SELECTION, ESTABLISHMENT AND *IN VIVO* DENITRIFICATION OF A *PROPIONIBACTERIUM* STRAIN IN BEEF CATTLE

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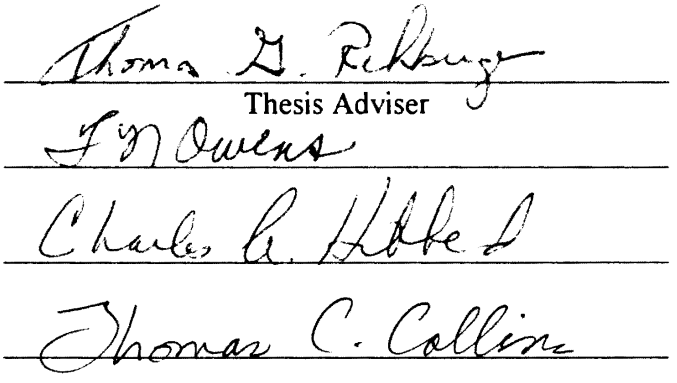

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CHAPTER I

INTRODUCTION

Since the report by Mayo in 1895, nitrate has been recognized as toxic to cattle. Excessive levels of nitrate accumulate in plant material due to moderate drought conditions, over-fertilization, successive cloudy days during the early growing season, frost damage or any other condition that prevents or slows the plants ability to convert nitrate to plant proteins. The name "nitrate" toxicity is actually a misnomer for the nitrate ion is itself innocuous. The actual toxicosis results from the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) by rumen microbes using the nitrate ion as a terminal electron acceptor during anaerobic respiration. Nitrite accumulates in the rumen as the normal rumen microbiota are unable to further reduce or utilize nitrite. As nitrite levels rise in the rumen, the nitrite ion readily passes through the rumen wall and enters the blood stream. Once in the blood stream, the nitrite ions bind to the oxygen binding sites of hemoglobin molecules which converts the hemoglobin to methemoglobin. This methemoglobin is unable to carry oxygen. Some methemoglobin is reconverted back to hemoglobin by NADPH reductase but the rate of reconversion is slow and ineffectual in toxic situations. As methemoglobin concentration increase, the blood takes on a characteristic chocolate brown color as the animal begins to experience the symptoms of "nitrate" toxicity. As methemoglobin concentration reach 30 to 40%, the animal becomes lethargic, develops a staggering

gait and begins to pant while experiencing a rapid pulse. When the methemoglobin concentration exceeds 50% the animal develops labored breathing, muscle tremors, collapse, fall into a coma and die due to anoxia. The entire course of symptoms can occur in as little as 30 minutes or as long as 12 hours after the ingestion of a toxic dose of nitrate. Onset of toxicosis is dependent on the concentration of nitrate ingested, the availability of that nitrate, condition of the animal, stress factors and individual animal variation.

One possible solution for " nitrate" toxicity would be to reduce the amount of nitrite that accumulates in the rumen. This could be accomplished through the establishment of a naturally occurring, denitrifying bacteria in the rumen. The feeding of specific viable microorganisms to livestock with the intent to alter microbial balance within the gastrointestinal tract has been practiced for many years. Although the modes of action and beneficial effects of these direct-fed microbial products has not always been scientifically demonstrated, the importance of microbes in fermentation and digestion is well recognized.

One group of bacteria capable of reducing nitrate and nitrite to non-toxic nitrogenous compounds are propionibacteria. The microaerophilic propionibacteria are an industrially important group of microorganisms primarily used in the dairy industry as starter cultures for Swiss-type cheeses. Other industrial applications include the production of vitamin B_{12} and propionic acid as well as their use as direct-fed microbial inoculates for silage and grains. The objectives of this study were then to (1.) identify all strains of propionibacteria capable of denitrification, (2.) select a strain capable of rapid, large scale reduction of nitrate and nitrite, (3.) determine the capacity of the selected strain to establish in the rumen and (4.) determine if the

established strain is able to lessen the occurrence and severity of "nitrate" toxicity *in vivo*.

CHAPTER II

"NITRATE" TOXICITY-OVERVIEW

The consumption of excessive levels of nitrate is toxic to livestock was first reported by Mayo in 1895. Nitrate (NO_3) is now recognized as the non-toxic precursor with its derivative nitrite (NO_2) being the toxic agent. To date, nitrate toxicity has been demonstrated in such widely diverse livestock species as beef cattle, dairy cattle, sheep, swine (Deeb and Sloan, 1975), goats (Prasad, 1983), reindeer (Nordkrist *et al.*, 1984) and water buffalo (Prasad *et al.*, 1984).

Nitrate can accumulate in the environment and enter the animal's food supply through two different avenues. The first is through the water supply. Across Oklahoma, the nitrate concentration of ground water ranges from 0.5 and 26.5 ppm (J. Duncan, State Environmental Laboratory, personal communication). Local nitrate concentrations may be much higher due to run-off and leaching of nitrate from over-fertilized fields, feedlots or other animal waste sources into the surface or ground water supplies. Nitrate in drinking water can be particularly dangerous as it is immediately available in the rumen while feed sources of nitrate must be released from plant cells first. This increased availability results in the increased toxic affect of water-borne nitrate over nitrate contained in feed. The second and more common source of nitrate is from forages via the soil. Accumulations of nitrate in the soil can occur due to over-fertilization of fields, leaching of nitrate into the soil from feedlots and other sources of animal waste or inhibited plant growth due to severe drought or herbicide treatment. (Pfister, 1988) on fields moderately fertilized over several seasons.

Nitrate is a key component in plant growth acting as the inorganic nitrogen source for the production of plant proteins (Wright and Davidson, 1964). Certain species of plants are more prone than others to accumulate nitrate. Common forages and feedstuffs known to accumulate nitrate under certain conditions include alfalfa, annual brome,

clovers, fescue, kikuyugrass, orchardgrass, pearl millet, sorghum, sorghum x sudan crosses, sunflower, sweetclover, switchgrass, timothy, wheatgrasses, wild rye, witchgrass, barley forage, beet pulp, corn forage, kale, molasses, oat forage, rape, turnips and wheat forage. Certain weeds common in pastures and crop fields also accumulate nitrate these include such species as dock, goldenrod, jimson weed, johnsongrass, kochia, lamb's quarter, nightshade and pigweed.

Normally found in plants, nitrate seldom accumulates because it is rapidly reduced and combined with carbohydrates to form amino acids and plant proteins (O'Hara and Fraser, 1975). Nitrate, however, does accumulate in plants under certain environmental conditions and in certain stages of plant growth. Environmental conditions that result in nitrate accumulation are those in which the plant continues to absorb nitrate from the soil but is unable to photosynthesize. These conditions include during and immediately after a moderate drought in which the plant continues to take up nitrate from the soil but nitrate reductase activity is reduced due to leaf stress caused by low water availability (Pfister, 1988). Other conditions include frost damage to leaves and shading of the plant, particularly during the early growth phases (Pfister, 1988).

Nitrate also accumulates in certain plants parts. Highest nitrate concentrations will be found in the stems followed by the leaves with the lowest concentrations found in the grain (Krejsa *et al.*, 1987; Pfister, 1988; Fjell *et al.*, 1991). Forage maturity also plays a role in nitrate accumulation with early growth and regrowth having higher accumulation than older more mature plants (Pfister, 1988; Fjell *et al.*, 1991).

The form and manner in which the forage is consumed also affects its toxicity. Hays become more permeable during rehydration in the rumen and release their nitrate more quickly (80% in 20 minutes) than fresh plant material (30% in 20 minutes). This makes dry hay more toxic than lush, green forage (Geurink *et al.*, 1979) even though they may contain the same concentration of nitrate.

Once the nitrate enters the rumen of pre-gastric fermenters or the cecum of post-gastric fermenting species it is reduced by the normal microflora, in most cases, to nitrite. The nitrite formed may or may not be reduced further depending on the species of microbe and its requirements.

Nitrate may be reduced for one of two reasons. First, reduction of nitrate is an energetically favorable process in propionibacteria as well as some other species (Van Gent-Ruijters *et al.*, 1975; Kaspar, 1982; Allison and Macfarlane, 1989). In species of bacteria capable to anaerobic respiration, nitrate can increase production of ATP through increased citric acid cycle activity and by oxidative phosphorylation coupled to nitrate reduction (Van Gent-Ruijters *et al.*, 1975).

Nitrate also may be reduced to nitrite as the first step in the production of ammonia for later use in the synthesis of microbial protein (Lewis, 1951; Kaspar and Tiedje, 1981).

The key enzyme responsible for nitrate reduction is nitrate reductase. It catalyses the reduction of nitrate (NO_3) to nitrite (NO_2). Nitrate reductase is a constitutive enzyme whose production is stimulated by nitrate (Van Gent-Ruijters *et al.*,1976; Kaspar, 1982) in some bacteria including certain strains of propionibacteria. Many gastrointestinal microbes produce nitrate reductase so that when large quantities of nitrate are consumed, nitrite can accumulate rapidly in the gastrointestinal tract. The pH optimum of nitrate reductase is 6.5 (Tillman *et al.*, 1965) which is within the normal pH range of the rumen of ruminants fed forage (6.2-6.5); hence, nitrite production within the rumen is more favorable when host animals are fed forage.

A limited number of species of bacteria in the gastrointestinal tract reduce nitrite further to other less toxic nitrogenous compounds such as nitrous oxide (N_2O) or ammonia (NH_4^+) (Lewis, 1951; Cheng *et al.*, 1988). Normally, reduction of nitrite by the gastrointestinal microflora is much slower than the reduction of nitrate. The rate of nitrite reduction can be as much as two times slower than that of nitrate reduction (Allison and

Reddy, 1984). The rate of nitrite reduction may be slower because nitrite reduction is an energetically unfavorable process (Hasan and Hall, 1977; Cole, 1978). The nitrite ion is unable to replace nitrate as the electron acceptor in phosphorylation-coupled electron transfer. In addition, the nitrite ion can be toxic to some bacteria (Kaspar, 1982). This information suggests that the nitrite reductase enzyme is a detoxifying mechanism.

Another factor contributing to the slower nitrite reduction is that the pH optimum of nitrite reductase is 5.6 (Tillman *et al.*, 1965) well below that of the rumen of ruminants fed forage (6.2-6.5). Slower reduction than production of nitrite leads to the accumulation of nitrite in the rumen.

The enzymes nitrate and nitrite reductase require a number of cofactors; these include such minerals as copper, iron, magnesium and manganese. In addition, molybdenum is essential for nitrate reductase activity (Korzeniowski *et al.*, 1980; 1981). Coupling of nitrate reductase to nitrite reductase in propionibacteria and other species may be attributed to the need to detoxify nitrite.

The nitrate-nitrite reductase enzyme system is stimulated by the presence of nitrate in the gastrointestinal tract because the bacteria that can adapt to a high nitrate diet must be able to detoxify nitrite (Allison and Reddy, 1984; Alaboudi and Jones, 1985). This increase in nitrate and nitrite reductase synthesis can occur in as little as four hours but may require as long as three to six days for optimum adaptation of the community. This communal adaptation, however, can be lost just as quickly with the removal of nitrate from the diet of the host animal (Allison and Reddy, 1984; Alaboudi and Jones, 1985).

THE PHYSIOLOGY OF "NITRATE" TOXICITY

While nitrate can be absorbed through the rumen wall and into the bloodstream there is no evidence to indicate that this circulating nitrate can be reduced to nitrite in the bloodstream (Wang *et al.*, 1961, Winter, 1962). Up to 27% of nitrate can be excreted in the urine within a few hours of dosing (Wang *et al.*, 1961; Setchell and Williams, 1962), with some being recycled into the gut from the bloodstream via salivary and gastrointestinal secretions (Deeb and Sloan, 1975).

As the nontoxic nitrate is being absorbed, the nitrite accumulating in the rumen also is passing readily through the ruminal wall into the bloodstream. Once in the bloodstream, the nitrite ion oxidizes the ferrous iron of hemoglobin to ferric iron producing methemoglobin, a chocolate-colored pigment that is unable to carry oxygen. Signs of "nitrate" toxicity will begin to appear when 40 to 60% of the hemoglobin is converted to methemoglobin (Deeb and Sloan, 1975). While methemoglobin can be converted back to hemoglobin by NADPH reductase (Vertregt, 1977), this capacity is limited and easily exceeded. When concentrations of methemoglobin exceed 70-80%, death from methemoglobinemia usually occurs. However, individuals vary widely in susceptibility.

Ruminants and post-gastric fermenters are more susceptible to "nitrate" poisoning than monogastrics because gastrointestinal microorganisms are responsible for essentially all the reduction of nitrate and nitrite (Lewis, 1951). Species susceptibility to methemoglobinemia also is related to its capacity to reduce methemoglobin (O'Hara and Fraser, 1975). In studies of the rate of methemoglobin formation in man, goats, sheep, horses, cattle and pigs, the rate of methemoglobin reduction of ruminants was highest in sheep and lowest in cattle (Smith and Beutler, 1966). Hemoglobin from ruminants also was more easily oxidized to methemoglobin than was the hemoglobin of nonruminants (Smith and Beutler, 1966).

ACUTE AND CHRONIC "NITRATE" TOXICITY

Nitrate toxicity can be classified as acute or chronic (Wright and Davison, 1964). In acute cases, death may occur 2 to 3 hours after symptoms of a lethal dose appear or the animal may collapse and recover spontaneously. Collapse and death, when it occurs, is the result of hypoxia brought about by methemoglobinemia causing the oxygen transport capacity of blood to drop below that required for life (Deeb and Sloan, 1975). Symptoms of acute nitrate poisoning may include one or more of the following: staggered gait, excessive salivation, lethargy, accelerated pulse, labored breathing, muscle tremors, frequent urination, brownish discoloration of nonpigmented skin and vaginal membranes, collapse, coma and death. Blood samples taken within this time frame will be chocolate brown in color due to its high concentration of methemoglobin.

Additional problems have been associated with chronic nitrate toxicity, including abortion, decreased weight gain, decreased milk production, vitamin A deficiency and hyperthyroidism (Wright and Davison, 1964; Deeb and Sloan, 1975). Much contradicting data surrounds these problems and attempts to verify or refute them have had variable results (Deeb and Sloan, 1975).

Sublethal levels of nitrate have been reported to cause abortions in cattle. Abortions have been noted in cows eating high nitrate silage (Pfander *et al.*, 1964) and in cows grazing high nitrate weeds in Wisconsin (Sund *et al.*, 1957; Simon *et al.*, 1958, 1959a, 1959b). Abortions have been induced in pregnant heifers by feeding nitrate or placing nitrate into their rumens (Simon *et al.*, 1959a; Davison *et al.*, 1964). Johnson *et. al.* (1984) analyzed the nitrate contents of 227 stillborn or aborted fetuses and found that 83 had high nitrate levels in their aqueous humor; 54 of those fetuses also had high nitrate concentrations in their blood. Conversely, Winter and Hokanson (1964) found no adverse effect to pregnancy from feeding nitrate to 15 heifers.

The mechanism of abortion due to nitrate is not known exactly. Studies with guinea pigs suggest that fetal death is from hypoxia due to maternal methemoglobinemia (Sinha and Sleight, 1971). This hypoxia may be due to insufficient oxygen transfer to the fetus from the dam (Malestein *et al.*, 1980). Reduced oxygen transfer from the dam to the fetus results in intrauterine death.

Effects of nitrate on weight gain and milk production is much more variable. Reduced weight gains attributed to chronic nitrate poisoning have been reported by researchers in cattle, sheep and swine (Weichenthal *et al.*, 1963; Pfander *et al.*, 1964; O'Hara and Fraser, 1975). However, researchers have failed to demonstrate decrease in weight gain (Sokolowski *et al.*, 1961; Cline *et al.*, 1962; Smith *et al.*, 1962; Crawford *et al.*, 1966). Milk yields generally have not decreased in cases of chronic nitrate toxicity (Davison *et al.*, 1963; Crawford *et al.*, 1966; Farra and Satter, 1971). However, Wright and Davison (1964) found that milk production was reduced by nitrate consumption when feed consumption was depressed or cows were near collapse. Nielson (1974) found that milk yields were lowered consistently in cows on high nitrate forages.

While nitrates impair thyroid function in rats by competing with iodine for receptors and interfering with thyroxine synthesis (Lee, 1970; Deeb and Sloan, 1975), such effects have not been detected in cattle (Jainudeen *et al.*, 1965; Wright and Davison, 1964).

In research with rats, nitrites oxidized vitamin A and its precursor carotene in the gastrointestinal tract (Wright and Davison, 1964; Deeb and Sloan, 1975). Similar studies with nitrates showed no influence on carotene, vitamin A or vitamin A storage in the liver. Therefore, nitrites may destroy vitamin A in ruminants under certain conditions, but considering the amount of nitrite required, acute "nitrate" toxicity would be a far greater concern (Deeb and Sloan, 1975).

TOXIC LEVELS OF NITRATE AND VARIABILITY IN SUSCEPTIBILITY

The consequences of nitrate toxicity on beef cattle varies with the concentration of nitrate present and the reproductive state of the animal. For pregnant cattle, NO_3 concentrations below 1,500 ppm are considered safe for both cow and calf. Nitrate concentration in the range of 1,500 to 5,000 ppm may cause early term abortions and reduce breeding performance. When nitrate concentrations rise to 5,000 to 10,000 ppm, mid to late term abortions can occur, calves that are born can be weak, and growth and milk yields by cows are reduced. When nitrate concentrations in susceptible animals exceed 10,000 ppm, abortions, acute nitrate toxicity symptoms and death can occur.

Safe levels for non-pregnant beef cattle range form 0 to 5,000 ppm NO_3. Milk yields and growth rate are decreased with subacute symptoms starting at 5,000 ppm up to 10,000 ppm NO_3. Depending on the animal's susceptibility, acute nitrate toxicity symptoms and death can occur at NO_3 concentrations of 10,000 ppm or above.

An animal's susceptibility to nitrate toxicity can be altered based the animal's feeding behavior, previous adaptation and the type and form of its diet. Hungry animals eat more and are more likely to ingest a toxic dose of nitrate from marginally toxic feed than well-fed animals (Kretschmer, 1958). Environmental factors also can affect hunger; snow and ice cover can create hunger conditions that will increase intake when animals next receive feed. Many high nitrate feeds are highly palatable and digestible; this increases their consumption over that of less palatable, lower nitrate feeds.

Another factor which alter an animal's susceptibility to nitrate is adaptation. Adaptation can occur on two levels, one is the adaptation of the ruminal microorganisms to subtoxic levels of nitrate and nitrite. This occurs due to the inducible nature of the nitrate/nitrite reductase enzyme system; once adapted, microbes detoxify a greater amount

of nitrite than if they were not adapted. This allows the animal to tolerate a higher level of nitrate.

A second level of adaptation can occur in the animal's physiological response to long term subtoxic nitrate concentrations. These physiological adaptions include increased hemoglobin and blood volumes (Jainudeen *et al.*, 1964). The increased hemoglobin concentration helps to compensate for hemoglobin lost via methemoglobin formation and helps to maintain the animal's oxygen supply. The increased blood volume also helps to compensate for the vasodilation and the resulting low blood pressure caused by the presence of nitrite in the bloodstream.

Diet can affect susceptibility in several ways. High energy feeds may stimulate the increased growth of rumen microorganism which lowers the rumen pH into a range closer to the optimum pH of nitrite reductase and away from the optimum of nitrate reductase. This may increase the rate of reduction of both nitrate and nitrite in the rumen to non-toxic nitrogenous compounds (Burrows *et al.*, 1987). This allows the animal to consume a higher level of nitrate in the diet without experiencing ill effects. Conversely, research conducted by Smith *et al.* (1992) indicated that energy supplementation, in levels tested, had no effect on methemoglobin concentration. However, they demonstrate that a dietary protein supplement, two to four pounds of 16% CP daily, reduced maximum methemoglobin levels and increased the rate of reconversion back to normal hemoglobin (Smith *et al.*, 1992).

The form in which the nitrate is consume also is important. Nitrate in water is available immediately in the rumen and therefore is more toxic than nitrate released gradually from plant materials. Dry hays release their nitrate more quickly in the rumen (80% in 20 minutes) than lush, green forages (30% in 20 minutes) (Geurink *et al.*, 1979).

Finally, diets that produce elevated amounts of ammonia, such as feeds high in protein or nonprotein nitrogen, may inhibit the reduction of nitrate through negative

feedback mechanisms. This inhibition is the result of ammonia being one of the end products of nitrite reduction in certain bacteria.

CONVENTIONAL MANAGEMENT STRATEGIES

Feeds that are high in energy may speed nitrate and nitrite reduction by stimulating microbial growth and lowering rumen pH. This method of feeding a supplementary high energy feed has the additional benefit of diluting the nitrate.

Adaptation exploits the elevated levels of nitrate and nitrite reductase produced by rumen microbes due to the presence of elevated but non-toxic levels of nitrate in the diet. For this, nitrate is fed at an increasing level in the diet for several days before the animals are released on to the potentially toxic feed source.

A third method is to blend in a low nitrate feed with the high nitrate feed source so that the total dietary nitrate concentration is reduced to a manageable level.

A newer management strategy include the incorporation of tungsten into the diets of cattle on high nitrate feeds. Korzeniowski *et al.* (1980; 1981) showed that tungsten was incorporated in place of molybdenum into the nitrate reductase enzyme forming an inactive analog. Consequently, tungsten inhibits nitrite formation in the rumen. While this experimental method inhibited nitrite formation, tungsten has several adverse affects. Feeding tungsten inhibits sulfite and xanthine oxidase and decreases cellulose digestion and copper metabolism (Korzeniowski *et al.*, 1980). Other problems include accumulation of tungsten in the bones, organs, and other tissues of the animal's body as well as the excretion of tungsten into the milk of treated animals. Fertilizer produced from the manure of treated animals also might have an adverse affect on soil and root microbes and higher plants.

The major problem associated with these conventional methods is that the nitrate content of the animal's diet must be assessed beforehand. This requires that the nitrate content of various components of the animal's diet (feed, forage and water) be tested. In addition, samples to be tested must represent the variability in nitrate concentrations found

in the feed. For example, in order to accurately sample hay bales, individual core samples must be tested from between 20 and 40% of the total number of bales. Sampling of green, standing forages and hays has to be extensive due to the uneven accumulation of nitrate across a given field creating what are known as "hot spots". Animals fed hay baled from one of these spots may experience nitrate toxicity while other animals fed on bales from other areas of the same field may be perfectly safe. In addition, animals grazing these "hot spots" as green forage are in danger.

A final strategy for combating nitrate toxicity is a direct-fed microbial product. In order for this product to be effective, the microbe used would have to survive and establish in the gastrointestinal tract in such numbers so as to reduce the concentration of nitrite present. Additional goals of such a product would be long term survival and establishment with long term protection based on constitutive or low threshold stimulation of the nitrate and nitrite reductase enzymes. Such a product, once established, might protect an animal indefinitely and reduce that animal's susceptibility to nitrate toxicity for the life to the animal.

DIRECT-FED MICROBIALS

The rumen is a complex ecosystem with a diverse community of microbes. The ruminal bacteria are adapted to grow and reproduce at a pH between 5.5 and 7.0 in the absence of oxygen at temperatures of between 37 to 40°C. The ruminal bacteria grow in a constantly changing environment of fermentation products and substrate from feed ingesta. The steady supply of nutrients from feed ingesta and continuous removal of fermentation products maintains a relatively constant condition for dense populations of bacteria to develop (Hungate, 1966).

Microbes play an essential role in the nutrition of ruminants. The bacteria in the rumen produce energy for their life processes largely by fermenting organic compounds found in feedstuffs ingested by the host or produced from the host's cells. The process of fermentation yields organic acids which are excreted by the bacteria and absorbed into the bloodstream of the host where they are utilized as the primary energy source for the ruminant. In addition, bacteria are a significant source of protein for the host.

Into this complex and important ecosystem, a direct-fed microbial product must successfully compete and adequately perform its job. Direct-fed microbial products can be defined as live bacterial or yeast preparations that are administered orally or added to feeds or premixes. The theory behind direct-fed products is that the selected cultures might augment normal ruminal microbiotic function making the system utilize available nutrients more efficiently, or make the host animal more resistant to stress-induced diseases or even help the microbiota detoxify noxious compounds detrimental to the host. These tasks can be performed by the direct-fed organism in the lumen of the gastrointestinal tract or by their establishment on the epithelial surfaces of the tract or in the mucosal biofilm associated with that tract.

