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PROPIONIBACTERIA AS INOCULANTS TO HIGH MOISTURE CORN

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PROPIONIBACTERIA AS INOCULANTS TO HIGH MOISTURE CORN

By

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ABSTRACT

PROPIONIBACTERIA AS INOCULANTS TO HIGH MOISTURE CORN

By

Ruby V. Bato

Two studies were conducted to evaluate the efficacy of propionibacteria as silage inoculants. In the first study, the performance of propionibacteria with or without lactic acid bacteria were evaluated. Results showed that propionibacteria enhanced the fermentation of reconstituted corn only up d 21 of ensiling. The combination of *P. acidipropionici* DH42 with lactic acid bacteria as inoculants reduced silage pH and butyric acid and increased propionic, acetic and lactic acids. LAB inoculation did not significantly increase the LAB population in the treated silages. During aerobic exposure, all the silages appeared well-preserved. Organic acid levels remained stable throughout the exposure period. Propionibacteria inoculation did not significantly reduce the yeast and mold population. However, the silages with *P. acidipropionici* DH42 + LAB had higher propionic, acetic and lactic acids and lower pH.

In the second study, the effect of moisture on the efficacy of propionibacteria as silage inoculants was tested. Rolled corn of moisture contents ranging from 22-35% were used. The 22-28% moisture levels appeared to favor the growth of the *P. acidipropionici* DH42 in silage. After 120 d of ensiling, PAB-inoculated high moisture corn gave higher propionic and acetic acids and lower pH and butyric acid at 22-28%. *P. acidipropionici* DH42 inoculated at 10^6cfu/g better

gave results as compared to the 10^5cfu/g. During aerobic exposure, higher propionic and acetic acids and lower pH were observed with the PAB-treated silages from the 22-28% moisture levels. Propionibacteria inoculation did not significantly reduce yeast and mold counts.

The vitamin B_{12} production capability of *P. acidipropionici* DH42 was also evaluated in comparison with *P. shermanii*. Results showed comparable vitamin B_{12} production of the two propionibacteria strains. After 72 h of incubation, the *P. acidipropionici* DH42 and *P. shermanii* cultures grown at 30°C had vitamin B_{12} contents of 852.85 and 840.69 ng/ml, respectively. Both strains grew better at 30°C than at 40°C. *P. acidipropionici* DH42 cultures tend to have higher propionic and acetic acids while the *P. shermanii* cultures had higher succinic and malic acids.

A PCR-based detection of *P. acidipropionici* DH42 was developed. Nested PCR was used with DH42-specific primers dhb1 and dhb2 for the secondary amplification of a 1,267 bp-fragment. Using the established protocols for PCR amplification, as low as 10^2 cfu/ml and 10^3cfu/ml of *P. acidipropionici* DH42 in silage extracts and rumen fluid, respectively, were detected.

The silage studies had shown that moisture level affects the efficacy of *P. acidipropionici* DH42. The 22-28% moisture content appears to favor its growth. An inoculation rate of 10^6 cfu/g of ensiling material is recommended. *P. acidipropionici* DH42 can produce vitaminB_{12} that is comparable to *P. shermanii*'s producing capability. Moreover, *P. acidipropionici* DH42 can be detected in silage and rumen fluid samples using PCR technology.

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INTRODUCTION

Ensiling of feedstuffs is a means by which the livestock producer is assured of a year-round feed supply. In the 1990's, advances in silage technology have made silage the principal method of forage preservation for dairy and beef cattle producers in North America (Bolsen, 1998). The main goal of ensiling is to retain as much of the nutritional value of the original crop as possible. The interaction of various biological and technological factors during the ensiling process determines silage quality.

Silage is the product formed by the fermentation of grass or other material of sufficient moisture content (Woolford, 1984). In addition to the oxygen-free environment, the production of acids (mainly lactic and acetic acids) through the fermentation of carbohydrates by anaerobic bacteria produces an environment that is unfavorable for most spoilage microorganisms. The increase in acidity or decline in pH depends on many factors such as the amount of fermentable carbohydrate in the crop to be ensiled, its buffering capacity and dry matter (DM) content and the type and amount of microorganisms that are present.

Silage additives had been used to either improve the nutritional value of the silage or enhance the fermentation thereby reducing dry matter losses. Various silage additives include fermentation stimulants such as bacterial inoculants and enzymes; fermentation inhibitors such as organic acids (propionic, formic and sulfuric acids) and substrates or nutrient sources, such as ammonia, urea, and anhydrous ammonia (Bolsen, 1998). Propionic acid is one of the acids that are commonly used in silage due to its antifungal properties. It is normally used at the rate of 1.5% of dry matter (Pitt, 1990). Propionic

acid is used in the storage of grains (Patkar et al., 1995) and in silage preservation (McDonald et al., 1991) to prevent mold growth. The occurrence of mycotoxins in foods and feeds due to fungal colonization is a worldwide problem. In the United States, corn is of a major concern because it is most susceptible to aflatoxin contamination (Wood, 1992). Even silage may also contain aflatoxin if corn is ensiled with the mycotoxin present. Ground high moisture corn is potentially more hazardous because grinding and the high moisture content favors the growth of toxigenic molds (Pier et al., 1992).

Propionic acid is commercially produced by petrochemical routes making its use expensive (Playne, 1986). It is also corrosive. For these reasons, and the increasing consumer preference for natural products, propionibacteria appear a viable alternative for propionic acid production in situ. Propionibacteria has many industrial applications (Boyaval and Corre, 1995) although they are most commonly used as starter cultures in cheese in which they contribute to the development of the typical flavor and the characteristic holes in Emmental, Gruyere and other Swiss-type cheeses (Perez-Chaia et al., 1988). Propionibacteria have been used as natural sources of propionic acid in the production of bakery products to improve shelf life (Javainen and Linko, 1993; Linko et al., 1997). Mantere-Alhonen (1995) and Perez-Chaia (1999) reviewed studies in the use of propionibacteria as probiotics. Their beneficial effects are derived from the production of propionic acid, bacteriocins, vitamin B_{12}, and ability to grow and survive gastric digestion (Perez-Chaia et al., 1995a).

The use of propionic acid-producing bacteria (PAB) appears beneficial as an inoculant for ensiling high moisture feeds. Improved fermentation was observed in PAB-treated silages but the effect on the aerobically exposed silages was variable (Florez-

Galarza et al., 1985; Alio et al., 1994; Dawson, 1994; Weinberg et al., 1995, Higginbothan et al., 1996 and 1998; Kreikemier et al 1998, Bolsen, 1998; Merry and Davies, 1999). There is not enough information on the factors affecting the efficacy of propionibacteria as silage inoculants.

Chapter 1 presents a review of literature on propionibacteria, their metabolism and properties, and the use of their metabolic products such as propionic and acetic acids as antimicrobial agents. The use of propionibacteria as silage inoculants is also discussed. Chapter 2 deals with the use of propionibacteria and lactic acid bacteria as inoculants for ensiling reconstituted high moisture corn. Chapter 3 evaluates the effect of moisture on the efficacy of propionibacteria as silage inoculants in high moisture corn. Chapter 4 presents a study on the vitamin B_{12} production of *P. acidipropionici* DH42 in a batch culture system. Chapter 5 highlights the development of a PCR-based assay for the detection of *P. acidipropionici* DH42 in silage and rumen fluid samples. Chapter 6 gives an overview of the project findings and the recommendations from the results of the studies.

The overall hypothesis of this project is that the addition of *P. acidipropionici* DH42 to silage will increase the production of propionic acid in the ensiled high moisture corn. The increased propionic acid is expected to improve the aerobic stability of the resulting silage by reducing yeast and mold counts, which is believed to be responsible for aerobic instability.

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CHAPTER 1

REVIEW OF LITERATURE

Propionibacteria: Properties and Metabolism

Propionibacteria are pleomorphic, non-sporeforming, gram-positive rods that frequently forms irregular clumps with the "Chinese-character" arrangements. They are nonmotile, anaerobic to aerotolerant and form small raised colonies that are cream, yellow, orange or deep red (Glatz, 1992). Their nutritional requirements have been well-studied (Hettinga and Reinbold, 1972).

Propionibacteria have proteolytic activity. They contain at least two weak proteinases, one is cell wall-associated and one membrane-bound (Langsrud et al., 1995). The cell wall-bound enzyme acted preferentially on the β-casein while the second is released at the stationary phase possibly by autolysis or excretion (Dupuis et al., 1995). The caseinolytic activity is however low which is not sufficient for effective growth; thus it is believed the enzyme has other functions. They also have a variety of peptidases and can degrade many amino acids but large variations in species and strains were noted (Perez-Chaia, et al., 1990; El-Soda et al., 1992; Langsrud et al., 1995).

Genetics of Propionibacteria

Studies on the genetic systems of propionibacteria are limited. Genetic studies have been hampered by the lack of sufficient gene transfer systems and convenient cloning vector (Gautier et al., 1993). Manipulation of propionibacteria have been limited to selection of spontaneous mutants and those generated by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (Glatz and Anderson, 1988). High propionic acid-producing

strains have also been selected by growing *P. acidipropionici* and transferring the strain into growth medium with increasing levels of propionic acid (Glatz and Anderson, 1988).

Several plasmids in the propionibacteria have been characterized (Rehberger and Glatz, 1990). Plasmid designated pRG01 is found alone in *P. acidipropionici* and *P. freudenreichii,* and either alone or with other plasmid in *P. jensenii.* Another plasmid, pRG02 is also found in *P. jensenii.* Plasmid pRG03 found in *P. freudenreichii* has been linked to lactose fermentation while pRG05 found in *P. jensenii*, is associated with the clumping phenomenon.

Bacteriophages have also been found in propionibacteria. In *P. freudenreichii* isolated from Swiss cheese, bacteriophage concentration is about 7 x 10^5 phage forming units and depends on the sample and strain used for detection (Gautier, et al., 1995a). Bacteriophages were detected only when the population of propionibacteria reached 10^8 to 10^9 colony forming units per gram of cheese.

Many strains of propionibacteria are resistant to lysozyme (Johnson and Cummins, 1972). Instead of protoplast formation, Gautier et al. (1995b) used electroporation in which cells are exposed to rapid pulses of a high-strength electrical field. The electrotransfection of *P. freudenreichii* with DNA phage produced 7x10^5 transfectants per μg of DNA under optimal conditions. For lysozyme-sensitive strains, osmotically fragile cells are produced by exposure to 20 mg/ml of lysozyme for at least 15 min (Baehman and Glatz, 1989). Cells regenerate in 21 days from protoplasts overlaid with soft agar that had 0.5 M sucrose and 2.5% gelatin.

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Molecular Detection of Propionibacteria

Differentiation of the four classical species of *Propionibacteria (P. acidipropionici, P. freudenreichii, P. jensenii,* and *P. thoenii)* is based on five biochemical characteristics, which include ability to ferment sucrose or maltose, and reduce nitrate, and differences in β-hemolysis, color of pigment and isomer of diaminopimelic acid in cell wall (Cummins and Johnson, 1986). However, distinction between the species is often difficult since variation in phenotyphic characteristics is not always reproducible (Grimont and Grimont, 1986; Riedel et al., 1994). Other species identification techniques include analyses of cellular protein profiles (Baer, 1987; Riedel and Britz, 1992), and plasmids (Rehberger and Glatz, 1990), immunoblotting (Baer and Ryba, 1991), and genome analysis (Gautier et al., 1992; Rehberger, 1993). These studies re-emphasized the problems associated with identification of propionibacteria.

The use of molecular methods in identification and detection of microorganisms has been widely used in recent years. Ribosomal ribonucleic acid (rRNA) gene restriction patterns are useful identification tools when biochemical tests are poor or atypical (Grimont and Grimont, 1986). The rRNAs are ubiquitous and extremely conserved molecules, which can be sequenced and compared to published or computer-based data banks for species identification. The polymerase chain reaction (PCR) has been used to amplify 16S rDNA of the four classical *Propionibacteria* species (Riedel et al., 1994) and distinct patterns are observed for each species. Charfreitag and Stackebrandt (1989) determined the intra-and intergeneric positions of *Propionibacterium* by comparing 16S rRNA. *P. acidipropionici, P. jensenii* and *P. thoenii* constitute a phylogenetically tight cluster, while *P. freudenreichii, P. acnes* and

P. propionicus are loosely related to each other. Likewise, they are not closely related to the *P. acidipropionici-P. jensenii-P. thoenii* cluster either. De Carvalho et al. (1994) and Riedel and Britz (1996) used rRNA gene restriction patterns (ribotyping) to differentiate the classical propionibacteria. Their study showed that the four species of propionibacteria, *P. freudenreichii, P. jensenii, P. acidipropionici* and *P. thoenii*, gave restriction patterns with species-specific fragments. Moreover, de Carvalho (1994) found that ribotyping also allowed differentiation of *P. freudenreichii* sub. *freudenreichii* from *P. freudenreichii* sub. *shermanii.* Riedel and Britz (1996) on the other hand, found only a single ribotype profile for the *P. freudenreichii* group and indicated that there is no need to separate the group into subspecies. Rossi et al. (1997) reported the PCR-amplification of the 16S-23S spacer region to distinguish 67 strains of dairy propionibacteria. Their findings validated the four current groupings of classical propionibacteria. They also indicated that after *Hinf* I digestion of the 16S-23S spacer region, *P. freudenreichii, P. jensenii* and *P. acidipropionici* are easily recognized. Using the Randomly Amplified Polymorphic DNA (RAPD)-PCR and Conventional Gel Electrophoresis Restriction Endonuclease Analysis (CGE-REA), Rossi et al. (1998) found a clear distinction of the current four clusters of propionibacteria. The techniques highlighted the presence of particular phenotypic characters and allowed intra-specific differentiation. A recent study developed a method to differentiate the *Propionibacterium* from other genera using a modified multiplex-PCR approach (Dasen, et al., 1998). This method detects both classical and cutaneous propionibacteria species by the amplification of a *Propionibacterium*-genus specific 900-bp. Rossi et al. (1999) had developed a genus- and species-specific PCR-based detection of dairy propionibacterium in milk, cheese, soil

and forage samples. They observed that in soil and forage samples, one-step PCR amplification cannot detect cells lower than 10^5 and they recommended a double-step amplification or nested PCR to improve detection.

Antimicrobial Action of Propionic Acid

In the USA, propionic acid and sodium and calcium propionates are considered GRAS (Generally Recognized as Safe). The sodium and calcium salts are the forms mainly used in food preservation. In breads and cakes, they are used against molds and *Bacillus mesentericus*, the bacterium that causes ropiness in breads (Lueck, 1980). In cheeses, where propionibacteria are used as starter cultures in combination with lactic acid bacteria, propionic acid is produced and provides some protection against molds (Eklund, 1989).

Propionic acid had been widely used in the storage of grains, nuts, hay, and silage to control molding. Ranzani and Fonseca (1995) used ammonium propionate to determine its effect on the growth of potentially aflatoxigenic fungi in unshelled peanuts in comparison with grapefruit seed extract and fungicides such as sodium orthophenylphenate and thiabendazole. They observed significant reduction in total fungal and *Aspergillus flavus parasiticus* counts when ammonium propionate was used at 5000mg/kg. Patkar et al. (1995) noted that the incidence of *Aspergillus flavus* was greatly reduced when propionic acid was added at 2µl/g in rice and sorghum and at 3µl/g in groundnut. *Eurotium* sp was found in the propionic acid-treated grains and presumed to be acid-tolerant. The effect of propionic acid to *Gibberella zeae*, a common fungi infecting wheat and maize residues has also been studied. Khonga and Sutton (1991) observed with a 5% (w/v) solution, propionic acid suppressed production of perithecia

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under laboratory condition. Under field conditions, it reduced inoculum (spore) production of *G. zeae* and encouraged its colonization by saprothrops. Toxicity of propionic acid to fungi depends on pH. Strider and Winstead (1960) observed that propionic acid is toxic to *Cladosporim cucumerinum* at 0.01 M at pH 5.0 but not at pH 6.0 indicating that the undissociated acid is the toxicant not its cation. Knapp et al. (1976) observed that propionic acid added at 1% to alfalfa hay showed no visible molding, prevented heating, and reduced dry matter loss. The effect propionic acid has also been tested in *Salmonella*. Since feeds are believed to be important source of infection of food animals, chemical treatments are being explored to reduce the recontamination of feeds after heat treatment (Ha et al., 1998).

Several researchers have described the membrane-directed mode of action of propionic acid against bacteria (Sheu and Freese, 1972; Sheu et al. 1972; Freese, et al. 1973), and yeasts (Moon, 1983). Using whole cells of *Bacillus subtilis* or membrane vesicles, Sheu and Freese (1972) and Sheu et al. (1972) observed that propionic acid and other short chain fatty acids inhibited cell growth which could be due to their inhibitory effect on the uptake of amino acids and other compounds necessary for growth or that ATP generation which depends on the electron transport system is inhibited by fatty acids. Moon (1989) also observed reduction in the cellular growth efficiency Y_{ATP} (defined as µg cells/mole glucose consumed) of *Saccharomyces ovarum*. Salmond et al. (1984) suggested that the growth inhibition of organic acids to *E. coli* consists of two components, the inhibition of a metabolic function by the undissociated acid (HA), and a generalized inhibition due to the acidification of the cytoplasm due to the accumulation of the acid (as A^-).

Moon (1989) further observed synergistic effects of mixtures of acetic, lactic and propionic acids against acid-tolerant yeasts. In fungi, Strider and Winstead (1960) suggested that propionic acid appears to act within the cell with *C. cucumerinum,* but with *Aspergillus flavus*, it appears to be at the cell surface.

In addition to propionic acid, antimicrobial proteins or bacteriocins have also been detected in propionibacteria. *P. jensenii* 126 produces a bacteriocin, Jenseniin G that has inhibitory properties against dairy propionibacteria and several lactic acid bacteria (Grinstead and Barefoot, 1992). Jenseniin G is stable at 100°C for 15 minutes and to pH values ranging from 3 to 12. Activity is detected in 50 to 100-fold concentrated 10-day old culture supernatants (Grinstead and Barefoot, 1992). Another bacteriocin is Propionicin PLG-1which is produced by *P. thoenii* 127 (Lyon and Glatz, 1991 and 1993). Propionicin PLG-1 inhibits gram-positive bacteria including *P. thoenii, P. acidipropionici, P. jensenii* and lactic acid bacteria, gram-negative bacteria (*Pseudomonas, Vibrio,* and *Campylobacter* spp. and *E. coli*) and selected yeast and molds (Lyon and Glatz, 1991). The bacteriocin is stable to temperature ≤ 85°C and to pH between 3-9 and is produced at the late stationary phase in agar and broth cultures (Lyon and Glatz, 1993). A more recent bacteriocin has been identified as being produced by *P. jensenii* B1264 (Ratnam et al., 1999). The bacteriocin inhibited propionibacteria and lactic acid bacteria and was bactericidal to *Lactobacillus delbrueckii* subs. *lactis* ATCC 4797. Its maximum activity was detected after 10 days of growth and 10-fold concentration. It is stable at 100°C for 60 minutes and to pH ranging from 2 to 10 (Ratnam et al., 1999). Bacteriocins had been examined as food preservatives (Lyon et al., 1993).

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Interactions of propionibacteria with lactic acid bacteria

Propionibacteria and lactobacilli can be found in mixed culture in many environments such as in cheese, fermented milk, bakery products, and silage. In most cases, a commensalistic form of interaction between propionibacteria and lactic acid bacteria had been observed in co-cultures (Lee et al., 1976; Liu and Moon, 1982, Parker and Moon, 1982; Perez-Chaia et al., 1987; Javainen and Linko, 1993; Perez-Chaia, 1994; Piveteau et al., 1995; Jimeno et al., 1995). Lactobacilli can ferment glucose into lactic acid. Propionibacteria can either use glucose or lactate as substrate although would prefer glucose to the latter (Hettinga and Reinbold, 1972). The stimulatory effect of LAB on PAB depends on the strains involved. Perez-Chaia et al. (1995b) observed that propionibacteria strains are inhibited in mixed cultures that rapidly reached low pH values. When grown in mixed cultures with different propionibacteria strains, only *L. helveticus* ATCC 15009 showed the highest pH value allowing the pH sensitive propionibacteria to grow (Perez-Chaia et al., 1995b). Piveteau et al. (1995) observed that among five LAB strains, only *L. helveticus* and *Streptococcus thermophilus* stimulated *P. freudenreichii* and *P. acidipropionici.* They found that the increase in growth rate and cell yield of *P. freudenreichii* in the presence of *L. helveticus* RR coincided with an increased conversion of lactate to propionate and acetate. Increased cell yields and growth rates were also observed when *P. shermanii* and *L. acidophilus* were grown together, which may be related to the availability or concentration of lactate (Lui and Moon, 1982). Thierry et al. (1999) observed that when five strains of *P. freudenreichii* were grown in media where different thermophilic lactic acid-producing bacteria had

been previously grown, stimulatory effect to propionibacteria were observed with a low proportion of L(+) lactate.

Propionibacteria can use the lactate produced by LAB from the fermentation of glucose (Lee et al., 1976; Liu and Moon, 1982). LAB produce different isomers of lactate. *L. delbrueckii* subsp. *lactis* produces D lactate. *S. thermophilus* produces L lactate while *L. helveticus* produces both D and L lactate forms (Piveteau et al., 1995). Some strains of PAB prefer the L lactate to the D lactate (Crow, 1986; Piveteau et al., 1995). The consumption of lactate by propionibacteria can slow down the increase in acidity, which inhibits the growth of the LAB.

Interactions between PAB and LAB are not limited to lactate production and utilization. Lactobacilli can also benefit from mixed culturing with the added carbon dioxide produced by propionibacteria (Friedman and Gaden, 1970). Moreover, in Emmental cheeses, Baer (1995) observed that the growth of propionibacteria was enhanced by the amino acids released due to the proteolytic activity of starter cultures such as *S. thermophilus* and *L. delbruickii*. In whey, *L. helveticus* increased the levels of amino acids and peptideswhich stimulated the growth of *P. freudenreichii* (Piveteau et al., 1995). However, when the concentration of amino acids is too high, which were produced when highly proteolytic lactic bacteria were used, the growth of propionibacteria were inhibited (Baer, 1995). Thierry et al. (1999) observed similar results where *P. freudenreichii* were stimulated by high peptide levels and low free amino acid levels. This, however, conflicts with the results of Baer and Ryba (1999) who observed that high amino acids stimulated the growth of PAB, regardless of the lactobacilli strain. Moreover, contrary to earlier observations of Jimeno et al. (1995),

Baer and Ryba (1999) concluded that neither acetate, formate nor diacetyl, which is formed when PAB is co-cultured with *L. rhamnosus*, inhibited the growth of propionibacteria.

Inhibitory effect of propionibacteria on lactic acid bacteria has also been observed. When glucose concentration is low, propionic acid has an inhibitory effect on the growth of LAB (Perez-Chaia et al., 1994 a,b). The researchers suggested that the propionic acid could diffuse inside the cells, increasing the inward leak of H^+ into the cells. To compensate for this, extrusion of the excess H^+ by H^+-ATPase is needed but when glucose is limiting, metabolism cannot supply the ATP required for the process. Consequently, growth rate and biomass production of the lactic acid bacteria is reduced.

Production of Vitamin B_{12}

In addition to propionic acid, vitamin B_{12} is another major product of *Propionibacterium spp.* that has commercial importance. Among the propionibacteria strains, the *P. freudenreichii* and *P. shermanii* are commonly used. Industrial production of vitamin B_{12} uses *Propionibacterium spp.* and *Pseudomonas denitrificans*. However, since propionibacteria are slow-growers, they have not been used widely for the commercial production of the vitamin.

Vitamin B_{12} is an essential part of enzyme systems that carry out basic metabolic functions. Humans and animals depend on microbial synthesis for their supply of the vitamin. In ruminants, dietary cobalt appears to be the main limiting factor in its synthesis by ruminal microflora (Mc Dowell, 1989). However, Sutton and Elliot (1972) found that on high concentrate diets, vitamin B_{12} synthesis is decreased and analogues, which have little or no vitamin B_{12} activity are produced. The ruminal microorganisms

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such as *Prevotella ruminicola* (Strobel, 1992) and *Bacteroides spp* (Varel and Bryant, 1974; Chen and Wolin, 1981) require vitamin B_{12} for growth and propionate production. Strobel (1992) found that cell protein yields were reduced by 15 to 25% in the absence of vitamin B_{12}. In the synthesis of propionic acid, the enzyme methylmalonyl-CoA mutase that catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA, is a coenzyme B_{12}-dependent enzyme (Wood, 1981).

P. acidipropionici produces vitamin B_{12} intracellularly in the form of 5,6 dimethylbenzimidazoylcobamide (Quesada-Chanto et al., 1994a). Propionibacteria strain ar1 AKU1251 can excrete the vitamin (mainly in the form of hydroxycobalamin) to the culture medium (Yongsmith et al., 1982).

Different substrates have been used in the production of vitamin B_{12}. Using 10% whey solids and 1.5% yeast extract, Bullerman and Berry (1966) found vitamin B_{12} production of 8.43 µg/ml using *P. shermanii*. Using molasses, Quesada-Chanto et al. (1994a) observed 45 mg/l vitamin B_{12} produced by *P. acidipropionici*.

Many factors affect the production of vitamin B_{12}. Bullerman and Berry (1966) observed increased vitamin B_{12} levels with the addition of cobalt and the vitamin B_{12} precursor 5,6 dimethybenzimidazole (DMBI). Quesada-Chanto et al. (1994b) indicated that for optimum production of vitamin B_{12}, cobalt ions, betaine and 5,6 DMBI must be added to the growth medium at the rates of 5 mg/l, 5 g/l and 2 mg/l , respectively. Cobalt ions are important in the formation of cobalamin. Betaine (trimethyglycine), on the other hand, provides the methyl groups of the corrin ring in the synthesis of vitamin B_{12}. DMBI is necessary for the formation of 5,6 dimethylbenzimidazoyl cobamide. Yeast

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extract was found to stimulate growth and product formation of propionibacteria (Quesada-Chanto et al., 1994b).

Culture conditions such as pH, temperature and aeration also affect vitamin B_{12} production. *P. acidipropionici* cultures are highly pH-dependent (Hsu and Yang, 1991). Quesada-Chanto et al. (1994b) found that the optimal value for the production of propionic acid and vitamin B_{12} is pH 6.5-6.8. They also recommended growing cultures at 40^0C for optimal production of vitamin B_{12}. While propionibacteria can be grown under anaerobic conditions, they are oxygen-tolerant. With aeration, Bullerman and Berry (1966) observed increased vitamin B_{12} yield and that the addition of precursor had no effect on vitamin production with aeration. Quesada-Chanto et al. (1994b) found optimum propionic acid production under completely anaerobic conditions while aeration was required for vitamin B_{12} production. On the other hand, Yongsmith et al. (1982) using *Propionibacterium sp* trapped in urethane prepolymers found that vitamin B_{12} production is less with aeration compared to static culture.

Silage and Silage Additives

Silage is the product formed by the fermentation of grass or other material of sufficient moisture content, generally greater than 50% (Woolford 1984; Bolsen, et al., 1995). The main objectives of ensiling are to maintain anaerobiosis and to discourage the proliferation of undesirable microorganisms such clostridia and enterobacteria (McDonald et al., 1991). The latter is mainly accomplished by production of lactic acid, which results in the pH reduction and deters the growth of undesirable microorganisms during ensiling.

The ensiling process involves several phases (Pitt, 1990; Bolsen et al., 1995). In the aerobic phase, two plant activities occur: respiration and proteolysis. In addition, aerobic microorganisms from the fresh crop predominate using up the oxygen trapped in the ensiled material. In this phase, excessive heat production can occur reducing the digestibility of protein and fiber constituents due to browning and Maillard reactions. In the lag phase, plant cell membranes break down releasing cell juices that provide medium for microbial growth. Facultative and obligate anaerobes proliferate in the fermentation phase. The lactic acid bacteria rapidly grow and consume the water-soluble carbohydrates producing mainly lactic acid. Acetic acid, ethanol, carbon dioxide and some other minor products are also produced depending on the predominating LAB strain. The production of lactic acid reduces pH to 3.5-5.0, which limits microbial activity. In the stable phase, the availability of nutrients can affect the growth of lactic acid bacteria. Limited water-soluble carbohydrates will slow down the rate of pH decline. Oxygen infiltrating the silo through openings in silo walls and plastic covers can cause yeast and molds and other aerobic microorganisms to proliferate causing heating and substantial dry matter loss. In the feedout phase, aerobic microbial activity predominates in the silage that is exposed to air. This is undesirable since significant loses in dry matter can occur through the consumption of residual soluble sugars, organic acids and other fermentation products. Yeast and molds have been implicated in the aerobic deterioration of silages.

The ideal crop for ensiling should contain sufficient amount of fermentable substrate in the form of water-soluble carbohydrates, low buffering capacity and a dry

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matter content above 200 g kg^{-1}. Since many crops used for ensiling do not fulfill these requirements, material pretreatment and additives are often used (McDonald et al., 1991).

The moisture content of ensiling material affects not only the production of effluent, which can affect silage quality, but also the availability of nutrients for microbial growth and the rate of fermentation. If soluble carbohydrates are very high, lactic acid bacteria rapidly proliferate and rapid pH decline is observed. Dry matter affects the production of organic acids in high moisture corn as well. Faber et al. (1989) observed higher concentrations of lactic acid, acetic acid, ammonia nitrogen and lower pH in low DM corn (68 and 67% shelled corn, 71 and 64% ear corn) compared to high dry matter corn (76% shelled corn and 79% ear corn). They also observed significant interaction between dry matter and inoculation. In alfalfa silage, Garcia et al. (1989) observed that lactic, acetic, and propionic acids are higher with low dry matter (46% DM) as compared to the high dry matter silages (62%). The observed effects of moisture on acid production can be attributed to the its effect on microbial metabolic activities. All chemical reactions of cells require an aqueous environment although bacteria require relatively higher levels of moisture for their growth than yeast and molds (Jay, 1998).

Microbial Inoculants

In the early studies, additives were used to ensure that lactic acid bacteria dominate the fermentation process, although more recent developments have focused on improving the nutritive value of the silage and reducing dry matter losses (McDonald et al., 1991). Silage additives are classified into fermentation stimulants, fermentation inhibitors, aerobic deterioration inhibitors, nutrients and absorbents (Woolford, 1985; McDonald et al., 1991).

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Among the fermentation stimulants, it is the microbial inoculants that had received much of the attention in recent years. Commercial microbial inoculants for silage mainly include different strains of lactic acid-producing bacteria (LAB) such as *Lactobacillus* sp., *Pediococcus* sp. and *Enterococcus* sp. (Weinberg et al., 1995a). Lower pH, and higher lactic acid content in LAB-inoculated silages have been observed (Florez-Galarza et al., 1985; Rust et al., 1989; Faber et al., 1989; Fitzsimons et al., 1992; Wardynski et al., 1993; Weinberg, et al., 1995a). The increased production of lactic acid causes rapid decline in pH, which retards the growth of undesirable microorganisms. However, the stability of the ensiled material once the silos are opened has been inconsistent. Upon exposure, the LAB-inoculated silages had heating, lower dry matter recovery, faster deterioration than the uninoculated silages with no effect on the nutritive value (Rust et al., 1989; Stokes, 1992; Sanderson, 1993; Wardynski et al., 1993). Inoculation rate and the LAB species also affected silage stability. *Lactobacillus buchneri* inoculated at 1x 10^6 cfu/g gave more stable corn silages compared to those inoculated at the rate of 1x 10^5 cfu/g. *Lactobacillus plantarum* had minimal effects on silage fermentation and aerobic stability compared to *L. buchneri*–inoculated corn silages (Ranjit et al., 1998). Reduced aerobic stability was also observed when a mixture of *L. plantarum* and *Enterococcus faecium* were used to ensile high moisture ear corn (Sebastian et al., 1995).

The effect of bacterial inoculants in aerobic stability is unclear. Aerobic instability has been mainly associated with the yeasts and molds. Lower yeast and mold numbers were observed in the more stable high moisture corn silages (Hara and Ohyama, 1979; Rust and Yokoyama, 1992). Propionic acid has been used to prevent aerobic

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deterioration in rye grass silage (Hara and Ohyama, 1978), and high moisture ear corn silage (Sebastian et al., 1995).

Propionibacteria as inoculants

Recent studies explored the use of propionic acid bacteria to improve the aerobic stability of silages. Lindgren et al. (1983) evaluated the use of *P. shermanii* with LAB as inoculants to grass silage. No increase in propionic and acetic acids in silages were observed. Using 27% dry matter high moisture corn, Florez-Galarza et al. (1985) observed lower pH and yeast population in those inoculated with *P. shermanii*. Moreover, mold growth was prevented in all inoculated corn samples. Alio et al. (1994) on the other hand, observed that PAB inoculation did not influence the aerobic stability of 24% DM orange pulp silage. Weinberg et al. (1995a) in their study with pear millet and maize silages observed marginal effect of *P. shermanii* inoculation with or without LAB in the aerobic stability of the silages. Kreikeimeier et al. (1997) reported increased propionate in the propionibacteria inoculated high-moisture corn after 90 d of ensiling. They also observed increased growth performance of the finishing cattle fed with the inoculated silage. Higginbotham et al. (1998), using whole plant corn inoculated with *P. acidipropionici* alone or in combination with *P. cerevisiae* noted little effect of inoculation on pH, concentration of water-soluble carbohydrates, lactic acid, concentration of volatile fatty acids and on the aerobic stability of the exposed silages. Dawson et al. (1998) evaluated the effects of *P. acidipropionici* DH42, a bacterium that was isolated from high moisture corn (Dawson, 1994) on fermentation characteristics and aerobic stability of ensiled high moisture corn. After 42 d of ensiling, increased propionic acid (0.35 vs. 0.03 g/100 g of dry matter), acetic acid, lower pH, lower yeast

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and mold counts and higher DM recovery were observed in the inoculated silages. During aerobic exposure, the inoculated silages had higher propionic acid and recovery of organic matter and the temperature remained unchanged indicating aerobic stability. The inconsistent results on the use of propionibacteria as a silage inoculant only stress the need for more studies on factors their efficacy. In a simulation model done by Pitt (1997) on the growth and fermentation of propionic acid bacteria in silage, factors such as pH, water activity, temperature, and concentration of organic acids were considered.

Objectives

The current research is aimed at conducting further studies on the use of *P. acidipropionici* DH42 as an inoculant for high moisture corn. Specifically, the project will be conducted to:

1. compare DH42 with commercial propionic acid-producing bacteria with or without lactic acid-producing bacteria as inoculants to reconstituted high moisture corn silage;
2. evaluate the effects of moisture content on the fermentation characteristics and aerobic stability of DH42-inoculated high moisture corn silage;
3. determine vitamin B_{12} production of DH42 using batch cultures at two incubation temperatures;
4. develop a PCR-based method of detecting DH42 in silage and rumen fluid samples.

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CHAPTER 2

PROPIONIBACTERIA AND LACTIC ACID-PRODUCING BACTERIA AS INOCULANTS FOR ENSILING RECONSTITUTED CORN

Abstract

The study was conducted to determine the effects of two propionibacteria (*Propionibacterium* sp. P42 and *P. acidipropionici* DH42) and lactic acid bacteria as inoculants to reconstituted rolled corn. Treatments were as follows: control (sterile distilled water), DH42, P42, DH42+LAB, DH42+P42, DH42+P42+LAB, and autoclaved DH42 culture. Microbial inoculants were added at the rate of 10^5cfu/g material. Silos were opened after 7, 21, and 90 d post-ensiling. Samples from the 90-d ensiling period were used for aerobic stability evaluation. Fresh, fermented and exposed corn samples were taken for chemical and microbial analyses.

Inoculation enhanced the fermentation of the reconstituted corn up to 21 d of ensiling. The combination of *P. acidipropionici* DH42 with lactic acid bacteria as inoculants reduced silage pH and butyric acid and increased propionic, acetic and lactic acids. LAB inoculation did not significantly increase the LAB population in the treated silages.

During aerobic exposure, all the silages appeared well-preserved. Organic acid levels remained stable throughout the exposure period. However, the silages treated with *P. acidipropionici* DH42 + LAB had higher average propionic and lactic acids and lower pH than the control. Propionibacteria inoculation did not significantly reduce the yeast and mold counts since the control silages also appeared well-preserved.

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Introduction

In recent years, ensiling high moisture corn has gained wide acceptance for several reasons. Ensiling eliminates the added cost of drying and corn can be harvested 2 to 3 weeks earlier thereby reducing field losses. However, microbial deterioration due to mold and spoilage organisms can reduce the quality of high moisture corn.

Propionic acid has been used as a preservative to improve storage life of high moisture corn (Deyoe et al., 1973; Burrell et al., 1973; Jones et al., 1974). In silage, the benefits of propionic acid are attained with the addition of 1-1.5% to the material (Thomas, 1978). More recently, the use of propionibacteria has been explored as an alternative to chemical treatments. Lindgren et al. (1983) observed no propionic acid in the silages inoculated with *P. shermanii* and lactic acid bacteria. On the other hand, Flores-Galarza (1985) observed improved reduction in the yeast and mold counts of ensiled high moisture corn with *P. shermanii*. Weinberg et al. (1995), Kreikeimer et al. (1997) and Higginbotham et al. (1998) observed marginal improvement in fermentation and aerobic stability of propionibacteria-inoculated silages. Dawson et al. (1998) observed improved fermentation and aerobic stability in high moisture corn inoculated with *P. acidipropionici* DH42.

This study was conducted to determine the efficacy of *P. acidipropionici* DH42 as a silage inoculant with or without lactic acid bacteria and to determine whether the co-inoculation with another propionibacterium strain, *Propionibacterium* sp. P42 and LAB would be advantageous.

The hypothesis of this study is that the addition of propionibacteria is expected to enhance the fermentation of high moisture corn and reduce the yeast and mold counts in

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the silage particularly during aerobic exposure. Moreover, the addition of LAB as a co-inoculant to PAB is expected to enhance the performance of PAB.

Materials and Methods

Inoculants

The three inoculants used were: *P. acidipropionici* DH42 (ATCC 55737) which was taken from cultures maintained at the microbiology laboratory of the Department of Animal Science, Michigan State University, East Lansing, Michigan. (MSU, East Lansing, MI), *Propionibacterium* sp. P42 (Laporte Biochem International, Milwaukee, WI) and lactic acid-producing bacteria (Laporte Biochem International, Milwaukee, WI). *Propionibacterium* sp. P42 and the lactic acid-producing bacteria were received as freeze-dried cultures and rehydrated with sterile distilled water according to the manufacturer's instructions about 30 min before use.

P. acidipropionici DH42 was grown in 0.5X Lactobacilli MRS broth (Difco). Before use, at least three successive transfers were done to ensure its activation. *P. acidipropionici* DH42 was incubated for 18 h at 39°C. The amount of *P. acidipropionici* DH42 needed to meet the required inoculation rate of 10^5 cfu/g material was calculated from the linear regression formula which had been established beforehand. Based on the OD value of the culture, estimated cfu/ml was calculated. Serial dilutions of the culture were also made and plated in Lactobacilli MRS agar (Difco) to verify the calculated counts.

Silage Preparation

Dried corn was rolled and reconstituted by adding distilled water to adjust the moisture content to 28%. Separate gloves and mixing tubs were used for each treatment to prevent cross-contamination. Laboratory silos (47.5 x 10.2 cm diameter) were used. The silos were made of polyvinyl chloride (PVC) pipes with rubber caps fitted with rubber policeman (Bolsen, 1992). There were seven inoculant treatments: control (sterile distilled water), DH42, P42, DH42+LAB, DH42+P42, DH42+P42+LAB, and autoclaved DH42 cultures. All inoculants were added at the rate of 10^5cfu/g of material. The latter treatment was used to determine the effect of the culture medium that was added with the DH42 cultures. It was prepared by autoclaving the DH42 culture for 15 min at 17 psi to kill all bacteria. The appropriate amounts of inoculants were measured and the volume adjusted to 100 ml by adding sterile distilled water. The inoculants were added to the corn and mixed by hand in a tub. For d 0, two samples were taken from each treatment. Three silos were prepared for each treatment and ensiling period combination. Silos were opened after 7, 21 and 90 d of ensiling. Silos were weighed before and after ensiling and the dry matter recovery was determined. Fresh, fermented and exposed corn samples were taken for chemical and microbial analyses.

Aerobic Stability

The silage from the 90-d ensiling period was used for the aerobic stability evaluation. About 1 kg from each silo was measured into a plastic bag, placed in a Styrofoam container and exposed to air for 5 d. Samples for chemical and microbial analyses were taken after 1, 3, and 5 d of exposure. Cooking thermometers were inserted into the silage to monitor temperature. Temperatures were taken daily.

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Chemical Analyses

The dry matter content was determined by drying the samples in a forced air oven (55°C) for 48h. Aqueous extracts from each sample were obtained by mixing 50 g of corn in 450 ml of sterile 0.9% saline using a Stomacher (Model 3500, Tekmar) for 5 min and strained through four layers of cheese cloth into 50-ml centrifuge tubes. The pH of the extracts was taken. Glucose, ethanol and the organic acids were determined by HPLC as described by Dawson (1994) with slight modification. Instead of filtering the samples, they were centrifuged (26,000 x g) for 30 min and the supernatant used for analyses.

Microbial Analyses

Serial dilutions of the extracts were prepared in 1% peptone broth (Difco) and appropriate dilutions were plated in selective media. Rogosa SL Agar (Difco) was used to enumerate lactic acid bacteria and Rose Bengal Agar (Difco) with Antimicrobic Supplement C (Difco) was used to estimate yeast and molds. Culture plates were incubated at 39°C.

Statistical Analyses

Data for the fermentation phase were analyzed as a one-way completely randomized design using the General Linear Model subroutine of the Statistical Analysis System (SAS, 1990). Microbial counts were analyzed using the transformed data ($\log_{10}$ [Y], where Y is the microbial count). Due to the increasing variability of the various parameters over time, the data were analyzed separately for each collection period (i.e. d 0, 7, 21, 90). At a given collection period, the model used for each parameter (e.g. pH, propionic acid, acetic acid) was as follows:

$Y_i = \mu + \alpha_i + e_i$

Where:

Y	=	individual variable measured (e.g. pH, propionic acid, glucose)
μ	=	overall mean
α_i	=	effect of treatment
e_i	=	random residual error

For the aerobic stability phase, data were analyzed by PROC MIXED of the SAS, using the repeated measures analysis (SAS, 1990) since repeated samplings from the same sample were done. Treatment means were compared using the Bonferroni (SAS, 1990).

Results and Discussion

Fermentation Phase

The initial dry matter content (Table 2-1) differed ($P<0.05$) among treatments, ranging from 69.60-73.57%. This was possibly due to the uneven mixing of water when the corn was reconstituted. The control had the highest dry matter throughout the ensiling period. The control had 73.57% dry matter at the start of ensiling and 72.91% after 90 d of ensiling. The autoclaved DH42, on the other hand, had the lowest dry matter with slightly below 70% during the fermentation phase.

Propionic acid (Table 2-2) was detected in the silages after 7 d of ensiling. Propionic acid levels ranged from 0.02-0.04 g/100 g DM. The lowest ($P<0.05$) levels were observed with DH42 (autoclaved), P42+DH42+LAB and DH42 + LAB. The other treatments had similar propionic acid levels. After 21 d of ensiling, the combination of DH42 and P42 gave the highest level with 0.10 g/100 g DM followed by DH42 alone

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Table 2-1. Dry matter content (%) of fresh and ensiled HMC

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	69.60 [d]	69.79 [c]	69.38 [d]	69.65 [d]
DH42+P42+LAB	71.90 [c]	71.64 [b]	71.26 [b]	71.30 [bc]
DH42+LAB	72.01 [c]	72.00 [b]	71.78 [b]	72.21 [ab]
DH42+P42	72.45 [bc]	72.16 [b]	71.76 [b]	71.99 [ab]
DH42	70.20 [d]	70.44 [c]	70.15 [c]	70.56 [cd]
P42	72.80 [ab]	72.47 [ab]	71.82 [b]	71.82 [abc]
Control	73.57 [a]	70.32 [a]	73.03 [a]	72.91 [a]
S.E.M.[n]	0.05	0.07	0.05	0.10

[abcd]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

Table 2-2. Effect of inoculation on propionic acid content (g/100 g DM) of fresh and ensiled HMC

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	0.00	0.02 [b]	0.030 [bc]	0.11
DH42+P42+LAB	0.00	0.02 [b]	0.007 [d]	0.12
DH42+LAB	0.00	0.02 [b]	0.007 [d]	0.11
DH42+P42	0.00	0.03 [a]	0.098 [a]	0.12
DH42	0.00	0.04 [a]	0.053 [b]	0.11
P42	0.00	0.04 [a]	0.029 [cd]	0.07
Control	0.00	0.04 [a]	0.041 [bc]	0.07
S.E.M.[n]		0.001	0.002	0.004

[abcd]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

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(0.05 g/ 100 g DM). After 90 d, differences in the propionic acid levels among treatments were not significant (P>0.05). The silages treated with the combination of DH42 + P42 +LAB had 0.12 g/100 g DM propionic acid. The data suggest that addition of propionibacteria did not enhance the production of propionic acid in the silages and that there was no advantage of adding LAB. In a modeling study done by Pitt (1997), greater accumulation of propionate was predicted with the co-culture of LAB with propionibacteria due to the greater production of lactate increasing the substrate for propionibacteria. Results of this study did not verify or support this hypothesis. Lindgren et al. (1983) also observed no increase in propionic or acetic acid when a mix culture of *P. shermanii* and lactic acid bacteria were used as silage inoculants. Weinberg et al. (1995 a,b) observed similar results. Conversely, Kreikemier et al. (1997) observed increased propionate when LAB and propionibacteria were inoculated into high moisture corn but their values were much lower than the values observed in this study. Their silos were however stored at 16°C, which could have affected the growth of the inoculant.

Acetic acid (Table 2-3) was not observed until after d 7 of ensiling. On d 7, acetic acid levels were higher (P<0.05) in the HMC treated with the autoclaved DH42 than the control and the DH42+LAB. Differences among treatments were not significant after 21 and 90 d of ensiling. Since propionibacteria produce acetic acid in addition to propionic acid, higher levels of acetic acid are expected when propionibacteria are used as starter cultures. However, the control had acetic acid levels that were comparable to inoculated silages.

LAB inoculation increased lactic acid (Table 2-4) production in the treated silages. On d 7, the combination of DH42+P42+LAB had higher (P<0.05) lactic acid

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[a]Colum
[b]Standard

Table 2-3. Effect of inoculation on acetic acid content (g/100 g DM) of fresh and ensiled HMC

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	0.00	0.12 [a]	0.15	0.45
DH42+P42+LAB	0.00	0.10 [ab]	0.13	0.31
DH42+LAB	0.00	0.07 [b]	0.15	0.26
DH42+P42	0.00	0.07 [ab]	0.16	0.41
DH42	0.00	0.08 [ab]	0.15	0.35
P42	0.00	0.07 [ab]	0.12	0.32
Control	0.00	0.05 [b]	0.17	0.34
S.E.M.[n]		0.004	0.004	0.014

[ab]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

Table 2-4. Effect of inoculation on lactic acid content (g/100 g DM) of fresh and ensiled HMC

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	0.00	1.29 [ab]	1.60 [ab]	1.06
DH42+P42+LAB	0.00	1.36 [a]	1.59 [ab]	1.13
DH42+LAB	0.00	0.94 [abc]	1.74 [a]	1.46
DH42+P42	0.00	0.82 [abc]	1.48 [ab]	1.10
DH42	0.00	0.94 [abc]	1.84 [a]	1.15
P42	0.00	0.67 [bc]	1.17 [b]	0.80
Control	0.00	0.61 [c]	1.20 [b]	0.79
S.E.M.[n]		0.05	0.04	0.05

[abc]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

level than P42 and control silages. After 21 d of ensiling, DH42 + LAB and DH42 had higher ($P<0.05$) lactic acid levels than P42 and control. However, after 90 d of ensiling, differences among treatments were not significant. Lactic acid levels observed in this study are lower than that reported by Wardynski et al. (1993), but higher than that reported by Phillip and Fellner (1991). Differences in lactic acid production could be due to differences in grain processing (whether whole or rolled) and dry matter content of the ensiling material. Both influence the fermentation of lactic acid bacteria by affecting the substrate available for fermentation (Pitt et al., 1985; Muck, 1990) and water activity. Goodrich et al. (1975) also observed that corn ensiled at harvest had higher lactic acid content than corn that was dried and reconstituted. Moreover, de Vries et al. (1970) and Thomas et al. (1979) observed that homofermentative lactic acid bacteria shifts to a heterofermentative mode of fermentation when glucose is limiting. Instead of only lactic acid being produced, other metabolites such as acetate, ethanol and formate are formed. Table 2-6 shows that glucose was rapidly used by d 7 of ensiling.

The effect of lactic acid bacteria inoculation in high moisture corn silage is variable. While Schaeffer et al. (1989) observed higher lactate in the inoculated silages ($P<0.05$), Phillip and Fellner (1992) observed marginal increase ($P<0.10$) while Wardynski et al. (1993) observed higher lactate in the uninoculated silages. This could be due to differences in the epiphytic lactic acid bacteria numbers in the initial ensiling material. When there are sufficient bacteria in the material, the added bacterial inoculant does not appreciably affect the total LAB population. Bolsen et al. (1996) pointed out that strain selection is as important as the number of lactic acid bacteria applied per gram of crop.

Significant differences in initial pH (Table 2-5) of the corn samples were observed. The DH42+P42 and DH42 silages had the highest ($P<0.05$) pH values while the silages with autoclaved DH42 culture had the lowest pH. After 7 d of ensiling, the control and the P42 silages had the highest pH with 4.26. On d 21, all the inoculated silages had lower ($P<0.05$) pH values compared to the control. By d 90, DH42+LAB silages had lower pH than the silages treated with autoclaved DH42 culture but not different from the control. The decrease in pH observed in this study is consistent with results of other studies that used LAB as inoculants (Bolsen et al., 1996).

Glucose content (Table 2-6) of the corn varied initially and ranged from 0.16 to 0.42 g/100 g DM. After 7 d of ensiling, more than 90% of the glucose was utilized. Thereafter, glucose changed little until d 90 where it was hardly detectable in all treatments. Decrease in glucose is expected since bacteria use it as a substrate. However, decrease in pH also promotes hydrolysis of sugars from the cell walls (Jones et al., 1992). But, it is unlikely that the pH differences were sufficiently large to create a difference in glucose release from cell walls. All of the glucose was essentially utilized during the fermentation phase.

