are numerous reports of improved feed efficiency in ruminants supplemented with monensin. Feed efficiency is improved either by animals decreasing feed intake and maintaining production or increases in production without any increase in feed intake (Daenicke *et al.*, 1982). There are also reports that monensin supplemented cows have a higher intake under conditions of subacute ruminal acidosis (Mutsvangwa *et al*., 2002). The increased intake is probably related to decreased lactate production in monensin supplemented animals.

4.6 Negative effects of monensin supplementation

In addition to the positive effects of monensin supplementation to ruminants described above, there are some negative aspects of feeding monensin. There are fairly consistent reports that monensin depresses microbial protein production, due to its antimicrobial effect on the rumen (McGuffey *et al.* 2001). In some cases monensin has led to a depression in milk protein percentage (Broderick, 2004, Odongo *et al*., 2007). Some researchers have reported depression in fibre digestion *in vitro* (Russell and Strobel, 1989). However, others report no effect on fibre digestion (McGuffey *et al.* 2001). A common effect of monensin is depressed feed intake and this may be related to a decrease in the rate of digestion of fibrous materials in the rumen. Enhanced propionate to acetate ratio has lead to the depression in milk fat content of milk due to the lack of lipogenic precursors (Mutsvangwa *et al*., 2002, Broderick, 2004, Odongo *et al*., 2007). Duffield *et al.* (2003) reported in a trial with multiple dairy herds that milk fat depression only occurs when monensin was combined with a low fibre diet suggesting and interaction between monensin and dietary fibre.

5. Effects of combining yeast and monensin on animal production

There appears to be no interaction between yeast and monensin in batch culture fermentations with yeast and monensin (Sullivan and Martin, 1999). Yeast and monensin have differing modes of action and this indicates that their combination may have positive complimentary effects to benefit animal production. Yeast culture supplementation tends to stimulate the bacteria that are not inhibited by monensin (Dawson, 2005). Therefore monensin inhibits certain bacterial populations causing the rumen fermentation to produce propionate at the expense of acetate with the result that the fermentation process becomes more energy efficient. Yeast culture on the other hand stimulates certain bacterial populations improving nutrient digestion and microbial protein production (Dawson, 2005). There are reports that yeast culture progressively increased crude protein content of milk as the level of milk production increased, whereas monensin progressively decreased milk crude protein (Erasmus *et al.*, 2005). Monensin

is widely reported to have a depressive effect on feed intake while yeast culture tends to have the opposite effect due to its impact on the rate of digestion, especially of fibre (Dawson, 2005, Erasmus *et al.*, 2005). Monensin improves both the energy and protein status of the animals while addition of yeast culture may lessen the impact of monensin on the rumen bacterial population and animal intake. Monensin reduces incidence of rumen acidosis by inhibiting organisms that produce lactate (Dawson, 2005). Yeast culture may also aid in alleviating rumen acidosis as yeast supplementation has been shown to stimulate organisms that utilize lactate in the rumen (Callaway and Martin, 1997). Organisms that utilize lactate generally convert the lactate to propionate (Dawson, 2005). This suggests the potential for further complimentary effects of yeast and monensin on propionate production. An investigation into the complimentary effects between yeast culture and monensin was one of the objectives for this study, as stated in chapter one. This aspect will be discussed in chapter 5.

Chapter 3

The effect of ten strains of *Saccharomyces cerevisiae* on the lag time of three pure culture anaerobic rumen bacteria

1. Introduction

In 1980, Brown was one of the first researchers to develop and publish a method for measuring bacterial growth by means of standard spectrophotometry (Brown, 1980). This method involved using turbidimetric measurements of a growing bacterial culture and relating it to culture growth. It was a reliable and reproducible predictor of bacterial growth curve shape, onset of logarithmic growth (lag) and maximum growth rate. Based on Browns method, it was proposed that turbidimetric measurements could be used to evaluate the effects of certain additives on the growth of an anaerobic bacterial monoculture.

There are previous studies of the effects of yeast supplementation on pure culture rumen bacteria (Callaway and Martin, 1997, Sullivan and Martin, 1999). In these studies, the focus was mainly on substrate utilisation by the bacteria and how yeasts or yeast filtrate affects this substrate utilisation. Growth of bacteria was measured intermittently; however, no attempt has been made to capture the entire growth curve of the bacterial populations in order to determine what stage of bacterial growth is influenced by yeast culture supplementation. It is possible that if the growth of the bacterial population is followed closely, we may be able to identify the stage of growth that is altered by yeast supplementation. This may shed some light on the mode of action whereby yeast culture stimulates rumen fermentation.

The rumen bacteria studied in these experiments were: *Ruminococcus albus* strain 7, for its major role in cellulose digestion and production of ethanol, acetate, formate and lactate (Dehority, 2003); *Selenomonas ruminantium* strain GA192, for its role in the digestion of starch and other soluble carbohydrates and production of lactate, acetate and propionate (Dehority, 2003); *Ruminobacter amylophilus*, has a major role in starch fermentation and production of acetate, formate and succinate (Dehority, 2003). This bacterium was chosen due to previous work done with rumen simulating continuous cultures (Kozenski, unpublished). In his work, denaturing gradient gel electrophoresis (DGGE) was performed on cultures with and without yeast culture supplementation. Changes in banding patterns were noted with one set of bands being darker in the yeast treatments than in the control. The band was sent for DNA sequencing and subsequent analysis found that the band most likely represented *Ruminobacter amylophilus*. Lag time experiments were therefore performed with this bacterium to see if it would respond to yeast supplementation in pure culture.

The objectives of this series of experiments were to:

- Develop methodology for studying the effects of yeast culture on the lag time of pure culture anaerobic bacteria
- Evaluate the effects of ten specific yeast culture supplementations on the growth of pure rumen bacterial cultures *in vitro*.

2. Materials and Methods

2.1 Standard method for the evaluation of the effect of yeast on the lag time of pure culture bacteria

Experimental design: Trials were conducted consisting of a control and treatment groups. The numbers of treatments varied according to individual experiments and are described in Tables 3.3-3.5. Each treatment group and control group consisted of five replicates.

Bacterial growth media (Appendix A) was prepared in 4ml volumes in 16mm x 125mm Pyrex test tubes sealed with butyl rubber stoppers. Media was produced anaerobically by boiling to drive off excess oxygen followed by cooling under oxygen free carbon dioxide as well as the addition of the reducing agent cysteine. Media was sterilized by autoclaving at 120ºC for 15 minutes.

Five replicate tubes of bacterial growth media per treatment were inoculated with equivalent volumes of bacterial culture solution. The volume of bacterial culture solution added varied among individual experiments. The initial bacterial inoculum was estimated by measuring the absorbance at 600nm of control and treatment tubes and/or by enumeration of the parent culture by the Hungate roll tube technique (Hungate, 1969). Treatment tubes were inoculated with 0.2ml of yeast culture while control tubes received 0.2ml of fresh yeast growth media (TSB) to rule out any effects of yeast growth media on bacterial growth. All inoculations and additions to experimental tubes were done aseptically and anaerobically under oxygen free carbon dioxide. Control and treatment tubes were incubated in a water bath (37ºC). Experimental tubes were incubated at 37°C and absorbance at 600nm (Abs_{600nm}) was measured over time on a Spectronic 20 spectrophotometer.

Anaerobiosis was monitored by visual inspection of experimental tubes for colour changes related to the oxidation of the oxygen indicator Resazurin (Sigma, St Louis, MO). Figure 3.1 is a schematic representation of the basic experimental procedure as described above.

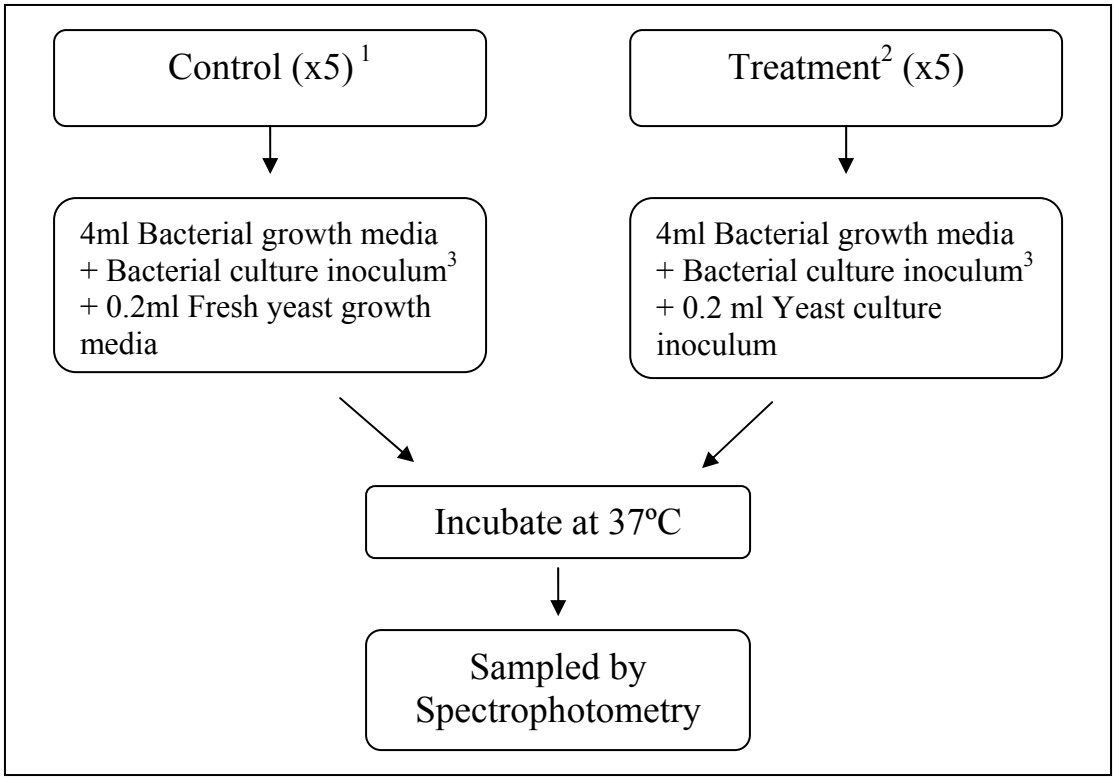

Figure 3.1 Schematic representation of inoculation procedure of experimental tubes in lag time experiments

[1] Represents the five replications per treatment in treatment and control vessels
[2] Represents one treatment group. Number of treatments varied according to individual experiments
[3] Volume of bacterial culture inoculum varied among individual experiments

Preparation of Bacterial Cultures:

Three species of rumen bacteria were used in this series of experiments: *Ruminococcus albus* strain 7; *Selenomonas ruminantium* strain GA192 and *Ruminobacter amylophilus* strain H18. Bacteria were resuscitated by anaerobically transferring the cells from stock culture slants into 4ml of media (media used varied between individual experiments) using a sterile platinum loop and incubated at 37°C for 24h. This liquid culture was used to inoculate another volume of media, which was incubated at 37°C for 24h. This second liquid culture was used to prepare the inocula used in the experimental tubes. This

method of preparation of experimental parent cultures will be referred to as the standard sub-culture technique (SSCT). Inoculum size of bacterial cultures was varied in an attempt to find the appropriate initial inoculum size for the determination of the lag phase.

Preparation of live yeast cultures:

A summary of the yeast strains evaluated is presented in Table 3.1. The yeast cultures used in the experiments were single colonies isolated from Dichloran-Rose Bengal-chloramphenicol agar (Difco, Sparks MD) plates and streaked onto Tryptic Soy agar (TSA)(Difco, Sparks MD) slants that were incubated at 30°C for approximately 48 h. Slants were stored at 4°C and used as the source of supplemental yeast for experimental cultures. Tryptic Soy Broth (TSB) (Difco, Sparks MD) was inoculated from TSA slants and incubated at 30°C for approximately 24 h before the beginning of each experiment. The absorbance of 24 h yeast cultures varied between 0.1 and 0.6. These cultures were diluted to an absorbance of Abs $_{600nm}$ = 0.1 and added at 0.2ml per treatment tube. Fresh TSB was added at the same volume to control tubes. One experimental tube from each treatment had a 1ml sample removed, prior to yeast supplementation, for enumeration of bacteria using the Hungate roll tube technique (Hungate, 1969). Only 0.15ml of fresh TSB or yeast culture solution was added to these sampled tubes to compensate for the difference in volume. TSB and yeast culture added to experimental tubes were first bubbled under oxygen free carbon dioxide for 5 min in order to avoid changes in optical density (OD) associated with oxidation and the concomitant colour change in the Resazurin.

Table 3.1 *Saccharomyces cerevisiae* yeast strains tested for their ability to stimulate rumen bacteria

Yeast Strain	Description
NCYC	Strain 1026 from the National collection of yeast cultures
DCL	Isolated from Yea-Sacc DCL batch # 04-FS050
LV	Isolated from Levucell SC20 batch # 02234
FE	Isolated from Yea-Sacc Fermin batch # 960A
LF	Isolated from La Florida batch # Prob 507
UNI	A yeast from the personal collection of Dr K. Dawson, Alltech Inc
NLV	Levucell SC20 obtained from Francois Ouellette on 21 April 2005
VH1,2,3&5	Yeasts isolated by V. Holder from rumen fluid

Lag time determination:

Measured absorbance values (culture density) were subjected to log transformation and plotted against time (Figure 3.2). A straight-line equation was obtained by linear regression of data points within the linear portion of the resulting curve (usually between Abs $_{600nm}$ = 0.1 and 0.4). Lag time was estimated by extrapolating the established regression line to the log of the absorbance at the beginning of the experiment.

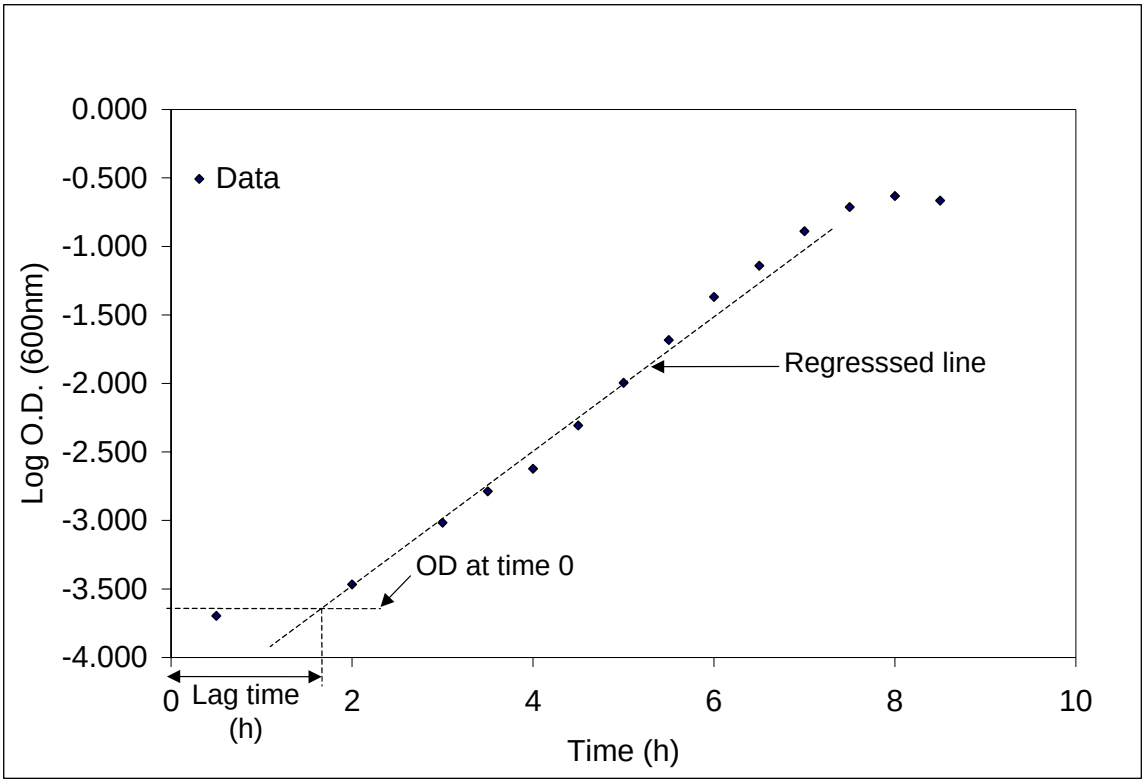

Figure 3.2 Lag time determination method applied to all pure culture experiments

Data (♦) were subject to log transformation and plotted against time. Regression was performed on the linear portion of the resulting curve. Lag was estimated by extrapolating the regression line to the log of mean absorbance at the start of the experiment

2.2 Modifications of the standard method used for the evaluation of the effect of yeast on the lag time of pure culture bacteria

Table 3.2 indicates the modifications that were made to growth media, carbohydrate source, initial inoculum size and sampling time in individual trials. Following the table is a brief description of other specific modifications made to particular trials. Experiment 1 (1.1-1.9) was conducted on the bacterium *Ruminococcus albus* strain 7. Experiment 2 (2.1-2.6) was conducted on the bacterium *Selenomonas ruminantium* and Experiment 3 (3.1-3.9) was conducted on the bacterium *Ruminobacter amylophilus.*

Trial 1.1-1.5

Trials were conducted according to the standard method (Section 2.2.1)

Trial 1.6

The purpose of this trial was to determine whether a culture prepared by the standard subculture technique (SSCT) and a continuously sub-cultured culture differ in their response to yeast supplementation.

- Growth of a culture prepared by SSCT (Std) is compared to a culture that has been sub-cultured daily for 4 weeks (Daily Subculture or D.S).

Trials 1.7-1.9

Trials were conducted according to the standard method (Section 2.2.1)

Trials 2.3- 2.6

A second control was included in these experiments to be sampled after the treatment groups. The purpose of this second control was as follows: It took approximately 15 min to complete sampling. This caused the final treatment to have about 10 min more incubation time in the water bath at the first time interval than the first treatment. Thereafter all incubation times remained constant. The second control treatment was therefore included to examine whether the time taken to sample the experimental tubes (15min) was affecting the calculated values for lag time.

Trial 3.1

The purpose of this experiment was to obtain representative growth curves for the bacterium *Ruminobacter amylophilus* strain H18. The bacteria were grown on either starch or maltose. Carbohydrate source and inoculum size were varied in order to find the appropriate combination for determining the lag time.

- Media used: Modified Medium A with 0.15% starch or maltose as the sole carbohydrate source.

- Parent cultures were prepared in the appropriate media for the experiment to be inoculated (e.g. Starch based parent media used to inoculate starch based experimental cultures).
- Treatments:
    - Low inoculum (Abs $_{600nm}$ = 0.012) in a starch based medium
    - High inoculum (Abs $_{600nm}$ = 0.029) in a starch based medium
    - Low inoculum (Abs $_{600nm}$ = 0.013) in a maltose based medium
    - High inoculum (Abs $_{600nm}$ = 0.021) in a maltose based medium

Trial 3.2-3.9

Trials were conducted according to the standard method (Section 2.2.1)

Table 3.2 Modifications to growth media, carbohydrate source, initial inoculum and sampling duration shown for individual trials

Trial number	Bacterial Growth Media	Carbohydrate Source	Initial Inoculum[1] (Abs $_{600nm}$)	Sampling Duration (min)
1.1	Modified RGCB	0.15% Cellobiose	0.056	15
1.2	Modified RGCB	0.15% Cellobiose	0.046	15
1.3	Modified Medium A	0.15% Cellobiose	0.032	15
1.4	Modified RGCB	0.15% Cellobiose	0.026	15
1.5	Modified RGCB	0.15% Cellobiose	0.026	15
1.6	Modified RGCB	0.15% Cellobiose	0.018	15
1.7	Modified Medium A	0.15% Cellobiose	0.074	15
1.8	Modified Medium A	0.15% Cellobiose	0.006	15
1.9	Modified Medium A	0.15% Cellobiose	0.011	15
2.1	Modified Medium A	0.15% Cellobiose	0.029	15
2.2	Modified Medium A	0.15% Cellobiose	0.022	15
2.3	Modified Medium A	0.15% Cellobiose	0.015	15
2.4	Modified Medium A	0.15% Cellobiose	0.014	15
2.5	Modified Medium A	0.15% Cellobiose	0.010	15
2.6	Modified Medium A	0.15% Cellobiose	0.010	15
3.1	Modified Medium A	0.15% Starch or Maltose[2]	[3]	10
3.2	Modified Medium A	0.15% Starch	0.054	10
3.3	Modified Medium A	0.15% Starch	0.087	10
3.4	Modified Medium A	0.15% Starch	0.001	10
3.5	Modified Medium A	0.15% Starch	0.010	10
3.6	Modified Medium A	0.15% Starch	0.038	10
3.7	Modified Medium A	0.15% Maltose	0.020	10
3.8	Modified Medium A	0.15% Maltose	0.020	10
3.9	Modified Medium A	0.15% Maltose	0.018	10

1 Average starting inoculum for all treatment and control tubes
2 Either starch or maltose was the sole carbohydrate source
3 Initial inoculum varied between treatments in experiment 3.1

2.3 Treatments used in the experiments on the effects of yeast on the lag time of pure culture bacteria

Treatment allocation for individual trials is summarized in Tables 3.3-3.5. Yeast treatments were chosen based primarily on their performance in previous trials.

Table 3.3 Allocation of yeast treatment[1] according to trial for experiment 1

TrialNumber	Control	NCYC	DCL	LV	FE	LF	UNI
1.1	X	X	X	X	X		
1.2	X	X	X	X	X	X	
1.3	X	X	X	X	X	X	
1.4	X	X				X	
1.5	X					X	X
1.6	X						X
1.7	X	X				X	X
1.8	X	X				X	X
1.9	X	X				X	X

1 Control = No yeast; NCYC = Strain 1026 from the National collection of yeast cultures; DCL = a Yea-Sacc DCL batch # 04-FS050 isolate; LV = a Levucell SC20 batch # 02234 isolate; FE = a Yea-Sacc Fermin batch # 960A isolate; LF = a La Florida batch # Prob 507 isolate; UNI = Yeast from personal collection of Dr. K Dawson.

Table 3.4 Allocation of yeast treatment[1] according to trial for experiment 2

Trial Number	Control	NCYC	LF	UNI	NLV
2.1	X	X	X	X	
2.2	X		X	X	X
2.3	X		X	X	
2.4	X		X	X	
2.5	X		X	X	
2.6	X		X	X	

1 Control = No yeast; NCYC = Strain 1026 from the National collection of yeast cultures; LF = a La Florida batch # Prob 507 isolate; UNI = Yeast from personal collection of Dr. K Dawson, NLV = a new Levucell SC20 isolate.

Table 3.5 Allocation of yeast treatment[1] according to trial for experiment 3

Trial Number	Control	LF	UNI	VH1	VH2	VH3	VH5
3.1	X						
3.2	X	X	X	X	X	X	X
3.3	X	X	X	X	X	X	X
3.4	X	X	X	X	X	X	X
3.5	X	X	X	X	X	X	X
3.6	X	X	X	X	X	X	X
3.7	X	X	X		X		
3.8	X	X	X			X	
3.9	X	X	X				X

1 Control = No yeast; LF = a La Florida batch # Prob 507 isolate; UNI = Yeast from personal collection of Dr. K Dawson; VH 1,2,3,5 = yeasts isolates isolated by V. Holder from rumen fluid.

3. Results

Results are presented as tables containing lag times as well as graphs showing both absorbance (Abs $_{600nm}$) and the log of absorbance plotted against time.

3.1 The effects of yeast supplementation on the lag time of *Ruminococcus albus* strain 7 using different yeast species and bacterial inoculum sizes

Trial 1.1 The effects of selected yeast culture supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.056) in modified RGCB

Table 3.6 Effects of selected yeast supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs $_{600nm}$ = 0.056) in modified RGCB

Treatment	Lag time (h)
Control	0.946
S. cerevisiae (NCYC)	0.993
S. cerevisiae (DCL)	0.766
S. cerevisiae (LV)	0.730
S. cerevisiae (FE)	0.744
SE (pooled)	0.121
P-value	0.425

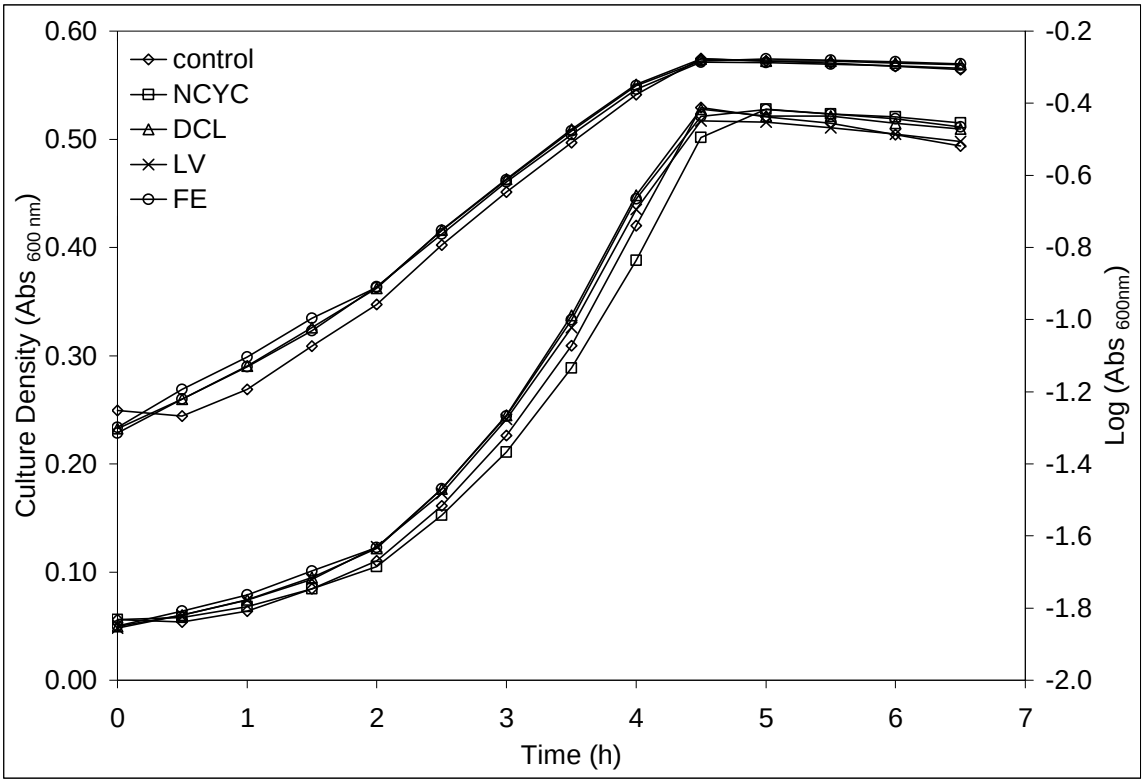

Figure 3.3 Effects of selected yeast supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs $_{600nm}$ = 0.056) in modified RGCB

The calculated lag time of *R. albus* supplemented with yeast strain NCYC was similar to that of the control. The average calculated lag times of *R. albus* supplemented with yeast strains DCL, LV and FE appear to be shorter than the control with treatments DCL, LV and FE supplemented *R. albus* having lag times 0.227 h, 0.216 h and 0.202 h shorter than the control treatment respectively. However, variability within the treatments groups rules out the possibility of any statistical differences between treatments (P=0.425). The average initial inoculum absorbance was Abs $_{600nm}$ = 0.056. Culture density increased between the first and second readings and therefore lag time is not apparent in the graph of culture density versus time. The initial inoculum size was probably too high resulting in rapid growth of the cultures and consequently, short lag times. The calculated values for lag time may have been too short with the result that variation among treatments was larger than the differences between treatments.