Although direct-fed products have been used for years, their exact modes of action are not known. Most products are assumed to work by competitive exclusion, antibiotic or bacteriocin production and immuno-stimulation.

Regardless of mode of action for a certain product, products ultimately succeed or fail based on the allogenic and autogenic factors present in the environment and in the strains selected as direct-fed microbial products. In brief, allogenic factors are those influences on the microbiota coming from the host, the host's ingesta and the environment (Savage, 1989). Autogenic factors, on the other hand, are influences of the resident and introduced microbes on themselves and on other microorganisms (Savage, 1989).

Competitive exclusion, also known as competitive antagonism or colonization resistance, is a natural defense system found in the host animal's gastrointestinal tract formed by the indigenous microbiota. The microbiotic barrier created makes target epithelial cells unavailable for pathogen colonization or creates an environment detrimental to pathogens or other novel microbes.

Target cells are rendered unavailable to pathogens or less efficient indigenous bacteria simply by a direct-fed organism being attached to the particular site preferred by the other organisms. Allogenic factors affecting this process include the types and number of binding sites presented by the host, the rate of cell sloughing of the host, the passage rate of ingesta through the host, the type of nutrients available from the ingesta and the pH maintained in the gastrointestinal tract by the host on a particular diet. Autogenic factors influencing this process include the ability of the direct-fed organism to bind to the proper sites on the epithelia and the ability of the direct-fed organism to compete and survive in the gastrointestinal tract on the nutrients and under the conditions present.

In addition, the doubling rate of direct-fed organisms is very important. Those direct-fed organisms with doubling rates slower than the rate at which epithelial cells are sloughed or slower than the passage rate of the ingesta in the gastrointestinal tract, must be continuously introduced into the gastrointestinal tract or their protection or efficiency

will be lost over time. On the other hand, those direct-fed organisms with doubling times equal to or greater than the host's sloughing or passage rates, once established, can maintain their population level and, in the latter case, increase it. This type of doubling rate would help maintain or increase the level of protection or efficiency over time assuming all other conditions remained constant.

The second major factor to consider in competitive exclusion, that of creating an environment detrimental to pathogens or other less desirable microbes, generally is controlled by autogenic factors. A pathogen or less desirable microbial population may be reduced or eliminated from the gastrointestinal tract by the active metabolism of selected direct-fed cultures. This metabolism could produce byproducts that inhibit the growth and survival of certain other microbes. Such byproducts include hydrogen peroxide, certain organic acids or bases, inefficient or harmful analogs of compounds required for the growth and survival of pathogens or less desirable microbes and compounds that alter the local redox potential. These inhibitory effects may be local, associated only with the binding site regions on the epithelia or in the biofilm, or systemic, inhibiting growth and survival of less desirable microbes throughout an entire region of the gastrointestinal tract.

Another theory, that of antibiotic or bacteriocin production by the direct-fed organism is based mostly on autogenic factors. Antibiotics and bacteriocins are compounds produced by bacteria solely for the purpose of inhibiting the growth or survival of other strains of bacteria. These compounds may be strain specific or broad ranged and generally work by inhibiting transcription, translation, and protein synthesis or by disrupting membrane permeability, nutrient transport or energy production in susceptible strains. (Mikolajcik and Hamdan, 1975; Savage, 1987; Gaya and Verhoef, 1988; Itoh and Freter, 1989). A direct-fed culture operating under this theory would need to survive and produce functional antimicrobial compounds under ruminal conditions. These antimicrobial compounds then would need to have contact with their target microorganisms. This contact might require that the direct-fed and target microorganisms

be in close proximity depending on the sensitivity of the target microbe, the ability of the antimicrobial to defuse through the surrounding substrates and the toxicity of the antimicrobial.

The final theory, that of immuno-stimulation by direct-fed microorganisms, is the most recently advanced. Simply stated, the direct-fed culture stimulates the host animal's immune system to produce more antibodies that recognize other microorganisms such as pathogens or cancerous cells of the host (Perdigon *et al.*, 1987). This stimulated immune response has been demonstrated to be initiated by increased production of gamma interferon, Y-IFN, in some host species due to certain direct-fed cultures (De Simone *et al.*, 1986). The allogenic and autogenic factors regulating immune stimulation have yet to be determined specifically. Germ-free animals have a much reduced immune system when compared to conventional animals; the introduction of any new strain of bacteria into a host may elicit an immune stimulation response (Abrams and Bishop, 1965).

DIRECT-FED MICROBIAL CULTURES

Direct-fed products have been marketed for virtually all domesticated animals. These animals include beef and dairy cattle, sheep, swine, horses, goats, poultry, dogs and cats. In addition, direct-fed products have been developed for humans.

Many livestock direct-fed microbial products have been marketed as a means to increase the survival rate or decrease the mortality in young animals experiencing nutritional, environmental or emotional stress or directly following antibiotic treatment. Other products have been marketed as a means to decrease the prevalences of certain disease states or gastrointestinal deficiencies such as scours, mastitis, metritis, lactose maldigestion and hypercholesterolemia. Finally, some direct-fed products have been marketed on the basis of increased live weight gains, increased daily gains and advantages in feed efficiency and utilization.

Regardless of the purpose or the host species involved, the number of microbial cultures used in direct-fed products is relatively small. Most are from the genus *Lactobacillus* and include such species as *L. acidophilus*, *L. brevis*, *L. bulgaricus*, *L. casei*, *L. cellobiosus*, *L. curvatus*, *L. delbrueckii*, *L. fermentum*, *L. lactis*, *L. plantarum* and *L. reuterii*. The second most common genus is *Streptococcus* and includes such species as *S. cremoris*, *S. diacetylactis*, *S. faecium*, *S. intermedius*, *S. lactis* and *S. thremophilus*. Closely following *Streptococcus* are the *Bifidobacterium* species *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. infantis*, *B. longum* and *B. thermophilum*. Next comes members of the genus *Bacteroides* with the strains *B. amylophilus*, *B. capillosus*, *B. ruminocola* and *B. suis*. Other bacterial strains include *B. coagulans*, *B. licheniformis*, *B. pumilus*, *B. lentus* and *B. subtilis* from the genus *Bacillus*. The genus *Pediococcus* also is represented in the direct-fed market by such strains as *P. acidilacticii*, *P. cerevisiae* and *P. pentosaceus*. Other bacterial strains include *Leuconostoc mesenteroides* and the

Propionibacterium strains *P. freudenreichii*, *P. shermanii* and *P. acidipropionici* (Hutcheson, 1991).

Several strains of yeast and fungi also have been marketed as direct-fed microbial products. These cultures include *Aspergillus niger*, *A. oryzae*, and *Saccharomyces cerevisiae* (Hutcheson, 1991).

Efficacy, particularly in the animal direct-fed area, has long been of concern for researchers and manufacturers of direct-fed products. In many cases, the data supporting the products in question are limited, flawed or non-existent. In most cases, particularly with the data gathered by manufacturers, the research consists of information collected from *in vitro* trials and/or poorly controlled feeding trials with limited numbers of animals with large variations in animal's size, sex, diet and environment. This is not to suggest that direct-fed products do not or cannot work, but to point out that more careful and extensive research is required to determine the true value of many direct-fed microbial products being marketed today.

One problem that can account for variable efficacy is the manufacturing and packaging of the products. Cultures that prove efficacious *in vitro* often do not survive the delivery system. Survival problem can result from the culture being unable to withstand one or more of the following processes; lyophilization, dehydration and high temperatures during pelleting process (as seen with top dressing and pellet type products); rehydration in the gastrointestinal tract; exposure to oxygen during processing; inability to escape from the final form into the gastrointestinal tract (as seen with both pellets and gels); survival with premixes and minerals that are present in the final form of the product. In a study conducted on fifteen lactobacilli feed supplements from eight different manufacturers in 1981, the numbers of lactobacilli contained in the products varied widely. All fifteen products were plated from their final forms onto lactobacillus selection agar (LBS) and LBS plus 0.2 percent oxgall (LBSO). While all products claimed to have counts from between $1x10^7$ to $1x10^9$ CFU/g, only two had counts greater than $1x10^7$

CFU/g. Two additional products had counts greater than $1x10^5$ CFU/g and the rest had counts of $6.9x10^4$ CFU/g or less; three of the products had counts less than 100 CFU/g on LBS agar (Gilliland, 1981).

Other factors that affect efficacy of a product and the dosage level required include the animal species tested, the animal's stage of maturity, plane of production, level of stress, and the environment. Because of this wide spectrum of variables, it is surprising that the range of responses to direct-fed microbial products is broad. What is noteworthy from the literature is not so much the lack of response, but the plethora of positive responses spanning a huge range of experimental protocols, animal species, geography and products tested (Fox, 1988).

In a brief review of literature on cattle studies, positive results have been reported with calves under production conditions. In one study, more lactobacilli and fewer coliforms were found in the small intestines of calves fed pasteurized whole milk containing *L. acidophilus* than in non-treated control calves (Gilliland, 1980). In another study, a 36.9% reduction in calf scours was attributed to the use of a *Lactobacillus* inoculant (Tournot, 1976). Under feedlot conditions, a 13.2% increase on average daily gain, a 6.3% increase in feed efficiency and a 27.7% decrease in morbidity was observed in cattle given Probios (Pioneer Hi-Bred), a lactic acid bacteria (LAB) combination, when compared to untreated cattle (Wren, 1987a,b). Two important factors that affect the performance of direct-fed products are dosage level and establishment.

Dosage level, in many cases, is fully as important as the species and strain of the direct-fed culture used. Factors that affect the minimum effective dose include product purity, batch to batch consistency and product shelf-life. In products developed for cattle and pigs to improve average daily gain and feed efficiency, direct-fed microbial manufacturers recommend a minimum dose in the range of $1x10^8$ to $1x10^{10}$ CFU/head/day. Those companies that offer guaranteed microbial counts employ dose ranges from $1.5x10^8$ to $1x10^{10}$ CFU/dose.

Site of establishment of a direct-fed culture also is very important to its performance *in vivo*. In general, a culture may establish in one or more of three areas of the gastrointestinal tract, the epithelial surface, the biofilm layer or the lumen of the gastrointestinal tract. In addition, the culture may establish one or more of the regions of the gastrointestinal tract. These regions include the rumen of ruminants or the intestines.

Cultures that do not establish in the biofilm layer or on the epithelial surface must reproduce quickly (faster than the rate of passage of the gastrointestinal tract) or be dosed on a continual basis. If not, the effects of these cultures will be lost over time as they are flushed through the digestive tract and eventually voided by the animal.

Most successful direct-fed cultures become established in the digestive tract. Both lactobacilli and streptococci are known to attach to the epithelial surface of the gastrointestinal tract (Fuller *et al.*, 1978). Factors affecting attachment to the epithelia include the sloughing rate of epithelial cells and, in some cases, host specificity of the direct-fed culture (Wesney and Tannock, 1979; Barrow *et al.*, 1980).

Other bacteria, such as members of the genus *Bacillus*, may establish and have their effect in the biofilm layer that covers the surface of gastrointestinal epithelia.

Finally, the region of the gastrointestinal tract where a direct-fed culture is to establish and have an effect can alter the efficacy of the product. Those cultures intended to establish and modify the functions of the lower digestive tract (small and large intestines, cecum) must be able to survive the high acidity of the gastric stomach and be bile tolerant. Cultures that are deficient in one or both of these characteristics may not survive to have an effect or will survive in such low numbers as to be ineffective.

The method of tracking a culture can affect its presumed efficacy. Most direct-fed cultures today are monitored using differential-selective media and confirmed through carbohydrate fermentations or other biochemical tests.

TRACKING INTRODUCED CULTURES IN COMPLEX ENVIRONMENTS

A major difficulty associated with efficacy studies of direct-fed cultures is the problem of accurate and consistent recovery and enumeration of the direct-fed organism *in vivo*. The indigenous population present in the normal gastrointestinal tract may exceed 100 billion CFU/g ($1x10^{11}$ CFU/g) (Wolin, 1981; Savage, 1986). Techniques used to detect a single species in this milieu and provide an accurate and consistent count have included selective-differential medias, plasmid profiling and DNA "fingerprinting".

The most common but least accurate method of identification of direct-fed microbial products is the use of selective-differential media (Tannock, 1988). In brief, differential media does not inhibit the growth of the vast majority of the microbes present but does allow an observer to identify a specific strain or genus of bacteria within that population. This usually is carried out through strain or genus specific chemical reactions with the media and is commonly detected as a color change. On the other hand, selective medias attempt to inhibit the growth of all microorganisms present except the species or genus of interest. This process usually is performed through the use of antibiotics, unique nutrient sources or other inhibitory compounds. Selected colonies on this type of media may or may not be distinguished based on colony morphology. A differential-selective media is a combination of the two types of media in which the genus or species to be enumerated can be differentiated from the background microbes that have survived the selective properties of the media.

Once colonies have been isolated on differential-selective media, the next step is to perform a series of biochemical reaction tests; plasmid profiling or DNA "fingerprinting" serves as the final confirmation tool.

While differential-selective media can be effective as an isolation tool, their use as a strain specific identification tool is limited, particularly when dealing with complex

environments. In most cases, especially for those strains currently being used as direct-fed cultures, current differential-selective medias are not strain specific. Therefore, in order to distinguish between the introduced strain and native strains that may be present in the indigenous population, additional confirmation steps must be performed.

Once suspected colonies are isolated, the most common confirmatory tests performed are biochemical tests (Tannock, 1988). These biochemical tests, which normally consist of specific carbohydrate fermentations and other strain specific reactions, are intended to identify specific strains of bacteria based on colorimetric reactions. One problem associated with biochemical testing is that even though two suspect colonies may share identical phenotypic characteristics and test reactions this doesn't necessarily indicate that they are genotypically identical. Closely or not so closely related indigenous strains may share identical phenotypes and reaction profiles with introduced strains. More accurate identification requires genetic analysis to distinguish between closely related strains.

Plasmid profiling is simply the comparison of isolated plasmid DNA patterns of suspected colonies with the patterns generated from pure isolates of the introduced strain. Plasmid profiling has been used to distinguish strains of lactobacilli in the digestive tract of piglets (Tannock *et al.*, 1990), to monitor populations of specific strains of *Lactobacillus plantorum* in silage fermentations (Hill and Hill, 1986) and to monitor *Bacillus pumilus* inoculant strains in hay (Hendrick *et al.*, 1991).

While plasmid profiling is a more specific tool to confirm the identity of a strain, plasmids often are transient genetic elements in a bacterial cell. For example, plasmids may be subject to conjugal transfer within or between species in an environment. Specifically, conjugal transfer of plasmids has been noted between strains of *Lactobacillus reuteri* and between lactobacilli and *Enterococcus faecalis* (Tannock, 1987) and a number of Gram-positive bacteria (Shrago *et al.*, 1986). Other reported cases include transfer between streptococci and gastrointestinal strains of lactobacilli and betweeen strains of

Lactobacillus plantorum (Vescova *et al.*, 1983; Shrago *et al.*, 1986). In addition, there may be a natural loss of plasmids within certain species of bacteria over time. This is due to incongruent replication rates between those plasmids and their host cells. A final problem associated with plasmid profiling is that the introduced strains may not have any plasmids or may lack a unique plasmid profiles. Thus plasmid profiling, while useful for identification of some strains, is not the most certain method of strain identification.

The most positive method of strain identification involves the profiling of the bacterial genome itself. This is accomplished by digesting the bacterial chromosome with a combination of restriction enzymes that produces a unique chromosomal profile. Once this is performed on suspected isolated colonies, the pattern generated can be compared to a parent profile to determine its identity. This method has been used successfully in the recent past to identify specific strains of bacteria including *Escherichia coli*, *Shigella* ssp. and *Propionibacterium freudenreichii* using pulsed-field gel electrophoresis (Ogram and Sayler, 1988; Venkatesan *et al.*1988; Rehberger, 1993).

Care must be taken to ensure that the combination of restriction enzymes used creates a truely unique chromosomal profile. Other problems include difficulties in isolating and extracting intact DNA for the chromosomal profiling proceedure. Other methods of strain identification used in the past include phage susceptibility, antibiotic resistance, cell wall fatty acid profiles and cell surface proteins. The disadvantage of these techniques is that they are slow and do not always discriminate between strains within a species.

PROPIONIBACTERIA

Propionibacteria are characterized as Gram-positive, catalase-positive, nonspore-forming, nonmotile, microaerophilic, pleomorphic, rod-shaped bacteria (Hettinga and Reinbold, 1971).

Industrially, the propionibacteria are an important group of organisms used primarily by the dairy industry as starter cultures for Swiss-type cheeses (Langsrud and Reinbold, 1973). Other industrial applications of propionibacteria include their use as direct-fed microbial products for humans (Kornyeva, 1981; Nabukhotnyi *et al.*, 1983), as inoculants for grain and silage (Woolford, 1975; Flores-Galarza *et al.*, 1985), and as producers of vitamin B_{12} and propionic acid (Perlman, 1978; Playne, 1985).

One of the distinguishing characteristics of propionibacteria listed in Bergey's Manual of Determinative Bacteriology is the ability of some strains to reduce nitrate and nitrite (Buchanan and Gibbons, 1974). In *P. acidipropionici*, nitrate reductase synthesis is constitutive that also is nitrate stimulated (Van Gent-Ruijters *et al.*, 1976; Kaspar, 1982).

The reduction of nitrate is an energetically favorable process in propionibacteria (Van Gent-Ruijters *et al.* 1975; Kaspar, 1982; Allison and MacFarlane, 1989). In studies with *P. acnes*, cell yields were higher (12-22%) in chemostats with nitrate added (Allison and MacFarlane, 1989) indicating that the metabolism of nitrate was energetically favorable. In work with *P. acidipropionici*, growth rates were increased 20-50% by the presence of 10mM nitrate (Kaspar, 1982). In other work with *P. acidipropionici* (Van Gent-Ruijters *et al.*, 1975), molar growth yields were increased in the presence of nitrate; this was attributed to an increased production of ATP through increased citric acid cycle activity and by oxidative phosphorylation coupled to nitrate reduction.

In contrast, the reduction of nitrite is energetically unfavorable in such species as *Clostridium tertium* (Hasan and Hall, 1977) and *Escherichia coli* (Cole, 1978). In some

species of propionibacteria, as little as 1mM of nitrite inhibited growth; these include *P. acidipropionici*, *P. freudenreichii*, *P. jensenii*, *P. shermanii* and *P. thoenii* (Kaspar, 1982). In the case of propionibacteria, the nitrite ion is unable to replace nitrate as the electron acceptor in phosphorylation-coupled electron transfer. The nitrite ion also shows, to varying degress, a toxic effect on propionibacteria (Kaspar, 1982). This evidence suggests that nitrite reductase is a detoxification mechanism and not an energy producing system in propionibacteria. The production of toxic nitrite from the energetically favorable nitrate reduction may account for the coupling of nitrate reductase to nitrite reductases in some species of propionibacteria.

Future prospects for the *in vivo* reduction of nitrite in beef cattle appears encouraging. Although Kaspar (1982) indicated that some propionibacteria strains were inhibited by nitrite, the strains he used were not selected for their ability to denitrify and were not nitrate stimulated before exposure to nitrite. Adequate strain selection is a key component in the successful reduction of nitrite *in vivo*. Therefore, our first consideration was to determine the best strains of propionibacteria to use based on *in vivo* studies of their individual abilities to reduce nitrite. Once the strains were screened based on quantitative nitrite reduction studies, the next factor we considered was the ability of the selected strains to survive and reduce nitrite in the rumen. Initial studies then were conducted using ruminal fluid *in vitro* as a screening process. Those strains that survived and reduced nitrite in rumen fluid *in vitro* were used for *in vivo* efficacy trials. Factors we considered from *in vivo* trials include survival in the functioning rumen, level of inoculum, establishment of the strain in the rumen, site of establishment, population level of established culture and length of establishment. The ability to track our culture was extremely important in gathering information on *in vivo* populations. Fortunately, our lab had developed a reliable differential-selective media capable of enumerating propionibacteria in complex environments.

The final and conclusive test was toxicity trials with test animals. These trials determined if the established population of propionibacteria were capable of reducing nitrite exposure to the animals and could protect them from "nitrate" toxicity. These and many other factors were considered in our studies.

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CHAPTER III

The selection and ruminal establishment of a denitrifying *Propionibacterium* strain in beef cattle

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ABSTRACT

Strains of *Propionibacterium* were screened for denitrification and ruminal establishment to examine their possible use as a direct-fed microbial for nitrate toxicity. Seventeen strains were found to be capable of denitrification and were tested for their ability to denitrify high levels of nitrate (20,000 ppm). Of those seventeen strains, only two strains, P5 and P42, reduced these higher levels of nitrate. When media was inoculated with strain P5 at 10^5 CFU/ml, 50% of the nitrate was reduced in 24 h. Nitrite accumulation started at 12 h and continued for the next 60 h, after which nitrite was reduced. Strain P42 reduced nitrate and nitrite at slower rates than P5. The enzymes for nitrate and nitrite reduction in strain P5 were produced under both aerobic and anaerobic conditions in the absence of nitrate. However, nitrate reductase activity was stimulated by the presence of nitrate. Stimulated cells were able to reduce 80% of the available nitrate in 6 h; non-stimulated cells required 16 h to reduce an equivalent amount of nitrate.

Ruminal establishment trials indicated animals inoculated daily with P5 at 10^5 CFU/ml of rumen volume had counts greater than 10^4 CFU/ml by day 30. In a second trial, animals inoculated daily with P5 at 10^7 CFU/ml of rumen volume had counts greater than 10^5 CFU/ml by day 30. At 115 days post-inoculation, P5 populations were maintained at their established levels. Propionibacteria populations were monitored using a selective-differential medium. Typical colonies were confirmed as P5 based on plasmid DNA analysis.

(Key Words: *Propionibacterium*, Beef Cattle, Denitrification)

INTRODUCTION

"Nitrate" toxicity, often seen in ruminants, is caused by the reduction of excessive levels of nitrate to nitrite by the normal rumen microbiota during anaerobic respiration. Nitrite accumulates in the rumen because the normal rumen microbiota reduce or utilize nitrite very slowly. The nitrite passes readily through the rumen wall into the bloodstream where it binds with hemoglobin to form methemoglobin. Methemoglobin is unable to carry oxygen and, in acute cases, may cause death in the host animal due to anoxia. Various method have been explored to reduce the impact of "nitrate" toxicity on ruminants. These methods include the incorporation of nitrate into the diet (Majak *et al.*, 1982) to adapt the rumen microbiota and the incorporation of a nitrate reductase inhibiting compound, tungsten, (Korzeniowski *et al.*, 1980, 1981) into the diets of ruminants. Other methods include feed management strategies that blend high nitrate feeds with low nitrate feed sources as well as energy (Burrows *et al.*, 1987) and protein (Smith *et al.*, 1992) supplementation. These methods require continual incorporation during the course of high nitrate exposure to ensure adequate protection from "nitrate" toxicity. Another unexplored possibility is to develop a direct-fed microbial product capable of reducing nitrite and thus preventing the formation of methemoglobin.

Propionibacterium are an industrially important group of bacteria used primarily as starter cultures for Swiss-type cheeses (Langsrud and Reinbold, 1973), grain and silage inoculants (Flores-Golarza *et al.* 1985; Tomes, 1989; Dawson *et al.*, 1991) and the production of vitamin B_{12} and propionic acid (Perlman, 1978; Playne, 1985). Propionibacteria are a normal constituent of the rumen accounting for 1.4% of the total rumen population ($1x10^9$-$1x10^{10}$ CFU/ml) (Oshio *et al.*, 1987). Some strains of propionibacteria also are known to reduce nitrite as well as nitrate to other non-toxic compounds (Buchanan and Gibbons, 1974). While the use of propionibacteria as direct-fed microbial cultures for livestock production has been limited, their ability to function in the rumen as well as to reduce nitrite makes them a potential candidate for use as a direct-fed microbial product for "nitrate" toxicity.

The purpose of this study was to identify those propionibacteria strains capable of nitrite reduction, to determine their survival and denitrification in an *in vitro* ruminal fluid system and to determine the survival and establishment *in vivo* of a selected propionibacteria strain in the rumen.