Butyric acid (Table 2-7) was detected only after 21 d of ensiling. In silage, butyrate production is expected at the latter stage of ensiling and is mainly attributed to clostridial fermentation. However, yeasts and *Bacillus* sp. can also produce butyrate (Mc Donald et al., 1991). On d 21, butyric acid was higher in the silages treated with autoclaved DH42 culture than the control, DH42+LAB and DH42+P42+LAB. On d 90, highest ($P<0.05$) butyric acid levels were detected in autoclaved DH42-treated silages.

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[a]Colum
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Table 2-5. Effect of inoculation on the pH of fresh and ensiled HMC

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	5.28 [d]	4.10 [d]	4.16 [cd]	4.34 [a]
DH42+P42+LAB	5.44 [c]	4.08 [d]	4.12 [d]	4.27 [ab]
DH42+LAB	5.55 [b]	4.10 [d]	4.14 [d]	4.13 [b]
DH42+P42	5.66 [a]	4.21 [b]	4.23 [bc]	4.26 [ab]
DH42	5.69 [a]	4.16 [c]	4.14 [d]	4.25 [ab]
P42	5.52 [b]	4.26 [a]	4.26 [b]	4.32 [ab]
Control	5.53 [b]	4.26 [a]	4.36 [a]	4.29 [ab]
S.E.M.[n]	0.01	0.01	0.01	0.01

[abcd]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

Table 2-6. Effect of inoculation on glucose content (g/ 100 g DM) of fresh and ensiled HMC

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	0.32 [b]	0.02	0.00 [b]	0.00
DH42+P42+LAB	0.16 [c]	0.01	0.02 [a]	0.00
DH42+LAB	0.42 [a]	0.01	0.02 [a]	0.01
DH42+P42	0.32 [b]	0.01	0.01 [ab]	0.00
DH42	0.40 [a]	0.02	0.01 [ab]	0.00
P42	0.42 [a]	0.01	0.00 [b]	0.00
Control	0.33 [b]	0.01	0.00 [b]	0.00
S.E.M.[n]	0.002	0.001	0.001	0.001

[abc]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

Table 2-7. Effect of inoculation on butyric acid content (g/100 g DM) of fresh and ensiled HMC.

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	0.00	0.00	0.055 [a]	0.16 [a]
DH42+P42+LAB	0.00	0.00	0.020 [d]	0.08 [b]
DH42+LAB	0.00	0.00	0.024 [cd]	0.08 [b]
DH42+P42	0.00	0.00	0.053 [ab]	0.10 [b]
DH42	0.00	0.00	0.050 [ab]	0.11 [ab]
P42	0.00	0.00	0.046 [ab]	0.11 [ab]
Control	0.00	0.00	0.039 [bc]	0.07 [b]
S.E.M.[n]			0.001	0.004

[abcd]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

Through out the ensiling period, ethanol levels (Table 2-8) were not affected by inoculation. This is in contrast to results of Dawson et al. (1998) where DH42-inoculated silages had lower ethanol levels. Bolsen et al. (1996) also reviewed studies on the use of lactic acid bacteria as silage inoculants and found that over 90% of the inoculated silages had lower ethanol contents. Ethanol is produced by yeast and heterofermentative lactic acid bacteria (Mc Donald et al., 1991). However, *L. brevis* which is the most common heterofermentative lactic acid bacteria found in high moisture corn does not ferment glucose anaerobically to ethanol (McDonald et al., 1991).

The initial citric acid levels (Table 2-9) were significantly different among treatments. P42 had the highest level (0.16 g/ 100 g DM) while the DH42+P42+LAB had the lowest (0.05 g/ 100 g DM). After 90 d of ensiling, the treatment with the highest citric acid content was the silages with the autoclaved DH42 culture (0.22 g/ 100 g DM)

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Table 2-8. Effect of inoculation on ethanol content (g/100 g DM) of fresh and ensiled HMC.

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	0.00	0.35	0.38	0.51
DH42+P42+LAB	0.00	0.28	0.32	0.28
DH42+LAB	0.00	0.21	0.37	0.38
DH42+P42	0.00	0.31	0.43	0.49
DH42	0.00	0.28	0.38	0.41
P42	0.00	0.26	0.42	0.46
Control	0.00	0.22	0.35	0.40
S.E.M.[n]		0.02	0.01	0.02

[n]Standard error of the mean.

Table 2-9. Effect of inoculation on citric acid content (g/100 g DM) of fresh and ensiled HMC.

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	0.11 [b]	0.00	0.17 [a]	0.22 [a]
DH42+P42+LAB	0.05 [e]	0.00	0.16 [ab]	0.17 [ab]
DH42+LAB	0.11 [b]	0.00	0.17 [a]	0.15 [b]
DH42+P42	0.08 [d]	0.00	0.17 [a]	0.15 [b]
DH42	0.09 [c]	0.00	0.15 [ab]	0.15 [b]
P42	0.16 [a]	0.00	0.11 [b]	0.13 [b]
Control	0.11 [b]	0.00	0.13 [ab]	0.13 [b]
S.E.M.[n]	0.001		0.004	0.004

[abcde]Column mean with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

which was not different with the DH42+P42+LAB (0.17 g/100 g DM). The inoculation did not affect dry matter recovery (Table 2-10) throughout the ensiling period. On d 90, dry matter recovery ranged from 96.14-99.26%. The initial, d 7 and d 90 LAB population (Table 2-11) were not affected by inoculation. On d 21, silages with DH42+LAB and DH42+P42+LAB had lower ($P<0.05$) LAB counts than control silage. The LAB counts in the control silages were high and may have limited the effectiveness of the LAB portion of the inoculant. In a similar study, Wardynski et al. (1993) did not observe differences in the LAB population between the control and inoculated silages and they indicated that the epiphytic bacterial population was much greater than the amount added by the inoculant.

The initial and d 21 yeast and mold counts (Table 2-12) did not significantly differ among treatments. On d 90, the control and P42 silages had lower ($P<0.05$) yeast and mold counts compared to the DH42+P42+LAB-treated silages. Propionibacteria inoculation is expected to increase the propionic acid content of the silages. However, the d 90 propionic acid levels were not significantly different among treatments (Table 2-2). Dawson (1994) and Wardynski et al. (1993) showed reduced yeast and mold counts with PAB-inoculated HMC. Levels of propionate in the HMC was much higher than in their studies, than seen in this study (0.12 g/100g DM). At low propionic acid concentration, acid-tolerant yeasts are able to metabolize the acid (Lord et al., 1981; Magan and Lacey, 1986) further reducing the acid available.

Aerobic Phase

Propionic acid contents (Table 2-13) of the exposed silages differed ($P<0.01$) among treatments. The DH42, DH42+P42+LAB, and autoclaved DH42-treated silages

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DH42+P
DH42
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Control
S.E.M.[b]

[a]Colum
[b]Standard

Table 2-10. Effect of inoculation on dry matter recovery (%) of ensiled HMC.

Treatment	Ensiling period (d)		
	7	21	90
DH42 (autoclaved)	98.72	99.96	98.86 [ab]
DH42+P42+LAB	99.84	99.88	96.14 [b]
DH42+LAB	100.15	101.04	98.91 [ab]
DH42+P42	100.17	101.56	98.08 [ab]
DH42	98.46	99.46	99.26 [a]
P42	99.90	102.06	97.97 [ab]
Control	100.32	101.26	97.94 [ab]
S.E.M.[n]	0.24	0.51	0.26

[ab]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

Table 2-11. Effect of inoculation on lactic acid bacteria counts (log cfu/ g DM) of fresh and ensiled HMC.

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	6.37	8.64	8.95 [a]	8.02
DH42+P42+LAB	6.32	8.62	8.63 [bc]	8.40
DH42+LAB	6.54	8.64	8.61 [c]	8.51
DH42+P42	6.33	8.60	8.86 [ab]	7.57
DH42	6.46	8.59	8.89 [a]	8.16
P42	6.34	8.58	8.84 [abc]	8.09
Control	6.20	8.62	8.93 [a]	8.45
S.E.M.[n]	0.05	0.02	0.02	0.13

[abc]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

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[a]Column
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[b]Standard

Table 2-12. Effect of inoculation on yeast and mold counts (log cfu/ g DM) of fresh and ensiled HMC.

Treatment	Ensiling period (d)			
	0	7	21	90
DH42 (autoclaved)	7.69	5.59 [b]	4.00	3.25 [ab]
DH42+P42+LAB	7.63	6.22 [ab]	4.11	3.78 [a]
DH42+LAB	7.76	5.89 [ab]	4.11	2.35 [ab]
DH42+P42	7.47	6.27 [ab]	4.01	2.97 [ab]
DH42	7.50	5.95 [ab]	4.15	2.44 [ab]
P42	7.62	6.18 [ab]	3.97	2.18 [b]
Control	7.48	6.69 [a]	4.18	1.98 [b]
S.E.M.[n]	0.05	0.04	0.08	0.11

[ab]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

Table 2-13. Effect of inoculation on propionic acid content (g/100 g DM) of exposed HMC silage

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	0.11	0.12	0.14	0.10	0.12 [a]
DH42+P42+LAB	0.12	0.11	0.12	0.11	0.12 [a]
DH42+LAB	0.11	0.09	0.12	0.11	0.11 [ab]
DH42+P42	0.12	0.11	0.09	0.13	0.11 [ab]
DH42	0.11	0.11	0.13	0.12	0.12 [a]
P42	0.07	0.08	0.09	0.09	0.08 [b]
Control	0.07	0.08	0.10	0.09	0.08 [b]
S.E.M.[n]	0.01	0.01	0.01	0.01	0.01

[ab]Column means with unlike superscripts differ(P<0.07) except DH42+P42+LAB vs. control, P<0.09; DH42 vs. control, P<0.10.
[n]Standard error of the mean.

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had significantly higher propionic acid than the control and P42-treated silages. The effects of day and treatment x day interaction on propionic acid level during aerobic exposure were not significant.

Lactic acid (Table 2-14) levels were also affected ($P<0.002$) by treatment. The average lactic acid content of the DH42+LAB silages was higher ($P<0.01$) compared to the control and P42 silages with 1.36 vs. 0.83 and 0.86 g/100 g DM, respectively. Lactic acid levels changed little during exposure period. The effects of day and treatment x day interaction were not significant. Dawson et al. (1998) reported higher residual lactic acid with inoculated silages after 5 d of exposure using *P. acidipropionici* DH42. It could be due to difference in the ensiling material. They used high moisture corn while this study used reconstituted corn. Phillip and Fellner (1992) observed a 51% decrease in lactic acid content of LAB-inoculated high moisture ear corn during aerobic exposure. Unlike the present study, they did not detect any of the volatile fatty acids whose antimicrobial properties would have reduced the assimilation of lactic acid by aerobic microorganisms. The effect of treatment on pH (Table 2-15) was significant ($P<0.03$). The effect of day was also significant ($P<0.001$) but not the day x treatment interaction. The silages inoculated with DH42+LAB had significantly lower pH than the P42 and autoclaved DH42-treated silages with 4.15 vs. 4.29 and 4.32, respectively.

Acetic acid (Table 2-16) levels were also relatively stable over time. HMC treated with autoclaved DH42 culture had higher average acetic acid content compared to the control, DH42+P42+LAB and DH42+LAB silages. Wardynski et al. (1993) had higher acetic acid values with at least 3 g/100 g DM. Differences in ensiling material could account for this discrepancy. This study used reconstituted corn and had a lower

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*Column
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*Standard

Table 2-14. Effect of inoculation on the lactic acid content (g/ 100 g DM) of exposed HMC silage.

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	1.06	0.95	0.95	0.87	0.96 [b]
DH42+P42+LAB	1.13	1.05	1.14	1.11	1.11 [ab]
DH42+LAB	1.46	1.22	1.33	1.44	1.36 [a]
DH42+P42	1.10	0.95	1.06	0.95	1.01 [b]
DH42	1.15	1.02	1.08	1.20	1.11 [ab]
P42	0.80	0.81	0.89	0.92	0.86 [b]
Control	0.79	0.77	0.87	0.88	0.83 [b]
S.E.M[n]	0.11	0.11	0.11	0.11	0.07

[ab]Column means with unlike superscripts differ (P<0.05) except DH42+LAB vs. P42 and DH42+LAB vs. control, P<0.01 and DH42+LAB vs DH42+P42, P<0.10.
[n]Standard error of the mean.

Table 2-15. Effect of inoculation on pH of exposed HMC silage.

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	4.34	4.32	4.30	4.34	4.32 [a]
DH42+P42+LAB	4.27	4.26	4.21	4.27	4.25 [ab]
DH42+LAB	4.13	4.16	4.13	4.18	4.15 [b]
DH42+P42	4.26	4.27	4.27	4.29	4.27 [ab]
DH42	4.25	4.25	4.23	4.28	4.25 [ab]
P42	4.32	4.31	4.29	4.33	4.31 [a]
Control	4.29	4.28	4.28	4.32	4.29 [ab]
S.E.M[n]	0.03	0.03	0.03	0.03	0.04

[ab]Column means with unlike superscripts differ (P<0.05) except DH42 (autoclaved) vs. DH42+LAB, P<0.03.
[n]Standard error of the mean.

Table 2-16. Effect of inoculation on acetic acid content (g/100 g DM) of exposed HMC silage

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	0.45	0.51	0.57	0.48	0.50 [a]
DH42+P42+LAB	0.31	0.30	0.31	0.33	0.31 [bc]
DH42+LAB	0.26	0.25	0.30	0.32	0.28 [c]
DH42+P42	0.41	0.37	0.45	0.40	0.40 [ab]
DH42	0.35	0.34	0.39	0.44	0.38 [abc]
P42	0.32	0.35	0.40	0.42	0.37 [abc]
Control	0.34	0.37	0.44	0.43	0.39 [bc]
S.E.M[n]	0.04	0.04	0.04	0.04	0.03

[abc]Column means with unlike superscripts differ (P<0.07) except DH42 (autoclaved) vs. DH42+P42+LAB and DH42 (autoclaved) vs. DH42+LAB, P<0.002.
[n]Standard error of the mean.

moisture content compared to that used by Wardynski et al. (1993). In general, high moisture enhances the production of organic acids in high moisture corn (Goodrich et al., 1975; Baron et al., 1986; Faber et al., 1989).

During aerobic exposure, some of the silages had trace amounts of residual glucose (Table 2-17). The effect of treatment x day interaction was significant (P<0.0001). From d 0 to 3, the silages treated with DH42+LAB had generally higher residual glucose compared to other treatments. This is also the treatment with the lowest pH. The effect of acid hydrolysis on the release of sugars from cell walls could have caused the higher glucose level in this treatment. The effects of treatment and day on butyric acid (Table 2-18) levels were highly significant (P<0.0001). The average butyric

Table 2-17. Effect of inoculation on the glucose content (g/ 100 g DM) of exposed HMC silage

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	0.00 [b]	0.00 [c]	0.00 [c]	0.00	0.00
DH42+P42+LAB	0.00 [b]	0.01 [bc]	0.01 [bc]	0.00	0.00
DH42+LAB	0.01 [a]	0.02 [a]	0.02 [a]	0.01	0.01
DH42+P42	0.00 [b]	0.01 [ab]	0.01 [ab]	0.00	0.01
DH42	0.00 [b]	0.00 [c]	0.00 [c]	0.00	0.00
P42	0.00 [b]	0.01 [c]	0.00 [c]	0.00	0.00
Control	0.00 [b]	0.01 [c]	0.01 [ab]	0.00	0.01
S.E.M[n]	0.00	0.00	0.00	0.00	0.00

[abc]Column means with unlike superscripts differ (P<0.08 for d 1 and P<0.05 for d 1 and 3).
[n]Standard error of the mean.

Table 2-18. Effect of inoculation on the butyric acid content (g/ 100 g DM) of exposed HMC silage.

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	0.16	0.15	0.17	0.13	0.15 [a]
DH42+P42+LAB	0.08	0.09	0.08	0.06	0.08 [d]
DH42+LAB	0.08	0.08	0.09	0.08	0.08 [cd]
DH42+P42	0.10	0.11	0.12	0.10	0.11 [bc]
DH42	0.11	0.13	0.13	0.12	0.12 [b]
P42	0.11	0.14	0.14	0.13	0.13 [ab]
Control	0.07	0.09	0.09	0.08	0.08 [cd]
S.E.M[n]	0.01	0.01	0.01	0.01	0.01

[abcd]Column means with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

acid of the silages treated with autoclaved DH42 culture and the P42 were the highest with 0.15 and 0.13 g/ 100 g DM, respectively. Ethanol (Table 2-19) levels were high but were not affected by inoculation. Yeast fermentation could account for the high levels of ethanol in the silages since the yeast and mold population (Table 2-22) remained relatively high during the exposure phase. A significant treatment x day interaction affected the citric acid content (Table 2-20) of the exposed silages. Citric acid content of the exposed silages with autoclaved DH42 culture was generally higher than other treatments from d 0 up to d3. LAB population remained high throughout the exposure period (Table 2-21). Treatment did not affect the LAB population of the silages. Likewise, inoculation did not affect the yeast and mold counts (Table 2-22) during the exposure period. Dawson et al. (1998) reported lower yeast and mold counts with DH42-inoculated high moisture corn silages during aerobic exposure. Their study however indicated higher propionic acid levels in the treated silages and lower initial yeast and mold counts. In this study, the initial yeast and mold counts were about 10^7 cfu/ g DM, while Dawson et al. (1998) reported 10^4cfu/g DM. The differences in inoculation rate and the quality of the ensiling material could account for the discrepancy in results.

Throughout the exposure period, the dry matter (Table 2-23) of the control silages remained highest. The inoculated HMC tended to have lower dry matter contents than the control. Table 2-24 shows the temperature of the silages during the 5-d aerobic exposure. The effect of treatment on temperature was significant ($P<0.01$). The control and P42-treated silages had higher temperature compared to DH42+LAB-treated silages. However, temperature differences were small that it is likely to have minimal effects on DM recovery or spoilage. Phillip and Fellner (1991) also observed no significant

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Table 2-20
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Table 2-19. Effect of inoculation on ethanol content (g/100 g DM) of exposed HMC silage.

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	0.51	0.40	0.32	0.20	0.36
DH42+P42+LAB	0.28	0.27	0.28	0.18	0.25
DH42+LAB	0.38	0.35	0.29	0.18	0.30
DH42+P42	0.49	0.42	0.29	0.21	0.35
DH42	0.41	0.36	0.29	0.20	0.32
P42	0.46	0.42	0.36	0.25	0.37
Control	0.40	0.38	0.31	0.20	0.32
S.E.M[n]	0.04	0.04	0.04	0.04	0.03

[n]Standard error of the mean.

Table 2-20. Effect of inoculation on citric acid content (g/100 g DM) of exposed HMC silage.

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	0.22 [a]	0.27 [a]	0.26 [a]	0.17	0.23
DH42+P42+LAB	0.17 [ab]	0.26 [ab]	0.17 [b]	0.14	0.18
DH42+LAB	0.15 [b]	0.21 [ab]	0.17 [b]	0.15	0.17
DH42+P42	0.15 [b]	0.22 [ab]	0.19 [b]	0.13	0.17
DH42	0.15 [b]	0.22 [ab]	0.16 [b]	0.15	0.17
P42	0.13 [b]	0.21 [ab]	0.14 [b]	0.13	0.15
Control	0.13 [b]	0.20 [b]	0.15 [b]	0.13	0.15
S.E.M[n]	0.01	0.01	0.01	0.01	0.01

[ab]Column means with unlike superscripts differ ($P<0.03$ for d 0, $P<0.04$ for d 1, $P<0.02$ for d 3).
[n]Standard error of the mean.

Table 2-21. Effect of inoculation on the lactic acid bacteria counts (log cfu/ g DM) of exposed HMC silage.

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	8.02	8.67	8.57	8.69	8.49
DH42+P42+LAB	8.40	8.49	8.46	8.60	8.49
DH42+LAB	8.51	8.44	8.32	8.30	8.39
DH42+P42	7.57	8.47	8.30	8.38	8.18
DH42	8.16	8.44	8.30	8.40	8.33
P42	8.09	8.36	8.20	8.32	8.24
Control	8.45	8.42	8.18	8.23	8.32
S.E.M[n]	0.18	0.18	0.18	0.18	0.10

[n]Standard error of the mean.

Table 2-22. Effect of inoculation on the yeast and mold counts (log cfu/ g DM) of exposed HMC silage.

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	3.25	1.43	1.63	2.30	2.15
DH42+P42+LAB	3.78	2.75	2.73	2.97	3.06
DH42+LAB	2.35	2.46	2.81	3.08	2.67
DH42+P42	2.97	2.56	1.98	3.01	2.63
DH42	2.44	2.84	2.09	3.24	2.65
P42	2.18	2.90	2.36	3.11	2.51
Control	1.98	2.80	2.27	3.02	2.63
S.E.M[n]	0.33	0.33	0.33	0.33	0.19

[n]Standard error of the mean.

Table 2-23. Effect of inoculation on the dry matter (%) of exposed HMC silage.

Treatment	Exposure period (d)				Average
	0	1	3	5	
DH42 (autoclaved)	69.65 [d]	70.18 [c]	70.16 [c]	71.88 [c]	70.48
DH42+P42+LAB	71.30 [bc]	71.74 [b]	71.84 [b]	73.25 [bc]	72.06
DH42+LAB	72.21 [ab]	72.42 [b]	72.42 [b]	74.20 [ab]	72.81
DH42+P42	71.99 [abc]	72.63 [b]	72.60 [b]	74.30 [ab]	72.88
DH42	70.56 [cd]	71.27 [bc]	71.27 [bc]	73.08 [bc]	71.54
P42	71.82 [abc]	72.64 [b]	72.64 [b]	74.28 [ab]	72.85
Control	72.91 [a]	74.34 [a]	74.32 [a]	75.47 [a]	74.26
S.E.M[n]	0.24	0.24	0.24	0.24	0.20

[abcd]Column means with unlike superscripts differ (P<0.01, except for d 5 with P<0.001).
[n]Standard error of the mean.

Table 2-24. Effect of inoculation on temperature (°C) of exposed HMC silage.

Treatment	Exposure period (d)						Average
	0	1	2	3	4	5	
DH42 (autoclaved)	22.41	22.41	21.67	21.67	21.48	21.48	21.85 [ab]
DH42+P42+LAB	22.41	22.50	21.85	21.85	21.67	21.48	21.96 [ab]
DH42+LAB	21.85	21.76	21.21	21.11	21.30	21.11	21.39 [b]
DH42+P42	22.78	22.60	21.57	21.67	21.67	21.48	21.96 [ab]
DH42	23.15	23.06	22.13	22.22	21.85	22.59	22.50 [ab]
P42	23.33	23.15	22.78	22.78	22.22	22.41	22.78 [a]
Control	23.33	23.33	22.97	23.15	22.78	22.78	23.06 [a]
S.E.M.[n]	0.34	0.34	0.34	0.34	0.34	0.34	0.27

[ab]Column means with unlike superscripts differ (DH42+LAB vs. control, P<0.01; DH42+LAB vs. P42, P<0.06).
[n]Standard error of the mean.

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correlation between the chemical criteria and temperature criteria of aerobic stability in high moisture corn silage. They concluded that any inferences regarding the effect of bacterial inoculation on aerobic stability depended on the criterion chosen.

Implications

This study shows that DH42 in co-culture with lactic acid bacteria enhanced the fermentation of reconstituted corn only up to 21 d of ensiling. P42 inoculation had marginal effect as an inoculant to the reconstituted corn.

The silages appeared stable based on the lack of changes in residual organic acids during aerobic exposure. The silages treated with DH42+LAB reconstituted had relatively higher organic acids than other treatments. However, yeast and mold counts were not effectively reduced by the inoculation of DH42. Since the control appeared well-preserved if based on the yeast and mold counts, the effect of propionibacteria inoculation in reducing yeast and mold counts was not observed in this study. Further studies need to be conducted to determine the effective inoculation rate and other factors that affect the growth of DH42 in silage.

CHAPTER 3

EFFECT OF MOISTURE ON PROPIONIBACTERIA AS INOCULANTS TO HIGH MOISTURE CORN SILAGE

Abstract

Rolled corn of different moisture contents (35.2%, 32.9%, 27.9%, 24.1%, 23.1%, and 21.9%) were inoculated with control (sterile distilled water), *P. acidipropionici* DH42 at 10^5 colony forming units (cfu) per gram of fresh corn, *P. acidipropionici* DH42 at 10^6 cfu/g, *P. jensenii* at 10^6 cfu/g and uninoculated sterile Reinforced Clostridial Medium broth. Silos were opened after 10, 21, and 120 days of ensiling. Samples from the 120-d silos were also taken and exposed to air for 5 days. Fresh, ensiled and exposed samples were taken for microbial and chemical analyses.

The moisture of the ensiling material affected the efficacy of propionibacteria as inoculants. PAB-inoculated high moisture corn gave significantly higher propionic and acetic acid production at 22-28% and 23% moisture levels, respectively after 120 d of ensiling. Lower pH at 24% and butyric acid at 28% was also observed with the PAB-treated silages. Inoculation did not affect the yeast and mold counts during ensiling. *P. acidipropionici* DH42 inoculated at 10^6cfu/g better gave results as compared to the 10^5cfu/g inoculation rate.

During aerobic exposure, higher propionic and acetic acids were observed with the PAB-inoculated silages at 22-28% moisture levels. On the other hand, lower pH and lactic acid were observed with the PAB-inoculated silages at 22-28% and 22%, respectively. While lower yeast and mold counts were observed in the PAB-treated

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silages at 22-23% moisture levels compared to the control, they did not differ with the RCM-treated silages.

Introduction

Propionic acid has been used considerably in preservation of stored grains (Patkar et al., 1995) and bakery products (Huitson, 1968; Javainen and Linko, 1993) due to its antifungal properties. In silage, the benefits of propionic acid treatment had been demonstrated (Britt et al., 1975; Hara and Ohyama, 1979; Ashbell et al., 1984; Rahnema and Neal, 1992). One drawback for its widespread use is that it is corrosive. The use of propionic acid-producing bacteria (PAB) appears advantageous particularly with increasing consumer preference for natural products. Improved fermentation of PAB-treated silages were observed but the effects on aerobically exposed silages were variable (Flores-Galarza et al., 1985; Alio et al., 1994; Weinberg, et al., 1995; Kreikemier et al., 1997; Higginbotham et al., 1998; Dawson et al., 1998;). More information is needed to determine the factors affecting efficacy of PAB as silage inoculants. This study evaluated the effects of the moisture content of corn grain in the efficacy of two *Propionibacteria* species as inoculants on the fermentation profile and aerobic stability of silage.

Materials and Methods

Inoculants

Propionibacterium jensenii (ATCC 53962) was purchased from American Type Culture Collection (ATCC) while *P. acidipropionici* DH42 (ATCC 55737) was obtained from cultures maintained in the microbiology laboratory of the Department of Animal Science, Michigan State University, East Lansing, Michigan. Both inoculants were

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grown anaerobically in Reinforced Clostridial Medium (Unipath, England). Before use, at least three transfers to a fresh medium were made to ensure that the cultures were active. The cultures were incubated anaerobically at 30°C for about 18 h before use.

Silage preparation

During the fall of 1996, corn was harvested weekly to gather ensiling materials of various moisture contents. The freshly harvested corn was rolled prior to ensiling. Laboratory silos made of polyvinyl chloride (PVC) pipes (47.5x10.2 cm diameter) with rubber caps fitted with policeman (Fisher) were used. The different moisture contents at ensiling were: 35.2%, 32.9%, 27.9%, 24.1%, 23.1%, and 21.9%. In each moisture level, corn was divided and allotted to five inoculant treatments: control (sterile distilled water), *P. acidipropionici* DH42 at 10^5 colony forming units (cfu) per gram of fresh corn, *P. acidipropionici* DH42 at 10^6 cfu/g, *P. jensenii* at10^6 cfu/g and uninoculated sterile Reinforced Clostridial Medium broth (Unipath, England). The latter treatment was added to determine the effects of the nutrients in the fermentation broth in the growth of epiphytic bacteria. The amounts of inoculants needed to meet the inoculation rate were estimated from optical density (OD) readings of the inocula prior to inoculation. Linear regression correlating the OD (at 600 nm) with colony forming unit counts had been prepared separately for each inoculant. Based on the OD value, estimated cfu/ml of the inoculum was calculated using the regression line. Serial dilutions of the starter cultures were also made and the appropriate dilutions plated in Reinforced Clostridial Medium. The plates were incubated anaerobically at 30°C for 5 d. This was done to validate the calculated estimates of the microbial counts. Prior to inoculation, sterile distilled water was added to bring the final volume of the inoculant to 100 ml so as not to alter the

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moisture level by adding different volumes of liquid. The inoculants were added to the corn in a plastic tub and mixed by hand. Different tubs and latex gloves were used for each treatment to prevent any cross-contamination. Four replicate silos were prepared for each treatment and time combination. However, the 35 and 22% moisture levels had three replicates since the amount of harvested corn was limited. In addition, 28% moisture level had eight replicates for the d 0 and 120 sampling periods since the initial moisture contents of the corn for two successive weeks were not significantly different from each other. Hence the data were pooled to reflect one moisture level. The silos were opened after 10, 21 and 120 d of ensiling. Silos were weighed before and after ensiling and the difference in dry matter weights was expressed as dry matter recovery.

Aerobic Stability Evaluation

Ensiled corn from the 120-d collection was used for aerobic stability evaluation. About 1 kg from each sample was weighed into a plastic bag, placed in a Styrofoam container and exposed to air. Cooking thermometers were inserted into each bag to monitor silage temperature. Morning and afternoon temperatures were taken and the average used for analyses. Temperatures were monitored for 5 d. Samples for laboratory analyses were collected after 1, 3, and 5 d of aerobic exposure.

Chemical and Microbial Analyses

Fresh, fermented and exposed corn samples were taken for chemical and microbial analyses. Aqueous extracts were prepared by adding 450 ml sterile, 0.9% NaCl to 50 g silage sample, homogenized in a Stomacher (Tekmar, Model 3500) for 5 min and

strained into sterile 50-ml centrifuge tubes. The pH of the extracts was determined using a pH meter (Cole Palmer, Model 05569-20).

The dry matter was determined by forced-air oven drying at 64°C for 48 h. The difference in weights before and after drying was expressed as the dry matter. Glucose, ethanol and organic acids were measured by ion exchange-exclusion high performance liquid chromatography (HPLC) as described by Dawson (1994) with slight modification. The extracts were centrifuged (26,000 x g) for 30 min and the supernatant used for the HPLC analyses.

Serial dilutions of the extracts were prepared in 1% Bacto-peptone broth (Difco) and appropriate dilutions were plated in various selective media. Rose Bengal Agar (Difco) with Antimicrobic Supplement C (Difco) was used to estimate yeast and molds and the plates were incubated aerobically at 39°C for 2 to 3 d. Pal propiobac (Standa Industrie, France) was used to estimate propionibacteria in 22, 28, 33 and 35% moisture levels. Plates were incubated anaerobically at 30°C for 5 d. Yellow colonies were counted and presumptively identified as propionibacteria (Thierry and Madec, 1995). Yeast and mold counts were done for fresh, ensiled and exposed corn samples while propionibacteria counts were done for d 0 and 120.

Statistical Analyses

Data for the fermentation phase were analyzed as a one-way completely randomized design using the General Linear Model subroutine of the Statistical Analysis System (SAS, 1990). Microbial counts were analyzed using the transformed data ($\log_{10}$ [Y], where Y is the microbial count). Data were analyzed by moisture level. Due to the increasing variability of the various parameters over time, the data were analyzed

separately for each collection period (i.e. d 0, 10, 21, 120). At a given moisture level and collection period, the model used for each parameter (e.g. pH, propionic acid, acetic acid) was as follows:

$Y_i = \mu + \alpha_i + e_i$

Where:

Y	=	individual variable measured (e.g. pH, propionic acid, glucose)
μ	=	overall mean
α_i	=	effect of treatment
e_i	=	random residual error

For the aerobic stability phase, data were analyzed by PROC MIXED of the SAS, using the repeated measures analysis (SAS, 1990) since repeated samplings from the same sample were done. Treatment means were compared using the Bonferroni (SAS, 1990).

Results and Discussion

Fermentation Phase

Initial propionic acid contents (Appendix Table A-1) of the corn were similar across treatments. This indicates that the amount of propionic acid in the propionibacteria-containing inoculants was low and had very little impact on the initial propionic acid content of the corn. Moisture and PAB inoculation (Figures 3-1 to 3-6) affected the propionic acid content of silages. On d 10, the silage treated with DH42 at 10^6 cfu had the highest propionic acid at 28 % moisture levels. At 24% moisture level, the same treatment had similar propionic acid level with the *P. jensenii*-treated silages but higher than the other treatments. On d 21, propionic acid of DH42-treated silages

inoculated at 10^6 cfu/g at 24 and 28% moisture levels were the highest ($P<0.01$) with 0.14% and 0.15%, respectively. At the end of the ensiling period, the same treatment

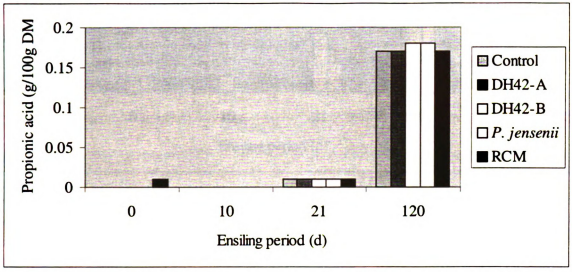

Figure 3-1. The effect of inoculation on the propionic acid of fresh and ensiled high moisture corn at 35% moisture level (Standard error of the means: d0=0, d10=0. d21=0, d120=0.01).

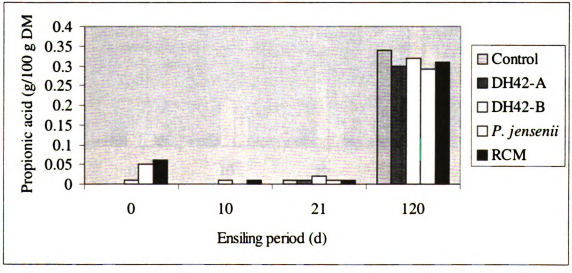

Figure 3-2. The effect of inoculation on the propionic acid of fresh and ensiled high moisture corn at 33% moisture level (Standard error of the means: d0=0.01, d10=0, d21=0, d120=0.01).

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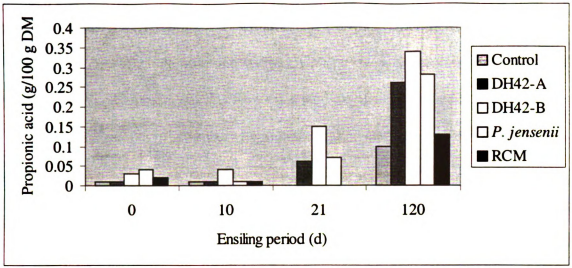

Figure 3-3. The effect of inoculation on the propionic acid of fresh and ensiled high moisture corn at 28% moisture level (Standard error of the means: d0=0, d10=0, d21=0, d120=0.01).

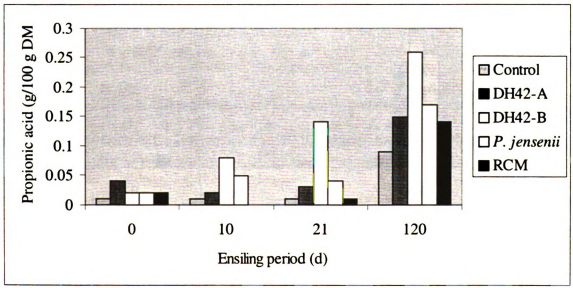

Figure 3-4. The effect of inoculation on the propionic acid of fresh and ensiled high moisture corn at 24% moisture level (Standard error of the means: d0=0, d10=0, d21=0.01, d120=0.01).

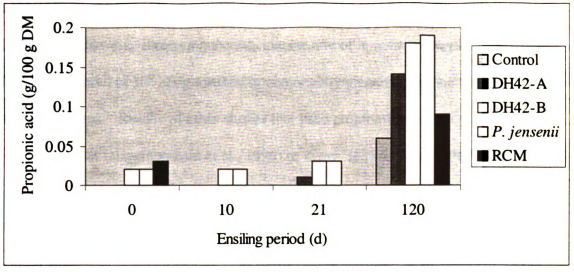

Figure 3-5. The effect of inoculation on the propionic acid of fresh and ensiled high moisture corn at 23% moisture level (Standard error of the means: d0=0.01, d10=0, d21=0, d120=0.01).

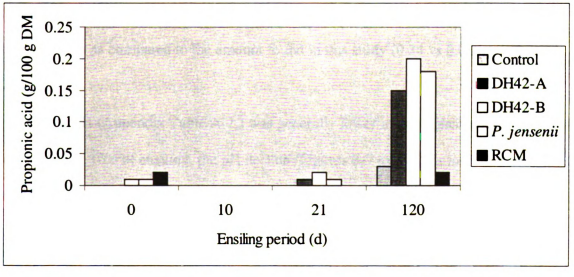

Figure 3-6. The effect of inoculation on the propionic acid of fresh and ensiled high moisture corn at 22% moisture level (Standard error of the means: d0=0, d10=0, d21=0, d120=0.01).

had higher ($P<0.05$) propionic acid than the control and RCM-inoculated silages at 22-28% moisture levels. Increasing the inoculation rate of *P. acidipropionici* DH42 from 10^5 cfu/g material to 10^6 cfu/g resulted in numerically greater propionic acid levels in the resulting silages. Results of other studies that used propionibacteria as silage inoculants, reported similar (Higginbotham et al., 1996) or lower (Kreikemeier, 1997) propionic acid levels. Higginbotham et al. (1998) indicated that propionic acid was undetectable in the corn silage inoculated with *P. acidipropionici.* Propionic acid levels found in the present study was lower compared to an earlier study (Dawson et al., 1998) using the same inoculant. Using high moisture corn with 74% dry matter, Dawson et al. (1998) reported propionic acid content of 0.35g/100 g DM after 42 d of ensiling. In the present study, the 28% moisture level had about 0.34g/100 g DM propionic acid after 120 d of ensiling. Differences in the quality of the ensiling material could account for this discrepancy. It was noted that in the earlier study, the high moisture corn had higher initial glucose as compared to the amount found in this study (0.34 vs 0.035 g/100 g DM).

The pH (Appendix Table A-2,) was generally lower in the higher moisture level silages. After 10 d of ensiling, the pH decline (Figures 3-7 to 3-12) is most apparent with the 33 and 35% moisture levels. The pH at 35% moisture level ranged from 3.89-3.92 while at 22% moisture level, the pH ranged from 5.28-5.30. The effect of inoculation was observed only after d 21 of ensiling at 22% moisture level. The DH42-10^6 inoculated silages had significantly lower pH compared to the control and RCM-inoculated silages but similar to the other PAB-inoculated silages. After 120 d of ensiling, the 35% moisture level had pH ranging from 3.70-3.75 while the 22% moisture level ranged from

4.55-4.67. The effect of PAB inoculation was observed at 22 and 24% moisture levels. The *P. jensenii* and DH42-10^6 inoculated silages at 24% moisture levels had lower ($P<0.05$) pH than the control and RCM-inoculated silages. At 22% moisture level, the

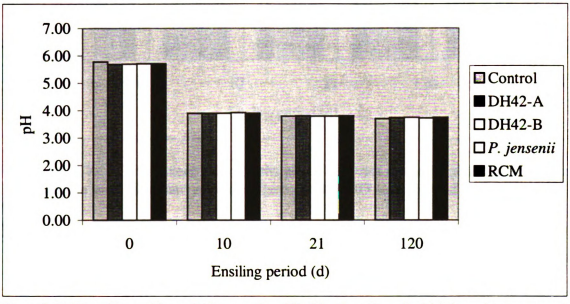

Figure 3-7. The effect of inoculation on the pH of fresh and ensiled high moisture corn at 35% moisture level (Standard error of the means: d0=0.02, d10=0.01, d21=0, d120=0.01).

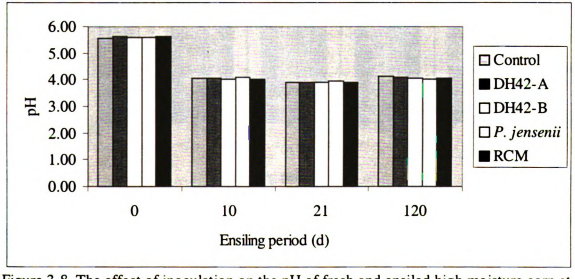

Figure 3-8. The effect of inoculation on the pH of fresh and ensiled high moisture corn at 33% moisture level(Standard error of the means: d0=0.02, d10=0.01, d21=0.01, d120=0.02).

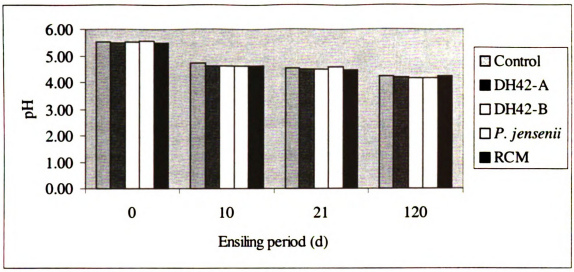

Figure 3-9. The effect of inoculation on the pH of fresh and ensiled high moisture corn at 28% moisture level (Standard error of the means: d0=0.03, d10=0.01, d21=0.01, d120=0.01)

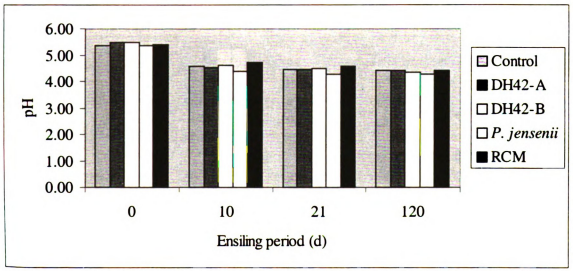

Figure 3-10. The effect of inoculation on the pH of fresh and ensiled high moisture corn at 24% moisture level (Standard error of the means: d0=0.02, d10=0.04, d21=0.03, d120=0.10)

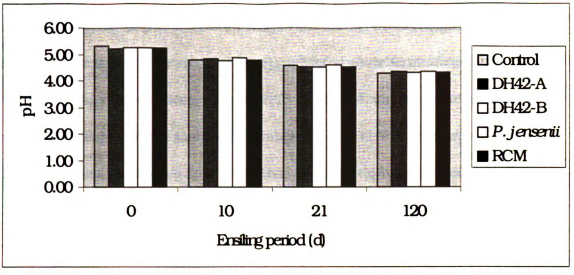

Figure 3-11. The effect of inoculation on the pH of fresh and ensiled high moisture corn at 23% moisture level (Standard error of the means: d0=0.02, d10=0.03, d21=0.02, d120=0.01)

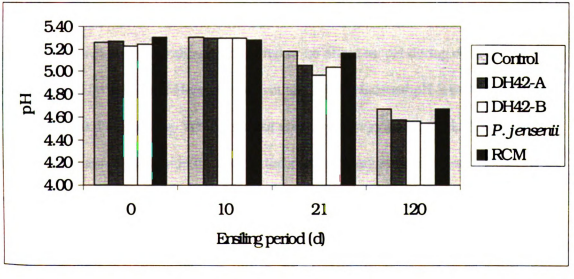

Figure 3-12. The effect of inoculation on the pH of fresh and ensiled high moisture corn at 22 % moisture level (Standard error of the means: d0=0.02, d10=0.02, d21=0.01, d120=0.01)

PAB-inoculated silages had significantly lower pH compared to the uninoculated silages (control and RCM). The 22% moisture silages had the highest pH ranging from 4.55-4.67. This indicates that the propionibacteria enhanced silage fermentation. Kreikemeir et al. (1997) also observed lower pH with PAB-inoculated silages compared to the uninoculated silages. In the study of Higginbotham et al. (1997), *P. acidipropionici* silages inoculated at 10^6 cfu/g fresh forage had lower pH than the uninoculated silages on d 30 but differences were not observed after 100 d of ensiling. Weinberg et al. (1995a, b) and Higginbotham et al. (1998) observed only marginal effect of propionibacteria inoculation on silage pH. The effect of moisture on propionibacteria is possibly due to its effect on the pH. The lowest propionic acid production was observed in silages with the lowest pH level indicating the possible inhibitory effect of acidic conditions to the growth of propionibacteria. Previous reports using *P. shermanii* as inoculant to pearl millet and maize silages (Weinberg et al., 1995a) and wheat and sorghum silages (Weinberg et al., 1995b), and *P. acidipropionici* to whole-plant corn silage (Higginbotham et al., 1998) showed marginal or no effect on pH during ensilement. Weinberg et al. (1995a) and Higginbotham et al. (1998) observed pH levels ranging from 3.7-4.0 after 90 d of ensiling. When used for ensiling high moisture corn, *P. shermanii* reduced the yeast population in the inoculated silages (Flores-Galarza et al., 1985). In this study, the pH of the resulting silages ranged from 4.53-4.73 after 60 d. Higginbotham et al. (1998) reported that propionic acid was undetectable indicating that the growth of propionibacteria was not sustained during the ensiling process. In the study of Florez-Galarza et al. (1985), the higher pH could have favored the growth of propionibacteria resulting in decreased yeast population. Pahlow and Honig (1994) indicated that the

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Figures 3-13

production of propionic acid by propionibacteria ceases below pH 4.8. Moreover, Weinberg et al. (1995b) recommended PAB inoculation in slow-fermenting grass silages. Perez-Chaia et al. (1995) observed that the growth of propionibacteria grown in mixed cultures with *Lactobacilli* was inhibited when there is rapid pH reduction, but not when there is slow pH reduction. In a modeling study done by Pitt (1997), he noted that there is a wide variation in studies about the optimum pH for growth of PAB which ranged from pH 3.2-7.0. This is probably due to differences in the sensitivity to pH among propionibacteria strains. Rehberger and Glatz (1998) indicated that while there are differences to pH sensitivity, none of the strains they tested was able to initiate growth at pH below 5. In the present study, the rate of pH decline is most apparent at 35% moisture level. After 10 d of ensiling, silages in this moisture level had pH ranging from 3.89-3.92. After 120 d of ensiling, most of the silages had pH of about 4.0 while the 35% moisture level had the lowest ($P<0.01$) pH. Even at this pH level, propionic acid was about 0.169 g/100g DM. Since *P. acidipropionici* DH42 had been isolated from high moisture corn silage, this strain is presumably more adapted to the acidic silage environment than other strains used in other studies. When grown in glucose, *P. acidipropionici* DH42 had acceptable growth rates between pH 4.9 to 7.8 (Dawson, 1994). Perez-Chaia et al. (1988) observed that among the propionibacteria strains they studied, *P. acidipropionici* was the only one that showed activity at pH values that were inhibitory for growth of propionibacteria indicating that this species could be more resistant to low pH.

The effect of PAB inoculation on the acetic acid content (Appendix Table A-3, Figures 3-13 to 3-18) of the silages was observed on d 21 at 24% moisture level. The

Acetic acid (g/100 g

0.25
0.20
0.15
0.10
0.05
0.00

Figure 3-13.
moisture con
d21=0, d120

Acetic acid (g/100 g

0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00

Figure 3-14.
moisture co
d21=0, d12

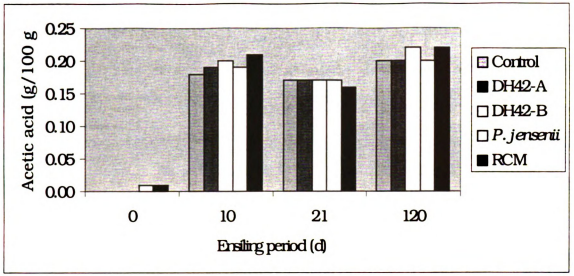

Figure 3-13. The effect of inoculation on the acetic acid of fresh and ensiled high moisture corn at 35% moisture level (Standard error of the means: d0=0, d10=0.01, d21=0, d120=0.01)

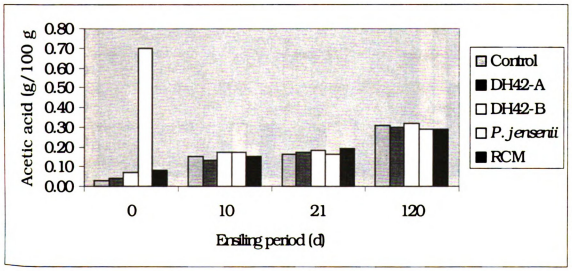

Figure 3-14. The effect of inoculation on the acetic acid of fresh and ensiled high moisture corn at 33% moisture level (Standard error of the means: d0=0, d10=0.01, d21=0, d120=0.01)

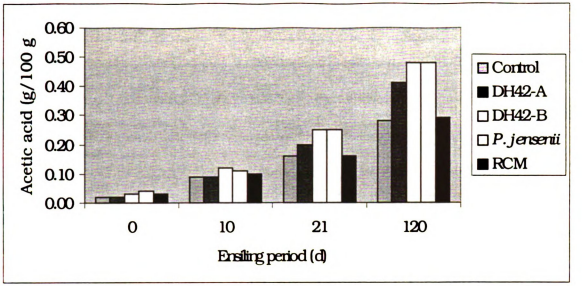

Figure 3-15. The effect of inoculation on the acetic acid of fresh and ensiled high moisture corn at 28% moisture level (Standard error of the means: d0=0, d10=0, d21=0.01, d120=0.03)

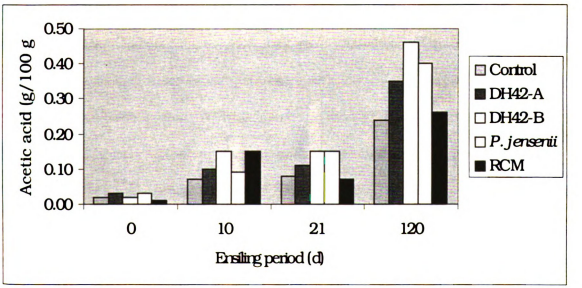

Figure 3-16. The effect of inoculation on the acetic acid of fresh and ensiled high moisture corn at 24% moisture level (Standard error of the means: d0=0.02, d10=0.02, d21=0.01, d120=0.01)

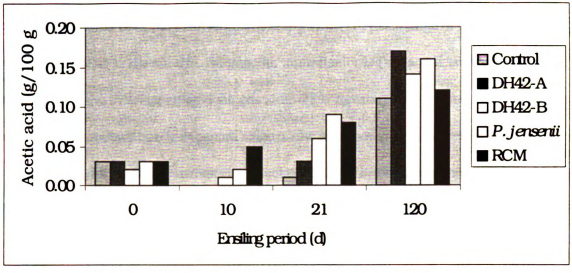

Figure 3-17. The effect of inoculation on the acetic acid of fresh and ensiled high moisture corn at 23% moisture level (Standard error of the means: d0=0.02, d10=0.02, d21=0.01, d120=0.01)

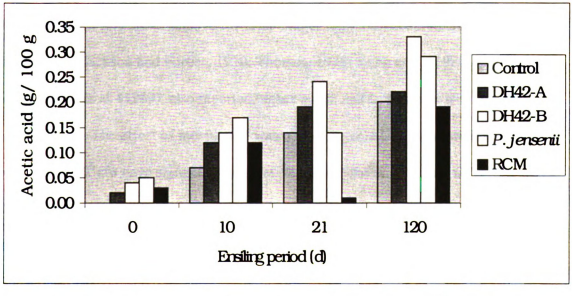

Figure 3-18. The effect of inoculation on the acetic acid of fresh and ensiled high moisture corn at 22% moisture level (Standard error of the means: d0=0, d10=0, d21=0.01, d120=0.01)

PAB-treated silages had higher acetic acid than the control and the RCM-treated silages. After 120 d of ensiling and at 23% moisture level, the DH42 with 10^6 cfu had higher acetic acid than the control (0.33 vs 0.20 g/100 g DM). *P. jensenii* gave 0.29 g/100 g DM which was similar to the control. In general, numerically higher acetic acid values were observed with the PAB-inoculated silages at 23-28% moisture levels. Increased acetic acid with PAB inoculation is expected since acetic acid is also produced in addition to propionic acid during the fermentation of lactate and glucose by propionic acid bacteria (Wood, 1981). High propionic acid and acetic acid levels in silage is beneficial because of their antifungal properties. Moon (1989) observed synergistic effects of mixtures of acetic, lactic and propionic against acid-tolerant yeasts.

