PROPIONIC ACID-PRODUCING BACTERIA AS BIOINOCULANTS FOR THE PRESERVATION OF ENSILED HIGH-MOISTURE CORN

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By

Tadd Everett Dawson

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ABSTRACT

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Aerobic instability is the major cause of spoilage, nutrient loss and feed refusal of ensiled, high-moisture corn. In addition, some organisms responsible for aerobic deterioration of ensiled, high-moisture corn can pose a health risk to livestock and agricultural workers through production of harmful compounds such as mycotoxins. A series of studies was conducted to address the hypothesis that addition of selected, propionic acid-producing bacteria will increase propionic acid content of ensiled, high-moisture corn and consequently decrease aerobic deterioration by controlling the growth of microorganisms implicated in causing aerobic deterioration of ensiled feeds. In experiment one, a mixture of two strains of cheese associated propionic acid-producing bacteria (*Propionibacterium acidpropionici* and *P. freudenreichii*) was applied with or without a commercial lactic acid-producing bacteria bioinoculant to reconstituted high-moisture corn. The mixture of *P. acidpropionici* and *P. freudenreichii* was effective in increasing propionic acid concentration to .26 g/ 100 g DM and decreasing numbers of yeasts and molds after 56 days of ensilement. Experiment two focused on enriching and isolating propionic acid-producing bacteria from ensiled feeds. A method was developed which facilitated isolation

of a rapid growing, high acid-producing strain of *P. acidpropionici* (designated DH42) from ensiled, high-moisture corn. Propionic acid production and growth rate of eight strains of propionic acid-producing bacteria, including *P. acidipropionici* DH42, when grown on substrates and competing with microorganisms found in ensiled, high-moisture corn were evaluated in experiment three. *Propionibacterium acidipropionici* DH42 had the greatest propionic acid production and growth rate indicating it was more competitive in ensiled, high-moisture corn. In the fourth experiment, *P. acidipropionici* DH42 was used as a bioinoculant in high-moisture corn. Addition of *P. acidipropionici* DH42 increased propionic acid content to .32 g/ 100 g DM after 42 days ensilement and to .61 g/ 100 g DM after five days aerobic exposure of the ensiled material compared to less than .05 g/ 100 g DM in control corn. Aerobic deterioration was dramatically reduced in the treated, high-moisture corn as evidenced by lower temperature, pH, numbers of yeasts and molds, total aerobic bacteria and *Acetobacter* during five days aerobic exposure of the ensiled material. A final study was conducted in which a non-radioactive nucleic acid probe to detect *P. acidipropionici* DH42 was developed. In conclusion, selected, propionic acid-producing bacteria are capable of being effective bacterial inoculants for preservation of ensiled, high-moisture corn.

This dissertation is dedicated to my Mother and the loving memories of my Father, Grandfather and Great-Grandmother whom all instilled the desire to pursue and to learn.

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INTRODUCTION

Storage of feedstuffs by ensilement is a major management practice used in ruminant livestock production. In 1992, over eight million hectares of land were used for corn production in Michigan producing greater than one hundred million bushels (approximately twenty billion kg) of corn. On a national scale, greater than ten billion bushels of corn were harvested with approximately half being fed to livestock. Three conventional methods of preserving the corn crop are whole crop silage, dry grain and as ensiled, high-moisture corn. Corn silage or ensiled, high-moisture corn is a major portion of the rations fed to feedlot and dairy cattle (Wilkens, 1981).

Ensiling high-moisture corn is popular because it allows producers to economically store and feed grain without added expenses incurred from drying. High-moisture corn is easily handled and allows for a more rapid fall harvest at an earlier date (two to three weeks), elimination of several rehandling operations, allows for greater mechanization, rodent free storage and decreased field losses when compared to corn stored as dry grain. However, some disadvantages associated with ensiling high-moisture corn are the need to determine moisture content at harvest, spoilage during feeding, approximately 25% more weight to handle and grain will heat (aerobic instability) if removed from the silo to soon before feeding (Jones, et al. 1974).

Aerobic deterioration, also called aerobic instability or poor bunk life, is due to the biological activities of aerobic microorganisms that metabolize substrates produced during the ensiling fermentation (e.g., lactic acid) or residual carbohydrates (e.g., glucose) of the original crop. The main products associated with this aerobic metabolism are carbon dioxide, water and heat. An inherent problem with the ensilement of high-moisture corn is the loss of nutrients or dry matter as carbon dioxide and heat when exposed to atmospheric oxygen. Loss of dry matter by aerobic deterioration is an inefficiency that depletes digestible nutrients and prevents maximum use of the feedstuff by ruminant animals. Previous research summarized by Henderson et al. (1979) and Honing and Woolford (1990) showed that aerobic instability and deterioration of ensiled feeds can account for losses in excess of 35 % of the original crop dry matter.

Generally, good silo management practices and maintaining anaerobic conditions during ensilement will help prevent aerobic deterioration during storage (Woolford et al., 1982). The natural fermentation that occurs in non-treated silage is a lactic acid fermentation similar to that found in sauerkraut. Production of lactic acid is generally sufficient to decrease pH of the ensiled crop below 4.5. Low pH generally yields a metabolically stable feed when stored anaerobically. However, lactic acid is a preferred substrate for microorganisms implicated in the aerobic deterioration process.

Antibiotics (Woolford and Cook, 1978) and direct acidification (Jones et al. 1974; Ohyama and McDonald, 1975) have been used to address problems associated with aerobic deterioration. Ohyama and coworkers (1975, 1979) directly acidified corn and grass silage with mixtures of formic, propionic, butyric and other volatile fatty acids. Results showed

success in preventing aerobic deterioration with increasing chain length of the volatile fatty acid used. Propionic acid is commonly used in the baking and dairy industries as a mycostatic and bacteriostatic agent to help preserve products such as breads and cheese (Glatz, 1992). Ohyama and McDonald (1975) showed that addition of propionic acid at low levels (1 to 3 percent) to ensiled crops reduced aerobic deterioration and increased dry matter intake when fed to dairy cattle. Bulk quantities of acetic, formic and propionic acid are currently obtained from petroleum products. Costs associated with these processes usually precludes their use as additives to ensiled crops for prevention of aerobic deterioration. In addition, direct use of acids has inherent problems associated with operator health and equipment damage.

Current research dealing with silage is focused on developing new methods to preserve the harvested crop in order to achieve the greatest feed quality possible. To accomplish this goal, new and innovative methods of controlling silage fermentation and aerobic instability need to be developed and implemented. The overall objective of this dissertation research was to develop and study a novel method of manipulating the fermentation and aerobic deterioration of high-moisture corn by using selected, propionic acid-producing bacteria as bioinoculants.

The specific aims of this dissertation research were:

1. Select species of propionic acid-producing bacteria capable of increasing propionic acid content of ensiled, high-moisture corn.

2. Study and optimize conditions that affect the performance of the selected, propionic acid-producing bacteria.
3. Develop nucleic acid probes for the selected, propionic acid-producing bacteria that will allow more precise evaluation of their ecological interaction.
4. Evaluate the use of the selected, propionic acid-producing bacteria as bioinoculants for ensiled, high-moisture corn.

Chapter 1 reviews pertinent literature related to losses associated with aerobic deterioration of ensiled crops and previous attempts to reduce these losses. In addition, Chapter 1 reviews interrelationships of various microbial ecosystems and niches as they apply to the fermentation and aerobic instability of ensiled feeds. A study addressing the first and third objective using two classical strains of propionic acid-producing bacteria is discussed in Chapter 2. Chapter 3 describes the methodology used for enrichment and isolation of propionic acid-producing bacteria from ensiled feeds. A strain of *Propionibacterium acidipropionici*, designated *P. acidipropionici* DH42, was isolated from ensiled, high-moisture corn and is characterized in Chapter 4. The ability of propionic acid-producing bacteria, with emphasis on *P. acidipropionici* DH42, to compete for substrates against the epiphytic microbial population of high-moisture corn is evaluated in Chapter 5. Chapter 6 discusses a study that uses results from previous chapters and evaluates *Propionibacterium acidipropionici* DH42 as a bioinoculant for ensiled, high-moisture corn. Chapter 7 deals with development and validation of a non-isotopic nucleic acid probe for *P. acidipropionici* DH42. Research findings are summarized and conclusions based on the objectives are discussed in Chapter 8. Pertinent data with

corresponding analysis of variance tables and supporting studies are presented in three appendices.

The overall hypothesis for this research is that addition of selected, propionic acid-producing bacteria as bioinoculants to ensiled, high-moisture corn will increase production of propionic acid during the ensiling fermentation. Consequently, increased concentration of propionic acid in ensiled, high-moisture corn will decrease extent of aerobic deterioration and instability by controlling growth of microorganisms responsible for aerobic deterioration.

CHAPTER 1

REVIEW OF THE LITERATURE

Principle objectives of any preservation method are adequate recovery of nutrients and production of a product that is acceptable to livestock (Barnett, 1954; McCullough, 1978). Previous strategies for efficient, silage fermentation encouraged high levels of lactic acid production and lower pH (below 4.2) during the anaerobic fermentation phase. This efficient fermentation usually produces silage that is stable under anaerobic storage conditions. However, once the ensiled mass is disturbed during feedout or other storage perturbations, aerobic microorganisms will begin to metabolize the lactic acid and residual carbohydrates. The resulting heat and mold formation has been defined as poor bunk stability or aerobic instability. Nutrient losses (as dry matter) during aerobic metabolism (instability) have been reported to be as high as 35% (Henderson et al., 1979). The Food and Agriculture Organization of the United Nations estimates that grain losses during storage are in excess of 5%.

Importance of ensiled feedstuffs

In a summary of past silage research, McCullough (1993) reported that over 120 million tons of ensiled crops are produced on an annual basis in the United States costing

over 2.25 billion dollars (McCullough, 1978). Of this amount, a vast majority is used for feeding ruminant animals (Wilkens, 1981). Importance of ensiled feedstuffs to animal agriculture has been recently discussed by Pitt (1990) and Bolsen (1991).

Phases of the ensilement process

Phases of the ensilement process that ultimately affect the resulting silage are generally broken down into five phases (Barnett, 1954; Muck, 1988). These phases are plant cellular respiration after harvest, initial acetic acid fermentation, initial lactic acid fermentation, major lactic acid fermentation and the quiescent storage phase. The quiescent phase can be interrupted by either a possible secondary fermentation initiated by either clostridia or aerobic deterioration often due to air infiltration. Besides these five phases, the quality of the silage preserved can be influenced by two additional factors or phases. These two additional phases are the interactions of the epiphytic microbial population with the crop in the field and resulting microbial growth at feedout. Other physical factors influence the seven phases of ensilement and therefore the quality of the silage fed by influencing the pattern of the fermentation. Major factors include crop moisture, packing density of the ensiled crop, ambient temperature at harvest, temperature during silo filling and unloading and crop maturity.

Microbiology and biochemistry of the ensilement process

The microbial ecosystem associated with ensilement is very diverse and dynamic. Pahlow (Figure 1-1 as referenced by Woolford, 1991) diagrammed the general interactions associated with the major microbial groups associated with the silage fermentation.

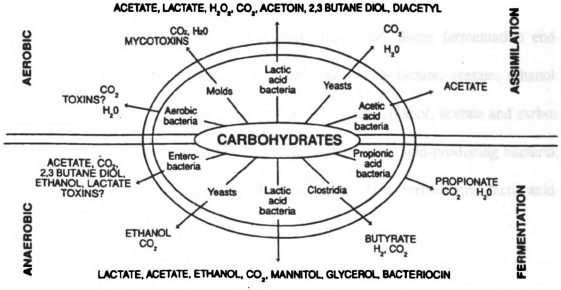

Figure 1-1. Aerobic and anaerobic metabolism of forage carbohydrates by various microorganisms associated with the ensilement fermentation (Pahlow, 1991 as referenced by Woolford, 1991).

Microorganisms of interest in silages include various aerobic bacteria, lactic acid-producing bacteria, acetic acid-producing bacteria, propionic acid-producing bacteria, endospore-forming bacteria, coliforms, listeria and yeasts and molds. Each of the above described microbial groups affects the fate of carbohydrates and fermentation products such as lactic acid and carbon dioxide.

Anaerobic fermentation of plant carbohydrates to lactic acid and the resulting decrease in pH is the biological basis to the preservation of ensiled feeds. Lactic acid-producing bacteria will produce a diverse array of end-products depending on species involved, presence of oxygen and carbohydrate fermented. Some fermentation end-products produced by lactic acid-producing bacteria include lactate, acetate, ethanol, carbon dioxide, mannitol, glycerol and bacteriocins. Lactate, ethanol, acetate and carbon dioxide are normally associated with heterofermentative lactic acid-producing bacteria, whereas lactic acid is the predominant end-product of the homofermentative lactic acid-producing bacteria (Seale, 1986).

Growth and metabolism of clostridia are generally not associated with high quality silage. Clostridia are normally associated with poor ensiling conditions where a sufficiently low pH was not attained. Fermentation products associated with clostridia are butyrate, carbon dioxide and hydrogen. Coliforms, commonly referred to as enterobacteria, are also associated with poor quality silage (Östling and Lindgren, 1991). Acetic acid, ethanol, 2,3-butanediol, carbon dioxide, lactic acid and toxins have been found in silages where coliforms dominated the ensiling fermentation. Yeasts have also been found to grow

anaerobically in silage and their main end-products of carbohydrate metabolism are ethanol and carbon dioxide.

Nitrogenous compounds, such as amino acids and proteins, can be metabolized by microorganisms associated with aerobic deterioration and poor ensilement to ammonia-nitrogen and volatile amines. These compounds have been shown to have detrimental effects on organoleptic properties and animal acceptance of resulting silages (McDonald, 1981; Woolford, 1984).

Influence of oxygen on ensilement and aerobic deterioration

Aerobic microbial metabolism in ensiled feedstuffs when exposed to oxygen has been the focus of many studies (Woolford, 1990; Ruxton and Gibson, 1993). Significant losses are attributed to aerobic activity, with the majority due to aerobic deterioration and surface wastage (Table 1-1). Aerobic instability, or poor bunk life, is characterized by increases in silage temperature and pH, decreases in lactic and acetic acid and a concomitant reduction in feeding value (Honing and Woolford, 1979).

Many physical changes occur to ensiled, high-moisture corn during aerobic deterioration. The germ, embryo, entire seed or kernel can become discolored (Jones et al., 1974). Heating and mustiness is generally accompanied with losses of dry matter during aerobic deterioration of ensiled, high-moisture corn (McDonald, 1981). In addition, biochemical changes occur within the grain which lead to a decrease in the digestibility of nitrogen containing nutrients, such as proteins and amino acids. Evidence of this phenomena is the increase in losses of nitrogen in urine due to the increase in ammonia

Table 1-1. Source, magnitude, and causative factors of dry matter losses during ensilement and aerobic exposure (Henderson et al., 1979; Woolford, 1990)

Source	Dry Matter Loss (%)	Causative Factor
Initial plant respiration	1 - 2	Plant enzymes and aerobic microorganisms
Primary fermentation	0 - 4	Lactic acid bacteria
Secondary fermentation	0 - 5	Clostridia
Effluent and/or wilting	2 - 7	Low dry matter or wilting technique
Surface waste	0 - 10	Aerobic microorganisms
Aerobic deterioration	0 - 35	Aerobic microorganisms

content from the deamination of amino acids (Pitt, 1986). In addition, the Maillard reaction can cause increases in the amount of nitrogen containing compounds found in feces, indicating decreased nitrogen retention (Pitt, 1986). Potential for production of harmful metabolites by the microflora associated with the deterioration process, such as mycotoxins, can be a problem (Lee et al., 1986; Woolford, 1984a; Widstrom, 1992.).

Majority of losses associated with aerobic deterioration occur on the surface during storage in the silo and feed-out losses from the surface (exposed silage face) as feed is removed (Woolford, 1984b). Several factors influence the extent of aerobic instability in ensiled feeds. Main factors are moisture content of the grain, temperature during storage, temperature of grain before storage, temperature at feeding, length of storage, oxygen infiltration during storage, tightness of packing, number of aerobic microorganisms and substrates available for metabolism by aerobic microorganisms (Jones et al., 1974, Ohyama et al., 1981; Muck, 1988). Pitt (1986) summarized additional factors that have an effect on poor bunk life. These factors are presence of oxygen, presence of carbon dioxide, silage dry matter content, forage species ensiled and concentration of fermentation acids.

Recent reports, reviews, and models relevant to aerobic deterioration of ensiled crops include Courtin and Spoelstra (1990), Woolford (1990), Pitt et al. (1991a,b), Muck and O'Keily (1992) and O'Keily and Muck (1992). The review by Woolford (1990) explains the extent of the problems associated with aerobic instability of ensiled feeds and is a good general reference for this area of research. Mathematical models have been developed that combine research results that identify the causes of aerobic instability and

simplify the prediction of possible aerobic deterioration in ensiled feeds. Courtin and Spoelstra (1990) developed a mathematical model which predicts the stability of grass and corn silages by simulating the growth of yeast and acetic acid bacteria (*Acetobacter spp.*), oxidation of fermentation acids, consumption of oxygen and concomitant production of carbon dioxide and rise in silage pH. Initiation of aerobic instability is attributed to the growth of acetic acid bacteria with or without the parallel growth of yeasts in the model of Courtin and Spoelstra (1990). In addition, this model and most other models associated with the silage fermentation consider the effects of initial number of yeasts and molds, pH, moisture content, lactic acid and acetic acid concentrations on stability. All of these factors directly influence which microbial population is given an environmental advantage for growth and therefore, which populations will predominate. Pitt and coworkers (1991b) developed a mathematical model to predict aerobic instability as affected by the growth of yeasts and molds. It was concluded that temperature, water-soluble carbohydrate concentration, fungal populations and concentration of undissociated acidic fermentation end-products had the largest effects on aerobic instability of corn silage.

Muck and O'Kiely (1992) studied the effect of fermentation end-products on the initiation and extent of aerobic deterioration of alfalfa and corn silages. The effect of yeasts on the aerobic deterioration of the same silages was reported in a companion paper, O'Kiely and Muck (1992). In alfalfa silage, a "stability factor" or deterioration inhibitor was found to be present in certain silos after the fermentation was completed and was not thought to be a major or principle product of the ensiling fermentation (O'Kiely and Muck, 1992).

Wardynski (1991) evaluated aerobic instability of high-moisture corn stored at different temperatures, moisture levels and inoculation rates. As moisture level increased from 22 to 37%, dry matter recovery from laboratory silos decreased. Additionally, as corn moisture increased, aerobic instability increased. High-moisture corn removed from laboratory silos stored at 4°C was less stable than corn stored at 20°C. Inoculation with lactic acid-producing bacteria increased dry matter recovery from the silo but did not alter aerobic instability.

Oxygen can dramatically influence the seven phases associated with ensilement during the field phase, initial aerobic phase in the silo, air infiltration during storage and aerobic deterioration after opening the silo before feeding. This is due to enhancement of the growth of microorganisms involved in processes associated with aerobic deterioration.

Microbiology associated with aerobic deterioration

Factors that determine the specific microorganisms that will proliferate in silage when exposed to air are not fully understood (Woolford, 1990; Courtin and Spoelstra, 1991). Simultaneously with the growth of these organisms, silage pH and temperature will increase (Courtin and Spoelstra, 1991). Increases in silage temperature are associated with the growth of yeasts, molds and certain bacteria (Woolford, 1990; Rust and Yokoyama, 1992). The microbes responsible for aerobic instability metabolize many substrates, some derived from the silage fermentation and others that are residues of the initial crop (Woolford, 1990).

In a recent survey of aerobic instability of high-moisture corn from 29 farms in Michigan (Rust and Yokoyama, 1992), aerobic instability was positively associated with yeast and mold populations. Neither fermentation end-products nor pH was associated with instability. In addition, propionic acid was not a major component of the fermentation end-products. Similarly, in corn silage, lower pH and greater lactic acid did not improve stability (Rust et al., 1989).

Aerobic respiration of microorganisms associated with instability of ensiled feeds can result in formation of an inedible crusty or slimy layer and the possible formation of toxins. Potentially pathogenic microorganisms have been found in aerobically deteriorating silages (Table 1-2; Woolford, 1990). These include microorganisms that pose a potential health hazard to livestock and agricultural workers (Ruxton and Gibson, 1993). Woolford (1990) summarized research findings that specifically found the occurrence of the organisms responsible for listeriosis, botulism and mycotoxin formation in aerobically unstable silages.

Aerobic metabolism of carbohydrates by silage microorganisms (Figure 1-1) leads to the production of diverse metabolic end-products. As a whole, the aerobic heterotrophic microbial population associated with silages will metabolize forage carbohydrates to carbon dioxide, water and heat. However, there are a few exceptions that produce other compounds such as propionic acid-producing bacteria. In addition, some toxins associated with enterobacteria and molds can be associated with the aerobic metabolism of forage carbohydrates (Woolford, 1990). Yeasts aerobically metabolize carbohydrates to carbon dioxide, water and heat as does the mixed heterotrophic aerobic microbial population.

Table 1-2. Microorganisms previously isolated from aerobically deteriorating corn silage and ensiled, high-moisture corn (Burmeister and Hartman 1966; Beck, 1978; and Seitz et al., 1982a,b)

Yeasts	**Molds**
Candida intermedia	*Geotrichium spp.*
Candida krusei	*Monascus spp.*
Candida parapilosis	*Monilia spp.*
Candida melini	*Mucor spp.*
Hanselua anomala	*Penicillium spp.*
Pichia fermentans	
Pichia membranefaciens	
Saccharomyces baili	
Saccharomyces exiquis	
Torulopsis candida	
Proteolytic bacteria	**Lactic acid-producing bacteria**
Bacillus cereus	*Lactobacillus buchneri*
Bacillus firmus	*Lactobacillus bulgaricus*
Bacillus lentus	*Lactobacillus viridescens*
Bacillus sphaericus	*Pediococcus cerevisiae*
Aerobic deterioration inititators	
Acetobacter spp.	

Lactic acid-producing bacteria can aerobically metabolize soluble forage carbohydrates to acetate, lactate, hydrogen peroxide, carbon dioxide, acetonin, 2,3-butanediol and diacetyl. Acetic acid-producing bacteria have been implicated as initiators of the aerobic deterioration process (Spoelstra, 1990). These microorganisms will metabolize carbohydrates to acetate and carbon dioxide.

Many problems are associated with the diverse end-products produced by the aerobic silage microbial population. This is mostly due to the concomitant production of heat associated with aerobic instability. Heat production during aerobic instability can easily cause the silo biomass to exceed 55 °C (Wardynski et al., 1993) which will decrease feed acceptance by cattle and can reduce the availability of nitrogen containing compounds.

Methods of preventing aerobic deterioration

Methods commonly used to reduce losses associated with aerobic deterioration deal with either increasing the rate of pH decline or by attempting to inhibit growth of aerobic microorganisms. Inorganic acids such as sulfuric, hydrochloric and phosphoric have been used to preserve harvested crops by rapidly decreasing the pH below 4 which will generally lead to a metabolically quiescent biomass during anaerobic storage (Woolford, 1978). Use of these additives is becoming less attractive because of hazards associated with the use of strong acids. Besides health risks to personnel, other negative factors include feed refusal by animals, destruction of harvesting and storage equipment and increased environmental liability from the effluent from silages treated with theses acids.

Organic acids have been effective in the inhibition of yeasts, molds, and bacteria that are responsible for the deterioration of stored crops such as hay and silages (Ekstrom, 1973; Jones et al., 1974; Raeker et al., 1992). Formic acid has been widely used in Europe with grass silages. Acetic acid is also effective in the preservation of ensiled feeds (Crawshaw et al., 1980). Many commercial hay and silage preservation amendments are based on mixtures of formic and acetic or acetic and propionic acid. However, demand for acetic acid as a preservative for human food generally precludes its use as a feed additive on a price basis.

As previously mentioned, inhibition of the silage fermentation can be a method used to reduce losses associated with aerobic deterioration. Fermentation inhibitors such as sulfites, sulfur dioxide and sodium metabisulfite have been used with some success in the preservation of feeds (Woolford, 1984). In addition, formaldehyde-containing additives have been evaluated (Woolford, 1975; Barry et al., 1980) for the prevention of aerobic instability in grass and corn silages.

One of the most common additives for silages is lactic acid-producing bacterial inoculants. Many commercial bacterial inoculants are based solely on use of lactic acid-producing bacteria as starter cultures. A multitude of research has been done on the efficacy of bioinoculants based on the use of lactic acid-producing bacteria for preserving ensiled feeds. It is generally accepted that the addition of lactic acid-producing bacteria will lead to a more rapid decrease in the pH of the ensiled material (Seale, 1986). This is accomplished by supplying a high amount, usually greater than 10^5 cfu$\cdot g^{-1}\cdot DM^{-1}$, of homofermentative lactic acid-producing bacteria at harvest or when placing into storage.

It is not the scope nor the intent of this review to focus on the use of lactic acid-producing bacteria due to many reviews on this subject. Seale (1986) conducted a comprehensive review addressing the use of lactic acid-producing bacteria as silage amendments.

Other additives have been used to help reduce aerobic deterioration. Antibiotics (Woolford and Cook, 1978) have been added to experimental silos to decrease proliferation of yeasts and molds. Compounds that buffer or increase the pH of the ensiled material, enhance the lactic acid production by extending the fermentation. Ammonia (Phillip et al., 1985) and limestone (Woolford, 1984) are two common compounds used in this manner. Ammonia serves two purposes in the ensilement process. Firstly, increasing pH of the ensiled material above 8 (providing limited bacteriostatic and mycostatic activity) and providing a source of non-protein nitrogen that can be used by some silage microorganisms. However, the use of ammonia is usually for the improvement of crude protein content of silages.

Increases in lactic acid concentration of ensiled feeds has not been consistent in enhancing aerobic stability. Rust et al. (1989) found that inoculation of corn silage with a homofermentative lactic acid-producing bacteria increased aerobic instability. Addition of lactic acid-producing bacterial inoculants to high-moisture corn has also had mixed results. Wardynski et al. (1993) showed an increase in aerobic instability of ensiled, high-moisture corn inoculated with a lactic acid-producing bacterial inoculant. However, Phillip and Fellner (1992) found that the addition of a lactic acid-producing bacterial inoculant decreased aerobic instability.

Effectiveness of propionic acid as a preservative

Propionic acid has been shown to be one of the most effective and economical mycostatic (mold inhibiting) and bacteriostatic (bacteria inhibiting) agents (Table 1-3; Voelker et al., 1989; Pitt et al., 1991). The effectiveness of propionic acid is based on the inhibitory properties of the undissociated acid and not the pH reducing properties (Woolford, 1975). Many commercial products based on propionic acid are available to increase the shelf life of bakery and dairy products, and enhance the preservation of hay and silages (Bolsen, 1991; Wittenberg, 1991; Glatz, 1992).

Table 1-3. Minimum inhibitory concentration of propionic and lactic acids at pH 5 for selected microorganisms found in ensiled feeds (Woolford, 1975)

Microorganisms	Propionic acid (mM)	Lactic acid (mM)
Homofermentative LAB	250	94
Clostridia	< 8	8
Yeasts	125	>250
Molds	63	>250

Propionic acid is commonly found in ensiled feeds at very low levels (less than .05%, compared to 2 to 4% for lactic acid). Addition of propionic acid at low levels (less than 1%) increased storage life of ensiled, high-moisture grains and resulted in similar or improved animal performance when compared to non-treated control corn (Jones et al., 1974; Voelker et al., 1989). Propionates are generally used for reducing losses within the silo, decreasing soluble protein losses and decreasing aerobic deterioration of silages (Pitt,

1986). Woolford and Cook (1977) showed that propionic acid, at 2.5% DM, inhibited aerobic instability of corn silage for 19 days. Britt and coworkers (1975) showed that propionic acid was effective in the inhibition of pH changes of aerobically-exposed corn silage for 36 days. Two different studies using propionic acid in corn silage (Huber and Soejono, 1976; Hara and Ohyama, 1979) showed reduced heating and fungal counts after 7 days of aerobic exposure. Aerobic stability of unwilted grass silages was also improved by addition of propionic acid as measured by more stable temperatures for 36 h (Mann and McDonald, 1976) and pH for 6 days (Crawshaw et al., 1980) of aerobic exposure.

