THE ISOLATION, CHARACTERIZATION AND

UTILIZATION OF *PROPIONIBACTERIUM*

AS A DIRECT-FED MICROBIAL

FOR BEEF CATTLE

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FORMAT OF THESIS

This thesis is prepared as outlined by the Oklahoma State University graduate style manual. This thesis is presented in the Journal of Animal Science style format. The use of this format allows for independent chapters to be suitable for submission to scientific journals. Each paper is complete in itself with an abstract, introduction, materials and methods, results and discussion, and literature cited section. In some instances an implications section was included.

CHAPTER I

INTRODUCTION

For many years, ruminant nutritionists and microbiologists have been interested in manipulating the rumen microbial ecosystem to improve production efficiency of domestic ruminants. As much as 12% of the energy in feed may be converted to methane and lost via eructation in ruminant animals; thus, much research has been conducted over the past 20 years aimed at reducing feed energy losses associated with methanogenesis in the rumen. Perhaps the most studied and widely used group of feed additives is the ionophores, which are thought to improve feed utilization by increasing the amount of metabolizable energy available to the animal as propionate, resulting in a decreased feed:gain ratio (Bergen and Bates, 1984). This theory is supported by the observation that propionate-producing ruminal bacteria (i.e., *Selenomonas ruminantium*) are more resistant to ionophores than other ruminal bacteria.

Growing concerns over the use of antibiotics and other growth stimulants in the animal feed industry increased interest in evaluating the effects of direct-fed microbials (DFM) on animal performance. However, compared to ionophores, little research has been accomplished to evaluate the usefulness of DFM on ruminal microbial fermentation and ruminant performance. The need for a safe food supply has motivated researchers to investigate and develop new non-antibiotic, or "natural" feed additives.

Consumers' demands for tender, flavorful meat has led producers to accelerate production practices of adapting cattle to high concentrate diets. In doing so, producers are fraught with increasing incidences of a metabolic disorder known as lactic acidosis. The acid-sensitive ruminal microorganisms are unable to assimilate the increasing concentrations of lactate quickly enough to prevent rumen pH from dropping as a result. As the pH drops, rumen function is inhibited, digestion comes to a halt, and the lactic

1

acid is absorbed into the blood stream. Substantial decreases in blood pH can cause death. Often animals that do not succumb to the disease, the low ruminal and blood pH, and the sloughing of the epithelial lining of the rumen, will have an increased likelihood of sepsis and bacterial colonization which can result in liver abscesses.

Producers have incorporated intensive management practices to prevent the occurrence of acidosis. Animals are adapted gradually by diluting grains with roughage. Another widely practiced strategy has been to add chemical or antimicrobial compounds to the feed, such as ionophores which inhibit lactic acid production (Muir et al., 1981). Much interest has been generated over the past few years in manipulating the ruminal microflora to enhance feedstuff utilization and alleviate problems associated with current feeding practices. Stabilization of ruminal fluid pH by enhancing the lactate-utilization capabilities of ruminal bacteria (*Sel. ruminantium*) without using antibiotics or ionophores has the potential to improve performance and overcome the economic losses associated with acidosis of the rumen (Martin et al., 1992). Direct-fed microbials such as *Megasphaera elsdenii* can reduce the incidence of the lactic acidosis by reducing accumulation (Greening et al., 1991). These approaches rely on the lactate-utilizing properties and ruminal survivability of specific probiotic cultures.

Historically, propionibacteria strains have been used in the Swiss cheese industry to utilize lactic acid and produce propionate. The objective of this study was to determine if selected strains of propionibacteria (Parrott, 1996) could survive in the rumen and inhibit the accumulation of lactic acid when cattle were adapted at an accelerated rate to a readily fermentable diet rich in carbohydrates. The indigenous ruminal strains of *Propionibacterium* were isolated and characterized to define population parameters and allow for identification of inoculated strains.

2

CHAPTER II

DIRECT-FED MICROBIALS

A diverse community of microorganisms comprise the rumen ecosystem. The ruminal bacteria have evolved to grow and reproduce at a pH between 5.5 and 7.0 under anaerobic conditions at temperatures between 37° and 40° C. The rumen environment is constantly changing due to fermentation products and substrates from ingested feeds. The continuous removal of fermentation products and steady supply of feed ingesta allows a relatively constant condition for the dense populations of bacteria to flourish (Hungate, 1966).

Microbes play an essential role in the nutrition of ruminants. The bacteria are responsible for producing essential metabolites which ferment the organic compounds found in the ingested feedstuffs. The process of fermentation yields organic acids excreted by the bacteria and then taken up by the absorptive epithelial cells that line the gastrointestinal tract. Once absorbed into the bloodstream, the organic acids are utilized as the primary energy source for the ruminant.

A direct-fed microbial (DFM) must successfully compete and adequately perform within this complex ecosystem. DFM products can be defined as live bacterial or yeast cultures that are either administered orally or ingested when mixed with feeds or premixes. In theory, direct-fed microbial preparations are meant to augment normal ruminal microbiota functions making the rumen more nutritionally efficient or more resistant to stress-induced diseases. Direct-fed microbials have also successfully been used to prevent toxicologically detrimental diseases such as nitrate toxicity (Swartzlander, 1994) and grass tetany (tricarballylate) (Cook et al., 1994). Therapeutic responses such as these mentioned can be accomplished with inoculation of direct-fed

organisms either into the lumen of the gastrointestinal tract or by their establishment along the surface epithelial cells of the tract or even in the associated mucosal biofilm.

While direct-fed microbials have been used for many years, the exact mode of action is still a mystery. Most likely the ability of the direct-fed microbial to competitively exclude other organisms or elicit an antimicrobial response allows them to work. Irregardless of the mode of action, the allogenic and autogenic factors present in the environment to which the product is inoculated will mandate the success or failure of the DFM. Savage (1989) noted that the allogenic factors include the responses of the microbiota from the host, host's ingesta and the environment. The autogenic factors include those influencing the resident and introduced microbes on themselves and on other microorganisms. Competitive exclusion is a natural defense mechanism found in the gastrointestinal tract created by the indigenous microbial populations. Essentially forming a barrier between pathogenic microorganisms and the surface epithelium.

DFM can render a target cell unavailable to pathogens or less efficient indigenous bacteria by occupying attachment sites. Some of the allogenic factors affecting attachment include the types and number of binding sites the host has available, the rate of cell sloughing, the rate of passage of ingested material, substrate type and availability of the diet and the pH of the gastrointestinal tract of the ruminant maintained on a particular diet. The autogenic factors include the ability of the direct-fed organism to attach itself to the surface of the epithelium and its ability to compete and survive in the gastrointestinal tract with the available nutrients.

Another important consideration for the use of DFM products is the rate at which they are capable of doubling their populations. A direct-fed organism with a doubling rate slower than the rate of passage and epithelial sloughing will need to be reintroduced into the gastrointestinal tract repeatedly in order to remain efficacious. Ideally, an organism that has the capability of doubling its population level rapidly would also be more capable of establishing and maintaining its populations, potentially

4

offering the ruminant an increased or maintained level of protection or efficiency over time.

DIRECT-FED MICROBIAL CULTURES

DFM products have been marketed for a wide variety of domesticated animals and even for humans. Many of the DFM products developed for livestock have been marketed to improve survival rates and decrease mortality rates in young animals easily compromised during nutritional, environmental or emotional stresses. Others have been developed to decrease the prevalence of certain diseases or gastrointestinal deficiencies such as scours, mastitis, metritis, lactose maldigestion and hypercholesterolemia. In addition, some direct-fed products have been useful for reestablishing balance to the intestinal flora after antibiotic therapy. Lastly, some direct-fed products have been developed to elicit increases in rate of gain, daily gain and feed efficiency.

Many of the marketed DFM products come from the genus *Lactobacillus* and include such species as *L. acidophilus*, *L. brevis*, *L. bulgaricus*, *L. casei*, *L. cellobiosus*, *L. delbruekii*, *L. fermentum*, *L. lactis*, *L. plantarum* and *L. reuterii*. The next most commonly used genus is *Streptococcus* which includes such species as *S. cremoris*, *S. diacetylactis*, *S. faecium*, *S. intermedius*, *S. lactis* and *S. thermophilus*. The genus *Bacteroides* includes several strains such as *B. adolescentis*, *B. anaimalis*, *B. bifidum*, *B. infantis*, *B. longum* and *B. thermophilum*. From the genus *Bacillus* strains COME *B. coagulans*, *B. lichenformis*, *B. pumilus*, *B. lentus* and *B. subtilis*. Other bacterial strains include *Pedicoccus acidilacticii*, *P. cerevisiae*, *P. pentosaceus*, *Leuconostoc mesenteroides*, *Propionibacterium freudenreichii*, *P. shermanii* and *P. acidipropionici* (Hutcheson, 1991). Also, several strains of yeast and fungi have been marketed which include *Aspergillus niger*, *A. oryzae* and *Saccharomyces cerevisiae*

(Hutcheson, 1991). These products have been used alone and in various combinations.

In respect to the area of animal direct-fed microbials, much of the data supporting the products appears to be limited, flawed or non-existent. Manufacturers have often presented data collected from *in vitro* trials and/or poorly constructed feeding trials containing limited numbers of animals that vary in size, sex, diet and environmental conditions. By limiting the variation, researchers and manufacturers will be able to paint a much clearer picture of the potential uses for and the true value of direct-fed microbials.

Often times cultures that appear to be efficacious *in vitro* do not survive the delivery system. Processing such as lyophilization, dehydration and pelleting along with rehydration in the gut can cause irreversible cell damage. During processing, cultures can be exposed to oxygen, ultraviolet light and adverse temperatures which can injure or rupture the cells.

A study conducted on fifteen feed supplements containing lactobacilli cultures from eight different manufacturers showed a wide variation in counts. All fifteen products were plated from the package onto *Lactobacillus* selection agar (LBS) and LBS plus 0.2% oxgall (LBSO). Each of the packages claimed to have counts between 1×10^7 and 1×10^9 CFU/g. However, only two of the fifteen had counts greater than 1×10^7. Two others had counts greater than 1×10^5 CFU/g and the remaining packages had counts of 6.9×10^4 CFU/g or less; three of the products had counts less than 100 CFU/g on LBS agar (Gilliland, 1981).

A variety of factors can influence efficacy of the direct-fed product and the dosage level which include the species of the animal, the maturity of the animal, the plane of production, level of stress and environmental conditions. A broad range of responses is cited in literature, not so much a lack of response, but a plethora of

6

positive responses which span a huge range of animal species, products, experimental protocols and geography (Fox, 1988).

Briefly, some positive results cited in the literature have been reported with calves fed pasteurized whole milk containing *L. acidophilus*. The treated calves had fewer intestinal coliforms and more lactobacilli than calves receiving the untreated pasteurized whole milk (Gilliland et al., 1980). Another study indicates a 36.9% reduction in calf scours attributed to the use of a *Lactobacillus* inoculant (Tournot, 1976). Under feedlot conditions using a lactic acid bacteria combination known as Probios (Pioneer Hi-Bred), a 13.2% increase in average daily gain was noted along with a 6.3% increase in feed efficiency and a 27.7% decrease in morbidity (Wren, 1987 a, b).

The performance of direct-fed microbial products is greatly influenced by the dosage level and the ability of the culture to establish in the gastrointestinal tract. In respect to dosage level, influences that can impact effectiveness include product purity, batch-to-batch consistency and product shelf life. Products developed to improve average daily gain and feed efficiency in cattle and pigs have a recommended minimum dose range of 1×10^8 to 1×10^{10} CFU/head/day. Those companies that offer a guaranteed microbial count employ dose ranges of 1.5×10^8 to 1×10^{10} CFU/dose.

The ability of the direct-fed culture to establish in one or more areas of the gastrointestinal tract is directly related to *in vivo* performance. Cultures can establish in either the epithelial surface, the biofilm layer or the lumen of the gastrointestinal tract. In order for a culture to establish within the lumen it is imperative that it reproduce quickly or repeated dosing is necessary. Most successful direct-fed microbial cultures become established in the digestive tract, both lactobacilli and streptococci attach to the surface epithelial (Fuller et al., 1978). Bacilli appear to effectively establish in the biofilm layer. The sloughing rate of the epithelial cells can greatly influence the attachment of direct-fed cultures in the gastrointestinal tract and, in some cases, the

host specificity (Wesney and Tannock, 1979; Barrow et al., 1980). Those cultures intended to modify the functions of the lower digestive tract must be acid and bile tolerant to be efficacious.

TRACKING DIRECT-FED CULTURES IN COMPLEX ENVIRONMENTS

While in the development stage of the direct-fed microbial it is advantageous to be able to accurately and consistently recover and enumerate the culture *in vivo*. The indigenous population of the normal gastrointestinal tract can exceed 100 billion CFU/g (1×10^{11} CFU/g) (Wolin, 1981; Savage, 1986). Techniques used to track a species in a complex environment include the use of selective-differential medias, plasmid profiling, DNA "fingerprinting" and PCR-RFLP. Unfortunately limited work has been accomplished on sequencing the genomic DNA of various propionibacteria.

The use of selective-differential media is a common, though limited, identification method. A selective-differential media exploits a species' unique properties and allows it to be easily identified from the other microbes that have survived. Once a colony can be isolated and purified, the next step would be utilizing a secondary method to confirm the identity of suspect colonies, such as biochemical reaction tests. Biochemical analysis of suspected colonies normally consists of specific carbohydrate fermentations and other strain specific reactions, intended to identify specific strains based on reactive color change. Two suspected colonies may share similar phenotypic characteristics and test reactions; however, this does not necessarily indicate their genotypic relatedness. Genetic analysis offers a more accurate method of identification by which related strains are more easily distinguished.

Plasmid profiling has been used to distinguish strains of lactobacilli in the intestinal tract of piglets (Tannock et al., 1990) to monitor populations of specific strains of *Lactobacillus plantarum* in corn-silage fermentations (Hill and Hill, 1986) and to

8

monitor the hay inoculant *Bacillus pumilus* (Hendrick et al., 1991). Simply by comparing the patterns produced by the isolated plasmid DNA from suspected colonies to that of pure isolates of the introduced strain, relatedness can be determined.

However, plasmid profiling is not with out its limitations. Plasmids are often transient genetic elements in bacterial cells due to their ability to be conjugally transferred within or between species in an environment. Conjugal transfer has been noted between strains of *Lactobacillus reuteri*, between lactobacilli and *Enterococcus faecalis* (Tannock, 1987), and a number of Gram-positive bacteria (Shrago et al., 1986). Furthermore, certain species of bacteria may naturally lose their plasmids over time due to incongruent replication rates or they may simply not contain any plasmid(s). Often if the bacteria does contain a plasmid the profile may not be unique to that strain alone.

A more precise method of identification is the method of DNA "fingerprinting". The genomic DNA can be isolated and digested with a combination of restriction enzymes which will produce a unique pattern, much like that of an individual's fingerprint. The direct-fed microbial culture will produce a unique chromosomal pattern which in turn can be compared with the suspected isolates. DNA fingerprinting has proved useful for identifying specific strains of enteric bacteria such as *Escherichia coli*, *Shigella ssp.* and *Lactobacillus ssp.* as well as nonenteric strains such as *Propionibacterium freudenreichii* using pulsed-field gel electrophoresis (Ogram and Sayler, 1988; Venkatesan et al., 1988; Parrott et al., 1994; Rehberger, 1993).

GENUS *PROPIONIBACTERIUM*

Bacteria from the genus *Propionibacterium* are classified as Gram positive, non-motile, non-spore forming, pleomorphic rod-shaped bacteria capable of producing propionate, acetate and carbon dioxide from carbohydrates. Propionibacteria are anaerobic to aerotolerant, generally catalase positive and grow best at 30-37°C (Cummins and Johnson, 1986). Another distinguishing characteristic is the ability of some strains to reduce nitrate and nitrite (Buchanan and Gibbons, 1974).

The genus *Propionibacterium* is commonly divided into two groups based upon their normal ecological niche. The first group known as "classical" or "dairy" propionibacteria are readily isolated from fermented dairy products, silage and olives (Cummins and Johnson, 1986). The "dairy" propionibacteria generally include four recognized and characterized species: *P. freudenreichii, P. jensenii, P. theonii* and *P. acidipropionici.* The second group consists of those strains typically found on human skin or in the intestinal tract, known as the "acnes group" or "cutaneous" propionibacteria. Included in this group are four strains: *P. acnes, P. avidum, P. granulosum* and *P. lymphophilum.*

Propionibacteria holds great prospect as a direct-fed microbial. Kornyeva (1981) reported that infants exhibiting various degrees of pathogenic staphylococci intestinal infections were able to establish populations of *Propionibacterium shermanii* within the intestinal tract. After 7 days of feeding acidophilus milk, 87.5% of the infants manifesting symptoms of the infection were excreting *P. shermanii.* When healthy infants were given the acidophilus milk, 95.5% excreted *P. shermanii.* The fecal concentration of *P. shermanii* for both groups increased 100-fold during the 14 day experiment. *Propionibacterium freudenreichii* subjected to gastric digestion survived without any loss of viability according to Mantere-Alhonen (1983). Nabukhotnyi et al. (1983) reported two fermented milk products, "Malyutka" and "Malyush", were modified

10

by adding *P. shermanii* and used to treat acute gastrointestinal disease in infants. The formulas containing propionibacteria proved to be more effective in treating the disease than the control formula.

Mantere-Alhonen (1982) inoculated fodder with propionibacteria and reported an increased growth rate in swine and a stabilized intestinal flora. The number of propionibacteria isolated from intestinal contents was greater in treated animals than control animals, with no apparent intestinal adhesion demonstrated. A study performed on dairy calves fed a whole milk mixture containing a co-culture of propionibacteria and a lactic acid culture, plus antibiotics, appeared to increase weight gains (Vladimirov et al., 1978).

Propionibacteria are industrially important to the production of Swiss type cheeses. The starter culture used in production contains a *Lactobacillus* strain which produces lactic acid, which is metabolized by propionibacteria to form propionic acid and CO_2. Propionic acid is responsible for the distinct flavor of Swiss cheese and the slow release of CO_2, forming the characteristic "eyes" (Lansgrud and Rienbold, 1973). Propionibacteria are capable of growing at temperatures as low as 4°C (Hofherr and Glatz, 1983) which is important for the manufacturing of Swiss cheese, since maximum CO_2 production occurs at 12.8°C (Hettinga and Reinbold, 1971).

RUMINAL ACIDOSIS

In the United States, feedlot operators have been feeding cattle diets rich in readily fermented carbohydrates (RFC) to provide consumers with tender, flavorful meat. Often producers are forced to adapt animals at an accelerated rate to meet demand and minimize cost. When the rumen environment is not adequately adapted to high concentrations of these RFC, the perturbation can cause microbial populations to shift and lactic acid to accumulate in the rumen. Lactic acid is a considerably stronger

acid compared to normal volatile fatty acids (VFA) with a pK of 3.9 vs. 4.8 for acetic acid, which when accumulated decreases pH and reduces salivary function (i.e. buffering capacity). If severe, lactate accumulation will disrupt the physiological acid-base balance, limiting the animals ability to oxygenate blood, resulting in death.

Most often feedlot producers attempt to minimize the effects of lactic acidosis by slowly and systematically adapting cattle to high concentrate rations. Performance is considered submaximal until the animal is adapted to the highest concentration. Normally, the pH of ruminal contents ranges from 5.5 to 7.5 (Blood and Radostis, 1989); however, the ruminal pH of an acidotic animal can fall to a range of 3.9 to 4.5. When the acid production rate exceeds the absorption rate, acid accumulation drives the pH down (Hungate et al., 1952). The low pH conditions inhibits carbohydrate digestion, but increases the rate of lactic acid absorption (Dunlop, 1972; Slyter, 1976).

The processing of cereal grains and other readily fermented carbohydrates such as immature, rapidly growing forage, tubers, and root crops contributes to the likelihood of acidosis occurring. Various methods of processing, such as flaking, rolling, heating and pressure treatments erode away the protein coating or endosperm making the starch more available to fermentation, in turn increasing the rate of acid production and the likelihood of ruminal acidosis. Acidosis occurs most often when animals are abruptly changed from a forage diet to concentrate or when unadapted animals gain access to grain storage bins or lush green pasture. Even adapted animals that engorge large amounts of grain are susceptible to acidotic events.

Other factors which can contribute to the risk of acidosis include the body condition. Ruminants in poor condition, lacking energy reserves, are more susceptible (Slyter, 1976). The ruminal digesta is greatly influenced by the form of roughage used in the diet. By diluting the RFC with roughage some buffering capacity is provided. When forage is finely chopped or pelleted, ruminants eat faster, chew less and dilute

the diet with less saliva (Brent, 1976; Utley et al., 1973). Consequently, ruminal digesta becomes more acidic.

FEEDS CAPABLE OF CAUSING ACIDOSIS

Abrupt changes from low energy forage diets to high energy grain diets often provokes an acidotic event (Huntington and Britton, 1978). Howard (1981) suggested that the primary cause of acidosis was the inadequate adaptation to highly digestable carbohydrate sources, although Mullen (1976) noted that clinical acidosis can occur in grain-adapted animals as well when fed excess carbohydrate. The feeds which can cause indigestion in ruminants have been reviewed (Dunlop and Hammond, 1965; Dunlop, 1972; Slyter, 1976). Due to the high starch content, grains such as wheat, barley, corn, oats, milo and rye all have the potential to cause acidosis when ingested in excess. Other foods such as molasses, fruit crops and sugar cane which are high in soluble sugars can also initiate an acidotic event (Dunlop and Hammond, 1965). Even high starch foods such as potatoes and beans can provoke acidosis (Slyter, 1976).

The amount of cereal grain necessary to induce acidosis is variable, depending on the type of grain and the physical form in which the grain is adminstered (Britton, 1985). McAllister et al. (1990) observed that the microbial colonization was substanially greater in various halved and quartered cereal grains compared to whole grains, thus indicating that the physical processing of grains does indeed influence digestability and the potentiality of acidosis.

PHYSIOLOGICAL EFFECTS OF RUMINAL ACIDOSIS

The symptoms associated with the metabolic disorder known as ruminal acidosis vary with severity and persistency of a low ruminal pH. Periods of feed engorgement are often followed by delayed and/or reduced feed consumption. During the acidotic episode, the animal becomes depressed and listless, often exhibiting dehydration, diarrhea and laminitis. Often acute acidosis results in death. More prevalent, though less frequently considered, is subacute, subclinical or "chronic" acidosis. The symptoms are subtle and seldom appear clinically. Cattle tend to "go off" feed or reduce intake, reducing potential gains or performance (Mullen, 1976; Slyter, 1976; Britton, 1987).

Previous studies suggested the cause of ruminal acidosis on D-lactic acid. Following the infusion of RFC, concentrations of lactic acid in the rumen and blood increased (Dunlop and Hammond, 1965; Huber, 1976; Kezar and Church, 1979; Slyter, 1976). Kezar and Church (1979) reported that ruminal lactic acid concentration exceeded 100 mM within 48 h after sucrose was infused into the rumen.

Harmon et al. (1985) compared ruminal changes in steers induced with acute ruminal acidosis (dosed intraruminally with glucose, 12 g/kg body weight) and subacute acidosis (switched directly from hay to a 70% concentrate diet). The results indicated higher levels of VFA produced and absorbed relative to lactic acid in subacutely acidotic steers. Steers experiencing acute acidosis demonstrated a predominately lactic acid fermentation. Not surprisingly, pH values were lower in the acutely acidotic steers (4.2, compared to 5.8 in the subacute animals). Fermentation patterns for lactic acid were similar in both groups until approximately 6 h post-engorgement, suggesting there is some similarity between subacute acidosis and the onset of acute acidosis.

Underwood (1992) summerized the clinical signs of ruminant acidosis. At the onset of acute acidosis, body temperature, pulse and respiratory rates become slightly

14

elevated. The increased respiratory rate is a direct result of lactate entering the blood stream. Lactate dissociates, causing hydrogen ions and bicarbonate to combine and then to dissociate producing water plus carbon dioxide; the carbon dioxide is removed via the increased respiration. As the disease progresses, the animal may become hypothermic with body temperatures 3° to 4° C below normal. Respiration drastically reduces to only 10 to 20 breathes per minute. Fecal matter changes from a light yellow-green color with a slightly sweet smell to a profuse, foul smelling, foamy consistency often tinged with blood.

Dehydration is a result of increased rumen osmolality (Huber, 1971). Increasing osmotic pressure occurs when small molecular weight compounds such as lactic acid, glucose and VFA accumulate in the rumen. When rumen osmotic pressure exceeds the osmotic pressure of the blood, water is drawn into the rumen from plasma, interstitial and intracellular fluids. Huber (1971) reported that body water was reduced by 8% in acidotic sheep due to high ruminal osmolality. The change in tonality of the rumen is compounded by the fact that animals consuming concentrate-rich diets produce less saliva, exacerbated by the extensive grain processing of feedlot rations. Ruminal buffering was reduced when dietary roughage, in this case peanut hulls, were ground or pelleted (Utley et al., 1973). Garza and Owens (1989) reported that 92-96% of the fluid in the rumen must originate from saliva and flux through the rumen wall, less than 10% was supplied by drinking water. Therefore, saliva must be responsible for the dilution of ruminal acids and maintenance of fluid in the rumen. The fluid makes it possible for the acids to be mixed, exposing them to the rumen wall for absorption as well as being flushed to the omasum.

As pH drops below 5.0, ruminal motility declines and stasis occurs (Dougherty et al., 1974). Crichlow and Chaplin (1985) manually engorged sheep with ground wheat (40 g/kg body/weight) and observed ruminal stasis within 8-12 h. Earlier studies by Juhasz and Szegedi (1968) indicated ruminal motility did not decline immediately after

15

adding lactic acid solutions to the rumen to decrease pH to 4.0; ruminal stasis didn't occur until approximately 2 h later. Bruce and Huber (1973) reported that motility was depressed within 10 min. of duodenal infusion of lactic acid. Crichlow (1988) substantiated these findings by isolating reticuloruminal epithelial receptors in 19 anesthetized sheep and determining that non-dissociated VFA, particularly butyric acid, were capable of stimulating a large portion of the receptors. Therefore, the system of intestinal receptor activation by lactic acid followed by hormonal depression of ruminal activity by secretin described by Bruce and Huber (1973) appears to be the most accurate.

Rumenitis and hyperkeratosis occur as a result of decreased ruminal pH (Brent, 1976; Howard, 1981; Huntington, 1988). Swelling and edema of the rumen papillae is evident, as are areas of detached mucosa and epithelial necrosis in acute acidosis (Howard, 1981). Rumenitis occurred in barley-fed calves at slaughter even though ruminal pH averaged 5.5 (Kay et al., 1969), suggesting the potential for damage to the ruminal tissues during subacute acidosis.

Bovine laminitis occurs more often among animals consuming a high concentrate diet, usually after an episode of ruminal acidosis (Brent, 1976). Dirkson (1969) reported that laminitis set in shortly after inducing acidosis. High concentrations of histamine found in the blood appear to be responsible for laminitis. Unfortunately, whether histamines originate from normal ruminal metabolites or from other bodily tissues stressed by dehydration or blood acidity is still unknown.

Ruminants that succumb to acidosis will usually exhibit liver abscesses, brain lesions and hemorrhagic inflammation of the rumen (Brent, 1976). Liver abscesses result in economic loss, since the liver makes up approximately 2% of the carcass weight (Tindall, 1983). In addition, ruminants with hepatic abscesses generally gain weight more slowly and less efficiently.

16

MICROBIAL CHANGES OCCURRING DURING RUMINAL ACIDOSIS

During acute acidosis, the rumen microbial changes which occur have been reviewed (Dunlop and Hammond, 1965; Hungate, 1966; Dirksen, 1969; Slyter, 1976; Coleman, 1979). Unfortunately, the influence of subacute acidosis on rumen microbes is not well documented. It is presumed that the microbial changes during subacute acidosis are similar to those observed during the onset of acute acidosis (Dirkson, 1970; Mullen, 1976).

Although lactate is the dominant end-product of many dairy fermentations such as yogurt and cheese, it is not usually a major end-product of ruminal fermentation. Acetate is produced by most of the carbohydrate-fermnenting bacteria found in the rumen. Hungate (1979) initially explained the preponderance of acetogenic ruminal bacteria by the amount of ATP produced (4 verses 2 ATP/hexose). However, experiments with acidotic cows contradicted this notion of maximum efficiency, therefore Hungate later modified his conclusion to include that ATP per time was a more important criterion than ATP per sugar (Russell, 1997 unpublished).

Ruminal bacteria like *Streptococcus bovis* and *Selenomonas ruminatium* are able to change their fermentation products (Russell, 1997 unpublished). Under normal feeding conditions, when pH is high and carbohydrate -fermentation rate is slow, lactate production declines, *S. bovis* converts glucose to acetate, formate and ethanol. However, when extra glucose is available *S. bovis* grows rapidly, producing lactate as the primary fermentation product (Russell and Hino, 1985).

When cells are metabolizing glucose slowly, intracellular fructose-1, 6-diphosphate (FDP) accumulates, lactate dehydrogenase (LDH) is then activated, and glucose can then be converted to lactate. Low extracellular pH resulting from rapid growth and lactate production inhibits growth of *S. bovis*, yet the fermentation does not return to the aforementioned state. The acidic environment causes intracellular pH to

decrease from near neutral or slightly alkaline to about 5.5. At this intracellular pH the requirement of LDH for FDP is reduced fourfold and maximum velocity of LDH is increased. Furthermore, pyruvate formate lyase (PFL) is sensitive to low pH and production of acetate, formate and ethanol is curtailed. Thus, a cascade or spiral effect toward greater lactate production and lower pH becomes apparent when S. bovis growth rate is increased.

Selenomonas ruminantium ferments carbohydrates to acetate, propionate and small amounts of butyrate (Hishinuma et al., 1968), but like S. bovis can also divert production to lactate when excess glucose is present . The ability to ferment lactate to acetate and formate, as well as specific production of L(+), D(-) or DL lactate isomers is strain dependent (Scheifinger et al., 1975; Wallace, 1978). However, Scheifinger et al. (1975) demonstrated increased lactate and decreased acetate and propionate production at faster growth rates in lactate-utilizing strains of S. ruminantium. Subsequent continuous culture experiments by Wallace (1978), using a lactilytic strain of S. ruminantium which produced only D(-) lactate, indicated a similar shift in fermentation products. Enzyme kinetics experiments indicated that the LDH of S. ruminantium is an allosteric enzyme possessing two binding sites for pyruvate, and that positively cooperative binding of pyruvate to the LDH molecule resulted in activation of the enzyme. Unlike the LDH of S. bovis, S. ruminantium LDH activity is unaffected by FDP, and is regulated by homotropic activation by its substrate, pyruvate (Wallace, 1978). Although Russell (1997, unpublished) reports that FDP accumulation does influence LDH activation in S. rumiantium, 'there has been little conclusive proof that increase in intracellular pyruvate is responsible for the increase in lactate production'. Lactate -producing cells had no more pyruvate than non-energized cells (Strobel and Russell, 1991).