Moisture level affected the lactic acid content of the silages (Appendix-Table A-4, Figures 3-19 to 3-24). Higher lactic acid production was observed with the high moisture silages. On d 120, the 35% moisture level gave lactic acid values ranging from 3.56-3.69 g/100 g DM while the 22% moisture level gave values ranging 0.18 to 0.54 g/100 g DM. The lower acetic and lactic acids in the drier silages tend to indicate restricted fermentation (Jackson and Forbes, 1970; Thomas, 1978; Kung et al., 1984; Luchini et al., 1997). Garcia et al. (1989) also reported higher acetic and lactic acids with low dry matter silages. The effect of inoculation was observed with 22% moisture level where the DH42 at 10^6 cfu gave significantly lower lactic acid than the control and RCM-treated silages. This could be due to the increased lactic acid utilization of the DH42 in this treatment. In addition to glucose, propionibacteria can metabolize lactic acid (Gottschalk, 1985).

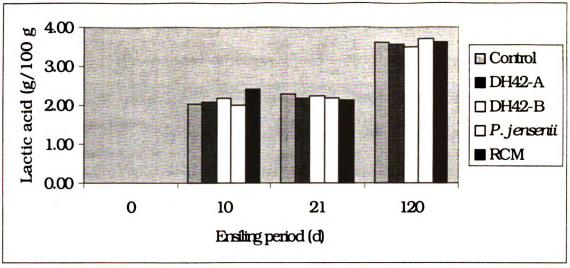

Figure 3-19. The effect of inoculation on the lactic acid content of fresh and ensiled high moisture corn at 35% moisture level (Standard error of the means: d0=0, d10=0.06, d21=0.03, d120=0.13).

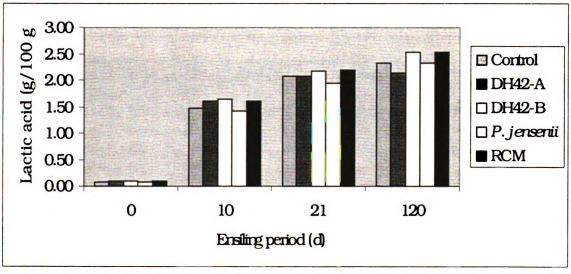

Figure 3-20. The effect of inoculation on the lactic acid content of fresh and ensiled high moisture corn at 33% moisture level (Standard error of the means: d0=0.01, d10=0.04, d21=0.03, d120=0.06).

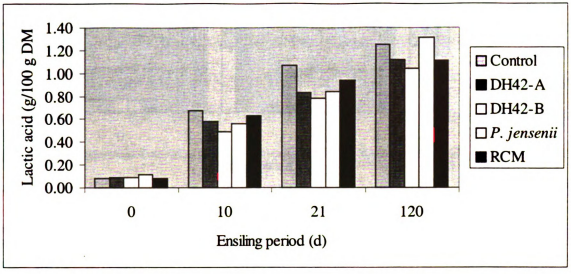

Figure 3-21. The effect of inoculation on the lactic acid content of fresh and ensiled high moisture corn at 28% moisture level (Standard error of the means: d0=0.01, d10=0.02, d21=0.02, d120=0.05).

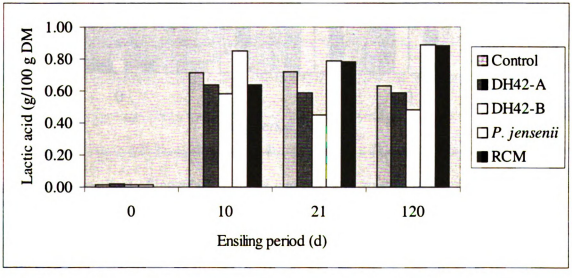

Figure 3-22. The effect of inoculation on the lactic acid content of fresh and ensiled high moisture corn at 24% moisture level(Standard error of the means: d0=0, d10=0.13, d21=0.05, d120=0.05).

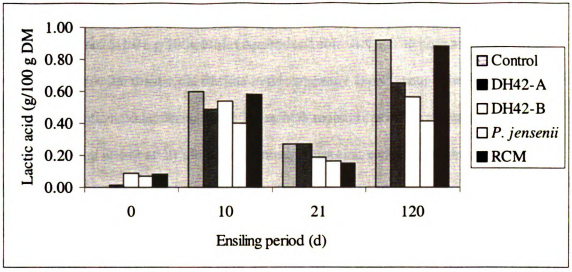

Figure 3-23. The effect of inoculation on the lactic acid content of fresh and ensiled high moisture corn at 23% moisture level(Standard error of the means: d0=0.01, d10=0.03, d21=0.02, d120=0.05).

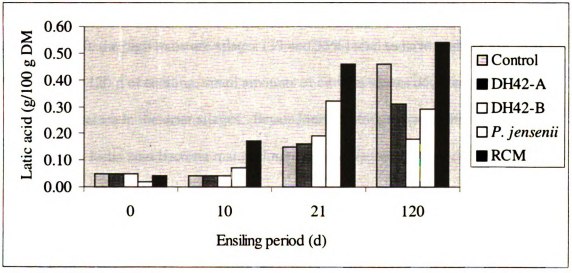

Figure 3-24. The effect of inoculation on the lactic acid content of fresh and ensiled high moisture corn at 22% moisture level(Standard error of the means: d0=0.01, d10=0.01, d21=0.02, d120=0.02).

The corn from the 35% moisture level had the highest initial glucose content ranging from 0.75-1.01 g/100g DM (Appendix Table A-5). The high available sugar may account for the drastic pH decline in this moisture level through the fermentation of glucose by lactic acid bacteria. On d 21, at 24% moisture level the silages with *P. acidipropionici* DH42 at 10^6cfu gave glucose values that were higher than *P. jensenii*-treated silages (0.12 vs 0.04 g/100 g DM). On d 120, the 33% moisture level had higher glucose levels regardless of inoculant. Glucose values ranged from 0.36-0.41g/100 g DM. Glucose, particularly in the high moisture silages appears to be high even after 120 d of ensiling. This could be due to the lower pH in the wetter silages. Jones et al. (1992) indicated that decrease in pH promotes hydrolysis of sugars from cell walls.

Citric acid (Appendix Table A-6) was also detected in the silage. McDonald et al. (1991) indicated that in herbage, citric and malic acids are quantitatively most important. On d 0 and 10, the high moisture silages (33 and 35%) tend to have higher citric acid levels. After 120 d of ensiling, small amounts of citric acid was detected in the silages with higher values in the drier silages. Bryan-Jones (1969) indicated that citric acid is fermented by lactic acid bacteria mainly *Enterococcus faecalis*.

Ethanol (Appendix Table A-7) tends to be higher with the high moisture silages. Differences among inoculants were observed only at 28% moisture level at d 120. The silages with DH42 at 10^6 cfu had lower ($P<0.05$) ethanol concentration than the control silages (0.49 vs 0.75 g/100 g DM). Weinberg et al. (1995b) and Kreikemeier et al. (1997) also observed lower ethanol values in PAB-inoculated silages. However, Dawson et al. (1998) found no difference in ethanol levels between DH42-treated silages

and uninoculated high moisture corn silages while Higginbotham et al. (1998) found higher ethanol concentration in the inoculated silages.

Butyric acid (Appendix Table A-8) was detected after 21 d of ensiling in silages from the 24% moisture level. The propionibacteria-inoculated silages from the 28% moisture levels gave lower butyric acid levels compared to the uninoculated silages. Butyric acid is usually caused by clostridial growth occurring at the latter stages of ensiling but other organisms such as yeasts and *Bacillus* spp. also produce small amounts (Mc Donald et al., 1991). Dry matter recovery (Appendix Table A-9) was affected by moisture but not by inoculation. The 28-33 % moisture levels gave the lowest dry matter recovery throughout the ensiling period. On d 120, 33% moisture level gave the lowest DM recovery values while the highest was with the 23% moisture level. Dawson et al. (1998) found higher DM recovery with inoculated high moisture corn silages while Kreikemeier et al. (1997) and Higginbotham et al. (1998) found the reverse. Higginbotham et al. (1998) indicated that the high initial moisture of the corn plant and bacterial inoculation allowed extensive fermentation of the silages reducing dry matter recovery in the inoculated silages.

Yeast and mold counts (Appendix Table A-10, Figures 3-25 to 3-30) decreased as ensiling progressed. After 10 d of ensiling, reduction in yeast and mold counts were observed at 24% moisture level. The DH42-10^6 cfu/g treated silages had lower yeast and mold counts than the RCM-treated silages but similar to the control. At d 21, the *P. jensenii*-treated silages had significantly lower yeast and mold counts than the control at 22 and 28% moisture levels. On d 120, regardless of moisture level, the *P.*

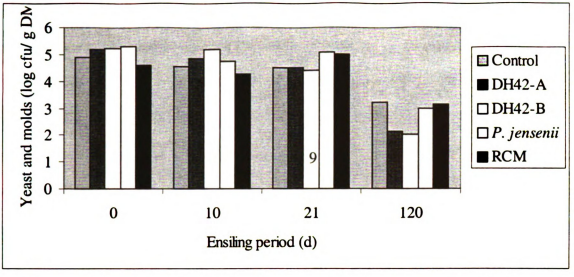

Figure 3-25. The effect of inoculation on the yeast and mold counts of fresh and ensiled high moisture corn at 35% moisture level (Standard error of the means:d0=0.08, d10=0.11, d21=0.28, d120=0.22).

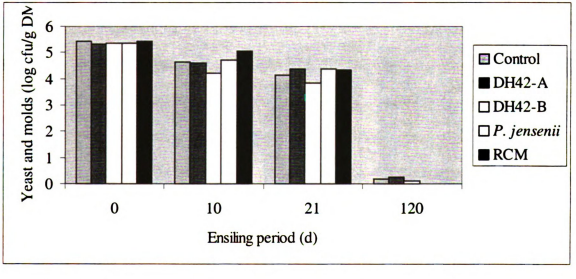

Figure 3-26. The effect of inoculation on the yeast and mold counts of fresh and ensiled high moisture corn at 33% moisture level (Standard error of the means:d0=0.05, d10=0.07, d21=0.06, d120=0.06).

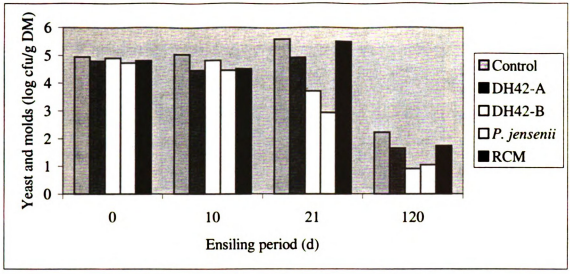

Figure 3-27. The effect of inoculation on the yeast and mold counts of fresh and ensiled high moisture corn at 28% moisture level (Standard error of the means:d0=0.07, d10=0.08, d21=0.17, d120=0.20).

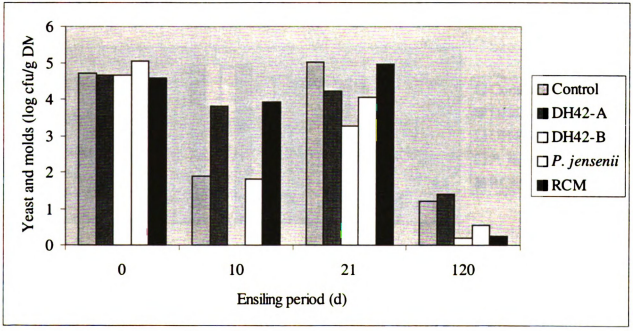

Figure 3-28. The effect of inoculation on the yeast and mold counts of fresh and ensiled high moisture corn at 24% moisture level (Standard error of the means:d0=0.06, d10=0.31, d21=0.22, d120=0.26).

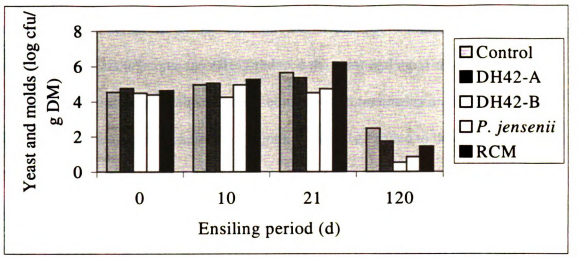

Figure 3-29. The effect of inoculation on the yeast and mold counts of fresh and ensiled high moisture corn at 23% moisture level (Standard error of the means:d0=0.10, d10=0.14, d21=0.18, d120=0.29).

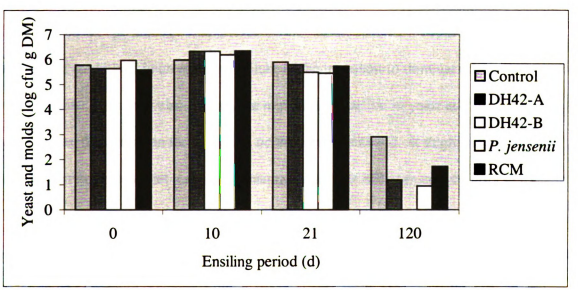

Figure 3-30. The effect of inoculation on the yeast and mold counts of fresh and ensiled high moisture corn at 22% moisture level (Standard error of the means:d0=0.07, d10=0.07, d21=0.04, d120=0.24).

acidipropionici DH42 silages with 10^6 cfu/g DM generally had numerically lower yeast and mold counts compared to the uninoculated control silages. The results indicate that inoculation of *P. acidipropionici* DH42 reduced the yeast and mold counts. As was observed in earlier silage trials, microbial counts within treatments are highly variable. This indicates that a greater number of samples would be needed to detect significant differences among treatments. Using propionic acid to preserve hay, Magan and Lacey (1986) indicated that the difficulty of mixing the material with the acid evenly allows yeasts and fungi to grow in under-treated pockets without competition from other microorganisms. It had been demonstrated that yeasts could use propionic acid as substrate when levels are low (Lord et al., 1981; Magan and Lacey, 1986). During the silage preparation for this study, thorough mixing had been done to ensure that the inoculants are well distributed, although it is also possible that there were areas or pockets that were uninoculated allowing the proliferation of yeast and molds. Weinberg and Muck (1992) pointed out that uneven mixing of the inoculant could be one of the factors that could lead to an apparent failure of the inoculant to dominate the fermentation. This may also explain the improved reduction in yeast and mold counts with increased inoculation rate of the *P. acidipropionici DH42*. It might be necessary to have more replicates to get a more representative sample of the microbial flora in the silage.

The amount propionic acid needed to prevent moulding of hay and silage depends on the level of pH and moisture content (Lacey et al., 1978; Hara and Ohyama, 1978; Lord et al., 1981). With a pK of 4.87, more of the propionic acid is in the undissociated form at lower pH hence, increased antimicrobial action. Rusul et al. (1987) showed that

with an initial pH of 5.5 of the medium, a maximum of 1% propionic acid permitted the growth and aflatoxin production of *Aspergillus parasiticus* after 3 d of incubation. However, when the initial pH is 4.5, the maximum concentration was reduced to 0.1%. The higher the moisture content of the material to be preserved, the more propionic acid is needed to prevent deterioration due to yeasts and molds. Sauer et al. (1986) indicated that 0.4% propionic acid is needed to preserve corn grain. Lacey et al. (1978) also observed that propionic acid treatment was less effective in preserving hay when baled with 41% moisture as compared to the drier hays. Lacey et al. (1983) further indicated that in hay, about 0.12 g propionate/ 100 g water is needed for every 1% moisture content above 20% to prevent molding. In a more recent findings, Magan and Lacey (1986) observed the higher tolerance of yeast to propionic acid when water exceeds 30%. This observation may explain the higher yeast and mold counts at higher moisture levels even though the propionic acid level were higher compared to the drier silages. Hara and Ohyama (1978) suggested that the effectiveness of propionic acid depends on the concentration of the acid in the moisture phase of the silages.

The propionibacteria counts (Appendix Table A-11) at d 0 indicate that even the uninoculated corn had propionibacteria although lower than the inoculated ones. After 120 d of ensiling, propionibacteria counts are high in all the silages at different moisture levels. The lowest count were observed at 35% moisture level, with the control silage having propionibacteria counts of $10^{4.8}$cfu/g DM. It also appears that propionibacteria counts did not change even after 120 d of ensiling except for the increase in the uninoculated silages. The low counts could also be due to the sampling technique. Microbial counts were taken using silage extracts prepared by stomaching the silage

samples for 5 min. Sharp et al. (1991) observed that recovery of added microbial cells from stomaching is only 9 % as compared to 109% using differential centrifugation technique.

Aerobic Phase

During the exposure period, average daily propionic acid (Appendix Table A-12, Figures 3-31 to 3-32) decreased only after 3 d of exposure. Significant effect of PAB inoculation was observed at 22-28% moisture levels. The DH42 10^6cfu/g-treated silages had significantly higher propionic acid levels than the control and RCM-treated silages. Moreover, this treatment had significantly higher propionic acid than the *P. jensenii*-treated silages at 24 and 28% moisture levels. The highest propionic acid was observed at 28% moisture level, with an average of 0.27 g/ 100 g DM for the duration of the exposure period. While there was no increase in propionic acid concentration during aerobic exposure as Dawson et al. (1998) observed, the propionic acid levels were higher than that reported by Kreikemeier et al. (1998). Other studies (Weinberg et al., 1995b; Higginbotham et al., 1996; 1998) found no propionic acid in the exposed silages. *P. acidipropionici* DH42 can be grown aerobically (Dawson, 1994) and thus, can continue to grow and produce propionic acid even when the silage is exposed to air. Propionibacteria are considered facultative anaerobes (Cummins and Johnson, 1992). However, Quesada-Chanto et al. (1997) observed that *P. shermanii* CDB 10014 can grow at high volumetric oxygen transfer coefficients (K_La).

The effect of inoculation on the average acetic acid content (Appendix Table A-13, Figures 3-33 to 3-34) levels in the exposed silages was observed at 22, 23 and 28%

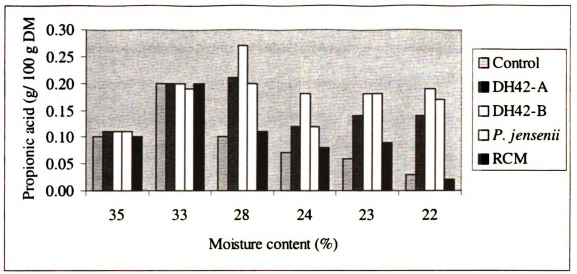

Figure 3-31. The effect of inoculation and moisture content on the average propionic acid of exposed high moisture corn silage (Standard error of the means: 35%=0.01, 33%=0.01, 28%=0.01, 24%=0.01, 23%=0.01, 22%=0.01).

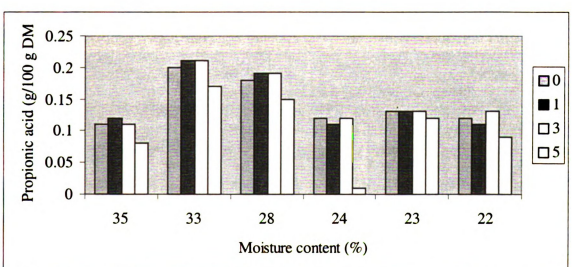

Figure 3-32. The effect of moisture content on the average propionic acid content of high moisture corn silage during 5-day exposure period.

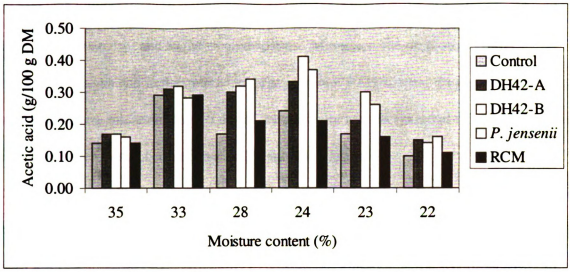

Figure 3-33. The effect of inoculation and moisture content on the average acetic acid content of exposed high moisture corn silage (Standard error of the means: 35%=0.02, 33%=0.02, 28%=0.02, 24%=0.03, 23%=0.02, 22%=0.01).

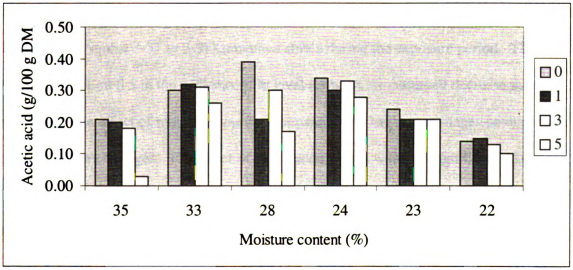

Figure 3-34. The effect of moisture content on the acetic acid content of high moisture corn silage during 5-day exposure period.

moisture levels. At these moisture levels, DH42-10^6cfu/g silages had higher acetic acid level than the control and RCM-treated silages. Moreover, the propionibacteria-inoculated silages had higher acetic acid than the non-PAB silages. In general, acetic acid levels were maintained up to d 3 of the exposure and decreased at d 5. Higher acetic acid levels were also observed at 23-22% moisture levels.

Moisture affected lactic acid levels (Appendix Table A-14, Figures 3-35 to 3-36) but not inoculation during the exposure period. Lactic acid was highest in the silages with 33-35% moisture level indicating extensive fermentation in the wetter silages. In contrast to earlier findings (Dawson et al., 1998), inoculation had no effect on the lactic acid levels. At 35% moisture level, lactic acid declined at d 5 of aerobic exposure, while at 24 and 33 % moisture levels, significant decline was noted at d 3. The decline in lactic acid is due to its metabolism by aerobic microorganisms (Wood et al., 1991).

Except of the silages from the 35% moisture level, the pH of silages (Appendix Table A-15, Figures 3-37 to 3-38) remained stable during the exposure period. The increase in pH on d 5 in the 35% moisture level supports the observed decrease in lactic acid level. The effect of moisture was most apparent with the wetter silages having lower pH than the drier silages. The effect of inoculation on pH was also significant. The DH42-10^6 cfu/g and *P. jensenii*-treated silages had lower pH compared to the control and RCM-treated silages at 22, 24, 28% moisture levels. During aerobic exposure, the increase in pH is attributed to consumption of organic acids by yeast, molds and aerobic bacteria (Mc Donald et al., 1991). Hence, maintenance of low pH indicates better preservation of the exposed silage. As discussed earlier, acetic and propionic acid levels tend to be higher at 22-28% moisture levels with the PAB-treated silages.

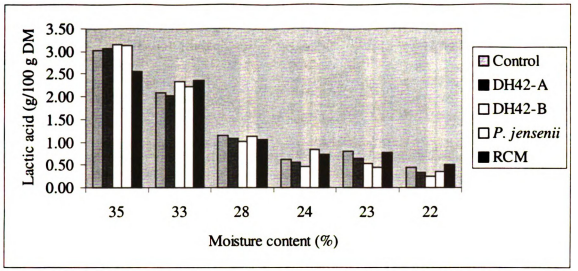

Figure 3-35. The effect of inoculation and moisture content on the average lactic acid content of exposed high moisture corn silage (Standard error of the means: 35%=0.42, 33%=0.11, 28%=0.08, 24%=0.09, 23%=0.09, 22%=0.02).

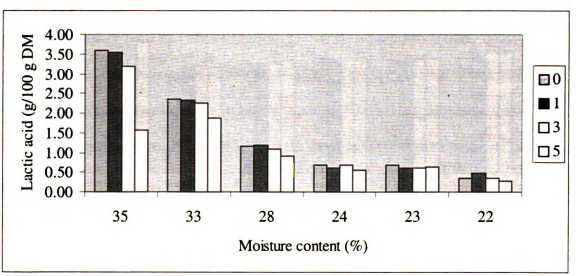

Figure 3-36. The effect of moisture content on the lactic acid content of high moisture corn silage during 5-day exposure period

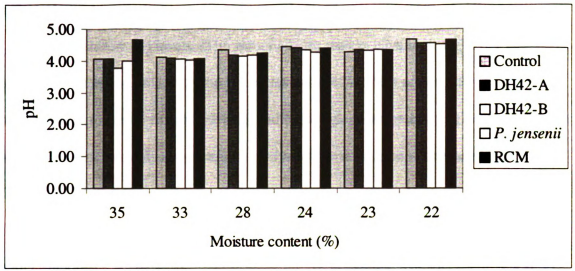

Figure 3-37. The effect of inoculation and moisture content on the average pH of exposed high moisture corn silage (Standard error of the means: 35%=0.38, 33%=0.03, 28%=0.04, 24%=0.02, 23%=0.03, 22%=0.02).

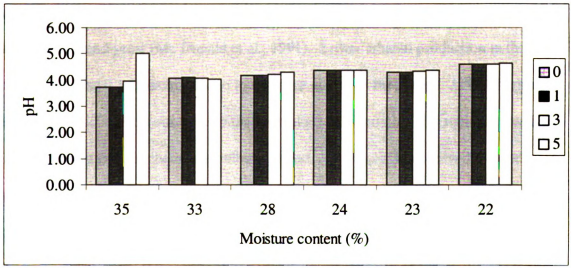

Figure 3-38. The effect of moisture content on the average pH of high moisture corn silage during 5-day exposure period.

Effect of moisture was also observed in the glucose (Appendix Table A-16) and ethanol (Appendix Table A-17) levels. The wetter silages tend to have higher glucose and ethanol than the drier silages. The pH seems to have an effect on the glucose level. The acidic silages (33-35% moisture levels) had higher glucose content. Higher glucose in the acidic silages is possibly due to acid hydrolysis of structural components in the silage. Jones et al. (1992) observed increased solubilization of cell wall sugars as pH decreased. They further indicated that the effect of inoculation on increased solubilization of cell wall components is due to its effect in reducing the silage pH. The glucose levels at 33 and 35% moisture levels decreased on d 5 of aerobic exposure. Contrary to the results of Dawson et al. (1998) inoculation did not increase glucose levels. However, at 22% moisture level, the *P. acidipropionici* DH42-10^5 had higher glucose values than the control silages. Ethanol values tend to be lower for the inoculated silages particularly at 28% moisture level. Ethanol is a product of fermentation by heterofermentative lactic acid bacteria and yeast (Mc Donald et al., 1991). Lower ethanol production in the inoculated silages is possibly due to lower yeast counts in the silages. At d 3, highest ethanol production was observed in the control silages from the 35% moisture level with 0.695 g/ 100 g DM. In chemostat cultures, Thomas et al. (1979) observed that the mainly homofermentative *Streptococcus lactis* shifts to heterolactic fermentation under low glucose availability. Instead of producing lactate, there is a shift to formation of ethanol, formate, and acetate. It would be noted that the drier silages had lower glucose levels. The 22-24% moisture levels had some amounts of citric acid (Appendix Table A-18), which were not affected by inoculation and exposure period. Butyric acid (Appendix Table A-19) production was highest at 33% moisture level ranging from 0.15-0.32 g/100

g DM. Small amounts were also detected at 23-28% moisture levels. Butyric acid production is mainly attributed to clostridial fermentation. Inoculation did not affect the residual butyric acid levels in the silages. The silages with 35% moisture level had the highest yeast and mold counts (Appendix Table A-20; Figures 3-39 to 3-40) after 5 d with about 10^8 cfu/g DM. Moreover, at this moisture level, yeast and mold counts progressively increased over the 5 d exposure period. At other moisture levels, the counts over the 5 d exposure period were variable with slight increases at d 5. While the control had the highest yeast and mold counts at 22 and 23% moisture levels, the PAB-treated silages had comparable counts with the RCM-treated silages. It is possible that the nutrients present in the latter inoculant favored the growth of lactic acid bacteria and propionibacteria whose growth is beneficial to ensiling. Dry matter recovery (Appendix Table A-21) was not affected by inoculation. There is no apparent trend in the effect of moisture in the temperature (Appendix Table A-22) of the silages. After 5 d of exposure,

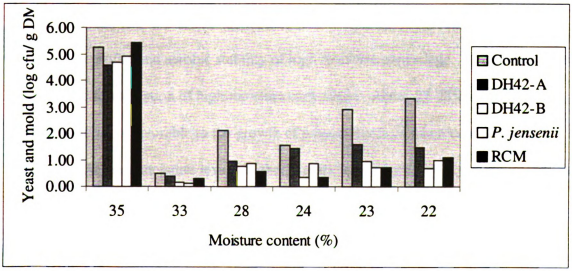

Figure 3-39. The effect of moisture content and inoculation on the yeast and mold counts of exposed high moisture corn silage ((Standard error of the means: 35%=0.40, 33%=0.12, 28%=0.44, 24%=0.36, 23%=0.42, 22%=0.33).

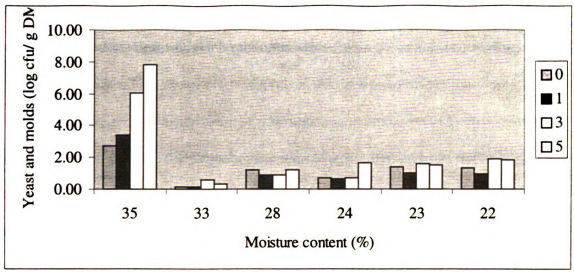

Figure 3-40. The effect of moisture content on the yeast and mold counts of high moisture corn silage during exposure period.

silages from the 28 and 35% moisture levels tend to have higher temperatures than silages from other moisture contents (Appendix Table A-23).

Implications

The study shows that both moisture content and inoculation affected the fermentation profile and aerobic stability of high moisture corn silage. Propionibacteria enhanced the fermentation of high moisture corn silage. About 22-28% moisture in the ensiling material is favorable to the growth of propionibacteria when used as silage inoculants. At these moisture levels, higher propionic and acetic acids and lower pH and butyric acid were observed with the PAB-treated silages after 120 d of ensiling. At higher moisture levels (33-35%), the sharp decline in pH appeared to restrict the growth of propionibacteria. While most of the silages appear stable when judged based on the temperature of the exposed silages, the propionibacteria-inoculated silages had higher

residual organic acids (propionic and acetic) and lower pH. The use of higher inoculation rate (10^6 cfu/g material) for DH42 is recommended.

CHAPTER 4

VITAMIN B_{12} PRODUCTION OF *P. ACIDIPROPIONICI* DH42 IN BATCH CULTURE SYSTEM

Abstract

P. acidipropionici DH42 and *P. shermanii* were grown in batch cultures at two incubation temperatures (30 and 40°C) using Reinforced Clostridial Medium (RCM) supplemented with 10 mg/l of $CoCl_2.6H_2O$. Samples were taken after 20, 40 and 72 h of incubation for analysis. True vitamin B_{12}, pH, optical density and organic acids of the cultures were determined. Results showed comparable vitamin B_{12} production of the two propionibacteria strains. After 72 h of incubation, the *P. acidipropionici* DH42 and *P. shermanii* cultures grown at 30°C had vitamin B_{12} contents of 852.85 and 840.69 ng/ml, respectively. Both strains grew better at 30°C than at 40°C. Poor growth of *P. shermanii* was evident at 40°C incubation. Lower pH was observed at lower incubation temperature but for the *P. acidipropionici* DH42 cultures, the difference of the pH between the two incubation temperatures was not significant. *P. acidipropionici* DH42 cultures tend to have higher propionic and acetic acids while the *P. shermanii* cultures had higher succinic and malic acids.

Introduction

Propionic acid and vitamin B_{12} are two major products of propionibacteria of commercial importance. Industrial production of vitamin B_{12} uses *Propionibacterium spp.* and *Pseudomonas denitrificans* (Glatz, 1992). Among the propionibacteria strains, *P. shermanii* and *P. freudenrechiii* are commonly used for this purpose. Vitamin B_{12} is an essential part of enzyme systems that carry out basic metabolic functions. Humans

and animals depend on microbial synthesis for their supply of the vitamin. In ruminants, dietary cobalt appears to be the main limiting factor in its synthesis by ruminal microflora (Mc Dowell, 1989). However, Sutton and Elliot (1972) found that in high concentrate diets, vitamin B_{12} synthesis is decreased and analogues, which have little or no vitamin B12 activity, are produced. The ruminal microorganisms such as *Prevotella ruminicola* (Strobel, 1992) and *Bacteroides spp.* (Varel and Bryant, 1974; Chen and Wolin, 1981) require vitamin B_{12} for growth and propionate production. Strobel (1992) found that microbial protein yields were reduced by 15 to 25% in the absence of vitamin B_{12}.

As a silage inoculant, *P. acidipropionici* DH42 has been shown to improve the stability of aerobically exposed silages (Dawson et al., 1998). As a feed additive, it may provide additional benefits to the animal as a source of vitamin B_{12}. However, the vitamin B_{12} production of *P. acidipropionici* DH42 has not been determined. This study was conducted to determine the vitamin B_{12} production of *P. acidipropionici* DH42 using two incubation temperatures under anaerobic condition of cultivation.

Materials and Methods

Cultures and Media

The two propionibacteria species used in this study were *Propionibacterium acidipropionici* DH42 (ATCC 55737) and *P. shermanii* (ATCC 13673). *Lactobacillus leichmannii* (ATCC 7830) was used for the microbial assay of vitamin B_{12}. *P. shermanii* (ATCC 13673) and *Lactobacillus leichmannii* (ATCC 7830) were purchased from the American Type Culture Collection. Both propionibacteria strains were maintained on Reinforced Clostridial Medium (Oxoid) agar slants held at 4°C. Before use as inoculants,

at least three sub-transfers of each propionibacterium in Reinforced Clostridial Medium (RCM) broth were done to activate the cultures. The headspace in the culture bottle was flushed with CO_2 passed through a hot copper column to eliminate any oxygen.

Lactobacillus leichmannii cultures were maintained in B_{12} Culture Agar USP (Difco) slants. Before the culture was used for the assay, sub-transfers using B_{12} Inoculum Broth USP (Difco) were done twice daily for a period of not less than one week. The culture was assumed to be active when turbidity was observed within 2 h after transfer.

Fermentation Media

Separate cultures were prepared for each propionibacterium strain at two incubation temperatures (30^0C and 40°C). The lower temperature setting (30°C) was used because this is the optimum temperature for the growth of *P. acidipropionci* DH42. The higher temperature setting (40°C) was used because this is the optimum temperature for vitamin B_{12} production based on the study of Quesada-Chanto et al. (1994b). After 48 h of growth, the cultures were transferred into 25-ml serum bottles at the rate of 10% under CO_2. Reinforced Clostridial Medium (Oxoid) was used and supplemented with 10 mg/l of $CoCl_2.6H_2O$. After 48 h, 5,6 dimethylbenzimidazole (DMBI, Sigma) was added at the rate of 10 mg/l. Samples were analyzed after 20, 40, and 72 h of incubation. Three vials were prepared for each strain x incubation temperature x incubation time combination. The pH of the cultures was measured using a pH meter (Cole Palmer). Growth was also monitored by the optical density of the cultures at 600 nm using a spectrophotometer (Spectronic 21D, Milton Roy). The cultures and the uninoculated medium were analyzed for their contents of true vitamin B_{12} using a microbial assay and

organic acids were analyzed using HPLC. The vitamin B_{12} and organic acid concentrations of the uninoculated medium were subtracted from the values determined in the inoculated cultures to represent that which was produced from the metabolism of the bacteria.

Vitamin B_{12} Assay

Vitamin B_{12} was determined by microbial assay using *Lactobacillus leichmannii* (ATCC 7830) as described by Okada et al (1985). This assay is considered to analyze for true vitamin B_{12} content. The assay extracts were prepared as follows: 1 ml of the culture was added to 5 ml of 0.2 M acetate buffer (pH 4.5), 0.2 ml of potassium cyanide (0.5mg/ml), and 30 ml of distilled water. The mixture was heated to 100°C for 30 min, cooled and 0.3 ml of 10% metaphosphoric acid solution was added. The solution was put in ice water for 30 minutes. Distilled water was added to the solution to bring the final volume to 50 ml. The solution was centrifuged. Two aliquots of 20 ml each were removed from the supernatant. One portion was adjusted to pH 6.0 and distilled water added to reach a volume of 40 ml. The solution was recentrifuged to supply test extract A. The other portion was adjusted to pH 11-12 and heated to 120°C for 30 min. Thereafter, the pH was adjusted to 6.0 and distilled water added to bring the final volume to 40 ml. The solution was recentrifuged to obtain test extract B in which the true vitamin B_{12} is destroyed.

Using a standard vitamin B_{12} (Sigma) solution, tubes (16x100 mm) of different concentrations (0.0 to 0.25 ng/assay tube) of vitamin B_{12} were prepared as described by USP (1995). About 2.5 ml of B_{12} Assay Medium USP (Difco) was measured into tubes and the standard B_{12} solution or sample extracts were added. Distilled water was added

to bring the final volume to 5 ml. The tubes were autoclaved at 121°C for 5 min. The tubes were allowed to cool and one drop of *Lactobacillus leichmannii* culture (OD_{600}=0.125) was added aseptically to all tubes except for two blank solutions. The tubes were covered with sterile rubber stoppers and incubated for 10-15 h at 37°C. After incubation, the tubes were stuck in the refrigerator for 15-20 minutes to stop the cultures from growing (Becton Dickenson). The optical density (OD 600nm) of the tubes was taken. Linear regression analysis was done using the absorbance of the standard vitamin B_{12} solutions at a given concentration of vitamin B_{12}. The vitamin B_{12} content of the cultures was calculated from the standard curve.

Statistical Analyses

The data were analyzed as a multifactorial design using the general linear model of the Statistical Analysis System (SAS, 1990). Treatment means were compared using the Tukey-Kramer test (SAS, 1990). The ANOVA model used was as follows:

$$Y_{ijkm} = \mu_{ijk}+\alpha_i+\beta_j+\gamma_k+(\alpha\beta\gamma)_{ijk}+(\alpha\beta)_{ij}+ (\alpha\gamma)_{ik}+(\beta\gamma)_{jk}+\varepsilon_{ijkm}$$

Where:

Y_{ijkm} = individual response variable measured (e.g. vitamin B_{12}, propionic acid etc.)

μ_{ijk} = overall mean

α_i = effect of propionibacteria strain (DH42, *P. shermanii*)

β_j = effect of temperature (30, 40°C)

γ_k = effect of time (20, 40, 72 h)

$(\alpha\beta\gamma)_{ijk}$ = interaction of strain, temperature and time

$(\alpha\beta)_{ij}$ = interaction of strain and temperature

$(\alpha\gamma)_{ik}$= interaction of strain and time

$(\beta\gamma)_{jk}$ = interaction of temperature and time

ε_{ijkm} = random residual error (assumed normally distributed)

Results and Discussion

The vitamin B_{12} concentration of the two propionibacteria cultures at different incubation times and different incubation temperatures is shown in Table 4-1. After 20 h of incubation, vitamin B_{12} production of *P. acidipropionici* DH42 grown at 30°C was higher ($P<0.01$) than the *P. shermanii* cultures at 30°C but this value was comparable (412.49 vs 406.13 ng/ml) to that of the vitamin B_{12} content of the *P. shermanii* culture at 40°C. Vitamin B_{12} content of the *P. acidipropionici* DH42 at 40°C after 20 h of incubation was the lowest ($P<0.01$). Significant decline in the vitamin B_{12} levels of the *P. shermanii* was also noted on the 40 h sampling time for the cultures incubated at 40°C.

Table 4-1. Effect of incubation temperature on the true vitamin B_{12} (ng/ml) of *P. acidipropionici* DH42 and *P. shermanii* at different incubation times.

Strains	Incubation time (h)					
	20		40		72	
	Incubation temperature (°C)					
	30	40	30	40	30	40
	ng/ml					
P. shermanii	221.44^{d}	406.13^{bc}	327.25^{c}	13.82^{e}	852.85^{a}	324.31^{c}
P. acidipropionici	412.49^{bc}	12.89^{e}	430.91^{b}	440.97^{b}	840.69^{a}	785.61^{a}
S.E.M.[n]	15.61 (19.11)					

[n]Standard error of mean. Value in parenthesis is the SEM of *P. shermanii* culture at 20 h incubation at 40°C.
Means with unlike superscripts differ ($P<0.01$)

An analysis of the uninoculated medium showed that it contained about 132.64 ng/ml of true vitamin B_{12}. For both strains, vitamin B_{12} contents of the cultures inoculated at

30°C increased ($P<0.01$) over time. For the cultures inoculated at 40°C, there was a significant decline in the vitamin B_{12} content of the *P. shermanii* cultures incubated at 40°C during the 40 h sampling time. Significant increase in vitamin B_{12} content of the cultures was observed from 40 to 72 h incubation time. This is possibly due to the addition of 5,6 DMBI after 48 h. This stimulates the production of vitamin B_{12} by the formation of 5,6 dimethylbenzimidazolyl cobamide (Yongsmith et al., 1982; Marwaha et al., 1983; Quesada-Chanto et al., 1994b). Marwaha et al. (1983) suggested the addition of 5,6 DMBI 24 h before the end of the fermentation. Propionibacteria can synthesize their own DMBI under anaerobic conditions and adding DMBI at the start of the fermentation inhibits the growth of the organisms (Friedman and Cagen, 1970).

Comparison of the vitamin B_{12} production of both strains is not easy since they have different cell densities at a given sampling time. At 30°C incubation temperature however, it appears that the two propionibacteria strains are comparable when the optical density and the vitamin B_{12} contents of their respective cultures are compared. However, at 40°C after 72 h of incubation *P. shermanii* appears to make more vitamin B_{12} than *P. acidipropionici* DH42 per unit of cell yield. The vitamin B_{12} content of the *P. shermanii* cultures was about 324.31 ng/ml with an OD of 0.265. On the other hand, *P. acidipropionici* DH42 had 786.61 ng/ml with an OD of 1.577. These values give an estimate of vitamin B_{12}/unit OD of 1223.8 for *P. shermanii*, and 498.8 for *P. acidipropionici*. Quesada-Chanto (1994b) observed increased vitamin B_{12} production as the temperature is increased with 40°C as the optimum temperature for vitamin B_{12} production. They used a *P. acidipropionici* strain. The vitamin B_{12} contents of the *P. acidipropionici* DH42 cultures at 30 and 40°C incubation temperatures after 72 h

incubation were not significantly different although the OD of the 40°C cultures was lower. At 48 h, the OD of the *P. acidipropionici* DH42 at 30°C incubation temperature was significantly higher than that incubated at 40°C but their vitamin B_{12} contents are comparable. Quesada-Chanto (1994b) noted that product formation might rapidly increase with increasing temperature even if cell concentration is decreased which was the case for the propionic acid and vitamin B_{12} production in this study. The increased vitamin B_{12} production at higher incubation temperature could be due to increased cobalt uptake with increased temperature. Scheneider et al. (1995) observed increased cobalt-binding affinity with *P. arabinosum* exposed to high temperature.

In comparing the vitamin B_{12} production of the strains, the effect of pH on vitamin B_{12} synthesis, particularly for the *P. acidipropionici* DH42 should be considered. Propionibacteria are strongly pH-dependent (Hsu and Yang, 1991). Quesada-Chanto (1994b) indicated that the optimum pH for propionic acid and vitamin B_{12} production is between pH 6.5-6.8. The pH of the *P. acidipropionici* DH42 cultures is significantly lower than that of *P. shermanii* (Table 4-3). Although *P. acidipropionici* DH42 appears to grow well even at low pH, vitamin B_{12} production could have been affected.

Result of this study is difficult to compare with other published results because of the differences in culture conditions, substrates and the assay method for vitamin B_{12}. Unlike most studies which used the continuous culture and methods are geared towards commercial production of vitamin B_{12}, this study used a batch culture method. Hence, with the accumulation of end products in the media, the resulting decrease in pH may have affected cell efficiency and vitamin B_{12} synthesis. Bullerman and Berry (1966) used the same strain *P. shermanii* (ATCC 13673) to determine its vitamin B_{12} production

using cheese whey and they reported about 8 μg/ml of vitamin B_{12} incubation of 168 h. In this study, 852.85 ng/ml was observed after 72 h of incubation. This present study however accounts for only the true vitamin B_{12} while their study measured total vitamin B_{12}. Moreover, their study used continuous cultures with higher cobalt level (20 ppm), longer incubation time (168 h) and with yeast supplementation. The continuous culturing method helps the cells in a steady state of growth and prevents the pH decline, which was a problem in this study. Hatanaka et al. (1988) observed that with pH-controlled batch cultures, about 2.14 mg/l vitamin B_{12} was produced. Using a hollow-fiber module that entrapped the cells and allowed the removal of organic acids produced, the vitamin B_{12} production rose to 52 mg/l. The growth promoting effect of yeast extract on propionibacteria had been reported (Quesada-Chanto et al., 1994b, 1997) which was attributed to vitamins present in the yeast extract (Hettinga and Reinbold, 1972).

Temperature affected the growth of both strains. Based on the OD of the cultures, it is evident that both strains grew better ($P<0.01$) at 30°C than at 40°C (Table 4-2). Moreover, the *P. acidipropionici* DH42 grew better than *P. shermanii* up until 72 h of incubation where *P. shermanii* grown at 30°C had comparable OD to the *P. acidipropionici* DH42 cultures. *P. shermanii* grew very poorly at 40°C. On the other hand, *P. acidipropionici* DH42 cultures appeared to be more tolerant of the higher incubation temperature. After 72 h of incubation, *P. acidipropionici* DH42 cultures grown at 30 and 40°C had comparable OD.

As expected, the pH of the cultures decreased with time (Table 4-3). At any sampling time, the pH of the *P. acidipropionici* DH42 was lower ($P<0.01$) than that of *P.*

Table 4-2. Effect of incubation temperature in the optical density of *P. acidipropionici* DH42 and *P.shermanii* at different incubation times.

Strain	Incubation time (h)					
	20		40		72	
	Incubation temperature (°C)					
	30	40	30	40	30	40
P. shermanii	0.803^{c}	0.114^{d}	1.382^{b}	0.176^{cd}	1.583^{a}	0.265^{c}
P. acidipropionici	1.700^{a}	1.507^{a}	1.627^{a}	1.497^{a}	1.617^{a}	1.577^{a}
S.E.M.[n]	0.014					

[n]Standard error of mean.
Means with unlike superscripts differ (P<0.01)

shermanii. Since *P. acidipropionici* DH42 grew faster than *P. shermanii*, the accumulation of organic acids (mainly propionic and acetic acids) in the growth medium could account for the lower pH in the *P. acidipropionici* DH42 cultures. The pH values of the *P. acidipropionici* DH42 cultures in the two incubation temperatures were the same. For the *P. shermanii* cultures, the pH was lower (P<0.01) in the cultures incubated at 30°C. With the inhibition of propionic acid on cell growth, Quesada-Chanto et al. (1994b) developed a two-stage fermentation process to produce vitamin B_{12} from

Table 4-3. Effect of incubation temperature on the pH of P. *acidipropionici* DH42 and *P. shermanii* at different sampling times.

Strain	Incubation time (h)					
	20		40		72	
	Incubation temperature (°C)					
	30	40	30	40	30	40
P. shermanii	5.79^{b}	5.99^{a}	5.25^{c}	5.89^{ab}	4.95^{d}	5.81^{b}
P. acidipropionici	4.72^{ef}	4.84^{de}	4.64^{f}	4.66^{f}	4.61^{f}	4.65^{f}
S.E.M.[n]	0.025					

[n]Standard error of mean.
Means with unlike superscripts differ (P<0.01).

molasses or sugar where the fermentation was switched from anaerobic to aerobic to reduce the propionic acid production and enhance the production of vitamin B_{12}.

Propionic and acetic acid are the main products of propionibacterial fermentation. Table 4-4 shows the net production of propionic acid in the cultures. Significant ($P<0.01$) three-way interaction of inoculant x incubation time x temperature was observed in the propionic acid levels. The propionic acid content of *P. acidipropionici* DH42 cultures was higher ($P<0.01$) than that of *P. shermanii.* Regardless of incubation temperature, the propionic acid levels in the *P. acidipropionici* DH42 cultures numerically increased over time but differences were not significant, while the levels in the *P. shermanii* cultures increased ($P<0.05$) during the 40 to 72 h but not from 20 to 40 h of incubation time. The significant increase could be due to the addition of 5,6 dimethylbenzimidazole at 48 h. Quesada-Chanto et al. (1994b) indicated that 5,6 DMBI is required for the optimal production of cells and propionic acid.

Unlike the decrease in cell biomass with increased in incubation temperature, the propionic acid production of *P. acidipropionici* DH42 was higher ($P<0.01$) than the *P. shermanii* in the 40°C incubation temperatures both at 40 and 72 h sampling times. Quesada-Chanto et al. (1994b) observed the high temperature dependence of *P. acidipropionici* strain. They noted increased product concentration as temperature increased with the optimum temperature of 37°C for propionic acid production. Unlike the *P. acidipropionici* DH42 cultures, the *P. shermanii* cultures had higher ($P<0.01$) propionic acid levels in the 30°C incubation temperature than at 40°C. As noted in Table 4-2, *P. shermanii* grew very poorly at 40°C and the propionic acid levels were also the lowest ($P<0.01$).