Mechanisms of antimicrobial action of propionic acid

The bacteriostatic and mycostatic properties of propionic acid are very beneficial in the preservation of high-moisture feedstuffs. The following description of the antimicrobial action mechanisms of propionic acid is adapted from Ray and Sandine (1992). Propionic acid affects different types of microorganisms in various ways. Extent of control (slowing or inhibiting) of microbial growth, is influenced by factors such as propionic acid concentration, pH of the environment, lipophilic properties, temperature, resistance of the microbes present, amount of microbes present and anaerobic conditions.

At high concentrations of propionic acid, the pH outside the cells is decreased. Propionic acid acts similarly to strong inorganic acids at these concentrations. Exposed cell wall components, the periplasmic space and outer surface of the cytoplasmic membrane are exposed to the low external pH. At low pH, enzymes associated with these components can become denatured therefore leading to destabilization of the cell membrane. This in

turn can lead to increased permeability of the membrane to the acids in the external space. Denatured cell membrane components and influx of copious amounts of acidic compounds can lead to cell death.

Bacteriostatic action of propionic acid is based on the lipophilic nature of the undissociated acid. Propionic acid's pK_a (4.87) allows a greater proportion of the undissociated acid at a given pH when compared to acetic and lactic acids (Table 1-4). The increased permeability of the lipid bilayer associated with bacterial cell membranes to propionic acid facilitates more undissociated acid to diffuse into the cytoplasm. Undissociated propionic acid within the aqueous environment of the cytoplasm dissociates into hydrogen ions and propionate ions. Once the concentration of the hydrogen ions exceeds the buffering capacity of the cytoplasm, the internal pH will begin to decrease and the excess hydrogen ions will need to be transported via membrane associated proton pumps. Transport of these ions out of the cell requires energy. Depletion of energy to remove excess hydrogen ions can lead to decreased internal energy stores that in turn limits nutrient transport processes that require energy.

Table 1-4. Influence of pH on proportion of undissociated molecules of lactic, acetic and propionic acids in aqueous solution (Ray and Sandine, 1992)

Acid	pK_a	Undissociated acid (% of total acid concentration)			
		pH 4	pH 5	pH 6	pH 7
Lactic	3.85	39.2	6.1	0.6	0.1
Acetic	4.75	84.5	34.9	5.1	0.5
Propionic	4.87	87.6	41.7	6.7	0.7

Besides depleting microbial energy stores, once the proton pumps cannot keep up with the excess hydrogen ions, the pH of the cytoplasm will further decrease. Low cytoplasmic pH can lead to similar effects as high concentrations of propionic acid or low external pH. Such effects as denaturation of structural and functional components of the microbial cell will greatly affect cellular growth and viability.

The effect of propionic acid on microbial growth is enhanced by elevated temperature, low microbial load and anaerobic conditions. As stated previously, propionic acid affects groups of microorganisms differently. Yeast and molds are more resistant to propionate followed in resistance by gram positive bacteria. The gram negative bacteria are the least resistant to the bacteriocidal effects of propionic acid. In addition, acidophilic and fermentative bacteria are more resistant to propionic acid than respiratory bacteria. This may be due to greater tolerance to low cytoplasmic pH or changes in cell membrane permeabilities to lipophilic compounds.

Why use propionic acid-producing bacteria?

The caustic nature of propionic acid is problematic for equipment and hazardous for personnel when added before storage. Production of propionic acid by propionic acid-producing bacteria will alleviate problems associated with the pre-storage application of caustic materials. Lactic acid-utilizing bacteria, which produce propionic acid, have been isolated from grass silage (Woolford, 1975). However, these organisms did not account for a large portion of the microbial population (Rosenberger, 1956; Woolford, 1975).

Beyond possibly affecting the microbial ecosystem associated with ensiled, high-moisture corn and decreasing aerobic instability, the addition of propionic acid-producing bacteria may serve as a vehicle to influence the rumen microbial ecosystem and possibly help alleviate some rumen maladies such as acidosis. Diets that contain high concentrations of fermentable sugars, such as 85% grain diets that are commonly fed to feedlot cattle, can lead to over-production of ruminal lactic acid that overwhelms the lactic acid-utilizing population of the rumen (Slyter, 1976). Extent of acidosis can range from temporary lack of appetite to animal death (Huber, 1976). *Megaspheara elsdenii* is currently being evaluated (Robinson et al., 1991) as a possible direct feed microbial which is thought to help reduce the amount of ruminal lactic acid accumulation. Additional in vitro studies have been conducted looking at the possible use of either *M. elsdenii* or *Propionibacterium shermanii* to modulate changes in ruminal lactic acid production (Kung et al., 1991).

Leng (1970) summarized research findings that found propionate to be the main glucose precursor in ruminants. Amino acids, lactate, pyruvate and glycerol are all possible gluconeogenic precursors. However, amino acids are generally required for protein synthesis. Carbon from propionic acid is metabolized by liver tissue more efficiently than lactic acid (Armento, 1992). Over 85% of propionic acid absorbed by the rumen is cleared by hepatic tissue (Armento, 1992). Theoretically, increasing the proportion of propionic acid absorbed from the rumen may significantly affect protein and carbohydrate usage by ruminants to the extent of improving use of dietary and microbial protein post-ruminaly.

Historical perspective for propionic acid-producing bacteria

Propionic acid-producing bacteria are commonly used in the production of dairy products such as Swiss and Emmental cheeses (Drinan and Cogan, 1992) and can produce up to 1% propionic acid in Swiss cheeses. Hettinga and Reinbold (1972a,b,c) provided a series of classical reviews on the growth, metabolism and miscellaneous metabolic activities of the propionic acid-producing bacteria which are very informative and the basis for the majority of recent information. Bacteria of the genus *Propionibacterium* (Table 1-5) are divided into two distinct categories based on the ecosystem. The classical (dairy) species are normally associated with cheese production whereas, the cutaneous (skin associated) are generally found on skin and can be responsible for facial acne (*P. acnes*) and infections following surgery. Both types are gram positive and occur in a diverse array of shapes. Most are branched or rod shaped (sometimes looking like Chinese alphabet characters). In addition, *Propionibacterium spp.* are generally resistant to lysozyme, sulfonamides and penicillin-G (Cummins and Johnson, 1990). Other bacteria which produce propionic acid include species within the genera *Bacteroides, Clostridium, Fusobacterium, Megasphaera, Prevotella, Selenomonas, Treponema,* and *Veillonella* (Johns 1951a,b; Holdeman et al., 1977).

Table 1-5. Species of genus *Propionibacterium* categorized by ecosystem (Cummins and Johnson, 1986)

Classical (dairy related)	Cutaneous (skin associated)
P. acidipropionici	*P. acnes*
P. freudenreichii subsp. *freudenreichii*	*P. avidium*
P. freudenreichii subsp. *shermanii*	*P. granulosum*
P. jensenii	*P. lymphopilium*
P. thoenii	

Previous research with propionic acid-producing bacteria

Early research in the 1930's was focused on using propionic acid-producing bacteria for the commercial production of propionic acid (Hettinga and Reinbold, 1972a) and vitamin B_{12} (Bullerman and Berry, 1965). Microbial fermentations became the popular method to produce propionic acid. In the 1950's, there was a shift to the chemical synthesis of propionic acid from petroleum products (Hettinga and Reinbold, 1972a) and production of vitamin B_{12} by *Pseudomonas* (Glatz, 1992). These production systems were less expensive and had higher yields of propionic acid and vitamin B_{12}.

The elucidation of the fermentation pathways of propionic acid-producing bacteria were by Johns (1951b,c; 1952), Wood et al. (1955) and Crow (1986; 1987a,b). There are two pathways used by different microorganisms for the production of propionic acid. Bacteria of the genus *Clostridium* and a other genus use the acrylate pathways which incorporates acrylic acid as an intermediate in the fermentation of lactic acid to propionic acid (Johns, 1952). In contrast, bacteria of the genus *Propionibacterium* use the succinate-

methylmalony-CoA pathway (Figure 1-2). For a complete description of the enzymatic pathways involved with production of propionic acid production by *Propionibacterium spp.* the review by Hettinga and Reinbold (1972a) is very comprehensive and follows the general diagram outlined in Figure 1-2. The general stoichiometry of the propionic acid fermentation is (Wood, 1981):

$$3 \text{ glucose} + 12 P_i + 12 \text{ ADP} \rightarrow 12 \text{ ATP} + 4 H_2O + 4 CO_2 + 2 \text{ acetate} + 4 \text{ propionate}$$

Hexoses are predominately converted to pyruvate by the Emden-Meyeroff pathway. Lactic acid is oxidized in the presence of fumarate by lactic dehydrogenase. Three of the six ATP are produced by the action of pyruvate kinase in which three mol of phosphoenolpyruvate are converted to 3 mol pyruvate and 3 mol ATP. An additional ATP is produced by the action of acetyl kinase in which acetyl-phosphate is converted to acetate and ATP. The final two ATP are produced by the action of fumarase in conjunction with flavoprotein and reduced NAD. Carbon dioxide arises in the fermentation from the activity of pyruvate dehydrogenase that produces acetyl-CoA from pyruvate.Intermediates of the propionic acid fermentation not mentioned above include methyl-malonyl-CoA, methyl-malonyl, propionyl-CoA, acetyl-CoA, oxaloacetate, malate, fumarate, succinate and succinyl-CoA. Biotin and cyanocobalamin (vitamin B_{12}) are two very important cofactors along with NAD(H_2) and FAD(H_2). A complex which include biotin (designated C1 in Figure 1-2) facilitates the transfer of one carbon intermediates.

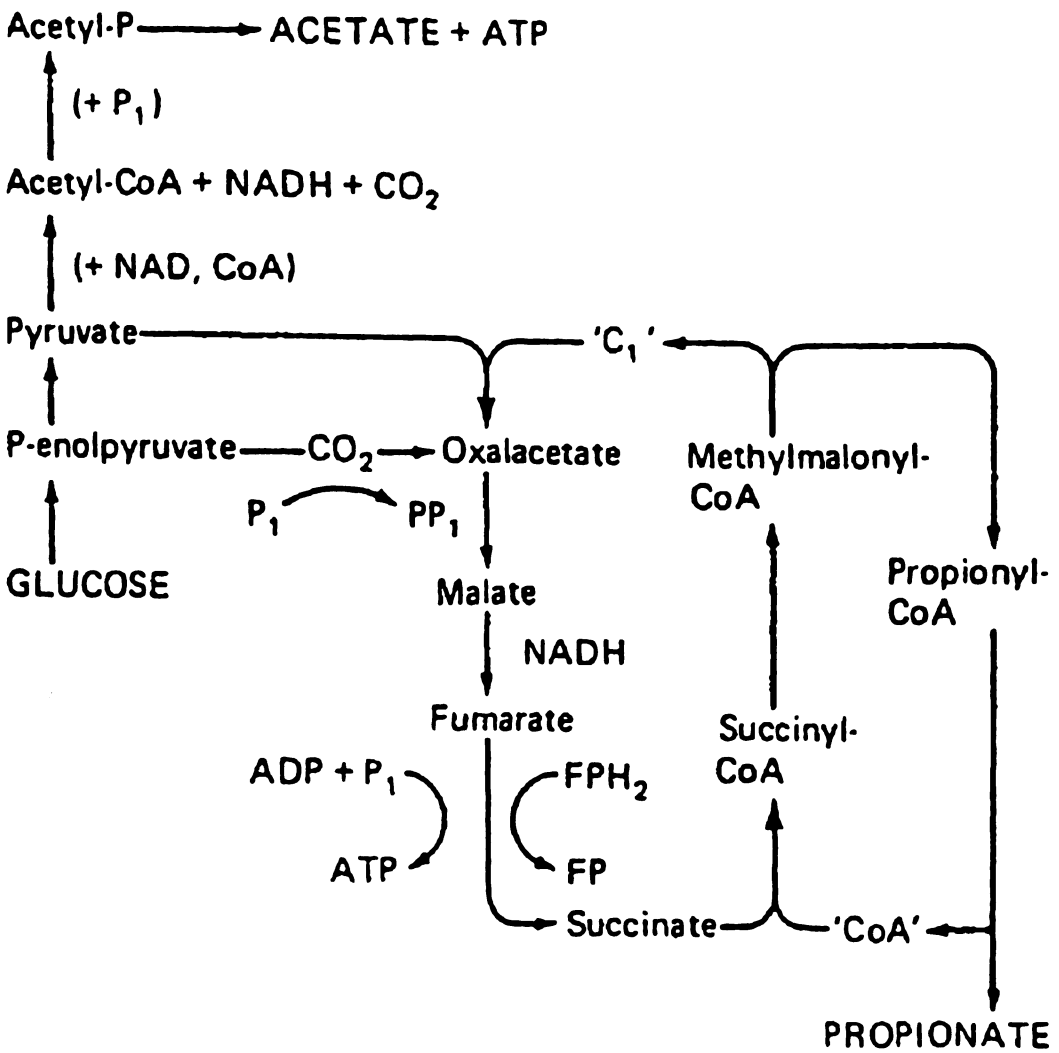

Figure 1-2. Production of ATP, acetate and propionate by *Propionibacterium spp.* utilizing reduced flavoprotein and biotin containing transfer complex (C1; Cummins and Johnson, 1990).

Current research is focused on increasing propionic acid production from corn co-products and the production of bacteriocins for food grade operations. Bayer and Ryba (1991) have studied the relatedness of *Propionibacterium spp.* by immunoblotting of cell-associated proteins which can be presumptive bacteriocins and other cell associated proteins. Research at Ohio State University (Lewis and Yang, 1992a,b,c) is currently focused on developing new methods to use propionic acid-producing bacteria and dairy byproducts for propionic acid formation. Propionic acid production by propionic acid-producing bacteria is both associated with exponential growth and with the stationary phase of growth. In addition, the optimal pH for propionic acid production is lower than the pH optimum for growth. These two points favor the possibility of propionic acid-producing bacteria's competitiveness in slightly acidic environments such as silages.

Parker and Moon (1982) showed a beneficial (commensalistic) interaction between lactic acid-producing bacteria and propionic acid-producing bacteria. It appears that propionic acid-producing bacteria produce metabolites that benefit the growth of the lactic acid bacteria (Parker and Moon, 1982; Flores-Galarza et al., 1985). Most propionic acid-producing bacteria prefer lactic acid to other substrates (Hettinga and Reinbold, 1972a) which partially alleviates the end-product inhibition of lactic acid on the growth of lactic acid-producing bacteria (Parker and Moon, 1982). As mentioned earlier, Bullerman and Berry (1965) and Hettinga and Reinbold (1972a) explained that propionic acid-producing bacteria produce vitamins and other cofactors that may further benefit the ensiling fermentation. Another advantage of the classical propionic acid-producing bacteria is inherent stability when grown in the laboratory. Commensalism of propionic acid-

producing bacteria with the epiphytic lactic acid-producing bacteria may serve as a major factor for improving the silage fermentation.

Some possible disadvantages associated with the known classical propionic acid-producing bacteria are slow growth, low acid tolerance, and low substrate affinities (Russel and Baldwin, 1979; Russel et al., 1979; Glatz, 1992). These disadvantages would possibly exclude their use as bioinoculants.

Enrichment and isolation of propionic acid-producing bacteria

Previous efforts at development of selective media for propionic acid-producing bacteria have been based on yeast extract, peptone and sodium lactate (Vedamuthu and Reinbold, 1967; Hettinga et al., 1968; Peberdy and Freyers, 1976). The selectivity of the above media was based on the fermentation of lactate by propionic acid-producing bacteria under anaerobic conditions. However, lactic acid-producing bacteria and other microorganisms commonly found in silages can use the same media components. Reddy and coworkers (1973) and Drinan and Cogan (1992) attempted to correct this problem by addition of two antibiotics, cloxacillin and kanamycin. This alleviated the growth of most lactic acid-producing bacteria except *Leuconostocs*. The concentration of antibiotics necessary to inhibit the growth of *Leuconostocs* was also inhibitory to the growth of propionic acid-producing bacteria of interest. Currently, enrichment and isolation of propionic acid-producing bacteria are still based on the ability of propionic acid-producing bacteria to use lactic acid in the production of propionic acid (Atlas and Parks, 1993).

Novel selection procedures (continuous or semi-continuous enrichment) and possible genetic engineering may simplify selection of new strains that can overcome the previously mentioned obstacles. Muck (1988) recommends that lactic acid-producing bioinoculants be added at 10^6 cfu•g^{-1}•DM^{-1} for best results. This number, 10^6 to 10^7 per g, of propionic acid-producing bacteria at the time of storage may allow propionic acid-producing bacteria a competitive advantage when used as a silage bioinoculant.

Use of nucleic acid probes in microbial ecology

Reviews by Hazen and Jimenez (1988) and Sayler and Layton (1990) discussed probe technology, radioactive and non-radioactive labeling, colony and plaque hybridization, environmental DNA recovery and hybridization as it pertains to the use of nucleic acid probes in environmental microbiology. Currently, double-stranded DNA, single-stranded DNA, RNA and oligonucleotides are used in the development of gene probes for detecting specific sequences, and therefore specific species or strains of microorganisms, in environmental applications.

Sensitivity and specificity of nucleic acid probes have been improved by the use of nonradioactive labeling and detection (Martin et al., 1990) with and without the use of the polymerase chain reaction (Lion and Haas, 1990). Specific sequences of 16S rRNA have been used for the amplification of species and strain specific nucleic acid probes (Barry et al. (1990).

Nucleic acid probes are becoming increasingly popular for the evaluation of interactions of different microorganisms in various ecosystems. Historically, procedures

for detection of nucleic acid sequences involved radioactive materials. Recently, emphasis has been placed on the development and use of non-isotopic methods for detection of nucleic acids. Digoxigenin is a steroid that can be cross-linked by a chemical spacer arm to dexoxyuridine-triphosphate (DIG-dUTP) and is the basis for a commercial nonradioactive labeling and detection kit (Genius-1, Boehringer Mannheim). The use of DIG-dUTP in a random primed DNA labelling method can lead to its incorporation every 20 to 25 nucleotides in the newly synthesized DNA. The DIG-dUTP can then be detected by immunological methods and detected by colorimetric, fluorescent or luminescent techniques depending on the substrate chosen for the alkaline phosphatase conjugated to the anti-DIG antibody fragment. Sensitivity of this method is comparable, if not better, to radioactive techniques and can be done more safely and quickly.

Specific applications using nucleic acid probes are numerous. Most applications are associated with pathogenic microorganisms such as the detection of *Listeria monocytogenes* (Peterkine et al., 1991; Wang, 1991). However, nucleic acid probes are commonly used in microbial ecology studies. Examples in soil microbiology include the detection of *Bradyrhizobium japonicum* (Holben et al., 1988) and denitrifying bacteria (Smith and Tiedje, 1992) in soil. Stahle et al. (1988) and Briesacher et al. (1992) have used nucleic acid probes to monitor the rumen microbial ecosystem with emphasis on *Fibrobacter succinogenes* S85. Nucleic acid probes have been developed using regions of the 16S rRNA DNA sequence that is specific to strains of *Streptococcus bovis*.

Very limited research has been done studying the use of nucleic acids for following the persistence or interactions of silage microorganisms. Hill and Hill (1986) used plasmid

profiles for a bacterial inoculant based on a specific plasmid containing *Lactobacillus plantarum* in corn silage. This approach was successful in differentiating lactic acid-producing bacteria which proliferated during ensilement and identifying the organisms as those which were added before ensilement as a lactic acid-producing bacterial inoculant. Hendrich and coworkers (1991) developed a radioactive nucleic acid probe to a cryptic plasmid of a strain of *Bacillus pumilus* that was being used as a bioinoculant for hay. This was done to simplify monitoring the persistence of the inoculated strain in hay, soil and plant material. In addition, the use of the probe was to relate efficacy of the product to organism numbers. Plasmid transfer between the inoculated strain of *Bacillus pumilus* and epiphytic *Bacillus pumilus* was considered a disadvantage to this method.

Non-radioactive nucleic acid probes were developed to the total genomic DNA of strains of lactic acid-producing bacteria involved in wine production by Lonvaud-Funel and coworkers (1991). This method showed that non-radioactive nucleic acid probes, based on total genomic DNA, were species specific for most of the *Lactobacillus spp.* involved in the fermentation of wine. In addition, Zarda and coworkers (1991) used non-radioactive nucleic acid probes to detect single cells based on rRNA sequences. Generation of nucleic acid probes from the genomic DNA of selected bacteria should allow for more precise measurement of their status in diverse microbial ecosystems such as ensiled feedstuffs.

CHAPTER 2

INOCULATION OF RECONSTITUTED HIGH-MOISTURE CORN WITH PROPIONIC ACID-PRODUCING BACTERIA WITH OR WITHOUT ADDITION OF LACTIC ACID-PRODUCING BACTERIA

Abstract

Dry corn (90% DM) was cracked in a roller mill and reconstituted with sterile distilled deionized water to 70% DM. Treatments consisted of control (no inoculum added); PAPB (*Propionibacterium acidipropionici* ATCC 4965 and *P. freudenreichii* ATCC 6207); LAPB (a commercial mixture of *Lactobacillus plantarum*, *Streptococcus faecium* and *Pediococcus acidilacticii)*; and PAPB+LAPB (a mixture of PAPB and LAPB). All inocula was applied at 10^6 colony forming units per g DM. Approximately 1.5 kg of the treated reconstituted, high-moisture corn was placed into a laboratory silo. Duplicate silos were prepared for each combination of treatment and storage interval. Silos were opened on d 0, 2, 7, 14, 28, and 56. Lactic acid-producing bacteria and non-mycotic lactic acid- utilizing microorganisms were enumerated on LBS agar or Na-lactate agar with amphotericin-B. Yeasts and molds were enumerated on rose bengal agar supplemented with chloramphenicol. The pH of the aqueous extract was lowest ($P < .05$) on d 56 for LAPB and PAPB+LAPB (4.02 and 3.99) as compared to control and PAPB (4.31 and

4.24). Yeasts and molds were lowest ($P < .05$) in treatment PAPB on d 56. Propionic acid content was highest ($P < .01$) in the PAPB treatment on all days except zero. In addition, propionic acid concentration was highly correlated ($r=-.81$; $P < .0001$) with the inhibition of the proliferation of yeasts and molds.

Introduction

Propionic acid is routinely used as a treatment for the prevention of mold proliferation in high-moisture (80% DM or less) stored grains (Jones et al., 1974). The undissociated form of propionic acid is an effective bacteriostatic and mycostatic agent (Crawshaw et al., 1980). Propionic acid-producing bacteria are used in cheese production and have been used as experimental amendments to silage fermentations. Lindgren and coworkers (1983) used *Propionibacterium shermanii* to manipulate the fermentation of grass silage; however, they were unsuccessful in changing the fermentation pattern. Flores-Galarza and coworkers (1985) used a combination of selected propionic acid-producing bacteria and lactic acid-producing bacteria to preserve high-moisture corn. A beneficial synergistic relationship between propionic acid-producing bacteria and lactic acid-producing bacteria was observed in the above study (Flores-Galarza et al., 1985) as well as in an other study (Parker and Moon, 1982). The objective of this study was to evaluate the effectiveness of a mixture of *P. acidipropionici* ATCC 4965 and *P. freudenreichii* ATCC 6207 with or without added lactic acid-producing bacteria as possible bioinoculants for the preservation of reconstituted, high-moisture corn.

Experimental Procedures

A 4 x 6 factorial arrangement of four treatments and six storage intervals (0, 2, 7, 14, 28, 56 d) was used to evaluate the effect of inoculation of reconstituted, high-moisture corn using propionic acid-producing bacteria with or without lactic acid-producing bacteria. Treatments were: 1) control (no inoculum added); 2) PAPB (a mixture of *P. acidipropionici* ATCC 4965 and *P. freudenreichii* ATCC 6207); 3) LAPB (HMF-10, American Farm Products, Ypsilanti, MI, composed of *Lactobacillus plantarum*, *Pediococcus acidilacticii*, and *Streptococcus faecium*); and 4) a mixture of treatments PAPB and LAPB. Duplicate silos were used for each treatment by storage interval combination. Dry corn (90% DM) was cracked in a roller mill and reconstituted with sterile deionized water to 70% DM by hand agitiation for 15 min in a 20 l plastic bucket. Inocula were grown aerobically for 12 h to 10^9 cfu per ml. The PAPB inoculum was grown in a medium modified from Malik et al. (1968) and Holdeman et al. (1977). This media contained (g/l) Na-lactate, 16.0; peptone, 5.0; trypticase, 10.0; yeast extract, 10.0; tween-80, .5; cysteine, .5; $CaCl_2$ (anhydrous), 0.018; $MgSO_4 \cdot 7H_2O$, 0.018; K_2HPO_4, 0.04; KH_2PO_4, 0.04; $NaHCO_3$, 0.4; NaCl, 0.08; $MnSO_4$, 0.002; and amphotericin-B, .001. The LAPB inoculum was reconstituted as directed by the manufacturer by diluting the dry inoculant in distilled water to achieve a final concentration of 10^9 cfu/ml. Approximately 1.5 kg of the reconstituted corn, along with 10^6 cfu/g DM of the appropriate organisms, was placed into a PVC laboratory silo equipped with a rubber policeman for gas release release (Wardynski, 1991). Silos were emptied on d 0, 2, 7, 14, 28 and 56 and a homogenous 25 g sample was removed from each silo on the appropriate

day and extracted in 225 ml sterile saline (0.9% w/v) using a stomacher lab blender (Tekmar 3500, Tekmar, Cincinnati, OH) to dislodge the microflora from the corn. A one ml aliquot was serially diluted from 10^{-2} to 10^{-6}. Lactic acid-producing bacteria were enumerated on LBS agar medium (Difco, Detroit, MI). Non-mycotic lactic acid-utilizing microorganisms were enumerated on the medium used for growth of the inoculum supplemented with 1.5% agar. A similar medium was used by Flores-Glalarza et al. (1985) for the presumptive enumeration of propionic acid-producing bacteria. However, initial laboratory tests showed that lactic acid-producing bacteria were able to grow on this media. Sterile agar media (12.5 ml molten agar at 45°C) was poured aseptically onto each petri dish and the plates were gently swirled to disperse the microflora. Yeast and molds were enumerated using rose bengal agar with chloramphenicol supplement (Difco, Detroit, MI). Agar pour plates of the lactate producers and utilizers were incubated at 37°C in an anaerobic glovebox (Coy laboratories, Troy, MI). Yeasts and molds plates were incubated at 37°C aerobically. Microbial counts were performed using a digital colony counter under a Quebec darkfield counting chamber (American Optical, Buffalo, NY). Results are expressed as log colony forming units per gram of dry matter (log cfu/g DM). On d zero, samples were processed for microbial counts within two hours of treatment application. Acetic and propionic acid were determined on aliquots of the aqueous extracts by gas-liquid chromatography as described by Baertsche et al. (1986). The pH of the aqueous extract was measured within five minutes of extraction at 23 °C using a combination glass electrode (AccupHast, Fisher Scientific, Chicago, IL) attached to a digital pH meter

(Fisher Scientific 825MP pH meter, Fisher Scientific, Chicago, IL) that was standardized from pH 4 to 7 using commercial buffers (Curtis Mattheason, Woodale, IL).

