HYDROGEN PEROXIDE PRODUCTION AT REFRIGERATION TEMPERATURE BY LACTOBACILLI FROM RAW MILK

by

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CHAPTER I

INTRODUCTION

Hydrogen peroxide (H_2O_2) has been used as part of the peroxidase-thiocyanate system for the short-term preservation of raw milk in some developing countries. The production of H_2O_2 by certain lactic acid bacteria can be beneficial to food preservation and prevent the growth of undesired microorganisms. Sufficient H_2O_2 is produced by some lactobacilli to inhibit growth of food borne pathogens and spoilage microorganisms in some refrigerated foods. For example, they produce enough to inhibit the growth of *Salmonella typhimurium* (Watson and Schubert, 1969), and psychrotrophic spoilage microorganisms in nonfermented food (Gilliland and Speck, 1975; Gilliland and Martin, 1980). Among the lactic acid bacteria, the species *Lb. delbrueckii* subsp *lactis* appears to produce the highest levels of H_2O_2 at refrigeration temperatures without growing (Premi and Bottazzi, 1972; Gilliland, 1980). Selection of strains of this microorganism that produce high level of H_2O_2 at refrigeration temperature could provide a useful means for increasing the shelf life of some refrigerated foods. The objective of this study was to isolate new strains of *Lb. delbrueckii* subsp *lactis* from raw milk that can produce higher level H_2O_2 at refrigerated temperature than strains currently available in our laboratory.

CHAPTER II

LITERATURE REVIEW

INTRODUCTION

For years, starter cultures containing lactic acid bacteria (LAB) have been used in the production of dairy products such as cheese, sour cream, yogurt, and other fermented foods. The involvement of such bacteria was important in making such foods long before it was known that the bacteria were involved at all. It was empirically found that raw milk that was incubated at ambient temperature for several hours became coagulated. In addition, it is believed that cheese formation was accidentally discovered when milk was placed for storage in bag made from the stomachs of domesticated animals such as cow, goat, and sheep (Board and Gould, 1991; Cogan *et al*, 1991). Due to the presence of enzymes contained in the animal's stomach (pepsin, chymosin) and lactic acid produced by lactic acid bacteria from the fermentation of lactose in the milk, the casein coagulated, forming cheese curd as the whey was expelled. This is the basis for the manufacture cheese. Cultured products from raw milk were made by inoculating the milk with a portion of a previous batch of fermented product to initiate the fermentation. This technique is referred to as "backslopping" (Gilliland, 1985). Fermented milk could be prepared in this manner since the required lactic acid bacteria were present in the raw milk. The variations among species, and strains of lactic acid bacteria, caused much variation in the characteristics of the resulting

fermented milks. Today, there are more varieties of cultured dairy foods, fermented meats, and fermented vegetables produced through the growth and the action of lactic acid bacteria which are known as starter cultures. Many of these lactic acid bacteria used as starter cultures for the manufacture of fermented milks originally came from raw milk. Compared to the original fermented products, the quality and consistency of fermented products has been improved. The advances of technology involving maintenance, freezing, lyophilization, and distribution of commercial starter cultures has provided greater flexibility, and reliability in the manufacture of cultured products (Codon and Accolas, 1990; Cogan *et al*, 1991).

TYPE OF STARTER CULTURES

The primary functions of dairy starter cultures is to produce lactic acid from lactose in milk. Some also produce acetic acid, formic acid, acetaldehyde, diacetyl, carbon dioxide (CO_2), polysaccharides, peptides, and free amino acids that contribute to the flavor, aroma, and texture of fermented dairy products. There are sixteen genera among lactic acid bacteria (LAB), but there are only 4 genera commonly involved in starter culture used in dairy industry: *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Lactobacillus*. These starter cultures are all Gram positive, catalase negative, and nonmotile rods and cocci. Even though they are facultative anaerobic bacteria, they grow better anaerobically than aerobically.

This is because they lack a cytochrome system for electron transport system to generate energy (ATP) aerobically (Monnet, 1995). Therefore, fermentation is used by these bacteria to generate the energy needed for growth.

Lactococcus

Lactococcus spp. are cocci that occur in pairs or in chains. Most ferment sugars homofermentatively, producing L-lactate and grows at 10°C but not at 45°C (Kandler, and Weiss, 1986). There are currently five species in the genus, which are *Lc. lactis*, *Lc. garviae*, *Lc. planatarum*, *Lc. piscium*, and *Lc. raffinolactis*. Two subspecies *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* are the primary ones used as starter cultures for dairy fermentation. The major phenotypic difference between these two subspecies are that *Lc. lactis* subsp. *lactis* grows at 40°C, at pH 9.2, in the presence of 4% sodium chloride (NaCl), and produces ammonia from arginine, whereas *Lc. lactis* subsp. *cremoris* does not (Sandine, 1985). The capability to produce acid through the fermentation of lactose allows these starter cultures to play an important role in manufacture of fermented dairy products.

Leuconostoc

Leuconostoc spp. also are cocci that occur in pairs and in chains (Kandler, and Weiss, 1986). They are catalase negative and form chains of cocci or oval-shaped cells. They normally can be distinguished from *Lactococcus* by

inoculating them in litmus milk. *Lactococcus* spp. generally coagulates the milk and reduces the litmus, whereas *Leuconostoc* spp. does not (Sandine, 1985; Cogan, 1985). The most important species in dairy starter cultures is *Leuc. mesenteroides* subsp. *cremoris*. The major role of *Leuconostoc* spp. in dairy fermentation is the production of volatile flavor components such as diacetyl, which contributes to the unique flavor of some cultured dairy products.

Streptococcus

This genus contains 27 species. Only one species is found in starter cultures—*Streptococcus salivarius* subsp. *thermophilus*. Like *Lactococcus* spp., and *Leuconostoc* spp., *Strep. salivarius* subsp. *thermophilus* are cocci that occurs in pairs and in chains. They ferment lactose to produce lactic acid and grow best at 45°C. *Streptococcus salivarius* subsp. *thermophilus* is one of two species in traditional cultures for yogurt manufacture (Sandine, 1985).

Lactobacillus

Lactobacillus is a genus composed of a large group of rod-shaped bacteria. *Lactobacillus* spp. are Gram-positive, nonspore forming, nonmotile rods, and are catalase negative (Kandler, and Weiss, 1986). Although, lactobacilli are facultative anaerobic bacteria, they lack a cytochrome system for electron transfer to generate energy (ATP), which makes it preferable for lactobacilli to grow

anaerobically. Based on the differences in the way they ferment carbohydrates, they are divided into two groups: homofermentative and heterofermentative (Cogan *et al*, 1991). The homofermentative species are the ones encountered in dairy starter cultures.

The primary lactobacilli in commercial dairy starter cultures included *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus*, *Lb. acidophilus*, and *Lb. casei*. *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, *Lb. acidophilus*, and *Lb. helveticus* are considered as thermophilic starter microorganisms and grow best at temperatures of 45°C or higher. They can be distinguished from each other by sugar fermentation and the isomers of lactate produced (Kandler, and Weiss, 1986; Gilliland, 1985).

Lactose fermentation by the lactobacilli involves the Embden-Meyerhof-Parnas (EMP) pathway in which lactose is translocated into the cell by lactose permease without chemical modification. The lactose is then hydrolyzed by Beta-galactosidase into glucose and galactose. An interesting observation for *Lb. delbrueckii* subsp. *bulgaricus*, and *Lb. delbrueckii* subsp. *lactis* is that when grown on lactose, the galactose portion of the molecule is excreted from the cell in proportion to the molecules of lactose utilized (Hickey *et al*, 1986). Thus, the model of lactose transport of these bacteria is that single transmembrane antiport premease simultaneously translocates lactose molecule into cytoplasm and galactose molecules out of the cell (Hickey, *et al*, 1986; Foucound and Poolman,

1992). The sugar is then further metabolized through the EMP in which each molecule of glucose is converted into two molecules of lactic acid with a net gain of two molecule of adenosine triphosphate (ATP) (Kandler and Weiss, 1986). The metabolism is referred to as homolactic fermentation.

