SELECTION OF *PROPIONIBACTERIUM* STRAINS CAPABLE OF UTILIZING LACTIC ACID FROM *IN VITRO* MODELS

By

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CHAPTER I

INTRODUCTION

The practice of feeding livestock grain such as corn with rapidly fermented carbohydrate has developed in response to consumers' demand for tender and flavorful meat. This demand has led to increasingly accelerated production schedules to produce meat at a minimal cost. During this process, however, producers have been forced to feed diets very rich in grain, to process the grain, and to adapt animals to such diets very rapidly. This has increased the incidence of metabolic disorders such as lactic acidosis of ruminants. Lactic acidosis is defined as accumulation of lactic acid in the rumen during fermentation of rapidly fermented carbohydrate(s). With this condition, ruminal microbes are not able to metabolize the lactic acid as fast as it is being produced. This condition is most prevalent when the diet being fed is switched rapidly from one rich in forage to one rich in grain. Ruminal concentrations of lactic acid increase resulting in low ruminal pH. This change in rumen environment inhibits many acid-sensitive strains of natural flora which can utilize lactic acid. Consequently, the pH drops further and most ruminal activities are inhibited. Not only is digestive function halted, but also lactic acid is absorbed into the blood stream. Substantial decreases in blood pH cause death. Even if acidotic animals do not die, the low ruminal and blood pH and sloughing of the epithelium lining of the rumen increases the incidence of liver abscesses due to sepsis and colonization of bacteria in the liver.

In the past, intensive management of feeding has been the only weapon to combat acidosis. More specifically, grains are diluted with roughage and the increase in dietary concentrate percentage is very gradual. Other strategies have been to add chemical or antimicrobial components such as ionophores to inhibit production of lactic acid in the rumen (Muir *et al.* 1981). Current consumer concerns about antibiotic residues in meat

and overall disdain for "unnatural" chemicals being used in production of meat have made new solutions to acidosis necessary. Direct-fed microbials such as *Megasphaera elsdenii* can reduce the accumulation of lactic acid in the rumen of acidosis induced beef cattle (Greening *et al.*, 1991). This approach to handling acidosis relies on the lactate-utilizing properties and ruminal survival of specific probiotic cultures. Propionibacteria strains also may be effective as direct-fed microbials to reduced the incidence of ruminal acidosis if strains can be identified that will survive in the rumen and utilize lactic acid under harsh ruminal conditions. This study was designed to select a group of *Propionibacterium* that could utilize lactic acid (80 mM/L) produced in the rumen of feedlot cattle in quantitative pure culture experiments and test ability of various strains to compete with ruminal bacteria in a batch fermentation system.

CHAPTER II

RUMINAL ACIDOSIS

Ruminal acidosis is a term used for disturbances that occur in the rumen and/or gastrointestinal tract, most often resulting from the engorgement of large amounts of readily fermented carbohydrate(s) (RFC) by unadapted animals. The carbohydrate portion of the feed is rapidly fermented resulting in accumulation of metabolic end products including lactate and other volatile fatty acids; these acids lower the pH of ruminal contents, reduce salivation, and eventually are absorbed into the blood stream (Dunlop, 1972). Normally, the pH of ruminal contents ranges from 5.5 to 7.5 (Blood and Radostis, 1989); diets rich in starch produce a lower pH while diets rich in cellulose produce a higher ruminal pH. Lactate and VFA endproducts are usually produced slowly in the rumen and never accumulate because they are absorbed at the rate they are produced. However, with consumption of large amounts of RFC, the number of amylolytic bacteria increases; when acid production rate exceeds absorption rate, acids accumulate resulting in a lower ruminal pH (Hungate *et al.* 1952). The ruminal pH of acidotic animals can fall to a range of 3.9 to 4.5; these low pH conditions inhibit further carbohydrate digestion but increase the rate of absorption of lactic acid (Dunlop, 1972; Slyter, 1976).

Sources of RFC include immature, rapidly growing forage, tubers, root crops, and, most commonly, cereal grains. Grain processing, through increasing exposure of the starch in grain, increases the rate of acid production following a meal and increases the likelihood of ruminal acidosis. Flaking or finely rolling grain increases the likelihood of acidosis by increasing the rate at which starch is fermented (Dunlop, 1972; Slyter, 1976). With most unprocessed cereal grains, a large proportion of the starch is encased with

protein of the endosperm. Heating or pressure treating grains disrupts the protein and causes the starch granules to explode creating sheets of rapidly fermented starch.

Ingestion of an abnormally high amount of RFC, by either non-adapted or adapted ruminants, can cause acid to accumulate. Hence, acidosis often results when the diet is changed abruptly from forage to concentrate or when unadapted animals gain access to grain storage bins or lush green pasture. Even for adapted animals, engorgement of a large amount of grain can result in lactic acidosis.

Several additional factors can influence the incidence of and risk from acidosis. Ruminants in poor condition, lacking energy reserves, are more susceptible (Slyter 1976). The form of roughage in the diet also is important because roughage itself provides some buffering capacity, it dilutes RFC of the diet, and it influences the amount of saliva produced. Saliva contains both water and bicarbonate; the former dilutes the acids and the latter neutralizes the acids. When forage is finely chopped or pelleted, ruminants eat faster, chew less, and dilute the diet with less salvia (Brent, 1976; Utley, *et al.*, 1973). Consequently, ruminal digesta becomes more acidic.

PHYSIOLOGICAL EFFECTS OF RUMINAL ACIDOSIS

Symptoms associated with ruminal acidosis vary with the severity and the persistency of the low ruminal pH. Periods of feed engorgement usually are followed by both delayed and reduced feed consumption. During acidosis, animals are depressed and listless; they often exhibit dehydration, diarrhea, and laminitis and can die from dehydration and anoxia.

Most of the early research blamed ruminal acidosis on one specific acid, D-lactic acid. Following infusion of RFC, concentrations of lactic acid in both ruminal fluid and blood often increase (Dunlop and Hammond, 1965; Huber, 1976; Kezar and Church,

1979 and 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.

Clinical signs of ruminant acidosis were summarized by Underwood (1992). In the earlier stages of acute acidosis, body temperature, pulse and respiratory rates become slightly elevated. The increased respiration is in direct response to lactate entering the blood. Lactate dissociates causing hydrogen ions and bicarbonate to combine which then dissociate to produce water plus carbon dioxide; carbon dioxide is removed through the lungs by increased respiration. As the disease progresses, animals may become hypothermic with body temperatures 3 to 4 °F degrees below normal values. Respiratory rate is drastically reduced, to only 10 to 20 breaths per minute. Fecal matter changes from soft, yellow-green, and sweet smelling to a profuse, foul smelling, foamy material often tinged or darkened due to blood.

Dehydration occurs as a result of increased rumen osmolality (Huber 1971). Accumulation of small molecular weight compounds such as lactic acid, glucose, and VFA, increases the osmotic pressure of ruminal contents. When osmotic pressure in the rumen is greater than osmotic pressure of blood, water is pulled into the rumen from plasma, interstitial, and intracellular fluids. Huber (1971) reported that body water was reduced by 8% by high ruminal osmolality in acidotic sheep. This change in the tonicity of the rumen is compounded by the fact that animals consuming concentrate-rich diets produce less saliva, a condition exacerbated by extensive grain processing in modern feedlot rations. Utley *et al.* (1973) found that ruminal buffering capacity was reduced when dietary roughage, in this case peanut hulls, were ground or pelleted. Garza and Owens (1989) reported less than 10% of ruminal water is supplied by drinking water and concluded that 92 to 96% of the fluid in the rumen of heifers must originate from saliva and flux through the ruminal wall. This means that saliva, not drinking water, must be responsible for diluting ruminal acids and for maintaining fluid in the rumen to aid

ruminal the mixing to expose acids to the ruminal wall absorption, and for flushing the acids to the omasum.

Bovine laminitis often occurs following ruminal acidosis. Its incidence is higher among animals consuming high concentrate diets (Brent, 1976). Dirksen (1969) observed signs of laminitis shortly after ruminal acidosis had been induced. High blood concentrations of histamine have been blamed for laminitis, but whether this histamine, which is a normal ruminal metabolite, actually originates from the rumen or from other tissues of the body due to the stress of dehydration or blood acidity, is not known.

The presence and type of ruminal microbes also are important. When ruminants are fed concentrate-rich diets, the ruminal prevalence of protozoa is greatly reduced (Slyter, 1976). Although numbers can decrease following engorgement with RFC, the decrease also occurs in ruminants adapted gradually to a concentrate-rich type (Slyter *et al.*, 1965). This decrease has been 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; with acidosis, ruminal pH drops below 5.5 (Dunlop, 1972; Slyter, 1976). Thereby, protozoa may not survive. 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, they release large amounts of amylase into the rumen environment which may enhance the conversion of starch to acid which can exacerbate the acidotic condition.

Ruminants that die from acidosis usually exhibit abscessed livers, brain lesions, and hemorrhagic, inflamed rumens (Brent, 1976). Abscesses in the livers results in an economic loss because liver makes up approximately 2% of carcass weight (Tindall,

1983); in addition, ruminants with hepatic abscesses generally gain weight slower and less efficiently.

SEVERITY OF RUMINAL ACIDOSIS

Based on severity of an individual case, ruminal acidosis often is classified as subacute and acute. This classification is based on the condition of the animal and dictates the strategy needed to manage the problem and the economic impact.

Acute acidosis. Acute acidosis usually results from diet mismanagement. Although it is not commonly observed in commercial feedlots, acute acidosis is most likely to cause death and thereby results in the highest monetary loss per animal.

Acute acidosis is easily recognized. Affected animals exhibit characteristic signs: abrupt loss of appetite, laminitis or death. All of these symptoms result from the extremely low rumen pH values. Ruminal pH, which can decrease to 5.0 or below, is a result of complete disruption of the rumen flora and death of most rumen bacteria and protozoa (Hungate *et al.*, 1952). Acute acidosis has two phases. First, when excess carbohydrate is available, rapidly growing *Streptococcus bovis* and lactobacilli proliferate. These microbes produce lactic acid and reduce pH which inhibits or kills cellulolytic bacteria and protozoa and decreases the diversity of nutrients available for microbes (Hungate *et al.*, 1952; Scheifinger and Wolin 1973; Slyter *et al.*, 1974). Secondly, continued presence of free glucose inhibits conversion of lactate to volatile fatty acids, further exacerbating the acid load. Hishinuma *et al.* (1968) demonstrated that conversion of lactate to acetate and propionate by a pure culture of *Selenomonas ruminantium* was inhibited when glucose was present. Nakamur, *et al.* (1971) reported that less lactate accumulated when the ratio of substrate to bacteria was low, perhaps a result of increased growth on glucose by bacteria that produce products other than lactate (Slyter, 1976).

As acids (lactic and VFA) and glucose accumulate in the rumen, the increased ruminal osmotic pressure begins to effect animal health. During periods of acute acidosis, osmolality can be doubled causing water to be pulled from the blood to offset this imbalance. Rapid influx of water through the ruminal wall erodes the ruminal epithelium (Eadie and Mann, 1970). Increased rumen osmolality results in tissue dehydration, further compounding the problem (Johnson, 1991).

Subacute Acidosis. More difficult to recognize and define, subacute or chronic acidosis reduces animal performance. Although lacking any physically noticeable symptoms, subacute acidosis generally is considered to be a greater economic problem for commercial feedlots than acute acidosis because managers may not detect the fact that feed intake and thereby performance is suboptimal. Reduced feed intake may be due to low pH and high ruminal acid concentrations; ruminal VFA have been reported to reduce rumen motility and parotid salivation (Slyter, 1976). Additionally, poor performance can be attributed largely to reduced feed intake and cyclic periods of engorgement followed by anorexia. This behavior and an increased incidence of ruminal acidosis results in more cases of intestinal ulcers, rumenitis, hepatitis, and laminitis, each of which decreases animal performance (MacLean, 1966).

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(pK 4.5 vs pK 3.6), their accumulation has detrimental effects on ruminal motility and function. During absorption, bicarbonate enters the rumen in exchange for the VFA being absorbed, so if absorption is decreased, this source of buffer also is decreased. An increased concentration of VFA alone can decrease ruminal pH sufficiently to cause acute acidosis (<5.0). In addition to lowering pH, VFA accumulation can reduce rumen motility by stimulating of epithelial receptors of the reticulo-rumen (Crichlow and Chaplin, 1985). Butyrate, acetate, and propionate all stimulated ruminal receptive fields isolated from anesthetized sheep, butyrate was more effective (stimulating 95% of the receptors) than acetate (76%) and propionate (53%). Surprisingly, lactic acid was relatively ineffective in substantially activating ruminal receptors. This evidence suggests that VFA may be more effective than lactic acid in causing ruminal stasis. However, further evidence linking high VFA concentrations to other symptoms associated with acidosis is unavailable.

Free ruminal glucose also permits opportunistic microorganisms to proliferate; such microbes are capable of producing toxic compounds such as endotoxins and amino acid decarboxylating enzymes(Huber, 1976). For example, decarboxylation of histidine yields histamine. Ruminal concentrations of histamine are increased in both cattle and sheep following grain engorgement (Dain *et al.*, 1955; Koers *et al.*, 1976). Dain *et al.* (1955) reported that concentrations of ruminal histamine and tyramine in sheep were correlated directly with illness. Toxic concentrations of histamine completely stopped rumen motility and this stasis was associated with animal illness. These data are consistent with the earlier observations of Dougherty (1942) regarding reduced ruminal motility. Toxic levels of the amines may be as low as 5 mg per ml of ruminal ingesta for animals that have recovered from acidosis during which concentrations reached 20 mg per ml. Concentrations as high as 70 mg per ml of ruminal fluid were observed in animals who died. Dain *et al.* (1955) added that as the pH of the rumen fluid decreased, concentration of histamine increased. These researchers suggested that acid-tolerating

populations of lactobacilli may be responsible for this increase. This idea was supported by earlier observations of Rodwell (1953) who isolated 8 species of lactobacilli from the rumen of over-fed sheep that could decarboxylate histidine. Generally, lactobacilli populations increase during and after grain engorgement or rapid adaptation to RFC; this tends to support the observations of Rodwell (1953) and Dain *et al.* (1955). However, other researchers have questioned these data; Huber (1976) suggested that the increase in histamine may be due simply to an increase in activity of the decarboxylase enzyme due to a low pH rather than to an increase in the number of organisms that produce decarboxylase.

Sjaastad and Stormorken (1963) and Ahrens (1967) suggested that histamine is not responsible for decreasing rumen motility as was suggested earlier by Dain *et al.* (1955). They detected a substantial lag time between histamine formation and the decrease in pH in both *in vitro* and *in vivo* experiments. Other evidence suggests that during the absorption process, histamine is inactivated by methylation or oxidation (Goth, 1974).

Increased concentrations of ruminal endotoxins have been reported to follow excess consumption of RFC (Allison *et al.*, 1975; Nagaraja *et al.*, 1978). Presumably, this increase is the result of changes in the microbial composition of the rumen. Shortly after ingestion of large amounts of grain, ruminal populations of Gram-positive microorganisms increase sharply while Gram-negative populations decrease (Mann, 1970; Dunlop, 1972; Allison *et al.*, 1975). Mullenax *et al.* (1966) isolated an endotoxin from the rumen of sheep and cattle suffering from acidosis. They reported that intravenous injection of this endotoxin reduced ruminal motility and decreased blood pressure. In addition, Mullenax *et al.*(1966) proposed that release and absorption of endotoxin from Gram-positive bacteria could play a role in the pathogenicity of ruminal acidosis.

Nagaraja *et al.* (1978) reported that the concentration of free ruminal endotoxin increased substantially within 12 hours post engorgement of grain and that microbial populations shifted from predominately Gram-negative to Gram-positive, consistent with earlier reports. However, they indicated from *in vitro* studies that the increase in free endotoxin was not correlated with the decrease in Gram-negative bacteria. Additionally, no substantial absorption of endotoxin from the rumen was detected.

While several researchers have detected other factors potentially associated with acidosis, most of the available literature suggests that accumulation of lactic acid following ingestion of RFC usually is responsible for initiating ruminal acidosis. But what permits lactic acid to accumulate remains unknown. The initial decline in pH may be the result of decreased numbers or activity of non-acid tolerant bacteria and protozoa; this permits competitors such as lactate producing *S. bovis* to thrive. This exacerbates the accumulation of lactic acid lowering pH further which eventually inhibits *S. bovis*. An acidic rumen environment favors lactobacilli resulting in more lactic acid production and a lower rumen pH. It seems logical to suggest that lactic acid is responsible for the reduction in ruminal pH because lactic acid is a stronger acid than other VFA (pK 3.9 vs 4.8 for acetate). As the molar concentration of lactate increases and pH decreases, the number of lactate utilizing bacteria declines which result causes lactate to accumulate further.

If severe, lactate accumulation disrupts normal ruminal function and acid-base balance. Blood acidosis interferes with the ability of hemoglobin to carry oxygen placing the animal at immediate risk of death. Those animals surviving severe acid accumulation often suffer keratinization of the rumen wall which causes a persistent (2-6 weeks) reduction in rate of VFA absorption. Additionally, sloughing of the ruminal epithelium permits ruminal bacteria to enter the blood stream whereafter pathogens can become embedded in the liver and cause liver abscesses. Also, acidotic animals may be crippled due to laminitis.

In less severe cases of acidosis, efficiency of production may be affected. Feeding behavior may be altered as animals cycle through periods of anorexia followed by engorgement; this prolongs the effects of the disease. This level of lactic acidosis may be the most important factor affecting the profitability of feedlot production because abnormal feeding behavior greatly affects total nutrient intake and feed efficiency.

Lactic acidosis occurs most frequently when an animal's diet is switched abruptly from roughage to concentrate. Although the number of starch-utilizing microbes may be low, they rapidly convert RFC in grains to lactic acid. Feedlot cattle often experience acidosis during the transition from roughage diets fed during growth to the concentrate diets fed in feedlots. Lactic acidosis occurs during this transition to the concentrate-rich feedlot diets.

RUMINAL PRODUCTION OF LACTIC ACID

Microbial inhabitants normally found in the rumen of adapted ruminants metabolize carbohydrates found in grain and produce acids (acetate, butyrate, propionate, and lactate) and gases (CO_2 and methane) while they grow and increase in microbial mass. Carbohydrates that are readily fermented include starch, maltose, sucrose, lactose, cellobiose, fructose, and glucose; starch comprises the largest percent (normally over 50%) of high energy diets. Because *S. bovis* is amylolytic and produces lactic acid, it has been suggested to be responsible for the initial decrease in ruminal pH following grain consumption. Indeed, in several early studies, *S. bovis* was indicated to be the predominant lactic acid producing microbe in the rumen of animals engorging large amounts of grain (Hungate *et al.* 1952, Mann *et al.* 1954). MacPherson (1955) showed that *S. bovis* could still ferment sugars even when the pH was below 4.5. However, at extremely low pH values, *S. bovis* numbers decline, so at low pH, the majority of lactic acid must be produced by species of the *Lactobacillus* genus. Lactobacilli have been reported to dominate the rumen microflora of animals fed large amounts of starch or

glucose (Briggs 1955, Jensen *et al.* 1956, Perry and Briggs 1957). Culture studies (J. Leedle, personal communication) indicate that *Streptococcus bovis* comprise a much smaller portion of the total ruminal population with concentrate than with roughage diets, so this organism may be more prevalent as a cause of acidosis with ruminants fed roughage than those fed concentrate diets.

Feed composition can alter the concentrations of ruminal end products. Total acid concentration in the rumen generally is lower with high roughage than high concentrate diets and lactic acid is very rarely detected with roughage diets. In contrast, following grain engorgement, lactic acid of grain-fed animals can occasionally exceed 100 mM.

Two forms of lactic acid are produced by ruminal bacteria, the D(-) and the L(+) form. Both will depress ruminal pH, but production rates and metabolism of these two isomers differ. Earlier workers believed that the D (-) isomer was solely responsible for the metabolic disorder; these researchers termed the disorder D(-) lactic acidosis (Dunlop and Hammond 1965). However, more recent literature indicates that both L(+) and D(-) can accumulate in the rumen of feedlot cattle consuming high concentrate feeds, especially early in the feeding period (Nakamura *et al.*, 1989). During the first 6 months of a feeding study, blood contained higher concentrations of L(+) than D(-) lactate, however after that time, concentrations of these two isomers were nearly equal. What might alter the ratio of D:L lactate in the rumen and in blood is not completely understood; production ratios may vary with substrate (feedstuff) and microbial type. Animals rapidly metabolize L-lactate, as it is produced from glucose by tissues and released into the blood stream; in contrast, D-lactate is metabolized slowly and is partially excreted in urine.

The conditions under which lactate accumulates are quite consistent. Increasing the dietary concentration of rapidly fermented carbohydrates such as processed grains results in very rapid fermentation with rate of lactate production exceeding it rate of use. Concentrations of lactate in the rumen of sheep being fed a good quality alfalfa hay prior

to engorgement of cracked wheat (Ryan 1964) were under 10 mM, but within 48 hours post-feeding, ruminal concentrations exceeded 100 mM, a 10-fold increase.

DISAPPEARANCE OF RUMINAL LACTIC ACID

Lactic acid produced in the rumen or consumed as part of ensiled forage has several fates: absorption through the ruminal wall, passage to the omasum and abomasum, or metabolism by ruminal microorganisms. Most lactate disappears by the latter route. However, more lactate is absorbed when ruminal pH is low. Williams and MacKenzie(1965) as summarized by Slyter (1976) reported that rate of absorption of lactic acid was twice as great at pH 4.0 than at pH 5.2; absorption rates were not different between D(-) and L(+) lactate.

Gill *et al.* (1986) observed that 90% of total lactate in sheep rumen was converted to VFA. The percentage of acetate and propionate produced were 55% and 31%, respectively. Counotte (1981) reported that the proportion of VFA produced depends on ruminal pH and the fractional outflow rate of ruminal water. Higher pH (6.8) and relatively high dilution rates resulted in productions of 64%, 33%, and 3% for acetate, butyrate, and propionate, respectively. At lower flow rates and pH, a higher proportion of propionate was produced from lactate. Infusion of lactate generally increases the proportion of ruminal propionate suggesting that propionate is the main end-product of lactate metabolism in the rumen (Emery *et al.* 1966, Chamberlin *et al.* 1983).

In general, two major lactic acid utilizers inhabit the rumen. Ruminal isolates of *Selenomonas ruminatium* (Bryant 1956) and *Megasphaera elsdenii* (Elsden *et al.*, 1956) have been characterized as the major lactic acid fermenters. *Anaerovibrio lipolytica* is the predominant lactic acid utilizing species in forage diets. However the inability *A. lipolytica* to survive at pH values as low as *M. elsdenii and S. ruminatium* casts doubt on its importance in lactate metabolism of grain-fed ruminants (Slyter, 1976).

CONVENTIONAL MANAGEMENT OF ACIDOSIS

Ruminal acidosis can be managed by keeping the ruminal fermentation under control thus reducing the probability for acid accumulation. Methods for control include gradual diet changes, limiting daily intake, special processing of grain, and including specific feed additives, e.g., antibiotics or buffers.

Diet control management centers on adaptation of animals to diets rich in RFC over a prolonged time period, often up to 21 days. During this time, the ratio of grain to roughage in the diet of newly arriving cattle is increased in several small steps. Most commercial feedlots formulate and deliver several "adaptation" diets that contain different ratios of grain to forage. This adaptation period ranges from two to four weeks depending on the aggressiveness of management. This method usually is quite effective in controlling acidosis but is very costly to the producer due to the high cost of producing, transporting, and chopping forage, disposing of increased animal waste, and lower production efficiencies.

Various workers have recommended specific diets and feeding times to control ruminal fermentation and thereby preventing acidosis. Elam (1976) suggested that newly received cattle that are hungry should be allowed to adjust by feeding a 40 to 60% roughage diet containing 40 Mcal NEg with *ad libitum* long stem hay. Once cattle are eating well, the hay is discontinued and the energy density of the diet is increased by no more than 10% at a single time.

Including ionophores in the diet of ruminants reduces the incidence of ruminal acidosis. Both *in vitro* and *in vivo* experiments have shown that ionophores control ruminal fermentation by reducing the ruminal populations of gram-positive, lactic acid-producing organisms such as *Streptococcus bovis* and *Lactobacillus spp.* The sensitivity of *S. bovis* to ionophores and effects of ionophores on ruminal fermentation have received the most attention. Most researchers believe *S. bovis* is responsible for the initial pH drop

following carbohydrate loading. With *in vitro* incubations, Newbold and Wallace (1988) found that during lactic acidosis, *S. bovis* populations increased dramatically followed by increases in acid tolerant populations of *Lactobacillus*.

Following induction of acidosis, cattle fed ionophores have lower ruminal lactic acid concentrations and higher pH values than cattle not fed ionophores (Newbold and Wallace, 1988; Muir and Barreto, 1979; Nagaraja *et al.*, 1981; Nagaraja *et al.*, 1985). However, the total extent of ruminal fermentation presumably is not changed because ruminal concentrations of VFA often are increased when ionophores are fed.

Although conventional management methods can greatly reduce the incidence of feedlot acidosis, other methods must be examined. This is because many consumers consider the addition of antibiotics to animal feeds to be an undesirable practice. Additionally, feedlot managers continually are searching for ways to reduce labor and feed costs while improving animal performance and carcass composition.

MANAGEMENT ALTERNATIVES

Various methods for controlling acidosis have received attention in the past few years. Techniques to modify ruminal fermentation have been the most effective; these generally have sought to prevent the accumulation of lactate and other acids by maintaining a healthy and "balanced" population of ruminal microorganisms rather than by trying to offset the effects of acid accumulation. The addition of succinate to the diet reduces the effects of acidosis by increasing the pH in acidotic animals (Smolenski *et al.*, 1991). The addition of succinate to *in vitro* and *in vivo* acidosis models significantly increased pH values even though lactate concentration was not altered. Smolenski *et al.* (1991) proposed that when succinate is converted to propionate, the propionate utilizers may have a competitive advantage over the lactate producers thereby avoiding the spiraling accumulation of lactic acid. The addition of dicarboxylic acids such as fumurate

and malate have been shown to stimulate the lactic acid utilization rates of *Selenomonas ruminatium,* a major ruminal inhabitant (Nisbet and Martin, 1990). They reported the addition of malate (10 mM) increased the rate of lactic acid at 24 h by 10-fold and the absence of malate lead to little or no lactic acid uptake by *Selenomonas ruminatium.*

Another method of rumen fermentation modification is the addition of specific ruminal organisms that utilize lactate. *Megasphaera elsdenii,* when added to the rumen of experimentally animals, reduced the concentration of lactic acid and inhibited the decline in pH (Greening *et al.*, 1991). Although this organism protected against pH decline and lactate accumulation when it was administered at the time of acidosis induction or 2 hours afterward, it was not effective when fed to the animals 8 hours before induction. Even though *Megasphaera elsdenii* has been reported to be one of the most important lactate utilizers in the rumen of animals consuming high concentrate diets (Elsden *et al.*, 1956), the viability of the introduced bacteria remains questionable if no activity was detected just 8 hours after it was dosed. Introduced cultures must have the ability to survive, function, and compete in the face of cutthroat competition in the rumen.