The decreased pH resulting from lactate overproduction subsequently inhibits growth of S. bovis (Russell and Dombrowski, 1980; Newbold and Wallace, 1988) and

18

provides a suitable environment for growth of anaerobic *Lactobacillus* species (Howard, 1981; Nagaraja et al., 1981). The proliferation of the lactobacilli results in still greater accumulations of lactic acid (Newbold and Wallace, 1988). Newbold and Wallace (1988) established glucose-limited continuous co-cultures of the lactate and sugar-utilizing bacteria *Megasphaera elsdenii* and *S. ruminantium*, with the lactate-producing bacteria *S. bovis* and *Lactobacillus spp.* Simulated acidosis was induced by addition of a 100-fold excess of glucose (40 g/l) to the fermenter. *S. bovis* quickly outgrew the other species, corresponding to increased VFA and lactate concentrations. Growth of *M.elsdenii* and *S. ruminantium* was depressed after 5 h. After 12 h *S. bovis* growth declined and *Lactobacillus* growth increased, becoming the dominant organism at 48 h. Increased lactate and decreased VFA were closely associated with an increase in lactobacilli and a decrease in the other bacterial species .

Decreased ruminal pH inhibits growth of cellulolytic and lactate-utilizing bacteria (Kistner et al,. 1979; Russell and Dombrowski, 1980; Therion et al., 1982; Newbold and Wallace, 1988) . The decreased activity of lactate-utilizing bacteria at low pH is reflected in the decreased VFA production and increased ruminal lactate accumulation observed during later stages of acute acidosis (Kezar and Church, 1979; Nagaraja et al,. 1981; 1982; 1985; Harmon et al., 1985). Peters et al. (1989) noted increased total VFA, acetate, propionate and butyrate at pH 6.7 than at pH 5.7. Total VFA, propionate and butyrate were also effected by osmolality.

Therefore, during acute acidosis there is a shift toward increased numbers of rapidly growing (low G+C) gram-positive bacteria, specifically rods (*Lactobacillus*) and cocci (*S. bovis*), and greatly reduced numbers of gram-negative bacteria (lactate-utilizing and cellulolytic bacteria) (Dirkson, 1969; Allison et al., 1975; Slyter, 1976; Howard, 1981). *S. ruminantium* , a bacterium once classified as gram-negative, has a 16S rRNA sequence that is more closely related to gram-positive bacteria (Olsen et al., 1994).

19

Bacterial changes during subacute acidosis include increased total bacterial counts and increased numbers of gram-positive bacteria (Dirkson, 1969; Mullen, 1976). Effects of subacute acidosis on lactate-utilizing and celluloytic bacteria have not been determined. However, *in vitro* studies relating pH to growth and activity of these bacterial groups suggest some depression may occur (Kistner et al., 1979; Russell and Dombrowski, 1980; Therion et al., 1982; Newbold and Wallace, 1988).

Ruminal ciliated protozoa are also adversely effected by acidotic rumen conditions (Slyter, 1976; Coleman, 1979; Howard, 1981). Ruminants fed concentrate-rich diets will have a reduced number of protozoa, whether engorgement or gradual adaptation occurs (Slyter et al., 1965). The decrease in protozoal numbers is attributed to low pH (Quin et al., 1962) and to high osmotic pressure (Ahrens, 1967) of ruminal contents. Quin et al. (1962) reported that ruminal protozoa did not survive prolonged exposure to pH values below 5.5; during acidosis pH can drop below 5.5 (Dunlop, 1972; Slyter, 1976). An osmotic pressure of 260 mOsm was reported by Quin et al. (1962) as the most favorable condition for ciliates. Following engorgement of wheat, heifers had ruminal osmotic pressures as high as 523 mOsm within 16 hours. Ruminal protozoa assimilate large amounts of free carbohydrate (Oxford, 1951 from Slyter, 1976), therefore active, viable protozoa should stabilize ruminal fermentation. Conversely, when protozoa lyse, large amounts of amylase are released into the rumen environment which may enhance the conversion of starch to acid, which exacerbates the acidotic condition.

Mackie et al. (1979) reported on changes in the number and types of lactate-producing and lactate-utilizing bacteria in the rumen of sheep subjected to a stepwise adaptation from a low- to a high-concentrate diet. A surge in the amylolytic bacteria always preceded an increase in lactate-utilizing bacteria, the final diet containing 71% grain and molasses, the two groups tended to balance each other, resulting in a low accumulation of lactic acid. Among the lactate-utilizing bacteria identified were acid

sensitive *Veillonella* and *Selenomonas*, which were superseded by the more acid-tolerant *Anaerovibrio* and *Propionibacterium*. The proportion of *Propionibacterium* accounted for 40 to 50% of the lactate-utilizing bacteria on 5 of 12 occasions of the experiment. *Megasphaera* appeared intermittently throughout the experiment, usually forming <22% of the lactate-utilizing bacteria.

The two major utilizers of lactic acid in the rumen are *Selemonas ruminatium* (Bryant, 1956) and *Megasphaera elsdenii* (Elsden et al., 1956). *Anaerovibrio lipolytica* has been reported as the predominant lactic acid utilizing strain on forage fed diets. However, Slyter (1976) cast doubt on its importance in lactate metabolism since it is not capable of surviving as low of pH values as *M. elsdenii* and *S. ruminatium*.

PROPIONIBACTERIUM STRAIN SELECTION

Forty-four strains of *Propionibacterium* were examined for their ability to utilize lactic acid in *in vitro* rumen fluid simulations (Parrott et al., 1996). The strains were tested for the ability to grow and utilize a nutrient broth supplemented with 80 mM lactic acid. Lactic acid utilization was examined at two pH levels- - one representative of the acidic rumen (5.0) and the other representative of the forage fed rumen (7.0). No difference was detected between strains at pH 7.0. However, at pH 5.0 two strains, P49 and P99, performed significantly better than the others--utilizing 76.90 mM and 78.79 mM of lactic acid respectively. These *P. freudenreichii* strains reached greater cell densities and utilized more lactic acid at pH 5.0 than the other three species examined. Only fifteen strains examined were capable of surviving in a simulated rumen fluid model and utilizing lactic acid produced by native lactate-producing bacteria.

When compared with the other strains, P42 appeared to have the greatest rate of lactate utilization (1.61mM/h), but was not statistically different (P<.05) from strains P63, P54, P25, P41, P111, P81 and P104. In addition, P42 had the highest rate of

pH increase (.0377 units /h) compared to P63, P54, P25 and P41, again there was no statistical difference (P<.05) between the strains mentioned. When the data was compared according to Gompertz non-linear curve fitting equation, a clear separation was revealed, P54 and P63 significantly increased the lag time for lactic acid accumulation and suppressed the rate of H+ concentration. Strains P54 and P63 were examined genetically by pulsed-field gel electrophoresis, with the intact chromosomal DNA suggesting that the two strains are closely related and may be genotypically identical (Parrott, 1997).

OBJECTIVES

This study was designed to define the ruminal populations of *Propionibacterium* in order to effectively track introduced cultures. Isolates were characterized phenotypically and genotypically in order to categorize relatedness. Utilizing strains selected by Parrott (1997), a mixed culture was examined for its ability to establish within the rumen of cattle during dietary challenged with readily fermented carbohydrates. The *in vivo* study would prove the efficacy of a mixed culture in utilizing lactic acid produced by indigenous microorganisms. In addition, the *in vivo* model would test the ability of the mixed culture to be enumerated and genotypically defined when isolated from a complex environment like the rumen. Ultimately, this study would define a direct-fed microbial product useful in preventing the occurrence of the metabolic disorder known as lactic acidosis.

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23

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CHAPTER III

CHARACTERIZATION OF *PROPIONIBACTERIUM* ISOLATED FROM THE BOVINE RUMEN

ABSRACT

Organisms of the genus *Propionibacterium* comprise a small portion of the ruminal microflora. They are slow growing, fastidious and difficult to isolate from ruminal fluid. Given the limited research on native ruminal strains, the purpose of this study was to characterize indiginous propionibacteria populations from the bovine rumen. Ruminal fluid from five fistulated cows was collected at three sampling times. The diversity of the propionibacteria isolates was characterized by biochemical tests, carbohydrate fermentation patterns, plasmid DNA profiling and pulsed-field gel electrophoresis analysis. Based on carbohydrate fermentation patterns and biochemical tests, 95% of the isolates were identified as *P. acidipropionici* and the remaining 5% were identified as P. jensenii. Overall, 35% of the 132 isolates examined contained plasmids. Two plasmid profiles were common, a single 2.5 kb plasmid and a single 7.0 kb plasmid. Only one strain was found to contain more than one plasmid. The predominant plasmid profile varied with different sampling times. Pulsed-field gel electrophoresis analysis of genomic DNA profiles produced by Xba I digests showed little diversity in the first two sampling periods. However, the genomic diversity increased form 2 and 3 profiles to more than 9 different profiles for the June sampling period. These results indicate that the native ruminal propionibacteria populations, though phenotypically similar, are indeed genotypically diverse and unstable.

(Key words: *Propionibacterium*, Rumen, Cattle)

INTRODUCTION

Anaerobic microbes in the rumen ferment carbohydrates to lactic acid and volatile fatty acids. Abrupt dietary increases of rapidly fermentable carbohydrates, i.e., during adaptation to high concentrate diets or following grain engorgemant, the supply of acids increases and an abundant amount of lactic acid is produced. Normally, lactic acid is present in the rumen at low concentrations, but after carbohydrate supply increases abruptly, ruminal contration of lactic acid can to 100 mM. This condition, known as lactic acidosis, continues to be one of the most prominent production problems facing the cattle feeding industry.

The organisms that produce lactic acid, namely *Streptococcus bovis* and *Lactobacillus* species, presumably are the primary culprits responsible for the decline in ruminal pH (Allison et al., 1975; Dunlop, 1972; Hungate et al., 1952; Mann 1970). Certain feed additives including lasalocid, monensisn, thiopeptin and virginiamycin are narrow spectrum antibiotics with activity against gram-positive organisms, i.e., lactate producing bacteria; these have been used to prevent lactic acidosis (Nagaraja et al., 1982; Tung and Kung, 1993; Muir et al., 1981). Nutrients that stimulate the lactate-utilizing bacteria, cultures that maintain ruminal protozoa, and supplements to reduce meal size also appear to be useful. Inoculation of the rumen with lactic acid-utilizing organisms is another logical prophylactic for ruminal acidosis.

Kung and Hession (1995), using *in vitro* fermentation with a mixed population of ruminal microorganisms, significantly reduced the accumulation of lactic acid by inoculating with *Megasphaera elsdenii*. Ruminal pH of beef cattle inoculated with *M. elsdenii* inoculation during induced acidosis remained above 5.51 while control cattle dropped to 4.65. Inoculating cattle with this same bacteria 8 h prior to inducing acidosis resulted in higher pH for treated than control cattle (Greening et al., 1991). Presumably the *M. elsdenii* strain introduced into the rumen did not become established. Huber et

31

al. (1976) reported that, although *M elsdenii* appeared to flourish during the initial stages of adaptation, population declined during acidosis, probably due to a lack of acid tolerance. One problem of using a *M. elsdenii* culture to alleviate lactic acidosis is that it is a gram-negative microorganism and therefore is not considered a GRAS (generally regarded as safe) organism.

Propionibacterium are normal inhabitants of the rumen and account for approximately 1.4% of the total microbial population (Oshio et al., 1987). Swartzlander (1994) showed that a denitrifying strain of *Propionibacterium acidipropionici* (P5) became established and an active population was maintained when it was introduced into the rumen of beef cattle. These data indicate that ruminal inoculation with propionibacteria may reduce the formation of undesirable endproducts such as lactic acid. This evidence was strong enough to prompt two investigators (Ware and Garner, 1996; Garner and Ware, 1996) to apply for patents on the use of P5 with *Lactobacillus acidophilus* ATCC 53545 to administer to ruminants to increase meat or milk production, or to prevent or minimize acidosis during the transition from a roughage diet to a feedlot diet.

During strain selection by Parrott (1997), rumen simulation models verified that certain strains of propionibacteria were well suited to modulate pH decreases and inhibit the accumulation of lactic acid. Parrot reported no significant difference between strains P54 and P63; both *P jensenii* strains increased pH and suppressed accumulation of lactic acid. Genetic analysis revealed that P54 and P63 were genotypically similar. Parrott concluded that P63 has the better chance of utilizing lactic acid in the rumen of beef cattle consuming large amounts of grain.

This study was designed to 1) to quantify the indigenous populations of *Propionibacterium* in the bovine rumen, 2) determine the effectiveness of a mixed strain inoculum of *Propionibacterium* in the rumen under abrupt dieatary challenges.

32

MATERIAL AND METHODS

Isolation of Propionibacteria. The propionibacteria strains examined in this study were obtained from rumen fluid collected from five fistulated Holstein cows at the Oklahoma State University Dairy Cattle Research Center. Rumen fluid was collected on three occasions over a five month period (150 days) of the 305 day lactation cycle. The cows were fed a quantity of diet (Table 1) daily in proportion to daily milk production .

Approximately 100-150 ml of rumen fluid was collected from beneath the raft of ingesta or squeezed from the particulate ingesta into 250 ml bottles. The rumen fluid was transported to the laboratory and strained through four layers of cheese cloth to remove remaining debris. Fluid at various dilutions was plated on a selective and differential agar. The plates were allowed to dry for maximal absorption and then placed into a 32°C incubator for 7-10 days under anaerobic conditions (GasPak).

After 7-10 days each plate was examined for distinctive colonies--smooth, round, or convex shaped colonies. Individual colonies were picked from the original dilution plates and streaked four consecutive times to ensure purity. Isolated colonies were picked from the plate and placed in 10 ml tubes of sodium lactate broth (NLB) and incubated statically for 36 to 48 hours at 32°C. The cultures were maintained by frequent transfers (1%) into NLB. Each isolate was grown and harvested by centrifugation, resuspended in NLB plus 10% glycerol and stored at -75°C.

Identification of Propionibacteria Isolates. Suspected propionibacteria colonies were grown in tubes of 10 ml NLB for 36 to 48 h at 32°C. Each isolate was gram-stained and tested for catalase activity. All isolates gram-staining positive, exhibiting pleomorphic rods, some coccoidal forms, occurring in the characteristic Chinese letter configurations (Bergy's, 1990) were biochemically analyzed for their ability to ferment lactose, mannitol and trehalose. Furthermore, each isolate was observed for its ability to reduce esculin (0.01%) and nitrate (0.2%) as well as for incomplete hydrolysis of

33

gelatin (12%). Gram positive isolates that reduced esculin and nitrate were classified as *P. acidipropionici.*. The gram positive, esculin reducing, but non-nitrate reducing isolates were classified as *P. jensenii.*

High Pressure Liquid Chromotography. Volatile fatty acid (VFA) analysis was performed on the Hewlett-Packard 1090AX High Pressure Liquid Chromatography (HPLC) (Hewlett Packard, Atlanta, Georgia). Isolates grown in NLB for 36 h at 32°C were harvested at 12,000 rpm (1500 x g) for 15 minutes. The supernatant was acidified by 1:1 dilution with 0.01 M H_2SO_4 to halt fermentation. Samples (1 ml) were filtered through 0.2μm Gelman filters into autosampler vials. A 5μl sample was injected and separated using a HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, California) preheated to 65°C with 0.005 M H_2SO_4 as the mobile phase at a flow rate of 1 ml/ min. Lactate, acetate, propionate and butyrate were detected using a diode array detector scanning wavelengths 210 to 254 nm. Concentrations were determined by calculating peak areas and comparing these to known areas of external standards using Hewlett-Packard software.

Plasmid DNA isolation. Plasmid DNA was isolated from the propionibacteria strains as follows: A 1% inoculum taken from a 36 h culture was placed into 10 ml of sterile NLB and incubated at 32°C until late log phase (between 36 and 48 h). Cell suspensions were harvested by centrifugation (1500 x g for 15 min). The supernatant was decanted and the pellet resuspended in 1 ml Tris-EDTA buffer containing 15% sucrose. Resuspended cells were stored in 1.5 ml centrifuge tubes at -20°C until plasmid DNA analysis was performed. Frozen samples were thawed at room temperature. Cells were washed and resuspended in 1 ml Tris-EDTA buffer containing 15% sucrose. The cells were washed again and resuspended to a final volume of 250 μl with fresh Tris-EDTA + 15% sucrose buffer, samples were mixed well by vortexing. Lysozyme (50 μl of a 100 mg/ ml solution) was added and the tubes were incubated for 1 h at 32°C. Pronase (10 mg/ ml preincubated at 37°C for 1 h) was added (35 μl) and the mixture

34

was incubated at 32°C for 1 h. Following incubation 0.25 M EDTA was added (111 μl) to the sample and the tubes were held for 15 min on ice. Tris-EDTA containing 20% SDS was added (111 μl) and held on ice for an additional 15 min. Sodium acetate (75 μl of a 3.0 M solution) was added and the mixture was incubated on ice for 30 min. The debris was pelleted by centrifugation (12000 x g for 15 min) and the supernatant was transferred to a clean 1.5 ml microcentrifuge tube. Cold ethanol (750 μl of 95%) was added to the supernatant and mixed well by gently inverting the tube several times. The samples were stored at -20°C for 1 h to precipitate the DNA. The DNA was then pelleted by centrifugation (12,000 g for 15 min) and allowed to dry. The DNA was then resuspended in 40 μl Tris-EDTA buffer and 5 μl tracking dye and loaded onto an agarose gel. The DNA was separated by gel electrophoresis using a 0.7% agarose gel at 50 volts for approximately 15 h or until the tracking dye reached 3/4 of the length of the gel. The agarose gels were examined after 45 min of staining with an ethidium bromide solution and photographed.

Preparation of intact genomic DNA. Intact genomic DNA from representative strains was isolated from cells embedded in agarose beads. Cultures were grown to mid-log stage in 10 ml NLB supplemented with various percentages of glycine. The cells were harvested by centrifugation (9000 x g for 10 min) and resuspended to one tenth the original volume in 10X ET buffer (*500 mM EDTA, 10 mM Tris-HCl, pH 8.0). The cell suspension was mixed with an equal volume of 1% low-melting point agarose (Beckman Instruments, Palo Alto, CA), loaded into a syringe and injected into Tygon tubing (ID-1/16", OD-1/8") to solidify. The solidified cell-agarose mixture was forced through the tubing by injecting 10 ml of cold 10X ET buffer, then gently chopped using a Teflon spatula into smaller pieces (beads). The beads were harvested (5500 g for 10 min) and resuspended in 10 ml 10X ET containing 5 mg/ ml lysozyme and incubated at 32 °C with gentle shaking for 2 h. After incubation the beads were harvested by centrifugation (5500 x g for 10 min) and resuspended in 10 ml of lysis buffer (10X ET buffer containing

35

100 µl/ ml of Proteinase K and 1% Sarkosyl), and incubated at 55°C for 15 hours to insure lysis and release of genomic DNA. Once lysed, the beads were harvested by centrifugation (5500 x g for 10 min) and resuspended in 10 ml of 1 mM phenylmethylsulfonyl fluoride to incubation at room temperature for 2 h with gentle shaking to remove contaminating protease activity. The beads of purified DNA were washed three times in TE buffer (10 mM Tris-HCl, 1 mM EDTA-Na$_2$, pH 7.5) and resuspended in 10 ml TE buffer, and stored at 4°C until restriction endonuclease digestion was performed.

***In situ* restriction endonuclease digestion and pulsed-field gel electrophoresis.**
Agarose beads containing DNA were equilibrated in 1X restriction endonuclease buffer for 1 h prior to enzyme digestion. After equilibration 10 to 20 units of the restriction enzyme, *Xba* I, was added to 90 µl of beads, incubated at 37°C for 6-8 h. Following digestion, the beads were heated to 65°C to melt the agarose and inactivate the enzyme prior to loading ointo wells.

The DNA fragments were separated on 1.0% agarose gels in 0.5X TBE buffer at 15°C for 23 h using a CHEF-DR III electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, California). Each set of restriction endonuclease digests were separated at different initial and final pulse times to provide maximum separation of small, medium and large fragments. To determine the molecular size of the DNA fragments 50 Kb lambda DNA multimers, intact yeast chromosomes and restriction fragments of lambda DNA were included as standards.

RESULTS AND DISCUSSION

A total of 132 strains isolated from the rumen fluid were identified as
Propionibacterium based on the reduction of esculin, hydrolysis of gelatin, production
of propionate and acetate from lactate and the Gram-positive, pleomorphic cell
morphology. All isolates examined in this study fermented mannitol and trehalose. All
but three of the isolates fermented lactose. A total of 126 strains reduced nitrate and 26
of these isolates reduced nitrite as well. Based on these results, 132 of the isolates
were characterized as *P. acidipropionici* (capable of nitrate reduction) and 5 were
characterized as *P. jensenii*.

Ruminal populations of propionibacteria ranged from 10^3 to 10^4 cfu/ml.
Propionibacteria populations varied among different cows at different sampling times.
Three of the five cows used in this study had detectable propionibacteria populations at
two of the sampling times; only one cow had detectable propionibacteria populations at
all three sampling times while one cow never had detectable populations of
propionibacteria at any of the sampling times. Those isolates characterized as *P.
acidipropionici* comprised the predominant population at all sampling times, accounting
for 96% of the total propionibacteria isolated from the rumen. Based on these findings,
phenotypic diversity within the ruminal propionibacteria isolates appears to be limited.

Plasmids were detected in 32.6% (43 out of 132) of the isolates screened (Table
2). Only one strain contained more than one plasmid. Of the 42 isolates that contained
a single plasmid, 31 strains contained a 2.7 kb plasmid whereas the remaining 11
strains contained a 7.0 kb plasmid. No relationship between plasmid presence and the
biochemical and fermentation activity of the isolates was apparent.. Whether plasmids
of the same molecular weight found in different isolates are identical is not known.
Restriction endonuclease analysis could be used to further characterize the relationship
among these plasmids.

The predominant plasmid profile differed at each sampling date (Table 2). Plasmids of a similar molecular weight were not detected on different sampling times. The absence of plasmids in the majority of strains (67.4%) and the lack of conserved plasmids among isolates at different sampling times indicates that the plasmids detected are not important for survival and function of this organism in the ruminal environment.

Genomic DNA profiles produced by Xba I digests were used to identify isolates that shared the same DNA digestion pattern (Figure 1). Isolates with a common digestion pattern (> 90% of the fragments comigrating) were assigned to the same genomic digestion profile group (Table 3). Overall, 13 different digestion profiles were observed for the 110 isolates. Eight profiles (2, 4,7, 8, 9, 11, 12, 13) were unique to only one isolate. The predominant genomic profile (group I) was shared by 48 isolates, accounted for 43.6 % of all these isolates.

Little diversity in the genomic digestion pattern was observed in isolates from the first two sampling periods. The group I digestion profile was observed for 77.4% of the isolates from the first two sampling periods. However, this digestion profile was never detected in isolates from the June sampling. Indeed, none of the genomic profiles observed in isolates from the first two sampling times were ever seen in the isolates obtained in June and the diversity of the genomic profiles increased from 2 and 3 profiles in the first and second sampling periods to 9 different profiles for the June sampling, with 8 of these isolates having unique profiles.

IMPLICATIONS

For some unknown reason, during the June sampling, it appears the genetic diversity of the ruminal propionibacteria had increased markedly. Differences in ruminal populations across time and among animals, as well as changes in plasmid presence and type and genomic characteristics indicates that the native ruminal propionibacteria

are diverse and unstable. This is not surprising considering the potential fluctuations in substrate supply, end-product concentrations available for microbial use. Whether continued inoculation would stabilize the ruminal population remains to be seen. Further characterization and analysis of the native ruminal propionibacteria populations from lactating dairy cows is currently underway at Agtech Products, Inc., Milwaukee, WI.

Table 1. Ration Content (% on an as fed basis)[a]

Ration Mix	Diet A	Diet B
Alfalfa	7.8	2.2
Sorghum	44.6	66.4
Whole Corn	7.5	2.1
Grain Mix:	38.0	27.1
Shelled corn (67%)		
Soybean meal (27%)		
Molasses (3%)		
Limestone (1%)		
Dicalcium phosphate (1%)		
Trace minerals (1%)		
Prairie Hay	2.1	2.2
Bypass protein	1 lb	

[a] Cow 179 fed diet A ; others diet B.

Table 2. Plasmid DNA analysis of propionibacteria isolates.

| Sampling Date	Number of Isolates Screened	Number with or without plasmid	Plasmid Content	
			Number of Plasmids	MW (Kb)
February	31	20	0	
		11	1	7.0
April	35	4	0	
		31	1	2.7
June	66	65	0	
		1	2	1.6, 1.8

Table 3. Analysis of 41 propionibacteria isolates shown in Figures 1-4.

| Isolate Number | Species Identification | Plasmid Content | | Genomic digestion profile |
		Number of Plasmids	MW (Kb)	
162	*P. acidipropionici*	0		1
166	*P. acidipropionici*	0		1
169	*P. jensenii*	0		1
170	*P. acidipropionici*	0		1
173	*P. acidipropionici*	0		1
176	*P. acidipropionici*	0		1
178	*P. acidipropionici*	0		1
179	*P. jensenii*	0		1
180	*P. acidipropionici*	0		1
182	*P. acidipropionici*	ND		1
188	*P. acidipropionici*	0		1
195	*P. jensenii*	1	7.0	2
233	*P. acidipropionici*	0		3
236	*P. acidipropionici*	1	2.7	4
238	*P. acidipropionici*	1	2.7	1
245	*P. acidipropionici*	1	2.7	1
246	*P. acidipropionici*	1	2.7	1
248	*P. acidipropionici*	1	2.7	1
249	*P. acidipropionici*	1	2.7	1
261	*P. acidipropionici*	1	2.7	1
266	*P. acidipropionici*	0		3
272	*P. acidipropionici*	1	2.7	1
277	*P. acidipropionici*	0		3
279	*P. acidipropionici*	1	2.7	1
345	*P. acidipropionici*	0		6
346	*P. acidipropionici*	0		10
347	*P. acidipropionici*	0		10
348	*P. acidipropionici*	0		6
349	*P. acidipropionici*	0		6
350	*P. acidipropionici*	0		5
351	*P. acidipropionici*	0		6
352	*P. acidipropionici*	0		6
354	*P. acidipropionici*	0		5
362	*P. acidipropionici*	0		U
365	*P. acidipropionici*	0		U
377	*P. acidipropionici*	0		U
381	*P. acidipropionici*	0		U
388	*P. acidipropionici*	0		U
393	*P. acidipropionici*	0		5
395	*P. acidipropionici*	0		U
400	*P. acidipropionici*	0		U

ND = Not Determined
U = Unique genomic profile

Figure 1. Pulsed-field gel electrophoresis profile of genomic DNA from *Propionibacterium* isolates collected in February.
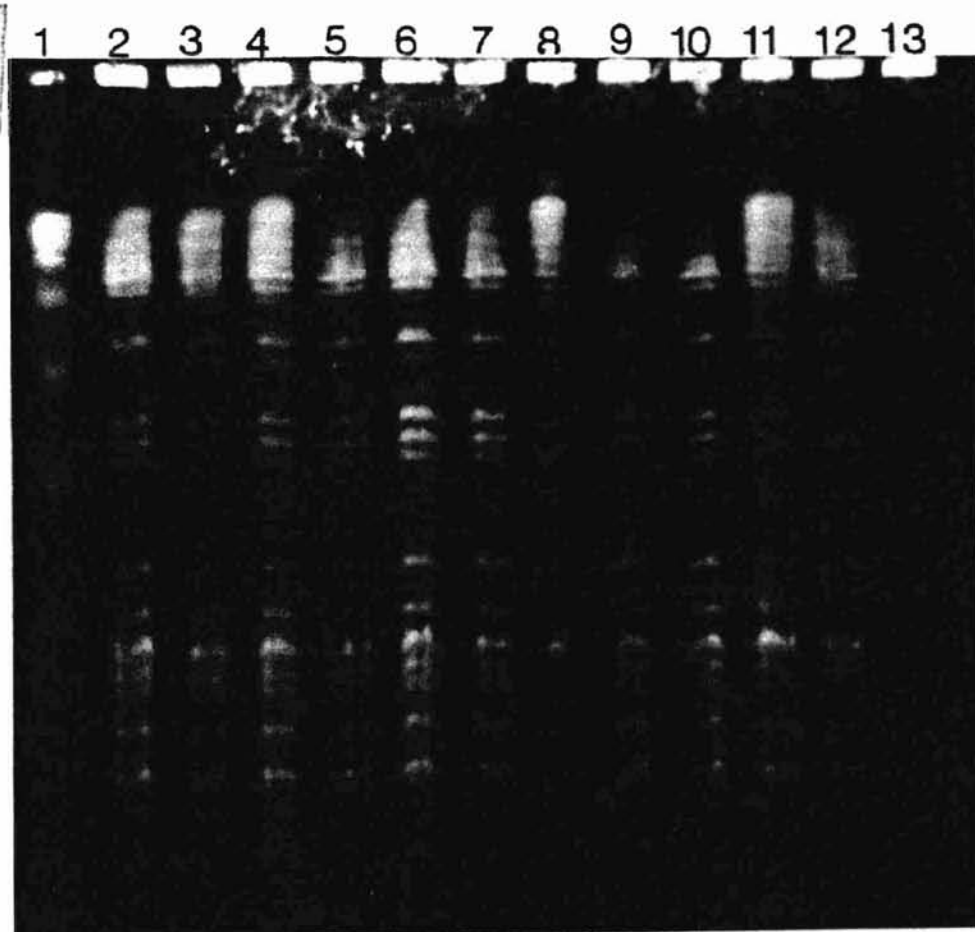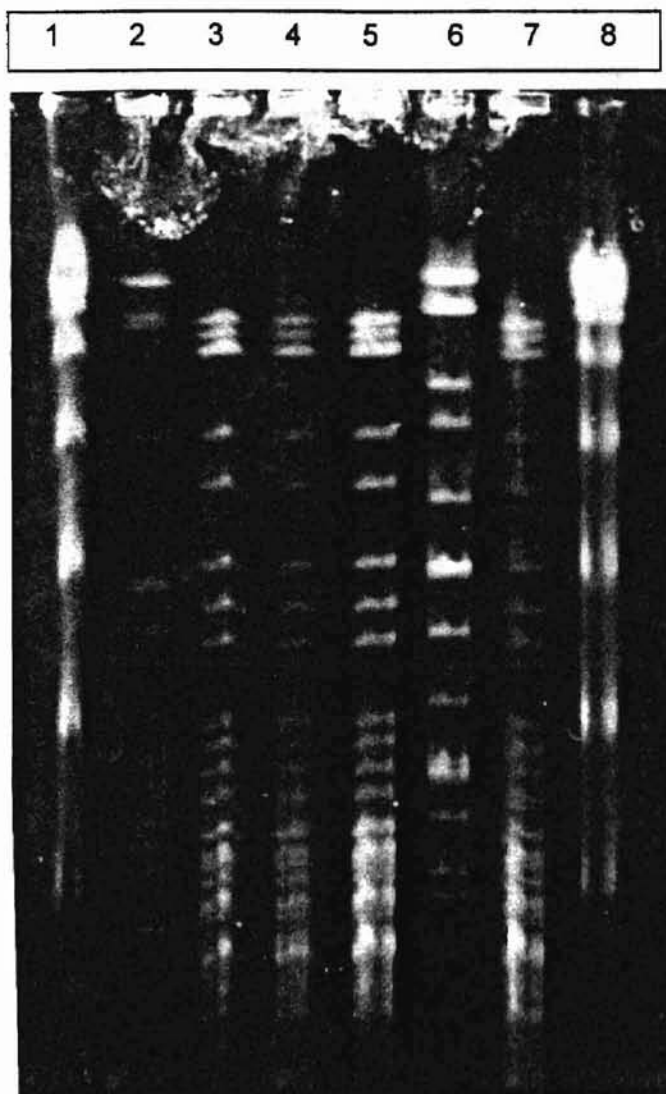

