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# Incorporation of probiotic bacteria into a Swiss cheese slurry model system

by

**Damian Paul Montoya**

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
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This is to certify that the master's thesis of

Damian Paul Montoya

has met the thesis requirements of Iowa State University

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# INTRODUCTION

The documented use of probiotic bacteria for its health benefits to humans has been known for hundreds of years (Oberman 1985). The research of these organisms did not start until the early 1900s. The investigative studies of Elie Metchnikoff and Henry Tisser reported high counts of certain bacteria in fecal matter through consumption of fermented dairy products. Research on the *Lactobacilli* and *Bifidobacterium* described by Metchnikoff and Tisser, respectively, initiated a new area of research of healthy bacteria (Metchnikoff 1908; Fuller 1992). These anaerobes, when incorporated in foods at levels greater than 6 $\log_{10}$ cfu/g, have demonstrated a number of beneficial effects based on clinical studies (Kurmann 1988). Scientific evidence has shown that a diet of certain probiotic bacteria has suppressed some symptoms of traveler's diarrhea, antibiotic-associated intestinal disorder, inflammatory bowel diseases, lactose intolerance, colon cancer, urogential infections and hypercholesterolemia (Marteau 2002).

The use of selected probiotic bacteria in food has been recognized by the FDA as Generally Recognized As Safe (GRAS) for use in dairy products and dietary supplements. The incorporation of probiotic bacteria into dairy foods has provided a carrier vehicle for transferring the probiotic bacteria to the gut. Probiotic bacteria have been incorporated into dairy products such as fresh milk, fermented milk, beverages, various cheeses, powdered milk, cookies, yogurt and other dairy desserts (Lourens-Hattingh and Vilijoen 2001).

Since probiotic bacteria are not naturally found in dairy products, there are some challenges associated with incorporating these bacteria into foods. Yogurt has been a common product with added probiotic bacteria; however, the low pH, oxygen content and storage period decreases the viability of the probiotic bacteria to a level that is not beneficial to one's health (Klaver and others 1993; Mattila-Sandholm and others 2002).

The incorporation of probiotic bacteria into cheese has provided a more suitable environment than other dairy products for delivery to the human stomach. The fat content of the cheese allows for a protective matrix for the probiotic bacteria to survive in the low pH conditions of the human gut. The pH and cheese composition can influence the survivability of probiotic bacteria. *Bifidobacterium infantis* inoculated into cream dressing of cottage cheese can remain viable for 29 days under a pH of 4.5 and salt content of 1.8%, but remains viable for only 15 days when inoculated cream dressing and curd are combined (Blanchette and others 1995, 1996). *B. bifidum* and *B. longum* were incorporated into both Crescenza and Canestrato Pugliese cheeses. *B. bifidum* was viable after storage in both cheeses, but only *B. longum* remained viable in Crescenza cheese to provide beneficial health effects (Gobbetti and others 1998; Corbo and others 2001). Argentinean Fresco, Gouda and white-brined cheese used combinations of bifidobacterium and lactobacilli. In Argentinean Fresco, *B. bifidum* and *B. longum* in combination with *Lactobacillus acidophilus* were able to survive to a level just above 6.0 $\log_{10}$ cfu/g (Vinderola and others 2000). The high salt content ranging from 2% to 4% of Gouda and white-brined cheeses affected the viability of probiotic bacteria. *B. lactis* combined with *L. acidophilus* remained viable in a high salt content after ripening and storage (Gomes and others 1995, 1998; Yilmaztekin and others 2004). Three studies were conducted on the incorporation of probiotic bacteria in Cheddar cheese. The long storage period of Cheddar cheese is a concern for the viability of the probiotic bacteria. Gardiner and other (1998) had questionable results in incorporating a mixture of bifidobacterium and lactobacilli because they used an initial inoculum level below the 6 $\log_{10}$ cfu/g. The added probiotic bacteria were viable after cheese production and remained viable for only 3 months of storage. The incorporation of bifidobacterium into Cheddar cheese showed more promising results. *B. bifidum* remained viable for 24 weeks when incorporated

into Cheddar cheese (Dinakar and Mistry 1994). *B. infantis* remained viable but was only monitored for 12 weeks (Daigle and others 1999). Salt concentration, pH and the right bacteria or mixtures of bacteria make a cheese with viable probiotic bacteria.

All of the above mentioned studies on probiotic cheeses were examined for cheese composition based on pH, moisture content and salt content. Proteolytic activity and lipolysis were examined through total Nitrogen (N) and pH 4.6-soluble nitrogen and free fatty acids, respectively. The probiotic bacteria were also monitored for lactic acid and acetic acid production and $\beta$-galactosidase activity. Little or no information on the sensory results or flavor analyses were provided to draw adequate conclusion about the effect that incorporated probiotic bacteria had on cheese quality. Fat, protein, moisture content, salt content and pH seemed to be main quality attributes that were tested in all probiotic cheeses. The increased $\beta$-galactosidase activity was influenced by the addition of probiotic bacteria to cheese (Gobbetti and others 1998). Studies on Crescenza, Canestrato Pugliese and Cheddar cheeses all reported increases in lactic and acetic acid. Proteolytic activity was increased in Crescenza and Cheddar cheese but not Canestrato Pugliese (Gardiner and others 1998; Gobbetti and others 1998; Daigle and others 1999; Corbo and others 2001).

Based on the Swiss cheese making process, there are several benefits for incorporating bifidobacteria into Swiss cheese. The pH of Swiss cheese remains close to 6.0. There is a drop in the pH to 5.2, but it is a gradual drop and the bifidobacteria are able to adjust to the acidic environment. As seen in previous papers on probiotic cheeses, the salt concentration can inhibit the growth the added probiotic bacteria. With a low salt concentration of 0.5%, bifidobacterium are able to withstand the salt and increase in bacterial counts. Unlike most cheeses, Swiss cheese contains propionibacterium which produce carbon dioxide for eyehole formation. The vacuum-packaged cheese and added carbon

dioxide production enhance the viability of bifidobacterium. The established conditions of Swiss cheesemaking provide a good candidate for a new functional food.

The objective of our study was to examine microbial and flavor attributes of Swiss cheese slurries with incorporated probiotic bacteria. Through a concurrent study, *Bifidobacterium breve*, *B. infantis*, *B. longum* and *Pediococcus acidilactici* were examined for microbial viability and flavor attributes and compared to a control Swiss cheese curd slurry. Titratable acidity, pH and free amino acids were also monitored. Our hypothesis is that *B. breve*, *B. infantis*, *B. longum* and *P. acidilactici* can be incorporated into Swiss cheese without adverse effects to the cheese flavor and remain viable to provide beneficial effects.

# Literature Review

## History of Probioitcs

The idea and use of probiotics is not novel in modern science. The earliest recorded use of probiotics dates back many centuries. Biblical scripture mentions the use of fermented milk for treatment of body ailments. In addition, Hippocrates wrote that, in addition to being a food product, fermented milk was also a medicine (Oberman 1985). The discovery of probiotics can be attributed to Louis Pasteur who first discovered anaerobic bacteria in the late 1800s. In his study of the origin of bacteria found in fermented products, Pasteur discovered bacteria that required an oxygen-free environment that he called anaerobic. Through studies of bacterium in fermented milk Pasteur isolated what we know as *Lactobacillus* (Pasteur 1909-1914). While Pasteur first isolated *Lactobacillus*, Elie Metchnikoff is credited with the discovery of the benefits of lactobacilli. While working at the Pasteur Institute in the early 1900s, Metchnikoff found that the Bulgarian peasants who consumed large quantities of fermented milk lived longer than the average person. When *Lactobacillus* was available in a pure form, Metchnikoff was able to ferment the milk to what is known as yogurt (Metchnikoff 1908; Hughes & Hoover 1991; O'Sullivan 1992). While it is difficult to know the exact bacterial strain that was used by Metchnikoff, *Lactobacillus bulgaricus* is closely related to the description of the bacterial strain in the1907 publication "The Prolongation of Life." Metchnikoff said, "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes." In a 1906 publication, French pediatrician Henry Tissier reported that children with diarrhea had low Y-shaped bacterial counts when compared to healthy children. Tissier thought that if these "bifid" Y-shaped bacteria could be given to patients with diarrhea there would be a restoration of a healthy microflora in the gut

(FAO/WHO 2001; Fuller 1992). With these first discoveries of probiotics, research has led to the understanding of the health benefits and incorporation of probiotics in food.