Table 4-4. Effect of incubation temperature in the propionic acid content of *P. acidipropionici* DH42 and *P. shermanii* at different incubation times.

Strain	Incubation time (h)					
	20		40		72	
	Incubation temperature (°C)					
	30	40	30	40	30	40
	%					
P. shermanii	0.048[bc]	0.009[c]	0.154[a]	0.015[c]	0.280[a]	0.033[bc]
P. acidipropionici	0.295[a]	0.344[a]	0.329[a]	0.371[a]	0.349[a]	0.388[a]
S.E.M.[n]	0.02					

[n]Standard error of mean.
Means with unlike superscripts differ (P<0.01).

In general, the acetic acid (Table 4-5) production of *P. acidipropionici* DH42 was significantly higher than that of *P. shermanii*. The acetic acid production of the cultures after 20 h of incubation did not differ among the cultures. However, after 40 and 78 h of incubation, the *P. acidipropionici* DH42 cultures inoculated at 30°C had the highest acetic acid production. For both propionibacteria strains, the lower incubation temperature enhanced acetic acid production.

Table 4-5. Effect of incubation temperature in the acetic acid content of *P. acidipropionici* DH42 and *P. shermanii* at different incubation times.

Strain	Incubation time (h)					
	20		40		72	
	Incubation temperature (°C)					
	30	40	30	40	30	40
	%					
P. shermanii	0.020[ab]	0.006[b]	0.051[ab]	0.010[b]	0.080[ab]	0.017[b]
P. acidipropionici	0.083[ab]	0.141[a]	0.096[ab]	0.076[ab]	0.104[ab]	0.079[ab]
S.E.M.[n]	0.024					

[n]Standard error of mean.
Means with unlike superscripts differ (P<0.01)

Although levels were low, measurable production of succinic acid (Table 4-6) was observed particularly for the *P. shermanii* cultures. Only trace amounts were detected with the *P. acidipropionici* DH42 cultures. There was no apparent effect of incubation temperature although the highest ($P<0.01$) level (0.034%) was observed in the *P. shermanii* cultures incubated at 40°C at 72 h sampling time. Quesada-Chanto et al. (1997) also observed increased succinate production in *P. shermanii* cultures with certain yeast extract brands. The increase in succinate formation correspondingly caused lower propionic acid production. They speculated that the accumulation of succinic acid could be due to the presence or absence of a substance interfering with the conversion of succinic acid to methylmalonyl-CoA in the metabolic pathway to produce propionic acid. As observed in this study, the *P. shermanii* cultures had significantly lower propionic acid levels.

Table 4-6. Effect of incubation temperature in the succinic acid content of *P. acidipropionici* DH42 and *P. shermanii* at different incubation times.

Strain	Incubation time (h)					
	20		40		72	
	Incubation temperature (°C)					
	30	40	30	40	30	40
	%					
P. shermanii	0.03^{ab}	0.01^{cd}	0.03^{ab}	0.02^{ab}	0.02^{b}	0.03^{a}
P. acidipropionici	0.00^{d}	0.01^{c}	0.00^{cd}	0.00^{d}	0.00^{cd}	0.00^{d}
S.E.M[n].	0.002					

[n] Standard error of mean.
Means with unlike superscripts differ ($P<0.01$).

Malic acid (Table 4-7) was also detected in the cultures. The uninoculated Reinforced Clostridial Medium did not contain malic acid, so it can be assumed that

malic acid was formed from the fermentation of the bacteria. Malic acid is one of the metabolites in the production of propionic acid via the succinate-propionate pathway (Gottschalk, 1985). In general, *P. shermanii* cultures had higher malic acid than the *P. acidipropionici* DH42 cultures, which as in the case of succinic acid, could also explain the lower propionic acid levels in the *P. shermanii* cultures. Incubation temperature had no consistent effect on the malic acid production in the cultures. Highest ($P<0.01$) malic acid level (0.097%) was observed in the *P. shermanii* cultures incubated at 40°C sampled at 40 h. There are no reports as what factors prevent the conversion of malate into fumarate (Gottschalk, 1985) during the metabolic cycle. It can only be assumed that the two propionibacteria strains are affected differently by the culture conditions. Using *P. shermanii*, Ye et al. (1999) observed increased production of malate and fumarate when culture condition is switched from anaerobic to aerobic conditions and thereby reducing propionic acid production. They attributed this to the effect of oxygen on the enzymes. It should be noted that *P. acidipropionici* could be grown aerobically (Dawson, 1994) with substantial production of propionic acid. Although propionibacteria are generally considered anaerobic microorganisms, some *Propionibacterium* species have been observed to possess the components of typical aerobic electron transport chain (Vries et al., 1972; Quesada-Chanto et al., 1998). The effect of the dissolved oxygen in the medium to the growth of the two strains could not be discounted. It is possible that the differences in both the succinic and malic acid contents of the cultures could be due to differences in the oxygen-sensitivity of the two propionibacteria strains.

Table 4-7. Effect of incubation temperature in the malic acid content of *P. acidipropionici* DH42 and *P. shermanii* at different incubation times.

Strain	Incubation time (h)					
	20		40		72	
	Incubation temperature (°C)					
	30	40	30	40	30	40
	%					
P. shermanii	0.092^{abc}	0.031^{abcd}	0.000^{d}	0.097 ^{a}	0.031^{abcd}	0.096^{ab}
P. acidipropionici	0.018 ^{c}	0.052^{abcd}	0.014^{d}	0.018^{bcd}	0.014 ^{d}	0.017^{cd}
S.E.M.[n]	0.012					

[n]Standard error of mean.
Means with unlike superscripts differ (P<0.01).

Implications

Results of the study showed that *P. acidipropionici* DH42 produce vitamin B_{12} levels that are comparable with *P. shermanii* at 30°C. With the *P. acidipropionici* DH42 cultures, higher cell yield was observed at 30°C but propionic acid production was higher with the 40°C incubation temperature. Since batch cultures had been used in this study, vitamin B_{12} production capacity of the cultures could have been limited by the growth conditions. Further studies along this line are recommended.

CHAPTER 5

PCR-BASED DETECTION OF *P. ACIDIPROPIONICI* DH42 IN CORN SILAGE AND RUMEN FLUID

Abstract

A polymerase chain reaction (PCR)-based method of detection for *P. acidipropionici* DH42 in silage and rumen fluid samples was developed. Nested PCR was used with DH42-specific primers dhb1 and dhb2 for the secondary amplification of a 1,267 bp-fragment. Using the established protocols for PCR amplification, as low as 10^2 cfu/ml and 10^3cfu/ml of *P. acidipropionici* DH42 in silage extracts and rumen fluid, respectively, were detected. Moreover, the 16S rDNA of *P. acidipropionici* DH42 was sequenced and BLAST search showed its high homology to *P. acidipropionici* and two other bacterial species. The results of an earlier study on its metabolic profile and the 16S rDNA sequence confirmed the earlier identification of DH42 as a propionibacterium of the *P. acidipropionici* strain.

Introduction

Detection of propionibacteria in the environment is difficult because the media that are currently used for their isolation are not sufficiently selective and colonies often appear only after 6 d of incubation (Thierry and Madec, 1995). In addition, many strains of propionibacteria are resistant to lysozyme (Johnson and Cummins, 1972). Hence, DNA recovery from a given sample can be limited. Rossi et al. (1999) observed that with forage and soil samples, cell numbers lower than 10^5 could not be detected and they recommended a double-step amplification or semi-nested amplification to improve sensitivity. Polymerase chain reaction (PCR) techniques have been used to identify and

distinguish different propionibacteria species (Riedel et al., 1994; Rossi et al., 1997; Riedel et al., 1998; Rossi et al., 1999), and to distinguish it from other genera (Dasen et al., 1998; Mielle et al., 1999).

Nested PCR can eliminate unwanted amplification products while at the same time dramatically increasing sensitivity (Mullis and Faloona, 1987; Zhang and Ehrlich, 1994). Nested PCR amplifies the DNA in two steps. In the first round of PCR, a pair of primers is used to generate a long segment that contains the target DNA. An aliquot of the PCR product is then subjected to another round of amplification using primers internal to the first set of primers to amplify the target DNA. This approach is often successful even if the desired product is initially below the level of detection by ethidium bromide staining and in the presence of visible spurious bands (Roux, 1995). The efficiency of the second round of PCR is enhanced because of the more rapid and more complete denaturation of the first reaction product as compared with the total genome (Porter-Jordan et al., 1990).

With the potential commercial application of *P. acidipropionici* DH42 as an inoculant, a system of detection and monitoring is needed to evaluate its persistence and efficacy. This study was conducted to develop a sensitive and more rapid detection of *P. acidipropionici* DH42 in silage and rumen fluid samples.

Materials and Methods

Bacterial Strains and Culture Conditions

Propionibacteria, lactobacilli and other bacterial strains as shown in Table 5-1 were used. *Eubacterium combesii* (ATCC 25545) was purchased from the American Type Culture Collection (ATCC). Propionibacteria and *E. combesii* were cultured using

Reinforced Clostridial Medium (Unipath) under CO_2 atmosphere and incubated at 30°C. The lactobacilli strains were cultured in Lactobacilli MRS broth (Difco) and incubated at 37°C. For propionibacteria strains, culture purity was initially checked by plating the cultures in Purple Broth Base (Difco) agar supplemented with 1% i-erythritol (Sigma). Some propionibacteria have the ability to ferment erythritol (Hetinga and Reinbold, 1972) and colonies are pigmented yellow in color. Rogosa SL agar (Difco) was used to check the lactobacilli strains. Cultures were also gram stained to check purity. Major products of fermentation such as propionic acid and acetic acid for the propionibacteria and lactic acid for the lactobacilli were determined using high performance liquid chromatography as described by Dawson (1994).

Table 5-1. Bacterial cultures used in the study.

Organism	Source
Propionibacterium acidipropionici DH42	ATCC 55737
Propionibacterium acidipropionici	ATCC 25562
Propionibacterium shermanii CDC 3094	Laboratory stock
Propionibacterium pentosaceum P11	Laboratory stock
Propionibacterium shermanii P92	Laboratory stock
Propionibacterium shermanii	ATCC 13673
Propionibacterium freudenreichii	ATCC 1382
Propionibacterium jensenii P25	Laboratory stock
Propionibacterium sp. P42	Laporte Biochem. Intl.
Lactobacillus sakei	ATCC 15521
Lactobacillus delbrueckii subs. *lactis*	ATCC 7830
Lactobacillus confusus	ATCC 27646
Bacillus subtilis	Laboratory stock
Bacillus sp.	Laboratory stock
Bacillus subtilis	ATCC 6633
Eubacterium combesii	ATCC 25545

16S rDNA Sequencing

Pure culture of *P. acidipropionici* DH42 was sent to Midilabs, Inc. (http://www.midilabs.com) for 16S rDNA sequencing. Partial sequencing of the 1,267

base pair fragment was also done in the Molecular Pathogenesis Laboratory of the Department of Animal Science, MSU. Secondary PCR product amplification from rumen fluid sample was verified by gel electrophoresis in a 0.8 % agarose gel. The remaining PCR product was then purified using Wizard® PCR Preps DNA Purification System (Promega). Sequencing was done using ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) with primers dhb1 and dhb2 as forward and reverse primers, respectively. The ABI 373 Automated Sequencer (Perkin Elmer) was used for in-house sequencing.

Primer Design and Synthesis

Primers dhb1 and dhb2 (Table 5-2) were designed using the ARB program (Strunk and Ludwig, 1996) from the alignment of the 16S rDNA of *P. acidipropionici* DH42 with other sequences in the program's database (as of July 1999). Regions apparently unique to DH42 were selected and primers complementary to these regions were designed. The universal primers bak11w and bak4 (Dasen et al., 1998) corresponding to *E. coli* 16S rRNA positions 8-25 and 1522-1540, respectively, were also used (Figure 5-1). Primers were synthesized by the Macromolecular Structure Facility of MSU. Primers were stored at –20°C until use.

Silage and Rumen Fluid Sampling and Inoculation

Corn silage was collected from the Beef Cattle Teaching and Research Center. Equal amounts (w/v) of silage (250g) and 0.9% saline solution (250 ml) were mixed using a Stomacher (Tekmar) for about 10 min. The mixture was filtered using four layers of cheesecloth. The homogenate was measured into five 50-ml centrifuge tubes. Each

tube was artificially inoculated with *P. acidipropionici* DH42 culture at the rates of 10^2,10^3,10^4, and 10^5cfu/ml silage extract. One tube was kept uninoculated. The silage homogenates were kept in ice until DNA extraction was performed.

Rumen fluid samples were collected from ruminally-fistulated cows fed high concentrate diets at the MSU Dairy Cattle Research Center. Rumen fluid was strained using four layers of cheesecloth. Homogenate samples were placed into 50-ml centrifuge tubes. Each tube was inoculated with DH42 as described above for the silage samples and one tube was kept uninoculated. The samples were kept in ice until DNA extraction was performed.

Aliquots of the corn silage and rumen fluid samples were frozen (-20°C). Due to the sensitivity of the PCR reaction, extra care was taken to prevent cross-contamination of inoculated and uninoculated samples. Gloves and sterilized glassware were used in preparation of all the samples.

DNA Extraction

DNA was extracted from pure bacterial cultures and from corn silage and rumen fluid samples using UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories). About 100 µl of sample was used for extraction. With the pure bacterial cultures, about 0.5 ml cultures incubated overnight were used in DNA extraction. DNA extracts were stored at –20°C until use.

DNA was quantified using Beckman DU600 spectrophotometer as described by Maniatis et al., 1982. DNA integrity and verification of spectrophotometric determination was checked by gel electrophoresis using a molecular weight marker (Bio-Rad) in a 0.8 % agarose gel containing 0.5µg/ml ethidium bromide. Approximately 8 µl

of DNA from each extract was mixed with 2 µl loading dye and electrophoresed at about 80 v for about 2-3 h.

Table 5-2. Alignment of 16S rDNA of propionibacteria and other bacterial species with primers dhb1 and dhb2 (ambiguous or non-matching positions are boxed).

Primer dhb1 (5'→3')	CCGGATATGAGCTCCTG
P. acidipropionici DH42	CCGGATATGAGCTCCTG
E. combesii	CCGNATATGAGCTTTCA
P. acidipropionici	CCGGATATNAGCTTTCA
P. thoenii	CCGGATATGAGCTCCTG
P. jensenii	CCGGATATGAGCTCTNA
P. granulosum	CTGGATATGTGCTCCTG
P. cyclohexanicum	CTGGATATGAACTGGGC
P. propionicus	CCGGATAGACATCCTTG
A. israelii	CCGGATAGGAGCTNCTN
F. prausnitzii	CCGGATAGGAGCTCCTN

Primer dhb2 (3'→5')	TTGTGCAAGACGCACCC
P. acidipropionici DH42	AACACGTTCTGCGTGGG
E. combesii	AACACGTTCTGCGTGGG
P. acidipropionici	AACACGTNCTGCGTG...G
P. thoenii	TTCCC...TNGT.....G...GGG
P. jensenii	NACCT...NTTG.....G...GGG
P. avidum	AACC.....TGTGT.....GGG
P. propionicus	AACC.....TGTGT.....GGG
P. granulosum	AACAC...TTTTT...GTGGG
P. cyclohexanicum	ATCC.....CTTGT.....GGGG
A. israelii	NACC.....GTTGT.....GG...G
F. prausnitzii	NACC.....GTTGT.....GG...G

Figure 5-1. DH42 16S rDNA sequence and the target sites of the four primers.

TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACAT
bak11
GCAAGTCGAACGGTAAGGCCCTTTCGGGGGTACACGAGTGGCGAACGGGTG
AGTAACACGTGAGTAACCTGCCCACTTCTTCGGGATAACGCTAGGAAACTGG
TGCTAATACCGGATATGAGCTCCTGCCGCATGGTGGGGGTTGGAAAGTGTTT
dhb1
GTGGTGGTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTC
ACCAAGGCGGTGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACATTGGG
ACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGCGGGATGACGGCCTTCG
GGTTGTAAACCGCTTTCACCAGGGGCGAAGGCATyCTTTTGGGGTGTTGACGG
TACCTGGAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTGATACG
TAGGGTGCGAGCGTTGTCCGGATTTATTGGGCGTAAAGGGCTCGTAGGCGGT
TGATCGCGTCGGAAGTGAAAACTTGGGGCTTAACCCTGAGCGTGCTTTCGAT
ACGGGTTGACTTGAGGAAGGTAGGGGAGAATGGAATTCCTGGTGGAGCGGT
GGAATGCGCAGATATCAGGAGGAACACCAGTGGCGAAGGCGGTTCTCTGGA
CCTTTCCTGACGCTGAGGAGCGAAAGCGTGGGGAGCAAACAGGCTTAGATAC
CCTGGTAGTCCACGCTGTAAACGGTGGGTACTAGGTGTGGGGTCCATTCCAC
GGATTCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCC
GCAAGGCTAAAACTCAAAGGAATTGACGGGGCCCCGCACAAGCGGCGGAGC
ATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGGA
TTGGTAACGGTCAGAGATGGCCGCCCCCCTTGTGGGCCGGTTCACAGGTGGT
GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTCGTCCACTGTTGCCAGCATTTGGTTGGGGACTCAGTGGAGA
CCGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCC
CTTATGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAAAGAGTGGCGA
CATCGTGAGGTGGAGCGAATCTCAGAAAGCCGGTCTCAGTTCGGATTGGGGT
CTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAAC
GCTGCGGTGAATACGTTCCCGGGGCTTGTACACACCGCCCGTCAAGTCATGA
AAGTCGGTAACACCCGAAGCCGGTGGCCCAACACGTTCTGCGTGGGGGAGTC
dhb2
GTCGAAGGTGGGACTGGTAATTAGGACTAAGTCGTAACAAGGTAGCCGTACC
GGAAGGTGCGGyTGGATCACCTCCTT
bak4

Polymerase Chain Reaction

Nested PCR reaction was done as described by Herman et al. (1995) and Rossi et al. (1999). The first round of PCR was done using 0.2 ml tubes in a 50 μl reaction mixture containing 5 μl of 10X PCR buffer (GibcoBRL), 2.0 μl of 50mM magnesium chloride ((GibcoBRL), 1 μl of 1.25 mM dNTPs mixture (GibcoBRL), 2.5 U of *Taq* polymerase (GibcoBRL), 20 pmol of each primer (bak4 and bak11), and 1 μl template DNA (100 ng). Distilled water (GibcoBRL) was added to make up a volume of 50 μl. PCR was done using a Perkin-Elmer 9600 thermal cycler as follows: 3 min of denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 1 min, then a final extension step at 72°C for 10 min.

For the second round of PCR, an aliquot of the dilution of the primary PCR product was used as template. A 50 μl reaction mixture was prepared containing 5 μl of 10X PCR buffer (GibcoBRL), 2.0 μl of 50mM magnesium chloride ((GibcoBRL) for the control and appropriate amount for the samples containing DNA, 1 μl of 1.25 mM dNTPs mixture (GibcoBRL), 2.5 U of *Taq* polymerase (GibcoBRL), 20 pmol of each primer (dhb1 and dhb2), and 1 μl template DNA. Distilled water (GibcoBRL) was added to make up a volume of 50 μl. The second amplification was done after an initial denaturation step at 94°C for 3 min, by 30 cycles of denaturation at 94°C for 30 sec, annealing at desired temperature for 30 sec, and extension at 72°C for 1 min then a final extension step at 72°C for 10 min.

A two-step PCR amplification was also done to compare it with sensitivity of the nested PCR in detecting the presence of *P. acidipropionici* DH42 in rumen fluid samples.

The first round of PCR was done using 0.2ml tubes in a 50 μl reaction mixture containing 5 μl of 10X PCR buffer (GibcoBRL), 1.50 μl of 50mM magnesium chloride (GibcoBRL), 1 μl of 1.25 mM dNTPs mixture (GibcoBRL), 2.5 U of *Taq* polymerase (GibcoBRL), 20 pmol of each primer (dhb1 and dhb2), and 1 μl template DNA (100 ng). Distilled water (GibcoBRL) was added to make up a volume of 50 μl. PCR was done as follows: 3 min of denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec and extension at 72°C for 1 min then a final extension step at 72°C for 10 min. Another round of amplification was done using the same primers and 5 μl of the 1:10 dilution of the primary PCR products. The annealing temperature was maintained at 64°C but magnesium chloride concentration was reduced to allow amplification of the target band in the DH42-inoculated samples.

Optimization of PCR Reaction

Primary PCR was done using standard conditions as described previously. For the secondary PCR, optimum annealing temperature and concentrations of Mg^{++}, primers, dNTPs, and Taq polymerase were determined. The calculated annealing temperature for primers dhb1 and dhb2 (54°C) was used as the starting temperature for the optimization (Table 5-3). Different concentrations of $MgCl_2$ (0.5, 0.75, 1.0, 1.25, and 1.5 mM), primers (7.5, 10, 20 pmols), dNTPs (12.5 μM, 25 μM) and Taq polymerase (2.0 and 2.5 units, U) were tested.

The optimum temperature and concentration were determined as that which generated the strongest band staining after UV illumination of the gel without the appearance of other bands. The target DNA in the secondary PCR product is a 1,267-base pair band when DH42 is present in the sample.

Table 5-3. Synthetic oligonucleotides sequences, positions and calculated melting temperature(T_m)

Primer	Sequence (5'→3')	*E. coli* position	T_m*(°C)
dhb1	CCGGATATGAGCTCCTG	172-188 F	54
dhb2	CCCACGCAGAACGTGTT	1429-1445R	54
bak11	AGGAGGTGATCCARCCGCA	8-25 F	50
bak4	AGTTTGATCMTGGCTCAG	1522-1540 R	58

*Calculated based on the formula: T_m = 4 (G+C) + 2 (A+T)

Primer Specificity Evaluation

DNA extracts from pure bacterial cultures and from rumen fluid and corn silage samples were used as templates. Nested and double-step PCR reactions were done as described previously using the optimized conditions for the primers. Specificity is determined by the appearance of the 1,267 bp band in the secondary PCR products.

Phylogenetic Analysis

Using DH42 16S rRNA sequence as a query sequence, a local alignment search was done using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) World Wide Web site (http://www.ncbi.nlm.nih.gov/BLAST/). BLAST uses a heuristic algorithm, which seeks local as opposed to global alignments and is therefore able to detect relationships among sequences that share only isolated regions of similarity (Altschul et al., 1990). Out of 377 BLAST hits on the query sequence, 16S rRNA sequences of 25 *Propionibacterineae*, 24 *Actinobacteria* and three environmental samples of unidentified eubacteria were selected for further analysis (Table 5-4).

Using the computer software package Windows 32 MegAlign© 1993-1999 (DNASTAR Inc.), multiple alignment of 16S rRNA sequences of DH42, 25

Propionibacterieae species, 23 other *Actinobacteria* species and *Escherichia coli* (as a non-related organism) was done and their phylogenetic relationships were estimated. The alignment of the sequences was performed using the Clustal V method described in Higgins and Sharp (1989). The Clustal V method groups sequences into clusters by examining sequence distances between all pairs. Clusters were aligned as pairs then collectively as sequence groups to produce the overall alignment. After the multiple alignment was completed, a Neighbor-Joining method was employed to reconstruct phylogeny for the putative alignment. A phylogenetic tree was generated on which branch distances (lengths) corresponded to sequence divergence.

Gel Electrophoresis

PCR products were run in 0.8 % agarose gel (GibcoBRL) stained with 0.5 μg/ml ethidium bromide (GibcoBRL). About 2 μl 10X Blue Juice™ gel loading buffer (GibcoBRL) was added to 8 μl of PCR product and electrophoresed at 80V for about 2-3 h. A 1kb DNA ladder (GibcoBRL) was used as a marker.

Results and Discussion

PCR Optimization

Figure 5-2 shows the combined effect of magnesium chloride concentration and annealing temperature on the appearance of the 1,267 bp band with DNA extracted from *P. acidipropionici* DH42. It shows that amplification of the target band is possible even at 69°C using 1.25 mM of $MgCl_2$. In addition to the magnesium chloride concentration, the optimum annealing temperature also depends on other factors such as the concentration of dNTPs, primer and Taq polymerase and amount of DNA. For the primer concentration, it was found that amplification was possible using 10 pmol of each primer.

The lowest concentration tested (7.5 pmol) showed no amplification product. Taq polymerase level was tested at 2.5, 2.0, and 1.5 U. No amplification product was observed at less than 2.0 U. With pure cultures, stringency was also attained by diluting the primary PCR products to 1:1,000. At 68°C annealing temperature and 1.5 mM $MgCl_2$, using 1 µl of a 1:1000 dilution of the primary PCR product as template for secondary PCR prevented the non-specific amplification of other non-propionibacteria and most propionibacterial species tested. Among the propionibacteria strains used, only *P. acidipropionici* (ATCC 25562) had the 1,267-bp fragment after secondary PCR under these PCR conditions. Dawson (1994) had used this microorganism as a reference in the initial identification of DH42 and had observed the similarity its metabolic profile with *P. acidipropionici* DH42. This indicates that the two microorganisms are closely related. The PCR optimization was then aimed at preventing the amplification of the 1,267 bp band with the *P. acidipropionici* (ATCC 25562) samples. This was done by adjusting the primer, dNTP and Taq polymerase concentrations. At 68°C annealing temperature, and using 10 pmols of each primer, 1.0 mM of $MgCl_2$, and 2.0 units of Taq polymerase, the amplification of *Propionibacterium acidipropionici* (ATCC 25562) and other bacterial species was prevented (Figs. 5-3 and 5-4).

Primer Specificity

Based on the alignment of the 16S rDNA of *P. acidipropionici* DH42 with the ARB (Strunk and Ludwig, 1996) program's database, *E. combesii* was found to have the closest match differing by only four nucleotides. During the secondary amplification using 68°C annealing temperature, the estimated 1,267bp fragment was not detected using *E. combesii* DNA. If it differed with DH42 by just four nucleotides, a less stringent

condition would have allowed amplification of the same size fragment in *E. combesii.* In the alignment of the specific-propionibacteria primer gd1, Dasen et al. (1998) observed that *E. combesii* showed 100% similarity with the target sequence. Morever, they also observed it clustered with *P. thoenii* (92.3%) and had only 78% similarity with other *Eubacterium* species. They also found that *A. israelii* is more similar to *P. acnes* than to other species from the genus *Actinomyces.* They concluded the need for re-evaluation of these strains or sequencing problems. Based on the HPLC analysis of *E. combesii* overnight culture medium, it produced iso-acids but did not produce significant amounts of either propionic acid or acetic acids, which would indicate that it is not a propionibacterium. Moreover, it did not form yellow colonies using Purple Base Agar with i-erythritol while all the propionibacteria species used in this study did. During PCR, *E.combesii* DNA did not form the 1,267 bp-fragment with primers dhb1 and dhb2. Under less stringent conditions, amplification of this fragment would have been possible if it differs with DH42 by only 4 nucleotides. At 68°C annealing temperature, it formed a fragment size that is about 1.5 kb (Fig. 5-5). A similar fragment size was observed in other bacteria when the amount of template for secondary amplification was high.

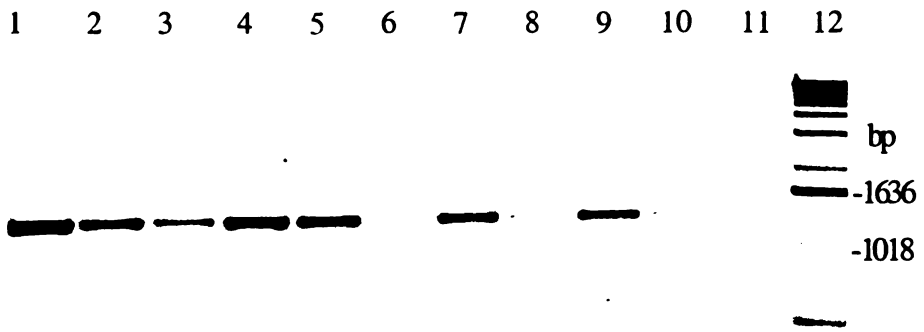

Figure 5-2. Effect of annealing temperature (lanes 1-3, 66°C; lanes 4-6, 67°C, lanes 7-8, 68°C, lanes 9-11, 69°C) and magnesium chloride concentration:1.5 mM (lanes1,4,7); 1.25 mM (lane 10); 1.0 mM (lanes 2, 5, 8, 9); and 0.5 mM (lanes 3 and 6); lane 12, 1kb DNA ladder.

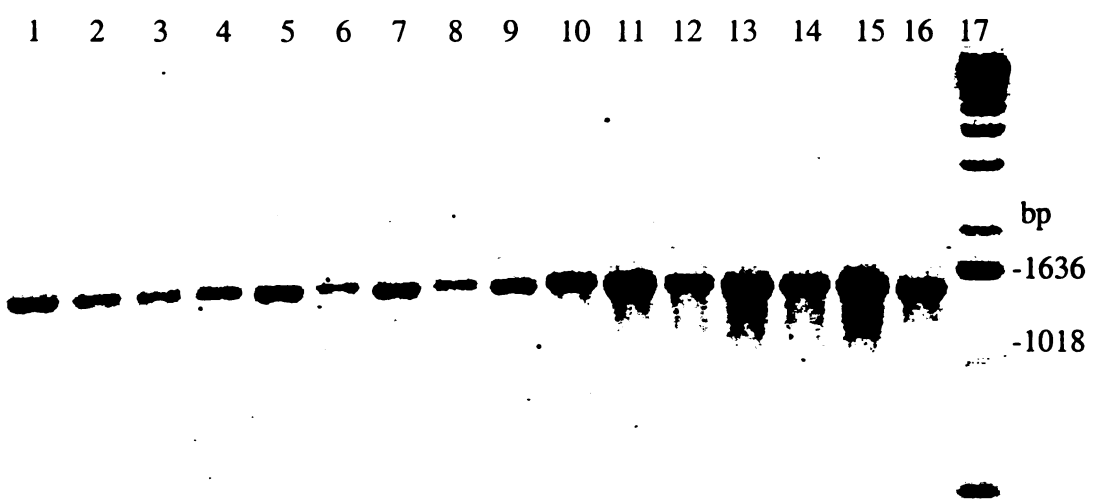

Figure 5-3. Primary PCR showing the 1.5 kb fragment using primers bak11 and bak4 with the following bacterial strains: 1, *Propionibacterium freudenreichii*; 2, *P. shermanii* CDC3094;3, *P. shermanii* P92; 4, *P. thoenii* P15; 5, *P. pentosaceum* P11; 6, *P. acidipropionici* (ATCC 25562); 7, *P. shermanii*; 8, *Propionibacterium sp.*; 9, *P. acidipropionici* DH42; 10, *Lactobacillus sakei*; 11, *Lactobacillus confusus*; 12, *Lactobacillus delbruickii*; 13, *Bacillus subtilis*; 14, *Bacillus sp.*; 15, *Bacillus subtilis* (ATCC 6633); 16, *Eubacterium combesii*; 17, 1 kb DNA ladder

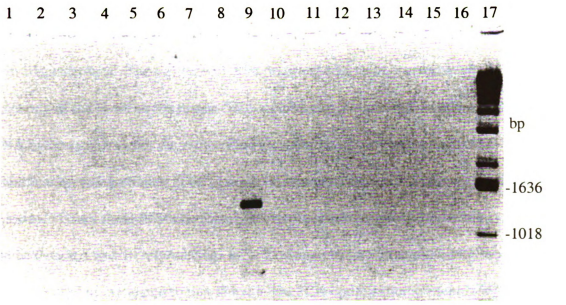

Figure 5-4. Secondary PCR showing the 1.3 kb fragment amplification using primers dhb1 and dhb2 with the following bacterial strains: 1, *Propionibacterium freudenreichii*; 2, *P. shermanii* CDC3094; 3, *P. shermanii* P92; 4, *P. thoenii* P15; 5, *P. pentosaceum* P11; 6, *P. acidipropionici* (ATCC 25562); 7, *P. shermanii*; 8, *Propionibacterium sp.*; 9, *P. acidipropionici* DH42; 10, *Lactobacillus sakei*; 11, *L. confusus*; 12, *L. delbruckii*; 13, *Bacillus subtilis*; 14, *Bacillus sp.*; 15, *B. subtilis* (ATCC 6633); 16, *Eubacterium combesii*; 17, 1 kb DNA ladder.

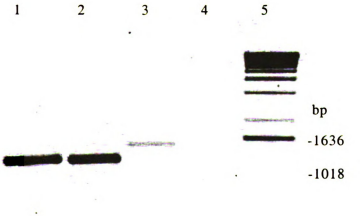

Figure 5-5. Secondary PCR amplification products showing the 1,267 bp-fragment size in *P. acidpropionici* DH42, lane 1; *P. acidipropionici* (ATCC 25563), lane 2; *Eubacterium combesii*, lane 3; negative control, lane 4; 1-kb DNA ladder, lane 5.

The detection limit of the assay was also determined using rumen fluid and corn silage extracts. Figure 5-6 shows the primary amplification products in the rumen fluid and corn silage extracts. The estimated 1.5 kb fragment size was observed in all the samples. Smearing of the bands in the rumen fluid samples can indicate the abundance of bacterial DNA in the samples. For the rumen fluid samples, stringency of the secondary amplification was sufficient at 66°C annealing temperature and 1.5 mM magnesium chloride. Under these PCR conditions, it was observed the lowest inoculation rate that can be detected was 10^3 cfu/ml (Fig. 5-7). Lower annealing temperature for secondary PCR was used as compared to that found in the PCR optimization protocol (66°C vs 68°C) because it was observed that at higher annealing temperature, the 10^4 cfu/ml was the lowest inoculation rate that could be detected. With the silage extracts, the same annealing temperature was used, but lower magnesium chloride concentration (1.0 mM) was used to prevent the non-specific amplification of the uninoculated samples. Under these conditions, as low as 100 cfu/ml of *P. acidipropionici* DH42 can be detected in the silage extracts. The difference in the detection limit in rumen fluid and corn silage samples could be due to their differences in their microbial load and how these affect amplification efficiency and the presence of contaminants that interfere with PCR. While it can be assumed that the amplification efficiency is the same for all 16S rDNA in the rumen fluid samples, the use of universal primers contain degeneracy that may influence the formation of primer-template hybrids (Wintzingerode et al., 1997). Moreover, varying molecular percent G + C composition of 16S rRNA genes can also cause differential amplification. Templates with lower G + C content will have more efficient strand separation, thus preferential amplification may result (Wintzingerode et al., 1997). With

the abundance of DNA in the rumen fluid samples, higher competition with non-DH42 DNA could have reduced the amplification of DH42 16S rDNA during primary PCR. Consequently, the initial template for secondary amplification may have been less in the rumen fluid samples as compared to the corn silage extracts. Increasing the amount of template for secondary PCR or using less stringent condition such as higher magnesium chloride concentration did not improve the sensitivity of the assay. It is also possible that certain amounts PCR inhibitors is present in the rumen fluid samples making amplification less efficient as compared to the silage extracts. While the DNA extraction kit included a solution to remove PCR inhibitors, it might have not been sufficient to remove all the inhibitors in the rumen fluid samples. The presence of humic acids and humic substances in environmental samples and their negative effect in lysis efficiency PCR amplification had been documented (Wintzingerode et al., 1997).

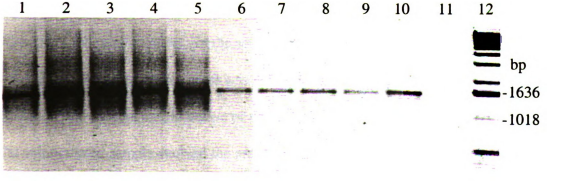

Figure 5-6. Primary PCR showing the amplification products of primers bak4 and bak11 using DNA extracts from rumen fluid (lanes 1-5, amplification from uninoculated rumen fluid, 10^2-10^3, 10^4, 10^5cfu/ml, respectively) and silage (lanes 6-10, amplification from uninoculated silage, 10^2-10^3, 10^4, 10^5cfu/ml, respectively); lane 11, negative control and lane 12, 1 kb DNA ladder.

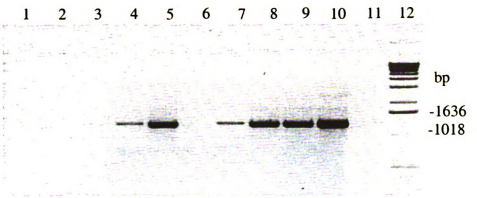

Figure 5-7. Secondary PCR showing the amplification products of primers dhb1 and dhb2 using DNA extracts from rumen fluid (lane 1-5, amplification from uninoculated rumen fluid, 10^2, 10^3, 10^4, 10^5cfu/ml, respectively) and silage (lanes 6-10, amplification from uninoculated silage, 10^2, 10^3, 10^4, 10^5cfu/ml, respectively); lane 9, negative control; lane 10, 1 kb DNA ladder.

A double-step PCR amplification for the rumen fluid samples was tried to check if selective amplification of DH42 and better detection could be achieved. Rossi et al. (1998) recommended this procedure to increase the sensitivity of detection of propionibacteria in silage or soil samples. In the first round of PCR, slightly lower annealing temperature (64°C) was used to allow better amplification since at higher temperature (66-68°C), no primary amplification product was observed in the samples with lower inoculation rates. In the second step, while the temperature was maintained at 64°C, magnesium chloride level was reduced 1.25 mM to provide a more stringent condition and prevent amplification of non-DH42 DNA. In the primary amplification, the uninoculated sample showed the presence of the 1,267 bp-band (lane 1). This is not unusual since a less stringent condition would allow amplification of non-specific hybridization products. The presence of other *P. acidipropionici* species in the rumen could have produced this band. In the second amplification, while the decreased amount

of magnesium chloride prevented the amplification in the uninoculated samples, the 10^3 cfu/ml of DH42 was the lowest inoculation rate that could be detected (Figure 5-8).

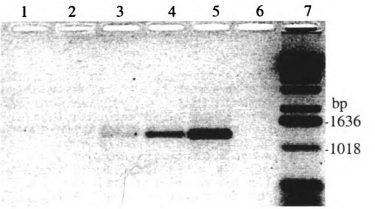

Figure 5-8. Secondary PCR of rumen fluid samples: lane 1, uninoculated; lane 2, 10^2; lane 3, 10^3; lane 4, 10^4; lane 5, 10^5 cfu/ml; lane 6, negative control, lane 7, 1kb DNA ladder.

Phylogenetic Analysis

Table 5-4 shows the *Propionibacteriacea* species that was used for the analysis. The highest BLAST scores (2387-2778) were found for three species: *P. microaerophilus, E. combesii*, and *P. acidipropionici*. This shows that the three organisms are the ones closely related to DH42 based on their 16S rRNA sequences. Dawson (1994) observed that the metabolic profile of *P. acidipropionici* (ATCC 25562) closely matched that of *P. acidipropionici* DH42. This confirms the identity of this species as *P. acidipropionici.*

Figure 5-9 shows that *P. acidipropionici* DH42 formed a cluster with the *P. acidipropionici, E. combesii*, and *P. microaerophilus*. *P. acidipropionici* DH42 is also

within the cluster of *P. jensenii* and *P. thoenii* which is in agreement of the findings of Charfreitag and Stackebrandt (1989).

Table 5-4. Taxonomy BLAST report for 13 propionibacteria species compared against DH42 using BLAST

Bacteria [eubacteria]	BLAST score (bits)
. Actinobacteria [Firmicutes, high GC Gram+]	
. . Actinomycetales [Actinobacteridae]	
. . . Propionibacterineae [actinomycetes]	
. . . . Propionibacteriaceae [actinomycetes]	
. . . . . Propionibacterium [actinomycetes]	
. . . . . Propionibacterium microaerophilus	2778
. . . . . Propionibacterium acidipropionici	2387
. . . . . Propionibacterium thoenii	2240
. . . . . Propionibacterium jensenii	2065
. . . . . Propionibacterium propionicus DSM 43307	1764
. . . . . Propionibacterium avidum DSM 4901	1756
. . . . . Propionibacterium acnes	1651
. . . . . Propionibacterium sp. V07/12348	978
. . . . . Propionibacterium cyclohexanicum	821
. . . . . Propionibacterium sp. LCDC-98A072	815
. . . . . Propionibacterium propionicus	794
. . . . . Propioniferax innocua	786
. . . . Eubacterium combesii	2680

DNA Sequencing

Figure 5-10 shows the alignment of the sequenced fragment with the DH42 16S rDNA starting from *E. coli* position 196 to 767. Of the 571 nucleotides in the DH42 DNA sequence, the sequence of the fragment from the rumen fluid sample differed by 6 nucleotides. Differences in the sequences of the DH42 and that of the fragment could be due to the differences in the primers used for the sequencing. Method of primer synthesis and approach to primer purification can affect the quality of the sequencing data obtained in the dye terminator cycle sequencing reactions (Perkin Elmer, 1995). BLAST search was also done using the partial sequence of the fragment. Table 5 shows the top ten out of the 200 BLAST hits in the query sequence. The highest BLAST scores (1005-1031)

were found for three species: *P. microaerophilus, E. combesii*, and *P. acidipropionici*, which agree with the earlier BLAST results of the DH42 sequence.

Implications

This study showed that as low as 10^2 and 10^3 cfu/ml of *P. acidipropionici* DH42 can be detected in corn silage and rumen fluid, respectively. While the PCR assay is not as sensitive in the rumen fluid as compared to the silage samples, it appears sufficient considering the current suggested inoculation rate of at least 10^5 cfu/g material in silage. Improvement in the detection of *P. acidipropionici* DH42 in rumen fluid samples might be achieved with a different DNA extraction method or the use of PCR enhancers.

Figure 5-9. Phylogenetic tree of the order *Actinomycetales*. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events

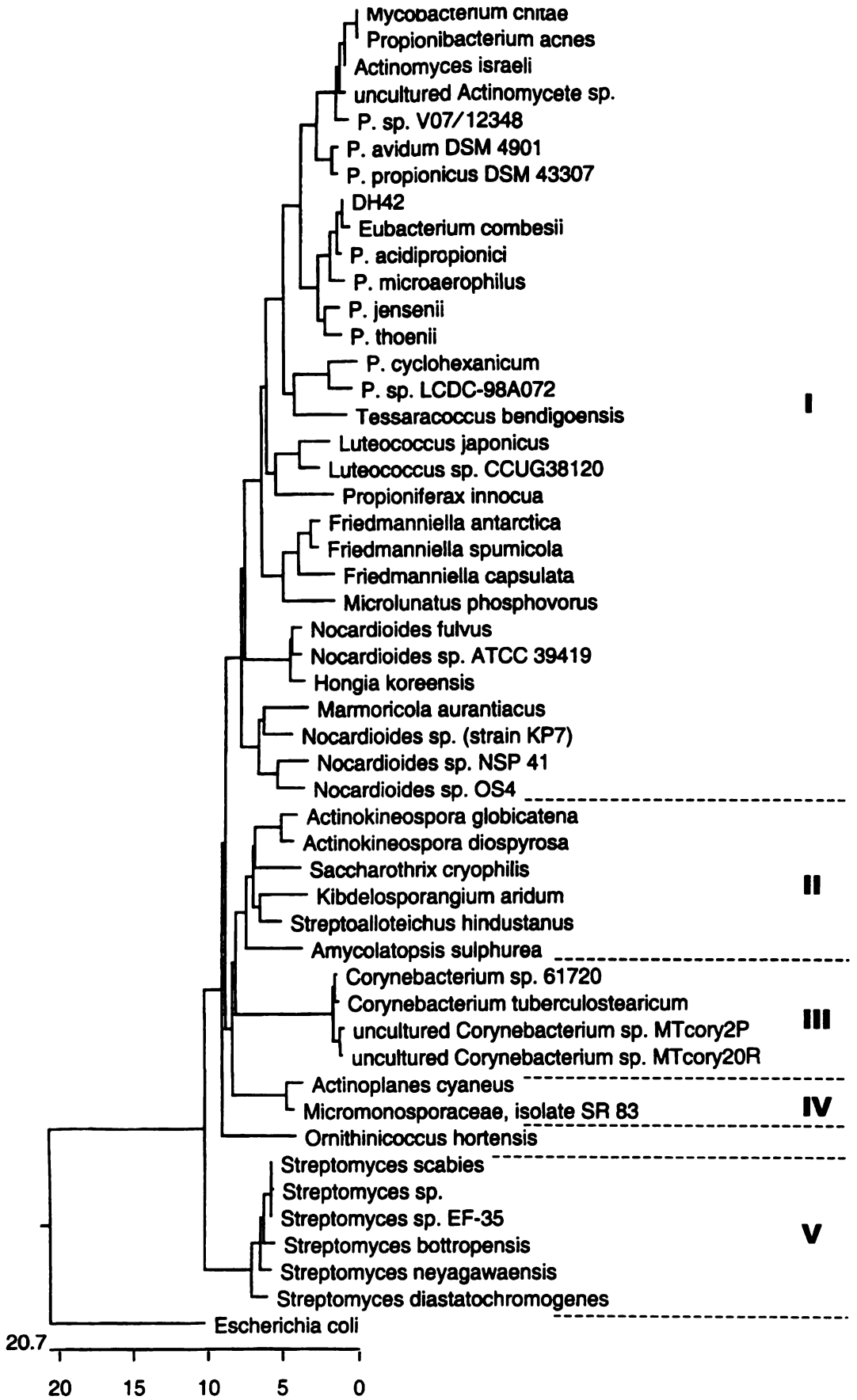
Mycobacterium chitae
Propionibacterium acnes
Actinomyces israeli
uncultured Actinomycete sp.
P. sp. V07/12348
P. avidum DSM 4901
P. propionicus DSM 43307
DH42
Eubacterium combesii
P. acidipropionici
P. microaerophilus
P. jensenii
P. thoenii
P. cyclohexanicum
P. sp. LCDC-98A072
Tessaracoccus bendigoensis
Luteococcus japonicus
Luteococcus sp. CCUG38120
Propioniferax innocua
Friedmanniella antarctica
Friedmanniella spumicola
Friedmanniella capsulata
Microlunatus phosphovorus
Nocardioides fulvus
Nocardioides sp. ATCC 39419
Hongia koreensis
Marmoricola aurantiacus
Nocardioides sp. (strain KP7)
Nocardioides sp. NSP 41
Nocardioides sp. OS4
Actinokineospora globicatena
Actinokineospora diospyrosa
Saccharothrix cryophilis
Kibdelosporangium aridum
Streptoalloteichus hindustanus
Amycolatopsis sulphurea
Corynebacterium sp. 61720
Corynebacterium tuberculostearicum
uncultured Corynebacterium sp. MTcory2P
uncultured Corynebacterium sp. MTcory20R
Actinoplanes cyaneus
Micromonosporaceae, isolate SR 83
Ornithinicoccus hortensis
Streptomyces scabies
Streptomyces sp.
Streptomyces sp. EF-35
Streptomyces bottropensis
Streptomyces neyagawaensis
Streptomyces diastatochromogenes
Escherichia coli
I
II
III
IV
V
20.7
20
15
10
5
0

Figure 5-9

196

DH42..... GGAAAGTGTTTGTGGTGGTGGATGGACTCGCGGCCTATCAGCTT
RF..... GGAAAGTGTTTGTGGTGGTGGATGGACTCGCGGCCTATCAGCTT

DH42.....GTTGGTGAGGTAGTGGCTCACCAAGGCGGTGACGGGTAGCCG
RF..... GTTGGTGAGGTAGTGGCTCACCAAGGCGGTGACGGGTAGCCG

DH42.....GCCTGAGAGGGTGACCGGCCACATTGGGACTGAGATACGGCC
RF..... GCCTGAGAGGGTGACCGGCCACATTGGGACTGAGATACGGCC

DH42.....CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
RF..... CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG

DH42.....GGCGGAAGCCTGATGCAGCAACGCCGCGTGCGGGATGACGGCC
RF..... GGCGGAAGCCTGATGCAGCAACGCCGCGTGCGGGATGACGGCC

DH42.....TTCGGGTTGTAAACCGCTTTCACCAGGGGCGAAGGCATyCTTTT
RF.....TTCGGGTTGTAAACCGCTTTCACCAGGGGCGAAGGCATCCTTTT

DH42.....GGGGTGTTGACGGTACCTGGAGAAGAAGCACCGGCTAACTAC
RF..... GGGGTGTTGACGGTACCTGGAGAAGAAGCACCGGCTAACTAC

DH42.....GTGCCAGCAGCCGCGGTGATACGTAGGGTGCGAGCGTTGTCCG
RF..... GTGCCAGCAGCCGCGGTGATACGTATGGTGCGAGCGTTGTCCG

DH42.....GATTTATTGGGCGTAAAGGGCTCGTAGGCGG'TTGATCGCGTCG
RF..... GATTTATTGGGCGTAAAGGGCTCGTANGCGG'TTGATCGCGTCG

DH42.....GAAGTGAAAACTTGGGGCTTAACCCTGAGCGTGCTTTCGATAC
RF..... GAAGTGAAAACTTGGGGCTTAACCCTGAGCGTGCTTTCGATAC

DH42.....GGGTTGACTTGAGGAAGGTAGGGGAGAATGGAATTCCTGGTGG
RF..... GGGTTGACTTGAGGAAGGTAGGGGAGAATGGAATTCCTGGTGG

DH42.....AGCGGTGGAATGCGCAGATATCAGGAGGAACACCAGTGGCGA
RF..... AGCGGTGGAATGCGCAGATATCAGGAGGAACACCAGTGGCGA

DH42.....AGGCGGTTCTCTGGACCTTTCCTGACGCTGAGGAGCGAAAGCG
RF..... AGGCGGTTCTCTGGAACTTTCCTGACGCTGAAGAGCGAAAGCG

767

DH42.....TGGGGAGCAAACAGGCTTAGATAC
RF..... TGGGGAGCAAACAGGCTTAAATAC

Figure 5-10. Partial nucleotide sequence of the 1,327 bp-fragment from rumen fluid (RF) sample using primers dhb1 and dhb2 as forward and reverse primers, respectively. Ambiguous or non-matching positions are boxed.

Table 5-5. Distribution of top ten Blast hits on the query sequence.