Lane #) strain number: 1) Lambda ladders 2) 162 3) 166 4) 169
5) 170 6) 173 7) 176 8) 178 9) 179
10) 180 11) 182 12) 188 13) 195

Figure 2. Pulsed-field gel electrophoresis profile of genomic DNA from *Propionibacterium* isolates colleted in April.
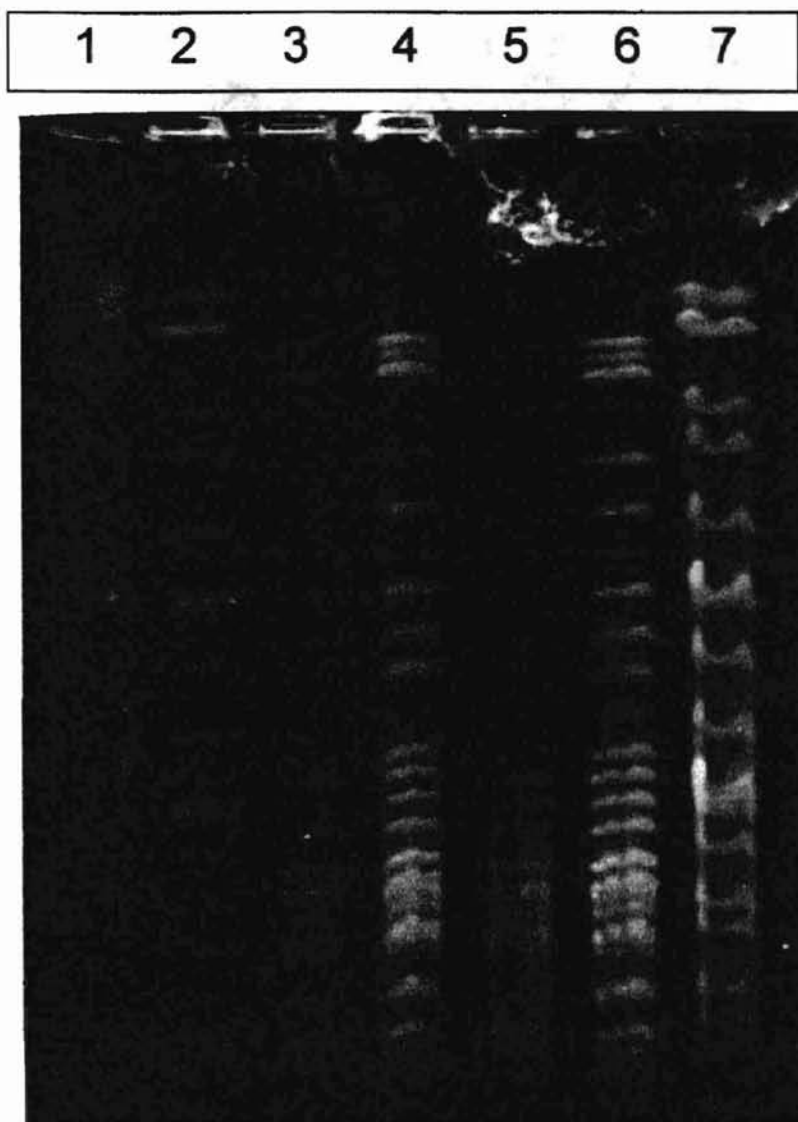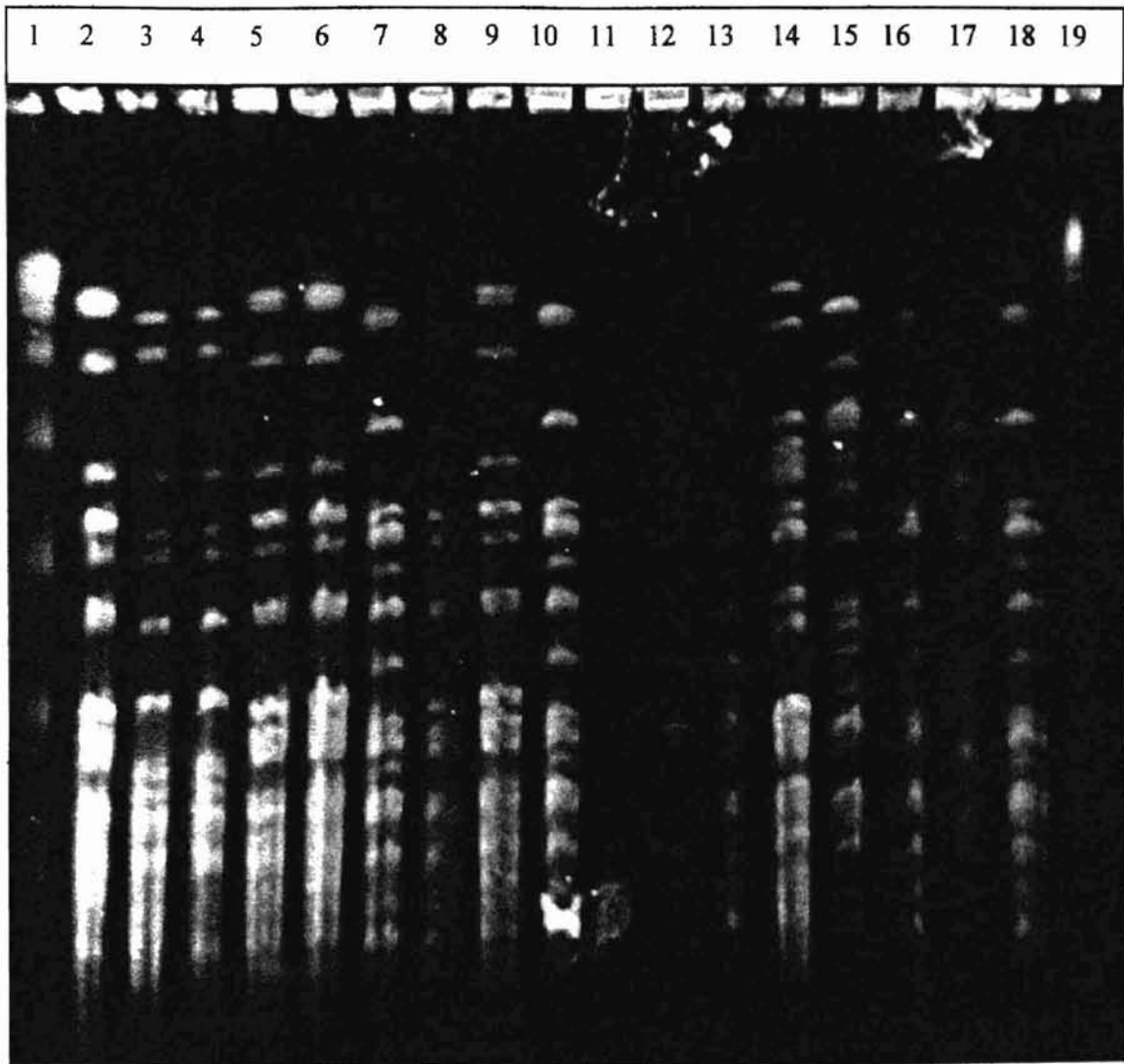

Lane #) strain number: 1) Lambda ladder 2) 266 3) 272
4) 249 5) 179 6) 248 7) 233

Figure 3. Pulsed-field gel electrophoresis profiles of genomic DNA from *Propionibacterium* isolates collected in April.

Lane #) strain number: 1) Lambda ladders 2) 236 3) 238 4) 245 5) 248 6) 277 7) 279 8) Lambda ladders

Figure 4. Pulsed-field gel electrophoresis of genomic DNA from Propionibacterium isolates collected in June.

Lane #) strain number: 1) Lambda ladders 2) 345 3) 346 4) 347 5) 348 6) 349
7) 350 8) 351 9) 352 10) 354 11) 362 12) 365 13) 377
14) 381 15) 388 16) 393 17) 395 18) 400
19) Lambda ladders

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CHAPTER IV

ACIDOSIS PREVENTION: IMPACT OF INOCULATION WITH A PROPIONIBACTERIUM MIXED CULTURE ON CATTLE RAPIDLY ADAPTATED TO A HIGH CONCENTRATE DIET

ABSRACT

A mixed culture of propionibacteria was inoculated daily during a 9 d period when heifers (500 kg) were being switched from a 0 to a 90 % concentrate diet. Each heifer received, via the cannula, a daily inoculation (6.3×10^{11} cfu) of a propionibacteria mixed culture consisting of three propionibacteria strains P54, P81 and P89. Ruminal fluid was collected to determine pH, total lactate, volatile fatty acid (VFA), and glucose concentrations, as well as ruminal osmolality and culture count. During the 9 day adaptation period, no acidotic episodes were observed, ruminal pH never dropped below 5.4 and no lactic acid accumulation was detected. However, following adaptation to a high concentrate diet, three acidotic events occurred, although lactic acid never accumulated to concentrations reported in the literature. During and after the inoculation period populations never exceeded dosage rates indicating that these cultures were not becoming established in the rumen. Perhaps lactate accumulation was being prevented while the animals were being inoculated with the mixed propionibacteria culture even though the mixed strain culture of propionibacteria never became established in the rumen.

(Key words: *Propionibacterium*, Acidosis, Cattle)

INTRODUCTION

Propionibacterium strains had been selected previously for their ability to utilize lactic acid in pure culture broth medium, and for preventing the accumulation of lactic acid and stabilizing pH in rumen fluid models (Parrott, 1997). Based on Parrott's (1997) results, three strains (P81, P89 and P54) were selected to be used in combination to prevent the ruminal acidosis in cattle being adapted rapidly to a high concentrate diet. Statistical re-analysis of Parrott's data fitting results to Gompertz non-linear equations, P63 and P54 were determined to be significantly better at utilizing lactic acid *in vitro* than any other strains. In addition, P54 and P63 were determined to be genetically identical based on the number and size of the DNA fragments produced by restriction endonuclease digestion on pulsed-field gel electrophoresis (Parrott, 1997).

Typically, the feedlot industry uses a 21 to 28 day adaptation period to adjust forage-fed cattle to high concentrate diets. Shortening this adaptation period to only 9 days would be advantageous economically to producers and would decrease the time the cattle need to be fed. The objective of this study was to determine if a mixed *Propionibacterium* culture would survive in the rumen and would stabilize pH through preventing accumulation of lactic acid in the rumen of cattle rapidly adapted (9 days) to a high concentrate diet.

MATERIAL AND METHODS

Three 500 kg ruminally fistulated heifers housed in metabolism stalls at the Oklahoma State University Nutrition-Physiology Unit in Stillwater, OK, had been maintained on a grass-hay diet for at least 10 wk. On days 1 and 2, a 30 % concentrate diet was fed. Subsequently the concentrate percentage of the ration was increased by 15% every second day such that a 90% concentrate was fed on and after

49

day 9 of the 21 day trial (Table 1). Diet composition is shown in Tables 2 and 3. The animals had ad libitum access to feed and water. Intake was monitored daily throughout the study.

Each animal was inoculated daily via its ruminal cannula for 21 days with 10 ml of the mixed culture suspended in Sodium Lactate Broth (NLB) with 10% added glycerol. The culture was grown in NLB from pure cultures supplied by AgTech Products, Inc. of Waukesha, Wisconsin. The cultures had grown in 1500 ml NLB for 48 h, they were plated at dilutions 10^{-7}, 10^{-8}, and 10^{-9} to determine the cfu/ ml and to examine purity. Cells were harvested by centrifugation in sterile 500 ml bottles at 5500 x g, and resuspended in NLB with 10% glycerol to a final volume 1/10 the initial volume (i.e., 150 ml). The three strains to be used then were mixed together, transferring 10 ml of each concentrated cell suspension to a tube for freezing. This resulted in a final volume of 30 ml of frozen cell suspension; of this 10 ml (6.3×10^{11} cfu/ml) was dispensed directly into the rumen via the cannula immediately after the 0800 sampling period each day for 21 d. Culture performance was monitored by serially diluting (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) rumen fluid and plating on a selective/differential media in order to enumerate the propionibacteria.

Ruminal fluid was sampled four times each day for 10 d (0, 2, 6, and 10 h after fresh feed) and pH, lactate and VFA concentrations were measured . For days 11 through 21, a single daily sample was taken at 0800 post-feeding. In addition, ruminal glucose and osmotic pressure were measured daily on days 0 through 3, 6 through 10, 15, 17 and 21. Approximately 50 ml of rumen fluid was removed from the rumen using a vacuum pump connected to an in-dwelling ruminal filter. Ruminal pH was measured immediately using a portable Digi-Sense[R] LCD pH meter (Cole Parmer Instrument Co., Chicago, IL). A 1.5 ml sample of rumen fluid was centrifuged at 5500 x g for 10 min to remove debris. The supernatant fluid was decanted and frozen (-20°C) until total (D + L) lactate and VFA analyses was performed using a Hewlett Packard 1090 High

Pressure Liquid Chromatography (HPLC) system equipped with a diode-array detector (Hewlett Packard, Atlanta, GA). Prior to analysis, these samples were thawed in cold water and 0.5 ml of the rumen fluid supernatant was transferred to a clean centrifuge tube and acidified with an equal volume of 0.01 M sulfuric acid to halt fermentation. The acidified sample was filtered through a 0.2 µm membrane directly into a 2 ml HPLC autosampler vial that then was capped. A 5 µl sample was injected and separated on a HPX-87H column (Bio-Rad Laboratories Inc., Hercules, CA) heated to 65 °C with a mobile phase of 0.005 M sulfuric acid at a flow rate of 1 ml/min. Lactate, acetate, propionate and butyrate were detected using a diode array detector scanning wavelengths 210 to 254 nm. Concentrations were determined by calculating peak areas at 210 nm and comparing peak size and times to known external standards using Hewlett Packard software.

Ruminal fluid glucose levels and osmotic pressure were measured using an Osmette Precision 2007 osmometer (Precision Systems, Inc., Sudbury, MA). Glucose concentrations were determined using a procedure adapted from the Rapid Stat [TM] Kit and the Glucose Rapid Stat Reagent (Pierce Chemical Co., Rockford, IL) using a single reagent, o-toluidine at 6% in glacial acetic acid, for a quantitative colorimetric determination. The o-toluidine reacts with the glucose in the presence of heat and acid to form a blue-green complex, Using a Gilford Response UV-VIS spectrophotometer (Gilford Instrumentation, Oberlin, OH) the glucose concentration could be determined by color intensity formed by the mixture.

RESULTS AND DISCUSSION

During the 9 d adaptation period (Table 7), as the diet increased in percent concentrate, dry matter (DM) intake decreased. Typically, intake decreased markedly on the day after ruminal pH dropped (Figures 5, 7, and 13). However, ruminal pH

51

remained above 5.4 throughout the 9 d adaptation period. In addition, lactic acid remained below detectable levels while total VFA concentration within the rumen increased from a mean of 14.9 mM on day 0 to a mean of 292.3 mM on day 10. Three acidotic events occurred during the 21 d study, all after the 9 d adaptation period. During each event, lactic acid concentration remained considerably lower than expected (Uhart and Carroll, 1967). Figure 19 illustrates the pH and lactate concentration of the three acidotic events to those levels reported by Uhart and Carroll.

On day 9, the first day of the 90% concentrate diet, animal 911 consumed nearly 4.0% of her body weight (20.2 kg) of feed. On day 10, her intake decreased considerably; six hours after being fed (1400), ruminal pH was 5.1 and lactic acid was 17.76 mM l. At 14 hours post-feeding (2200), rumen pH was 4.8 with a lactic acid concentration of 55.5 mM. At 2200, this animal was dosed intraruminally with sodium bicarbonate to prevent damage to the rumen wall and reduce absorption of the lactic acid to the blood stream. Uhart and Carroll (1967) reported that when rumen pH of their cattle reached 4.8, lactic acid concentrations were 99.96 mM. Though lactate was considerably higher than the 1 to 4 mM concentrations normally found in the rumen, its concentration was under 60% that reported by Uhart and Carroll. Ruminal glucose concentrations increased during the acidotic event from being unobservable on d 9 to 1.68 mM on d 10 while ruminal osmotic pressure increased from 319 to 374 mOsm/kg. Figures 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 18, 19, 20 and 21 illustrate the interaction between pH, feed intake, ruminal osmolarity and ruminal glucose for each animal.

The same animal (911) experienced a second acidotic event on d 19 during which ruminal pH dropped to 4.7. The night before this acidotic event, the cap on the ruminal cannula dislodged and a large amount of rumen content was expelled. A reduced quantity of buffered ruminal contents would make it difficult to compensate for acids produced from freshly consumed feed and enhance the potential for acidosis. At the

time of the event, ruminal lactate was 21.09 mM; however, no treatment was administered and ruminal pH returned to 5.6 with no traces of lactate on d 21.

On d 15, animal 528 became acidotic; ruminal pH dropped to 4.8 and lactic acid reached 20.6 mM at 0800. Approximately 9 h post-feeding, though intake was low (1.4 kg), ruminal pH reached 4.6. Sodium bicarbonate was administered and ruminal pH returned to normal (6.3 on d 16) with no detectable level of lactic acid. Oddly, this animal exhibited lactate accumulation prior to the acidosis event, although pH remained stable. On d 13 pH was 5.6 and lactate measured 28.2 mM while d 14, pH was 5.7 and lactate was 13.3 mM.

The remaining animal in the study never experienced an acidosis during the trial. This animal did experience one day of low intake during the adaptation period, ingesting only 4 kg of feed on d 6, compared to a mean of 14 kg/d the other 20 days. On d 6 of the trial, ruminal pH measured 5.3 at 0800 but no lactic acid was detected and intake rebounded with no detrimental effects.

Standard plate counts on a selective/ differential media showed that on average the rumen population of the mixed culture to be 2.0×10^6 cfu/ml. Table 8 shows the average plate counts for d 8, 9, 10, 14, 17 and 21. Values from d 0 through 7 plate counts were discarded due to media contamination. Total counts in the rumen, assuming ruminal liquid volume was 50 L and liquid dilation rate was 5%/ h, also are presented. Ruminal counts, taken 24 h after dosing, never exceeded the dose level. The culture was tracked for 21 d after the final inoculation; no inoculated organisms were detected; indicating that this mixed strain culture did not become established in the rumen. Although, some of the organisms may have become established among the rumen pilli on the ruminal wall, swabs of the ruminal surface yielded no propionibacteria based on conventional plating methods.

These data suggests that a mixed strain culture of *Propionibacterium* strains 54, 81, 89 did not become established in the rumen. Nevertheless, inoculation appeared

to stabilize ruminal pH during dietary challenge. Unfortunately, uninoculated animals were not equally challenged. Ruminal lactate concentrations never accumulated to the expected levels indicative of such a severe dietary challenge, even when pH had dropped. Most remarkably was the fact that none of these animals became acidotic during the rapid adaptation period, as both literature (Kezar and Church, 1979; Dunlop, 1972; Dunlop and Hammond, 1965; Huber, 1976; Slyter, 1976) and common sense would predict.

During this trial, all three animals exhibited variable feed intake, with six incidences occurring throughout the 21 d . As each incidence occurred an abnormally high propionate level was found during HPLC analysis to occur a few days before. An internal standard was injected into the sample to determine if it was truly propionate. The peak split and revealed a co-eluting compound. After intensive study, the compound was unable to be identified

IMPLICATIONS

Further experimentation is needed to evaluate culture efficacy in the ruminal environment. Future experimental models need to include animals that remain uninoculated, yet nutritionally challenged equally.

Table 4. Composition of concentrate diets (DM basis).

Ingredient, %	Concentrate Content				
	30%	45%	60%	75%	90%
Ground corn	22.1	36.9	51.8	66.9	82.0
Cottonseed hulls	35.1	26.2	17.7	7.6	0.0
Alfalfa pellets	35.1	29.2	22.7	17.7	10.2
B-075 Supplement	7.7	7.7	7.8	7.8	7.8

Table 5. Composition of B-075 supplement on a DM basis.

Ingredient%	
Cane molassed	2.37
Limestone 38%	14.26
Salt	3.79
Vitamin A-30,000	0.13
Cottonseed meal	59.22
Soybean meal 44%	11.72
Manganese oxide	0.08
Zinc oxide	0.06
Potassium chloride	1.93
Urea	6.44

Table 6. Nine day adaptation scheme.

Day	% Concentrate
0	0
1	30
2	30
3	45
4	45
5	60
6	60
7	75
8	75
9-21	90

55

Table 7. Mean intakes during the adaptation period.

Day	% Concentrate	Mean DM intake (kg)
1	30	22.7
2	30	10.0
3	45	11.0
4	45	14.0
5	60	12.3
6	60	8.9
7	75	11.8
8	75	10.3
9	90	13.6

Table 8. Enumeration of the mixed *Propionibacterium* culture (P54, P81 and P89) from rumen fluid.

Day	Count (Mean)cfu/ml
1-7	ND
8	2.1×10^6
9	3.8×10^6
10	1.3×10^6
14	1.7×10^6
17	1.2×10^6
21	3.1×10^5

*ND not determined due to contamination

Table 9. Daily mean ruminal pH and total VFA concetrations.

Day	pH	[VFA] (mM/L)
0	7.14	14.9
1	7.01	32.0
2	6.53	33.3
3	6.53	35.7
4	6.19	44.3
5	5.92	44.3
6	5.74	132.3
7	5.98	46.3
8	5.86	152.4
9	6.07	191.4
10	5.78	292.3
11	6.27	122.0
12	6.67	89.5
13	6.37	142.0
14	6.07	159.4
15	5.50	166.5
16	6.00	132.1
17	5.97	169.0
18	6.13	116.4
19	5.23	82.3
20	5.40	97.0
21	5.53	78.6

Figure 5. Comparison of Feed Intake vs. Ruminal pH for Animal 911 Experiment One.
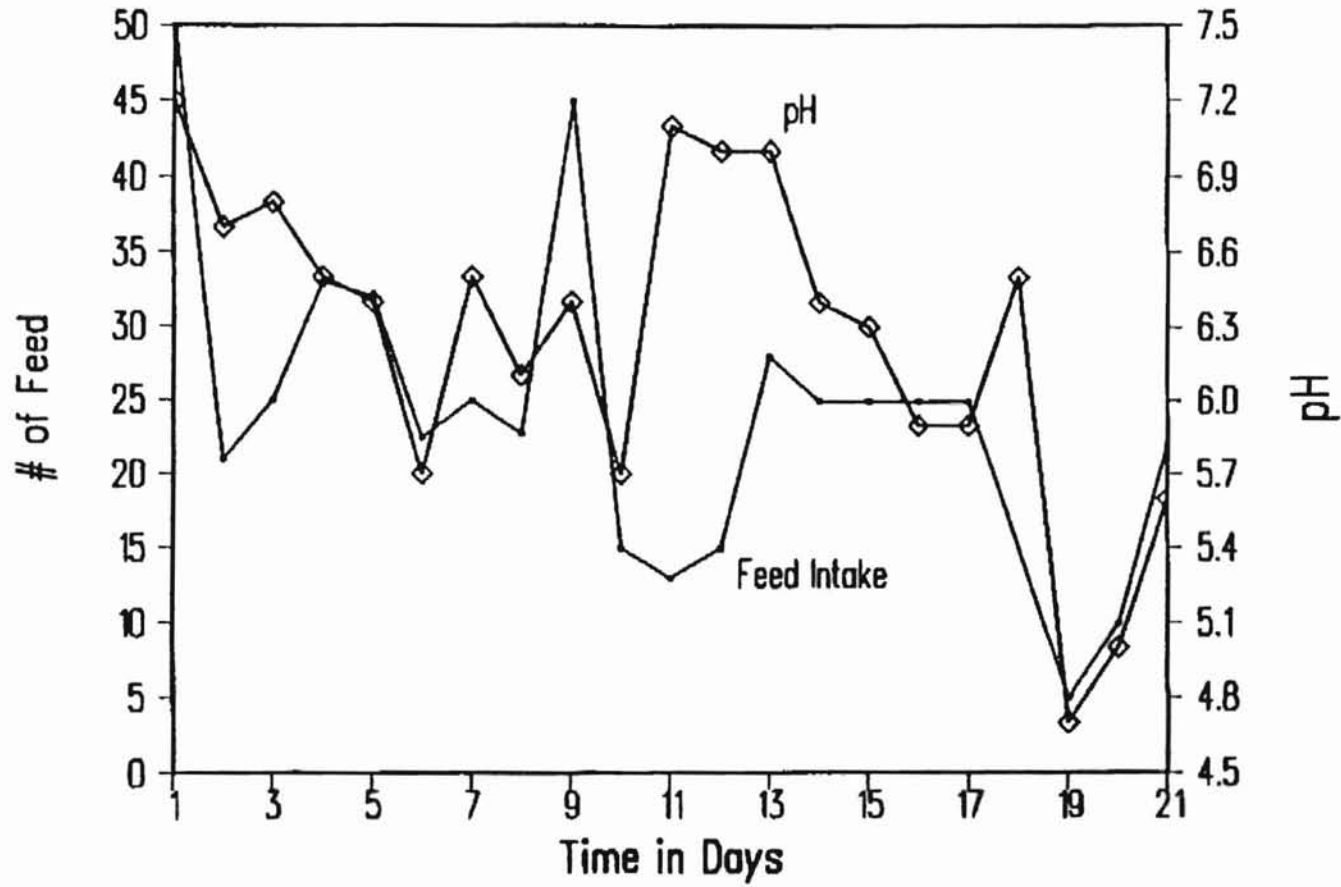
Figure 6. Comparison of Feed Intake vs. Ruminal Osmolarity for Animal 911 Experiment One
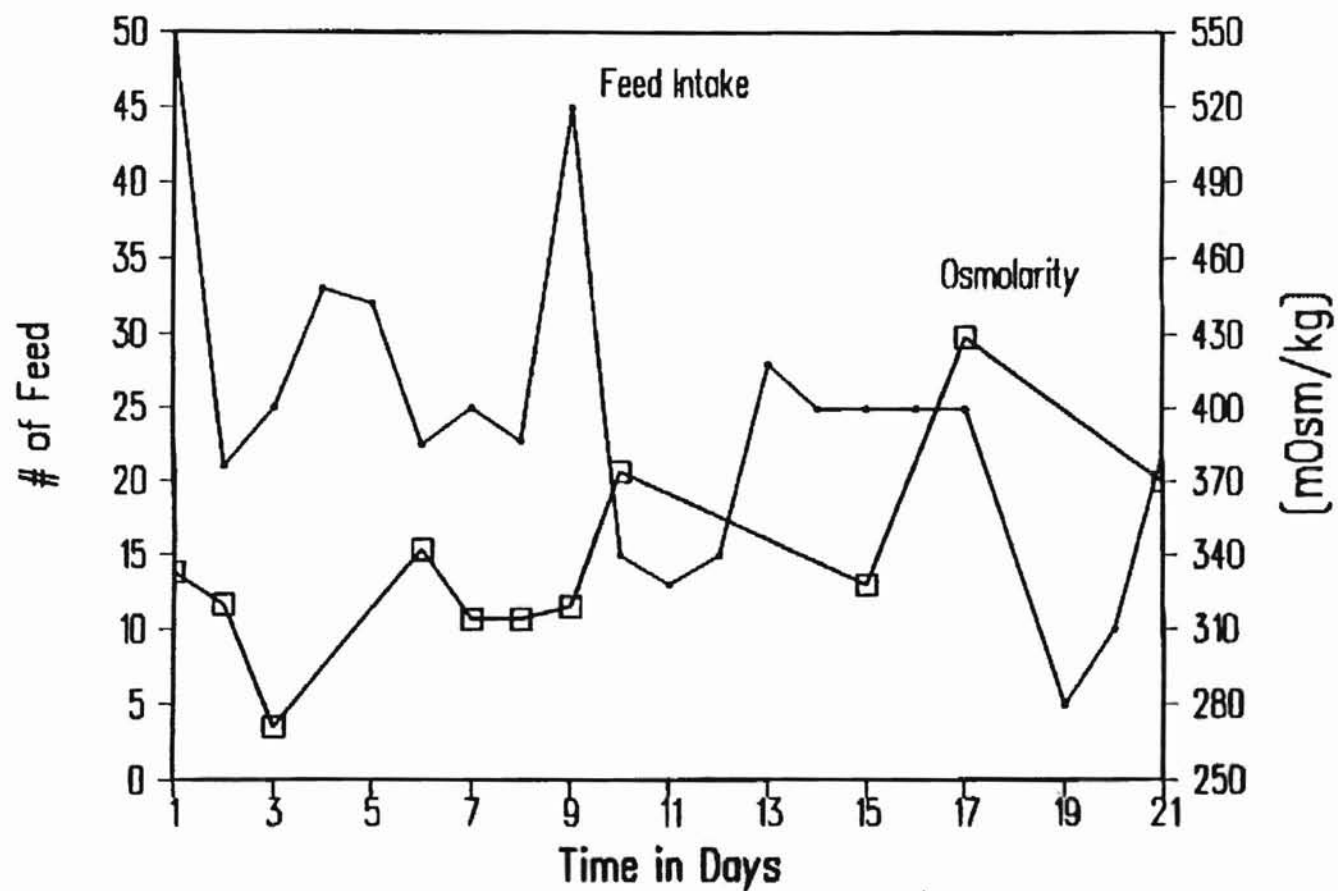
Figure 7. Comparison of Feed Intake vs. Ruminal Glucose Concentration for Animal 911 Experiment one.
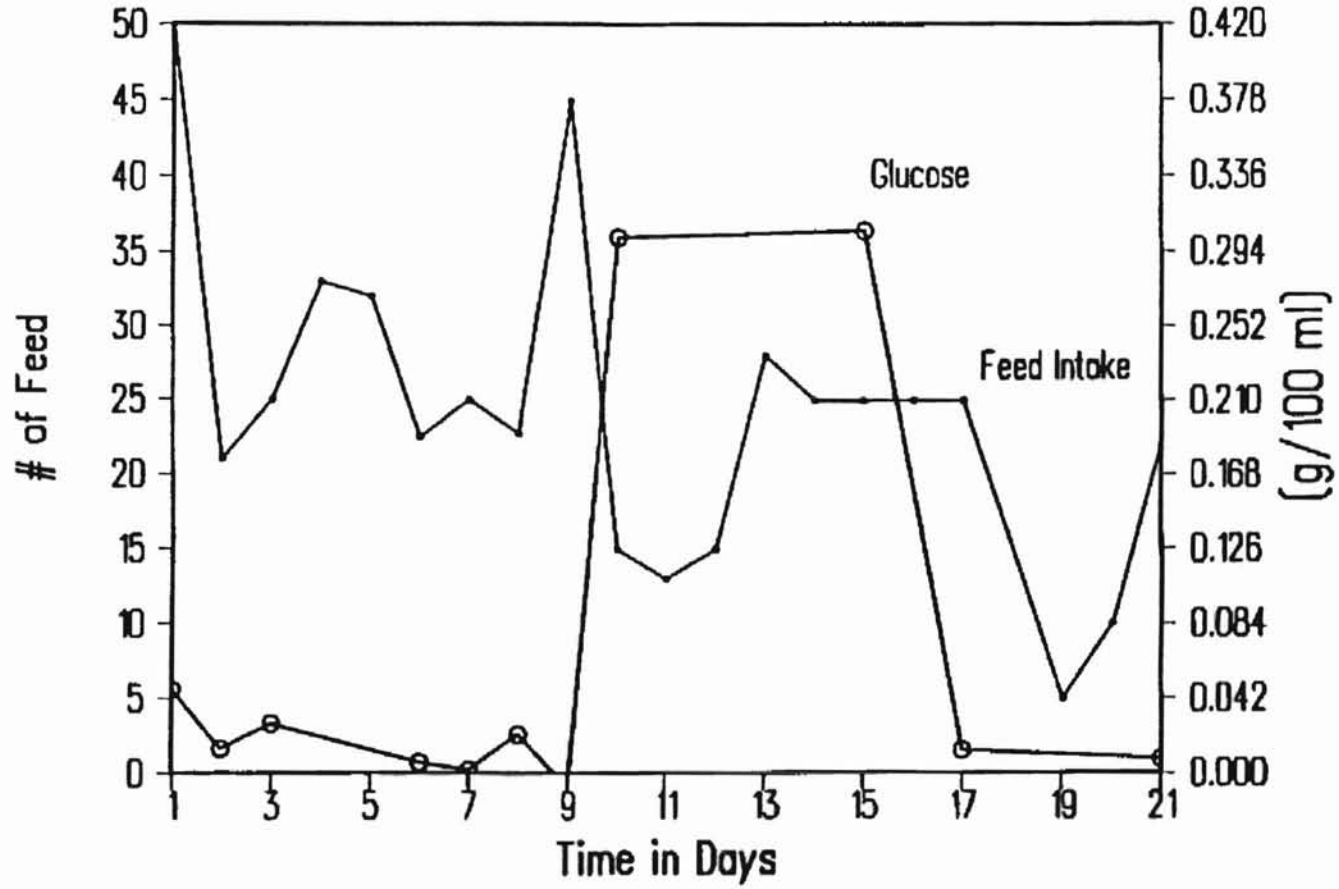
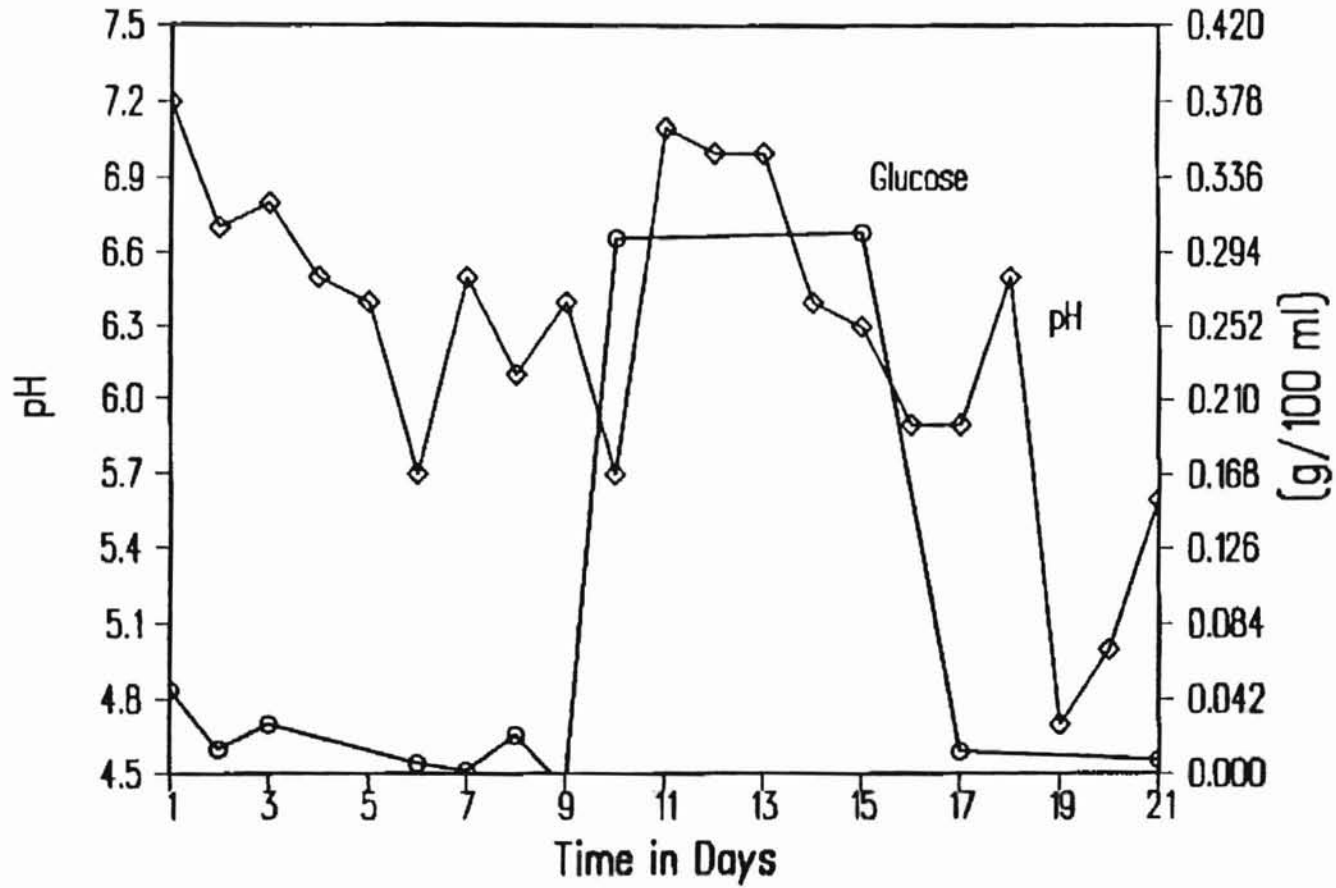Figure 8. Comparison of Ruminal pH vs. Ruminal Glucose Concentration for Animal 911 Experiment One.