The addition of *Aspergillus oryzae* and *Saccharomyces cerevisiae* has been reported to stimulate the lactic acid utilization rates of ruminal organisms. Martin and Nisbet (1992) suggested the production of soluble dicarboxylic acids malate and fumurate by the introduced organisms stimulated *Selenomonas ruminatium.*

Propionibacteria have promise as a probiotic for both humans and animals. Although *Propionibacterium* occur naturally in the rumen (Gutierrez, 1953; Hungate, 1966), the current literature makes no mention of their survival or growth when introduced into the rumen. Recent work in our laboratory has demonstrated both survival in the bovine rumen and prophylactic value of *Propionibacterium acidipropionici* (P5) for preventing nitrate toxicity when administered as a direct-fed microbial for cattle (Swartzlander, 1994).

Propionibacterium have been shown to 1) utilize lactic acid in a mixed environment producing energetically favorable propionic acid as an end-product, 2) be a natural inhabitant of the rumen capable of becoming established when fed as a freeze-dried concentrate, 3) have selective qualities which allow them to be enumerated and identified from competing microflora. However, it is imperative to determine if strains of propionibacteria can survive, compete, and utilize lactic acid when the ruminal environment is acidotic.

TRACKING SPECIFIC CULTURES INTRODUCED INTO A DIVERSE MICROBIAL POPULATION

One of the major hurdles in studying the usefulness of direct-fed microbials is the difficult task of recovering a culture from the complex and microbial diverse environment into which it has been introduced. Establishment of the culture at a given location, e.g., the rumen, intestine, must be shown before treatment with the culture can be considered efficacious. Specific techniques have been developed to aid in the isolation and identification of target organisms in order to solve this dilemma. Selective-differential media, plasmid profiling , and DNA "fingerprinting" have been used for this purpose.

Due to its simplicity, selective-differential media has been the most common method of isolating direct-fed microbials. Unfortunately, the accuracy of this technique is less than that of other available techniques (Tannock, 1988). Selective media makes use of antibiotics, unique nutrients, or other compounds that inhibit the growth of competing microorganisms while enhancing or having no affect on growth of the microbe of interest. Differential media allows an observer to identify a specific organism when found within a population of competing microflora. Most commonly, color changes are produced by strain or genus specific chemical reaction. Because differential media do not inhibit the competing organisms, selective media must be used. Thus selective-differential media can be used to enumerate a certain genus or species by allowing for differentiation of the specific organism from background microbes that survive the selection process.

Although the selective-differential media technique is a useful tool for isolating a genera or species from a complex mixture, most current formulations are not strain specific. This presents a problem when dealing with a population as extremely diverse as found in the rumen. Because other strains of the same genus may be present, one must be able to identify accurately the presence of a specific strain. Colonies isolated by selective-differential media methods can be identified and confirmed as a specific strain

by biochemical testing and or genetic identification such as plasmid profiling or DNA "fingerprinting".

Biochemical testing is the most common method used to confirm the identification of isolated colonies (Tannock, 1988). Most commonly, tests measuring carbohydrate fermentations or other chemical reactions that are strain-specific. Because biochemical tests are based on phenotypic responses, they do not necessarily reflect the genetic identity of an isolate. Native microbes, closely related or not, may share the same phenotypic profiles as introduced microbes. The more definitive methods for confirmation involve genetic profiles.

For plasmid profiling, migration patterns of plasmids isolated from suspect colonies are compared with those from an introduced culture. Plasmid profiling was used by Tannock *et al.* (1990) to distinguish between strains of lactobacilli isolated from the gastrointestinal tract of swine. It also has been used to monitor *L. plantarum* -inoculated corn silage (Hill and Hill, 1986).

While plasmid profiling is useful for confirming suspect colonies isolated with selective-differential media, this technique has limitations. Conjugal transfer of plasmid DNA between species and even genera has been reported (Tannock, 1987). Such transfer will cause very distantly related species to exhibit similar plasmid profiles and just because plasmids are of the same molecular weight does not mean they are the same. In addition, bacterial cells may lose plasmid DNA since replication rates may differ between the cell and the extrachromosomal DNA; this leaves some daughter cells without the plasmid. Finally, many strains lack plasmid DNA or do not posses a unique plasmid DNA profile. Consequently, although plasmid profiling can be more useful for confirmation than carbohydrate tests, it is not the most accurate method for strain identification.

A more accurate method of strain identification requires profiling of the bacterial chromosome. Following digestion with specific restriction endonucleases, chromosomal

DNA fragments from identified and unknown isolates can be compared. Each fragment has a specific migration. This procedure has been used to identify *Escherichia coli, Shigella spp., Propionibacterium freudenreichii* and many others using pulsed-field gel electrophoresis (Ogram and Sayler, 1988; Rehberger, 1993).

GENUS OF *PROPIONIBACTERIUM*

Propionibacteria are classified as Gram positive, non-motile, non-spore forming, pleomorphic rods whose fermentation products include largely propionic and acetic acids. They are anaerobic to aerotolerant, generally catalase positive, and grow best at 30-37 °C (Cummins and Johnson, 1986).

The *Propionibacterium* genus commonly is divided into two groups depending on their normal habitat. The first group, "classical propionibacteria" or "dairy propionibacteria" are readily isolated from cheese and dairy products. This group has also been found in other fermented products such as silage and olives (Cummins and Johnson, 1986). The "dairy propionibacteria" generally include four recognized and characterized species: *P. freudenreichii, P. jensenii, P. thoenii, and P. acidipropionici.* The second group of propionibacteria strains are those found typically on human skin or in the intestine. These are called the "acnes group strains" or "cutaneous propionibacteria" and include four strains: *P. acnes, P. avidum, P. granulosum, and P. lymphophilum.*

Kornyeva (1981) reported that *Propionibacterium shermanii* became established in the intestines of infants. Infants showing various pathogenic staphylococci intestinal infections were fed acidophilus milk made with *P. shermanii* for 14-21 days. After just 7 days, 87.5% of the treated infants were excreting *P. shermanii.* When healthy infants were given the milk, 95.5% were found to excrete *P. shermanii.* The fecal concentration of *P. shermanii* for both groups increased 100-fold during the 14-day experiment.

Mantere-Alhonen (1983) examined the effect of gastric digestion on *P. freudenreichii*; he found that the culture survived well without any loss of viability. Two fermented milk products, "Malyutka" and "Malyush," both modified by adding *P. shermanii*, were used as treatment for acute infant gastrointestinal disease. Formulas containing the added propionibacteria culture were more effective in treating the disease than the products without added probiotics (Nabukhotnyi *et al.,* 1983).

Mantere-Alhonen (1982) reported that propionibacteria added to fodder increased the growth rate of swine and stabilized the intestinal microflora. Although no apparent intestinal adhesion could be demonstrated, numbers of propionibacteria isolated from the intestinal contents were higher for treated than control animals. In a study with dairy calves, a whole milk mixture was treated with a combination of propionibacteria, a lactic acid culture, and antibiotics; addition of this mixture increased weight gains (Vladimirov *et al.* 1978).

The genus *Propionibacterium* has been used in a number of processes. Currently the most common use is in production of Swiss-type cheeses. Lactic acid produced by *Lactobacillus* starter culture strains is metabolized by propionibacteria to form propionic acid and CO_2. Propionic acid is a major flavor component; slow release of CO_2 forms the characteristic "eyes" of this type of cheese (Lansgrund and Reinbold, 1973). Propionibacteria can grow at temperatures as low as 4 °C (Hofherr and Glatz. 1983). This is important for manufacturing of Swiss cheese because maximum CO_2 production occurs at 12.8 °C (Hettinga and Reinbold, 1972).

OBJECTIVES

This study was designed to select strains of *Propionibacterium* that could utilize lactic acid (80 mM/L) produced in the rumen of feedlot cattle in quantitative pure culture experiments and test ability of various strains to compete with ruminal bacteria in a batch fermentation system. This *in vitro* rumen simulation should be useful to identify strains of *Propionibacterium* that can survive the in rumen and utilize the lactic acid produced by indigenous microorganisms. Strains also were tested for their ability to be enumerated and genotypically identified when isolated from a complex mixed culture, e.g., the rumen.

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CHAPTER III

SELECTION OF *PROPIONIBACTERIUM* STRAINS CAPABLE OF UTILIZING LACTIC ACID FROM *IN VITRO* MODELS

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ABSTRACT

Forty-four strains representing four species of *Propionibacterium* were screened for lactic acid utilization to examine their potential for use in a direct-fed microbial to prevent lactic acidosis in feedlot cattle. Strains were tested for utilization of lactic acid and growth in a nutrient broth supplemented with 80 mM lactic acid at two different pH values - one representing the pH of an acidic rumen (5.0) and the other that of a forage-fed ruminant (7.0). No differences in growth and lactic acid utilization were detected among strains at pH 7.0. Data from pH 5.0 experiments showed *P. freudenreichii* strains P49 and P99 utilized 76.90 mM and 78.59 mM of lactic acid respectively, which was significantly more compared to other strains. Compared with strains of *P. acidipropionici, P. jensenii and P. thoenii, P. freudenreichii* strains reached significantly higher cell densities and utilized more lactic acid at pH 5.0. Rumen fluid simulation models were used to examine the ability of fifteen selected propionibacteria strains to survive and utilize lactic acid produced by native ruminal microorganisms. Eleven of the fifteen propionibacteria strains tested utilized lactic acid in the rumen model. Compared with other strains, P42 had the highest rate of pH increase (.0377 units/h), but was not statistically ($P<.05$) different from strains P63, P54, P25, and P41. Strain P42 also had the highest rate of lactic acid utilization (1.61 mM/h) compared to others but was not statistically ($P<.05$) different from strains P63, P54, P25, P41, P111, P81, and P104. Gompertz non-linear curve fitting equation revealed that strains P54 and P63 significantly increased the lag time for lactic acid accumulation and suppressed the rate of H+ concentration

(Key words: *Propionibacterium,* rumen, lactic acid utilization,)

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INTRODUCTION

Feeding of grains such as corn, to cattle improves the rate and efficiency of gain and improves the tenderness and flavor of the meat by increasing the intramuscular fat deposits (marbling). However, feeding grains and other rapidly fermented carbohydrates to ruminants has increased the incidence of metabolic disorders such as lactic acidosis.

Over consumption of readily fermented carbohydrates leads to an accumulation of ruminal lactic acid; its accumulation perturbs the normal ruminal flora (Dunlop, 1972; Slyter 1976; Elam, 1976). Lactic acid producing microorganisms, namely *Streptococcus bovis* and *Lactobacillus* species that produce lactate, presumably are primarily responsible for the decline in rumen pH (Allison *et al.*, 1975; Dunlop, 1972; Hungate *et al.*, 1952; Mann, 1970).

Treatments which inhibit the growth of *S. bovis* can help to prevent lactic acidosis. Antibiotics such as lasalocid, monensin, thiopeptin, and virginiamycin have a narrow spectrum of activity against gram-positive organisms and generally help to prevent the decrease in pH seen with cattle and sheep engorged with readily fermented carbohydrates (Nagaraja *et al.*, 1982; Tung and Kung, 1993; Muir *et al.*, 1981). The sensitivity of *S. bovis* to thiopeptin and monensin has been demonstrated using *in vitro* experiments (Muir and Barreto, 1979; Tung and Kung, 1993).

Inoculation of the rumen with lactic acid-utilizing organisms is a logical alternative prophylactic for ruminal acidosis due to current consumer perceptions of antibiotic residues in the food supply. Using *in vitro* fermentation with a mixed population of ruminal microorganisms, lactic acid accumulation was significantly reduced by inoculating with *Megasphaera elsdenii* (Kung and Hession, 1995). *M. elsdenii* inoculation of beef cattle at the time of experimentally inducing acidosis resulted in ruminal pH values of 5.51 compared to control values of 4.65. However, inoculating cattle with the same bacteria 8 h prior to inducing acidosis resulted in no significant

difference between treated and control ruminal pH values(Greening, *et al.*, 1991). These data suggest that the *M. elsdenii* introduced do not have the ability to become sufficiently established to significantly inhibit pH reductions in ruminal acidosis.

Propionibacterium are normal inhabitants of the rumen and account for approximately 1.4% of the total microbial population (Oshio *et al.*, 1987), therefore may provide an additional biological approach to help prevent the accumulation of lactic acid in the rumen. A denitrifying strain of *Propionibacterium* was shown to established an active population when introduced into the rumen of beef cattle (Swartzlander, 1994). This data suggest that the ruminal inoculation of propionibacteria can affect the formation of undesirable endproducts such as lactic acid.

The purpose of this study were 1) to identify those propionibacteria strains capable of reducing sub-acute levels (80 mM/L) of lactic acid at pH values similar to the acidic rumen (pH 5.0), and 2) to determine the ability of selected strains to inhibit the accumulation of lactic acid and subsequent pH reduction of a rumen simulation model supplemented with glucose.

Bacterial strains. *Propionibacterium* cultures used in this study were obtained from the culture collection of Agtech Products, Inc., Waukesha, WI. Cultures were maintained at -75 °C in a sodium lactate broth (NLB) supplemented with 10% glycerol (Hofherr and Glatz, 1983). The specific propionibacteria strains used in this study are listed in Table 1.

Culture conditions. Strains were activated by placing a portion of the frozen suspension in 10 ml of NLB and incubated at 32 °C for 36-48 hours. Strains were sub-cultured by transferring a 1% volume of the culture at mid-log growth to fresh NLB. Cultures were transferred a minimum of three times before being tested. The purity of tested strains was monitored by regularly streaking cultures onto a sodium lactate agar (NLA).

***In vitro* acidified and neutralized broth medium.** Primary strain selection involved testing the growth and lactic acid utilization of cultures in a basal broth media. The acidified medium was prepared by including 80 mM L(+) lactic acid in a basal broth containing 1% yeast extract, 1% tryptone, dipotassium phosphate and distilled water. The pH of the broth medium was raised to pH 5.0 using 5.0 M NaOH. Following filter sterilization (Gelman Sciences, Ann Arbor, Michigan), the medium was dispensed at a volume of 10 ml into sterile screw cap test tubes. Neutralized broth medium was prepared the same as acidified media except that the pH of broth was raised to 7.0 with 5.0 M NaOH prior to filter sterilization.

Rumen fluid simulation medium. Ruminal fluid was collected via ruminal cannula 2 h post feeding from a cross-bred beef heifer fed a high roughage diet. The ruminal fluid was strained through four layers of cheesecloth and transported to the laboratory in an insulated container. Test ruminal fluid media contained 250 ml of strained ruminal fluid, 62.5 ml McDougall's buffer (McDougall, 1948), and 1.5% dextrose. The added dextrose served as a readily fermented carbohydrate to simulate conditions found in the rumen of animals following grain engorgement. Strained ruminal fluid, buffer, and dextrose were

dispensed into sterilize 500 ml bottles and allowed to equilibrate in a water bath at 39 °C for approximately 15 minutes prior to inoculation. Initial pH of the rumen fluid model ranged from 6.6 to 6.9 depending on date of collection

High Pressure Liquid Chromatography. Samples were prepared for HPLC analysis by aseptically removing 1.0 ml from the test medium at the appropriate sampling times. Samples were placed in a 1.5 ml microcentrifuge tube and the cells were pelleted by centrifugation (10 minutes, at 12,500 rpm). A sample of 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 stop fermentation. These samples were stored at -20 C until analysis was performed. For analysis, frozen tubes were allowed to thaw at room temperature and filtered through 0.2 um filters directly into 2 ml HPLC autosampler vials and capped.

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 (Bio-Rad 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, but 210 nm was the optimum setting for determining peak height with minimum background noise. Peak areas were used to determine compound concentrations by comparison with external standards. Peak purity was monitored by UV scanning techniques as an aid in identifying abnormal wavelength patterns present in a single peak

Pulsed-field gel electrophoresis. Strains were aseptically transferred to 10 ml sodium lactate broth containing 2.0% glycine and incubated at 32 °C until an optical density (600 nm) of 0.8-1.0 was reached, usually 36-42 hours. Intact genomic DNA was isolated from cell suspensions (Rehberger, 1993). Intact bacterial genomic DNA was digested by restriction endonucleases *Xba I, Hpa I, Hind III,* and *SnaB I.* Restriction fragments were separated using a continuous homogeneous electrophoresis field (CHEF) (Bio-Rad

Laboratories, Inc. Hercules, California). Conditions were optimized for maximum separation of DNA fragments ranging in size from 50 to 250 kb when using a 0.8% agarose gel. CHEF Instrument settings included: current (6 V/cm), initial switch time (5.55 sec.), final switch time (17.89 sec.), TBE buffer (45 mM Tris, 45 mM Boric acid, 1.25 mM EDTA disodium salt, adjusted to a pH of 8.0), temperature (14 °C) and angle (120°). The molecular weight of DNA fragments were determined by comparing the migration distance to a 50 KB lambda ladder standard.

***In vitro* broth medium experimental procedures**. For each propionibacteria strain, duplicate tubes were inoculated with a 1% inoculum of a 48 h culture for each propionibacteria strain. Tubes were incubated under static conditions at 32 °C for 48 h.. Growth was determined by measuring increases in optical density at 0 h and ever 8 hours beginning with 16 h using a Milton Roy Spectronic 601 spectrophotometer (Milton Roy, Rochester, New York) set to a wavelength of 600 nm. Samples (1 ml) were aseptically removed from each tube at the time optical density readings were taken and prepared for organic analysis by HPLC.

Rumen model experimental procedures. Duplicate bottles were inoculated with the appropriate propionibacteria strain to be tested at a level of 1 x 10^7 cfu/ml. Bottles were flushed with CO_2, capped, and incubated at 39 °C for 48 h. Every 6 h during the 48 h incubation period, samples were collected and analyzed for pH, lactic acid and volatile fatty acid (VFA) concentrations. Additional samples were collected at 16 h and 48 h for use in microbiological analysis. Lactic acid and VFA samples were prepared by aseptically collecting a 1 ml sample in a 1.5 ml microcentrifuge tube. Cells were pelleted by centrifugation (10 minutes at 12,500 x g). One-half ml of supernatant was mixed with an equal volume of 10 mM H_2SO_4 and filtered through a 0.2 um membrane filter.

Microbiological analysis consisted of plating serial dilutions (10^{-3}, 10^{-4} and 10^{-5}) of the *in vitro* rumen fluid medium on a propionibacteria selective-differential medium

(PSA). Colonies with typical propionibacteria morphology were confirmed using pulsed-field gel electrophoresis (PFGE).

Differences in pH and lactic acid concentration between inoculated and uninoculated controls at each sampling time were calculated and regressed against incubation time up to 24 h in order to select the best lactic acid utilizing strains. Strains for which a change over time in lactate or pH was detected (an $R > 0.50$ against sampling time) were compared using Duncan's Multiple Range procedures (SAS, 1985). Additionally, Gompertz equation was used to analyze the sigmoidal curves for pH decrease and lactic acid concentration increase (Zwietering *et al.* 1990).

RESULTS

Broth medium supplemented with 80 mM lactic acid, pH 5.0 The levels of lactic acid utilization at 24 and 48 h of incubation are listed in Tables 2 and 3, respectively. Twenty-five of the forty-four strains tested utilized 8 mM (10%) or more by 24 h. Seven of the twenty-five strains; P111, P106, P99, P44, P53, P31, and P54, utilized over 20.0 mM (25%) of the available lactic acid. Means for lactic acid utilization of each species are shown in Figure 1. *P. acidipropionici* strains had the lowest utilization (8 mM) while *P. jensenii* strains utilized the most lactic acid (16 mM). By 48 h of incubation, twenty-four strains had utilized over 20 mM (25%), with eight strains utilizing more than 40 mM (50%). The utilization of lactic acid ranged from 1.10 mM to 78.59 mM. *P. freudenreichii* strains P49 and P99 utilized 76.90 mM and 78.59 mM respectively, which was significantly more lactic acid compared to other strains. Two strains, P106 and P86 utilized less lactic acid at 48 h when compared to 24 h. This may be due to a shift in lactic acid production by the strains or in experimental error since all other strains had utilized more lactic acid at 48 h compared to 24 h. Six of the eight most active lactic acid utilizers were classified as *P. freudenreichii.* Means of lactic acid utilization for each species (Figure 1) indicate *P. freudenreichii* strains utilized the most (49 mM). while *P. thoenii* strains utilized the least (14 mM).

Optical density values for 24 and 48 h of incubation are presented in Tables 4 and 5. After 24 h only two strains had optical density values over 0.30. *P. jensenii* strain P88 had the highest value (.385), but was not significantly different from that of *P. freudenreichii* strain P99 (.335). By 48 h, strain P99 exhibited the highest level of growth with an optical density of 2.4. All species had similar lag time as suggested by similar strain means at 24 h (Figure 2). By 48 h, *P. freudenreichii* strains had reached a higher maximum optical density when compared to other species.

Broth medium supplemented with 80 mM lactic acid, pH 7.0 Lactic acid utilization results for 24 and 48 h are listed in Tables 6 and 7. Five strains had utilized over 40 mM lactic acid by 24 h. These strains which were from *P. acidipropionici*, *P freudenreichii*, and *P. thoenii* species included P90, P104, P49, P99, and P85. By 48 h, no significant differences were observed for lactic acid utilization among strains. Comparison of the mean lactic acid utilization for each species at 24 hours indicated *P. freudenreichii* strains utilized the highest concentrations with a mean of 40 mM while *P. jensenii* strains utilized the lowest concentration with a mean of 17 mM (Figure. 3). However, all species had similar lactic acid utilization values by 48 h.

Data for 24 and 48 h optical density values of strains grown at pH 7.0 are listed in Tables 8 and 9. *P. freudenreichii* strain P104 had the highest optical density at 24 h but was not significantly different from *P. acidipropionici* strain P90. Only thirteen strains failed to reach an optical density of 1.0 by 24 h. Of those thirteen, six strains were classified as *P. jensenii*, suggesting that this species tended to have longer lag times. By 48 h, most strains had optical density readings above 2.0. The means of species optical densities suggests only minimal differences after 24 or 48 h of incubation (Figure 4).

The variation for both optical density and lactic acid utilization within species was lower for strains grown in pH 7.0 broth when compared to pH 5.0 at both 24 and 48 h. Most all strains had increased amounts of lactic acid utilization and higher optical densities when grown at pH 7.0. Most of the top ranking strains (P99, P49, P104, P90) were not greatly affected by the decreased pH condition. However many strains were inhibited by the lower pH level (i.e. P101, P68, P3, P5, P96, P69) and had decreased lactic acid utilization at pH 5.0 by as much as 88%.

Rumen simulation. High variability was observed among strains tested in the rumen simulation model. Strain performance across and within experiments was quite variable. This may have been due to variation in the rumen fluid collected on different days from the donor animal; since similar fluctuations were noted in control tubes.

The rate of change in pH and lactic acid concentration was determined by regressing the difference between inoculated and control rumen fluid incubations against time. Only when the regression coefficient for rate of change in pH and lactate was greater than 0.50 for an inoculated flask was the data included in the statistical analysis (Table 10). Compared with other strains, P42 had the highest rate of pH increase (0.0377 units/h), but was not statistically ($P<0.05$)different from strains P63, P54, P25, and P41. Strain P42 also had the highest rate of lactic acid utilization (1.61 mM/h) compared to others but was not statistically ($P<0.05$) different from strains P63, P54, P25, P41, P111, P81, and P104. Since linear regression analysis did not adjust for differences in lag times, other non-linear methods were employed

Ruminal fluid simulation data was analyzed using the Gompertz non-linear equation technique. Values up to 24 h were used in the analysis since a decrease in lactic acid concentration was observed after 24 h in all controls. Flasks inoculated with strains P54 and P63 had significantly lower rates of hydrogen ion accumulation (Table 11). When the rate of H+ increase of inoculated flasks was compared to the control (0.00018), only strains P54 and P63 had significantly different values of -1.45 and 2.18 respectively. Strains P54, P63 and also P25 had a significant impact on the lactic acid production lag time. P54 and P63 increased the lag time of lactic acid accumulation by 2.06 and 2.63 (h) respectively, thereby slowing the accumulation of acid. On the other hand, strain P25 decreased the lag time of inoculated samples thus resulting in faster lactic acid accumulation. Strain P111 was the only strain found to significantly increase the lag time of H+.

Strain Survival. The viable plate counts of strains at 16 h and 48 h of incubation in the rumen simulation model are presented in Table 12. Nine strains maintained a population of at least 1.0×10^4 cfu/ml for 48 hours. Six of the nine strains exceeded 1.0×10^5 cfu/ml; strains P25 and P63 had the highest rates of survival at 6.0×10^5 and 1.0×10^6 cfu/ml, respectively.

Pulsed-field gel electrophoresis Genetic analysis of genomic DNA was used to identify differences and similarities among propionibacteria strains. Results of *Xba I* digests of intact genomic DNA isolated from propionibacteria strains are shown in Figure 5. Differences in the number and size of fragments were observed for strains P41, P63, P81, P89, and P104. This data suggest the five strains are not closely related. Two strains of propionibacteria used in this study, P54 and P63, were found to have many co-migrating *Xba I* fragments suggesting they may be closely related (Data not shown). Further analysis of strains P54 and P63 was performed using three additional restriction endonucleases (*Hind III, Hpa I, and SnaB I).* For each enzyme, the number and size of DNA fragments were identical for each strain, confirming strain P54 and P63 are closely related if not identical (Data not shown).

Based on the differences in *Xba I* digestion patterns observed for the majority of strains tested, pulsed-field gel electrophoresis was determined to be useful for strain identification. Genetic analysis was performed on isolates obtained from rumen simulation models at the end of the 48 h incubation to confirm the identity of propionibacteria present in the test medium. The identity of isolates was confirmed by comparing the DNA migration patterns of suspect colonies to that of the specific inoculated strain of propionibacteria.