The energy yielding fermentative pathway is important in dairy manufacture because lactic acid, which is the primary end product, is necessary for the development of the characteristic flavor and texture of cultured products such as yogurt, swiss cheese, and kefir.

Lactobacillus delbrueckii subsp. *lactis*

Lactobacillus delbrueckii subsp. *lactis* grows well at 45°C and frequently at 50-52°C with an optimum at 40-44°C and no growth at 15°C. *Lactobacillus delbrueckii* subsp. *lactis* is homofermentative and produce a D-lactic acid as the primary end product of fermentation. *Lactobacillus delbrueckii* subsp. *lactis* fermented glucose, fructose, lactose, maltose, mannose, sucrose, and trehalose. They do not ferment ribose, arabinose, xylose, rhamnose, mannitol, sorbitol, melezitose, and gluconate (Kandler, and Weiss, 1986). They were originally isolated from milk.

While *Lactobacillus delbrueckii* subsp. *lactis* has played a role in the food industry as starter cultures for certain cultured products, it also has a potential as a biopreservative for refrigerated food products, due to its capability of producing

high levels of hydrogen peroxide (H_2O_2) at refrigeration temperature (Gilliland, and Speck, 1975). This has potential benefit to the food industry for use in food preservation (Gilliland, 1980; Watson and Schubert, 1969; Gilliland and Speck, 1975; Gilliland and Martin, 1980; Gilliland, and Ewell, 1983; Brashears *et al*, 1998). For years, the microorganism was known as *Lb. lactis*. However, Weiss *et al* (1983) changed its taxonomic name into *Lb. delbrueckii* subsp. *lactis*. In regards to the respective description from the early investigations, *Lb. delbrueckii* (Leichman 1896), *Lb. leichmannii* (Hennebery 1903), *Lb. lactis* (Orla-Jensen 1919), and *Lb. bulgaricus* (Orlan-Jensen 1919) have been found to have a great number of basic characteristics in common. They all produce D-lactic acid from glucose, they all grow at the same optimum temperature, contain identical mole percentage of guanine plus cytosine (% G+C) in their DNA (Gasser and Mandel, 1968), all have the the same cell wall composition (Kandler, 1970), and their NDA-dependent D-Lactic acid hydrogenases (LDH) migrate similarly in starch gel electrophoresis (Gasser, 1970). Based on immunological studies, they were found completely homologous to each other (Gasser and Gasser, 1971). Miller *et al* (1971) reported *Lb. lactis* and *Lb. leichmannii* had complete nucleic acid homology based on DNA-RNA hybridization. The strains of *Lb. delbrueckii*, *Lb. bulgaricus*, *Lb. lactis*, and *Lb. leichmannii* were found to have more than 80% DNA/DNA homology (Weiss *et al*, 1983) and the phenotypical differences among the four species were found to be variations in ability to ferment various

carbohydrates. Thus, Weiss *et al* (1983) divided the four species into three subspecies: *Lb. delbrueckii* subsp. *delbrueckii*, *Lb. delbrueckii* subsp. *bulgaricus*, and *Lb. delbrueckii* subsp. *lactis*. *Lactobacillus delbrueckii* subsp. *lactis* is comprised of the strains formerly assigned to *Lb. lactis*, and *Lb.leichmannii* (Weiss *et al*, 1983).

ANTAGONISMS OF LACTOBACILLI TOWARD UNDESIRABLED MICROORGANISMS ASSOCIATED WITH FOODS

The practice of fermentation is among the oldest forms of food preservation. Raw products such as milk, meats, and vegetables were allowed to undergo fermentation so they could be saved for later consumption (Gould, 1989). Lactic acid fermentation has played a main role in food preservation since acidification of the food can help to prevent the growth of food borne pathogens and spoilage microorganisms. In developed countries, starter cultures have been developed and are selected for their ability to produce consistent, high quality cultured foods more than for preservation for food. One of the main preservative techniques used in developed countries is storing food at low temperature. There has, however, been renewed interest in the role of starter cultures in the preservation of foods. Fermented foods are considered to be a relatively safe form of food preservation and are not commonly associated with food-poisoning

outbreaks (Gombas, 1989). The preservation of foods by lactic acid bacteria was thought to be entirely due to the low pH resulting from metabolism of sugar to produce organic acids (Gould, 1989). However, the increase in the concentration of acids, which leads to the retardation or elimination of spoilage microorganisms is not the only inhibitory mechanism possible from the starter culture bacteria. The overall preservative effect probably depends on a series interacting antimicrobial compounds (Gould, 1989; Board and Gould, 1991). This is because in addition to organic acid, lactic acid bacteria also can produce small amounts of other antimicrobial compounds such as hydrogen peroxide, ethanol, diacetyl, and bacteriocins (Bank *et al*, 1986).

Bacteriocins

Bacteriocins are low-molecular weight proteins, which usually have a narrow spectrum of antimicrobial activity. The bacteriocin-producing bacteria most extensively studied have been the lactic acid bacteria, particularly *Lc. lactis* subsp. *lactis*, which produces the bacterocin nisin. This bacteriocin has received more attention than any other. Nisin is relatively stable to heat and low pH and active against Gram-positive bacteria. Nisin is a lantibiotic, which is an antimicrobial polycyclic peptide that inhibits vegetative cells of Gram-positive bacteria (Stiles and Hasting, 1991). Bacteriocins produced by *Lactobacillus* spp., *Leuconostoc* spp., and *Pediococcus acidilactici* have been reported to inhibit

Listeria monocyctogenes (Bhunia *et al*, 1988; Harris *et al*, 1989; Raccach *et al*, 1989). However, nisin has been the main one adopted for use as a preservative by the food industry. Because of their rather narrow spectrum of antibacterial activity, bacteriocins may have limited use as biopreservatives. They are active primarily against other Gram positive bacteria while most spoilaage and pathogenic bacteria in refrigerated are Gram negative bacteria.

Lactic Acid and Acetic Acid

The major antagonistic effects of lactic acid bacteria in fermented foods is primarily due to the lowered pH resulting from the fermentation of sugar to organic acids particularly lactic, and acetic acids (Board and Gould, 1991). Although, lactic acid (pK_a 3.86) is stronger than the acetic acid (pK_a 4.75), acetate produced better antimicrobial potential in those foods where the pH was between 4 to 6 (Adam and Hall, 1988). Adam and Hall (1988) also found that a mixture of lactic acid and acetic acid enhanced inhibition of *Salmonella enteritidis* and *Escherichia coli* compared with using the individual acids. Board and Gould (1991) suggested that lactic acid bacteria could be genetically engineered to produce increased amounts of acetic acid, which could be more effective in preserving foods. However, this could adversely affect the flavor of some of the cultured products. Even though organic acids do not necessarily kill the undesirable microorganisms, they may have a pronounced effect on their growth.