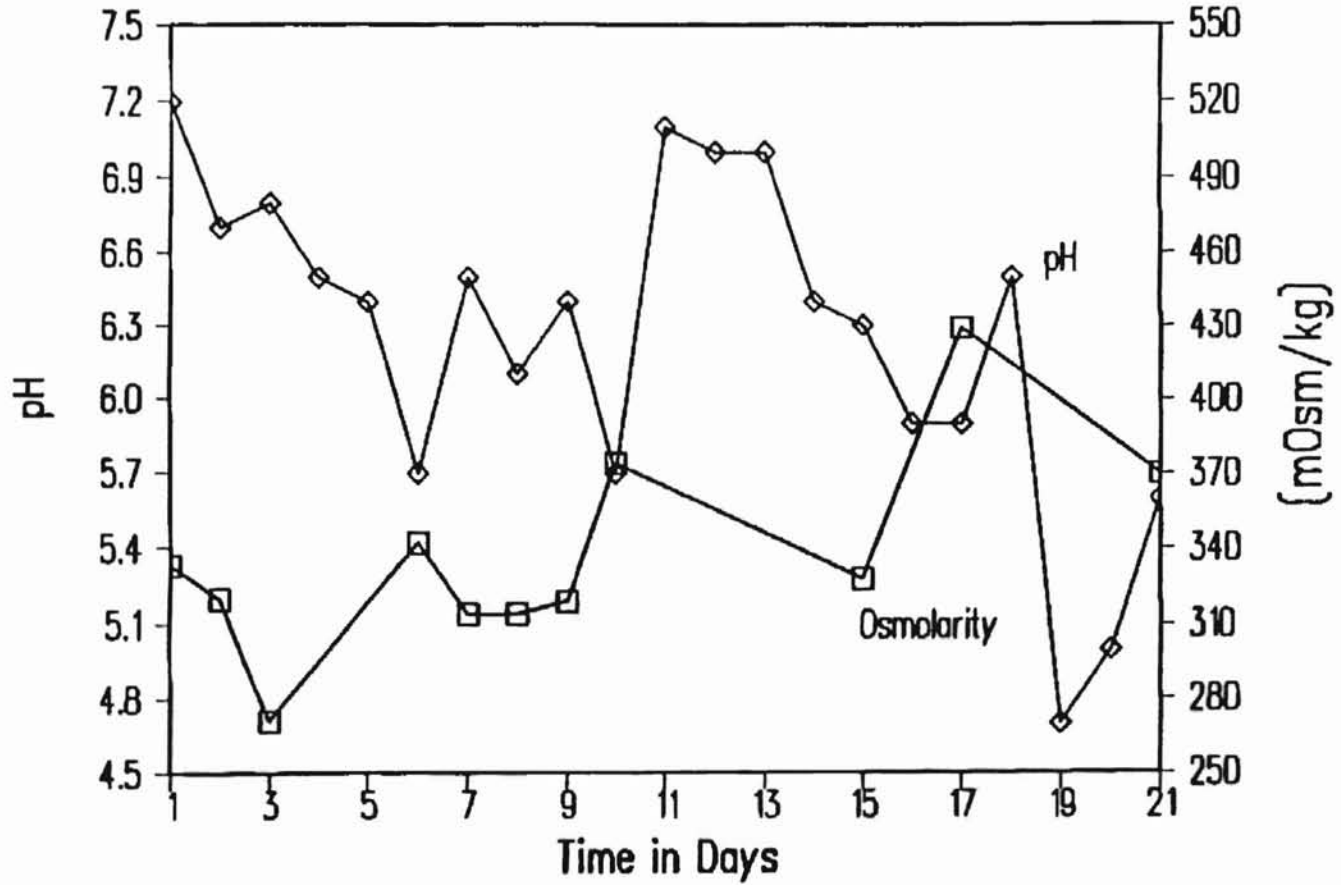Figure 9. Comparison of Ruminal pH vs. Ruminal Osmolarity for Animal 911 Experiment One

Figure 10. Comparison of Ruminal Glucose Concentration vs. Ruminal Osmolarity for Animal 911 Experiment One.
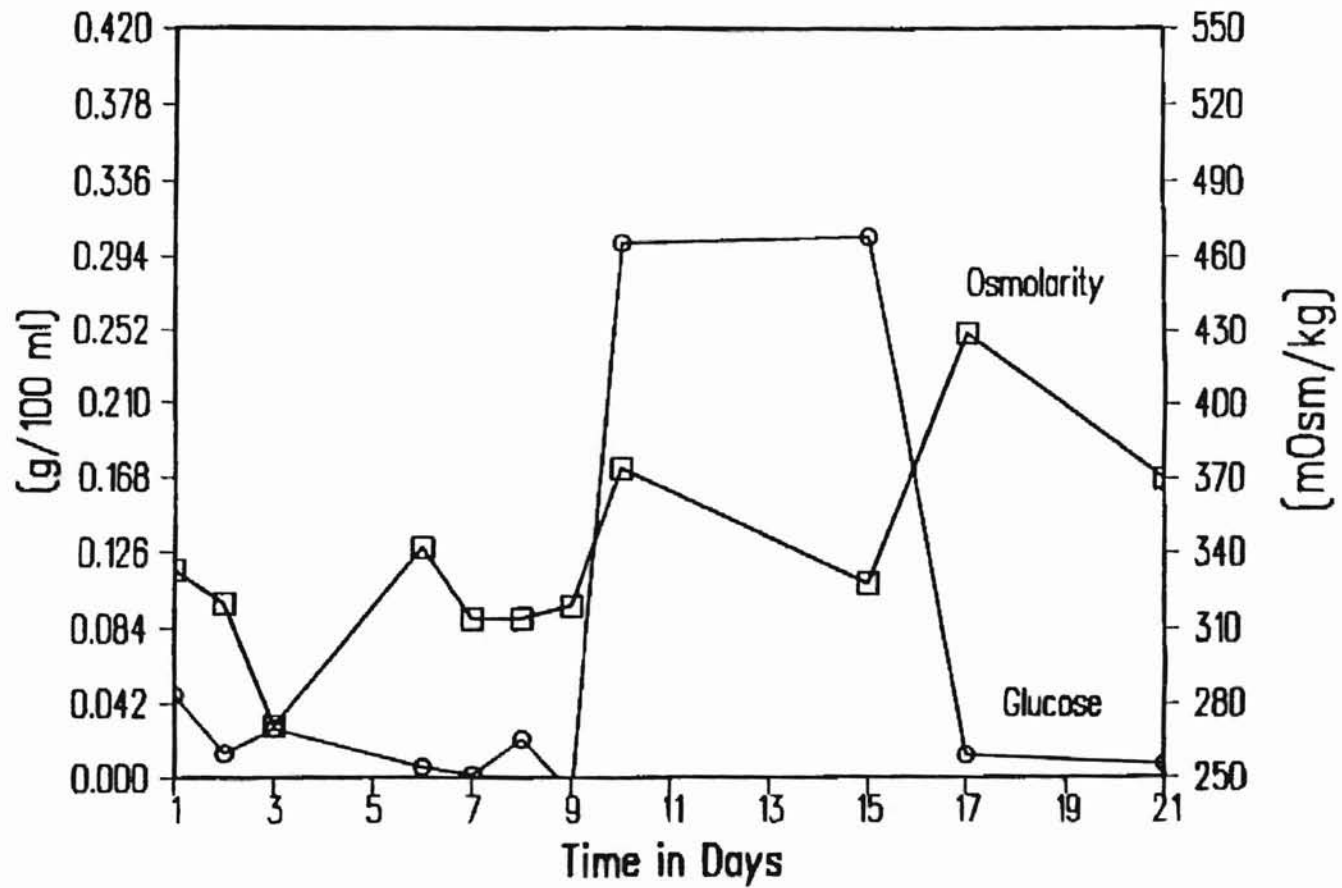
63

Figure 11. Comparison of Feed Intake vs Ruminal pH for Animal 528 Experiment One.
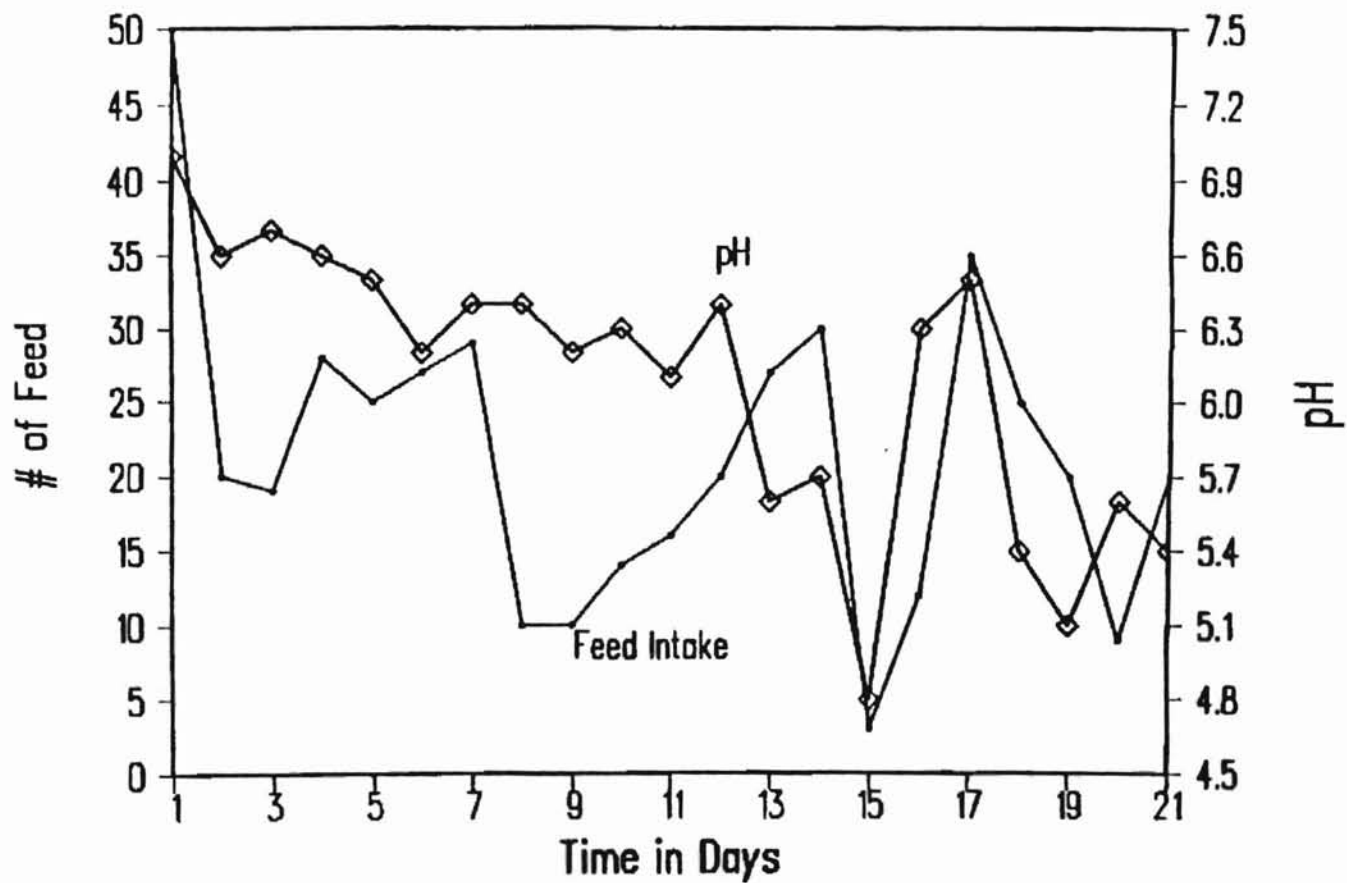
Figure 12. Comparison of Feed Intake vs Ruminal Glucose Concentrations for Animal 528 Experiment One.
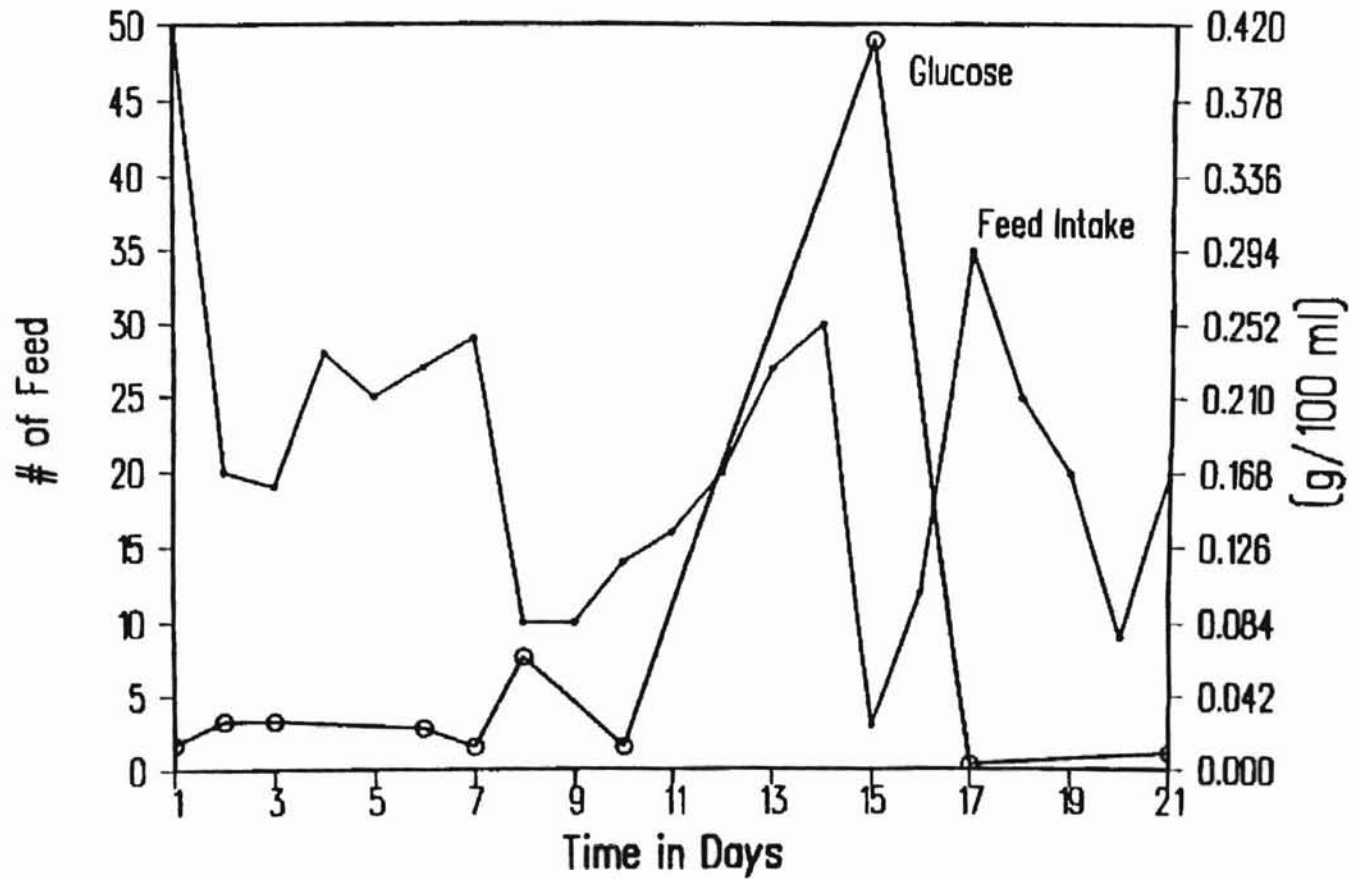
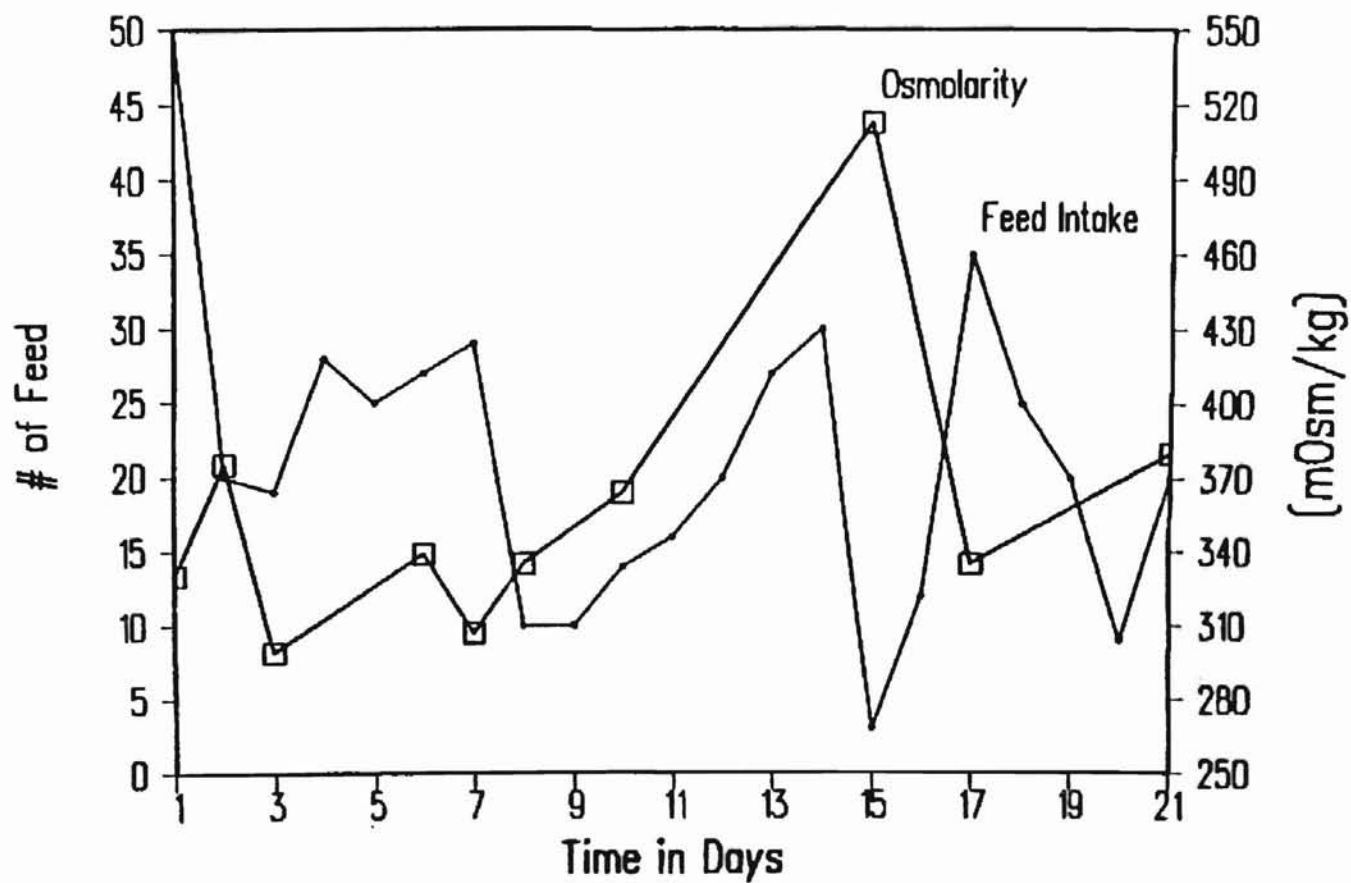Figure 13. Comparison of Feed Intake vs. Ruminal Osmolarity for Animal 528 Experiment One.

Figure 14. Comparison of Ruminal pH vs. Ruminal Glucose Concentration for Animal 528 Experiment One.

Figure 15. Comparison of Ruminal pH vs. Ruminal Osmolarity for Animal 528 Experiment One.

89

Figure 16. Comparison of Ruminal Glucose Concentration vs. Rumial Osmolarity for Animal 528 Experiment One.