Even though probiotic bacteria and their benefits had been discovered in the early 20th century, the term 'probiotic' was not coined until the middle 1960s. At that time, it was described how one bacterium would help stimulate the growth of another bacterium. As advances in probiotic research came about, the definition for probiotics has also changed. In 2001, the FAO/WHO described probiotics as "Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host." The latest definition, coined in 2002 by Marteau and others described probiotics as "microbial cell preparations or components of microbial cells that have a beneficial effect on health and well-being" (Fioramonti and others 2003). While each of the definitions may differ, the benefits that probiotics organisms provide are generally linked to health improvement.

## Bifidobacterium

### History

The genus *Bifidobacterium* was discovered by Orla-Jensen and was described in a 1924 publication *La classification des bacteries* lactiques. The bacterium was named on the basis of its appearance of Y or V forms when freshly isolated from fecal matter from humans and animals. Reuter (1963) described seven new species of *Bifidobacterium* when performing a comparative study of microflora of infant and adults stools for this organism. These species include *Bifidobacterium adolescentis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium lactentis, Bifidobacterium liberorum, Bifidobacterium longum* and *Bifidobacterium parvulorum*. Species of *Bifidobacterium* were not described in Orla-Jensen's article in accordance with the bacteriological code of 1924.

## Characteristics of Bifidobacterium species used in the present study

### Morphology

*Bifidobacterium breve*

*B. breve* are short, slender or thick, commonly club-shaped rods. They are not in a bifid shape that is characteristic of *Bifidobacterium*. The bacteria are gram-positive and may autoagglutinate in saline. The original strain was isolated from an infant's intestine. Colonies of this organism are convex, smooth, 2-3 mm in diameter and exhibit a paste-like consistency (Reuter 1971; Buchanan and Gibbons 1974).

*Bifidobacterium infantis*

*B. infantis* are short, thin, spherically-shaped cells and contain central granules. Generally, they show no bifurications. *B. infantis* differs in morphology from other strains based on media type and testing conditions. *B. infantis* was said to originate in feces of infants that were breast-fed. The bacterium is gram-positive when stained and do not clump together in a saline solution. The colonies are convex, 2-3 mm in diameter, and soft, moist or paste-like in consistency (Reuter 1971, Buchanan and Gibbons 1974).

*Bifidobacterium longum*

*B. longum* is characterized by their long, curved, club-shaped, swollen or dumb-bell shaped rods which may show bifid shape. The bacterium can give a variable reaction to gram staining but are noted to be gram-positive. Colonies of this organism are convex, soft, moist and shiny or slimy in nature. *B. longum* are also well dispersed in nature when in a saline solution. The bacteria were isolated from the intestine of an adult (Reuter 1971; Buchanan and Gibbons 1974).

### Growth Requirements

All three strains of *Bifidobacterium* grow best under anaerobic conditions with a slight tolerance to oxygen when exposed. The bacterium grows best at 37°C and will not grow at above 46.5°C or below 20°C (Reuter 1971; Buchanan and Gibbons 1974).

## Pediococci

### History

*Pediococcus acidilactici* was isolated from fermented plant material. It is commonly found in spoiled beer, and its presence is dependent upon certain fermentation characteristics. While the existence of the *acidilactici* species had been known for some time, there was no real differentiation between *Pediococus pentosaceus* and *Pediococcus acidilactici.* With the development of technology for studying the DNA of bacteria, researchers were finally able to give a genetic distinction between the two species (Mundt and others 1969; Garrity 2001).

### Morphology

*P. acidilactici* show similar characteristics to all *Pediococci.* The bacterium is spherical in shape and is never elongated. Division of the cells occurs in an alternating pattern of two planes at right angles. The smooth, round, grayish-white colonies of the cocci range in size from 1.0 to 2.5 mm diameter. *Pediococci* are gram-positive and nonmotile (Garrity 2001; Mundt and others 1969).

### Growth Requirements

*Pediococcus acidilactici* is an anaerobic organism but is quite tolerant of oxygen. Its growth rate is seen to be similar in aerobic and anaerobic environments. Amino acids are important factors for growth. Methionine is the only amino acid that is not required. There are also several essential vitamins required for growth which include riboflavin, pyridoxine,

pantothenic acid, niacin and biotin. The optimal growth temperature is 40°C, but the bacteria can withstand temperatures up to 52°C (Garrity 2001, Mundt and others 1969).

## Clinical Characteristics and Functionality of Probiotic Foods

Marteau (2002) published a review of studies and health claims from periodic probiotic consumption. The health claims included reduction of diarrhea for antibiotic-associated disorders, gastroenteritis, lactose intolerance, intestinal infection and colonization by pathogenic bacteria, traveler's diarrhea, irritable bowel disease and inflammatory bowel diseases. In addition, there were health claims for other clinical conditions such as colon cancer, urogenital infection and tumors, allergies and hypercholesterolemia. Although there have been many health claims, none of the health claims have been supported by strong scientific evidence.

With the discovery of probiotics, researchers have analyzed probiotic mechanistic and physiological properties within the human gut. Whether in the form of a dietary supplement or food, the probiotic bacteria must survive the physiological conditions of the human body. Proteolytic enzymes, decreases in pH, bile and other salts and mucus can adversely affect the survival of the probiotic bacteria. To initiate a physiological effect, the probiotic bacteria must adhere to the intestinal wall where normal microflora is found (Widmaier and others 2004).

### Inhibition of Helicobacter pylori

The use of probiotics in a clinical setting has provided scientific proof of the health benefits that can be obtained through a probiotic diet. While clinical trials take time, researchers have proven their objectives through both *in vivo* and *in vitro* studies. Felly and Michetti (2003) performed cellular studies with *Helicobacter pylori* in the presence of

*Lactobacillus* spp. The results of the study demonstrated that lactic acid produced from *Lactobacillus johnsonii* La I was responsible for the suppression of *H. pylori* both *in vitro* and *in vivo.*

**Lactose Intolerance**

Lactose intolerance affects millions of people around the world. Many babies are diagnosed with lactose intolerance after weaning. This group of people lacks the enzyme for breaking down the milk sugar, lactose, into a form, glucose and galactose, which can be used by the body. Typical symptoms of lactose intolerance include bloating, cramping, flatulence, diarrhea and an increase in breath hydrogen (Suarez and others 1997). Studies have shown that through the addition of certain starter cultures to dairy products, lactose intolerant individuals can consume dairy products with reduced symptoms (Scheinbach 1998). It is not certain of how probiotics aid in relief of lactose intolerance, but it is know that certain strains of probiotics bacteria ferment lactose (Ouwehand and other 2002). Studies have shown variable results on the role of probiotics in lactose intolerance. One consistent factor is that *Streptococcus thermophilus* and *Lactobacillus bulgaricus* have shown positive results for breakdown of lactose. Martini and others (1991) reported no effect for *L. acidophilus* and *B. bifidus* for reduction of symptoms of lactose intolerance. Previous research reported that sonication of *Lactobacilli* and *Bifidobacterium* cells prior to addition into dairy products significantly promoted lactose breakdown (McDonough and others 1987a, 1987b).