MATERIALS AND METHODS

Bacterial strains

Propionibacterium strains were obtained from the Oklahoma State University Department of Animal Science culture collection. Strains were grown at 32°C in sodium lactate broth (NLB) or on sodium lactate agar (NLA) (Hofherr and Glatz, 1983). The culture collection and subsequent ruminal isolates were maintained in NLB supplemented with 10% glycerol at -75°C.

Experimental designs and procedures

Qualitative strain selection

Propionibacterium strains were grown in 10 ml tubes of NLB supplemented with 1% KNO_3 for 36 hours at 32°C. After 36 hours, qualitative nitrate and nitrite reduction was determined in the 10 ml tubes using the colormetric assay developed by Schneider and Yeary (1973).

Quantitative strain selection

Propionibacterium strains capable of denitrification were grown in 100 ml of NLB supplemented with KNO_3 (20,000 ppm). Nitrate concentrations were monitored over time using a nitrate ion specific electrode (Hach Company, Ames, Iowa). Nitrite concentrations were monitored over time using a colormetric assay (Schneider and Yeary, 1973).

In vitro ruminal fluid survival

Propionibacterium strains capable of reducing high levels of nitrate (20,000 ppm) were inoculated into 200 ml of freshly collected ruminal fluid supplemented with 20,000 ppm KNO_3. Strains were inoculated at $1x10^7$ CFU/ml of rumen fluid in duplicate flasks with two additional flasks maintained as uninoculated controls. The ruminal fluid was incubated at 39°C with agitation. Samples were taken at 0, 12, 24, 36 and 48 hours and analyzed for propionibacteria populations. Serial dilutions of each sample were plated on propionibacteria selective agar (PSA). Plates were incubated for seven days at 32°C anaerobically to enumerate propionibacteria populations.

Inducibility of denitrification

To determine if denitrification could be induced in the selected strain an additional *in vitro* experiment was conducted. The selected strain was grown in either NLB or NLB supplemented with 0.1% KNO_3 at 32°C. These *Propionibacterium* treatments were then used as the inoculum at $1x10^7$ CFU/ml for duplicate 500 ml flasks of NLB supplemented with 500 ppm KNO_3 and incubated at 39°C to resemble concentrations and conditions found in the rumen. Samples were collected every hour

for the first four hours with two additional samples at 6.5 and 19.5 h for the determination of nitrate concentration using an ion specific electrode.

In vivo establishment studies

Two *in vivo* establishment trials were conducted using a freeze-dried preparation of P5 at two different inoculation levels (10^5 CFU/ml of rumen fluid and 10^7 CFU/ml of rumen fluid). In both trials, 10 crossbred heifers fitted with ruminal cannulas were fed a low nitrate 50:50 concentrate: roughage diet balanced for protein, minerals and vitamins. Animals were separated spatially to avoid cross-contamination; two animals were maintained as uninoculated controls. Experimental animals received a daily dose of propionibacteria culture as a top dressing on their feed. Ruminal samples (250 ml) were collected 6 h after daily inoculation via rumen cannulas on days 0, 1, 2, 3, 4, 5, 7, 10, 14, 18, 21, 24, 29, and 32. These samples were serially diluted and plated (10^{-3}, 10^{-4}, 10^{-5}) on PSA. Plates were incubated for 7 days under anaerobic conditions at 32°C. Following incubation, suspected colonies were enumerated and isolated. Plasmid DNA from suspected colonies was isolated and purified by a rapid mini-scale procedure (Rehberger and Glatz, 1990) and analyzed by agarose gel (0.7%) electrophoresis (Maniatis *et al.*, 1982). Plasmid profiles generated from isolated colonies were compared to the profile of the inoculated strain (Rehberger and Glatz, 1990) to confirm the strain identification.

RESULTS

Strain selection

Seventeen of 154 *Propionibacterium* cultures reduced nitrate and nitrite. The denitrifying strains were *P. acidipropionici* strains P3, P5, P5-3, P5-23, P11, P42, P58, P90, P105, P108, P111; *P. freudenreichii* strains P22, P75 P80, P104, P120; *P. jensenii* strains P9. Of those 17, two cultures, *P. acidipropionici* strains P5 and P42, were capable of reducing high levels of nitrate (20,000 ppm). *In vitro* studies indicated that P5 was able to reduce nitrate from 20,000 to 2,000 ppm in 84 h; P42 took 108 h. NLB supplemented with 20,000 ppm nitrate inoculated with P5 at $1x10^5$ CFU/ml showed a 50% reduction in nitrate concentration at 24 h. Nitrite accumulation began at 12 h, continued to 60 h, but had decreased by 90% by 84 h (figure 1). *In vitro* ruminal fluid survival of P5, inoculated at $1x10^7$ CFU/ml, was $8.7x10^6$ CFU/ml at 1 h (87%), $5.6x10^6$ CFU/ml at 24 h (56%) and $8.0x10^5$ CFU/ml at 36 h (1%). Therefore P5 was chosen for *in vivo* establishment studies.

Inducibility of denitrification

The P5 culture grown in the presence of 0.1% KNO_3 reduced 80% of the nitrate in 6 h. In contrast, a non-induced culture required 16 h to reduce an equivalent amount of nitrate (figure 2). However, nitrate concentrations before 4 h or at 20 h were not different.

In vivo establishment studies

Trial 1

Five of eight heifers fed P5 at $1x10^5$ CFU/ml rumen fluid/day had detectable counts of propionibacteria ($>10^3$ CFU/ml) by day 18. By day 30 of feeding, eight of eight heifers had counts greater than 10^4 CFU/ml. Neither of the two control animal had detectable propionibacteria counts. Counts taken two and ten days after inoculation had ceased showed similarly high populations indicating that the organism had been retained.

Trial 2

Eight of eight heifers fed P5 at $1x10^7$CFU/ml rumen fluid/day had propionibacteria counts greater than 10^3 CFU/ml by day 10. Again, neither control animal showed a detectable propionibacteria count. Counts taken two and ten days after inoculation had ceased showed that populations remained constant. In trials 1 and 2, plasmid profiles generated from all colonies recovered were identical to the profile of strain P5 (figure 3) which exhibits a single 6.7 kb plasmid (pRGO1).

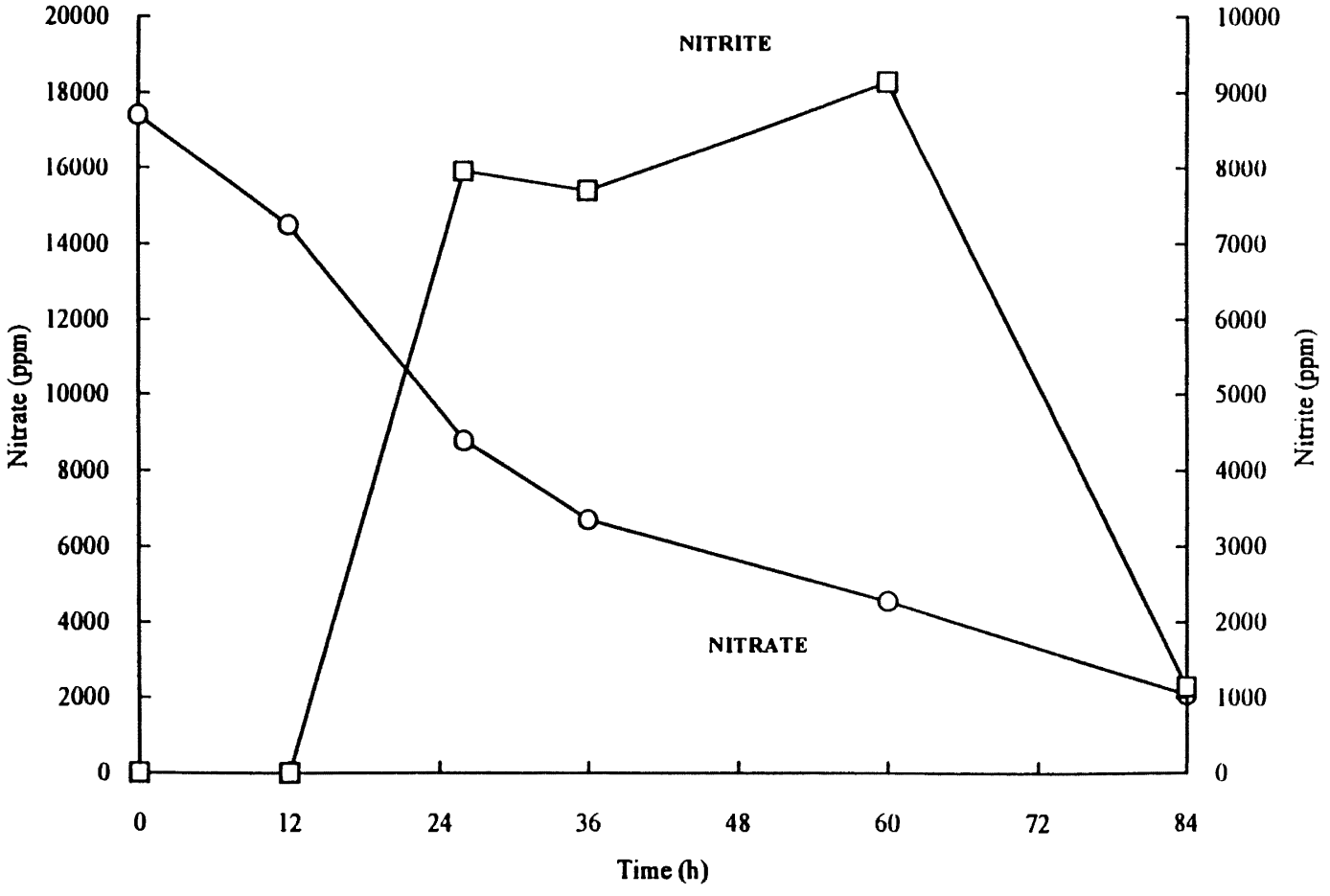

Figure 1. ***In vitro*** **denitrification of P5 in NLB.**

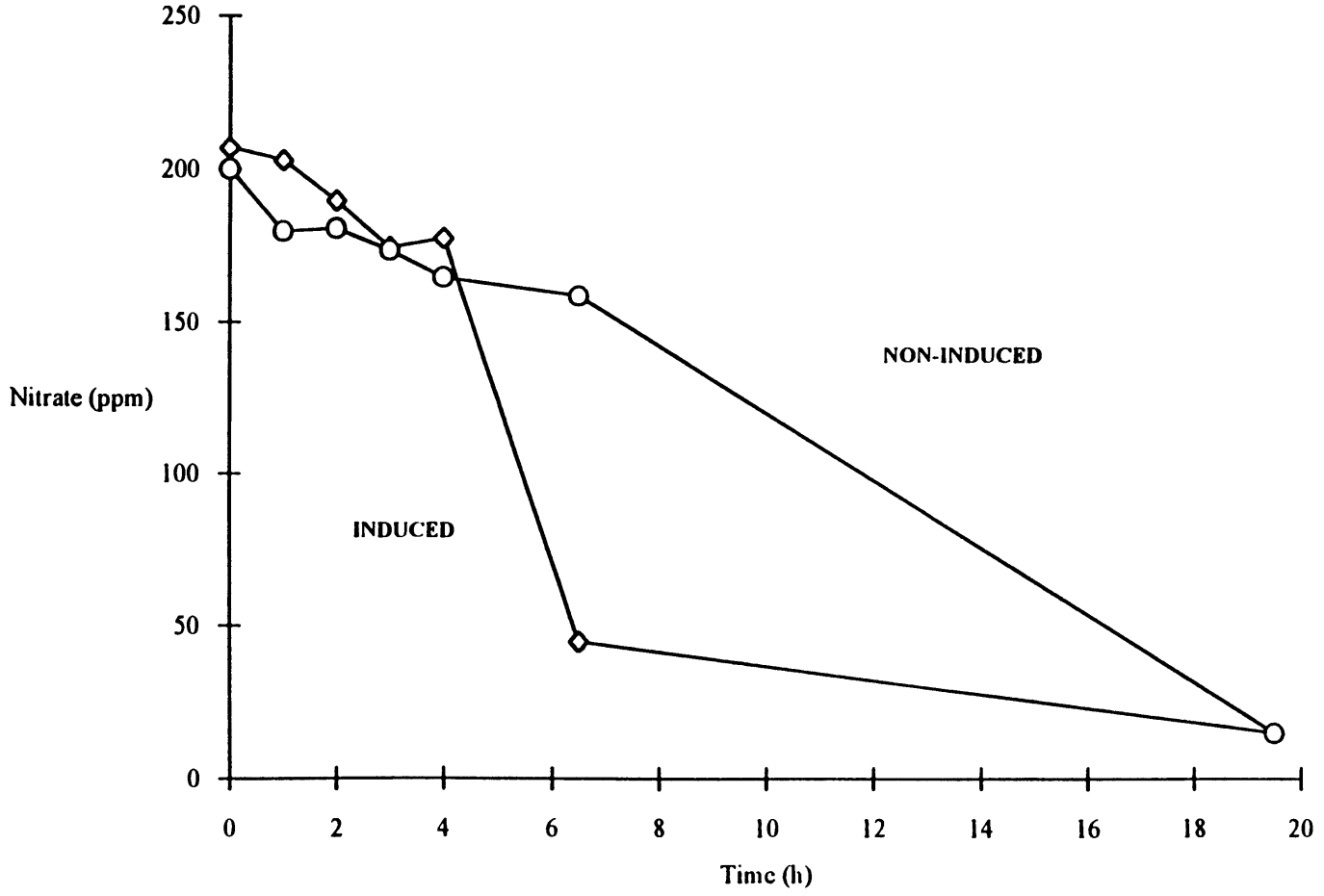

Figure 2. Induced vs. non-induced *in vitro* denitrification of P5.

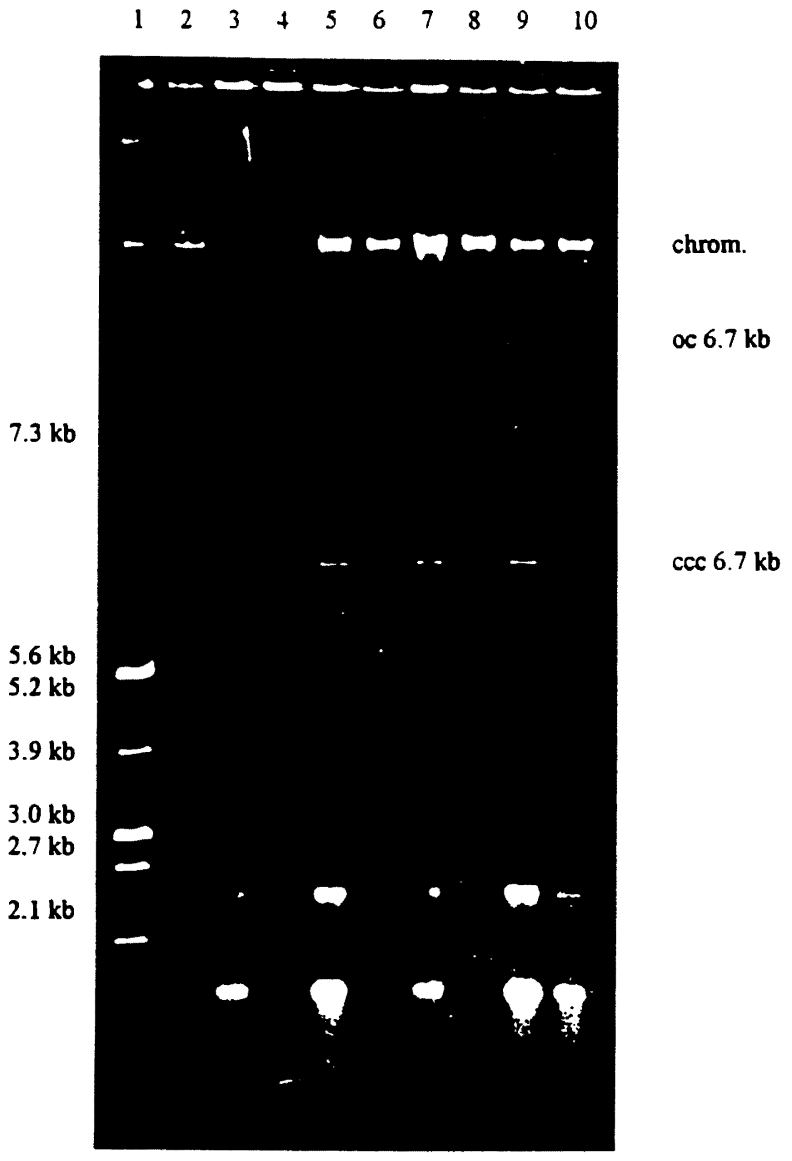

Figure 3. Plasmid DNA profiles of propionibacteria ruminal isolates: (1) *Escherichia coli* V517; (2) strain P5; (3) strain P5; (4) 641-1; (5) 610-1; (6) 610-2; (7) 610-3; (8) 634-1; (9) 634-2; (10) 634-3.

DISCUSSION

Of the 154 propionibacteria strains screened, 11% exhibited the capacity to denitrify. Of that 11%, the 11 strains of *P. acidipropionici* accounted for 65% of denitrifiers and 7% of total strains tested. The 5 *P. freudenreichii* strains accounted for 29% of denitrifiers and 3% of total strains tested. Finally, the *P. jensenii* strain represented 6% of denitrifiers and 1% of total strains tested. These results agree with those reported in Bergey's Manual of Determinative Bacteriology (8th ed.) as well as previous studies (Kaspar, 1982; Van Gent-Ruijters *et al.*, 1975). High rates of nitrate and nitrite reduction of *Propionibacterium acidipropionici* have been reported by other researchers, in particular, Kaspar (1982) noted that strains of *P. acidipropionici* had higher nitrite reduction activities than other strains of propionibacteria species tested.

The inducibility of denitrification in *P. acidipropionici* was reported previously. Kaspar (1982) observed that the incorporation of 1 mM nitrate into the growth medium of *P. acidipropionici* increased the denitrification activity by 40-fold compared to unadapted cultures.

While the results of *in vitro* experiments were encouraging, successful establishment of populations of P5 in the rumen is more difficult to explain. One of the factors that may have aided the survival and establishment of strain P5 in the rumen of beef cattle is constant presence of low levels of nitrate in the forage portion of the ruminant diet. All plant materials contain detectable amounts of nitrate (Selk, 1993). In *P. acidipropionici* nitrate reductase synthesis is both constitutive and nitrate stimulated (Van Gent-Ruijters *et al.*, 1976; Kaspar, 1982). In addition, the reduction of nitrate is an energetically favorable process in propionibacteria (Van Gent-Ruijters *et al.*, 1975; Kaspar, 1982; Allison and MacFarlane, 1989). For *P. acidipropionici*, growth rates were increased by 20-50% by including 10 mM nitrate in the media (Kaspar, 1982). In other work with *P. acidipropionici* (Van Gent-Ruijters *et al.*, 1975), molar growth yields were increased by the presence of nitrate; this was attributed to an increased production of ATP through increased citric acid cycle activity and by oxidative phosphorylation coupled to nitrate reduction.

The presence of nitrite reductase in P5 gives it an advantage for survival and establishment over other propionibacteria strains and the normal rumen microbiota that are unable to reduce nitrite and therefore are inhibited or killed by nitrite.

Plasmid profiles to monitor specific populations in complex environments is used as a final confirmation tool in strain identification. Plasmid profiling has been used to distinguish strains of lactobacilli in the digestive tract of piglets (Tannock *et al.*,

1990), to monitor populations of specific strains of *Lactobacillus plantarum* in silage fermentations (Hill and Hill, 1986) and to monitor *Bacillus pumilus* inoculant strains in hay (Hendrick *et al.*, 1991). While plasmid profiling is one positive step toward confirmation of strain identification, plasmids are subject to conjugal transfer within and between species in an environment as well as to spontaneous loss during cell division. This makes strain identification less than 100% positive even after its plasmid profile has been determined. To be more certain, a chromosomal profile unique to the strain to be monitored must be developed. This project currently is underway for OSU's propionibacteria culture collection utilizing pulsed field gel electrophoresis (PFGE) (Rehberger, 1993).

While the results of this initial research look promising for the development of a direct-fed microbial product for "nitrate" toxicity, further research is needed to answer additional questions about the population levels required to reduce a toxic level of nitrite *in vivo* and how closely the *in vitro* nitrite reduction results compare with requirements by animals produced under various conditions.

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CHAPTER IV

In vivo Denitrification In Beef Cattle By a Selected Strain of *Propionibacterium acidipropionici*[1]

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ABSTRACT

Eight crossbred beef heifers fitted with ruminal cannulas were used to evaluate the effect of inoculation with ruminal propionibacteria on nitrate toxicity. Four heifers were dosed daily with *Propionibacterium acidipropionici* strain P5 (10^7 CFU/ml of ruminal fluid); the remaining four heifers served as uninoculated controls. Ruminal populations of P5 were monitored using differential-selective media and plasmid DNA profiling. Heifers were fed coarsely chopped native grass hay for 7 d prior to the nitrate challenge. In trial 1, heifers were challenged with pearl millet hay (21,766 ppm nitrate). Ruminal nitrate concentrations peaked at approximately 450 ppm, 2 h post-challenge for both groups and were not affected (P=.68) by inoculation. Ruminal nitrite concentrations increased more rapidly and declined sooner in inoculated heifers than in control heifers. Compared to controls, inoculated heifers had 43% less ruminal nitrite. Blood nitrite concentrations in inoculated heifers peaked at 8.9 ppm, 4 h post-nitrate challenge and decreased to 3.2 ppm at 5.5 h. In contrast, blood nitrite concentrations of controls continued to increase throughout the sampling period to 10.2 ppm at 5.5 post-feeding. In trial 2, heifers were challenged with pearl millet hay containing 23,850 ppm nitrate. Ruminal nitrate concentrations were not affected by P5 inoculation and peaked at 759 ppm at 2 h post-feeding; however, hay intake was 17% higher (P=.18) for inoculated heifers. Ruminal nitrite concentrations peaked at 1,743 ppm for control heifers; ruminal nitrite was lower (P<.0001) at 6 and 8 h (552 ppm) for inoculated heifers. Blood nitrite concentrations were reduced by 38% (21.0 vs. 13.6 ppm) at 6 (P=.02) and 8 (P=.009) h in heifers inoculated with propionibacteria. Inoculation also reduced percent methemoglobin by 35% (40.54 vs. 26.72%) at 8 (P=.04) and 12 (P=.06) h. Compared to controls, P5 inoculation reduced ruminal nitrite concentration by 46%, blood nitrite by 38% and percent methemoglobin by 35%. This study illustrates that the effects of consumed nitrate were reduced when beef cattle were pre-inoculated with *Propionibacterium acidipropionici* strain P5.

Introduction

"Nitrate" toxicity in ruminants is caused by the reduction of excessive levels of nitrate to nitrite by the normal rumen microbiota (Lewis, 1951). Nitrite is absorbed into the bloodstream where it binds with hemoglobin to form methemoglobin (Deeb and Sloan, 1975). Methemoglobin is unable to carry oxygen and, in acute cases, the resulting anoxia may cause death (Wright and Davison, 1964).

Propionibacterium, an industrially important group of bacteria, are used as starter cultures for Swiss-type cheeses (Langsrud and Reinbold, 1973) and in the production of vitamin B_{12} and propionic acid (Perlman, 1978; Playne, 1985). Propionibacteria also have been used as grain and silage inoculants (Flores-Golarza *et al.*, 1985; Tomes, 1989; Dawson *et al.*, 1991). In addition, some strains of propionibacteria reduce nitrite and nitrate to non-toxic compounds (Buchanan and Gibbons, 1974). While the use of propionibacteria as direct-fed microbial cultures for livestock production has been limited, this additional property justifies research into their development as a direct-fed microbial product for preventing "nitrate" toxicity. Previous work identified a *Propionibacterium* strain (P5) which rapidly denitrifies *in vitro* and becomes established as a viable population (10^5 CFU/ml ruminal fluid) in the rumen (Swartzlander and Rehberger, unpublished data) .

The objective of this study was to determine the prophylactic value of an established population of P5 for animals challenged with a potentially toxic dose of feed containing a high nitrate concentration.

Materials and Methods

Eight crossbred beef heifers (236 kg) were fitted with ruminal cannulas and employed in research following procedures outlined by the Oklahoma State University Animal Care and Use Committee. All animals were maintained individually in 2.44 m by 3.81 m slatted floor pens in an environmentally controlled building. Animals were given free access to water throughout the study.