The experiment was analyzed as a completely randomized design by analysis of variance (Steele and Torrie, 1980) using the general linear models (GLM) procedure of the Statistical Analysis System (SAS, 1987). Individual treatment means were compared using the Bonferroni t-test (SAS, 1987). The ANOVA model for the fermentation study was as follows:

$$Y_{ijk} = \mu + T_i + D_j + (T \bullet D)_{ij} + e_{ijk}$$

Where:

Y_{ijk} = Individual response variable measured (e.g., pH, numbers of lactic acid-producing bacteria, propionic acid).

μ = Overall mean.

T_i = Effect of treatment (Control, PAPB, LAPB or PAPB+LAPB).

D_j = Effect of period of ensiling (0, 2, 7, 14, 28 or 56 d).

$(T \bullet D)_{ij}$ = Interaction of treatment and period of ensiling.

e_{ijk} = Random residual effect (assumed normally distributed).

Results and Discussion

The pH of the aqueous extract of the ensiled, high-moisture corn (Table 2-1) decreased over time in all treatments. An interaction of treatment and period of ensiling ($P < .0001$) was observed. The interaction of treatment with period of ensiling is

generally observed in fermentation experiments due to the rapid growth and acid production of the homofermentative lactic acid-producing bacteria as found in treatments LAPB and PAPB+LAPB. Phillip and Fellner (1992), Schaefer et al. (1989) and Wardynski et al. (1993) showed similar decreases in pH with the addition of homofermentative lactic acid-producing bacteria. The pH decreased to a greater extent in the LAPB and LAPB + PAPB treatments when compared to control or PAPB after 56 d of ensilement. This indicates the PAPB did not increase acid production of the epiphytic lactic acid-producing bacteria or the homofermentative lactic acid-producing bacteria in treatment LAPB. The pH of PAPB treated corn was greater ($P < .05$) than corn inoculated with LAPB from d 2 to 56.

As seen with the change in silage pH there was a treatment by ensiling period ($P < .0001$) interaction on numbers of lactic acid-producing bacteria (Table 2-2). All treatments, including control, provided dramatic increases in numbers of lactic acid-producing bacteria during the first seven days of storage. Many studies have shown the same effect when measuring the growth of added lactic acid-producing bacteria. Lactic acid-producing bacteria in the control silage numbers stabilized after d 7. Treatments LAPB and LAPB + PAPB had decreases ($P < .05$) in numbers of lactic acid-producing bacteria by d 56 to less than control or PAPB. Since the distribution or number of homofermentative lactic acid-producing bacteria was not determined, care must be taken in stating the decrease in numbers is only due to a switch in the type of lactic acid-producing bacteria which were growing and predominating later in the fermentation.

Lactic acid-utilizing bacteria (Table 2-3) followed the same general trend as observed for lactic acid-producing bacteria. The lactic acid-utilizing bacteria were enumerated using the medium that Flores-Galarza et al. (1986) which was presumptively used for enumeration of propionic acid-producing bacteria in ensiled, high-moisture corn. However, lactic acid-producing bacteria are capable of growing on this media as seen with high numbers of lactic acid-utilizing bacteria in the LAPB treatment at d 0. Therefore, the numbers of lactic acid-utilizing bacteria (as measured by this medium) followed the same general trend as that followed by the lactic acid-producing bacteria. Control and PAPB numbers of lactic acid-utilizing bacteria stabilized after d 7. By d 28, LAPB and LAPB + PAPB had lactic acid-utilizing numbers less than ($P < .01$) control or PAPB.

The PAPB treatment was effective in decreasing the number of yeast and molds (Table 2-4) when compared to all other treatments ($P < .01$) at d 14, 28, and 56. Yeasts and molds were not enumerated on d 0, 2 or 7 due to problems in obtaining the rose bengal agar. A treatment effect ($P < .0001$) was observed and attributable to the decreased numbers of yeasts and molds observed in PAPB. The effect of adding propionic acid-producing bacteria on decreasing the growth of yeasts and molds was also reported by Flores-Galarza et al. (1985). However, Flores-Galarza et al. (1985) showed a much greater decrease in yeasts to values less than 10^4 cfu$\cdot g^{-1}\cdot DM^{-1}$. There was no estimate of propionic acid production in that study and *P. shermanii* was used as an inoculant. In addition, Flores-Galarza et al. (1985) also reported decreases in yeasts and molds by using lactic acid-producing inoculants. This was not found the case in this study or by Wardynski

(1991). Since initial numbers of yeasts and molds are not attainable for this experiment, comparisons to the initial load of yeasts and molds cannot be made.

Acetic acid is a co-product in the fermentation pathways of propionic acid-producing bacteria (Wood, 1981). Therefore, the increase in the concentration of acetic acid (Table 2-5) in the PAPB treated corn after 14 d of ensilement was expected. A tendency ($P < .13$) was observed for an increase in acetic acid concentration during ensilement for all treatments. Production of acetic acid within the silo can be advantageous due to the antimycotic activities it possesses which is slightly less than that of propionic acid. Amount of acetic acid produced during the ensiling fermentation was lower than that reported by Wardynski et al. (1993). This may be attributed to slight differences in moisture content of the corn, the fact that the corn in this study was reconstituted and possibly the length of ensilement. Decrease in pH and increases in the number of lactic acid-producing bacteria tends to preclude the effect of reconstitution for it appears that reconstituted corn follows a similar fermentation pattern as corn harvested at similar moisture levels.

Propionic acid production (Table 2-6) was greatest ($P < .0001$) in corn treated with the mixture of *P. acidipropionici* ATCC 4965 and *P. freudenreichii* ATCC 6207 after 2, 7, 14, 28 and 56 d of ensilement when compared to all other treatments. No propionic acid was measured in either control or LAPB treated corn. Previous studies have found that the propionic acid concentration of non-treated ensiled, high-moisture corn is generally less than .1 g/100 g DM. A period of ensilement effect ($P < .0001$) indicates production of propionic acid by treatment PAPB was time dependent and increased with

increasing length of storage as commonly seen in the lactic acid fermentations associated with silage. Treatment PAPB+LAPB had higher ($P < .05$) propionic acid concentration from d 2 to d 56 than did control or LAPB. As expected, enhanced production of propionic acid was not observed in the PAPB+LAPB treatment as compared to PAPB. The expectation was based on co-culture work of Parker and Moon (1982) and Lee et al. (1976). Both of these studies showed increased propionic acid production when co-cultures of lactic acid-producing bacteria and propionic acid-producing bacteria were grown in liquid media. Differences in substrates available for growth, water activity, pH of environment and other factors associated with competition in the silage microbial ecosystem probably did not allow for the same proliferation of the propionic acid-producing bacteria when added in combination with LAPB.

Production of propionic acid within the silo is possible based on the results presented. Recommendations for preserving corn containing 70% DM (moisture level used in this study) state that 1.1% propionic acid should be added to preserve the corn for six months (Jorgensen et al., 1980). Even though PAPB treated corn did not reach the 1.1% propionic acid, there was a noticeable effect on the numbers of yeasts and molds. Further studies are needed to select propionic acid-producing bacteria capable of competing for substrates found in ensiled, high-moisture corn. In addition, future studies should address what concentration is necessary to be produced within the silo to yield a stable product. Many variables will need to be addressed such as temperature of storage, moisture level of the ensiled crop, amount of oxygen ingression during storage, feedout rates and many

Table 2-1. Effect of propionic acid-producing bacteria (PAPB) with or without addition of lactic acid-producing bacteria (LAPB) on the pH of ensiled reconstituted, high-moisture corn

Time (d)	Treatment				SEM
	Control	PAPB	LAPB	PAPB + LAPB	
	pH of aqueous extract				
0	5.33[a]	5.52[b]	5.69[b]	5.52[b]	.063
2	4.83[b]	4.96[b]	3.90[a]	4.01[a]	
7	4.84[c]	4.35[b]	3.83[a]	3.95[a]	
14	4.57[c]	4.31[b]	3.90[a]	3.99[a]	
28	4.46[b]	4.28[b]	3.93[a]	3.92[a]	
56	4.31[b]	4.24[b]	4.02[a]	3.99[a]	

[abc] Means within a row lacking a common superscript differ ($P < .05$)

Table 2-2. Effect of propionic acid-producing bacteria (PAPB) with or without addition of lactic acid-producing bacteria (LAPB) on the number of lactic acid-producing bacteria of ensiled reconstituted, high-moisture corn

Time (d)	Treatment				SEM
	Control	PAPB	LAPB	PAPB + LAPB	
	log cfu•g^{-1}•DM^{-1}				
0	5.10^{b}	4.64^{a}	8.11^{d}	7.81^{c}	.091
2	7.94^{a}	8.54^{b}	9.34^{c}	9.48^{c}	
7	9.00^{a}	9.02^{ab}	9.38^{c}	9.27^{bc}	
14	9.13^{b}	8.57^{a}	8.80^{a}	8.56^{a}	
28	8.96^{c}	8.63^{b}	8.18^{a}	8.33^{a}	
56	8.79^{b}	8.61^{b}	7.19^{a}	7.39^{a}	

^{abcd} Means within a row lacking a common superscript differ ($P < .05$)

Table 2-3. Effect of propionic acid-producing bacteria (PAPB) with or without addition of lactic acid-producing bacteria (LAPB) on the number of lactic acid- utilizing microorganisms of ensiled reconstituted, high-moisture corn

Time (d)	Treatment				SEM
	Control	PAPB	LAPB	PAPB + LAPB	
	log cfu•g^{-1}•DM^{-1}				
0	5.74^{a}	8.77^{c}	8.36^{b}	8.49^{b}	.092
2	8.33^{a}	8.61^{b}	9.14^{c}	9.39^{c}	
7	8.94^{a}	8.97^{a}	9.30^{b}	9.20^{ab}	
14	9.14^{b}	8.99^{ab}	8.84^{a}	8.76^{a}	
28	8.86^{c}	8.58^{b}	8.17^{a}	8.24^{a}	
56	8.74^{c}	8.73^{c}	7.09^{a}	7.73^{b}	

^{abc} Means within a row lacking a common superscript differ ($P < .05$)

Table 2-4. Effect of propionic acid-producing bacteria (PAPB) with or without addition of lactic acid-producing bacteria (LAPB) on the number of yeasts and molds of ensiled reconstituted, high-moisture corn

Time (d)	Treatment				SEM
	Control	PAPB	LAPB	PAPB + LAPB	
	log cfu•g^{-1}•DM^{-1}				
14	6.55^{b}	4.81^{a}	6.15^{b}	6.45^{b}	.207
28	6.37^{b}	5.49^{a}	6.35^{b}	6.16^{b}	
56	6.01^{b}	5.37^{a}	6.18^{b}	6.08^{b}	

^{ab} Means within a row lacking a common superscript differ ($P < .05$)

Table 2-5. Effect of propionic acid-producing bacteria (PAPB) with or without addition of lactic acid-producing bacteria (LAPB) on the acetic acid content of ensiled reconstituted, high-moisture corn

Time (d)	Treatment				SEM
	Control	PAPB	LAPB	PAPB + LAPB	
	g/ 100 g DM				
0	.10	.13	.14	.06	.037
2	.18	.20	.10	.16	
7	.14	.20	.11	.17	
14	.13[a]	.25[b]	.10[a]	.16[ab]	
28	.11	.16	.11	.15	
56	.15	.22	.15	.20	

[ab] Means within a row lacking a common superscript differ ($P < .05$)

Table 2-6. Effect of propionic acid-producing bacteria (PAPB) with or without addition of lactic acid-producing bacteria (LAPB) on the propionic acid content of ensiled reconstituted, high-moisture corn

Time (d)	Treatment				SEM
	Control	PAPB	LAPB	PAPB + LAPB	
	g/ 100 g DM				
0	0	.00	.00	.00	.015
2	.00[a]	.13[c]	.00[a]	.05[b]	
7	.00[a]	.23[c]	.00[a]	.05[b]	
14	.00[a]	.21[c]	.00[a]	.05[b]	
28	.00[a]	.14[c]	.00[a]	.05[b]	
56	.00[a]	.26[c]	.00[a]	.07[b]	

[abc] Means within a row lacking a common superscript differ ($P < .05$)

other factors addressed which impact the ensiling fermentation and aerobic stability of ensiled feeds.

Implications

Addition of a mixture *Propionibacterium acidipropionici* ATCC 4965 and *P. freudenreichii* ATCC 6207 as a bioinoculant to reconstituted, high-moisture corn was effective in decreasing the numbers of yeast and molds when compared to control, lactic acid-producing bacteria and the lactic acid-producing bacteria + propionic acid-producing bacteria mixture. Addition of *P. acidipropionici* ATCC 4965 and *P. freudenreichii* ATCC 6207 was not effective in decreasing silage pH when compared to control, and reached a higher terminal pH when compared to the lactic acid-producing bacterial inoculant. Addition of propionic acid-producing bacteria to ensiled, high-moisture corn shows promise as a bacterial inoculant based on decreased proliferation of yeasts and molds. More studies need to be conducted to isolate propionic acid-producing bacteria that are more suitable for use in ensiled crops.

CHAPTER 3

ENRICHMENT AND ISOLATION OF PROPIONIC ACID-PRODUCING BACTERIA FROM ENSILED FEEDSTUFFS

Abstract

Propionic acid is an effective mold and bacterial-inhibiting agent commonly used in human food products and animal feeds. Propionic acid-producing bacteria are commonly used for cheese production. The objective of this study was to develop methods to isolate indigenous propionic acid-producing bacteria from ensiled feedstuffs. Thirty-eight samples of various ensiled feeds (corn silage, ensiled, high-moisture corn, alfalfa haylage and oatlage) were obtained from Michigan farms. Samples were homogenized and enrichments made in a medium containing: sodium lactate, peptone, yeast extract, minerals, buffers and amphotericin-B. Primary enrichments were incubated at 30°C until turbid growth was attained. Enrichments were analyzed for propionic acid concentration by high performance liquid chromatography and primary enrichments containing greater than 10 mM propionic acid were transferred to a secondary enrichment medium containing sodium propionate. Progressive enrichments were allowed to grow until turbid and analyzed for propionate production. Enrichments with greater than 10 mM propionate production were serially diluted and plated on a homologous agar. Single colony isolates were transferred to

homologous agar with sterile toothpicks and streaked for purity and characterized by fermentation profiles, catalase production, and gram stain. A bacterium was isolated from ensiled, high-moisture corn that produced propionic acid and acetic acid from either lactate or glucose and is further characterized in Chapter 4.

Introduction

Propionic acid is routinely used as a treatment for the prevention of mold proliferation in high-moisture (i.e., less than 80% DM) stored grains (Jones et al., 1974). The undissociated form of propionic acid is an effective bacteriostatic and mycostatic agent (Crawshaw et al., 1980). Propionic acid-producing bacteria are used in cheese production and limited commercial production of propionic acid. Lindgren and coworkers (1983) used *Propionibacterium shermanii* to manipulate the fermentation of grass silage. However, they were unsuccessful in changing the fermentation pattern. Flores-Galarza and coworkers (1985) used a combination of selected propionic acid-producing bacteria and lactic acid bacteria to preserve high-moisture corn. A synergistic relationship between propionic acid-producing bacteria and lactic acid-producing bacteria was observed in the above study (Flores-Galarza et al., 1985). Similarly, Parker and Moon (1982), reported enhance growth of lactic acid-producing bacteria and increased production of propionic acid by propionic acid-producing bacteria when grown in co-culture with lactic acid-producing bacteria. Lactic acid-utilizing bacteria, which produce propionic acid, have been isolated from grass silage (Woolford, 1975c). However, these organisms did not account for a large portion of the microbial population (Rosenberger, 1951; Woolford, 1975c). Currently, enrichment and

isolation of propionic acid-producing bacteria are still based on their ability to use lactic acid (Atlas and Parks, 1993). Selectivity of the above media is based on the fermentation of lactate by propionic acid-producing bacteria under anaerobic conditions. However, lactic acid-producing bacteria and other microorganisms commonly found in silages can use the same media components. Reddy and coworkers (1973) and Drinan and Cogan (1992) attempted to correct this problem by addition of two antibiotics, cloxacillin and kanamycin. This alleviated the growth of most lactic acid-producing bacteria except *Leuconostocs*. The concentration of antibiotics necessary to inhibit the growth of *Leuconostocs* was also inhibitory to the growth of propionic acid-producing bacteria of interest. The objective of this study was to develop methods to simplify isolation of indigenous propionic acid-producing bacteria from ensiled feedstuffs that might be useful as bioinoculants for ensiled, high-moisture corn.

Experimental Procedures

Sample description. Thirty-eight samples (approximately 500 g) of various ensiled feedstuffs were obtained from different Michigan farms. The geographical source and type of ensiled material obtained for inoculum source for enrichment cultures are shown in Table 3-1.

Media composition. Enrichment and isolation media were similar to that of Malik et al. (1968) and Holdeman and coworkers (1977). Modifications included the addition of amphotericin-B (Sigma A-9528, St. Louis, MO) to decrease proliferation of yeasts and molds in enrichment cultures and use of Hungate tubes with nitrogen as gas phase. Primary

enrichment media contained (g/L in distilled deionized water): Na-lactate syrup (60% syrup), 16.0; peptone, 1.0; yeast extract, 1.0; Tween-80, .5; cysteine, .5; $CaCl_2$, .018; $MgSO_4 \cdot 7H_2O$, .018; K_2HPO_4, .04; KH_2PO_4, .04; $NaHCO_3$, .4; NaCl, .08; $MnSO_4$, .002 and Amphotericin-B, .001. Secondary enrichment media contained (g/L in distilled deionized water): Na-lactate syrup (60% syrup), 16.0; peptone, 1.0; Na-propionate, 10.0; yeast extract, 1.0; Tween-80, .5; cysteine, .5; $CaCl_2$, .018; $MgSO_4 \cdot 7H_2O$, .018; K_2HPO_4, .04; KH_2PO_4, .04; $NaHCO_3$, .4; NaCl, .08; $MnSO_4$, .002 and Amphotericin-B, .001. Primary and secondary enrichment media were prepared using nitrogen as the gas phase and 10 ml of primary or secondary enrichment media was pipetted into 16 x 125 mm Hungate tubes (Bellco 2047-00125, Vineland, NJ) which were sealed by a manual crimper (Bellco, Vineland, NJ). Both the primary and secondary enrichment media used lactic acid as the main carbon source for fermentation. Sodium propionate was added to the secondary enrichment medium to reduce competition of non-propionic acid-producing bacteria from the primary enrichment meduim. Isolation media contained (g/L in distilled deionized water): Na-lactate syrup (60% syrup), 16.0; peptone, 5.0; Na-propionate, 10.0; trypticase, 10.0; yeast extract, 1.0; Tween-80, .5; cysteine, .5; $CaCl_2$, .018; $MgSO_4 \cdot 7H_2O$, .018; K_2HPO_4, .04; KH_2PO_4, .04; $NaHCO_3$, .4; NaCl, .08; $MnSO_4$, .002; Amphotericin-B, .001 and agar, 15.0.

Enrichment and isolation procedures. Twenty-five g of ensiled material described in Table 3-1 was homogenized in 225 ml of autoclaved .9% NaCl (pH 7.0) in a Stomacher (Model 3500, Tekmar, Cincinnati, OH) for 5 min. A 100 μl aliquot was removed by a sterile one ml tuberculin syringe fitted with a 5/8 inch 25 gauge needle

(Becton Dickinson 9626, Rutherford, NJ). This aliquot was transferred to a Hungate tube containing 10 ml of primary enrichment media. Primary enrichments were incubated at 30 °C (optimum growth temperature for classical *Propionibacterium spp.*, Glatz, 1992) until turbidity was observed. Primary enrichment media contained sodium lactate as an selective agent for the growth of propionic acid-producing bacteria (Atlas and Parks, 1993). Since yeasts and molds also metabolize lactic acid, growth of lactic acid-utilizing yeasts and molds was reduced by the inclusion of the mycostatic agent, amphotericin-B. One ml aliquots of enrichments were removed using one ml syringes (Becton Deckinson 9626, Rutherford, NJ) and aseptic technique. Samples were analyzed for propionic acid production by HPLC procedures as described later. Primary enrichment cultures containing greater than 10 mM propionic acid were transferred to Hungate tubes containing 10 ml secondary enrichment media to help dilute non-propionic acid-producing bacteria from the enrichment cultures. Secondary enrichment media contained sodium propionate as an agent for the inhibition of non-desired microorganisms and to enhance the growth of propionic acid-producing bacteria. Enrichments were incubated as before and HPLC analysis was done on cultures that had turbid growth.

Secondary enrichments which had propionic acid content greater than 10 mM above the added sodium propionate were serially diluted from 10^{-1} to 10^{-6} in autoclaved .9% NaCl. A 500 μl aliquot of each dilution was spread on the surface of 10 ml of isolation media that was poured aseptically into sterile 100 x 15 mm Petri dishes (Baxter D1096, Deerfield, IL). The plates were incubated in an anaerobic glovebox (Coy laboratories, Troy, MI) and visually checked for colony formation. Single colonies were aseptically

removed and streaked onto fresh isolation media plates to evaluate culture purity. Single colonies were transferred to peptone-yeast extract-lactate media (Holdeman et al., 1977) and incubated as before. Culture extracts were analyzed by HPLC for propionic acid production as were enrichment cultures. Pure cultures which produced propionic acid were stored in peptone-yeast extract-lactate media mixed with 50% glycerol at -20°C. Gram stain and catalase reaction were performed as described by Holdeman and coworkers (1977).

HPLC analysis of enrichment cultures. Analysis of progressive enrichment media for propionic acid was done by ion exchange-exclusion HPLC (BIORAD aminex HPX-87H, Richmond, CA). Mobile phase consisted of .005 N H_2SO_4 at a flow rate of .9 ml/min. Column temperature was maintained at 65°C by an external column heater (Waters Millipore, Milford, MA). Three ml of enrichment cultures were filtered through .2 μm ion chromatography syringe filters (Gelman Acrodisks, 25 mm, Ann Arbor, MI) into 3 ml HPLC sample vials (National Scientific, Atlanta, GA). Filtered samples were stored at -20 °C until analysis. Twenty μl of each filtered sample was injected by an autoinjector (Waters WISP 712, MIlford, MA) and analytes were detected by refractive index (Waters 410 refractive index detector, Milford, MA). Peak heights were quantified by a commercial HPLC software package (Turbochrom 3, PE Nelson, Cupertino, CA) and compared to standards of lactic, acetic and propionic acid (Supelco, Bellefonte, PA).

Table 3-1 Source and type of sample used as enrichment sources for propionic acid-producing moisture bacteria from ensiled feeds

Farm or Location	Sample Type
Michigan State University Beef Cattle Research Unit	corn silage
	ensiled, high-moisture corn
Michigan State University Dairy Research Unit	alfalfa haylage
	corn silage
	ensiled, high-moisture corn
Galesburg, MI	alfalfa haylage
	corn silage
	ensiled, high-moisture corn
Parma, MI	corn silage
Big Rapids, MI	alfalfa haylage
	oatlage
Falmouth, MI	alfalfa haylage
	alfalfa haylage + corn silage
	corn silage
Reed City, MI	corn silage
Chatam, MI	alfalfa haylage
Muir, MI	alfalfa haylage
	ammoniated corn silage
Litchfield, MI	alfalfa haylage
	corn silage
Mason, MI	alfalfa haylage, corn silage
	ensiled, high-moisture corn

Results and Discussion

Few samples of ensiled feeds had primary enrichments that produced propionic acid. A total of 219 primary enrichments was prepared and analyzed using 38 different inoculum sources. Only 34% of the primary enrichments had propionic acid production above the threshold limit. Eight of the primary enrichments yielded secondary enrichments with propionic acid production. Growth of epiphytic microorganisms was observed in all enrichments suggesting that the enrichment media used was not selective only for propionic acid-producing bacteria. Many enrichments that did not produce propionic acid were characterized by copious gas production and very putrid odor production. Analysis of these primary enrichments by HPLC indicated high amount of n-butyric and i-butyric acids with higher (unidentified) chain length volatile fatty acids. Production of butyric acid in enrichments would suggest possible enrichment for lactate-utilizing clostridia.

Previous attempts by Woolford (1975c) were not successful in isolating propionic acid-producing bacteria from fresh forages. Rosenberger (1956) found that numbers of *Propionibacterium spp.* were very low in silage. Woolford (1975c) was able to isolate a limited number of propionic acid-producing bacteria resembling *Propionibacterium spp.* from (two out of six) ensiled samples. A recent review of the literature did not find any published studies regarding the isolation of propionic acid-producing bacteria from ensiled feeds. Very few colonies of bacteria grew on the isolation media after transfer from secondary enrichments that had propionic acid production. Although an antimycotic agent was used, amphotericin-B, overgrowth of molds was observed on many plates of isolation media.

Use of the techniques described facilitated the isolation of a bacterium from ensiled, high-moisture corn (from the MSU Dairy Research and Teaching Facility) that produced propionic acid and acetic acid from either lactate or glucose. The bacterium is a gram-positive, facultative anaerobe, non-sporeforming, nonmotile, pleiomorphic rod which forms clumps of cells when grown in peptone-yeast extract-lactate medium. Surface colonies, when grown aerobically on agar plates are pin-point (approximately 1 mm) in size, yellow, circular and possess a strong catalase reaction. End-products from the fermentation of lactate and glucose are acetate and propionate (1:3 and 1:5 ratio, respectively). This bacterium is further characterized in Chapter 4.

Implications

The use of progressive enrichments in a semi-selective media allows for the selection and screening of many different types of microorganisms. Using the methods discussed, a rapid growing propionic acid-producing bacteria was isolated from ensiled, high-moisture corn. Adaptation of this selection process, or use of improved selection media, will simplify enrichment and isolation of novel, propionic acid-producing bacteria from ensiled feeds that should enhance selection of new strains of propionic acid-producing bacterial inoculants.

CHAPTER 4

CHARACTERIZATION OF *PROPIONIBACTERIUM ACIDIPROPIONICI* DH42, A PROPIONIC ACID-PRODUCING BACTERIA ISOLATED FROM ENSILED, HIGH-MOISTURE CORN

Abstract

A bacterium was isolated from ensiled, high-moisture corn that produced propionic acid and acetic acid from either lactate or glucose. The bacterium is a gram-positive, non-sporeforming, non-motile, pleiomorphic rod, facultative anaerobe which forms clumps of cells when grown in peptone-yeast extract-lactate medium. Surface colonies are pin-point size after five days aerobic incubations at 30 °C. In addition, surface colonies are yellow, circular, and possess a strong catalase reaction. The end-products from the fermentation of lactate and glucose are acetate and propionate (1:3 and 1:5 ratio, respectively). The bacterium grows rapidly using glucose under anaerobic and aerobic conditions in liquid media. However, the organism grows very slowly on the surface of aerobically incubated solid media. The majority of the phenotypic characteristics of the isolated bacterium match those of the ATCC type strain of *Propionibacterium acidipropionici*, therefore the name *Propionibacterium acidipropionici* DH42 is proposed for the identification of the isolated propionic acid-producing bacterium.

Introduction

Classical propionic acid-producing bacteria, those associated with dairy products, are generally characterized as being gram-positive, catalase-positive, non-sporeforming, rod-shaped facultative anaerobes with pH optimum for growth between 6 and 7 (Hettinga and Reinbold, 1972a; Hsu and Yang, 1991). *Propionibacterium shermanii* (Lindgren et al., 1983; Flores-Galarza et al., 1985) and *P. jensenii* (Tomes, 1991) have been used with mixed results as inoculants in corn silage, grass silage and ensiled, high-moisture corn. In addition, *P. acidipropionici* and *P. freudenreichii* were used in Chapter 2 as inoculants for reconstituted ensiled, high-moisture corn. However, most of the above mentioned *Propionibacterium spp.* were previously isolated from dairy products. The objective of this study was to phenotypically characterize and identify a fast growing, propionic acid-producing bacteria, designated DH42, isolated from ensiled, high-moisture corn in Chapter 3.