Table 1. *Propionibacterium* strains

OSU strain number	Species designation	Strain designation	Source
P2	*P. acidipropionici*	128	B
P3	*P. acidipropionici*	E14	A
P4	*P. thoenii*	TH25	A
P5	*P. acidipropionici*	E214	A
P9	*P. acidipropionici*	129	B
P10	*P. thoenii*	R9611	A
P15	*P. thoenii*	TH20	A
P20	*P. thoenii*	TH21	A
P21	*P. thoenii*	R6	A
P25	*P. jensenii*	J17	A
P26	*P. thoenii*	8266	B
P31	*P. freudenreichii*	1294	E
P35	*P. acidipropionici*	1505	E
P38	*P. acidipropionici*	13	D
P41	*P. jensenii*	14	D
P42	*P. acidipropionici*	10	D
P44	*P. jensenii*	363	E
P46	*P. jensenii*	E.1.2	F
P48	*P. freudenreichii*	E.11.3	F
P49	*P. freudenreichii*	E.15.01	F
P50	*P. acidipropionici*	E.7.1	F
P52	*P. acidipropionici*	E.5.1	F
P53	*P. acidipropionici*	E.5.2	F
P54	*P. jensenii*	E.1.1	F
P63	*P. jensenii*	PJ54	G
P68	*P. jensenii*	PJ53	G
P69	*P. jensenii*	PJ23	G
P74	*P. jensenii*	PZ99	G
P78	*P. acidipropionici*	PA62	G
P79	*P.thoenii*	PT52	G
P81	*P. acidipropionici*	PP798	G
P85	*P.thoenii*	20	H
P86	*P. jensenii*	11	H
P88	*P. jensenii*	22	H
P89	*P. freudenreichii*	5571	I
P90	*P. acidipropionici*	5578	I
P96	*P. freudenreichii*	8903	I
P99	*P. freudenreichii*	ATCC 9615	J
P101	*P. freudenreichii*	ATCC 9617	J
P104	*P. freudenreichii*	ATCC 6207	J
P105	*P. thoenii*	ATCC 4871	J
P106	*P. jensenii*	ATCC 4964	J
P108	*P. acidipropionici*	ATCC 14072	J
P111	*P. acidipropionici*		O

Sources: (A). Cornell Iniversity, Ithaca, NY; (B). Iowa State University, Ames IA; (C). Dr. K.W. Sahli, Station Federale D'Industrie Laitiere Liebefeld-Bern, Switzerland; (D). Dr. W. Kundrat, University of Munich, Munich, Germany; (E). Dr. V.B.D. Skerman, University of Queensland, Brisbane, Australia; (F) Dr. C.B. van Neil, Hopkins Marine Station, Pacific Grove, CA; (G) Communicable Disease Laboratory, Atlanta, GA; (H). Isolated from Gruyere cheese imported from France; (J). Amercian Tyoe Culture Collection, Rockville, MD; (O). Origin unknown.

Table 2. Lactic acid utilization of propionibacteria strains grown in 80 mM broth at pH 5.0 for 24 hours.

Species	strain	24 h lactate utilization (mM)*
P. acidipropionici		
	111	29.97 ab
	53	21.47 bcdef
	38	14.91 bcdefghi
	90	10.68 cdefghi
	81	9.44 cdefghi
	35	8.33 defghi
	52	8.29 defghi
	2	7.88 defghi
	5	7.57 defghi
	78	5.92 efghi
	9	5.44 efghi
	42	4.72 efghi
	50	3.66 fghi
	3	0.74 i
P. freudenreichii		
	99	26.36 bc
	31	22.60 bcde
	89	19.15 bcdefgh
	48	11.21 cdefghi
	49	10.08 cdefghi
	104	9.91 cdefghi
	96	5.14 efghi
	101	0.74 i
P. jensenii		
	106	43.49 a
	44	25.00 bcd
	54	20.19 bcdefg
	86	18.33 bcdefghi
	88	17.60 bcdefghi
	41	14.16 bcdefghi
	69	10.96 cdefghi
	74	10.51 cdefghi
	46	8.55 defghi
	63	7.88 defghi
	68	7.33 defghi
P. thoenii		
	79	11.01 cdefghi
	10	10.51 cdefghi
	85	9.51 cdefghi
	26	6.86 efghi
	105	6.03 efghi
	21	3.70 fghi
	4	2.72 ghi
	15	1.92 hi
	20	1.55 hi

*Values with the same letter are not significantly different

Table 3. Lactic acid utilization of propionibacteria strains grown in 80 mM broth at pH 5.0 for 48 hours.

Species	strain	48 h lactate utilization (mM)*
P. acidipropionici		
	90	58.70 bc
	111	38.85 ef
	81	36.45 fg
	78	35.05 fgh
	52	26.21 ghijk
	53	24.82 hijkl
	38	23.68 ijklm
	2	17.44 klmnop
	35	15.21 lmnopq
	9	13.24 mnopqr
	42	12.45 nopqr
	50	10.27 opqrs
	5	9.29 pqrs
	3	8.74 pqrs
P. freudenreichii		
	99	78.59 a
	49	76.90 a
	48	65.26 b
	104	61.68 b
	89	51.22 cd
	31	48.68 d
	96	10.27 opqrs
	101	1.10 s
P. jensenii		
	88	46.51 de
	41	36.08 fg
	54	34.18 fghi
	106	30.14 fghi
	44	27.54 ghijk
	63	26.67 ghijk
	74	24.13 ijklm
	46	23.74 ijklm
	69	15.21 lmnopq
	86	8.46 pqrs
	68	7.49 pqrs
P. thoenii		
	26	21.25 jklmn
	105	21.20 jklmn
	10	20.49 jklmno
	85	17.95 klmnop
	21	17.83 klmnop
	79	14.43 lmnopq
	20	11.19 nopqrs
	4	5.53 qrs
	15	3.19 rs

*Values with the same letter are not significantly different

Figure 1 . Mean lactic acid utilization by each species of Propionibacterium at pH 5.0

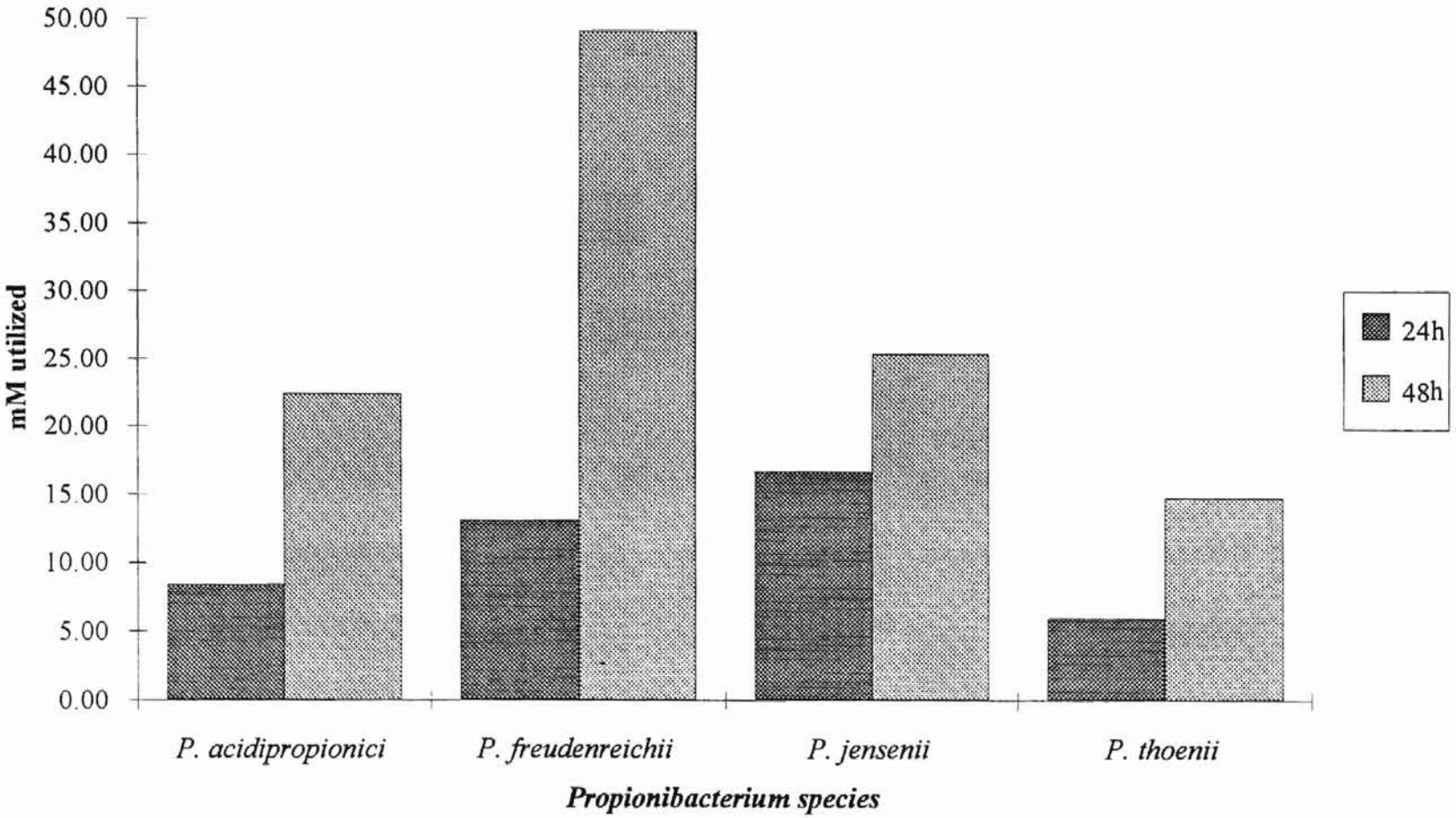


Table 4. Optical density of propionibacteria strains grown in 80 mM broth at pH 5.0 for 24 hours

Species	strain	24 h optical density (600 nm)*
P. acidipropionici		
	111	.299 abc
	78	.267 bcde
	3	.257 bcde
	81	.256 bcde
	90	.234 bcdefghi
	53	.196 cdefghij
	52	.189 cdefghijk
	38	.134 ghijklm
	2	.130 hijklm
	5	.100 jklmn
	9	.085 jklmn
	50	.084 jklmn
	35	.076 klmn
	42	.056 mn
P. freudenreichii		
	99	.335 ab
	49	.269 bcd
	104	.248 bcdef
	48	.230 bcdefghi
	31	.135 fghijklm
	89	.111 jklmn
	96	.102 jklmn
	101	.003 n
P. jensenii		
	88	.385 a
	106	.244 bcdefg
	41	.241 bcdefgh
	86	.238 bcdefgh
	44	.227 bcdefghi
	74	.163 defghijklm
	46	.159 defghijklm
	54	.154 efghijklm
	69	.138 fghijklm
	63	.123 ijklm
	68	.122 ijklm
P. thoenii		
	105	.253 bcde
	26	.188 cdefghijkl
	10	.166 defghijklm
	85	.162 defghijklm
	79	.135 fghijklm
	21	.107 jklmn
	4	.082 jklmn
	20	.074 lmn
	15	.070 mn

*Values with the same letter are not significantly different

Table 5. Optical density of propionibacteria strains grown in 80 mM broth at pH 5.0 for 48 hours

Species	strain	48 h optical density (600 nm)*
P. acidipropionici		
	111	1.195 ef
	90	1.133 ef
	78	1.033 efg
	81	1.033 efg
	52	.936 fgh
	53	.579 hijklm
	2	.567 hijklm
	3	.479 ijklmn
	38	.479 ijklmn
	9	.405 ijklmno
	50	.264 jklmno
	5	.196 lmno
	35	.133 mno
	42	.130 no
P. freudenreichii		
	99	2.439 a
	104	1.883 b
	48	1.605 bc
	49	1.586 bcd
	31	.978 efgh
	89	.581 hijklm
	96	.220 klmno
	101	-.003 o
P. jensenii		
	88	1.368 cde
	44	1.215 def
	41	.830 fghi
	106	.703 ghij
	46	.690 ghij
	86	.669 ghij
	74	.628 ghijkl
	54	.625 ghijkl
	63	.440 ijklmn
	68	.320 jklmno
	69	.319 jklmno
P. thoenii		
	105	.665 ghij
	10	.655 ghijk
	26	.629 ghijkl
	21	.457 ijklmn
	79	.432 ijklmn
	85	.424 ijklmno
	20	.307 jklmno
	4	.192 lmno
	15	.152 mno

*Values with the same letter are not significantly different

Figure 2 . Mean optical density of each species of Propionibacterium at pH 5.0

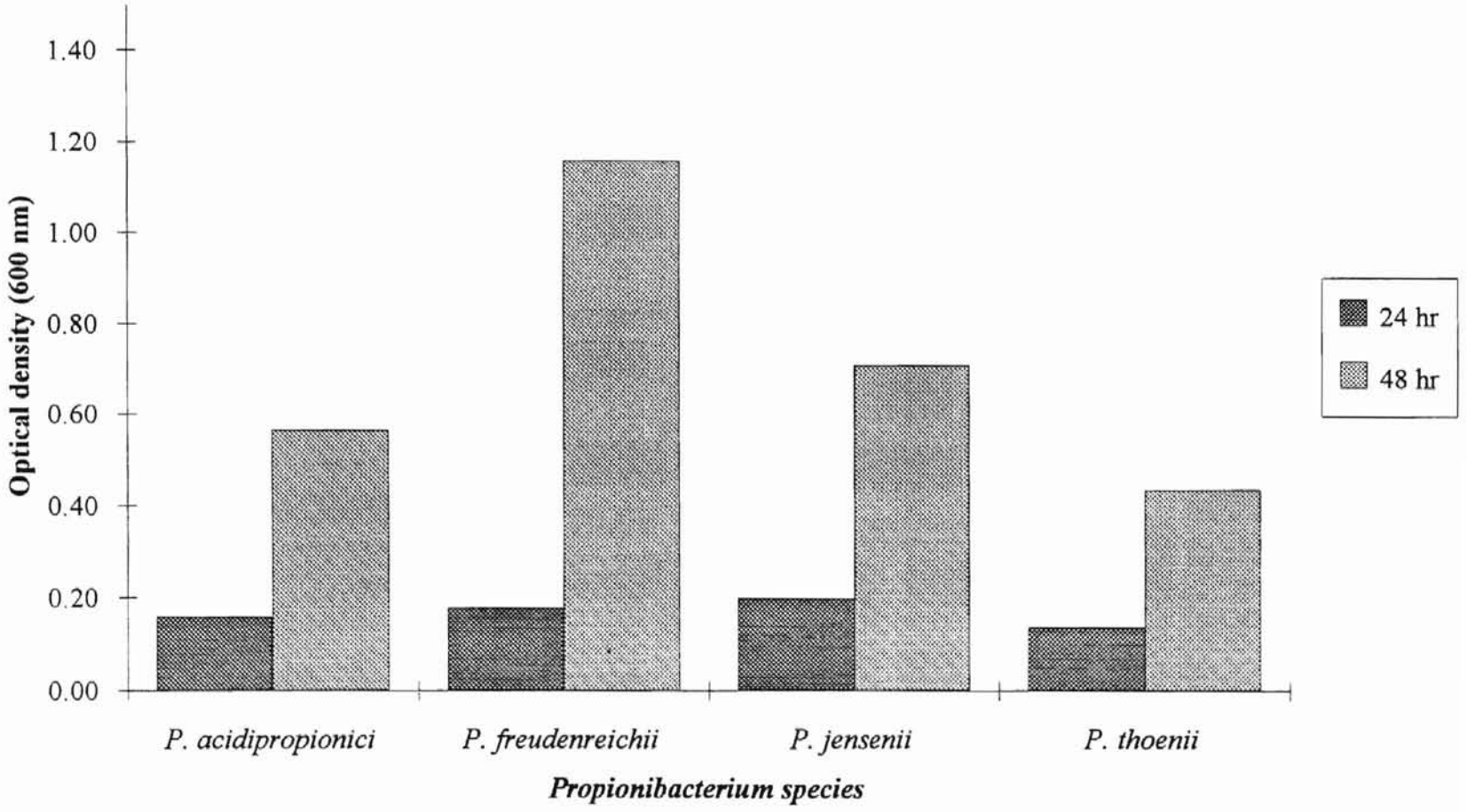


Table 6. Lactic acid utilization of propionibacteria strains grown in 80 mM broth at pH 7.0 for 24 hours.

Species	strain	24 h lactate utilization (mM)*
P. acidipropionici		
	90	71.82 a
	35	33.52 bcd
	81	30.86 bcd
	3	29.86 bcd
	50	24.75 cd
	78	21.53 cd
	108	21.42 cd
	52	16.43 cd
	53	8.33 cd
	5	6.66 d
P. freudenreichii		
	104	70.49 a
	49	61.49 ab
	99	44.51 abc
	101	38.30 abcd
	96	17.65 cd
	31	13.32 cd
P. jensenii		
	54	34.85 bcd
	74	27.08 bcd
	63	26.53 bcd
	88	11.88 cd
	68	10.77 cd
	44	6.99 d
	69	5.33 d
P. thoenii		
	85	41.07 abcd
	79	39.07 abcd
	105	19.31 cd
	26	7.99 cd

*values with same letter are not significantly different

Table 7. Lactic acid utilization of propionibacteria strains grown in 80 mM broth at pH 7.0 for 48 hours

Species	strain	48 h lactate utilization (mM)*
P. acidipropionici		
	90	77.15 a
	3	77.15 a
	81	67.27 a
	50	62.38 a
	78	62.38 a
	53	58.94 a
	35	57.39 a
	52	57.39 a
	108	57.39 a
	5	56.72 a
P. freudenreichii		
	104	77.15 a
	49	77.15 a
	101	67.27 a
	99	67.27 a
	96	64.05 a
	31	60.72 a
P. jensenii		
	54	77.15 a
	74	77.15 a
	63	62.38 a
	68	56.61 a
	69	54.72 a
	44	49.17 a
	88	47.51 a
P. thoenii		
	79	77.15 a
	85	77.15 a
	105	62.38 a
	26	62.38 a

*values with same letter are not significantly different

Figure 3. Mean lactic acid utilization by each species of Propionibacterium at pH 7.0

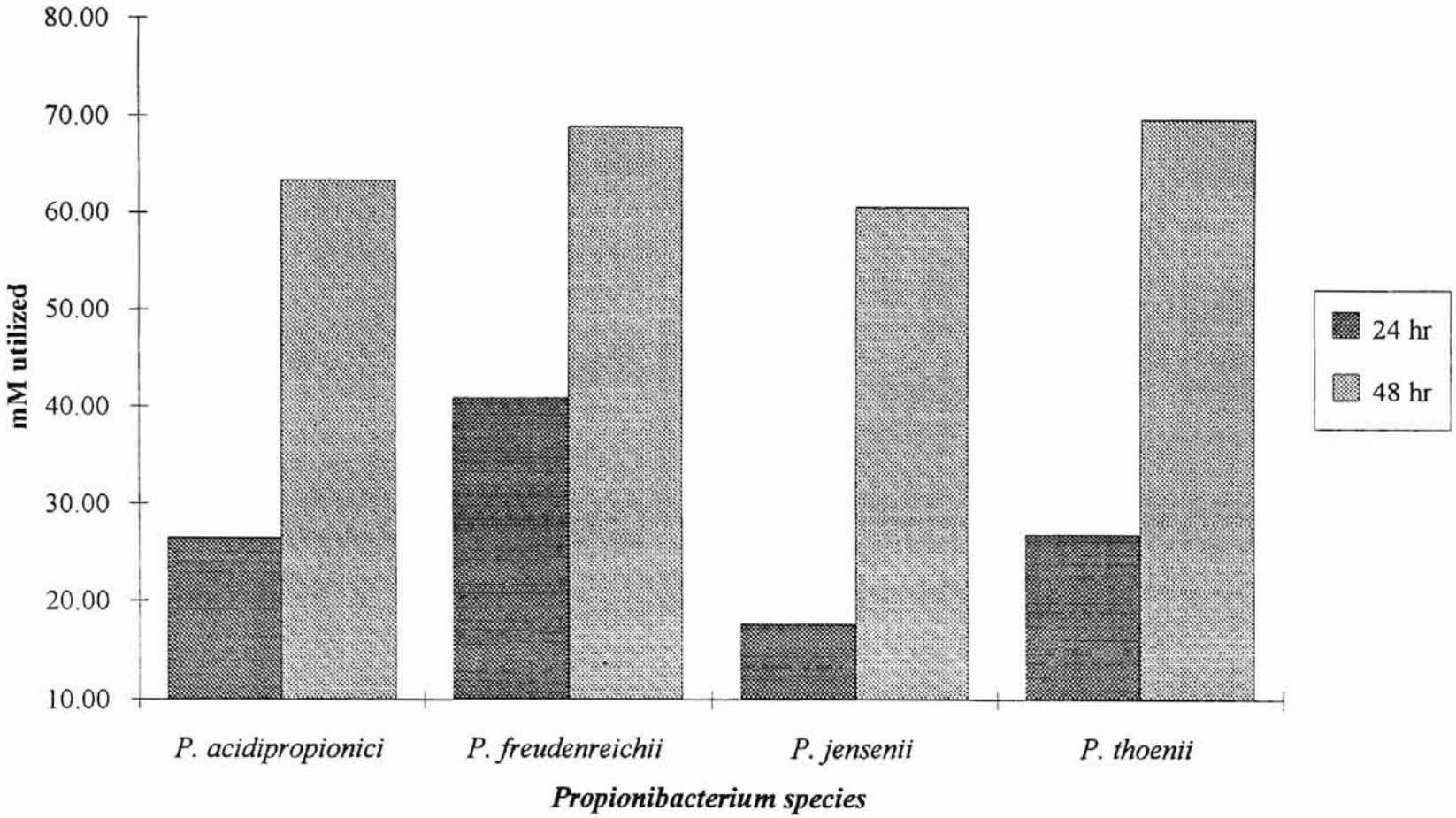


Table 8 . Optical density of propionibacteria strains grown in 80 mM broth at pH 7.0 for 24 hours

Species	strain	24 h optical density (600 nm)*
P. acidipropionici		
	90	1.53 ab
	81	1.37 bcd
	50	1.11 cdefg
	52	1.10 cdefg
	78	1.05 cdefghi
	3	1.01 defghij
	35	0.91 efghijk
	5	0.78 fghijkl
	108	0.71 ghijkl
	53	0.55 kl
P. freudenreichii		
	104	1.88 a
	99	1.47 bc
	101	1.23 bcde
	49	1.18 bcdef
	31	0.63 jkl
	96	0.60 jkl
P. jensenii		
	88	1.05 cdefghi
	68	0.80 fghijkl
	63	0.79 fghijkl
	54	0.77 fghijkl
	74	0.65 hijkl
	69	0.46 l
	44	0.42 l
P. thoenii		
	79	1.35 bcd
	105	1.14 bcdefg
	85	1.07 cdefgh
	26	0.64 ijkl

*values with same letter are not significantly different

Table 9. Optical density of propionibacteria strains grown in 80 mM broth at pH 7.0 for 48 hours.

Species	strain	48 h optical density (600 nm)*
P. acidipropionici		
	5	2.835 abc
	52	2.750 abcde
	53	2.640 abcdef
	108	2.450 cdefg
	90	2.425 cdefg
	35	2.300 fgh
	81	2.297 fgh
	3	2.135 gh
	50	1.903 h
	78	1.897 h
P. freudenreichii		
	96	2.810 abc
	31	2.750 abcde
	99	2.643 abcdef
	49	2.355 defg
	101	2.190 gh
	104	2.175 gh
P. jensenii		
	69	2.938 ab
	68	2.775 abcd
	74	2.700 abcedf
	44	2.697 abcedf
	54	2.525 bcdefg
	63	2.300 fgh
	88	2.290 fgh
P. thoenii		
	105	3.005 a
	26	2.915 ab
	85	2.345 efg
	79	2.135 gh

*values with same letter are not significantly different

Figure 4. Mean optical density of each species of Propionibacterium at pH 7.0

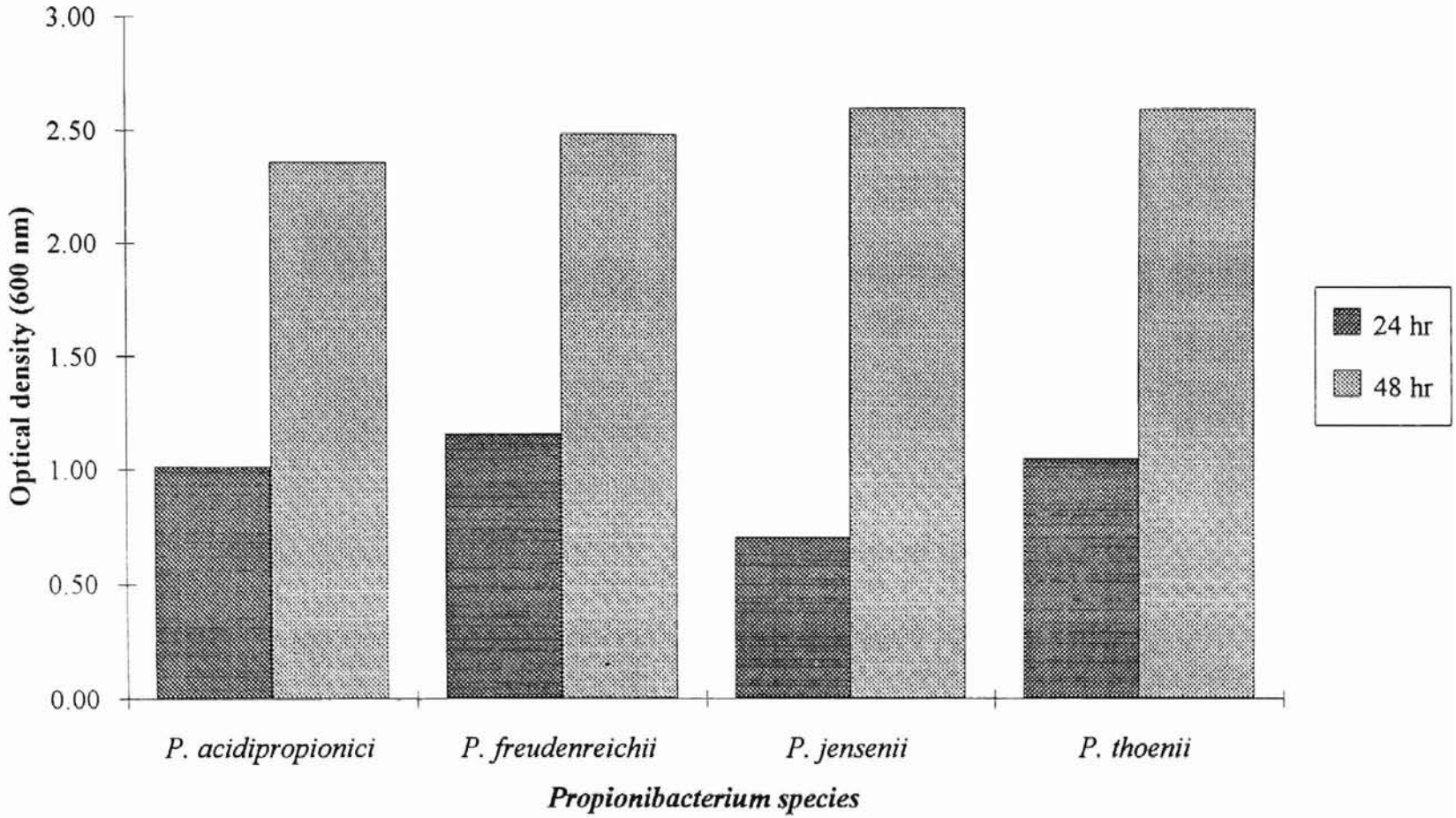


Table 10. Impact of added *Propionibacterium* strains on rates of change in pH and lactate concentration of incubated rumen fluid models.