Hydrogen peroxide (H_2O_2)

In the dairy industry, H_2O_2 has been used to treat milk for making certain cheeses due to its ability to kill undesired microorganisms (Luck, 1956; Teply *et al*, 1958; Gilliland, 1969). Hydrogen peroxide produced by lactic acid bacteria in raw milk can react with thiocyanate (SCN^-), in the presence of lactoperoxidase to form hypothiocyanite that can inhibit microorganisms (Daeschel, 1989). Accumulation of H_2O_2 by itself also play an important role in food preservation (Gilliland, 1980). It also has the potential for minimizing of food borne illness due to its repression of the growth of food borne pathogens in foods (Watson and Schubert, 1969; Gilliland and Speck, 1975; Gilliland and Martin, 1980; Gilliland and Ewell, 1983; Brashears *et al*, 1998). In lactic acid bacteria such as lactococci and lactobacilli, H_2O_2 is formed as an end product from the oxidation of lactate, oxidation of pyruvate, and/or the oxidation of nicotinamide adenine dinucleotide (NADH) (Kandler, 1983). Lactate can be oxidized to pyruvate which yield H_2O_2 as a byproduct by L-lactate oxidase or NAD-independent D-lactate dehydrogenase, and pyruvate can be oxidized into acetyl-phosphate and carbon dioxide yielding H_2O_2 as a byproduct by pyruvate oxidase. Nicotinamide adenine dinucleotide is oxidized to NAD yielding H_2O_2 by NADH oxidase. The later reaction is stimulated by flavin adenine dinucleotide (FADH) (Anders *et al.*, 1970; Collin and Aramaki, 1980, Codon, 1987).

Several studies have shown the antimicrobial action of H_2O_2 produced by lactobacilli. *Lactobacillus delbrueckii* subsp *lactis* and *Lb. delbrueckii* subsp *bulgaricus* produced enough H_2O_2 to inhibit the growth of *Staphylococcus aureus* when stored at 5°C for several hours (Dahiya and Speck, 1968). They further reported that at higher temperature the concentration H_2O_2 produced by *Lb. delbrueckii* subsp *lactis* was lower that at 5°C. In other studies, lactobacilli produced enough H_2O_2 to inhibit the growth of *Listeria monocytogenes* (Tharrington and Sorrells, 1992), *Salmonella typhimurium* (Watson and Schubert, 1969), and psychrotropic spoilage bacteria (Gilliland and Speck, 1975, Gilliland and Martin, 1980; Gilliland and Ewell, 1983). The addition of catalase was found to reduce or eliminate the inhibitory action of H_2O_2. Raccach and Baker (1978) indicated that the levels of H_2O_2 produced under experimental condition were usually not enough to provide antimicrobial action. However, *Lb. delbrueckii* subsp *lactis* produced 5.5-12.5 ug H_2O_2 ml^{-1} (Dahiya and Speck, 1968), whereas, *Lb. plantarum* and *Pediococcus acidilactici* (meat starter cultures) produced only 0.85 ug H_2O_2 ml^{-1} (Raccach and Baker, 1978). The lack of inhibitory action in the study of Raccach and Baker (1978) may have been due to the differences in species of lactic acid bacteria used and lack of production of suffeicient H_2O_2. Brashears *et al* (1998) reported production of H_2O_2 by *Lactobacillus delbrueckii* subsp *lactis* resulted in decreases in the number of *Escherichia coli* 0157:H7 during refrigerated storage of chicken meat.

OBJECTIVE OF PRESENT STUDY

The capability of *Lb. delbrueckii* subsp. *lactis* to produce enough H_2O_2 to be antagonistic towards spoilage microorganisms and food borne pathogen is well recognized and documented. Furthermore this can occur without growth of *Lb. delbrueckii* subsp. *lactis* at refrigerated temperature. This provides the potential of adding cells of this microorganism to refrigerated foods to exert control over undesirable microorganisms during refrigerated storage without fermentation, which would alter the taste of the food. This process could be costly if too high a number of lactobacilli were required. The work of Brashear *et al* (1998) involved the use of 5×10^7 lactobacilli per ml in the treatment solution to cause the antagonistic action. Thus, the objective of our experiment was to isolate strains of *Lb. delbrueckii* subsp. *lactis* from raw milk that produce higher levels of hydrogen peroxide at low temperature (5°C) than did those in the study of Brashears *et al* (1998).

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CHAPTER III

COMPARISON OF NEWLY ISOLATED STRAINS OF *LACTOBACILLUS DELBRUECKII* SUBSP. *LACTIS FOR* HYDROGEN PEROXIDE PRODUCTION AT 5°C

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ABSTRACT

Isolates of *Lactobacillus delbrueckii* subsp. *lactis* obtained from raw milk samples were compared for the ability to produce hydrogen peroxide (H_2O_2) at refrigeration temperature (5°C). Nineteen out of 101 lactobacilli isolated were identified as *Lb. delbrueckii* subsp. *lactis*. In most cases, the isolates of *Lb. delbrueckii* subsp. *lactis* from a given raw milk sample produced more H_2O_2 than did other isolates of lactobacilli from the same sample. Seven isolates of *Lb. delbrueckii* subsp. *lactis*, which produced the highest levels of H_2O_2 at 5°C were selected for comparison with *Lb. delbrueckii* subsp. *lactis* I (Brashears *et al*, 1998) for H_2O_2 production at 5°C in phosphate buffer containing 5 mM glucose and phosphate buffer containing 5 mM sodium lactate. In 24 hours, strain T2-5 produced 7.0 ug/10^9 CFU on sodium lactate and 4.4 ug/10^9 CFU on glucose. Three other strains also produced more H_2O_2 on sodium lactate than on glucose. However, three remaining new isolates produced more H_2O_2 on glucose than on sodium lactate. The seven new isolates of *Lb. delbrueckii* subsp. *lactis* produced significantly ($P < 0.05$) higher concentrations of H_2O_2 than did *Lb. delbrueckii* subsp. *lactis* I in both solutions. Strain T2-5 produced more H_2O_2 than did the other six newly isolated strains of *Lb. delbrueckii* subsp. *lactis* in this comparison.

INTRODUCTION

Some lactobacilli produce enough hydrogen peroxide to inhibit *Pseudomonas* spp., *Bacillus* spp., and *Proteus* spp. (Price and Lee, 1970), *Listeria monocyctogenes* (Tharrington and Sorrells, 1992), *Staphylococcus aureus* (Dahiya and Speck, 1968), and *Salmonella typhimurium* (Watson and Schubert, 1969). Studies also indicated that H_2O_2 produced by *Lb. delbrueckii* subsp. *bulgaricus*, and *Lb. delbrueckii* subsp. *lactis* in milk inhibited the growth of psychrotophic bacteria when stored at 5 to 7°C (Gilliland and Speck, 1975; Gilliland and Ewell, 1983). At 5-7°C, no change in pH of the milk was noted. In recent research reported by Brashears *et al* (1998), the cells of *Lb. delbrueckii* subsp. *lactis* I produced sufficient H_2O_2 to inhibit *Escherichia coli* 0157:H7 on refrigerated raw chicken. However, high number of the lactobacilli were used to produce this effect.

The above studies indicate that *Lb. delbrueckii* subsp. *lactis* has potential as a biopreservative for some refrigerated foods. The ability of this organism to produce enough H_2O_2 without growing at refrigeration temperature should enable them to extend the self life of some refrigerated foods without altering the acidity of the foods.

The objective of this study was to isolate new strains of *Lb. delbrueckii* subsp. *lactis* from raw milk that produce higher levels of H_2O_2 than *Lb.*

delbrueckii subsp. *lactis* I used by Brashears *et al* (1998) at refrigeration temperature (5°C). This should make their use as a biopreservative even more feasible.

MATERIALS AND METHODS

Source of Cultures

Lactobacillus delbrueckii subsp. *lactis* I used in this study was from the stock culture collection of the Food Microbiology Laboratory in the Department of Animal Science at Oklahoma State University. It was maintained by weekly subculture in MRS broth (1% inoculum and 18 hours incubation at 37°C) and stored in a refrigerator (5°C) between subcultures. It was subcultured three times immediately prior to each experimental use.