Propionibacterium acidipropionici strain P5 was grown in pure culture in large scale fermenters and lyophilized by a commercial company (FAR-MOR Biochem Inc., Milwaukee, WI). Four heifers were assigned randomly to receive daily doses (10^7 CFU/ml of ruminal fluid) of lyophilized *Propionibacterium acidipropionici* strain P5 delivered via the ruminal cannula throughout the adaptation and sampling periods. The remaining heifers served as uninoculated controls. Treatment groups were separated by a feed alley (4.27 m) to avoid cross-inoculation.

The heifers were fed a low nitrate diet (50:50 roughage:concentrate) balanced for protein, minerals and vitamins (NRC, 1984) for 14 d. Heifers then were fed a low nitrate, low quality native grass hay for seven additional days. A nitrate challenge was given on day 22 by offering all animals coarsely chopped (2.54 cm screen) high nitrate pearl millet hay containing 21,766 ppm nitrate as NO_3^- (DM basis) in trial 1 and 23,850 ppm in trial 2. Hay refusals were weighed to determine hay intake and orts were discarded. Two nitrate toxicity trials, separated temporally by a 90 d de-adaption period, were conducted to determine the efficacy of the inoculated strain.

Prior to the nitrate challenge, all animals were fitted with in-dwelling jugular catheters. In addition, cylindrical stainless steel filters (76.2 mm by 25.4 mm) attached to a 2 m length of rubber vacuum hose (12.7 mm interior diameter) were inserted through the ruminal cannula plug of each heifer. On day 22 (nitrate challenge), ruminal fluid and blood samples were taken at 0, 1, 2, 3, 3.5, 4 and 6 h post-feeding for trial 1. For trial 2, ruminal

fluid and blood sampling for trial 2 was extended to 12 h (0, 1, 2, 3.5, 4, 5, 6, 8, 10 and 12 h post-feeding). At each sampling time, approximately 50 ml of ruminal fluid were removed using a vacuum pump connected to the in-dwelling ruminal filters. Samples were analyzed immediately for nitrate concentration with a pH/ISE meter equipped with a nitrate-ion specific electrode (Hach Co., Ames, IA). Three ml then were transferred to a microcentrifuge tube and the debris was pelleted by centrifugation at 12,000 rpm for five min. Following centrifugation, the supernatant was decanted and frozen (-20^0 C). At a later time, the nitrite concentration of the sample was determined in duplicate utilizing a colormetric assay (Schneider and Yeary, 1973).

Three ml of blood were collected at each sampling time utilizing a 3 cc heparinized syringe via the in-dwelling jugular catheter. In trial 2, duplicate .1 ml subsamples were removed for methemoglobin determination (Evelyn and Malloy, 1938). The remaining sample was separated by centrifugation and frozen (-20^0 C) for later nitrite analysis as in trial 1.

STATISTICAL ANALYSIS

Data were analyzed as a split plot in time with treatment, time, animal(treatment) and treatment(time) included in the model. Treatment differences were evaluated at each sampling time with a t-test (Steel and Torrie, 1980). A repeated measures procedure was used to determine the statistical probability of the time x treatment interaction being random (Ott, 1988).

RESULTS AND DISCUSSION

In trial 1, prairie hay intake during the adaption period was 41% higher (P=.02) for the propionibacteria-inoculated heifers than control heifers (figure 1). On the nitrate challenge day, intake of high nitrate pearl millet hay was greater than with prairie hay (2.0% of BW) but was not affected by inoculation (P=.27). This level of pearl millet hay intake represents a nitrate challenge of 435 mg NO_3/kg BW. This exceeds the toxic level of 265 mg NO_3/kg BW proposed by Hibberd *et al.* (1993).

Ruminal nitrate concentrations for all cattle increased rapidly and peaked at approximately 450 ppm at 2 h post-feeding (figure 2). For the entire sampling period, ruminal nitrate concentrations for the inoculated heifers were not different (time x treatment, P=.68) from the controls although ruminal nitrate concentration at 5.5 h was lower (P<.001) for inoculated heifers than control heifers.

For ruminal nitrite, the time x treatment interaction evaluated over the entire sampling period (figure 3) was not significant (P=.90). Ruminal nitrite concentrations for inoculated heifers tended to increase more rapidly than the control heifers and to decline more rapidly. By 5.5 h post-nitrate challenge, ruminal nitrite concentrations tended (P=.16) to be lower for inoculated heifers. If the nitrite curves are extrapolated to zero, the total quantity of ruminal nitrite (estimated by the area under the curve) was 43% less in inoculated heifers. Deeb and Sloan (1975) noted that ruminal nitrate and nitrite concentrations peaked at 4 to 8 h after test animals were dosed with near toxic nitrate concentrations (1000 ppm NO_3).

Blood nitrite concentrations for control heifers (figure 4) increased at 3.5 h post-feeding and continued to increase through the remainder of the sampling period reaching a concentration of 10.2 ppm at 5.5 h post-nitrate challenge. In contrast, blood nitrite concentrations in treated heifers increased at 3 h post-feeding and peaked (8.9 ppm) at 4 h post-feeding. By 5.5 h post-feeding, blood nitrite concentrations for the inoculated heifers had decreased to 3.2 ppm, a concentration lower (P=.03) than in the control heifers.

The results of trial 1 suggested that propionibacteria inoculation could reduce ruminal and blood nitrite concentrations in heifers challenged with high nitrate pearl millet hay. In

trial 2, sampling times were extended to 12 h post-feeding and methemoglobin concentrations were determined to gain a better understanding of the toxicosis process and confirm the results of trial 1.

As in trial 1, prairie hay intake during the adaption period of trial 2 was 43% higher (P=.005) for the inoculated than the control heifers (figure 5). On the nitrate challenge day, control heifers consumed 1.63% of their BW as pearl millet hay. This level of pearl millet hay intake represents 389 mg NO_3/kg BW which again exceeds the proposed toxic level of 265 mg NO_3/kg BW. On the challenge day, inoculated heifers consumed 17% more pearl millet hay (P=.18) than the controls; this increased their nitrate intake to 456 mg NO_3/kg BW. Thus, inoculated heifers were subjected to a more severe nitrate challenge than were the control heifers.

Ruminal nitrate concentrations increased rapidly and peaked 2 h post-feeding (figure 6). Ruminal nitrate concentrations did not differ (time x treatment, P=.77) between the treated and the controls even though hay intake was 17% higher (P=.18) for inoculated heifers (figure 5). Ruminal nitrate concentrations peaked at 760 ppm; this was 62% higher than peak concentrations observed in trial 1.

Ruminal nitrite concentrations in control heifers peaked 6 h post-feeding at 1,743 ppm (figure 7). In contrast, ruminal nitrite concentrations in inoculated heifers increased at a slower rate and peaked at a lower level (552 ppm). Ruminal nitrite concentrations were lower (P=.0001) at 6 and 8 h post-feeding in heifers inoculated with P5. Compared to controls, total ruminal nitrite (estimated as the area under the nitrate concentration curve) was 46% lower for treated than control heifers. This response was similar to the 43% reduction in total ruminal nitrite projected from trial 1.

Blood nitrite concentrations mirrored changes in ruminal nitrite concentrations (figure 8). Blood nitrite concentrations increased slowly in both groups until increasing sharply at 3.5 h post-feeding. Blood nitrite concentrations for control heifers peaked at 21 ppm at 6 h post-feeding. In contrast, blood nitrite concentrations for inoculated heifers peaked at 5 h post-feeding (13.6 ppm) and decreased to presampling concentrations by 8 h post-feeding.

Although time x treatment interaction for the entire sampling period was not significant (P=.40), inoculation significantly reduced blood nitrite concentrations at 6 h (P=.01) and 8 h (P=.009) post-feeding.

Percent methemoglobin for both groups remained low until 3 h post-feeding (figure 9). Methemoglobin percent for inoculated heifers increased from 3 to 6 h post-feeding and peaked 6 h post-feeding at 26.7% of hemoglobin. Burrows *et al.* (1987) noted that in acute toxicosis cases (50 to 69% methemoglobin), methemoglobin concentrations and their accompanying symptoms of nitrate toxicity occurred within 3 to 6 h of ingestion of a toxic nitrate dose. Percent methemoglobin for control heifers continued to increase from 3 to 8 h post-feeding to 40.5% of hemoglobin at 8 h post-feeding. Inoculation reduced percent methemoglobin at 6 (P=.15), 8 (P=.04) and 12 (P=.06) h post-feeding compared to control heifers. The apparent decrease in percent methemoglobin in control animals at 10 h post-nitrate challenge may, in part, be attributed to one control animal that exhibited symptoms of acute nitrate toxicity including muscle tremors, labored breathing and lethargy. This animal was revived with methylene blue and was removed from the study at 10 and 12 h. Thus, the percent methemoglobin values for control heifers at 10 and 12 h may be biased downward because data from this heifer were not included.

In both trials, established ruminal populations of P5 ($>10^5$ CFU/ml of ruminal fluid) were confirmed in inoculated heifers using selective-differential media (PSA) and plasmid profiling (Rehberger and Glatz, 1990). In neither trial did control heifers have detectable populations of P5.

Various methods have been developed to reduce the impact of nitrate toxicity on ruminants. Majak *et al.* (1982) and Allison and Reddy (1984) illustrated that ruminal microbiota adapt to continuously high levels of dietary nitrate by increased ruminal nitrite reduction. The supposition that the continual incorporation of nitrate into a diet would maintain an elevated level of nitrite reduction without ill effects has yet to be tested. The limitations associated with this method of toxicity prevention would be that the adaptation is necessary and the de-adaptation period is as few as four days (Allison and Reddy, 1984).

Allison and Reddy (1984) also noted that as dietary nitrate exceeds 18,000 ppm, nitrate reduction by the adapted rumen microbiota was twice as rapid as nitrite reduction allowing nitrite to accumulate. Another prophylatic strategy is to incorporate of tungsten as a nitrate reductase inhibiting compound into the diets of ruminants (Korzeniowski *et al.*, 1980, 1981). While this method does inhibit nitrite formation (86-100% inhibition with dosage levels of 20-100 micromoles of Na_2WO_4) some adverse effects have been associated with the use of Tungsten (Korzeniowski *et al.*, 1980).

Other methods of nitrate toxicity control include feed management practices. For example, the addition of 3.2 kg of corn into the diet of beef cattle challenged with .3 g $NaNO_3$/kg BW reduced percent methemoglobin by 66% (Burrows *et al.*, 1987). This effect may be due to increased growth of ruminal microorganisms and reduced ruminal pH closer to the optimum of nitrite reductase and away from the optimum for nitrate reductase. In addition, Smith *et al.* (1992) noted that supplementing the crude protein content of a high nitrate forage diet (40,000-50,000 ppm NO_3) to 16% CP reduced percent methemoglobin from 30% to 14% to 19%. They postulated that the increased protein may have supplied ruminal microbiota with alternative nitrogen sources.

In the current study, a population of *Propionibacterium acidipropionici* strain P5 was established in the rumen of beef cattle that reduced blood nitrite by 38% and percent methemoglobin by 35%. Once an efficacious population is established in the rumen (10^7 CFU/ml of ruminal fluid for 8 d), the P5 population (10^5 CFU/ml of ruminal fluid) remains stable for over 7 months (Swartzlander and Rehberger, unpublished data) eliminating the need for daily dosing and the cost, management problems and adverse effects associated with nitrate, energy, and protein adaptation or Tungsten feeding methods. The most effective utilization of a direct-fed microbial product such as *Propionibacterium acidipropionici* strain P5 would be its incorporation into a comprehensive nitrate management program utilizing one or more of the conventional management methods to minimize nitrate levels in the ration and reduce nitrite formation in the rumen.

IMPLICATIONS

Nitrate toxicity trials suggest that established populations of *Propionibacterium acidipropionici* strain P5 at levels of $1x10^5$ CFU/ml of ruminal fluid can reduce nitrite accumulation in the rumen and blood of beef cattle consuming large quantities of nitrate. Consequently, production and death losses due to nitrate toxicity could be reduced through the use of a direct-fed microbial product developed from strain P5.

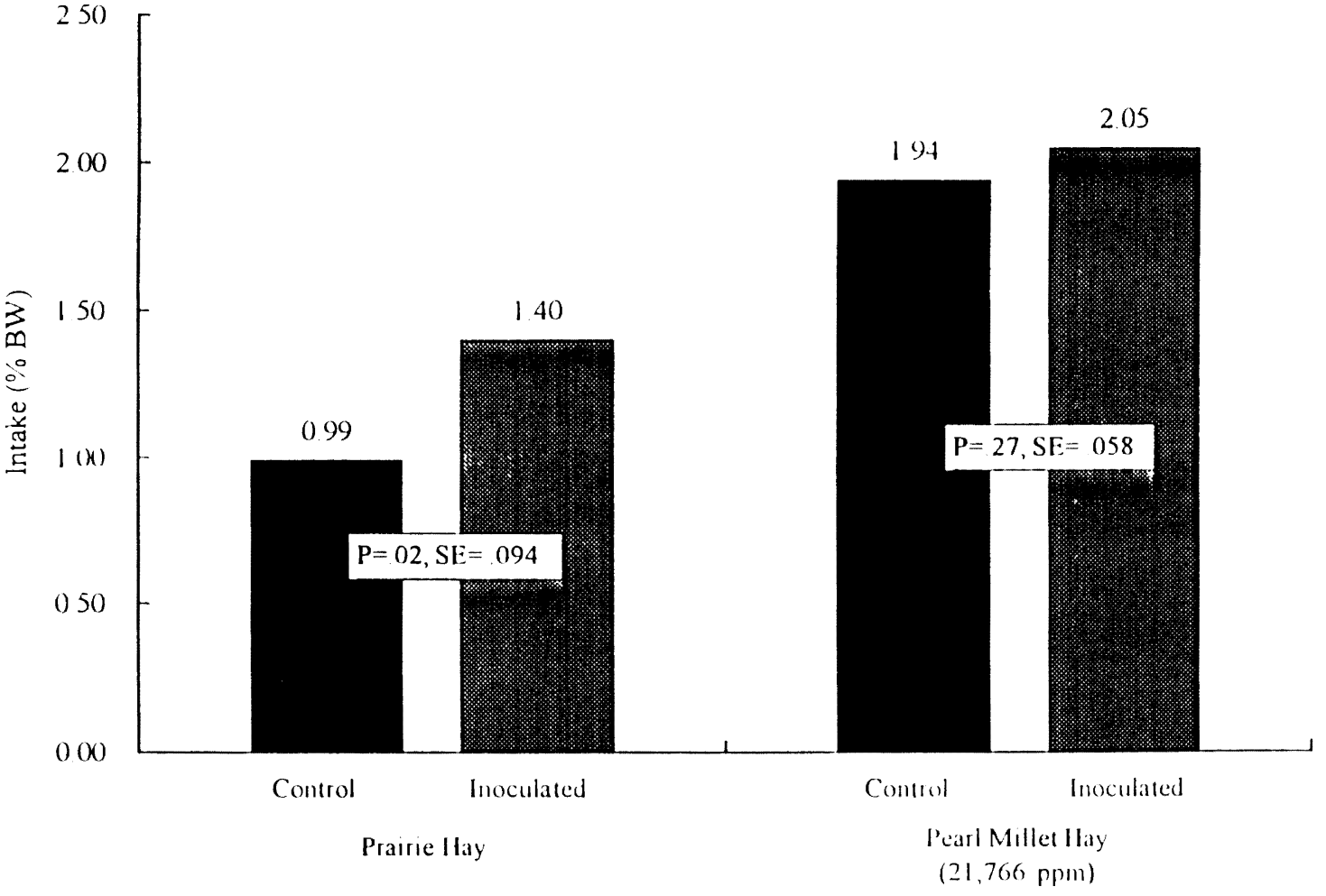

Figure 1. Forage intake of inoculated and control heifers during trial 1.

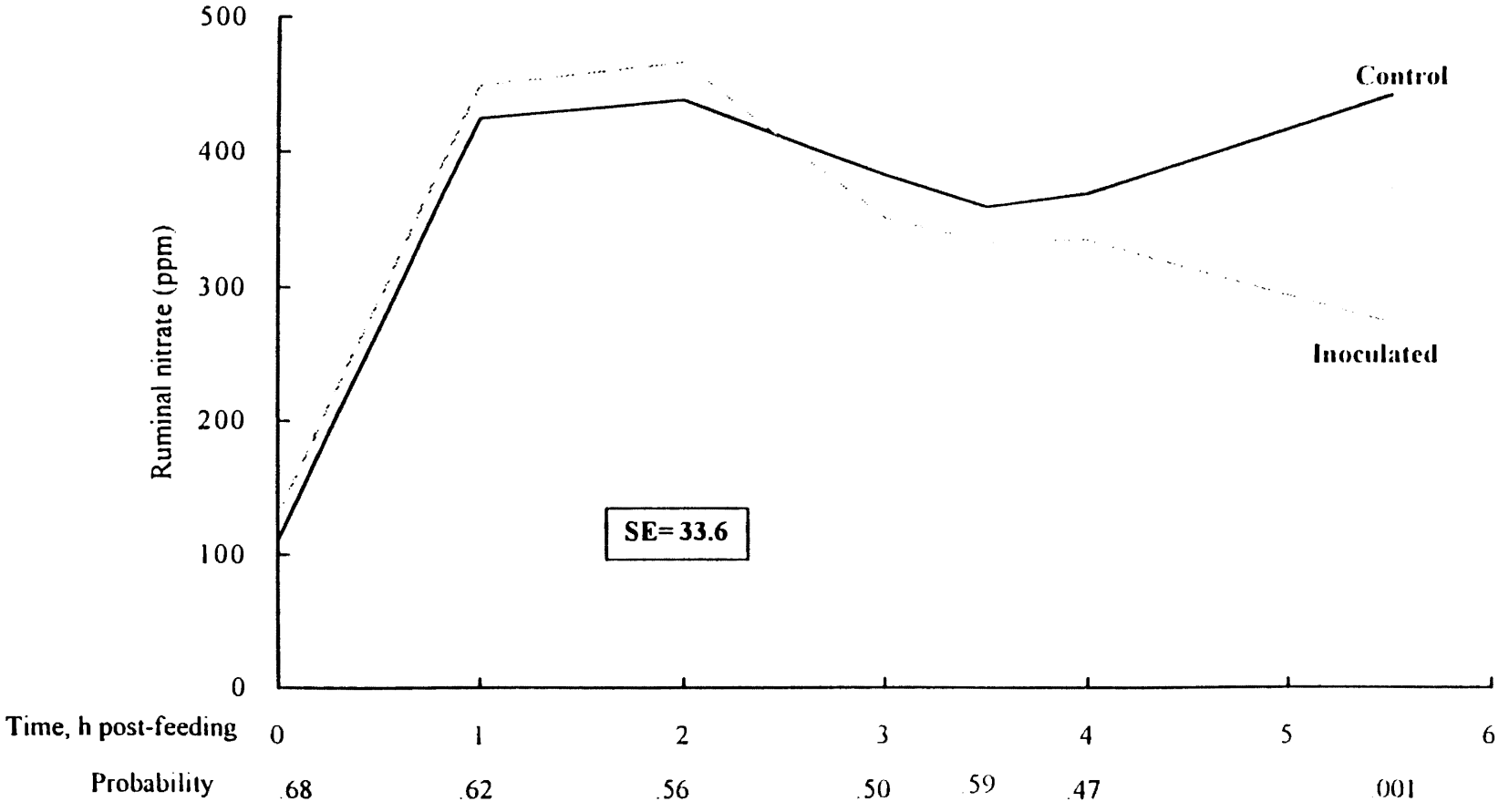

Figure 2. Effect of propionibacteria inoculation on ruminal nitrate concentrations in beef heifers challenged with high nitrate pearl millet hay (Trial 1).

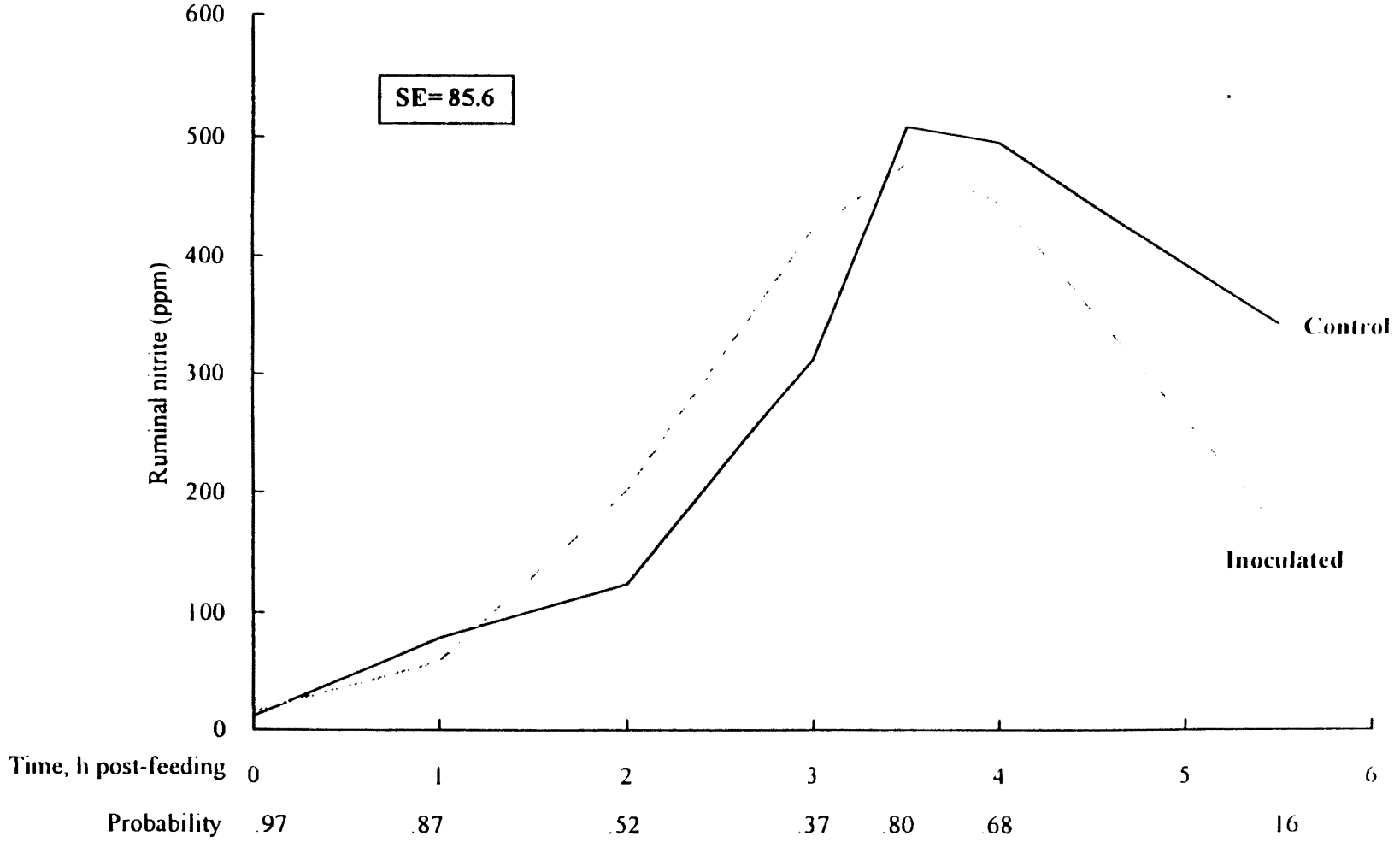

Figure 3. Effect of propionibacteria inoculation on ruminal nitrite concentrations in beef heifers challenged with high nitrate pearl millet hay (Trial 1).