**Traveler's Diarrhea**

A common problem for world travelers is diarrhea from food or water from another country. Native people have developed an immunity to microbial contaminants in the food and water of their native country, but this is not always the case for foreigners. Travelers will often have diarrhea that is not always treatable with antibiotics. Oksanen and others

(1990) focused their study on 820 travelers to southern Turkey. When returning from their vacations, each subject filled out a survey related to diarrhea and other symptoms. There was an 11.8% decrease in diarrhea for those subject consuming *Lactobacillus rhamnosus* GG. This study did not provide the variability that consuming *L. rhamnosus* GG would reduce diarrhea for travelers to other countries or that *L. rhamnosus* GG will be effective for all travelers to Turkey. While consuming *L. rhamnosus* GG was effective for some travelers to Turkey, *L. rhamnosus* GG may not be effective for the same traveler with diarrhea when visiting another country. Destination of travel and strain of probiotic bacterium may contribute to variabilities in the effectiveness of the probiotics treatment. To determine the effect of probiotic bacteria on reducing the incidence diarrhea, 400 American travelers were given either *Lactobacillus rhamnosus* GG or a placebo to consume during the duration of their travel in a non-controlled trial. For the travelers that reported back, the incidence of diarrhea was only 3.9% for subjects taking the probiotics compared to 7.4% for subjects taking the placebo (Marteau 2002; Hilton and other 1997).

**Antibiotic-Associated Intestinal Disorders**

With many forms of antibiotics available to relieve infections or disease in the gastrointestinal tract, there is concern about the overuse of antibiotics and the development of immunity to these anitbotics. With this recent trend, there is a renewed interest in studying an alternative form of medicine or a safer form of an antibiotic. Specific emphasis has been placed on determining the effects of probiotics within the gastrointestinal tract and the identification of strains that are best suited to treat gastrointestinal disorders (Novorgrudsky and Plant 2003). Within the last 20 years, there has been an influx of research to address these points. Diarrhea is a symptom of resistance to antibiotics when overused. The results

have shown a reduction of diarrhea following the regular consumption of probiotics (Elmer and other 1996).

D'Souza and others (2002) reviewed clinical studies where a probiotic bacterium was used to prevent or treat diarrhea resulting from antibiotic usage. From the nine studies that were reviewed, *Clostridium difficile* was identified the causative agent for the diarrhea symptoms. *Saccharomyces boulardii* or *Lactobacillus* GG were the probiotics used to counter the effects of *C. difficile*. Although these probiotics may prevent antibiotic-associated diarrhea, further studies need to be performed on other probiotic species.

**Inflammatory Bowel Diseases**

Chronic intestinal inflammation and diarrhea from inflammatory bowel diseases (IBD), such as Crohn's disease, ulcerative colitis and pouchitis, are the common diseases requiring suppression through antibiotics. The cause of IBD is unknown; the inflammation is thought to have occurred in response to changes in endogenous microflora (Shanahan 2001). Although there is currently not a cure for these diseases, suppression of the inflammation can be a relief to many patients. In a blind study, 28 patients suffering from Crohn's disease were given either *E. coli* Nissle 1917 or a placebo. The results showed that 33% of the patients consuming probiotics went into relapse in comparison to the 66% of the patients consuming a placebo (Malchow 1997). A recently developed dietary supplement called VSL #3 is under clinical trials in the United States. VSL#3 is a freeze-dried mixture of probiotics which includes *Streptococcus*, *Bifidobacterium* and *Lactobacilli*. VSL #3 has shown some promising results in the treatment of IBD (Madsen 2001).

**Colon Cancer**

Studies have shown that high fat diets increase the incidence of colon cancer through stimulating increased production of bile acids. Bacterial metabolism of bile acids has been

the proposed factor for promoting colon cancer (Reddy and others 1977). Rafter (2003) reviewed studies addressing probiotics and colon cancer. In animal studies, a diet with *Bifidobacterium longum* and *B. inulin* showed a decrease in azoxymethane (AOM)-induced colonic small aberrant crypt foci (ACF) (Rowland and other 1998). Reddy and others (1993) reported that lyophilized cultures of *B. longum* fed to rats inhibited liver, colon and mammary tumors induced by 2-amino-3-methyl-3H-imidazo (4, 5-f) quinoline (IQ). *B. infantis* and *B. adolescentis* inhibited 3-methylcholanthrene-induced tumors when the probiotic bacterium was injected subcutaneously or intraperitroneally into BALB/c mice (Kohwi and other 1978).

The potential health benefits of probiotics have been studied in humans on a 3-day fried meat diet. A difference was examined in subjects given *L. acidophilus* in comparison to fermented milk. Fecal matter from the subjects consuming fermented milk at day 3 had higher levels of mutagenic bacteria than the subjects consuming *L. acidophilus* during the 3 day period (Lidbeck and other 1992). Because the probiotic diet reduced the amount of mutagens, there may be a reduction in the risk of colon cancer. While it is not known how the bacteria inhibit cancer, it is suggested that probiotic bacteria compete with the intestinal flora for nutrients and reduce the growth of mutagenic bacteria and toxins. Probiotic bacteria are able to survive in the presence of the normal gut flora without affecting the normal colony counts (Rafter 2003).

**Atopic Eczema**

While many of the previous studies are related to symptoms associated with the GI tract, the use of probiotics in immunology is being further investigated and has shown some promising results. Previous studies demonstrated protection from the development of certain allergies but the exact mechanism is still unknown (Kalliomaki 2001). Isolauri and others

(2000) were interested in giving probiotic bacteria to expecting mothers that have an immediate relative with atopic eczema, allergic rhinitis or asthma. *Lactobacillus rhamnosus* GG or placebo was randomly given to the expecting mother for the full term of their pregnancy and to the infant from birth until 6 months of age. In the study, 46 of the 132 children were diagnosed with atopic eczema at age 2. Those children taking the placebo were twice as likely to develop eczema in comparison to those with a probiotic diet.

**Urogenital infection**

Although most studies have focused on disorders associated with the GI tract, probiotic research has been conducted in other areas of medicine. Urogential infections can be a common problem in any healthy woman. Recent studies have shown that a probiotic diet has relieved the symptoms of urogenital infection. Reid and others (2001) chose 10 women based on their history of recurrent yeast vaginitis, bacterial vaginosis and urinary tract infections. The women were fed *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14, resuspended in milk, twice a day for 14 days. Daily vaginal swabs were plated on MRS agar and colonies were subcultured to examine bacterial DNA to test for vaginitis and *Lactobacilli* strains. All women in the study showed a relief in their symptoms. In addition, *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamosus* GR-1 were shown to provide healthy microflora for reduction of the infection.

**Hypercholesterolemia**

Probiotic bacteria may also reduce cholesterol levels in hypercholesterolemic patients. Scheinbach (1998) reviewed studies on the reduction of cholesterol showing conflicting or variable results in animals and humans. Probiotic strain, patient symptoms and dosage were not controlled in these studies. The administration of probiotic bacterium has reduced the cholesterol content in pigs but not in rats (Fletcher 1995). A recent study

examined the cholesterol level of hypocholesterolemic women when fed 300 g of yogurt with or without probiotics. There was no significant difference in cholesterol levels of either test group (Kießling and others 2002). Richelsen and others (1996) discovered that not all subjects demonstrated reduced LDL-cholesterol levels when consuming probiotics on a regular basis. There are two proposed mechanism for the lowering of cholesterol through probiotic bacteria, but there is no proof that a probiotic bacterium reduces cholesterol levels in humans. One mechanism proposes cholesterol is used in the formation of bile salts. Probiotic bacteria will function like bile-salt hydrolase (BSH) and breakdown the bile salts into a form that can be excreted. The body then has to use serum cholesterol to make bile salts to compensate for this decrease in the bile salt pool.