Enumeration of Bacteria

Microorganisms were enumerated by pour plate technique using MRS agar. The MRS agar was prepared by adding 1.5% agar (Difco Laboratories, Detroit, MI) to lactobacilli MRS broth (Difco Laboratories). Appropriate decimal dilutions were prepared using 99 ml sterile dilution blanks containing 0.1% peptone (Difco Laboratories) and 0.001% antifoam emulsion (Sigma Chemical Co., St. Louis, MO) in distilled water. Dilutions were prepared following procedures in the Compendium of Methods for the Microbiological Examination of Foods (Swanson *et al*, 1992). Petri dishes containing the dilutions were poured with molten MRS agar (45°C). After solidification, the plates were overlayed and incubated at 37°C for 48 hours. Colonies were counted with the aid of a Quebec Colony Counter.

Isolation and Identification of Lactobacilli

Raw milk samples were obtained from individual cattle at the Oklahoma State University Dairy Cattle Center and from private farms in the Stillwater area. Samples also were obtained from receiving tankers at a commercial dairy plant. The raw milk samples were placed in ice during transport to laboratory. In the laboratory, 1 ml of each raw milk sample was transferred into 100 ml of LBS broth prepared from individual ingredients using the manufacturer's formulation (Baltimore Biological Laboratories, Cockeysville, Md) containing 0.1% agar for selective preenrichment. The remainder of each sample was aseptically transferred into a sterile bottle. The inoculated broth and raw milk were incubated at 37°C. Each sample was checked by Gram stain daily until Gram-positive rod shaped bacteria became predominant.

After Gram-positive rod shaped bacteria became predominant, appropriate dilutions to yield isolated colonies were plated by the pour plate method using LBS agar prepared from individual ingredients using the manufacturer's (Baltimore Biological Laboratories) formulation. After solidification, the LBS plates were overlayed and incubated for 48 hours at 37°C. Up to ten isolated colonies were randomly picked from the LBS plates using a flame-sterilized needle and inoculated into separate tubes of MRS broth. The tubes were then incubated at 37°C until growth was evidenced by turbidity (usually 24 hours).

To ensure the purity of the isolates, appropriate dilutions were plated

by pour plate method with MRS agar. They were overlayed with the same medium and incubated at 37°C for 48 hours. Then the purity of the culture was ascertained by observing the morphology of the colonies on the plates. If more that one type of colony was observed, one of each colony type was picked into sterile MRS broth and incubated at 37°C for 24 hours. Purity of each was then confirmed.

All cultures that were Gram-positive, catalase negative rods were assumed to be lactobacilli. Further identity tests involved testing the ability to grow at 45°C and 15°C, and fermentation patterns determined using API 50CH kits (bioMerieux Vitek, Inc., Hazelwood, MO). Identity was based on the ability of the isolates to ferment the following sugars: amygdalin, arabinose, cellobiose, esculin, galactose, gluconate, glucose, fructose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, surcose, trehalose, and xylose. Each isolate was identified by comparing the test results to the characteristics reported of each species of lactobacilli in Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986).

Maintenance of Isolated Cultures

All isolates were maintained by weekly subculture in MRS broth using 1% inocula and incubation at 37°C for 18 hours. They were stored at refrigeration temperature (5°C) between transfers. Each isolate was subcultured at least three

times in MRS broth just prior to experimental use. Stock cultures of each isolate were maintained by subculture in MRS agar stabs every two weeks.

Hydrogen Peroxide Production

Cells of the lactobacilli were grown in 10 ml of MRS broth (1% inoculum for 18 hours at 37°C). A 1 ml portion of the broth culture was taken for plate count on MRS agar. The cells from the remainder of the culture were harvested by centrifugation at 12,000 x g at 4°C for 10 minutes. The cells were washed twice with 9 ml volumes of cold sodium phosphate buffer (1 M, pH 6.5) and resuspended in 9 ml of cold 1 M sodium phosphate buffer (pH 6.5) containing 1% glucose. The cell suspension was inoculated (0.5 ml) into each of two tubes containing 9.5 ml of the buffer containing 1% glucose. The tubes were incubated at 5°C. After 1 hour and 24 hours of incubation, the cells were removed by centrifugation at 12,000 x g at 4°C for 10 minutes and the supernatants were assayed for hydrogen peroxide.

Hydrogen Peroxide Assay.

Five ml of sample were mixed with 0.1 ml of 1% aqueous O-dianisidine (Sigma Chemical Co.), and 1ml of 0.001% aqueous peroxidase (Horseradish Type VI-A; Sigma Chemical Co.) in clean test tubes. A blank was prepared containing 5 ml of sodium phosphate buffer containing 1% glucose instead of the sample.

The tubes were incubated for 10 minutes at 37°C. The reaction was stopped by adding 0.2 ml of 4 N HCl to each tube. Absorbance reading (A_{400nm}) of each sample was determined and peroxide content was determined by comparing the A_{400nm} to a standard curve (Gilliland, 1969).

Selection of Most Active Isolates

The isolates from each source were compared for H_2O_2 production in groups of 3 to 10 depending on the number obtained from each enrichment procedure for each sample of raw milk. The best isolates identified *as Lb. delbrueckii* subsp. *lactis* from each group were selected for further comparison to select the isolates capable of producing the most H_2O_2 at 5°C. Only those identified *as Lb. delbrueckii* subsp. *lactis* were considered since preliminary observations indicated they were the most active isolates at producing H_2O_2. Because of a limitation on the number of isolates that could be compared in a group, these were evaluated in two groups to narrow the selection of the best isolates. From each of these two groups the best 3-4 were selected for comparison with *Lb. delbrueckii* subsp. *lactis* I used in an earlier study (Brashears *et al*, 1998).

The selected isolates of *Lb. delbrueckii* subsp. *lactis* were tested for H_2O_2 production following the procedure outlined in a previous paragraph except 5 mM glucose and 5 mM sodium lactate were used in place of 1% glucose. Two tubes for each substrate (i.e. glucose and sodium lactate) were prepared and incubated

for 24 at 5°C.

Statistical Analysis

For purposes of statistical analysis, each raw milk sample was considered as a treatment. The isolates obtained from each sample were considered as an experimental unit. The two or three replicate experimental tests were considered as subsampling since they were conducted using the same isolates.

The data from the replications of experiments were analyzed using general linear model (GLM) procedures from SAS® (SAS® Institute Inc., 1985) to determine if differences existed among isolates or treatments. The least significant different (LSD) procedure was used to separate means.

RESULTS

Incidence of *Lactobacillus delbrueckii* subsp. *lactis*

Gram-positive, catalase negative, rod-shaped bacteria were assumed to be lactobacilli. One hundred and one isolates of lactobacilli were obtained from thirty-six raw milk samples (Table 1). Only nineteen of these isolates were identified as *Lb. delbrueckii* subsp. *lactis* (Listed in appendix A). All but two of the remaining isolates were identified as *Lb. fermentum*, and the other two were identified as *Lb. casei*. No isolates obtained from raw milk samples from private dairy farms were identified as *Lb. delbrueckii* subsp. *lactis*. Eighteen isolates out of forty-eight isolates of lactobacilli from the tankers of the commercial dairy processor were identified as *Lb. delbrueckii* subsp. *lactis*. One out of fifty-one lactobacilli isolated from the dairy cattle center was identified as *Lb. delbrueckii* subsp. *lactis*.