Trial 1.2 The effects of selected yeast supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.054) in modified RGCB

Table 3.7 Effects of selected yeast supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs $_{600nm}$ = 0.054) in modified RGCB

Treatment	Lag time (h)
Control	0.078
S. cerevisiae (NCYC)	-0.208
S. cerevisiae (DCL)	-0.107
S. cerevisiae (LV)	-0.027
S. cerevisiae (FE)	0.071
S. cerevisiae (LF)	0.190
SE (pooled)	0.078
P-value	0.020

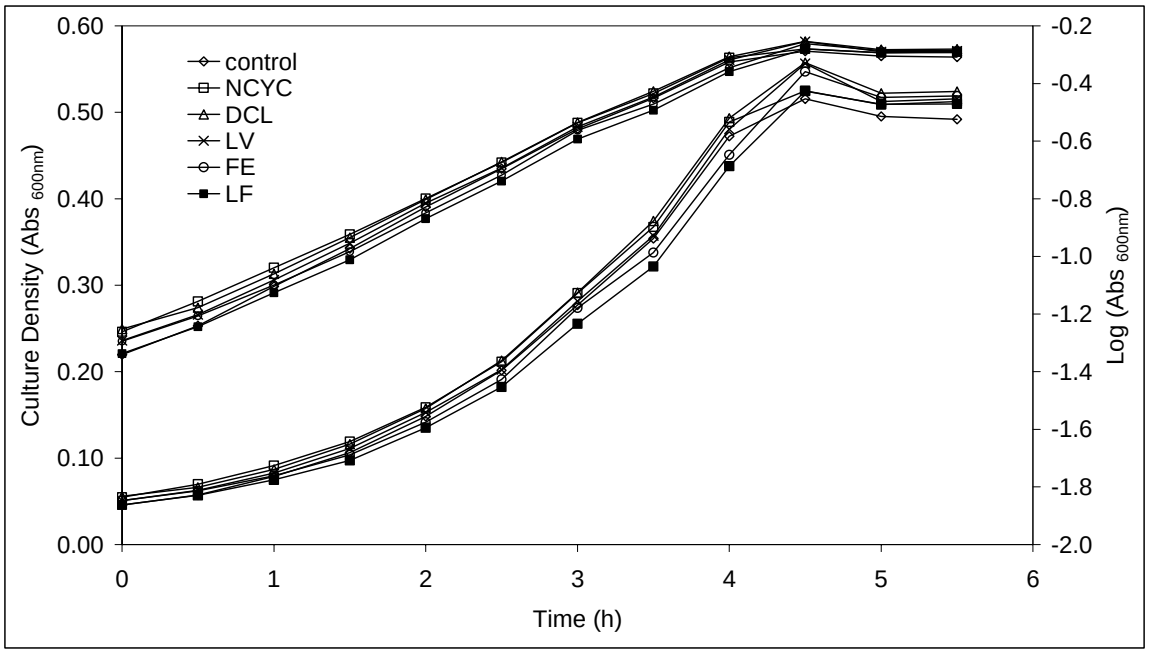

Figure 3.4 Effects of selected yeast supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs $_{600nm}$ = 0.054) in modified RGCB

R. albus supplemented with NCYC yeast culture had shorter lag times than control cultures (P = 0.020). The lag times of the *R. albus* supplemented with yeasts DCL, LV, FE and LF did not differ statistically from the control treatment. However, the lag time calculation yielded negative values for lag time when performed on the data for *R. albus* supplemented with yeasts NCYC, DCL and LV. The cultures were already increasing in absorbance rapidly at the first reading (Figure 3.4). This caused the regressed line to intersect the initial inoculum at a negative time value. It may be that the initial inoculum in the experiment, i.e. Abs_{600nm} = 0.054, was too high and this caused the rapid initial culture growth. Reducing the initial inoculum of the experiment may allow for a more prolonged period of lag, which should allow positive calculated lag time values.

Trial 1.3 The effects of selected yeast supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.032) in modified RGCB

Table 3.8 Effects of selected yeast supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.032) in modified RGCB

Treatment	Lag time (h)
Control	0.075
S. cerevisiae (NCYC)	-0.084
S. cerevisiae (DCL)	-0.170
S. cerevisiae (LV)	-0.257
S. cerevisiae (FE)	-0.319
S. cerevisiae (LF)	-0.044
SE (pooled)	0.099
P-value	0.103

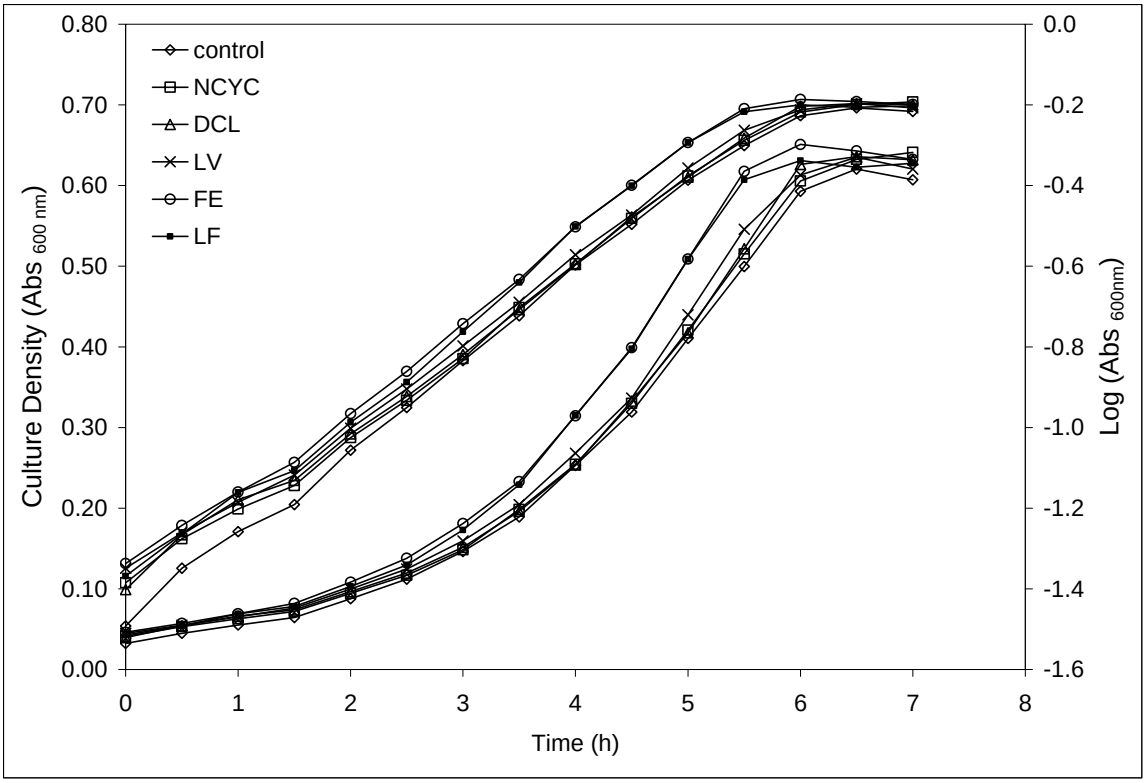

Figure 3.5 Effects of selected yeast supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.032) in modified RGCB

The average calculated lag times of *R. albus* supplemented with yeast strains NCYC, DCL, LV, FE and LF were shorter than the control. Treatment cultures had lag time values of between 0.1 and 0.4 h shorter than that of the control. However, significant variability within the treatments groups rules out the possibility of any statistical differences between treatments (P=0.103). Culture density increased between the first and second readings and therefore lag time is not apparent in the graph of culture density versus time (Figure 3.5). The initial inoculum size may have been too high resulting in rapid growth of the cultures and consequently, very short lag times. The calculated values for lag were too small with the result that variation between treatments was larger than the differences between treatments. The technique should be revised by reducing the initial inoculum to increase the length lag time.

Once again the lag time calculation yielded negative values for lag time. The cultures were already increasing in absorbance at the first reading (Figure 3.5). This caused the regressed line to intersect the initial inoculum at a negative time value. It may be that the

initial inoculum in the experiment, i.e. Abs $_{600nm}$ = 0.032, was still too high and this caused the rapid initial culture growth. Reducing the initial inoculum of the experiment may allow an extended lag time, which would allow positive lag time values to be calculated.

Trial 1.4 The effects of *S. cerevisiae* strains NCYC and LF supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.026) in modified RGCB

Table 3.9 Effects of *S. cerevisiae* strains NCYC and LF supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.026) in modified RGCB

Treatment	Lag time (h)
Control	1.996
S. cerevisiae (NCYC)	1.992
S. cerevisiae (LF)	1.461
SE (pooled)	0.164
P-value	0.063

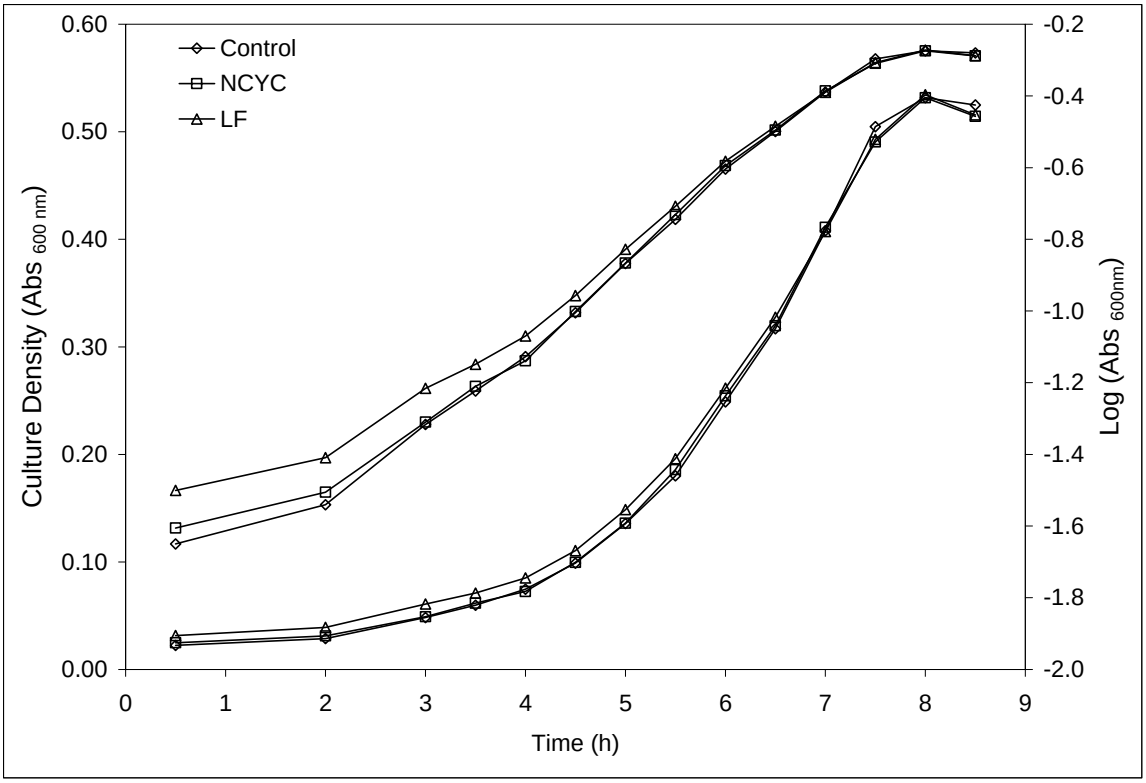

Figure 3.6 Effects of *S. cerevisiae* strains NCYC and LF supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.026) in modified RGCB

The calculated average lag time of *R. albus* supplemented with LF yeast was 0.535 h shorter than that of the control cultures. Lag time data was subjected to three different statistical analyses: Tukey's Studentized Range test (most stringent), Dunnett's t test and LSD t test (least stringent). The minimum significant difference for Tukey's Studentized Range was 0.619 h while 0.581 h and 0.506 h were the minimum differences required for Dunnett's t test and LSD t test respectively. The difference between LF and control for this experiment was 0.535 h and was therefore significant according to the LSD t test (least stringent) (Table 3.9). The initial inoculum in this experiment was Abs_{600nm} = 0.026. The first two data points on the curves of culture growth versus time are at a similar absorbance indicating that little culture growth occurred in this time period (Figure 3.6). Calculation of lag time yielded positive values (Table 3.9).

Trial 1.5 The effects of *S. cerevisiae* strains LF and UNI supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.026) in modified RGCB

Table 3.10 Effects of *S. cerevisiae* strains LF and UNI supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.026) in modified RGCB

Treatment	Lag time (h)
Control	1.823
S. cerevisiae (LF)	1.395
S. cerevisiae (UNI)	1.198
SE (pooled)	0.142
P-value	0.026

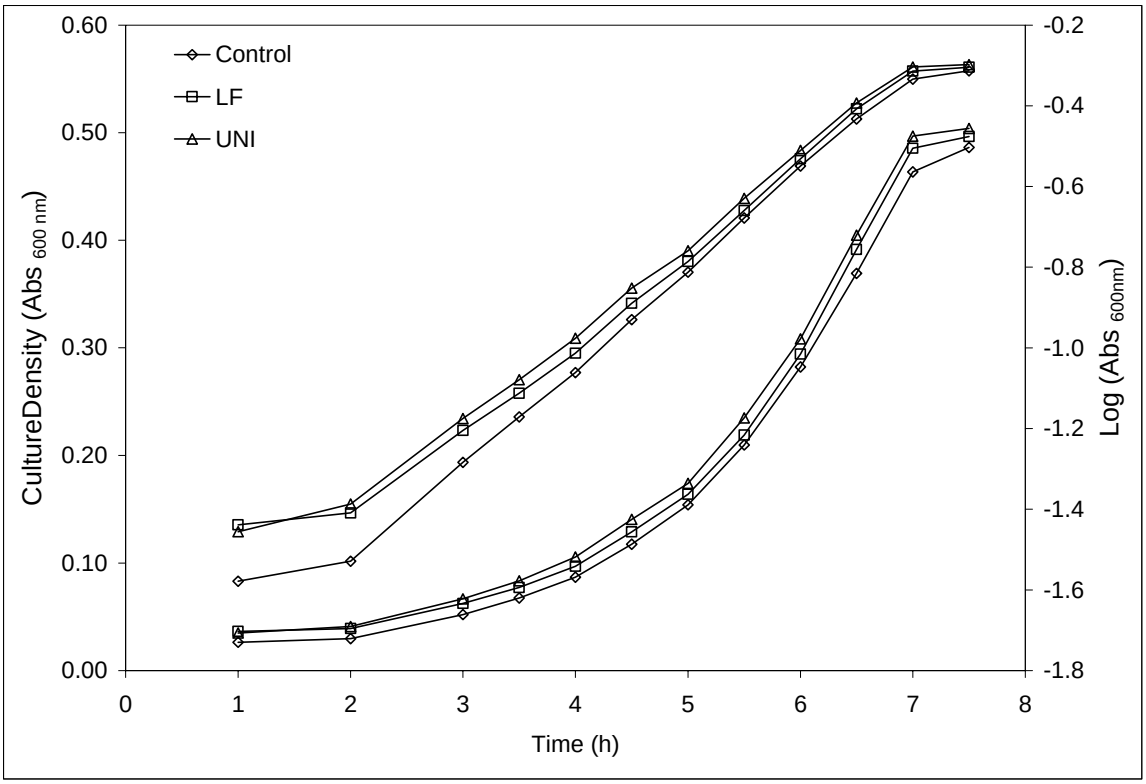

Figure 3.7 Effects of *S. cerevisiae* strains LF and UNI supplementation on the lag time of *Ruminococcus albus* strain 7 (Starting inoculum - Abs_{600nm} = 0.026) in modified RGCB

The calculated average lag times were 0.428 h and 0.625 h shorter than control for *R. albus* supplemented with LF and UNI yeasts respectively. Lag time data was subjected to three different statistical analyses: Tukey's Studentized Range test (most stringent), Dunnet's t test and LSD t test (least stringent). The minimum significant difference for Tukey's Studentized Range was 0.537 h while 0.503 h and 0.438 h were the minimum differences required for Dunnett's t test and LSD t test respectively. The difference between LF and control was 0.428 h for this experiment and was not significant for any of the three tests (Table 3.10). Supplementation with the UNI strain significantly reduced the lag time of *R. albus* by 0.625 h and was therefore significant according to all three tests. The initial inoculum in this experiment was Abs $_{600nm}$ = 0.026. The first two data points on the curves of culture growth versus time are at a similar absorbance indicating that little culture growth occurred in this time period, allowing lag time to be visualized (Figure 3.7). The initial inoculum used in this experiment was appropriate for visualising and comparing lag times between treatments.

Trial 1.6 The effects culture age and supplementation with *S. cerevisiae* strain UNI on the lag time of *Ruminococcus albus* strain 7 in modified RGCB

Table 3.11 Effects of culture age and supplementation with *S. cerevisiae* strain UNI on the lag time of *Ruminococcus albus* strain 7 in modified RGCB

Treatment[1]	Lag Time (h)
'D.S' Control	0.957
'D.S' UNI	0.580
'SSCT' Control	0.776
'SSCT' UNI	0.417
SE (pooled)	0.104
P-values	
Treatment effect	0.003
Culture age effect	0.116
Treatment/culture age interaction effect	0.931

1 Treatments: D.S = Daily subcultured for 4 weeks; SSCT = standard subculture technique

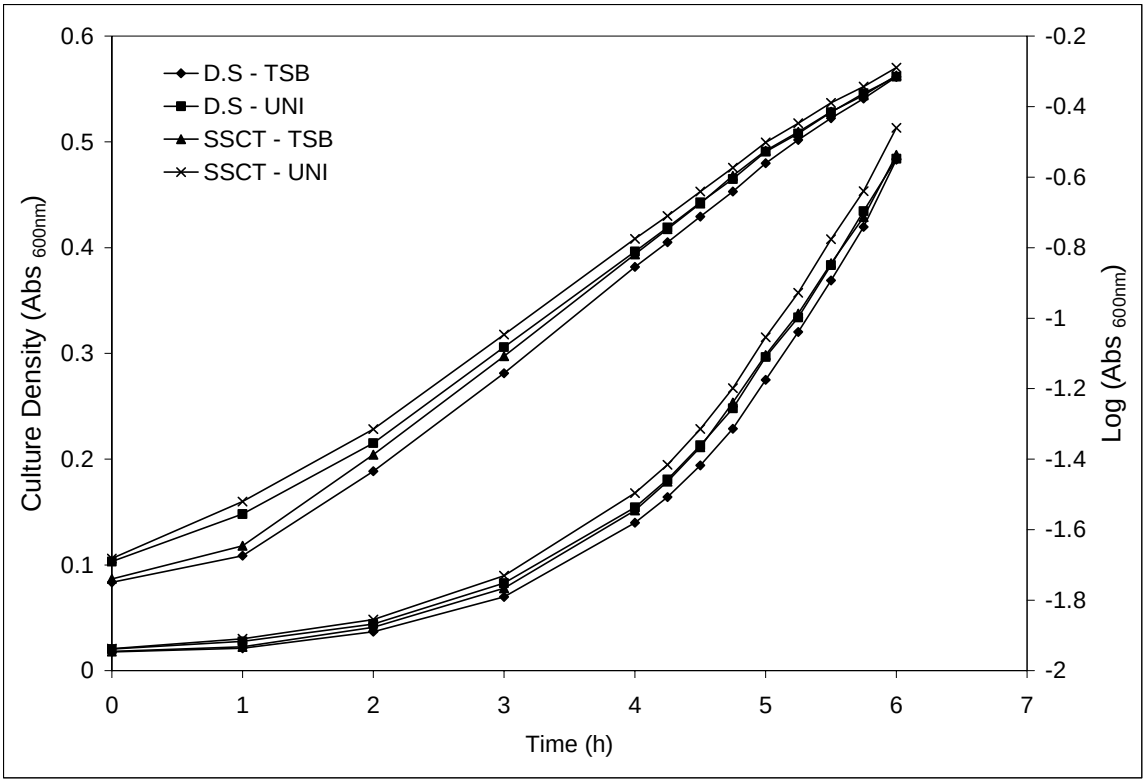

Figure 3.8 Effects of culture age and supplementation with *S. cerevisiae* strain UNI on the lag time of *Ruminococcus albus* strain 7 in modified RGCB

Treatment with UNI yeast reduced the lag time of the *Ruminococcus albus* strain 7 cultures prepared by both the standard subculture technique (SSCT) and the Daily sub-culture technique (D.S) (P = 0.003) (Table 3.11). *R. albus* supplemented with UNI yeast had significantly shorter lag times regardless of 'age' of the culture used as inoculum. This is apparent by the P-value associated with the culture age effect (P=0.116). There was no detectable interaction between the number of times the parent culture was sub-cultured and the effect of treatment with UNI (P=0.931).

Trial 1.7-1.9 The effects of yeast supplementation on the lag time of *Ruminococcus albus* strain 7 in modified medium A

The repeatability was poor between tubes within treatments. The experimental units showed no culture growth in medium A. There were problems with repeatability of growth of *Ruminococcus albus* in Medium A at inoculum levels where the bacterial count was low. The average initial bacterial count in experiments 1.7-1.9 varied between 0.9 and 1.8 x10^6 CFU/ml (Table 3.12). The variability in bacterial growth did not seem to be associated with differences in viable counts between tubes within each treatment as variability in initial inoculum absorbance between individual tubes was similar to that recorded in experiments with RGCB as the growth media. Variability was low when modified RGCB was the growth media. The repeatability of the growth of *Ruminococcus albus* strain 7 in medium A at a low inoculum level (1.37 x 10^6 CFU/ml) was poor (Figure 3.9). However, at a high inoculum (4.29 x 10^6 CFU/ml) the growth was repeatable (Figure 3.10). The repeatability issues with medium A disappeared as the inoculum size was increased.