## Incorporation of Probiotic Bacteria into Yogurt

### Functional Foods

Numerous laboratory and clinical trials have demonstrated many health benefits of probiotics. The major questions from many of the clinical studies is which strains of probiotics work best for suppressing or inhibiting a particular disease. While FDA regulations are strict, there are probiotic strains that are approved for use in foods. Nestle (Lausanne, Switzerland) has gained FDA approval for the use of *Streptococcus thermophilus* and *Bifidobacterium lactis* in formula for infants older than 4 months. Actimel, a fermented milk beverage containing *Lactobacillus casei*, is available in natural grocery stores around the U.S. (Sanders 2003). The safety of probiotics is a concern to consumers since it is a live organism that is consumed for health benefit. Few cases have reported septicemia and endocarditis from consumption of lactobacilli, bifidobacterium or other lactic acid bacteria. The majority of safety concerns entail *Enterococci faecium* and *Enterococci faecalis*, which

have been incorporated into probiotic mixtures (Marteau 2001). While there have been documented cases of infections associated with probiotic treatments; in each case, the source of contamination was from environmental sources.

The application of probiotics in functional foods has provided a carrier system for delivery of probiotic bacteria to the human gut. There are numerous dairy products that function as vehicles for transferring the probiotics. These products include fresh milk, fermented milk, beverages, various cheeses, powdered milk, cookies, yogurt and other dairy desserts (Lourens-Hattingh and Vilijoen 2001).

**Challenges**

Yogurt is a product that has become popularly known for containing probiotics. Consumer acceptability plays a role in the acceptance of this product, but a greater factor is the quality characteristics of yogurt that are affected by the incorporation of probiotics. There are challenges presented to researchers and producers to meet the demand of the consumer when providing a probiotic product. Most probiotic yogurts contain *Lactobacillus acidophilus* and bifidobacteria because of their perceived benefits to human health (Daly and Davis 1998). The viability of various probiotics during production and storage are critical to providing health benefits (Mattila-Sandholm and others 2002). Another problem is developing a product were the probiotic bacteria will survive the human gut and provide health benefits without altering flavor or texture attributes (Mattila-Sandholm and others 1999).

**Yogurt production**

In recent years, there has been an increasing trend in consumer acceptability of probiotic yogurt. *Lactobacillus acidophilus* and *Bifidobacterium bifidum* are added to yogurt cultures to provide health benefits. Traditional yogurt starter cultures, which are lactic acid

bacteria, do not survive processing in high enough numbers to provide health benefits to the consumer (Gilliland 1979). Conventional yogurt production consists of heating of milk and milk powder, cooling, addition of cultures, incubation, fermentation, addition of fruit and sugars, packaging and storage (Tamime and Robinson 1999).

The fermentation process is a critical step for providing the final product. The addition of probiotic bacteria may alter the fermentation process for a consumer accepted product that is health beneficial. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are critical for sharp flavor characteristics and lowering the pH of yogurt (Davis and others 1971; Hamann and Marth 1983). The lactic acid bacteria use $\beta$-galactosidase to breakdown lactose into glucose and galactose that is easy for absorption (Thomas and Crow 1984). The free amino acid content is higher in yogurt than in milk because of proteolytic activity of the bacterial cultures (Rasic and Kurmann 1983).

The production of probiotic yogurt has a slight variation from the conventional process. Homogenized milk with increased protein content (3.6-3.8%) is heated to 80-90°C. The traditional starter cultures are added at a different temperature than the conventional method. A similar temperature (45°C) for traditional yogurt is used for the starter cultures and probiotic bacteria when added to the milk. Incubation, fermentation and packaging occur similarly to conventional process. The probiotic cultures are added with the starter cultures or after fermentation (Anon 1994).

**Factors Affecting Survivability**

The level and survivability of probiotics incorporated into yogurt can be affected by environmental and production conditions. There are several studies showing the effect of yogurt processing on the survivability of probiotics. Klaver and others (1993) reported low-acid tolerance of some strains of probiotic bacteria. The pH changes are critical for the

activity of the starter cultures. The rapid pH drop to an acidic environment reduces the viable count of probiotics. Several strains of *L. acidophilus* have shown a rapid decline of viable cells at pH 2.0, but no significant changes occur at pH 4.0 (Hood and Zottola 1988). *B. bifidum* has demonstrated higher acid tolerance than *L. acidophilus*. *B. longum* and *B. pseudolongum* are able to survive acidic conditions of pH 1.5 – 3.0 (Lankaputhra and Shah 1995).

Bifidobacteria are strict anaerobes and oxygen levels in dairy products can create low viable counts. Low levels of oxygen in milk promote increased bacterial counts of bifidobacteria (Klaver and other 1993). Ishibashi and Shimamura (1993) reported a solution to the oxygen problems during packaging and storage. Since *Streptococcus thermophilus* can take up large amounts of oxygen, *S. thermophilus* and *Bifidobacterium* are inoculated together. The result is high oxygen uptake by *S. thermophilus* and enhanced viability of *Bifidobacterium*.

## Incorporation of Probiotic Bacteria into Cheese

There are many dairy products containing probiotics being produced around the world. One probiotics-containing product that is not commercially found within the United States is cheese. Cheesemaking is characterized by the steps of coagulation, draining, and ripening (Brulé and others 2001). The difference in cheese varieties are characterized by texture and hardness in addition to flavor (Banks 1998). These variations in ripening time, pressing and starter cultures are critical to providing the uniqueness of each cheese.

### Unripened cheeses

Unripened cheeses consist of cheeses that are fresh and have not undergone the ripening process. Unripened cheeses include cottage cheese, cream cheese and baker's

cheese. Cottage cheese is a soft, unripened cheese in which the curd is coated with a salted cream dressing. It is typically made from skim milk with inoculated lactic acid starter cultures (Fox and others 2000). Currently, the only unripened cheese with incorporated probiotics is cottage cheese. Blanchette and others (1995) incorporated various amounts of inoculum containing *Bifidobacterium infantis* into cultured cottage cheese dressing. Cream dressing of 8, 11 or 14% milk fat was prepared with added mixtures of polypeptides of tryptic casein hydrolyzate. *B. infantis* was inoculated at 9.3 $\log_{10}$ cfu/g in cream dressing at 1, 3, or 5 % (wt/wt) and fermented. In addition to bacterial viability, $\beta$-galactosidase activity in the presence of salt and pH conditioning during fermentation were tested. A non-fermented control was also analyzed for *B. infantis* viability (Blanchette and others 1995).

The enriched mixture of tryptic casein hydrolyzate only affected the increased bacterial population of *B. infantis* especially in 3 and 5% inoculum. Proulx and others (1994) reported the growth of probiotic bacteria was enhanced through the addition of tryptic casein hydrolyzate. The reduction of pH was not seen because of buffering capacity of retentate powder of freeze dried bacteria (Ventling and Mistry 1993). A pH of 4.5 could not be obtained in samples with added salt. A pH of 4.5 with no added salt showed the highest bacterial counts of 9.15 $\log_{10}$ CFU/g. Based on the results, 3% of inoculum was able to show the best viability and reach a pH 4.5. A salt concentration of 1.8% increased bifidobacterium growth and $\beta$-galactosidase activity (Blanchette and others 1995).

A follow up study used the best conditions of *Bifidobacteria infantis* incorporated into cream dressing and combined it with dry curd. The cottage cheese was examined for survivability of *B. infantis* for a 29-day storage period. The pH and $\beta$-galactosidase activity were monitored in cottage cheese with incorporated *B. infantis*. Fermentation showed little difference in $\beta$-galactosidase activity in comparison to unfermented cream dressing at pH

greater than 5.0. There was good lactose hydrolysis from *B. infantis* during the storage period but the viability of the bacteria only lasted through 10 to 15 days of storage. When performing untrained sensory panels, panelists preferred the control cottage cheese and probiotics cottage cheese fermented to pH 5.5. *B. infantis* at pH 4.5 showed the best viability, but it did not have the best consumer acceptance. *B. infantis* can contribute to $\beta$-galactosidase activity in cottage cheese but the bacteria would not survive storage conditions to provide other health benefits (Blanchette and others 1996).