Comparison of Isolates for Hydrogen Peroxide Production at 5°C

The amount of H_2O_2 produced varied among isolates of lactobacilli from each sample of raw milk. As example, the results obtained for isolates from tanker 2 at the commercial dairy processing plant are shown in Table 2. The amounts of H_2O_2 detected after 24 hours were considerably higher than at 1 hour for all isolates except T2-6, which produced very little. The levels of H_2O_2 ranged from

0.1 ug/10^9 CFU to 0.5 ug/10^9 CFU at 1 hour and from 0.1 ug/10^9 CFU to 3.4 ug/10^9 CFU at 24 hours. Isolate T2-5 produced significantly ($P < 0.05$) more H_2O_2 than did T2-2, T2-3, T2-4, and T2-6. Isolates T2-2, and T2-3 both produced more ($P < 0.05$) than did isolates T2-4, and T2-6. In addition, T2-2 and T2-5 were identified as *Lb. delbrueckii* subsp. *lactis*, and the others were identified as *Lb. fermentum*.

Similar variations were obtained for other isolates of lactobacilli from other sources (Tables 7-20 in appendix B). Generally speaking, the isolates identified as *Lb. delbrueckii* subsp. *lactis* from individual samples, produced more ($P < 0.05$) H_2O_2 than did those isolates identified as other species of *Lactobacillus*. Thus all nineteen isolates identified as *Lb. delbrueckii* subsp. *lactis* were selected for further comparison.

The isolates were arbitrarily divided into two groups for initial comparison. The first group contained ten isolates and the second group contained nine isolates (Table 3 and 4). In the first group (Table 3) the levels of H_2O_2 ranged from 0.2 ug/10^9 CFU to 3.4 ug /10^9 CFU after 24 hours at 5°C. Strain T1-9 produced significantly ($P < 0.05$) more H_2O_2 than all the other isolated strains except T4-8. Strain T4-8 did not produce significantly ($P > 0.05$) more H_2O_2 than T4-7, and T4-1 but did produce more ($P < 0.05$) than did strains T1-3, T1-5, T4-4, T1-1, T6-1, and T6-5. Isolates T1-9, T4-8, T4-7, and T4-1 were selected for comparison with the best isolates from group 2. In the second group (Table 4), isolate T2-5

produced 4.7 ug/10^9CFU in 24 hours, which was significantly greater ($P < 0.05$) than the other strains. Strain C4409-16 and T5-2 produced the second and third most H_2O_2, although isolate T5-2 was not significantly better than isolate T2-2. Isolates T2-5, C4409-16, and T5-2 were selected for comparison with the best isolates of *Lb. delbrueckii* subsp. *lactis* in group 1.

The best isolates from the two groups of *Lb. delbrueckii* subsp. *lactis* (C4409-16, T1-9, T2-5, T4-1, T4-7, T4-8, and T5-2) were compared along with *Lb. delbrueckii* subsp. *lactis* I for H_2O_2 production in phosphate buffer containing either 5 mM glucose or 5 mM sodium lactate at 5°C (Table 5). Villegas, and Gilliland (1998) reported that *Lb. delbrueckii* subsp. *lactis* I produced H_2O_2 in buffer containing either sodium lactate or glucose. Isolate T2-5 produced significantly more ($P < 0.05$) H_2O_2 than the other six isolates and *Lb. delbrueckii* subsp. *lactis* I in buffer containing either glucose or sodium lactate. Strain T2-5 produced 7.0 ug/10^9 CFU in phosphate buffer containing 5 mM sodium lactate, which was significantly ($P < 0.05$) more than it produced (4.4 ug/10^9 CFU) in buffer containing 5 mM glucose. *Lactobacillus delbrueckii* subsp. *lactis* I produced 1.1 ug/10^9 CFU in phosphate buffer containing 5 mM glucose and buffer containing 5 mM sodium lactate. All of the strains except *Lb. delbrueckii* subsp. *lactis* I showed a significant difference ($P < 0.05$) in the amount of H_2O_2 produced in buffer containing glucose or sodium lactate. Isolates T2-5, T4-1, T4-7, and T1-9, all produced higher amount of H_2O_2 in buffer containing 5 mM sodium lactate

than in the buffer containing 5 mM glucose. Isolates T4-8, T5-2, and C4409-16, all produced significantly higher level of H_2O_2 in the buffer containing glucose than in the buffer containing sodium lactate.

TABLE 1

INCIDENCE OF *LACTOBACILLUS DELBRUECKII* SUBSP. *LACTIS* AMONG LACTOBACILLI ISOLATED FROM RAW MILK SAMPLES OBTAINED FROM DAIRY CATTLE CENTER AT OSU, A COMMERCIAL DAIRY PROCESSOR, AND PRIVATE FARMS

Sources	**Number of Raw Milk Sample**	**Numbers of Isolates**	
		Lactobacilli	***Lactobacillus delbrueckii* subsp. *lactis***
Dairy Cattle Center at OSU	18	51	1
Commercial Dairy Processor	10	48	18
Private farms	8	2	0
Total	36	101	19

TABLE 2

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM RAW MILK[1] INCUBATED AT 37°C FROM MILK TANKER NUMBER 2

Isolates	**Identity**	**H_2O_2 Produced [2] (ug /10^9 CFU)**	
		1 Hour	**24 Hours**
T2-5	*Lb. delbrueckii* subsp. *lactis*	0.5 [a]	3.4 [a]
T2-2	*Lb. delbrueckii* subsp. *lactis*	0.2 [b]	2.1 [b]
T2-3	*Lb. fermentum*	0.3 [b]	1.9 [b]
T2-4	*Lb. fermentum*	0.2 [b]	0.6 [c]
T2-6	*Lb. fermentum*	0.1 [c]	0.1 [c]

[1] Raw milk samples incubated at 37°C until Gram-positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour} = 0.28$; $SE_{24\ hours} = 0.03$)from two replicate experiments.

[a, b, c] Values in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 3

COMPARISON OF ISOLATES OF *LACTOBACILLUS DELBRUECKII* SUBSP. *LACTIS* STRAINS (GROUP 1) FOR THE PRODUCTION OF HYDROGEN PEROXIDE AT 5°C[1]

Isolates	H_2O_2 Produced [2] (ug /10^9 CFU)
	24 Hours
T1-9	3.4 [a]
T4-8	2.9 [a, b]
T4-7	2.7 [b]
T4-1	2.5 [b, c]
T1-3	2.0 [c, d]
T1-5	1.8 [d, e]
T4-4	1.3 [e, f]
T1-1	1.2 [f]
T6-1	1.1 [f]
T6-5	0.2 [g]

[1] In 1M phosphate buffer (pH 6.5) containing 1 % glucose incubation; 24 hours incubation

[2] Each value is a mean ($SE_{24\ hours} = 0.14$) form three replicate experiments.

[a, b, c, d, e, f, g] Values without common superscript letters differ significantly ($P < 0.05$).

TABLE 4

COMPARISON OF ISOLATES OF *LACTOBACILLUS DELBRUECKII* SUBSP. *LACTIS* STRAINS (GROUP 2) FOR THE PRODUCTION OF HYDROGEN PEROXIDE AT 5°C [1]

Isolates	**H_2O_2 Produced [2] (ug /10^9 CFU)**
	24 Hours
T2-5	4.7 [a]
C4409-16	3.9 [b]
T5-2	2.8 [c]
T2-2	2.2 [c, d]
T2-9	2.0 [d]
T3-2	1.7 [d, e]
T3-9	1.1 [e]
T5-4	1.1 [e]
T3-1	1.0 [e]

[1] In 1M of phosphate buffer (pH 6.5) containing 1 % glucose; 24 hours incubation.