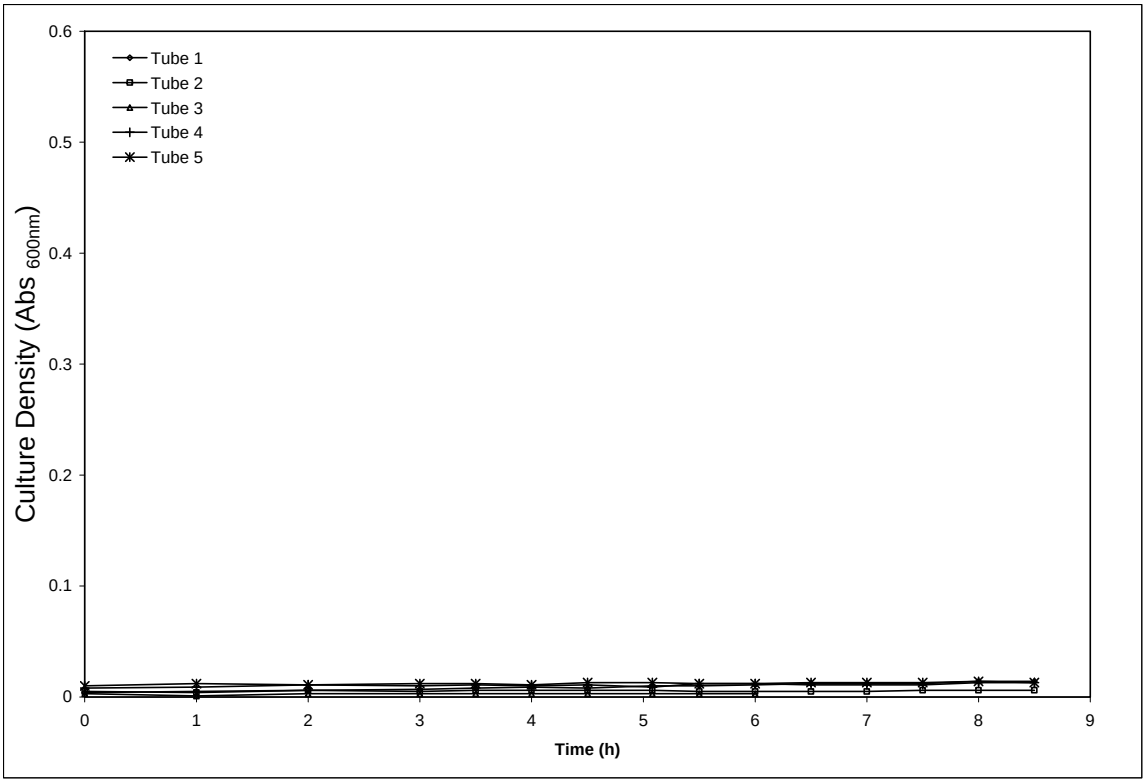

Figure 3.9 Absorbance *vs.* time in replicate tubes within the control treatment group with a low inoculum (1.37 x 10^6 CFU/ml) in medium A

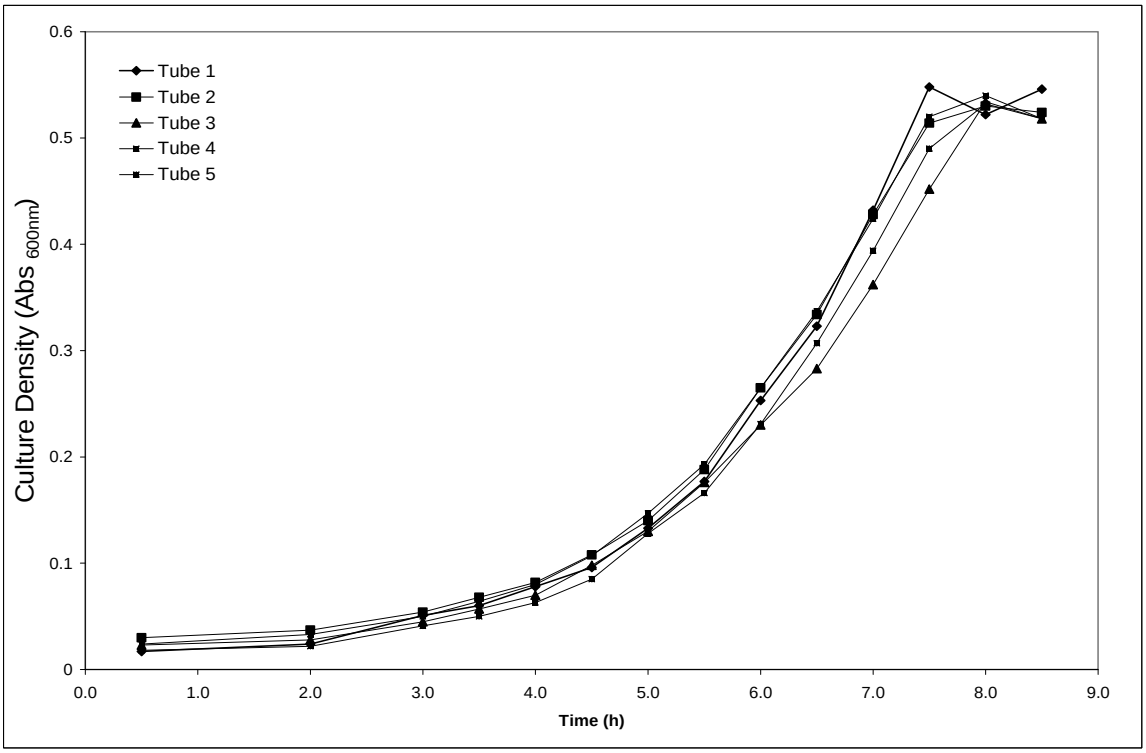

Figure 3.10 Absorbance *vs.* time in replicate tubes within the control treatment group with high inoculum (4.29 x 10^6 CFU/ml) in medium A

Table 3.12 Summary of viable count analyses (n=3) for experiments in modified Medium A

Inoculum size	Parent culture (CFU/ml)	SD	Experimental cultures	
			Estimated counts (CFU/ml)[1]	Actual counts (CFU/ml)[2]
High	17.56×10^7	2.639	4.29×10^6	NC[3]
Low	7.13×10^7	0.493	1.74×10^6	1.68×10^6
Low	5.63×10^7	0.306	1.37×10^6	1.03×10^6
Low	0.85×10^7	0.047	0.93×10^6	1.38×10^6

[1] Bacterial numbers in the experimental culture estimated from viable cell counts in the parent culture.

[2] Bacterial numbers in the experimental culture estimated from viable cell count enumeration on the experimental cultures.

[3] NC= not counted.

Experiments in Medium A with a higher inoculum showed good repeatability (Figure 3.10). Subsequent experiments in Medium A had about one third the inoculum and showed poor repeatability or no culture growth (Figure 3.9). Estimated counts in the experimental cultures were calculated by measuring the viable count of the parent culture and calculating its dilution in the experimental cultures. Estimated counts compared well to actual counts (Roll tube method) on the experimental cultures (Table 3.12).

3.2 The effects yeast supplementation on the lag time of *Selenomonas ruminantium GA192* using different yeast species and bacterial inoculum sizes

Trial 2.1 The effects selected yeast supplementation on the lag time of *Selenomonas ruminantium* (Starting inoculum - Abs_{600nm} = 0.029) in modified Medium A

Table 3.13 Effects of selected yeast supplementation on the lag time of *Selenomonas ruminantium* (Starting inoculum - Abs_{600nm} = 0.029) in modified Medium A

Treatment	Lag time (h)	Dunnett's test[1]	% Difference
Control	0.590		
S. cerevisiae (NCYC)	0.378	No difference	-35.96
S. cerevisiae (LF)	0.285	Different	-51.64
S. cerevisiae (UNI)	0.242	Different	-58.97
SE (pooled)	0.070		
P-value	0.013		

[1] The minimum significant difference for Dunnett's t-test = 0.268 h

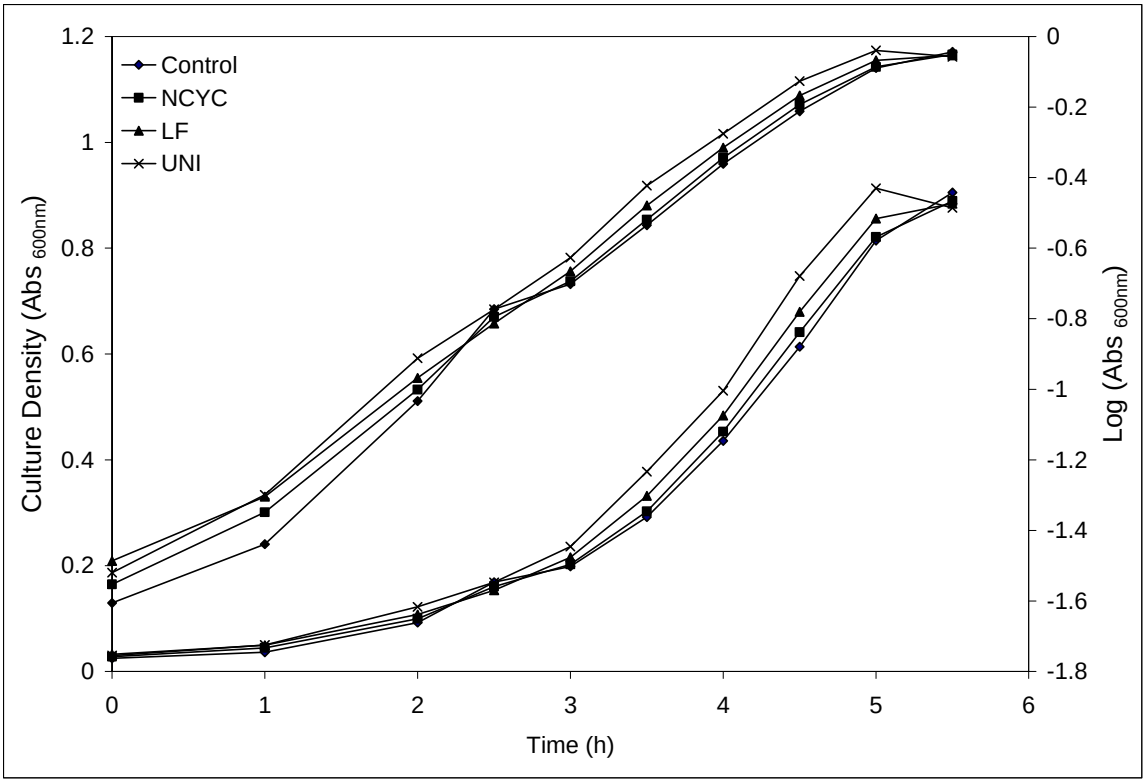

Figure 3.11 Effects of selected yeast supplementation on the lag time of *Selenomonas ruminantium* (Starting inoculum - Abs_{600nm} = 0.029) in modified Medium A

On average, the lag time of *S. ruminantium* supplemented with yeast NCYC was approximately 0.2h shorter than that of the control. The lag time of *S. ruminantium* supplemented with LF yeast was approximately 0.3h shorter than the control. The lag time of *S. ruminantium* supplemented with yeast UNI was approximately 0.35h shorter than the control cultures. The average initial concentration of *S. ruminantium* in experimental cultures as calculated by Hungate roll tube enumeration on the parent culture was 2.29 x 10^6 CFU/ml. According to Dunnett's t-test the lag time of *S. ruminantium* supplemented with LF was 51.64% shorter than the control and the lag time of *S. ruminantium* supplemented with UNI was 58.97% shorter than the control. Yeast NCYC had no significant effect on the lag time of *S. ruminantium*. Lag time on the graph of Culture Density *vs.* time was not obvious (Figure 3.10). Absorbance measurements at the first reading were slightly higher than the initial readings. It was suggested that the initial inoculum be reduced to extend the lag time in order to better

visualize lag. It is also important to extend the lag time to avoid the difficulties associated with statistical calculations with smaller lag time values.

Trial 2.2 The effects of selected yeast supplementation on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs600nm = 0.022) in modified Medium A

Table 3.14 Effects of selected yeast supplementation on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs_{600nm} = 0.022) in modified Medium A

Treatment	Lag time (h)	Dunnett's test[1]	% Difference
Control	0.616		
S. cerevisiae (NLV)	0.485	No difference	-21.28
S. cerevisiae (LF)	0.335	Different	-45.59
S. cerevisiae (UNI)	0.280	Different	-54.49
SE (pooled)	0.055		
P-value	0.002		

[1] The minimum significant difference for Dunnett's t-test = 0.210 h

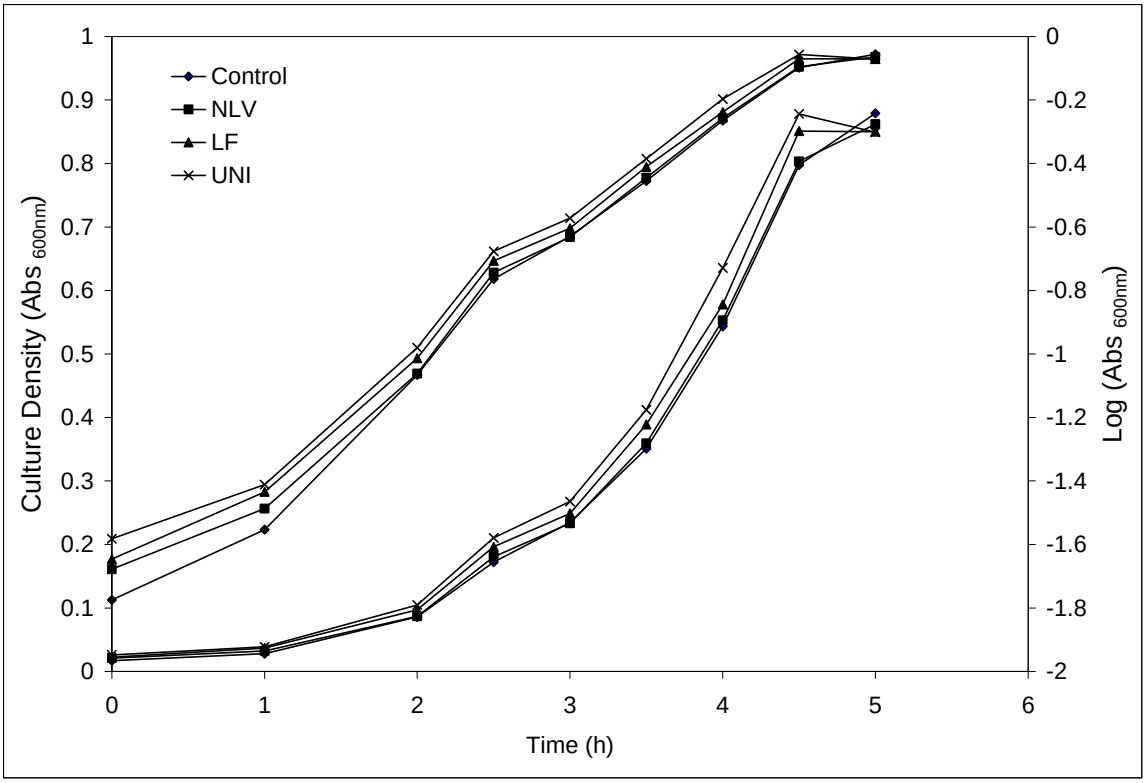

Figure 3.12 Effects of selected yeast supplementation on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs_{600nm} = 0.022) in modified Medium A

Based on averages, the lag time of *S. ruminantium* supplemented with yeast NLV did not differ from the control. The lag time of *S. ruminantium* supplemented with yeast LF was approximately 0.13h shorter than the control and *S. ruminantium* supplemented with yeast UNI, approximately 0.34h shorter. The average initial concentration of bacteria in experimental cultures as calculated by Hungate roll tube enumeration on the parent culture was 1.20 x 10^6 CFU/ml. According to Dunnet's t test the lag time of *S. ruminantium* supplemented with yeast LF was 45.59% shorter than the control and the lag time of *S. ruminantium* supplemented with yeast UNI was 54.49% shorter than the control. NLV had no significant effect on lag time. Lag time on the graph of Log Abs_{600nm} *vs.* time was still not obvious (Figure 3.11). Absorbance measurements at the first reading were slightly higher than the initial readings. It was suggested that the initial inoculum be reduced further to extend the lag time in order to better visualize lag. It is also important to extend lag time to avoid the difficulties associated with statistical calculations with smaller lag time values.

Trial 2.3 The effects of selected yeast supplementation and sampling time on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs_{600nm} = 0.015) in modified Medium A

Table 3.15 Effects of selected yeast supplementation and sampling time on the lag time of *Selenomonas ruminantium* (Starting inoculum - Abs_{600nm} = 0.015) in modified Medium A

Treatment	Lag time (h)	Dunnett's test[1]	% Difference
Control1	1.235		
S. cerevisiae (LF)	0.832	Different	-32.65
S. cerevisiae (UNI)	0.832	Different	-32.65
Control2	0.995	Different	
SE (pooled)	0.050		
P-value	0.0001		

[1] The minimum significant difference for Dunnett's t-test = 0.086 h

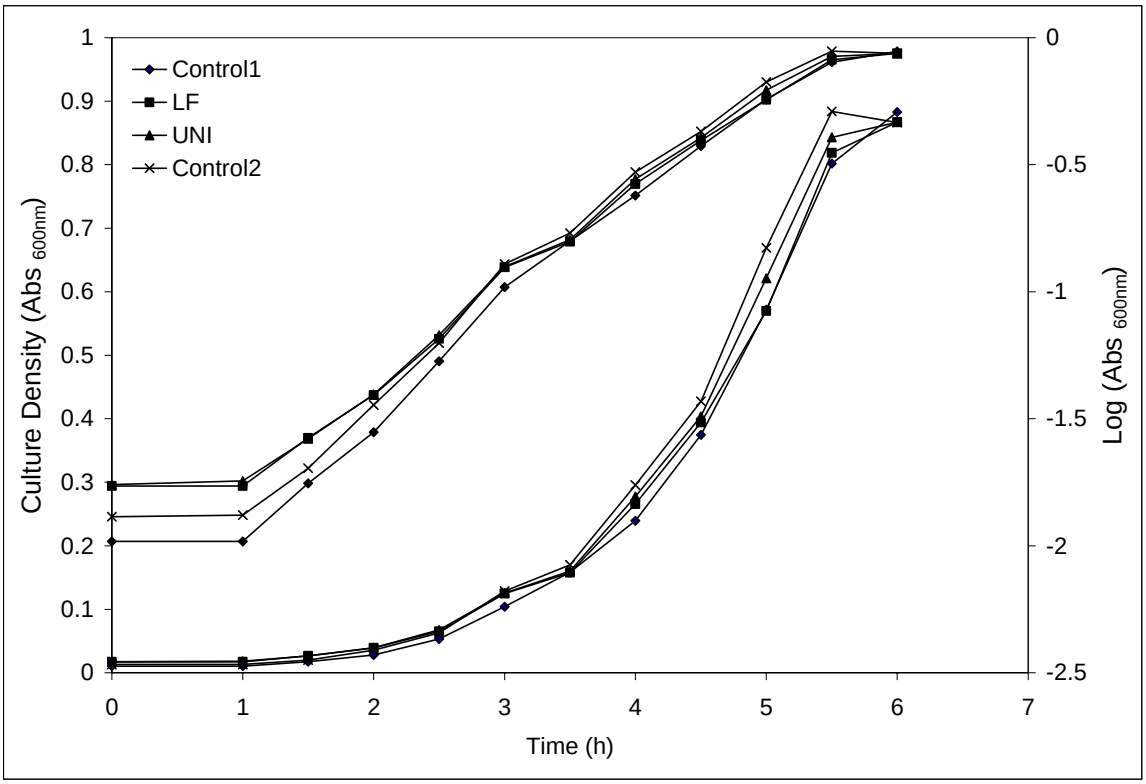

Figure 3.13 Effects of selected yeast supplementation and sampling time on the lag time of *Selenomonas ruminantium* (Starting inoculum - Abs_{600nm} = 0.015) in modified Medium A

Based on averages, the lag times of *S. ruminantium* supplemented with yeasts LF and UNI were approximately 0.4h shorter than Control 1. The lag time of Control 2 was about 0.24h shorter. The average initial concentration of bacteria in experimental cultures as calculated by Hungate roll tube enumeration on the parent culture was 5.93 x 10^5 CFU/ml. According to Dunnet's t test the lag times of *S. ruminantium* supplemented with yeasts LF and UNI were both 32.65% shorter than Control1. The lag time of Control2 was 19.4% shorter than the control. It appeared from this experiment that the time duration of sampling was influencing the calculation of lag time. The experiment was repeated (Trial 2.4) to validate this theory. Lag time was clearly visible on the graph of Culture density *vs.* time (Figure 3.13). The initial inoculum used in this experiment was therefore appropriate for visualizing lag.

Trial 2.4 The effects of selected yeast supplementation and sampling time on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs_{600nm} = 0.014) in Modified Medium A

Table 3.16 Effects of selected yeast supplementation and sampling time on the lag time of *Selenomonas ruminantium* (Starting inoculum - Abs_{600nm} = 0.014) in Modified Medium A

Treatment	Lag time (h)	Dunnett's test[1]	% Difference
Control1	1.213		
S. cerevisiae (LF)	0.897	Different	-26.00
S. cerevisiae (UNI)	1.039	No difference	-14.30
Control2	1.226	No difference	1.08
SE (pooled)	0.065		
P-value	0.008		

[1] The minimum significant difference for Dunnett's t-test = 0.252 h

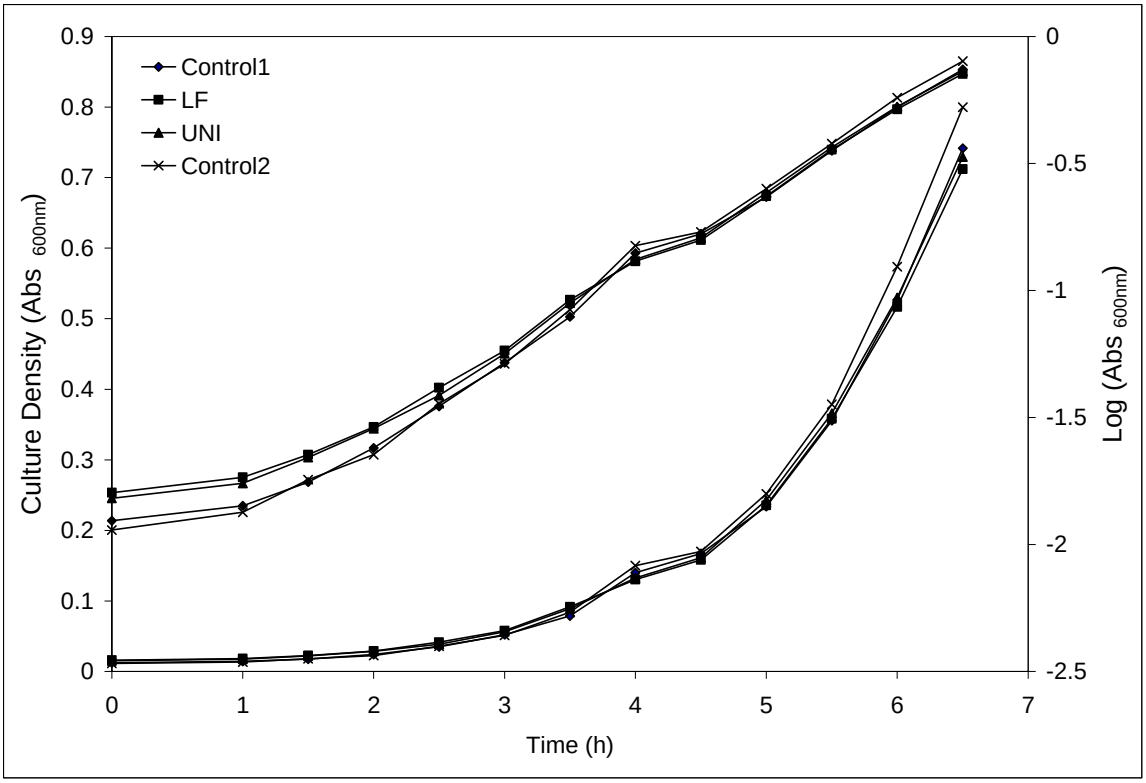

Figure 3.14 Effects of selected yeast supplementation and sampling time on the lag time of *Selenomonas ruminantium* (Starting inoculum - Abs_{600nm} = 0.014) in Modified Medium A

Based on averages, the lag time of *S. ruminantium* supplemented with yeast LF was about 0.3 h shorter than Control1. The lag time of *S. ruminantium* supplemented with yeast UNI was 0.17 h shorter than Control1. The lag times of Control 1 and Control 2 were similar. The average initial concentration of *S. ruminantium* in the experimental cultures as calculated by Hungate roll tube enumeration on the parent culture was 3.77 x 10^5 CFU/ml. According to Dunnet's t test the lag time of *S. ruminantium* supplemented with yeast LF was 26% shorter than Control1. The lag time of *S. ruminantium* supplemented with yeast UNI was not significantly different from Control1. The lag time of Control 2 and Control 1 were similar. Lag time calculations produced a similar value for lag for the control treatments both at the beginning and at the end of the sampling period. This suggests that the period that lapsed between reading the absorbance of the first set of treatment samples and the last set of treatment samples did not affect the calculation of lag time. The experiment was repeated to validate these results (Trial 2.6).

Trial 2.5 The effects of *S. cerevisiae* strains LF and UNI supplementation on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs_{600nm} = 0.010) in modified Medium A

Table 3.17 Effects of *S. cerevisiae* strains LF and UNI supplementation on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs_{600nm} = 0.010) in modified Medium A

Treatment	Lag time (h)	Dunnett's test[1]	% Difference
Control	0.688		
S. cerevisiae (LF)	0.491	Different	-28.62
S. cerevisiae (UNI)	0.207	Different	-69.92
SE (pooled)	0.063		
P-value	0.0006		

[1] The minimum significant difference for Dunnett's t-test = 0.122 h

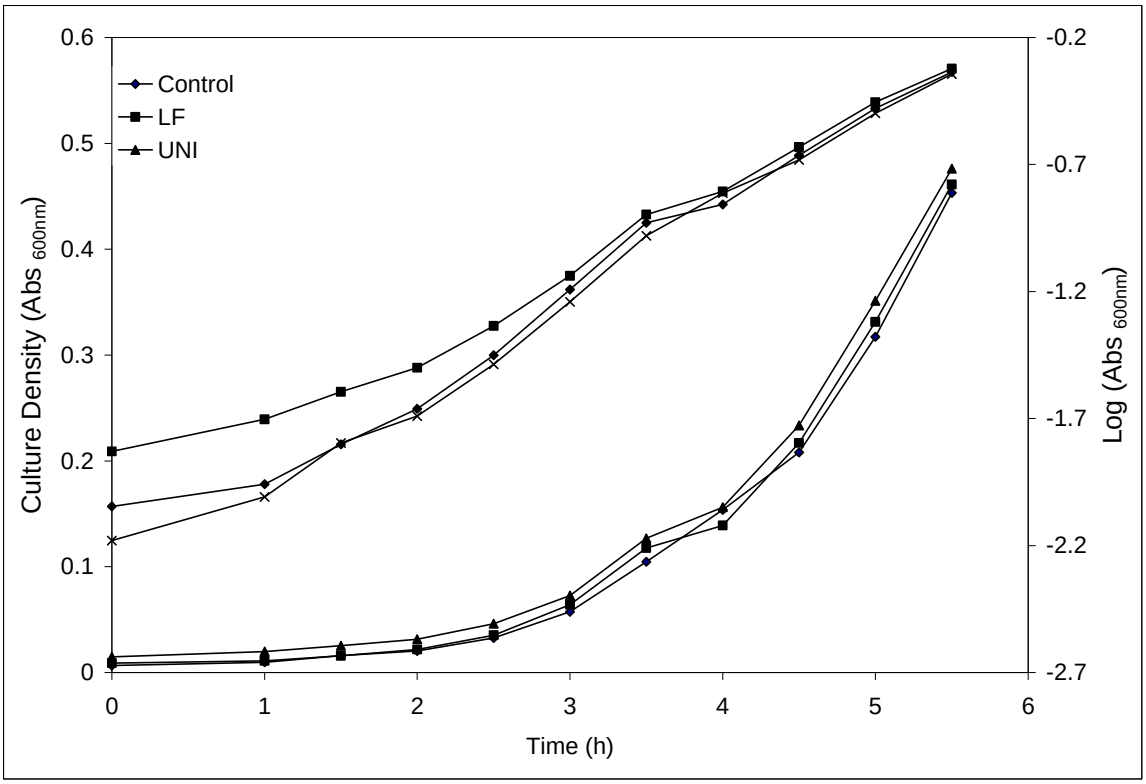

Figure 3.15 Effects of *S. cerevisiae* strains LF and UNI supplementation on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs_{600nm} = 0.010) in modified Medium A

Based on averages, the lag time of *S. ruminantium* supplemented with yeast LF was 0.2 h shorter than the Control. The lag time of *S. ruminantium* supplemented with yeast UNI was 0.5 h shorter than the Control. The average initial concentration of *S. ruminantium* in experimental cultures as calculated by Hungate roll tube enumeration on the parent culture was 4.54x 10^5 CFU/ml. According to Dunnet's t test the lag time of *S. ruminantium* supplemented with yeast LF was 28.62% shorter than Control and *S. ruminantium* supplemented with yeast UNI showed a reduction in lag time of nearly 70%.