Figure 17. Comparison of Feed Intake vs. Ruminal pH for Animal 531 Experiment One.
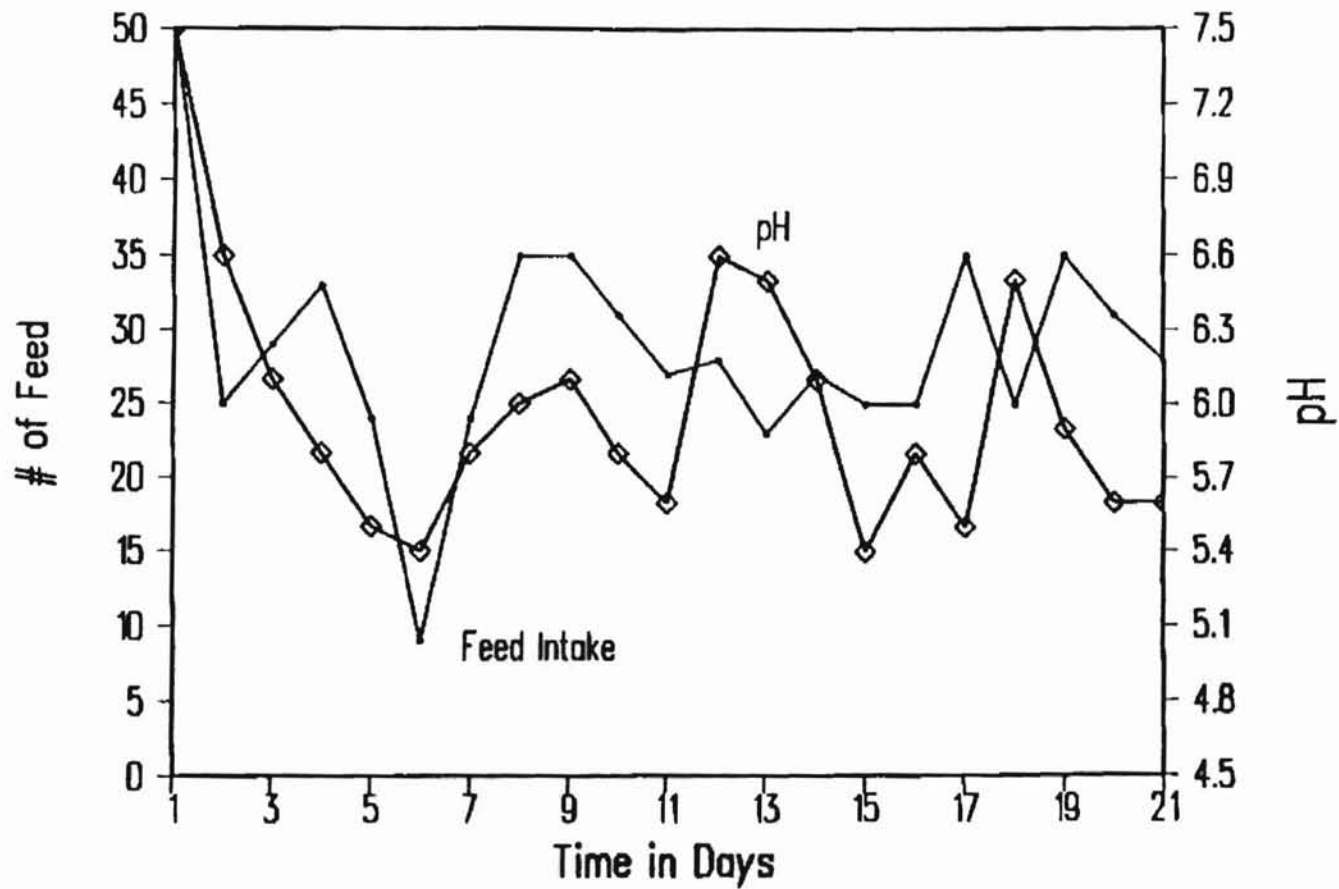
Figure 18. Comparison of Feed Intake vs. Ruminal Glucose Concentrations for Animal 531 Experiment One.
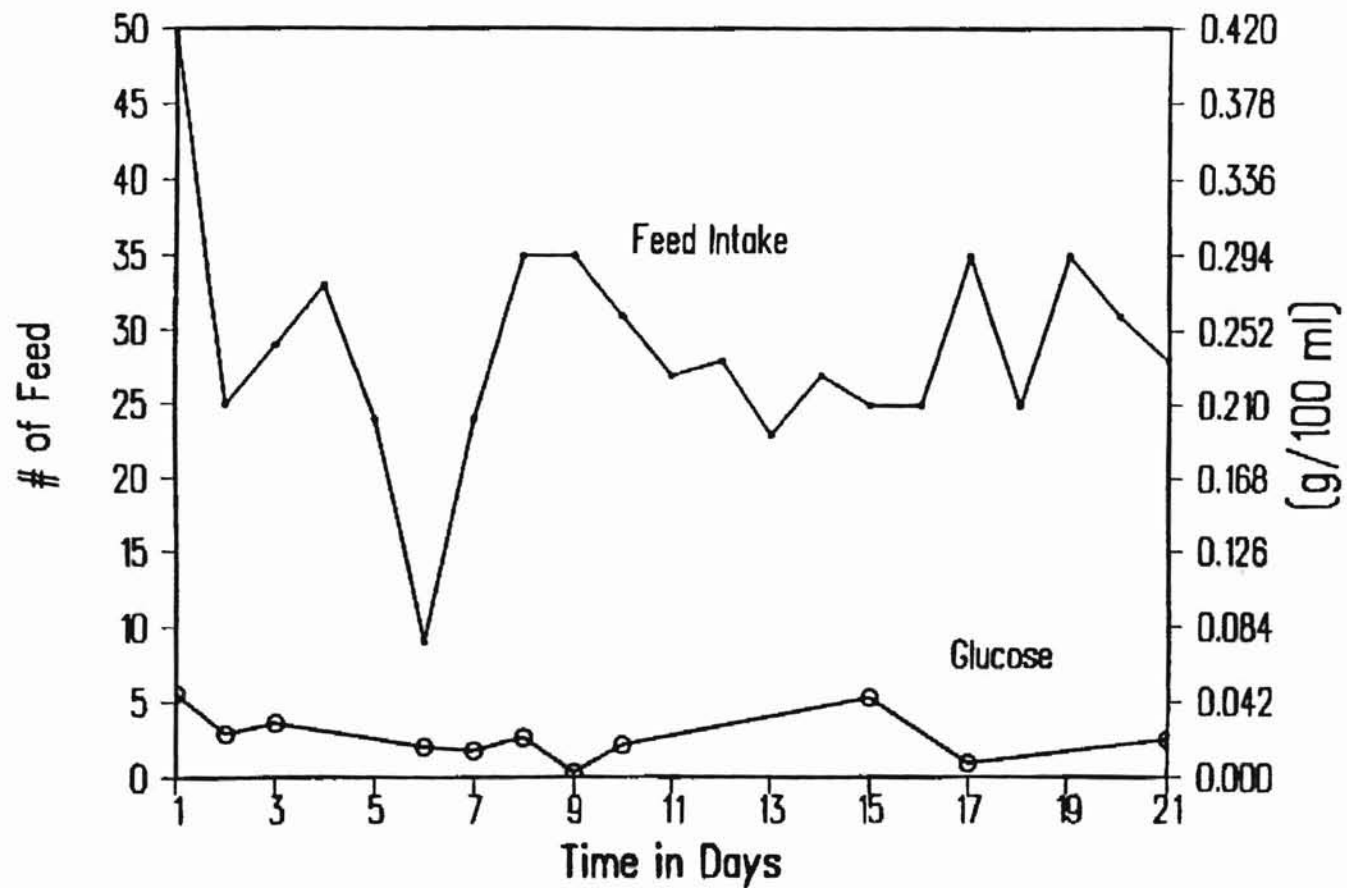
Figure 19. Comparison of Feed Intake vs. Ruminal Osmolarity for Animal 531 Experiment One.
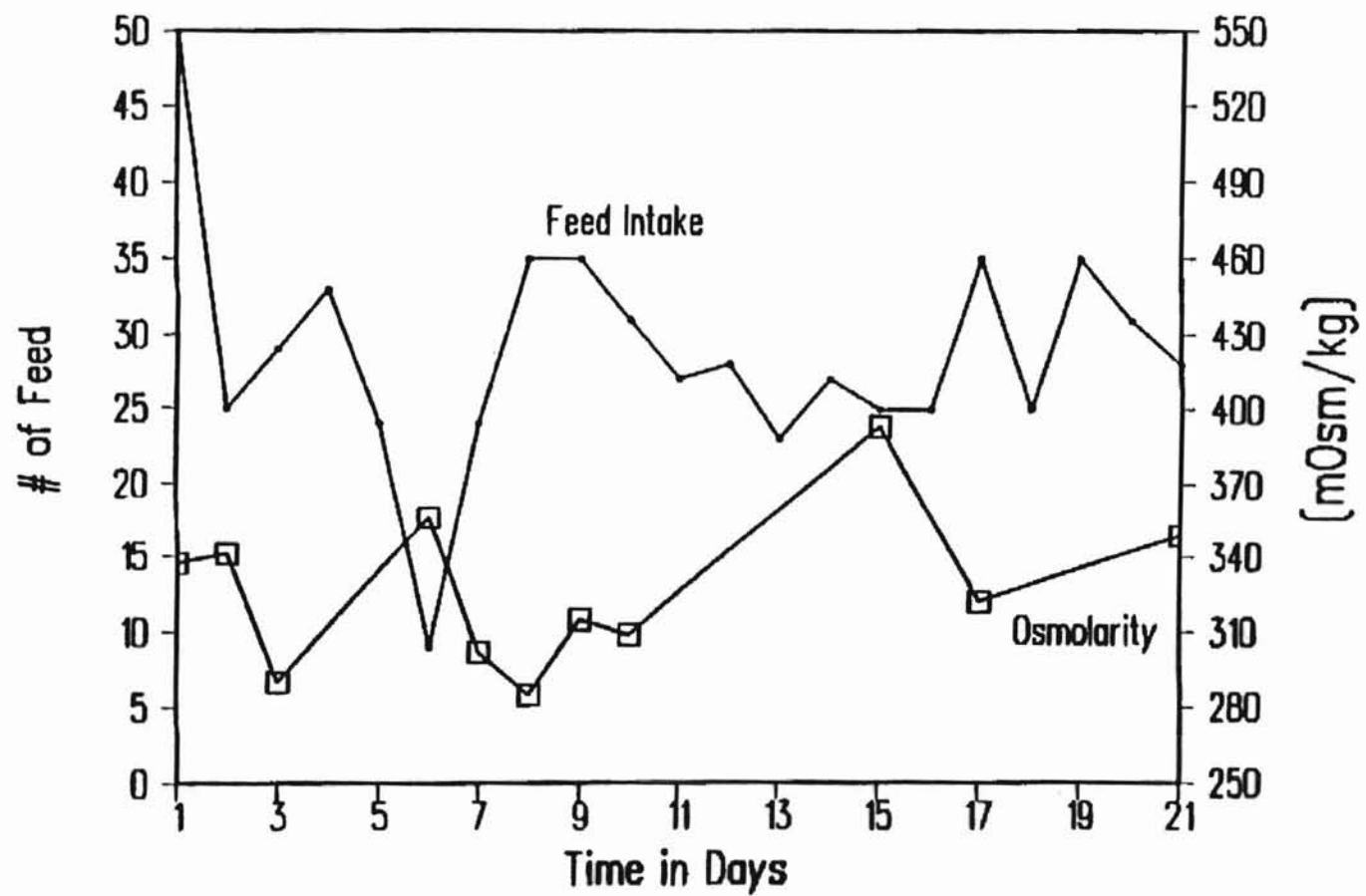
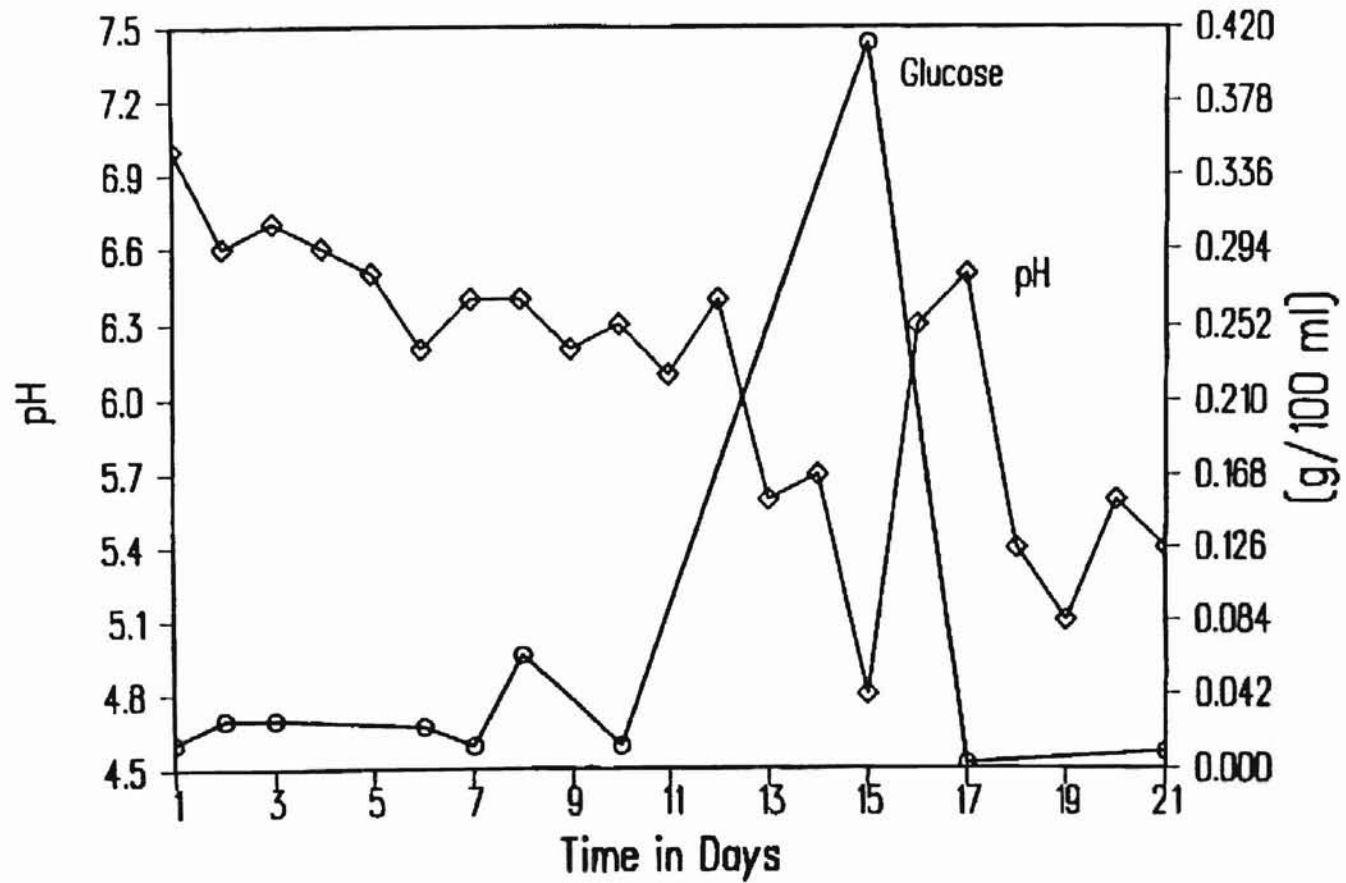Figure 20. Comparison of Ruminal pH vs. Ruminal Glucose Concentrations for Animal 531 Experiment One.
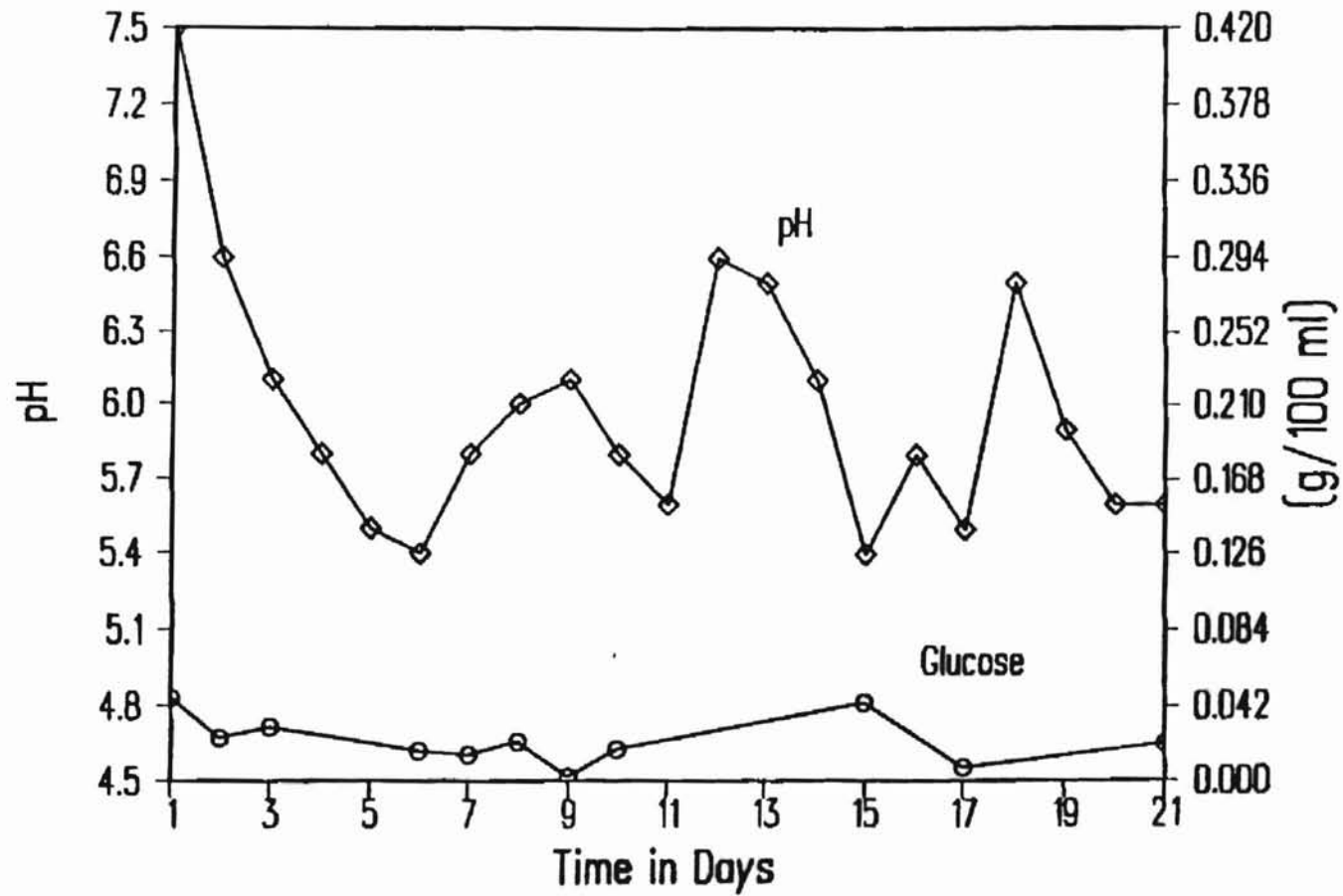
Figure 21. Comparison of Ruminal pH vs. Ruminal Osmolarity for Animal 531 Experiment One.
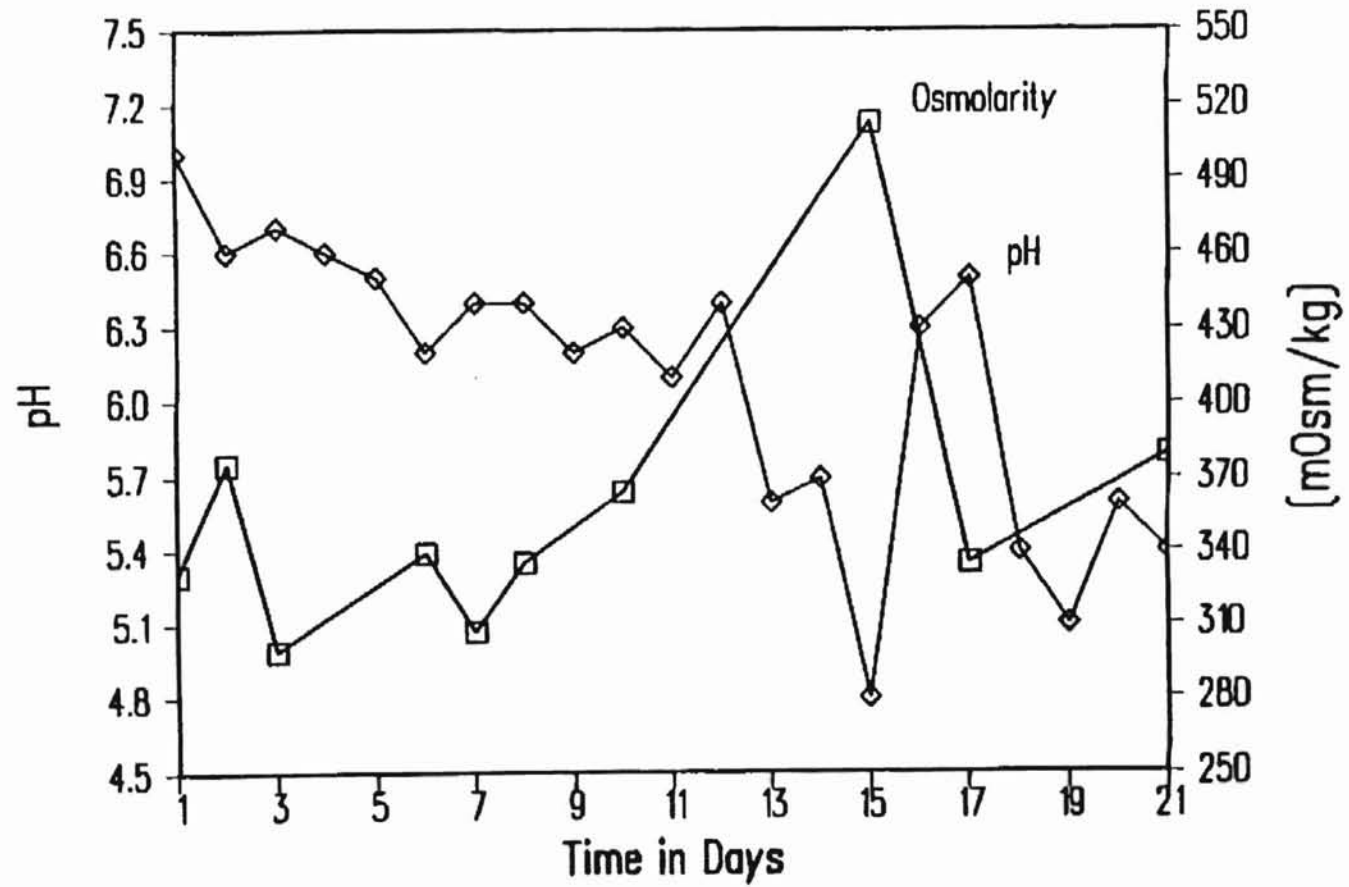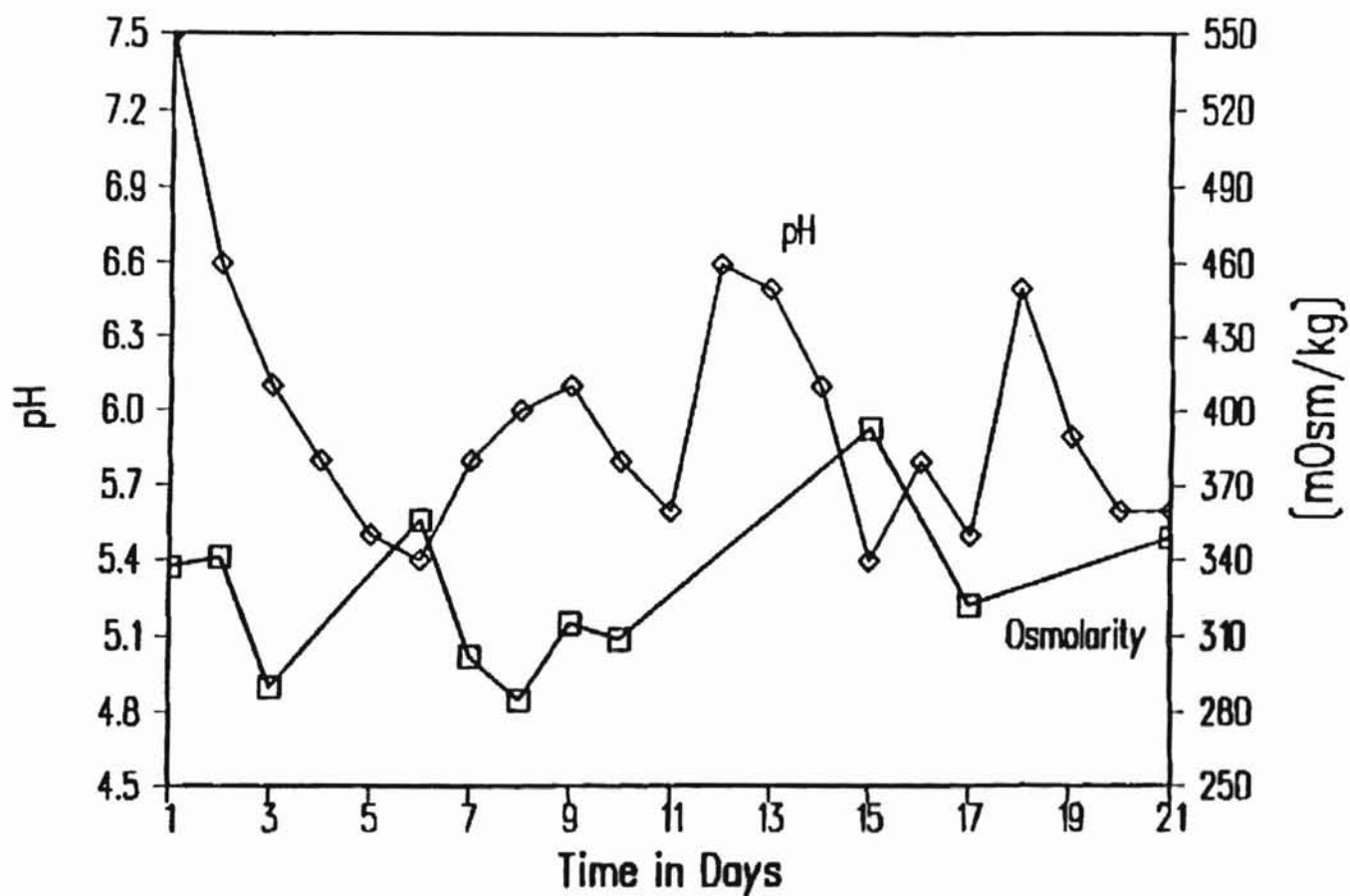
Figure 22. Comparison of Ruminal Glucose Concentrations vs. Ruminal Osmolarity for Animal 531 Experiment One.
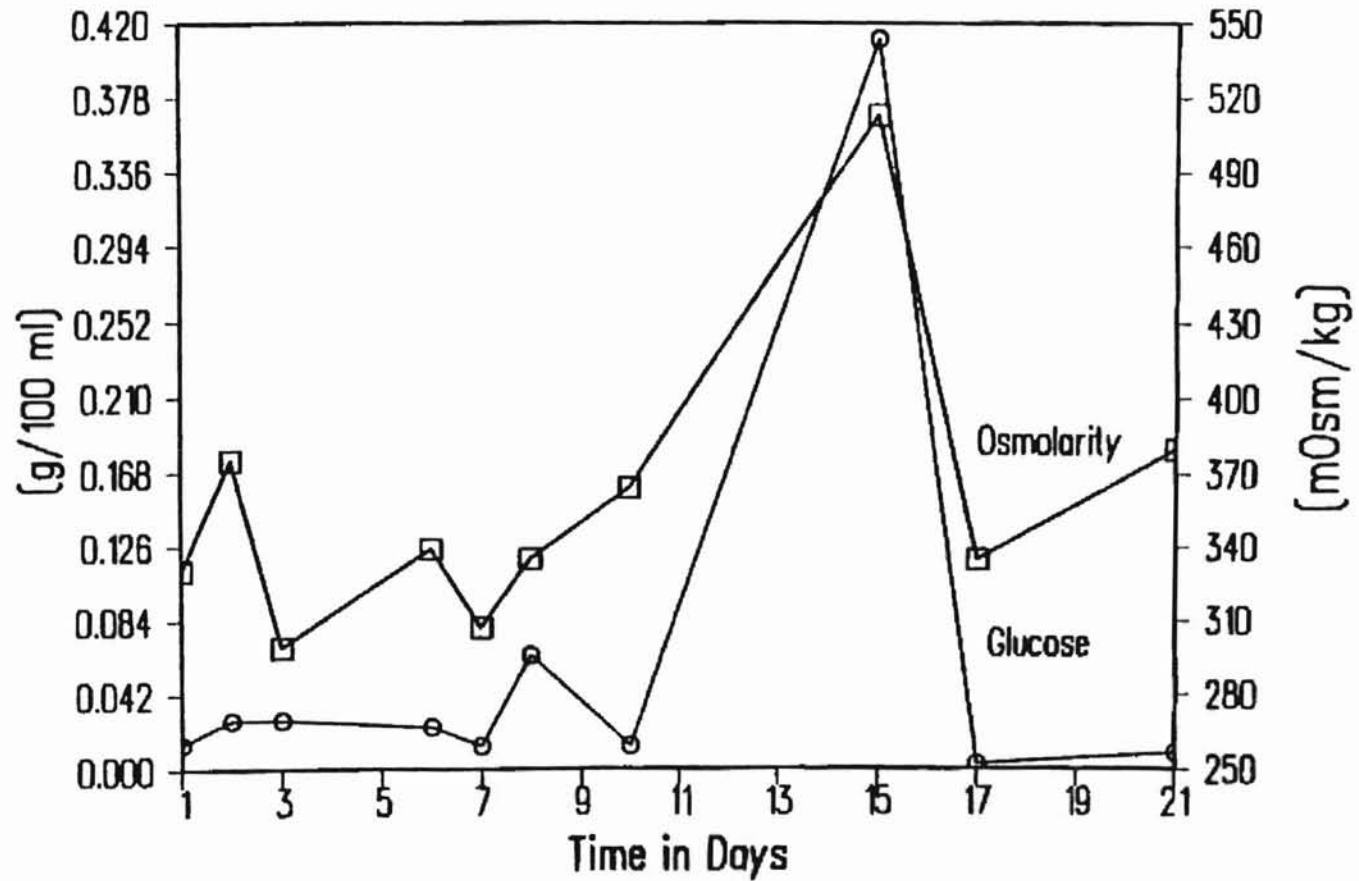
75
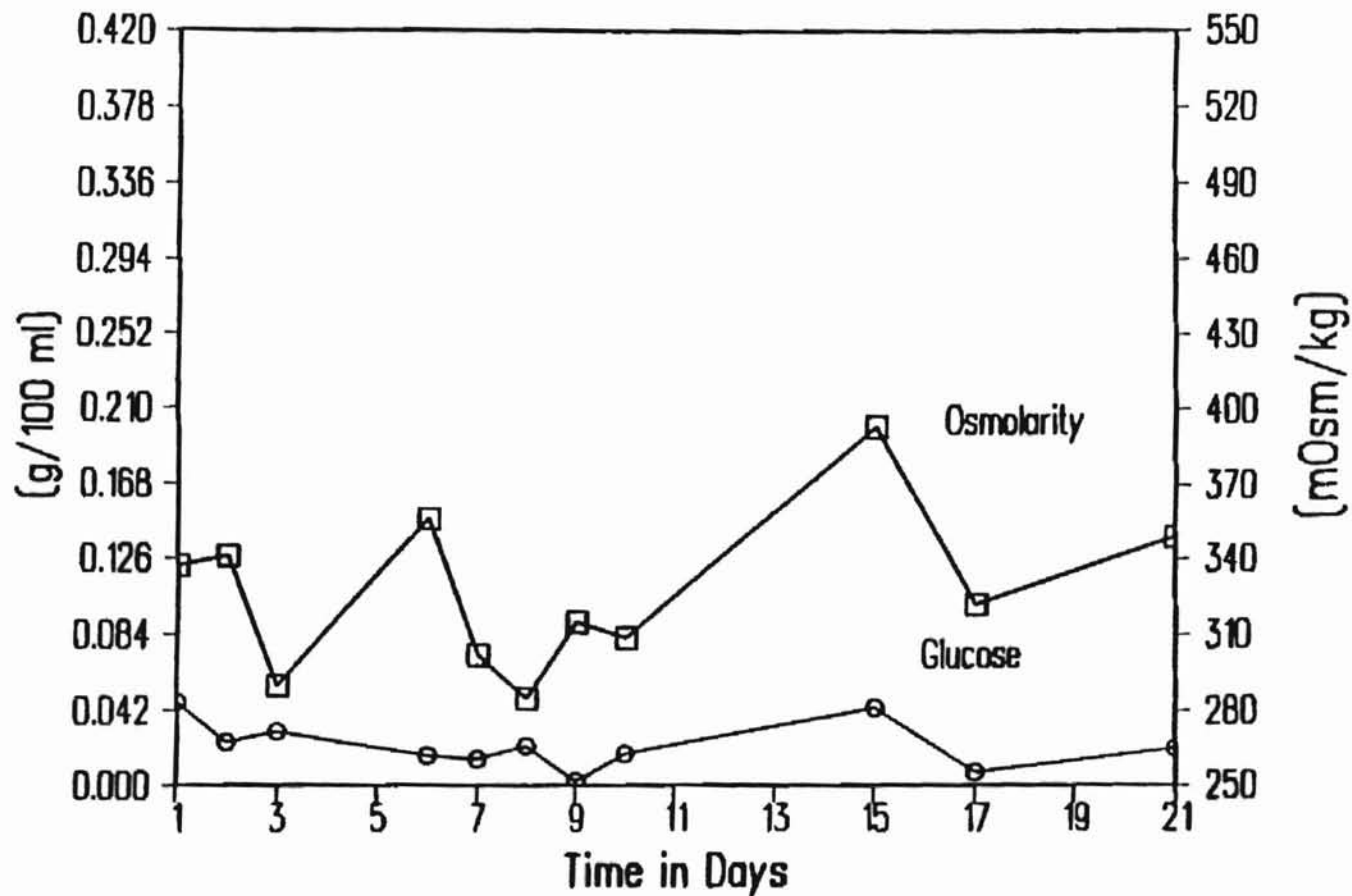
Figure 23. Comparison of Ruminal pH and Relative Lactate Concentrations for Experiment 1 (animal 911) and Literature.
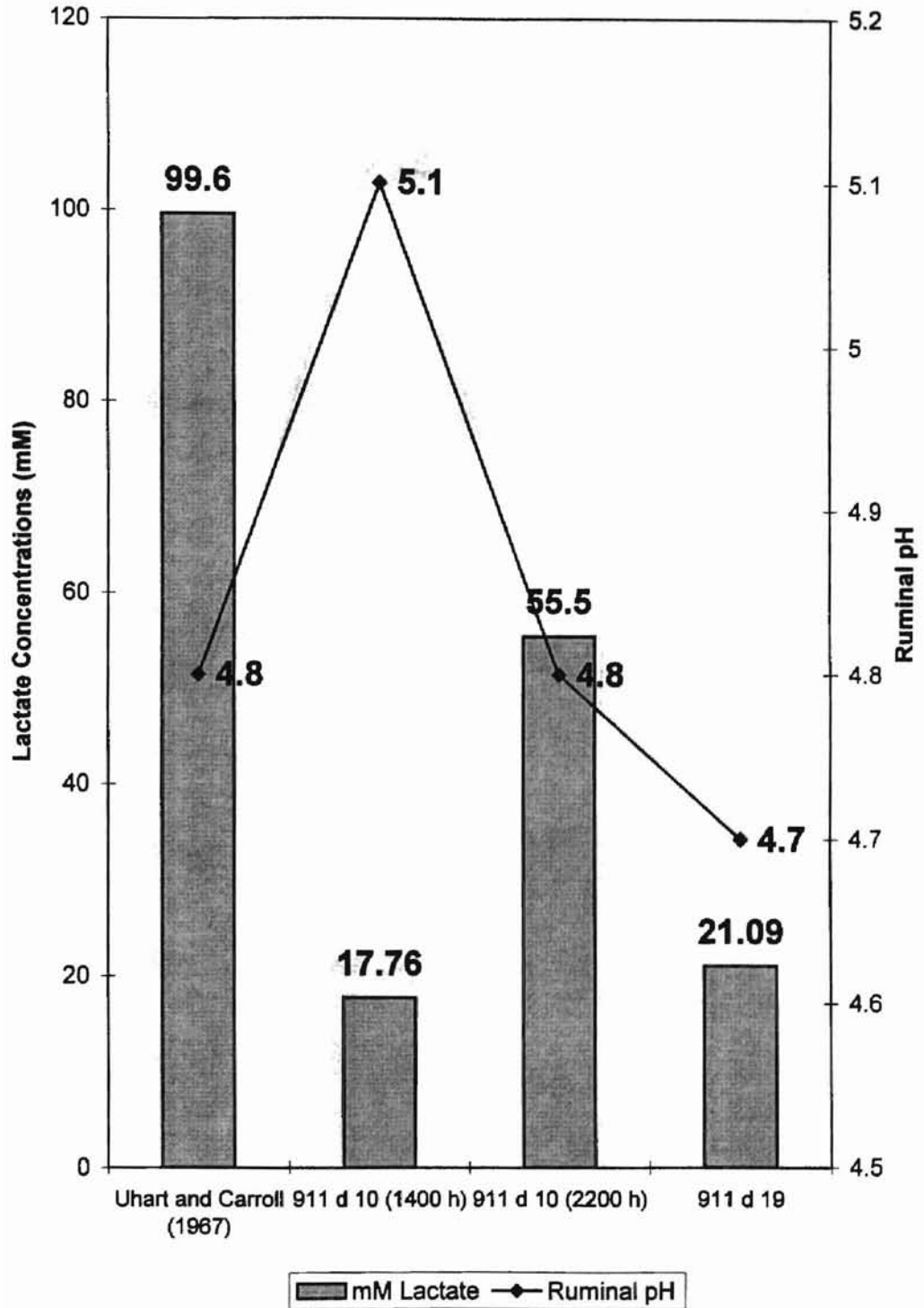
Figure 24. Comparison of Ruminal pH and Relative Lactate Concentrations for Experiment 1 (animal 528) and Literature.
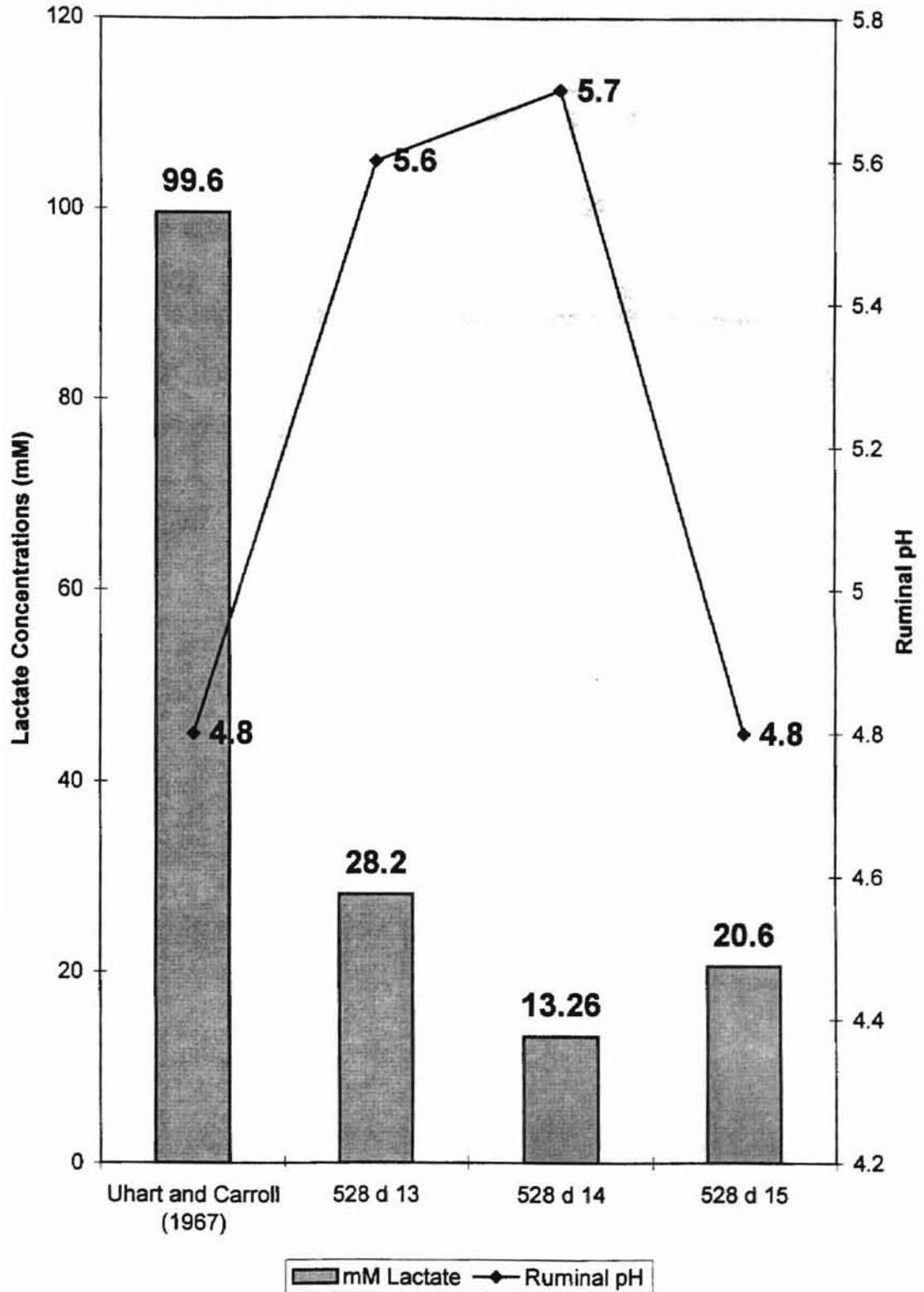
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CHAPTER V