**Soft cheeses**

Soft cheeses consist of a large number of varieties with variable production processes. Characteristics of soft cheese include mixed coagulation of enzymatic and acid pathways and short ripening periods (Ramet 2000).

**Crescenza**

Crescenza cheese is a soft, rindless, Italian cheese with a high count of lactic acid bacteria. These conditions make this cheese a good candidate for incorporation of probiotic bacteria. Six individual treatments of Crescenza cheese were made consisting of *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, a mixture of all three cultures, an immobilized mixture of all three cultures and a control. Viability of the probiotic bacteria, influence of starter cultures, proteolysis, enzymatic activity and sensory attributes were tested with each Crescenza cheese treatment (Gobbetti and others 1998).

The viability of bifidobacterium added to Crescenza cheese is greater if added individually into a batch of cheese rather than adding a mixture. The final cell count of *B. bifidum, B. longum* and *B. infantis* in Crescenza was 8.05, 7.12 and 5.23 $\log_{10}$ cfu/g, respectively, when the cultures were added to the cheese. *B. infantis* decreased in viability during the storage period but *B. bifidium* and *B. longum* were able to maintain high enough

bacterial counts to meet the 6.0 $\log_{10}$ cfu/g therapeutic minimum for providing health benefits. The bifidobacterium were able to coexist with the *Streptococcus thermophilus* starter culture without any adverse effects; however, the rapid acid production by the starter cultures inhibited the probiotic bacteria growth of the bifidobacteria to high bacterial counts (Gobbetti and others 1998).

There were minor quality changes seen in Crescenza cheese with added probiotics. Lactic acid and acetic acid contents of the bifidobacterium cheese were higher than the control cheese. In addition, $\alpha$- and $\beta$-galactosidase activity was increased in Crescenza cheese with incorporated bifidobacteria. Similar biochemical activity results were seen in previous research with cheeses (Blanchette and others 1996). No significant differences were shown in proteolytic activity of the control cheese and the various Crescenza cheeses with incorporated bifidobacteria. The different Crescenza cheeses were acceptable to an experienced group of panelists. No significant differences were seen in sensory attributes. Based on a 10-point hedonic scale, the texture and flavor of each cheese sample was scored around 7.4 except for *Bifidobacterium bifidum*, which had a slightly lower flavor score of 6.6. It was concluded that *B. bifidum* and *B. infantis* demonstrated the greatest viability but only *B. infantis* demonstrated overall consumer acceptability for Crescneza cheese in comparison to the control (Gobbetti and others 1998).

**Argentinean Fresco Cheese**

Fresco cheese is a soft cheese that is generally found in Latin and South American countries. It is a soft cheese that is made from goat's milk but can be made from cow's milk. With the high pH and protein content, it was considered to be a good candidate for incorporation of probiotics. Vinderola and others (2000) wanted to determine the viability of nine different combinations of bifidobacteria, *Lactobacillus acidophilus* and *Lactobacillus*

*casei* during storage of Argentinean Fresco cheese. In addition, the authors wanted to see if the bifidobacteria in the newly developed Fresco cheeses could survive the acid condition of the stomach.

Results showed that all nine combinations were able to survive the 60-day storage period with counts above 6.0 $log_{10}$ cfu/g, except for the combination *B. longum* A2 and *L. acidophilus* A1. When combining three bacteria, there was greater survivability. *B. bifidum* or *B. longum*, *L. acidophilus* and *L. casei* were combined in different combinations. The end result was higher viability of Bifidobacterium when combined with 2 strains of *Lactobacillus*. The natural microflora of the cheese was not affected by the addition of the various combinations of probiotic bacteria. For certain combinations, the natural microflora counts were lowered in the presence of the probiotic bacteria (Vinderola and others 2000).

The viability of each pure probiotic bacteria culture and starter culture bacteria was tested at pH conditions of 2 and 3 in 1 N hydrochloric acid after 30, 60, 120 and 180 minutes. After 3 hours, *B. bifidum*, *L. acidophilus*, and *L. casei* were able to survive at counts above 6.0 $log_{10}$ cfu/g. *Streptococcus thermophilus* counts dropped to 0 logs after 30 minutes. When a pH of 2 was examined, only *B. bifidum* was able to survive the acidic conditions. The Fresco cheese would act as a protective barrier against the acidic conditions of the human gut and deliver the probiotics at levels that are considered beneficial (Vinderola and others 2000).

**Semi-soft cheeses**

**Gouda cheese**

Gouda is a semi-soft cheese that is characterized with a yellow interior dotted with a few tiny holes and a mild, nutlike flavor. It can have a bitter flavor that is due to lower milk pH (Stadhouders and Hep 1975).

The influence of salt diffusion on the cheese matrix and probiotic viability was examined during a 9 week ripening period of Gouda cheese. *Bifidobacterium lactis* and *Lactobacillus acidophilus* strain Ki replaced the conventional starter culture of Gouda cheese. Various Gouda cheese treatments were made containing starter cultures of 3.5% and 7% in addition to salt concentrations ranging from 2 to 4%. The cheese was tested on a weekly basis (Gomes and others 1995, 1998).

After the first week, the Gouda cheese contained $\sim 10^9$ cfu/g *B. lactis* and $\sim 10^8$ cfu/g *L. acidophilus* in both 7 and 3.5% inoculated cheeses. *B. lactis* decreased at a rate of 1 log cycle/week during the 9 week period and *L. acidophilus* decreased by two log cycles by the sixth week in a 4% salted cheese. As the salt concentration decreased, the viability of the probiotic bacteria increased. The authors concluded that salt concentration influences the ability of probiotic bacteria to survive in the cheese matrix (Gomes and others 1995, 1998).

**White-brined cheese**

White-brined cheese is a semi-soft cheese that traditionally has a high salt content. White-brined cheeses include Feta, Akawi or Lighvan. White-brined cheeses are popular in some countries because the high salt content prevents spoilage due to high temperature and lack of storage conditions. Traditional manufacturing processes need to be modified to allow the viability of the probiotics bacteria and still maintain product quality (Gomes and Malcata 1998).

White-brined cheese is a staple food in the Middle East (Ahmed and others 1995). With a surge of food from Western countries, there was a renewed interest in developing a probiotic dairy product. *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, *Bifidobacterium bifidum*, *Bifidobacterium adolescentis*, were individually added with starter cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* in equal

amounts in skim milk. The survivability of the bacteria in four different probiotic cheeses during a 60-day shelf life was monitored. In addition, pH, fat and protein content, salt content and moisture were examined in the cheese samples (Ghoddusi and Robinson 1996).

Out of the four probiotic bacteria added, only *B. bifidum* survived the full storage period and maintained the therapeutic level for providing health benefits. The levels of all bacteria were low in white-brined cheese. The pH of the white-brined cheese started around 4.6 and rose to 4.85 after 30 days of storage. It was also reported that a moisture content of 59.5%, protein content of 12.0%, fat content of 20.5% and salt content of 7.15% were within the range for standard white-brined cheese conditions. There is further work needed for white brined cheese to become a functional food (Ghoddusi and Robinson 1996).