Trial 2.6 The effects of selected yeast supplementation and sampling time on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs_{600nm} = 0.010) in Modified Medium A

Table 3.18 Effects of selected yeast supplementation and sampling time on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs_{600nm} = 0.010) in modified Medium A

Treatment	Lag time (h)	Dunnett's test[1]	% Difference
Control1	1.098		
S. cerevisiae (LF)	0.709	Different	-35.41
S. cerevisiae (UNI)	0.721	Different	-34.30
Control2	0.994	No difference	-9.45
SE (pooled)	0.059		
P-value	0.0003		

[1] The minimum significant difference for Dunnett's t-test = 0.226 h

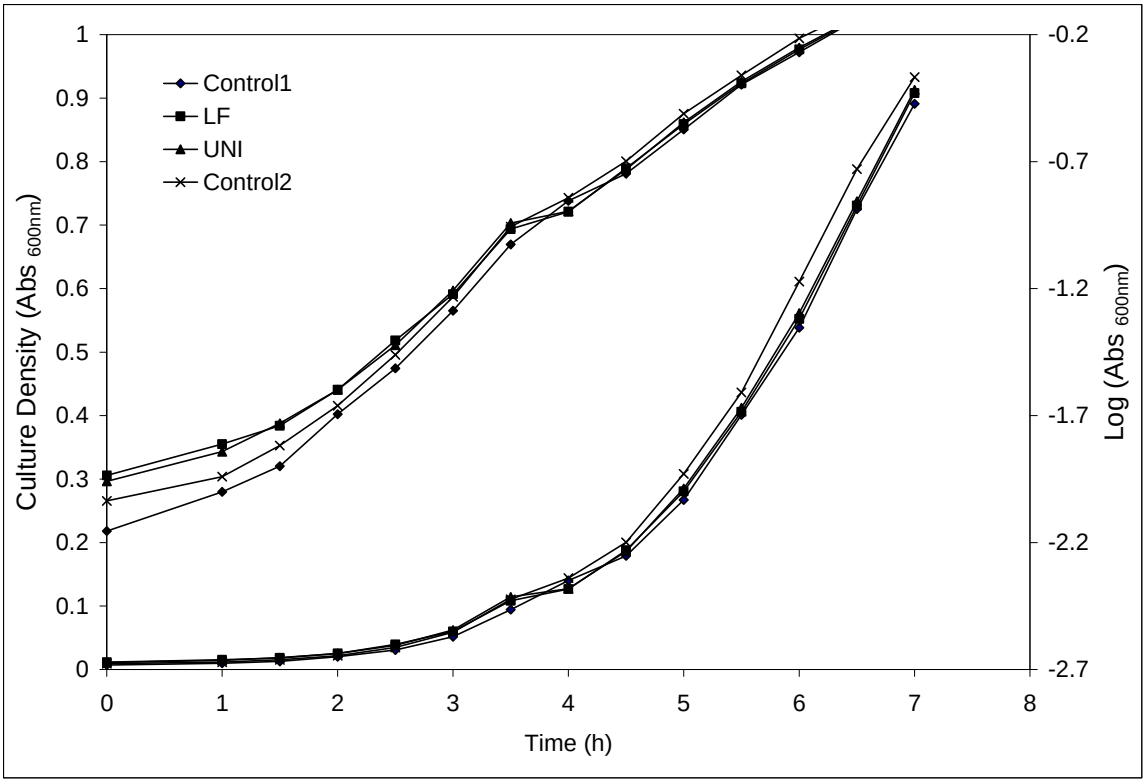

Figure 3.16 Effects of selected yeast supplementation and sampling time on the lag time of *Selenomonas ruminantium* (Starting inoculum – Abs_{600nm} = 0.010) in modified Medium A

Based on averages, the lag times of *S. ruminantium* supplemented with yeasts LF and UNI were approximately 0.38 h shorter than the control cultures. The lag times of the Control1 and Control2 cultures were similar. The average initial concentration of *S. ruminantium* in experimental cultures calculated by Hungate roll tube enumeration on the parent culture was 5.54 x 10^5 CFU/ml. According to Dunnet's t test the lag times *S. ruminantium* supplemented with LF and UNI were both approximately 35% shorter than Control1. Control1 and Control2 did not differ with respect to their lag times. Lag time calculations produced similar values for the control treatments at the beginning and at the end of the sampling period indicating that the period lapsing between reading the absorbance of the first treatment sample set and the last treatment sample set did not affect the calculation of lag time.

3.3 The effects yeast supplementation on the lag time of *Ruminobacter amylophilus* H18 with different yeast species, inoculum sizes and carbohydrate sources

Trial 3.1 Evaluation of carbohydrate source and inoculum level for determination of the lag time of *Ruminobacter amylophilus*

Figure 3.16 represents the culture growth of *Ruminobacter amylophilus* over time with different initial inoculum levels and on different carbohydrates sources.

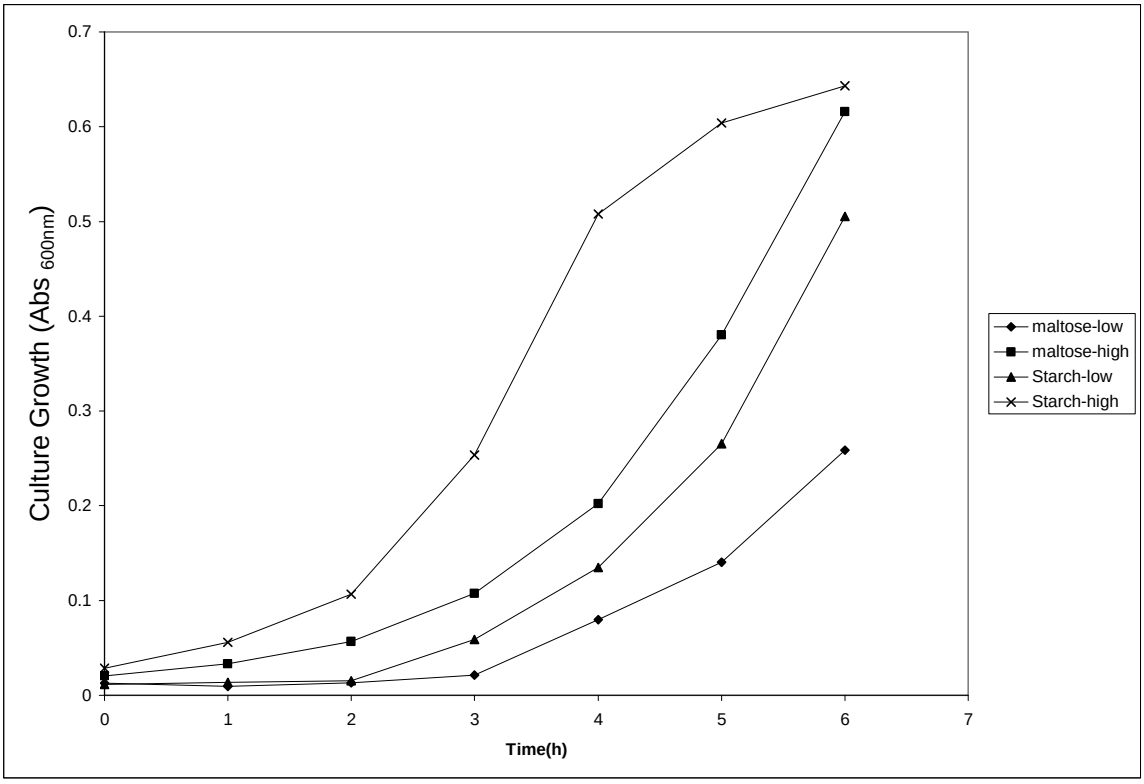

Figure 3.17 Evaluation of carbohydrate source and inoculum level for determination of the lag time of *Ruminobacter amylophilus*

The cultures of *Ruminobacter amylophilus* grown on starch based media with a high initial inoculum grew most rapidly. These were followed in turn by *R. amylophilus* grown on maltose based media with a high inoculum, *R. amylophilus* grown on starch based media with a low initial inoculum and finally *R. amylophilus* grown on maltose with a low initial inoculum. *R. amylophilus* grown in starch-based media with a low

initial inoculum (Initial inoculum - Abs $_{600nm}$ = 0.028) grew more rapidly than *R. amylophilus* with a similar inoculum level (maltose-low) on a maltose-based medium (Figure 3.17). *R. amylophilus* cultures with a high initial inoculum that were grown on starch-based media (Starch-High) completed their growth curve in approximately 6h. There would be insufficient data points in the linear portion of the log (Abs $_{600nm}$) *vs.* time curve to accurately calculate lag time. *R. amylophilus* grown from a low initial inoculum in starch based media or cultures grown from a high initial inoculum in maltose media would result in bacterial growth curves similar to those seen in previous experiments for determining lag time. In theory, this would allow sufficient data points in the linear portion of the curve to calculate lag time.

Trials 3.2-3.6 The effects of yeast supplementation on the lag time of *Ruminobacter amylophilus* in modified Medium A with starch as the carbohydrate source

The turbidimetric data produced when the bacterium *R. amylophilus* was grown on starch based media was highly variable. The following table shows the average pooled standard error for Trial 3.2-3.6 with different bacteria and different carbohydrate sources (Table 3.19).

Table 3.19 Average pooled standard error for three series of lag time determination experiments using different bacteria and different carbohydrate sources

Bacterium	Carbohydrate source	Average pooled SE
Selenomonas ruminantium	0.15 % cellobiose	0.060[1]
Ruminobacter amylophilus	0.15 % starch	0.121[2]
Ruminobacter amylophilus	0.15 % maltose	0.086[3]

1 Average pooled standard error for 6 trials
2 Average pooled standard error for 4 trials
3 Average pooled standard error for 3 trials

Variation from experiments with *R. amylophilus* in starch-based media was greater than in similar experiments with the bacterium *Selenomonas ruminantium* with cellobiose as the carbohydrate source. Later experiments undertaken with *Ruminobacter amylophilus*

grown with maltose as the carbohydrate source (Trial 3.7-3.9) showed less variation (Table 3.19). Starch was less soluble in the media than maltose or cellobiose. Precipitation was readily visible in starch containing media whilst media containing cellobiose or maltose at the same concentration showed no precipitation. It appeared that the precipitation of starch in the media caused turbidimetric fluctuation and resulted in the increase in variation in Trials 3.2-3.6. Media containing maltose was therefore more appropriate for determining lag time of cultures of *R. amylophilus* and was used for the remainder of the experiments on this organism (Trial 3.7-3.9).

Trial 3.7 The effects of selected yeast supplementation on the lag time of *Ruminobacter amylophilus* (Starting inoculum – Abs_{600nm} = 0.020) in modified Medium A with maltose as the carbohydrate source

Table 3.20 Effects of selected yeast supplementation on the lag time of *Ruminobacter amylophilus* (Starting inoculum – Abs_{600nm} = 0.020) in modified Medium A

Treatment	Lag time (h)	Dunnett's test[1]	% Difference
Control	1.633		
S. cerevisiae (LF)	1.109	Different	-32.12
S. cerevisiae (UNI)	1.184	Different	-27.49
S. cerevisiae (VH2)	1.307	No difference	-16.12
SE (pooled)	0.085		
P-value	0.002		

[1] The minimum significant difference for Dunnett's t-test = 0.329 h

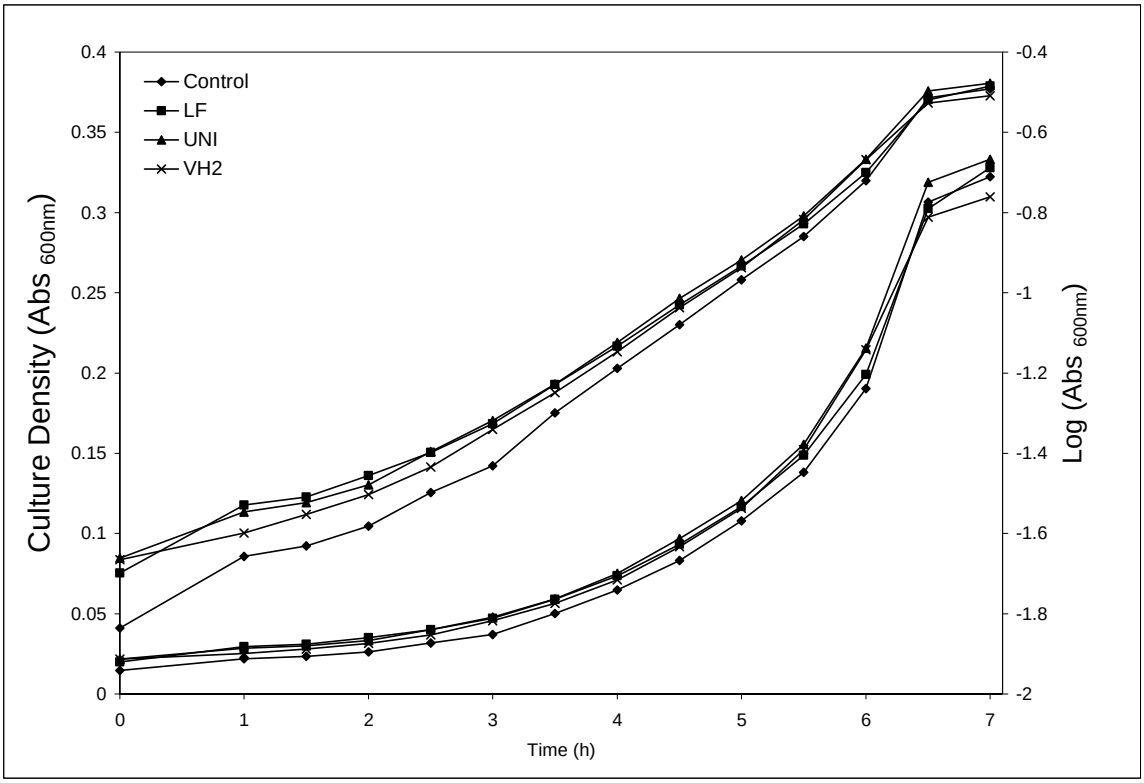

Figure 3.18 Effects of selected yeast supplementation on the lag time of *Ruminobacter amylophilus* (Starting inoculum – Abs_{600nm} = 0.020) in modified Medium A

Based on averages, the lag time of *R. amylophilus* supplemented with yeast LF was 0.53 h shorter than the control. The average lag time of *R. amylophilus* supplemented with yeast UNI was 0.45 h shorter than the control. The average initial concentration of *R. amylophilus* in experimental cultures as calculated by Hungate roll tube enumeration on the parent culture was 1.20×10^7 CFU/ml. According to Dunnet's t test the lag time of *R. amylophilus* supplemented with LF was 32.12% shorter than the control. The lag time of *R. amylophilus* supplemented with UNI was 27.49% shorter than the control. VH2 had no significant effect on lag time. Lag time was fairly obvious on the curve of culture density *vs.* time (Figure 3.18). The initial inoculum size used in this experiment appeared to be appropriate for visualizing and calculating lag times of *Ruminobacter amylophilus*.

Trial 3.8 The effects of selected yeast supplementation on the lag time of *Ruminobacter amylophilus* (Starting inoculum – Abs_{600nm} = 0.020) in modified Medium A with maltose as the carbohydrate source

Table 3.21 Effects of selected yeast supplementation on the lag time of *Ruminobacter amylophilus* (Starting inoculum – Abs_{600nm} = 0.020) in modified Medium A

Treatment	Lag time (h)	Dunnett's test[1]	% Difference
Control	1.546		
S. cerevisiae (LF)	0.978	Different	-36.74
S. cerevisiae (UNI)	0.979	Different	-36.70
S. cerevisiae (VH3)	1.516	No difference	-1.92
SE (pooled)	0.142		
P-value	0.007		

[1] The minimum significant difference for Dunnett's t-test = 0.488

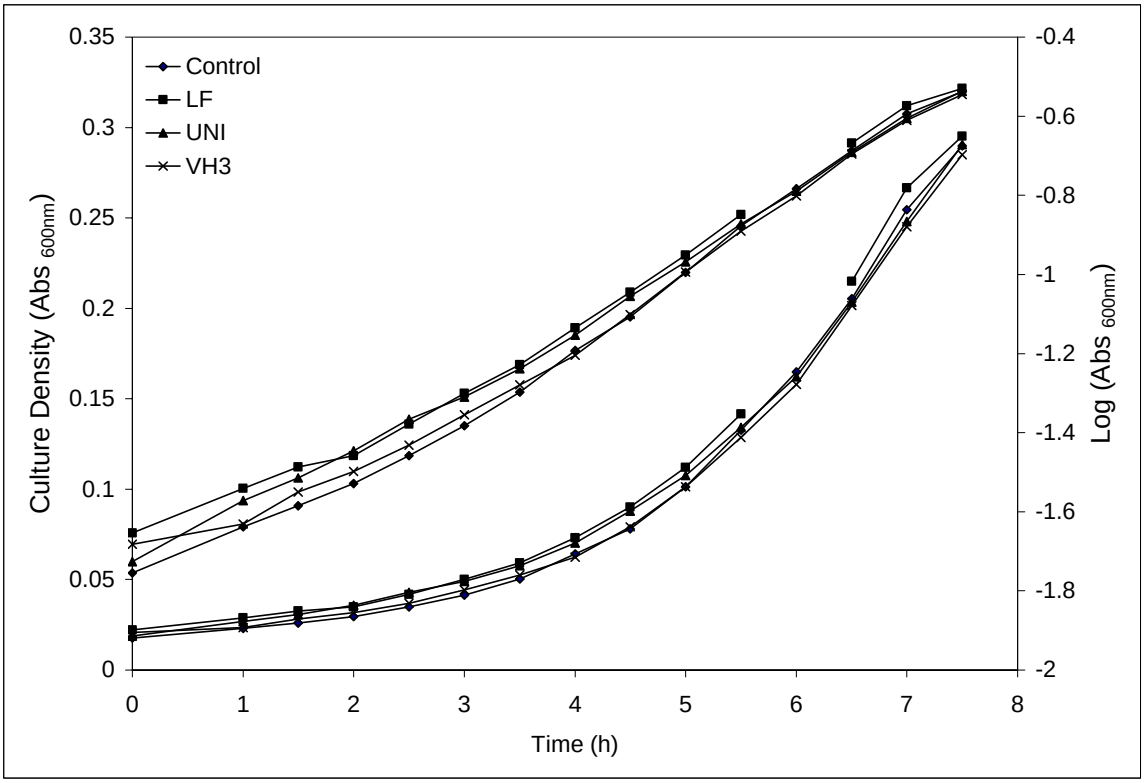

Figure 3.19 Effects of selected yeast supplementation on the lag time of *Ruminobacter amylophilus* (Starting inoculum – Abs_{600nm} = 0.020) in modified Medium A

Based on averages, the lag times of *R. amylophilus* supplemented with yeasts LF and UNI were approximately 0.57 h shorter than the control. The average initial concentration of *R. amylophilus* in experimental cultures as calculated by Hungate roll tube enumeration on the parent culture was 9.34 x 10^6 CFU/ml. According to Dunnet's t test the lag time of *R. amylophilus* supplemented with LF was 36.74% shorter than Control and the lag time of *R. amylophilus* supplemented with UNI was 36.70% shorter than the Control treatment. VH3 yeast had no effect on lag time. At the 6 h sampling, cultures in the LF treatment could not be sampled due to technical difficulties; consequently, the 6 h sampling was not included in the calculation of lag time for the LF treatment.

Trial 3.9 The effects of selected yeast supplementation on the lag time of *Ruminobacter amylophilus* (Starting inoculum – Abs600nm = 0.018) in Modified Medium A with maltose as the carbohydrate source

Table 3.22 Effects of selected yeast supplementation on the lag time of *Ruminobacter amylophilus* (Starting inoculum – Abs600nm = 0.018) in Modified Medium A

Treatment	Lag time (h)	Dunnett's test[1]	% Difference
Control	1.504		
S. cerevisiae (LF)	1.147	Different	-23.71
S. cerevisiae (UNI)	1.009	Different	-32.93
S. cerevisiae (VH5)	1.351	No difference	-10.18
SE (pooled)	0.045		
P-value	<0.00001		

[1] The minimum significant difference for Dunnett's t-test = 0.174 h

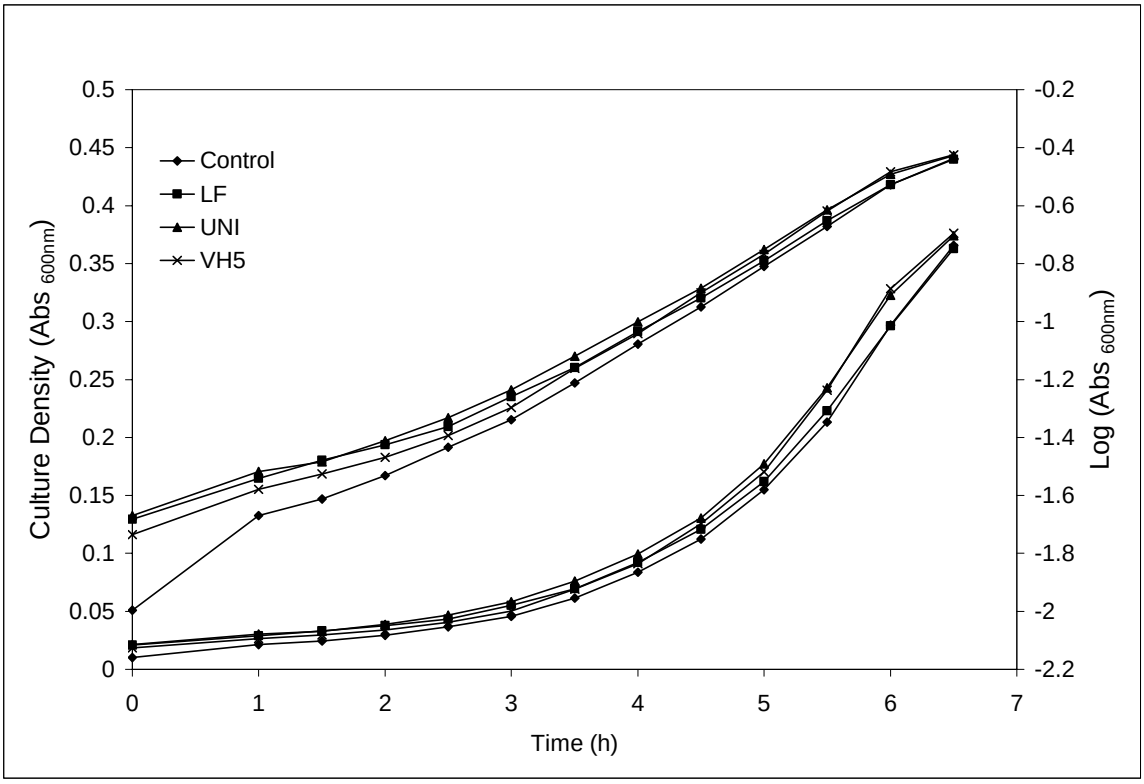

Figure 3.20 Effects of selected yeast supplementation on the lag time of *Ruminobacter amylophilus* (Starting inoculum – Abs600nm = 0.018) in Modified Medium A

Based on averages, the lag time of *R. amylophilus* supplemented with yeast LF was 0.36 h shorter than the control. The average lag time of *R. amylophilus* supplemented with yeast UNI was 0.5 h shorter than the control. The average initial concentration of *R. amylophilus* in experimental cultures as calculated by Hungate roll tube enumeration on the parent culture was 1.20 x 10^7 CFU/ml. According to Dunnett's t test the lag time of *R. amylophilus* supplemented with LF was 23.7% shorter than the Control. The lag time of *R. amylophilus* supplemented with UNI was 32.9% shorter than the Control (Table 3.22). The lag time of the VH5 supplemented *R. amylophilus* did not differ from the Control treatment.

4. Discussion

4.1 The effect of yeast culture supplementation on the lag time of *Ruminococcus albus* strain 7

Supplementation with yeast strains LF and UNI consistently reduced the lag time of pure cultures of *Ruminococcus albus* strain 7 in RGCB. Cultures of *Ruminococcus albus* supplemented with LF yeast had lag times that were 24.9 ± 2.7% shorter on average (average of Trial 1.4-1.5) than the control cultures. Cultures of *Ruminococcus albus* supplemented with UNI yeast had lag times that were 40.0 ± 6.0% shorter on average than the control cultures (average of Trials 1.5-1.6).