IN VIVO ESTABLISHMENT AND ACTIVITY OF SELECTED STRAINS WITH TWO ADAPTATION SCHEDULES (5 AND 9 DAY)

ABSTRACT

Three trials were conducted to determine the effects of ruminal inoculation with a mixed strain propionibacteria culture on the ruminal pH of cattle rapidly adapted to a highly fermentable diet (90% concentrate). Sixteen cannulated beef cattle were fed grass hay for 21 d and separated into gender groups (8 steers, 8 heifers) for two of three feeding trials. The original mixed propionibacteria inoculum of P81, P89 and P104 was injected directly into the rumen at a rate of 6×10^{11} cfu/d of 4 of the 8 steers (400 kg). The diet was rapidly adapted over either 5 or 9 d, from grass hay to 90% concentrate. The ruminal pH, lactate and VFA concentrations were virtually the same for the treated and untreated groups. No acidotic events occurred during adaptation for either group, though three calves did demonstrate low ruminal pH (< 5.3) post-adaptation. Despite the low ruminal pH, lactic acid never accumulated above 20.87 mM concentrations. Trial 2, consisted of the 8 heifers (438 kg) adapted over a 5 d period from hay to 90% concentrate; half were inoculated with a modified mixed strain culture (P41, P63, P81, P89, and P104) at 1.3×10^{12} cfu/d. Although ruminal pH values were not significantly different between the treated and untreated animals, the treated animals did maintain a more stable pH; dropping only 1.29 pH units compared to 1.46 pH units for the untreated animals during the first 9 d of the study. All 8 of the animals experienced acidosis during the trial, one animal did exceed 15 mM concentrations of lactic acid when ruminal pH fell below 4.7. In trial 3, 14 of animals (7 steers, 7 heifers) (437 kg) were subjected to an abrupt dietary change from 50% concentrate to 90% concentrate after. Seven were inoculated with the modified mixed strain culture, as in trial 2, prior to the abrupt switch in diet. After 1 d on 90% concentrate these animals

79

were fasted for 24 h. Following the fasting period, animals were presented with 40 lb of 90% concentrate (approximately 3% of mean body weight). Animals were allowed to consume feed for 1.5 h before the remaining feed was placed directly into the rumen via the cannula. Feed was withdrawn again and the animals were forced fed via the cannula 3.0% of body weight. Ruminal pH indicative of acidosis was detected in both the inoculated and uninoculated cattle. Lactate concentrations again remained below 20 mM. Prior to the ration change, *in vitro* fermentation's were performed to screen the animals for lactic acid production. Mean levels of lactic acid in the *in vitro* ruminal models ranged from 16.2 to 69.9 mM. Ruminal fluid from all but two of the animals accumulated more than a mean of 40 mM lactic acid within 24 h. These results indicate that an effective lactic acid producing population of microorganisms was present in the ruminal fluid of all animals. Furthermore, these 14 head were screened for and found to have a substantial lactate utilizing populations (> 1.21×10^8 cfu/ml) present in the rumen.

(Key words: *Propionibacterium*, Rumen, Acidosis)

INTRODUCTION

Feedlot cattle often exhibit signs of acute and (or) chronic acidosis after ingesting large amounts of readily fermentable carbohydrate both during adaptation to concentrate-rich diets and during the feeding period (Owens et al., 1996). Feeding more dietary roughage, less thoroughly processed grains, and limiting the quantity of feed provided may reduce the likelihood of acidosis, but are economically undesirable alternatives. The time allocated for adaptation is often prolonged from 21 to 28 d. Decreasing the length of time for adaptation would decrease the number of days the cattle would need to be fed prior to marketing.

In vitro strain selection studies at OSU have shown that inoculating with propionibacteria can prevent the accumulation of lactic acid and stabilize pH in ruminal fluid models (Parrott, 1997), therefore, potentially reducing the time needed for ruminal adaptation to high concentrate diets. The objective of this study was to evaluate the effectiveness of a mixed strain culture of propionibacteria for preventing ruminal acidosis in cattle when rapidly adapted to a readily fermentable carbohydrate diet.

MATERIAL AND METHODS

Sixteen cannulated beef cattle were fed grass hay for 21 d and separated into gender groups (8 steers, 8 heifers). The gender groups were tested separately to ensure adequate time for sample collection and preparation.

Trial 1. Eight steers (400 kg) were randomly assigned to one of two adaptation periods, 5 or 9 d (Table 10). Half the animals were inoculated with the original propionibacteria mixed strain culture (P81, P89, and P104), the other half were not inoculated. The animals were separated by the aisle, to prevent inoculated animals from mixing with the control animals. The animals were inoculated intraruminally at

81

0800 h (0 h post-feeding), after the first daily sample of rumen fluid was taken. The 10 ml of inoculum contained 2×10^{11} cfu/ml of each strain of *Propionibacterium*. Inoculation began on d 1 of the 21 d trial. The animals were fed 13.6 kg of DM or 3.4% of mean body weight. Intake was measured daily.

Ruminal pH, lactate and VFA concentrations were measured 6 times each day (0, 4, 8, 10, 12 and 14 h post-feeding) on days 0 through 10; on day 11 samples were collected at 0, 4, 8 and 12 h post-feeding. Two daily samples were collected on days 12 through 14, d 17, and d 21 at 0 and 8 h post-feeding; samples were taken for the remaining days at 8 h post-feeding (the most likely time for a pH drop). The culture was enumerated on the selective/ differentiating media (see Chapter III) by using the 0 h ruminal fluid sample serially diluted (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}).

Trial 2. The eight heifers were housed two to a pen to initiate competition for feed and increase feed intake. The animals had ad libitum access to feed and water over the 5 d adaptation period and throughout the study. Diet composition is outlined in Tables 11 and 12. Again, half of the animals were inoculated, a modified mixed propionibacteria culture was used (P41, P63, P81, P89 and P104). The 25 ml inoculum contained 2.5×10^{11} cfu of each strain, giving a final volume of 1×10^{7} cfu/ml in the rumen.

Ruminal pH was measured at 0 and 8 h post-feeding on days 0 through 3, at 0, 4, 8, 10 and 12 h post –feeding on days 4 through 7, and at 0, 4, and 8 h post-feeding during d 8 and 9. Ruminal VFA and lactate concentrations were analyzed for all samples on days 4 through 7. Rumen fluid was serially diluted out to 10^{-4} and plated onto the selective/ differentiating media to determine populations of the propionibacteria mixed stain culture on days 0, 3, 5 and 7. On day 5 a second sample was collected to enumerate the culture 4 h post-feeding.

Trial 3. Fourteen of the sixteen head of cattle were used in this trial. Once again divided into treated and control groups, the treated received 25 ml (2.5×10^{11} cfu/ml or

1.0×10^9 cfu/hd/d) of the modified mixed strain inoculum(P41, P63, P81, P89 and P104) daily. However, inoculation began 14 days prior to the dietary challenge. In addition, prior to the dietary changes to 50% concentrate and then to 90% concentrate, 50 ml ruminal fluid was collected for *in vitro* evaluation of lactic acid producing bacteria. Since data from previous trials showed no real accumulation of lactic acid, 2 ml of a 26% glucose stock solution was added to 33 ml ruminal fluid to provide the native lactic acid producing bacteria sufficient amounts of glucose to produce an 80 mM concentration of lactic acid. Samples were collected every 8 h for 24 h. Previous work by Parrott (1997) showed that *in vitro* rumen fluid models remain viable for 24 h.

Sample collection. All ruminal samples were collected by attaching a vacuum pump to an indwelling ruminal tube fitted with a stainless steel filter tip. Approximately 100 ml of ruminal fluid was drawn to determine pH and dispensed appropriately to sterile tubes. On the days when microbial analysis was performed 50 ml of fluid was transported on ice to the laboratory for plating. HPLC samples were drawn once pH was determined; 1.5 ml of ruminal fluid was centrifuged at 5500 x g for 10 min. The supernatant was transferred to a fresh tube and frozen for analysis.

In preparation for HPLC analysis, 0.5 ml of supernatant was diluted with an equal portion of 10 mM sulfuric acid, filtered through 0.2 um filters (Gelman) into 2 ml HPLC autosampler vials. The samples were analyzed on an HPLC 1090 (Hewlett Packard, Atlanta, GA) equipped with a diode array detector, Five ul of prepared sample was injected at a flow rate of 1.0 ml/min through an HPX87 column using a mobile phase of 5 mM sulfuric acid. Concentrations of lactic acid and VFA were calculated based on retention times and peak areas of known external standards measured at 210 nm.

Lactate Utilizing Populations. All 14 animals were evaluated for indigenous populations of lactate utilizing bacteria in ruminal fluid. Rumen fluid from each animal was strained through 4 layers of cheesecloth into a sterile bottle (200 ml) and

83

transported. The analysis was performed while the animals were on the 50% concentrate diet, prior to the abrupt switch to 90% concentrate.

Plating method. Using the media developed by Mackie and Heath, (1979) (Recipe contained in Table 13), both the control and treated animal ruminal fluid was plated to 10^{-6}. A total plate count was performed. The populations present were considered to be representative of the native lactate utilizing bacteria in the rumen since relatively high concentrations of lactic acid in the media provided the sole carbon source on the plates. All plates were grown under anaerobic conditions using GasPak containers.

In vitro Rumen Fluid Models. In addition, rumen fluid *in vitro* models were used to evaluate the populations with added glucose and lactic acid. Thirty-three ml of rumen fluid was dispensed to a clean sterile 50 ml tube. To each tube was added 2 ml of a 12% sodium lactate stock solution and/or 2 ml of a 26% glucose stock solution. All tubes were incubated in a 38°C waterbath for 24 h. Samples were collected every 8 hours of the 24 h incubation period for plating and HPLC analysis. A second *in vitro* was performed under similar parameters with one exception, an additional 2 ml of the mixed strain culture was added to all the treated rumen fluid.

Culture Interaction. The mixed strain culture (P41, P63, P81, P89, P104) was evaluated using the reverse phase passage diffusion method (Kekessy and Piguet, 1970) and the direct supernatant spot assay method (Mayr-Harting et al., 1972).

Reverse Phase Passage Diffusion. The reverse phase passage diffusion procedure is as follows. The 0.1 ml of 48 h producer strain is inoculated onto the surface of a sodium lactate agar (NLA) plate. After a 5 d incubation period under anaerobic conditions (GasPak) the agar was detached from the edges of the petri dish with a sterile spatula and inverted. The 0.1 ml of an immature culture (18-24 h) of indicator strain was seeded into tempered molten NLA, 10 ml of the seeded agar is poured over the inverted producer strain creating a lawn of growth. Plates were examined every 24 h for 48 h to determine if any inhibition zones occurred.

Direct Supernatant Spot Assay. Twenty ul of prepared cell-free supernatant was spotted onto to a (1.0%) seeded indicator plate of NLA. Cell-free supernatant was prepared from late log phase (36-48 h) pure cultures by centrifugation at 10,000 rpm for 10 min. The pH was adjusted to 6.5 with 5N sodium hydroxide, and filter sterilized to remove all cells.

Statistical Analysis. In trial 1, all measurements were analyzed using a 2 x 2 factorial split plot design. The data from trials 2 and 3 were analyzed using a split plot design. Treatment differences were evaluated using a t-test. (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Trial 1. Table 14 depicts daily DM intake by treatment during the adaptation periods. Maximum DM intake was never greater than 13.6 kg. Mean intakes were not different (P>0.05) due to either inoculation or adaptation periods. Daily intakes fluctuated among days, although, DM intakes tended to decrease earlier for those animals on the 9 d adaptation scheme than for those on the 5 d adaptation scheme.

No significant difference (P>0.05) was detected in ruminal pH between treatment and adaptation period. Ruminal pH never achieved acidotic conditions during either adaptation period (Figure 21). However, after adaptation, ruminal pH did indicate that all of the animals, except one treated steer, did indeed become acidotic. Of the seven steers that exhibited acid pH, two maintained ruminal pH values below 5.2 for several days; one being a control animal on the 9 d adaptation scheme, the other being an inoculated animal on the 5 d scheme. Figure 20 shows that during the first ten days mean pH appears to be higher for the 9 day adaptation. However, Figure 21 depicts the 5 d adaptation as actually maintaining a higher pH during adaptation to 90% concentrate. Mean ruminal pH reached its lowest point on days 14 and 18. A linear decrease in ruminal pH for the first 10 d was attributed to the increased concentrate

level of the diet. Mean ruminal pH was shown to be lower (5.88) at 10 h post feeding than at 4 h post-feeding (6.06).

Inoculation had no significant (P>0.05) impact on ruminal lactate or VFA concentrations between treatment groups. During adaptation ruminal lactate never exceeded 3.0 mM, and through the remainder of the trial despite indicative ruminal pH levels, the highest lactate level was only 20.87 mM. Of the seven animals exhibiting low ruminal pH levels only three had detectable levels of lactic acid with a mean of 8.71 mM. The literature has consistently shown that when ruminal pH becomes acidic, that lactate levels should be in excess of 50 mM (Dunlop, 1972; Uhart and Carroll, 1967).

Trial 2. Once again, inoculation appeared to have no significant (P>0.05) impact on Dry Matter (DM) intake between treatment groups. DM intake dropped on day 6 of the trial, the second day of 90% concentrate. Mean daily DM intake (Table 15) dropped from 23 kg on d 4 to 18 kg on d 9. This data is consistent with the literature (Slyter, 1976; Leedles et al., 1995).

While inoculation appeared to have no significant (P>0.05) effect on ruminal pH or ruminal concentrations of VFA or lactate, pH levels were slightly higher for treated animals than control after the switch to 90% concentrate (Figure 22). While ruminal pH dropped 1.46 units in the control heifers, treated heifers showed a 1.29 decrease, although the difference was determined not to be significant (P>0.05). Each heifer in this trial exhibited a ruminal pH indicative of acidosis at some point during this trial, yet in only one instance was lactate detectable. A control animal on d 6 became acidotic (ruminal pH 4.68), although lactate concentrations were only 19 mM. The HPLC analysis was determined to be accurate since with each run fresh external standards are prepared and included randomly throughout the samples. Once again these values are inconsistant with the literature. Briggs et al., (1957) reported that lactic acid concentrations in the rumen of sheep exceeded 20 mM when ruminal pH fell below 5.0.

86

Trial 3. Ruminal pH values during this study were not significantly affected (P>0.05) by the modified mixed strain inoculum. During the period when 90% concentrate was fed each of the 14 animals exhibited ruminal pH values below 5.3, indicative of acidotic conditions. Two treated animals did not have to be forced fed during the d 8 engorgement, they were able to consume the entire ration on their own. The second day of engorgement, mean ruminal pH for the control cattle was 5.44 vs. 5.20 for the treated cattle. Despite the low ruminal pH, ruminal lactate never exceeded 20 mM concentrations. Figure 23 depicts mean daily pH for treated vs control during dietary challenge.

While no evidence of propionibacteria establishing in the rumen was detected, it was determined that several of the control animals did have substantial populations of lactate utilizers as high as 3.5×10^8 cfu/ ml, with a mean population of $>1.21 \times 10^8$ cfu/ml. The most common lactic acid producing bacteria in the rumen are *Streptococcus bovis* and *Lactobacillus spp.* Mackie and Heath (1979) developed their media to enumerate lactate-utilizing bacteria in the rumen of sheep when fed varying levels of readily fermentable carbohydrates. The media supports the growth of all species of lactate-utilizing bacteria reported to inhabit the rumen. They reported finding counts ranging from 4.2×10^8 to 1.9×10^9/g of ingesta, suggesting that our counts may have been even higher if the fluid had not been strained.

When the rumen fluid from each animal was supplemented with 26% glucose stock solution the lactate-utilizing counts went from a mean count of 6.4×10^8 cfu/ml at 8 h to a high of 1.6×10^8 cfu/ml at 16 h and maintained that level out to 24 h. Theoretically, if lactate populations are about 10^8 then the inoculation with propionibacteria is only about 1% if the total lactate utilizing population. Parrott (1997) reported that when strains were inoculated into *in vitro* rumen fluid models that 1×10^7 cfu/ml appeared to be the optimal level, thus suggesting that more is not always better.

HPLC analysis on the *in vitro* rumen fluid models also revealed that when glucose was added that lactic acid did accumulate (Table 16), suggesting that the microflora present in the animals did have the ability to produce lactic acid. Mean levels of lactic acid in the in vitro ruminal models ranged from 16.2 to 69.9 mM. Ruminal fluid from all but two of the animals accumulated more than a mean of 40 mM lactic acid within 24 h. Furthermore, when the treated animals rumen fluid models were inoculated with additional mixed strain culture, 4 of the 7 animals did utilize 100% of the lactic acid supplemented.

Only one (treated) animal was able to utilize all detectable amounts of lactate (80 mM) by 16 h. When the additional culture was added (*in vitro*) this same animal was capable of utilizing 64% of lactate in just 8 h. Prior to inoculation this animal had no detectable levels of lactate-utilizers, suggesting that the culture must have been responsible for utilization. The two control animals that also utilized 100% of the lactate were also found to have high counts of lactate-utilizing bacteria (3.5×10^9 cfu/ml and 2.7×10^8 cfu/ml).

Table 17 shows the results of the culture interaction. The reverse phase passage diffusion method resulted in P63, P89 and P104 having the greatest inhibitory response. The best zone of inhibition was produced by P63 (the producer strain) on P41 and P89 as indicators. The direct supernatant spot assay showed no signs of inhibition, suggesting that the antimicrobial response may be dependent on the presence of cells. According to Mao (1994), P5 (*P.acidipropionici*) did indicate a zone of inhibition when seeded over P63 (*P.jensenii*) a producer strain. These data suggests that the mixed strain culture may not be suitable for inoculation into the rumen due to the antimicrobial potential between strains.

IMPLICATIONS

The mixed strain inoculum used in these trials doesn't appear to stabilize ruminal pH *in vivo*. Other ruminal intermediaries may need to be further evaluated, such as the

88

production of glyoxal and methylglyoxal in the rumen and its role in ruminal acidosis and the effects on a direct-fed microbial. Since recovery of the inoculated propionibacteria free-floating in the rumen was poor, the ruminal biofilm or papillae needs to be better evaluated—possibly biopsied. A single strain such as P63, which appears to have some antimicrobial activity, makes it a better candidate for survival in the ruminal microbial ecosystem.

Table 10. Adaptaion schemes for trial 1 and trial 2.

Day	5 Day Scheme	9 Day scheme
	% Concentrate	
1	30	30
2	45	30
3	60	45
4	75	45
5	90	60
6	90	60
7	90	75
8	90	75
9	90	90
10-21	90	90

Table 11. Composition of diets (DM basis) for trials 1, 2 and 3..

Feed Ingredients (%)	30	45	50	60	75	90
Ground corn	22.1	36.9	42.2	51.8	66.9	82.0
Cottonseed Hulls	35.1	26.2	20.8	17.7	7.6	0.0
Alfalfa pellets	35.1	29.2	29.2	22.7	17.7	10.2
B-075 supplement[a]	7.7	7.7	7.8	7.8	7.8	7.8

[a] composition of the suppliment presented below (Table 2 of chapter IV)

Table 12. Composition of B-075 supplement on a DM basis.

Ingredient%	
Cane molassed	2.37
Limestone 38%	14.26
Salt	3.79
Vitamin A-30,000	0.13
Cottonseed meal	59.22
Soybean meal 44%	11.72
Manganese oxide	0.08
Zinc oxide	0.06
Potassium chloride	1.93
Urea	6.44

Table 13. Composition of LH media for enumeration of lactate utilizing bacteria
(Mackie and Heath, 1979).

Component	% weight/ volume (L)
20% Sodium Lactate (10% solution)	2.0
Trypticase	2.0
Yeast Extract	2.0
Volatile fatty acids[a]	0.31
Trace elements[b,c]	2.0
Hemin (0.05% solution)	2.0
$NaHCO_3$ (9.1% solution)	0.7
Agar	1.5

[a] VFA composition (Caldwell and Bryant, 1966)

Acetic acid	17 ml
Propionic acid	6 ml
Butyric acid	4 ml
Isobutyric	1 ml
n- valeric	1 ml
isovaleric	1 ml
DL-α-methylbutyric	1 ml

[b] Trace elements (Kogut and Podoski, 1953)

K_2HPO_4	1.0 g
NH4Cl	1.0 g
MgSO4, 7H2O	0.5 g
FeSO4	100 ug
(NH4)2SO4, 5H2O 100 ug	
H3BO3	200 ug
CoSO4, 7H2O	100 ug
CuSO4, 5H2O	10 ug
MnSO4, 4H2O	10 ug
(NH4)6Mo7O24, 4H2O	100 ug
ZnSO4, 7H2O	200 ug

[c] Trace elements in 10x Stock solution

Table 14. Mean DM intakes for trial 1 during adaptation (kg/d).

Day	5 Day Adaptation		9 Day Adaptation	
	Control	Treated	Control	Treated
1	13.6	13.6	13.6	13.6
2	13.6	13.6	13.0	13.6
3	13.6	13.6	10.9	12.5
4	10.1	10.0	12.7	12.4
5	10.2	13.4	10.9	12.3
6	11.8	11.1	13.5	13.4
7	10.6	9.3	12.7	13.6
8	9.8	7.7	4.8	10.9
9	10.5	11.8	11.5	9.3

Table 15. Mean daily DM intakes for trial 2 (kg/d).

Day	Control	Treated
0	27.3	27.3
1	27.3	27.3
2	31.8	31.8
3	19.3	21.0
4	27.0	19.7
5	23.6	22.5
6	11.5	13.3
7	15.9	14.5
8	17.7	18.7
9	19.8	16.1

Table 16. Lactic acid concentration (mM) of in vitro rumen fluid models during trial 3.

| Animal | Hours | | | |
	0	8	16	24
C1	0.0	0.0	18.3	28.9
C2	0.0	1.7	33.3	46.1
C3	0.0	0.0	17.2	40.0
C4	0.0	12.2	43.3	45.0
C5	0.0	0.6	45.5	46.1
C6	0.0	48.3	55.5	48.8
C7	0.0	27.2	66.0	69.9
T1	0.0	1.7	38.9	52.2
T2	0.0	0.0	14.4	16.1
T3	0.0	49.4	40.5	50.5
T4	0.0	25.0	55.5	64.4
T5	0.0	18.3	45.0	53.3
T6	0.0	36.1	37.2	45.5
T7	0.0	8.3	32.2	47.7

Table 17. Inhibitory activity of *Propionibacterium* strains used in mixed strain inoculum.

| Producer strain | Indicator strain | | | | |
	P41	P63	P81	P89	P104
P41		-	-	-	-
P63	+		-	+	-
P81	-	-		-	-
P89	+	-	-	-	-
P104	-	+	-	-	

Figure 25. Comparison of Mean Ruminal pH of Cattle during the first 10
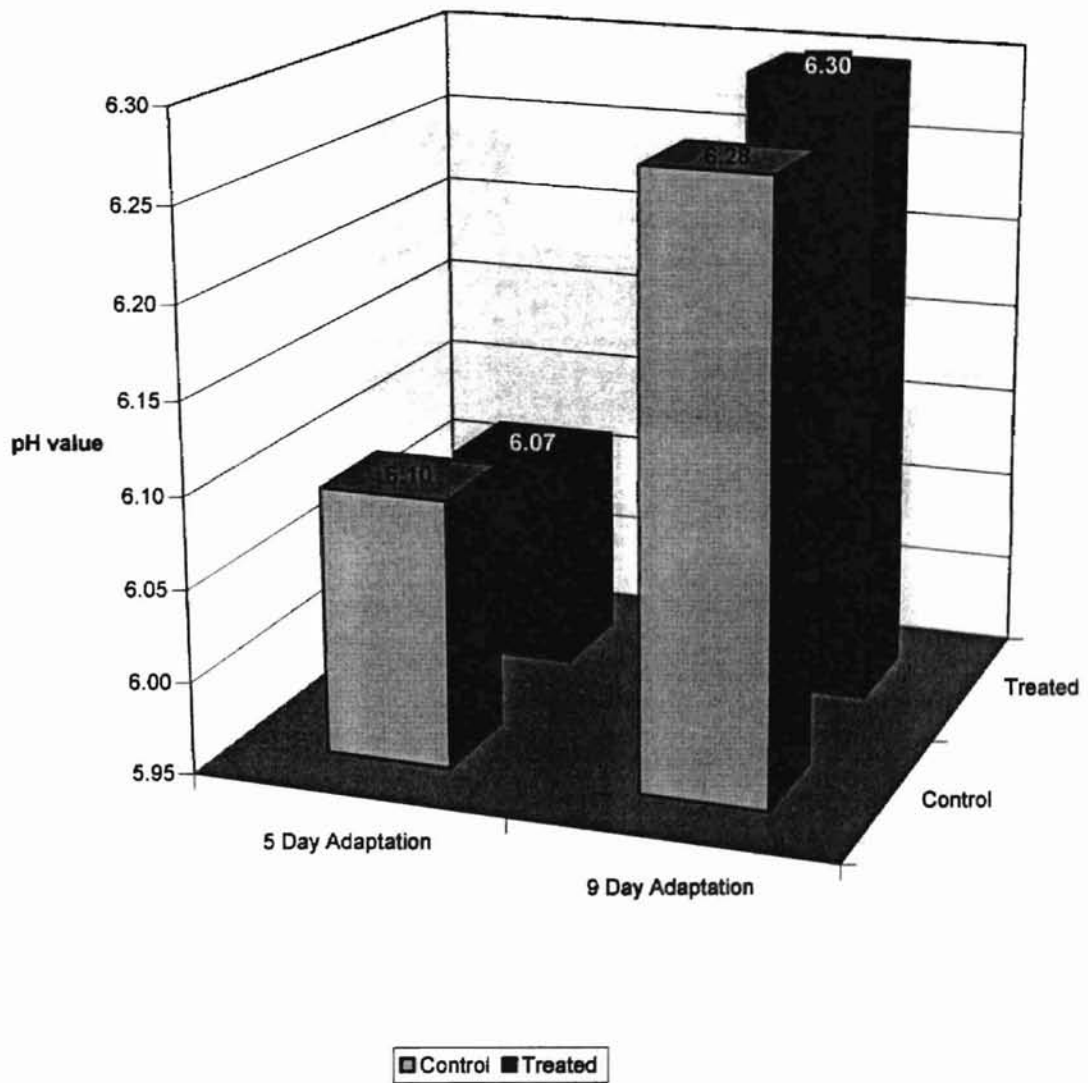days of Trial 1.