Experimental Procedures

Bacterial strains. A propionic acid-producing bacteria previously isolated from ensiled, high-moisture corn was maintained by growing in .5X (half-strength) MRS (Difco, Detroit, MI) and storing at -20° in glycerol. Reference strains of propionic acid-producing bacteria were obtained from the following sources to facilitate phenotypic comparisons of the isolated bacterium to known strains. *Propionibacterium jensenii* P25, *P. shermanii* P92 and *P. thoenii* were kind gifts from Chr. Hansen's Biosystems (Milwaukee, WI). *Propionibacterium acidipropionici* 22562 and *P. freudenreichii* 1382

were obtained from Michigan Biotechnology Institute (Lansing, MI). All cultures were grown in 16 x 125 mm Hungate tubes (Bellco 2047-00125, Vineland, NJ) sealed with a manual crimper (Bellco, Vineland, NJ) under ambient gas phase at 30 °C in .5X MRS (Difco, Detroit, MI) and maintained at -20 °C stored in glycerol.

Media composition. General media used for this study included .5X MRS (Difco, Detroit) for fermentation profiles and maintenance of cultures. Cellobiose, erythritol, lactose, maltose, mannitol, sorbitol, soluble starch, sucrose and trehalose were added to a final concentration of 1% as sterile solutions to purple broth base (BBL 11558, Cockeysville, MD) to assess fermentation characteristics. Peptone-yeast extract-lactate and peptone-yeast extract-glucose media were used for pH optima determination (Holdeman et al., 1977).

HPLC analysis of fermentation end-products. Aliquots of fermentation media were analyzed for acidic end-products by ion exchange-exclusion HPLC (BIORAD aminex HPX-87H, Richmond, CA). Mobile phase consisted of .005 N H_2SO_4 at a flow rate of .9 ml/min. Column temperature was maintained at 65°C by an external column heater (Waters Millipore, Milford, MA). Three ml of enrichment cultures were filtered through .2 μm ion chromatography syringe filters (Gelman Acrodisks, 25 mm, Ann Arbor, MI) into 3 ml HPLC sample vials (National Scientific, Atlanta, GA). Filtered samples were stored at -20 °C until analysis. Twenty μl of the filtered samples was injected by an autoinjector (Waters WISP 712, Milford, MA) and analytes were detected by refractive index (Waters 410 refractive index detector, Milford, MA). Peak heights were quantified

by a commercial HPLC software package (Turbochrom 3, PE Nelson, Cupertino, CA) and compared to standards of lactic, acetic and propionic acid (Supelco, Bellefonte, PA).

Metabolic profile determination. BIOLOG-GP (BIOLOG systems, gram positive microplate, Hayward, CA) was used to evaluate the metabolic profile of the propionic acid-producing bacteria isolated from high-moisture corn and reference strains of *Propionibacterium spp.* listed previously. The following procedure was modified for liquid cultures as compared to cultures on solid agar described in the BIOLOG user's manual (Hayward, CA). Cultures were grown in .5X MRS (Difco, Detroit) in Hungate tubes at 30 °C to mid-exponential phase (OD_{600} = .8) and transferred to fresh .5X MRS. These cultures were grown for 12 h and 1 ml was aseptically removed and placed into a sterile microcentrifuge tube. Cultures were harvested by centrifugation (2 min @ 16,000 x *g*) and resuspended in 18 ml sterile .9% NaCl. An eight-channel multipipetter was used to dispense 150 μl of the resuspended culture per well of the BIOLOG-GP microplate. BIOLOG-GP microplates were incubated at 30 °C for 24 h. Optical density (OD_{590}) of each well was measured by a BIOLOG microstation plate reader and results transformed to compare to known strains of gram positive bacteria using the BIOLOG microstation gram positive database and the reference strains of *Propionibacterium spp.*

Cellular membrane fatty acid composition determination. Fatty acid content of the cellular membranes was analyzed as fatty acid-methyl esters using the MIDI Microbial Identification System of bacteria by gas chromatography of cellular fatty acids (MIDI, Newark, DE) at Michigan Biotechnology Institute (Lansing, MI). The profile of fatty acid-

methyl esters was compared to known profiles of reference strains of different bacteria to help in identification.

Growth characteristics. Optimal pH for growth on either glucose or lactate was determined using a 1% inoculum of the isolated bacterium taken from an exponentially growing culture and inoculated into peptone-yeast extract-lactate and peptone-yeast extract-glucose (Holdeman et al., 1977) at pH values of 4.9, 5.3, 5.7, 5.8, 6.2, 6.5, 6.7, 6.9, 7.4 and 7.9. The pH of the media was measured after autoclaving. Initial pH values before autoclaving were 4.5, 5.0, 5.5, 6.0, 6.5, 6.8, 6.9, 7.0, 7.5 and 8.0. Duplicate tubes of each media and pH were incubated at 30 °C and OD_{600} was measured in a Spectronic 70 (Bausch and Lomb, Newark, NJ) at 60 min intervals during growth. Specific growth rate was determined for each pH and carbon source (glucose or lactate) by determining the slope of the natural log of OD_{600} against time by regression procedures (PROC REG) of SAS (SAS, 1987) as described by Russel et al. (1979). Therefore, the specific growth rate of the isolated organism is reported with the units or h^{-1}. These units correspond to the change in absorbance at 600 nm (a unitless measurement) per change in time (h) which is the slope of the line which describes exponential growth. Nitrate reduction, esculin hydrolysis, catalase production, gelatin digestion and gram stain were performed using the procedures described by Holdeman and coworkers (1977).

Results and Discussion

Propionic acid was the major fermentation product from glucose and lactic acid as previously discussed in Chapter 3 of the fast growing isolate (DH42) from ensiled, high-

moisture corn. The differential characteristics of classical species of the genus *Propionibacterium* are listed in Table 4-1. The propionic acid-producing bacteria (DH42) isolated from ensiled, high-moisture corn hydrolyzed esculin, reduced nitrate and produced acidic end-products from cellobiose, erythritol, lactose, maltose, mannitol, sorbitol, soluble starch, sucrose and trehalose. In addition to these fermentation characteristics, the isolated organism did not digest gelatin. This allowed for the classification of isolate DH42 as a classical propionic acid-producing bacteria and not a cutaneous strain. These characteristics of the isolated bacteria, DH42, matched the differential characteristics of *P. acidipropionici* 22562. Based on these characteristics, DH42 was presumptively identified as *P. acidipropionici*. Future discussion of results will refer to the organism as *Propionibacterium acidipropionici* DH42.

Phase contrast microscopy (Figure 4-1) revealed that *P. acidipropionici* DH42 was a pleiomorphic rod that followed the description of *Propionibacterium spp.* (Holdeman et al., 1977). *Propionibacterium acidipropionici* DH42 was approximately .5 to .9 μm wide and up to 10 μm in length. Cells of *P. acidipropionici* DH42 generally appeared in pairs or were aggregated into small clumps containing up to 100 cells when grown in .5X MRS. The general morphology was similar to that of the type strain of *P. acidipropionici* (ATCC 22562).

Propionibacterium acidipropionici DH42 grew slowly on the surface of aerobic tryptic soy agar plates supplemented with 1% glucose. Colonies were pinpoint in size after five days growth at 30 °C. In addition, surface colonies were a translucent yellow, circular with entire margins and were catalase positive. *Propionibacterium acidipropionici* DH42

grew well in liquid media aerobically or anaerobically. Maximum growth was generally attained after 12 to 24 h in .5X MRS incubated at 30 °C. No noticeable gas was produced, as measured by inverted Durham tubes, after five d aerobic incubation in .5X MRS. Terminal pH (48 h growth) of cultures of *P. acidipropionici* DH42 was 3.9 and 4.1 for cultures grown in purple base-glucose and .5X MRS, respectively. The sediment of cells of *P. acidipropionici* DH42 after 72 h growth was ropy with some granularity. This deviates from the type strain of *P. acidipropionici* ATCC 22562 that had a ropy sediment with no noticeable granularity. No noticeable pigment was produced by *P. acidipropionici* DH42 when grown in .5X MRS. All cultures were a creamy white color in contrast to the dark reddish-brown color of *P. theonii* P15.

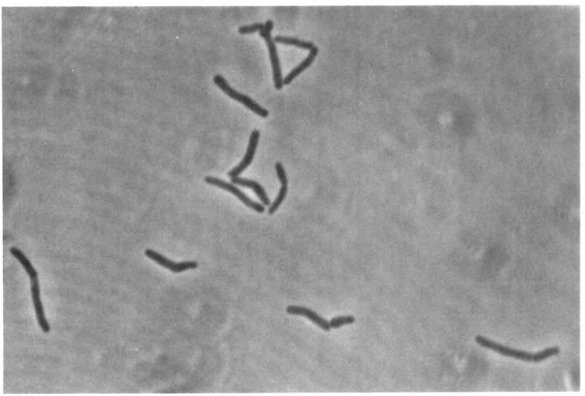

Figure 4-1. Phase contrast photomicrograph of *P. acidipropionici* DH42 grown in .5X MRS under ambient gas phase in sealed Hungate tubes (1500X magnification, each bacterium approximately .7 μm wide).

Table 4-1 Differential characteristics of *Propionibacterium acidipropionici* DH42, *P. acidipropionici* 22562, *P. freudenreichii* 1382, *P. jensenii* P25, *P. thoenii* P15 and *P. shermanii* P92

Characteristic	DH42	acidiprop-ionici	freuden-reichii	jensenii	thoenii	shermanii
esculin hydrolysis	+	+	+	+	+	-
gelatin digestion	-	-	-	-	-	+
nitrate reduced	+	+	+	-	-	-
Acidic end-products from:						
cellobiose	+	+	-	+	+	-
erythritol	+	+	+	+	+	+
lactose	+	+	-	+	+	+
maltose	+	+	-	+	+	+
mannitol	+	+	-	+	-	-
sorbitol	+	+	-	-	+	-
soluble starch	+	+	-	+	+	-
sucrose	+	+	+	+	+	-
trehalose	+	+	-	+	+	-

The BIOLOG-GP library (gram positive database) does not contain metabolic information for *Propionibacterium spp.* However, use of the microplate reader and the pre-filled BIOLOG-GP allows for a rapid determination of metabolic characteristics of isolates. Since *P. acidipropionici* DH42 grew slowly on the surface of agar plates, cells were harvested from liquid culture and washed in saline. Procedures followed from that step are identical to those used for the BIOLOG system as described in the experimental procedures section. Reference strains of *Propionibacterium spp.* and *P. acidipropionici* DH42 were capable of metabolizing a vast array of compounds found in the BIOLOG-GP microplate (Table 4-2). The BIOLOG software could discriminate the different *Propionibacterium spp.* evaluated, but was unable to differentiate a species identification from its database. *Propionibacterium acidipropionici* DH42 matched the metabolic profile of *P. acidipropionici* 22562 with few discrepancies. *Propionibacterium acidipropionici* DH42 metabolized D-arabitol, α-D-lactose, mono-methyl-succinate, alaniniamide, putrescine, adenosine-5'-monophosphate and uridine-5'-monophosphate, whereas *P. acidipropionici* 22562 was unable to fully metabolize these compounds. In contrast, *P. acidipropionici* 22562 was able to metabolize acetic acid and α-hydroxybutyric acid better than *P. acidipropionici* DH42.

Fatty acid compositional analysis of the cellular fatty acids (Table 4-3.) confirmed the preliminary identification of DH42 as *P. acidipropionici.* Most of the cellular fatty acids consisted of the isomers a 15-carbon fatty acid. Greater than 80% of the total cellular fatty acids were composed of saturated fatty acids of 15 carbons in length. This matches the description of the cellular lipids seen in classical propionic acid-producing

bacteria (Hettinga and Reinbold, 1972a). Comparison of the fatty acid composition to the database of the MIDI system found that *P. acidipropionici* DH42 had a similarity index of .58 to the type strain of *P. acidipropionici*. An index greater than .6 is considered an excellent match using the MIDI system, whereas indices greater than .5 are considered to be good matches. Slight differences in the cellular fatty acids are to be expected between different isolates, especially when the two strains are from drastically different ecosystems. Strains of *Propionibacterium spp.* in the MIDI database are based on the *Propionibacterium spp.* from Virginia Polytechnical Institute Anaerobe Laboratory that are predominately associated with dairy products.

Growth characteristics of *P. acidipropionci* DH42 using glucose or lactate are shown in Figure 4-2. Previous research by Hsu and Yang (1992) using *P. acidipropionici* ATCC 4875 had similar results for growth characteristics when grown on glucose or lactate at pH 6.6. In that study, *P. acidipropionici* ATCC 4875 grew slower on lactic acid than glucose, however it was capable of producing similar amounts of propionic acid which indicated a higher specific substrate consumption rate. The use of glucose and lactic acid over a range of media pH is described in Table 4-4. *Propionibacterium acidipropionici* DH42 grew more rapidly ($P < .01$) on glucose than lactic acid at all pH values evaluated. The effect of pH on the specific growth rate of *P. acidipropionici* DH42 was greater when the cells were grown on lactic acid. The optimal pH for growth on lactic acid was from 6.2 to 7.8, whereas maximal growth was obtained at pH 5.3 when grown on glucose with acceptable growth rates between pH 4.9 and 7.8.

Table 4-2 Metabolic profile of *Propionibacterium acidipropionici* DH42, *P. acidipropionici* 22562, *P. freudenreichii* 1382, *P. jensenii* P25, *P. shermanii* P92 and *P. thoenii* P15 as measured by BIOLOG-GP microplates

Substrate	DH42	acidiprop-ionici	freuden-reichii	jensenii	shermanii	thoenii
α-cyclodextrin	-	-	-	-	-	-
β-cyclodextrin	-	-	/	-	-	-
dextrin	+	+	+	+	+	+
glycogen	+	/	/	/	/	/
inulin	-	-	-	-	-	-
mannan	-	-	-	-	-	-
tween 40	+	+	+	+	+	+
tween 80	+	+	+	+	+	+
N-acetyl-D-glucosamine	+	+	+	+	+	+
N-acetyl-D-mannosamine	+	+	+	+	+	+
amygdalin	-	-	-	-	-	-
L-arabinose	+	+	+	+	+	+
D-arabitol	+	-	+	+	+	+
arbutin	+	+	+	+	+	+
cellobiose	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+
L-fucose	+	+	+	+	+	+
D-galactose	+	+	+	+	+	+
D-galacturonic acid	+	+	+	+	+	+
gentiobiose	+	+	+	+	+	+

Table 4-2 (cont'd).

Substrate	DH42	acidiprop-ionici	freuden-reichii	jensenii	shermanii	thoenii
D-gluconic acid	+	+	+	+	+	+
α-D-glucose	+	+	+	+	+	+
m-inositol	+	+	+	+	+	+
α-D-lactose	+	/	-	/	+	/
lactulose	-	-	-	-	+	-
maltose	+	+	+	+	+	+
maltotriose	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+
D-melezitose	-	-	+	-	-	-
D-melibiose	+	+	-	+	+	+
α-methyl D-galactoside	+	+	+	+	+	+
β-methyl D-galactoside	+	+	+	+	+	/
3-methyl glucose	/	/	/	/	/	-
α-methyl D-glucoside	+	+	/	-	/	-
β-methyl D-glucoside	+	+	+	+	+	+
α-methyl D-mannoside	-	-	-	-	-	-
palatinose	+	+	+	+	+	+
D-psicose	+	+	/	+	+	/
D-raffinose	+	+	+	+	+	+

Table 4-2 (cont'd).

Substrate	DH42	acidiprop-ionici	freuden-reichii	jensenii	shermanii	thoenii
L-rhamnose	+	+	+	+	+	+
sedoheptulosan	-	-	-	-	-	-
D-sorbitol	+	+	+	+	+	+
stachyose	+	+	+	+	+	+
sucrose	+	+	+	+	+	+
D-tagatose	-	-	-	-	-	-
D-trehalose	+	+	+	+	+	+
turanose	+	+	+	+	-	-
xylitol	-	-	-	-	-	-
D-xylose	+	+	+	+	+	+
acetic acid	/	+	/	+	+	/
α-hydroxybutyric acid	-	/	/	-	+	-
β-hydroxybutyric acid	+	+	+	+	+	+
γ-hydroxybutyric acid	-	-	-	-	-	-
p-hydroxyphenyl acetic acid	+	+	+	+	+	+
α-keto glutaric acid	-	-	-	-	-	-
α-keto valeric acid	-	-	-	-	-	-
lactamide	-	-	-	-	/	-
D-lactic methyl ester	+	+	+	+	+	+

Table 4-2 (cont'd).

Substrate	DH42	acidiprop-ionici	freuden-reichii	jensenii	shermanii	thoenii
L-lactic acid	+	+	+	+	+	+
methyl pyruvate	+	+	+	+	+	+
mono-methyl succinate	+	/	+	+	+	+
propionic acid	-	-	-	/	+	-
pyruvic acid	+	+	+	+	+	+
succiniamic acid	-	-	-	-	/	-
succinic acid	+	+	+	+	+	+
N-acetyl L-glutamic acid	+	+	+	+	+	+
alaniniamide	+	/	-	/	-	-
D-alanine	+	+	+	+	+	+
L-alanine	+	+	+	+	+	+
L-alanyl-glycine	+	+	+	+	+	+
L-asparagine	+	+	+	+	+	+
L-glutamic acid	+	+	+	+	+	+
glycyl-L-glutamic acid	/	/	+	/	/	/
L-pyroglutamic acid	-	-	-	-	-	-
L-serine	+	+	+	+	+	+
putrescine	+	-	-	-	-	-
2,3-butanediol	-	-	-	-	-	-
glycerol	+	+	+	+	+	+

Table 4-2 (cont'd).

Substrate	DH42	acidiprop-ionici	freuden-reichii	jensenii	shermanii	thoenii
adenosine	+	+	+	+	+	+
thymidine	+	+	+	+	+	+
uridine	+	+	+	+	-	+
adenosine-5'-monophosphate	+	/	-	/	/	/
thymidine-5'-monophosphate	-	-	-	-	-	-
uridine-5'-monophosphate	/	-	-	-	-	/
fructose-6-phosphate	+	+	+	+	+	+
glucose-1-phosphate	+	+	+	+	+	+
glucose-6-phosphate	+	+	+	+	+	+
D-L-α-glycerol phosphate	+	+	+	+	+	+

+ = organism able to metabolize compound
/ = organism metabolizes compound very weakly
- = organism does not metabolize compound

Table 4-3 Cellular membrane fatty acid composition of *Propionibacterium acidipropionici* DH42

Fatty Acid	% of total cellular fatty acids	Standard Deviation
13:0 ISO FAME	0.35	0.01
13:0 ANTEISO FAME	0.28	0.00
13:0 FAME	0.20	0.01
14:0 FAME	0.44	0.03
15:0 ISO FAME	38.28	0.07
15:0 ANTEISO FAME	22.68	0.02
15:0 FAME	12.15	0.25
15:0 ISO DMA	5.86	0.08
15:0 ANTEISO DMA	1.81	0.02
16:0 FAME	0.99	0.01
16:0 DMA	0.70	0.01
17:0 ISO FAME	2.68	0.14
17:0 ANTEISO FAME	0.55	0.01
17:0 FAME	1.65	0.07
un 17.103 17:0i DMA	0.69	0.01
17:0 ANTEISO FAME	0.32	0.01
17:0 DMA	0.81	0.01
18:1 CIS 9 FAME	0.35	0.05

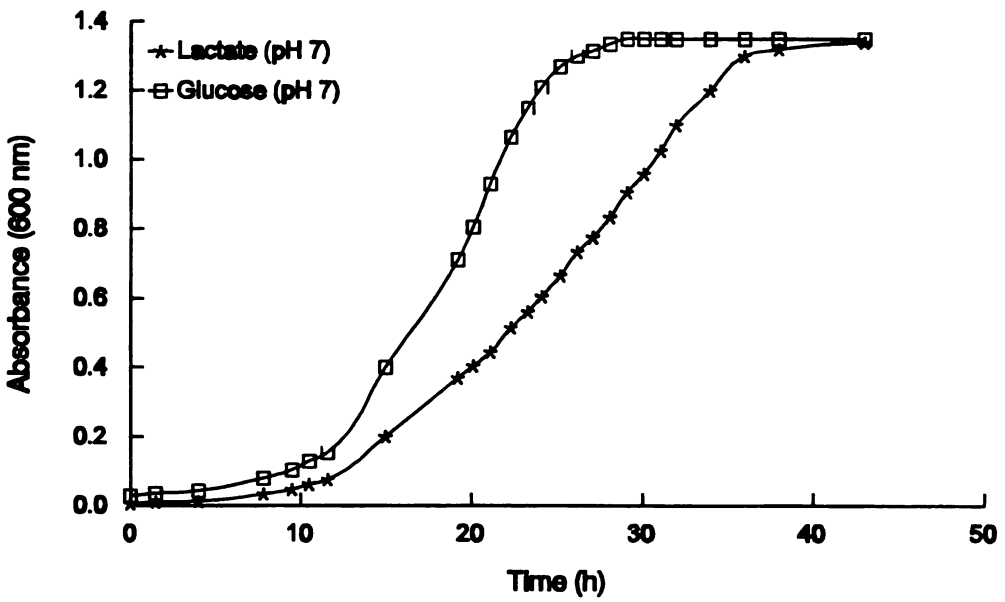

Figure 4-2. Growth characteristics of *P. acidipropionici* DH42 grown in either peptone-yeast extract-lactate or peptone-yeast extract-glucose at pH 7 under nitrogen.

Table 4-4. Effect of pH on specific growth rate of *P. acidipropionici* DH42 grown on glucose or lactate under nitrogen

Media pH	Carbon source		SEM	$P <$
	Glucose	Lactate		
	Specific growth rate (h^{-1})			
4.9	.124^{bcd}	.025^{a}	.0056	.0001
5.3	.141^{e}	.087^{d}		.0001
5.7	.134de	.068^{bc}		.0001
5.8	.114^{ab}	.055^{b}		.0001
6.2	.112^{ab}	.076^{cd}		.0002
6.5	.132^{cde}	.080^{cd}		.0001
6.7	.101^{a}	.080^{cd}		.0135
6.9	.117^{abc}	.080^{cd}		.0002
7.4	.116^{abc}	.085^{d}		.0008
7.8	.107^{a}	.085^{d}		.0125

^{abcde} Means within a column lacking a common superscript differ ($P < .05$)

Implications

A propionic acid-producing bacterium previously isolated from ensiled, high-moisture corn had similar phenotypic characteristics as *Propionibacterium acidipropionici* and was designated *P. acidipropionici* DH42. *Propionibacterium acidipropionici* DH42 had slight dissimilarities from the type strain of *P. acidipropionici* in the content of cellular lipids and the ability to metabolize putrescine. *Propionibacterium acidipropionici* DH42 can grow aerobically and anaerobically on lactic acid or glucose. Further studies need to be done to evaluate its competitiveness in the microbial ecosystem from which it was isolated to help predict efficacy as a bacterial inoculant for the preservation of high-moisture corn.

CHAPTER 5

COMPETITION OF PROPIONIC ACID-PRODUCING BACTERIA IN A MIXED MICROBIAL COMMUNITY FROM ENSILED, HIGH-MOISTURE CORN

Abstract

Substrate competition is a major factor influencing the diversity or proportional distribution of microbial communities. A study was conducted to evaluate competitiveness of propionic acid-producing bacteria when introduced into an environment with a mixed microbial community and substrates commonly found in ensiled, high-moisture corn. Eight strains of propionic acid-producing bacteria (*Propionibacterium acidipropionici, P. freudenreichii* (2 strains), *P. jensenii, P. pentosaceum, P. shermanii, P. thoenii* and *P. acidipropionici* DH42, an isolate from ensiled, high-moisture corn) were inoculated into three media (peptone-yeast extract-lactate and two aqueous extracts from ensiled, high-moisture corn samples) with and without the addition of the mixed epiphytic microbial community. All strains were capable of metabolizing at least 80% of the lactic acid present to propionic acid and acetic acid in all three media within 7 d. *Propionibacterium acidipropionici* DH42 was the most competitive for lactic acid and substrates found in extracts of ensiled, high-moisture corn when compared to the other strains. These results

suggest propionic acid-producing bacteria possess features that may enhance production of propionic acid and decrease aerobic deterioration of ensiled crops. Therefore, these microorganisms may prove beneficial as bacterial inoculants to ensiled crops.

Introduction

Propionic acid-producing bacteria have been isolated from ensiled crops by Woolford (1975c) and in Chapter 3. Selected strains of propionic acid-producing bacteria previously isolated from cheese have been used in experimental silos (Flores-Galarza et al., 1992) with limited success. A problem addressed for the classical propionic acid-producing bacteria, which might limit their use as silage bioinoculants, is their slow growth rate (Glatz, 1992) and possible poor substrate affinity (Russel et al., 1979). The objective of this study was to evaluate propionic acid production and growth rate of propionic acid-producing bacteria, with emphasis on *P. acidipropionici* DH42, when introduced into an environment similar to a mixed microbial community and substrates commonly found in ensiled, high-moisture corn.

Experimental Procedures

Media and bacterial strains. Three media were used to evaluate growth rate, propionic acid production and competitiveness of eight propionic acid-producing bacteria. Peptone-yeast extract-lactate broth (pH 6.9, 1.1% lactic acid; Holdeman, 1977) and filtered (.2 μm) aqueous extracts of two ensiled, high-moisture corn samples (Extract 1: pH 4.1, 0.11% lactic acid; Extract 2: pH 4.6, 0.02% lactic acid) were prepared in

Hungate tubes under N_2 and sealed with serum stoppers. *Propionibacterium acidipropionici* DH42 was isolated from ensiled, high-moisture corn in Chapter 3 and characterized in Chapter 4. *Propionibacterium acidipropionici* 4965 and *P. freudenreichii* 6207 were purchased from American Type Culture Collection (ATCC, Rockville, MD). *Propionibacterium pentosaceum* P11, *P. thoenii* P15, *P. jensenii* P25, *P. shermanii* CDC 3094 and *P. freudenreichii* P72 were kind gifts from Chr. Hansen's Biosystems (Milwaukee, WI). Cultures were inoculated in duplicate (.1 ml per 10 ml media, approximately 10^7 organisms) with and without the addition of .1 ml of a mixed microbial community from ensiled, high-moisture corn. An aqueous homogenate of two different samples of ensiled, high-moisture corn was prepared by homogenizing 25 g of the sample in 225 ml sterile .9% NaCl for 5 min. The mixed microbial community was harvested by centrifugation (15 min @ 400 x *g*) of 50 ml of the aqueous extracts of the ensiled, high-moisture corn samples.

Acetic and propionic acid were quantified by ion exchange-exclusion HPLC (BIORAD aminex HPX-87H, Richmond, CA). Mobile phase consisted of .005 N H_2SO_4 at a flow rate of .9 ml/min. Column temperature was maintained at 65°C by an external column heater (Waters Millipore, Milford, MA). Three ml of culture extracts after 150 h of growth was filtered through .2 μm ion chromatography syringe filters (Gelman Acrodisks, 25 mm, Ann Arbor, MI) into 3 ml HPLC sample vials (National Scientific, Atlanta, GA). Filtered samples were stored at -20 °C until analysis. Twenty μl of the filtered samples was injected by an autoinjector (Waters WISP 712, Milford, MA) and analytes were detected by refractive index (Waters 410 refractive index detector, Milford,

MA). Peak heights were quantified by a commercial HPLC software package (Turbochrom 3, PE Nelson, Cupertino, CA) and compared to standards of lactic, acetic and propionic acid (Supelco, Bellefonte, PA).