[2] Each value is a mean ($SE_{24\ hours}$ = 0.19) from three replicate experiments.

[a, b, c, d, e] Values without common superscript letters differ significantly ($P < 0.05$).

TABLE 5

HYDROGEN PEROXIDE PRODUCTION BY CELLS OF *LACTOBACILLUS DELBRUECKII* SUBSP *LACTIS* AT 5°C IN BUFFER CONTAINING EITHER 5 mM GLUCOSE OR 5 mM SODIUM LACTATE[1]

Isolates [2]	H_2O_2 Produced (ug /10^9 CFU)	
	5mM Glucose	5mM Sodium Lactate
T2-5	4.4 [b]	7.0 [a]
T1-9	2.8 [g]	3.4 [c]
T4-7	2.9 [f]	3.2 [d]
T4-1	2.0 [j]	3.0 [e]
T5-2	3.4 [c]	2.4 [i]
C4409-16	2.5 [h, i]	1.7 [k]
T4-8	2.5 [h, i]	1.4 [l]
Lb. delbrueckii subsp *lactis* I	1.1 [m]	1.1 [m]

[1] 1 M sodium phosphate buffer (pH 6.5) containing 5 mM glucose or 5 mM sodium lactate; 24 hours at 5°C.

[2] Isolates T1-9, T4-7, T4-1, and T4-8 were from Group 1; isolates T2-5, T5-2, and C4409-16 were from Group 2. Each value is a mean ($SE_{24\ hours}$ = 0.02) from two replicate experiments.

[a, b, c, d, e, f, g, h, i, j, k, l, m] Values in rows and/or column followed by common superscript letter do not differ significantly ($P < 0.05$).

DISCUSSION

There was variation in the species of lactobacilli isolated from different raw milk sources. More *Lb. delbrueckii* subsp *lactis* cultures were isolated from the raw milk tankers of a commercial dairy processor than from individual cows on the dairy farms. This may have been due to the milk in the tankers having come from dairy farms from different geographical regions and from many individual cows. Whereas, the raw milk samples obtained from the local dairy farms and OSU Dairy Cattle Center were from different individual cattle.

Among isolates of lactobacilli, *Lb. delbrueckii* subsp *lactis* generally produced higher levels of H_2O_2 than did species of other lactobacilli from the raw milk. This species of *Lactobacillus* has been recognized as producing greater amounts of H_2O_2 at refrigeration temperature than other lactobacilli (Gilliland, 1980).

Results from this study showed tremendous variation among strains of *Lb. delbrueckii* subsp. *lactis* in the production of H_2O_2 at 5°C. Among them, strain T2-5 produced significantly ($P < 0.05$) more than the others. Strain T2-5 produced approximately seven times more H_2O_2 than did *Lb. delbrueckii* subsp *lactis* I when inoculated in phosphate buffer containing 5 mM sodium lactate, and four times more H_2O_2 than did *Lb. delbrueckii* subsp *lactis* I in buffer containing 5 mM glucose was used. *Lacbacillus delbrueckii* subsp *lactis* I produced enough H_2O_2 in

an earlier study to inhibit *Escherichia coli* O157:H7 in refrigerated raw chicken (Brashears *et al*, 1998). Thus, since strain T2-5 produced more H_2O_2 than strain *Lb. delbrueckii* subsp *lactis* I, it has potential of being even more effective than strain *Lb. delbrueckii* subsp *lactis* I as bioperservation agent for refrigerated foods.

Four strains of *Lb. delbrueckii* subsp *lactis* produced greater amounts of H_2O_2 on sodium lactate than on glucose. Just the opposite was observed for three other strains in that more was formed on glucose than on lactate. This suggests that different enzymatic systems may be dominant in the strains. Villegas and Gilliland (1998) reported at least two enzymes to be active in *Lb. delbrueckii* subsp *lactis* I resulting in the accumulation of H_2O_2 at refrigeration temperature. Nicotinamide adenine dinucleotide (reduced form, NADH) oxidase was present. It forms H_2O_2 during its oxidation of NADH. Lactate oxidase which forms H_2O_2 during its oxidation of lactate also was indicated.

In the study by Villegas and Gilliland (1998) little H_2O_2 was produced by the lactobacilli in phosphate buffer alone. Thus to maximize H_2O_2 production by *Lb. delbrueckii* subsp *lactis* on refrigerated foods the addition of glucose or sodium lactate may be advantageous. Since it produced more H_2O_2 on sodium lactate than on glucose, selection of *Lb. delbrueckii* subsp *lactis* T2-5 as a biopreservative agent in conjunction with sodium lactate could offer greater potential. Furthermore, lactate would be less likely to serve as an energy source for growth other bacteria present on the food that might grow and cause spoilage at

storage temperature.

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APPENDIX A

IDENTIFICATION OF ISOLATES

TABLE 6

IDENTIFY OF CHARACTERISTIC OF ISOLATES IDENTIFIED AS *LACTOBACILLUS DELBRUECKII* SUBSP. *LACTIS* [1]

Test	Bergey [2]	*Lb. lactis* I	C4409-16	T1-1	T1-3	T1-5	T1-9
Amygdalin	+	-	-	+	+	-	+
Arabinose	-	-	-	-	-	-	-
Cellobiose	d	-	-	-	+	-	+
Esculin	+	+	-	+	+	-	+
Galactose	d	-	-	+	+	+	-
Gluconate	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-
Mannose	+	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-	-
Melibiose	-	-	-	+	-	-	-
Raffinose	-	-	-	+	-	-	-
Rhamnose	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-
Salicin	+	-	-	-	+	-	-
Sorbitol	-	-	-	-	-	-	-
Surcose	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-

[1] All isolates were Gram positive, catalase negative rod shaped bacteria that grew at 45°C but not 15°C

[2] Reaction for *Lb. delbrueckii* subsp. *lactis* as listed in Bergey's Manual of Systematic Bacteriology

TABLE 6

IDENTIFY OF CHARACTERISTIC OF ISOLATES IDENTIFIED AS *LACTOBACILLUS DELBRUECKII* SUBSP. *LACTIS* [1]

Test	T2-2	T2-5	T2-9	T3-1	T3-2	T3-9	T4-1
Amygdalin	-	-	-	+	+	+	-
Arabinose	-	-	-	-	-	-	-
Cellobiose	-	-	-	+	+	+	-
Esculin	+	+	+	+	+	+	-
Galactose	+	+	+	+	+	+	+
Gluconate	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-
Mannose	+	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-	-
Melibiose	+	+	+	+	+	+	-
Raffinose	+	+	+	+	+	+	-
Rhamnose	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-
Salicin	-	-	-	+	+	+	-
Sorbitol	-	-	-	-	-	-	-
Surcose	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-

[1] All isolates were Gram positive, catalase negative rod shaped bacteria that grew at 45°C but not 15°C

TABLE 6

IDENTIFY OF CHARACTERISTIC OF ISOLATES IDENTIFIED AS *LACTOBACILLUS DELBRUECKII* SUBSP. *LACTIS* [1]

Test	T4-4	T4-7	T4-8	T5-2	T5-4	T6-1	T6-5
Amygdalin	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	+
Esculin	-	-	+	+	+	+	+
Galactose	+	+	+	+	+	-	+
Gluconate	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-
Mannose	+	+	+	+	+	+	+
Melezitose	-		-	-	-	-	-
Melibiose	-	-	+	+	+	+	-
Raffinose	-	-	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-
Ribose	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	+
Sorbitol	-	-	-	-	-	-	-
Surcose	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+
Xylose	-	-	-	-	-	-	-

[1] All isolates were Gram positive, catalase negative rod shaped bacteria that grew at 45°C but not 15°C