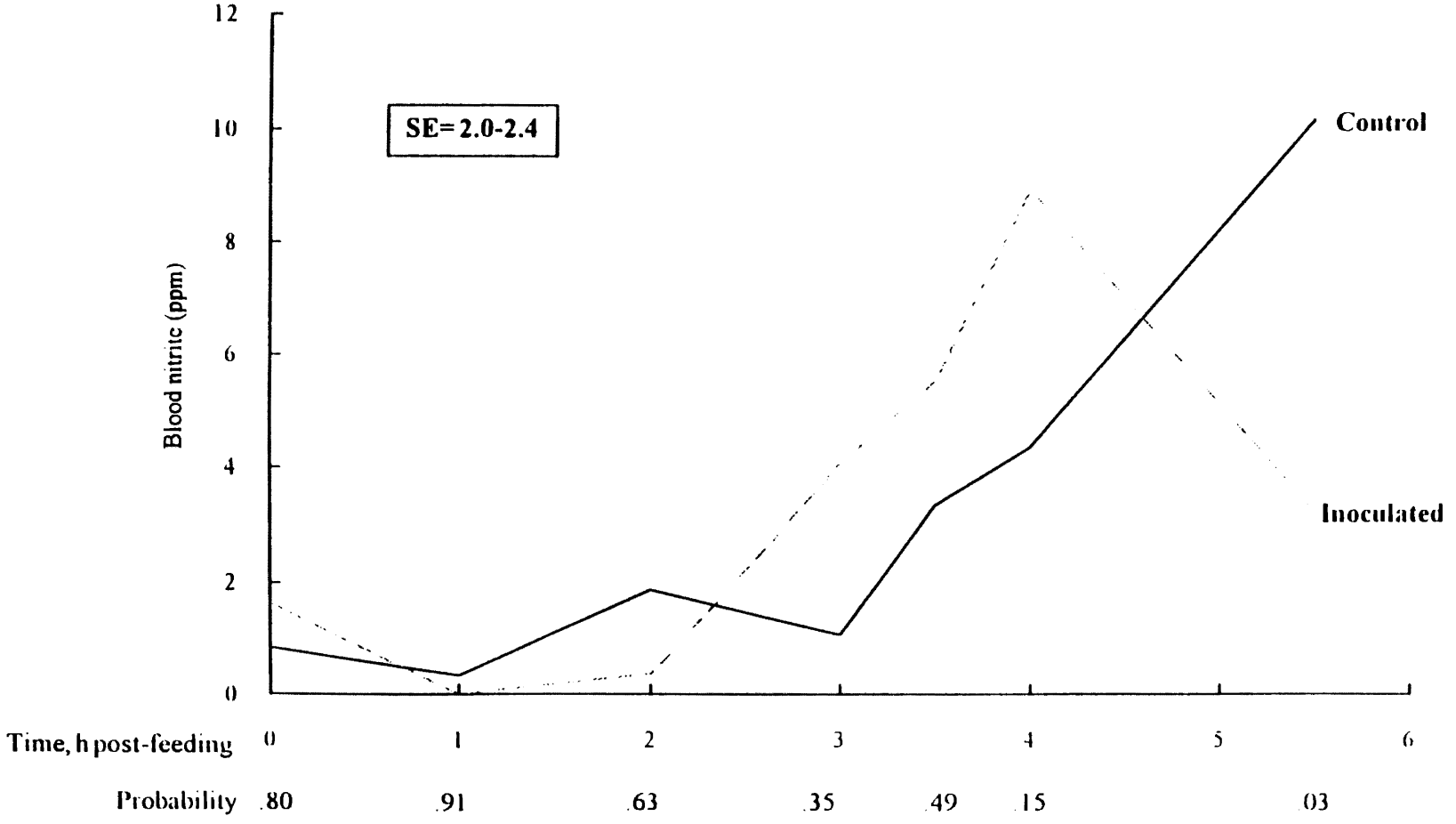

Figure 4. Effect of propionibacteria inoculation on blood nitrite concentrations in beef heifers challenged with high nitrate pearl millet hay (Trial 1).

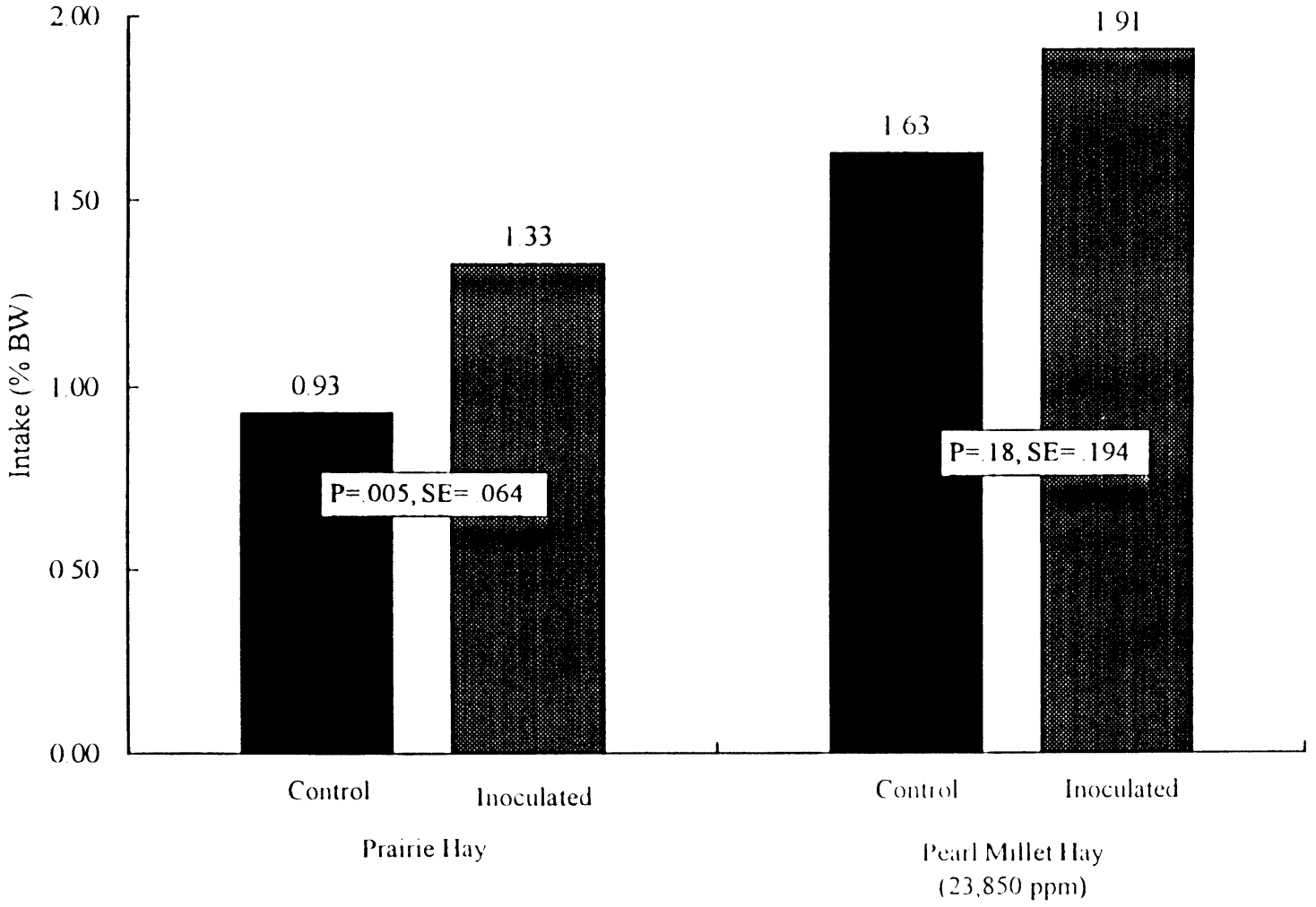

Figure 5. Forage intake of inoculated and control heifers during trial 2.

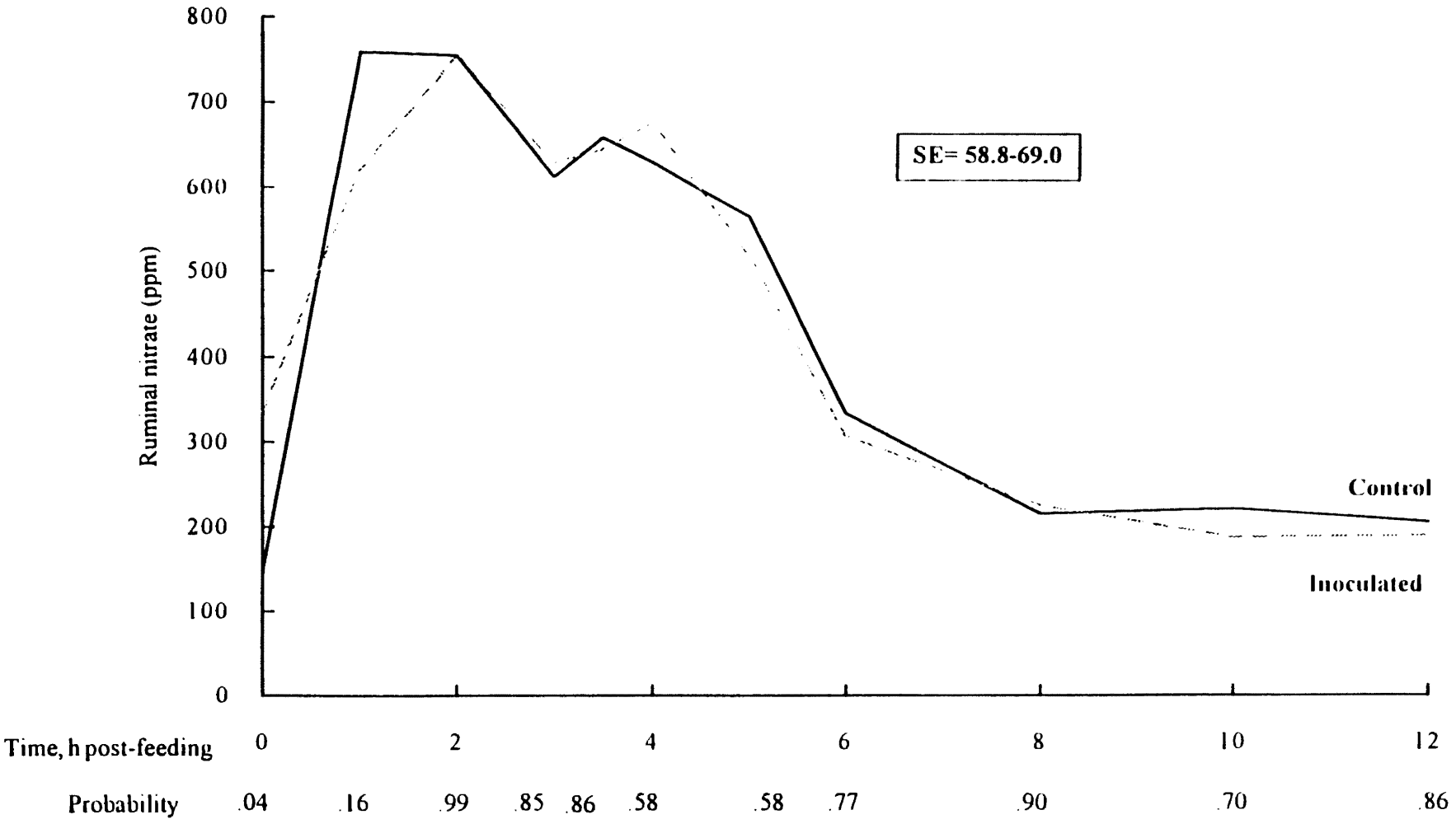

Figure 6. Effect of propionibacteria inoculation on ruminal nitrate concentrations in beef heifers challenged with high nitrate pearl millet hay (Trial 2).

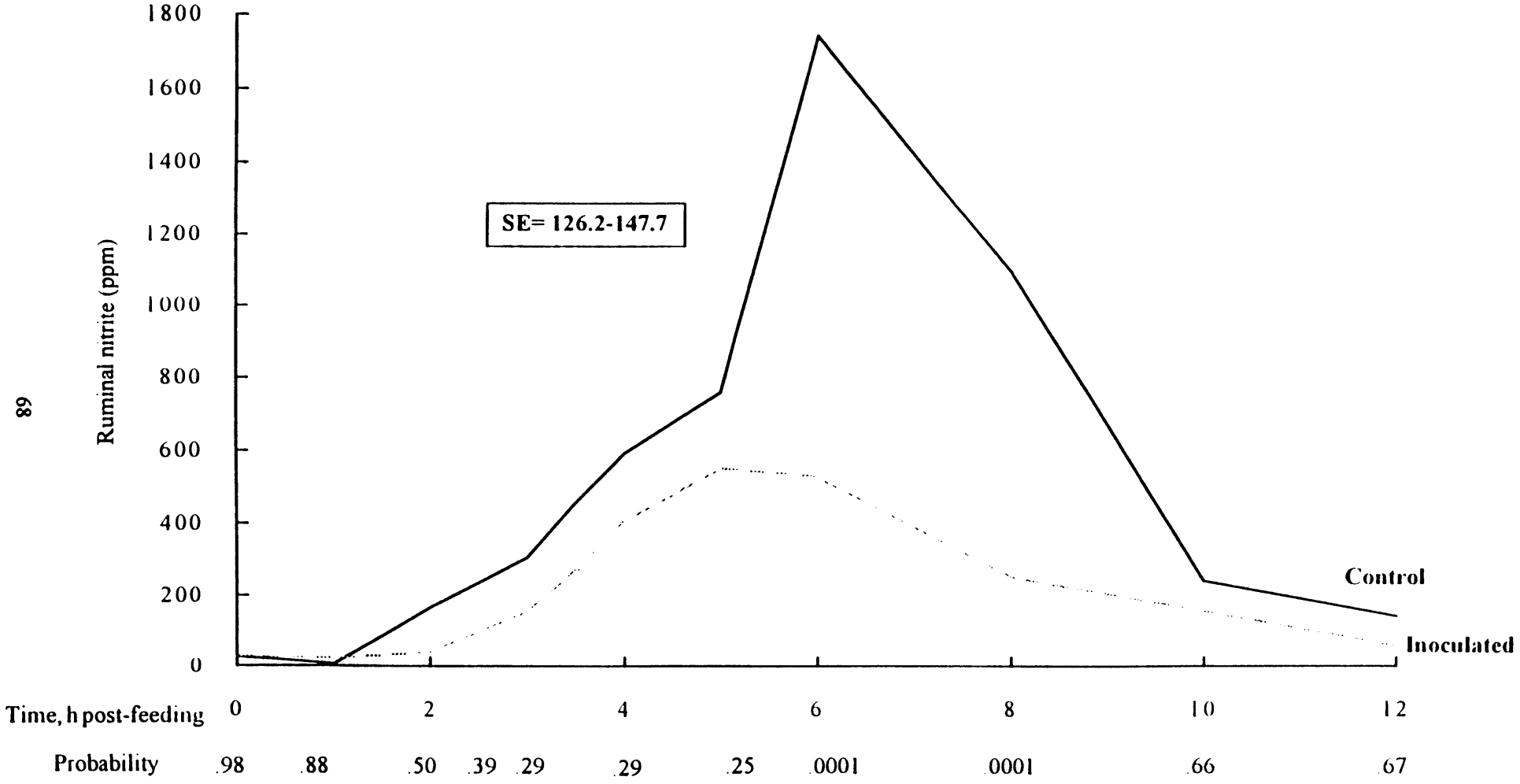

Figure 7. Effect of propionibacteria inoculation on ruminal nitrite concentrations in beef heifers challenged with high nitrate pearl millet hay (Trial 2).

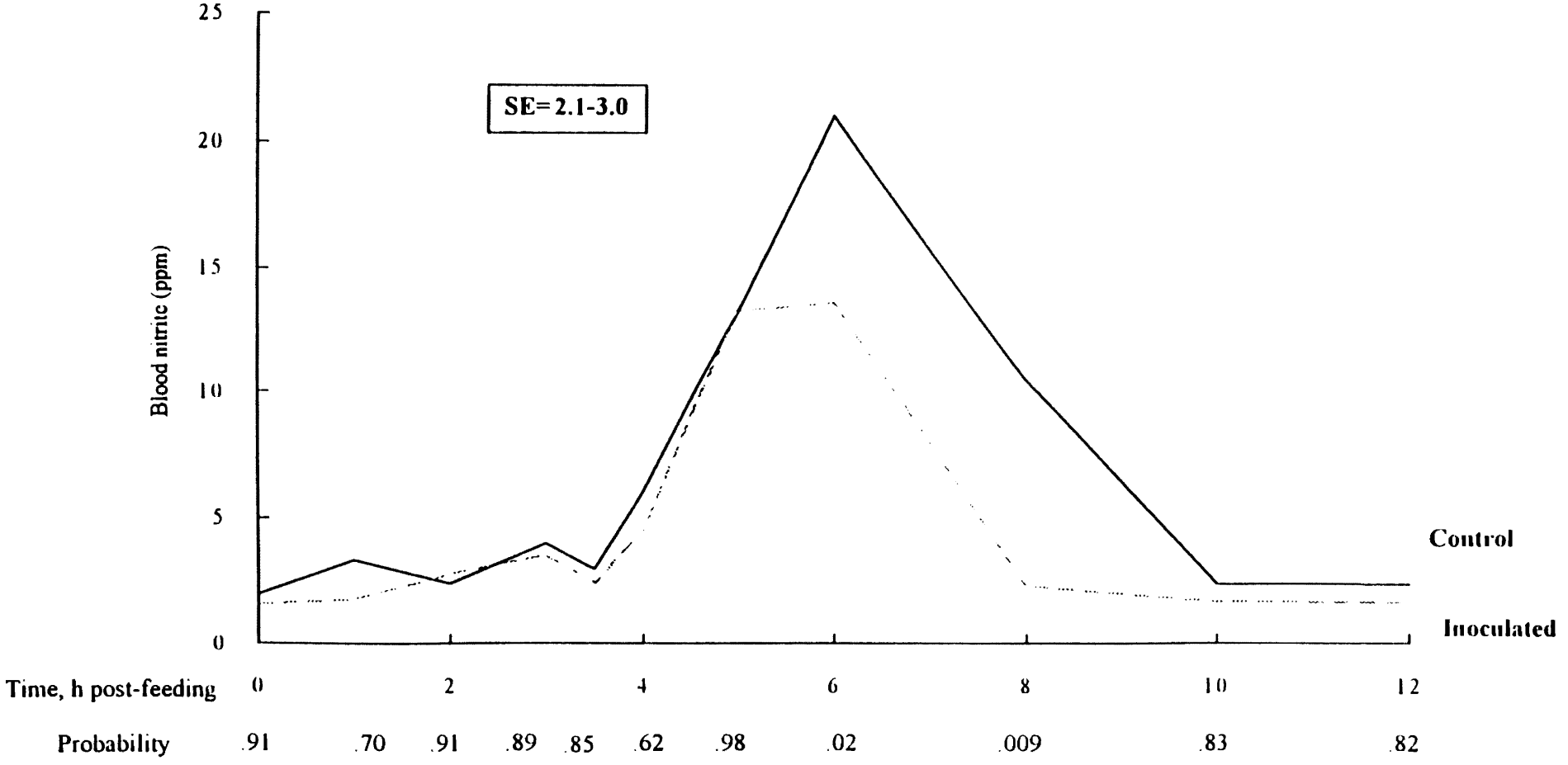

Figure 8. Effect of propionibacteria inoculation on blood nitrite concentrations in beef heifers challenged with high nitrate pearl millet hay (Trial 2).

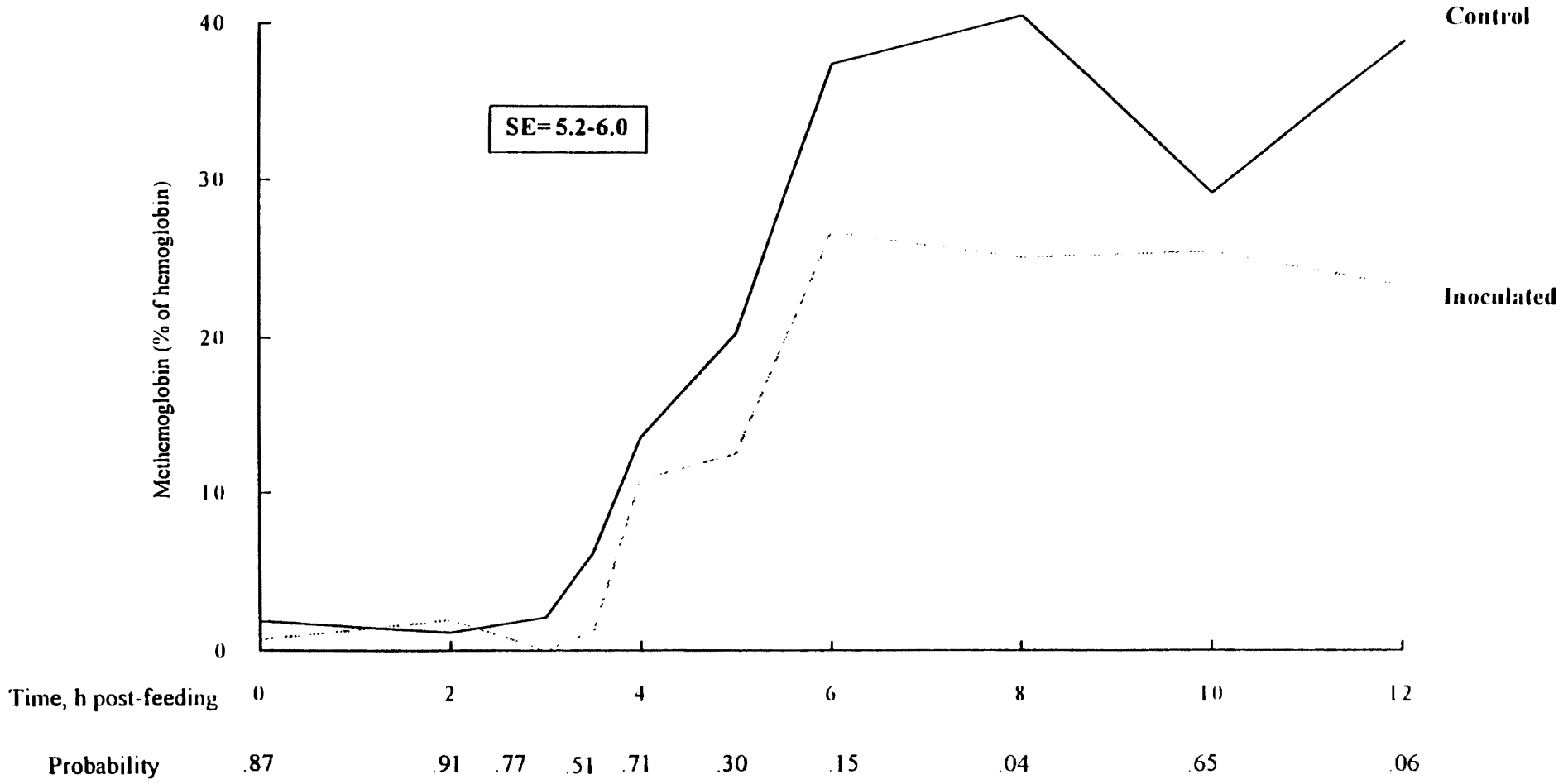

Figure 9. Effect of propionibacteria inoculation on percent methemoglobin in beef heifers challenged with high nitrate pearl millet hay (Trial 2).

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CHAPTER V

SUMMARY AND CONCLUSIONS

The toxic effect of high levels of nitrate in the diet of ruminants has long been recognized. "Nitrate" toxicity is caused by the reduction of excessive levels of nitrate to nitrite by the normal rumen microbiota. Nitrite passes readily through the rumen wall in to the blood stream where it binds with hemoglobin to form methemoglobin. The methemoglobin, being unable to transport oxygen and may cause death in the host animal due to anoxia. One possible solution to this problem is the development of a direct-fed microbial product for alleviating "nitrate" toxicity.

The aim of this work was to identify strains of propionibacteria capable of nitrite reduction. Identified strains would have their denitrification abilities quantified for selection purposes. Selected strains would be tested for survival in ruminal fluid *in vitro*. One strain would then be tested for survival and establishment *in vivo* at two different inoculation levels. The selected strain would be tested to see if it had a significant prophylactic value for animals fed a toxic level of nitrate from a high nitrate feed. Response would be evaluated from ruminal nitrate/nitrite, blood nitrite and methemoglobin concentrations in inoculated animals versus uninoculated control animals.