Specific growth rate of the mixed microbial community was determined by measuring OD_{600} in a Spectronic 70 spectrophotometer (Bausch and Lomb, Newark, NJ) during incubation at 30° C for 150 h. The slope of the linear portion of the growth curve was determined using regression procedures (PROC REG) of SAS (SAS, 1987) and compared using the general linear models method of SAS (SAS, 1987). Specific growth rate of the mixed microbial community is presented as change in OD_{600} (a unit-less measurement) per h during the linear portion of growth.

The experiment was analyzed as a completely randomized design by analysis of variance (Steele and Torrie, 1980) using the general linear models (GLM) procedure of the Statistical Analysis System (SAS, 1987). Preplanned comparisons of individual treatment means within strain of *Propionibacterium spp.* with or without the addition of the epiphytic microbial population were done using the PDIFF procedure (SAS, 1985).

The ANOVA model for the fermentation study was as follows:

$$Y_{ijk} = \mu + S_i + C_j + (S \bullet C)_{ij} + e_{ijk}$$

Where:

Y_{ijk} = Individual response variable measured (e.g., specific growth rate, acetic acid and propionic acid).

μ = Overall mean.

S_i = Effect of strain of *Propionibacterium spp.* (*P. acidipropionici* DH42, *P. acidipropionici* 4965, *P. freudenreichii* 6207, *P. freudenreichii* P72, *P. shermanii* CDC 3094, *P. pentosaceum* P11, *P. thoenii* P15 and *P. jensenii* P25).

C_j = Effect of epiphytic microbial population (added epiphytic population or pure).

$(S \bullet C)_{ij}$ = Interaction of strain and presence of epiphytic population.

e_{ijk} = Random residual effect (assumed normally distributed).

Results and Discussion

Acetic acid production by the propionic acid-producing bacteria in the first extract (Table 5-1, pH 4.1, .11% lactic acid) was similar for all strains tested. All of the lactic acid was metabolized by the strains evaluated with 150 h. The amount of acetic acid produced was greater than what could be expected based on the initial amount of lactic acid indicating metabolism of other substrates not detected by HPLC analysis. The extract

of ensiled, high-moisture corn with pH 4.6 (Table 5-2) had similar values for acetic acid production with or without addition of the epiphytic microbial population which was also seen in Table 5-1. *Propionibacterium shermanii* CDC 3094 tended to have the highest concentration of acetic acid production in the extract with the higher pH (Table 5-2). *Propionibacterium acidipropionici* DH42 had the highest concentration of acetic acid after 150 h when grown with the epiphytic microbial population in peptone-yeast extract-lactate media (Table 5-3). *Propionibacterium jensenii* P25 did not grow in peptone-yeast extract-lactate media. This was unanticipated since transfers were made from exponential growth in the same media. With the exception of *P. freudenreichii* 6207, all of the other propionic acid-producing bacteria had greater production of acetic acid when grown with the epiphytic microbial population extracted from ensiled, high-moisture corn. Interaction of propionic acid-producing bacteria with lactic acid-producing bacteria has been shown to increase growth of both species (Parker and Moon, 1982). This may explain the enhanced production of acetic acid which could be attributed to either the increased growth of the epiphytic bacteria and(or) the propionic acid-producing bacteria themselves. Amounts of acetic acid production in peptone-yeast extract-lactate media corresponds with concentrations of acetic acid obtained for *P. acidipropionici* DH42 when grown on this media in previous experiments.

Table 5-1. Acetic acid production of selected propionic acid-producing bacteria grown in an aqueous extract of ensiled, high-moisture corn (pH 4.1, .11% lactic acid) with (mixed culture) or without (pure culture) epiphytic microorganisms found in ensiled, high-moisture corn

Strain	Culture		SEM	$P <$
	Mixed	Pure		
	Acetic acid (% w/v)			
P. acidipropionici DH42	.094	.087	.008	.5265
P. acidipropionici 4965	.096	.103		.5761
P. freudenreichii 6207	.114	.107		.5789
P. shermanii CDC 3094	.099	.089		.4398
P. pentosaceum P11	.101	.080		.0933
P. thoenii P15	.084	.072		.3180
P. jensenii P25	.071	.114		.0024
P. freudenreichii P72	.104	.115		.3584

Table 5-2. Acetic acid production of selected propionic acid-producing bacteria grown in an aqueous extract of ensiled, high-moisture corn (pH 4.6, .02% lactic acid) with (mixed culture) or without (pure culture) epiphytic microorganisms found in ensiled, high-moisture corn

Strain	Culture		SEM	$P <$
	Mixed	Pure		
	Acetic acid (% w/v)			
P. acidipropionici DH42	.022	.016	.002	.1146
P. acidipropionici 4965	.023	.018		.1656
P. freudenreichii 6207	.026	.017		.0161
P. shermanii CDC 3094	.031	.032		.8099
P. pentosaceum P11	.020	.027		.0718
P. thoenii P15	.024	.028		.3495
P. jensenii P25	.026	.025		.6019
P. freudenreichii P72	.021	.016		.1299

Table 5-3. Acetic acid production of selected propionic acid-producing bacteria grown in peptone-yeast extract-lactate media (pH 6.9, 1.1% lactic acid) with (mixed culture) or without (pure culture) epiphytic microorganisms found in ensiled, high-moisture corn

Strain	Culture		SEM	$P <$
	Mixed	Pure		
	Acetic acid (% w/v)			
P. acidipropionici DH42	.502	.326	.023	.0001
P. acidipropionici 4965	.385	.328		.1004
P. freudenreichii 6207	.338	.292		.1764
P. shermanii CDC 3094	.373	.268		.0062
P. pentosaceum P11	.426	.289		.0009
P. thoenii P15	.363	.258		.0059
P. jensenii P25	ND	ND		
P. freudenreichii P72	.346	.278		.0574

Propionibacterium acidipropionici DH42 had the greatest production of propionic acid in extract with .11% lactic acid and pH 4.1 (Table 5-4) with the epiphytic microbial population. The low pH of the extract was well below the optimal pH (between 6.5 and 6.9) determined for *P. acidipropionici* DH42 in Chapter 3. *Propionibacterium jensenii* P25 was able to grow in this extract as indicated by the higher ($P < .01$) production of propionic acid without the addition of the epiphytic microbial population (.075 vs .048%). All other cultures evaluated had similar values for production of propionic acid with or without the addition of the epiphytic microbial population. Propionic acid production was similar for all strains evaluated in the extract with .02% lactic acid and pH 4.6 (Table 5-5). As seen in Table 5-4, *P. jensenii* P25 was the only propionic acid-producing bacteria evaluated which had differences in the amount of propionic acid produced. However, the addition of the epiphytic bacterial population enhanced production of propionic acid in this extract.

Propionibacterium acidipropionici DH42 had the greatest amount of propionic acid produced when grown with the epiphytic microbial population in peptone-yeast extract-lactate media (Table 5-6). As stated previously, *P. jensenii* P25 did not grow in this peptone-yeast extract-lactate media. *P. freudenreichii* P72 had similar amount of propionic acid produced with or without the addition of the epiphytic microbial population. All of the other strains evaluated showed enhanced ($P < .10$) production of propionic acid when grown with the epiphytic microbial population.

Table 5-4. Propionic acid production of selected propionic acid-producing bacteria grown in an aqueous extract of ensiled, high-moisture corn (pH 4.1, .11% lactic acid) with (mixed culture) or without (pure culture) epiphytic microorganisms found in ensiled, high-moisture corn

Strain	Culture		SEM	$P <$
	Mixed	Pure		
	Propionic acid (% w/v)			
P. acidipropionici DH42	.073	.066	.007	.4384
P. acidipropionici 4965	.050	.041		.3396
P. freudenreichii 6207	.050	.045		.6384
P. shermanii CDC 3094	.062	.055		.4781
P. pentosaceum P11	.063	.052		.2898
P. thoenii P15	.055	.054		.9175
P. jensenii P25	.048	.075		.0103
P. freudenreichii P72	.052	.036		.1126

Table 5-5. Propionic acid production of selected propionic acid-producing bacteria grown in an aqueous extract of ensiled, high-moisture corn (pH 4.6, .02% lactic acid) with (mixed culture) or without (pure culture) epiphytic microorganisms found in ensiled, high-moisture corn

Strain	Culture		SEM	$P <$
	Mixed	Pure		
	Propionic acid (% w/v)			
P. acidipropionici DH42	.018	.017	.002	.7939
P. acidipropionici 4965	.017	.012		.1461
P. freudenreichii 6207	.018	.019		.6599
P. shermanii CDC 3094	.022	.023		.7441
P. pentosaceum P11	.017	.017		.8446
P. thoenii P15	.016	.013		.3037
P. jensenii P25	.017	.009		.0161
P. freudenreichii P72	.018	.022		.1821

Table 5-6. Propionic acid production of selected propionic acid-producing bacteria grown in peptone-yeast extract-lactate media (pH 6.9, 1.1% lactic acid) with (mixed culture) or without (pure culture) epiphytic microorganisms found in ensiled, high-moisture corn

Strain	Culture		SEM	$P <$
	Mixed	Pure		
	Propionic acid (% w/v)			
P. acidipropionici DH42	.688	.573	.019	.0008
P. acidipropionici 4965	.601	.519		.0093
P. freudenreichii 6207	.597	.497		.0022
P. shermanii CDC 3094	.589	.541		.0940
P. pentosaceum P11	.574	.526		.0946
P. thoenii P15	.546	.504		.1401
P. jensenii P25	ND	ND		
P. freudenreichii P72	.472	.487		.5652

Addition of the epiphytic microbial population with the propionic acid-producing bacteria tended to increase specific growth rate of the total mixed microbial community in extract 1 (Table 5-7). *Propionibacterium pentosaceum* P11 and *P. jensenii* P72 showed the greatest increases in specific growth rate of the mixed microbial community when grown in combination with the epiphytic microbial population. Specific growth rate of the propionic acid-producing bacteria and the total mixed community in extract 2 (Table 5-8) was lower than that seen for extract 1 (Table 5-7) with the exception of *P. thoenii* P15 which showed a marked increase in specific growth rate when grown with the epiphytic microbial population.

Addition of the epiphytic microbial population decreased ($P < .01$) specific growth rate of the propionic acid-producing bacteria when grown in peptone-yeast extract-lactate media (Table 5-9). The specific growth rate for *P. acidipropionici* DH42 was the greatest for the strains evaluated when grown in pure culture. The specific growth rate in peptone-yeast extract-lactate media in Chapter 3 was slightly higher than what was obtained by *P. acidipropionici* DH42 in this study. When grown in combination with the epiphytic microbial population, all strains had similar growth rates. As shown in Table 5-3 and 5-6, *P. jensenii* P25 did not grow in peptone-yeast extract-lactate media.

Table 5-7. Specific growth rate of selected propionic acid-producing bacteria grown in an aqueous extract of ensiled, high-moisture corn (pH 4.1, .11% lactic acid) with (mixed culture) or without (pure culture) epiphytic microorganisms found in ensiled, high-moisture corn

Strain	Culture		SEM	$P <$
	Mixed	Pure		
	Specific growth rate (h^{-1})			
P. acidipropionici DH42	.0167	.0144	.0011	.1548
P. acidipropionici 4965	.0162	.0134		.0959
P. freudenreichii 6207	.0164	.0148		.3180
P. shermanii CDC 3094	.0167	.0158		.5406
P. pentosaceum P11	.0173	.0156		.2845
P. thoenii P15	.0167	.0139		.0900
P. jensenii P25	.0175	.0147		.0922
P. freudenreichii P72	.0167	.0160		.6595

Table 5-8. Specific growth rate of selected propionic acid-producing bacteria grown in an aqueous extract of ensiled, high-moisture corn (pH 4.6, .02% lactic acid) with (mixed culture) or without (pure culture) epiphytic microorganisms found in ensiled, high-moisture corn

Strain	Culture		SEM	$P <$
	Mixed	Pure		
	Specific growth rate (h^{-1})			
P. acidipropionici DH42	.0107	.0122	.0008	.1898
P. acidipropionici 4965	.0126	.0123		.7952
P. freudenreichii 6207	.0108	.0100		.4494
P. shermanii CDC 3094	.0121	.0119		.8735
P. pentosaceum P11	.0123	.0113		.3359
P. thoenii P15	.0183	.0082		.0316
P. jensenii P25	.0118	.0115		.8376
P. freudenreichii P72	.0112	.0103		.4184

Table 5-9. Specific growth rate of selected propionic acid-producing bacteria grown in peptone-yeast extract-lactate media (pH 6.9, 1.1% lactic acid) with (mixed culture) or without (pure culture) epiphytic microorganisms found in ensiled, high-moisture corn

Strain	Culture		SEM	$P <$
	Mixed	Pure		
	Specific growth rate (h^{-1})			
P. acidipropionici DH42	.0098	.0301	.0013	.0001
P. acidipropionici 4965	.0105	.0159		.0122
P. freudenreichii 6207	.0099	.0234		.0001
P. shermanii CDC 3094	.0111	.0273		.0001
P. pentosaceum P11	.0111	.0215		.0001
P. thoenii P15	.0111	.0252		.0001
P. jensenii P25	.0111	.0000		.0001
P. freudenreichii P72	.0120	.0238		.0001

Implications

Selected strains of propionic acid-producing bacteria can metabolize substrates commonly found in ensiled, high-moisture corn. *Propionibacterium acidipropionici* DH42 (a propionic acid-producing bacteria isolated from ensiled, high-moisture corn) and *P. shermanii* CDC 3094 were more capable of competing and producing elevated amounts of propionic acid in peptone-yeast extract-lactate and aqueous extracts of ensiled, high-moisture corn than the other strains of *Propionibacterium spp.* evaluated. With these factors in consideration, it is reasonable to expect bioinoculants for ensiled crops to start using selected strains of propionic acid-producing bacteria to improve the quality of ensiled feedstuffs.

CHAPTER 6

MANIPULATION OF THE FERMENTATION AND AEROBIC STABILITY OF ENSILED, HIGH-MOISTURE CORN BY ADDITION OF *PROPIONIBACTERIUM ACIDIPROPIONICI* DH42

Abstract

Aerobic instability is the major cause of spoilage, nutrient loss and feed refusal of ensiled, high-moisture corn. A 2 x 6 factorial arrangement with two inoculant treatments: *Propionibacterium acidipropionici* DH42 (previously isolated from ensiled, high-moisture corn) added at 10^6 cfu•g^{-1}•DM^{-1} and control (sterile water) over six periods of ensiling (0, 2, 7, 14, 28 and 42 d) was used to evaluate the effect of a propionic acid-producing bacterial bioinoculant on fermentation characteristics and aerobic stability of ensiled, high-moisture corn. Addition of *P. acidipropionici* DH42, increased propionic acid content (.35 vs .03 g/100 g DM, $P < .01$) after 42 d ensilement when compared to non-treated ensiled, high-moisture corn. In addition, corn treated with *P. acidipropionici* DH42 had a higher acetic acid concentration (.63 vs .29 g/100 g DM, $P < .01$), lower pH (4.17 vs 4.32, $P < .01$), lower numbers of yeasts and molds ($10^{4.2}$ vs $10^{5.0}$ cfu•g^{-1}•DM^{-1}, $P < .05$) and higher dry matter recovery (98.7 vs 96.8%, $P < .05$) after 42 d ensilement when compared to non-treated ensiled, high-moisture corn. Temperature increased ($P < .01$) to 30 °C in the control

corn after three days of aerobic exposure, whereas the temperature of *P. acidipropionici* DH42 treated corn remained unchanged from ambient temperature (21°C). After five days of aerobic exposure, increases ($P < .01$) in pH (6.6 vs 4.4), numbers of yeasts and molds ($10^{7.8}$ vs $10^{5.2}$ cfu•g^{-1}•DM^{-1}), numbers of *Acetobacter* ($10^{7.9}$ vs $10^{5.2}$ cfu•g^{-1}•DM^{-1}) and numbers of total aerobic bacteria ($10^{8.6}$ vs $10^{8.0}$ cfu•g^{-1}•DM^{-1}) were observed in control indicating poorer aerobic stability compared to treated, high-moisture corn. Propionic acid concentration was higher (.61 vs 0 g/100 g DM, $P < .01$) after five days of aerobic exposure in high-moisture corn ensiled with *P. acidipropionici* DH42. In addition, organic matter recovery of inoculated corn was greater (94.4 vs 93.5%, $P < .01$) after five days aerobic exposure. Presence and persistence of *P. acidipropionici* DH42 in the treated, high-moisture corn were verified using a non-radioactive nucleic acid probe discussed in Chapter 7. Inoculation of high-moisture corn with *P. acidipropionici* DH42 improves the ensiling fermentation and reduces aerobic instability of ensiled, high-moisture corn.

Introduction

Aerobic deterioration of ensiled feeds is detrimental to the production of livestock feeds for many reasons. Aerobic microorganisms such as yeasts, molds and certain bacteria metabolize the highly-digestible fraction of the dry matter and can account for losses of dry matter in excess of 30% (Woolford, 1990). In addition, the microbial activity associated with aerobic deterioration can increase the temperature of the feed and generate feed that is less palatable and reduced digestibility (Ruxton and Gibson, 1993). Propionic acid has been used quite successfully in reducing losses associated with aerobic instability (Lindgren et al.,

1985). Flores-Galarza and coworkers (1985) added strains of propionic acid-producing bacteria commonly found in cheese and decreased proliferation of yeasts and molds associated with high-moisture corn. However, Lindgren and coworkers (1983) were unsuccessful in manipulating the fermentation of grass silage by the addition of *P. shermanii*. A strain of *P. acidipropionici* (DH42) was isolated from high-moisture corn and characterized in Chapter 3. The objective of this study was to evaluate the effectiveness of *P. acidipropionici* DH42 on the fermentation and aerobic stability of ensiled, high-moisture corn.

Experimental Procedures

High-moisture corn (74% DM) was obtained from the Mason elevator (Mason, MI) and ensiled in laboratory silos (45.7 x 10.2 cm dia.) constructed of PVC pipe and fitted with rubber caps as described by Wardynski (1991). The time delay from harvest until ensiling was not determined since the corn came from a commercial elevator. The corn appeared fresh and did not have any characteristics associated with pre-fermented corn. A rubber policeman (Fisher Scientific, Chicago, IL) was slit and inserted with copper tubing into the top cap to allow for gas release during ensiling. Thirty silos were used in a 2 x 5 factorial arrangement of treatments consisting of two treatments (control and *P. acidipropionici* DH42) and six periods of ensilement (0, 2, 7, 14, 28 and 56 d) with three silos for each treatment and period of ensilement combination.

Propionibacterium acidipropionici DH42, a propionic acid-producing bacteria isolated using methods described in Chapter 3 and characterized in Chapter 4, was grown

aerobically in a shaker water bath (60 rpm, 30 °C) to late log phase in Actinomyces broth (BBL, Cockeysville, MD). A 1.5 ml aliquot was applied to the corn using a 3 ml syringe with a 22 gauge needle while hand mixing, to assure homogenous application. The culture was applied to a final concentration of 10^6 cfu•g^{-1}•DM^{-1}. Inoculation rate was based on the recommended amounts of lactic acid-producing bacteria to be added as silage inoculants (Muck, 1988). Culture density of the inoculum was 10^9 cfu / ml as determined by decimal dilutions in purple base broth (BBL 11558, Cockeysville, MD) with 1% erythritol (Sigma, St. Louis, MO). Bacteria in the genus *Propionibacterium* are unique in their ability to ferment erythritol to propionic and acetic acids (Holdeman et al., 1977). Therefore, use of erythritol as a fermentation substrate and bromocresol purple as an acidic pH indicator dye allows for estimating numbers of propionic acid-producing bacteria when used as a most probable number dilution scheme.

Aqueous extracts of the ensiled, high-moisture corn were obtained by homogenizing 50 g of sample in 450 ml sterile .9% NaCl (pH 7.0) using a stomacher (Tekmar 3500, Cincinnati, OH) for 5 min. The pH of the aqueous extract was measured within five minutes of extraction at 23 °C using a combination glass electrode (AccupHast, Fisher Scientific, Chicago, IL) attached to a digital pH meter (Fisher Scientific 825MP pH meter, Fisher Scientific, Chicago, IL) that was standardized from pH 4 to 7 using commercial buffers (Curtis Mattheason, Woodale, IL). Serial dilutions of the aqueous extract of corn after addition of the inoculum in the same media supplemented with amphotericin-B (Sigma), a mycostatic agent used in mammalian cell culture, allowed estimation of the number of added *P. acidipropionici* DH42. Serial dilutions were incubated aerobically at 30

°C and the highest dilution that had growth and yellow color (indication of erythritol fermentation) was used as an estimate of the number of propionic acid-producing bacteria. An appropriate control consisted of 1.5 ml of sterile .9% NaCl. Lactic acid-producing bacteria were enumerated on pour plates of LBS agar medium (Difco, Detroit). Yeasts and Molds were enumerated on pour plates of Rose Bengal agar medium base supplemented with chloramphenicol (Difco, Detroit, MI). The media described by Spoelstra (1988) was used as pour plates to enumerate *Acetobacter spp.* Total aerobic bacteria were enumerated on pour plates of tryptic soy agar (Difco, Detroit, MI).

Dry matter and ash were determined according to AOAC (1984) by drying at 60°C for dry matter and ignition at 550 °C for ash. Glucose, lactic acid, acetic acid, propionic acid and ethanol were determined by ion exchange-exclusion HPLC (BIORAD aminex HPX-87H, Richmond, CA) following the general procedures of Canale et al. (1984). Mobile phase consisted of .005 N H_2SO_4 at a flow rate of .9 ml/min. Column temperature was maintained at 65°C by an external column heater (Waters Millipore, Milford, MA). Three ml of aqueous extracts of each sample of ensiled, high-moisture corn were filtered through .2 μm ion chromatography syringe filters (Gelman Acrodisks, 25 mm, Ann Arbor, MI) into 3 ml HPLC sample vials (National Scientific, Atlanta, GA). Filtered samples were stored at -20 °C until analysis. Twenty μl of the filtered samples was injected by an autoinjector (Waters WISP 712, Milford, MA) and analytes were detected by refractive index (Waters 410 refractive index detector, Milford, MA). Peak heights were quantified by a commercial HPLC software package (Turbochrom 3, PE Nelson, Cupertino, CA) and compared to standards of lactic, acetic and propionic acid (Supelco, Bellefonte, PA).

Statistical analyses. The fermentation experiment was analyzed as a completely randomized design by analysis of variance (Steele and Torrie, 1980) using the general linear models (GLM) procedure of the Statistical Analysis System (SAS, 1985). Individual treatment means were compared using the Bonferroni t-test (SAS, 1985). The ANOVA model for the fermentation study was as follows:

$$Y_{ijk} = \mu + T_i + D_j + (T \bullet D)_{ij} + e_{ijk}$$

Where:

Y_{ijk} = Individual response variable measured (e.g., pH, numbers of lactic acid-producing bacteria, propionic acid).

μ = Overall mean.

T_i = Effect of treatment (Control or *P. acidipropionici* DH42)

D_j = Effect of period of ensilement (0, 2, 7 , 14, 28 or 42 d).

$(T \bullet D)_{ij}$ = Interaction of treatment and period of ensilement .

e_{ijk} = Random residual effect (assumed normally distributed).

Aerobic stability was analyzed as above but using the repeated measure option of the general linear models procedure (SAS, 1987) since repeated measurements were made on each silo opened after 42 d ensilement and not on individual silos used in the fermentation experiment.

Results and Discussion

Fermentation characteristics. Both the control corn and corn treated with *P. acidipropionici* DH42 showed the normal fermentation pattern observed in ensiled, high-moisture corn except increased propionic acid in corn inoculated with *P. acidipropionici* DH42. The pH (Table 6-1) showed the characteristic decrease ($P < .0001$) seen during typical ensilement of high-moisture corn. However, addition of *P. acidipropionici* DH42 resulted in high-moisture corn with a lower pH ($P < .05$) after 14 d ensiling. The pH values obtained in this study followed the same pattern observed in the study reported in Chapter 2 and the general values reported for the fermentation of similar corn by Wardynski (1991).

The hypothesis to be tested was that the addition of *P. acidipropionici* DH42 would increase production of propionic acid during ensilement . A treatment by ensiling period interaction ($P < .0001$) was observed for propionic acid concentration (Table 6-2). No propionic acid was detected in aqueous extracts of control or treated high-moisture corn during the first 2 d of ensiling. Propionic acid concentration increased in *P. acidipropionici* DH42 to values greater ($P < .0001$) than control corn after 14 d ensiling. Control corn had measurable amounts (.03 g/100 g DM) of propionic acid after 42 d ensilement which has also been observed in control and corn treated with a lactic acid-producing bacterial inoculant (Wardynski et al., 1993). Propionic acid production was greatest after 14 d in reconstituted, high-moisture corn treated with *P. acidipropionici* ATCC 4965 and *P. freudenreichii* ATCC 6207 in the study reported in Chapter 2. This was not the case with the addition of *P. acidipropionici* DH42 in which the maximum amount of propionic acid was

reached after 42 d. Future studies may need to be conducted to evaluate the effect of longer periods of ensilement on the production of propionic acid in ensiled, high-moisture corn.

Acetic acid (Table 6-3) increased ($P < .01$) during ensilement and was greatest ($P < .01$) in corn treated with *P. acidipropionici* DH42 after 42 d ensilement . The higher amount of acetic acid is to be expected in the treated corn because acetic acid is a co-product of the fermentation of carbohydrates and lactic acid by *P. acidipropionici* DH42. Another fermentation acid which is highly correlated to decreases in silage pH is lactic acid. The production of lactic acid (Table 6-4) increased ($P < .01$) through 14 d in both the control and treated corn following the same general fermentation pattern observed in non-treated ensiled, high-moisture corn. Glucose (Table 6-5) was rapidly fermented (period of ensiling $P < .0001$) within the first 2 d post ensiling. Very little residual glucose remained in either the control or treated corn after 42 d ensiling. Ethanol (Table 6-6) increased throughout the first 14 d (period of ensiling $P < .0001$) of ensiling in both control and treated corn. No differences ($P > .1$) were observed between treatments, possibly due to the large variation in ethanol content within treatments and periods of ensiling.

Numbers of lactic acid-producing bacteria (Table 6-7) increased (period of ensiling $P < .0001$) at an exponential rate during the first 7 d ensilement . The increase in numbers of lactic acid-producing bacteria corresponds to the decreases in pH and increases in lactic acid concentration. Addition of *P. acidipropionici* DH42 prior to ensilement resulted in lower ($P < .01$) number of lactic acid-producing bacteria after 42 d ensilement . It can only be surmised about what type of lactic acid-producing bacteria were present. Therefore,

conclusions of the effect of *P. acidipropionici* DH42 on homofermentative lactic acid-producing bacteria cannot be made.

Yeast and mold numbers (Table 6-8) increased throughout the first 14 d ensilement (period of ensiling $P < .0001$) to maximum values of $10^{6.2}$ for both control and treated corn. Numbers of yeasts and molds are important because they have been implicated as having a major role in the aerobic deterioration of ensiled, high-moisture corn (Rust and Yokoyama, 1992). The increase in numbers of yeasts and molds could help explain the observed increase in ethanol, a major end-product of their metabolism. However, numbers of yeasts and molds decreased after 14 d and tended ($P < .1$) to be lower after 28 d in the corn treated with *P. acidipropionici* DH42. Addition of *P. acidipropionici* DH42 was effective in decreasing number of yeasts and molds after 42 d ensiling.