Sequences producing significant alignments	Score (bits)
gb|AF234623.1|AF234623 *Propionibacterium microaerophilus* 16...	1031
gb|L34614.1|EUBRRDH *Eubacterium combesii* 16S ribosomal RNA	1019
emb|X53221.1|PACP116S *Propionibacterium acidi-propionici* pa...	1005
emb|X53220.1|PTH16S *Propionibacterium thoenii* partial 16S rRNA	862
emb|X53219.1|PJ16S *Propionibacterium jensenii* partial 16S rRNA	813
emb|AJ003058.1|PPAJ3058 *Propionibacterium propionicus* DSM 4...	549
emb|AJ003055.1|PAAJ3055 *Propionibacterium avidum* DSM 4901 1...	549
emb|Y17821.1|PRSP17821 *Propionibacterium* sp. V07/12348 16S ...	535
gb|AF154099.1|AF154099 Uncultured hydrocarbon seep bacteriu...	519
gb|AF154832.1|AF154832 *Propionibacterium acnes* 16S ribosoma...	519
gb|AF145256.1|AF145256 *Propionibacterium acnes* 16S ribosoma...	519

CHAPTER 6

SUMMARY AND CONCLUSION

Two studies were conducted to evaluate the efficacy of propionibacteria as silage inoculants. In the first study, the performance of propionibacteria with or without lactic acid bacteria were evaluated using reconstituted corn. Results showed that propionibacteria enhanced the fermentation of reconstituted corn only up d 21 of ensiling. The combination of *P. acidipropionici* DH42 with lactic acid bacteria as inoculants reduced silage pH and butyric acid and increased propionic, acetic and lactic acids. LAB inoculation did not significantly increase the LAB population in the treated silages. During aerobic exposure, all the silages appeared well preserved. Organic acid levels remained stable throughout the exposure period. Propionibacteria inoculation did not significantly reduce the yeast and mold population. However, the silages with *P. acidipropionici* DH42 + LAB had higher propionic, acetic and lactic acids and lower pH.

In the second study, the effect of moisture on the efficacy of propionibacteria as silage inoculants was tested. Rolled corn of moisture contents ranging from 22-35% were used. The moisture of the ensiling material affected the efficacy of propionibacteria as inoculants. The 22-28% moisture levels appeared to favor the growth of the *P. acidipropionici* DH42 in silage. After 120 d of ensiling, PAB-inoculated high moisture corn gave significantly higher propionic and acetic acids at 22-28% moisture levels. Lower pH at 24 % and butyric acid at 28% moisture levels was also observed with the PAB-treated silages. Inoculation did not affect the yeast and mold counts during ensiling. *P. acidipropionici* DH42 inoculated at 10^6cfu/g better gave results as compared to the 10^5cfu/g inoculation rate. During aerobic exposure, higher propionic and acetic

acids and lower pH were observed with the PAB-treated silages from the 22-28% moisture levels. Propionibacteria inoculation did not significantly reduce yeast and mold counts.

The vitamin B_{12} production capability of *P. acidipropionici* DH42 was also evaluated in comparison with *P. shermanii*. Results showed comparable vitamin B_{12} production of the two propionibacteria strains. After 72 h of incubation, the *P. acidipropionici* DH42 and *P. shermanii* cultures grown at 30°C had vitamin B_{12} contents of 852.85 and 840.69 ng/ml, respectively. Both strains grew better at 30°C than at 40°C. Poor growth of *P. shermanii* was evident at 40°C incubation. Lower pH was observed at lower incubation temperature but for the *P. acidipropionici* DH42 cultures, the differences of the pH between the two incubation temperatures were not significant. *P. acidipropionici* DH42 cultures tend to have higher propionic and acetic acids while the *P. shermanii* cultures had higher succinic and malic acids.

A PCR-based detection of *P. acidipropionici* DH42 was developed. Nested PCR was used with DH42-specific primers dhb1 and dhb2 for the secondary amplification of a 1,267 bp-fragment. Using the established protocols for PCR amplification, as low as 10^2 cfu/ml and 10^3cfu/ml of *P. acidipropionici* DH42 in silage extracts and rumen fluid, respectively, were detected.

The silage studies had shown that moisture level affects the efficacy of *P. acidipropionici* DH42. The 22-28% moisture contents appear to favor its growth. An inoculation rate of 10^6 cfu/g of ensiling material is recommended. It has been shown that *P. acidipropionici* DH42 can produce vitaminB_{12} that is comparable to *P. shermanii*

producing capability. *P. acidipropionici* DH42 can easily be detected in silage and rumen fluid using PCR technology.

APPENDICES

APPENDIX A

Data Used for Analyses in Chapter 3

Table A-1. Effect of inoculation and moisture on the propionic acid content (g/100 g) of fresh and ensiled HMC.

Moisture level	Treatment	Ensiling Period (d)			
%		0	10	21	120
	Control	0.00	0.00	0.01 [ab]	0.17
	DH42 10^5	0.00	0.00	0.01 [b]	0.17
35	DH42 10^6	0.00	0.00	0.01 [a]	0.18
	P. jensenii	0.00	0.00	0.01 [b]	0.18
	RCM	0.01	0.00	0.01 [b]	0.17
	S.E.M	0.00	0.00	0.00	0.01
	Control	0.00	0.00	0.01	0.34 [a]
	DH42 10^5	0.00	0.00	0.01	0.30 [ab]
33	DH42 10^6	0.01	0.01	0.02	0.32 [ab]
	P. jensenii	0.05	0.00	0.01	0.29 [b]
	RCM	0.06	0.01	0.01	0.31 [ab]
	S.E.M.[n]	0.01	0.00	0.00	0.01
	Control	0.01	0.01 [b]	0.00 [c]	0.10 [b]
	DH42 10^5	0.01	0.01 [b]	0.06 [b]	0.26 [a]
28	DH42 10^6	0.03	0.04 [a]	0.15 [a]	0.34 [a]
	P. jensenii	0.04	0.01 [b]	0.07 [b]	0.28 [a]
	RCM	0.02	0.01 [b]	0.00 [c]	0.13 [b]
	S.E.M.[n]	0.00	0.00	0.00	0.01
	Control	0.01	0.01 [c]	0.01 [b]	0.09 [b]
	DH42 10^5	0.04	0.02 [bc]	0.03 [b]	0.15 [b]
24	DH42 10^6	0.02	0.08 [a]	0.14 [a]	0.26 [a]
	P. jensenii	0.02	0.05 [ab]	0.04 [b]	0.17 [ab]
	RCM	0.02	0.00 [c]	0.01 [b]	0.14 [b]
	S.E.M.[n]	0.00	0.00	0.01	0.01
	Control	0.00	0.00	0.00 [b]	0.06 [c]
	DH42 10^5	0.00	0.00	0.01 [ab]	0.14 [ab]
23	DH42 10^6	0.02	0.02	0.03 [a]	0.18 [a]
	P. jensenii	0.02	0.02	0.03 [a]	0.19 [a]
	RCM	0.03	0.00	0.00 [b]	0.09 [bc]
	S.E.M.[n]	0.01	0.00	0.00	0.01
	Control	0.00	0.00	0.00	0.03 [b]
	DH42 10^5	0.00	0.00	0.01	0.15 [a]
22	DH42 10^6	0.01	0.00	0.02	0.20 [a]
	P. jensenii	0.01	0.00	0.01	0.18 [a]
	RCM	0.02	0.00	0.00	0.02 [b]
	S.E.M.[n]	0.00	0.00	0.00	0.01

[abc]Column means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n]Standard error of the mean.

Table A-2. Effect of inoculation and moisture on the pH of fresh and ensiled HMC.

Moisture level	Treatment	Ensiling period (d)			
%		0	10	21	120
	Control	5.78	3.89	3.79	3.70
	DH42 10^5	5.69	3.90	3.80	3.73
35	DH42 10^6	5.70	3.89	3.79	3.74
	P. jensenii	5.71	3.92	3.79	3.72
	RCM	5.71	3.90	3.80	3.75
	S.E.M.[n]	0.02	0.01	0.00	0.01
	Control	5.56	4.04	3.90	4.12
	DH42 10^5	5.63	4.06	3.89	4.10
33	DH42 10^6	5.59	4.01	3.90	4.04
	P. jensenii	5.57	4.08	3.92	4.02
	RCM	5.64	4.03	3.89	4.05
	S.E.M.[n]	0.02	0.01	0.01	0.02
	Control	5.52	4.72	4.51	4.24
	DH42 10^5	5.48	4.62	4.50	4.18
28	DH42 10^6	5.52	4.61	4.48	4.14
	P. jensenii	5.53	4.62	4.55	4.16
	RCM	5.49	4.61	4.46	4.22
	S.E.M.[n]	0.03	0.01	0.01	0.01
	Control	5.35	4.55	4.46	4.43 [a]
	DH42 10^5	5.48	4.53	4.46	4.40 [ab]
24	DH42 10^6	5.46	4.60	4.49	4.33 [bc]
	P. jensenii	5.36	4.36	4.27	4.27 [c]
	RCM	5.41	4.70	4.56	4.40 [ab]
	S.E.M.[n]	0.02	0.04	0.03	0.10
	Control	5.32	4.79	4.55	4.27
	DH42 10^5	5.22	4.84	4.54	4.33
23	DH42 10^6	5.26	4.76	4.53	4.30
	P. jensenii	5.23	4.88	4.62	4.34
	RCM	5.24	4.80	4.51	4.31
	S.E.M.[n]	0.02	0.03	0.02	0.01
	Control	5.26	5.30	5.18	4.67
	DH42 10^5	5.27	5.29	5.06	4.57
22	DH42 10^6	5.22	5.29	4.97	4.56
	P. jensenii	5.24	5.29	5.04	4.55
	RCM	5.30	5.28	5.16	4.67
	S.E.M.[n]	0.02	0.02	0.01	0.01

[a]Column means within same moisture level with unlike superscripts differ (P<0.05).
[n]Standard error of the mean.

Table A-3. Effect of inoculation and moisture on the acetic acid content (g/100g DM) of fresh and ensiled HMC.

Moisture level	Treatment	Ensiling period (d)			
%		0	10	21	120
	Control	0.00 [b]	0.18	0.17	0.20
	DH42 10^5	0.00 [b]	0.19	0.17	0.20
35	DH42 10^6	0.00 [b]	0.20	0.17	0.22
	P. jensenii	0.01 [b]	0.19	0.17	0.20
	RCM	0.01 [a]	0.21	0.16	0.22
	S.E.M.[n]	0.00	0.01	0.00	0.01
	Control	0.03 [c]	0.15	0.16	0.31
	DH42 10^5	0.04 [bc]	0.13	0.17	0.30
33	DH42 10^6	0.07 [a]	0.17	0.18	0.32
	P. jensenii	0.70 [ab]	0.17	0.16	0.29
	RCM	0.08 [a]	0.15	0.19	0.29
	S.E.M.[n]	0.00	0.01	0.00	0.01
	Control	0.02 [b]	0.09	0.16	0.28 [c]
	DH42 10^5	0.02 [ab]	0.09	0.20	0.41 [abc]
28	DH42 10^6	0.03 [ab]	0.12	0.25	0.48 [a]
	P. jensenii	0.04 [a]	0.11	0.25	0.47 [ab]
	RCM	0.03 [ab]	0.10	0.16	0.29 [abc]
	S.E.M.[n]	0.00	0.00	0.01	0.03
	Control	0.02	0.07 [c]	0.08 [b]	0.24
	DH42 10^5	0.03	0.10 [abc]	0.11 [ab]	0.35
24	DH42 10^6	0.02	0.15 [ab]	0.15 [a]	0.46
	P. jensenii	0.03	0.09 [bc]	0.15 [a]	0.40
	RCM	0.01	0.15 [a]	0.07 [b]	0.26
	S.E.M.[n]	0.00	0.01	0.01	0.03
	Control	0.00 [c]	0.07	0.14 [ab]	0.20 [bc]
	DH42 10^5	0.02 [bc]	0.12	0.19 [ab]	0.22 [bc]
23	DH42 10^6	0.04 [ab]	0.14	0.24 [a]	0.33 [a]
	P. jensenii	0.05 [a]	0.17	0.14 [ab]	0.29 [ab]
	RCM	0.03 [ab]	0.12	0.01 [b]	0.19 [c]
	S.E.M.[n]	0.00	0.01	0.02	0.01
	Control	0.03	0.00 [b]	0.01	0.11
	DH42 10^5	0.03	0.00 [b]	0.03	0.17
22	DH42 10^6	0.02	0.01 [b]	0.06	0.14
	P. jensenii	0.03	0.02 [b]	0.09	0.16
	RCM	0.03	0.05 [a]	0.08	0.12
	S.E.M.[n]	0.00	0.00	0.01	0.01

[a,b,c] Column means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n] Standard error of the mean.

Table A-4. Effect of inoculation and moisture on the lactic acid content (g/100g DM) of fresh and ensiled HMC.

Moisture level	Treatment	Ensiling period (d)			
%		0	10	21	120
	Control	0.00	2.02	2.28	3.61
	DH42 10^5	0.00	2.08	2.19	3.56
35	DH42 10^5	0.00	2.19	2.22	3.49
	P. jensenii	0.00	1.99	2.19	3.69
	RCM	0.01	2.40	2.12	3.62
	S.E.M.[n]	0.00	0.06	0.03	0.13
	Control	0.07	1.48	2.07	2.32
	DH42 10^5	0.09	1.61	2.08	2.13
33	DH42 10^6	0.10	1.64	2.17	2.53
	P. jensenii	0.08	1.42	1.95	2.32
	RCM	0.10	1.60	2.19	2.53
	S.E.M.[n]	0.01	0.04	0.03	0.06
	Control	0.08	0.67	1.07	1.25
	DH42 10^5	0.09	0.58	0.83	1.12
28	DH42 10^6	0.09	0.49	0.78	1.04
	P. jensenii	0.11	0.56	0.84	1.31
	RCM	0.08	0.63	0.94	1.11
	S.E.M.[n]	0.01	0.02	0.02	0.05
	Control	0.01	0.71	0.72	0.63
	DH42 10^5	0.02	0.64	0.59	0.59
24	DH42 10^6	0.01	0.58	0.45	0.48
	P. jensenii	0.01	0.85	0.79	0.89
	RCM	0.00	0.64	0.78	0.88
	S.E.M.[n]	0.00	0.13	0.05	0.05
	Control	0.00	0.60	0.27	0.92
	DH42 10^5	0.01	0.49	0.27	0.65
23	DH42 10^6	0.09	0.54	0.19	0.56
	P. jensenii	0.07	0.40	0.16	0.41
	RCM	0.08	0.58	0.15	0.88
	S.E.M.[n]	0.01	0.03	0.02	0.05
	Control	0.05	0.04 [b]	0.15 [b]	0.46 [ab]
	DH42 10^5	0.05	0.04 [b]	0.16 [b]	0.31 [bc]
22	DH42 10^6	0.05	0.04 [b]	0.19 [b]	0.18 [b]
	P. jensenii	0.02	0.07 [b]	0.32 [ab]	0.29 [bc]
	RCM	0.04	0.17 [a]	0.46 [a]	0.54 [a]
	S.E.M.[n]	0.01	0.01	0.02	0.02

Column means within the same moisture content with unlike superscripts differ ($P<0.05$).
[n]Standard error of the mean

Table A-5. Effect of inoculation and moisture on glucose content (g/100 g DM) of fresh and ensiled HMC.

Moisture level	Treatment	Ensiling period (d)			
%		0	10	21	120
	Control	1.00 [a]	0.15	0.14	0.26
	DH42 10^5	0.92 [ab]	0.18	0.15	0.21
35	DH42 10^6	1.02 [a]	0.19	0.15	0.21
	P. jensenii	1.01 [a]	0.14	0.15	0.26
	RCM	0.75 [b]	0.18	0.17	0.29
	S.E.M.[n]	0.02	0.01	0.00	0.01
	Control	0.10	0.17	0.23	0.41
	DH42 10^5	0.10	0.18	0.24	0.36
33	DH42 10^6	0.11	0.18	0.24	0.40
	P. jensenii	0.09	0.16	0.23	0.36
	RCM	0.09	0.18	0.28	0.39
	S.E.M.[n]	0.01	0.00	0.00	0.01
	Control	0.08	0.10	0.11	0.19
	DH42 10^5	0.10	0.08	0.09	0.20
28	DH42 10^6	0.06	0.08	0.09	0.17
	P. jensenii	0.06	0.07	0.09	0.16
	RCM	0.06	0.08	0.09	0.18
	S.E.M.[n]	0.01	0.00	0.00	0.01
	Control	0.06	0.03	0.08 [ab]	0.05
	DH42 10^5	0.06	0.04	0.06 [ab]	0.05
24	DH42 10^6	0.05	0.05	0.12 [a]	0.10
	P. jensenii	0.05	0.04	0.04 [b]	0.03
	RCM	0.05	0.05	0.07 [ab]	0.09
	S.E.M.[n]	0.00	0.00	0.41	0.01
	Control	0.27 [a]	0.05	0.04	0.09
	DH42 10^5	0.25 [ab]	0.05	0.04	0.08
23	DH42 10^6	0.13 [bc]	0.04	0.03	0.06
	P. jensenii	0.12 [c]	0.04	0.03	0.10
	RCM	0.08 [c]	0.04	0.03	0.06
	S.E.M.[n]	0.01	0.00	0.00	0.01
	Control	0.05	0.16 [a]	0.12 [a]	0.06
	DH42 10^5	0.04	0.17 [a]	0.08 [ab]	0.11
22	DH42 10^6	0.02	0.13 [a]	0.06 [ab]	0.09
	P. jensenii	0.04	0.10 [ab]	0.05 [b]	0.11
	RCM	0.05	0.03 [b]	0.05 [b]	0.05
	S.E.M.[n]	0.00	0.01	0.01	0.76

[a,b,c] Column means within same moisture content with unlike superscripts differ ($P<0.05$).
[n] Standard error of the mean.

Table A-6. Effect of inoculation and moisture on the citric acid content (g/100 g DM) of fresh and ensiled HMC.

Moisture level	Treatment	Ensiling period (d)			
%		0	10	21	120
	Control	0.04	0.04	0.01	0.00
	DH42 10^5	0.05	0.05	0.03	0.00
35	DH42 10^6	0.05	0.06	0.03	0.00
	P. jensenii	0.06	0.02	0.03	0.00
	RCM	0.05	0.05	0.05	0.00
	S.E.M.[n]	0.01	0.01	0.00	0.00
	Control	0.12	0.01	0.02 [ab]	0.00
	DH42 10^5	0.04	0.03	0.01 [b]	0.00
33	DH42 10^6	0.05	0.02	0.02 [ab]	0.00
	P. jensenii	0.03	0.02	0.02 [ab]	0.00
	RCM	0.03	0.01	0.04 [a]	0.00
	S.E.M.[n]	0.01	0.00	0.00	0.00
	Control	0.04	0.01	0.01	0.00
	DH42 10^5	0.09	0.01	0.01	0.00
28	DH42 10^6	0.01	0.01	0.00	0.00
	P. jensenii	0.01	0.01	0.01	0.00
	RCM	0.01	0.01	0.01	0.00
	S.E.M.[n]	0.01	0.00	0.00	0.00
	Control	0.01	0.01	0.01	0.00
	DH42 10^5	0.01	0.01	0.01	0.00
24	DH42 10^6	0.01	0.01	0.01	0.01
	P. jensenii	0.01	0.01	0.01	0.00
	RCM	0.01	0.01	0.01	0.01
	S.E.M.[n]	0.00	0.00	0.00	0.00
	Control	0.04	0.01	0.01 [a]	0.01
	DH42 10^5	0.05	0.01	0.01 [a]	0.01
23	DH42 10^6	0.03	0.01	0.00 [b]	0.01
	P. jensenii	0.01	0.01	0.00 [ab]	0.02
	RCM	0.02	0.01	0.00 [b]	0.01
	S.E.M.[n]	0.00	0.00	0.00	0.01
	Control	0.02	0.02	0.02 [a]	0.02
	DH42 10^5	0.02	0.02	0.02 [ab]	0.02
22	DH42 10^6	0.02	0.02	0.02 [ab]	0.02
	P. jensenii	0.02	0.01	0.01 [b]	0.02
	RCM	0.02	0.01	0.01 [b]	0.02
	S.E.M.[n]	0.00	0.00	0.00	0.00

Column means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n]Standard error of the mean.

Table A-7. Effect of inoculation and moisture on the ethanol content (g/100g DM) of fresh and ensiled HMC.

Moisture level	Treatment	Ensiling period (d)			
%		0	10	21	120
	Control	0.00	0.61	0.56	0.48
	DH42 10^5	0.00	0.68	0.55	0.56
35	DH42 10^6	0.00	0.63	0.54	0.52
	P. jensenii	0.00	0.59	0.55	0.49
	RCM	0.01	0.75	0.54	0.51
	S.E.M.[n]	0.00	0.02	0.00	0.02
	Control	0.13	0.69	0.84	0.85
	DH42 10^5	0.18	0.69	0.85	0.88
33	DH42 10^6	0.20	0.68	0.83	0.92
	P. jensenii	0.16	0.63	0.83	0.86
	RCM	0.17	0.69	0.82	0.86
	S.E.M.[n]	0.00	0.01	0.01	0.02
	Control	0.03	0.60	0.66	0.75
	DH42 10^5	0.05	0.53	0.55	0.54
28	DH42 10^6	0.07	0.55	0.54	0.49
	P. jensenii	0.07	0.53	0.49	0.53
	RCM	0.07	0.52	0.50	0.67
	S.E.M.[n]	0.00	0.02	0.02	0.03
	Control	0.04	0.55	0.59	0.42
	DH42 10^5	0.03	0.45	0.52	0.41
24	DH42 10^6	0.04	0.47	0.59	0.48
	P. jensenii	0.03	0.40	0.42	0.32
	RCM	0.03	0.50	0.63	0.55
	S.E.M.[n]	0.00	0.02	0.01	0.03
	Control	0.00	0.44	0.15	0.15
	DH42 10^5	0.01	0.40	0.23	0.26
23	DH42 10^6	0.03	0.41	0.22	0.34
	P. jensenii	0.04	0.37	0.14	0.26
	RCM	0.05	0.36	0.00	0.42
	S.E.M.[n]	0.01	0.02	0.03	0.02
	Control	0.06	0.26	0.63	0.56
	DH42 10^5	0.05	0.32	0.57	0.44
22	DH42 10^6	0.04	0.31	0.55	0.37
	P. jensenii	0.03	0.29	0.59	0.36
	RCM	0.04	0.35	0.60	0.59
	S.E.M.[n]	0.00	0.02	0.02	0.03

Column means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n]Standard error of the mean.

Table A-8. Effect of inoculation and moisture on the butyric acid content (g/100g DM) of fresh and ensiled HMC.

Moisture level	Treatment	Ensiling period (d)			
%		0	10	21	120
	Control	0.00	0.00	0.00	0.00
	DH42 10^5	0.00	0.00	0.00	0.00
35	DH42 10^6	0.00	0.00	0.00	0.00
	P. jensenii	0.00	0.00	0.00	0.00
	RCM	0.00	0.00	0.00	0.00
	S.E.M.[n]	0.00	0.00	0.00	0.00
	Control	0.00	0.00	0.00	0.32 [a]
	DH42 10^5	0.00	0.00	0.00	0.23 [ab]
33	DH42 10^6	0.00	0.00	0.00	0.27 [ab]
	P. jensenii	0.00	0.00	0.00	0.19 [b]
	RCM	0.00	0.00	0.00	0.26 [ab]
	S.E.M.[n]	0.00	0.00	0.00	0.01
	Control	0.00	0.00	0.00	0.10 [a]
	DH42 10^5	0.00	0.00	0.00	0.03 [b]
28	DH42 10^6	0.00	0.00	0.00	0.01 [b]
	P. jensenii	0.00	0.00	0.00	0.01 [b]
	RCM	0.00	0.00	0.00	0.10 [a]
	S.E.M.[n]	0.00	0.00	0.00	0.01
	Control	0.00	0.00	0.03	0.10 [ab]
	DH42 10^5	0.00	0.00	0.01	0.07 [ab]
24	DH42 10^6	0.00	0.00	0.00	0.03 [b]
	P. jensenii	0.00	0.00	0.00	0.03 [b]
	RCM	0.00	0.00	0.01	0.12 [a]
	S.E.M.[n]	0.00	0.00	0.00	0.01
	Control	0.00	0.00	0.00	0.00 [b]
	DH42 10^5	0.00	0.00	0.00	0.00 [b]
23	DH42 10^6	0.00	0.00	0.00	0.00 [b]
	P. jensenii	0.00	0.00	0.00	0.00 [b]
	RCM	0.00	0.00	0.00	0.03 [a]
	S.E.M.[n]	0.00	0.00	0.00	0.00
	Control	0.00	0.00	0.00	0.00
	DH42 10^5	0.00	0.00	0.00	0.00
22	DH42 10^6	0.00	0.00	0.00	0.00
	P. jensenii	0.00	0.00	0.00	0.00
	RCM	0.00	0.00	0.00	0.00
	S.E.M.[n]	0.00	0.00	0.00	0.00

[a,b,ab]Column means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n]Standard error of the mean.

Table A-9. Effect of moisture and inoculant on the dry matter recovery (%) of ensiled HMC

Moisture level	Treatment	Ensiling period (d)		
%		10	21	120
	Control	98.65	99.33	98.03
	DH42 10^5	99.96	99.15	97.49
35	DH42 10^6	98.46	98.66	98.21
	P. jensenii	99.02	99.27	97.13
	RCM	97.22	98.85	96.62
	S.E.M.[n]	0.41	0.17	0.21
	Control	97.69	96.88 [ab]	95.93
	DH42 10^5	96.27	97.61 [ab]	95.53
33	DH42 10^6	96.74	96.99 [ab]	95.69
	P. jensenii	96.45	98.09 [a]	96.38
	RCM	96.39	95.95 [b]	94.52
	S.E.M.[n]	0.61	0.18	0.29
	Control	92.95	93.07	96.77
	DH42 10^5	95.09	93.76	97.17
28	DH42 10^6	93.68	95.46	97.23
	P. jensenii	93.36	92.70	97.58
	RCM	90.03	95.69	97.34
	S.E.M.[n]	0.55	0.00	0.12
	Control	98.67	97.91	97.59
	DH42 10^5	97.17	97.95	97.24
24	DH42 10^6	97.96	97.45	97.34
	P. jensenii	97.78	97.81	97.70
	RCM	97.65	97.42	97.41
	S.E.M.[n]	0.23	0.13	0.09
	Control	99.53	99.47 [a]	99.11
	DH42 10^5	99.02	98.95 [ab]	98.91
23	DH42 10^6	99.19	99.12 [a]	98.33
	P. jensenii	99.05	99.16 [a]	98.78
	RCM	98.47	97.46 [b]	98.22
	S.E.M.[n]	0.13	0.15	0.13
	Control	99.00	97.93	97.84
	DH42 10^5	99.12	97.85	98.05
22	DH42 10^6	99.47	99.21	98.54
	P. jensenii	99.16	98.15	97.92
	RCM	99.49	99.52	98.30
	S.E.M.[n]	0.16	0.40	0.16

Column means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n]Standard error of the mean.

Table A-10. Effect of moisture and inoculant on the yeast and molds counts (log cfu/g DM) of fresh and ensiled HMC.

Moisture level	Treatment	Ensiling period (d)			
%		0	10	21	120
	Control	4.87	4.56	4.52	3.20
	DH42 10^5	5.17	4.84	4.51	2.11
35	DH42 10^6	5.23	5.17	4.40	2.02
	P. jensenii	5.31	4.72	5.05	2.98
	RCM	4.60	4.25	4.99	3.13
	S.E.M.[n]	0.08	0.11	0.28	0.22
	Control	5.44	4.64	4.13	0.18
	DH42 10^5	5.31	4.58	4.37	0.25
33	DH42 10^6	5.36	4.23	3.82	0.13
	P. jensenii	5.35	4.71	4.35	0.00
	RCM	5.44	5.05	4.33	0.00
	S.E.M.[n]	0.05	0.07	0.06	0.06
	Control	4.94	5.03	5.59	2.22
	DH42 10^5	4.79	4.45	4.92	1.65
28	DH42 10^6	4.89	4.81	3.70	0.91
	P. jensenii	4.72	4.45	2.93	1.05
	RCM	4.80	4.51	5.49	1.73
	S.E.M.[n]	0.07	0.08	0.17	0.20
	Control	4.71	1.89	5.02	1.21
	DH42 10^5	4.66	3.80	4.21	1.39
24	DH42 10^6	4.67	0.00	3.26	0.19
	P. jensenii	5.04	1.82	4.06	0.56
	RCM	4.58	3.92	4.97	0.26
	S.E.M.[n]	0.06	0.31	0.22	0.26
	Control	4.54	4.95	5.62	2.46
	DH42 10^5	4.76	5.05	5.34	1.73
23	DH42 10^6	4.48	4.24	4.48	0.53
	P. jensenii	4.38	4.94	4.72	0.85
	RCM	4.62	5.27	6.22	1.45
	S.E.M.[n]	0.10	0.14	0.18	0.29
	Control	5.78	5.98	5.89	2.91
	DH42 10^5	5.63	6.32	5.79	1.19
22	DH42 10^6	5.64	6.32	5.48	0.00
	P. jensenii	5.97	6.18	5.44	0.94
	RCM	5.59	6.34	5.73	1.72
	S.E.M.[n]	0.07	0.07	0.04	0.24

Column means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n]Standard error of the mean.

Table A-11. Effect of inoculation and moisture on propionibacteria counts (log cfu/g DM) of fresh and ensiled HMC.

Moisture level	Treatment	Ensiling period (d)	
%		0	120
	Control	1.84 [bc]	4.80
	DH42 10^5	3.52 [abc]	5.56
35	DH42 10^6	5.90 [ab]	6.15
	P. jensenii	6.51 [a]	6.20
	RCM	0.00 [c]	5.28
	S.E.M.[n]	0.41	0.16
	Control	1.68	5.53
	DH42 10^5	5.10	5.79
33	DH42 10^6	5.74	5.88
	P. jensenii	6.25	5.80
	RCM	1.68	5.40
	S.E.M.[n]	0.29	0.04
	Control	1.96 [b]	4.88 [b]
	DH42 10^5	5.62 [a]	5.19 [ab]
28	DH42 10^6	6.32 [a]	6.09 [a]
	P. jensenii	6.55 [a]	6.16 [a]
	RCM	4.32 [ab]	5.47 [ab]
	S.E.M.[n]	0.16	0.07
	Control	0.00 [d]	5.88
	DH42 10^5	5.51 [b]	6.12
22	DH42 10^6	6.11 [ab]	6.12
	P. jensenii	6.35 [ab]	5.89
	RCM	3.67 [c]	6.20
	S.E.M.[n]	0.07	0.07

Column means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n]Standard error of the mean.

Table A-12. Effect of inoculation and moisture on the propionic acid content (g/100g DM) of exposed HMC silage

Moisture level	Treatment	Exposure period (d)				
%		0	1	3	5	Average[n]
	Control	0.17	0.19	0.17	0.11	0.10
	DH42 10^5	0.17	0.19	0.17	0.13	0.11
35	DH42 10^6	0.18	0.18	0.16	0.14	0.11
	P. jensenii	0.18	0.18	0.16	0.14	0.11
	RCM	0.18	0.17	0.15	0.09	0.10
	Average	0.11 [a]	0.12 [a]	0.11 [a]	0.08 [b]	(0.01)
	Control	0.34	0.32	0.32	0.25	0.20
	DH42 10^5	0.30	0.33	0.31	0.26	0.20
33	DH42 10^6	0.32	0.34	0.32	0.26	0.20
	P. jensenii	0.29	0.30	0.30	0.24	0.19
	RCM	0.31	0.32	0.31	0.27	0.20
	Average	0.20	0.21	0.21	0.17	(0.01)
	Control	0.10	0.11	0.11	0.10	0.10 [c]
	DH42 10^5	0.26	0.23	0.24	0.20	0.21 [b]
28	DH42 10^6	0.34	0.30	0.28	0.25	0.27 [a]
	P. jensenii	0.28	0.23	0.24	0.21	0.20 [b]
	RCM	0.13	0.12	0.12	0.11	0.11 [c]
	Average	0.18	0.19	0.19	0.15	(0.01)
	Control	0.09	0.08	0.10	0.09	0.07 [c]
	DH42 10^5	0.15	0.16	0.17	0.14	0.12 [b]
24	DH42 10^6	0.26	0.24	0.25	0.20	0.18 [a]
	P. jensenii	0.17	0.15	0.17	0.14	0.12 [b]
	RCM	0.14	0.11	0.11	0.08	0.08 [bc]
	Average	0.12	0.11	0.12	0.01	(0.01)
	Control	0.06	0.05	0.06	0.05	0.06 [c]
	DH42 10^5	0.14	0.14	0.14	0.13	0.14 [b]
23	DH42 10^6	0.19	0.18	0.17	0.18	0.18 [a]
	P. jensenii	0.19	0.17	0.20	0.15	0.18 [a]
	RCM	0.09	0.09	0.07	0.11	0.09 [c]
	Average	0.12	0.11	0.12	0.01	(0.01)
	Control	0.03	0.02	0.03	0.02	0.03 [c]
	DH42 10^5	0.15	0.15	0.16	0.11	0.14 [b]
22	DH42 10^6	0.20	0.21	0.19	0.15	0.19 [a]
	P. jensenii	0.18	0.17	0.18	0.14	0.17 [a]
	RCM	0.02	0.02	0.03	0.01	0.02 [c]
	Average	0.12 [a]	0.11 [ab]	0.13 [a]	0.09 [b]	(0.01)

Column or row means within the same moisture level with unlike superscripts differ ($P<0.05$).

[n]Number in parenthesis denotes the standard error of the mean of the column.

Table A-13. Effect of inoculation and moisture on the acetic acid content (g/100g DM) of exposed HMC silage.

Moisture level	Treatment	Exposure period (d)				
%		0	1	3	5	Average[n]
	Control	0.20	0.22	0.14	0.00	0.14
	DH42 10^5	0.20	0.22	0.20	0.05	0.17
35	DH42 10^6	0.22	0.21	0.21	0.03	0.17
	P. jensenii	0.20	0.21	0.18	0.04	0.16
	RCM	0.22	0.16	0.16	0.03	0.14
	Average	0.21 [a]	0.20 [a]	0.18 [a]	0.03 [b]	(0.02)
	Control	0.31	0.29	0.31	0.25	0.29
	DH42 10^5	0.30	0.34	0.32	0.27	0.31
33	DH42 10^6	0.32	0.35	0.35	0.28	0.32
	P. jensenii	0.29	0.30	0.31	0.24	0.28
	RCM	0.29	0.31	0.30	0.26	0.29
	Average	0.30 [a]	0.32 [a]	0.31 [a]	0.26 [b]	(0.02)
	Control	0.28	0.13	0.18	0.11	0.17 [c]
	DH42 10^5	0.41	0.25	0.33	0.21	0.30 [ab]
28	DH42 10^6	0.48	0.26	0.32	0.22	0.32 [a]
	P. jensenii	0.48	0.27	0.39	0.22	0.34 [a]
	RCM	0.29	0.16	0.31	0.09	0.21 [bc]
	Average	0.39 [a]	0.21 [c]	0.30 [b]	0.17 [c]	(0.02)
	Control	0.24	0.23	0.27	0.24	0.24 [ab]
	DH42 10^5	0.35	0.34	0.35	0.29	0.33 [ab]
24	DH42 10^6	0.46	0.39	0.44	0.36	0.41 [a]
	P. jensenii	0.40	0.35	0.40	0.33	0.37 [a]
	RCM	0.26	0.21	0.21	0.16	0.21 [b]
	Average	0.34 [a]	0.30 [ab]	0.33 [a]	0.28 [b]	(0.03)
	Control	0.20	0.16	0.17	0.15	0.17 [c]
	DH42 10^5	0.22	0.21	0.20	0.20	0.21 [bc]
23	DH42 10^6	0.33	0.30	0.30	0.28	0.30 [a]
	P. jensenii	0.29	0.24	0.27	0.23	0.26 [ab]
	RCM	0.19	0.16	0.12	0.18	0.16 [c]
	Average	0.24	0.21	0.21	0.21	(0.02)
	Control	0.11	0.11	0.10	0.07	0.10 [c]
	DH42 10^5	0.17	0.15	0.16	0.12	0.15 [ab]
22	DH42 10^6	0.14	0.17	0.15	0.12	0.14 [abc]
	P. jensenii	0.16	0.20	0.18	0.13	0.16 [a]
	RCM	0.12	0.15	0.12	0.07	0.11 [b]
	Average	0.14 [a]	0.15 [a]	0.13 [a]	0.10 [b]	(0.01)

Column or row means within the same moisture level with unlike superscripts differ ($P<0.05$).

[n]Number in parenthesis denotes the standard error of the mean of the column.

Table A-14. Effect of inoculation and moisture on the lactic acid content (g/100g DM) of exposed HMC silage

Moisture level	Treatment	Exposure period (d)				
%		0	1	3	5	Average[n]
	Control	3.61	3.79	3.51	1.14	3.01
	DH42 10^5	3.56	3.68	3.45	1.57	3.07
35	DH42 10^6	3.49	3.38	3.39	2.32	3.14
	P. jensenii	3.69	3.63	3.38	1.79	3.12
	RCM	3.62	3.25	2.29	1.06	2.56
	Average	3.59 [a]	3.55 [a]	3.20 [a]	1.58 [b]	(0.42)
	Control	2.32	2.16	2.14	1.73	2.09
	DH42 10^5	2.13	2.21	2.04	1.71	2.02
33	DH42 10^6	2.53	2.53	2.38	1.93	2.34
	P. jensenii	2.32	2.35	2.36	1.92	2.23
	RCM	2.53	2.44	2.34	2.08	2.35
	Average	2.36 [a]	2.34 [a]	2.25 [b]	1.87 [c]	(0.11)
	Control	1.25	1.28	1.14	0.94	1.15
	DH42 10^5	1.12	1.12	1.15	0.92	1.08
28	DH42 10^6	1.04	1.08	1.10	0.87	1.02
	P. jensenii	1.31	1.18	1.12	0.89	1.13
	RCM	1.11	1.24	0.88	0.96	1.05
	Average	1.17	1.18	1.08	0.91	(0.08)
	Control	0.63	0.56	0.68	0.60	0.62
	DH42 10^5	0.59	0.58	0.60	0.48	0.56
24	DH42 10^6	0.48	0.48	0.52	0.41	0.47
	P. jensenii	0.89	0.80	0.88	0.75	0.83
	RCM	0.88	0.70	0.73	0.55	0.72
	Average	0.69 [a]	0.62 [b]	0.68 [b]	0.56 [b]	(0.09)
	Control	0.92	0.73	0.79	0.76	0.80
	DH42 10^5	0.65	0.64	0.61	0.63	0.63
23	DH42 10^6	0.56	0.53	0.46	0.57	0.53
	P. jensenii	0.41	0.38	0.51	0.47	0.44
	RCM	0.88	0.82	0.61	0.76	0.77
	Average	0.68	0.62	0.60	0.64	(0.09)
	Control	0.46	0.46	0.44	0.44	0.45 [a]
	DH42 10^5	0.31	0.35	0.36	0.27	0.32 [b]
22	DH42 10^6	0.18	0.43	0.20	0.15	0.24 [c]
	P. jensenii	0.29	0.60	0.32	0.25	0.36 [b]
	RCM	0.54	0.61	0.54	0.34	0.51 [a]
	Average	0.36 [b]	0.49 [a]	0.36 [b]	0.29 [b]	(0.02)

Column or row means within the same moisture level with unlike superscripts differ ($P<0.05$).

[n]Number in parenthesis denotes the standard error of the mean of the column.

Table A-15. Effect of inoculation and moisture on the pH of exposed HMC silage.

Moisture level	Treatment	Exposure period (d)				
%		0	1	3	5	Average[n]
	Control	3.70	3.69	4.82	5.15	4.06
	DH42 10^5	3.73	3.72	3.72	5.00	4.05
35	DH42 10^6	3.74	3.72	3.74	3.91	3.78
	P. jensenii	3.72	3.71	3.75	4.82	3.99
	RCM	3.75	3.76	3.73	6.26	4.65
	Average	3.73 ^{b}	3.72 ^{b}	3.95 ^{b}	5.03 ^{a}	(0.38)
	Control	4.12	4.14	4.13	4.10	4.12
	DH42 10^5	4.10	4.11	4.10	4.07	4.09
33	DH42 10^6	4.04	4.08	4.05	4.02	4.05
	P. jensenii	4.02	4.03	4.02	4.01	4.02
	RCM	4.05	4.07	4.06	4.01	4.05
	Average	4.06 ^{b}	4.09 ^{a}	4.07 ^{ab}	4.04 ^{c}	(0.03)
	Control	4.24	4.24	4.27	4.56	4.33 ^{a}
	DH42 10^5	4.18	4.17	4.20	4.20	4.19 ^{bc}
28	DH42 10^6	4.14	4.13	4.16	4.16	4.15 ^{c}
	P. jensenii	4.16	4.17	4.19	4.20	4.18 ^{c}
	RCM	4.22	4.23	4.24	4.37	4.26 ^{ab}
	Average	4.19 ^{b}	4.19 ^{b}	4.21 ^{b}	4.30 ^{a}	(0.04)
	Control	4.43	4.42	4.42	4.44	4.43 ^{a}
	DH42 10^5	4.40	4.40	4.39	4.42	4.40 ^{a}
24	DH42 10^6	4.33	4.33	4.32	4.35	4.33 ^{b}
	P. jensenii	4.27	4.26	4.25	4.29	4.27 ^{c}
	RCM	4.40	4.38	4.39	4.41	4.39 ^{a}
	Average	4.37 ^{b}	4.36 ^{c}	4.36 ^{c}	4.38 ^{a}	(0.02)
	Control	4.27	4.26	4.28	4.33	4.28
	DH42 10^5	4.33	4.31	4.34	4.39	4.34
23	DH42 10^6	4.30	4.30	4.32	4.38	4.32
	P. jensenii	4.34	4.33	4.34	4.39	4.35
	RCM	4.31	4.32	4.33	4.39	4.33
	Average	4.31 ^{c}	4.30 ^{c}	4.32 ^{b}	4.38 ^{a}	(0.03)
	Control	4.67	4.66	4.66	4.72	4.68 ^{a}
	DH42 10^5	4.57	4.55	4.54	4.56	4.55 ^{b}
22	DH42 10^6	4.56	4.57	4.57	4.59	4.57 ^{b}
	P. jensenii	4.55	4.52	4.53	4.55	4.54 ^{b}
	RCM	4.67	4.65	4.63	4.70	4.66 ^{a}
	Average	4.60 ^{ab}	4.59 ^{b}	4.58 ^{b}	4.62 ^{a}	(0.02)

Column or row means within the same moisture level with unlike superscripts differ ($P<0.05$).

[n]Number in parenthesis denotes the standard error of the mean of the column.

Table A-16. Effect of inoculation and moisture on the glucose content (g/100g DM) of exposed HMC silage

Moisture level	Treatment	Exposure period (d)				
%		0	1	3	5	Average[n]
	Control	0.26	0.27	0.26	0.15	0.23
	DH42 10^5	0.21	0.22	0.21	0.18	0.20
35	DH42 10^6	0.21	0.21	0.21	0.19	0.21
	P. jensenii	0.26	0.25	0.24	0.18	0.23
	RCM	0.29	0.25	0.22	0.16	0.23
	Average	0.25 [a]	0.24 [a]	0.23 [a]	0.17 [b]	(0.02)
	Control	0.41	0.39	0.39	0.31	0.38
	DH42 10^5	0.36	0.38	0.36	0.30	0.35
33	DH42 10^6	0.40	0.40	0.39	0.32	0.38
	P. jensenii	0.36	0.37	0.37	0.30	0.35
	RCM	0.39	0.39	0.38	0.34	0.38
	Average	0.38 [a]	0.39 [a]	0.38 [a]	0.32 [b]	(0.01)
	Control	0.19	0.17	0.16	0.12	0.16
	DH42 10^5	0.20	0.19	0.15	0.13	0.17
28	DH42 10^6	0.17	0.15	0.13	0.10	0.14
	P. jensenii	0.16	0.17	0.14	0.15	0.16
	RCM	0.18	0.16	0.15	0.14	0.16
	Average	0.18 [a]	0.17 [b]	0.15 [a]	0.13 [c]	(0.02)
	Control	0.05	0.04	0.05	0.05	0.05
	DH42 10^5	0.05	0.05	0.07	0.05	0.06
24	DH42 10^6	0.10	0.08	0.10	0.07	0.09
	P. jensenii	0.03	0.02	0.02	0.02	0.02
	RCM	0.09	0.06	0.07	0.05	0.07
	Average	0.06 [a]	0.05 [b]	0.06 [a]	0.05 [b]	(0.02)
	Control	0.09	0.07	0.08	0.06	0.08
	DH42 10^5	0.08	0.08	0.08	0.07	0.08
23	DH42 10^6	0.06	0.06	0.07	0.07	0.06
	P. jensenii	0.10	0.08	0.10	0.07	0.09
	RCM	0.06	0.05	0.05	0.06	0.05
	Average	0.08 [a]	0.07 [bc]	0.07 [ab]	0.06 [c]	(0.01)
	Control	0.06	0.05	0.06	0.07	0.06 [b]
	DH42 10^5	0.11	0.10	0.14	0.09	0.11 [a]
22	DH42 10^6	0.09	0.05	0.08	0.08	0.07 [b]
	P. jensenii	0.11	0.00	0.12	0.10	0.08 [b]
	RCM	0.05	0.00	0.00	0.00	0.01 [c]
	Average	0.08 [a]	0.04 [b]	0.08 [a]	0.07 [a]	(0.01)

Column or row means within the same moisture level with unlike superscripts differ ($P<0.05$).

[n]Number in parenthesis denotes the standard error of the mean of the column.

Table A-17. Effect of inoculation and moisture on the ethanol content (g/100g DM) of exposed HMC silage.

Moisture level	Treatment	Exposure period (d)				
%		0	1	3	5	Average[n]
	Control	0.48	0.46	0.70	0.00	0.30
	DH42 10^5	0.56	0.55	0.28	0.06	0.38
35	DH42 10^6	0.52	0.48	0.35	0.02	0.34
	P. jensenii	0.49	0.48	0.33	0.02	0.33
	RCM	0.51	0.36	0.32	0.01	0.27
	Average	0.51 [a]	0.46 [a]	0.29 [b]	0.02 [c]	(0.03)
	Control	0.85	0.81	0.20	0.35	0.66
	DH42 10^5	0.88	0.86	0.65	0.37	0.69
33	DH42 10^6	0.92	0.79	0.67	0.33	0.68
	P. jensenii	0.86	0.79	0.68	0.32	0.67
	RCM	0.86	0.77	0.68	0.33	0.66
	Average	0.87 [a]	0.80 [b]	0.67 [c]	0.34 [d]	(0.03)
	Control	0.75 [a]	0.55 [a]	0.38 [ab]	0.14	0.45
	DH42 10^5	0.54 [b]	0.39 [b]	0.37 [ab]	0.14	0.36
28	DH42 10^6	0.49 [b]	0.36 [b]	0.28 [b]	0.11	0.31
	P. jensenii	0.53 [b]	0.35 [b]	0.34 [ab]	0.13	0.34
	RCM	0.67 [a]	0.55 [a]	0.42 [a]	0.13	0.45
	Average	0.60	0.44	0.36	0.13	(0.04)
	Control	0.42	0.34	0.29	0.13	0.29
	DH42 10^5	0.41	0.38	0.32	0.16	0.32
24	DH42 10^6	0.48	0.39	0.34	0.18	0.35
	P. jensenii	0.32	0.26	0.24	0.13	0.24
	RCM	0.55	0.39	0.33	0.15	0.35
	Average	0.44 [a]	0.35 [b]	0.30 [c]	0.15 [d]	(0.04)
	Control	0.15 [c]	0.26 [ab]	0.21	0.09	0.18
	DH42 10^5	0.26 [b]	0.16 [b]	0.15	0.05	0.15
23	DH42 10^6	0.34 [ab]	0.24 [ab]	0.17	0.06	0.20
	P. jensenii	0.26 [b]	0.18 [b]	0.17	0.05	0.16
	RCM	0.42 [a]	0.30 [a]	0.16	0.11	0.25
	Average	0.29	0.23	0.17	0.07	(0.02)
	Control	0.56	0.33	0.22	0.07	0.29
	DH42 10^5	0.44	0.31	0.25	0.12	0.28
22	DH42 10^6	0.37	0.26	0.15	0.08	0.22
	P. jensenii	0.36	0.27	0.22	0.10	0.24
	RCM	0.59	0.37	0.23	0.06	0.31
	Average	0.46 [a]	0.31 [b]	0.21 [c]	0.08 [d]	(0.03)

Row means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n]Number in parenthesis denotes the standard error of the mean of the column.

Table A-18. Effect of inoculation and moisture on the citric acid content (g/100g DM) of exposed HMC silage

Moisture level	Treatment	Exposure period (d)				
%		0	1	3	5	Average[n]
	Control	0.00	0.01	0.00	0.00	0.00
	DH42 10^5	0.00	0.00	0.00	0.00	0.00
35	DH42 10^6	0.00	0.00	0.00	0.00	0.00
	P. jensenii	0.00	0.00	0.00	0.00	0.00
	RCM	0.00	0.00	0.00	0.00	0.00
	Average	0.00	0.00	0.00	0.00	(0.00)
	Control	0.00	0.00	0.00	0.00	0.00
	DH42 10^5	0.00	0.00	0.00	0.00	0.00
33	DH42 10^6	0.00	0.00	0.00	0.00	0.00
	P. jensenii	0.00	0.00	0.00	0.00	0.00
	RCM	0.00	0.00	0.00	0.00	0.00
	Average	0.00	0.00	0.00	0.00	(0.00)
	Control	0.00	0.00	0.00	0.00	0.00
	DH42 10^5	0.00	0.00	0.00	0.00	0.00
28	DH42 10^6	0.00	0.00	0.00	0.00	0.00
	P. jensenii	0.00	0.00	0.00	0.01	0.00
	RCM	0.00	0.00	0.00	0.00	0.00
	Average	0.00	0.00	0.00	0.00	(0.00)
	Control	0.00	0.00	0.00	0.00	0.00
	DH42 10^5	0.00	0.01	0.01	0.00	0.00
24	DH42 10^6	0.01	0.01	0.01	0.01	0.01
	P. jensenii	0.00	0.00	0.00	0.00	0.00
	RCM	0.01	0.01	0.01	0.00	0.01
	Average	0.01	0.01	0.01	0.00	(0.00)
	Control	0.01	0.01	0.01	0.01	0.01
	DH42 10^5	0.01	0.01	0.01	0.01	0.01
23	DH42 10^6	0.01	0.01	0.01	0.01	0.01
	P. jensenii	0.02	0.01	0.01	0.01	0.01
	RCM	0.01	0.01	0.01	0.01	0.01
	Average	0.01	0.01	0.01	0.01	(0.00)
	Control	0.02	0.02	0.02	0.03	0.02
	DH42 10^5	0.02	0.02	0.02	0.02	0.02
22	DH42 10^6	0.02	0.02	0.02	0.01	0.02
	P. jensenii	0.02	0.01	0.02	0.02	0.02
	RCM	0.02	0.01	0.01	0.02	0.02
	Average	0.02	0.02	0.02	0.02	(0.00)

[n]Number in parenthesis denotes the standard error of the mean of the column.