Initial inoculum size in the bacteria growth experiments must be sufficiently low that lag may be visible and that calculations of lag times yield positive values. On the other hand, in modified Medium A at least, the inoculum should be high enough to ensure repeatability of culture growth between tubes within treatments. Lag time determination of *Ruminococcus albus 7* in medium A was not possible because the inoculum level required for repeatable growth was too high, resulting in very short lag times and subsequently, negative calculated values for lag time.

Reduction of the lag time of *Ruminococcus albus* means that bacterial populations will establish more quickly and may reach higher numbers in the rumen after feeding. This may have important effects on animal production. *Ruminococcus albus* is one of the major fibre digesting species in the rumen (Dehority, 2003). Clearance of fibre from the rumen is one of the major rate limiting steps to digesta flow rate, and thus animal intake (Lechner-Doll *et al.*, 1991). If live yeast culture speeds up the initial growth of fibre digesting species (reduces lag) it may lead to an increase in the rate of digestion of forages. An increase in the rate of digestion of fibre would lead to an increased clearance rate of fibre from the rumen. This would usually result in increased feed intake and thus improved animal production. Increased breakdown of forage material may also lead to

increased VFA production from fermented fibre making more energy available to the animal. Faster growth of bacteria will result in more bacterial cell mass which will make more microbial protein available for digestion in the small intestine.

4.2 The effect of yeast culture supplementation on the lag time of *Selenomonas ruminantium* strain GA192

Supplementation with yeast strains LF and UNI consistently reduced the lag time of pure cultures of *Selenomonas ruminantium* in modified Medium A. Yeasts LF and UNI strain caused reductions in average population lag times of 36.9±11.16% and 46.066±22.34% respectively (average of Trials 2.1-2.6). It appears that the time period that occurred between sampling the first and sampling the last treatments (±15min) had no effect on estimating the differences in lag times between treatment cultures.

Selenomonas ruminantium strain GA192 plays a role in the digestion of starch and other soluble carbohydrates as well as production of lactate, acetate and propionate (Dehority, 2003). If live yeast culture addition speeds up the initial growth of these bacteria (reduces lag), it may lead to an increase in the rate of degradation of starches and other soluble carbohydrates. Rapid degradation of carbohydrate may lead to excessive production and subsequent accumulation of lactate in the rumen. Accumulation of lactate will lead to digestive disturbance related to depression in the pH of the rumen. However, there are reports that yeast supplementation may stimulate lactate utilizing bacteria such as Megaphaera elsdenii (Dehority, 2003) and stimulation of such bacterial populations may lead to moderation of lactate and pH levels in the rumen (Callaway and Martin, 1997). Faster growth of bacteria will also result in more bacterial cell mass which will make more microbial protein available for digestion in the small intestine.

4.3 The effect of yeast culture supplementation on the lag time of *Ruminobacter amylophilus* strain H18

Supplementation with yeast strains LF and UNI consistently reduced the lag time of pure cultures of *Ruminobacter amylophilus* in modified Medium A. *R. amylophilus* cultures showed reductions in average population lag times of 30.8 ± 6.6% for LF and 32.4 ± 4.6% for UNI in modified medium A with maltose as the carbohydrate source (average of Trials 3.7-3.9). Results for the starch-based media were variable. Precipitation of starch in the media caused fluctuation in the turbidimetric readings from the spectrophotometer. The yeasts VH1, 2, 3 &5 had no effect on the lag time of cultures *Ruminobacter amylophilus* indicating that the strain used affects the ability of yeasts to stimulate rumen bacteria.

Ruminobacter amylophilus has a major role in starch fermentation and the production of acetate, formate and succinate. Stimulation (reduced lag) of this bacterial population may result in more rapid digestion of starch in the rumen. However, unlike *Selenomonas ruminantium*, *Ruminobacter amylophilus* does not produce lactate (Dehority, 2003), and therefore will be less likely to cause pH depression in the rumen. Faster growth of bacteria will result in more bacterial cell mass which will make more microbial protein available for digestion in the small intestine.

5. Conclusion

The yeasts UNI and LF were consistent in their ability to stimulate (reduce lag) pure bacterial cultures of *Ruminococcus albus* strain 7; *Selenomonas ruminantium* strain GA192 and *Ruminobacter amylophilus* strain H18. Shorter lag times are, however, not evident in the graphs of culture growth *vs.* time. It may be that the sampling frequency is to low to visualize lag time in the early periods of growth. The mechanism by which the stimulation of culture growth occurred is unclear. However, if the same thing happens in the rumen, reduced bacterial lag times could mean that bacteria would grow on feed in the rumen more quickly, with the possibility of an increase in the rate of digestion. Increased rate of digestion, especially of fibre, may also result in increased feed intake due to increased rate of clearance of digesta from the rumen, as well as an increase in the amount of nutrients available to ruminants. It is important to note that the effects of yeast culture supplementation on lag time may only be relevant to those animals that are fed in discrete meals, as it is in this situation that bacterial populations of the rumen may experience lag. Animals that are fed *ad libitum* will more likely have a continuous fermentation in the rumen with little lag. In this situation it may be more relevant to measure culture growth rates, cell yield or cell output rate.

Yeast strains differ in their ability to stimulate bacteria. In this series of experiments, only two of the 11 yeast strains studied were able to consistently shorten bacterial lag time. This confirms the hypothesis that the yeast strain used in the yeast supplement is one of the factors that causes variability in response of ruminant animals to yeast supplementation.

The lag time determination method as discussed in this chapter may be used to screen yeast strains for their ability to reduce lag time in specific bacterial populations. However, experimental method as well as laboratory skill has to be refined sufficiently to give repeatable results for this type of experiment. It may be useful to combine this technique with some measure of culture growth rate or cell output rate as discussed above.

A combination of different yeasts strains may stimulate specific rumen bacterial populations. This could be used to optimize digestion and production or stabilize rumen fermentation from specific diet (Dawson *et al.*, 1995).

A shorter lag phase of bacterial populations will result in a more rapid proliferation of bacteria in the rumen. This may result in an increased rate of digestion as well as more microbial protein being available for digestion in the small intestine. This is consistent with one of the most consistently reported modes of action of yeast culture which indicates that yeast culture supplementation increases the total and cellulolytic bacterial populations in the rumen (Dawson *et al.*, 1990, Newbold *et al.*, 1995, Lila *et al.*, 2004).

Chapter 4

In vitro gas production to evaluate the rumen fermentation of feed supplemented by yeast culture

1. Introduction

Anaerobic digestion of feed components by rumen bacteria produces the following gaseous products: CO_2, CH_4, and small amounts of H_2. Volatile fatty acids are produced during the anaerobic fermentation of feeds. These volatile fatty acids react with bicarbonate buffer to yield CO_2 under *in vivo* and *in vitro* conditions. Gasses produced by the fermentation are liberated at the same time and to the same degree that fermentation of feed occurs. Measurement of *in vitro* gas production could therefore provide researchers with a real time quantitative measurement of the rumen fermentation process (Schofield *et al.*, 1994) and may provide a novel means of predicting the effects of certain additives, such as yeast culture, on the rumen fermentation.

A number of workers have used gas production to analyze aspects of the fermentation of single feed ingredients (Getachew *et al.*, 1998, Grings *et al.*, 2005) or to predict the effect of feed additives on the fermentation of single feed ingredients (Prasad *et al.*, 1994, Davies *et al.*, 2000). Few researchers have used this technique to analyze fermentations of complete animal diets or to evaluate the effect of certain feed additives on the fermentation of these complete feeds. This is due partly to the complicated modelling process needed to evaluate the data of such trials. However, if the method can be used to predict the effect of additives on the complete diet, it may provide a more accurate predictor of the effects of these additives on rumen fermentation and animal production.

The techniques used to measure gas production range from simply measuring the volume of gas collected in glass gas syringes to high tech automated pressure transducers that relay data directly to a computer terminal. These devices described measure essentially

the same property of the fermentation i.e. the volume of gas produced by the fermentation. It is this property that may be used to quantify the rate of the fermentation.

The objective of this series of experiments was to determine whether the addition of certain live yeast cultures (LF and UNI) would affect the fermentation of a complete ruminant feed by a mixed rumen microbial population.

2. Materials & Methods

The experiments were set up using 250ml Pyrex bottles as the vessels containing the experimental cultures. Standard bottle caps were fitted with one-way valves, which were sealed with a silicone sealant. An automatic pressure transducer was connected to the one-way valves *via* master flex tubing. One-way valves were fitted to only allow movement of gasses in the direction from the Pyrex vessel to the pressure transducer.

400ml of fresh rumen fluid was collected and strained through 4 layers of cheesecloth. 1.6 l of McDougall's artificial saliva (Appendix A) was bubbled under oxygen free carbon dioxide for 30 minutes to remove excess oxygen from the fermentation medium. The 1.6 l of McDougall's artificial saliva and 400ml fresh rumen fluid were combined and placed in a 3L conical flask under an oxygen free carbon dioxide gas phase. While bubbling with carbon dioxide the artificial saliva/rumen fluid mixture was put on a magnetic stirrer plate to keep it homogenous.

In the first experiment (with LF yeast) there were six treatment vessels and six control vessels. The number of experimental vessels was reduced to 10 (5 control and 5 treatment) in the second experiment (with UNI yeast) in an attempt to reduce variation by removing faulty vessels. Control vessels contained 0.5 g feed with no yeast. Treatment bottles contained 0.5g of a feed/yeast mixture containing $5x10^9$ CFU yeast (LF or UNI) per kg of feed. 0.5g samples of feed or feed/yeast mixture were sealed in Ankom F57 filter bags. Feed samples were randomly allocated and placed in their respective bottles.

The bottles were gassed under carbon dioxide for a few moments in order to displace the air and to create anaerobiosis. 100ml of the McDougall's/Rumen Fluid mixture was added anaerobically and sealed off with the cap. After all bottles were sealed, they were placed in a shaking water bath (2 oscillations/sec, 37°C) for 15 min and allowed to come to temperature. Bottles were then vented by briefly opening all of the one-way valves simultaneously. The purpose of venting was so that all experimental units started the fermentation at the same pressure and so that increases in pressure were due only to the fermentation gasses generated and not to expansion of gasses from increased temperature. Pressure transducers were then connected to the vessels and pressure was recorded and reported to a computer terminal automatically over a 72-h period.

3. Results

3.1 Assessing the linearity of pressure probes used to measure gas pressure

Gas pressure probes were tested for their ability to show accurate linear measurements throughout the measurement range. This was done by injecting increment volumes of air into the bottles and testing the pressure with the probes. This data was used to construct a curve to assess the linearity of the pressure readings.

The curves in Figure 4.2 show the linearity of the gas pressure measurement to increment additions of known volumes of air. All probes measured the same pressure for the volume of air added. All probes measure pressure linearly across the range of pressure in which the data were expected. This rules out the possibility of any bias that may have been introduced by the equipment.

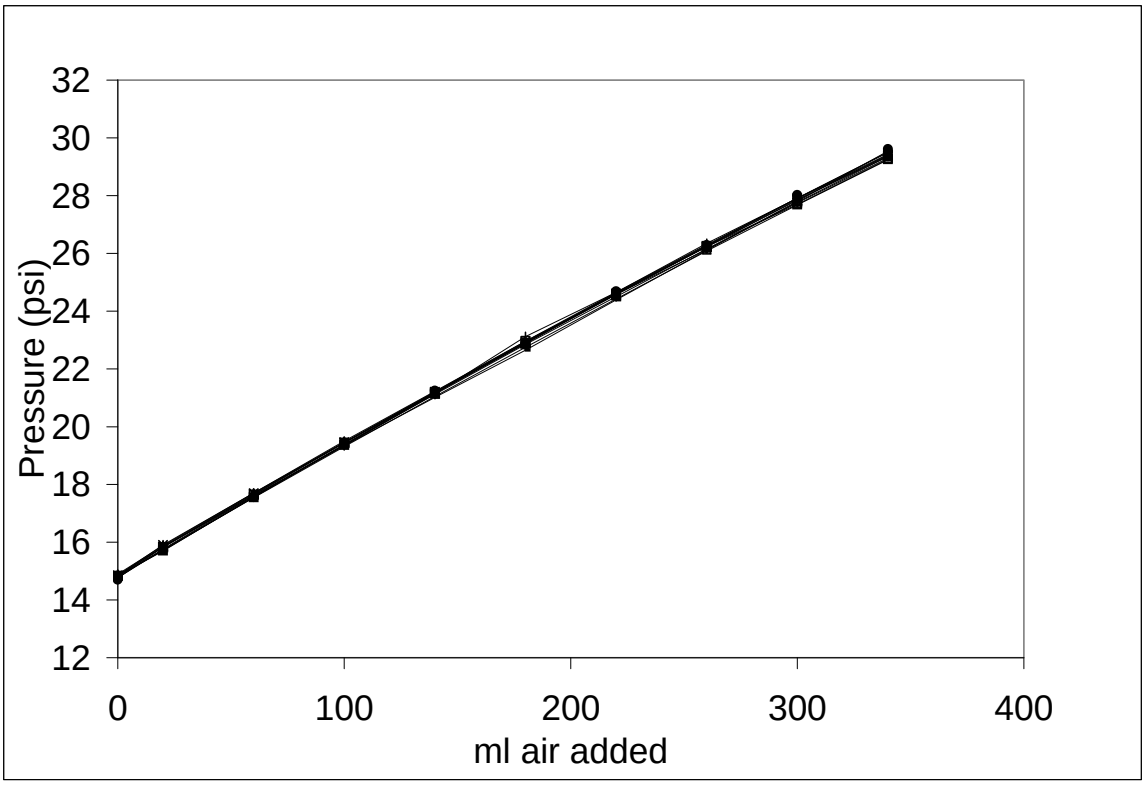

Figure 4.1 Linearity and precision of pressure probes – Increment additions of volume gave proportionate increments in pressure (PSI) for all probes

3.2 Using gas production profiles to model the fermentation process

A problem arises when one tries to describe the fermentation of a complete diet. Compound feeds are made up of a mixture of different substrates, each of which will give off different proportions of gasses and VFA when fermented. Each substrate will produce a certain amount of gas based on how much gas is produced by fermentation directly and how much is produced by buffering of the VFA that result from the fermentation of that substrate. Gas production must therefore be divided into different pools (or sources) of gas production in order to more accurately estimate the effect of certain factors on the fermentation. This may be done by performing non-linear regression on the curve of total volume of gas produced (Volume of gas is calculated directly from gas pressure measurements). It was found that a 2 pool logistic model (Schofield *et al.*, 1994) provided the best fit to the gas production curves ($R^2 > 0.99$).

This model divides the gas produced into two different pools or sources of gas production (Figure 4.2). The first pool may be described by the equation: y = A {1-exp (-B (x-C))} with A: The amount of gas produced by this pool; B: The rate at which this gas was produced and C: The lag time associated with this pool. The first pool has a faster rate of degradation and describes gas production from fermentation of starch and other soluble carbohydrates and proteins. There is also no lag time for this pool so the last term in the equation becomes 'x' instead of (x-C). The second pool may be described by the equation: y = D {1-exp (-E (x-C))} with D: The amount of gas produced by this pool; E: The rate at which this gas was produced and C: The lag time associated with this pool. This pool has a much slower rate of gas production and produces more gas over time than the first pool. This pool results from the fermentation of fibrous carbohydrates. We can then estimate the effects of treatment on the fermentation by measuring changes in the parameters that describe these pools.

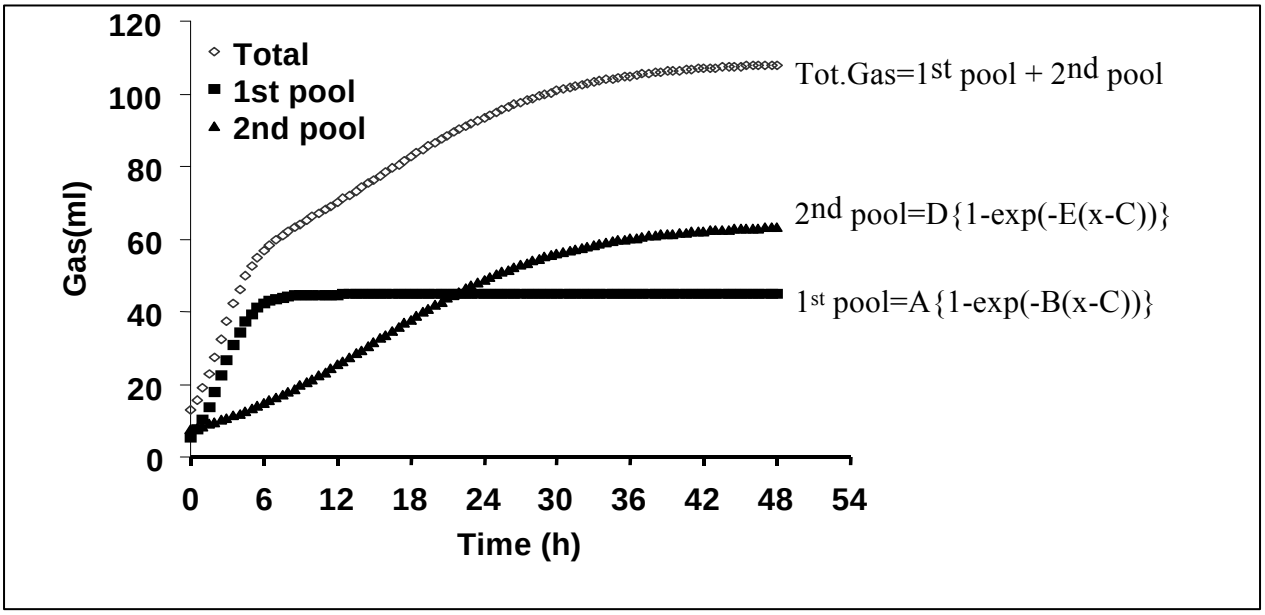

Figure 4.2 Two pool logistics model to describe the fermentation of a complete diet

3.3 The effects of yeast LF on fermentation parameters estimated by gas production

During the study of the effects of LF yeast on the fermentation of a complete feed, gas pressure built up to a maximum of between 21 and 22 PSI in the majority of the experimental vessels (Figure 4.3). Variation increased substantially after approximately 40 h and the pressure in these experimental vessels decreased towards the end of the experiment (Figure 4.3). This possibly indicated that the experimental vessels may have been leaking or losing pressure in some way although the source of this variation could not be identified. The variation made it difficult to draw conclusions from the data generated.

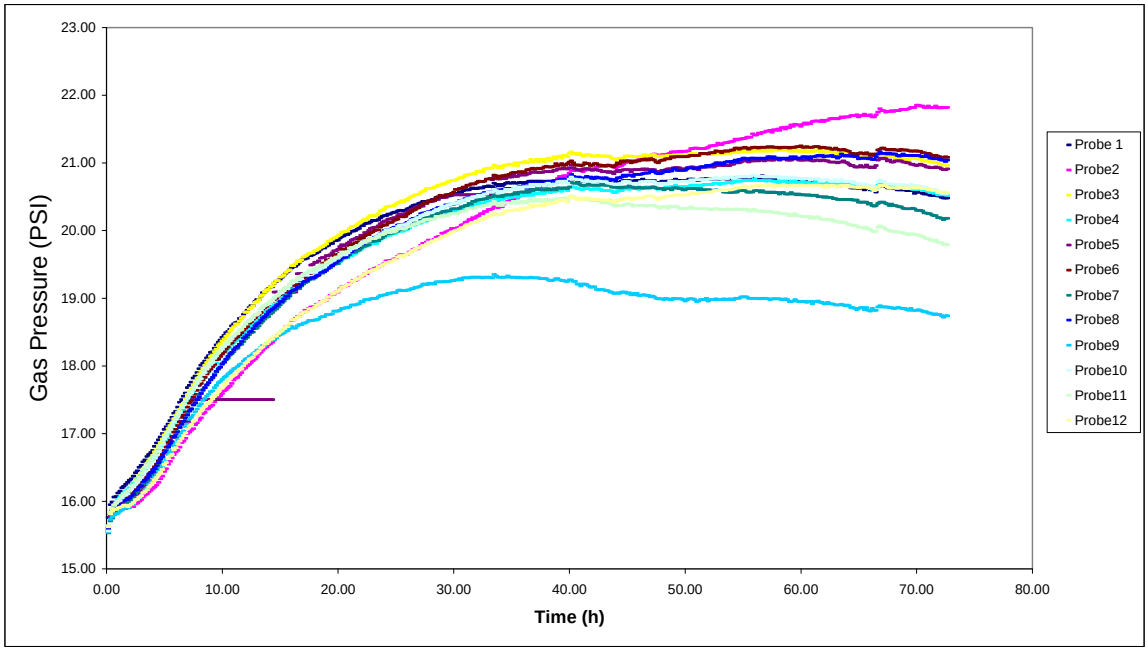

Figure 4.3 Gas pressure generated over time by cultures with and without the addition of LF yeast culture
Treatments: Control = Probes 1, 2, 3, 8, 10, 12; LF = Probes 4, 5, 6, 7, 9, 11

When pressure (PSI) data were converted to gas volume and subject to non-linear regression analysis there were no statistical differences between the volumes of gas produced (A), the rate at which the gas was produced (B) or the lag time (C) for either of the two pools of gas production (Table 4.1).

Table 4.1 Parameters describing gas production with/without LF yeast as estimated by non linear regression analysis using the 2-pool logistics model

	Control	LF
Total Gas produced	= 1st pool + 2nd pool	
1st pool	= A{1-exp(-B(x-C))}	
A (ml of gas)	22.51	23.35
B (fractional rate)	0.15	0.16
C (lag time, h)	0	0
2nd pool	= D{1-exp(-E(x-C))}	
C (lag time, h)	3.32	3.45
D (ml of gas)	49.29	53.15
E (fractional rate)	0.034	0.035

3.4 The effects of yeast UNI on fermentation parameters estimated by gas production

In the experiment on the effects of UNI yeast on the fermentation of a complete feed, variation tended to increase as the pressure increased in the experimental vessels (Figure 4.4). The source of increased variation could not be identified but may be from loss of pressure or leakage from the bottle caps, connections, masterflex tubing or even through the pressure probes themselves. Variation in pressure of fermentation gasses in the experimental units made it difficult to draw any conclusions from the data generated.

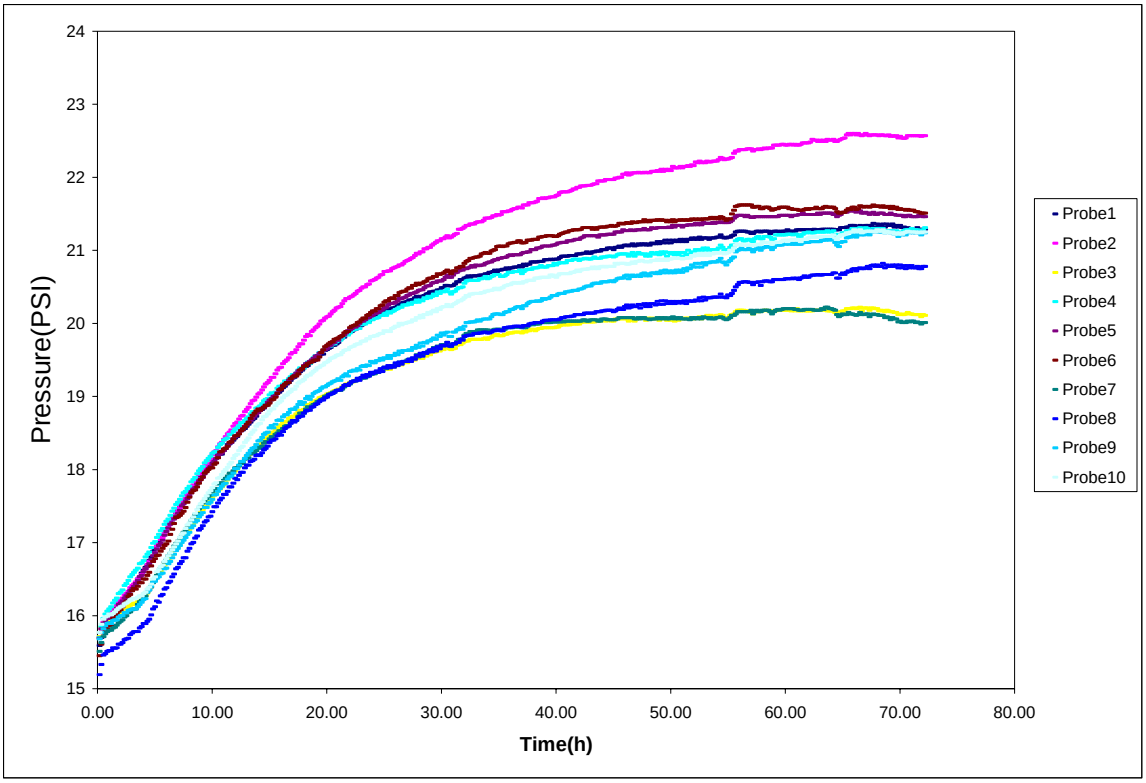

Figure 4.4 Gas pressure generated over time by cultures with and without the addition of UNI yeast culture
Treatments: Control = Probes 2, 3, 4, 6, 7; UNI = Probes 1, 5, 8, 9, 10

When pressure (PSI) data are converted to gas volume and subject to non-linear regression analysis there were no statistical differences between the volumes of gas produced (A), the rate at which the gas was produced (B) or the lag time (C) for either of the two pools of gas production (Table 4.2).