Strain	pH elevation, (Units/h)		Lactate decrease (mM/h)	
42	.03770	a	1.61	a
63	.03627	a	1.30	abc
54	.02433	ab	1.26	abc
25	.02380	ab	1.12	abc
41	.02372	abc	1.55	ab
111	.01691	bcd	1.05	abc
81	.01064	bcd	.71	abcdef
104	.00923	bcde	.88	abcd
89	.00785	bcde	.53	bcdef
88	.00590	bcde	.76	abcde
49	.00425	cde	.65	abcdef
48	.00366	de	NA	
99	.00051	de	-.17	def
31	.00026	de	-.22	ef
90	-.00917	e	-.32	f

Calculated by regressing the difference between inoculated and control fluid against incubation time.
Means in a column with the same superscript are not different (P<.05).

Table 11. Contrasts of maximum lactate accumulation and minimum pH of rumen models inoculated with various propionibacteria strains.

Strain	Lactate production rate (mM/h)	H+ increase rate (x 10^{-5})	Time lag of lactate production (h)	Time lag of H+ increase(h)
P25	18.87	4.65	4.41+	4.20
P31	38.85	11.63	5.15	3.81
P41	23.31	11.15	4.65	3.29
P42	24.42	7.46	5.52	3.99
P48	38.85	1.45	5.45	3.27
P49	6.67	6.45	5.89	3.28
P54	21.09	-1.45**	8.08**	3.56
P63	9.99	2.18*	6.47+	2.68
P78	12.21	9.34	5.30	3.02
P81	1.11	9.86	4.91	2.99
P88	14.43	11.49	5.76	2.87
P89	9.99	13.57	5.71	3.13
P90	14.43	5.18	5.00	3.28
P99	4.44	7.87	4.94	3.59
P104	-2.22	8.02	4.94	2.67
P111	14.43	5.17	4.97	5.74*
Control	38.85	17.99	5.45	4.72

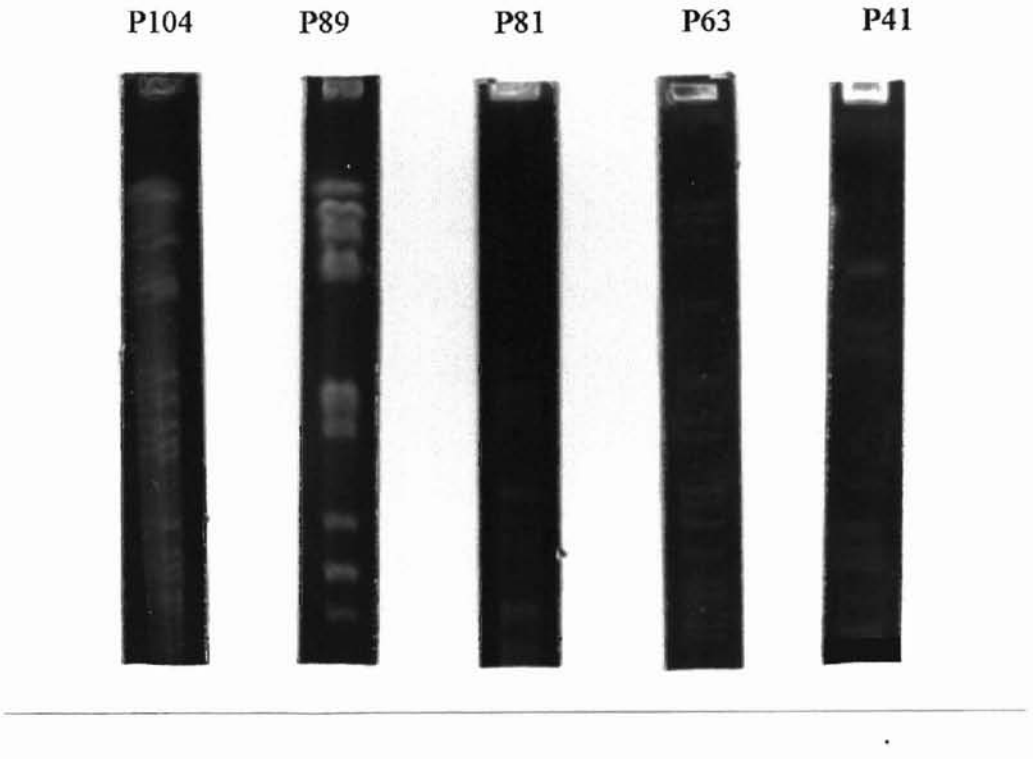
* Values significantly different when compared to controls (P<.05)
\+ Values significantly different when compared to controls (P<.01)
** Values significantly different when compared to controls (P<.001)

Table 12. Survival of *Propionibacterium* strains in the rumen model after 16 and 48 hours of incubation.*

Strain	*Propionibacterium* (cfu/ml)	
	16 h	48 h
63	7.4×10^6	1.0×10^6
25	2.5×10^5	6.0×10^5
81	5.0×10^6	3.0×10^5
90	1.0×10^4	1.0×10^5
88	8.3×10^6	1.0×10^5
54	1.0×10^5	1.0×10^5
111	2.0×10^6	1.0×10^4
99	1.0×10^4	1.0×10^4
41	4.7×10^6	1.0×10^4
104	5.0×10^6	1.0×10^3
89	1.0×10^3	1.0×10^3
48	1.0×10^5	1.0×10^3
42	1.1×10^6	1.0×10^3
31	1.0×10^3	1.0×10^3

*Propionibacteria count at 0 hour was 1×10^7 cfu/ml

Figure 5. Pulsed-field gel electrophoresis analysis of genomic DNA isolated from propionibacteria strains.



Xba I digestion

DISCUSSION

Slyter and Rumsey (1991) reported L-lactate levels increase to concentrations as high as 90 mM in the rumen of beef cattle 24 h after the diet was changed from 90% forage to 100% concentrate. In this study, experiments were performed to test the ability of propionibacteria strains to grow and utilize lactic acid when grown in conditions similar to those found in the acidotic rumen.

The differences observed in growth and lactic acid utilization at pH 5.0 indicated some species were better able to function at the lower pH. *P. freudenreichii* strains were clearly the better species for growth and lactic acid utilization under low pH conditions. Values for growth and lactic acid utilization at pH 7.0 were consistent with those reported in literature. Crow (1986) examined the substrate preference of *P. freudenreichii* strains grown in a complex media supplemented with 176 mM of DL-lactic acid at pH 6.5 and found that L-(+) lactic acid was reduced by 73 mM (83%) at 24 h.

Maximum values for growth and lactic acid utilization were generally much lower at pH 5.0 than at pH 7.0. However, many strains had reduced growth and lactic acid utilization when the pH was reduced to 5.0. Strains P49, P99 and P104 had none or only slight decreases in lactic acid utilization and growth under acid conditions, while strains P3, P5, P68, P69, and P101 represent a group of propionibacteria that were severely affected by the low pH conditions.

The inability of certain strains within a species group to tolerate pH and ruminal fluid typical of an acidotic rumen confirms the importance of studying and selecting organisms under environmental conditions in which they will be used. While *P. freudenreichii* strains were determined to grow well and utilize more lactic acid at pH 5.0 in broth experiments, strains failed to function in the competitive environment of rumen simulation models. A lack of strain survival may account for the inadequate performance of strains of this species in rumen simulation models. That is why it was important to

screen the propionibacteria strains selected from pure culture experiments in rumen simulation models. The rumen simulation models tested the strains ability to utilize lactic acid produced by native ruminal microorganisms, compete for nutrients in a complex microbial system and survive increased osmotic pressure and microbial predation.

Many strains could not be isolated from the rumen simulation models following 48 h of incubation. One assumption is that strains not detected were not able to survive in the rumen based media, however other possibilities do exists. Those strains not recovered may have been impossible to enumerate from the highly diverse community of ruminal organisms using the selective-differential medium employed. In addition, rumen models were maintained as batch culture systems with no outflow of endproducts. Thus endproduct accumulation which may injure cells, rendering them non-culturable on the selective-differential media. Transferring an amount of contents after 24 h of incubation to fresh rumen fluid media may help reduce this problem as suggested by Theodorou, *et al.*, (1987).

The ruminal fluid model used for this experiment was much less effective as a screening tool for selecting lactic acid utilizing strains after 24 h of incubation. In control flasks even when no propionibacteria strains were added, concentrations of lactic acid concentrations decreased and pH increased after 24 h of incubation. As a result, only the first 24 h of the incubation was considered in statistical analysis. Kung and Hession (1995) observed similar reductions following 24 h and attributed this to the accumulation of metabolic endproducts.

A non-linear curve fitting technique was employed to detected differences in pH and lactic acid lag periods between treated and control flasks. Gompertz equation was successful in predicting a non-linear curve for 24 h incubation data. Observed values were located on the predicted curve more than 95% of the time. Gompertz analysis revealed that strains P54 and P63 significantly increased the lag time of lactic acid accumulation and suppressed rate of H+ accumulation.

The use of pulsed-field gel electrophoresis proved to be a successful method for identifying propionibacteria strains isolated from rumen simulation models. This method will be useful in differentiating between inoculated and indigenous strains of propionibacteria thus allowing us to monitor the inoculated strains in the rumen in future *in vivo* experiments.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

Ruminal acidosis following consumption of excessive amounts of grain has been well described in the literature. The decrease in ruminal pH has been attributed to proliferation of starch fermenting microorganisms such as *Streptococcus bovis* and *Lactobacillus spp*, which outgrow other microorganisms when free glucose is present. Increased concentrations of total acid, usually lactic acid and volatile fatty acids, generally is considered to be responsible for acidosis.

Common management practice to stabilize ruminal fermentation and prevent acidosis consist of principle approaches, i). the use of extended feed adaptation periods and ii). the addition of ionophores. However, current consumer concerns of anitbiotic residues in meat products has increased the demand for alternative methods of controling lactic acidosis. The idea of a natural solution to the problem has been developed by feeding highly concentrated populations of viable lactic acid utilizing strains of bacteria normally found in the rumen , such as *Megasphera elsdenii,* to animals to control ruminal acidosis.

The goal of this study was to select strains of propionibacteria for the ability to utilize lactic acid under conditions similar to those found in the rumen. Models were used to examine growth and lactate use of cultures in both acidic (pH 5.0) and neutral (pH 7.0) media and under mixed culture conditions simulating ruminal acidosis. Strains were selected for their ability to utilize lactic acid and maintain active populations in each experimental model.

In pure culture experiments at pH 5.0 and 7.0, large differences between and within species of propionibacteria were detected. Lactic acid utilization at pH 5.0 ranged from 78.59 to 1.10 mM by 48 h. *P. freudenreichii* were determined to utilize

significantly more lactic acid with compared with other stains. In contrast, all strains metabolized lactic acid pH 7.0. No differences in optical density was detected at pH 7.0, but

P. freudenreichii strains had higher optical density reading at pH 5.0.

Ruminal acidosis simulation experiments determined the ability of selected strains of propionibacteria to modulate pH decreases and inhibit the accumulation of lactic acid produced by native ruminal microorganisms presented with a load of glucose. *P. jensenii* strains P54 and P63 were determined to increase pH and suppress the accumulation of lactic acid in a rumen simulation model.

Genetic analysis using pulsed-field gel electrophoresis of most propionibacteria strains resulted in genomic DNA differences for most strains. This method will be useful in differentiating between inoculated and indigenous strains of propionibacteria thus allowing us to monitor the inoculated strains in the rumen in future *in vivo* experiments. While most strains were determined to be genotypically unique, two strain were found to be similar. Strains P54 and P63 had similar DNA fragments following restriction endonuclease digestion.

In conclusion, the selection procedures used in this study determined *P. jensenii* strain P63 would have the best chance of utilizing lactic acid in the rumen of beef cattle consuming large amounts of grain.

APPENDIX A

FIGURES OF STRAIN GROUPS FOR LACTIC ACID UTILIZATION AND GROWTH FOR PH 5.0 AND PH 7.0 *IN VITRO MODELS*

Figure 6. Optical density of *P. acidipropionici* strains grown in lactate at pH 5.0.

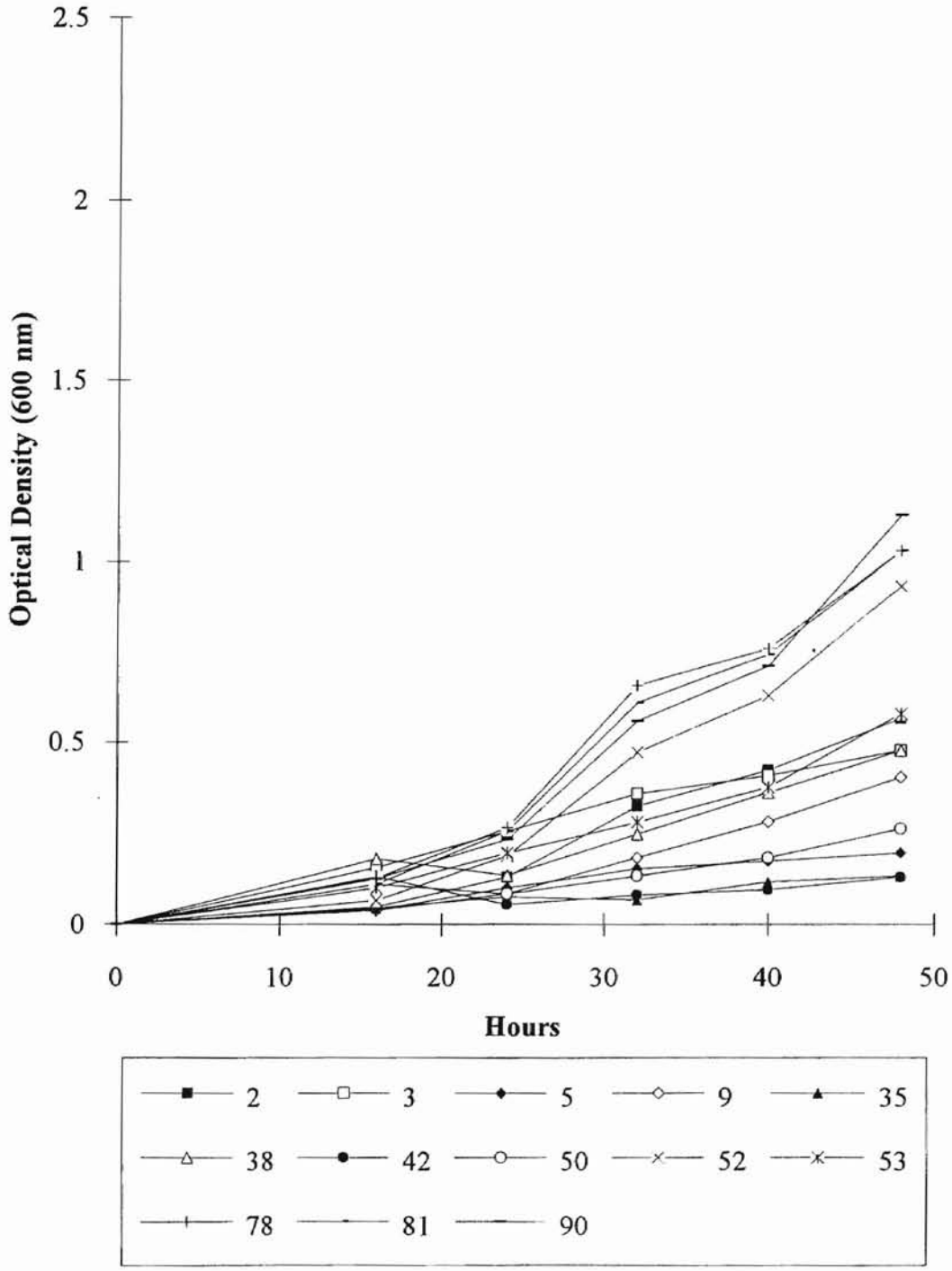


Figure 7. Optical density of *P. freudenreichii* strains grown in lactate at pH 5.0.

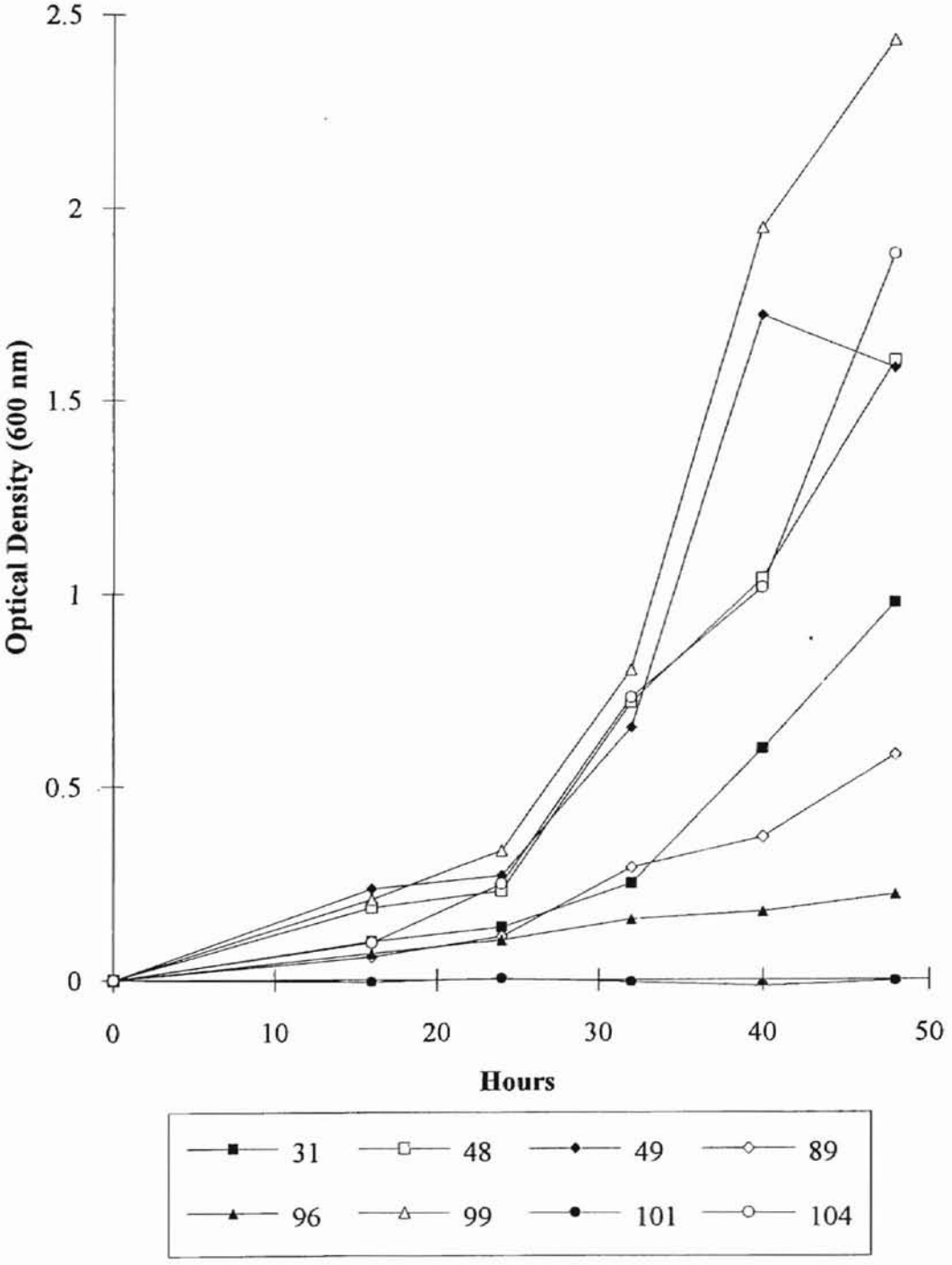


Figure 8. Optical density of *P. jensenii* strains grown in lactate at pH 5.0.

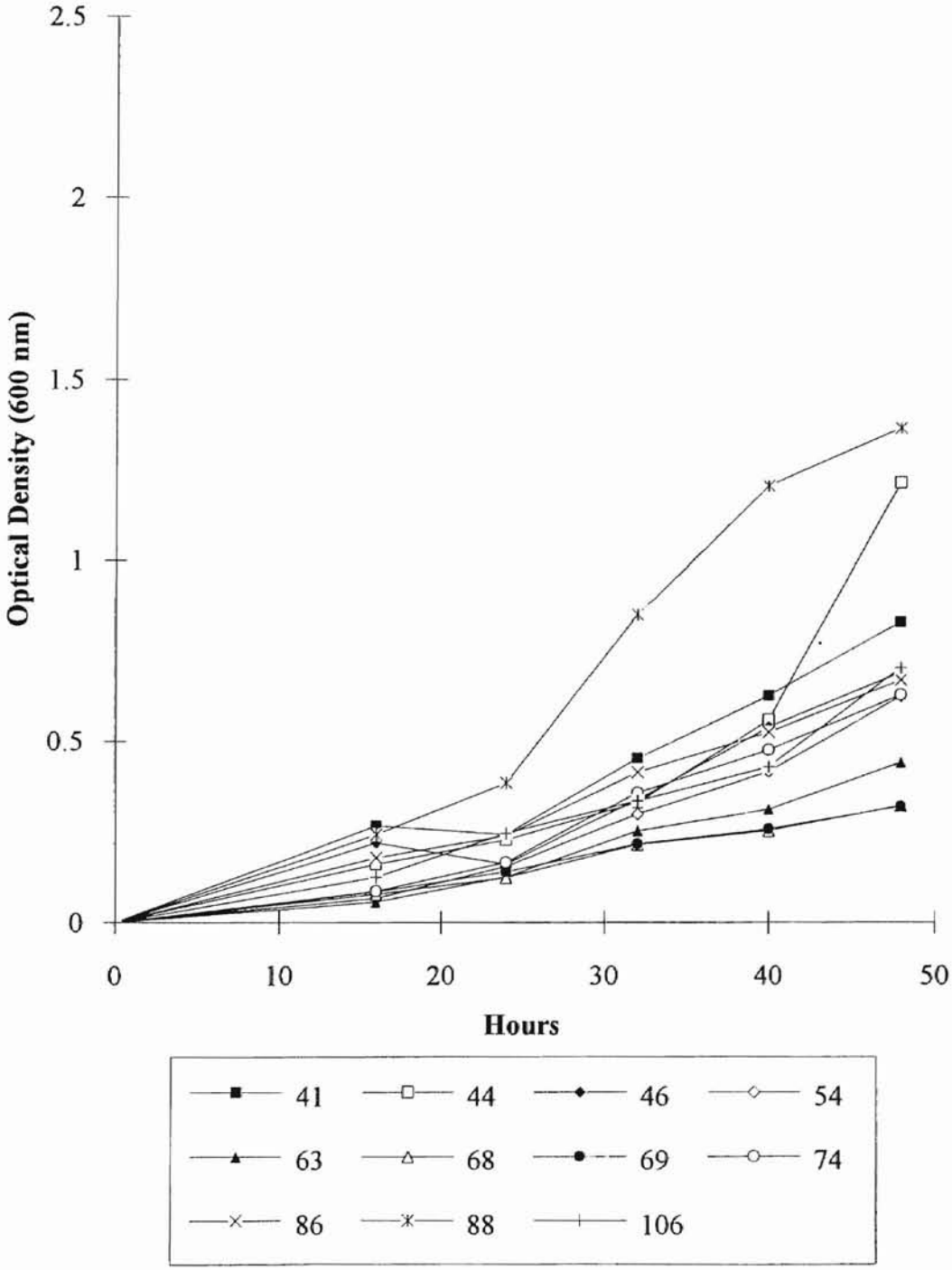


Figure 9. Optical density of *P. thoenii* strains grown in lactate at pH 5.0.

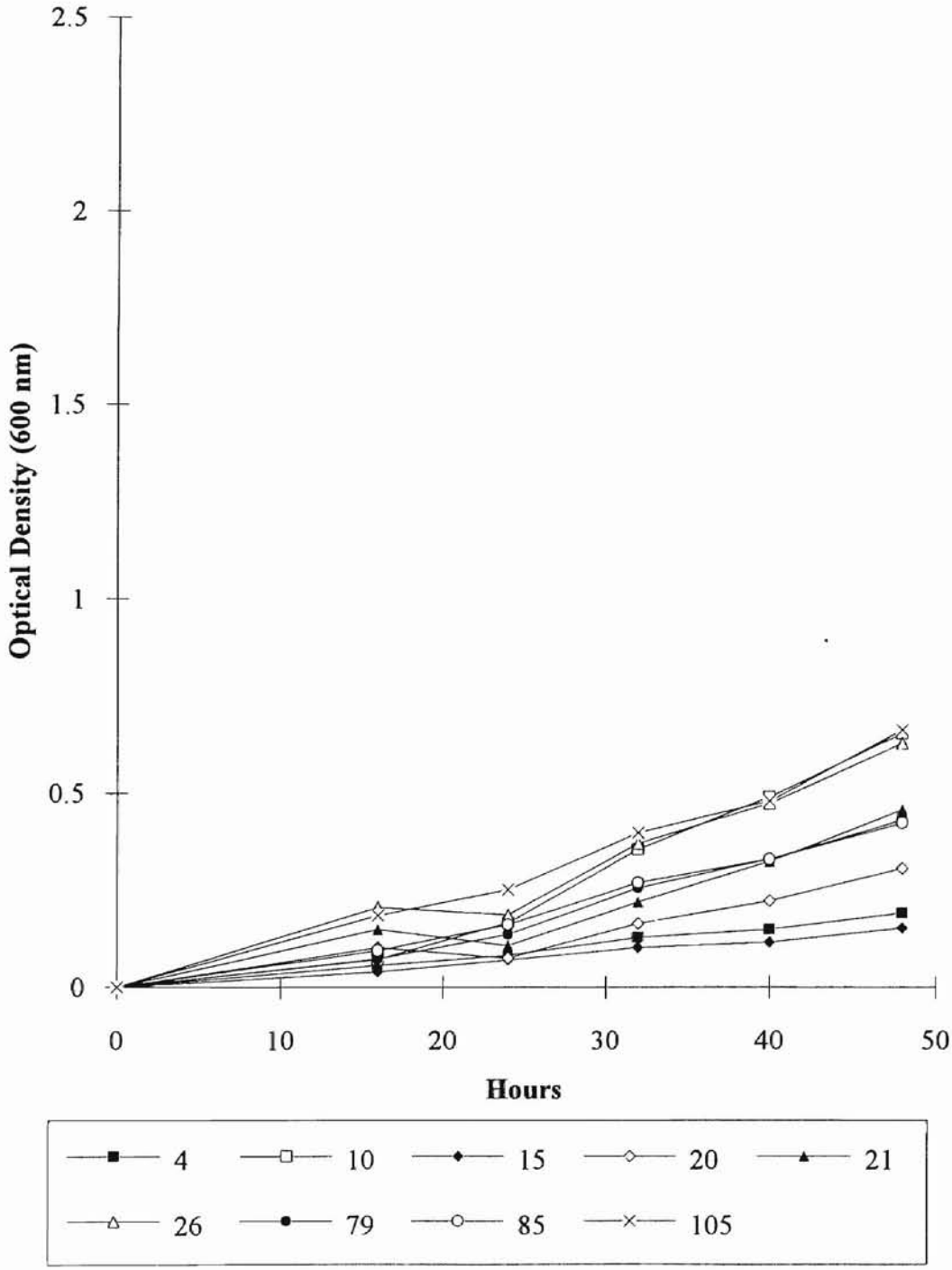


Figure 10. Optical density of *P. acidipropionici* strains grown in lactate at pH 7.0.