Table A-19. Effect of inoculation and moisture on the butyric acid content (g/100g DM) of exposed HMC silage.

Moisture level	Treatment	Exposure period (d)				
%		0	1	3	5	Average[n]
	Control	0.00	0.00	0.00	0.00	0.00
	DH42 10^5	0.00	0.00	0.00	0.00	0.00
35	DH42 10^6	0.00	0.00	0.00	0.00	0.00
	P. jensenii	0.00	0.00	0.00	0.00	0.00
	RCM	0.00	0.00	0.00	0.00	0.00
	Average	0.00	0.00	0.00	0.00	(0.00)
	Control	0.32	0.29	0.29	0.23	0.28
	DH42 10^5	0.23	0.24	0.22	0.18	0.22
33	DH42 10^6	0.27	0.27	0.25	0.20	0.25
	P. jensenii	0.19	0.19	0.18	0.15	0.17
	RCM	0.26	0.26	0.24	0.22	0.24
	Average	0.25	0.25	0.24	0.19	(0.02)
	Control	0.10	0.07	0.10	0.10	0.09
	DH42 10^5	0.03	0.01	0.03	0.04	0.03
28	DH42 10^6	0.01	0.00	0.01	0.02	0.01
	P. jensenii	0.01	0.00	0.01	0.02	0.01
	RCM	0.10	0.08	0.09	0.09	0.09
	Average	0.05	0.03	0.05	0.05	(0.01)
	Control	0.10	0.10	0.12	0.11	0.01
	DH42 10^5	0.07	0.07	0.08	0.06	0.07
24	DH42 10^6	0.03	0.03	0.04	0.03	0.03
	P. jensenii	0.03	0.03	0.04	0.03	0.03
	RCM	0.12	0.11	0.11	0.08	0.11
	Average	0.07	0.07	0.08	0.06	(0.02)
	Control	0.00	0.01	0.00	0.01	0.01
	DH42 10^5	0.00	0.00	0.00	0.00	0.00
23	DH42 10^6	0.00	0.00	0.00	0.00	0.00
	P. jensenii	0.00	0.00	0.00	0.00	0.00
	RCM	0.03	0.03	0.01	0.02	0.02
	Average	0.01	0.01	0.00	0.01	(0.00)
	Control	0.00	0.00	0.00	0.00	0.00
	DH42 10^5	0.00	0.00	0.00	0.00	0.00
22	DH42 10^6	0.00	0.00	0.00	0.00	0.00
	P. jensenii	0.00	0.00	0.00	0.00	0.00
	RCM	0.00	0.00	0.00	0.00	0.00
	Average	0.00	0.00	0.00	0.00	(0.00)

[n]Number in parenthesis denotes the standard error of the mean of the column.

Table A-20. Effect of inoculation and moisture on the yeast and molds counts (log cfu/g DM) of exposed HMC silage.

Moisture level	Treatment	Exposure period (d)				
%		0	1	3	5	Average[n]
	Control	3.20	3.67	6.40	7.67	5.24
	DH42 10^5	2.11	2.10	5.63	8.04	4.58
35	DH42 10^6	2.02	3.16	5.85	7.68	4.68
	P. jensenii	2.98	3.35	5.74	7.60	4.92
	RCM	3.13	4.21	6.50	7.89	5.43
	Average	2.68 [d]	3.39 [c]	6.02 [b]	7.78 [a]	(0.40)
	Control	0.16	0.16	0.73	0.88	0.48
	DH42 10^5	0.24	0.40	0.88	0.00	0.38
33	DH42 10^6	0.09	0.00	0.49	0.00	0.14
	P. jensenii	0.00	0.09	0.32	0.00	0.10
	RCM	0.00	0.00	0.55	0.68	0.31
	Average	0.10 [b]	0.13 [b]	0.59 [a]	0.31 [ab]	(0.12)
	Control	2.29	2.09	2.29	1.81	2.12
	DH42 10^5	1.38	0.52	0.37	1.58	0.96
28	DH42 10^6	0.93	0.71	0.65	0.72	0.75
	P. jensenii	0.91	0.74	0.74	1.03	0.86
	RCM	0.44	0.48	0.49	0.86	0.57
	Average	1.19	0.91	0.91	1.20	(0.44)
	Control	1.14	0.88	0.58	3.55	1.53
	DH42 10^5	1.39	1.27	1.11	1.91	1.42
24	DH42 10^6	0.17	0.00	0.34	0.86	0.34
	P. jensenii	0.53	0.53	1.01	1.42	0.87
	RCM	0.25	0.35	0.39	0.42	0.35
	Average	0.70	0.60	0.69	1.63	(0.36)
	Control	2.43	2.68	3.25	3.26	2.91 [a]
	DH42 10^5	1.73	1.33	1.87	1.48	1.60 [ab]
23	DH42 10^6	0.54	0.37	1.50	1.45	0.96 [b]
	P. jensenii	0.85	0.27	0.87	0.84	0.71 [b]
	RCM	1.45	0.52	0.49	0.45	0.72 [b]
	Average	1.40 [ab]	1.03 [b]	1.59 [a]	1.50 [ab]	(0.42)
	Control	2.90	2.97	3.41	3.95	3.31 [a]
	DH42 10^5	1.19	0.91	1.96	1.78	1.46 [b]
22	DH42 10^6	0.00	0.00	1.72	1.05	0.69 [b]
	P. jensenii	0.92	0.30	1.20	1.56	1.00 [b]
	RCM	1.72	0.70	1.06	0.91	1.10 [b]
	Average	1.35 [bc]	0.97 [c]	1.87 [a]	1.85 [ab]	(0.33)

Column or row means within the same moisture level with unlike superscripts differ ($P<0.05$).

[n]Number in parenthesis denotes the standard error of the mean of the column.

Table A-21. Effect of inoculation and moisture on the dry matter recovery (%) of exposed HMC silage.

Moisture level	Treatment	Exposure period (d)				
%		0	1	3	5	Average[n]
	Control	98.03	101.43	99.56	99.10	99.53
	DH42 10^5	97.49	101.31	101.49	98.94	99.81
35	DH42 10^6	98.21	101.56	100.36	101.11	100.31
	P. jensenii	97.13	100.47	99.90	100.04	99.38
	RCM	96.62	99.08	100.87	98.24	98.71
	Average	97.49 ^{c}	100.77 ^{a}	100.43 ^{ab}	99.49 ^{b}	(0.35)
	Control	95.93	99.61	99.16	99.75	98.61
	DH42 10^5	95.53	100.43	99.22	100.11	98.82
33	DH42 10^6	95.69	100.84	98.75	100.62	98.98
	P. jensenii	96.38	100.25	99.33	100.93	99.22
	RCM	94.52	101.10	98.79	100.57	98.74
	Average	95.61 ^{c}	100.45 ^{a}	99.05 ^{b}	100.39 ^{a}	(0.20)
	Control	96.77	101.43	97.54	101.41	99.29
	DH42 10^5	97.17	101.01	96.69	99.81	98.67
28	DH42 10^6	97.23	101.61	97.77	101.51	99.53
	P. jensenii	97.58	101.82	96.95	101.33	99.42
	RCM	97.34	101.34	97.34	101.30	99.33
	Average	97.22 ^{b}	101.44 ^{a}	97.26 ^{b}	101.07 ^{a}	(0.23)
	Control	97.59	100.81	99.21	99.89	99.37
	DH42 10^5	97.24	100.93	98.99	100.30	99.36
24	DH42 10^6	97.38	95.63	99.17	99.27	97.85
	P. jensenii	97.70	100.98	97.68	99.59	99.71
	RCM	97.41	100.91	98.92	100.09	99.38
	Average	98.67 ^{c}	100.37 ^{a}	98.79 ^{b}	99.20 ^{b}	(0.21)
	Control	99.11	100.00	100.00	99.52	99.65
	DH42 10^5	98.91	100.79	100.79	98.96	99.86
23	DH42 10^6	98.33	100.46	100.47	99.35	99.65
	P. jensenii	98.78	100.29	100.27	99.47	99.71
	RCM	98.22	100.31	100.33	98.68	99.38
	Average	98.67 ^{c}	100.37 ^{a}	100.37 ^{a}	99.20 ^{b}	(0.21)
	Control	97.83	99.26	102.52	101.53	100.29
	DH42 10^5	98.05	100.61	102.00	100.41	100.27
22	DH42 10^6	98.54	100.67	100.20	99.29	99.68
	P. jensenii	97.92	102.43	100.51	99.04	102.48
	RCM	98.29	100.50	99.67	99.84	99.58
	Average	98.13 ^{b}	102.69 ^{a}	100.98 ^{a}	100.02 ^{ab}	(1.21)

Row means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n] Number in parenthesis denotes the standard error of the mean of the column.

Table A-22. Effect of inoculation and moisture on the temperature (°C) of exposed HMC silage.

Moisture level (%)	Treatment	Exposure period (d) 0	1	2	3	4	5	Avg.[n]
	Control	18.52 [ab]	19.54	18.89	19.63	21.67	27.96	21.03
	DH42 10^5	17.78	19.35	18.52	17.78	20.74	25.00	19.86
35	DH42 10^6	18.89	19.63	19.26	18.33	19.26	25.28	20.11
	P. jensenii	18.70	19.44	19.54	18.70	23.89	23.98	20.71
	RCM	19.07	22.87	21.57	19.63	26.48	25.56	22.53
	Average	18.59 [c]	20.17 [bc]	19.56 [c]	18.81 [c]	22.41 [b]	25.56 [a]	(0.98)
	Control	21.81	20.21	19.51	19.65	19.79	20.28	20.21
	DH42 10^5	21.53	20.00	19.31	19.44	19.51	20.00	19.97
33	DH42 10^6	21.94	20.42	19.72	19.79	19.86	20.14	20.31
	P. jensenii	21.67	20.14	19.51	17.99	19.44	20.14	19.81
	RCM	21.11	19.44	18.82	18.68	19.10	19.58	19.46
	Average	21.61 [a]	20.04 [b]	19.36 [cd]	19.11 [d]	19.54 [c]	20.03 [b]	(0.25)
	Control	24.44	24.83	22.99	24.38	26.25	27.01	24.98
	DH42 10^5	24.03	24.34	22.64	23.33	25.69	25.69	24.29
28	DH42 10^6	24.51	24.86	22.85	23.85	25.83	26.04	24.66
	P. jensenii	24.65	24.86	22.85	24.03	25.97	26.18	24.76
	RCM	24.58	24.97	22.92	24.06	26.11	26.60	24.87
	Average	24.44 [bc]	24.77 [b]	22.85 [d]	23.93 [c]	25.97 [a]	26.31 [a]	(0.33)
	Control	23.89	20.76	20.56	20.76	20.56	21.39	21.32
	DH42 10^5	23.06	20.83	20.69	20.56	20.14	20.97	21.04
24	DH42 10^6	23.19	20.56	20.42	20.69	20.56	21.39	21.13
	P. jensenii	23.47	20.83	20.69	20.69	20.42	21.11	21.20
	RCM	23.19	20.90	20.42	20.83	20.56	21.39	21.22
	Average	23.36 [a]	20.78 [bc]	20.56 [c]	20.71 [c]	20.44 [c]	21.25 [b]	(0.34)
	Control	21.25	20.76	20.49	19.72	19.72	20.42	20.39
	DH42 10^5	21.11	20.42	20.63	19.72	19.44	19.58	20.15
23	DH42 10^6	21.25	21.11	20.69	20.42	20.00	20.28	20.63
	P. jensenii	21.67	21.53	21.60	20.56	20.97	21.11	21.24
	RCM	21.53	21.18	21.32	20.56	20.56	20.42	20.93
	Average	21.36	21.00	20.94	20.19	20.14	20.36	(0.22)
	Control	21.67	21.48	22.04	21.02	21.67	21.30	21.53
	DH42 10^5	21.67	21.67	22.22	21.20	21.48	21.48	21.62
22	DH42 10^6	21.48	21.02	21.48	20.74	21.30	20.56	21.10
	P. jensenii	21.85	21.94	21.85	21.57	21.48	21.11	21.64
	RCM	22.22	21.85	22.22	20.83	21.48	20.93	21.59
	Average	21.78 [ab]	21.59 [b]	21.96 [a]	21.07 [c]	21.48 [b]	21.07 [c]	(0.13)

Row means within the same moisture level with unlike superscripts differ ($P<0.05$).
[n]Number in parenthesis denotes the standard error of the mean of the column.

APPENDIX B

Data Used for Analyses in Chapter 2

Table B-1. Data used for analyses for chapter 2-chemical and microbial analyses*.

Trt	Rep	Time	Pa	La	Gl	Ac	Et	Bu	Ca	Lab	Ym	pH	Dm	Dmr
G	1	0	0.000	0.000	0.321	0.000	0.000	0.000	0.106	6.29	7.54	5.29	69.59	ND
G	2	0	0.000	0.000	0.310	0.000	0.000	0.000	0.106	6.30	7.52	5.27	69.60	ND
H	1	0	0.000	0.000	0.249	0.000	0.000	0.000	0.079	6.01	7.25	5.74	70.52	ND
H	2	0	0.000	0.000	0.251	0.000	0.000	0.000	0.079	6.05	7.30	5.70	70.47	ND
I	1	0	0.000	0.000	0.160	0.000	0.000	0.000	0.055	6.30	7.51	5.45	71.80	ND
I	2	0	0.000	0.000	0.161	0.000	0.000	0.000	0.055	6.28	7.50	5.42	72.00	ND
J	1	0	0.000	0.000	0.419	0.000	0.000	0.000	0.113	6.76	7.61	5.54	72.22	ND
J	2	0	0.000	0.000	0.419	0.000	0.000	0.000	0.112	6.75	7.60	5.55	71.80	ND
K	1	0	0.000	0.000	0.319	0.000	0.000	0.000	0.082	6.20	7.29	5.66	72.39	ND
K	2	0	0.000	0.000	0.321	0.000	0.000	0.000	0.081	6.20	7.30	5.65	72.50	ND
L	1	0	0.000	0.000	0.406	0.000	0.000	0.000	0.092	6.59	7.44	5.68	70.19	ND
L	2	0	0.000	0.000	0.389	0.000	0.000	0.000	0.094	6.60	7.41	5.69	70.20	ND
M	1	0	0.000	0.000	0.335	0.000	0.000	0.000	0.111	6.05	7.61	5.53	73.54	ND
M	2	0	0.000	0.000	0.333	0.000	0.000	0.000	0.111	6.05	7.62	5.53	73.60	ND
N	1	0	0.000	0.000	0.432	0.000	0.000	0.000	0.155	6.22	7.53	5.52	72.60	ND
N	2	0	0.000	0.000	0.416	0.000	0.000	0.000	0.160	6.20	7.54	5.51	73.00	ND
G	1	7	0.018	1.035	0.012	0.099	0.307	0.000	0.002	8.73	6.10	4.11	70.04	97.92
G	2	7	0.017	1.418	0.016	0.127	0.351	0.000	0.002	8.64	5.37	4.10	69.66	99.07
G	3	7	0.017	1.410	0.019	0.133	0.395	0.000	0.002	8.55	5.30	4.10	69.66	99.17
H	1	7	0.039	1.205	0.011	0.099	0.392	0.000	0.002	8.80	6.52	4.04	70.54	100.66
H	2	7	0.036	1.201	0.006	0.073	0.275	0.000	0.002	8.82	6.44	4.01	70.08	100.58
I	1	7	0.009	1.230	0.011	0.089	0.215	0.000	0.002	8.49	6.47	4.09	71.61	100.16
I	2	7	0.018	1.801	0.012	0.133	0.407	0.000	0.002	8.71	6.40	4.08	71.68	99.89
I	3	7	0.022	1.053	0.011	0.077	0.229	0.000	0.002	8.66	5.79	4.06	71.62	99.46
J	1	7	0.019	0.861	0.009	0.068	0.195	0.000	0.002	8.60	6.21	4.10	72.01	100.13
J	2	7	0.007	0.821	0.010	0.060	0.205	0.000	0.002	8.63	5.51	4.10	72.42	98.67
J	3	7	0.021	1.123	0.008	0.067	0.226	0.000	0.002	8.68	5.95	4.10	71.56	101.64
K	1	7	0.033	0.938	0.009	0.077	0.324	0.000	0.002	8.54	5.88	4.21	72.02	100.75
K	2	7	0.027	0.588	0.010	0.053	0.255	0.000	0.002	8.63	6.62	4.21	72.16	100.07
K	3	7	0.037	0.938	0.017	0.090	0.361	0.000	0.002	8.63	6.31	4.21	72.30	99.69
L	1	7	0.039	0.916	0.010	0.083	0.264	0.000	0.002	8.51	5.82	4.17	70.13	99.57
L	2	7	0.040	1.183	0.021	0.102	0.384	0.000	0.002	8.58	5.65	4.15	70.47	98.32
L	3	7	0.039	0.708	0.021	0.057	0.185	0.000	0.002	8.67	6.38	4.17	70.72	97.50

*Abbreviations used: Trt-treatment, Rep-replication, Pa-propionic acid, La-lactic acid, Gl-glucose, Ac-acetic acid, Et-ethanol, Bu-butyric acid, Ca-citric acid, Lab-lactic acid bacteria, Ym-yeast and molds, Dm-dry matter, Dmr-dry matter recovery, G-autoclaved DH42, H-DH42+LAB(reconstituted with tap water), I-DH42+P42+LAB, J- DH42+LAB (reconstituted with distilled water), K-P42+DH42, L-DH42, M- control, N-P42, ND-not determined.

Table B-1 (contd).

Trt	Rep	Time	Pa	La	Gl	Ac	Et	Bu	Ca	Lab	Ym	pH	Dm	Dmr
M	1	7	0.038	0.521	0.010	0.043	0.190	0.000	0.002	8.57	6.66	4.27	73.03	101.38
M	2	7	0.038	0.660	0.016	0.060	0.224	0.000	0.002	8.63	6.86	4.26	73.62	99.20
M	3	7	0.039	0.637	0.016	0.052	0.261	0.000	0.002	8.67	6.56	4.26	73.31	100.38
N	1	7	0.043	0.763	0.017	0.076	0.314	0.000	0.002	8.69	5.92	4.25	72.46	100.01
N	2	7	0.040	0.671	0.012	0.064	0.249	0.000	0.002	8.53	6.03	4.26	72.04	101.39
N	3	7	0.043	0.583	0.010	0.062	0.217	0.000	0.002	8.52	6.60	4.28	72.90	98.30
G	1	21	0.025	1.280	0.000	0.118	0.390	0.049	0.132	8.87	3.85	4.18	69.08	100.94
G	2	21	0.030	1.761	0.000	0.155	0.376	0.059	0.187	8.98	4.09	4.17	69.36	100.02
G	3	21	0.035	1.771	0.000	0.179	0.371	0.058	0.196	9.00	4.07	4.14	69.70	98.91
H	1	21	0.010	1.379	0.023	0.076	0.398	0.074	0.159	8.67	3.92	4.12	70.20	99.20
H	2	21	0.006	1.374	0.022	0.075	0.404	0.074	0.153	8.50	3.80	4.12	70.30	99.15
I	1	21	0.008	1.738	0.017	0.142	0.380	0.017	0.162	8.63	4.14	4.11	71.20	104.52
I	2	21	0.012	1.562	0.021	0.118	0.233	0.020	0.162	8.62	4.13	4.13	71.16	101.49
I	3	21	0.000	1.478	0.013	0.124	0.343	0.024	0.143	8.63	4.05	4.13	71.41	93.64
J	1	21	0.000	1.646	0.023	0.140	0.325	0.024	0.173	8.65	4.19	4.15	71.94	100.37
J	2	21	0.010	1.916	0.018	0.157	0.414	0.024	0.184	8.61	4.23	4.15	71.61	101.68
J	3	21	0.011	1.664	0.007	0.158	0.378	0.025	0.161	8.57	3.90	4.11	71.78	101.08
K	1	21	0.107	1.514	0.014	0.176	0.437	0.054	0.188	8.89	4.12	4.25	71.64	101.94
K	2	21	0.084	1.467	0.010	0.170	0.390	0.050	0.158	8.92	4.12	4.25	71.94	100.87
K	3	21	0.104	1.453	0.014	0.141	0.465	0.054	0.154	8.77	3.79	4.18	71.70	101.87
L	1	21	0.045	2.066	0.006	0.154	0.318	0.054	0.155	8.99	3.87	4.13	70.58	98.08
L	2	21	0.061	1.937	0.007	0.144	0.396	0.052	0.152	8.78	4.29	4.15	70.02	99.90
L	3	21	0.051	1.517	0.000	0.137	0.436	0.044	0.132	8.89	4.38	4.14	69.84	100.40
M	1	21	0.040	1.129	0.000	0.203	0.246	0.033	0.127	8.83	4.24	4.38	72.94	101.57
M	2	21	0.042	1.242	0.007	0.135	0.437	0.045	0.132	9.04	3.96	4.35	73.04	101.23
M	3	21	0.040	1.241	0.000	0.159	0.376	0.038	0.136	8.90	4.26	4.35	73.12	100.97
N	1	21	0.038	1.269	0.000	0.129	0.480	0.057	0.122	8.94	4.01	4.22	71.66	102.68
N	2	21	0.023	1.141	0.000	0.122	0.347	0.040	0.106	8.82	3.90	4.28	72.14	100.93
N	3	21	0.026	1.101	0.000	0.113	0.423	0.042	0.110	8.75	4.00	4.27	71.67	102.58
G	1	90	0.091	0.868	0.000	0.389	0.456	0.130	0.202	8.47	3.57	4.36	69.79	98.90
G	2	90	0.107	1.103	0.000	0.426	0.530	0.168	0.216	7.68	3.04	4.33	69.52	98.71
G	3	90	0.123	1.199	0.000	0.519	0.556	0.184	0.248	7.91	3.13	4.33	69.65	98.97
H	1	90	0.080	0.953	0.008	0.209	0.477	0.236	0.191	8.36	4.15	4.36	70.62	99.41
H	2	90	0.068	0.854	0.007	0.151	0.413	0.238	0.161	8.27	2.73	4.40	70.51	99.08
I	1	90	0.118	1.579	0.000	0.287	0.394	0.088	0.162	8.32	3.41	4.16	71.96	93.29
I	2	90	0.094	1.134	0.000	0.226	0.391	0.078	0.137	8.36	3.35	4.18	71.83	99.31
I	3	90	0.160	0.667	0.000	0.416	0.051	0.072	0.199	8.51	4.58	4.47	70.10	95.83
J	1	90	0.117	1.636	0.008	0.276	0.473	0.108	0.173	8.66	2.58	4.14	72.18	98.83
J	2	90	0.121	1.628	0.009	0.300	0.360	0.077	0.156	8.47	2.58	4.13	71.92	98.58
J	3	90	0.081	1.125	0.010	0.198	0.298	0.049	0.134	8.41	1.88	4.13	72.52	99.31

Table B-1 (contd).

Table B-1 (contd).

Trt	Rep	Time	Pa	La	Gl	Ac	Et	Bu	Ca	Lab	Ym	pH	Dm	Dmr
K	1	90	0.122	1.109	0.000	0.402	0.502	0.100	0.136	8.57	2.18	4.27	72.08	98.22
K	2	90	0.130	1.177	0.008	0.439	0.518	0.105	0.160	6.88	3.24	4.24	72.07	98.12
K	3	90	0.112	1.018	0.000	0.378	0.438	0.088	0.148	7.25	3.47	4.27	71.82	97.91
L	1	90	0.110	1.120	0.000	0.345	0.393	0.106	0.152	8.77	3.07	4.27	70.59	99.20
L	2	90	0.107	1.144	0.000	0.332	0.333	0.097	0.135	8.78	2.37	4.24	70.56	99.21
L	3	90	0.113	1.190	0.000	0.377	0.509	0.121	0.158	6.93	1.89	4.25	70.54	99.38
M	1	90	0.080	0.901	0.000	0.399	0.432	0.077	0.134	8.47	2.18	4.29	73.03	98.07
M	2	90	0.047	0.555	0.008	0.266	0.336	0.053	0.110	8.50	1.88	4.31	72.66	97.64
M	3	90	0.080	0.918	0.000	0.364	0.416	0.076	0.137	8.37	1.88	4.28	73.04	98.12
N	1	90	0.076	0.847	0.000	0.295	0.487	0.109	0.125	7.81	2.18	4.33	72.13	98.09
N	2	90	0.073	0.780	0.006	0.401	0.492	0.114	0.135	8.47	2.18	4.32	71.89	97.96
N	3	90	0.061	0.768	0.000	0.256	0.415	0.105	0.114	7.99	2.19	4.31	71.45	97.85
G	1	91	0.115	0.838	0.000	0.491	0.457	0.151	0.289	8.74	2.19	4.35	70.17	ND
G	2	91	0.126	1.002	0.007	0.503	0.363	0.153	0.285	8.76	0.19	4.30	70.20	ND
G	3	91	0.124	1.005	0.000	0.529	0.365	0.139	0.241	8.52	1.89	4.31	70.18	ND
H	1	91	0.095	1.065	0.014	0.258	0.430	0.278	0.254	8.42	2.67	4.36	71.25	ND
H	2	91	0.095	1.117	0.016	0.233	0.398	0.283	0.224	8.45	2.37	4.39	71.03	ND
I	1	91	0.066	0.889	0.011	0.160	0.341	0.096	0.238	8.30	2.18	4.16	72.08	ND
I	2	91	0.106	1.324	0.013	0.281	0.401	0.090	0.247	8.22	2.58	4.21	72.57	ND
I	3	91	0.170	0.933	0.000	0.468	0.075	0.075	0.293	8.95	3.49	4.40	70.88	ND
J	1	91	0.071	1.010	0.018	0.193	0.373	0.098	0.223	8.46	2.65	4.16	72.82	ND
J	2	91	0.132	1.640	0.016	0.335	0.313	0.080	0.199	8.48	1.88	4.16	72.04	ND
J	3	91	0.070	1.005	0.014	0.206	0.360	0.072	0.212	8.38	2.83	4.15	72.40	ND
K	1	91	0.108	0.907	0.011	0.362	0.412	0.099	0.193	8.39	2.36	4.27	72.50	ND
K	2	91	0.112	0.941	0.016	0.366	0.430	0.114	0.231	8.57	1.88	4.25	72.50	ND
K	3	91	0.115	0.991	0.012	0.375	0.407	0.118	0.244	8.45	3.46	4.28	72.90	ND
L	1	91	0.125	1.119	0.000	0.393	0.343	0.124	0.217	8.44	3.47	4.27	71.50	ND
L	2	91	0.117	1.179	0.000	0.351	0.351	0.137	0.231	8.44	3.17	4.23	71.27	ND
L	3	91	0.076	0.774	0.000	0.275	0.382	0.128	0.206	8.43	1.89	4.24	71.03	ND
M	1	91	0.084	0.811	0.014	0.403	0.383	0.089	0.204	8.46	2.17	4.28	74.20	ND
M	2	91	0.048	0.521	0.016	0.261	0.391	0.080	0.196	8.37	3.65	4.30	74.28	ND
M	3	91	0.097	0.985	0.012	0.440	0.352	0.087	0.211	8.44	2.57	4.27	74.53	ND
N	1	91	0.074	0.843	0.012	0.318	0.413	0.140	0.208	8.29	3.28	4.33	72.97	ND
N	2	91	0.070	0.680	0.011	0.398	0.425	0.136	0.205	8.42	2.18	4.30	72.51	ND
N	3	91	0.081	0.908	0.011	0.333	0.435	0.144	0.202	8.38	3.26	4.30	72.45	ND
G	1	93	0.124	0.788	0.000	0.525	0.351	0.152	0.246	8.61	2.80	4.34	70.17	ND
G	2	93	0.131	0.886	0.000	0.540	0.330	0.165	0.249	8.51	1.89	4.30	70.21	ND
G	3	93	0.153	1.184	0.000	0.641	0.285	0.186	0.272	8.59	0.20	4.27	70.18	ND
H	1	93	0.095	0.881	0.018	0.241	0.280	0.293	0.251	8.44	2.67	4.34	70.97	ND
H	2	93	0.103	0.882	0.011	0.203	0.305	0.308	0.209	8.37	2.37	4.35	71.03	ND

Table B-1 (cont'd).

Trt	Rep	Time	Pa	La	Gl	Ac	Et	Bu	Ca	Lab	Ym	pH	Dm	Dmr
I	1	93	0.130	1.569	0.011	0.326	0.308	0.111	0.204	8.46	2.36	4.13	72.07	ND
I	2	93	0.098	1.179	0.007	0.239	0.265	0.082	0.146	8.34	2.36	4.16	72.57	ND
I	3	93	0.135	0.682	0.000	0.366	0.261	0.043	0.170	8.58	3.48	4.35	70.88	ND
J	1	93	0.118	1.289	0.014	0.275	0.315	0.100	0.164	8.37	2.72	4.14	72.83	ND
J	2	93	0.132	1.493	0.019	0.325	0.287	0.089	0.190	8.34	2.58	4.14	72.04	ND
J	3	93	0.112	1.195	0.018	0.289	0.268	0.069	0.169	8.24	3.14	4.12	72.40	ND
K	1	93	0.129	0.953	0.007	0.397	0.284	0.107	0.171	8.30	2.18	4.24	72.50	ND
K	2	93	0.130	0.910	0.008	0.413	0.269	0.110	0.173	8.34	1.88	4.25	72.50	ND
K	3	93	0.000	1.306	0.015	0.526	0.307	0.145	0.217	8.25	1.88	4.32	72.79	ND
L	1	93	0.116	0.856	0.000	0.359	0.258	0.115	0.151	8.33	1.89	4.26	71.50	ND
L	2	93	0.135	1.229	0.000	0.384	0.258	0.129	0.173	8.31	2.49	4.20	71.27	ND
L	3	93	0.134	1.155	0.000	0.437	0.364	0.131	0.158	8.27	1.89	4.22	71.03	ND
M	1	93	0.103	0.931	0.009	0.444	0.289	0.091	0.171	8.19	2.17	4.29	74.20	ND
M	2	93	0.091	0.715	0.012	0.426	0.337	0.079	0.125	8.22	2.47	4.28	74.27	ND
M	3	93	0.106	0.971	0.009	0.436	0.315	0.092	0.145	8.14	2.17	4.26	74.48	ND
N	1	93	0.092	0.948	0.000	0.359	0.349	0.132	0.117	8.24	2.35	4.32	72.97	ND
N	2	93	0.090	0.722	0.000	0.463	0.354	0.130	0.150	8.20	1.88	4.28	72.51	ND
N	3	93	0.094	0.999	0.000	0.388	0.389	0.148	0.160	8.15	2.83	4.28	72.45	ND
G	1	95	0.106	0.846	0.000	0.502	0.228	0.126	0.179	8.65	1.89	4.35	71.36	ND
G	2	95	0.099	0.853	0.000	0.449	0.170	0.124	0.158	8.72	2.18	4.34	72.08	ND
G	3	95	0.102	0.905	0.000	0.494	0.195	0.129	0.180	8.69	2.83	4.33	72.21	ND
H	1	95	0.070	0.900	0.000	0.207	0.141	0.223	0.155	8.60	2.57	4.39	73.90	ND
H	2	95	0.067	0.872	0.000	0.169	0.168	0.234	0.131	8.36	2.35	4.39	72.87	ND
I	1	95	0.087	1.298	0.000	0.257	0.153	0.073	0.117	8.38	2.47	4.19	73.54	ND
I	2	95	0.100	1.161	0.000	0.277	0.186	0.072	0.121	8.39	3.08	4.22	73.66	ND
I	3	95	0.144	0.870	0.000	0.468	0.187	0.047	0.167	9.02	3.36	4.40	72.54	ND
J	1	95	0.106	1.396	0.008	0.300	0.214	0.093	0.156	8.27	3.02	4.18	73.86	ND
J	2	95	0.108	1.462	0.009	0.314	0.154	0.074	0.148	8.23	3.39	4.18	74.25	ND
J	3	95	0.108	1.463	0.008	0.334	0.183	0.063	0.141	8.41	2.82	4.17	74.49	ND
K	1	95	0.115	0.983	0.000	0.408	0.221	0.101	0.132	8.28	2.65	4.29	74.44	ND
K	2	95	0.114	0.935	0.000	0.393	0.212	0.103	0.128	8.37	3.28	4.28	74.29	ND
K	3	95	0.157	0.927	0.000	0.387	0.207	0.095	0.130	8.49	3.10	4.29	74.18	ND
L	1	95	0.139	1.317	0.000	0.477	0.218	0.135	0.179	8.49	3.35	4.30	73.03	ND
L	2	95	0.118	1.173	0.000	0.399	0.191	0.118	0.136	8.38	3.29	4.24	72.55	ND
L	3	95	0.114	1.101	0.000	0.431	0.191	0.116	0.138	8.33	3.08	4.29	73.65	ND
M	1	95	0.085	0.862	0.000	0.426	0.168	0.080	0.135	8.27	3.12	4.31	75.82	ND
M	2	95	0.082	0.883	0.000	0.445	0.228	0.079	0.139	8.23	3.14	4.34	75.35	ND
M	3	95	0.087	0.895	0.000	0.421	0.189	0.074	0.126	8.17	2.82	4.30	75.25	ND
N	1	95	0.073	0.928	0.000	0.363	0.235	0.125	0.124	8.26	2.82	4.34	74.62	ND
N	2	95	0.089	0.809	0.000	0.494	0.257	0.126	0.132	8.36	3.27	4.33	74.16	ND
N	3	95	0.092	1.027	0.000	0.397	0.255	0.138	0.141	8.34	3.23	4.33	74.06	ND

Table B-2. Data used for analyses in chapter 2- temperature during aerobic stability test.

Trt	Rep	temp90	temp91	temp92	temp93	temp94	temp95
G	1	22.78	22.78	22.22	22.22	21.11	21.11
G	2	21.67	21.67	21.67	21.11	21.11	21.11
G	3	22.78	22.78	21.11	21.67	22.22	22.22
H	1	22.78	22.78	21.67	21.67	20.56	20.56
H	2	21.67	21.67	21.11	21.11	21.67	21.67
I	1	22.22	22.22	21.67	21.67	21.67	21.67
I	2	22.22	22.50	21.67	21.67	21.67	21.67
I	3	22.78	22.78	22.22	22.22	21.67	21.11
J	1	22.22	21.94	21.39	21.11	21.11	21.11
J	2	22.22	22.22	21.67	21.67	21.67	21.11
J	3	21.11	21.11	20.56	20.56	21.11	21.11
K	1	23.33	23.06	21.94	22.22	21.11	21.11
K	2	21.67	21.67	21.11	21.11	22.22	22.22
K	3	23.33	23.06	21.67	21.67	21.67	21.11
L	1	22.22	22.22	21.11	21.11	21.11	22.22
L	2	23.89	23.89	23.33	23.33	22.78	23.33
L	3	23.33	23.06	21.94	22.22	21.67	22.22
M	1	23.89	23.89	23.06	23.33	22.22	22.22
M	2	23.33	23.33	22.78	22.78	23.33	23.33
M	3	22.78	22.78	23.06	23.33	22.78	22.78
N	1	22.78	22.78	22.78	22.78	22.22	22.78
N	2	23.89	23.61	22.78	22.78	22.22	22.78
N	3	23.33	23.06	22.78	22.78	22.22	21.67

*Abbreviations used: Trt-treatment, Rep-replication, temp-temperature

APPENDIX C

Data Used for Analyses in Chapter 3

Table

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Table C-1. Data used for analyses in chapter 3-chemical analyses.

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
A	S	1	0	5.80	ND	4.33	0.000	0.001	0.000	0.000	1.036	0.000	0.000
A	S	2	0	5.76	ND	5.18	0.000	0.000	0.056	0.000	0.981	0.000	0.002
A	S	3	0	5.77	ND	5.11	0.000	0.001	0.058	0.000	0.980	0.002	0.000
A	T	1	0	5.63	ND	5.34	0.000	0.000	0.033	0.000	0.820	0.000	0.001
A	T	2	0	5.75	ND	5.24	0.000	0.000	0.055	0.002	0.970	0.000	0.000
A	T	3	0	5.69	ND	4.93	0.000	0.000	0.055	0.000	0.970	0.000	0.001
A	U	1	0	5.73	ND	5.18	0.000	0.000	0.059	0.000	1.052	0.000	0.000
A	U	2	0	5.81	ND	5.41	0.000	0.000	0.051	0.000	1.019	0.000	0.000
A	U	3	0	5.57	ND	5.11	0.000	0.000	0.052	0.000	0.973	0.000	0.000
A	W	1	0	5.73	ND	5.48	0.003	0.000	0.065	0.000	0.978	0.000	0.000
A	W	2	0	5.75	ND	5.11	0.002	0.000	0.059	0.000	1.031	0.000	0.001
A	W	3	0	5.66	ND	5.33	0.009	0.005	0.055	0.000	1.023	0.000	0.000
A	X	1	0	5.65	ND	4.34	0.007	0.000	0.050	0.000	0.900	0.000	0.000
A	X	2	0	5.69	ND	5.12	0.022	0.000	0.062	0.000	0.719	0.002	0.000
A	X	3	0	5.78	ND	4.34	0.009	0.000	0.049	0.025	0.622	0.029	0.035
B	S	1	0	5.56	ND	5.19	0.011	0.002	0.132	0.073	0.108	0.039	0.003
B	S	2	0	5.55	ND	5.92	0.037	0.000	0.195	0.138	0.098	0.041	0.000
B	S	3	0	5.61	ND	5.40	0.022	0.009	0.102	0.142	0.130	0.052	0.004
B	S	4	0	5.53	ND	5.25	0.056	0.000	0.048	0.146	0.079	0.141	0.002
B	T	1	0	5.63	ND	5.35	0.033	0.000	0.039	0.175	0.048	0.113	0.000
B	T	2	0	5.67	ND	5.22	0.033	0.000	0.044	0.172	0.108	0.066	0.000
B	T	3	0	5.54	ND	5.28	0.044	0.000	0.048	0.191	0.145	0.156	0.000
B	T	4	0	5.67	ND	5.38	0.054	0.000	0.029	0.184	0.090	0.040	0.000
B	U	1	0	5.63	ND	5.30	0.070	0.000	0.037	0.205	0.135	0.145	0.044
B	U	2	0	5.62	ND	5.42	0.080	0.000	0.052	0.200	0.112	0.053	0.001
B	U	3	0	5.46	ND	5.34	0.083	0.000	0.038	0.204	0.103	0.098	0.000
B	U	4	0	5.66	ND	5.38	0.063	0.000	0.052	0.190	0.103	0.105	0.000
B	W	1	0	5.68	ND	5.57	0.069	0.000	0.036	0.170	0.093	0.056	0.000
B	W	2	0	5.59	ND	5.45	0.081	0.000	0.031	0.143	0.089	0.063	0.052
B	W	3	0	5.55	ND	5.16	0.062	0.000	0.023	0.168	0.079	0.048	0.000
B	W	4	0	5.46	ND	5.21	0.063	0.000	0.042	0.162	0.099	0.152	0.138
B	X	1	0	5.55	ND	5.05	0.054	0.000	0.025	0.163	0.074	0.033	0.000
B	X	2	0	5.63	ND	5.60	0.069	0.000	0.030	0.183	0.094	0.190	0.228
B	X	3	0	5.68	ND	5.54	0.084	0.000	0.045	0.156	0.106	0.091	0.000
B	X	4	0	5.68	ND	5.57	0.097	0.000	0.031	0.158	0.094	0.089	0.000
C	S	1	0	5.61	ND	5.32	0.021	0.000	0.074	0.007	0.181	0.010	0.004

*Abbreviations used: Mc-moisture content, Inoc-inoculant, Rep-Replication, Dmr-dry matter recovery, Ym-yeast and molds, Ac-acetic acid, Bu-butyric acid, Ca-citric acid, Et-ethanol, Gl-glucose, La-lactic acid, Pa-propionic acid, ND- not determined.

Table

Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
C	S	2	0	5.63	ND	4.76	0.020	0.000	0.065	0.015	0.084	0.005	0.005
C	S	3	0	5.78	ND	5.76	0.021	0.000	0.082	0.018	0.086	0.026	0.009
C	S	4	0	5.50	ND	4.97	0.014	0.000	0.086	0.023	0.103	0.010	0.000
C	T	1	0	5.71	ND	4.86	0.020	0.000	0.076	0.046	0.058	0.010	0.000
C	T	2	0	5.60	ND	5.10	0.015	0.000	0.044	0.059	0.085	0.010	0.002
C	T	3	0	5.42	ND	4.96	0.016	0.000	0.062	0.055	0.065	0.008	0000
C	T	4	0	5.64	ND	5.14	0.021	0.000	0.475	0.074	0.381	0.018	0.000
C	U	1	0	5.60	ND	5.61	0.018	0.000	0.027	0.075	0.057	0.010	0.001
C	U	2	0	5.74	ND	5.27	0.042	0.000	0.011	0.077	0.047	0.034	0.024
C	U	3	0	5.81	ND	5.28	0.029	0.000	0.012	0.091	0.051	0.021	0.046
C	U	4	0	5.45	ND	5.12	0.035	0.000	0.016	0.104	0.071	0.033	0.034
C	W	1	0	5.58	ND	4.78	0.024	0.000	0.014	0.061	0.056	0.015	0.000
C	W	2	0	5.74	ND	4.78	0.036	0.000	0.012	0.093	0.062	0.020	0.027
C	W	3	0	5.81	ND	4.81	0.032	0.000	0.006	0.085	0.057	0.007	0.024
C	W	4	0	5.45	ND	4.49	0.037	0.000	0.018	0.069	0.068	0.027	0.097
C	X	1	0	5.58	ND	5.34	0.033	0.000	0.007	0.076	0.059	0.000	0.124
C	X	2	0	5.47	ND	5.32	0.027	0.000	0.011	0.053	0.066	0.007	0.000
C	X	3	0	5.62	ND	4.79	0.014	0.000	0.006	0.051	0.066	0.000	0.000
C	X	4	0	5.75	ND	4.74	0.018	0.000	0.005	0.078	0.066	0.016	0.000
C	S	5	0	5.35	ND	4.84	0.020	0.000	0.004	0.058	0.042	0.170	0.005
C	S	6	0	5.43	ND	4.81	0.010	0.000	0.006	0.022	0.048	0.130	0.010
C	S	7	0	5.45	ND	4.46	0.011	0.000	0.007	0.015	0.048	0.117	0.004
C	S	8	0	5.39	ND	4.63	0.015	0.000	0.008	0.044	0.046	0.162	0.005
C	T	5	0	5.39	ND	4.23	0.030	0.000	0.010	0.103	0.062	0.183	0.044
C	T	6	0	5.46	ND	4.46	0.027	0.000	0.010	0.053	0.066	0.156	0.004
C	T	7	0	5.24	ND	5.17	0.010	0.000	0.008	0.015	0.044	0.116	0.008
C	T	8	0	5.35	ND	4.38	0.027	0.000	0.008	0.022	0.050	0.181	0.046
C	U	5	0	5.50	ND	3.86	0.045	0.000	0.009	0.054	0.055	0.163	0.025
C	U	6	0	5.44	ND	4.83	0.023	0.000	0.011	0.098	0.059	0.178	0.053
C	U	7	0	5.28	ND	4.49	0.009	0.000	0.008	0.015	0.047	0.159	0.042
C	U	8	0	5.31	ND	4.66	0.010	0.000	0.009	0.020	0.053	0.137	0.052
C	W	5	0	5.40	ND	4.94	0.027	0.000	0.007	0.020	0.046	0.168	0.031
C	W	6	0	5.49	ND	4.99	0.052	0.000	0.011	0.070	0.071	0.180	0.063
C	W	7	0	5.42	ND	4.66	0.059	0.000	0.010	0.029	0.055	0.306	0.033
C	W	8	0	5.38	ND	4.33	0.009	0.000	0.012	0.091	0.052	0.161	0.053
C	X	5	0	5.37	ND	4.67	0.012	0.000	0.008	0.081	0.050	0.175	0.000
C	X	6	0	5.48	ND	4.99	0.049	0.000	0.013	0.102	0.075	0.177	0.000
C	X	7	0	5.36	ND	3.68	0.035	0.000	0.013	0.101	0.074	0.193	0.003
C	X	8	0	5.26	ND	4.90	0.026	0.000	0.008	0.040	0.049	0.082	0.000
E	S	1	0	5.43	ND	4.74	0.008	0.000	0.010	0.033	0.070	0.010	0.007
E	S	2	0	5.33	ND	4.73	0.017	0.000	0.017	0.043	0.064	0.023	0.007

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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
E	S	3	0	5.29	ND	4.92	0.020	0.006	0.010	0.023	0.067	0.011	0.005
E	S	4	0	5.36	ND	4.44	0.021	0.000	0.008	0.056	0.040	0.008	0.007
E	T	1	0	5.55	ND	4.80	0.051	0.000	0.018	0.053	0.058	0.008	0.007
E	T	2	0	5.47	ND	4.66	0.018	0.000	0.013	0.025	0.054	0.005	0.048
E	T	3	0	5.49	ND	4.28	0.043	0.000	0.014	0.017	0.065	0.028	0.107
E	T	4	0	5.42	ND	4.88	0.013	0.000	0.011	0.020	0.041	0.037	0.007
E	U	1	0	5.52	ND	4.40	0.020	0.000	0.010	0.043	0.058	0.003	0.021
E	U	2	0	5.39	ND	4.80	0.030	0.000	0.011	0.037	0.048	0.006	0.023
E	U	3	0	5.53	ND	4.68	0.013	0.000	0.012	0.052	0.053	0.012	0.014
E	U	4	0	5.40	ND	4.82	0.010	0.000	0.003	0.028	0.042	0.000	0.020
E	W	1	0	5.33	ND	5.20	0.065	0.000	0.013	0.034	0.050	0.000	0.031
E	W	2	0	5.29	ND	5.24	0.019	0.000	0.006	0.026	0.049	0.003	0.033
E	W	3	0	5.37	ND	4.80	0.016	0.000	0.008	0.029	0.045	0.015	0.018
E	W	4	0	5.45	ND	4.93	0.016	0.000	0.008	0.029	0.048	0.020	0.016
E	X	1	0	5.48	ND	4.58	0.000	0.000	0.014	0.032	0.046	0.003	0.013
E	X	2	0	5.45	ND	5.01	0.019	0.000	0.010	0.022	0.047	0.002	0.020
E	X	3	0	5.30	ND	4.55	0.029	0.000	0.014	0.061	0.056	0.000	0.024
E	X	4	0	5.42	ND	4.18	0.002	0.000	0.009	0.021	0.046	0.003	0.010
F	S	1	0	5.37	ND	4.41	0.000	0.000	0.028	0.000	0.295	0.005	0.000
F	S	2	0	5.44	ND	5.05	0.000	0.000	0.048	0.000	0.256	0.000	0.000
F	S	3	0	5.32	ND	5.00	0.007	0.000	0.046	0.000	0.267	0.001	0.000
F	S	4	0	5.14	ND	3.71	0.006	0.000	0.034	0.000	0.264	0.001	0.000
F	T	1	0	5.13	ND	4.41	0.034	0.000	0.033	0.022	0.207	0.001	0.004
F	T	2	0	5.39	ND	4.89	0.015	0.000	0.084	0.024	0.286	0.043	0.003
F	T	3	0	5.17	ND	5.26	0.001	0.000	0.044	0.000	0.288	0.000	0.000
F	T	4	0	5.18	ND	4.49	0.015	0.000	0.040	0.005	0.210	0.008	0.004
F	U	1	0	5.24	ND	4.18	0.031	0.000	0.057	0.008	0.238	0.063	0.005
F	U	2	0	5.27	ND	4.52	0.042	0.000	0.038	0.072	0.142	0.177	0.054
F	U	3	0	5.32	ND	4.84	0.040	0.000	0.007	0.017	0.035	0.067	0.007
F	U	4	0	5.21	ND	4.36	0.033	0.000	0.011	0.014	0.116	0.058	0.005
F	W	1	0	5.19	ND	3.89	0.035	0.000	0.009	0.025	0.141	0.025	0.000
F	W	2	0	5.24	ND	4.90	0.029	0.000	0.012	0.012	0.155	0.016	0.008
F	W	3	0	5.24	ND	4.19	0.061	0.000	0.013	0.059	0.091	0.079	0.003
F	W	4	0	5.24	ND	4.52	0.058	0.000	0.018	0.079	0.071	0.146	0.065
F	X	1	0	5.29	ND	5.05	0.046	0.000	0.040	0.114	0.081	0.245	0.109
F	X	2	0	5.28	ND	4.69	0.028	0.000	0.013	0.006	0.026	0.024	0.003
F	X	3	0	5.13	ND	4.01	0.025	0.000	0.015	0.002	0.076	0.011	0.005
F	X	4	0	5.24	ND	4.73	0.018	0.000	0.012	0.057	0.135	0.055	0.004
G	S	1	0	5.28	ND	5.78	0.010	0.000	0.024	0.066	0.047	0.033	0.005
G	S	2	0	5.21	ND	5.92	0.047	0.000	0.022	0.065	0.045	0.021	0.003
G	S	3	0	5.29	ND	5.63	0.022	0.000	0.018	0.050	0.048	0.107	0.006