Figure 26. Comparison of Mean Ruminal pH during the 5 d Adaptation and 9 d Adaptation in Trial 1.
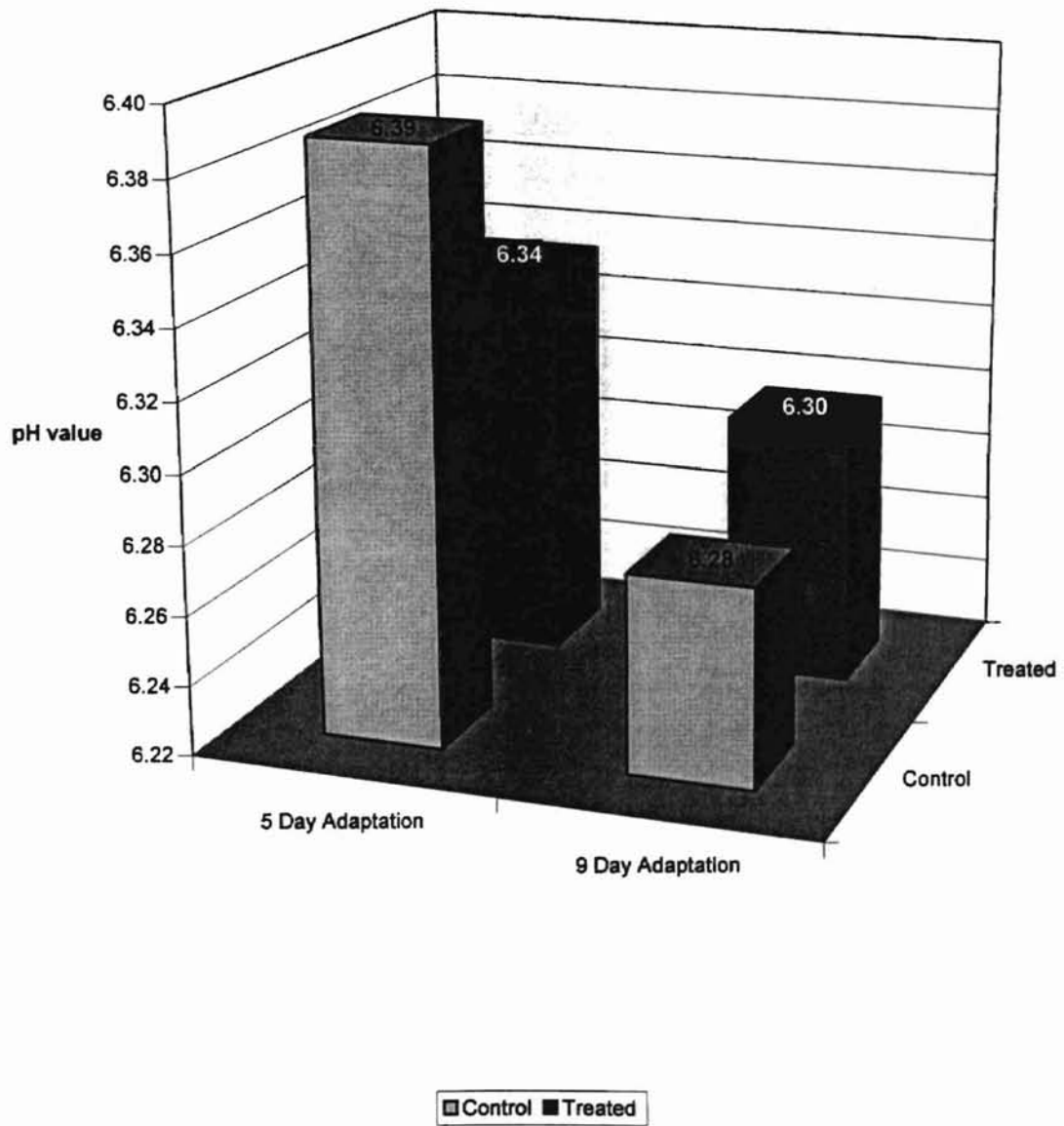
Figure 27. Comparison of Mean Ruminal pH of Control and Treated Cattle on 5 Day Adaptation Schedule during Trial 2.
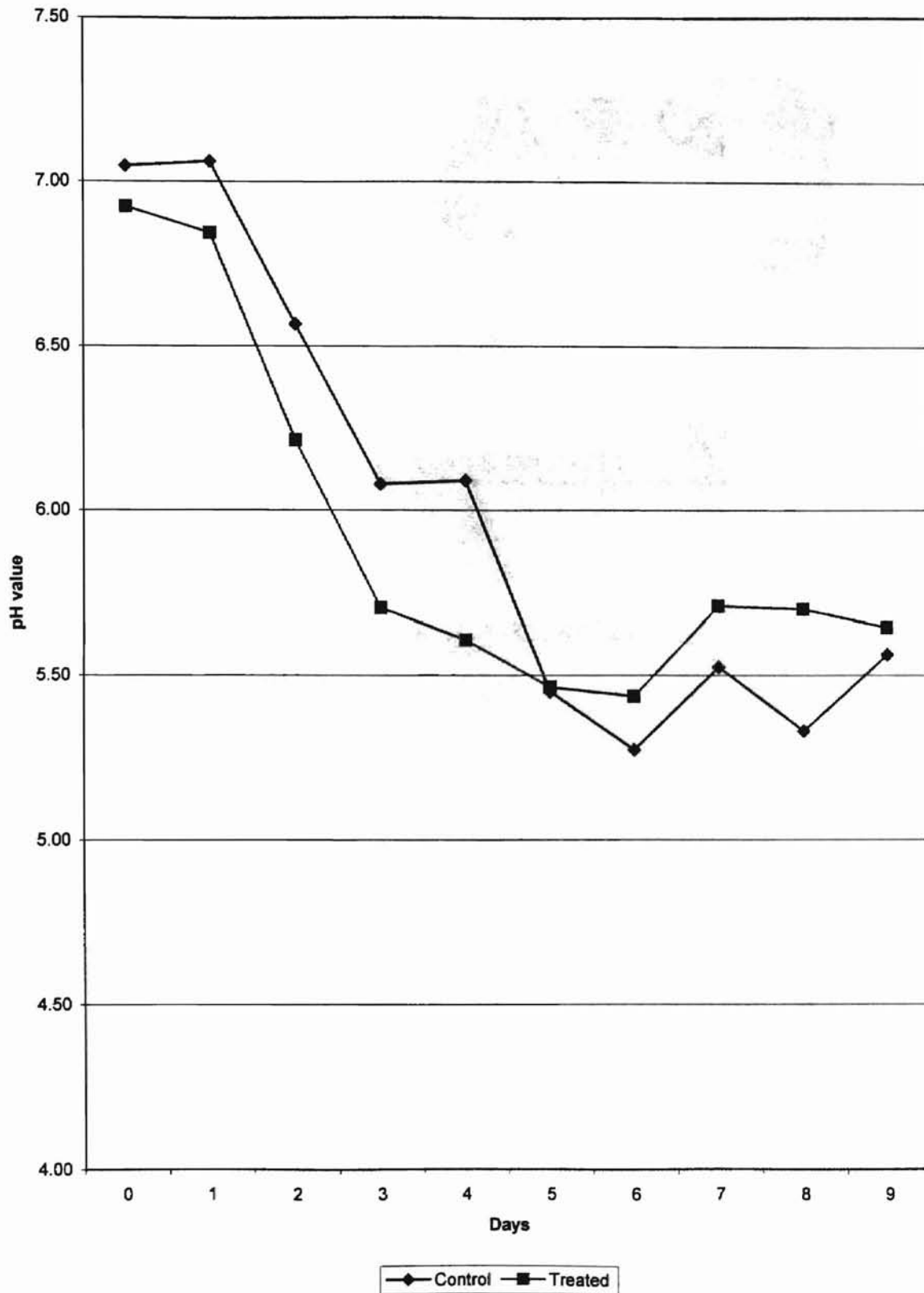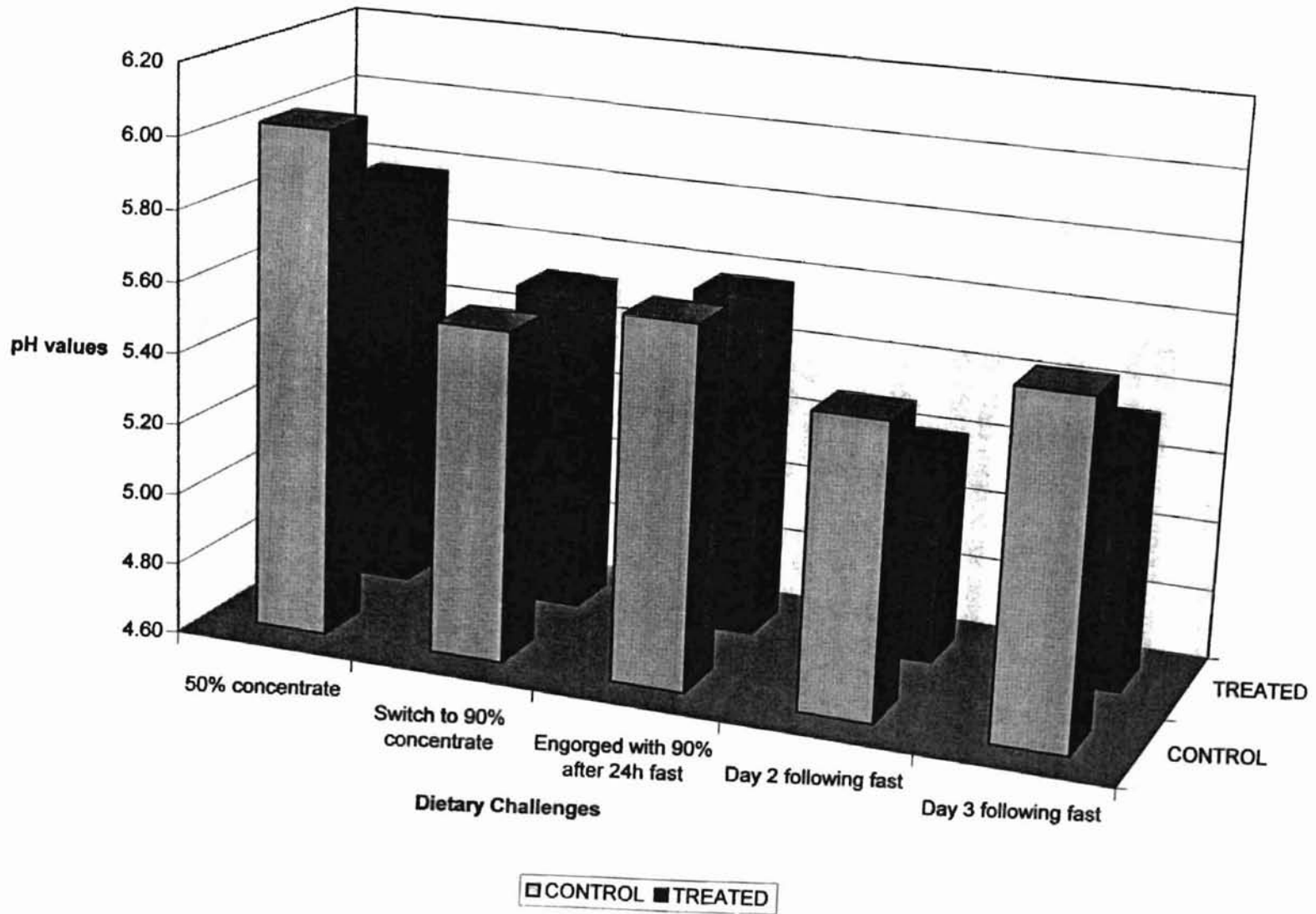
Figure 28. Comparison of Mean pH/day for Treated vs. Control Cattle during Trial 3 Dietary Challenge.

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CHAPTER VI

GENERAL CONCLUSIONS: THE CURRENT RESEARCH

Ruminal acidosis resulting from consumption of excessive amounts of grain has been well described in literature. The proliferation of starch fermenting microorganisms when free glucose is available causes a decrease in ruminal pH. Increased concentrations of lactic acid and volatile fatty acids has generally been considered to be the culprits. Industry has facilitated two primary practices to manage acidosis:, i, extending feed adaptation to 21 d and ii, adding ionophores to rations.

Research performed at OSU has shown that a select strain (P63) of propionibacteria has the ability to utilize lactic acid and maintain populations under acidic (pH 5.0) and neutral conditions (pH 7.0), in media and under mixed culture conditions, simulating ruminal acidosis *in vitro* (Parrott, 1997). The goal of this study was to determine and characterize indigenous populations of *Propionibacterium* in the bovine rumen and to determine efficacy of a mixed strain inoculum in the rumen when dietary challenges were induced. Simultaneously, research was performed to evaluate the efficacy of P63 when cattle were adapted to a highly fermentable carbohydrate diet at an accelerated adaptation. And lastly, production impacts of treating aggressively adapted cattle with a combination of glucose and lactic acid utilizing microorganisms were determined.

Two strains of *Propionibacterium* were phenotypically identified from the bovine rumen; *P. acidipropionici* (96%) and *P. jensenii* (4%). Genotypically, 13 different digestion profiles were observed for the 110 isolates analyzed. Eight other profiles were determined to be unique to one strain. Seasonal variations could be responsible

98

for the variation in genomic DNA profiles, further research is needed to verify this observation.

During the first four trials, when mixed strain cultures of *Propionibacterium* were used in cattle during short term adaptation from a high roughage diet to a high concentrate diet, no differences could be determined between treated and control cattle. Although little to no lactic acid accumulated *in vivo*, *in vitro* rumen models did verify that sufficient populations of lactic acid producing bacteria did exist in the rumen of cattle used in this study.

Furthermore, many of the control cattle were determined to have high populations of ruminal lactate utilizing bacteria, which presumably impeded their ability to accumulate lactic acid. Populations were determined to be as high as 3.5×10^9 cfu/ml, which was higher than our inoculum level of 1×10^7 cfu/ml. The culture was not detected through conventional plating during any of the trials.

The mixed strain culture was examined for antimicrobial activity, and P63 was found to cause inhibition of at least two strains. No determination was made as to the source of the inhibition, although many strains of *Propionibacterium* are capable of bacteriocin activity. Perhaps the antimicrobial activity of the mixed strains prevented establishment of the culture. All subsequent trials were performed with either the single strain of P63 or with a co-culture of P63 and *Lactobacillus acidophilus* ATCC 53545.

The use of dicarboxylic acids to promote P63 to more efficiently utilize lactate was evaluated. All of the additives evaluated (malate, aspartate, succinate and fumarate) did stimulate lactate utilization in P63. Despite the fact that the inoculum level was less $(1 \times 10^5$ cfu/ml) than other previous studies $(1 \times 10^7$ cfu/ml), all four of the additives proved to stimulate lactate utilization more efficiently than the control. Due to the repeated contamination of the 10 and 12 mM malate this study remains incomplete, but

it does offer great promise. It will be necessary to evaluate how well these additives work when ruminal microflora is present. If lactate utilization is stimulated, so too could lactate production.

The single most important finding of this research is that every decrease in ruminal pH was preceded by an increase in ruminal glucose levels (Chapter I; Swinney-Floyd, 1997). Russell (1997) reported that excess-carbohydrate in the rumen promotes lactate production, decreases growth efficiency and kills ruminal bacteria. Ruminal bacteria, such as *Fibrobacter succinogenes* and *Prevotella ruminicola*, in the presence of excess carbohydrate can produce methylglyoxal. Methylglyoxal is a highly reactive and extremely toxic end-product that disrupts DNA, inhibits protein synthesis, and kills bacteria. It is also toxic to mammals. Many of the lactic acid producing bacteria can survive increases in methylglyoxal concentration; therefore the "cure" for ruminal acidosis may not be in finding lactic acid utilizing bacteria only, but in finding those bacteria that can stabilize the glucose utilizing populations as well as utilizing lactic acid.

Related to this research, animal trials performed at OSU by Agtech Products, Inc., Milwaukee, WI, showed that feeding *Propionibacterium* strain P63 inhibited the accumulation of ruminal lactic acid by 38.5% compared to non-inoculated control cattle thus increasing ruminal pH by 0.2 to 0.5 units. In a large scale animal trial using 75 crossbred calves, averaging 317 kg, the effects of feeding *Propionibacterium* strain P63 alone and in combination with *Lactobacillus acidophilus* strain 53545 were evaluated. Three treatment groups were assigned i) no treatment, ii) P63 at 3.0×10^{11} cfu/hd/d, or iii) P63 at 1.0×10^9 cfu/hd/d in combination with *Lactobacillus acidophilus* strain 53545 at 1.0×10^8 cfu/hd/d. A 14 d establishment period was allowed while the cattle consumed a 50:50 ration at 1.5 to 2% of body weight. Following the 14 day establishment period cattle were fasted for 24 hours, then fed a 90% concentrate diet

100

(75% cracked wheat and 25% cracked corn) for 10 days. The ration was switched after day 10 to a 90% concentrate diet of 100% ground corn for the remainder of the trial.

According to Agtech's report, average daily gain (ADG) was higher (P<.04) during the wheat feeding period for cattle fed the combination of P63 and *Lactobacillus acidophilus* compared to the control cattle (3.58 lb/d vs. 2.45 lb/d = 46% increase). Feed conversion during the 120 d feeding period was improved by 6.6% in cattle fed the combination compared to the control (P<.04). Animals fed P63 alone only had a slightly higher feed efficiency though not significantly different. The combination culture showed a significant improvement in feed efficiency based on similar rates of gain and reduced feed intakes compared to control cattle.

The results of this study suggests that detrimental production losses associated with adapting cattle to highly fermentable diets can be reduced by pre-treating with the combination culture of P63 and lactobacilli 53545. Further studies should include an actual feedlot trial to determine the repeatability of these findings. In addition, the role of ruminal metabolic intermediaries like methylglyoxal and glyoxal in ruminal acidosis.

It is important to note that while this technology is promising, it is not expected to dramatically alter the production practices of cattle producers. However, it does offer a potential method of compounding the known effects of products known as ionophores, such as monensin. Monensin feeding increases average daily gain (+1.6 %), reduces feed intake (-6.4%) and improves feed efficiency (7.5%) in cattle fed high concentrate rations. Monensin causes increased propionate production, which translates into a $10/hd net profit for producers (Hibberd and Rehberger, 1994). Propionibacteria, in general, are monensin resistant. Therefore the additional propionate production in the rumen could potentially translate to an increase in profitability over and above monensin alone for feedlot cattle producers in Oklahoma.

The feedlot industry in Oklahoma generates in excess of $702 million gross income per year (USDA-ERS, Livestock Marketing Information Center website). The producers cost for a direct fed microbial product, like that outlined in this work, would average $.02 /head/ day (AgTech Products, Inc., verbal communication), roughly $2.84 for 142 day finishing time. Using Supreme Cattle Feeders (Liberal, KS) feedyard breakeven estimates for cattle going on feed 11/15/98, assuming half the gain (23% over control cattle) reported by Agtech, in a commercial feedlot setting for a 600 lb. (272 kg) steer, the total finished cost would be $703.94 at 142 d (with co-culture) compared to $713.79 at 175 d (without the co-culture). This translates to a $10 savings per head in total cost, but more importantly it translates to a 30 d faster finish with improved gains. According to the 1997 Oklahoma Agricultural Statistics (ODA, 1998), 907,000 head of cattle were marketed through feedlots in 1997. Total finished cost savings could translate to greater profits for Oklahoma cattle producers.

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APPENDIX A

USE OF DICARBOXYLIC ACIDS TO PROMOTE LACTATE UTILIZATION BY *PROPIONIBACTERIUM* STRAIN P63

USE OF DICARBOXYLIC ACIDS TO PROMOTE LACTATE UTILIZATION
BY *PROPIONIBACTERIUM* STRAIN P63

INTRODUCTION

The propionibacteria are important organisms used in several inducstrial

fermentations. Their metabolic properties and nutritional requirements have been

studied extensively (Hettinga and Reinbold, 1972 a, b, c). The most common

application of the propionibacteria in industrial fermentations is their use by the dairy

industry as starter cultures fro the manufacture of Swiss-type cheeses. As dairy starter

cultures, propionibacteria ferment the lactic acid generated by the lactic starter cultures

to produce the characteristic eyes and contribute to the typical flavor of Swiss cheese.

Smolenski and the Upjohn Company (1991) reported that cattle fed sodium

succinate accumulated less lactic acid (P<.07) than control cattle in in vitro and in vivo

models of acute acidosis. Sodium succinate may have a positive effect on acidosis by

modifying ruminal fermentation, thus producing less lactic acid. Since succinate is

converted to propionate in the rumen, propionate producers may have a competitive

advantage in succinate fed cattle by either diminishing lactate production or enhancing

lacte utilization.

Nisbet and Martin (1990) evaluated the effect of dicarboxylic acids on lactate

utilization by *Selenemonas ruminantium*. *Selenemonas ruminantium* is a Gram-negative

ruminal bacteria. Nisbet and Martin used four levels (4, 8, 10, and 12 mM) of malate,

fumarate, aspartate and succinate. Growth of *Sel. ruminantium* on lactate (2g/L) was

stimulated by 10 mM aspartate, fumarate, or malate after 24 h incubation. No growth

occurred on lactate alone. Different concentrations of malate stimulated lactate

utilization in a dose-response fashion; 10 mM malate gave the greatest response.

Martin and Nisbet (1992) suggested that because of the ability of *Sel. ruminantium* HD4

to grow on malate in the presence of hydrogen, malate may be providing an electron

sink for hydrogen that allows increased lactate utilization by this bacterium. Martin and Park (1996) noted that without hydrogen or lactate neither of the strains tested were capable of growth on malate. These findings suggest that electron transport plays a role in organic acid utilization by *Sel. ruminantium*.

Propionibacteria are anaerobic to aerotolerant and are generally catalase positive. Because anaerobic bacteria cannot use oxygen as a terminal electron acceptor, oxidation of the last electron carrier in the chain is used to reduce a terminal electron acceptor—sometimes known as an "electron sink compound" (Caldwell, 1995). Under these conditions, a net loss of electrons from the system occurs and a reduced compound is formed. In aerobic organisms, the compound is water, formed by the reduction of a half molecule of molecular oxygen with two protons and two electrons derived from the transport process. In some anaerobic organisms, organic compounds, particularly fumarate and acrylate, may serve functions as analogous to molecular oxygen. "Electron sink compounds" can greatly facilitate reactions such as decarboxylation catalyzed by pyruvate decarboxylase or pyruvate dehydrogenase in the synthesis of acetyl-CoA. Propionibacteria can reduce lactate to pyruvate using lactate dehydrogenase (Hettinga and Reinbold, 1972).

The purpose of this study to stimulate lactate utilization of *Propionibacterium jensenii* strain P63. P63 was selected by Parrott (1997) for its ability to utilize 80 mM lactic acid *in vitro* more effectively than 41 other strains analyzed, and for its ability to survive in in vitro rumen fluid models.

MATERIAL AND METHODS

Propionibacterium strain P63, previously selected for its ability to utilize lactic acid at pH 5.0 and 7.0 (Parrott, 1997), was grown in pure culture in 80 mM lactate broth supplemented with various levels of four dicarboxylic acids. The 80 mM lactic acid broth

was prepared using a basal media with D,L-lactic acid added. The pH was adjusted with 5 N NaOH to a final pH of 7.0 (v/v) and autoclaved.

The four additives used in this study were asparate, succinate, malate and fumarate. Each additive was prepared as a 24 mM stock solution in 80 mM lactic acid broth (pH adjusted to 7.0), The broth was filter sterilized through a 0.2 micron filter membrane into a sterile flask. The stock solutions were then diluted aseptically into four levels; 4 mM, 8 mM, 10 mM, and 12 mM. Forty ml was dispensed into 50 ml tubes and incubated overnight at 32°C to insure sterility. Each tube was inoculated at 1×10^5 cfu. The study was run in duplicate, including a control consisting of P63 in 80 mM lactic acid broth without any supplementation. All tubes were incubated at 32°C for a total of 48 h.

Samples were collected every 8 hours from the time of inoculation. Beginning at 16 h post-inoculation optical density was determined using a Milton Roy Spectronic 601 spectrophotometer (Milton Roy, Rochester, New York) set to a wavelength of 600 nm. Three ml was removed at each interval for HPLC analysis to determine lactate utilization. Samples were prepared for HPLC analysis by placing 1.0 ml of the sample into a 1.5 ml microcentrifuge tube. The cells were pelleted by centrifugation (10 minutes at 12,000 rpm). The supernatant fluid (0.5 ml) was transferred to a clean tube and acidified with an equal volume of 0.01 M sulfuric acid solution to halt fermentation. These samples were stored at -20°C until analysis was performed. For analysis the thawed sample was filtered through a 0.2 um filter into a autosampler vial.

Samples were analyzed using a Hewlett Packard 1090 HPLC system equipped with a diode-array detector (Hewlett Packard, Atlanta, Georgia). The sample was injected into 0.005 M H_2SO_4 mobile phase heated to 65°C and separated using a BioRad HPX-87H column (BioRad Laboratories, Inc., Hercules, California). The peaks

were detected with a diode-array detector at 210 nm. Other wavelengths were recorded and examined for peak purity, 210 nm was the optimum setting for determining peak height with minimal background noise. Peak areas were used to determine compound concentrations by comparison with external standards.

Fumaric acid supplemented samples were analyzed for lactate utilization using an enzyme assay for both D- and L-lactic acid (Boehringer Mannheim). The principle of the assay being that in the presence of D-lactate dehydrogenase, D- lactic acid is oxidized by NAD to pyruvate. L-lactic acid requires the presence of L-lactate dehydrogenase. The equilibrium of these reactions lies almost completely on the side of lactate. However, by trapping pyruvate in a subsequent reaction catalyzed by the enzyme glutamate-pyruvate transaminase in the presence of L-glutamate, the equilibrium can be displaced in favor of pyruvate and NADH. The amount of NADH formed in the above reactions is stoichiometric to the amount of D-, L-lactic acid, respectively. The increase in NADH is determined by means of its light absorbency at 340 nm.

RESULTS AND DISCUSSION

Figures 29 through 32 all show growth comparisons of P63 grown in broth with the various additives and the control. The spectral analysis showed that all tubes containing additive exceeded the growth of the control tubes. The 10 and 12 mM concentrations of malate repeatedly showed excessive growth at 16 h (>.500) and were discontinued. The 8 mM malate and 12 mM succinate, giving very similar results, showed the most advantageous response. Likewise the 12 mM concentrations of aspartate and fumarate each performed similarly. Figure 38 gives a growth comparison of all of the additives and control.

Lactate analysis showed that in most instances P63 with the dicarboxylic acids did indeed out perform the control. The 4 mM aspartate failed to exceed the control (Figure 33) and the 4 mM succinate only slightly exceeded the control (35). However, the 12 mM succinate was determined to utilize 74% of the lactic acid in 48 h, compared to only 19% by the control. The 12 mM fumarate supplemented samples showed a 50% reduction of lactate (Figure 36). The 4 and 8 mM malate (Figure 34) appears to hold the most promise. In comparison to the similar levels of the other additives, malate utilized nearly 50% more lactate at the 4 mM level and nearly 30% more at the 8 mM level. Figure 37 depicts lactate utilization of P63 by comparing the levels of additives with the greatest disappearance to the each other and the control. It appears that if higher concentrations of malate could be added without risk of contamination, lactate utilization would be greatly enhanced.

IMPLICATIONS

Martin and Nisbet (1992), reported the effects of aspartate, malate and fumarate on lactate uptake by *Selenomonas ruminantium* HD4. *Sel. ruminantium* is a common Gram negative ruminal bacterium which has the ability to utilize lactate, although not as a primary substrate. When HD4 is grown in the presence of malate or fumarate, L-lactate uptake is increased (Nisbet and Martin, 1994). Implementing this same principle, P63, a Gram-positive bacterium was able to enhance its ability to utilize lactate. The data from this study does indicate that *in vitro* lactic acid utilization by P63 can be improved. Before practical application can be made, it would be necessary to see how these dicarboxylic acids effect *in vitro* lactate production by the ruminal microflora. In addition, it would be interesting to see if P63 could utilize lactate if these same dicarboxylic acids when produced as metabolites from either *Aspergillus oryzae* or

Saccharomyces cerevisiae fermentation (Martin and Nisbet, 1992). Both are common nonbacterial direct-fed microbials available to producers and have been reported to improve ruminal and total tract digestibility, and have been reported to stimulate lactic acid utilization rates of ruminal organisms. Hettinga and Reinbold (1972) made reference to an increased yield of reduced products when hydrogen was donated by compounds occurring in yeast-water medium. Fumarate and malate are produced in a soluble form by *A. oryzae*, and malate has been found in fairly high concentrations in *S. cerevisiae* (Martin and Nisbet, 1992). More research is needed to elucidate the bioenergetics associated with electron transport in *Propionibacterium*.