Presence of coliform bacteria is generally an indicator of poor quality silage. Numbers of coliforms (Table 6-9) were slightly higher at d 0 for control corn. However, after 2 d ensilement both the treated and control corn had similar numbers of coliforms indicating the d 0 values did not cause a noticeable effect on their numbers after 2 d ensiling. Addition of *P. acidipropionici* DH42 decreased proliferation of coliforms as shown by lower ($P < .05$) numbers after 14, 28 and 56 d ensilement .

Dry matter and organic matter recovery were measured after 42 d ensilement . Dry matter recovery (Table 6-10) was improved ($P < .05$) from 96.8% for control corn to 98.7% by addition of *P. acidipropionici* DH42. Organic matter recovery (Table 6-10) was numerically higher ($P < .25$) in treated corn (99.6%) than in control corn (99.3%).

Table 6-1. Effect of *Propionibacterium acidipropionici* DH42 on the pH during the ensilement of high-moisture corn

Time (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	pH of aqueous extract			
0	5.39	5.45	0.033	0.2714
2	5.24	5.28		0.4068
7	4.84	4.78		0.2433
14	4.57	4.46		0.0338
28	4.35	4.20		0.0042
42	4.32	4.17		0.0042

Table 6-2. Effect of *Propionibacterium acidipropionici* DH42 on the propionic acid content during the ensilement of high-moisture corn

Time (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	g/100 g DM			
0	0.00	0.00	0.013	1.0000
2	0.00	0.00		1.0000
7	0.00	0.02		0.3059
14	0.00	0.12		0.0001
28	0.00	0.11		0.0001
42	0.03	0.35		0.0001

Table 6-3. Effect of *Propionibacterium acidipropionici* DH42 on the acetic acid content during the ensilement of high-moisture corn

Time (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	g/100 g DM			
0	0.00	0.00	0.079	1.0000
2	0.18	0.26		0.4910
7	0.18	0.32		0.2450
14	0.29	0.34		0.6249
28	0.14	0.15		0.9290
42	0.29	0.63		0.0051

Table 6-4. Effect of *Propionibacterium acidipropionici* DH42 on the lactic acid content during the ensilement of high-moisture corn

Time (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	g/100 g DM			
0	0.00	0.00	.146	1.0000
2	0.46	0.52		0.7593
7	0.68	0.74		0.8008
14	1.26	0.89		0.0871
28	0.64	0.43		0.3163
42	0.72	0.75		0.8832

Table 6-5. Effect of *Propionibacterium acidipropionici* DH42 on the glucose content during the ensilement of high-moisture corn

Time (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	g/100 g DM			
0	0.34	0.29	0.027	0.1914
2	0.01	0.02		0.9106
7	0.01	0.00		0.7043
14	0.01	0.01		0.9724
28	0.02	0.03		0.8090
42	0.00	0.04		0.2898

Table 6-6. Effect of *Propionibacterium acidipropionici* DH42 on the ethanol content during the ensilement of high-moisture corn

Time (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	g/100 g DM			
0	0.00	0.00	0.289	1.0000
2	0.85	0.84		0.9871
7	1.44	1.63		0.6581
14	2.21	1.60		0.1517
28	0.96	0.72		0.5478
42	1.67	1.13		0.1969

Table 6-7. Effect of *Propionibacterium acidipropionici* DH42 on the number of lactic acid-producing bacteria during the ensilement of high-moisture corn

Time (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	log cfu per g DM			
0	3.39	3.49	0.068	0.4034
2	6.39	6.35		0.6311
7	8.41	8.34		0.4939
14	8.57	8.47		0.3081
28	8.45	8.47		0.8366
42	8.46	8.19		0.0117

Table 6-8. Effect of *Propionibacterium acidipropionici* DH42 on the number of yeasts and molds during the ensilement of high-moisture corn

Time (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	log cfu per g DM			
0	3.57	3.9	0.199	0.3503
2	5.28	5.42		0.6331
7	4.43	4.68		0.3855
14	6.22	6.25		0.9163
28	5.34	4.80		0.0705
42	4.93	4.16		0.0126

Table 6-9. Effect of *Propionibacterium acidipropionici* DH42 on the number of coliforms during the ensilement of high-moisture corn

Time (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	log cfu per g DM			
0	6.9	6.63	.051	0.0048
2	7.06	7.02		0.5516
7	6.45	6.45		0.8903
14	6.38	5.98		0.0001
28	6.27	6.12		0.0529
42	8.05	7.51		0.0001

Table 6-10. Effect of *Propionibacterium acidipropionici* DH42 on the dry matter and organic matter recovery high-moisture corn after 42 days ensilement

Item	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	% recovered			
Dry matter	96.8	98.7	0.45	0.0403
Organic matter	99.3	99.6	0.17	0.2470

Aerobic stability. Since propionic acid is commonly used as a chemical aid to prevent aerobic deterioration, then the addition of *P. acidipropionici* DH42 prior to ensilement would be beneficial through an increase propionic acid content of ensiled, high-moisture corn. This was the case during 42 d of fermentation (Table 6-2) and during the aerobic exposure period of 5 d (Table 6-11). Propionic acid disappeared from the control corn after 5 d aerobic exposure. To the contrary, corn treated with *P. acidipropionici* DH42 showed increased propionic acid concentration with the greatest amount after 5 d (.61 g/100 g DM). The amount of propionic acid that is recommended for the addition to corn with 70% DM is 1.1% for 6 months storage. Future research will be needed to decide what concentration is necessary to be reached when propionic acid is being produced in the silo.

No trend was observed due to the variability of the amounts of acetic acid (Table 6-12) across treatments and time of aerobic exposure. The concentration of acetic acid was lower than that observed in the study by Wardynski et al. (1993) where concentration as high as 3 g/100 g DM were determined in high-moisture corn inoculated with lactic acid-producing bacteria. Ethanol (Table 6-13) is usually associated with coliform or yeast fermentations. There was a large variation in the amount of ethanol present in the ensiled, high-moisture corn and the only effect was due to time of aerobic exposure ($P < .01$). Ethanol concentration decreased during aerobic exposure.

An interaction ($P < .01$) of treatment and time of aerobic exposure was present for lactic acid concentration (Table 6-14) in the aerobically exposed high-moisture corn. Greater ($P < .05$) concentration of lactic acid was found in the control corn after 3 d exposure and less ($P < .01$) after 5 d as compared to the corn treated with *P. acidipropionici* DH42. Loss

of lactic acid is generally associated with aerobic instability and the differences observed in the *P. acidipropionici* DH42 treated corn at 5 d were anticipated. However, the lower amount of lactic acid at d 3 is puzzling. Glucose concentration (Table 6-15) was slightly increased ($P < .10$) by addition of *P. acidipropionici* DH42 after 3 d of aerobic exposure. Future studies should focus on changes in total carbohydrates present prior to and after ensilement or aerobic exposure.

A classical response seen in high-moisture corn undergoing aerobic deterioration is an increase in pH over time of aerobic exposure. The pH of control corn (Table 6-16) increased ($P < .0001$) from 4.32 to 6.62 after 5 d of aerobic exposure. Corn treated with *P. acidpropionici* DH42 had a stable pH (4.17) throughout the first 3 d of aerobic exposure and a slight increase to 4.38 after 5 d of aerobic exposure. Addition of *P. acidipropionici* DH42 prior to ensilement was effective in reducing the change in pH normally observed when ensiled feeds are exposed to oxygen.

Yeasts and molds have been implicated in the aerobic deterioration of ensiled feeds, especially ensiled, high-moisture corn (Rust and Yokoyama, 1992). *Propionibacterium acidpropionici* DH42 addition to ensiled, high-moisture corn was effective in maintaining lower ($P < .0001$) numbers of yeasts and molds (Table 6-17) in aerobically exposed ensiled, high-moisture corn. Control corn had initial yeast and mold numbers of $10^{4.93}$ cfu$\cdot$g$^{-1}\cdot$DM^{-1} and this increased ($P < .0001$) dramatically to $10^{7.83}$ cfu$\cdot$g$^{-1}\cdot$DM^{-1} after 5 d aerobic exposure. Numbers of yeasts and molds were 100 times lower ($P < .01$) in corn treated with *P. acidipropionici* DH42 after 3 and 5 days of aerobic exposure that was shown with a treatment by period of aerobic exposure interaction ($P < .0001$). Besides yeasts and molds,

acetic acid bacteria (*Acetobacter*) have been implicated in the initiation of aerobic deterioration (Spoelstra, 1990). Numbers of *Acetobacter* followed the same trends observed with the proliferation of yeasts and molds in the control corn.

Numbers of total aerobic bacteria over 5 d of aerobic exposure (Table 6-19) were decreased ($P < .01$) by addition of *P. acidipropionici* DH42. Whereas, the number of total aerobic bacteria increased in the control corn. Total aerobic bacteria are also indicators of the inherent stability of the ensiled feedstuffs. Therefore, the greater number of aerobic bacteria present, the higher the chance the ensiled material will be unstable.

Temperature of aerobically exposed silage will increase rapidly in unstable silages. The temperature of high-moisture corn ensiled with a lactic acid-producing bacterial inoculant increased to 50 °C within 5 d aerobic exposure in a study by Wardynski et al. (1993). The temperature of the control corn in this study (Table 6-20) increased to 30 °C within 3 d of aerobic exposure. Even though this indicated poor stability, it was not as unstable as the corn treated with a lactic acid-producing bacteria in the study by Wardynski et al. (1993).

Overall stoichiometry cannot be directly evaluated using the techniques presented in this study. All substrates and end-products must be accounted for in order to precisely estimate the overall balance of carbon. Dry matter (Table 6-21) and organic matter recoveries (Table 6-22) were reduced ($P < .05$) when ensiled, high-moisture corn was exposed to oxygen. In other words, dry matter and organic matter are being metabolized to carbon dioxide, water and heat which is decreasing the amount recovered. Addition of *P. acidipropionici* DH42 did not change the amount of dry matter loss after 5 d of aerobic

exposure (Table 6-21). However, organic matter recovery was increased by addition of *P. acidipropionici* DH42 after 5 d aerobic exposure indicating a slower rate of nutrient loss.

The aerobic stability of the control corn would have been considered adequate since temperature of the aerobically exposed ensiled, high-moisture corn was stable over 48 h. Temperature reached a peak (30.6 °C) at 3 d and then declined near ambient. Additionally, the maximum temperature recorded for the treated corn was 5 °C less (25.4 °C vs 30.6 °C) than control corn. The control corn in the study by Wardynski et al. (1993) did not reach a maximal temperature until 9 d of aerobic exposure. Addition of *P. acidipropionici* DH42 prior to ensilement delayed increases in temperature, pH, numbers of yeasts, molds, aerobic bacteria and *Acetobacter* indicating a dramatic improvement in aerobic stability over control corn that would have been considered normal ensiled, high-moisture corn.

Table 6-11. Effect of *Propionibacterium acidipropionici* DH42 on the propionic acid content during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	g/100 g DM			
0	0.03	0.35	0.034	0.0001
1	0.04	0.44		0.0001
3	0.07	0.28		0.0008
5	0.00	0.61		0.0001

Table 6-12. Effect of *Propionibacterium acidipropionici* DH42 on the acetic acid content during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	g/100 g DM			
0	0.29	0.63	0.153	0.1332
1	0.49	0.65		0.4654
3	0.12	0.32		0.3540
5	0.17	0.48		0.1743

Table 6-13. Effect of *Propionibacterium acidipropionici* DH42 on the ethanol content during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	g/100 g DM			
0	1.67	1.13	0.325	0.2624
1	1.44	1.42		0.9725
3	0.02	0.49		0.3298
5	0.05	0.30		0.5980

Table 6-14. Effect of *Propionibacterium acidipropionici* DH42 on the lactic acid content during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	g/100 g DM			
0	0.72	0.75	0.215	0.9208
1	1.31	1.20		0.7317
3	1.38	0.58		0.0179
5	0.50	1.65		0.0016

Table 6-15. Effect of *Propionibacterium acidipropionici* DH42 on the glucose content during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	g/100 g DM			
0	0.00	0.04	0.021	0.1924
1	0.05	0.08		0.3929
3	0.09	0.04		0.0671
5	0.04	0.10		0.1104

Table 6-16. Effect of *Propionibacterium acidipropionici* DH42 on the pH during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	pH of aqueous extract			
0	4.32	4.17	0.040	0.0184
1	4.37	4.22		0.0184
3	4.63	4.22		0.0001
5	6.62	4.38		0.0001

Table 6-17. Effect of *Propionibacterium acidipropionici* DH42 on the number of yeasts and molds during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	log cfu per g DM			
0	4.93	4.17	.223	0.0272
1	5.23	4.00		0.0012
3	6.93	4.67		0.0001
5	7.83	5.17		0.0001

Table 6-18. Effect of *Propionibacterium acidipropionici* DH42 on the number of *Acetobacter* during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	log cfu per g DM			
0	5.00	4.00	.182	.0013
1	5.00	3.93		.0008
3	6.93	4.37		.0001
5	7.93	5.20		.0001

Table 6-19. Effect of *Propionibacterium acidipropionici* DH42 on the number of total aerobic microorganisms during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	log cfu per g DM			
0	8.33	8.13	0.061	0.0346
1	8.00	8.00		1.0000
3	8.17	7.63		0.0001
5	8.57	8.03		0.0001

Table 6-20. Effect of *Propionibacterium acidipropionici* DH42 on temperature changes during aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	°C			
0	23.9	23.7	.88	.8958
1	21.7	21.5		.8953
2	21.7	21.8		.9580
3	30.6	21.4		.0001
4	27.6	21.7		.0001
5	23.7	25.4		.1862
6	21.9	21.1		.5289

Ambient temperature averaged 21.4

Table 6-21. Effect of *Propionibacterium acidipropionici* DH42 on dry matter recovery during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	% recovered			
1	100.5	99.1	0.40	0.0285
3	100.7	99.8		0.1449
5	99.7	99.5		0.7186

Table 6-22. Effect of *Propionibacterium acidipropionici* DH42 on organic matter recovery during the aerobic exposure of ensiled, high-moisture corn

Aerobic Exposure (d)	Treatment		SEM	$P <$
	Control	*P. acidipropionici* DH42		
	% recovered			
1	98.7	99.1	0.21	0.2497
3	98.5	98.8		0.3765
5	93.5	94.4		0.0108

Care should be exercised in extrapolating the effect of *P. acidipropionici* DH42 in laboratory silos on the fermentation of high-moisture corn. Future research is needed to evaluate the optimal inoculation rate, moisture levels, growth media, environmental (i.e., temperature, relative humidity) and method of application. Other additives such as lactic acid-producing bacteria inoculants and ammonia addition may affect the efficacy of propionic acid-producing bacteria when grown in high-moisture corn. In addition, the effect of *P. acidipropionici* DH42 on the fermentation and aerobic stability of different ensiled crops (i.e., alfalfa haylage, corn silage) cannot be directly estimated from this research since it was performed using only high-moisture corn.

Implications

Addition of *Propionibacterium acidipropionici* DH42 as a bacterial inoculant to high-moisture corn was successful in manipulating the ensiling fermentation and improving aerobic stability. Propionic acid content was increased to .35g/100 g DM during 42 d ensilement and to .61g/100 g DM during five d of aerobic exposure after ensilement. Aerobic stability was increased dramatically as evidenced by a more stabilized temperature and pH, reduced numbers of yeasts, molds and *Acetobacter* in high-moisture corn inoculated with *P. acidpropionici* DH42 prior to ensilement. Farm-scale silo experiments should be conducted to determine if *P. acidipropionici* DH42 can have the same effect in a less controlled environment.

CHAPTER 7

NON-RADIOACTIVE NUCLEIC ACID PROBES FOR PROPIONIC ACID-PRODUCING BACTERIA IN ENSILED, HIGH-MOISTURE CORN

Abstract

Use of nucleic acid probes for assessment of ecological interactions in microbial ecosystems is becoming popular with the advent of non-radioactive detection methods for nucleic acids. The objective of this study was to develop a protocol for a non-radioactive nucleic acid probe to monitor the persistence of a propionic acid-producing bacterial inoculant. Total DNA extracted from a propionic acid-producing bacteria previously isolated from ensiled, high-moisture corn, designated *Propionibacterium acidipropionici* DH42, was randomly labeled by the incorporation of digoxigenin-labeled deoxyuridine-triphosphate and used for detection of the organism previously added as a bioinoculant to ensiled, high-moisture corn. Specificity of the probe was evaluated by slot-blotting extracted DNA from exponential growth of nine *Propionibacterium spp.* and six *Lactobacillus spp.* No cross-hybridization was observed with DNA extracted from *Propionibacterium jensenii*, *P. thoenii*, *Lactobacillus bulgaricus*, *L. confusus*, *L. delbrukii*, *L. fructovorans*, *L. helveticus*, or *L. sake*. However, there was slight cross-hybridization with DNA extracted from *P. freudenreichii* and *P. shermanii*. The probe was used

successfully in detecting the presence and persistence of *P. acidipropionici* DH42 when added to high-moisture corn after 2, 7, 14, 28, and 42 d of ensiling. In addition, DNA extracted from ensiled, high-moisture corn treated with *P. acidipropionici* DH42 after three d of aerobic exposure, hybridized to the probe. Nucleic acids extracted from the mixed, microbial community associated with non-treated, high-moisture corn did not hybridize to the probe on any of the samples taken from the ensiling fermentation or aerobic exposure phases. These results infer that non-radioactive DNA probes can be used to monitor persistence of a propionic acid-producing bacterial inoculant in ensiled, high-moisture corn and support the fermentation data presented in chapter 6, in which addition of the propionic acid-producing bacteria increased propionic acid content after 14 days of ensiling. Further optimization needs to be performed to increase the sensitivity of detection for this probe.

Introduction

Nucleic acid probes are becoming popular for the evaluation of interactions of different microorganisms in various ecosystems. Historically, procedures for detection of nucleic acid sequences involved radioactive materials. Recently, emphasis has been placed on the development and use of non-radioactive methods for detection of nucleic acids. Digoxigenin is a steroid which can be cross-linked by a chemical spacer arm to deoxyuridine-triphosphate (DIG-dUTP) and is the basis for a commercial nonradioactive labelling and detection kit (Genius-1, Boehringer Mannheim, Indianapolis, IN). The use of DIG-dUTP in a random primed DNA labeling method can lead to incorporation of

DIG-dUTP every 20 to 25 nucleotides in the newly synthesized DNA. The DIG-dUTP can then be detected by immunological methods and visualized by colorimetric, fluorescent or luminescent techniques. The method of visualization is dependent on the substrate chosen for the alkaline phosphatase which is conjugated to the anti-DIG antibody fragment. Sensitivity of this method is comparable, if not better, to radioactive techniques and can be done in a safer and quicker manner.

Previous efforts at development of selective media for propionic acid-producing bacteria have been based on yeast extract, peptone and sodium lactate (Vedamuthu and Reinbold, 1967; Hettinga et al., 1968; Peberdy and Freyers, 1976). The selectivity of the above media was based on the fermentation of lactate by propionic acid-producing bacteria under anaerobic conditions. However, lactic acid-producing bacteria and other microorganisms commonly found in silages can metabolize lactic acid. Reddy and coworkers (1973) and Drinan and Cogan (1992) attempted to correct this problem by addition of two antibiotics, cloxacillin and kanamycin. This alleviated the growth of most lactic acid-producing bacteria except *Leuconostocs*. The concentration of antibiotics necessary to inhibit the growth of *Leuconostocs* was also inhibitory to the growth of propionic acid-producing bacteria.

Hendrich and coworkers (1991) developed a radioactive nucleic acid probe to a cryptic plasmid of a strain of *Bacillus pumilus* that was being used as a bioinoculant for hay. This was done to simplify monitoring the persistence of the inoculated strain in hay, soil and plant material. Furthermore, the probe was used to relate efficacy of the product

to organism numbers. Plasmid transfer between the inoculated strain of *Bacillus pumilus* and epiphytic *Bacillus pumilus* was considered a disadvantage to this method.

Non-radioactive nucleic acid probes were developed to the total genomic DNA of strains of lactic acid-producing bacteria involved in wine production by Lonvaud-Funel and coworkers (1991). This method showed that non-radioactive, nucleic acid probes, based on total genomic DNA, were species specific for most of the *Lactobacillus spp.* involved in fermentation of wine. Generation of nucleic acid probes from the genomic DNA of selected bacteria should allow for more precise measurement of their status in diverse microbial ecosystems such as ensiled feedstuffs.

A propionic acid-producing bacterium capable of competing for substrates against epiphytic microorganisms found in ensiled, high-moisture corn was isolated using methods described in chapter 3 and characterized in chapter 4. This organism, designated *Propionibacterium acidipropionici* DH42, was successfully used as a bioinoculant to improve the fermentation and aerobic stability of ensiled, high-moisture corn in the experiment described in chapter 6. The objective of this study was to develop a protocol for use of a non-radioactive nucleic acid probe to detect the presence of *P. acidipropionici* DH42 when used as a bioinoculant for ensiled, high-moisture corn.

Experimental Procedures

Bacterial strains. *Propionibacterium acidipropionici* DH42 was previously isolated from ensiled, high-moisture corn and stored in 8 ml of 50% glycerol and .5X (half strength) MRS (Difco, Detroit, MI) in 10 ml serum bottles (Bellco, Vineland, NJ)

at -20 °C. *Propionibacterium acidipropionici* 4965 and *P. freudenreichii* 6207 were purchased from American Type Culture Collection (ATCC, Rockville, MD). *Propionibacterium jensenii* P25, *P. "pentosaceum"* P11 (strain of *P. acidipropionici*), *P. shermanii* CDC 3094, *P. shermanii* P92 and *P. thoenii* were kind gifts from Chr. Hansen's Biosystems (Milwaukee, WI). *Propionibacterium acidipropionici* 22562, *P. freudenreichii* 1382, *Lactobacillus bulgaricus* 8144, *L. confusus* 27646, *L. delbrukii* 9649, *L. fructovorans* 8288, *L. helviticus* 12046 and *L. sake* 15521 were obtained from Michigan Biotechnology Institute (Lansing, MI). All cultures were grown in crimper sealed 16 x 125 mm Hungate tubes (Bellco 2047-00125) under ambient gas phase at 30 C in .5X MRS (Difco, Detroit, MI) and stored as previously described.

Nucleic acid extraction. The freeze-thaw DNA extraction method described by Tsai and Olson (1991) was modified by using a commercial DNA purification system (Wizard Minipreps DNA Purification System, Promega, Madison, WI). One ml of mid-exponential growth of *P. acidipropionici* DH42 was harvested by centrifugation (16,000 x *g* for 2 min) in a sterile 1.5 ml microcentrifuge tube. Supernatant was carefully decanted and the pellet was resuspended in 200 μl cell resuspension solution (50 mM Tris-HCL pH 7.5, 10 mM EDTA, 100 μg/ml RNase A; Wizard Minipreps DNA Purification System, Promega, Madison, WI). Three cycles of freezing in a -70 °C dry ice-ethanol bath and thawing in a 65 °C water bath were used to disrupt the cell membrane and release DNA. Two hundred μl of cell lysis solution (.2 M NaOH, 1% SDS; Wizard Miniprep DNA Purification system, Promega, Madison, WI) was added with complete mixing of the lysis solution achieved by repeated inversion of the microcentrifuge tube. Cell membrane

disruption was facilitated by heating the lysis mixture in a 95 °C water bath for 10 min. Potassium acetate (1.32 M, pH 4.8; Wizard Miniprep, Promega, Madison, WI) was added to the lysis solution to neutralize the cellular lysis material. Cellular debris was removed by centrifugation (16,000 x *g* for 5 min). A 500 μl aliquot of the supernatant was carefully removed and transferred to a sterile 1.5 ml microcentrifuge tube. One ml of DNA purification resin (Wizard Minipreps DNA Purification Resin, Promega, Madison, WI) was added and mixed by inversion of the microcentrifuge tube. A DNA purification minicolumn (Wizard Miniprep, Promega, Madison, WI) was affixed to a 3 ml disposable syringe and the resin mixture was slowly passed through the column with pressure applied by the syringe plunger. Two ml of column wash solution (200 mM NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EDTA in 50% ethanol; Wizard Miniprep, Promega, Madison, WI) were passed through the column. The DNA purification minicolumn was transferred to a microcentrifuge tube and centrifuged (16,000 x *g* for 20 s) to remove residual column wash solution and then transferred to a sterile microcentrifuge tube. Fifty μl of autoclaved water (heated to 80 °C) was placed into the DNA purification column and allowed to sit for 60 sec. Genomic DNA was then harvested by centrifugation of the DNA purification column (16,000 x *g* for 20 s) to a final volume of 50 μl.

Synthesis of non-radioactive probes for genomic DNA of *P. acidipropionici* DH42. Total DNA extracted from *P. acidipropionici* DH42 was randomly labeled with digoxigenin (Genius-1 Digoxigenin-dUTP labeling and detection kit, non-radioactive Cat. No. 1093 057, Boehringer Mannheim, Indianapolis, IN) using the general procedures outlined by Lonvaud-Funel and coworkers (1991) with modifications to the purification

of the labeled probe using a commercial DNA purification kit (Wizard Miniprep, Promega). A scaled up labeling reaction was performed as described in the Genius (Boehringer Mannheim # 101 023, Indianapolis, IN) manual. Thirty-five μl of the extracted DNA was denatured by heating in a 95 °C water bath for 10 min and then placed in a -70 °C dry ice-ethanol bath for 30 s. Denatured DNA template was added to a 1.5 ml microcentrifuge tube on ice. Ten μl of hexanucleotide mix (all possible combinations of hexanucleotides to serve as primers for DNA strand synthesis, Boehringer Mannheim # 127 081, Indianapolis, IN), 10 μl dNTP labeling mix (dATP, dCTP, dGTP, dTTP and DIG-dUTP, Boehringer Mannheim # 1277 065, Indianapolis, IN), 40 μl sterile water and 5 μl Klenow enzyme (DNA polymerase 1, labeling grade, Boehringer Mannheim # 1008 404, Indianapolis, IN) were added to the denatured DNA template. The reaction mixture was incubated in a 37 °C water bath for 20 h. Synthesis of the nucleic acid probe was terminated by addition of 10 μl EDTA (.2 M, pH 8.0). A 5 μl aliquot of a glycogen solution (20 mg/ml, Boehringer Mannheim #901 393, Indianapolis, IN) was added to facilitate DNA recovery. Nucleic acid purification was done as previously described in the extraction procedure using a commercial DNA purification kit (Wizard Miniprep, Promega, Madison, WI).

Yield of DIG-dUTP labeled genomic DNA was estimated by spotting 1 μl of DIG-dUTP standard DNA (DNA labeling and detection kit, nonradioactive Genius-1, Boehringer Mannheim 1093 657, Indianapolis, IN) at concentrations of 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl and .1 pg/μl on a 5 cm square piece of nylon membrane (Zeta-Probe GT membrane, Bio-Rad # 162-0190, Richmond, CA). One μl of the dilutions (1:5,

1:50, 1:500, 1:5,000 and 1:50,000) of DIG-dUTP labeled DNA were spotted on the same membrane. The membrane was dried in a vacuum oven at 80 °C for 30 min to affix the DNA to the membrane. Blocking areas of the membrane where DNA was not attached was accomplished by incubating the membrane in a heat sealed bag with 20 ml blocking solution (2% Boehringer Mannheim # 1096 176 blocking reagent , Indianapolis, IN; in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) on a shaker (Gyrotory water bath shaker G-76, New Brunswick Scientific, New Brunswick, NJ) at 150 rpm for 30 min. Blocking solution was removed and 30 ml of a 1:5000 dilution (in blocking solution) of anti-digoxigenin Fab fragment (anti-DIG antibody-alkaline phosphatase conjugate, 150 U/200 μl, Boehringer Mannheim #1093 274, Indianapolis, IN) was added then incubated for 5 min at 23 °C (ambient temperature). The membrane was washed twice in 25 ml buffer (Genius buffer 1, 100 mM Tris-HCl, 150 mM NaCl, pH 7.5) to remove unbound antibody. Alkaline phosphatase was then activated by incubating the membrane in alkaline buffer (Genius buffer 3, 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min. Forty-five μl NBT solution (75 mg/ml nitroblue tetrazolium salt in 70% v/v dimethyl formamide, Boehringer Mannheim #1383 213, Indianapolis, IN) and 35 μl X-phosphate (50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate, toluidinium salt in 100% dimethyl formamide, Boehringer Mannheim # 1383 221, Indianapolis, IN) were added to alkaline buffer (Genius buffer 3, 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) and color development allowed to proceed for 30 min in the dark. A blue-black precipitate, formazine, started to form immediately and the color development was normally sufficient within 30 to 60 min. Amount of DIG-dUTP labeled probe was estimated by comparing the

intensity of the color to the standard DIG-dUTP DNA. Approximately 30 ng of genomic DNA was labeled with DIG-dUTP using these conditions.