*Bifidobacterium bifidum* BB-02 and *Lactobacillus acidophilus* LA-5 were incorporated into white-brined cheese and examined during a 90-day storage period. The initial concentration of each probiotic bacteria was ~9 $\log_{10}$ cfu/g and ~9.30 $\log_{10}$ cfu/g based of inoculum rates of 2.5 and 5%, respectively. The salt concentration, probiotic viability and proteolytic activity were examined in the three white-brined cheese samples (Yilmaztekin and others 2004).

The salt penetration was greatest during the first 30 days of storage in brine. After 90 days of storage, the salt concentration was 5.52 g salt/100g, 5.59 g salt/100g and 5.23g salt/100g for the control, 2.5% inoculum and 5% inoculum, respectively. The pH also decreased with the increased amount of inoculum. The decreasing trend in proteolytic activity was reported based on storage time and increasing percentage of probiotics inoculum. After the 90 days of storage the natural microflora had bacterial counts of 7 $\log_{10}$ cfu/g, while inoculum rates of 2.5% and 5.0% had probiotic bacterial counts of 7.51 $\log_{10}$ cfu/g and 4.3 X $10^7$ cfu/g, respectively. There was approximately 1 to 2 log cycle reduction

in probiotic bacteria over the 90-day storage period. While there are higher counts initially for *L. acidophilus*, *B. bifidum* shows greater survivability. Preliminary data concluded that white-brined cheese with added probiotics could be a possible functional food (Yilmaztekin and others 2004).

**Hard cheeses**

**Canestrato Pugliese**

Canestrato Pugliese is a hard Italian cheese made from ewe's milk. It has a 56-day ripening period, and the milk only comes from the Foggia region. Despite the high cost of production, the consumer market is quite large. The incorporation of *Bifidobacterium bifidum* and *Bifidobacterium longum* into Canestrato Pugliese cheese was examined for microbiological and biochemical characteristics. The four different cheeses were made contained no added probiotics, *B. bifidum* only, *B. longum* only and a mixture of *B. bifidum* and *B. longum*. The pH, moisture, salt concentration, enzymatic activity and sensory characteristics of the cheeses were determined (Corbo and others 2001).

There was a decrease in counts of probiotic bacteria during the 56-day storage period. Initially, *B. bifidum* and *B. longum* were inoculated into the cheese at 7.0 and 8.0 $\log_{10}$ cfu/g, respectively. After 56 day, the bacterial counts of *B. bifidum* and *B. longum* decreased to 6.0 and 5.0 $\log_{10}$ cfu/g, respectively in all cheese samples. There were no significant differences seen in pH which ranged from 5.4 to 5.7. Canestrato Pugliese cheese containing probiotics had a final salt concentration of 7.60% and water activity of 0.903. There was also an increase in acetic acid concentration while lactic acid remained fairly stable during the ripening period. Based on biochemical results of the Canestrato Pugliese cheese, there was an increase in $\beta$-galactosidase activity in the bifidobacteria cheeses; however, proteolytic activity was similar to the control cheese sample. An untrained panel of 13 judges examined

the cheese for appearance, color, smell, taste and texture. No significant differences were observed between the control cheese sample and the three cheese samples containing probiotics. The authors concluded that, although *B. bifidum* survived the storage period at a therapeutic level, some technological modifications need to be made to the cheesemaking process. If the Canestrato Pugliese can be produced with bifidobacterium at a therapeutic level, it would provide health benefits to a large consumer market (Corbo and others 2001).

**Uncooked pressed cheeses**

**Cheddar**

Cheddar cheese was first developed in Cheddar, Somersetshire, England. There are now varieties such as American Cheddar. It is a hard, uncooked pressed cheese with a variable ripening period based on desired pH. Cheddar can be aged up to 24 months and have a final pH of 5.58. The pH is typically low during the cheesemaking process (Foster and others 1957).

Studies on the viability of probiotics in Cheddar cheeses have not shown satisfactory results based on shelf-life, low pH and aerobic conditions (Rogers 1991; Laroia and Martin 1991). Bifidobacterium was introduced to Cheddar cheese to study the viability of the bacteria, cheese flavor and texture and biochemical activity during ripening and storage. *Bifidobacterium bifidum* were incorporated as a commercially prepared sample or as an immobilized culture from ATCC that was prepared in lactobacilli MRS media. The characteristics of cheese composition, proteolytic activity, metabolic activity and microbial viability were examined in the prepared Cheddar cheese samples. To ensure consumer acceptability, an experienced taste panel evaluated the cheese every six weeks during the ripening and storage periods (Dinakar and Mistry 1994).

There were no significant differences in protein and moisture contents, but the immobilized bifidobacteria prepared cheese had lower fat and salt contents. The age of the cheese rather than bacterial treatment was the most significant factor in affecting proteolytic activity. Both *B. bifidum* incorporated cheeses showed increases in lactic acid but not acetic acid or ethanol as seen in other probiotic cheeses. The metabolic activity was not significantly different between the control Cheddar cheese and the probiotic cheeses. The natural microflora were not affected by the incorporation of *B. bifidum* in Cheddar cheese; however, aerobic microflora showed slight increases during the 24-week period. Both forms of *B. bifidum* showed increases over the 24 weeks, but the immobilized culture showed the greatest growth over the 24 weeks. The final counts of commercially prepared and immobilized prepared bifidobacterium were 7.15 $\log_{10}$ and 7.41 $\log_{10}$ cfu/g, respectively. The sensory analysis showed positive results. After 12 weeks, the appearance, flavor intensity, body and texture improved in *B. bifidum* Cheddar cheese in comparison to the control sample. Cheddar cheese was viable for at least 6 months with added bifidobacteria and a possible functional food for consumers (Dinakar and Mistry 1994).

Gardiner and others (1998) were interested in incorporating probiotics into Cheddar cheese at a level that would remain high during a long storage period. The research was to improve on the results obtained in the incorporation of *Bifidobacterium bifidum* in Cheddar cheese (Dinakar and Mistry 1994). *Lactobacillus salivarius* strains NFBC 310, NFBC 321 and NFBC 348; *Lactobacillus paracasei* strains NFBC338 and NFBC 364 and NSLAB *Lactobacillus* strains were incorporated into Cheddar cheese and examined during an 8-month ripening period. The probiotic cheddar cheese was examined for bile and temperature tolerance, cheese composition, probiotic bacteria viability, proteolytic activity and sensory evaluation (Gardiner and others 1998).

The *Lactobacillus* strains showed variable results for bile and temperature tolerance based on strain. The results were inconclusive based on the variation seen and the inability to distinguish certain strains. No differences were seen in salt, moisture content and pH of the cheese. NSLAB *Lactobacillus* and *L. salivarius* strains showed low viability during ripening. After 8 months, *L. salivarius* strains NFBC 348 and NFBC 321 had final counts of 5.54 $\log_{10}$ and 6.04 $\log_{10}$ cfu/g, respectively. The only bacteria that showed positive results were *L. paracasei* strains NFBC 338 and NFBC 364 with 8.46 $\log_{10}$ and 8.18 $\log_{10}$ cfu/g, respectively, after 3 months. Flavor and texture were similar to the control Cheddar cheese sample but some flavor defects were detected. High proteolytic activity was detected in all Cheddar cheese samples with incorporated *Lactobacillus* strains. The high proteolytic activity was in contrast to previous results of Cheddar cheese with incorporated bifidobacterium. The authors concluded that Cheddar cheese is a potential vehicle for carrying the tested strains to consumers (Gardiner and others 1998).

Daigle and others (1999) tried to produce a Cheddar-like cheese through the incorporation of *Bifidobacterium infantis*. An improvement of previous probiotics Cheddar cheeses was made by culturing cream with *B. infantis* before adding to skim milk. Different concentrations of *B. infantis* in cream were made with initial concentrations of 5 X $10^7$ and $10^8$ cfu/g, respectively. The cheese was analyzed for *B. infantis* viability during processing and storage, cheese composition, proteolysis, metabolic activity and sensory traits.