Table 4.2 Parameters describing gas production with/without UNI yeast as estimated by non-linear regression analysis using the 2-pool logistics model

	Control	UNI
Total Gas Produced	= 1st pool + 2nd pool	
1st pool	= A{1-exp(-B(x-C))}	
A (ml of gas)	28.81	35.25
B (fractional rate)	0.11	0.09
C (lag time, h)	0	0
2nd pool	= D{1-exp(-E(x-C))}	
C (lag time, h)	3.10	3.57
D (ml of gas)	49.25	46.68
E (fractional rate)	0.026	0.023

4. Discussion and Conclusion

There was no detected effect of LF or UNI yeast culture supplementation on the volume of gas produced (A), the fractional rate at which it was produced (B) or the lag time (C) for either of the two pools of gas production. The variation that was present between experimental units made it difficult to observe differences between treatments. The source of variation could not be identified. It may have come from different degrees of leaking of gas from the pressure vessels producing curves that look similar but move progressively away from each other toward the end of the time period. Some experimental units decreased in pressure towards the end of the experiments. Once fermentation is completed, gas pressure should remain constant at constant temperature. If the source of variation or decrease in pressure within the gas pressure system could be identified (this may be in the bottle caps, fittings in the caps connections to caps and probes, or even within the probes themselves), the method may be of some value in evaluating complete diets and the effect of certain additives on fermentation.

Chapter 5

The effects of the yeast UNI and monensin on rumen fermentation and digestion in rumen simulating continuous cultures

1. Introduction

The use of *in vivo* trials for analysis of animal feeds and feed additives is an expensive, time consuming and labour intensive process. This has lead researchers to look for alternative methods for analysis of animal feeds and feed additives and their potential effects on the ruminant animals. One such procedure is the development of artificial rumen technology. Early rumen simulations consisted of "batch' fermentation systems in which a quantity of feed was fermented in an enclosed vessel until a specified endpoint. These systems were followed by the development of continuous single-flow systems, simulating overflow of ruminal contents (Slyter and Putnam, 1967). The dual system was then developed with superior simulation of ruminal overflow (Garret and Yoon, 1997). Although there are fundamental differences between the fermentation that takes place in the rumen and that which occurs in *in vitro* fermenters, comparison of results from *in vivo* trials and rumen simulator trials indicate that rumen simulators can provide a reliable estimate of the rumen fermentation process (Bergen, 1977, Garret and Yoon, 1997).

UNI yeast was chosen for this study, as it was the most effective of the yeast strains studied at consistently reducing the lag time of select anaerobic bacteria. Furthermore the effect of UNI has not been studied in rumen simulating continuous cultures.

Monensin was used in this trial because it has recently received FDA approval for use in dairy diets in the United States. The effects of monensin on rumen fermentation are well documented (Daenicke *et al.*, 1982, Russell and Strobel, 1988, Sullivan and Martin,

1999, McGuffey *et al.* 2001, Shinzato *et al.*, 2006). Yeast and monensin have different modes of action and may have a complementary effect on rumen fermentation.
The objective of this experiment was to examine the effects of UNI yeast, monensin and their combination on rumen fermentation, bacterial protein synthesis and efficiency in rumen-simulating cultures.

2. Materials and Methods

2.1 Experimental Protocol

The rumen simulating continuous culture experimental units used in this experiment were single flow vessels as described by Slyter and Putnam, (1967). Twelve (12) cultures were used in a completely randomized 2x2 factorial design with 3 replicates per treatment. The two factors were UNI yeast and monensin.

The cultures were charged with approximately 1 l of fresh rumen fluid that was strained through 4 layers of cheesecloth and collected from a steer fed a 50% forage diet (DM basis).

Cultures were fed 20g DM of experimental diets per day split into two feedings at 8:00 am and 4:00 pm during the ten (10) day experimental period. The flow rate of McDougall's artificial saliva solution with no urea was maintained at approximately 30 ml/hr.

2.2 Base diet

The base diet used was formulated and balanced using CPM Dairy (Version 3.0.7a) (www.cpmdairy.com) for 40 kg milk, 3.5% fat, and 3.1% true protein for a 650 kg cow in 2nd lactation (Table 5.1).

Table 5.1 Base diet used for the experiment on UNI yeast and monensin in continuous culture

Feed Ingredient	Diet Composition %(as fed)
Grass Hay	30.00
Alfalfa Hay	10.00
Corn Grain, Ground	43.44
Soybean Meal	9.47
Distillers grains	3.30
Hallway Gainbooster mineral	1.20
Limestone	1.32
Salt	0.26
Vitamin Premix	1.01
Total	**100.00**

2.3 Treatments used in the study of the effects of yeast and monensin on rumen fermentation in continuous cultures

Experimental diets were prepared by adding treatment-specific premixes at a rate of 1% to the base diet. The treatments were as follows:

- Control: Ground corn added at 1% of diet
- UNI: 1.0 g/kg UNI-yeast (providing 5 x 10^9 CFU per kg feed)
- Monensin: 33.0 mg/kg feed of monensin.
- UNI + Monensin: 1.0 g/kg feed of UNI-yeast (providing 5 x 10^9 CFU per kg feed) and 33.0 mg/kg feed of monensin.

2.4 Sampling protocol and response variables

A 10 ml culture fluid sample was collected from all cultures immediately prior to the morning and evening feedings. This 10ml sample was used to measure pH and 1 ml from each morning sample was stored frozen for ammonia and VFA analysis. pH was measured with a standard pH meter. Ammonia was measured by the phenol-hypochlorite reaction for determining ammonia described by Weatherburn (1967). VFA analysis was performed by gas chromatography.

Flow rate was monitored by measuring the effluent weights daily at 8:30 am. Dry matter disappearance (DMD) was measured by collection of effluent samples (250.0 g) on days 8, 9 and 10. Samples were composited, subsampled (200.0 g) frozen, and freeze-dried. Apparent DMD is calculated as the dry matter fed minus the dry matter content of the effluent.

Microbial protein produced was estimated by collecting bacterial cells from each culture (separated by centrifugation) and effluent samples. Bacterial nitrogen was differentiated from feed nitrogen by determining the purine content of cultures and culture effluent. Purines were estimated according to the technique described by Ushida *et al.* (1985). On day 10, the total number of anaerobic and cellulolytic bacteria was estimated by the most probable numbers technique (Dehority, 2003).

2.5 Statistical Analysis

Digestion and microbial protein synthesis data were analyzed as a completely randomized design using the General Linear Model of SAS. Pre-planned contrasts were used to partition the main effects of UNI yeast and monensin, and the interaction term.

3. Results

None of the treatments had any effect on culture pH. Monensin increased the ammonia concentration in the Monensin treatment cultures, but not cultures with both UNI and Monensin (Interaction effect, *P*=0.049). UNI yeast alone had no detectible effect on ammonia concentration. Total VFA was elevated in the monensin only treatment but not in combination with UNI yeast (Interaction effect, *P*=0.075).

Whether supplemented alone, or in combination with UNI yeast, monensin increased the molar proportion of propionate (*P*=0.002) and reduced the acetate to propionate ratio (*P*=0.010). Monensin also reduced the molar proportion of butyrate (*P*=0.001).

There was a tendency for UNI yeast to increase apparent dry matter digestibility (*P*=0.083).

Bacterial yield (g/d) and microbial efficiency (g bacterial nitrogen produced per g dry matter digested or g Bacterial N/g DMD) were highest for cultures supplemented with UNI yeast alone, followed by the UNI-monensin combination treatment, the control, and then the monensin treatment.

UNI yeast tended to increase total anaerobic bacteria (*P*=0.071). Monensin depressed total anaerobic bacteria (*P*=0.028). Detailed results are summarised in Table 5.2.

Table 5.2 The effects of UNI yeast and monensin on rumen fermentation, digestion, microbial output and microbial efficiency

	UNI Yeast	Monensin	U+M	Control	SE	Contrasts		
						Y	M	I
Flow Rate (ml/h)	30.2	29.8	33.1	30.5	1.2	0.245	0.397	0.166
Dilution Rate (h^{-1})	0.030	0.030	0.033	0.031	0.001			
pH	6.32	6.27	6.44	6.34	0.062	0.289	0.700	0.153
Ammonia (mg/dl)	7.00	8.17	6.39	6.36	0.520	0.303	0.285	0.049
Total VFA (mM)	129.03	137.73	116.22	128.35	5.416	0.091	0.760	0.075
Proportions (mol/100mol)								
Acetate	62.11	62.42	62.26	62.72	0.632	0.558	0.913	0.733
Propionate	16.96	18.21	18.71	16.64	0.370	0.301	0.002	0.815
Isobuterate	1.78	1.67	1.73	1.78	0.039	0.463	0.091	0.421
Buterate	14.66	13.03	12.12	14.54	0.396	0.351	0.001	0.230
Isovalerate	2.91	3.07	3.46	2.74	0.132	0.068	0.011	0.432
Valerate	1.59	1.60	1.72	1.58	0.043	0.188	0.117	0.202
Acetate:Propionate	3.67	3.43	3.34	3.77	0.100	0.357	0.010	0.976
DM Digestibility (%)	48.44	53.46	49.52	47.20	1.855	0.487	0.083	0.199
True DM Digestibility (%)	68.60	69.39	67.97	62.77	4.362	0.626	0.512	0.430
Bacterial N (g/day)	0.345	0.261	0.305	0.275	0.064	0.404	0.689	0.847
g Bact N / DMTD	23.67	18.56	21.60	21.30	3.167	0.418	0.469	0.919

Y = yeast effect, M = Monensin effect, I = Yeast/Monensin interaction effect

4. Discussion

The increased molar proportion of propionate and the depressed Acetate: Propionate ratio in both monensin treatments indicates a more energetically efficient fermentation as the formation of propionate conserves more energy than the formation of acetate (Russell and Strobel, 1989).

There were higher ammonia concentrations in the monensin only treatment compared to the control. This could indicate that bacterial ammonia uptake was reduced or that de-amination of protein was increased. This is an uncharacteristic effect of monensin and is not consistent with the mode of action as described in the literature, which generally indicates that monensin has a protein sparing effect by reducing de-amination and therefore results in decreased ammonia concentrations in the rumen (Russell and Strobel, 1988, McGuffey *et al.*, 2001). There was lower bacterial N output and microbial efficiency (g Bacterial N/g DMD) was poorer for the monensin only treatment. This is consistent with the increased ammonia levels, suggesting that ammonia uptake by bacteria was reduced (i.e. less microbial protein was produced). Monensin decreased the number of total anaerobic bacteria while UNI yeast increased total anaerobic bacteria. However, when monensin was combined with UNI yeast, the negative effects of monensin on bacterial N output and efficiency are alleviated to some degree yet the proportion propionate remained higher in the combination treatment.

5. Conclusion

The effects of UNI yeast and monensin appear to be complementary on rumen fermentation. Yeast products could be used when feeding monensin to alleviate some of the negative effects of monensin on microbial protein production and efficiency while retaining the positive effects of monensin on animal feed efficiency. UNI yeast may be fed alone if the objective is to improve bacterial yield and efficiency and can be used in combination with monensin to prevent reductions in bacterial yield and bacterial efficiency if feed efficiency is a priority.

Chapter 6

Investigation into possible methods for developing a quality control assay for yeast culture products

1. Introduction

Traditionally, live yeast culture products intended for use in animal diets were controlled for quality by determining the concentration of live yeast cells or CFU's in the product. However, there has been dispute in the literature about whether live yeast cells are required for yeast supplementation to have its effect on ruminant production (Dawson *et al*., 1990, Newbold *et al*., 1996). There are reports that the presence of metabolically active yeast cells, and not the amount of yeast cells that can reproduce (i.e. CFU's) is required for yeasts to have their effect on rumen fermentation (Dawson, 2000). There is also evidence that the yeast strain used, and not just the number of yeast cells present, affects the response of ruminants to yeast supplementation (Dawson *et al*., 1995, Newbold *et al*., 1995). For this reason it was decided to find a quality control procedure that would not rely on counting the viable cells present in the yeast supplement but rather to measure the degree to which the yeast supplement stimulates rumen fermentation.

The objective of the study was to find a way to measure the degree to which cultures of *R. albus* 7 would be stimulated by yeast culture products as a quality control method. The initial assay development was performed with live yeast UNI, as UNI yeast decreased the lag time of *R. albus* 7 cultures grown on RGCB medium containing 0.15% (w/v) cellobiose as the sole carbohydrate source (Chapter 3). The *R. albus* 7 cultures were grown on Medium A, a defined growth medium for these experiments instead of RGCB, a rumen fluid containing medium (Appendix A). The properties that an ideal assay would possess:

- It should be sensitive to the parameter in question (Primary importance)
- It should be repeatable (Primary Importance)

- It should be rapid enough to be performed routinely and robust enough that results are consistent among multiple technicians

Three different analyses were assessed to determine their suitability as an assay for testing yeast culture products:

- Cellulase activity was measured. *R. albus* is a predominant cellulose digester and a measure of its cellulase activity may be an indication of how much certain yeast strains stimulate *R. albus* 7.
- Cellobiose digestion or more specifically, cellobiose disappearance from the media was measured. This could be a measure of the culture activity or rate of digestion and may be an indicator of stimulation of *R. albus* by the yeast culture.
- Culture density (Abs $_{600nm}$) was measured as an indirect measure of culture growth.

An additional experiment was performed to evaluate the effects of anaerobiosis and the presence or absence of bacterial cells on the cellulase assay. It is possible that the presence oxygen in the standard cellulase assay may inhibit cellulolysis due to the fact that the organisms responsible for cellulolytic activity are strictly anaerobic. This may limit the ability to observe the effects of yeast culture on the cellulolytic activity of *R. albus.* The cellulase activity of culture supernatant was evaluated to determine whether the cellulolytic activity of *R. albus* was directly associated with the bacterial cell or whether the cellulase is released into the medium.

The objective of the study was to determine whether the analysis of the cellulase activity, cellobiose digestion or culture density of a *R. albus* culture could served as a quality control measure of the relative ability of yeast cultures to stimulate rumen fermentation.

2. Materials and Methods

Three experiments were performed. Each of the three experiments consisted of two treatments (Control *vs.* UNI yeast) with three replicates per treatment. Experimental units were cultures of *R. albus* 7 in medium A with or without the addition of UNI yeast. The three experiments differed only in the concentration of the initial inoculum used. Initial inoculum was prepared by diluting an overnight parent culture of *R. albus* with fresh Medium A in the following ratios: 1:2, 1:5 and 1:10 in experiment 1, 2 and 3, respectively (Table 6.1). Experimental units were incubated in a water bath at 37ºC for 24 h. Samples (3 ml) from experimental units were taken at 0, 2, 4, 6 and 23.5 h after inoculation and used for the three different analyses: cellulase activity (0.2 ml x 3), cellobiose digestion/disappearance (1 ml) and culture density (Abs_{600nm}) (1 ml). The experimental cultures (25 ml) were grown in 50ml serum bottles. The growth medium did not contain Resazurin as the indicator colour would interfere with the cellulase assay (Abs_{569nm}) and culture density (Abs_{600nm}) measurements.

Table 6.1 *R. albus* inoculum levels for yeast culture quality control assay experiments

Experiment Number	Inoculum Level	Volume (ml) of overnight culture per bottle	Volume (ml) of fresh medium A per bottle
1	1:2	12.5	12.5
2	1:5	5	20
3	1:10	2.5	22.5

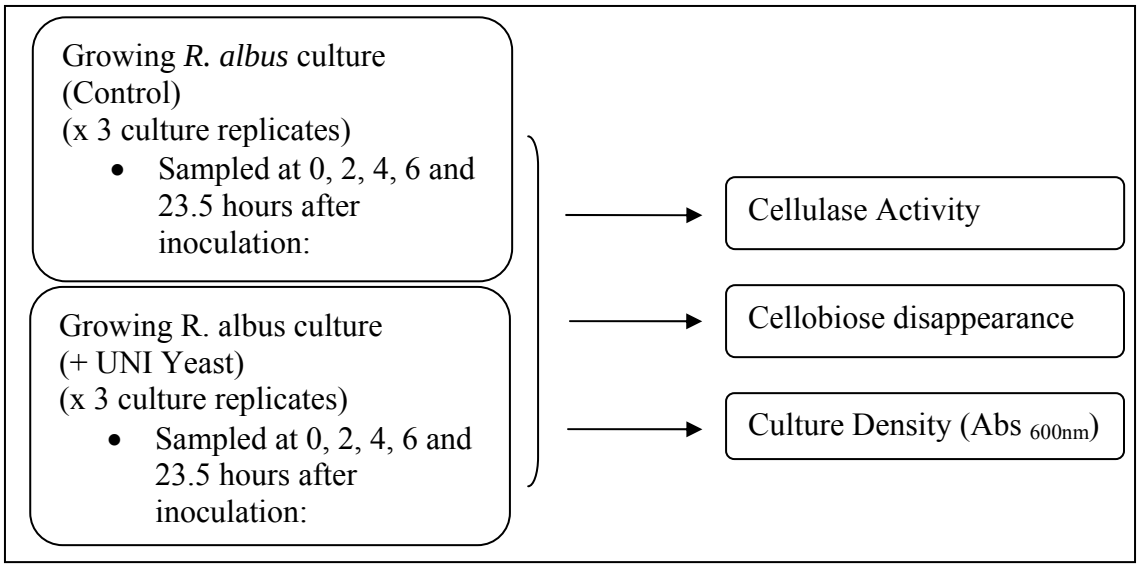

Figure 6.1 Schematic representation of experimental procedure for yeast culture quality control assay experiments

Yeast preparation: An overnight culture of UNI yeast in TSB was diluted by adding 2 ml of yeast culture to 8 ml of fresh TSB to produce a yeast preparation with an Abs_{600nm} of approximately 0.10. Each experimental bottle (25ml) in the UNI and Control treatments received 1.25 ml of the yeast preparation or TSB, respectively at the same time as inoculation with *R. albus*. The yeast preparation and TSB broth were bubbled with CO_2 for approximately 15 min to make them anaerobic prior to addition to the experimental cultures.

2.1 The determination of cellulase activity of a growing culture of *Ruminococcus albus* in Medium A

An attempt was made to detect cellulase activity in the growing culture of *R. albus* with and without the addition of UNI yeast culture. Cellulase activity was determined with the aid of the degradation marker Cellulose-Azure. Cellulose azure that is digested by *R. albus* liberates a blue colour into solution that is detectable by spectrophotometry.

Cellulase assay procedure *(As adapted from Sigma: 'Suitability assay for Cellulose Azure as a substrate for cellulase'):*

0.2 ml of experimental culture was added to 0.2 ml cellulose azure suspension (1 g in 100 ml sodium acetate buffer, pH 4.8). For the positive control, cellulase stock solution (Appendix A) was used in place of experimental culture. For the negative control, fresh medium A was used in place of experimental culture. Experimental tubes and tubes for positive and negative control were incubated in a water bath at 37ºC for 30 min.

After incubation, 0.8 ml of absolute ethanol was added to each tube and mixed thoroughly by vortexing. The purpose of the ethanol addition was to stop the cellulase activity reaction. Tubes were left undisturbed for ten minutes followed by centrifuging at 13,000 RPM for 5 minutes.

Cellulase activity was estimated by determining the absorbance of the resulting supernatant at 569nm. The blank solution used to zero the spectrophotometer was a mixture of 0.2ml of fresh Medium A, 0.2ml sodium acetate buffer solution and 0.8ml absolute ethanol (The blank was prepared in a 10ml volume and used for all experiments). The cellulase assay procedure was performed in triplicate for each time period.

2.1.1 Effect of anaerobiosis or absence of bacterial cells on cellulase activity assay

Ruminococcus albus 7 was grown on Medium A + 0.15% (w/v) cellobiose with no Rezazurin as discussed in 2.1. The cellulase activity on an overnight culture was measured by the cellulase–azure method (2.1) (Jones, 1993). Treatments were modifications of the standard method for cellulase activity (2.1) as follows:

1. Standard method – Assay was performed aerobically with *Ruminococcus albus* cell culture
2. Anaerobic – Cellulase assay was performed anaerobically in an anaerobic chamber
3. Aerobic/No cells – Assay was performed aerobically with culture supernatant (no bacterial cells present)

Cellulase assay was performed in triplicate on each of the three treatments.

Modifications to standard method:

The standard cellulose azure suspension was made anaerobic by bubbling the suspension under nitrogen gas for 30 min. Labelled Eppendorf tubes and an overnight culture of *R. albus* were put into an anaerobic chamber.

The culture for the Anaerobic treatment was added (0.2ml/tube) to triplicate Anaerobic treatment assay tubes which remained in the anaerobic chamber. The remainder of overnight culture, along with tubes for the Standard method and Aerobic/No cells treatments were removed from anaerobic chamber. The culture for Standard method was then added (0.2ml/tube) to triplicate cellulase assay tubes for the Standard method treatment.

Culture for Aerobic/No cells treatment was centrifuged at 13,000 rpm for 5 minutes and the supernatant removed. The supernatant was then added (0.2ml/tube) to triplicate

Aerobic/No cells treatment tubes. Standard method and Aerobic/No cells treatment cultures were vortexed in the presence of air in order to aerate them.

Cultures were then returned to the anaerobic chamber and cellulase activity was measure as in 2.1.

2.2 Determination of cellobiose digestion/disappearance from a culture of *Ruminococcus albus* in Medium A by HPLC analysis

The disappearance of substrate from the media may be used as a measure of bacterial growth or metabolic activity. Differences in growth or metabolic activity of *R. albus* in response to yeast supplementation may be detected by monitoring the level of cellobiose in the media by HPLC analysis.

At each sampling period, approximately 1 ml from each culture was filtered through a 0.22µm syringe filter into HPLC vials for analysis. Manoj Kudupoje performed the analyses using the standard conditions for alcohol fermentation samples (mobile phase, 0.002 M H_2SO_4; flow rate, 0.6 ml/min) and the refractive index detector. A 35-minute run time was required to allow all of the peaks associated with the medium to clear the detector. A cellobiose standard solution was analyzed first to obtain the retention time of the cellobiose peak (7.2-7.3 minutes). Cellobiose digestion was determined by comparison of the cellobiose peak area of each sample to the cellobiose peak area of Medium A (with 0.15% cellobiose).

2.3 Culture density measurement – Absorbance at 600nm

A similar method for monitoring bacterial growth was used in experiments to determine the effect of yeasts on the lag time of pure culture rumen bacteria (Chapter 2). Bacterial growth results in turbidity in the media. This can be measured by spectrophotometry. A difference in bacterial growth due to yeast supplementation may be reflected in the turbidity measurement.

The optical density of each sample (no replication) was measured at 600 nm by spectrophotometry at each time period. Media was made without Resazurin in order to make it possible to sub-sample the growing culture and measure the absorbance aerobically without causing fluctuation in the absorbance reading related to the colour change that would result if the oxygen indicator were present.

2.4 Statistical analysis

Data were analysed by the single factor analysis of variance (Anova) function of Microsoft Excel (2003).

3. Results

3.1 Effects of UNI yeast supplementation on cellulase activity, cellobiose digestion and culture growth of *R. albus* (Inoculum dilution = 1:2)

3.1.1 Cellulase activity

The cellulase activity of the experimental *R. albus* culture at 0 h was between 0.110 and 0.115 (Abs $_{569nm}$). The cellulase activity decreased after 2 h (Abs $_{569nm}$ < 0.090) for both control and UNI treatments. Cellulase activity increased to a maximum of 0.113 for the control and 0.108 for UNI yeast supplemented cultures at 6 h. The cellulase activity in *R. albus* cultures supplemented with UNI yeast was significantly higher than the Control at 4 h (Δ Abs $_{569nm}$ = 0.004; P = 0.02). The Control treatment had higher activity (Δ Abs $_{569nm}$ = 0.005; p = 0.06) than the UNI treatment at h 6. There was a numerical difference in mean cellulase activity at 23.5 h (Control = 0.121, UNI = 0.110; Figure 6.2). The differences in cellulase activity between *R. albus* culture with and without the addition of UNI yeast were minor and were only seen in the initial period. Higher cellulase activity at 4 h and lower cellulase activity at 23.5 h of *R. albus* supplemented with UNI yeast may indicate that UNI supplemented *R. albus* cultures had a more rapid initial growth (4 h) and were thus further into death phase at 23.5 h.