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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
G	T	1	0	5.15	ND	5.39	0.029	0.000	0.025	0.023	0.047	0.042	0.004
G	T	2	0	5.41	ND	5.69	0.016	0.000	0.019	0.048	0.033	0.055	0.008
G	T	3	0	5.24	ND	5.81	0.057	0.001	0.016	0.070	0.049	0.041	0.002
G	U	1	0	5.28	ND	6.17	0.014	0.000	0.020	0.063	0.037	0.109	0.002
G	U	2	0	5.20	ND	5.33	0.015	0.000	0.011	0.012	0.009	0.034	0.002
G	U	3	0	5.19	ND	5.41	0.035	0.001	0.017	0.044	0.023	0.011	0.035
G	W	1	0	5.31	ND	6.07	0.010	0.000	0.028	0.046	0.035	0.039	0.013
G	W	2	0	5.24	ND	5.93	0.025	0.001	0.020	0.034	0.044	0.019	0.003
G	W	3	0	5.17	ND	5.91	0.039	0.000	0.014	0.021	0.039	0.003	0.003
G	X	1	0	5.44	ND	5.94	0.047	0.000	0.016	0.054	0.048	0.022	0.031
G	X	2	0	5.23	ND	5.37	0.023	0.000	0.012	0.037	0.050	0.071	0.038
G	X	3	0	5.22	ND	5.46	0.029	0.000	0.021	0.029	0.037	0.011	0.002
A	S	1	10	3.88	99.34	4.54	0.187	0.000	0.017	0.618	0.139	2.061	0.004
A	S	2	10	3.91	99.59	4.66	0.189	0.000	0.102	0.658	0.190	2.210	0.003
A	S	3	10	3.89	97.01	4.48	0.165	0.000	0.014	0.556	0.116	1.800	0.001
A	T	1	10	3.91	98.18	4.85	0.169	0.000	0.032	0.657	0.148	1.780	0.000
A	T	2	10	3.90	100.00	4.26	0.194	0.001	0.068	0.681	0.209	2.211	0.001
A	T	3	10	3.88	100.00	5.41	0.215	0.000	0.036	0.691	0.174	2.243	0.004
A	U	1	10	3.89	99.27	5.47	0.202	0.002	0.039	0.630	0.160	2.146	0.006
A	U	2	10	3.87	97.01	5.41	0.193	0.001	0.040	0.575	0.159	2.105	0.000
A	U	3	10	3.92	99.12	4.64	0.216	0.000	0.099	0.676	0.247	2.317	0.006
A	W	1	10	3.89	96.57	4.70	0.165	0.000	0.013	0.553	0.116	1.768	0.002
A	W	2	10	3.94	100.00	5.26	0.183	0.000	0.012	0.604	0.127	1.962	0.003
A	W	3	10	3.92	99.62	4.21	0.215	0.001	0.034	0.617	0.161	2.243	0.003
A	X	1	10	3.90	97.13	4.57	0.242	0.000	0.070	0.892	0.230	2.766	0.007
A	X	2	10	3.87	98.46	4.01	0.194	0.000	0.036	0.683	0.155	2.214	0.001
A	X	3	10	3.93	96.08	4.16	0.203	0.000	0.033	0.679	0.160	2.227	0.002
B	S	1	10	4.04	94.09	4.80	0.136	0.000	0.009	0.635	0.142	1.349	0.001
B	S	2	10	4.01	100.50	4.65	0.145	0.002	0.011	0.673	0.163	1.424	0.000
B	S	3	10	4.07	98.31	4.65	0.159	0.000	0.014	0.759	0.183	1.514	0.003
B	S	4	10	4.03	97.90	4.47	0.155	0.000	0.011	0.703	0.178	1.617	0.004
B	T	1	10	4.02	91.78	4.84	0.118	0.002	0.042	0.624	0.171	1.495	0.004
B	T	2	10	4.11	97.03	4.48	0.117	0.000	0.015	0.700	0.171	1.675	0.002
B	T	3	10	4.06	98.32	4.55	0.098	0.001	0.061	0.671	0.172	1.634	0.002
B	T	4	10	4.06	97.96	4.45	0.168	0.002	0.014	0.769	0.192	1.620	0.004
B	U	1	10	4.02	97.35	4.52	0.163	0.000	0.012	0.691	0.184	1.601	0.002
B	U	2	10	3.96	93.41	4.56	0.178	0.001	0.013	0.647	0.179	1.787	0.006
B	U	3	10	4.05	98.27	3.41	0.164	0.000	0.051	0.650	0.166	1.412	0.008
B	U	4	10	4.01	97.95	4.42	0.186	0.000	0.014	0.736	0.198	1.753	0.007
B	W	1	10	4.12	96.66	4.77	0.136	0.000	0.010	0.515	0.111	1.087	0.004
B	W	2	10	4.09	92.06	4.69	0.215	0.000	0.012	0.624	0.153	1.397	0.003

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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
B	W	3	10	4.05	98.26	5.20	0.168	0.001	0.015	0.674	0.185	1.623	0.003
B	W	4	10	4.05	98.82	4.16	0.166	0.000	0.045	0.716	0.196	1.552	0.005
B	X	1	10	4.00	96.92	5.01	0.158	0.001	0.013	0.681	0.178	1.697	0.003
B	X	2	10	3.97	98.43	5.10	0.176	0.000	0.014	0.645	0.192	1.754	0.009
B	X	3	10	4.07	97.52	4.89	0.129	0.000	0.016	0.764	0.187	1.466	0.004
B	X	4	10	4.06	92.69	5.20	0.154	0.000	0.013	0.653	0.166	1.497	0.007
C	S	1	10	4.69	91.14	4.90	0.078	0.000	0.010	0.485	0.070	0.536	0.008
C	S	2	10	4.66	87.52	5.11	0.072	0.000	0.009	0.435	0.073	0.453	0.004
C	S	3	10	4.93	94.81	5.63	0.132	0.000	0.021	0.861	0.160	1.007	0.009
C	S	4	10	4.61	98.34	4.48	0.087	0.000	0.014	0.636	0.089	0.682	0.002
C	T	1	10	4.62	95.47	5.01	0.084	0.000	0.009	0.467	0.089	0.610	0.003
C	T	2	10	4.63	92.07	4.27	0.090	0.000	0.009	0.525	0.076	0.496	0.007
C	T	3	10	4.60	94.87	4.89	0.110	0.000	0.014	0.540	0.087	0.598	0.009
C	T	4	10	4.61	97.96	3.63	0.088	0.000	0.010	0.575	0.076	0.596	0.002
C	U	1	10	4.68	92.02	4.80	0.094	0.000	0.008	0.494	0.062	0.336	0.028
C	U	2	10	4.65	93.29	5.33	0.115	0.000	0.009	0.486	0.073	0.383	0.022
C	U	3	10	4.57	91.53	5.26	0.138	0.000	0.010	0.621	0.091	0.566	0.053
C	U	4	10	4.52	97.89	3.87	0.138	0.000	0.014	0.597	0.090	0.665	0.040
C	W	1	10	4.66	95.75	4.85	0.101	0.000	0.011	0.521	0.038	0.440	0.012
C	W	2	10	4.67	87.49	4.59	0.092	0.000	0.009	0.471	0.072	0.458	0.004
C	W	3	10	4.64	92.20	4.20	0.121	0.000	0.012	0.544	0.076	0.641	0.028
C	W	4	10	4.51	98.00	4.14	0.118	0.000	0.015	0.575	0.081	0.685	0.014
C	X	1	10	4.63	89.34	4.84	0.090	0.000	0.012	0.467	0.067	0.558	0.013
C	X	2	10	4.62	89.64	4.36	0.084	0.000	0.010	0.490	0.068	0.540	0.002
C	X	3	10	4.66	88.65	4.42	0.101	0.000	0.014	0.509	0.076	0.674	0.007
C	X	4	10	4.52	92.50	4.43	0.112	0.000	0.016	0.614	0.092	0.765	0.002
E	S	1	10	4.95	99.07	3.87	0.089	0.000	0.011	0.601	0.049	0.510	0.004
E	S	2	10	4.41	97.79	0.00	0.079	0.000	0.007	0.518	0.025	0.743	0.010
E	S	3	10	4.48	98.09	3.70	0.062	0.000	0.007	0.539	0.035	0.793	0.006
E	S	4	10	4.36	99.72	0.00	0.061	0.000	0.006	0.520	0.022	0.792	0.005
E	T	1	10	4.83	94.66	3.40	0.149	0.000	0.009	0.596	0.049	0.445	0.043
E	T	2	10	4.38	98.39	4.30	0.096	0.000	0.007	0.474	0.055	0.804	0.015
E	T	3	10	4.65	98.08	3.40	0.080	0.000	0.005	0.458	0.039	0.559	0.029
E	T	4	10	4.24	97.56	4.10	0.074	0.001	0.005	0.289	0.018	0.730	0.001
E	U	1	10	4.67	98.24	0.00	0.172	0.000	0.008	0.566	0.053	0.647	0.100
E	U	2	10	4.48	98.49	0.00	0.113	0.000	0.008	0.483	0.052	0.821	0.075
E	U	3	10	4.65	97.39	0.00	0.181	0.000	0.005	0.469	0.044	0.400	0.092
E	U	4	10	4.59	97.74	0.00	0.118	0.000	0.005	0.340	0.037	0.469	0.034
E	W	1	10	4.33	96.48	3.88	0.092	0.000	0.006	0.416	0.050	0.985	0.041
E	W	2	10	4.27	99.03	3.40	0.083	0.000	0.006	0.370	0.047	1.050	0.033
E	W	3	10	4.48	98.17	0.00	0.103	0.000	0.008	0.430	0.041	0.678	0.069

Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
E	W	4	10	4.36	97.43	0.00	0.075	0.000	0.006	0.367	0.037	0.684	0.046
E	X	1	10	4.79	97.37	4.24	0.148	0.000	0.004	0.386	0.035	0.502	0.000
E	X	2	10	4.53	97.99	3.70	0.138	0.000	0.009	0.532	0.052	0.789	0.003
E	X	3	10	4.87	97.40	3.40	0.174	0.000	0.008	0.497	0.044	0.438	0.007
E	X	4	10	4.59	97.82	4.35	0.131	0.000	0.007	0.573	0.050	0.829	0.001
F	S	1	10	4.59	99.59	4.44	0.010	0.000	0.010	0.443	0.044	0.709	0.000
F	S	2	10	4.84	99.20	5.21	0.029	0.000	0.014	0.460	0.051	0.648	0.002
F	S	3	10	4.91	99.94	5.29	0.143	0.000	0.012	0.399	0.047	0.486	0.004
F	S	4	10	4.81	99.38	4.87	0.108	0.000	0.012	0.475	0.041	0.567	0.004
F	T	1	10	4.66	98.78	4.69	0.024	0.000	0.006	0.453	0.040	0.676	0.000
F	T	2	10	4.93	99.79	5.06	0.149	0.000	0.011	0.350	0.044	0.352	0.003
F	T	3	10	4.97	98.85	6.18	0.178	0.000	0.011	0.363	0.052	0.351	0.006
F	T	4	10	4.81	98.65	4.27	0.131	0.000	0.010	0.423	0.042	0.578	0.003
F	U	1	10	4.61	99.15	5.13	0.044	0.000	0.008	0.350	0.031	0.536	0.004
F	U	2	10	4.75	99.26	3.81	0.181	0.000	0.010	0.427	0.044	0.456	0.047
F	U	3	10	4.96	99.28	3.77	0.199	0.000	0.012	0.380	0.046	0.425	0.013
F	U	4	10	4.72	99.07	4.24	0.149	0.000	0.009	0.472	0.046	0.737	0.035
F	W	1	10	4.85	98.72	5.27	0.110	0.000	0.010	0.409	0.045	0.408	0.020
F	W	2	10	4.91	99.51	4.81	0.188	0.000	0.010	0.368	0.042	0.391	0.024
F	W	3	10	4.88	98.76	5.75	0.176	0.000	0.010	0.268	0.044	0.320	0.003
F	W	4	10	4.89	99.21	3.95	0.191	0.000	0.007	0.426	0.041	0.483	0.018
F	X	1	10	4.72	99.17	4.90	0.139	0.000	0.009	0.421	0.044	0.631	0.004
F	X	2	10	4.83	98.74	5.20	0.100	0.000	0.010	0.393	0.043	0.533	0.002
F	X	3	10	4.81	96.86	5.72	0.059	0.000	0.008	0.118	0.031	0.437	0.009
F	X	4	10	4.85	99.12	5.26	0.166	0.000	0.009	0.513	0.046	0.708	0.004
G	S	1	10	5.37	98.27	5.82	0.002	0.000	0.015	0.269	0.155	0.029	0.000
G	S	2	10	5.29	99.07	6.12	0.005	0.000	0.017	0.298	0.155	0.036	0.000
G	S	3	10	5.23	99.66	6.01	0.002	0.000	0.012	0.214	0.166	0.042	0.001
G	T	1	10	5.39	99.32	5.99	0.006	0.001	0.020	0.263	0.226	0.040	0.000
G	T	2	10	5.24	99.79	6.05	0.002	0.000	0.014	0.303	0.164	0.048	0.000
G	T	3	10	5.25	98.26	6.90	0.001	0.000	0.020	0.384	0.110	0.026	0.001
G	U	1	10	5.37	99.94	6.17	0.005	0.000	0.014	0.224	0.166	0.028	0.000
G	U	2	10	5.30	99.45	6.63	0.011	0.000	0.021	0.375	0.113	0.043	0.002
G	U	3	10	5.20	99.01	6.16	0.009	0.000	0.012	0.318	0.116	0.056	0.003
G	W	1	10	5.40	99.08	6.36	0.010	0.000	0.014	0.271	0.092	0.041	0.000
G	W	2	10	5.21	99.95	6.07	0.013	0.000	0.013	0.240	0.124	0.069	0.002
G	W	3	10	5.26	98.45	6.11	0.023	0.001	0.011	0.355	0.071	0.108	0.001
G	X	1	10	5.38	99.16	6.36	0.043	0.000	0.007	0.336	0.032	0.157	0.001
G	X	2	10	5.26	99.38	6.46	0.041	0.000	0.011	0.362	0.033	0.154	0.001
G	X	3	10	5.21	99.94	6.20	0.057	0.000	0.006	0.348	0.030	0.188	0.002

Table C

Mc	In
A	S
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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
A	S	1	21	3.79	100.00	4.25	0.176	0.000	0.009	0.546	0.139	2.308	0.006
A	S	2	21	3.80	99.06	5.65	0.155	0.000	0.007	0.563	0.126	2.218	0.007
A	S	3	21	3.79	98.89	3.68	0.176	0.000	0.025	0.568	0.156	2.304	0.010
A	T	1	21	3.79	98.32	4.10	0.150	0.000	0.027	0.554	0.137	2.078	0.006
A	T	2	21	3.80	99.88	5.96	0.165	0.000	0.008	0.543	0.134	2.218	0.006
A	T	3	21	3.80	99.27	3.48	0.182	0.000	0.043	0.555	0.182	2.285	0.005
A	U	1	21	3.79	98.88	4.08	0.181	0.000	0.032	0.538	0.146	2.283	0.011
A	U	2	21	3.79	99.30	4.97	0.150	0.000	0.008	0.523	0.123	2.035	0.009
A	U	3	21	3.79	97.79	4.15	0.190	0.000	0.060	0.572	0.169	2.341	0.010
A	W	1	21	3.80	98.69	4.54	0.165	0.000	0.039	0.555	0.169	2.193	0.005
A	W	2	21	3.80	99.57	6.21	0.152	0.000	0.026	0.552	0.141	2.087	0.006
A	W	3	21	3.77	99.54	4.40	0.183	0.000	0.008	0.535	0.139	2.276	0.006
A	X	1	21	3.82	98.35	6.38	0.163	0.000	0.040	0.533	0.160	2.136	0.006
A	X	2	21	3.81	98.78	4.94	0.145	0.000	0.049	0.549	0.169	2.056	0.006
A	X	3	21	3.76	99.43	3.65	0.168	0.000	0.052	0.532	0.178	2.161	0.005
B	S	1	21	3.90	96.86	4.04	0.154	0.000	0.009	0.904	0.210	1.986	0.008
B	S	2	21	3.90	97.90	4.29	0.167	0.000	0.012	0.797	0.231	2.120	0.015
B	S	3	21	3.93	96.84	4.26	0.165	0.000	0.013	0.844	0.256	2.093	0.008
B	S	4	21	3.88	95.91	3.91	0.159	0.000	0.038	0.821	0.228	2.067	0.028
B	T	1	21	3.86	98.19	4.16	0.192	0.000	0.009	0.803	0.224	2.205	0.014
B	T	2	21	3.93	97.05	4.57	0.142	0.000	0.010	0.851	0.226	1.830	0.005
B	T	3	21	3.88	97.08	4.26	0.168	0.000	0.013	0.810	0.264	2.166	0.014
B	T	4	21	3.90	98.13	4.48	0.167	0.000	0.012	0.928	0.247	2.102	0.008
B	U	1	21	3.91	97.11	3.45	0.175	0.000	0.011	0.791	0.229	2.193	0.009
B	U	2	21	3.83	97.30	3.42	0.199	0.000	0.019	0.747	0.236	2.269	0.020
B	U	3	21	3.94	96.69	4.38	0.159	0.000	0.010	0.854	0.233	1.998	0.013
B	U	4	21	3.91	96.89	4.04	0.176	0.000	0.037	0.917	0.254	2.201	0.027
B	W	1	21	3.92	97.94	4.28	0.151	0.000	0.012	0.841	0.207	1.845	0.006
B	W	2	21	3.92	98.83	4.65	0.165	0.000	0.026	0.793	0.251	1.986	0.012
B	W	3	21	3.89	96.90	4.24	0.163	0.000	0.011	0.778	0.238	2.079	0.010
B	W	4	21	3.93	98.69	4.22	0.159	0.000	0.011	0.893	0.220	1.906	0.011
B	X	1	21	3.88	94.42	4.17	0.180	0.000	0.034	0.839	0.259	2.188	0.008
B	X	2	21	3.83	96.70	4.48	0.225	0.000	0.069	0.773	0.322	2.436	0.022
B	X	3	21	3.93	96.82	4.35	0.163	0.000	0.033	0.792	0.255	2.028	0.005
B	X	4	21	3.91	95.87	4.30	0.170	0.000	0.034	0.870	0.268	2.091	0.019
C	S	1	21	4.51	94.76	5.55	0.164	0.000	0.010	0.683	0.098	0.924	0.004
C	S	2	21	4.55	93.26	5.85	0.185	0.000	0.009	0.627	0.122	1.047	0.001
C	S	3	21	4.46	92.07	5.43	0.106	0.000	0.014	0.699	0.112	1.237	0.002
C	S	4	21	4.50	92.18	5.51	0.170	0.000	0.006	0.643	0.107	1.050	0.002
C	T	1	21	4.44	92.14	4.98	0.165	0.000	0.006	0.482	0.080	0.721	0.058

Table

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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
C	T	2	21	4.58	92.93	5.09	0.209	0.001	0.009	0.540	0.092	0.688	0.046
C	T	3	21	4.47	95.11	4.46	0.213	0.000	0.004	0.555	0.090	0.908	0.089
C	T	4	21	4.49	94.88	5.16	0.214	0.000	0.007	0.624	0.101	1.016	0.041
C	U	1	21	4.49	96.26	5.17	0.199	0.000	0.001	0.442	0.089	0.722	0.164
C	U	2	21	4.50	96.94	3.46	0.304	0.000	0.005	0.630	0.099	0.883	0.163
C	U	3	21	4.47	94.94	3.33	0.253	0.000	0.004	0.537	0.071	0.634	0.151
C	U	4	21	4.44	93.69	2.85	0.257	0.000	0.007	0.549	0.092	0.872	0.120
C	W	1	21	4.59	92.69	3.15	0.137	0.000	0.005	0.219	0.067	0.657	0.066
C	W	2	21	4.63	92.78	0.00	0.269	0.000	0.013	0.503	0.076	0.693	0.070
C	W	3	21	4.49	90.42	3.75	0.308	0.000	0.007	0.657	0.098	1.028	0.089
C	W	4	21	4.48	94.93	4.82	0.302	0.000	0.008	0.598	0.098	0.964	0.068
C	X	1	21	4.46	95.39	5.49	0.087	0.000	0.007	0.290	0.076	0.770	0.003
C	X	2	21	4.51	91.88	5.73	0.178	0.000	0.005	0.573	0.100	0.919	0.007
C	X	3	21	4.41	97.38	5.08	0.237	0.000	0.006	0.679	0.110	1.195	0.002
C	X	4	21	4.47	98.11	5.65	0.124	0.000	0.008	0.463	0.091	0.882	0.005
E	S	1	21	4.70	98.42	4.99	0.035	0.002	0.009	0.648	0.124	0.507	0.005
E	S	2	21	4.39	97.04	4.74	0.117	0.087	0.005	0.551	0.057	0.753	0.011
E	S	3	21	4.38	97.93	4.79	0.085	0.043	0.006	0.565	0.067	0.783	0.011
E	S	4	21	4.37	98.26	5.56	0.092	0.000	0.007	0.587	0.074	0.827	0.008
E	T	1	21	4.69	97.92	3.00	0.093	0.000	0.009	0.571	0.089	0.212	0.076
E	T	2	21	4.37	97.98	6.31	0.134	0.022	0.006	0.505	0.039	0.765	0.011
E	T	3	21	4.51	97.69	3.84	0.107	0.030	0.006	0.529	0.055	0.448	0.015
E	T	4	21	4.25	98.23	3.68	0.103	0.005	0.005	0.484	0.045	0.927	0.007
E	U	1	21	4.57	98.12	2.87	0.157	0.000	0.008	0.551	0.174	0.264	0.144
E	U	2	21	4.37	97.58	2.87	0.155	0.019	0.007	0.533	0.085	0.682	0.116
E	U	3	21	4.60	96.59	2.87	0.146	0.000	0.012	0.623	0.152	0.290	0.181
E	U	4	21	4.43	97.51	4.45	0.153	0.000	0.007	0.639	0.057	0.579	0.122
E	W	1	21	4.25	98.09	5.52	0.146	0.002	0.005	0.390	0.041	0.827	0.021
E	W	2	21	4.14	98.58	3.78	0.119	0.000	0.006	0.393	0.044	1.099	0.050
E	W	3	21	4.37	97.86	2.87	0.135	0.000	0.006	0.431	0.042	0.551	0.014
E	W	4	21	4.31	96.71	4.09	0.185	0.003	0.006	0.447	0.045	0.669	0.067
E	X	1	21	4.57	96.70	5.38	0.065	0.008	0.008	0.649	0.066	0.719	0.006
E	X	2	21	4.48	97.83	3.68	0.050	0.036	0.007	0.657	0.082	0.826	0.015
E	X	3	21	4.66	97.59	5.41	0.084	0.000	0.007	0.532	0.052	0.639	0.005
E	X	4	21	4.53	97.57	5.39	0.074	0.012	0.010	0.670	0.066	0.928	0.006
F	S	1	21	4.39	99.86	5.36	0.061	0.001	0.007	0.092	0.036	0.361	0.002
F	S	2	21	4.57	99.42	5.54	0.201	0.000	0.006	0.244	0.038	0.319	0.001
F	S	3	21	4.70	99.24	5.74	0.246	0.000	0.003	0.253	0.043	0.236	0.004
F	S	4	21	4.54	99.35	5.85	0.047	0.000	0.008	0.026	0.025	0.149	0.002
F	T	1	21	4.43	99.06	5.11	0.176	0.002	0.005	0.393	0.045	0.470	0.007

Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
F	T	2	21	4.57	99.16	5.07	0.258	0.001	0.006	0.278	0.042	0.226	0.016
F	T	3	21	4.62	99.19	5.43	0.288	0.000	0.006	0.221	0.040	0.203	0.018
F	T	4	21	4.55	98.36	5.74	0.044	0.000	0.005	0.017	0.029	0.168	0.011
F	U	1	21	4.51	99.63	5.16	0.159	0.000	0.001	0.164	0.032	0.239	0.033
F	U	2	21	4.48	98.64	4.55	0.257	0.000	0.001	0.233	0.035	0.161	0.026
F	U	3	21	4.66	99.79	4.91	0.279	0.000	0.004	0.128	0.025	0.085	0.018
F	U	4	21	4.46	98.44	3.31	0.278	0.001	0.000	0.333	0.043	0.276	0.052
F	W	1	21	4.65	99.17	5.90	0.000	0.000	0.003	0.000	0.024	0.131	0.009
F	W	2	21	4.61	99.82	3.45	0.259	0.000	0.003	0.272	0.034	0.203	0.050
F	W	3	21	4.64	98.80	3.45	0.277	0.000	0.006	0.282	0.037	0.141	0.042
F	W	4	21	4.57	98.83	6.07	0.005	0.000	0.002	0.002	0.020	0.154	0.024
F	X	1	21	4.51	95.79	5.96	0.009	0.001	0.001	0.000	0.031	0.126	0.001
F	X	2	21	4.54	97.36	5.90	0.016	0.001	0.002	0.004	0.027	0.141	0.000
F	X	3	21	4.50	98.23	6.41	0.000	0.001	0.003	0.002	0.026	0.152	0.002
F	X	4	21	4.50	98.46	6.63	0.000	0.001	0.002	0.002	0.026	0.168	0.004
G	S	1	21	5.22	96.14	6.02	0.007	0.000	0.016	0.580	0.096	0.115	0.000
G	S	2	21	5.20	98.68	5.92	0.006	0.000	0.021	0.641	0.120	0.143	0.000
G	S	3	21	5.13	98.97	5.73	0.028	0.000	0.024	0.664	0.131	0.193	0.000
G	T	1	21	5.09	95.08	5.89	0.008	0.000	0.014	0.509	0.072	0.110	0.001
G	T	2	21	5.09	100.18	5.74	0.015	0.000	0.021	0.623	0.127	0.193	0.005
G	T	3	21	5.01	98.31	5.75	0.074	0.000	0.020	0.590	0.054	0.173	0.009
G	U	1	21	5.00	99.81	5.24	0.040	0.000	0.016	0.449	0.080	0.167	0.008
G	U	2	21	4.98	99.19	5.59	0.050	0.000	0.018	0.588	0.043	0.143	0.012
G	U	3	21	4.92	98.64	5.61	0.095	0.000	0.013	0.602	0.053	0.263	0.034
G	W	1	21	5.00	96.49	5.41	0.059	0.000	0.010	0.446	0.037	0.226	0.004
G	W	2	21	5.10	99.50	5.55	0.100	0.000	0.012	0.659	0.048	0.344	0.007
G	W	3	21	5.01	98.45	5.36	0.111	0.000	0.012	0.678	0.052	0.389	0.016
G	X	1	21	5.22	99.14	5.77	0.078	0.000	0.008	0.607	0.043	0.311	0.002
G	X	2	21	5.17	99.87	5.87	0.052	0.000	0.010	0.630	0.052	0.516	0.003
G	X	3	21	5.08	99.54	5.54	0.117	0.000	0.013	0.556	0.053	0.540	0.000
A	S	1	120	3.70	98.36	2.47	0.224	0.000	0.006	0.599	0.307	4.112	0.200
A	S	2	120	3.70	98.14	4.73	0.189	0.000	0.004	0.413	0.241	3.563	0.165
A	S	3	120	3.70	97.59	2.41	0.171	0.000	0.002	0.422	0.231	3.140	0.141
A	T	1	120	3.80	96.86	3.16	0.234	0.000	0.004	0.622	0.106	3.260	0.163
A	T	2	120	3.73	98.58	1.81	0.183	0.000	0.002	0.546	0.249	3.233	0.153
A	T	3	120	3.67	97.02	1.33	0.196	0.000	0.003	0.521	0.267	4.191	0.206
A	U	1	120	3.69	97.70	1.63	0.171	0.000	0.004	0.499	0.220	3.173	0.151
A	U	2	120	3.74	98.51	1.81	0.223	0.000	0.002	0.459	0.270	3.860	0.195
A	U	3	120	3.79	98.41	2.61	0.259	0.000	0.002	0.591	0.153	3.430	0.181
A	W	1	120	3.72	97.54	2.28	0.191	0.000	0.003	0.491	0.244	3.265	0.157

Table C-

Mc	Inc
A	W
A	W
A	X
A	X
A	X
B	S
B	S
B	S
B	S
B	T
B	T
B	T
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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
A	W	2	120	3.77	98.08	3.08	0.212	0.000	0.005	0.444	0.284	3.368	0.159
A	W	3	120	3.66	95.75	3.57	0.192	0.002	0.002	0.530	0.256	4.425	0.217
A	X	1	120	3.78	96.65	3.56	0.220	0.000	0.001	0.462	0.298	3.400	0.169
A	X	2	120	3.78	97.35	3.06	0.249	0.002	0.005	0.540	0.310	3.410	0.158
A	X	3	120	3.69	95.85	2.76	0.186	0.000	0.002	0.512	0.247	4.041	0.197
B	S	1	120	4.11	95.78	0.64	0.333	0.306	0.000	0.734	0.396	2.401	0.354
B	S	2	120	4.06	97.56	0.00	0.278	0.301	0.000	0.796	0.393	2.484	0.323
B	S	3	120	4.17	94.70	0.00	0.292	0.328	0.000	0.887	0.434	2.244	0.345
B	S	4	120	4.12	95.66	0.00	0.339	0.348	0.000	0.984	0.412	2.130	0.326
B	T	1	120	4.07	95.76	0.94	0.346	0.187	0.000	0.713	0.306	2.136	0.307
B	T	2	120	4.05	95.15	0.00	0.241	0.211	0.000	0.833	0.381	2.271	0.303
B	T	3	120	4.05	95.65	0.00	0.295	0.237	0.000	1.012	0.397	2.339	0.303
B	T	4	120	4.22	95.57	0.00	0.315	0.288	0.000	0.948	0.363	1.776	0.297
B	U	1	120	4.04	96.00	0.00	0.330	0.268	0.000	0.883	0.388	2.510	0.341
B	U	2	120	3.94	96.18	0.34	0.354	0.210	0.000	0.908	0.393	2.951	0.300
B	U	3	120	4.06	95.30	0.00	0.246	0.281	0.000	0.916	0.398	2.425	0.315
B	U	4	120	4.13	95.28	0.00	0.364	0.316	0.000	0.961	0.407	2.233	0.312
B	W	1	120	3.99	96.84	0.00	0.182	0.136	0.000	0.744	0.315	2.181	0.245
B	W	2	120	4.13	96.63	0.00	0.322	0.266	0.000	0.905	0.379	2.076	0.298
B	W	3	120	4.00	95.90	0.00	0.338	0.192	0.000	0.906	0.373	2.381	0.304
B	W	4	120	3.96	96.14	0.00	0.326	0.145	0.000	0.890	0.354	2.624	0.297
B	X	1	120	4.04	95.70	0.00	0.293	0.320	0.000	0.929	0.413	2.724	0.335
B	X	2	120	3.97	96.54	0.00	0.317	0.224	0.000	0.873	0.422	2.966	0.312
B	X	3	120	4.11	94.95	0.00	0.267	0.241	0.000	0.631	0.364	2.143	0.302
B	X	4	120	4.07	90.90	0.00	0.262	0.263	0.000	1.000	0.377	2.288	0.288
C	S	1	120	4.18	96.09	3.84	0.123	0.162	0.000	0.849	0.287	1.640	0.230
C	S	2	120	4.36	96.21	0.97	0.087	0.072	0.002	0.773	0.122	1.051	0.030
C	S	3	120	4.19	95.83	0.00	0.127	0.184	0.000	0.844	0.264	1.930	0.158
C	S	4	120	4.23	96.75	4.35	0.168	0.090	0.005	0.745	0.324	1.516	0.024
C	T	1	120	4.08	97.41	2.79	0.298	0.023	0.000	0.787	0.251	1.658	0.370
C	T	2	120	4.22	97.18	1.22	0.292	0.000	0.007	0.607	0.373	1.106	0.314
C	T	3	120	4.11	96.60	0.85	0.256	0.018	0.000	0.635	0.255	1.318	0.315
C	T	4	120	4.24	96.32	0.67	0.270	0.015	0.006	0.665	0.160	1.396	0.270
C	U	1	120	4.06	96.85	0.85	0.350	0.000	0.000	0.670	0.235	1.401	0.445
C	U	2	120	4.04	96.36	0.97	0.268	0.000	0.002	0.516	0.191	1.378	0.456
C	U	3	120	4.08	96.94	0.32	0.307	0.000	0.000	0.628	0.211	1.195	0.416
C	U	4	120	4.16	96.45	1.57	0.247	0.004	0.002	0.380	0.162	1.143	0.315
C	W	1	120	3.97	96.32	2.04	0.281	0.000	0.002	0.523	0.284	1.816	0.390
C	W	2	120	4.33	97.35	1.61	0.380	0.000	0.000	0.603	0.187	1.591	0.290

Table C

Mc	In
C	W
C	W
C	X
C	X
C	X
C	X
C	S
C	S
C	S
C	S
C	1
C	1
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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
C	W	3	120	4.03	96.90	0.00	0.226	0.000	0.000	0.567	0.138	1.367	0.285
C	W	4	120	4.22	97.21	0.00	0.393	0.000	0.000	0.505	0.191	1.602	0.231
C	X	1	120	4.17	96.84	0.00	0.192	0.137	0.000	0.821	0.206	1.465	0.183
C	X	2	120	4.27	97.99	0.38	0.128	0.193	0.001	0.887	0.210	1.365	0.156
C	X	3	120	4.19	96.52	0.00	0.215	0.122	0.000	0.807	0.210	1.373	0.164
C	X	4	120	4.18	95.67	1.36	0.216	0.095	0.000	0.857	0.202	1.435	0.158
C	S	5	120	4.29	96.72	1.07	0.448	0.150	0.000	0.694	0.144	0.736	0.111
C	S	6	120	4.23	98.16	3.07	0.356	0.079	0.000	0.554	0.112	0.751	0.069
C	S	7	120	4.22	97.06	0.68	0.544	0.076	0.000	0.717	0.150	1.055	0.109
C	S	8	120	4.18	97.37	3.61	0.356	0.012	0.001	0.846	0.124	1.279	0.088
C	T	5	120	4.17	97.12	1.82	0.446	0.102	0.000	0.444	0.118	0.666	0.183
C	T	6	120	4.23	97.81	0.98	0.623	0.007	0.000	0.522	0.134	0.713	0.232
C	T	7	120	4.18	97.15	2.74	0.589	0.065	0.000	0.297	0.136	0.960	0.212
C	T	8	120	4.18	97.81	1.94	0.535	0.014	0.000	0.384	0.146	1.146	0.210
C	U	5	120	4.17	97.48	0.00	0.518	0.024	0.001	0.402	0.114	0.680	0.223
C	U	6	120	4.18	98.26	1.49	0.675	0.019	0.000	0.393	0.153	0.895	0.369
C	U	7	120	4.19	97.13	0.00	0.706	0.013	0.000	0.393	0.151	0.860	0.271
C	U	8	120	4.20	98.39	1.75	0.786	0.013	0.000	0.539	0.123	0.781	0.253
C	W	5	120	4.22	97.87	0.38	0.731	0.012	0.000	0.651	0.132	1.120	0.305
C	W	6	120	4.17	97.66	3.13	0.448	0.038	0.000	0.210	0.123	0.963	0.223
C	W	7	120	4.17	98.20	0.00	0.609	0.011	0.000	0.563	0.122	1.024	0.244
C	W	8	120	4.20	99.13	1.07	0.737	0.013	0.000	0.595	0.128	1.024	0.295
C	X	5	120	4.27	98.13	2.09	0.515	0.116	0.000	0.764	0.167	0.993	0.111
C	X	6	120	4.26	98.68	2.89	0.218	0.103	0.000	0.550	0.117	0.853	0.088
C	X	7	120	4.18	97.35	4.95	0.105	0.032	0.001	0.092	0.108	0.506	0.062
C	X	8	120	4.22	97.58	2.00	0.735	0.007	0.000	0.606	0.178	0.925	0.106
E	S	1	120	4.38	98.03	0.39	0.155	0.061	0.009	0.444	0.117	0.584	0.114
E	S	2	120	4.48	96.68	3.76	0.266	0.116	0.000	0.225	0.034	0.510	0.083
E	S	3	120	4.46	97.54	0.40	0.202	0.123	0.000	0.371	0.022	0.476	0.060
E	S	4	120	4.39	98.11	0.00	0.325	0.101	0.004	0.618	0.044	0.934	0.109
E	T	1	120	4.42	97.34	2.73	0.240	0.008	0.007	0.482	0.099	0.234	0.177
E	T	2	120	4.41	96.91	0.00	0.403	0.109	0.005	0.474	0.037	0.810	0.150
E	T	3	120	4.40	96.95	0.00	0.319	0.078	0.001	0.419	0.032	0.330	0.125
E	T	4	120	4.38	97.75	2.84	0.424	0.068	0.002	0.270	0.030	0.970	0.157
E	U	1	120	4.33	97.84	0.00	0.554	0.032	0.011	0.545	0.125	0.433	0.294
E	U	2	120	4.32	97.62	0.00	0.697	0.067	0.005	0.497	0.056	0.559	0.240
E	U	3	120	4.38	96.98	0.00	0.293	0.011	0.017	0.596	0.188	0.463	0.362
E	U	4	120	4.30	96.91	0.69	0.306	0.014	0.003	0.287	0.034	0.481	0.149
E	W	1	120	4.25	97.66	0.00	0.315	0.028	0.001	0.215	0.020	0.876	0.144
E	W	2	120	4.24	97.53	0.00	0.439	0.035	0.001	0.344	0.021	1.214	0.158

Table

Mc
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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
E	W	3	120	4.28	97.69	1.70	0.398	0.028	0.005	0.349	0.042	0.866	0.210
E	W	4	120	4.32	97.92	0.40	0.433	0.025	0.002	0.387	0.031	0.596	0.171
E	X	1	120	4.36	97.39	0.00	0.366	0.136	0.009	0.706	0.101	1.053	0.160
E	X	2	120	4.41	97.40	0.00	0.297	0.176	0.004	0.548	0.049	0.814	0.124
E	X	3	120	4.40	97.07	0.00	0.165	0.077	0.009	0.539	0.121	0.795	0.139
E	X	4	120	4.41	97.49	1.00	0.195	0.107	0.012	0.394	0.077	0.864	0.129
F	S	1	120	4.17	99.03	2.68	0.177	0.000	0.006	0.194	0.059	1.142	0.057
F	S	2	120	4.27	99.51	2.90	0.197	0.010	0.010	0.251	0.091	0.950	0.082
F	S	3	120	4.32	99.25	3.58	0.209	0.005	0.016	0.166	0.123	0.706	0.030
F	S	4	120	4.31	98.64	0.58	0.213	0.000	0.012	0.000	0.074	0.875	0.084
F	T	1	120	4.20	99.57	1.66	0.219	0.002	0.010	0.327	0.067	1.213	0.142
F	T	2	120	4.43	98.77	2.47	0.246	0.002	0.013	0.194	0.103	0.453	0.164
F	T	3	120	4.36	99.12	0.00	0.255	0.002	0.013	0.289	0.110	0.405	0.142
F	T	4	120	4.31	98.18	2.77	0.168	0.012	0.006	0.221	0.042	0.531	0.108
F	U	1	120	4.25	98.83	0.00	0.347	0.000	0.008	0.436	0.058	0.838	0.197
F	U	2	120	4.30	97.28	0.00	0.342	0.007	0.009	0.320	0.058	0.732	0.241
F	U	3	120	4.36	98.79	0.00	0.343	0.000	0.011	0.256	0.086	0.178	0.139
F	U	4	120	4.29	98.43	2.14	0.266	0.002	0.005	0.360	0.038	0.508	0.162
F	W	1	120	4.35	98.59	0.00	0.356	0.000	0.012	0.223	0.104	0.402	0.151
F	W	2	120	4.34	98.91	1.58	0.274	0.002	0.026	0.320	0.122	0.456	0.206
F	W	3	120	4.36	99.09	1.82	0.255	0.002	0.016	0.195	0.110	0.405	0.228
F	W	4	120	4.29	98.53	0.00	0.276	0.002	0.011	0.303	0.075	0.383	0.158
F	X	1	120	4.31	97.04	0.00	0.214	0.057	0.011	0.443	0.076	0.966	0.085
F	X	2	120	4.29	98.87	3.26	0.239	0.017	0.006	0.340	0.057	0.848	0.109
F	X	3	120	4.34	98.67	2.54	0.134	0.013	0.005	0.348	0.031	0.737	0.067
F	X	4	120	4.29	98.30	0.00	0.151	0.043	0.010	0.562	0.055	0.971	0.096
G	S	1	120	4.71	98.20	2.59	0.093	0.002	0.016	0.536	0.044	0.372	0.027
G	S	2	120	4.62	97.38	2.76	0.112	0.000	0.019	0.602	0.068	0.514	0.024
G	S	3	120	4.69	97.93	3.36	0.138	0.000	0.018	0.529	0.062	0.492	0.036
G	T	1	120	4.56	98.19	1.37	0.114	0.000	0.015	0.225	0.081	0.285	0.108
G	T	2	120	4.61	98.62	2.19	0.166	0.000	0.020	0.589	0.092	0.306	0.150
G	T	3	120	4.54	97.35	0.00	0.219	0.000	0.023	0.502	0.147	0.330	0.203
G	U	1	120	4.57	98.94	0.00	0.114	0.000	0.012	0.379	0.062	0.140	0.167
G	U	2	120	4.53	98.58	0.00	0.120	0.000	0.012	0.385	0.090	0.205	0.157
G	U	3	120	4.57	98.10	0.00	0.182	0.000	0.019	0.342	0.102	0.194	0.267
G	W	1	120	4.55	98.29	0.00	0.108	0.000	0.010	0.302	0.066	0.200	0.149
G	W	2	120	4.54	97.98	1.28	0.168	0.000	0.018	0.509	0.162	0.341	0.198
G	W	3	120	4.55	97.48	1.49	0.205	0.000	0.017	0.261	0.104	0.340	0.186
G	X	1	120	4.69	97.14	0.00	0.106	0.000	0.018	0.546	0.045	0.415	0.008
G	X	2	120	4.68	99.13	2.97	0.109	0.000	0.024	0.689	0.048	0.656	0.018
G	X	3	120	4.64	98.62	2.19	0.138	0.001	0.027	0.530	0.070	0.560	0.027

Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
A	S	1	121	3.66	103.31	3.29	0.226	0.000	0.006	0.487	0.301	3.979	0.200
A	S	2	121	3.68	100.59	4.80	0.216	0.000	0.005	0.397	0.260	3.835	0.185
A	S	3	121	3.72	100.39	2.93	0.206	0.000	0.005	0.489	0.255	3.564	0.176
A	T	1	121	3.77	102.48	4.03	0.260	0.000	0.002	0.544	0.105	3.295	0.170
A	T	2	121	3.73	99.63	3.60	0.214	0.002	0.002	0.537	0.273	3.521	0.176
A	T	3	121	3.66	101.82	0.00	0.197	0.000	0.002	0.553	0.267	4.236	0.215
A	U	1	121	3.68	102.75	2.93	0.194	0.000	0.004	0.477	0.246	3.566	0.183
A	U	2	121	3.69	102.35	2.79	0.180	0.000	0.002	0.452	0.243	3.382	0.175
A	U	3	121	3.78	99.58	3.77	0.250	0.000	0.002	0.501	0.132	3.179	0.168
A	W	1	121	3.70	99.95	3.19	0.221	0.004	0.002	0.489	0.269	3.741	0.183
A	W	2	121	3.75	100.96	3.96	0.203	0.000	0.000	0.460	0.254	3.100	0.151
A	W	3	121	3.67	100.51	2.89	0.191	0.000	0.003	0.490	0.231	4.049	0.193
A	X	1	121	3.81	99.45	4.81	0.059	0.002	0.004	0.045	0.248	2.513	0.168
A	X	2	121	3.78	94.88	4.22	0.234	0.000	0.002	0.501	0.272	3.321	0.157
A	X	3	121	3.68	102.91	3.61	0.174	0.000	0.002	0.518	0.237	3.913	0.189
B	S	1	121	4.11	99.57	0.00	0.251	0.232	0.000	0.657	0.327	1.946	0.289
B	S	2	121	4.09	99.16	0.00	0.289	0.301	0.000	0.854	0.403	2.441	0.335
B	S	3	121	4.16	100.20	0.00	0.290	0.304	0.000	0.834	0.424	2.174	0.339
B	S	4	121	4.18	99.52	0.64	0.334	0.325	0.000	0.890	0.405	2.061	0.317
B	T	1	121	4.11	100.77	0.00	0.432	0.208	0.000	0.752	0.362	2.359	0.377
B	T	2	121	4.05	101.10	1.60	0.239	0.221	0.000	0.809	0.393	2.365	0.307
B	T	3	121	4.04	100.16	0.00	0.335	0.254	0.000	1.028	0.424	2.494	0.334
B	T	4	121	4.23	99.68	0.00	0.338	0.266	0.000	0.834	0.347	1.609	0.291
B	U	1	121	4.09	100.80	0.00	0.401	0.267	0.000	0.702	0.391	2.414	0.359
B	U	2	121	3.97	100.79	0.00	0.318	0.223	0.000	0.830	0.390	2.894	0.329
B	U	3	121	4.12	101.30	0.00	0.296	0.280	0.000	0.767	0.427	2.608	0.344
B	U	4	121	4.12	100.49	0.00	0.379	0.308	0.000	0.875	0.406	2.205	0.316
B	W	1	121	4.00	100.82	0.00	0.161	0.123	0.000	0.639	0.297	2.124	0.232
B	W	2	121	4.12	100.15	0.34	0.335	0.285	0.000	0.853	0.406	2.208	0.327
B	W	3	121	4.02	101.00	0.00	0.339	0.176	0.000	0.798	0.374	2.309	0.302
B	W	4	121	3.99	99.04	0.00	0.346	0.168	0.000	0.871	0.388	2.752	0.343
B	X	1	121	4.06	102.11	0.00	0.312	0.303	0.000	0.709	0.400	2.565	0.335
B	X	2	121	4.01	100.88	0.00	0.379	0.224	0.000	0.720	0.408	2.755	0.323
B	X	3	121	4.12	100.61	0.00	0.259	0.235	0.000	0.691	0.351	2.029	0.300
B	X	4	121	4.10	100.79	0.00	0.277	0.284	0.000	0.951	0.409	2.396	0.318
C	S	1	121	4.22	100.17	1.28	0.129	0.158	0.000	0.627	0.272	1.545	0.229
C	S	2	121	4.35	101.95	3.59	0.141	0.074	0.005	0.463	0.150	1.258	0.071
C	S	3	121	4.21	103.52	0.00	0.093	0.153	0.000	0.466	0.235	1.727	0.155
C	S	4	121	4.24	101.48	3.49	0.139	0.080	0.005	0.565	0.218	1.444	0.147
C	T	1	121	4.07	101.00	1.34	0.269	0.020	0.000	0.537	0.224	1.550	0.335

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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
C	T	2	121	4.22	100.81	0.38	0.263	0.015	0.003	0.439	0.343	0.981	0.296
C	T	3	121	4.12	101.81	0.00	0.234	0.000	0.000	0.427	0.239	1.282	0.310
C	T	4	121	4.25	102.25	0.34	0.261	0.015	0.006	0.413	0.271	1.247	0.250
C	U	1	121	4.07	101.80	0.99	0.292	0.000	0.000	0.479	0.215	1.273	0.422
C	U	2	121	4.06	102.01	0.86	0.242	0.000	0.002	0.320	0.180	1.229	0.399
C	U	3	121	4.09	100.61	0.98	0.308	0.000	0.000	0.457	0.211	1.123	0.400
C	U	4	121	4.16	102.28	0.00	0.239	0.000	0.002	0.301	0.262	1.155	0.363
C	W	1	121	3.98	104.30	1.59	0.247	0.000	0.000	0.308	0.273	1.596	0.329
C	W	2	121	4.35	100.10	0.68	0.388	0.000	0.000	0.375	0.192	1.458	0.257
C	W	3	121	4.06	101.38	0.68	0.257	0.000	0.001	0.381	0.209	1.293	0.265
C	W	4	121	4.24	102.00	0.00	0.312	0.000	0.000	0.345	0.183	1.631	0.284
C	X	1	121	4.20	101.61	0.68	0.153	0.137	0.000	0.647	0.206	1.488	0.185
C	X	2	121	4.29	100.57	0.38	0.109	0.193	0.000	0.629	0.199	1.246	0.138
C	X	3	121	4.19	101.89	0.00	0.129	0.109	0.000	0.497	0.174	1.217	0.146
C	X	4	121	4.21	102.52	0.86	0.217	0.047	0.000	0.732	0.223	1.622	0.173
C	S	5	121	4.28	100.58	0.00	0.131	0.114	0.001	0.588	0.183	0.978	0.085
C	S	6	121	4.23	101.65	0.00	0.157	0.001	0.001	0.590	0.092	1.108	0.077
C	S	7	121	4.20	100.47	0.38	0.181	0.000	0.000	0.563	0.142	1.071	0.071
C	S	8	121	4.17	101.62	3.59	0.103	0.000	0.000	0.552	0.061	1.110	0.053
C	T	5	121	4.20	100.76	0.38	0.227	0.039	0.000	0.239	0.116	0.897	0.162
C	T	6	121	4.21	100.99	0.86	0.261	0.000	0.003	0.340	0.131	0.859	0.190
C	T	7	121	4.15	100.03	1.64	0.263	0.000	0.001	0.430	0.078	1.144	0.144
C	T	8	121	4.16	100.41	1.49	0.211	0.000	0.001	0.267	0.086	1.017	0.150
C	U	5	121	4.16	101.08	0.00	0.250	0.000	0.002	0.314	0.082	0.913	0.208
C	U	6	121	4.17	101.49	0.00	0.210	0.000	0.003	0.348	0.139	1.038	0.253
C	U	7	121	4.17	102.58	0.00	0.231	0.000	0.000	0.265	0.066	0.986	0.179
C	U	8	121	4.18	101.04	2.19	0.297	0.005	0.001	0.411	0.072	0.911	0.191
C	W	5	121	4.16	101.65	1.59	0.257	0.015	0.001	0.231	0.090	0.982	0.171
C	W	6	121	4.22	102.69	0.00	0.252	0.000	0.005	0.396	0.211	0.708	0.206
C	W	7	121	4.16	100.81	0.38	0.192	0.000	0.001	0.359	0.062	0.870	0.143
C	W	8	121	4.19	101.67	2.21	0.255	0.002	0.001	0.366	0.161	0.918	0.199
C	X	5	121	4.28	100.46	0.00	0.133	0.076	0.001	0.560	0.086	1.080	0.089
C	X	6	121	4.27	100.40	2.23	0.110	0.079	0.002	0.600	0.062	0.963	0.067
C	X	7	121	4.17	101.05	4.38	0.176	0.015	0.000	0.368	0.130	1.068	0.066
C	X	8	121	4.20	102.21	2.23	0.240	0.013	0.001	0.399	0.173	1.223	0.071
E	S	1	121	4.39	100.33	0.00	0.122	0.043	0.003	0.308	0.095	0.493	0.089
E	S	2	121	4.48	100.82	2.62	0.263	0.116	0.000	0.212	0.019	0.498	0.069
E	S	3	121	4.44	101.13	0.88	0.296	0.178	0.000	0.427	0.024	0.601	0.096
E	S	4	121	4.38	100.96	0.00	0.233	0.071	0.003	0.424	0.027	0.642	0.066
E	T	1	121	4.42	100.82	3.34	0.258	0.013	0.012	0.420	0.131	0.254	0.201