Seventeen of 154 *Propionibacterium* cultures were able to reduce nitrate and nitrite *in vitro*. Of those seventeen, two cultures, P5 and P42 reduced high levels of nitrate and nitrite (15,000 - 20,000 ppm). *In vitro* ruminal fluid survival studies indicated that P42 reduced nitrate and nitrite at a slower rate (108 vs. 80 hours) than P5 did. *In vivo* establishment trials with strain P5 indicated that daily inoculations with $1x10^7$ CFU/ml of ruminal fluid gave ruminal counts greater than $1x10^3$ CFU/ml by day10; daily inoculation with $1x10^6$ CFU/ml gave ruminal counts of $1x10^3$ CFU/ml by day 18. The efficacy trials with strain P5 inoculated daily at $1x10^7$ CFU/ml of ruminal fluid did not

significantly reduce ruminal nitrate peaks although inoculated heifers consumed 17 to 21% more high nitrate hay. No reason for this increase is apparent. Compared with control heifers, total ruminal nitrite concentrations in inoculated heifers were 43-45% lower. Blood nitrite concentrations were 38% lower in inoculated heifers. In the second efficacy trial, total methemoglobin concentrations were 33% lower in inoculated than control heifers.

An established population of *Propionibacterium acidi-propionici*, strain P5 in the rumen can exert a measure of prophylaxis when cattle consume large quantities of high nitrate feed. Consequently, production losses due to "nitrate" toxicity could be markedly reduced through the use of a direct-fed microbial product developed from strain P5. The increased hay intake with inoculation may suggest that these microorganisms exert some additional physiological effect to stimulate appetite. Whether the response observed was due to increased fermentation of the diet or simply animal to animal variation is not yet known. This intake response portends even greater applications for propionibacteria in the beef cattle industry. The tools and procedures developed in this study could be used to monitor naturally occurring propionibacteria populations *in vivo* to enhance the understanding of the ecology of the rumen.

APPENDICES

APPENDIX A

DATA TABLES FOR MEASURED VARIABLES OF CHAPTER IV

TABLE 1. TRIAL 1 MEAN +/- SE RUMINAL NITRATE CONCENTRATIONS AT EACH SAMPLING PERIOD IN HEIFERS INOCULATED WITH P5 (T) OR NOT (C)

Sampling period	Treatment	Nitrate, ppm	P > |T|
0 h post-feeding	C	110.75	
of high nitrate hay	T	130.45	0.6812
1 h post-feeding	C	424.75	
of high nitrate hay	T	448.50	0.6206
2 h post-feeding	C	438.75	
of high nitrate hay	T	467.00	0.5563
3 h post-feeding	C	383.00	
of high nitrate hay	T	350.75	0.5021
3.5 h post-feeding	C	358.75	
of high nitrate hay	T	332.75	0.5880
4 h post-feeding	C	369.25	
of high nitrate hay	T	334.50	0.4698
5.5 h post-feeding	C	443.00	
of high nitrate hay	T	273.75	0.0011
Pooled SEM		+\-25.88	

TABLE 2. TRIAL 1 MEAN +/- SE RUMINAL NITRITE CONCENTRATIONS AT EACH SAMPLING PERIOD IN HEIFERS INOCULATED WITH P5 (T) OR NOT (C)

Sampling period	Treatment	Nitrite, ppm	P > |T|
0 h post-feeding	C	11.64	
of high nitrate hay	T	16.28	0.9696
1 h post-feeding	C	78.55	
of high nitrate hay	T	59.34	0.8749
2 h post-feeding	C	123.97	
of high nitrate hay	T	201.84	0.5243
3 h post-feeding	C	311.68	
of high nitrate hay	T	422.28	0.3672
3.5 h post-feeding	C	508.47	
of high nitrate hay	T	477.96	0.8025
4 h post-feeding	C	494.60	
of high nitrate hay	T	444.74	0.6830
5.5 h post-feeding	C	342.19	
of high nitrate hay	T	168.90	0.1611
Pooled SEM		+\- 34.92	

TABLE 3. TRIAL 1 MEAN +/- SE BLOOD NITRITE CONCENTRATIONS AT EACH SAMPLING PERIOD IN HEIFERS INOCULATED WITH P5 (T) OR NOT (C)

Sampling period	Treatment	Nitrite, ppm	P > |T|
0 h post-feeding	C	0.840	
of high nitrate hay	T	1.650	0.7974
1 h post-feeding	C	0.340	
of high nitrate hay	T	0.000	0.9142
2 h post-feeding	C	1.883	
of high nitrate hay	T	0.375	0.6332
3 h post-feeding	C	1.080	
of high nitrate hay	T	4.028	0.3536
3.5 h post-feeding	C	3.337	
of high nitrate hay	T	5.530	0.4886
4 h post-feeding	C	4.353	
of high nitrate hay	T	8.928	0.1541
5.5 h post-feeding	C	10.177	
of high nitrate hay	T	3.245	0.0344
Pooled SEM		+\-1.318	

TABLE 4. TRIAL 2 MEAN +/- SE RUMINAL NITRATE CONCENTRATIONS AT EACH SAMPLING PERIOD IN HEIFERS INOCULATED WITH P5 (T) OR NOT (C)

Sampling period	Treatment	Nitrate, ppm	P > |T|
0 h post-feeding	C	148.44	
of high nitrate hay	T	336.00	0.0435
1 h post-feeding	C	758.84	
of high nitrate hay	T	621.10	0.1634
2 h post-feeding	C	755.25	
of high nitrate hay	T	754.00	0.9881
3 h post-feeding	C	612.75	
of high nitrate hay	T	628.50	0.8506
3.5 h post-feeding	C	659.25	
of high nitrate hay	T	644.50	0.8600
4 h post-feeding	C	630.25	
of high nitrate hay	T	676.00	0.5848
5 h post-feeding	C	563.75	
of high nitrate hay	T	517.75	0.5828
6 h post-feeding	C	333.84	
of high nitrate hay	T	307.50	0.7727
8 h post-feeding	C	215.25	
of high nitrate hay	T	225.25	0.9048
10 h post-feeding	C	221.20	
of high nitrate hay	T	186.50	0.7034
12 h post-feeding	C	205.20	
of high nitrate hay	T	189.00	0.8588
Pooled SEM		+\-21.48	

TABLE 5. TRIAL 2 MEAN +/- SE RUMINAL NITRITE CONCENTRATIONS AT EACH SAMPLING PERIOD IN HEIFERS INOCULATED WITH P5 (T) OR NOT (C)

Sampling period	Treatment	Nitrite, ppm	P > |T|
0 h post-feeding	C	26.58	
of high nitrate hay	T	30.05	0.9845
1 h post-feeding	C	6.88	
of high nitrate hay	T	0.00	0.8823
2 h post-feeding	C	161.38	
of high nitrate hay	T	39.75	0.4965
3 h post-feeding	C	308.48	
of high nitrate hay	T	156.11	0.3945
3.5 h post-feeding	C	458.89	
of high nitrate hay	T	270.66	0.2932
4 h post-feeding	C	593.24	
of high nitrate hay	T	404.18	0.2911
5 h post-feeding	C	758.93	
of high nitrate hay	T	551.56	0.2471
6 h post-feeding	C	1743.48	
of high nitrate hay	T	529.20	0.0001
8 h post-feeding	C	1095.79	
of high nitrate hay	T	250.90	0.0001
10 h post-feeding	C	239.84	
of high nitrate hay	T	155.42	0.6645
12 h post-feeding	C	139.62	
of high nitrate hay	T	56.67	0.6700
Pooled SEM		+\-162.00	

TABLE 6. TRIAL 2 MEAN +/- SE BLOOD NITRITE CONCENTRATIONS AT EACH SAMPLING PERIOD IN HEIFERS INOCULATED WITH P5 (T) OR NOT (C)

Sampling period	Treatment	Nitrite, ppm	P > |T|
0 h post-feeding	C	1.997	
of high nitrate hay	T	1.578	0.9131
1 h post-feeding	C	3.344	
of high nitrate hay	T	1.788	0.6967
2 h post-feeding	C	2.414	
of high nitrate hay	T	2.790	0.9096
3 h post-feeding	C	4.024	
of high nitrate hay	T	3.564	0.8897
3.5 h post-feeding	C	3.001	
of high nitrate hay	T	2.445	0.8514
4 h post-feeding	C	6.018	
of high nitrate hay	T	4.526	0.6158
5 h post-feeding	C	13.195	
of high nitrate hay	T	13.264	0.9815
6 h post-feeding	C	20.995	
of high nitrate hay	T	13.609	0.0177
8 h post-feeding	C	10.445	
of high nitrate hay	T	2.308	0.0092
10 h post-feeding	C	2.389	
of high nitrate hay	T	1.684	0.8276
12 h post-feeding	C	2.342	
of high nitrate hay	T	1.615	0.8223
Pooled SEM		+\-1.203	

TABLE 7. TRIAL 2 MEAN +/- SE METHEMOGLOBIN CONCENTRATIONS AT EACH SAMPLING PERIOD IN HEIFERS INOCULATED WITH P5 (T) OR NOT (C)

Sampling period	Treatment	Methemoglobin, mg/dl	P > |T|
0 h post-feeding	C	0.135	
of high nitrate hay	T	0.045	0.8593
2 h post-feeding	C	0.080	
of high nitrate hay	T	0.133	0.9177
3 h post-feeding	C	0.143	
of high nitrate hay	T	0.000	0.7791
3.5 h post-feeding	C	0.445	
of high nitrate hay	T	0.090	0.4855
4 h post-feeding	C	0.968	
of high nitrate hay	T	0.713	0.6159
5 h post-feeding	C	1.435	
of high nitrate hay	T	0.810	0.2217
6 h post-feeding	C	2.663	
of high nitrate hay	T	1.760	0.0799
8 h post-feeding	C	2.843	
of high nitrate hay	T	1.670	0.0243
10 h post-feeding	C	2.090	
of high nitrate hay	T	1.715	0.4994
12 h post-feeding	C	2.810	
of high nitrate hay	T	1.580	0.0299
Pooled SEM		+\-0.140	

APPENDIX B

FIELD TRIAL 1

FIELD TRIAL 1

Sixty crossbred commercial feedlot cattle were used to evaluate the efficacy of a commercial preparation of strain P5, tradename BOVA-PRO (FAR-MOR BIOCHEM, Milwaukee, WI). Thirty animals were assigned randomly to one of two treatments. Both groups were allowed access to *ad libitum* low nitrate hay and water for a period of 23 d. In addition, both groups received 2 lb/head/d of protein supplement. One group was fed daily a pelleted protein supplement which contained $1x10^7$ CFU/ml of ruminal fluid of BOVA-PRO. The control group received an identical supplement formulated without BOVA-PRO. At the end of the initial 23 d period, both groups were released onto sorghum-sudan hybrid green pasture. Ten animals from each group were monitored for ruminal nitrate, rumen and blood nitrite and methemoglobin concentrations at 4, 6, or 8 h post-release (via tail bleeding and stomach tube) and then removed from the pasture.

FORAGE ANALYSIS:

The pasture utilized in this trial was gridded and samples analyzed for nitrate content were collected at intervals of 18.29 m. Nitrate analysis was conducted on dried samples with an ion specific electrode. Nitrate concentrations in the pasture ranged from 4,180 to 28,800 ppm with an average concentration of 12,020 ppm nitrate.

RESULTS:

Ruminal nitrate concentrations increased for both groups throughout the trial (figure 1). Control animals had an average concentration of 883 ppm at 4 h which increased to 2277 ppm by the 8 h sample. Similarly, treated animals began at a somewhat higher concentration (943 ppm) at 4 h but at 8 h post-release had a lower mean concentration (1790 ppm) of nitrate.

Ruminal nitrite concentrations for field trial 1 were exceedingly low when compared to toxicity trials (figure 2). Control animals peaked at 4 h (36 ppm) then dropped to 22 ppm at 6 h before rebounding somewhat to 25 ppm at 8 h post-release. Conversely, treated animals peaked at 6 h post-release (26 ppm) and decreased to 23 ppm by 8 h post-release after an initial 4 h sample average of 21 ppm.

Blood nitrite concentrations for field trial 1 were exceedingly low when compared to toxicity trials (figure 3). Control animals exhibited 3.12 ppm at 4 h, a peak at 6 h of 3.65 ppm and a decrease by 8 h post-release to 2.41 ppm. Meanwhile, treated animals had

an average blood nitrite concentration at 4 h of 2.44 ppm, a peak at 2.97 ppm and a decrease to 2.49 ppm by 8 h post-release.

Percent methemoglobin showed the greatest separation of treatments although it too was extremely low compared to toxicity trials (figure 4). Control animals exhibited their highest concentration of methemoglobin at 4 h (1.37%) and it decreased steadily throughout the trial to 0.64% at 8 h post-release. Treated animals also showed their highest percent methemoglobin (1.21%) at 4 h which dropped sharply by 6 h to 0.47% and ended the trial at 0.54% of hemoglobin.

SUMMARY AND CONCLUSIONS:

Animals treated with BOVA-PRO showed numerically lower ruminal and blood nitrite concentrations as well as percent methemoglobin, particularly during the 4 and 6 h post-release sample periods when compared to controls. The concentrations of nitrite and methemoglobin exhibited in this trial were extremely low considering the high concentration of nitrate present in the forage and in the rumen. The discrepancy in nitrite release, particularly when compared to toxicity trials, may, in part, be explained by the lower nitrate concentration in the forage and the form of forage consumed. During our toxicity trials the high nitrate forage was in the form of 20,000 ppm nitrate hay where in the current trial, the lush, growing pasture contained 12,000 ppm nitrate. Lush, green forages release their nitrate more slowly than hays and nitrate may be in a form or complex in which the nitrate ion, contained in a fully hydrated plant cell, resists microbial degradation.

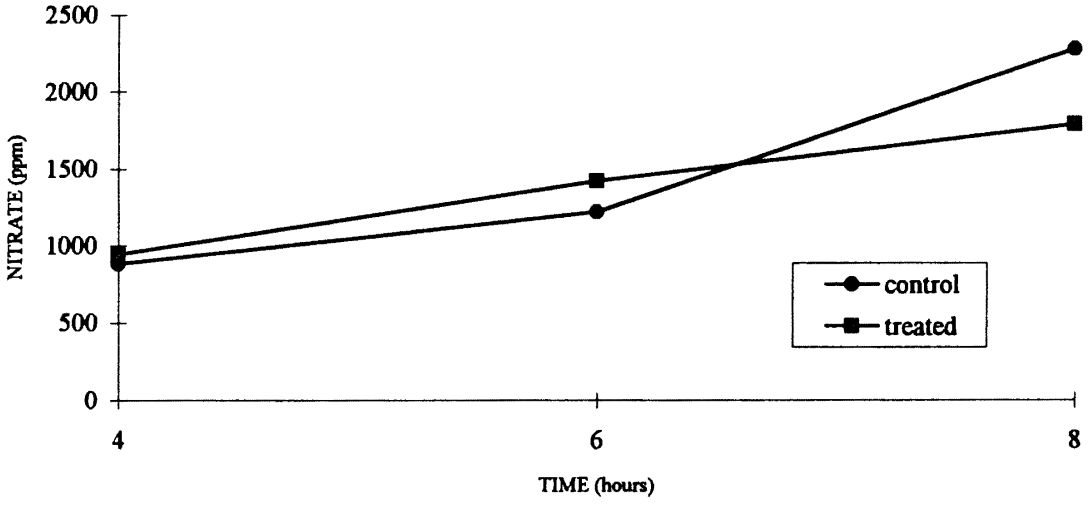


Figure 1. RUMEN NITRATE CONCENTRATIONS FOR FIELD TRIAL 1

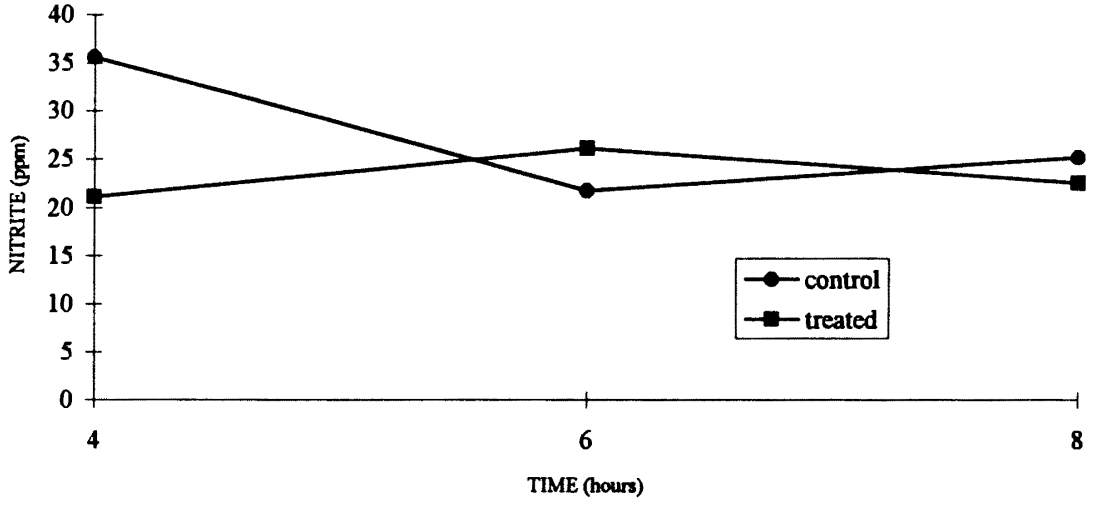


Figure 2. RUMEN NITRITE CONCENTRATIONS FOR FIELD TRIAL 1

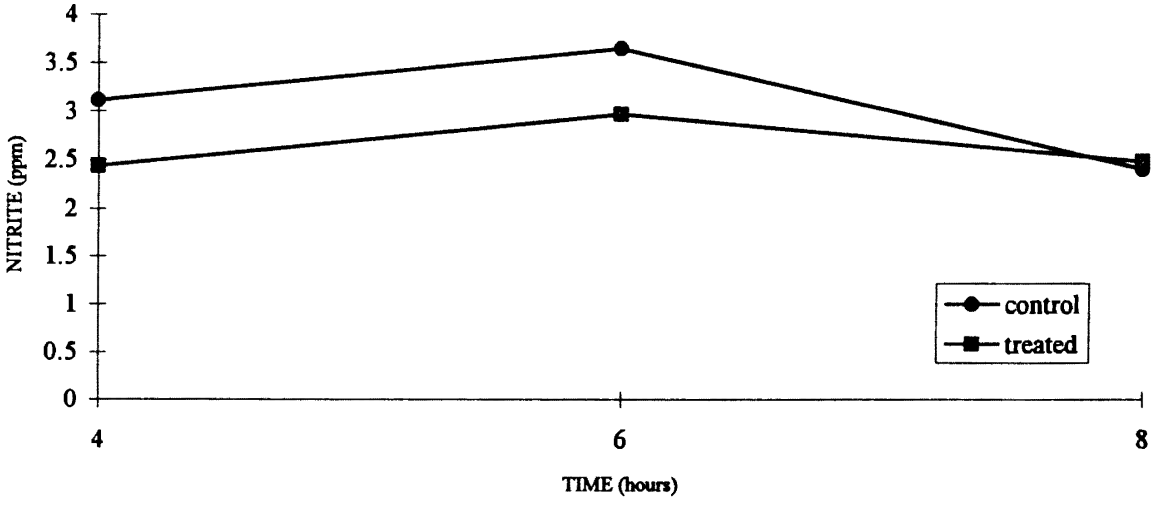


Figure 3. BLOOD NITRITE CONCENTRATIONS FOR FIELD TRIAL 1

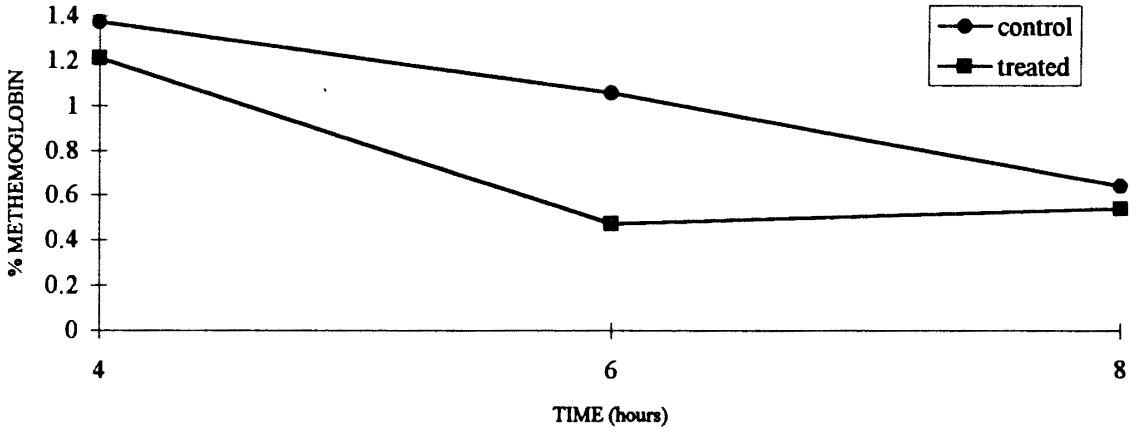


Figure 4. % METHEMOGLOBIN CONCENTRATIONS FOR FIELD TRIAL 1

APPENDIX C

FIELD TRIAL 2

FIELD TRIAL 2

Fifty-four crossbred commercial feedlot cattle were used to evaluate the efficacy of a commercial preparation of strain P5, tradename BOVA-PRO (FAR-MOR BIOCHEM, Milwaukee, WI). Twenty-seven animals were assigned randomly to one of two treatments. Both groups were allowed access to *ad libitum* low nitrate hay and water. In addition, both groups received 2 lb/head/d of protein supplement. One group was fed daily a pelleted protein supplement which contained $1x10^7$ CFU/ml of ruminal fluid of BOVA-PRO. The control group received an identical supplement formulated without BOVA-PRO. At the end of the initial establishment period, both groups were released onto sorghum-sudan hybrid green pasture. Six to eight animals from each group were monitored for rumen nitrate, rumen and blood nitrite and methemoglobin concentrations at 4, 6, 8 and 10 h post-release (via tail bleed and stomach tube) and then removed from the pasture.

FORAGE ANALYSIS:

The pasture utilized in this trial was gridded and samples analyzed for nitrate content were collected at intervals of 9.15 m. Nitrate analysis was conducted on dried samples with a ion specific electrode. Results indicated that nitrate concentrations in the pasture ranged from 4,930 to 39,600 ppm with an average concentration of 16,943 ppm nitrate.

RESULTS:

Ruminal nitrate concentrations decreased for both groups from 4 to 6 h but then treatment groups began to diverge; treated animals showed an increase in nitrate concentration while control animals continued to decrease (figure 1). Control animals had a mean concentration of 2378 ppm at 4 h which decreased to 1443 ppm by the 10 h sample. Conversely, treated animals began at a somewhat lower concentration (2010 ppm) at 4 h but at 10 h post-release had a higher mean concentration (2375 ppm) of rumen nitrate.

Ruminal nitrite concentrations for field trial 2 were exceedingly low when compared to toxicity trials (figure 2). Control animals peaked at 4 h (30 ppm) then dropped to 26 ppm at 6 h before leveling off at 25 ppm at 8 and 10 h post-release. Similarly, treated animals peaked at 4 h post-release (30 ppm). Ruminal nitrite concentrations in treated animals decreased continually to 17 ppm by 10 h post-release.

Again, blood nitrite concentrations for field trial 2 were exceedingly low when compared to toxicity trials (figure 3). Nitrite in control animals peaked at 4 h at 4.9 ppm and then leveled off at 3.34 ppm at 6 h and 10 h with a slight increase to 3.66 ppm at 8 h post-release. Meanwhile, treated animals had a peak mean blood nitrite concentration at 4 h of 3.80 ppm, which decreased to 3.47 and 2.86 ppm at 6 and 8 h respectively followed by a slight increase to 3.15 ppm by 10 h post-release.

Percent methemoglobin values for treatments parallel each other with treated animals being consistently lower. The values exhibited in this trial were extremely low compared to toxicity trials (figure 4). Both treatments began this trial at a similar point 1.63% for controls and 1.67% for treated at 4 h. Control animals exhibited their highest concentration of methemoglobin at 6 h (3.19%) which decreased to 1.56% at 8 h and 1.66% at 10 h post-release. Treated animals also showed their highest percent methemoglobin (2.74%) at 6 h which dropped sharply by 8 h to 1.11% and ended the trial at 1.25% of hemoglobin.