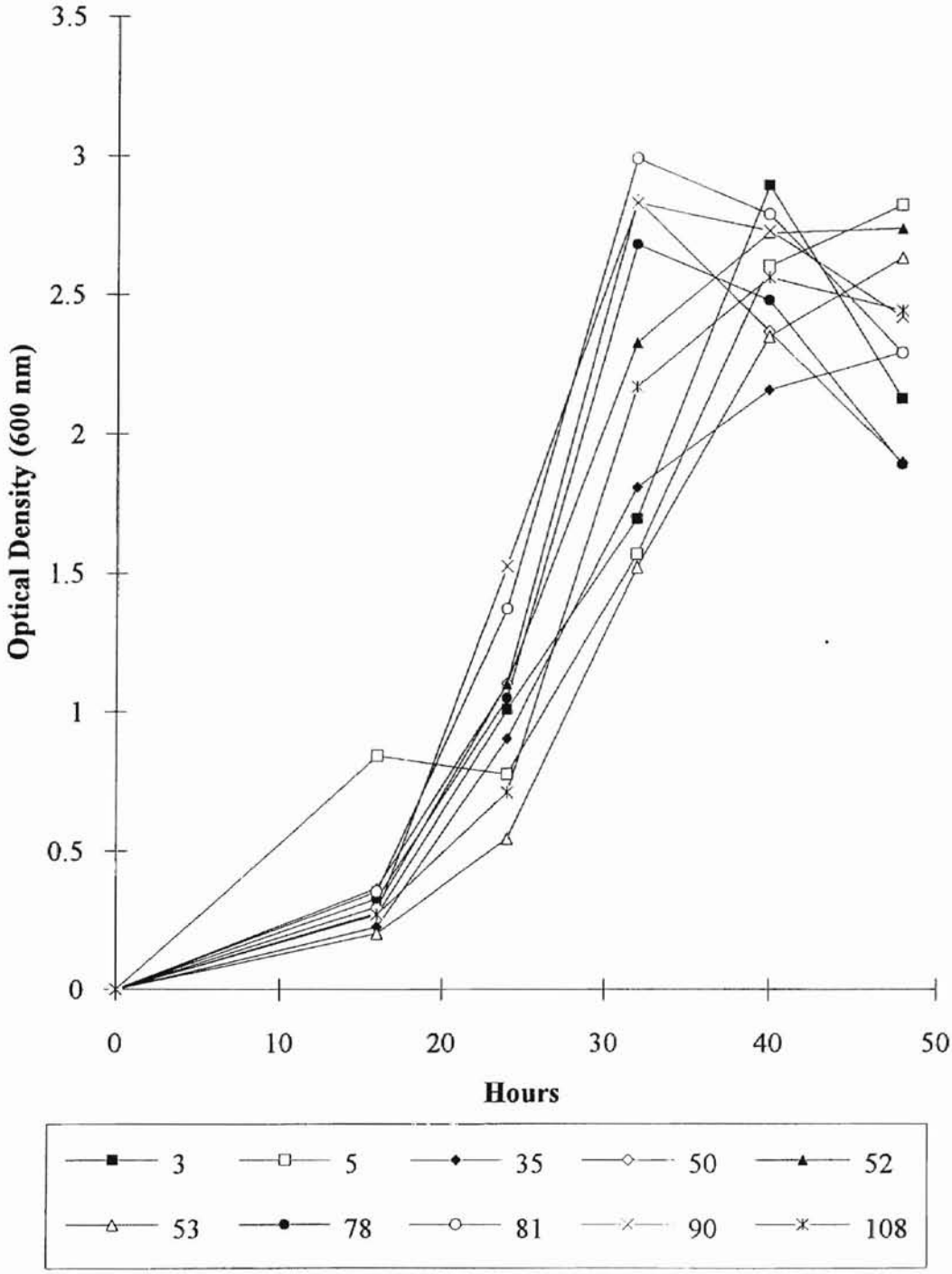


Figure 11. Optical density of *P. freudenreichii* strains grown in lactate at pH 7.0.

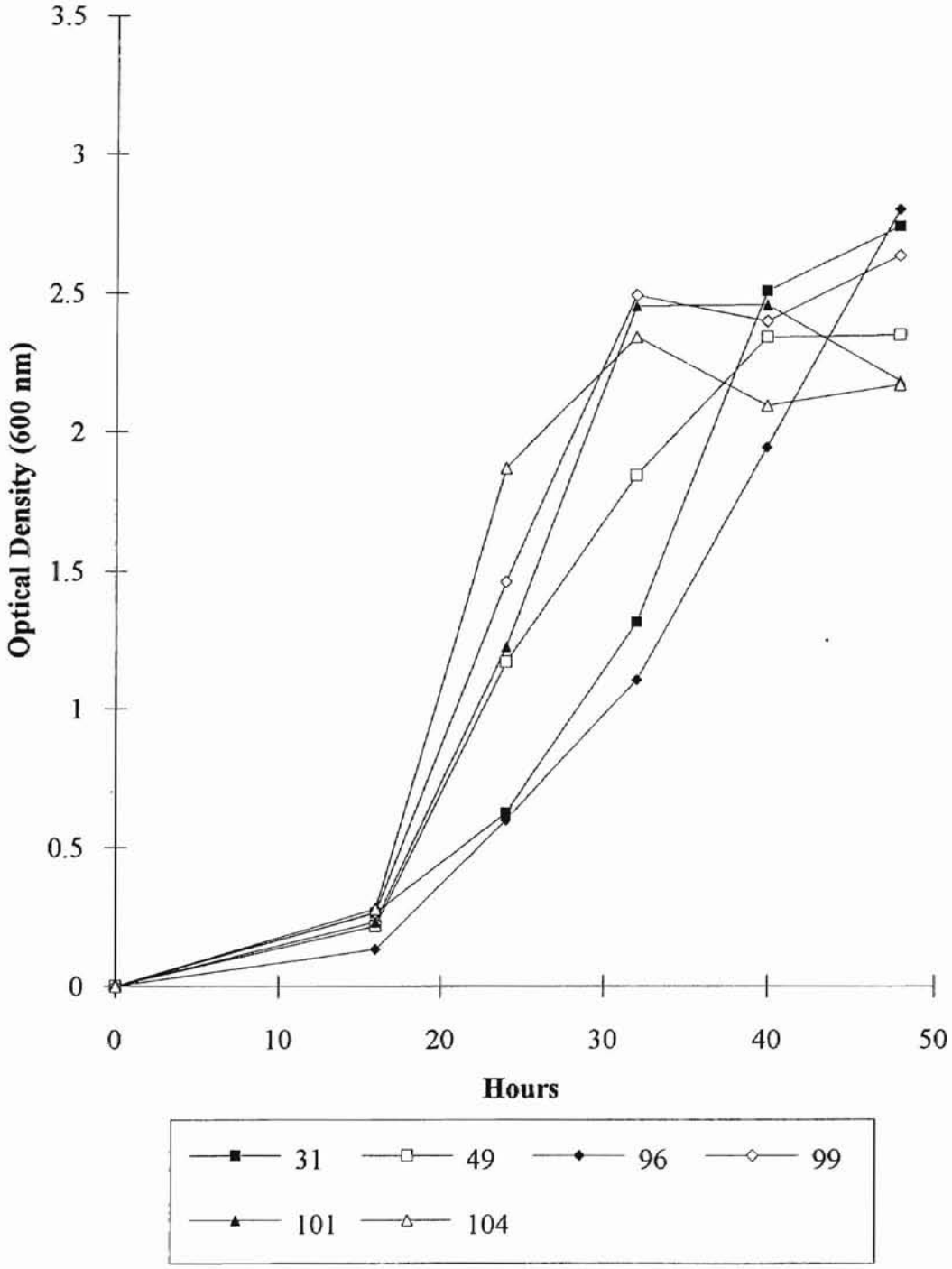


Figure 12. Optical density of *P. jensenii* strains grown in lactate at pH 7.0.

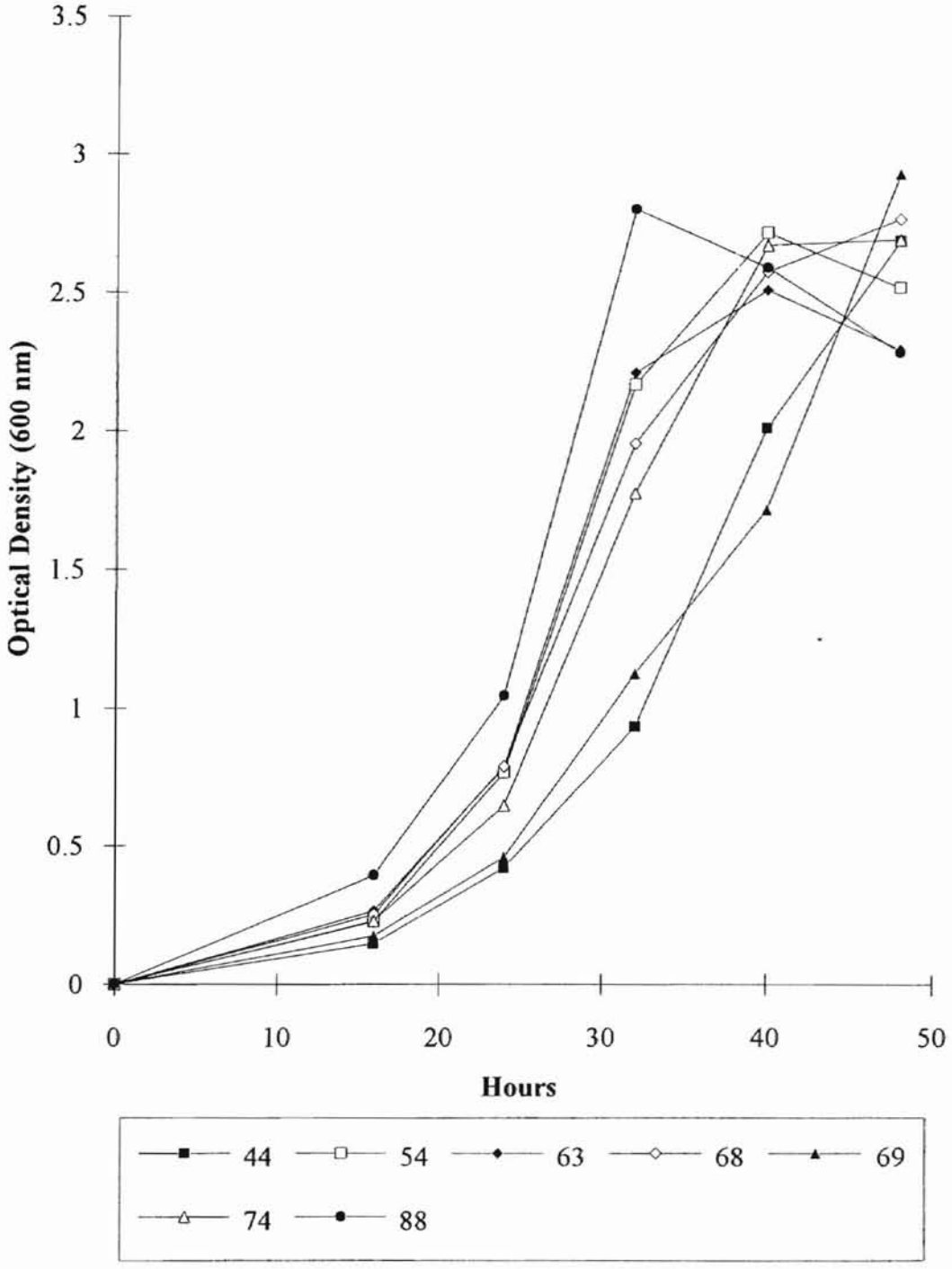


Figure 13. Optical density of *P. thoenii* strains grown in lactate at pH 7.0.

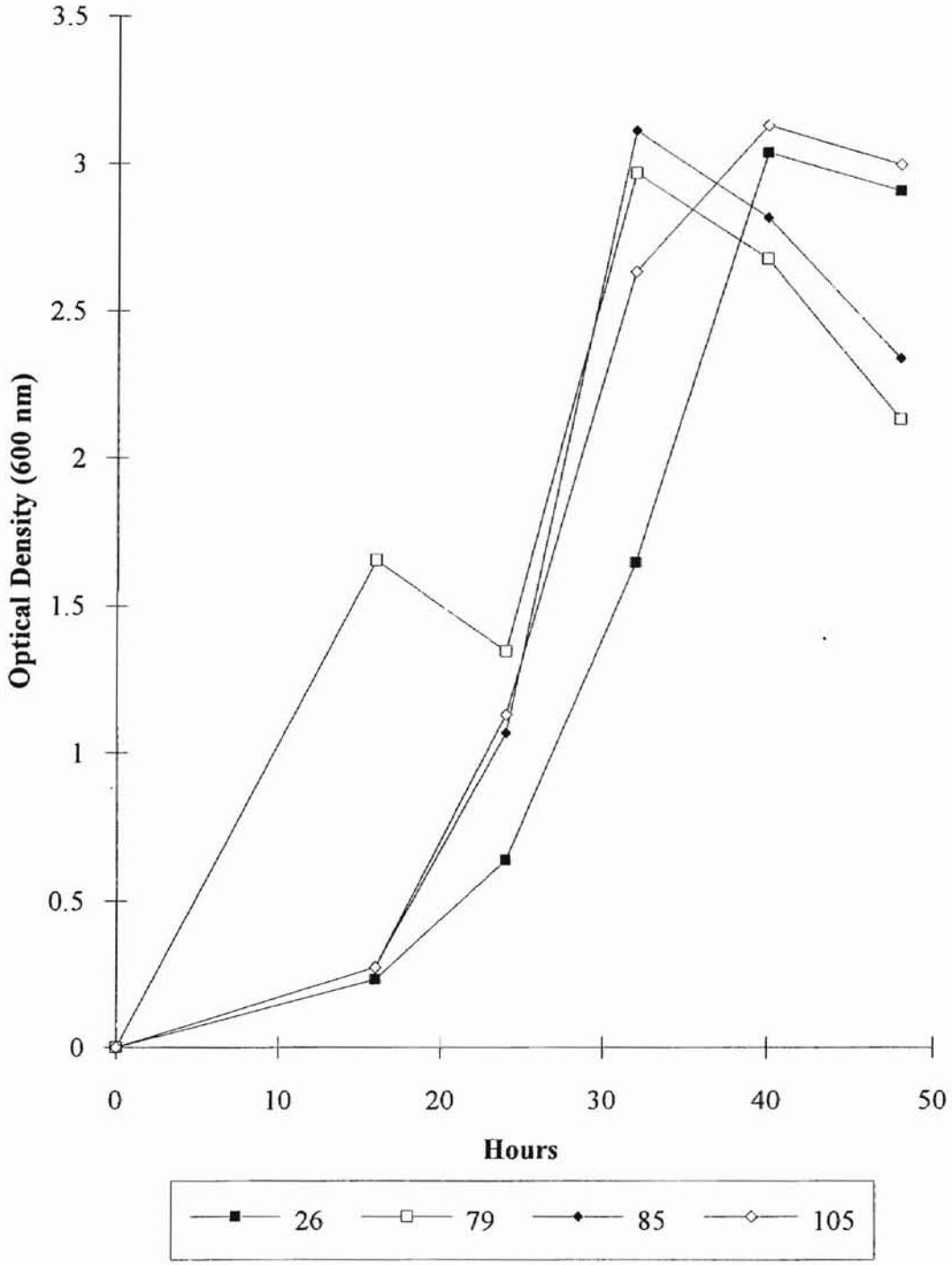


Figure 14. Lactic acid utilization of *P. acidipropionici* strains grown in lactate at pH 5.0.

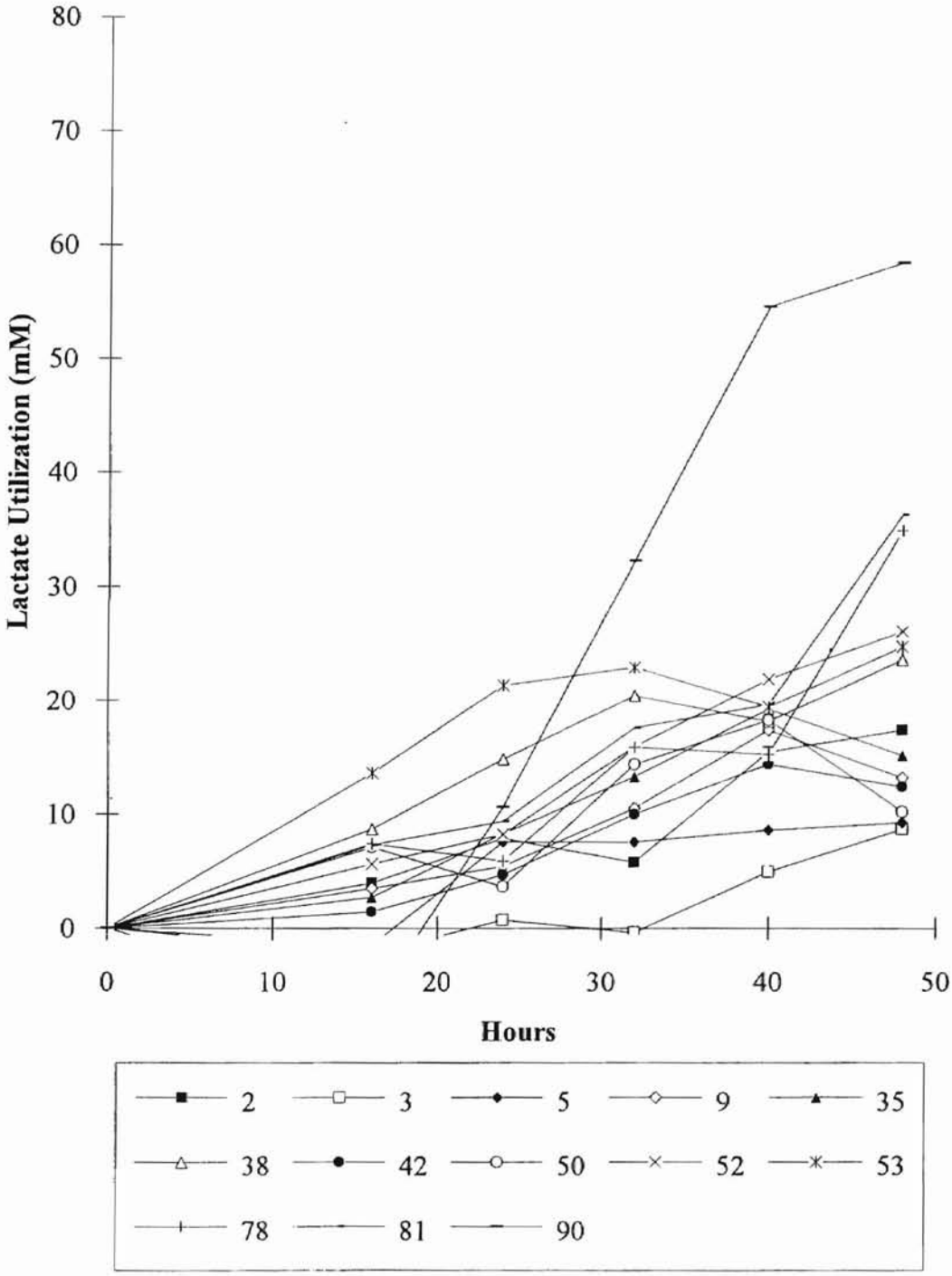


Figure 15. Lactic acid utilization of *P. freudenreichii* strains grown in lactate at pH 5.0.

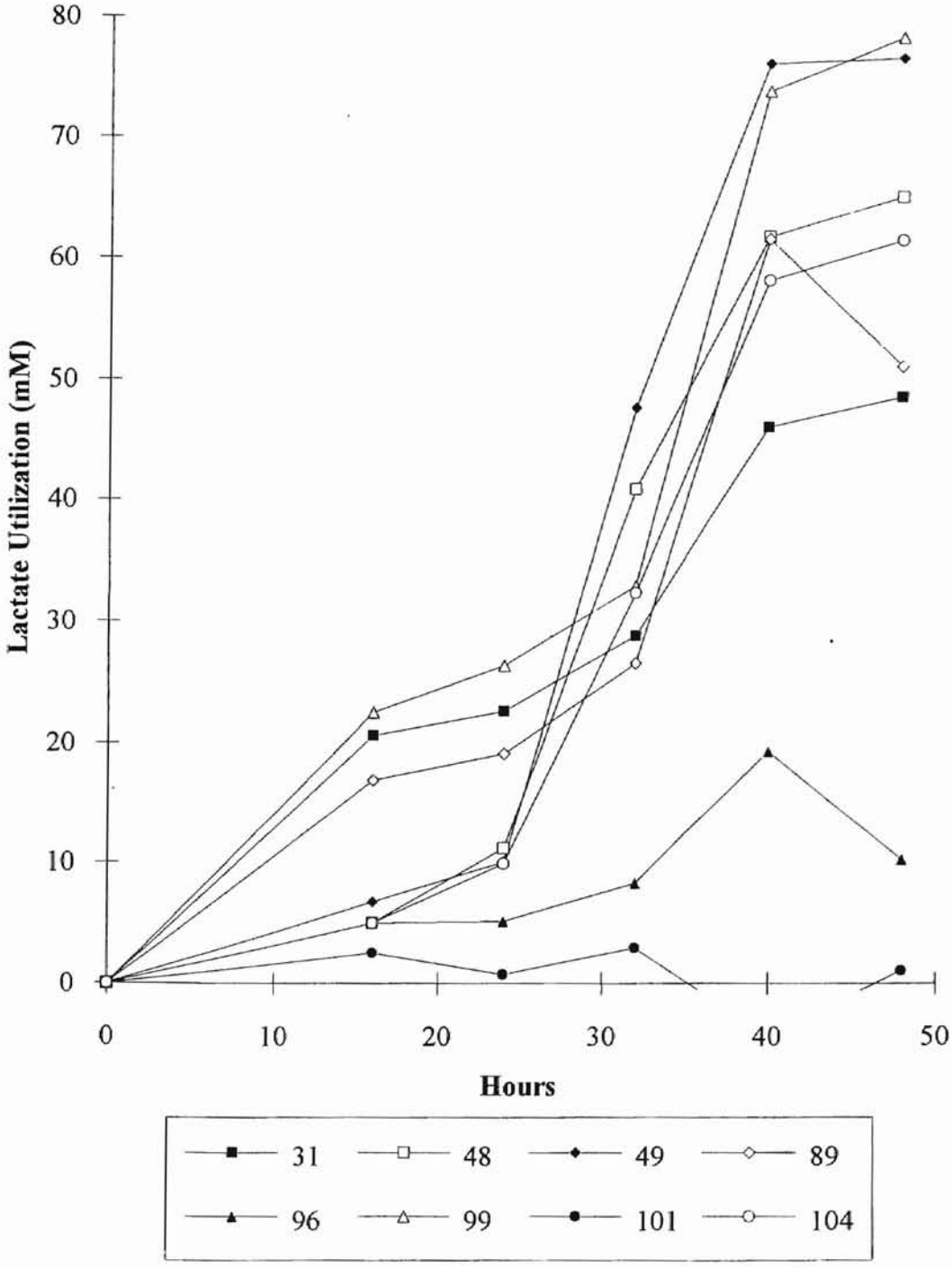


Figure 16. Lactic acid utilization of *P. jensenii* strains grown in lactate at pH 5.0.

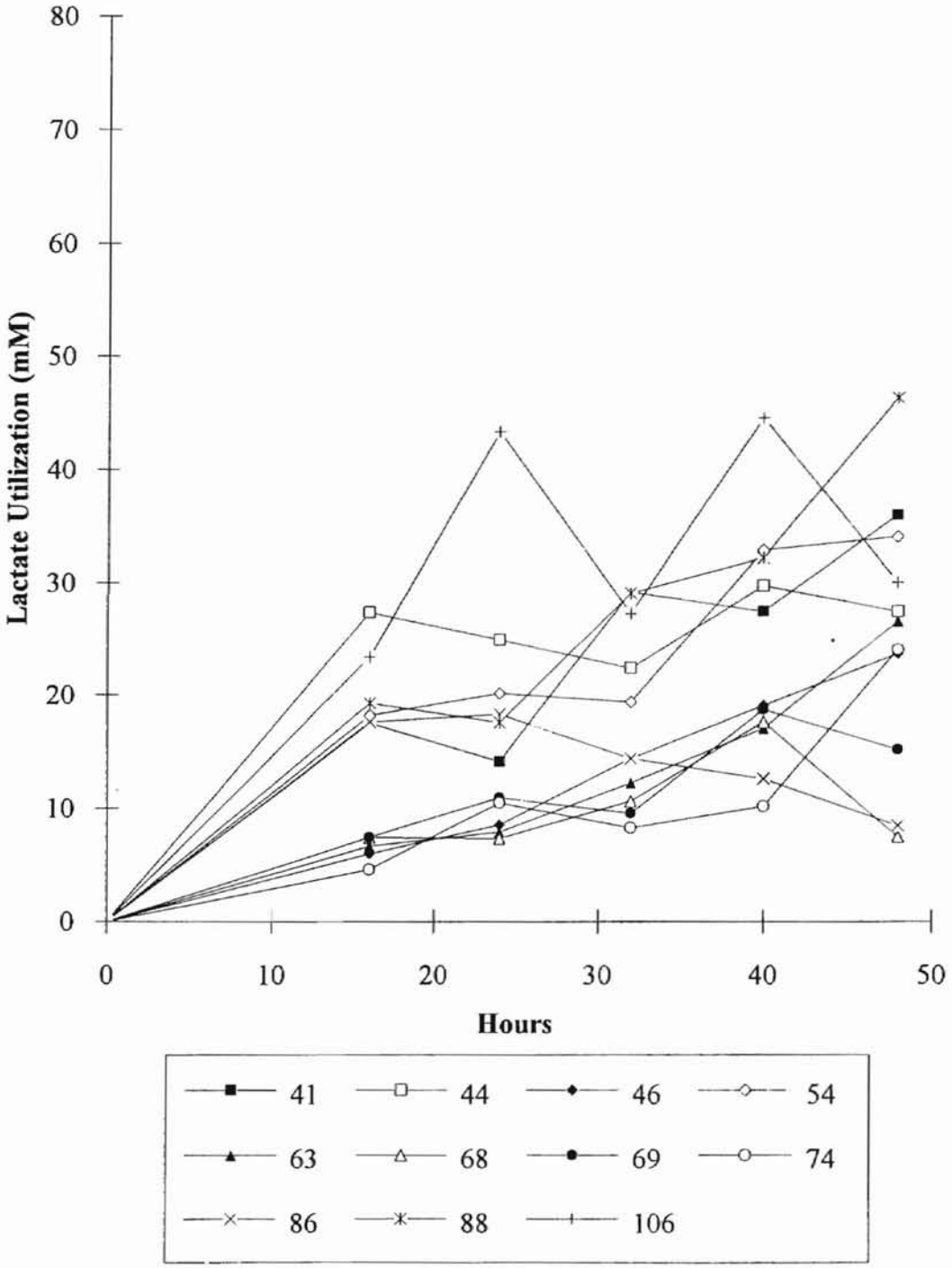


Figure 17. Lactic acid utilization of *P. thoenii* strains grown in lactate at pH 5.0.