APPENDIX B

DATA FROM ISOLATES FROM EACH RAW MILK SAMPLE

TABLE 7

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM INOCULATED RAW MILK[1] FROM DAIRY COW NUMBER 4609

Isolates	Identity	H_2O_2 Produced[2] ($ug/10^9$ CFU)	
		1 Hour	24 Hours
C4609-7	*Lb. fermentum*	0.10 [a]	0.11 [a]
C4609-5	*Lb. fermentum*	0.08 [a, b]	0.09 [a, b]
C4609-1	*Lb. fermentum*	0.07 [a, b]	0.09 [a, b]
C4609-9	*Lb. fermentum*	0.08 [a, b]	0.08 [a, b]
C4609-2	*Lb. fermentum*	0.09 [a, b]	0.08 [a, b]
C4609-6	*Lb. fermentum*	0.07 [a, b, c]	0.07 [b]
C4609-8	*Lb. fermentum*	0.05 [c]	0.06 [b]
C4609-4	*Lb. fermentum*	0.06 [a, b, c]	0.06 [b]
C4609-3	*Lb. fermentum*	0.04 [c]	0.06 [b]
C4609-10	*Lb. fermentum*	0.05 [b, c]	0.06 [b]

[1] Raw milk samples incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour} = 0.01$; $SE_{24\ hours} = 0.01$) from two replicate experiments.

[a, b, c] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 8

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM INOCULATED RAW MILK[1] FROM DAIRY COW NUMBER 4558, 4628, AND 4464

Isolates	**Identity**	**H_2O_2 Produced[2] ($ug/10^9$ CFU)**	
		1 Hour	**24 Hours**
C4558-4	*Lb. casei*	4.26 [a]	4.88 [a]
C4558-2	*Lb. casei*	2.55 [b]	3.03 [b]
C4628-1	*Lb. fermentum*	0.28 [a]	1.67 [a]
C4629-10	*Lb. fermentum*	0.19 [b]	0.51 [b]
C4464-5	*Lb. fermentum*	0.16	0.85

[1] Raw milk samples incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour\ for\ C4558} = 0.39$; $SE_{24\ hours\ for\ C4558} = 0.47$; $SE_{1\ hour\ for\ C4628} = 0.02$; $SE_{24\ hours\ for\ C4628} = 0.24$) from two replicate experiments.

[a, b] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 9

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM INOCULATED RAW MILK[1] FROM DAIRY COW NUMBER 4533

Isolates	Identity	H_2O_2 Produced[2] (ug/10^9 CFU)	
		1 Hour	24 Hours
C4533-10	*Lb. fermentum*	0.80 [a]	5.87 [a]
C4533-1	*Lb. fermentum*	0.39 [b]	1.38 [b]
C4533-2	*Lb. fermentum*	0.34 [b]	1.26 [b]
C4533-3	*Lb. fermentum*	0.33 [b]	0.99 [b]
C4533-9	*Lb. fermentum*	0.28 [b]	0.91 [b]

[1] Raw milk samples incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour} = 0.06$; $SE_{24\ hours} = 0.69$) from two replicate experiments.

[a, b] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 10

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM INOCULATED RAW MILK[1] FROM DAIRY COW NUMBER 4638

Isolates	**Identity**	**H_2O_2 Produced[2] (ug/10^9 CFU)**	
		1 Hour	**24 Hours**
C4638-4	*Lb. fermentum*	0.09 [a]	0.11 [a]
C4638-3	*Lb. fermentum*	0.06 [b]	0.08 [b]
C4638-6	*Lb. fermentum*	0.04 [c]	0.05 [c]

[1] Raw milk samples incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour} = 0.01$; $SE_{24\ hours} = 0.01$) from two replicate experiments.

[a, b, c] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 11

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM INOCULATED RAW MILK[1] FROM DAIRY COW NUMBER 4409

Isolates	Identity	H_2O_2 Produced[2] (ug/10^9 CFU)	
		1 Hour	24 Hours
C4409-6	*Lb. fermentum*	0.03 [c]	0.25 [a]
C4409-7	*Lb. fermentum*	0.09 [b]	0.10 [b]
C4409-9	*Lb. fermentum*	0.10 [a]	0.10 [b]
C4409-1	*Lb. fermentum*	0.09 [b]	0.09 [b]
C4409-2	*Lb. fermentum*	0.07 [b]	0.08 [b]
C4409-5	*Lb. fermentum*	0.07 [b]	0.08 [b]
C4409-4	*Lb. fermentum*	0.03 [c]	0.04 [c]
C4409-8	*Lb. fermentum*	0.03 [c]	0.04 [c]

[1] Raw milk samples incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour}$ = 0.01; $SE_{24\ hours}$= 0.01) from two replicate experiments.

[a, b, c] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 12

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM LACTOBACILLUS BROTH INOCULATED WITH RAW MILK[1] FROM DAIRY COW NUMBER 4409

Isolates	Identity	H_2O_2 Produced[2] ($ug/10^9$ CFU)	
		1 Hour	24 Hours
C4409-16	*Lb. delbrueckii* subsp. *lactis*	3.76 [a]	9.39 [a]
C4409-11	*Lb. fermentum*	0.23 [b]	0.47 [b]
C4409-14	*Lb. fermentum*	0.05 [b]	0.33 [b]
C4409-17	*Lb. fermentum*	0.08 [b]	0.12 [b]
C4409-20	*Lb. fermentum*	0.07 [b]	0.12 [b]
C4409-15	*Lb. fermentum*	0.04 [b]	0.08 [b]
C4409-12	*Lb. fermentum*	0.06 [b]	0.07 [b]
C4409-19	*Lb. fermentum*	0.05 [b]	0.07 [b]
C4409-18	*Lb. fermentum*	0.07 [b]	0.06 [b]
C4409-13	*Lb. fermentum*	0.04 [b]	0.05 [b]

[1] Broth (100 ml) inoculated 1 ml raw milk. Then incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour} = 0.21$; $SE_{24\ hours} = 0.48$) from two replicate experiments.

[a, b] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 13

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM INOCULATED RAW MILK[1] FROM DAIRY COW NUMBER 5

Isolates	Identity	H_2O_2 Produced[2] ($ug/10^9$ CFU)	
		1 Hour	24 Hours
C5-10	*Lb. fermentum*	3.22 [a]	4.43 [a]
C5-8	*Lb. fermentum*	0.78 [b]	2.22 [b]
C5-9	*Lb. fermentum*	0.68 [b]	2.07 [b]
C5-3	*Lb. fermentum*	0.52 [b]	1.59 [c]
C5-5	*Lb. fermentum*	0.22 [b]	0.33 [d]
C5-4	*Lb. fermentum*	0.28 [b]	0.31 [d]
C5-6	*Lb. fermentum*	0.20 [b]	0.28 [d]
C5-7	*Lb. fermentum*	0.13 [b]	0.26 [d]
C5-2	*Lb. fermentum*	0.16 [b]	0.24 [d]
C5-1	*Lb. fermentum*	0.11 [b]	0.22 [d]

[1] Raw milk samples incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour} = 0.18$; $SE_{24\ hours} = 0.39$) from two replicate experiments.

[a, b, c, , d] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 14

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM INOCULATED RAW MILK[1] FROM MILK TANKER NUMBER 1

Isolates	**Identity**	**H_2O_2 Produced[2] (ug/10^9 CFU)**	
		1 Hour	**24 Hours**
T1-1	*Lb. delbrueckii subsp. lactis*	0.30 [b]	1.77 [a]
T1-6	*Lb. fermentum*	0.42 [a]	1.32 [b]
T1-5	*Lb. delbrueckii subsp. lactis*	0.35 [b]	1.30 [b]
T1-4	*Lb. fermentum*	0.35 [b]	1.24 [b]
T1-3	*Lb. delbrueckii subsp. lactis*	0.15 [c]	0.71 [c]
T1-2	*Lb. fermentum*	0.15 [c]	0.03 [c]

[1] Raw milk samples incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour} = 0.02$; $SE_{24\ hours} = 0.10$) from two replicate experiments.