Specificity of probe. Total nucleic acids were extracted from 1 ml of exponential growth (OD_{600} >= .8) of cultures previously described, grown in .5X MRS, denatured by boiling in 500 ul .4 M NaOH and slot blotted (Bio-Dot SF Microfiltration Apparatus 170-6542, Bio-Rad laboratories, Richmond, CA) onto nylon membranes (Zeta Probe GT membrane 162-0190, Bio-Rad laboratories, Richmond, CA). The membrane was washed with 12.5 ml of 2X SSC (20X SSC = 3M NaCl, .3 M Na-citrate, pH 7.0) and allowed to air dry. The dried membrane was placed into a heat sealable bag with 30 ml of the standard hybridization solution (5X SSC, 1% blocking reagent, .1% Na-lauroylsarcosine, .02% SDS) and incubated at 65 °C for 2 h. An aliquot of the labeled probe (25 ng) was denatured by heating at 95 °C for 10 min and placing in a -70 °C dry ice-ethanol bath for 30 s. Denatured probe was added to fresh hybridization solution and transferred into the sealed bag with the membrane. Hybridization of the probe with homologous sequences was allowed to proceed for 12 h at 65 C. Unbound probe was removed by washing the membrane twice in 2X wash solution (2X SSC, .1% SDS) for 5 min and then twice in .5X wash solution (.5X SSC, .1% SDS) for 15 min at 65 C. Non-specific binding of the anti-DIG antibody was reduced by incubating the membrane in 30 ml Genius buffer 2 on a shaker (150 rpm) for 60 min. Anti-DIG antibody was diluted 1:5000 in Genius buffer 2 and used to replace the blocking buffer by incubating for 30 min. The membrane was transferred to a new heat sealable bag. Unbound anti-DIG antibody was removed by two washes with 100 ml of Genius buffer 1 for 15 min per wash. Alkaline phosphatase

conjugated to the bound antibody was activated by incubation of the membrane in 10 ml Genius buffer 3 (pH 9.5) for 10 min. Forty-five μl of NBT solution (75 mg/ml nitroblue tetrazolium salt in 70% v/v dimethyl formamide, Boehringer Mannheim #1383 213, Indianapolis, IN) and 35 μl X-phosphate (50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate, toluidinium salt in 100% dimethyl formamide, Boehringer Mannheim # 1383 221, Indianapolis, IN) were added to alkaline buffer (Genius buffer 3, 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) and color development allowed to proceed for 30 min in the dark. A blue-black precipitate, formazine, started to form immediately and the color development was sufficient for visualization after 30 to 60 min.

Detection of *P. acidipropionici* DH42 in ensiled, high-moisture corn. Aqueous extracts obtained from ensiled, high-moisture corn samples from the study reported in chapter 6 were stored at -20 °C until extraction of total microbial DNA. The DNA from the microbial community associated with the ensiled, high-moisture corn samples was extracted and slot blotted using methods previously described for the specificity experiment. The color reaction was allowed to proceed for 60 min.

Results and Discussion

Specificity of probe. Using the scaled up procedure outlined in the Genius kit user's manual (Boehringer Mannheim #101 023, , Indianapolis, IN), a total of 25 ng DIG-dUTP labeled probe was obtained. Lonvaud-Funel and coworkers (1991) were able to obtain 300 ng DIG-dUTP labeled probe from *Lactobacillus spp.* used in wine production. *Propionibacterium spp.* are very recalcitrant to lysis by lysozyme as used in the procedure

of Lonvaude-Funel and coworkers (1991). Less than 25 pg DIG-dUTP labeled probe for *P. acidipropionici* DH42 was obtained using their procedure. The freeze thaw method of cellular lysis, as outlined by Tsai and Olson (1991), was effective in increasing yield of DNA extracted from *P. acidipropionici* DH42 to 30 ng when used in combination with heating the lysate for 10 min at 95 °C.

The slot blot of samples for the specificity experiment is shown in Figure 7-1. The probe developed from *P. acidipropionici* DH42 hybridized with DNA extracted from a subculture of itself as expected. *Propionibacterium acidipropionici* 22562 showed the strongest hybridization of the cultures evaluated. Cross-hybridization was not surprising since *P. acidipropionici* 22562 is the type strain for *P. acidipropionici* at ATCC. *Propionibacterium "pentosaceum"* P11 is a strain of *P. acidipropionici* and showed hybridization with the probe but not to the same extent of *P. acidipropionici* 22562. The cultures of *P. acidipropionici* 4965 and *P. freudenreichii* 6207 did not grow well (OD_{600} = .1) in this experiment. The pellets of cells harvested by centrifugation were hardly visible. Therefore, the lack of hybridization of the probe to the DNA extracted from *P. acidipropionici* 4965 and *P. freudenreichii* 6207 was expected. All other cultures grew well (OD_{600} >= .8 after 20 h).

Nucleic acids extracted from *P. shermanii* P92, *P. shermanii* CDC 3094 and *P. freudenreichii* 1382 showed slight color development indicating hybridization less than the hybridization of the probe to *P. acidipropionici* 22562. Since the probe was based on total genomic DNA, cross-hybridization of the two species to the probe developed from *P. acidipropionici* DH42 may not be such an anomaly. These three species, *P.*

acidipropionici, *P. freudenreichii* and *P. shermanii* are common isolates from dairy products and share many physicochemical characteristics based on fermentation profiles (Holdeman et al., 1977). *Propionibacterium jensenii* P25 and *P. thoenii* P15 did not hybridize to the probe from *P. acidipropionici* DH42.

None of the *Lactobacillus spp.* used in this study showed any hybridization under the conditions evaluated. A concern that may arise is the absence of *L. plantarum*, *Pediococcus acidilactici* and *Streptococcus faecium* from the list of microorganisms evaluated. Future studies should look at the possible cross-hybridization with these species since they are commonly used as bioinoculants in ensiled feedstuffs. Lack of hybridization to *P. jensenii* P25, *P. thoenii* P15 and the diverse array of the *Lactobacillus spp.* evaluated may refute the possibility of cross hybridization with the three species commonly used as silage inoculants based on the diversity of the strains evaluated.

Future experiments should address improving the specificity of the probe toward *P. acidipropionici* DH42. Manipulation of the hybridization and washing conditions (higher stringency washes) may alleviate or control some of the species cross-hybridization. Other methods, such as development of probes based on variable regions of the 16S rRNA genes have been quite successful in being species and strain specific to organism such as *Streptococcus bovis* (Odelson et al., 1993). Future work is warranted in development of strain specific probes for silage bioinoculants to address efficacy and persistence of the introduced strains.

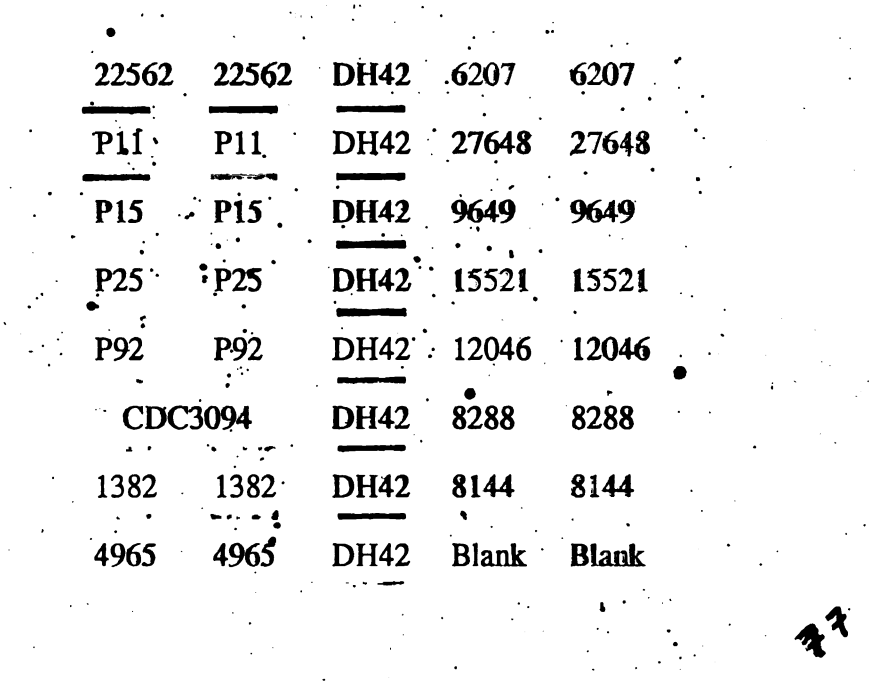

Figure 7-1 Template for the hybridization of a non-radioactive nucleic acid probe developed from *Propionibacterium acidipropionici* DH42 to total nucleic acids extracted from 1 ml of exponential growth of ***P. acidipropionici*** (American Type Culture Collection; ATCC 22562, ATCC 4965), *P. pentosaceum* (Chr. Hansen's Lab; CH P11), *P. thoenii* (CH P15), *P. jensenii* (CH P25), *P. shermanii* (CH P92, CH CDC 3094), *P. freudenreichii* (ATCC 1382, ATCC 6207), *Lactobacillus confusus* (ATCC 27646), *L. delbruckii* (ATCC 9649), *L. sake* (ATCC 15521), *L. helveticus* (ATCC 12046), *L. fructovorans* (ATCC 8288) and *L. bulgaricus* (ATCC 8144).

Detection of *P. acidipropionici* DH42 in ensiled, high-moisture corn. The slot blot corresponding to DNA extracted from the ensiled, high-moisture corn treated with *P. acidipropionici* DH42 or without (control) is shown in Figure 7-2. *Propionibacterium acidipropionici* DH42 was added to achieve a concentration of 10^6 cfu per g DM at the time of ensiling. A low intensity band of color was observed after 60 min color reaction in samples from 0 and 2 d post-ensilement in the *P. acidipropionici* DH42 treated corn indicating hybridization. The slight hybridization was probably due to insufficient amount of probe in the hybridization solution or inadequate time for color development. The color reaction was stopped after 60 min due to the concomitant development of background color development which could have been attributed to insufficient blocking. A culture of *P. acidipropionici* DH42 was diluted and extracted to give equivalents of 10^8, 10^7, 10^6, 10^5 and 10^4 cfu per g DM. The hybridization conditions and color development allowed for detection of approximately 10^6 cfu *P. acidipropionici* DH42 per g DM of ensiled, high-moisture corn.

Nucleic acids extracted from the microbial community associated with the control corn did not show any hybridization at any of the ensiling times. This suggested that the presence of *P. acidipropionici* DH42 or similar organisms was below detection levels using the conditions presented. Samples of corn treated with *P. acidipropionici* DH42 revealed easily noticed color indicating hybridization of the probe after 7, 14, 28 and 42 days of ensiling. The results presented from the fermentation experiment in chapter 6 showed that propionic acid content did not increase until 7 days of ensiling. Hybridization of the probe after 7 days of ensiling supports the observation that the production of

propionic acid in the high-moisture corn inoculated with *P. acidipropionici* DH42 was due to the addition of the organism and not some other factor. If all the propionic acid was present at the time of addition of the organism (d 0), one could possibly conclude that the fermentation was influenced by the media added with the bioinoculant and not the microorganism being added. Intensity of the bands in the slot blot increased from d 7 to d 42 suggesting an increase in the number of *P. acidipropionici* DH42 participating in the fermentation. The increase in growth of *P. acidipropionici* DH42 after 2 days of ensiling suggests that the organism is not a major participant in the early phase of the ensiling fermentation as would be the case with most *Lactobacillus spp.*

Propionic acid content increased after aerobic exposure in the corn treated with *P. acidipropionici* DH42 as reported in chapter 6. Aqueous extracts of the ensiled, high-moisture corn were obtained from samples exposed to air for 3 days after 42 days of anaerobic storage. As seen during the fermentation, the probe developed from *P. acidipropionici* DH42 did not hybridize to any of the control samples (Figure 7-2). However, DNA extracted from corn treated with *P. acidipropionici* DH42 did hybridize with the probe, indicating the presence and possible participation of *P. acidipropionici* during the production of propionic acid seen during the aerobic exposure of the ensiled, high-moisture corn.

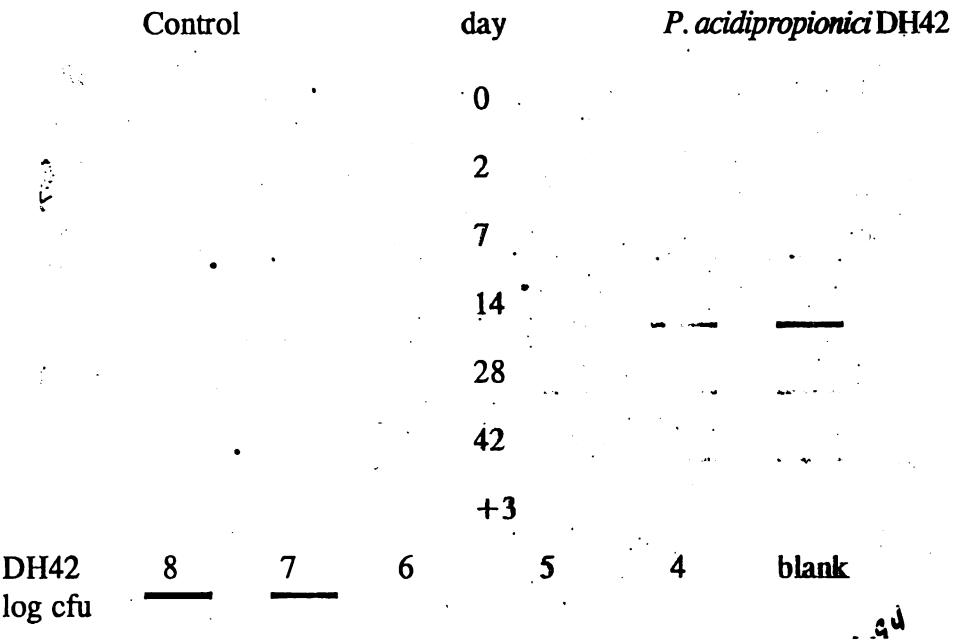

Figure 7-2 Template for the hybridization of a nonradioactive probe developed from *P. acidipropionici* DH42 to total microbial DNA extracted from aqueous extracts of ensiled, high-moisture corn inoculated with 10^6 cfu per g DM of *P. acidipropionici* DH42 or without (control) at day 0 after 0, 2, 7, 14, 28 and 42 days of ensiling and 3 days of aerobic exposure and slot blotted onto a nylon membrane.

Extracts of ensiled, high-moisture corn contain substances which clogged the nylon membranes used for slot blotting. The commercial DNA preparation system (Wizard Miniprep, Promega, Madison, WI) allowed for a rapid extraction and cleaning procedure that yielded DNA free of other contaminants that interfered with blotting. Some of thepropionic acid-producing bacteria evaluated had very noticeable colors. *Propionibacterium thoenii* P15 has a very distinguishing reddish-yellow pigment which interfered with prior steps when trying to visualize the color development associated with the procedure. All DNA preps were clear and amendable to be blotting onto the nylon membranes after the extraction and purification methods described.

Implications

A non-radioactive DNA probe was developed for a strain of *Propionibacterium acidipropionici* (DH42) previously isolated from ensiled, high-moisture corn. The probe detected the presence and proliferation of *Propionibacterium acidipropionici* DH42 when added as a bioinoculant to high-moisture corn. The probe did not hybridize to six strains of *Lactobacillus spp.*, *Propionibacterium thoenii* or *Propionibacterium jensenii*. However, there was limited cross-hybridization with DNA from two related propionic acid-producing bacteria, *Propionibacterium freudenreichii* and *Propionibacterium shermanii*. The use of non-radioactive DNA probes will allow more precise evaluation of the efficacy of silage bioinoculants.

CHAPTER 8

SUMMARY AND CONCLUSIONS

Studies conducted to evaluate selected, propionic acid-producing bacteria as bioinoculants for ensiled, high-moisture corn were successful in manipulating the ensiling fermentation and reducing the extent of aerobic deterioration. Specific aims of this research were to 1) select species of propionic acid-producing bacteria capable of increasing propionic acid content of ensiled, high-moisture corn; 2) study and optimize conditions that affect performance of the selected, propionic acid-producing bacteria; 3) develop nucleic acid probes for the selected, propionic acid-producing bacteria to allow for more precise evaluation of the ecological interactions; and 4) evaluate the use of the selected, propionic acid-producing bacteria as bioinoculants for ensiled, high-moisture corn.

The study reported in Chapter 2 showed that a mixture of *Propionibacterium acidipropionici* ATCC 4965 and *P. freudenreichii* ATCC 6207 was able to increase propionic acid content of ensiled, reconstituted, high-moisture corn to .26 g/100 g DM after 56 d. Increased propionic acid concentration was highly correlated to decreased numbers of yeasts and molds. Because *P. acidipropionici* ATCC 4965 and *P. freudenreichii* ATCC 6207 are isolates from dairy products, progressive enrichment

techniques were developed and used in Chapter 3 to isolate propionic acid-producing bacteria from ensiled feeds. A rapid growing propionic acid-producing bacteria was isolated from ensiled, high-moisture corn and characterized in Chapter 4. The isolated propionic acid-producing bacterium had similar phenotypic characteristics to *P. acidipropionici* and was designated *P. acidipropionici* DH42. *Propionibacterium acidipropionici* DH42 was then evaluated for its competitiveness and growth on substrates commonly found in ensiled, high-moisture corn in Chapter 5. Eight strains of propionic acid-producing bacteria were screened in the competition study. *Propionibacterium acidipropionici* DH42 was the most competitive as seen by higher growth rates and greater propionic acid production in two different aqueous extracts of samples of ensiled, high-moisture corn and in peptone-yeast extract-lactate medium.

Manipulation of the fermentation and aerobic stability of high-moisture corn by *P. acidipropionici* DH42 was evaluated in Chapter 6. Addition of *P. acidipropionici* DH42, as a bacterial inoculant, increased propionic acid concentration to .35 g/100 g DM after 42 days of ensiling and .61 g/100 g DM after five days of aerobic exposure after ensiling. Increased propionic acid was most likely responsible for decreased yeasts and molds during the ensiling fermentation and aerobic exposure. Consequently, dry matter recovery and aerobic stability were improved as evidenced by stable pH and temperature during aerobic exposure. Total DNA extracted from *P. acidipropionici* DH42 was used to generate a non-radioactive nucleic acid probe to monitor persistence of *P. acidipropionici* DH42 when added as a silage bioinoculant by a protocol described in Chapter 7. The non-radioactive, nucleic acid probe detected the presence of *P. acidipropionici* DH42 in extracts of ensiled,

high-moisture corn inoculated with *P. acidipropionici* DH42 prior to ensiling (Chapter 6). Nucleic acids extracted from the microbial community associated with the non-treated corn did not hybridize with the probe. This confirmed those changes in the fermentation and aerobic stability of the treated corn were due to the addition of *P. acidipropionici* DH42.

Limitations of experimental results are inherent to all biological experimentation. The isolation of *P. acidipropionici* DH42 was not meant to be the end to isolation of propionic acid-producing bacteria from ensiled feedstuffs. Further replication of each study would further validate results. It is quite possible that more competitive strains of propionic acid-producing bacteria are present in diverse types of silages. Future experiments need to address efficacy of propionic acid-producing bacteria in farm scale silos. In addition to manipulation of the ensiling fermentation, animal performance and feed acceptability should be evaluated.

Previous experimental results obtained using laboratory scale silos have generally correlated with responses observed in large scale silos. Extrapolation of results obtained in the two small scale silos experiments after d 42 and d 56 may not occur after 100 or more days of ensiling. This point should be verified by future experiments. Care should also be exercised is trying to directly apply results from these studies to fermentation of corn silage, alfalfa haylage or other silages treated with additives.

Positive experimental results with *P. acidipropionici* DH42 and use of non-radioactive nucleic acid probes to monitor population levels will allow further study of ecological interactions of microbial species in silage and rumen microbial ecosystems. The largest daily source of microbial matter to ruminant animals being fed ensiled feedstuffs

is the microbial population associated with the ensiled feeds. Many efforts have been made trying to develop direct-fed microbial feed-additives that may influence the rumen microbial population. An interesting future study could involve following persistence or establishment of *P. acidipropionici* DH42, or possible more competitive propionic acid-producing bacteria, in the rumen of cattle being fed a diet consisting of a large proportion of ensiled, high-moisture corn that was treated with *P. acidipropionici* DH42. Besides possible establishment of *P. acidipropionici* DH42, future studies should look at its influence on feedlot problems such as ruminal lactic acidosis.

Enrichment and isolation conditions detailed in Chapter 3 were successful in the isolation of propionic acid-producing bacteria from ensiled crops. However, in view of recent findings on the use of erythritol for selective growth of propionic acid-producing bacteria, the author recommends evaluation of different minimal media based on erythritol and selected antibiotics for the isolation of new propionic acid-producing bacteria from ensiled feeds. Sequential enrichment schemes discussed in Chapter 3 will still prove helpful if used in a controlled manner. Limiting the source of ensiled feeds to those that contain high levels of propionic acid may be an alternative to decreasing the number of enrichments to be made. However, this may also limit the likelihood of isolating a diverse array of propionic acid-producing bacteria that may not be dominating the crop due to unknown factors.

Use of the non-radioactive nucleic acid probe used in Chapter 7 in future silage fermentation studies should prove quite useful. Depending on what aspects of the fermentation need to be addressed, the nucleic acid probe can be used with few

modifications. Cross-hybridization with different strains of *Propionibacterium spp.* should be addressed in future studies. Use of higher stringency washes for removal of unbound nucleic acids or use of selected sequences of DNA may help address the cross-hybridization of related species. In addition, a larger array of common silage microorganisms should be evaluated for similar cross-hybridization.

The overall hypothesis for this research was that the addition of selected, propionic acid-producing bacteria as bioinoculants to ensiled, high-moisture corn would increase production of propionic acid during the ensiling fermentation and, consequently, decrease the extent of aerobic deterioration and instability by controlling the growth of the microorganisms responsible for the aerobic deterioration process. Addition of *P. acidipropionici* DH42 was capable of increasing propionic acid in ensiled, high-moisture corn sufficiently to increase aerobic stability. In addition, the persistence of *P. acidipropionici* DH42 was confirmed with a non-radioactive nucleic acid probe.

APPENDICES

APPENDIX A

DIVERSITY OF THE MICROBIAL COMMUNITY ASSOCIATED WITH AEROBICALLY-EXPOSED CORN SILAGE INOCULATED WITH *PROPIONIBACTERIUM ACIDIPROPIONICI DH42*

Abstract

The concentration of lactic acid in ensiled feedstuffs is generally sufficient to exclude the growth of detrimental microorganisms during anaerobic storage. Once the ensiled biomass is exposed to air, lactic acid is quickly metabolized resulting in aerobic deterioration. Propionic acid has been shown to be effective in inhibiting the growth of microorganisms responsible for the aerobic deterioration of silages. An experiment was conducted to evaluate the effects of a propionic acid-producing bacteria on the diversity of the microbial community associated with aerobically exposed corn silage. Treatments included sterile water (control), PAPB-24 (10^6 cfu•g^{-1}•DM^{-1} after 24 h growth of *Propionibacterium acidipropionici* DH42), PAPB-72 (10^6 cfu•g^{-1}•DM^{-1} after 72 h growth of *P. acidipropionici* DH42) and C3 (1% propionic acid added) were applied to 50 g of fresh chopped whole plant corn (28.6% DM) and stored in 75 ml test tubes with gas release valves (10 tubes per treatment). Five tubes were opened on d-25 and d-170 for each treatment. Aerobic bacteria, yeast and molds, lactic acid-utilizing bacteria, and lactic

acid-producing bacteria were enumerated at opening and on 7 and 14 d after opening. During ensiling, weight loss was minimized ($P < .01$) during the initial 25 d by C3. All treatments had similar ($P > .10$) weight loss and temperature changes when exposed to air after 25 d storage and similar pH values (3.88 $\pm$ .07). Yeasts and molds were decreased ($P < .01$) in PAPB-72 ($10^{5.1}$ cfu/g) and C3 ($10^{3.8}$ cfu/g) when compared with control ($10^{5.7}$ cfu/g) after 25 d of storage. The diversity of the microbial community as measured by the proportion of substrates metabolized by the mixed population of the BIOLOG-GN plate decreased ($P < .05$) in C3 and PAPB-72 (55%) compared with control (74%) and the epiphytic population (79%) after 25 d of ensiling. Addition of *P. acidipropionici* DH42 and propionic acid decreased the diversity of substrates metabolized by the microbial community after ensiling.

Introduction

Previous strategies for efficient silage fermentation encouraged high levels of lactic acid production and lower pH development (below 4.2) during the fermentation phase. This type of fermentation generally produces silage which is stable under anaerobic storage conditions. However, once the ensiled mass is exposed to oxygen, large amounts of nutrients can be metabolized or destroyed. Resulting heat and mold formation is generally defined as aerobic instability or poor bunk life. Propionic acid is a very effective mycostatic (mold inhibiting) and bacteriostatic (bacteria inhibiting) agent which has been shown to be beneficial in decreasing the losses associated with aerobic instability of ensiled feeds (Voelker et al., 1989). Propionic acid is more inhibitory than lactic acid to the

organisms generally considered to be initiators of silage spoilage. Objectives of this study were to evaluate the effects of *P. acidipropionici* DH42 on the microbial diversity of corn silage and the use of the BIOLOG microplate system for assessing changes in microbial communities associated with ensiled feeds.

Experimental Procedures

Four treatments, control (sterile water), PAPB-24 (24 h growth of *P. acidipropionici* DH42 grown in peptone-yeast extract-lactate) , PAPB-72 (72 h growth of *P. acidipropionici* DH42) and C3 (1% propionic acid added) were applied to 50 g chopped whole plant corn (28.6% DM). The material was ensiled in 75 ml test tubes with a gas release valve. Five tubes per treatment were stored for 25 and 170 d. Microbial enumeration was performed utilizing pour plates of LBS (Difco, Detroit, MI) for lactic acid-producing bacteria, Rose Bengal agar supplemented with chloramphenicol (Difco, Detroit, MI) for yeasts and molds, tryptic soy agar (Difco, Detroit, MI) for total aerobic bacteria and peptone-yeast extract-lactate agar for lactic acid-utilizing microbes (Holdeman et al., 1977). Volatile fatty acids and lactic acid were quantified by ion exchange HPLC (Bio-Rad aminex HPX-87H, Richmond, CA) using refractive index detection as described in previous chapters (Canale et al., 1984).