Fat protein, moisture, salt content and pH were not significantly different between the control cheese with unfermented cream and the two *B. infantis* cheeses. A difference in proteolytic activity was only seen in the highest inoculated probiotic cheese when compared with the other samples. No significant differences were seen in proteolytic activity during the storage period. As seen in many bifidobacterium cheeses, there was an increase in acetic acid

and lactic acid and $\beta$-galactosidase activity when compared with the control cheese sample. During the cheesemaking process, differences in viability were seen between the two *B. infantis* cheese samples were attributed to the inoculation levels. Before pressing, the Cheddar-like cheese made with initial concentrations of 7.7 $\log_{10}$ and 8 $\log_{10}$ cfu/g contained 8.16 and 7.71 $\log_{10}$ cfu/g *B. infantis*, respectively. Counts of the lactococci and lactobacilli starter cultures were not affected during the different treatments. During the 12-week storage period, no significant differences were seen between the two probiotic cheeses. The *B. infantis* cream inoculations of 7.7 $\log_{10}$ and 8 $\log_{10}$ cfu/g contained final *B. infantis* bacterial counts of 6.71 and 6.59 $\log_{10}$ cfu/g, respectively. The sensory panel could not distinguish between the control and *B. infantis* Cheddar-like cheeses (Daigle and others 1999).

The previous studies have provided insight on the incorporation of probiotic bacteria into cheese, but some of the results and methods demonstrated need for improvement to provide a quality product. Some articles reported data that was unclear or contradictory to other research. All probiotic cheese studies performed the same test of microbial viability, proteolytic and metabolic activity, cheese composition, and sensory evaluation. Cottage cheese showed viable results but only for a given time period (Blanchette and others 1995, 1996). Soft cheese such as Crescenza and Fresco had high levels of *bifidobacterium* during ripening but barely met the minimal standard of 6.0 $\log_{10}$ cfu/g during storage (Gobbetti and others 1998; Vinderola and others 2000). The high salt content of some cheese was detrimental to microbial viability. Gouda and white-brined cheese used high inoculum levels, but the high salt content killed some of the probiotic microorganisms. Those that survived adapted or were more tolerable of salt (Gomes and other 1995, 1998; Ghoddusi and Robinson 1996; Yilmaztekin and others 2004). The concern with some hard cheeses was the long ripening period. Corbo and others (2001) demonstrated that Canestrato Pugliese cheese

needs a high inoculum concentration to survive the long storage and ripening periods. Even with a high level of inoculum, not all *bifidobacterium* strains will survive processing and storage at a level to be considered therapeutic. Three studies were performed on Cheddar cheese, and the results were variable. *Bifidobacterium bifidum* was inoculated into Cheddar cheese at an initial level of 6 $log_{10}$ cfu/ml. A one log increase was seen during aging, but changes during storage were not reported (Dinakar and Mistry 1994). Another study incorporated *Lactobacilli* in Cheddar cheese. The initial level of the inoculum (~5.0 $log_{10}$ cfu/ml) was below the standard therapeutic level, and it was expected to survive storage and ripening. One *Lactobacilli* strain was able to survive ripening and storage (Gardiner and others 1998). Daigle and others (1999) were able to make a probiotics Cheddar cheese, but their sensory results were not explained. The previous research has provided the opportunity to improve the incorporation of probiotic bacteria into a different type of cheese.

## Swiss Cheese

Emmental, developed in Bern, Switzerland, was termed "Swiss cheese" when a publication referred to Emmental as Swiss cheese, thus becoming the American name for Emmental. Swiss cheese is a rindless block cheese that is characterized by its eyeholes. It is a hard cheese that is cooked and pressed. The milk is pasteurized and warmed to temperatures of 50-53°C. The starter cultures are then added. The traditional starter cultures are *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Propionibacterium freudenreichii*. If a ripening period is not required for the starter cultures, the rennet is added for coagulation. Once coagulation occurs, the cheese curds are cut to approximately the size of a rice or wheat grains. The curd is heated for 30 minutes. The cheese curd is "foreworked" to cause the separation of the curd and whey. The curd will then be cooked, and the whey will be drained.

The time from heating of the milk to draining of the whey is 3 hours. The pH drops from 6.6 to 6.3 during this time period. The Swiss cheese curd is then sent to a press where it is set for 18 hours. Pressed Swiss cheese has a pH of 5.2. The cheese is cut into blocks and placed in vats of brine. Following the brine treatment, the Swiss cheese should have a salt content of 0.5%. Swiss cheese blocks are then vacuum-packaged and sent to a 7°C cool room for precooling for 10 to 14 days. The precooling process allows for control of microbial and enzymatic activity. The Swiss cheese blocks are then brought into a warm room at 24.4°C for up to 21 days to allow eyehole formation to occur. Swiss cheese is periodically examined for size and number of eyeholes in a block. Once the cheese block meets the industry criterium, it will be sent to a 7°C cool room for ripening. The Swiss cheese block will remain in the cool room for 2 to 3 months. The Swiss cheese will then be sent to market for consumer purchase. The Swiss cheese has moisture and fat contents of 37.1 and 27.8%, respectively and a final pH of 5.5-5.7 (Reinbold 1957).

Based on the Swiss cheese making process, there are several benefits for incorporating bifidobacterium into Swiss cheese. The pH of Swiss cheese remains close to 6.0. There is a drop in the pH to 5.2 but it is a gradual drop, and the bifidobacteria are able to adjust to the acidic environment. As seen in previous papers on probiotics cheeses, the salt concentration can destroy the added probiotic bacteria. With a low salt concentration of 0.5%, bifidobacterium are able to withstand the salt and continue to maintain or increase in bacterial counts. Unlike most cheeses, Swiss cheese contains propionibacterium which produce carbon dioxide for eyehole formation. The vacuum-packaged cheese and added carbon dioxide production is an added benefit for the viability of bifidobacterium. The established conditions of Swiss cheesemaking provide a good candidate for a new functional food.

# VIABILITY OF BIFIDOBACTERIUM SPP. AND *PEDIOCOCCUS ACIDILACTICI* IN A SWISS CHEESE SLURRY MODEL

A paper to be submitted for publication in the Food Microbiology and Safety section of the Journal of Food Science

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**Abstract**

Gradient plating allowed for distinguishing concentrations of streptomycin where Swiss cheese background microflora but not the probiotic bacteria were inhibited. A final concentration of 1500 $\mu$g/mL Streptomycin was used throughout the experiment. With the use of rapid methods and a modified MRS agar, the incorporation of probiotic bacteria could be replicated in a short period. *Bifidobacterium breve*, *B. infantis*, *B. longum* and *Pediococcus acidilactici* were successfully incorporated into Swiss cheese at levels ranging from 9 to 10 $\log_{10}$ cfu/g. There was no significant difference seen in the viability of the four probiotic bacteria. There probiotic bacteria showed little growth based on the days of ripening at 0, 7 and 10 d. The Swiss cheese background microflora in the presence of each individual probiotic remained viable during the 10-d ripening period. No significant differences were seen in bacterial treatments nor ripening time for the viability of

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background microflora of Swiss cheese in the presence of probiotic bacteria. The Swiss cheese background microflora in the presence of each probiotic bacteria was comparable with the control. No significant differences were in the pH reading of each sample. The results of this study show that the probiotics bacteria were viable in the Swiss cheese curd slurry without adversely affecting the background microflora.

**Introduction**

Numerous health claims from regular consumption of probiotics have been demonstrated through clinical studies. Suppression of traveler's diarrhea, antibiotic-associated intestinal disorders and inflammatory bowel diseases has been demonstrated through consumption of various probiotic bacteria (Oksanen and others 1990; Malchow 1997; D'Souza and others 2002). The consumption of probiotics may also have beneficial effects for lactose intolerant individuals, colon cancer, urogenital infections and hypercholesterolemia (Richelsen and others 1996; Scheinbach 1998; Reid and others 2001; Rafter 2003). A therapeutic level of greater than $10^6$ cfu/g of probiotics must be consumed on a periodic basis to provide these health benefits (Kurmann 1988).