[a, b, c] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 15

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM LACTOBACILLUS BROTH INOCULATED WITH RAW MILK[1] FROM MILK TANKER NUMBER 1

Isolates	**Identity**	**H_2O_2 Produced[2] ($ug/10^9$ CFU)**	
		1 Hour	**24 Hours**
T1-9	*Lb. delbrueckii subsp. lactis*	0.41 [a]	2.27 [a]
T1-10	*Lb. fermentum*	0.06 [b]	0.06 [b]
T1-8	*Lb. fermentum*	0.04 [c]	0.04 [c]
T1-7	*Lb. fermentum*	0.04 [c]	0.04 [c]
T1-11	*Lb. fermentum*	0.04 [c]	0.03 [c]

[1] Broth (100 ml) inoculated with 1 ml raw milk. Then incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour} = 0.03$; $SE_{24\ hours} = 0.20$) from two replicate experiments.

[a, b, c] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 16

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM INOCULATED RAW MILK[1] FROM MILK TANKER NUMBER 3

Isolates	**Identity**	**H_2O_2 Produced[2] ($ug/10^9$ CFU)**	
		1 Hour	**24 Hours**
T3-9	*Lb. delbrueckii subsp. lactis*	0.29 [b]	1.79 [a]
T3-2	*Lb. delbrueckii subsp. lactis*	0.27 [b]	1.71 [a]
T3-10	*Lb. fermentum*	0.42 [a]	1.70 [a]
T3-1	*Lb. delbrueckii subsp. lactis*	0.23 [b, c]	1.61 [a]
T3-5	*Lb. fermentum*	0.30 [b]	1.44 [a, b]
T3-4	*Lb. fermentum*	0.24 [b, c]	1.36 [a, b]
T3-7	*Lb. fermentum*	0.24 [b, c]	1.29 [a, b]
T3-3	*Lb. fermentum*	0.26 [b, c]	1.18 [a, b]
T3-8	*Lb. fermentum*	0.16 [c]	0.76 [b]

[1] Raw milk samples incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour} = 0.02$; $SE_{24\ hours} = 0.09$) from two replicate experiments.

[a, b, c] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 17

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM LACTOBACILLUS BROTH INOCULATED WITH RAW MILK[1] FROM MILK TANKER NUMBER 2 AND 3

Isolates	**Identity**	**H_2O_2 Produced[2] ($ug/10^9$ CFU)**	
		1 Hour	**24 Hours**
T2-7	*Lb. fermentum*	0.46 [a]	2.29 [a]
T2-9	*Lb. delbrueckii subsp. lactis*	0.33 [a, b]	1.59 [b]
T2-8	*Lb. fermentum*	0.32 [a. b]	0.74 [c]
T2-10	*Lb. fermentum*	0.04 [b]	0.72 [c]
T3-11	*Lb. fermentum*	0.51	1.16

[1] Broth (100 ml) inoculated with 1 ml raw milk. Then incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour\ for\ T2} = 0.07$; $SE_{24\ hours\ for\ T2} = 0.17$) from two replicate experiments.

[a, b, c] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 18

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM INOCULATED RAW MILK[1] FROM MILK TANKER NUMBER 4 AND 6

Isolates	Identity	H_2O_2 Produced[2] ($ug/10^9$ CFU)	
		1 Hour	24 Hours
T4-7	*Lb. delbrueckii subsp. lactis*	1.25 [a]	3.61 [a]
T4-4	*Lb. delbrueckii subsp. lactis*	1.00 [b]	3.56 [a]
T4-1	*Lb. delbrueckii subsp. lactis*	0.61 [d]	3.03 [a, b]
T4-2	*Lb. fermentum*	0.77 [c]	2.39 [b]
T4-6	*Lb. fermentum*	0.46 [e]	1.42 [c]
T4-5	*Lb. fermentum*	0.18 [f]	1.07 [c]
T4-3	*Lb. fermentum*	0.23 [f]	0.84 [c]
T6-1	*Lb. delbrueckii subsp. lactis*	0.19	0.86

[1] Raw milk samples incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour\ for\ T4} = 0.07$; $SE_{24\ hours\ for\ T4} = 0.23$) from two replicate experiments.

[a, b, c, d, e, f] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 19

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM INOCULATED RAW MILK[1] FROM MILK TANKER NUMBER 5

Isolates	**Identity**	**H_2O_2 Produced[2] ($ug/10^9$ CFU)**	
		1 Hour	**24 Hours**
T5-2	*Lb. delbrueckii subsp. lactis*	0.23 [b, c]	2.91 [a]
T5-4	*Lb. delbrueckii subsp. lactis*	0.31 [a, b]	2.00 [a]
T5-5	*Lb. fermentum*	0.18 [c, d]	1.01 [b]
T5-1	*Lb. fermentum*	0.38 [a]	0.87 [b]
T5-6	*Lb. fermentum*	0.17 [d]	0.54 [b]

[1] Raw milk samples incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour} = 0.02$; $SE_{24\ hours} = 0.24$) from two replicate experiments.

[a, b, c, d] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 20

HYDROGEN PEROXIDE PRODUCTION AT 5°C BY ISOLATES OF LACTOBACILLI FROM LACTOBACILLUS BROTH INOCULATED WITH RAW MILK[1] FROMMILK TANKER NUMBER 4, 5 AND 6

Isolates	Identity	H_2O_2 Produced[2] ($ug/10^9$ CFU)	
		1 Hour	24 Hours
T4-8	*Lb. delbrueckii subsp. lactis*	0.50 [a]	3.63 [a]
T4-9	*Lb. fermentum*	0.33 [b]	0.62 [b]
T5-7	*Lb. fermentum*	0.04	0.05
T6-5	*Lb. delbrueckii subsp. lactis*	0.38 [a]	1.85 [a]
T6-2	*Lb. fermentum*	0.09 [b]	0.09 [b]

[1] Broth (100 ml) inoculated with 1 ml raw milk. Then incubated at 37°C until Gram positive rods predominated.

[2] Each value is a mean ($SE_{1\ hour\ for\ T4} = 0.36$; $SE_{24\ hours\ for\ T4} = 0.57$; $SE_{1\ hour\ for\ T6} = 0.06$; $SE_{24\ hours\ for\ T6} = 0.33$) from two replicate experiments.

[a, b.] Value in the same column followed by different superscript letters differ ($P < 0.05$).

TABLE 21

ANALYSIS OF VARIANCE FOR HYDROGEN PEROXIDE PRODUCTION BY CELLS OF *LACTOBACILLUS DELBRUECKII* SUBSP. *LACTIS* AT 5°C IN BUFFER CONTAINING EITHER 5 mM GLUCOSE OR 5 mM SODIUM LACTATE

Sources	**Degree of Freedom (DF)**	**Sum of Square (SS)**	**Mean Squares (MS)**	**F-Value**	**Pr > F**
Model	31	142.7322	4.6043	687.04	0.0001
Error	32	0.2145	0.0067		
Corrected Total	63	142.9466			
Strains	7	100.04452	14.2921	11.45	0.0001
Trt (Treatment)	1	0.6704	0.67035	0.54	0.4742
Strains * Trt	7	22.0507	3.1501	2.52	0.0593

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