Figure 29. Growth Comparison of P63 in 80 mM Lactic Acid Broth with Varying levels of Aspartic Acid.
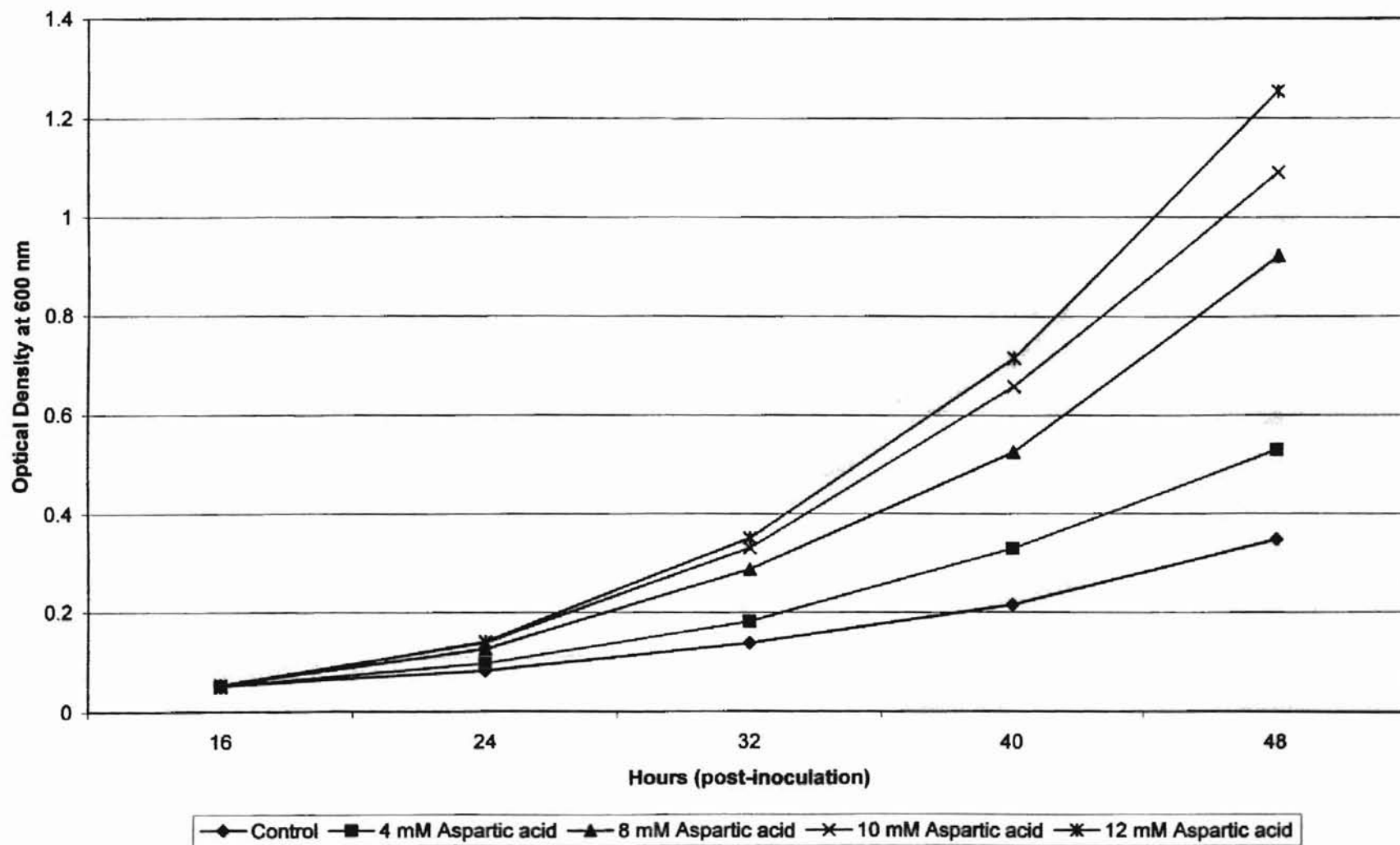
Figure 30. Growth Comparison of P63 in 80 mM Lactic Acid Broth with Varying levels of Fumaric Acid
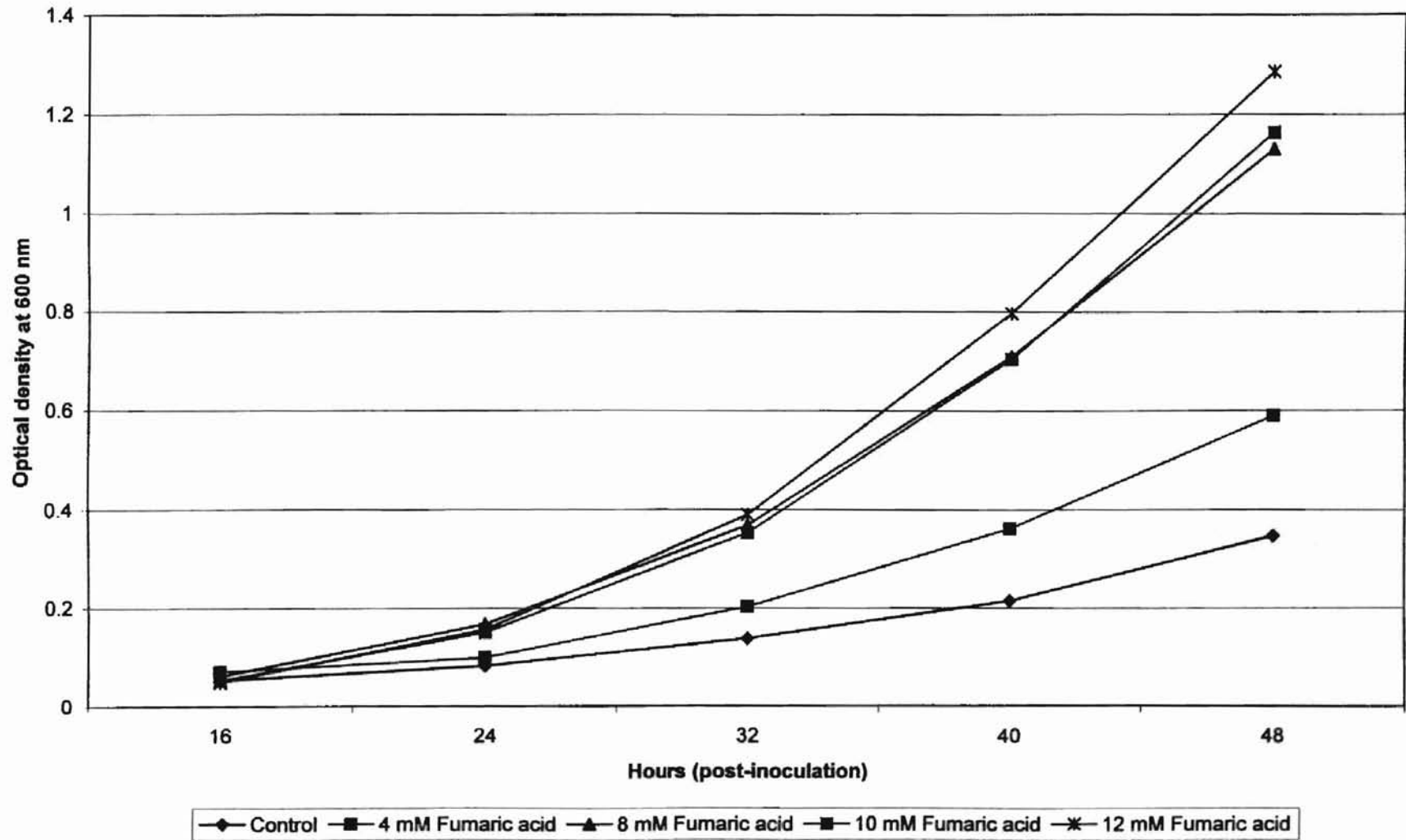
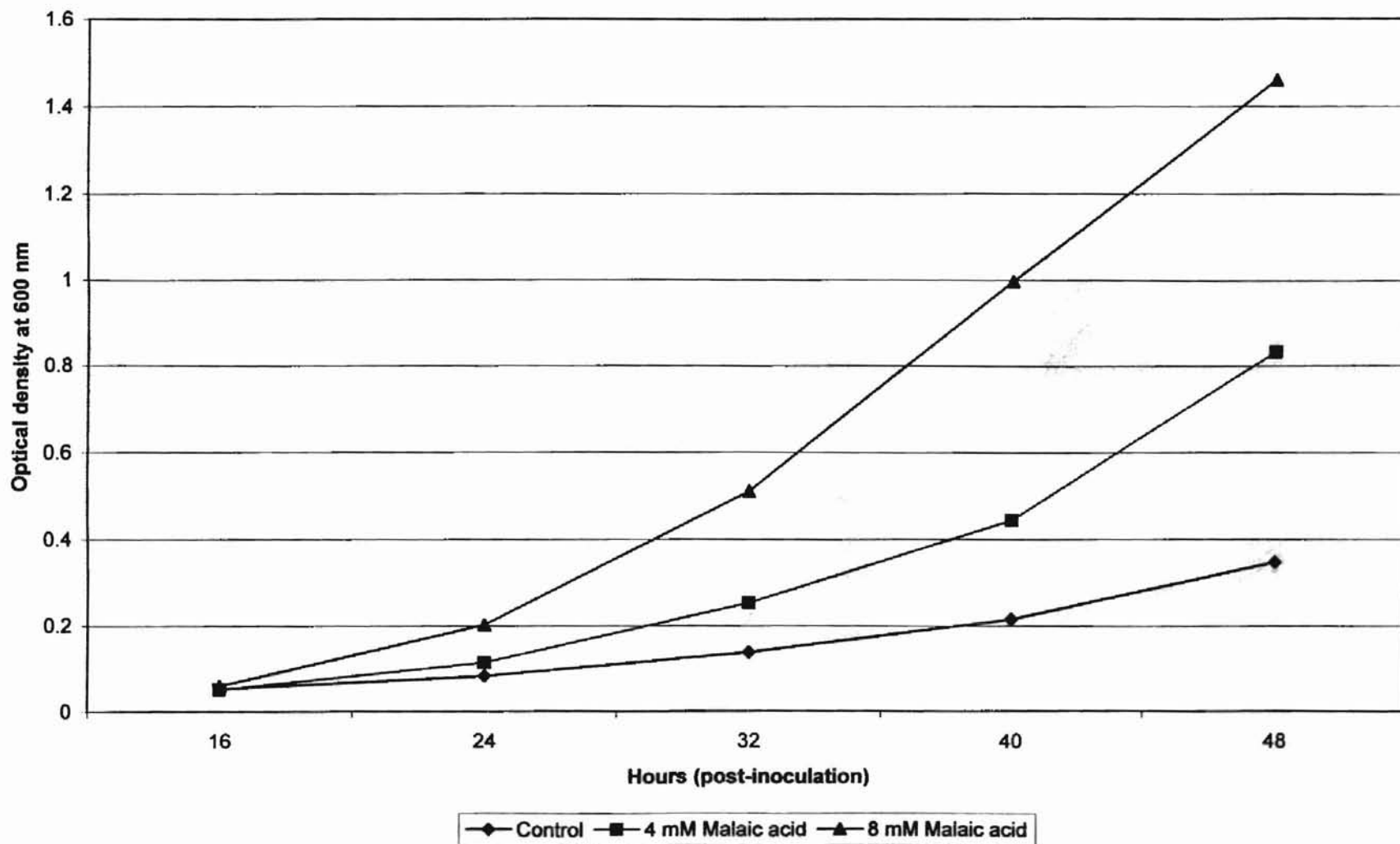Figure 31. Growth Comparison of P63 in 80 mM Lactic Acid Broth with Varying Levels of Malaic Acid

Figure 32. Growth Comparison of P63 in 80 mM Lactic Acid Broth with Varying Levels of Succinic Acid
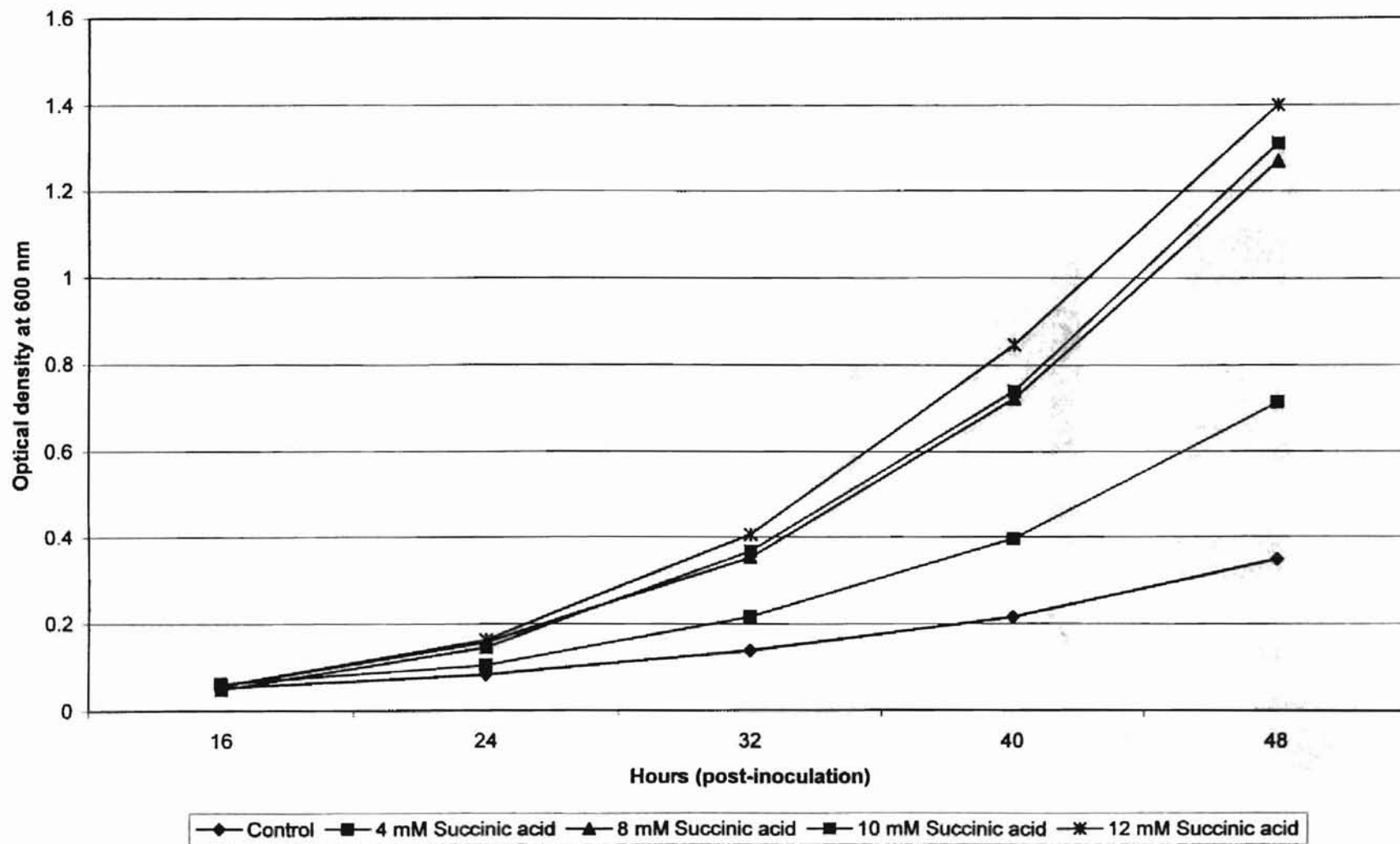
Figure 33. Mean utilization of Lactic Acid by P63 Supplimented with Varying Levels of Aspartic acid

115
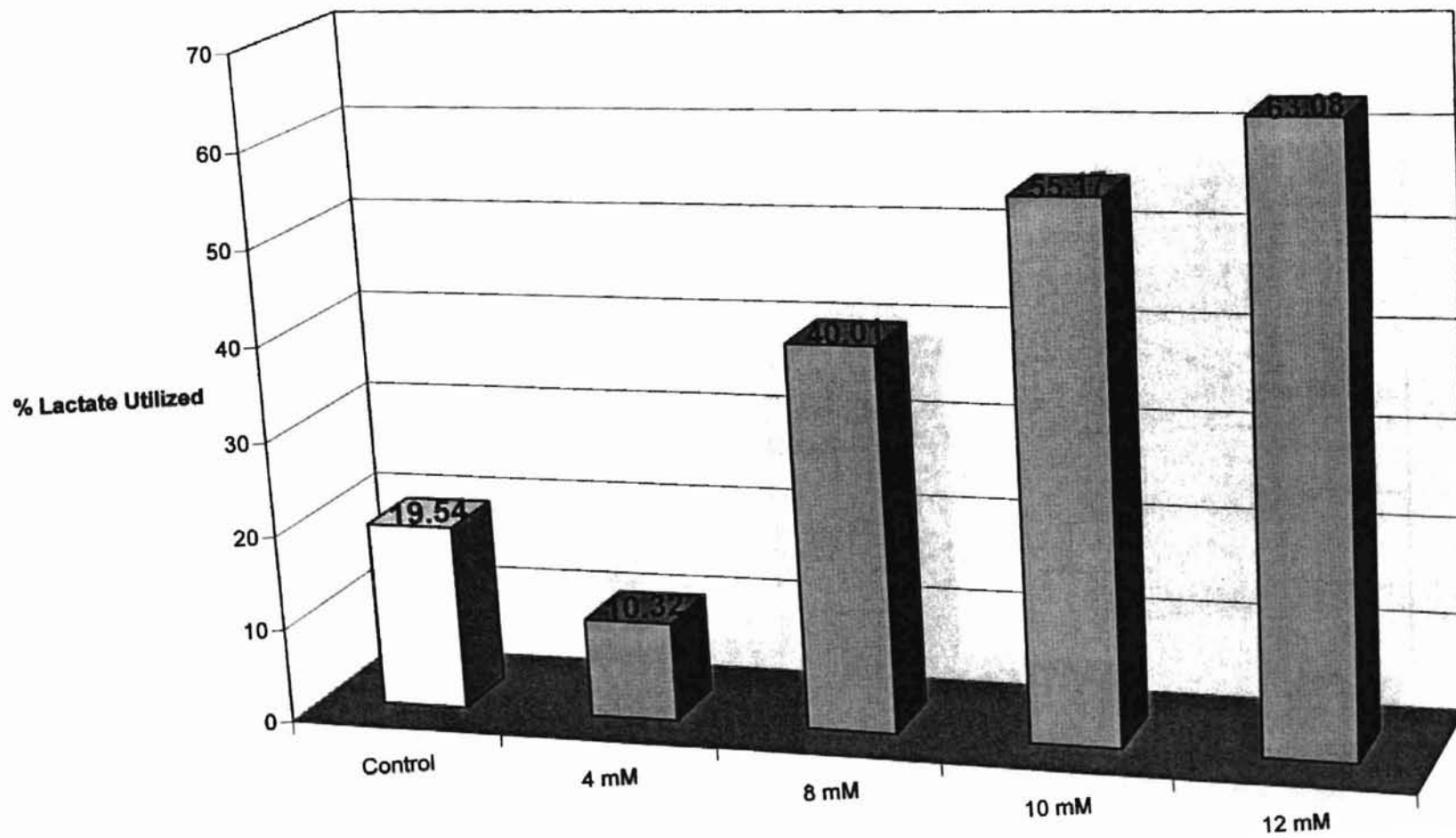
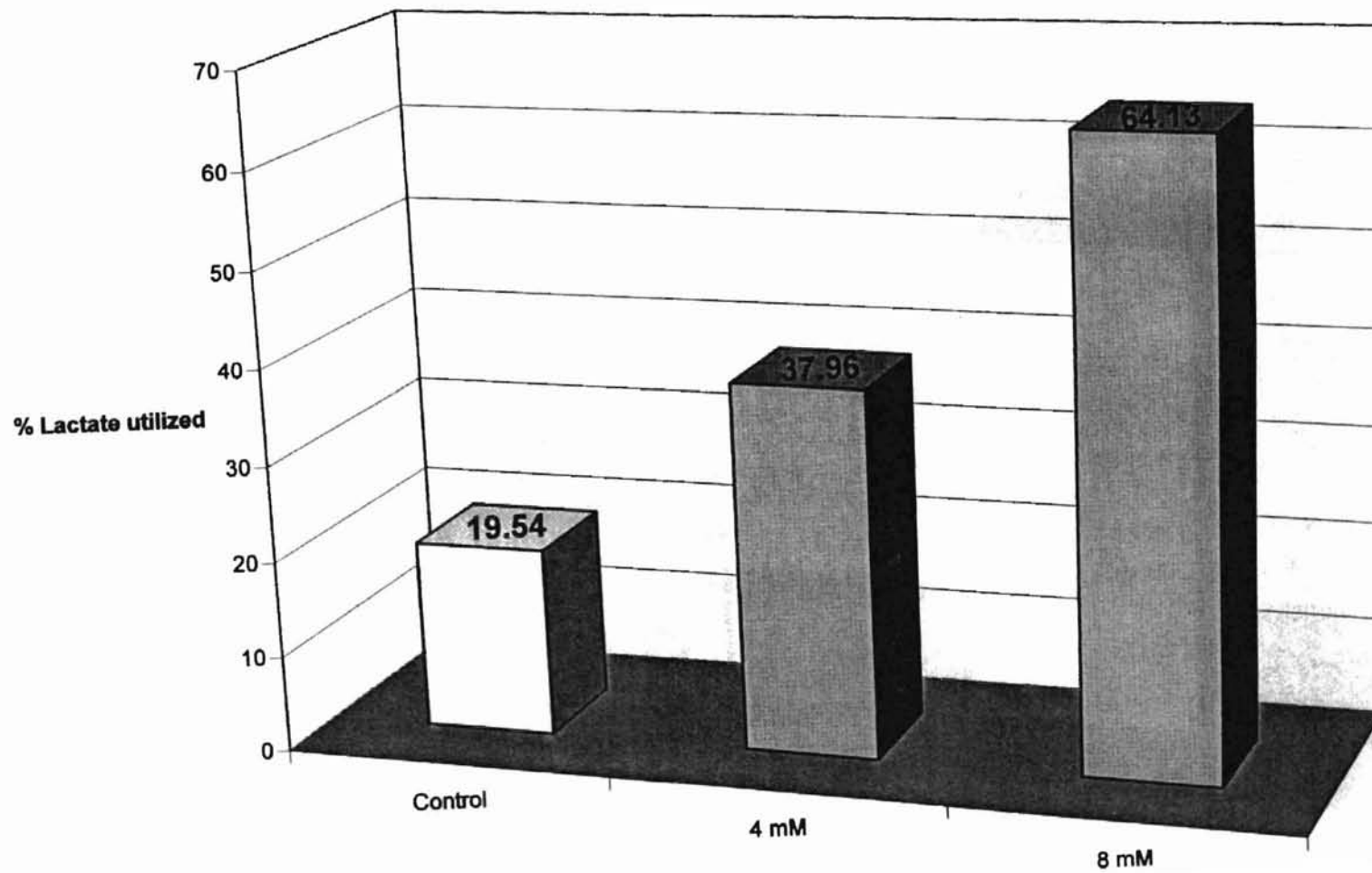Figure 34. Mean Utilization of Lactic Acid by P63 Supplimented with 2 Levels of Maleic Acid

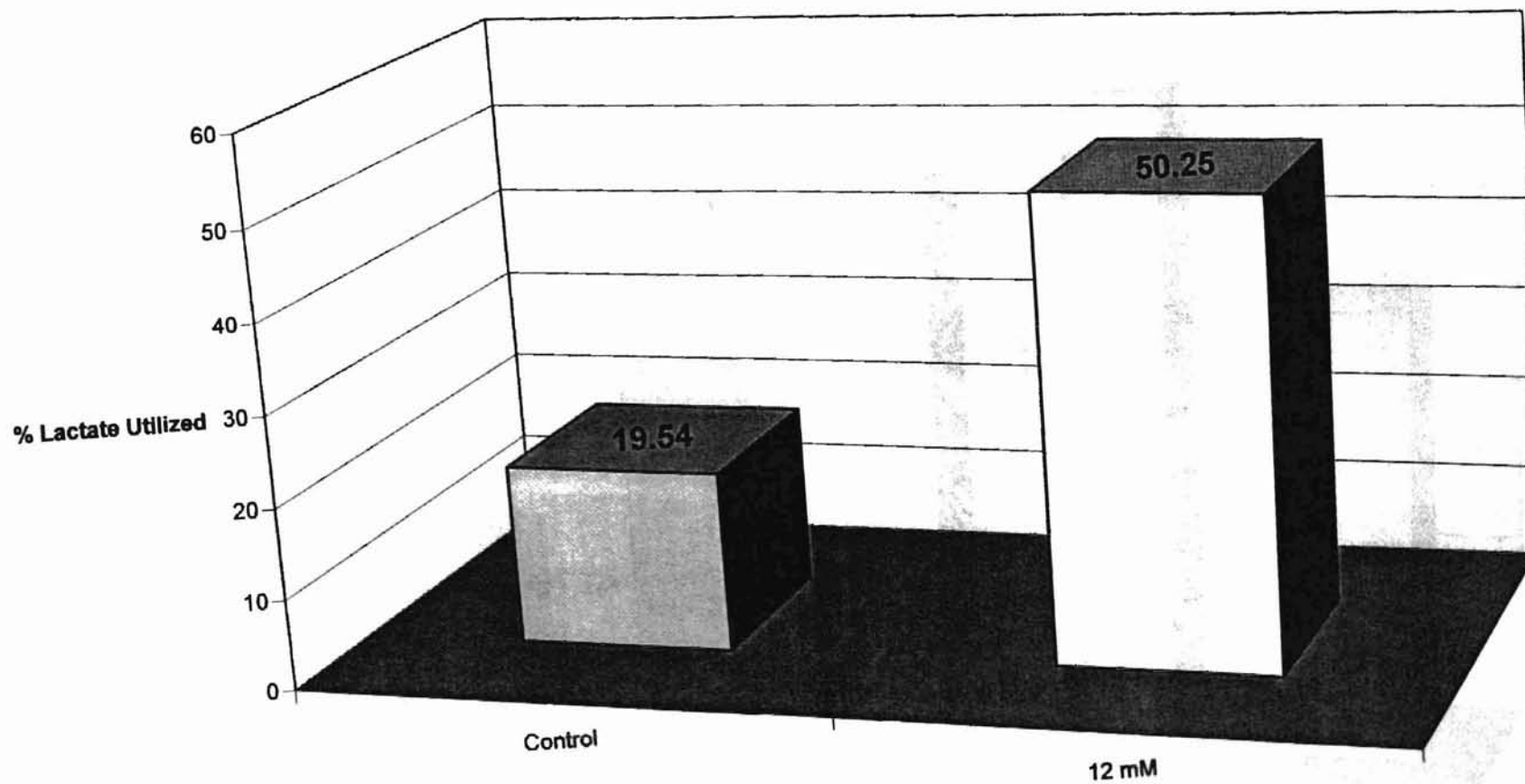Figure 36. Mean Lactate Utilization by P63 Supplimented with 12 mM Fumaric Acid

118

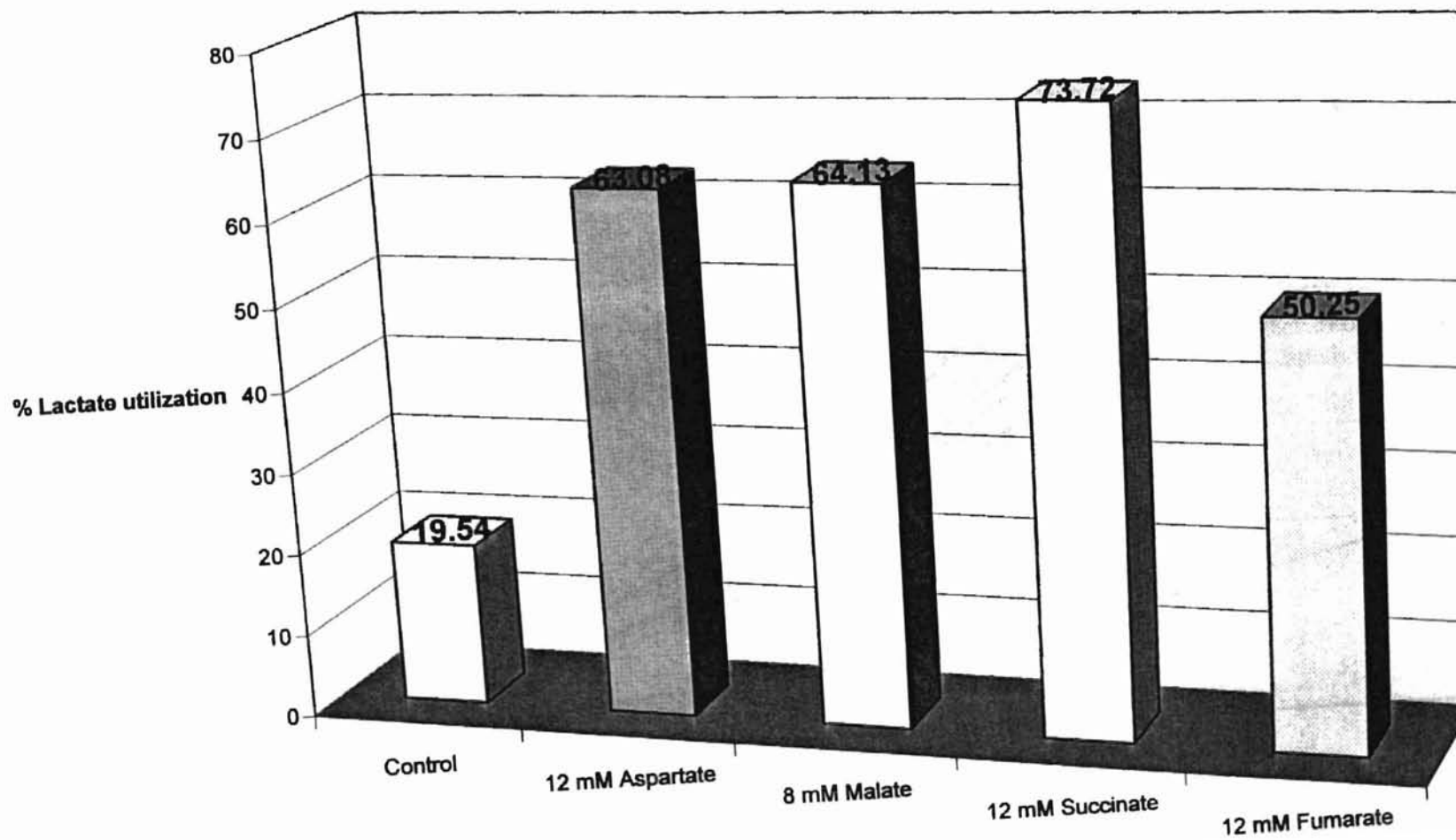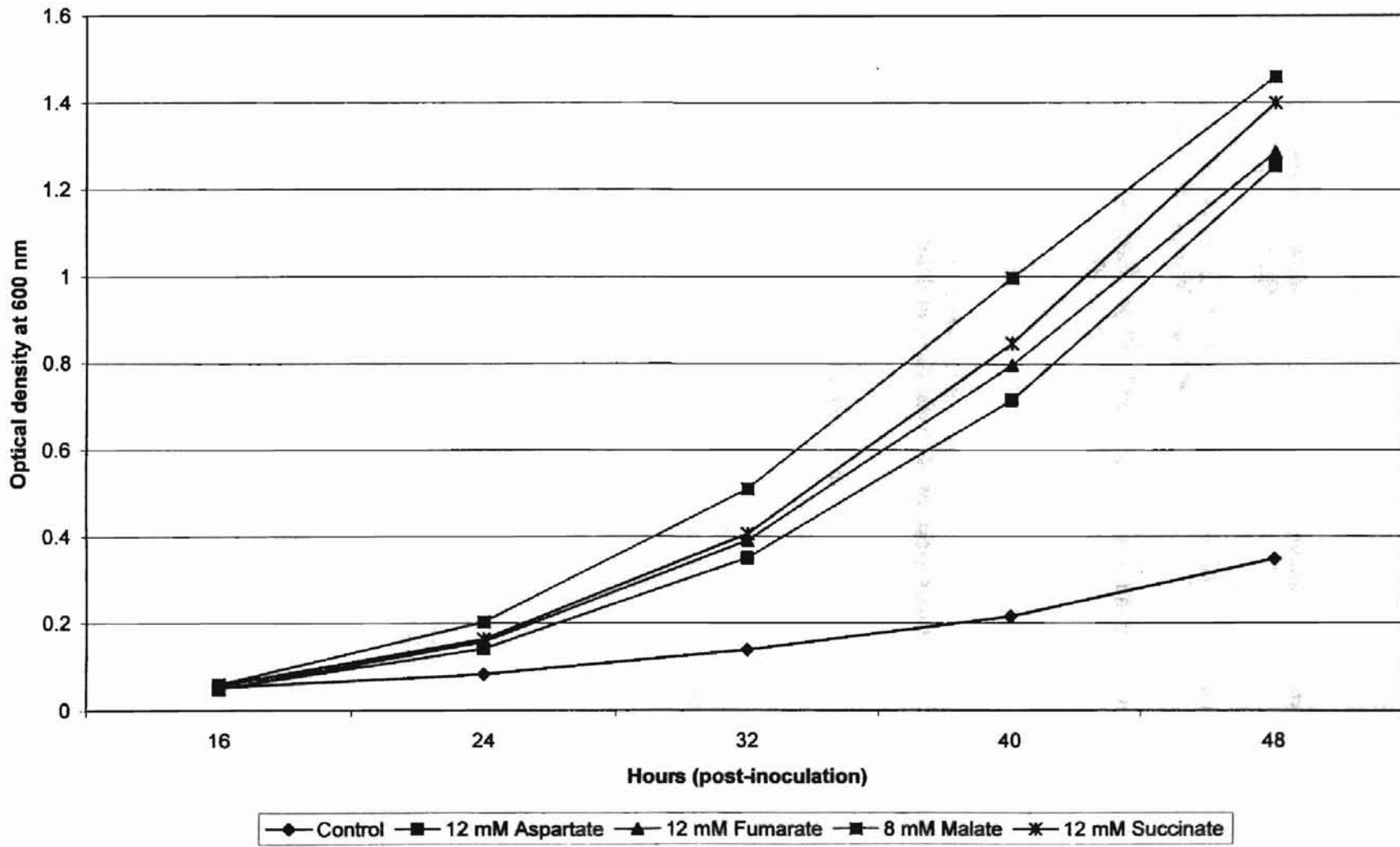Figure 37. Comparison of Percent Lactate Utilization by P63.

Figure 38. Growth Comparison of P63 in 80 mM Lactic Acid Broth with Dicarboxylic acids

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