SUMMARY AND CONCLUSIONS:

Compared with uninoculated controls, animals treated with BOVA-PRO showed numerical decreases in rumen nitrite concentrations as well as percent methemoglobin, particularly during the 6, 8 and 10 h post-release sample periods. The concentrations of nitrite and methemoglobin in this trial were extremely low considering the concentration of nitrate present in the forage and the rumen. The discrepancy in nitrite release, particularly when compared to toxicity trials, may, be explained, in part, by the lower nitrate concentration in the forage and the form of forage consumed. In our toxicity trials, the high nitrate (20,000 ppm) forage was in the form of hay while in the current trial the lush, growing pasture averaged 17,000 ppm. Lush, green forages releases its nitrate more slowly than hays, possibly because nitrate is in a form or complex contained in a fully hydrated plant cell that resists microbial degradation.

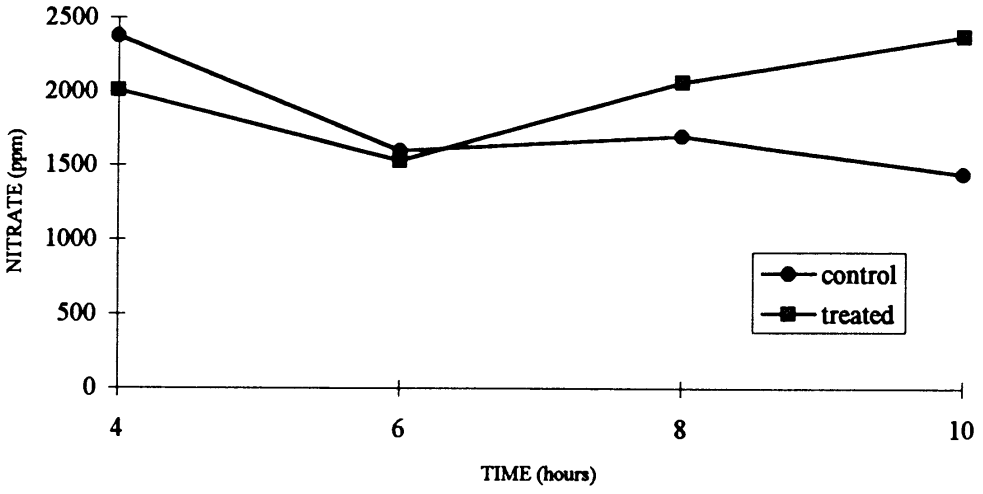


Figure 1. RUMEN NITRATE CONCENTRATIONS FOR FIELD TRIAL 2

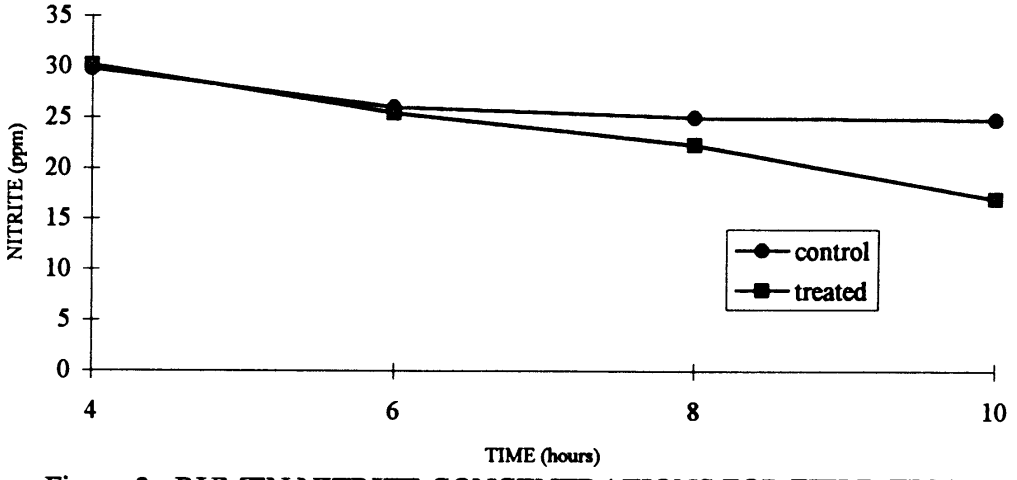


Figure 2. RUMEN NITRITE CONCENTRATIONS FOR FIELD TRIAL 2

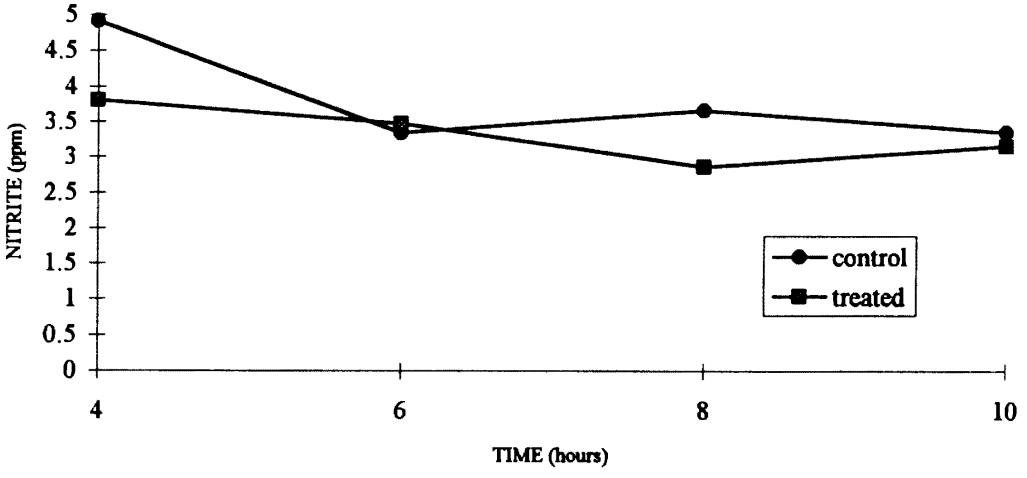


Figure 3. BLOOD NITRITE CONCENTRATIONS FOR FIELD TRIAL 2

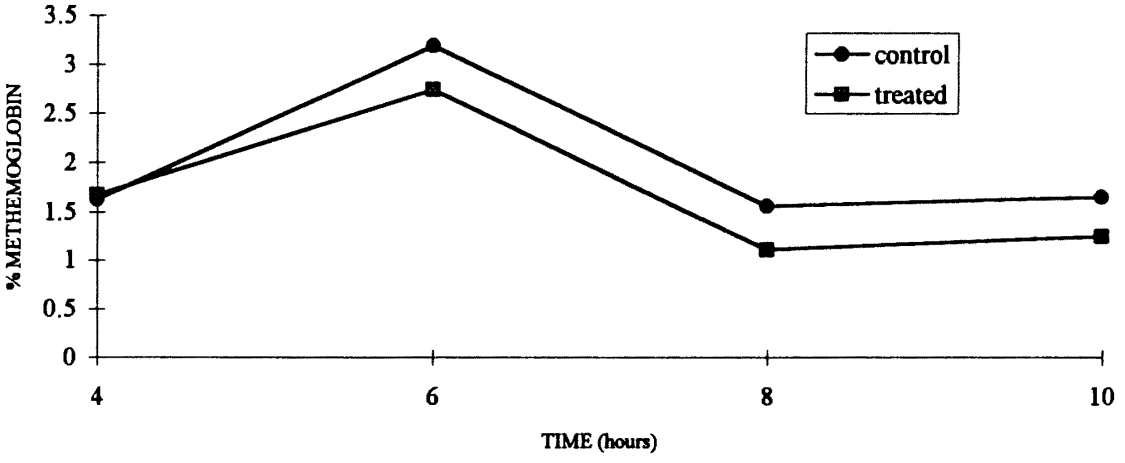


Figure 4. % METHEMOGLOBIN CONCENTRATIONS FOR FIELD TRIAL 2

APPENDIX D

DOSE RESPONSE TRIAL

Taken from: RESEARCH UPDATE-Use of Propionibacteria as a Direct Fed Microbial: Applications for Nitrate Toxicity.

By: T.G. Rehberger, C.A. Hibberd and J.H. Swartzlander

Dose Response Trial

Twenty-five crossbred heifers (500-600 lb) were used to evaluate the dosage effect of strain P5 on the response to a high nitrate challenge. Five heifers were randomly assigned to each of the five treatments. Doses tested included 0, 36,000, 130,000 and 2,900,000 CFU/ml of rumen fluid. In addition, one group received a one time dose of gel tongue paste. Blood nitrite and methemoglobin concentrations were monitored for a 12 hour period during consumption of the high nitrate feed to determine the relationship between the dose of strain P5 and the prophylactic response.

Treatments:

Prior to the high nitrate challenge, all animals received 2 pounds of supplement daily containing 0, 790,000, 2,800,000 or 63,000,000 CFU/g of strain P5 for eight days. These concentrations of strain P5 provided 0, 36,000, 130,000 and 2,900,000 CFU/ml of rumen fluid for establishment. Hay (low nitrate) and water were provided ad libitum. The treatments and the dosing rates for each group are summarized below.

Group 1: Feed- 790,000 CFU/g
P5 dose- 36,000 CFU/ml of rumen fluid daily for 8 days.

Group 2: Feed- 2,900,000 CFU/g
P5 dose- 130,000 CFU/ml of rumen fluid daily for 8 days.

Group 3: Feed- 63,000,000 CFU/g
P5 dose- 2,900,000 CFU/ml of rumen fluid daily for 8 days.

Group 4: Gel Tongue Paste- 15 g dose containing 3.3×10^{11} CFU/g administered 12 hour prior to challenge.

Group 5: Controls- no P5 added

Forage Analysis and Intake:

Following the eight day establishment period, heifers were given free access to coarsely chopped pearl millet hay as the high nitrate challenge. Composite hay samples collected from the treatment groups were analyzed for nitrates using an ion-specific electrode. Results indicate the pearl millet hay ranged from 31,200 to 32,400 ppm nitrate.

Treatment groups consumed on average 8.08 to 11.08 lbs per head of high nitrate hay. The individual intake for each treatment group is listed below. There was no relationship between the dose of strain P5 and feed intake.

Treatment	Intake (lbs/head)
Group 1 (36,000 CFU/ml)	8.8
Group 2 (130,000 CFU/ml)-	11.1
Group 3 (2,900,000 CFU/ml)-	8.1
Group 4 (GTP)	10.1
Group 5 (controls)	10.1

Results

Blood nitrite concentrations increased slowly and peaked at 10 hours post-feeding for four of the five treatment groups (see figure). Blood nitrite concentrations for group 1 (lowest dose) continued to increase from 8 to 12 hours post-feeding and did not peak by the termination of the trial. Heifers treated with the gel tongue paste had the highest peak blood nitrite concentration (26 ppm) while heifers in group 3 (highest dose) had the lowest peak nitrite concentration (13 ppm). The concentrations of blood nitrite were similar to those observed in trial 3 (20,000 ppm chalenge).

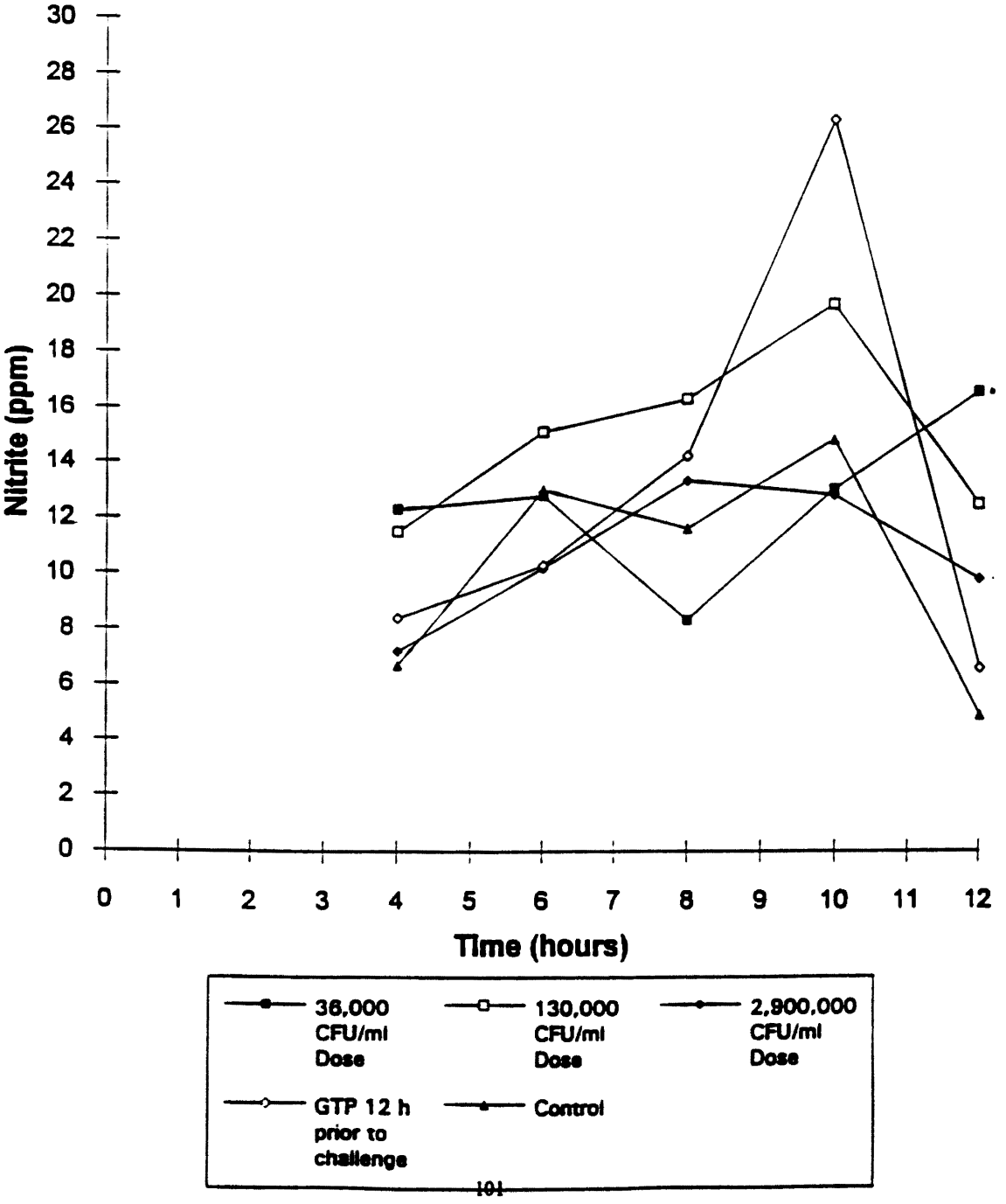
Methemoglobin concentrations increased over the 12 hour sampling period (see figure). At 12 hours post-feeding, heifers in four of the groups (group 1, 2, 4 and control) had higher percentages of methemoglobin than heifers receiving the highest dose of strain P5 (group 3). However, since the percentage of methemoglobin continued to increase over the sampling period the true peak values are unknown. Therefore, it is not possible to determine if this represents a prophylaxis response of the highest dose or a shifted methemoglobin response.

Summary and Conclusions

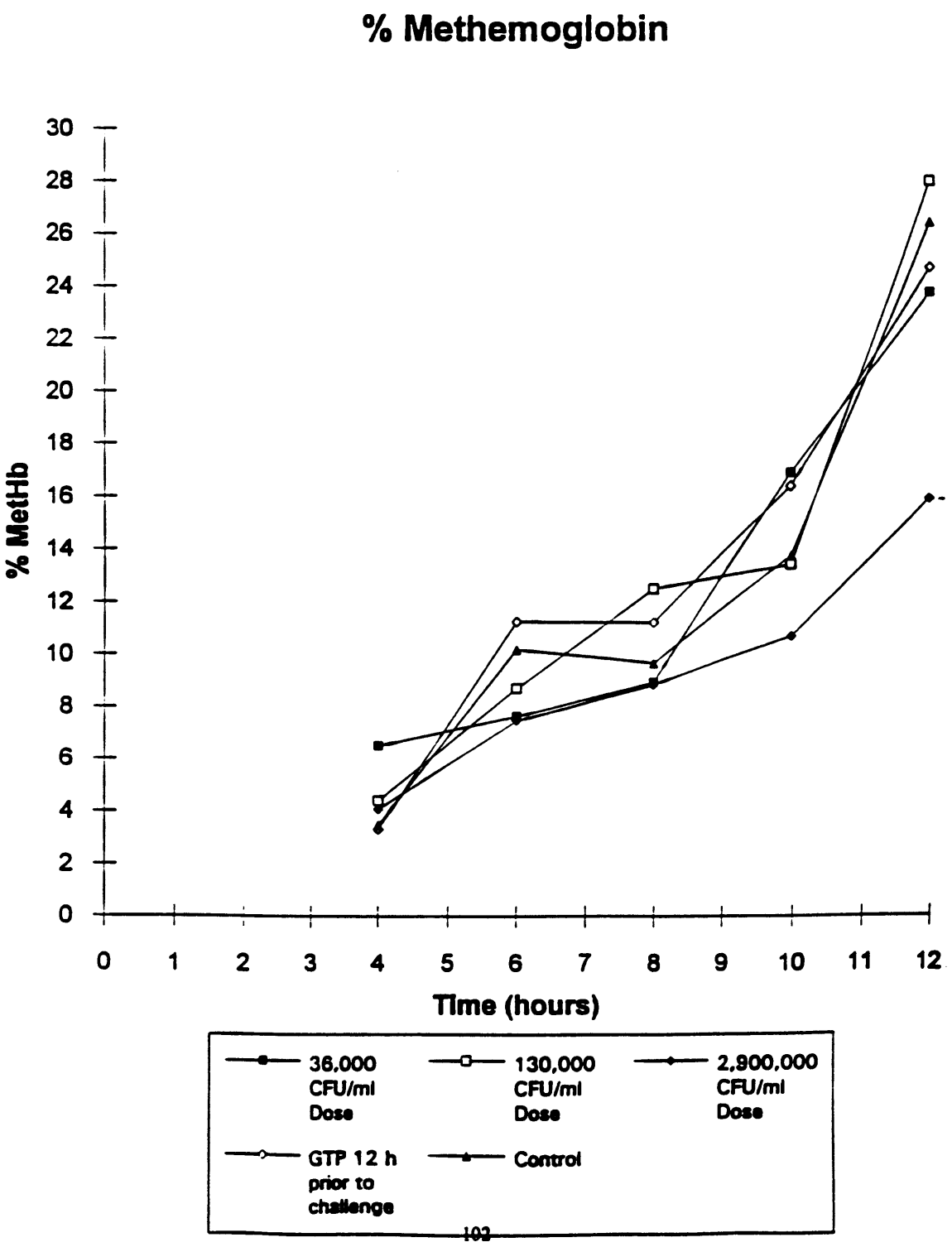
Heifers treated with the gel tongue paste had the highest peak blood nitrite concentration and a mean percentage of methemoglobin similar to controls. Therefore, the risk of nitrate toxicosis was not significantly reduced with the gel tongue paste. Heifers receiving the highest dose of strain P5, which was 3.5 times lower than the current recommended daily dose, had the lowest peak nitrite concentration and a percentage of methemoglobin much lower than controls at 12 hours post-feeding. An additional animal trial is necessary to confirm the efficacy of this lower dose since maximum values of methemoglobin were not definitively determined within the 12 hour sampling time.

Dose Response Trial

Blood Nitrite Concentration



Dose Response Trial



APPENDIX E

WITHDRAWAL TRIAL

Taken from: RESEARCH UPDATE-Use of Propionibacteria as a Direct Fed Microbial: Applications for Nitrate Toxicity.

By: T.G. Rehberger, C.A. Hibberd and J.H. Swartzlander

Withdrawal Trial

Eight crossbred heifers (700 lb) fitted with ruminal cannulas were used to evaluate the effect of withdrawal time from dosing strain P5 on the response to feeding a high nitrate forage. Heifers were withdrawn from dosing strain P5 180 days or 210 days prior to the high nitrate challenge. Heifers were fed coarsely chopped low-quality native grass hay and given 2 pounds of soybean meal daily prior to the nitrate challenge. Ruminal nitrate/nitrite and blood nitrite and methemoglobin concentrations were monitored for 12 hours during which time heifers were given free access to coarsely chopped pearl millet hay containing 31,000 ppm nitrate.

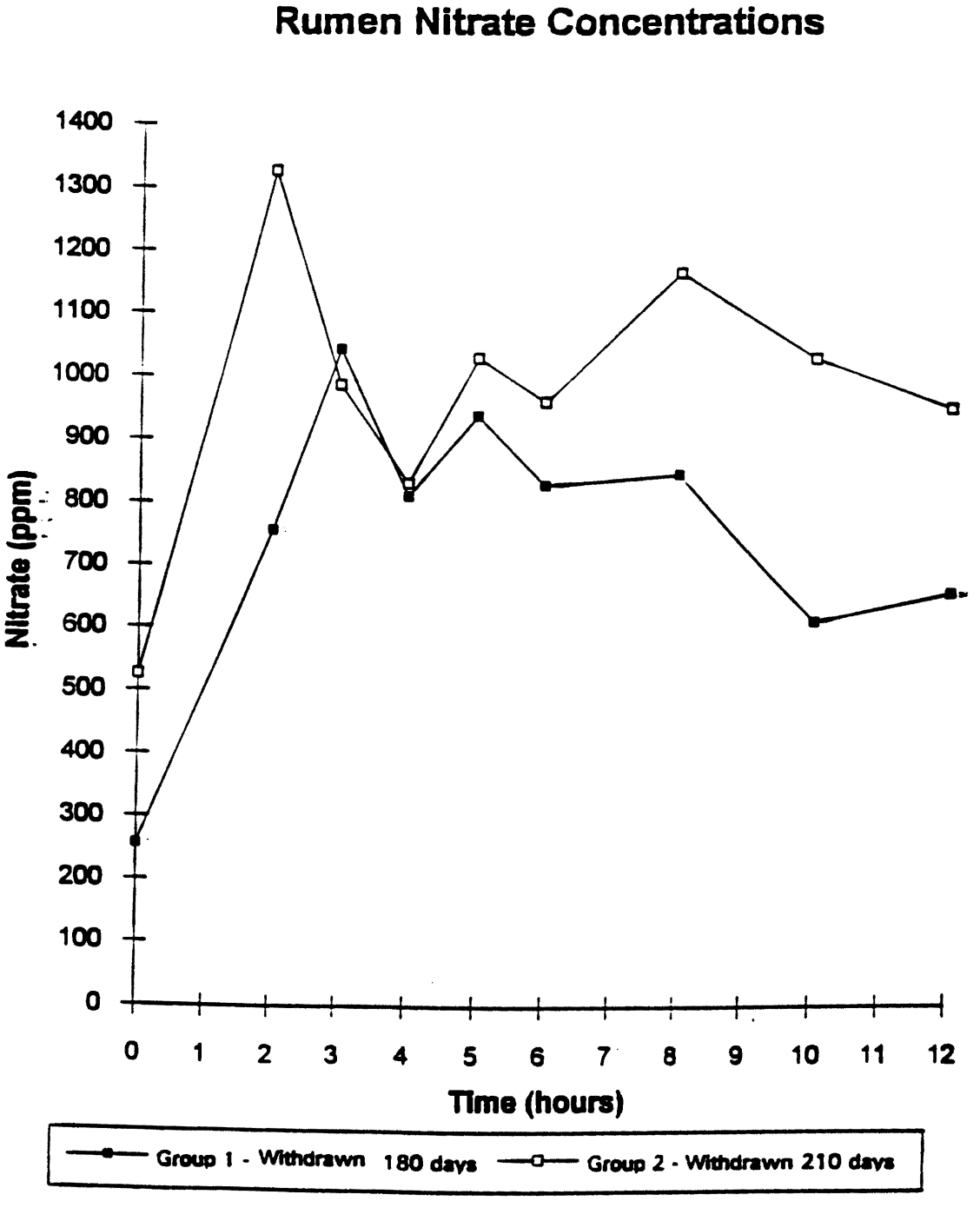
Results

Ruminal nitrate concentrations increased rapidly and peaked two to three hours post-feeding (see figure). Heifers withdrawn 210 days had nitrate concentrations that were higher than heifers withdrawn for 180 days. The mean ruminal nitrate concentration for heifers withdrawn for 210 days peaked at 1325 ppm which was 70% higher than ruminal nitrate concentrations observed in trial 3 (20,000 ppm challenge). Ruminal nitrite concentrations peaked at 5 hours post-feeding for heifers withdrawn 210 days and at 11 hours post-feeding for heifers withdrawn for 180 days (see figure). Heifers withdrawn for 210 days had a peak nitrite level of 580 ppm which was 1/2 of the peak ruminal nitrite concentration observed in trial 3 (20,000 ppm challenge). Heifers withdrawn for 180 days had a much lower ruminal nitrite level (270 ppm). The total quantity of ruminal nitrite for both groups, based on the total area under the curves, was much less than controls or inoculated heifers in trial 3 (20,000 ppm nitrate). These results indicate that heifers in both withdrawal groups had significantly reduced the nitrite to nitrous oxide or other nontoxic nitrogen compounds. Alternatively, it could be proposed that limited amounts of nitrate were reduced to nitrite resulting in the lower concentrations of ruminal nitrite. However, given the large number of nitrate reducing microorganisms in the rumen it is unlikely that nitrate reduction was limited.