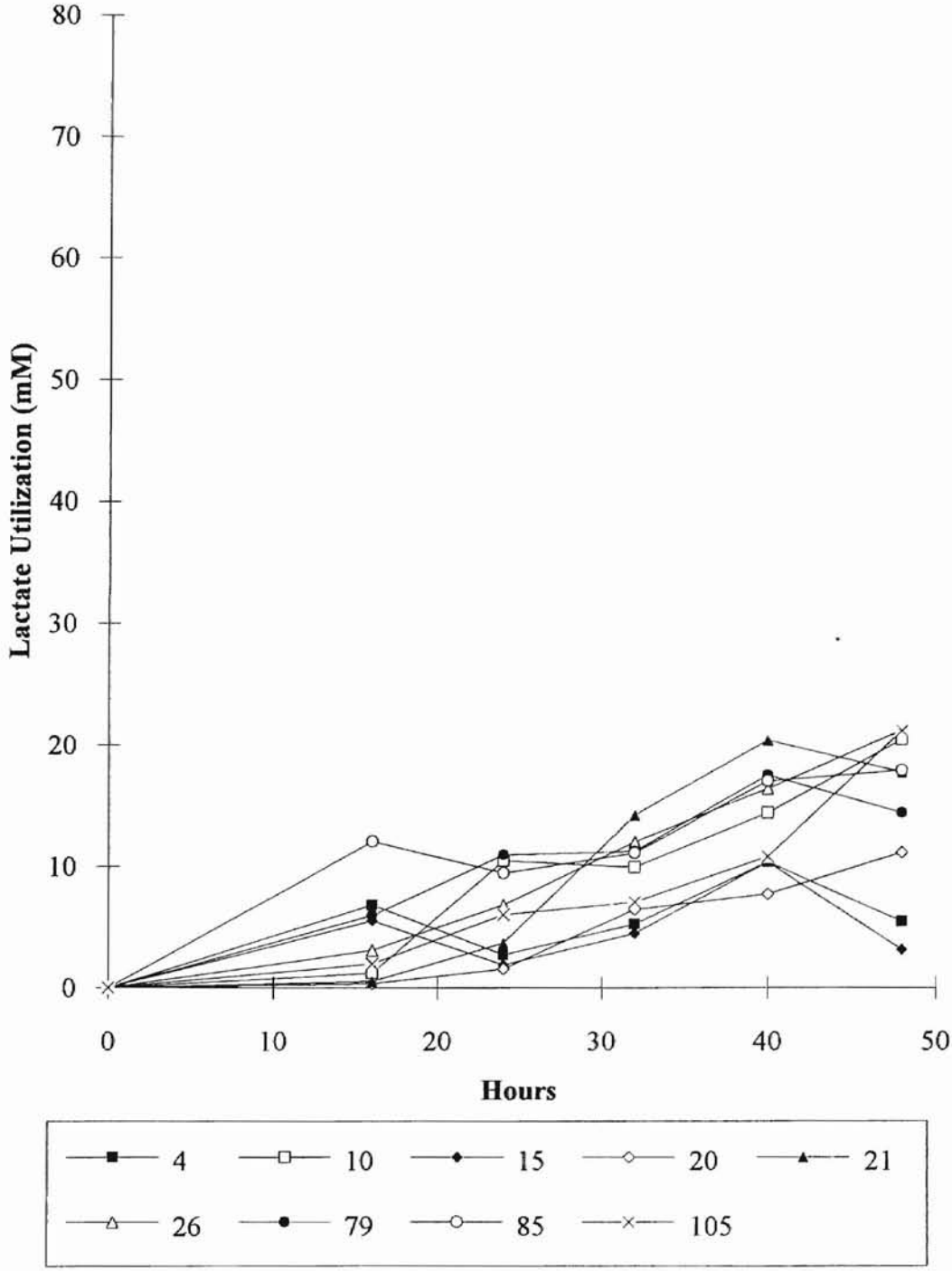


Figure 18. Lactic acid utilization of *P. acidipropionici* strains grown in lactate at pH 7.0.

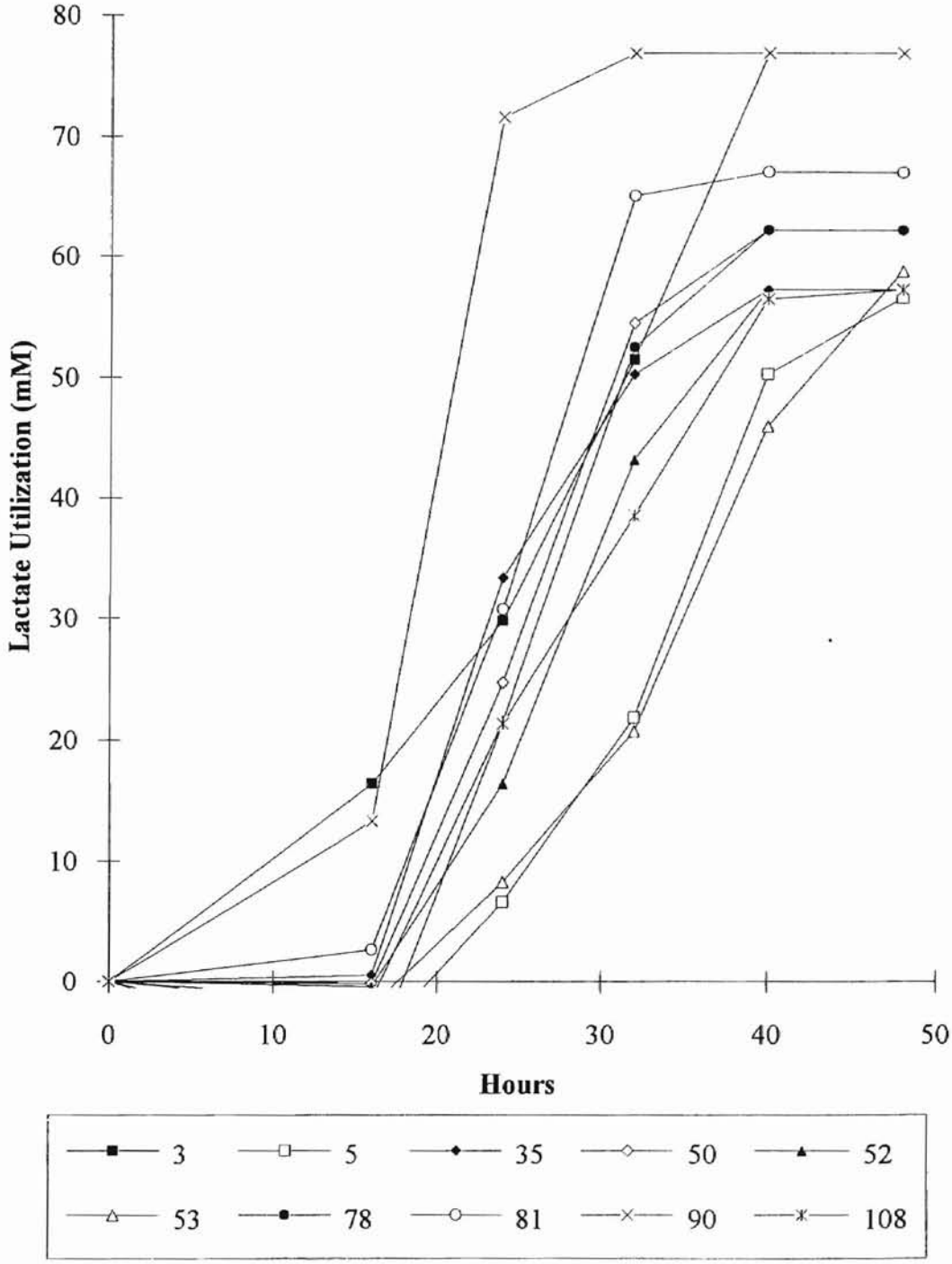


Figure 19. Lactic acid utilization of *P. freudenreichii* strains grown in lactate at pH 9.0.

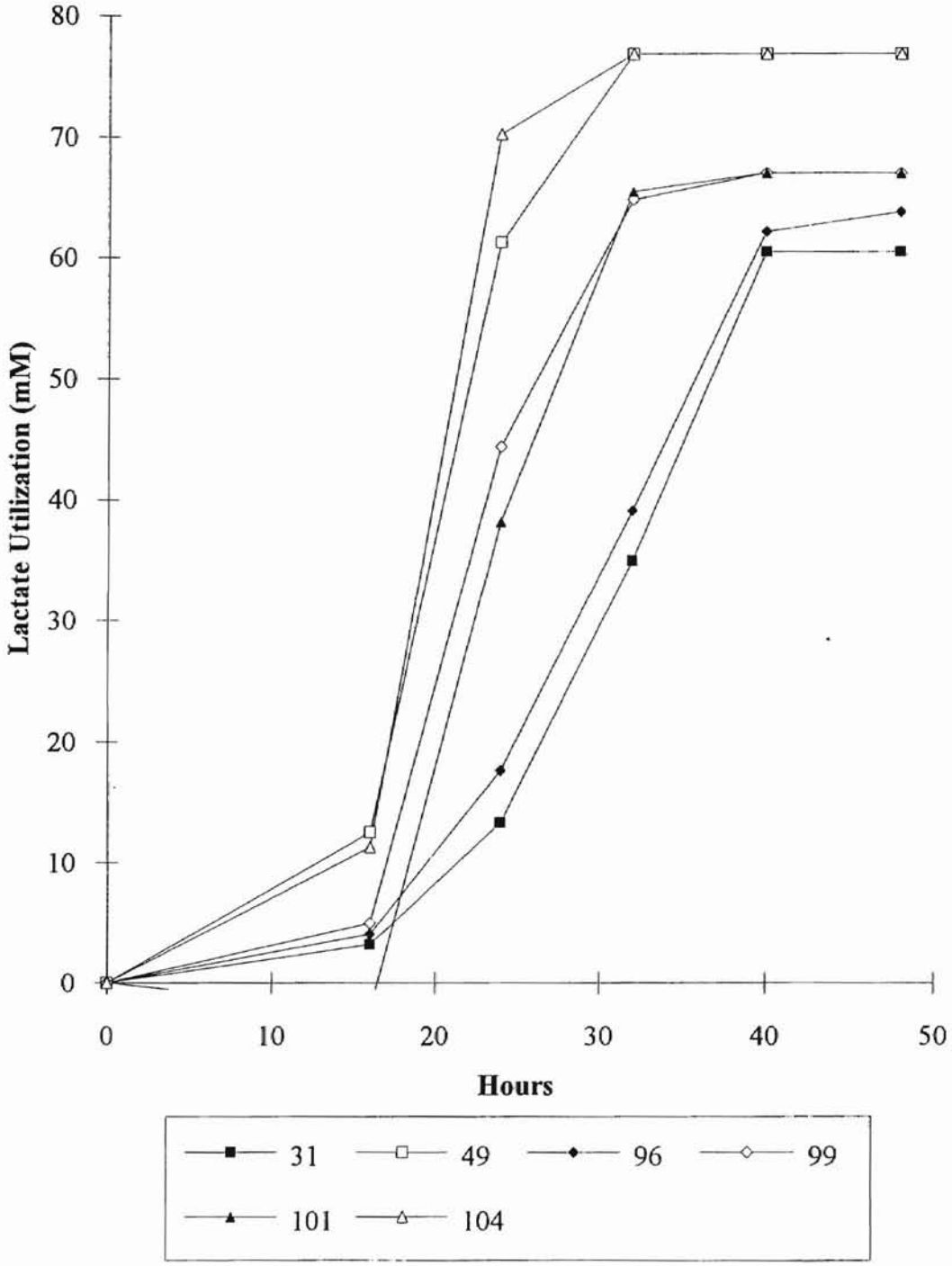


Figure 20. Lactic acid utilization of *P. jensenii* strains grown in lactate at pH 7.0.

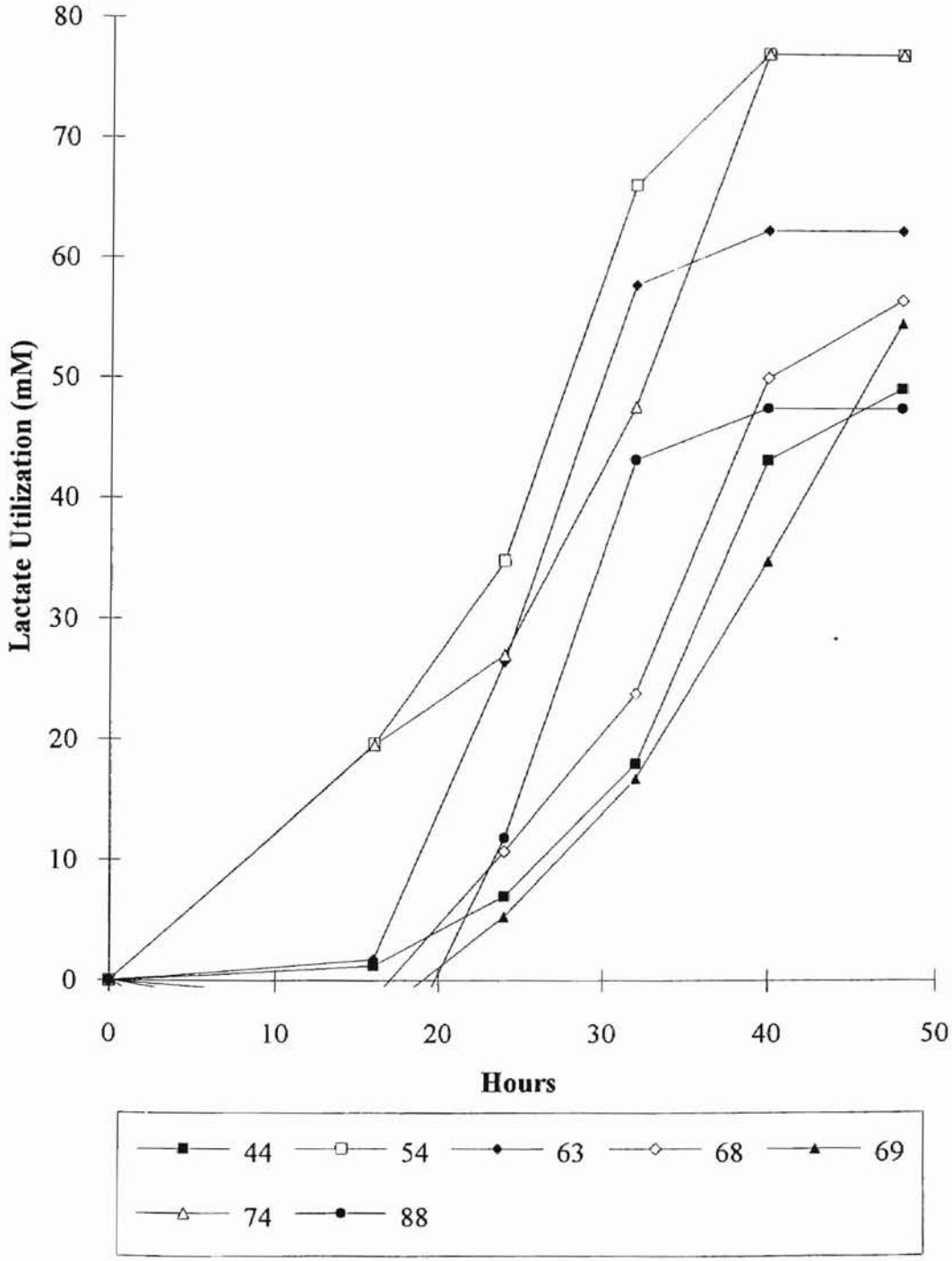
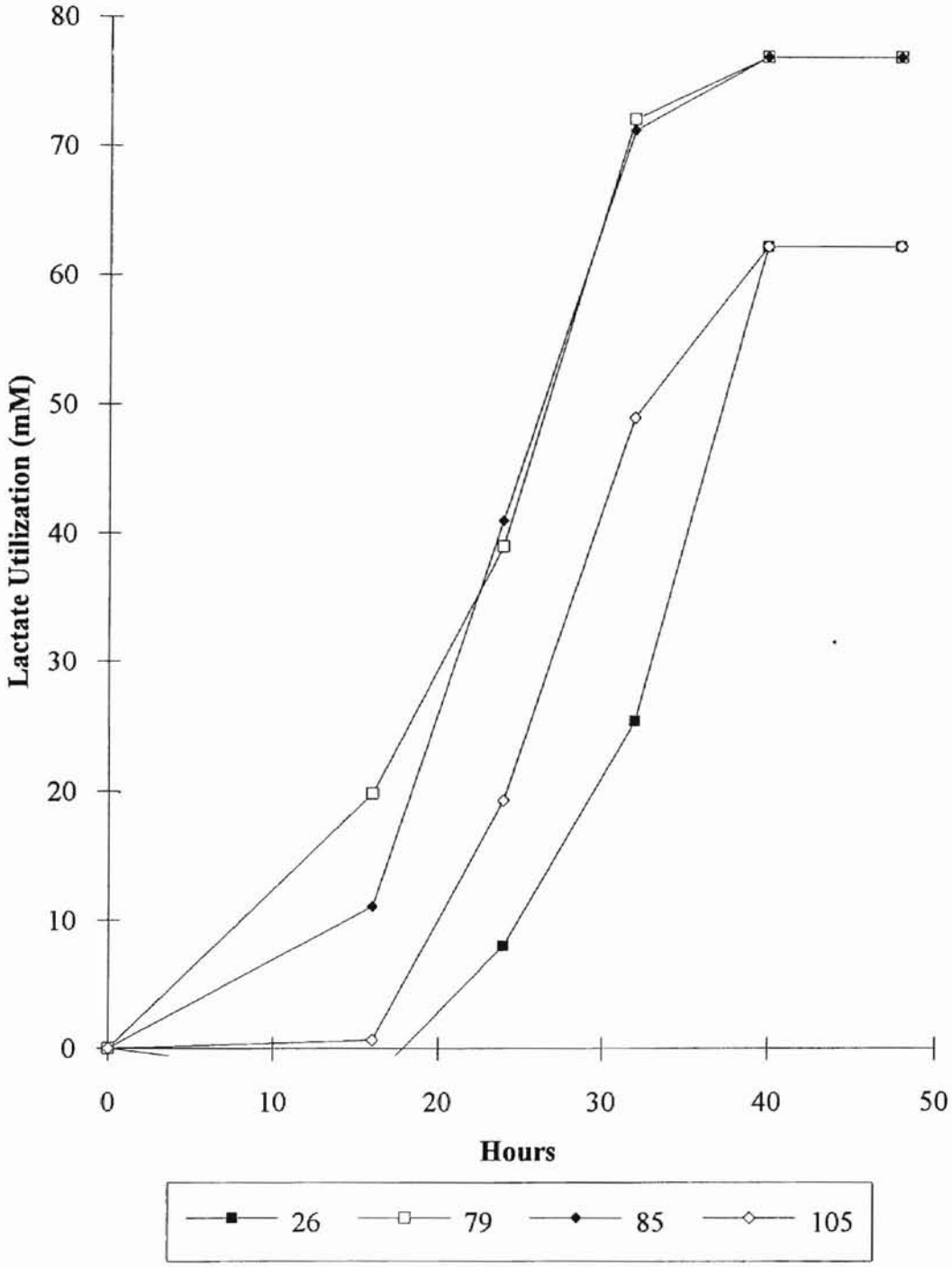


Figure 21. Lactic acid utilization of *P. thoenii* strains grown in lactate at pH 7.0.



APPENDIX B

FIGURES OF STRAIN EFFECT ON LACTIC ACID ACCUMULATION AND pH SUPPRESSION OF *IN VITRO* RUMEN FLUID MODEL

Figure 22. Rumen fluid simulation pH and lactic acid concentration for P25 and Control.

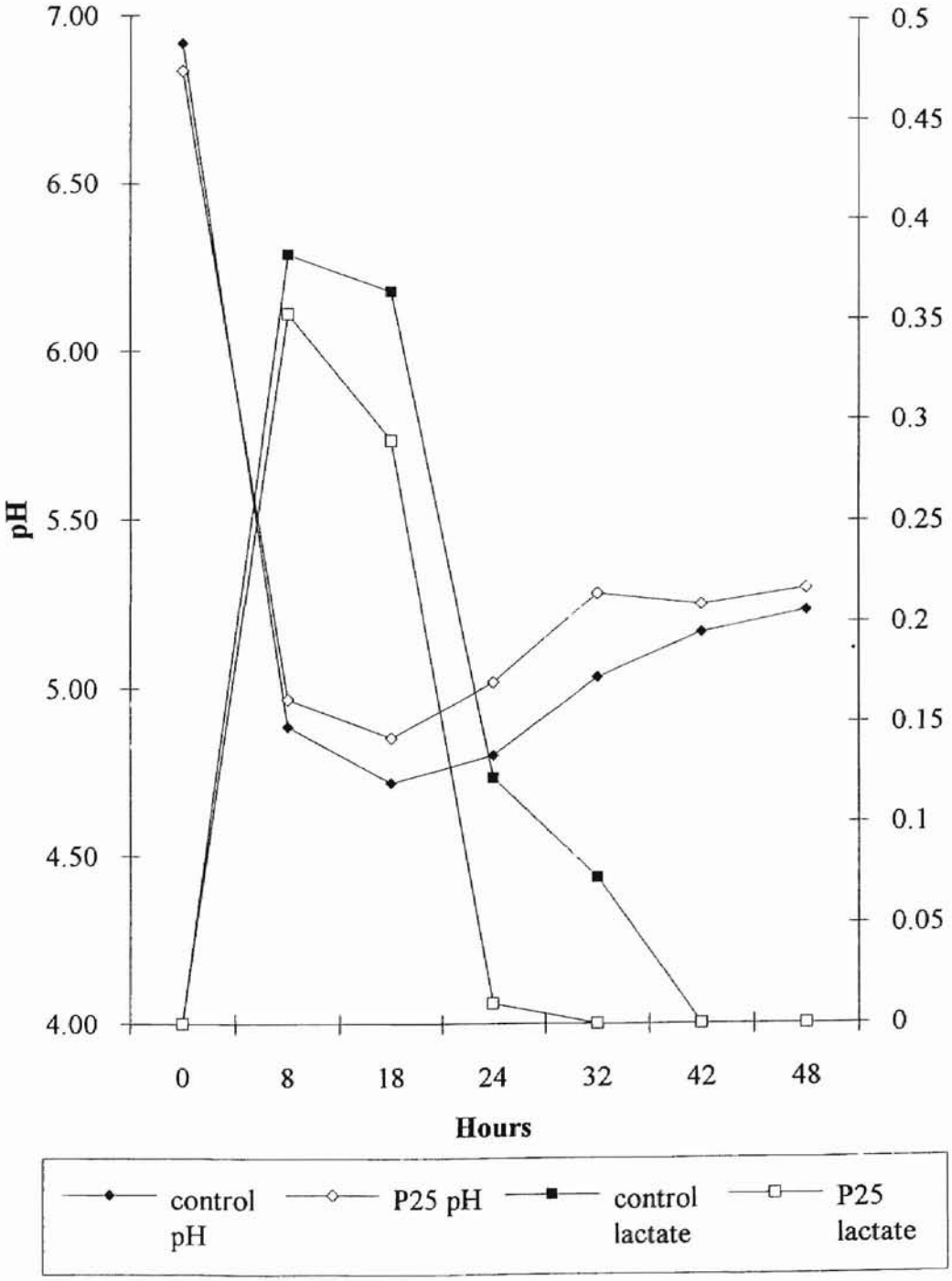


Figure 23. Rumen fluid simulation pH and lactic acid concentration for P31 and Control.

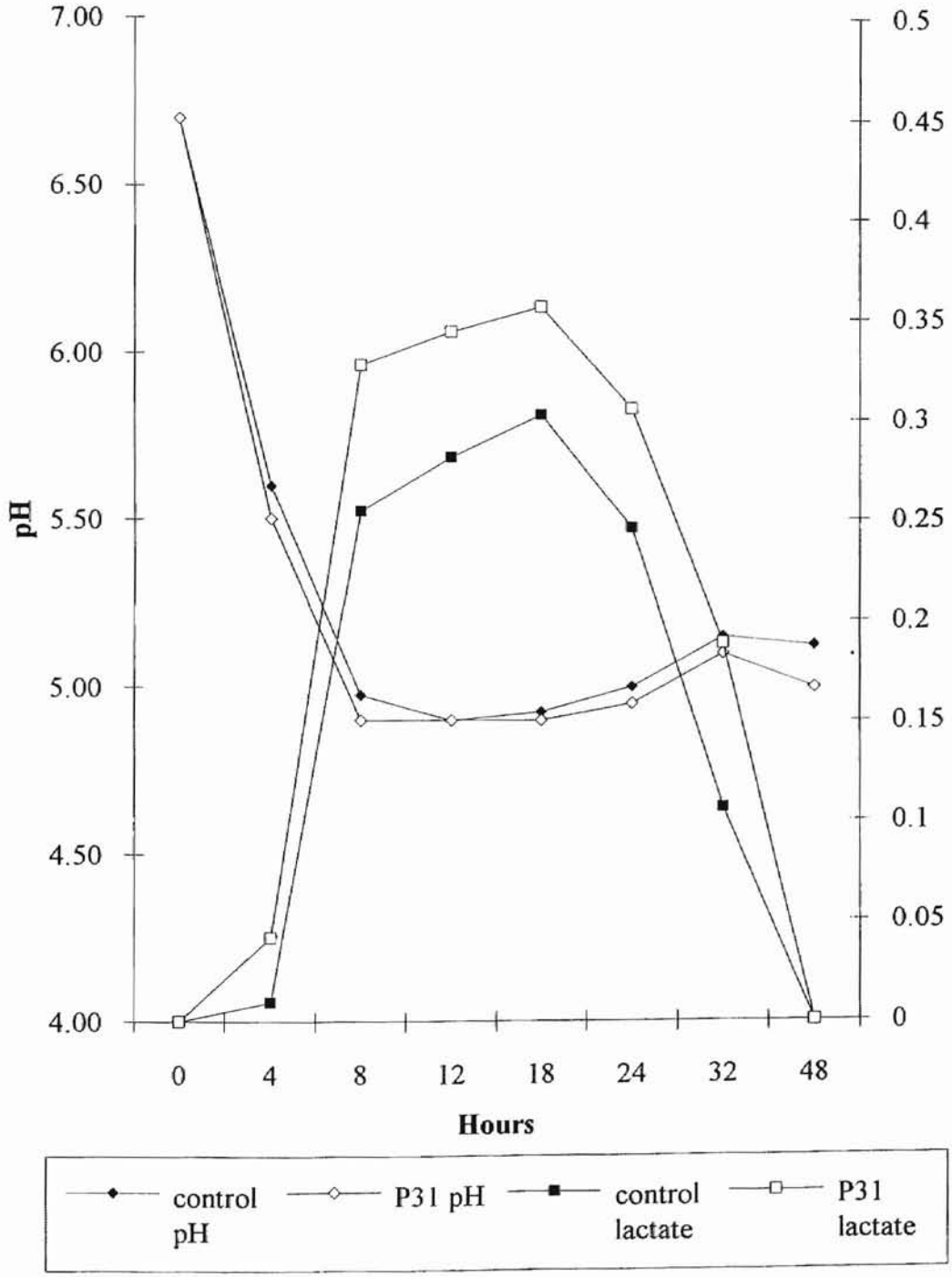


Figure 24. Rumen fluid simulation pH and lactic acid concentration for P41 and Control.