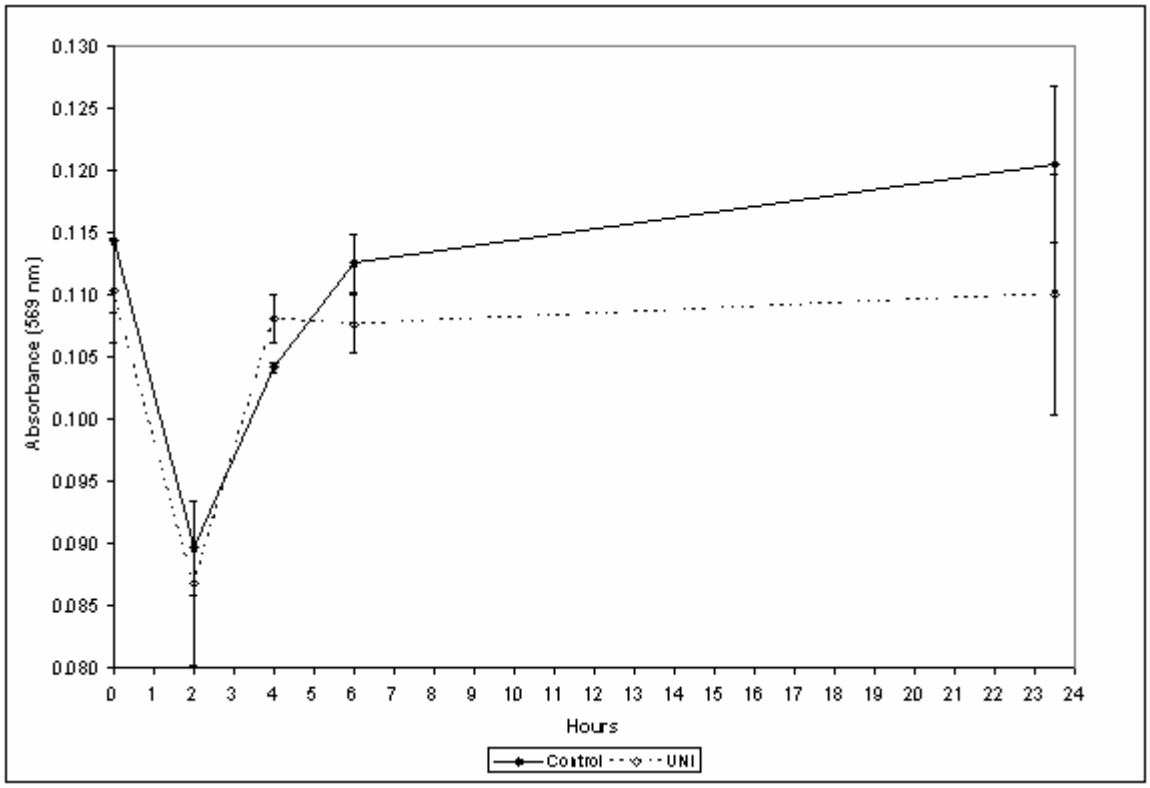

Figure 6.2 Cellulase activity (Abs $_{569nm}$) of a *R. albus* culture with an initial inoculum dilution of 1:2

Each point on the graph represents the mean of all assay replicates per treatment. Error bars represent the standard deviation

3.1.2 Cellobiose digestion/disappearance

There were no differences in cellobiose digestion/disappearance in *R. albus* cultures with or without the addition of UNI yeast at 0, 2, 4, 6 or 23.5 h (Figure 6.3). The percentage of cellobiose in the medium was approximately 80% of the initial concentration in fresh media at 0 h. This is probably due to the high level of initial bacterial inoculum used (1:2). The cellobiose content was decreased to approximately 55% by 2 h and remained above 50% after 6 h. Nearly 50% of the cellobiose remained after 23.5 h. It is unclear why nearly half of the cellobiose remained in the medium after 23.5 h. It is possible that end product accumulation inhibited bacterial growth before more cellobiose could be digested.

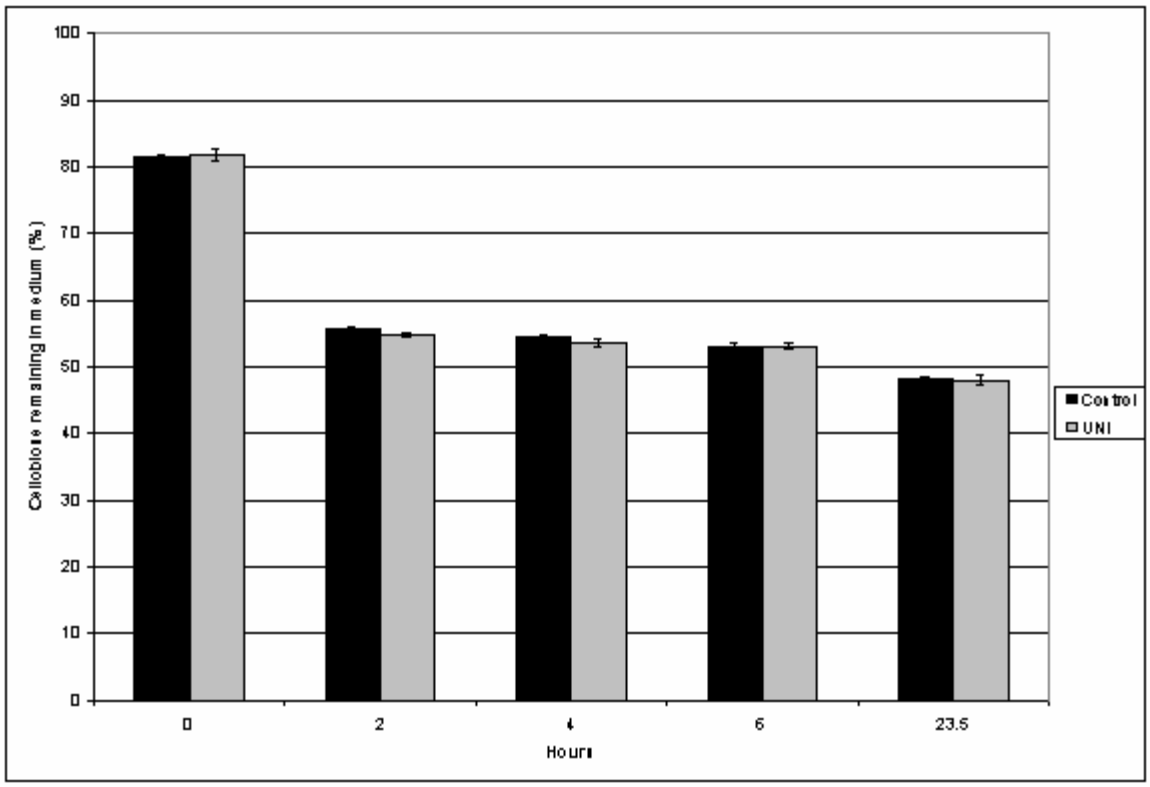

Figure 6.3 Cellobiose (%) remaining in medium of a *R. albus* culture with an initial inoculum dilution of 1:2

Each bar represents mean of three treatment replicates. Error bars represent the standard deviation

3.1.3 Culture Density (Absorbance at 600nm)

Differences in culture density were observed at 4 and 23.5 h. The UNI treatment had a higher mean culture density (0.460 *vs.* 0.448) at 4 h (P = 0.012), while the Control treatment had a higher mean culture density at 23.5 h (0.423 *vs.* 0.405) (P = 0.016). The cultures, regardless of treatment, appeared to be in stationary phase by 2 h, as indicated by the maximum culture density recorded at this time point (Figure 6.4). Cultures of *R. albus* grew rapidly due to the high initial bacterial inoculum (1:2). Higher culture density at 4 h and lower culture density at 23.5 h of *R. albus* supplemented with UNI yeast may indicate that UNI supplemented cultures had a more rapid initial growth (4 h) and were thus further into death phase at 23.5 h.

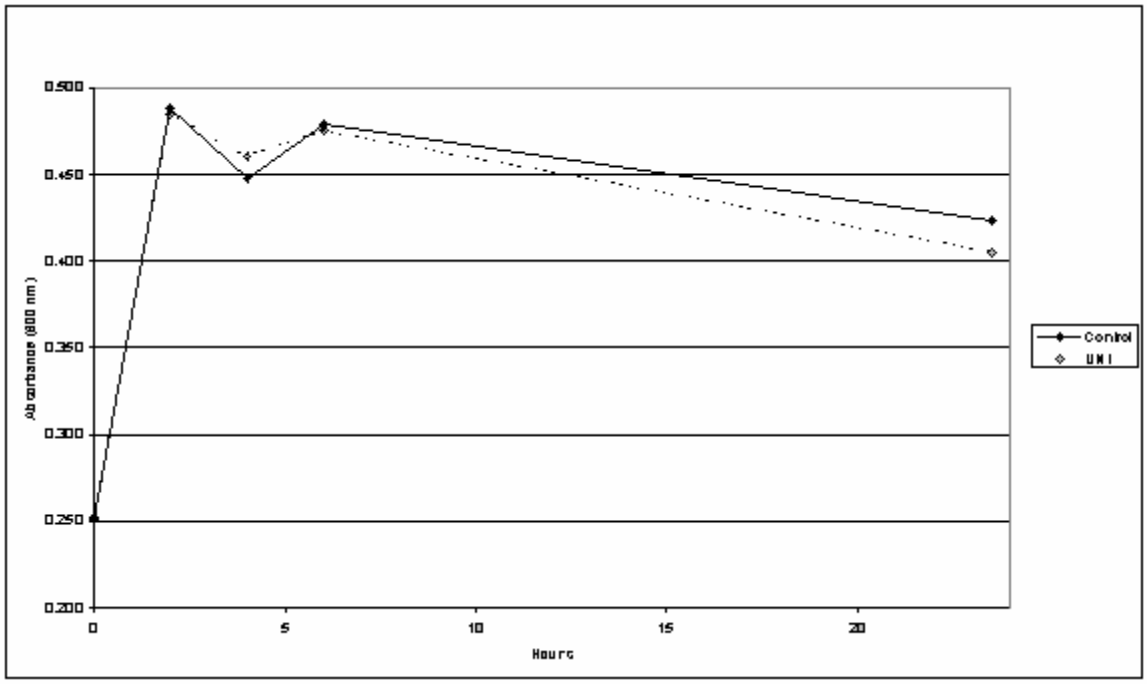

Figure 6.4 Culture density (Abs $_{600nm}$) *vs.* time of a *R. albus* culture with an initial inoculum dilution of 1:2
Each point on the graph represents the mean of treatment replicates (Abs $_{600nm}$)

3.2 Effects of UNI yeast supplementation on cellulase activity, cellobiose digestion and culture growth of *R. albus* (Inoculum dilution = 1:5)

3.2.1 Cellulase activity

The cellulase activity of cultures of *R. albus* increased to approximately 0.075 (Abs $_{569nm}$) at 2 h and to approximately 0.105 at 4 h. The maximum recorded cellulase activity was at 6 h (0.115 and 0.113 for Control and UNI treatments, respectively). The cellulase activity was numerically lower at 23.5 h for the control treatment (mean cellulase activity was 0.106 and 0.104 for UNI and Control treatments respectively). The cellulase activities were statistically similar between treatments at 0, 2, 4, 6 and 23.5 h. The increase in cellulase activity appeared to reflect the increase in culture density (Figure 6.5 and Figure 6.7).

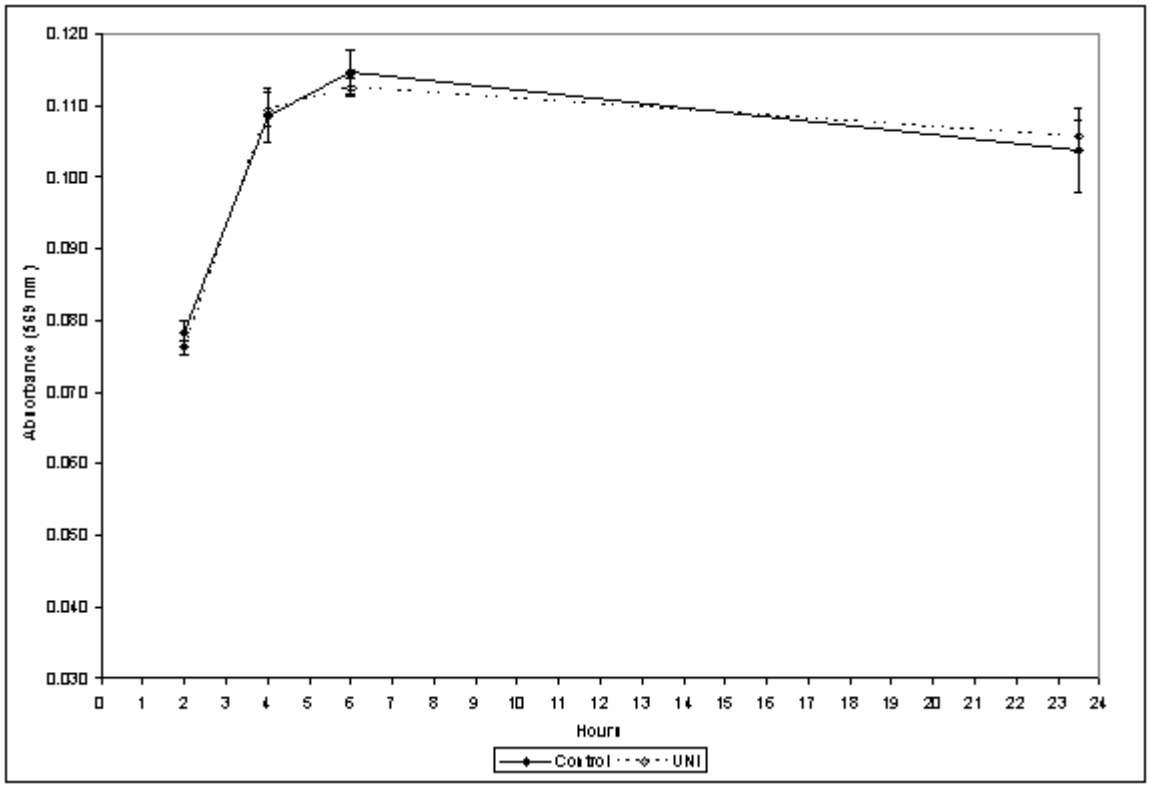

Figure 6.5 Cellulase activity (Abs $_{569nm}$) of a *R. albus* culture with an initial inoculum dilution of 1:5

Each point on the graph represents the mean of all assay replicates per treatment. Error bars represent the standard deviation

3.2.2 Cellobiose digestion/disappearance

Cellobiose digestion/disappearance was similar between treatments at 0, 2, 4, 6 and 23.5 h (Figure 6.6). The initial (0 h) relative amount of cellobiose remaining in the media was higher than in experiment 1 (approximately 98% *vs.* 80% of cellobiose present in uninoculated media) due to the lower bacterial inoculum used in this experiment (1:5). The percentage of cellobiose remaining in the medium decreased from approximately 98% at 0 h to around 83-84% at 2 h, 57-58% at 4 h, 53% at 6 h and approximately 47% at 23.5 h. Cellobiose disappearance was slightly higher in the UNI treatments up to 4 h and slightly higher in the Control treatments at 6 h and 23.5 h. There were no statistical differences in cellobiose digestion between the treatments.

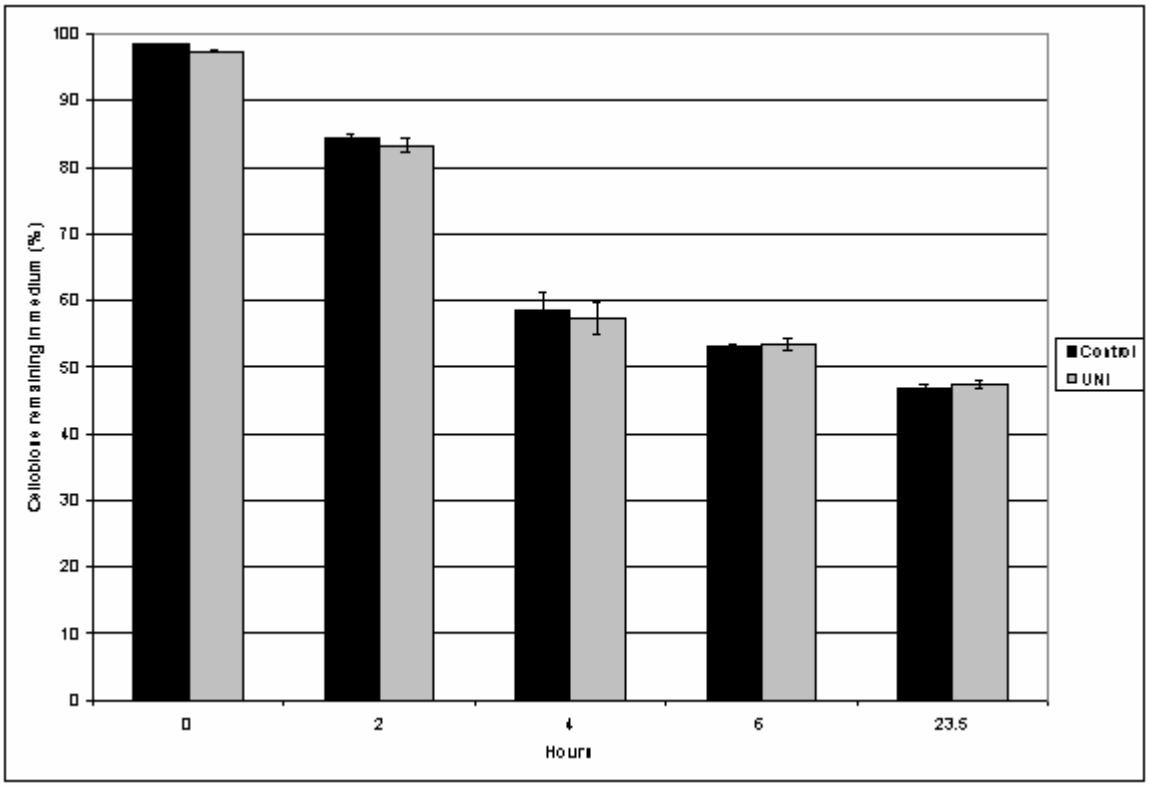

Figure 6.6 Cellobiose (%) remaining in medium of a *R. albus* culture with an initial inoculum dilution of 1:5
Each bar represents mean of three treatment replicates. Error bars represent the standard deviation

3.2.3 Culture density (Absorbance at 600nm)

The *R. albus* culture density increased from an average initial culture density of 0.107 at 0 h to a maximum of 0.515 and 0.507 for UNI and Control respectively at 6 h. The culture density was slightly higher for the UNI treatment at 2, 4 and 6 h. *R. albus* culture density was numerically higher in the control treatment at 23.5 h (mean Abs $_{600nm}$ was 0.474 and 0.428 for Control and UNI, respectively) (Figure 6.9). There were no statistically significant differences in culture growth for this experiment.

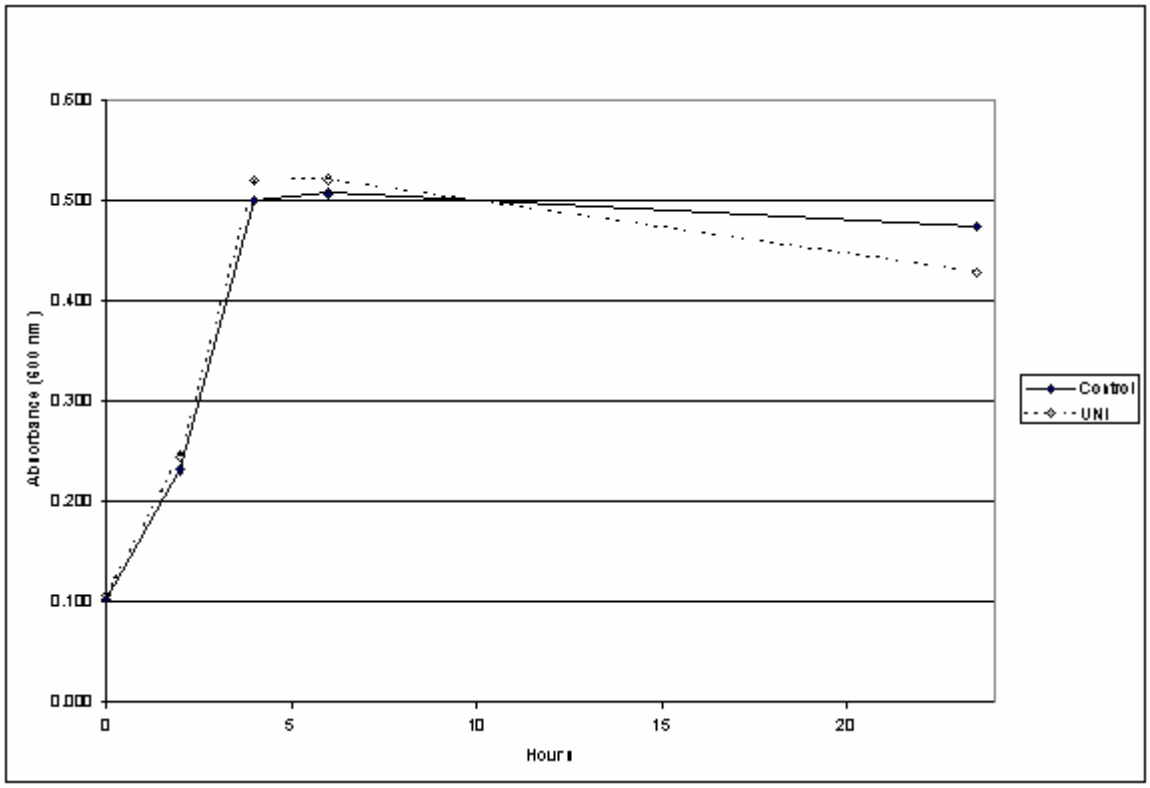

Figure 6.7 Culture density (Abs $_{600nm}$) *vs.* time of a *R. albus* culture with an initial inoculum dilution of 1:5
Each point on the graph represents the mean of the three treatment replicates (Abs $_{600nm}$)

3.3 Effects of UNI yeast supplementation on cellulose activity, cellobiose digestion and culture growth of *R. albus* (Inoculum dilution = 1:10)

3.3.1 Cellulase activity

The cellulase activity of cultures of *R. albus* increased with time from approximately 0.034 at 0 h to a maximum of 0.115 for the Control and 0.107 for the UNI treatments at 6 h. The cellulase activity of *R. albus* supplemented with UNI yeast was lower than the control at 6 h (P = 0.01). The cellulase activity of *R. albus* was not different between treatments at 23.5 h (23.5 h measurements were not included in Figure 6.8 so that differences in early cellulase activity may be visualised on the graph).

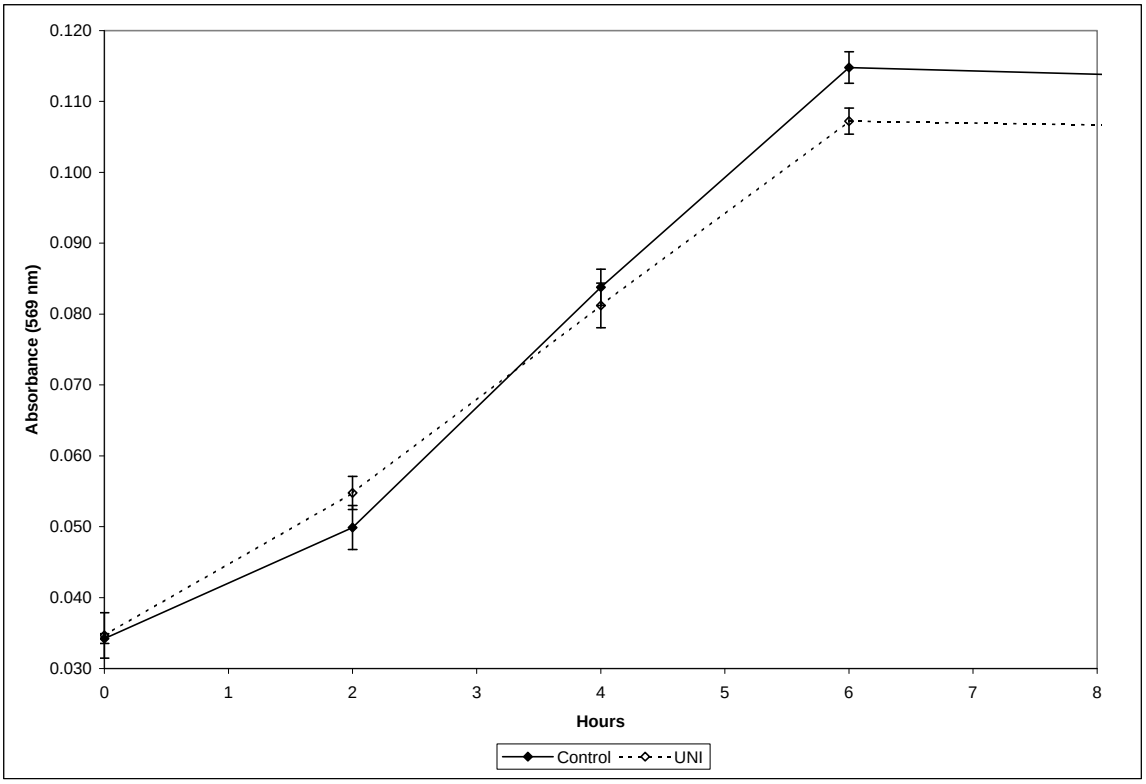

Figure 6.8 Cellulase activity (Abs $_{569nm}$) of a *R. albus* culture with an initial inoculum dilution of 1:10

Each point on the graph represents the mean of all assay replicates per treatment. Error bars represent the standard deviation

3.3.2 Cellobiose digestion/disappearance

There were no statistical differences due to treatment in the percentage cellobiose remaining in the medium at 0, 2, 4, 6 or 23.5 h. The percentage of cellobiose remaining in the medium decreased progressively from approximately 100% at 0 h, about 98% at 2 h, 75-78% at 4 h, approximately 50% at 6 h. Cellobiose remaining in the media at 23.5 h was approximately 45%. The largest numerical difference in cellobiose disappearance between treatments was observed at 4 h when the percentage cellobiose remaining in the medium was 75% and 78% for the Control and UNI treatments respectively.