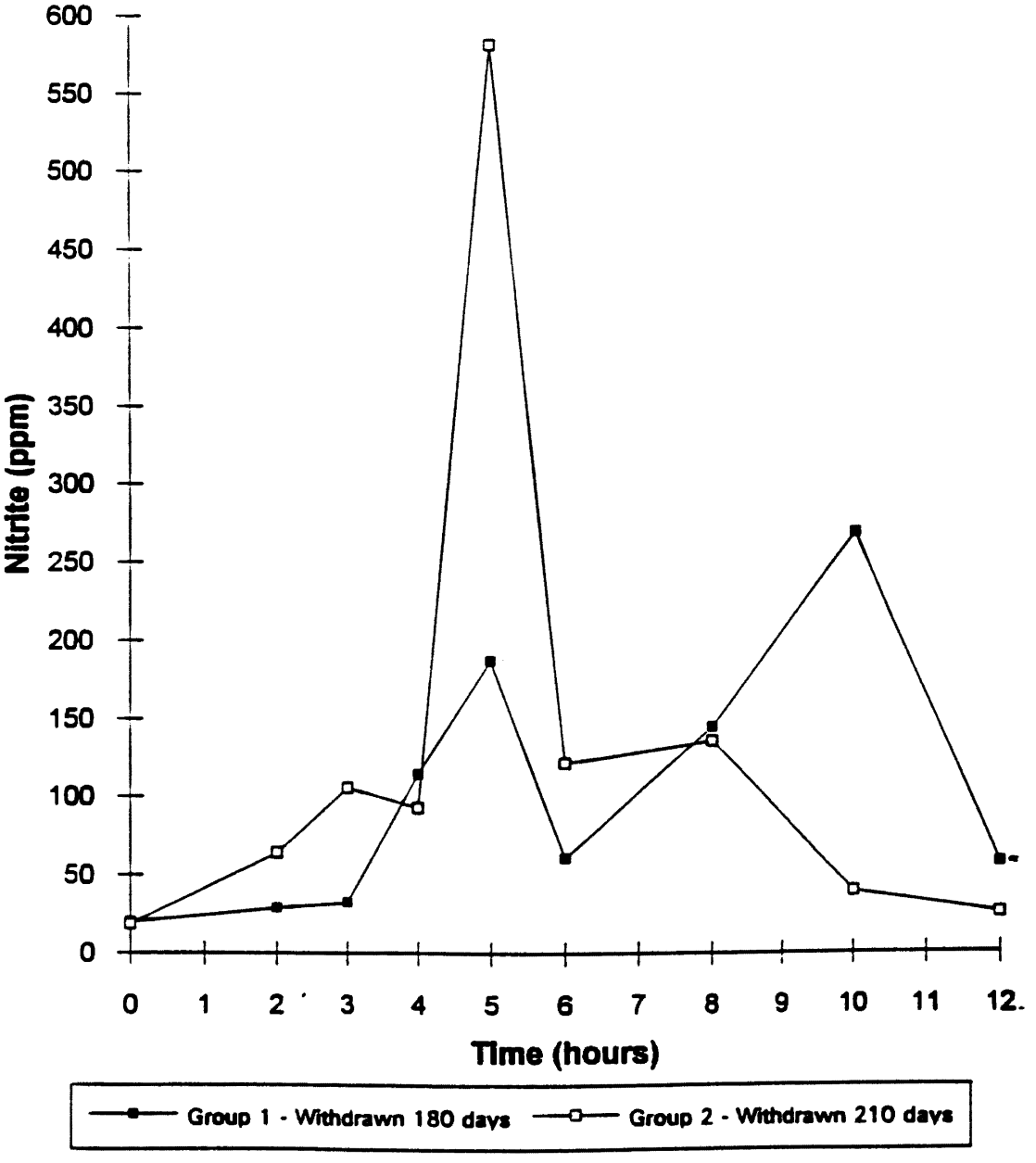
Blood nitrite concentrations showed a similar pattern to ruminal nitrite concentrations; heifers withdrawn for 210 days peaked at 5 hours post-feeding and heifers withdrawn for 180 days peaked 11 hours post-feeding (see figure). Again the peak values and total blood nitrite concentrations for both groups were lower than the blood nitrite concentrations for control and inoculated heifers observed in trial 3. The methemoglobin concentration, as determined as a percentage of the total hemoglobin concentration, peaked at 6 hours post-feeding and decreased to less than 1% by 10 hours post-feeding (see figure). Heifers withdrawn for 210 days had a higher peak and total methemoglobin during the trial than heifers withdrawn for 180 days however, this level is not considered to be high enough to result in toxicosis.

Implications

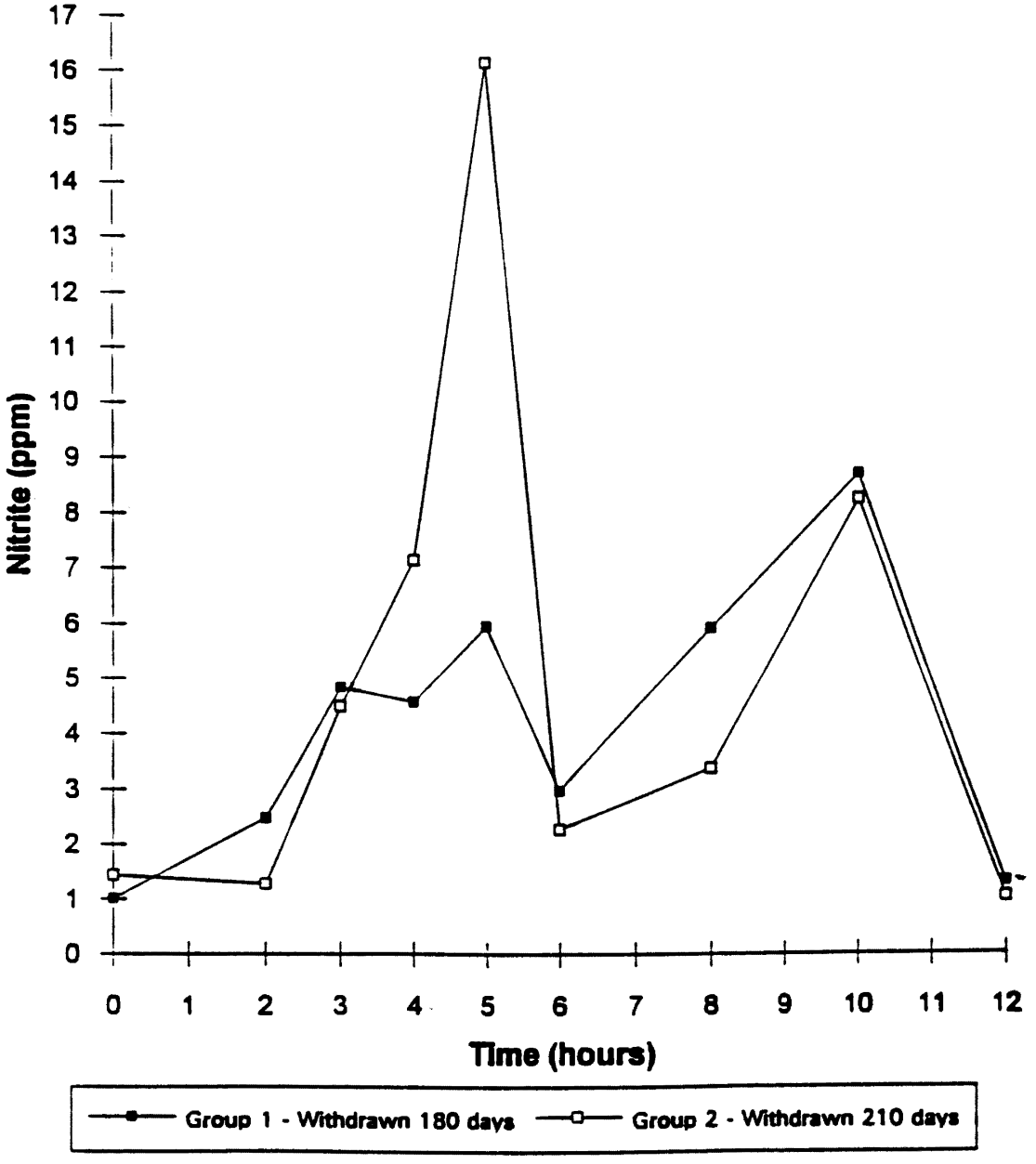
The nitrate toxicity studies with heifers withdrawn from dosing strain P5 for 180 and 210 days suggest that remaining populations of strain P5 will exert a measure of prophylaxis for cattle that consume large quantities of nitrate. In fact, the results indicate that heifers withdrawn from P5 dosing may reduce toxic nitrate concentrations more effectively than newly established heifers. Consequently, production losses to nitrate toxicity may be prevented for the duration of the stocker phase if producers initially establish an effective population of strain P5 in the rumen. Ultimately, this should simplify inoculation programs and provide extended protection after a single, successful establishment dose period.

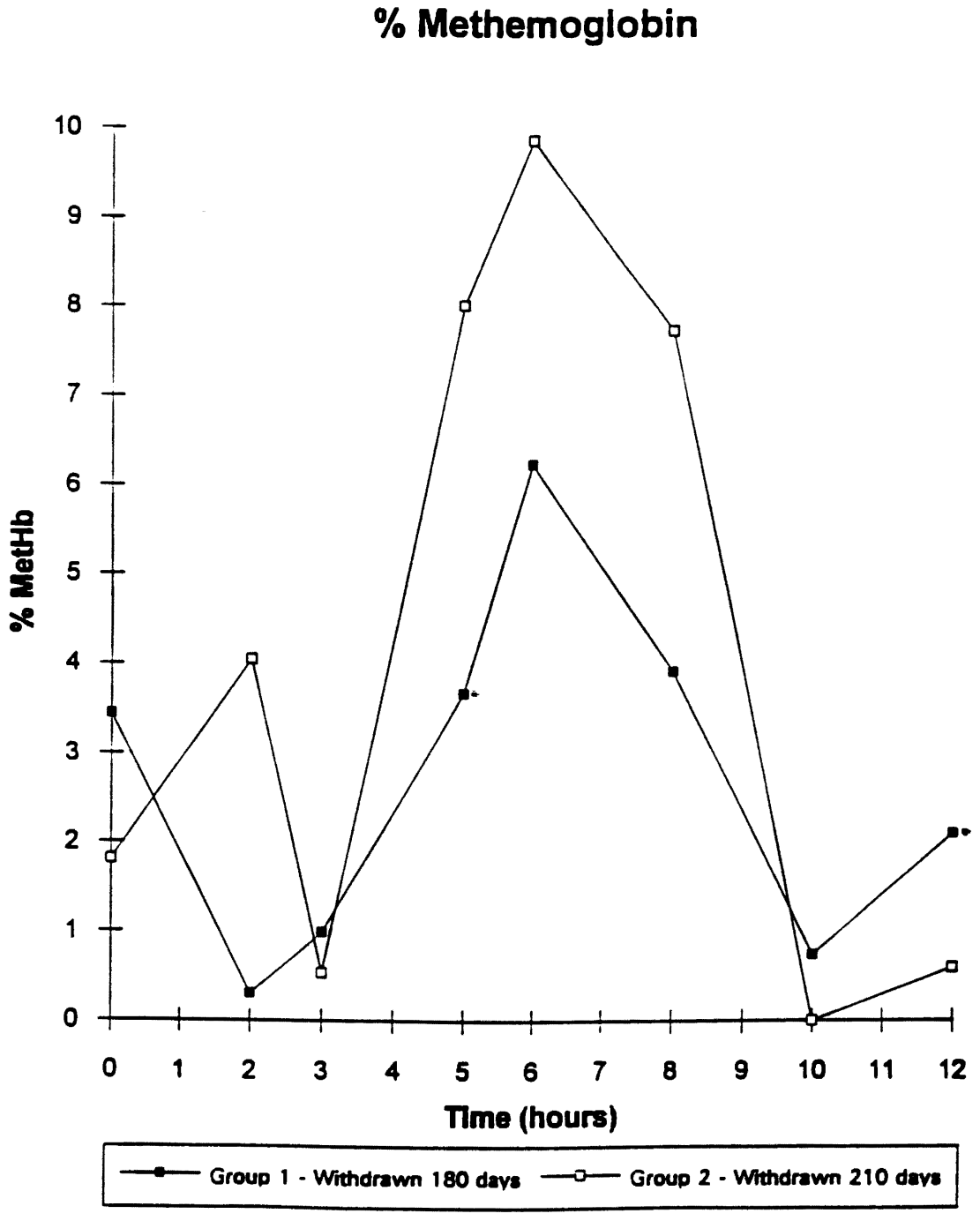

Rumen Nitrate Concentrations
Nitrate (ppm)
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (hours)
Group 1 - Withdrawn 180 days
Group 2 - Withdrawn 210 days

Rumen Nitrite Concentrations



Blood Nitrite Concentrations




% Methemoglobin
% MetHb
10
9
8
7
6
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (hours)
Group 1 - Withdrawn 180 days
Group 2 - Withdrawn 210 days

APPENDIX F

GEL TONGUE PASTE TRIAL

Taken from: RESEARCH UPDATE-Use of Propionibacteria as a Direct Fed Microbial: Applications for Nitrate Toxicity.

By: T.G. Rehberger, C.A. Hibberd and J.H. Swartzlander

Gel Tongue Paste Trial

The initial nitrate toxicity studies with heifers treated with a gel tongue paste 12 hours prior to the nitrate challenge provided conclusive evidence that a gel tongue paste was not effective at reducing the toxic effects of high nitrate consumption. Therefore, an additional study was conducted to determine if dosing the gel tongue paste at extended intervals prior to the nitrate challenge would establish strain P5 in the rumen and provide an effective population to exert a measure of prophylaxis.

Sixteen crossbred heifers weighing approximately 350 to 500 lb were randomly assigned to eight pens. Two pens of two heifers each were allocated to one of four treatments. Treatments consisted of the time of administration of the gel tongue paste prior to the high nitrate challenge (36 hours, 3 days or 7 days) and control heifers which did not receive a gel tongue paste dose. Heifers were given ad libitum access to low-quality native grass hay prior to feeding high nitrate hay. The high nitrate challenge was accomplished by providing ad libitum access to coarsely chopped pearl millet hay that contained a mean concentration of 20,380 ppm nitrate. Blood nitrite and methemoglobin concentrations were monitored for a 12 hour period during consumption of the high nitrate feed.

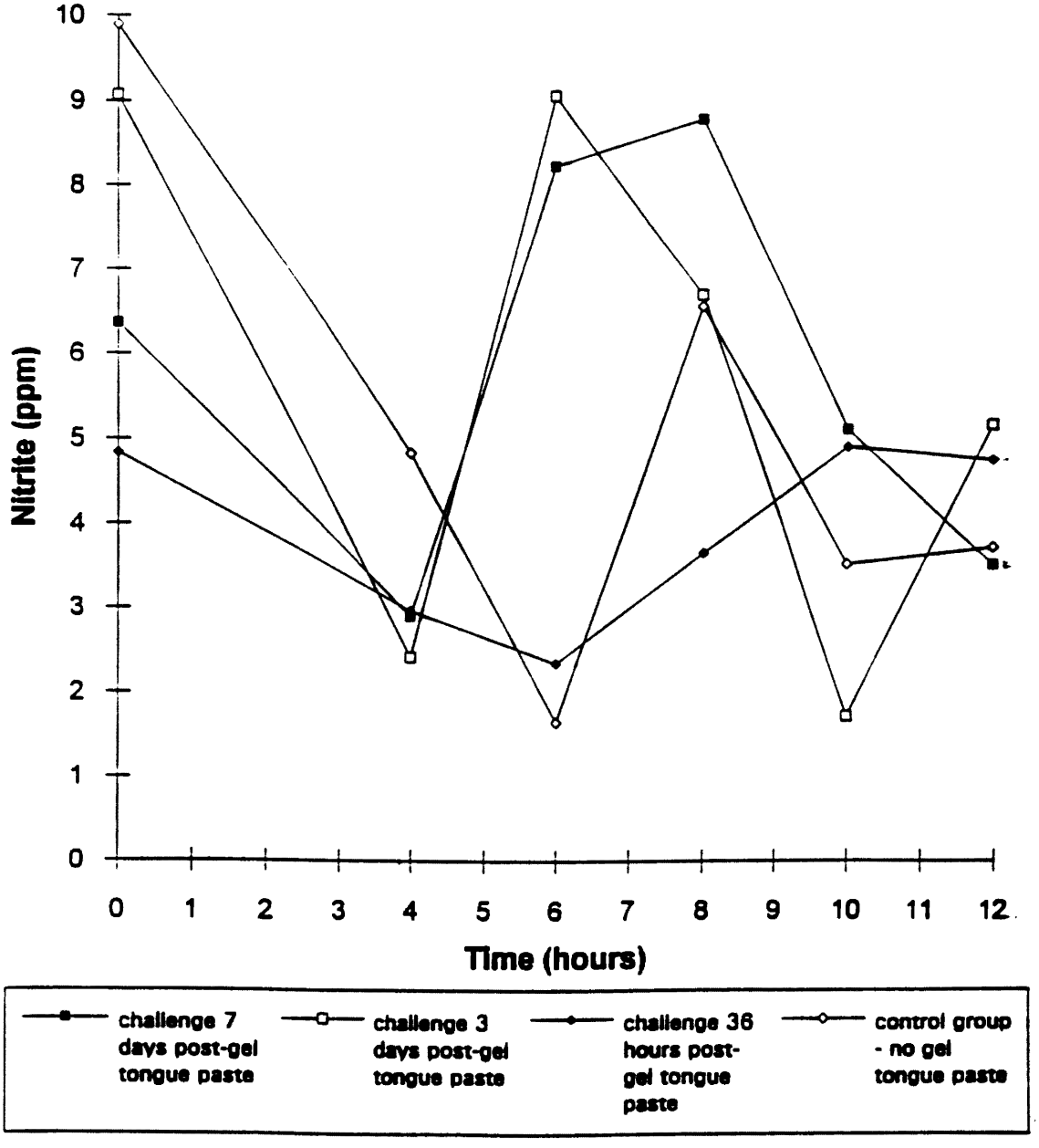
Results

Blood nitrite concentrations for all treatment and control heifers peaked at less than 10 ppm (see figure). Blood nitrite concentrations of control heifers were more than 50% lower than observed for trials 2 and 3 with a similar nitrate challenge (20,000 ppm). The percentage of methemoglobin increased slowly over the 12 hour sampling period and did not peak within the sampling period (see figure). However, the total percent methemoglobin for all treatment groups and controls were extremely low (<7%). Analysis of the feed intake data revealed that heifers consumed less than 1% of their total body weight (2.5 to 5 lbs). Therefore, due to the low intake levels, none of the heifers consumed a significant quantity of nitrate.

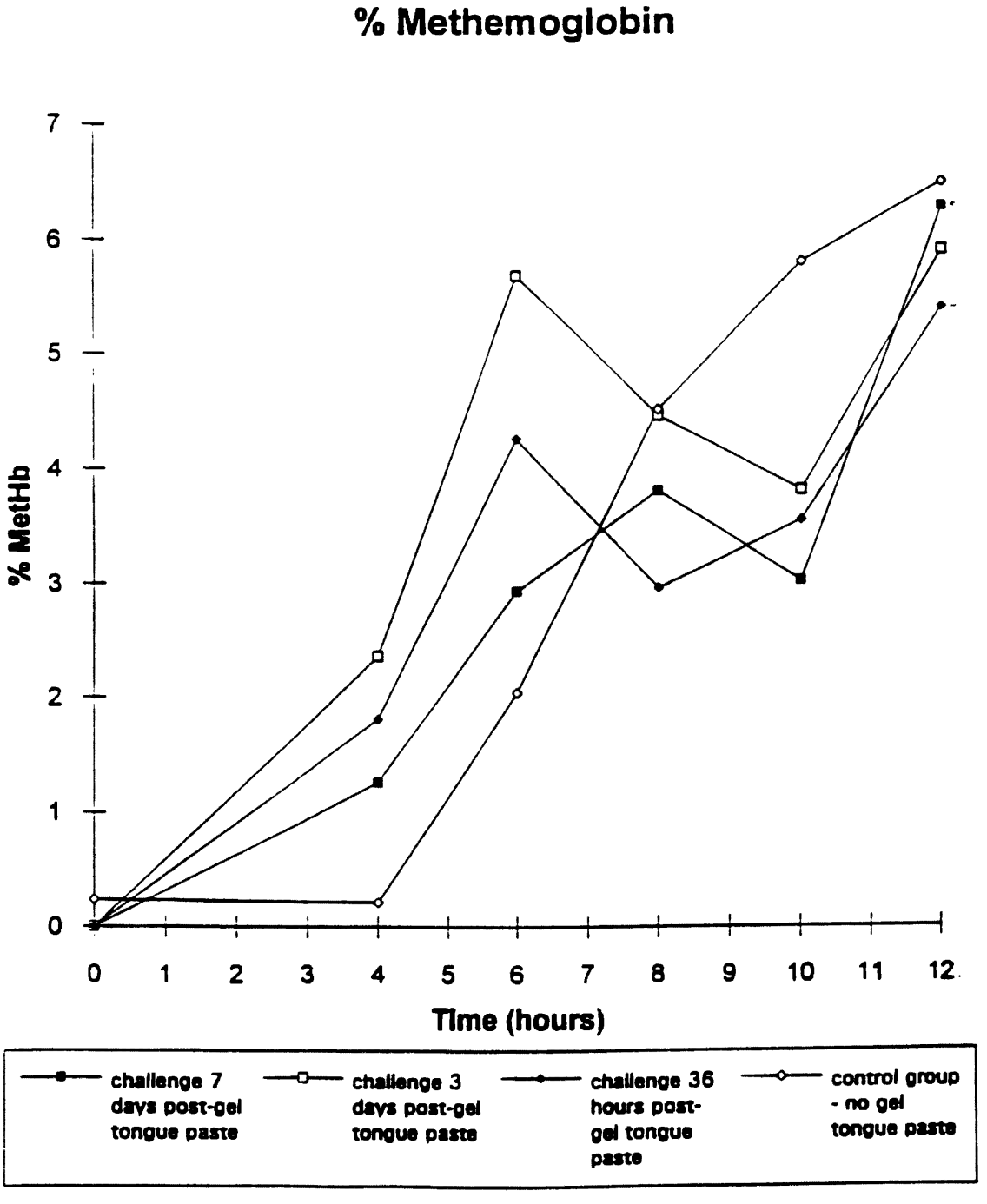
Conclusions and Implications

In conclusion, this trial will not be useful to determine if a gel tongue paste will effectly at provide a population of strain P5 capable of reducing the effects of nitrate toxicosis. In addition, given the results from earlier trials, the current working theory of the mode of action of strain P5 suggests that a one time dose of strain P5 will not provide an effective population to reduce nitrate toxicosis. Therefore, we strongly recommend alternative methods of delivery to prevent production losses due to nitrate toxicity.

Blood Nitrite Concentrations



Gel Tongue Paste Trial



VITA

JEFFERY HOWARD SWARTZLANDER

Candidate for the degree of

Master of Science

Thesis: SELECTION, ESTABLISHMENT AND *IN VIVO* DENITRIFICATION OF A *PROPIONIBACTERIUM* STRAIN IN BEEF CATTLE

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Professional Organizations: American Society for Microbiology