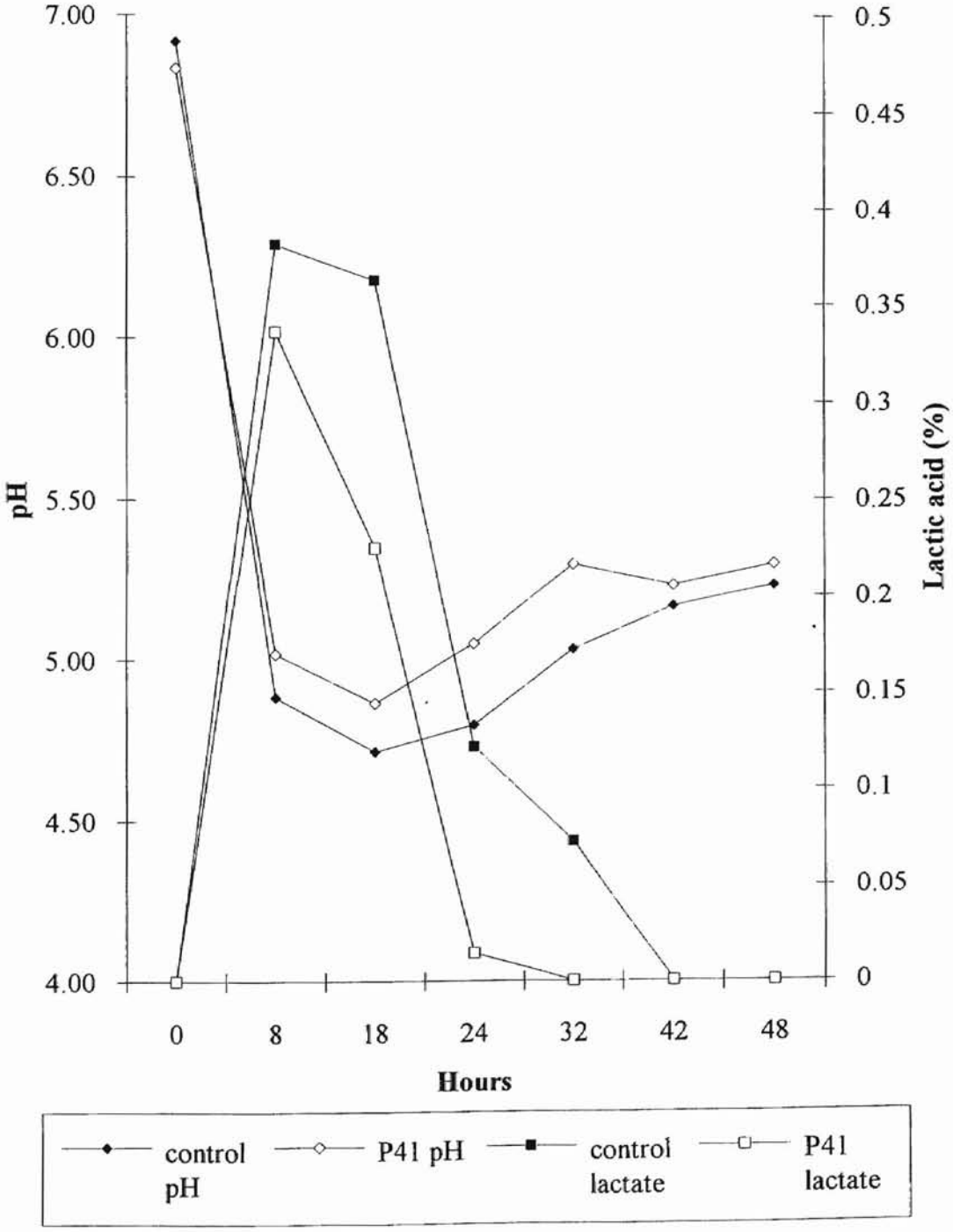


Figure 25. Rumen fluid simulation pH and lactic acid concentration for P42 and Control.

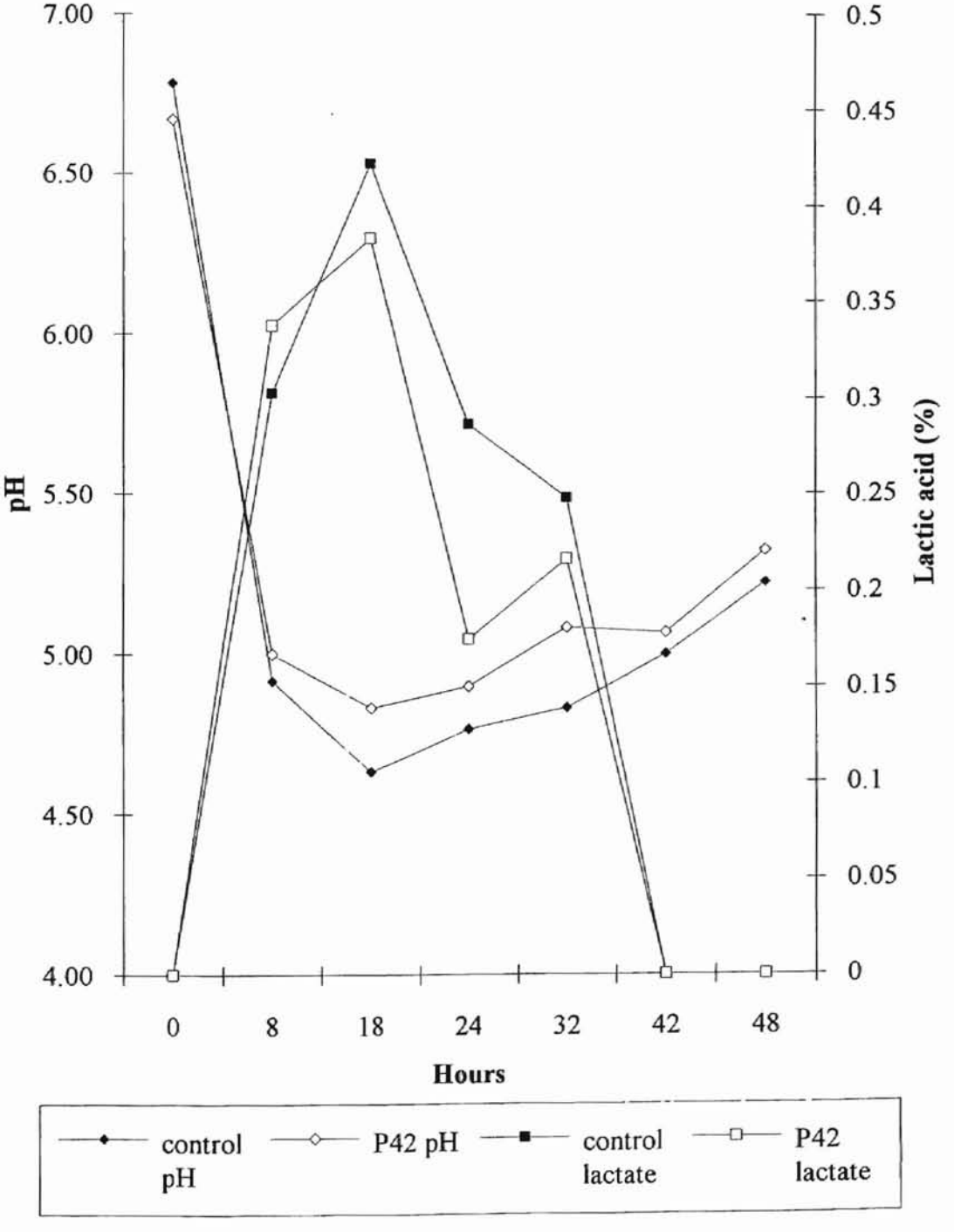


Figure 26. Rumen fluid simulation pH and lactic acid concentration for P48 and Control.

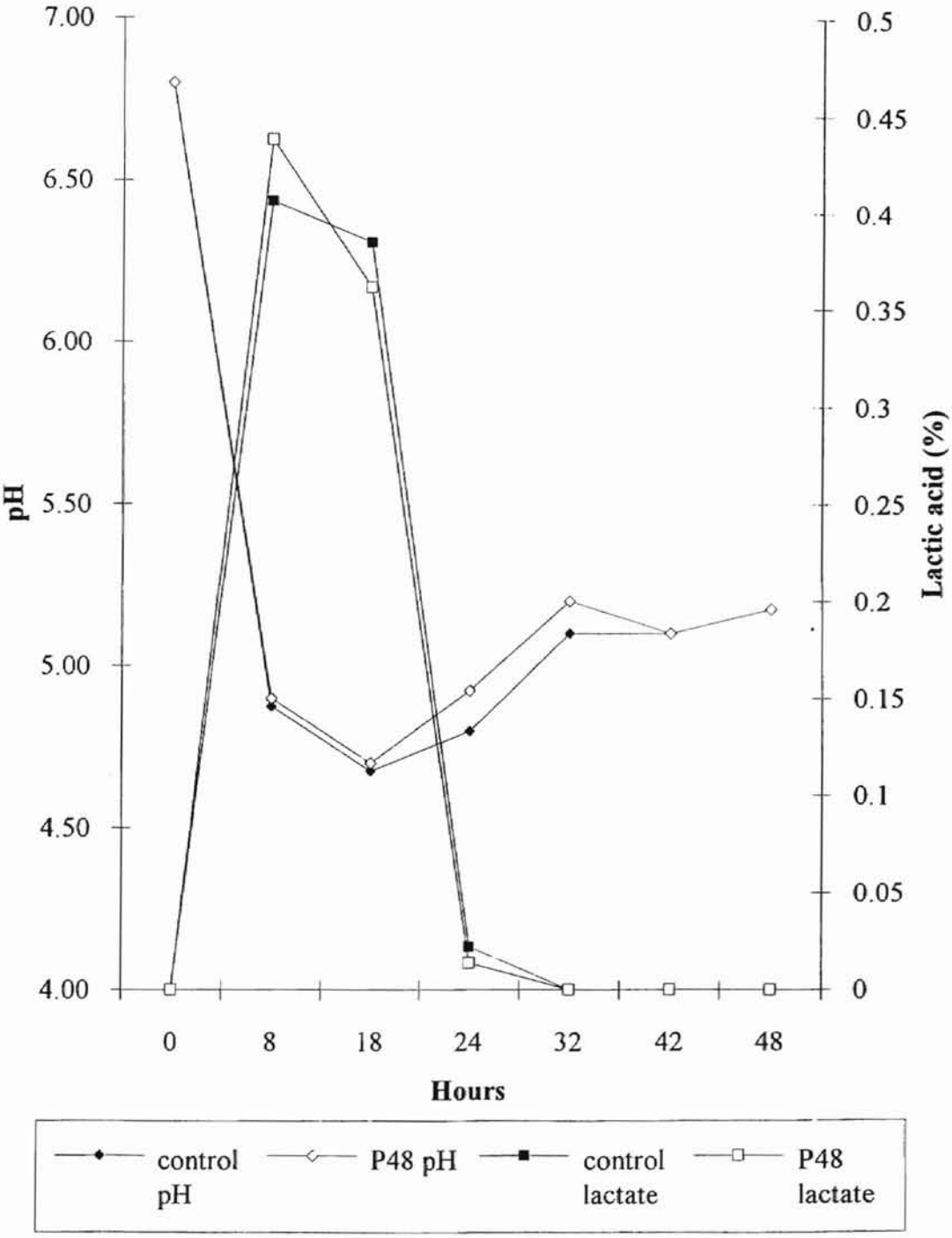


Figure 27. Rumen fluid simulation pH and lactic acid concentration for P49 and Control.

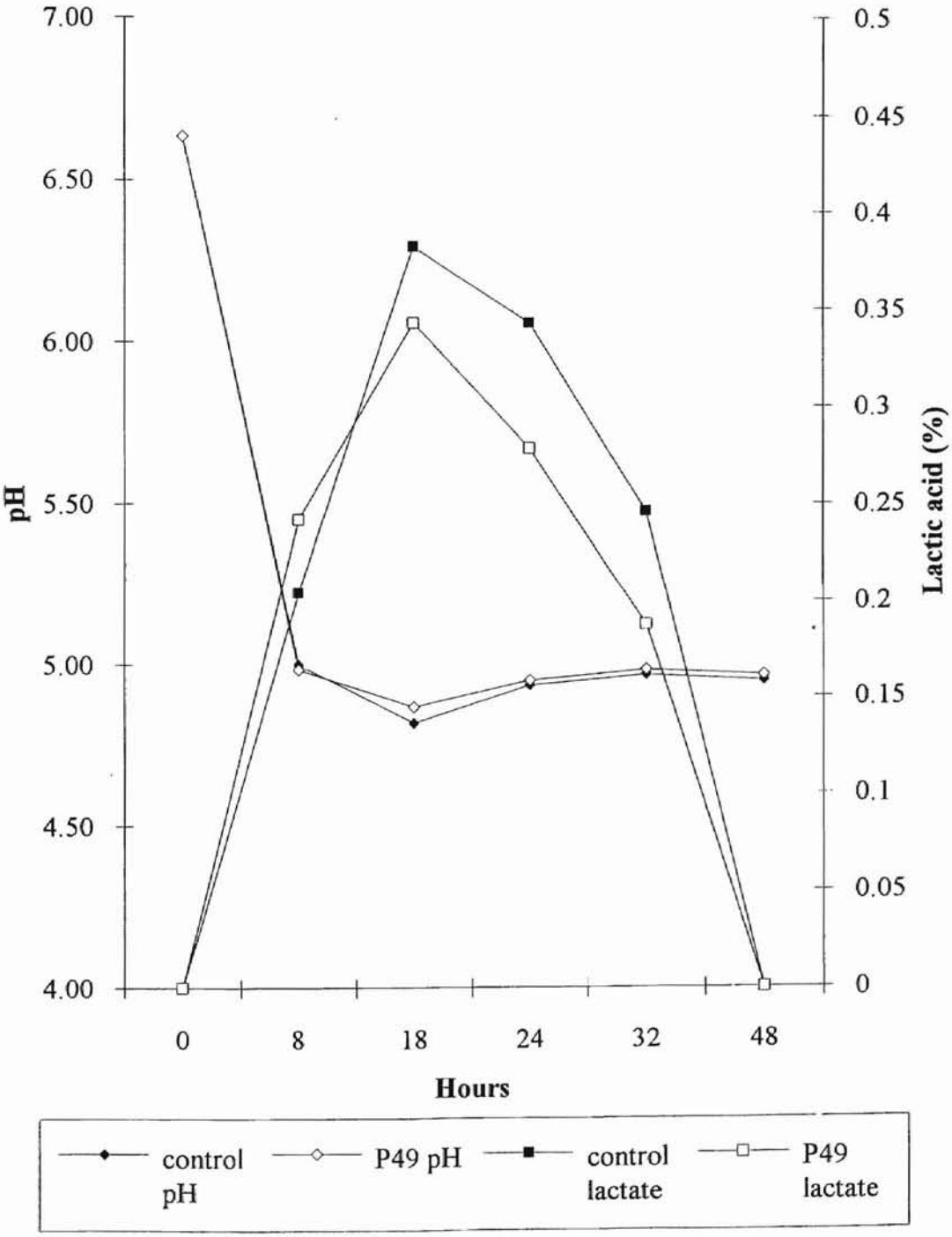


Figure 28. Rumen fluid simulation pH and lactic acid concentration for P54 and Control.

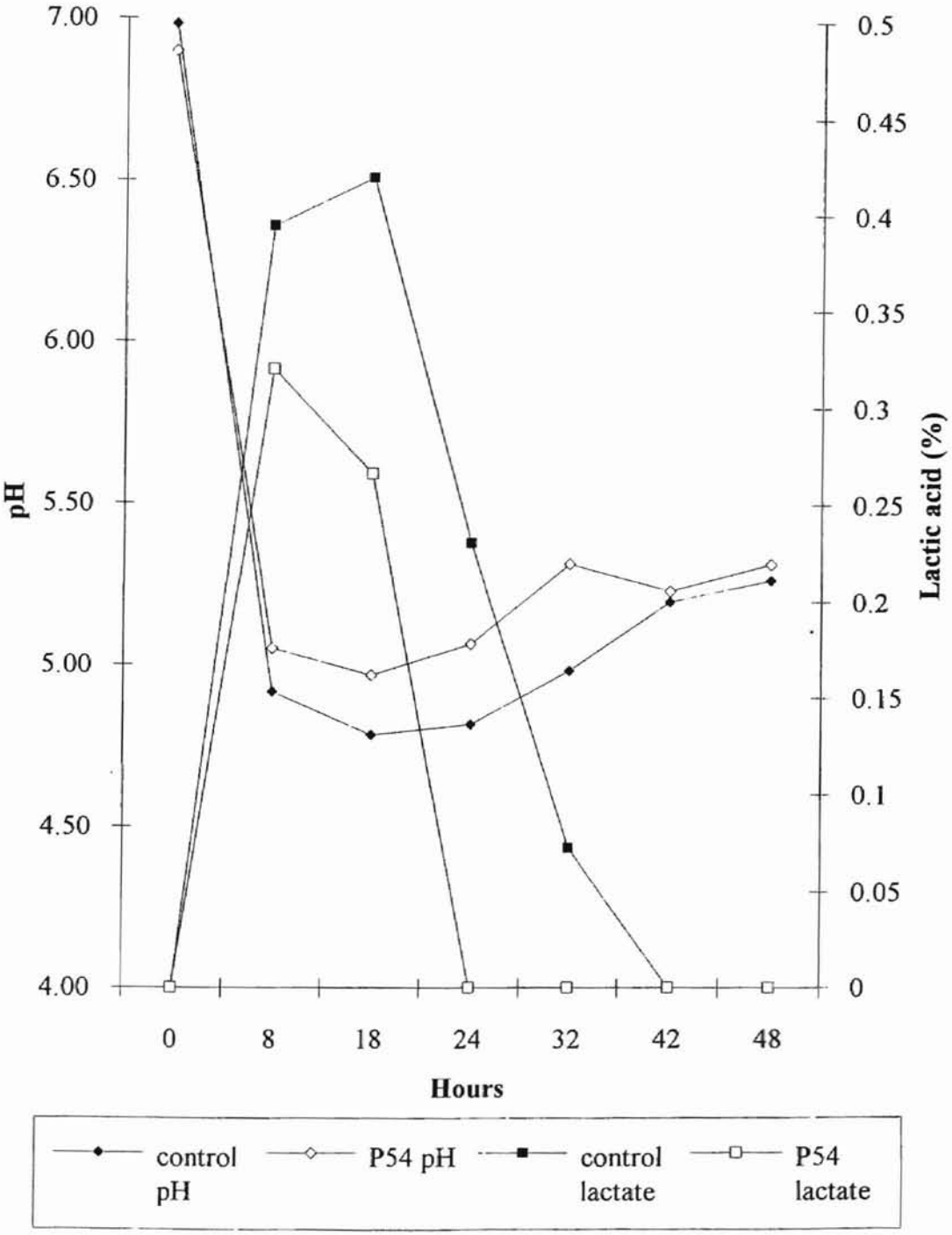


Figure 29. Rumen fluid simulation pH and lactic acid concentration for P63 and Control.

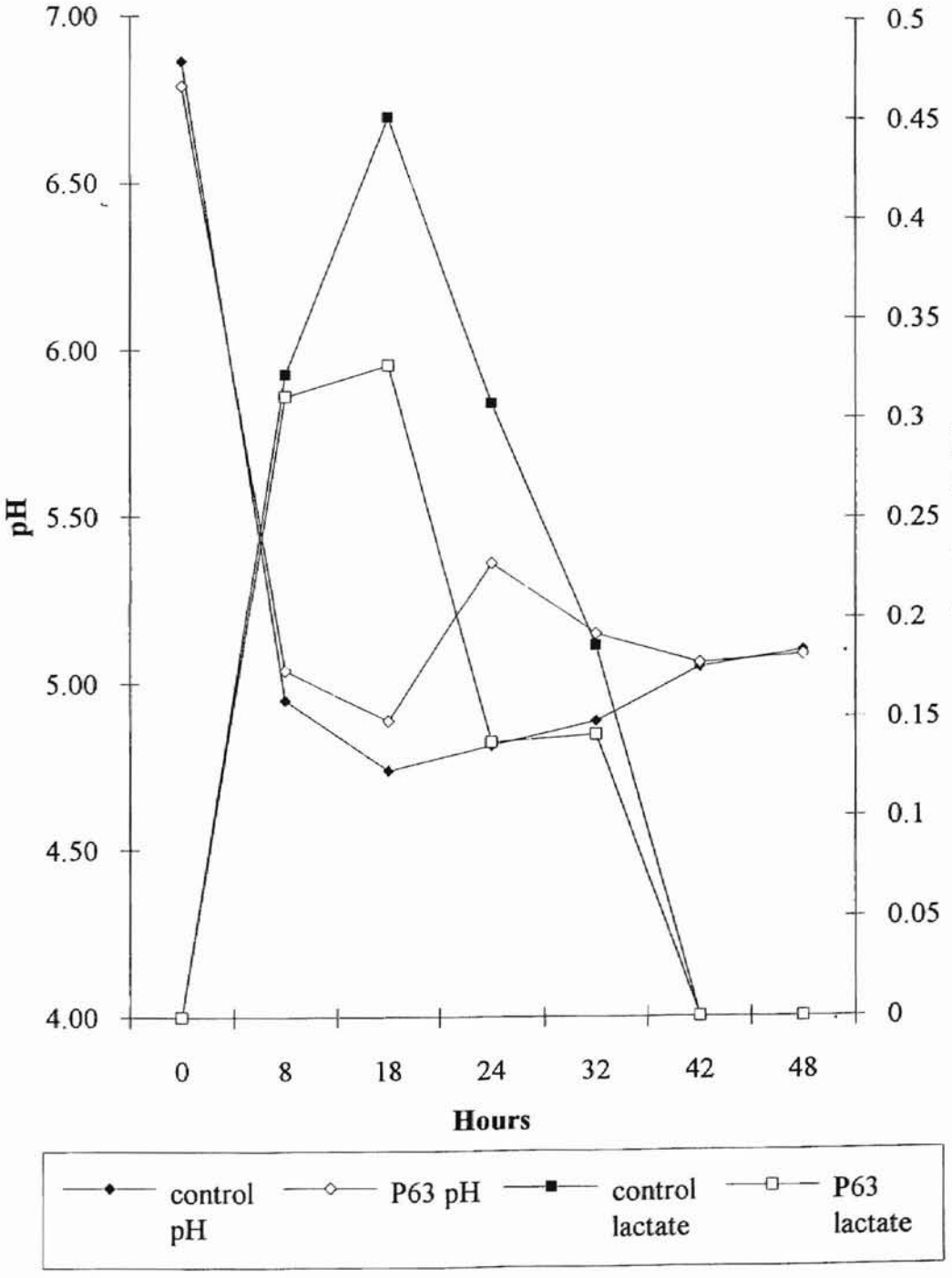


Figure 30. Rumen fluid simulation pH and lactic acid concentration for P78 and Control.

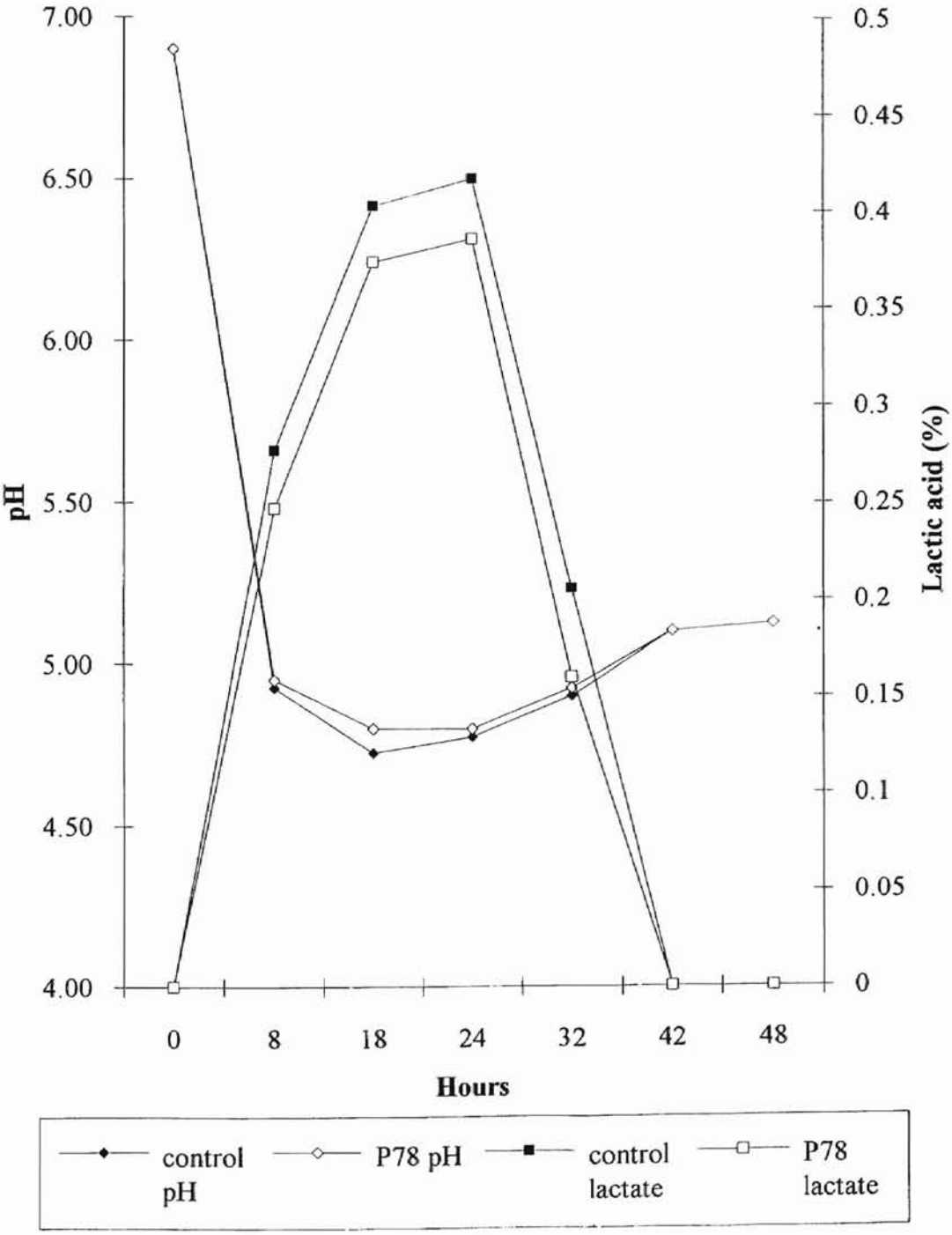


Figure 31. Rumen fluid simulation pH and lactic acid concentration for P81 and Control.