Table C- 1 (c

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Table C- 1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
E	T	2	121	4.39	100.74	0.00	0.365	0.099	0.003	0.393	0.024	0.705	0.124
E	T	3	121	4.41	100.71	0.00	0.407	0.117	0.004	0.486	0.039	0.469	0.168
E	T	4	121	4.37	101.43	1.73	0.326	0.066	0.000	0.236	0.021	0.877	0.126
E	U	1	121	4.33	100.77	0.00	0.440	0.031	0.010	0.400	0.082	0.337	0.241
E	U	2	121	4.31	100.22	0.00	0.388	0.044	0.003	0.372	0.022	0.443	0.164
E	U	3	121	4.39	79.79	0.00	0.267	0.016	0.010	0.413	0.145	0.409	0.319
E	U	4	121	4.30	101.74	0.00	0.474	0.031	0.007	0.359	0.057	0.748	0.230
E	W	1	121	4.25	99.53	0.40	0.293	0.029	0.000	0.198	0.016	0.825	0.134
E	W	2	121	4.20	101.67	0.00	0.375	0.043	0.004	0.323	0.014	1.131	0.147
E	W	3	121	4.29	100.34	1.00	0.391	0.025	0.004	0.296	0.035	0.816	0.189
E	W	4	121	4.31	102.37	0.71	0.337	0.022	0.000	0.236	0.023	0.437	0.125
E	X	1	121	4.36	101.31	0.00	0.236	0.098	0.006	0.440	0.065	0.674	0.115
E	X	2	121	4.41	101.26	0.00	0.267	0.161	0.004	0.428	0.038	0.698	0.101
E	X	3	121	4.38	100.48	0.70	0.139	0.057	0.009	0.345	0.077	0.637	0.098
E	X	4	121	4.37	100.58	0.70	0.189	0.117	0.013	0.325	0.077	0.793	0.130
F	S	1	121	4.15	99.61	3.37	0.115	0.000	0.004	0.217	0.033	0.787	0.038
F	S	2	121	4.27	99.02	2.40	0.132	0.014	0.006	0.290	0.055	0.644	0.053
F	S	3	121	4.30	100.50	3.89	0.189	0.000	0.014	0.229	0.124	0.641	0.035
F	S	4	121	4.30	100.86	1.06	0.197	0.019	0.009	0.283	0.071	0.864	0.085
F	T	1	121	4.20	101.77	0.00	0.165	0.000	0.005	0.158	0.049	1.001	0.112
F	T	2	121	4.39	100.11	3.01	0.203	0.000	0.009	0.088	0.077	0.345	0.127
F	T	3	121	4.35	100.79	0.00	0.218	0.000	0.013	0.189	0.115	0.451	0.173
F	T	4	121	4.30	100.48	2.32	0.233	0.016	0.009	0.215	0.065	0.769	0.164
F	U	1	121	4.23	100.81	0.00	0.302	0.000	0.005	0.226	0.039	0.733	0.166
F	U	2	121	4.30	101.34	0.00	0.212	0.000	0.005	0.140	0.040	0.458	0.148
F	U	3	121	4.36	99.72	0.00	0.346	0.000	0.011	0.234	0.105	0.216	0.183
F	U	4	121	4.29	99.99	1.48	0.334	0.000	0.007	0.358	0.052	0.708	0.220
F	W	1	121	4.33	100.36	0.00	0.202	0.000	0.006	0.128	0.063	0.315	0.135
F	W	2	121	4.34	100.14	0.00	0.218	0.000	0.008	0.209	0.079	0.364	0.154
F	W	3	121	4.38	100.16	1.06	0.213	0.000	0.010	0.143	0.091	0.357	0.198
F	W	4	121	4.28	100.49	0.00	0.344	0.000	0.010	0.219	0.080	0.496	0.181
F	X	1	121	4.32	101.53	0.00	0.126	0.027	0.005	0.199	0.023	0.626	0.055
F	X	2	121	4.31	99.54	0.88	0.178	0.020	0.005	0.241	0.045	0.690	0.088
F	X	3	121	4.32	100.18	1.19	0.169	0.024	0.008	0.328	0.063	0.902	0.089
F	X	4	121	4.32	99.98	0.00	0.171	0.054	0.007	0.447	0.057	1.073	0.110
G	S	1	121	4.72	98.84	3.07	0.132	0.000	0.023	0.484	0.089	0.549	0.040
G	S	2	121	4.60	100.11	2.08	0.086	0.001	0.014	0.165	0.039	0.424	0.009
G	S	3	121	4.67	98.81	3.76	0.102	0.000	0.018	0.342	0.018	0.404	0.017
G	T	1	121	4.59	101.00	0.00	0.170	0.000	0.023	0.337	0.104	0.400	0.156
G	T	2	121	4.57	100.86	2.72	0.130	0.000	0.017	0.353	0.086	0.362	0.133
G	T	3	121	4.48	99.96	0.00	0.160	0.000	0.011	0.243	0.102	0.274	0.147

Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
G	U	1	121	4.63	100.60	0.00	0.224	0.000	0.024	0.335	0.135	0.506	0.231
G	U	2	121	4.53	101.82	0.00	0.145	0.000	0.017	0.265	0.019	0.426	0.184
G	U	3	121	4.55	99.60	0.00	0.141	0.000	0.018	0.189	0.000	0.369	0.199
G	W	1	121	4.53	101.85	0.00	0.179	0.000	0.013	0.248	0.000	0.598	0.195
G	W	2	121	4.55	101.50	0.00	0.244	0.000	0.012	0.322	0.000	0.810	0.192
G	W	3	121	4.49	133.93	0.89	0.161	0.000	0.014	0.253	0.000	0.381	0.130
G	X	1	121	4.70	100.26	0.00	0.187	0.000	0.012	0.443	0.000	0.725	0.036
G	X	2	121	4.64	100.37	0.89	0.135	0.004	0.011	0.443	0.000	0.652	0.013
G	X	3	121	4.62	100.86	1.20	0.126	0.000	0.006	0.217	0.000	0.463	0.019
A	S	1	123	3.69	99.37	5.89	0.211	0.000	0.006	0.364	0.305	3.937	0.191
A	S	2	123	3.76	100.28	7.47	0.028	0.000	0.004	0.135	0.218	3.216	0.166
A	S	3	123	3.72	99.03	5.84	0.182	0.000	0.002	0.343	0.241	3.381	0.159
A	T	1	123	3.80	101.68	6.39	0.213	0.002	0.005	0.326	0.108	3.239	0.157
A	T	2	123	3.74	102.35	6.07	0.207	0.002	0.002	0.359	0.263	3.307	0.155
A	T	3	123	3.68	100.43	4.42	0.176	0.001	0.003	0.356	0.251	3.806	0.184
A	U	1	123	3.71	100.69	5.84	0.213	0.002	0.005	0.433	0.267	3.705	0.176
A	U	2	123	3.72	99.87	5.58	0.200	0.000	0.002	0.330	0.257	3.603	0.168
A	U	3	123	3.82	100.52	6.14	0.210	0.000	0.002	0.225	0.118	2.854	0.134
A	W	1	123	3.72	99.79	5.75	0.212	0.002	0.002	0.343	0.288	3.771	0.185
A	W	2	123	3.78	100.00	6.38	0.154	0.000	0.002	0.310	0.232	2.813	0.121
A	W	3	123	3.70	99.90	5.08	0.168	0.001	0.002	0.309	0.207	3.560	0.166
A	X	1	123	6.95	101.44	8.08	0.000	0.002	0.002	0.000	0.190	0.272	0.147
A	X	2	123	3.80	101.91	6.43	0.195	0.000	0.003	0.285	0.261	3.131	0.143
A	X	3	123	3.72	99.26	5.00	0.290	0.003	0.002	0.301	0.211	3.478	0.164
B	S	1	123	4.10	97.91	0.34	0.299	0.242	0.000	0.559	0.355	2.029	0.313
B	S	2	123	4.07	98.67	0.82	0.271	0.273	0.000	0.752	0.384	2.353	0.324
B	S	3	123	4.16	100.67	0.95	0.284	0.302	0.000	0.572	0.416	2.113	0.328
B	S	4	123	4.18	99.41	0.82	0.364	0.321	0.000	0.698	0.410	2.082	0.315
B	T	1	123	4.08	98.03	0.94	0.388	0.185	0.000	0.565	0.321	2.119	0.332
B	T	2	123	4.05	99.35	0.35	0.229	0.193	0.000	0.649	0.356	2.149	0.288
B	T	3	123	4.04	99.34	2.23	0.308	0.226	0.000	0.739	0.395	2.273	0.313
B	T	4	123	4.23	100.17	0.00	0.358	0.271	0.000	0.715	0.356	1.630	0.299
B	U	1	123	4.07	98.11	0.34	0.401	0.253	0.000	0.618	0.394	2.411	0.357
B	U	2	123	3.97	98.53	0.64	0.301	0.189	0.000	0.695	0.341	2.544	0.293
B	U	3	123	4.04	98.77	0.64	0.286	0.254	0.000	0.677	0.401	2.395	0.320
B	U	4	123	4.12	99.61	0.34	0.390	0.303	0.000	0.733	0.406	2.180	0.314
B	W	1	123	3.96	97.67	0.00	0.207	0.147	0.000	0.671	0.369	2.579	0.287
B	W	2	123	4.10	99.44	0.95	0.344	0.270	0.000	0.761	0.404	2.202	0.322
B	W	3	123	4.01	98.73	0.34	0.384	0.189	0.000	0.759	0.402	2.448	0.332
B	W	4	123	3.99	101.48	0.00	0.290	0.118	0.000	0.589	0.315	2.200	0.273
B	X	1	123	4.04	97.04	0.00	0.297	0.260	0.000	0.733	0.362	2.326	0.311

Table C

Mc	In
B	X
B	X
B	X
C	S
C	S
C	S
C	S
C	T
C	T
C	T
C	T
C	
C	
C

Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
B	X	2	123	4.00	99.16	1.85	0.368	0.217	0.000	0.705	0.412	2.769	0.322
B	X	3	123	4.11	99.42	0.34	0.270	0.253	0.000	0.633	0.395	2.247	0.328
B	X	4	123	4.09	99.53	0.00	0.251	0.224	0.000	0.649	0.353	2.013	0.276
C	S	1	123	4.24	98.74	0.38	0.247	0.157	0.001	0.478	0.185	1.094	0.191
C	S	2	123	4.36	96.84	3.85	0.105	0.101	0.003	0.176	0.176	0.698	0.090
C	S	3	123	4.22	95.65	0.98	0.199	0.208	0.002	0.546	0.237	1.890	0.190
C	S	4	123	4.27	97.51	3.93	0.360	0.115	0.003	0.528	0.261	1.548	0.132
C	T	1	123	4.12	97.74	1.16	0.314	0.030	0.000	0.478	0.158	1.253	0.315
C	T	2	123	4.23	97.14	0.00	0.489	0.040	0.003	0.395	0.213	1.389	0.327
C	T	3	123	4.12	96.43	0.32	0.394	0.029	0.000	0.555	0.195	1.469	0.390
C	T	4	123	4.25	98.27	0.00	0.459	0.024	0.003	0.430	0.194	1.301	0.269
C	U	1	123	4.08	96.42	0.85	0.325	0.008	0.000	0.388	0.126	0.922	0.343
C	U	2	123	4.07	96.49	0.98	0.278	0.008	0.003	0.352	0.119	0.968	0.299
C	U	3	123	4.09	99.07	0.38	0.450	0.016	0.000	0.460	0.173	1.206	0.455
C	U	4	123	4.18	97.42	0.38	0.503	0.019	0.002	0.330	0.204	1.408	0.401
C	W	1	123	4.00	95.79	1.22	0.354	0.023	0.001	0.297	0.145	1.048	0.261
C	W	2	123	4.35	95.62	0.68	0.621	0.010	0.003	0.471	0.208	1.130	0.291
C	W	3	123	4.05	96.90	0.37	0.472	0.024	0.001	0.468	0.190	1.617	0.352
C	W	4	123	4.26	96.89	0.68	0.608	0.014	0.002	0.476	0.195	1.264	0.324
C	X	1	123	4.20	98.71	1.23	0.397	0.150	0.001	0.433	0.171	0.685	0.157
C	X	2	123	4.28	96.39	0.37	0.402	0.121	0.002	0.642	0.209	0.809	0.150
C	X	3	123	4.21	96.92	0.00	0.365	0.156	0.001	0.536	0.186	0.903	0.170
C	X	4	123	4.22	96.74	0.37	0.484	0.115	0.002	0.620	0.215	1.039	0.187
C	S	5	123	4.30	97.43	1.72	0.125	0.109	0.001	0.323	0.120	0.841	0.065
C	S	6	123	4.28	97.13	3.93	0.140	0.055	0.000	0.425	0.133	1.032	0.068
C	S	7	123	4.27	98.03	1.50	0.179	0.022	0.000	0.332	0.117	1.028	0.064
C	S	8	123	4.25	99.02	3.63	0.060	0.013	0.001	0.205	0.081	0.987	0.053
C	T	5	123	4.23	98.57	0.00	0.201	0.061	0.001	0.258	0.120	0.773	0.134
C	T	6	123	4.27	99.70	1.47	0.253	0.000	0.002	0.271	0.158	0.975	0.186
C	T	7	123	4.19	99.71	1.16	0.233	0.014	0.001	0.250	0.146	1.081	0.133
C	T	8	123	4.22	85.98	3.58	0.290	0.004	0.000	0.291	0.042	0.936	0.187
C	U	5	123	4.20	98.71	0.99	0.249	0.010	0.000	0.179	0.103	1.002	0.205
C	U	6	123	4.21	98.23	0.00	0.236	0.017	0.003	0.235	0.114	1.113	0.221
C	U	7	123	4.21	98.11	1.29	0.247	0.011	0.000	0.138	0.104	1.082	0.178
C	U	8	123	4.20	97.73	3.24	0.233	0.006	0.001	0.143	0.115	1.102	0.146
C	W	5	123	4.20	97.69	1.16	0.250	0.015	0.000	0.131	0.090	0.882	0.148
C	W	6	123	4.22	98.03	0.38	0.353	0.007	0.001	0.323	0.108	0.960	0.211
C	W	7	123	4.20	98.81	1.96	0.216	0.000	0.001	0.293	0.114	1.017	0.159
C	W	8	123	4.22	95.88	1.55	0.265	0.003	0.001	0.280	0.090	1.026	0.184
C	X	5	123	4.30	96.82	2.73	0.219	0.062	0.001	0.383	0.101	0.758	0.070
C	X	6	123	4.31	98.04	4.21	0.213	0.078	0.000	0.389	0.117	1.009	0.084

Table

Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
C	X	7	123	4.20	97.13	5.74	0.137	0.038	0.000	0.173	0.090	0.958	0.065
C	X	8	123	4.22	97.99	4.41	0.236	0.024	0.000	0.217	0.102	0.897	0.062
E	S	1	123	4.39	99.25	0.00	0.160	0.067	0.008	0.280	0.116	0.678	0.127
E	S	2	123	4.49	99.91	1.92	0.310	0.137	0.000	0.155	0.021	0.606	0.089
E	S	3	123	4.43	97.99	0.40	0.300	0.166	0.002	0.305	0.030	0.586	0.091
E	S	4	123	4.37	99.69	0.00	0.306	0.094	0.005	0.408	0.045	0.848	0.103
E	T	1	123	4.41	97.84	2.85	0.300	0.024	0.011	0.396	0.169	0.321	0.220
E	T	2	123	4.40	101.30	0.00	0.348	0.094	0.002	0.291	0.023	0.663	0.125
E	T	3	123	4.39	99.36	0.88	0.402	0.121	0.004	0.373	0.044	0.505	0.177
E	T	4	123	4.36	97.44	0.70	0.360	0.070	0.003	0.225	0.026	0.926	0.140
E	U	1	123	4.31	98.74	0.00	0.467	0.034	0.010	0.344	0.096	0.370	0.247
E	U	2	123	4.30	99.01	0.00	0.539	0.071	0.004	0.354	0.037	0.592	0.222
E	U	3	123	4.38	98.88	0.00	0.293	0.027	0.012	0.378	0.198	0.447	0.346
E	U	4	123	4.30	100.05	1.36	0.439	0.030	0.006	0.262	0.055	0.668	0.200
E	W	1	123	4.24	98.34	0.70	0.296	0.039	0.000	0.145	0.016	0.881	0.144
E	W	2	123	4.20	98.13	1.76	0.421	0.048	0.003	0.263	0.015	1.196	0.155
E	W	3	123	4.27	98.03	0.70	0.414	0.019	0.005	0.217	0.035	0.829	0.191
E	W	4	123	4.30	96.23	0.88	0.467	0.035	0.000	0.342	0.031	0.612	0.191
E	X	1	123	4.36	98.42	0.00	0.205	0.083	0.005	0.329	0.059	0.555	0.081
E	X	2	123	4.41	98.40	0.00	0.278	0.172	0.004	0.388	0.048	0.757	0.113
E	X	3	123	4.38	99.58	0.00	0.173	0.078	0.008	0.364	0.105	0.807	0.134
E	X	4	123	4.41	99.26	1.55	0.185	0.099	0.007	0.249	0.066	0.805	0.108
F	S	1	123	4.18	100.66	3.54	0.139	0.000	0.005	0.186	0.045	0.917	0.046
F	S	2	123	4.27	100.43	3.13	0.126	0.000	0.005	0.241	0.053	0.612	0.050
F	S	3	123	4.35	99.69	4.82	0.189	0.003	0.016	0.172	0.133	0.649	0.034
F	S	4	123	4.31	99.87	1.49	0.216	0.007	0.010	0.239	0.085	0.999	0.097
F	T	1	123	4.21	98.67	1.63	0.152	0.000	0.005	0.146	0.049	0.848	0.096
F	T	2	123	4.43	100.21	3.23	0.174	0.000	0.010	0.083	0.077	0.284	0.112
F	T	3	123	4.38	99.09	0.59	0.237	0.000	0.014	0.194	0.117	0.473	0.176
F	T	4	123	4.35	100.95	2.01	0.244	0.006	0.010	0.156	0.067	0.827	0.166
F	U	1	123	4.25	99.95	1.37	0.256	0.000	0.005	0.154	0.040	0.617	0.143
F	U	2	123	4.32	99.88	1.59	0.180	0.000	0.005	0.101	0.039	0.386	0.129
F	U	3	123	4.38	101.00	3.03	0.368	0.000	0.015	0.196	0.117	0.254	0.209
F	U	4	123	4.33	100.86	0.00	0.386	0.000	0.012	0.216	0.090	0.571	0.189
F	W	1	123	4.31	99.38	1.29	0.223	0.000	0.008	0.115	0.077	0.339	0.148
F	W	2	123	4.34	99.01	0.59	0.269	0.000	0.017	0.190	0.118	0.473	0.198
F	W	3	123	4.38	101.20	1.59	0.222	0.000	0.013	0.104	0.126	0.385	0.219
F	W	4	123	4.31	98.76	0.00	0.357	0.002	0.009	0.265	0.064	0.839	0.236
F	X	1	123	4.33	99.84	1.37	0.052	0.003	0.014	0.046	0.014	0.246	0.018
F	X	2	123	4.31	100.99	0.00	0.180	0.018	0.006	0.165	0.051	0.710	0.091

Tab

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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
F	X	3	123	4.34	99.49	0.00	0.198	0.015	0.010	0.331	0.092	1.066	0.110
F	X	4	123	4.32	100.04	0.59	0.062	0.017	0.022	0.111	0.027	0.433	0.040
G	S	1	123	4.71	102.84	3.45	0.103	0.000	0.015	0.194	0.059	0.393	0.025
G	S	2	123	4.59	101.79	2.90	0.088	0.000	0.015	0.254	0.056	0.491	0.027
G	S	3	123	4.67	102.93	3.88	0.107	0.000	0.022	0.199	0.066	0.439	0.030
G	T	1	123	4.55	102.05	1.60	0.123	0.000	0.017	0.219	0.117	0.298	0.115
G	T	2	123	4.57	102.37	2.38	0.141	0.000	0.021	0.282	0.107	0.371	0.142
G	T	3	123	4.50	101.59	1.90	0.222	0.000	0.021	0.258	0.181	0.398	0.213
G	U	1	123	4.60	101.19	1.20	0.129	0.000	0.014	0.143	0.061	0.127	0.163
G	U	2	123	4.54	101.38	1.38	0.161	0.000	0.016	0.193	0.105	0.286	0.193
G	U	3	123	4.56	98.04	2.59	0.145	0.000	0.014	0.119	0.059	0.171	0.203
G	W	1	123	4.54	100.99	1.20	0.125	0.000	0.013	0.142	0.084	0.241	0.152
G	W	2	123	4.54	100.33	0.90	0.185	0.000	0.021	0.253	0.138	0.352	0.200
G	W	3	123	4.51	100.22	1.50	0.215	0.000	0.025	0.275	0.125	0.358	0.200
G	X	1	123	4.60	100.00	1.38	0.074	0.000	0.008	0.143	0.002	0.344	0.015
G	X	2	123	4.64	99.46	0.89	0.121	0.000	0.022	0.342	0.000	0.615	0.024
G	X	3	123	4.64	99.56	0.90	0.149	0.000	0.008	0.196	0.000	0.656	0.035
A	S	1	125	4.40	100.12	7.95	0.002	0.000	0.000	0.002	0.114	0.808	0.062
A	S	2	125	7.31	96.04	7.54	0.000	0.000	0.002	0.000	0.144	0.058	0.105
A	S	3	125	3.75	101.13	7.54	0.000	0.001	0.002	0.000	0.192	2.566	0.152
A	T	1	125	4.97	98.08	8.21	0.005	0.000	0.002	0.000	0.161	0.956	0.109
A	T	2	125	6.40	98.66	8.36	0.005	0.004	0.002	0.000	0.158	0.289	0.113
A	T	3	125	3.62	100.07	7.54	0.132	0.000	0.002	0.175	0.224	3.461	0.162
A	U	1	125	3.73	102.16	7.59	0.103	0.004	0.002	0.055	0.209	3.011	0.152
A	U	2	125	3.78	100.46	7.55	0.000	0.000	0.002	0.000	0.192	2.473	0.135
A	U	3	125	4.22	100.70	7.91	0.000	0.000	0.003	0.007	0.175	1.482	0.130
A	W	1	125	4.06	101.02	7.90	0.007	0.000	0.005	0.000	0.172	1.783	0.137
A	W	2	125	6.74	97.79	8.26	0.016	0.000	0.000	0.000	0.164	0.260	0.132
A	W	3	125	3.66	101.31	6.64	0.106	0.000	0.001	0.071	0.197	3.317	0.151
A	X	1	125	8.16	94.88	8.35	0.028	0.005	0.002	0.000	0.153	0.025	0.015
A	X	2	125	6.95	98.02	8.26	0.002	0.002	0.000	0.000	0.144	0.169	0.108
A	X	3	125	3.67	101.96	7.07	0.060	0.000	0.000	0.039	0.179	2.998	0.145
B	S	1	125	4.08	99.96	0.00	0.243	0.206	0.000	0.279	0.307	1.793	0.264
B	S	2	125	4.08	99.88	1.83	0.233	0.230	0.000	0.385	0.320	1.926	0.266
B	S	3	125	4.10	97.74	0.00	0.232	0.222	0.000	0.383	0.315	1.620	0.242
B	S	4	125	4.12	101.42	1.67	0.270	0.245	0.000	0.334	0.311	1.580	0.245
B	T	1	125	4.05	101.43	0.00	0.336	0.154	0.000	0.302	0.282	1.855	0.288
B	T	2	125	4.08	100.95	0.00	0.179	0.150	0.000	0.334	0.284	1.722	0.225
B	T	3	125	3.97	99.70	0.00	0.270	0.206	0.000	0.453	0.344	1.990	0.265
B	T	4	125	4.18	98.34	0.00	0.301	0.212	0.000	0.399	0.287	1.287	0.241

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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
B	U	1	125	4.04	100.16	0.00	0.338	0.212	0.000	0.320	0.326	1.958	0.291
B	U	2	125	3.96	101.13	0.00	0.250	0.166	0.000	0.347	0.316	2.263	0.250
B	U	3	125	3.98	99.24	0.00	0.217	0.201	0.000	0.307	0.319	1.893	0.246
B	U	4	125	4.09	101.94	0.00	0.296	0.218	0.000	0.340	0.309	1.611	0.233
B	W	1	125	3.93	102.56	0.00	0.155	0.116	0.000	0.268	0.299	2.080	0.235
B	W	2	125	4.11	101.50	0.00	0.256	0.213	0.000	0.349	0.305	1.660	0.238
B	W	3	125	3.96	99.97	0.00	0.294	0.163	0.000	0.352	0.321	1.964	0.255
B	W	4	125	4.04	99.70	0.00	0.250	0.115	0.000	0.323	0.286	1.969	0.245
B	X	1	125	4.00	102.37	0.00	0.254	0.239	0.000	0.328	0.347	2.213	0.282
B	X	2	125	4.02	100.55	1.36	0.313	0.179	0.000	0.317	0.349	2.311	0.264
B	X	3	125	4.07	100.19	1.36	0.237	0.228	0.000	0.290	0.344	1.936	0.280
B	X	4	125	3.95	99.16	0.00	0.233	0.212	0.000	0.389	0.326	1.855	0.253
C	S	1	125	4.25	102.52	0.40	0.114	0.131	0.000	0.197	0.198	0.885	0.165
C	S	2	125	4.60	103.55	4.66	0.010	0.073	0.007	0.000	0.062	0.596	0.076
C	S	3	125	4.28	100.35	0.00	0.120	0.141	0.000	0.200	0.194	1.313	0.131
C	S	4	125	4.23	102.12	2.18	0.178	0.148	0.011	0.366	0.279	1.516	0.178
C	T	1	125	4.12	99.97	0.69	0.229	0.051	0.001	0.256	0.179	1.172	0.285
C	T	2	125	4.19	101.21	3.27	0.170	0.018	0.005	0.124	0.231	0.647	0.195
C	T	3	125	4.16	101.55	0.69	0.202	0.025	0.004	0.241	0.081	1.103	0.275
C	T	4	125	4.27	99.78	1.67	0.153	0.027	0.005	0.192	0.230	0.745	0.186
C	U	1	125	4.11	102.02	0.00	0.219	0.029	0.002	0.126	0.063	0.968	0.307
C	U	2	125	4.08	101.89	1.99	0.174	0.019	0.003	0.179	0.113	0.864	0.284
C	U	3	125	4.14	101.84	0.00	0.246	0.010	0.002	0.222	0.067	0.881	0.317
C	U	4	125	4.20	99.83	0.87	0.179	0.009	0.005	0.114	0.207	0.845	0.277
C	W	1	125	4.03	101.05	0.98	0.160	0.013	0.002	0.085	0.090	1.029	0.224
C	W	2	125	4.37	100.93	1.88	0.188	0.009	0.006	0.175	0.285	0.540	0.193
C	W	3	125	4.08	102.27	0.87	0.222	0.035	0.001	0.175	0.073	1.125	0.245
C	W	4	125	4.28	101.61	0.40	0.213	0.011	0.008	0.196	0.213	0.951	0.229
C	X	1	125	4.22	99.31	1.23	0.104	0.107	0.000	0.186	0.172	0.792	0.127
C	X	2	125	4.30	102.09	1.81	0.055	0.177	0.001	0.326	0.212	0.942	0.117
C	X	3	125	4.25	100.81	0.39	0.126	0.143	0.001	0.202	0.239	1.255	0.174
C	X	4	125	4.24	103.05	0.00	0.102	0.033	0.000	0.194	0.185	0.939	0.121
C	S	5	125	4.31	102.97	0.00	0.136	0.136	0.003	0.116	0.057	0.886	0.082
C	S	6	125	4.26	100.08	1.36	0.144	0.090	0.004	0.202	0.080	1.015	0.069
C	S	7	125	4.24	102.71	0.42	0.156	0.039	0.000	0.006	0.041	0.979	0.059
C	S	8	125	6.28	96.99	1.19	0.002	0.015	0.005	0.000	0.023	0.289	0.038
C	T	5	125	4.23	99.55	2.91	0.292	0.113	0.004	0.089	0.079	1.050	0.210
C	T	6	125	4.26	97.87	0.41	0.256	0.034	0.004	0.141	0.109	0.831	0.194
C	T	7	125	4.20	98.30	0.42	0.229	0.035	0.000	0.086	0.058	0.982	0.132
C	T	8	125	4.18	100.24	1.37	0.171	0.004	0.000	0.003	0.062	0.806	0.115
C	U	5	125	4.19	104.23	0.70	0.264	0.015	0.003	0.009	0.074	0.831	0.210

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Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
C	U	6	125	4.18	99.46	1.59	0.220	0.020	0.004	0.094	0.121	0.950	0.251
C	U	7	125	4.19	101.30	0.00	0.231	0.009	0.002	0.030	0.082	0.908	0.181
C	U	8	125	4.20	101.51	0.00	0.246	0.005	0.000	0.090	0.054	0.724	0.159
C	W	5	125	4.18	100.27	0.71	0.275	0.033	0.002	0.037	0.083	0.973	0.179
C	W	6	125	4.24	99.64	1.73	0.305	0.014	0.012	0.216	0.234	0.826	0.258
C	W	7	125	4.18	101.24	0.00	0.209	0.021	0.006	0.100	0.070	0.895	0.166
C	W	8	125	4.22	103.60	0.71	0.218	0.001	0.002	0.065	0.137	0.802	0.175
C	X	5	125	4.29	102.68	1.52	0.130	0.088	0.003	0.096	0.068	0.975	0.083
C	X	6	125	4.36	101.13	0.00	0.051	0.104	0.009	0.000	0.091	1.140	0.095
C	X	7	125	5.05	100.80	0.71	0.009	0.037	0.004	0.000	0.127	0.646	0.073
C	X	8	125	4.25	100.51	0.41	0.135	0.007	0.002	0.016	0.039	0.966	0.062
E	S	1	125	4.40	100.20	4.15	0.146	0.073	0.007	0.075	0.119	0.712	0.128
E	S	2	125	4.50	99.83	3.97	0.305	0.138	0.003	0.132	0.023	0.564	0.100
E	S	3	125	4.47	100.84	4.26	0.281	0.157	0.002	0.136	0.024	0.563	0.078
E	S	4	125	4.39	98.68	1.80	0.210	0.058	0.000	0.164	0.029	0.576	0.060
E	T	1	125	4.45	101.08	1.20	0.257	0.023	0.012	0.270	0.132	0.265	0.199
E	T	2	125	4.42	99.15	1.68	0.315	0.079	0.000	0.165	0.017	0.580	0.102
E	T	3	125	4.41	100.40	2.69	0.348	0.101	0.003	0.116	0.040	0.455	0.156
E	T	4	125	4.39	100.57	2.06	0.243	0.051	0.001	0.075	0.014	0.624	0.101
E	U	1	125	4.35	99.56	0.89	0.513	0.044	0.008	0.224	0.104	0.412	0.270
E	U	2	125	4.35	99.65	0.60	0.415	0.052	0.003	0.147	0.026	0.458	0.171
E	U	3	125	4.40	100.84	1.37	0.213	0.015	0.011	0.202	0.119	0.317	0.240
E	U	4	125	4.29	97.03	0.60	0.295	0.018	0.000	0.148	0.035	0.445	0.127
E	W	1	125	4.26	101.21	3.19	0.303	0.037	0.001	0.086	0.018	0.887	0.146
E	W	2	125	4.23	100.40	0.59	0.372	0.039	0.003	0.179	0.012	1.082	0.137
E	W	3	125	4.32	102.35	1.29	0.297	0.006	0.004	0.088	0.024	0.591	0.140
E	W	4	125	4.34	100.54	0.59	0.343	0.022	0.007	0.148	0.022	0.440	0.129
E	X	1	125	4.37	100.04	0.77	0.210	0.076	0.007	0.134	0.049	0.619	0.087
E	X	2	125	4.44	100.14	0.00	0.206	0.127	0.001	0.240	0.026	0.540	0.067
E	X	3	125	4.41	101.01	0.89	0.127	0.063	0.006	0.184	0.078	0.569	0.093
E	X	4	125	4.43	99.54	0.00	0.098	0.057	0.003	0.035	0.033	0.481	0.070
F	S	1	125	4.23	100.02	4.04	0.132	0.000	0.007	0.098	0.047	0.972	0.055
F	S	2	125	4.31	99.41	3.72	0.149	0.009	0.006	0.142	0.054	0.771	0.060
F	S	3	125	4.40	99.13	4.08	0.144	0.003	0.009	0.056	0.098	0.507	0.011
F	S	4	125	4.38	99.53	1.21	0.171	0.017	0.006	0.054	0.055	0.777	0.081
F	T	1	125	4.28	99.88	1.60	0.158	0.004	0.001	0.089	0.041	0.950	0.104
F	T	2	125	4.47	99.69	2.50	0.192	0.001	0.010	0.017	0.072	0.341	0.116
F	T	3	125	4.42	98.56	0.91	0.204	0.001	0.020	0.103	0.096	0.430	0.149
F	T	4	125	4.40	97.71	0.90	0.229	0.005	0.010	0.000	0.052	0.778	0.148
F	U	1	125	4.33	97.89	1.21	0.313	0.003	0.006	0.063	0.046	0.817	0.160
F	U	2	125	4.36	100.32	2.78	0.235	0.004	0.011	0.077	0.061	0.549	0.174

Table C-1 (cont'd).

Mc	Inoc	Rep	Time	pH	Dmr	Ym	Ac	Bu	Ca	Et	Gl	La	Pa
F	U	3	125	4.43	99.26	0.60	0.307	0.000	0.012	0.087	0.102	0.234	0.183
F	U	4	125	4.39	99.94	1.21	0.281	0.000	0.008	0.000	0.049	0.673	0.201
F	W	1	125	4.39	98.91	1.78	0.219	0.000	0.008	0.044	0.069	0.332	0.152
F	W	2	125	4.38	100.62	0.00	0.152	0.003	0.004	0.024	0.033	0.646	0.075
F	W	3	125	4.43	98.44	0.91	0.221	0.002	0.012	0.044	0.094	0.420	0.211
F	W	4	125	4.37	99.89	0.69	0.335	0.002	0.008	0.075	0.070	0.484	0.156
F	X	1	125	4.41	98.25	0.84	0.175	0.034	0.006	0.045	0.038	0.777	0.073
F	X	2	125	4.38	98.98	0.00	0.211	0.000	0.012	0.095	0.079	0.396	0.152
F	X	3	125	4.38	98.18	0.94	0.165	0.003	0.010	0.130	0.046	0.842	0.094
F	X	4	125	4.37	99.30	0.00	0.166	0.049	0.024	0.157	0.059	1.039	0.114
G	S	1	125	4.73	101.32	4.04	0.068	0.000	0.021	0.058	0.055	0.326	0.017
G	S	2	125	4.67	101.75	3.72	0.091	0.000	0.028	0.138	0.105	0.602	0.036
G	S	3	125	4.75	101.52	4.08	0.053	0.000	0.042	0.000	0.044	0.386	0.020
G	T	1	125	4.58	103.27	1.24	0.094	0.000	0.011	0.074	0.083	0.237	0.082
G	T	2	125	4.58	99.36	1.61	0.107	0.000	0.016	0.182	0.081	0.297	0.101
G	T	3	125	4.52	98.61	2.51	0.154	0.000	0.017	0.116	0.116	0.279	0.135
G	U	1	125	4.60	100.26	0.96	0.083	0.000	0.011	0.038	0.048	0.088	0.104
G	U	2	125	4.55	99.08	0.95	0.158	0.000	0.016	0.180	0.117	0.236	0.186
G	U	3	125	4.62	98.53	1.24	0.121	0.000	0.015	0.012	0.084	0.138	0.168
G	W	1	125	4.58	99.50	2.78	0.121	0.000	0.019	0.096	0.101	0.260	0.151
G	W	2	125	4.55	99.21	0.70	0.134	0.000	0.015	0.109	0.131	0.265	0.136
G	W	3	125	4.52	98.42	1.23	0.138	0.000	0.012	0.083	0.072	0.234	0.133
G	X	1	125	4.71	99.28	1.79	0.076	0.000	0.016	0.060	0.006	0.320	0.012
G	X	2	125	4.70	100.98	0.00	0.064	0.000	0.022	0.070	0.000	0.340	0.013
G	X	3	125	4.68	99.27	0.96	0.078	0.000	0.021	0.038	0.000	0.357	0.016

Table C-2. Data used for analyses in chapter 3- temperature during aerobic stability test.

Mc	Inoc	Rep	temp0	temp1	temp2	temp3	temp4	temp5
A	S	1	18.33	19.44	18.61	18.33	18.89	29.44
A	S	2	17.78	18.61	18.61	21.11	26.67	25.56
A	S	3	19.44	20.56	19.44	19.44	19.44	28.89
A	T	1	18.33	20.00	19.44	18.33	21.11	24.72
A	T	2	17.22	19.17	17.78	17.78	23.33	29.44
A	T	3	17.78	18.89	18.33	17.22	17.78	20.83
A	U	1	17.78	18.89	18.33	17.78	17.78	22.22
A	U	2	20.00	20.56	20.56	19.44	21.11	28.06
A	U	3	18.89	19.44	18.89	17.78	18.89	25.56
A	W	1	17.78	19.44	19.44	18.33	18.33	23.61
A	W	2	19.44	19.72	19.72	19.44	35.00	26.39
A	W	3	18.89	19.17	19.44	18.33	18.33	21.94
A	X	1	19.44	29.44	26.67	21.67	23.89	27.22
A	X	2	18.89	19.44	18.89	18.89	37.78	26.39
A	X	3	18.89	19.72	19.17	18.33	17.78	23.06
B	S	1	22.22	20.83	20.00	20.00	20.28	20.56
B	S	2	20.56	19.44	18.89	18.89	19.17	19.44
B	S	3	22.78	20.83	20.00	20.28	20.00	20.56
B	S	4	21.67	19.72	19.17	19.44	19.72	20.56
B	T	1	21.67	20.00	19.44	19.44	19.44	20.00
B	T	2	21.67	20.28	19.44	19.72	19.44	20.00
B	T	3	22.22	20.56	19.72	20.00	20.28	20.56
B	T	4	20.56	19.17	18.61	18.61	18.89	19.44
B	U	1	21.67	20.28	19.44	19.44	19.44	20.00
B	U	2	21.67	20.28	19.44	19.72	19.44	19.44
B	U	3	21.11	19.44	19.17	19.17	19.44	19.44
B	U	4	23.33	21.67	20.83	20.83	21.11	21.67
B	W	1	21.67	20.28	19.44	13.89	19.44	20.56
B	W	2	21.67	20.28	19.44	19.17	19.44	20.00
B	W	3	21.67	19.72	19.17	19.17	18.89	19.44
B	W	4	21.67	20.28	20.00	19.72	20.00	20.56
B	X	1	21.67	19.44	18.89	18.89	19.44	20.00
B	X	2	21.11	19.72	18.89	18.61	18.89	19.44
B	X	3	20.00	18.61	18.06	18.06	18.33	18.89
B	X	4	21.67	20.00	19.44	19.17	19.72	20.00

Abbreviations used: Mc-moisture content, Inoc-inoculant, Rep-replication, temp0-temperature at d0, temp1- temperature at d1, temp2- temperature at d2, temp3-temperature at d3, temp4- temperature at d4, d5- temperature at d5.

Mc	Inoc
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C	S
C	S
C	S
C	T
C	T
C	
C	
C

Mc	Inoc	Rep	temp0	temp1	temp2	temp3	temp4	temp5
C	S	1	26.11	23.89	20.56	23.89	25.56	25.56
C	S	2	24.44	23.89	21.11	23.89	25.00	26.67
C	S	3	24.44	22.78	20.56	23.06	23.89	27.78
C	S	4	23.33	22.78	19.44	22.78	23.89	25.00
C	T	1	23.89	22.78	20.00	23.06	25.00	25.00
C	T	2	25.00	23.89	21.11	23.61	25.00	25.56
C	T	3	23.89	22.78	19.44	22.50	23.89	23.89
C	T	4	25.00	23.89	21.11	23.89	25.00	26.11
C	U	1	24.44	23.33	20.00	24.44	23.33	24.44
C	U	2	25.00	23.89	20.00	23.33	24.44	24.44
C	U	3	25.00	23.89	21.11	24.17	25.00	26.11
C	U	4	25.00	23.33	20.00	23.33	25.00	25.56
C	W	1	25.00	23.33	20.00	23.33	23.89	25.00
C	W	2	25.00	23.89	20.56	23.61	24.44	25.00
C	W	3	26.67	25.00	21.67	24.72	25.00	26.11
C	W	4	26.11	24.44	21.11	23.89	26.11	26.67
C	X	1	24.44	23.33	20.56	23.06	23.89	24.44
C	X	2	25.00	23.33	20.56	23.89	24.44	24.44
C	X	3	26.67	25.00	21.11	24.72	25.00	26.11
C	X	4	26.11	25.00	21.11	24.44	26.11	26.67
C	S	5	24.44	26.39	25.56	24.44	27.22	27.22
C	S	6	23.89	25.56	25.00	24.17	26.67	27.78
C	S	7	23.89	26.11	25.56	24.44	27.22	26.67
C	S	8	25.00	27.22	26.11	28.33	30.56	29.44
C	T	5	23.89	25.83	25.56	23.61	27.22	26.67
C	T	6	23.89	25.56	25.00	23.89	26.67	26.67
C	T	7	23.89	25.83	25.00	24.44	27.22	27.22
C	T	8	22.78	24.17	23.89	21.67	25.56	24.44
C	U	5	24.44	26.11	25.56	23.33	27.22	26.67
C	U	6	23.89	25.56	25.00	23.61	26.67	26.67
C	U	7	23.33	25.28	24.44	23.33	26.67	26.11
C	U	8	25.00	27.50	26.67	25.28	28.33	28.33
C	W	5	23.89	25.56	25.00	23.61	26.67	27.22
C	W	6	23.89	25.83	25.00	24.44	26.67	27.22
C	W	7	22.78	24.44	23.89	23.89	26.67	25.56
C	W	8	23.89	26.39	25.56	24.72	28.33	26.67
C	X	5	23.89	25.56	25.00	23.61	26.67	27.22
C	X	6	22.78	25.00	24.44	23.06	26.67	26.67
C	X	7	22.78	25.56	25.00	25.00	27.78	28.33
C	X	8	25.00	26.94	25.56	24.72	28.33	28.89

Mc	Inoc	Rep	temp0	temp1	temp2	temp3	temp4	temp5
E	S	1	23.33	20.56	20.00	19.17	18.89	20.00
E	S	2	24.44	20.83	20.56	20.56	20.56	21.11
E	S	3	24.44	20.28	20.00	21.11	20.56	22.22
E	S	4	23.33	21.39	21.67	22.22	22.22	22.22
E	T	1	21.67	20.00	20.56	20.56	20.56	21.11
E	T	2	23.89	20.56	20.00	19.72	18.89	20.00
E	T	3	22.78	21.11	20.56	20.00	20.00	20.56
E	T	4	23.89	21.67	21.67	21.94	21.11	22.22
E	U	1	22.78	20.83	21.11	20.83	20.56	21.11
E	U	2	22.78	21.11	21.11	20.28	20.56	21.11
E	U	3	24.44	20.28	18.89	21.11	20.56	21.67
E	U	4	22.78	20.00	20.56	20.56	20.56	21.67
E	W	1	23.33	20.00	18.89	20.00	20.00	20.56
E	W	2	23.89	21.39	21.11	21.11	21.11	21.11
E	W	3	23.33	20.56	21.11	19.44	18.33	20.56
E	W	4	23.33	21.39	21.67	22.22	22.22	22.22
E	X	1	21.67	20.00	20.00	20.28	20.00	20.56
E	X	2	23.89	21.11	20.00	21.94	22.22	22.78
E	X	3	22.22	20.83	21.11	21.11	20.56	21.67
E	X	4	25.00	21.67	20.56	20.00	19.44	20.56
F	S	1	21.11	20.28	19.72	19.44	19.44	20.56
F	S	2	21.11	20.00	20.28	19.44	19.44	20.00
F	S	3	20.56	20.56	20.56	19.44	19.44	20.00
F	S	4	22.22	22.22	21.39	20.56	20.56	21.11
F	T	1	21.11	20.28	21.11	20.00	20.00	20.00
F	T	2	20.56	20.00	19.72	19.44	18.89	18.89
F	T	3	21.67	20.83	21.11	20.00	19.44	20.00
F	T	4	21.11	20.56	20.56	19.44	19.44	19.44
F	U	1	21.11	21.11	20.83	21.11	20.00	20.56
F	U	2	21.67	21.39	21.39	21.11	21.11	21.11
F	U	3	21.11	21.11	20.83	20.00	19.44	20.00
F	U	4	21.11	20.83	19.72	19.44	19.44	19.44
F	W	1	21.67	21.67	21.67	20.00	20.00	20.56
F	W	2	21.67	21.11	21.67	21.11	21.11	21.11
F	W	3	22.22	22.22	22.22	21.67	21.67	21.67
F	W	4	21.11	21.11	20.83	19.44	21.11	21.11
F	X	1	21.67	21.39	21.39	20.56	21.11	20.56
F	X	2	21.67	21.39	21.67	20.56	20.56	20.56
F	X	3	21.67	20.83	20.56	20.00	20.00	20.00
F	X	4	21.11	21.11	21.67	21.11	20.56	20.56

Mc	Inoc
G	S
G	S
G	S
G	T
G	T
G	T
G	U
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G	

Mc	Inoc	Rep	temp0	temp1	temp2	temp3	temp4	temp5
G	S	1	22.22	21.94	22.78	20.83	22.22	20.56
G	S	2	21.67	21.39	21.67	21.11	21.67	22.22
G	S	3	21.11	21.11	21.67	21.11	21.11	21.11
G	T	1	22.22	21.94	22.78	20.56	21.67	21.67
G	T	2	21.11	21.11	21.67	21.11	21.11	21.11
G	T	3	21.67	21.94	22.22	21.94	21.67	21.67
G	U	1	21.67	20.56	21.67	20.28	21.67	20.00
G	U	2	21.11	21.11	21.67	20.56	21.11	21.11
G	U	3	21.67	21.39	21.11	21.39	21.11	20.56
G	W	1	21.67	22.22	22.22	21.39	21.11	20.56
G	W	2	22.22	22.22	21.67	22.22	22.22	21.67
G	W	3	21.67	21.39	21.67	21.11	21.11	21.11
G	X	1	22.22	22.22	22.22	20.83	21.67	20.56
G	X	2	22.22	21.39	22.22	20.56	21.11	21.11
G	X	3	22.22	21.94	22.22	21.11	21.67	21.11

APPENDIX D

Data Used for Analyses in Chapter 4

Table D-1. Data used for analyses in chapter 4*.

Inoc	Temp	Time	Rep	Ace	Mal	Prop	Suc	OD	pH
DH42	30	20	1	0.082	0.019	0.294	0.000	1.70	4.72
DH42	30	20	2	0.086	0.016	0.298	0.000	1.70	4.72
DH42	30	20	3	0.081	0.020	0.292	0.000	1.70	4.72
DH42	40	20	1	0.308	0.062	0.485	0.021	1.43	4.95
DH42	40	20	2	0.061	0.047	0.279	0.006	1.54	4.78
DH42	40	20	3	0.055	0.048	0.270	0.005	1.55	4.78
SHER	30	20	1	0.023	0.092	0.052	0.028	0.82	5.81
SHER	30	20	2	0.021	0.094	0.051	0.028	0.80	5.73
SHER	30	20	3	0.015	0.090	0.043	0.027	0.79	5.84
SHER	40	20	1	0.002	0.093	0.008	0.009	0.11	6.09
SHER	40	20	2	0.007	0.000	0.011	0.011	0.11	5.96
SHER	40	20	3	0.007	0.000	0.009	0.010	0.13	5.91
DH42	30	40	1	0.092	0.014	0.325	0.007	1.63	4.64
DH42	30	40	2	0.096	0.014	0.330	0.002	1.62	4.64
DH42	30	40	3	0.101	0.014	0.332	0.002	1.63	4.64
DH42	40	40	1	0.077	0.019	0.374	0.000	1.50	4.66
DH42	40	40	2	0.072	0.018	0.364	0.000	1.49	4.66
DH42	40	40	3	0.079	0.019	0.376	0.000	1.50	4.66
SHER	30	40	1	0.054	0.000	0.156	0.026	1.38	5.25
SHER	30	40	2	0.047	0.000	0.152	0.025	1.39	5.24
SHER	30	40	3	0.051	0.000	0.154	0.026	1.38	5.26
SHER	40	40	1	0.009	0.096	0.016	0.020	0.16	5.91
SHER	40	40	2	0.009	0.096	0.014	0.026	0.19	5.87
SHER	40	40	3	0.012	0.099	0.014	0.024	0.18	5.89
DH42	30	72	1	0.104	0.014	0.349	0.005	1.61	4.60
DH42	30	72	2	0.102	0.014	0.347	0.005	1.62	4.64
DH42	30	72	3	0.106	0.014	0.351	0.002	1.62	4.60
DH42	40	72	1	0.078	0.018	0.385	0.000	1.57	4.65
DH42	40	72	2	0.079	0.017	0.388	0.000	1.57	4.65
DH42	40	72	3	0.081	0.018	0.392	0.000	1.59	4.64
SHER	30	72	1	0.081	0.000	0.280	0.021	1.58	4.95
SHER	30	72	2	0.076	0.000	0.276	0.024	1.59	4.95
SHER	30	72	3	0.085	0.093	0.283	0.021	1.58	4.95
SHER	40	72	1	0.016	0.096	0.039	0.034	0.23	5.83
SHER	40	72	2	0.017	0.098	0.027	0.033	0.30	5.81
SHER	40	72	3	0.016	0.096	0.034	0.034	0.26	5.79

*Abbreviations used: Inoc-inoculant, temp-temperature, rep-replication, ace-acetic acid, mal-malate, prop-propionic acid, suc-succinic acid, OD-optical density, SHER-*P. shermanii*

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