Community diversity was assessed by a BIOLOG-GN (96 well microplate, Biolog Systems, Hayward, CA) using a procedure modified from Drasar and Roberts (1991) and Garland and Mills (1991) with modifications. Ten g of a homogenous sample of silage was removed from each experimental silo and homogenized in 90 ml of sterile .9% NaCl

in a Stomacher (Tekmar 3500, Cincinnati, OH) for 5 min. The homogenate was centrifuged (5,000 x *g* for 5 min) to remove plant debris. Supernatant was removed and the mixed microbial community was harvested by centrifugation (25,000 x *g* for 10 min). The mixed microbial community was resuspended in 30 ml sterile .9% NaCl to an OD_{600} of approximately .5. The suspension was vortexed and 150 μl was pipetted into each well of a BIOLOG-GN microplate. Microplates were incubated for 24 h at 30 °C and color reactions in each well were visually observed and recorded. BIOLOG-GN microplates contain ninety-five different carbon substrates with an indicator dye and a blank control in each well of a standard ninety-six well microplate. A metabolic "fingerprint" is generated by the different substrates which are utilized by either a pure culture of bacteria or a mixed microbial community

Results and Discussion

Weight loss of the corn silage (Table A-1) was slightly reduced by C3 and PAPB-72 after 25 d ensiling. The variation in the small scale silos was such that no treatment differences were observed after 170 d ensiling. In addition, the silos used in this experiment did not provide good ensiling conditions after 60 days of ensiling as evidenced by mold proliferation. Fermentation characteristics after 170 d ensiling (Table A-2) indicated similar pH and concentrations of lactic acid and ethanol. Propionic acid was greatest ($P < .05$) in C3 and slightly higher in PAPB-24 and PAPB-72. Acetic acid was lowest in C3. Microbial characteristics after 25 d ensiling (Table A-3) indicated lower numbers of yeasts and molds in C3 and PAPB-72 ($P < .05$). In addition there were

higher numbers of lactic acid-producing bacteria and lactic acid-utilizing bacteria in control silages than the three treated silages. All microbial characteristics were similar after 170 d ensiling (Table A-4). The microbial communities were different as evaluated by the BIOLOG-GN microplates after 25 d ensiling (Table A-5). As seen with silage weight loss, variation in the small silos after 170 d ensiling was sufficiently great to negate possible treatment effects.

Table A-1 Weight loss (% of DM) of corn silage inoculated with *P. acidipropionici* DH42 after 24 h or 72 h growth or treated with 1% propionic acid after 25 or 170 d ensilement

Treatment	Period of Ensiling (d)	
	25	170
Control	6.36[ab]	19.37
P. acidipropionici DH42 (24 h growth)	7.26[a]	21.29
P. acidipropionici DH42 (72 h growth)	6.33[ab]	19.27
Propionic acid (1%)	4.55[b]	23.36
SEM	0.56	3.53

[ab] Means within a column lacking a common superscript differ ($P < .05$).

Table A-2. Fermentation characteristics of corn silage inoculated with *P. acidipropionici* DH42 after 24 or 72 h growth or treated with 1% propionic acid after 170 d ensilement

Fermentation characteristic	Treatment				SEM
	Control	PAPB-24	PAPB-72	C3	
Lactic acid (% DM)	5.85	3.7	4.27	5.67	1.44
Acetic acid (% DM)	.66[a]	.53[ab]	.56[ab]	.33[b]	0.06
Propionic acid (% DM)	.07[a]	.23[bc]	.14[ab]	.95[c]	0.04
Ethanol (% DM)	1.29	1.25	1.66	0.27	0.47
pH	3.59	4.09	3.8	3.44	0.28

[abc] Means within a row lacking a common superscript differ ($P < .05$).

Table A-3. Microbial characteristics of corn silage inoculated with *P. acidipropionici* DH42 after 24 h or 72 h growth or treated with 1% propionic acid after 25 d ensiling

Treatment	Lactic acid-producing bacteria	Yeasts and Molds	Total aerobic bacteria	Lactic acid-utilizing bacteria
	log cfu•g^{-1}•DM^{-1}			
Control	6.87[a]	4.90[a]	6.03	6.56[a]
PAPB-24	6.50[b]	4.58[ab]	5.68	6.43[b]
PAPB-72	6.72[a]	4.31[b]	5.67	6.49[ab]
C3	6.18[c]	3.00[c]	5.55	6.34[c]
SEM	0.05	0.17	0.14	0.03

[abcd] Means within a column lacking a common superscript differ ($P < .05$).

Table A-4. Microbial characteristics of corn silage inoculated with *P. acidipropionici* DH42 after 24 h or 72 h growth or treated with 1% propionic acid after 170 d ensiling

Treatment	Lactic acid-producing bacteria	Yeasts and molds	Total aerobic bacteria	Lactic acid-utilizing bacteria
	log cfu•g^{-1}•DM^{-1}			
Control	4.58	4.35	6.02	5.91
PAPB-24	4.82	3.35	5.25	5.41
PAPB-72	5.77	4.04	6.02	6.04
C3	5.87	3.87	5.51	5.86
SEM	.51	.62	.51	.53

Table A-5. Percent of substrates in the BIOLOG-GN plate utilized by the mixed microbial community of corn silage inoculated with a *P. acidipropionici* DH42 or treated with propionic acid

Treatment	Ensiling Period (d)	
	25	170
	substrates used (%)	
Control	77[a]	68
P. acidipropionici DH42 (24 h)	71[a]	52
P. acidipropionici DH42 (72 h)	55[b]	76
Propionic acid (1%)	55[b]	17
SEM	5	24

[ab] Means within a column lacking a common superscript differ ($P < .05$).

Summary

The treatments PAPB-72 (72 h growth of *P. acidipropionici* DH42) and C3 (1% propionic acid) decreased yeast and mold number in corn silage after 25 d of ensiling. In addition, PAPB-72 and C3 decreased the diversity of the microbial community as measured by the diversity of substrates metabolized by the total microbial community.

Implications

Propionibacterium acidipropionici DH42 is capable of decreasing the diversity of the microbial community in corn silage ensiled in laboratory scale silos. In addition, BIOLOG microplates allow rapid assessment of changes in silage microbial communities.

APPENDIX B

SUPPORTING SMALL AND LARGE SCALE STUDIES EVALUATING *PROPIONIBACTERIUM ACIDIPROPIONICI* DH42 AS A BIOINOCULANT FOR ENSILED HIGH-MOISTURE CORN

A pilot study was conducted using high-moisture corn from the Michigan State University Dairy Research and Teaching Center. Two PVC lab-scale silos were filled for the control and *P. acidipropionici* DH42 treatments with 1.5 kg corn (73 %DM) obtained from the bunker silos on March 3, 1993. This pilot study was conducted to evaluate the effect of *P. acidipropionici* DH42 on the fermentation of corn harvested and placed into bunker silos in the winter (January). The corn was harvested late in the season because of immaturity, low test weight and high-moisture content the previous fall. Analysis of aqueous extracts was conducted as in previous chapters. High-moisture corn ensiled with *P. acidipropionici* DH42 had greater residual glucose, less lactic acid, greater acetic acid, ethanol and propionic acid that did the control non-treated corn (Table B-1).

A large scale study was performed using high-moisture corn (75% DM) inoculated with *P. acidipropionici* DH42. Two truck loads of high-moisture corn were purchased from the Mason Elevator Company (Mason, MI) and delivered to the Michigan State University Beef Cattle Research and Teaching Center. *Propionibacterium acidipropionici*

DH42 was applied to 35,900 kg corn and stored in silo 3, whereas water was added as a control to 38,200 kg and stored in silo 4. This high-moisture corn will be used in a feeding study to evaluate feed acceptability and animal performance. Initial results indicated less surface losses during ensiling. However, variation existed in aerobic stability of the ensiled, high-moisture corn. Cattle were consuming both control and treated corn at similar amounts indicating acceptable feed quality. Problems in obtaining the corn, freesing temperatures at during silo filling and partial filling of the silos possibly lead to the variation within the silos. Future experiments should be performed using uniform corn in numerous types of silos.

Table B-1. Fermentation characteristics of high-moisture corn ensiled with *P. acidipropionici* DH42 (10^6 cfu•g^{-1}•DM^{-1}) for 180 d

Fermentation characteristic	Treatment	
	Control	*P. acidipropionici* DH42
Glucose (% DM)	.14	.19
Acetic acid (% DM)	.26	.46
Ethanol (% DM)	.10	.18
Lactic acid (% DM)	.68	.43
Propionic acid (% DM)	.02	.32

Values are averages of two replicate per treatment

APPENDIX C

DATA TABLES

Table C-1. Data used for analysis in Chapter 2

DAY	TRT	REP	LAB	LAU	YM	PH	PRO	ACE
0	CON	A	5.11	5.88	ND	5.50	.000	.037
0	CON	B	5.08	5.59	ND	5.15	.000	.170
0	PAPB	A	4.76	8.84	ND	5.71	.000	.112
0	PAPB	B	8.08	8.40	ND	5.69	.000	.146
0	LAPB	A	8.08	8.40	ND	5.69	.000	.134
0	LAPB	B	8.13	8.32	ND	5.69	.000	.155
0	PA+LA	A	7.74	8.36	ND	5.51	.000	.037
0	PA+LA	B	7.87	8.62	ND	5.52	.000	.077
2	CON	A	7.67	8.10	ND	4.94	.000	.238
2	CON	B	8.21	8.55	ND	4.72	.000	.121
2	PAPB	A	8.63	8.73	ND	4.92	.171	.277
2	PAPB	B	8.44	8.48	ND	5.00	.080	.121
2	LAPB	A	9.34	9.12	ND	3.86	.000	.102
2	LAPB	B	9.34	9.16	ND	3.94	.000	.096
2	PA+LA	A	9.60	9.48	ND	4.04	.046	.164
2	PA+LA	B	9.36	9.29	ND	3.97	.056	.148
7	CON	A	8.93	8.94	ND	4.85	.000	.145
7	CON	B	9.07	8.93	ND	4.82	.000	.130
7	PAPB	A	9.02	8.91	ND	4.34	.227	.154
7	PAPB	B	9.02	9.02	ND	4.37	.225	.237
7	LAPB	A	9.43	9.34	ND	3.86	.000	.112
7	LAPB	B	9.33	9.25	ND	3.80	.000	.102
7	PA+LA	A	9.21	9.17	ND	3.94	.053	.179
7	PA+LA	B	9.33	9.22	ND	3.96	.047	.165

Table C-1. (cont.)

DAY	TRT	REP	LAB	LAU	YM	PH	PRO	ACE
14	CON	A	9.34	9.24	6.58	4.49	.000	.142
14	CON	B	8.92	9.04	6.52	4.65	.000	.118
14	PAPB	A	8.57	8.96	4.46	4.32	.238	.327
14	PAPB	B	8.56	9.02	5.15	4.30	.191	.182
14	LAPB	A	8.90	8.84	6.02	3.90	.000	.103
14	LAPB	B	8.69	8.83	6.27	3.89	.000	.099
14	PA+LA	A	8.60	8.89	6.65	3.99	.037	.152
14	PA+LA	B	8.51	8.63	6.25	3.99	.053	.163
28	CON	A	9.01	8.87	6.35	4.47	.000	.108
28	CON	B	8.90	8.84	6.39	4.44	.000	.110
28	PAPB	A	8.62	8.64	5.64	4.27	.184	.197
28	PAPB	B	8.63	8.52	5.34	4.29	.104	.115
28	LAPB	A	8.22	8.29	6.20	3.93	.000	.115
28	LAPB	B	8.13	8.05	6.49	3.93	.000	.102
28	PA+LA	A	8.30	8.15	6.05	3.92	.060	.187
28	PA+LA	B	8.36	8.33	6.26	3.91	.037	.112
56	CON	A	8.80	8.76	6.51	4.33	.000	.145
56	CON	B	8.77	8.72	5.50	4.28	.000	.155
56	PAPB	A	8.59	8.81	5.30	4.25	.294	.299
56	PAPB	B	8.62	8.64	5.43	4.23	.229	.150
56	LAPB	A	7.14	6.97	6.29	4.02	.000	.186
56	LAPB	B	7.23	7.20	6.07	4.02	.000	.109
56	PA+LA	A	7.32	7.68	5.96	3.97	.068	.207
56	PA+LA	B	7.46	7.78	6.19	4.01	.065	.189

Abbreviations:Day: Ensiling period (d); TRT: Treatment; CON = Control, LAPB = Lactic acid-producing bacteria, PAPB = Propionic acid-producing bacteria, PA+LA = PAPB+LAPB REP: replicate; LAB: lactic acid-producing bacteria (log cfu•g^{-1}•DM^{-1}), LAU: lactic acid-utilizing bacteria (log cfu•g^{-1}•DM^{-1}); YM: yeasts and molds (log cfu•g^{-1}•DM^{-1}); PH = pH of aqueous extract; PRO = propionic acid (g/100 g DM); ACE = acetic acid (g/100 g DM)

Table C-2. Analysis of variance of the effects of propionic acid-producing bacteria with or without lactic acid-producing bacteria and period of ensiling on results reported in Chapter 2

Characteristic	Source	df	Sum of Squares	*P*
pH of aqueous extract	Trt	3	2.46	.0001
	Time	5	11.41	.0001
	Trt*Time	15	1.86	.0001
	Error	24	.189	
Lactic acid-producing bacteria	Trt	3	2.16	.0001
	Time	5	39.60	.0001
	Trt*Time	15	25.72	.0001
	Error	24	.39	
Lactic acid-utilizing bacteria	Trt	3	1.55	.0001
	Time	5	10.31	.0001
	Trt*Time	15	16.71	.0001
	Error	24	.41	
Yeasts and molds	Trt	3	4.83	.0001
	Time	2	.14	.4760
	Trt*Time	6	.91	.1904
	Error	12	1.03	
Acetic acid	Trt	3	.037	.0110
	Time	5	.025	.1346
	Trt*Time	15	.031	.7021
	Error	24	.065	
Propionic acid	Trt	3	.211	.0001
	Time	5	.033	.0001
	Trt*Time	15	.061	.0001
	Error	24	.011	

Table C-3. Data used in the analysis presented in Chapter 6 fermentation study

TRT	DAY	REP	PH	GLC	LAC	ACE	PRO	ETH	LAB	YM	COL	DM REC	OM REC
C	0	A	5.5	0.38	0.00	0.00	0.00	0.00	3.5	3.2	6.9	ND	ND
C	0	B	5.3	0.44	0.00	0.00	0.00	0.00	3.3	3.9	6.9	ND	ND
C	0	C	5.4	0.20	0.00	0.00	0.00	0.00	ND	ND	ND	ND	ND
D	0	A	5.3	0.19	0.00	0.00	0.00	0.00	3.5	4	6.8	ND	ND
D	0	B	5.5	0.33	0.00	0.00	0.00	0.00	3.5	3.8	6.5	ND	ND
D	0	C	5.5	0.35	0.00	0.00	0.00	0.00	ND	ND	ND	ND	ND
C	2	A	5.3	0.01	0.28	0.11	0.00	0.59	6.2	5.2	7.1	ND	ND
C	2	B	5.2	0.02	0.55	0.24	0.00	1.13	6.3	5.2	7	ND	ND
C	2	C	5.3	0.00	0.54	0.20	0.00	0.82	6.7	5.4	7	ND	ND
D	2	A	5.2	0.05	0.49	0.18	0.00	0.92	6.2	5	6.9	ND	ND
D	2	B	5.3	0.00	0.53	0.38	0.00	0.84	6.3	5.4	7.1	ND	ND
D	2	C	5.4	0.00	0.54	0.23	0.00	0.77	6.6	5.8	7	ND	ND
C	7	A	4.8	0.00	0.42	0.15	0.00	0.96	8.4	4.9	6.4	ND	ND
C	7	B	4.8	0.00	0.72	0.20	0.00	1.63	8.5	4.6	6.4	ND	ND
C	7	C	4.8	0.04	0.91	0.19	0.00	1.74	8.4	3.8	6.5	ND	ND
D	7	A	4.8	0.00	0.70	0.26	0.03	1.49	8.4	4.7	6.4	ND	ND
D	7	B	4.8	0.00	0.74	0.33	0.00	1.68	8.3	4.7	6.4	ND	ND
D	7	C	4.8	0.00	0.77	0.35	0.03	1.70	8.3	4.7	6.6	ND	ND
C	14	A	4.6	0.00	0.88	0.18	0.00	1.43	8.6	6.1	6.4	ND	ND
C	14	B	4.5	0.00	1.80	0.40	0.00	3.12	8.5	6.3	6.3	ND	ND
C	14	C	4.5	0.02	1.09	0.28	0.00	2.07	8.6	6.3	6.4	ND	ND
D	14	A	4.5	0.00	0.99	0.48	0.13	1.64	8.4	6.6	5.9	ND	ND
D	14	B	4.5	0.01	1.12	0.29	0.14	2.08	8.5	5.8	6	ND	ND
D	14	C	4.5	0.01	0.56	0.25	0.08	1.09	8.5	6.4	6	ND	ND

Table C-3. (cont.)

TRT	DAY	REP	PH	GLC	LAC	ACE	PRO	ETH	LAB	YM	COL	DM REC	OM REC
C	28	A	4.3	0.02	0.68	0.14	0.00	1.17	8.4	5.2	6.3	ND	ND
C	28	B	4.3	0.01	0.59	0.08	0.00	0.50	8.5	5.7	6.3	ND	ND
C	28	C	4.3	0.02	0.66	0.20	0.00	1.23	8.4	5.1	6.3	ND	ND
D	28	A	4.3	0.02	0.31	0.12	0.08	0.64	8.5	4.4	6.2	ND	ND
D	28	B	4.2	0.04	0.59	0.19	0.14	0.87	8.5	4.6	6.1	ND	ND
D	28	C	4.2	0.02	0.40	0.14	0.10	0.63	8.4	5.4	6	ND	ND
C	42	A	4.3	0.00	0.57	0.57	0.04	3.05	8.4	4.9	8.2	95.8	99.3
C	42	B	4.3	0.00	1.20	0.22	0.06	1.29	8.5	4.9	8.1	98.0	99.4
C	42	C	4.3	0.00	0.41	0.09	0.00	0.67	8.5	5	7.9	96.7	99.2
D	42	A	4.2	0.05	1.00	0.42	0.29	1.21	8.2	4.6	7.5	98.7	99.3
D	42	B	4.2	0.08	0.98	0.47	0.36	1.31	8.2	3.9	7.6	98.7	99.5
D	42	C	4.2	0.00	0.28	1.01	0.39	0.87	8.3	4	7.5	98.8	100.1

Abbreviations:

TRT : C = control ; D = *P. acidipropionici DH42* DAY: Ensiling period (d)
REP: Replicate per treatment by ensiling period combination
PH: pH of aqueous extract GLC: Glucose content (g /100 g DM)
LAC: Lactic acid (g/100 g DM) ACE: Acetic acid (g/100 g DM)
ETH: Ethanol (g/100 g DM)
LAB: Lactic acid-producing bacteria (log $cfu \cdot g^{-1} \cdot DM^{-1}$)
YM: Yeast and molds (log $cfu \cdot g^{-1} \cdot DM^{-1}$) COL: Coliforms (log $cfu \cdot g^{-1} \cdot DM^{-1}$)
DMREC: Dry matter recovery (g/100 g DM)
OMREC: Organic matter recovery (g/100 g DM)
ND: Not determined

Table C-4. Analysis of variance of the effects of *P. acidipropionici* DH42 and period of ensiling on results reported in Chapter 6

Characteristic	Source	df	Sum of Squares	*P*
pH of aqueous extract	Trt	1	.034	.0040
	Time	5	7.517	.0001
	Trt*Time	5	.062	.0131
	Error	24	.081	
Glucose	Trt	1	.00002	.9185
	Time	5	.457	.0001
	Trt*Time	5	.007	.6728
	Error	24	.052	
Lactic acid	Trt	1	.047	.4011
	Time	5	3.780	.0001
	Trt*Time	5	.235	.6035
	Error	24	1.535	
Acetic acid	Trt	1	.095	.0326
	Time	5	.730	.0002
	Trt*Time	5	.120	.2967
	Error	24	.444	
Propionic acid	Trt	1	.077	.0001
	Time	5	.157	.0001
	Trt*Time	5	.109	.0001
	Error	24	.012	
Ethanol	Trt	1	.372	.2347
	Time	5	13.613	.0001
	Trt*Time	5	.761	.6944
	Error	24	6.003	

Table C-4. (cont.)

Characteristic	Source	df	Sum of Squares	*P*
Lactic acid-producing bacteria	Trt	1	.040	.1014
	Time	5	94.160	.0001
	Trt*Time	5	.099	.2490
	Error	22	.303	
Yeasts and molds	Trt	1	.118	.3320
	Time	5	18.789	.0001
	Trt*Time	5	1.428	.0711
	Error	22	2.631	
Coliforms	Trt	1	.448	.0001
	Time	5	11.211	.0001
	Trt*Time	5	.336	.0001
	Error	22		
Dry matter recovery	Trt	1	5.549	.0403
	Error	4	2.481	
Organic matter recovery	Trt	1	.150	.2470
	Error	4	.328	

Table C-5. Data used in Chapter 6 aerobic stability study

TRT	DAY	REP	PH	TMP	YM	TSA	EYA	GLC	LAC	ACE	PRO	ETH	DMR	OMR
C	0	A	4.3	23.3	4.9	8.5	4.9	0.00	0.57	0.57	0.04	3.05	ND	ND
C	0	B	4.3	24.4	4.9	8.2	5.0	0.00	1.20	0.22	0.06	1.29	ND	ND
C	0	C	4.3	23.9	5.0	8.3	5.1	0.00	0.41	0.09	0.00	0.67	ND	ND
D	0	A	4.2	22.8	4.6	8.2	4.0	0.05	1.00	0.42	0.29	1.21	ND	ND
D	0	B	4.2	24.4	3.9	8.1	4.0	0.08	0.98	0.47	0.36	1.31	ND	ND
D	0	C	4.2	21.7	4.0	8.1	4.0	0.00	0.28	1.01	0.39	0.87	ND	ND
C	1	A	4.4	21.7	5.5	7.9	5.1	0.03	1.04	0.48	0.06	0.75	101.4	98.5
C	1	B	4.3	22.2	5.3	8.0	5.0	0.08	1.54	0.50	0.07	1.87	100.6	98.3
C	1	C	4.3	21.1	4.9	8.1	4.9	0.05	1.35	0.48	0.00	1.69	99.6	99.3
D	1	A	4.3	21.1	4.7	8.1	3.9	0.09	1.12	0.60	0.38	1.22	99.8	99.1
D	1	B	4.2	20.6	3.5	8.0	4.0	0.05	0.94	0.53	0.37	1.12	99.3	98.9
D	1	C	4.2	22.8	3.8	7.9	3.9	0.10	1.53	0.83	0.56	1.93	98.2	99.2
C	2	A	ND	21.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C	2	B	ND	22.8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C	2	C	ND	20.6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D	2	A	ND	21.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D	2	B	ND	20.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D	2	C	ND	22.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C	3	A	4.8	31.1	7.2	8.3	7.1	0.16	0.85	0.10	0.06	0.00	101.2	98.3
C	3	B	4.7	35.0	6.4	8.1	7.1	0.05	1.37	0.00	0.08	0.00	98.7	98.4
C	3	C	4.5	25.6	7.2	8.1	6.6	0.08	1.92	0.26	0.07	0.06	102.7	98.4
D	3	A	4.2	21.1	4.4	7.6	4.0	0.06	1.03	0.56	0.37	0.85	100.0	98.4
D	3	B	4.3	18.9	5.0	7.7	4.8	0.04	0.69	0.41	0.28	0.61	100.4	98.9
D	3	C	4.2	21.7	4.6	7.6	4.3	0.00	0.00	0.00	0.17	0.00	101.2	98.3

Table C-3. (cont.)

TRT	DAY	REP	PH	TMP	YM	TSA	EYA	GLC	LAC	ACE	PRO	ETH	DMR	OMR
C	4	A	ND	26.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C	4	B	ND	28.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C	4	C	ND	27.8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D	4	A	ND	21.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D	4	B	ND	18.9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D	4	C	ND	21.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C	5	A	6.7	23.9	7.7	8.7	7.9	0.00	0.30	0.46	0.00	0.00	97.8	98.0
C	5	B	6.6	24.4	7.9	8.6	8.0	0.07	0.70	0.02	0.00	0.15	98.7	97.3
C	5	C	6.5	22.8	7.9	8.4	7.9	0.06	0.49	0.02	0.00	0.00	97.50	98.23
D	5	A	4.5	26.7	5.5	8.1	5.8	0.04	1.61	0.10	0.59	0.00	97.1	98.1
D	5	B	4.3	20.0	4.6	8.0	4.4	0.13	1.62	0.97	0.62	0.90	100.7	98.8
D	5	C	4.3	26.7	5.4	8.0	5.4	0.11	1.71	0.36	0.61	0.00	101.0	98.9
C	6	A	ND	22.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C	6	B	ND	21.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C	6	C	ND	21.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D	6	A	ND	21.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D	6	B	ND	18.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D	6	C	ND	21.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Abbreviations

TRT : C = control ; D = *P. acidipropionici DH42* DAY: Aerobic exposure period (d)

REP: Replicate per treatment by aerobic exposure period combination

TMP: Temperature of biomass

PH: pH of aquoeus extract GLC: Glucose content (g /100 g DM)

LAC: Lactic acid (g/100 g DM) ACE: Acetic acid (g/100 g DM)

ETH: Ethanol (g/100 g DM) TSA: Total aerobic bacteria (log $cfu \cdot g^{-1} \cdot DM^{-1}$)

YM: Yeast and molds (log $cfu \cdot g^{-1} \cdot DM^{-1}$) COL: Acetobacter (log $cfu \cdot g^{-1} \cdot DM^{-1}$)

DMR: dry matter recovery (g/100 g DM) OMR: organic matter recovery (g/100 g DM)

ND Not determined

Table C-6. Analysis of variance of the effects of *P. acidipropionici* DH42 and period of aerobic exposure results reported in Chapter 6

Characteristic	Source	df	Sum of Squares	*P*
pH of aqueous extract	Trt	1	3.263	.0001
	Time	3	6.379	.0001
	Trt*Time	3	4.546	.0001
	Error	16	.078	
Glucose	Trt	1	.001	.3406
	Time	3	.009	.1179
	Trt*Time	3	.011	.0741
	Error	16	.022	
Lactic acid	Trt	1	.027	.6651
	Time	3	.834	.1529
	Trt*Time	1	2.943	.0030
	Error	16	2.210	
Acetic acid	Trt	1	.388	.0317
	Time	3	.423	.1531
	Trt*Time	3	.032	.9265
	Error	16	1.121	
Propionic acid	Trt	1	.862	.0001
	Time	3	.063	.0064
	Trt*Time	3	.132	.0002
	Error	16	.056	
Ethanol	Trt	1	.010	.8647
	Time	3	8.686	.0011
	Trt*Time	3	.853	.4753
	Error	16	5.210	

Table C-6. (cont.)

Characteristic	Source	df	Sum of Squares	*P*
Yeasts and molds	Trt	1	18.027	.0001
	Time	3	16.210	.0001
	Trt*Time	3	3.510	.0019
	Error	16	2.387	
Total aerobic bacteria	Trt	1	.602	.0001
	Time	3	.645	.0001
	Trt*Time	3	.312	.0009
	Error	16	.180	
Acetobacter spp.	Trt	1	13.368	0.0001
	Time	3	20.350	0.0001
	Trt*Time	3	3.945	0.0001
	Error	16	1.587	
Temperature	Trt	1	44.847	.0002
	Time	6	116.61	.0001
	Trt*Time	6	139.71	.0001
	Error	28	66.107	
Dry matter recovery	Trt	1	3.059	.0255
	Time	2	1.268	.2967
	Trt*Time	2	1.062	.3557
	Error	12	5.646	
Organic matter recovery	Trt	1	1.171	.0117
	Time	2	93.981	.0001
	Trt*Time	2	.342	.3115
	Error	4	1.594	

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