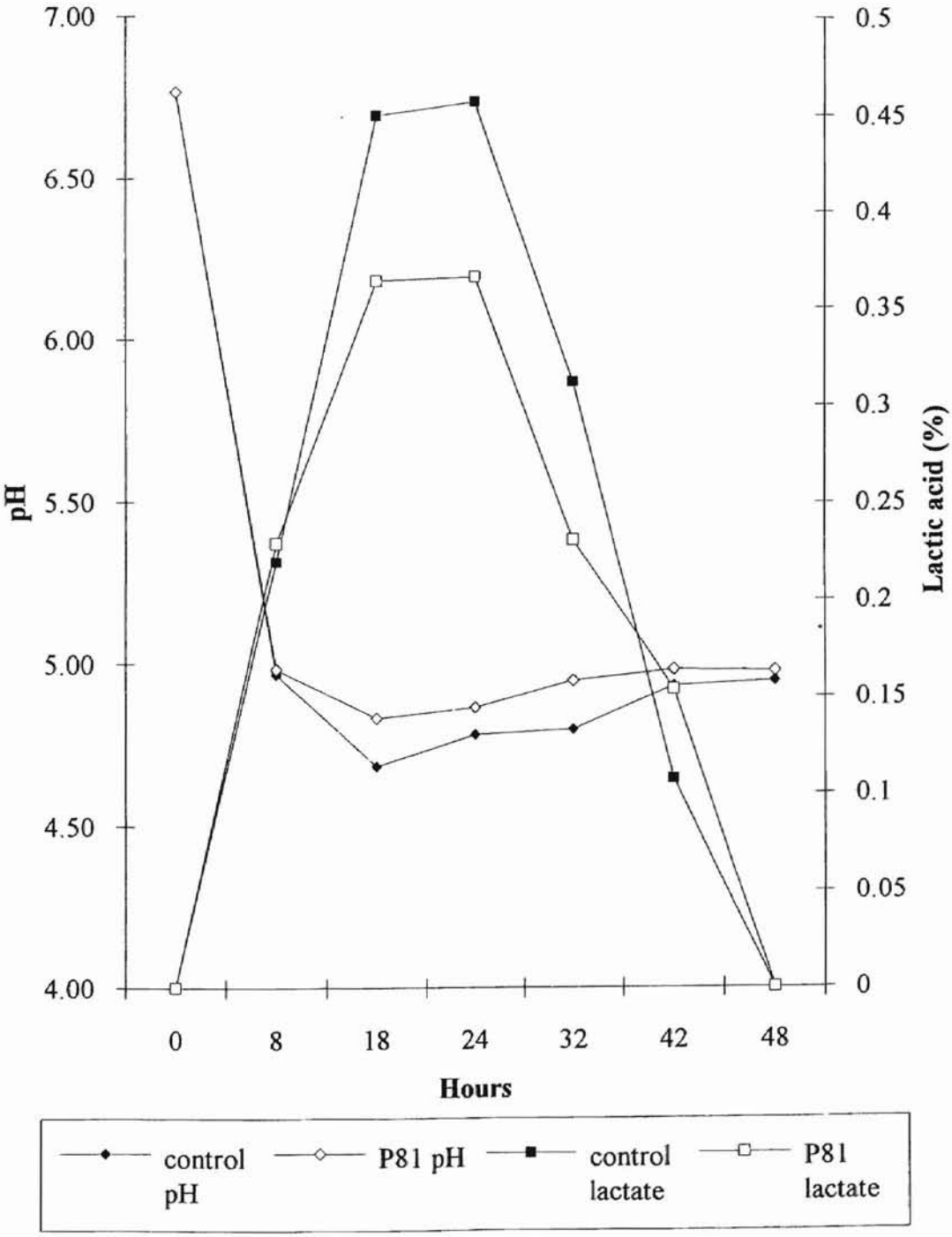


Figure 32. Rumen fluid simulation pH and lactic acid concentration for P88 and Control.

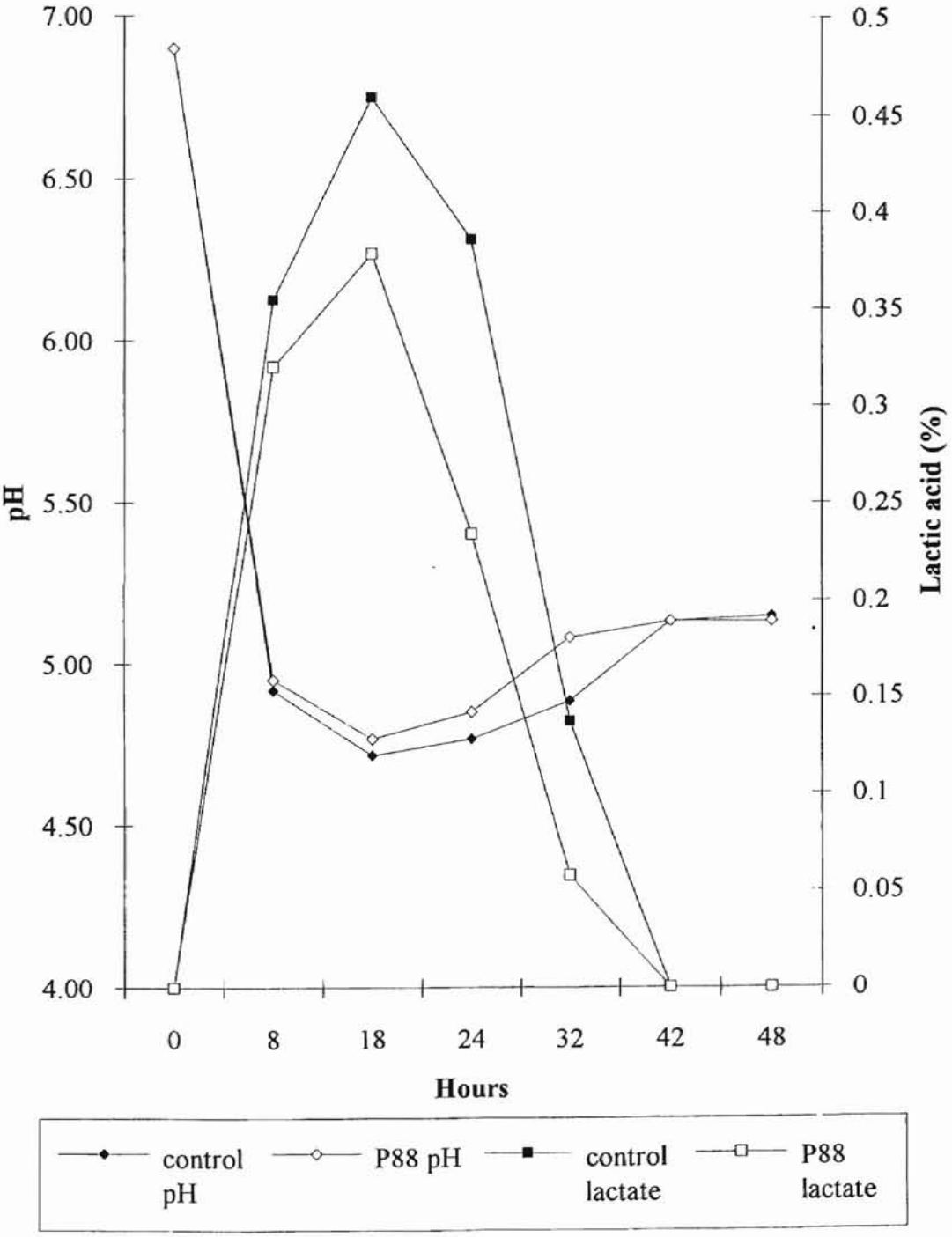


Figure 33. Rumen fluid simulation pH and lactic acid concentration for P89 and Control.

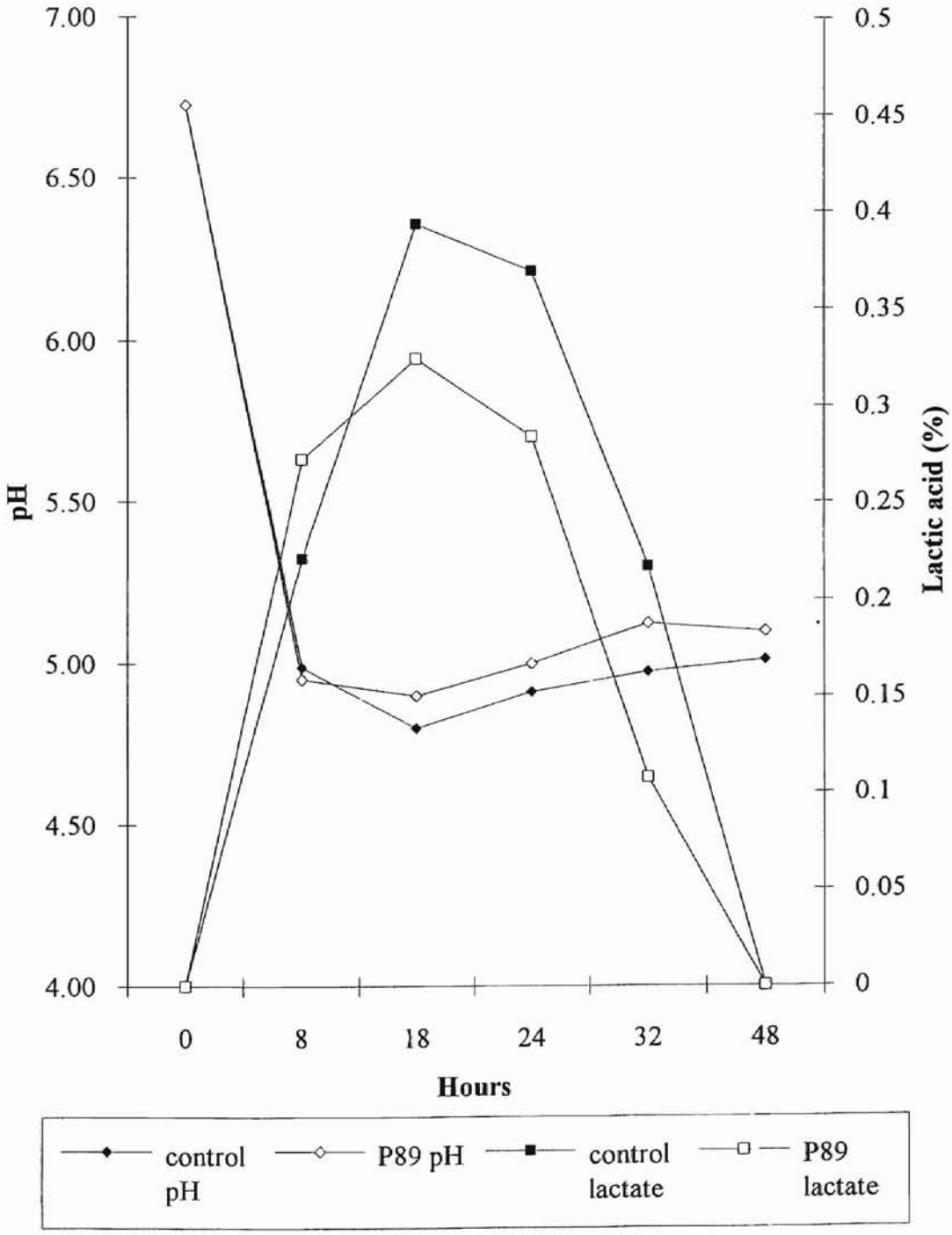


Figure 34. Rumen fluid simulation pH and lactic acid concentration for P90 and Control.

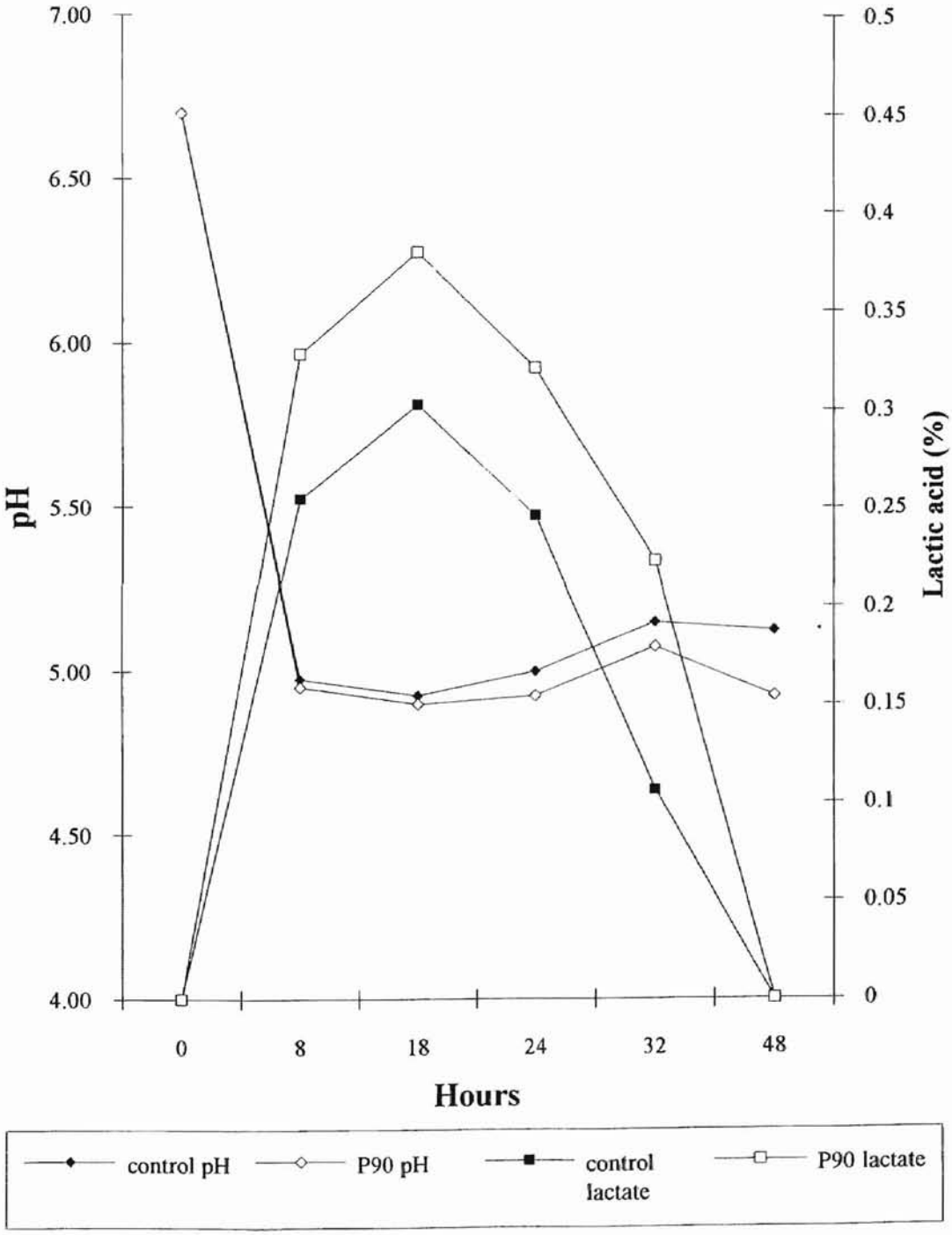


Figure 35. Rumen fluid simulation pH and lactic acid concentration for P99 and Control.

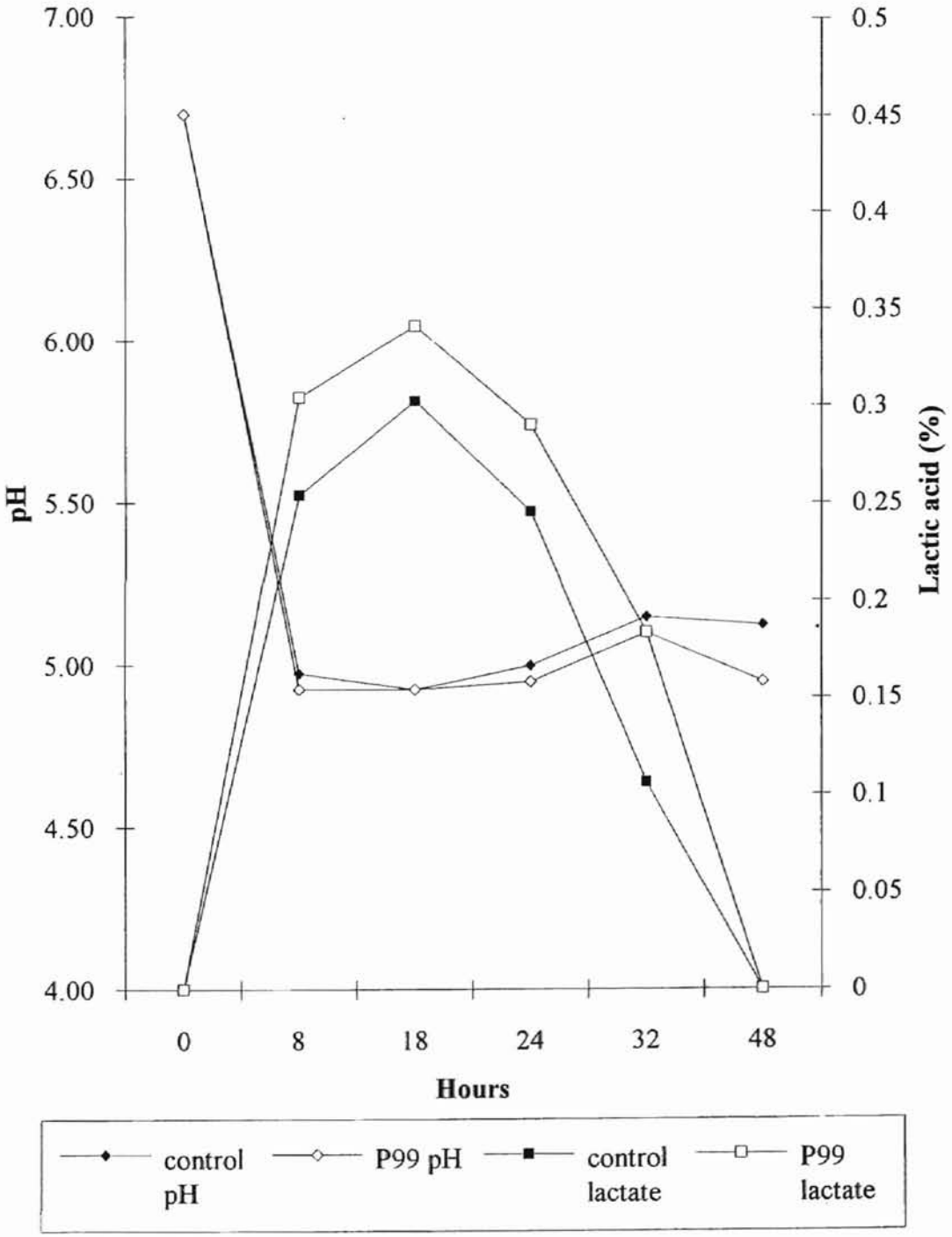


Figure 36. Rumen fluid simulation pH and lactic acid concentration for P104 and Control.

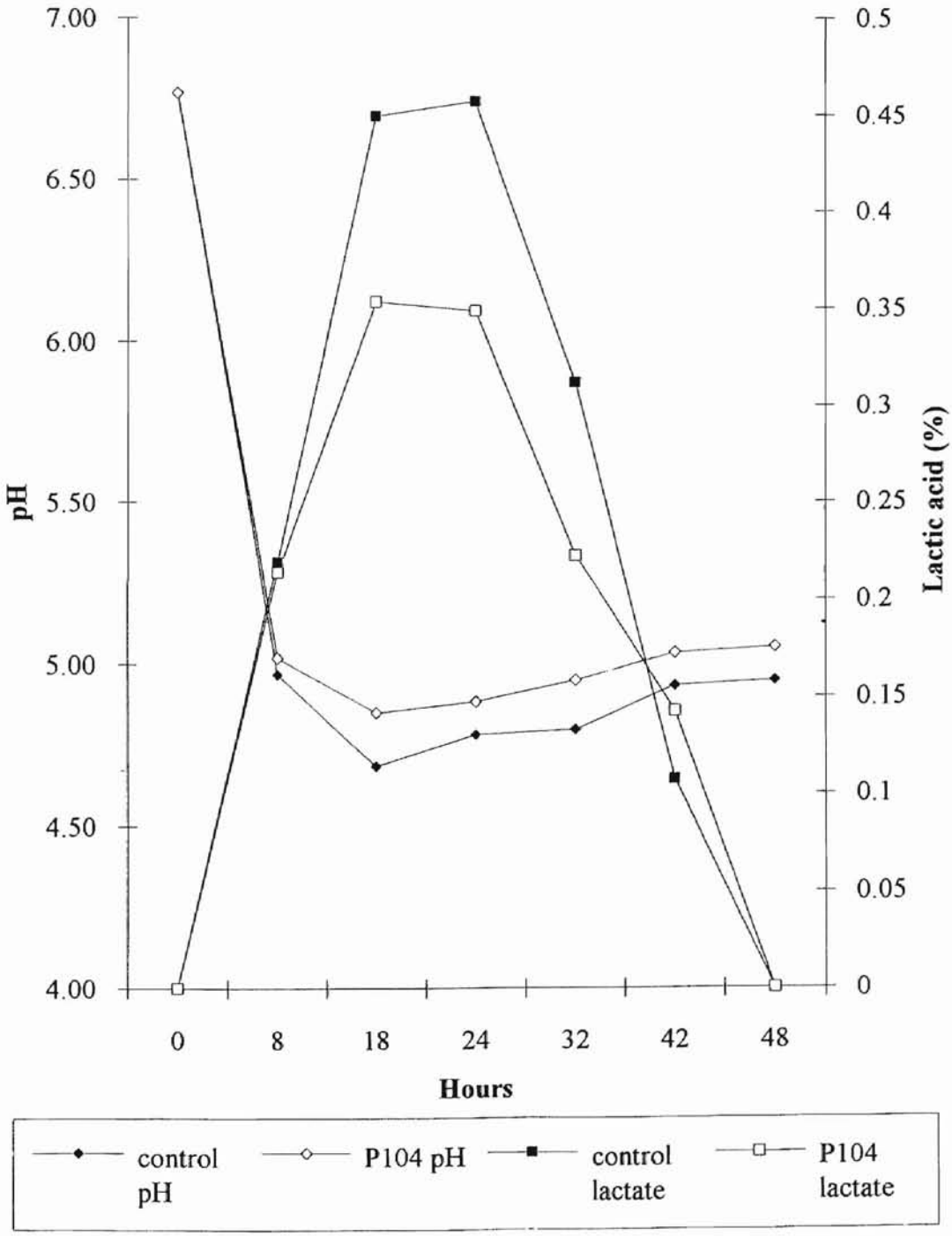
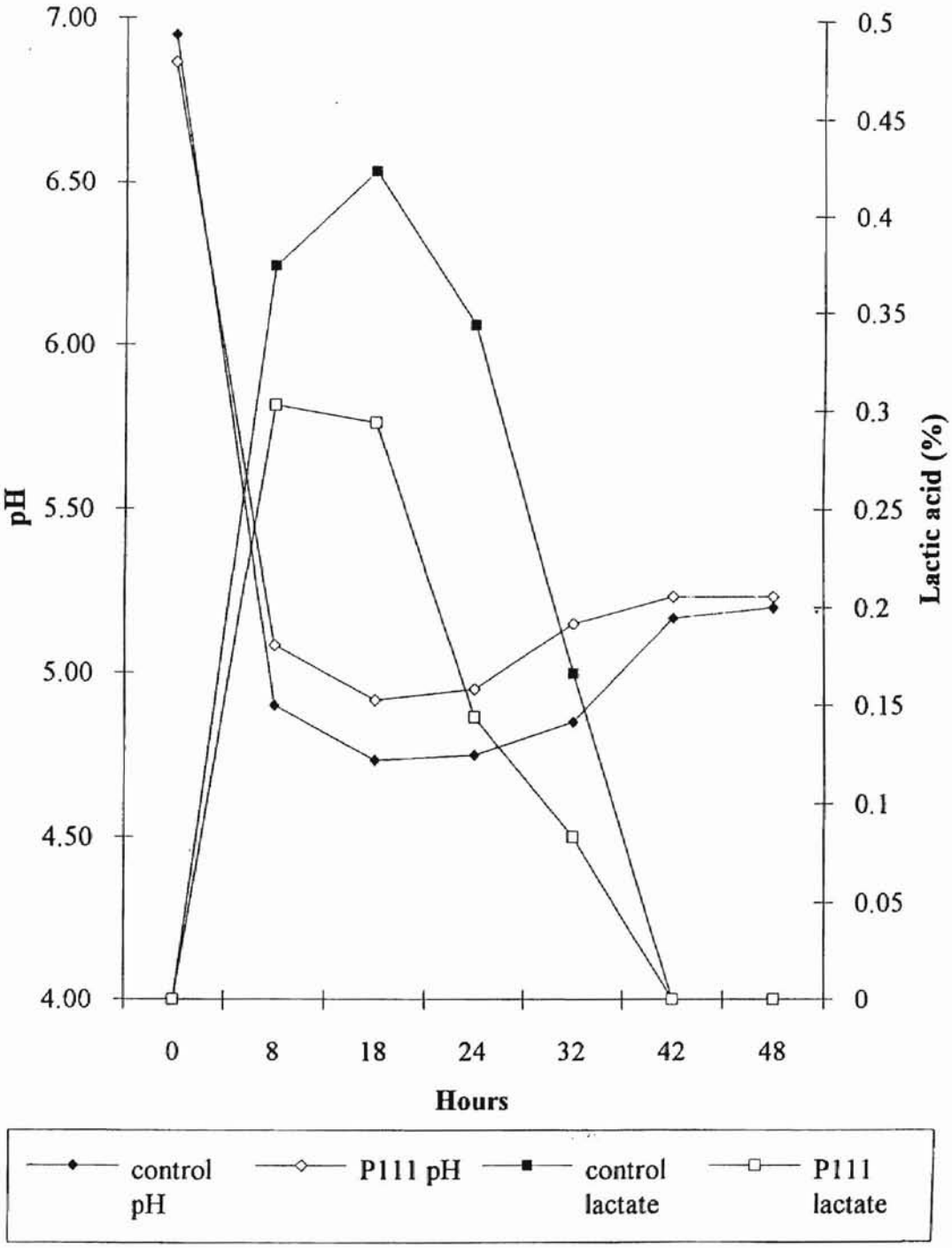


Figure 37. Rumen fluid simulation pH and lactic acid concentration for P111 and Control.



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