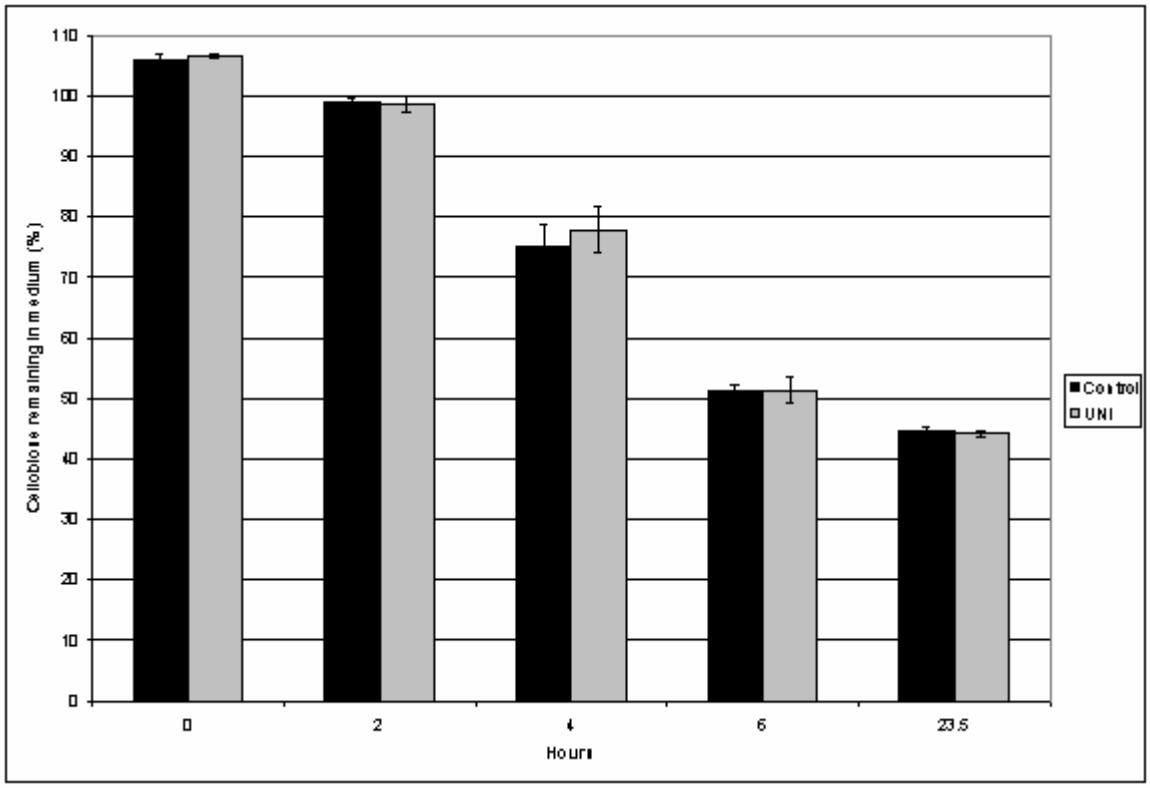

Figure 6.9 Cellobiose (%) remaining in medium of a *R. albus* culture with an initial inoculum dilution of 1:10
Each bar represents mean of three treatment replicates. Error bars represent the standard deviation

3.3.3 Culture density (Absorbance at 600nm)

The initial culture density (Abs $_{600nm}$) of *R. albus* at 0 h was low (~0.03) and increased to a maximum at 6 h of 0.545 for control and UNI yeast supplemented cultures. Although it is not clear if the cultures reached maximum density before or after 6 h, the Abs $_{600nm}$ of 0.545 at 6 h is comparable to the highest measurements recorded in the experiments with initial inoculum of 1:2 and 1:5. The mean culture density *R. albus* in the Control treatment was numerically greater than the UNI treatment at 4 h (0.285 *vs.* 0.269), and significantly greater at 23.5 h (0.484 *vs.* 0.436; $P = 0.04$).

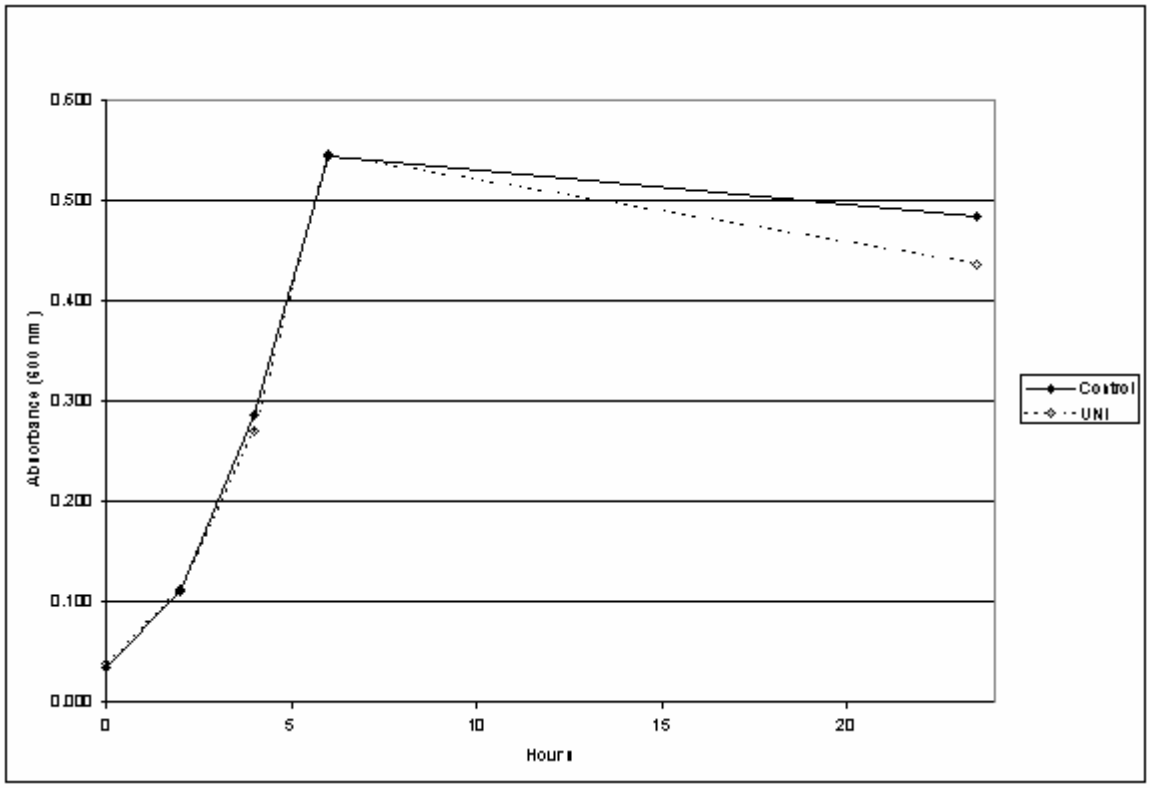

Figure 6.10 Culture Growth (Abs $_{600nm}$) *vs.* time of a *R. albus* culture with an initial inoculum dilution of 1:10
Each point on the graph represents the mean of treatment replicates (Abs $_{600nm}$)

3.4 Effect of anaerobiosis or absence of bacterial cells on cellulase activity

There was no difference in the cellulase activity recorded by the standard method and cellulase activity recorded when the bacterial culture supernatant was used in the place of whole cell culture. Anaerobic conditions reduced cellulase activity by approximately 25% (0.053 *vs.* 0.071) (Table 6.2). The replicate assays were repeatable with all values for co-efficient of variation being under 5%.

Table 6.2 The effects of anaerobiosis and absence of bacterial cells on the cellulase activity (Abs 569nm) assay

Treatment	Average A569nm	CV
Std. Method[1]	0.071	1.40845
Anaerobic[2]	0.053	2.86411
Aerobic/NoCells[3]	0.069	3.0024
Negative Control[4]	0.013	
Positive Control[5]	0.385	

1 Triplicate cellulase assay according to standard procedure. Aerobic and containing cells
2 Triplicate cellulase assay performed anaerobically but containing cells
3 Triplicate cellulase assay under aerobic conditions using culture supernatant (no cells)
4 Negative Control using medium A instead of culture
5 Positive control using cellulase standard (2.8 CMC units/ml activity)

3.5 Variability of analyses

3.5.1 Cellulase activity assay

The method of analysis exhibited acceptable variability based on the calculated coefficients of variation (CV). The coefficient of variation represents the standard deviation of the data as a percentage of the mean (CV = Standard Deviation / Mean * 100). The cellulase assays generally displayed a CV below 10 (and often near or below 5) (Table 6.3). The CVs were significantly lower when the measurements were combined by treatment replicate instead of assay replicate (Table 6.3 and 6.4). The variability was generally higher in the early time periods (0, 2 h) and significantly higher in the negative controls due to the low absorbance readings.

Table 6.3 Coefficients of variation (CV) in cellulase activity assay due to assay replication

Experiment Number	Treatment	Treatment Replicate	Hs				
			0	**2**	**4**	**6**	**23.5**
1	Control	1	2.20	2.13	3.47	1.53	5.32
		2	3.82	12.35	9.94	0.50	5.46
		3	7.15	8.01	5.09	8.67	1.68
	UNI	1	7.99	11.26	4.32	6.26	6.16
		2	9.71	4.72	4.15	6.93	8.30
		3	6.90	6.98	3.20	8.17	10.19
	Neg. Control		67.36	148.46	118.09	81.97	5.59
	Pos. Control		3.92	4.37	5.10	1.27	0.38
2	Control	1	*	8.00	5.80	3.06	4.31
		2	*	3.95	1.85	3.22	0.56
		3	*	2.60	4.89	0.51	3.06
	UNI	1	*	0.77	0.93	0.89	1.48
		2	*	4.16	1.05	1.75	5.74
		3	*	6.03	3.62	0.52	7.45
	Neg. Control		*	124.90	6.66	12.50	96.44
	Pos. Control		*	2.62	1.70	1.38	0.14
3	Control	1	2.94	5.43	6.15	1.77	4.69
		2	1.71	4.06	3.27	1.02	3.94
		3	4.95	5.77	2.47	3.56	2.14
	UNI	1	10.86	1.10	4.20	10.96	1.48
		2	11.36	0.00	1.86	4.59	1.55
		3	10.54	4.64	1.97	1.42	0.55
	Neg. Control		45.83	49.49	35.25	20.00	22.91
	Pos. Control		1.52	10.62	3.35	1.51	1.69

*** 0 h values were not available for trial 5.2 due to technical difficulties**

Table 6.4 Coefficients of variation (CV) in cellulase activity assay due to treatment replication

Experiment Number	Treatment	Hs				
		0	**2**	**4**	**6**	**23.5**
1	Control	4.96	4.19	0.37	2.10	5.20
	UNI	3.77	7.58	1.75	2.14	8.78
2	Control	*	2.35	3.42	2.70	5.63
	UNI	*	1.34	2.14	1.12	2.07
3	Control	2.03	6.21	3.04	1.93	3.26
	UNI	9.27	4.27	3.88	1.71	3.48

*** 0 h values were not available for trial 2 due to technical difficulties**

3.5.2 Cellobiose digestion/disappearance assay

This method of analysis exhibited acceptable variability based on the calculated coefficients of variation (CV). The CVs of the cellobiose peak areas (HPLC) were very low (often below 2 or 1) indicating very low variability (Table 6.5).

Table 6.5 Coefficients of variation (CV) in cellobiose remaining in culture medium (cellobiose peak area) due to treatment replication

Experiment number	Treatment	Hs				
		0	**2**	**4**	**6**	**23.5**
1	Control	0.48	0.74	0.70	0.76	0.70
	UNI	1.09	0.76	1.16	0.94	1.62
2	Control	0.09	1.03	4.65	0.49	1.20
	UNI	0.15	1.17	4.23	1.54	1.10
3	Control	0.71	0.67	4.78	1.81	1.27
	UNI	0.34	1.27	4.81	4.44	1.35

4. Discussion

Cellulase activity: The treatments were statistically different with respect to cellulase activity at only a few time points across the three experiments. However, the differences in cellulase activity were extremely small (e.g. maximum Δ ABS_{569} of 0.008) and do not appear to be biologically significant. In order to use this procedure as an assay, there would need to be a larger range of values in order to compare various yeast species' effect on the *R. albus* culture. The analysis as performed is not sensitive enough for measuring the parameter in question. The variability of the assay was acceptable (CV < 10%) and future experiments will require only one assay reaction per treatment replicate (rather then triplicate reactions). It is not clear why the cellulase activity in experiment 1 decreased significantly between 0 h and 2 h, in spite of the significant increase in bacterial culture density.

Cellobiose digestion/disappearance: There was no difference between treatments at any time for any of the 3 experiments. The procedure as performed was either performed at the wrong stage of culture growth or is not sensitive to the required parameter. The repeatability of replicates in this analysis is very good, with no treatment combination having a co-efficient of variation greater than 5%. It is not clear why the cellobiose in the medium remained around 50% even after 23.5 h in all experiments. It is possible that growth of the culture was curbed by end product accumulation before all of the carbohydrate source could be degraded.

Culture Growth Measurement: There were statistically significant treatment differences in *R. albus* culture density (Abs $_{600nm}$) at 23.5 h in experiments 2 and 3, but only a numerical difference in experiment 1 ($P = 0.16$). Bacterial culture density in the Control treatment was greater at 23.5 h than in the UNI treatments at all three inoculum levels (1:2, 1:5 and 1:10), suggesting a treatment effect at this time point. It is not clear why the Control treatments had consistently higher bacterial culture densities at 23.5 h but it may reflect a more rapid initial growth of *R. albus* cultures supplemented with UNI yeast. This may have resulted in UNI yeast supplemented bacterial cultures being further into

death phase at 23.5 h. It is possible that the early higher growth rate of *R. albus* supplemented with UNI yeast was undetected under the experimental conditions used. Another possibility is that yeast cultures may have produced by-products that inhibited the growth of the *R. albus* culture. It appears therefore that the sampling of growing *R. albus* cultures only intermittently by spectrophotometry is not appropriate for measuring the effects of yeasts on *R. albus*.

Effect of anaerobiosis or absence of bacterial cells on cellulose digestion: The cellulolytic activity of a *R. albus* culture was higher under aerobic conditions and it is therefore unlikely that air in the cellulase assay was affecting our ability to detect the stimulation of cellulase activity of *R. albus* by yeast containing products. The absence of bacterial cells had no effect on the cellulase activity of the culture. This may indicate that the cellulolytic activity is not directly associated with the cell but was released into the media. Culture supernatant may therefore be used in the place of whole cell culture when evaluating the effects of yeast on cellulase activity of *R. albus* in medium A.

5. Conclusion

The experiments in this report did not suggest which initial inoculum level or which assay candidate would be best for future yeast culture quality control assay development. It should be noted that the lowest inoculum size used in these experiments (1:10) was approximately five times more concentrated than the inocula used in the lag time determination assays that have shown the effects of UNI on the lag time of cultures of *R. albus* 7 (Chapter 3). It is not clear if the addition of UNI yeast did not stimulate the *R. albus* 7 cultures in these experiments or if the analyses as performed were unable to detect the stimulation using the selected response variables.

Conditions of anaerobiosis resulted in reduced cellulase activity in the cellulase assay, indicating that it may not be beneficial to attempt the assay anaerobically. However it should be noted that this experiment was not conducted with yeast addition. One could

speculate, for instance, that yeast improves cellulase activity under anaerobic conditions. There was no difference in cellulase activity when bacterial cells were absent. This may suggest that the cellulase activity is not directly associated with the *R. albus* cell.

Chapter 7

General Discussion and Conclusion

It is necessary to indicate the relevance of each section of the work done to the potential effects on production in ruminants.

The yeasts UNI and LF are consistent in their ability to stimulate (reduce lag) pure bacterial cultures of *Ruminococcus albus* strain 7, *Selenomonas ruminantium* GA192 and *Ruminobacter amylophilus* H18. Although the mechanism of action of this stimulation is unclear, if the same thing happens in the rumen, reduced bacterial lag means that bacteria will grow on feed in the rumen more quickly, with the possibility of an increase in the rate of digestion and therefore increased intake. Also, it should be stressed that not just any yeast can be used to alter production, as yeast cultures differ in their ability to stimulate anaerobic rumen bacteria. The method developed may be used to screen yeasts for the ability of specific yeast strains to stimulate specific bacterial populations. It has exciting prospects for manipulation of rumen fermentation. Combination of different yeasts strains may stimulate specific rumen bacterial populations. This could be used to optimize digestion and production or stabilize rumen fermentation from a specific diet.

Stimulation of rumen bacteria by yeast culture may indicate that bacteria grow more rapidly (decreased lag). Therefore they will colonize feed more quickly and increase both the rate of digestion and the amount of bacteria in the rumen at any give time. Increased bacterial levels in the rumen have the potential to provide more high quality microbial protein to animals supplemented with yeast culture.

Other methods for measuring the ability of yeast culture to stimulate bacteria or fermentation such as the gas pressure analyses, and Yea-Sacc assay techniques may be valuable techniques for rapid analysis of new strains of yeast. The modeling of the rumen fermentation process by monitoring gas production by the fermentation offers the

exciting prospect of analyzing diets or the effect of certain additive to diets with a simple analysis that does not require expensive equipment and that can be performed on multiple feeds in a short period of time.

It appears form the work done on UNI yeast and monensin in the rumen simulating continuous cultures that UNI yeast and Monensin may have a complementary effect on rumen fermentation. The increased molar proportion of propionate and depressed ratio of Acetate: Propionate is seen in monensin treatments alone and in combination with UNI yeast. This indicates a more energetically efficient fermentation as the formation of propionate conserves more energy than the formation of acetate (Russel and Strobel, 1989). There were numerically lower bacterial N output and microbial efficiency for the monensin only treatment. Monensin depressed total anaerobic bacteria. This is a commonly observed effect of Monensin due to the fact that it is an antimicrobial agent. UNI yeast increased total anaerobic bacteria. This is consistent with the literature, which indicates that increased bacterial populations may be central to the mode of action of yeasts on rumen fermentation/production. When monensin was combined with UNI yeast, the negative effects of monensin on bacterial N output and efficiency appear to be alleviated. The proportion of propionate remained higher in the treatment with both UNI yeast and monensin. The effects of UNI yeast and monensin therefore appear to be complementary on rumen fermentation. Yeast products could be used to alleviate some negative effects on microbial protein production and microbial efficiency seen when monensin is fed to ruminants while retaining the positive effects of monensin on animal efficiency. UNI yeast may be fed alone if the objective is to improve bacterial yield and efficiency and can be used in combination with monensin to prevent reductions in bacterial yield and efficiency if feed efficiency is a priority.

Chapter 8

Critical Evaluation

The effect of ten strains of Saccharomyces cerevisiae on the lag time of three pure culture anaerobic rumen bacteria (Chapter 3): The trials to evaluate the effects of various yeast cultures in rumen bacteria were mostly successful. The biggest hurdle to the successful completion of these trials was learning the laboratory skill required to keep anaerobic bacterial cultures both pure and anaerobic. If the trials were to be redone, it may be beneficial to evaluate more species of rumen bacteria that are responsible for the fermentation of major feed components in the rumen. It may be important to evaluate major lactate utilizing species such as *Megaspheara elsdenii*. This may provide a more complete picture of how yeast culture addition may affect the rumen fermentation as a whole. As mentioned previously, lag time of bacterial cultures may not be the most appropriate parameter to measure the effects of yeast culture on rumen bacteria. It may be a good idea to combine lag time data with data on culture growth rates or cell output rates especially if one is modelling the continuous fermentation that occurs when an animal is fed *ad libitum*. It may also be beneficial to analyse the amino acid content of these bacterial populations to determine how changes in these populations would affect the amino acid balance of the animal.

In vitro gas production to evaluate the rumen fermentation of feed supplemented by yeast culture (Chapter 4): The trials evaluating the gas pressure that was produced by the fermentation of animal feed in rumen fluid were unsuccessful due excessive variation between experimental units. The source of variation could not be identified but loss of gas or gas pressure from the units seemed likely. It was later found that the silicone sealant used to assemble the gas-measuring units is partially gas permeable and may have contributed to the excessive variation. If these experiments were to be re-attempted it may help use a gas tight sealant to construct the apparatus or to use experimental vessels that are designed for holding and measuring gas pressure instead of attempting to

construct these from existing laboratory equipment. This would allow workers to evaluate data that is not obscured by the experimental equipment.

Chapter 9

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Chapter 10

Appendices

Appendix A: Preparation of Media

1. RGCA

Rumen Fluid-Glucose-Cellobiose Medium (RGCA)

(Modified medium 98-5 from Bryant and Robinson. 1961. J. Dairy Sci. 44:1446-1456)

	Amount per			
Ingredient	100 ml	250 ml	500 ml	1 L
Glucose	0.05 g	0.13 g	0.25 g	0.50 g
Cellobiose	0.05 g	0.13 g	0.25 g	0.50 g
Soluble starch	0.05 g	0.13 g	0.25 g	0.50 g
Trypticase	0.5 g	1.25 g	2.5 g	5.0 g
Agar	2.0 g	5.0 g	10.0 g	20.0 g
Mineral solution 1	7.5 ml	18.75 ml	37.5 ml	75.0 ml
Mineral solution 2	7.5 ml	18.75 ml	37.5 ml	75.0 ml
Clarified rumen fluid	40.0 ml	100 ml	200 ml	400 ml
Reasazurin solution	0.10 ml	0.25 ml	0.50 ml	1.0 ml
Distilled water	38.0 ml	95.0 ml	190 ml	380 ml
Sodium carbonate solution	5.0 ml	12.5 ml	25.0 ml	50.0 ml
Cysteine solution	2.0 ml	5.0 ml	10.0 ml	20.0 ml

Mix all the ingredients except for the agar, sodium carbonate and cysteine solutions. Adjust the pH to 6.8 with sodium hydroxide. Place the mixture in a round bottom flask (or an erlenmayer flask), add the agar and boil to drive off the oxygen (resazurin turns pink). Place the flask in a water bath at 55°C to prevent agar from solidification. Cool the flask under CO_2. Add the sodium carbonate and cysteine solutions. Sparge the medium

with CO_2 until it turns golden-brown (pink or red indicate presence of oxygen). Dispense the medium under CO_2 in 4 ml volumes (13x100 mm tubes, #00 stoppers) for slants or 7 ml volumes (18x150 mm tubes, #1 stoppers) for roll tubes. Place the tubes in a press (use a metal rack) and sterilize at 120°C (15 lb) for 15 min. Store the medium at room temperature.

2. Media A

Preparation of Media A

Component	Amount/100ml
Glucose	0.05 g
Cellobiose	0.05 g
Starch	0.05 g
Trypticase	0.50 g
Mineral solution 3A	5.0 ml
Vitamin solution	1.0 ml
Hemin solution	1.0 ml
VFA solution	1.0 ml
Ferrous sulfate solution	1.0 ml
Methionine solution	1.0 ml
Ammonium sulfate solution	1.0 ml
10% Sodium hydroxide solution	1.9 ml
Resazurin solution	0.1 ml
Distilled water	80 ml
Sodium Carbonate solution	5.0 ml
Cysteine solution	2.0 ml

Combine all components except the cysteine and carbonate solutions. Boil the mixture to drive off the oxygen. Cool the mixture under oxygen free carbon dioxide. Adjust the pH to 6.8 while bubbling with CO_2. Add cysteine and carbonate solutions. Tube the medium under CO_2 gas phase. Stopper with butyl rubber stoppers and sterilize in a tube press. Media will turn yellow after autoclaving.

3. Anaerobic Dilution solution

Pre-reduced Anaerobic Dilution Solution

(Bryant and Robinson. 1961. J. Dairy Sci. 44:1446-1456)

	Amount (ml) per			
Ingredient	100 ml	500 ml	1 L	3 L
Mineral solution 1	3.75	18.75	37.5	112.5
Mineral solution 2	3.75	18.75	37.5	112.5
Rezasurin solution	0.1	0.5	1.0	3.0
Distilled water	87.0	435.0	870.0	2610.0
Cysteine solution	2.0	10.0	20.0	60.0
Sodium carbonate solution	5.0	25.0	50.0	150.0

Mix all the ingredients except the cysteine and sodium carbonate solutions. Bring the mixture to a boil to drive off the oxygen (maximum 5 min). Cool under CO_2 (use an ice bath for quicker cooling). Add the cysteine and sodium carbonate solutions and continue bubbling with CO_2 until the solution is colorless (rezasurin is reduced). Dispense the solution under CO_2 in 9 (18x150 mm, #1 stoppers) and 99 (square dilution bottles, #3 stoppers) ml volumes. Place the tubes/bottles in a press and sterilize at 120°C (15 lb) for 15 min. Store the solution at room temperature inside the anaerobic chamber.

4. TSB

Bacto

Tryptic Soy Broth: Soybean-casein Digest Medium

Becton, Dickinson and Company

Sparks, MD 21152

USA

5. TSA

Bacto

Tryptic Soy Agar: Soybean-casein Digest Medium

Becton, Dickinson and Company

Sparks, MD 21152

USA

6. Yeast Peptone

(Alltech – methods of analysis)

Component	Amount (g/l)
Bacto Peptone	1.0
Sodium Chloride	8.5
Tween 80	1.0

7. DRBC agar

Difco

Dichloran-Rose Bengal-Chloramphenicol Agar

Becton, Dickinson and Company

Sparks, MD 21152

USA

8. McDougall's Artificial Saliva

McDougall's Artificial Saliva Solution

(McDougall 1948. Biochem. J. 43:99-109)

Component[1]	g/liter	4 liters (g)	20 liters (g)
$NaHCO_3$	9.80	39.2	196.0
Na_2HPO_4	3.71	14.84	74.2
NaCl	0.47	1.88	9.4
KCl	0.57	2.28	11.4
$CaCl_2$ (anhydrous)	0.04	0.16	0.8
$MgSO_4*7H_2O$	0.12	0.48	2.4
Urea[2]	0.5	2.0	10

[1] Component: $NaHCO_3$ = sodium bicarbonate; Na_2HPO_4 = sodium phosphate (dibasic anhydrous); NaCl = sodium chloride; KCl = potassium chloride; $CaCl_2$ = calcium chloride (anhydrous) (can replace with 1 ml of a 4% (w/v) $CaCl_2$ solution per 1 L); $MgSO_4*7H_2O$ = magnesium sulfate (heptahydrate).

[2] Add Urea only when preparing solution to be used in rumen-simulating continuous cultures.

Weigh all ingredients except $CaCl_2$ into a flask, add half the volume of distilled water (eg: 500 ml when preparing 1 L) and stir until dissolved. Adjust to volume with distilled water and store. Immediately prior to use add $CaCl_2$ (solid or solution), keep at 39°C and bubble CO_2 until the pH is 6.8 to7.0.

9. Cellulase Solution

Cellulase solution (for cellulase assay positive control): Cellulase concentrate (Ultimase ACP-1; #BRACP-100305; 14,000 CMC units/g) was diluted in Medium A (0.01 g cellulase in 50 ml medium) to produce a stock solution with 2.8 CMC units/ml activity. The stock solution was aliquoted into Eppendorf tubes and frozen at -20ºC. The cellulase solution (0.2 ml) was added as a positive control in place of the culture in the cellulase assays.