Previous studies have provided insight on the incorporation of probiotic bacteria into cheese, but these studies have demonstrated the need for improvement in viability of these organisms to provide health benefits. Cottage cheese had a final bacterial count of 9.15 $\log_{10}$ cfu/g of *B. infantis* but dropped below 6 $\log_{10}$ cfu/g after 15 days of storage at 4°C (Blanchette and others 1995, 1996). Soft cheese such as Crescenza and Fresco had approximately 8 $\log_{10}$ cfu/g levels of *bifidobacterium* for their best samples but barely met the minimal standard of 6.0 $\log_{10}$ cfu/g during storage (Gobbetti and others 1998; Vinderola and others 2000). The high salt content of some cheeses was also detrimental to microbial viability. Gouda and white-brined cheese used high inoculum concentrations of probiotic

bacteria but bacterial survival was poor due to high salt content (Gomes and other 1995, 1998; Ghoddusi and Robinson 1996; Yilmaztekin and others 2004). Corbo and others (2001) demonstrated that Canestrato Pugliese cheese needs a high inoculum (8.0 $\log_{10}$ cfu/g) concentration to enhance probiotics survival during the long storage and ripening periods. *Bifidobacterium bifidum* was inoculated into Cheddar cheese at an initial concentration of 6 $\log_{10}$ cfu/ml. A one log increase was seen during aging, but changes during storage were not reported (Dinakar and Mistry 1994). Another study incorporated *Lactobacilli* in Cheddar cheese with an initial concentration of the inoculum (~5.0 $\log_{10}$ cfu/ml). One *Lactobacilli* strain was able to survive ripening and storage, but viability was still low (Gardiner and others 1998). Daigle and others (1999) were able to make a probiotic Cheddar cheese with final counts of 6.71 and 6.59 $\log_{10}$ cfu/g, respectively, for *B. infantis* after 12 weeks of storage.

Swiss cheese is a rindless block cheese that is characterized by its eyeholes. It is a hard cheese that is cooked and pressed. The traditional starter cultures are *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Propionibacterium freudenreichii*. During processing, the pH of the milk drops from 6.6 to 6.3 through the curd formation. The final product of Swiss cheese has moisture and fat contents of 37.1 and 27.8%, respectively, a salt content of 0.5% and the final pH between 5.5 and 5.7 (Reinbold 1957).

Based on the Swiss cheese making process, there are several benefits for incorporating bifidobacterium into Swiss cheese. The pH of Swiss cheese remains close to 6.0 in comparison to other cheese that range in pH from 4.85 to 5.55 (Boylston and others 2004). There is a drop in the pH to 5.2 but it is a gradual drop and the bifidobacteria are able to adjust to the acidic environment. As seen in previous papers on probiotic cheeses, many probiotic bacteria are unable to survive the salt concentration greater than 3.5% (Yilmaztekin

and others 2004). The lower salt concentration of Swiss cheese (0.5%) allows the bifidobacterium to maintain or increase in bacterial counts. Unlike most cheeses, Swiss cheese contains propionibacterium that produce carbon dioxide for eyehole formation. The vacuum-packaged cheese and added carbon dioxide production is an added benefit for the viability of bifidobacterium. The objective of this study was to determine the viability of *Bifidobacterium breve, B. infantis, B. longum* and *Pediococcus acidlactici* in a Swiss cheese slurry model system. The natural cheese microflora and pH were also monitored for significant effects that may occur when the probiotic bacteria are incorporated into the Swiss cheese slurry.

## Methods and Materials

### Bacterial Culture Preparation

Freeze-dried cultures of *Bifidobacterium breve, B. infantis, B. longum* and *Pediococcus acidilactici* were kindly obtained from Institut Rosell/Lallemand (Montreal, Canada).

Because bifidobacterium are strict anaerobes, an oxygen-free environment is needed for optimal growth. A simple broth using filter sterilized cysteine and Nitrogen purging was used to create a strict anaerobic environment. The apparatus consisted of a nitrogen tank (Linweld, Des Moines, IA), rubber tubing, glass wool and a canula (Becton-Dickinson, San Jose, CA). The nitrogen flowed through the tubing to a canula with glass wool acting as a filter for impurities from the nitrogen tank.

The needle of the canula was flame sterilized to prevent contamination. Modified MRS broth (Difco Laboratories, Detroit, MI) containing 0.05% L-cysteine·HCl (Sigma Chemical Co., St. Louis, MO) and 0.02% resazurin (Sigma Chemical Co.) was purged under a low nitrogen flow for 30 sec in a 50 ml roll tube. The tube was sealed using a screw top cap

and wrapped with parafilm prior to incubation at 37°C. The resazurin functioned as an oxygen indicator. Two tubes were prepared for each probiotic bacteria. After 24 h, 1 g of probiotic bacteria was aseptically added to one roll tube with reduced MRS broth under $N_2$ purging than incubated at 37°C for 24 h. A 1-ml aliquot of probiotic bacteria was transferred to a fresh modified MRS broth roll tube, nitrogen purged and incubated at 37°C for 24 h. Cells were harvested by centrifugation (Sorvall Super T21, Newton, CT) at 10,000 X g for 10 min at 4°C then resuspended in 10 ml of 0.1% buffered peptone water (Difco Laboratories, Detroit, MI). The final concentrations of Bifidobacterium species and *Pediococcus acidilactici* were 10.0 $\log_{10}$ cfu/ml.

**Swiss Cheese Curd Slurry Preparation**

Unsalted Swiss cheese curd obtained from Swiss Valley Farms (Luana, IA) was used for the cheese slurry model system. The Swiss cheese curd slurry was prepared according to a method by Madkor and others (1999). Swiss cheese curd (100 g), 40 ml sterile water and 0.5 g NaCl were aseptically placed into a Stomacher bag and homogenized in a stomacher (Stomacher 400; Seward Lab Systems, Thetford, Norfolk, UK) for 2 min. A control sample of uninoculated Swiss cheese curd slurry was also prepared. A 1-ml aliquot of an individual probiotic culture was added to an appropriately labeled bag at final cell concentration ranging from 9 to 10 $\log_{10}$ cfu/mL. The cheese slurries containing probiotic bacteria were pummeled for 1 min in a laboratory stomacher (Stomacher 400) to ensure the added probiotics culture thoroughly mixed with the Swiss cheese having a final concentration of 8 $\log_{10}$ cfu/g. Each bag was vacuum sealed and placed in a 37 °C incubator for 0, 7 and 10 d.

**Microbial Analysis**

The distinction between natural cheese microflora and each probiotic bacteria was determined through incorporation of streptomycin (Sigma Chemical Co.) with MRS agar

(Difco Laboratories). The gradient plating technique was used determine the concentration of streptomycin that would inhibit the natural Swiss cheese microflora without inhibiting the viability of the probiotic bacteria. The first layer of the gradient plate consisted of MRS agar. The plates were placed at a 45° angle to allow the agar to harden. The second layer consisted of MRS agar with streptomycin, ranging from 500 μg/mL to 1500 μg/mL, which was poured into the Petri dish on a flat surface. The final result was a plate with a variable amount of steptomycin. At 1500 μg/mL streptomycin, all four probiotic bacteria could grow but the natural Swiss cheese microflora were inhibited. Therefore, MRS agar with 1500 μg/mL Streptomycin was used to selectively enumerate probiotic bacteria added to the Swiss cheese slurry.

For microbial analysis, 10 g of Swiss cheese curd slurry was diluted in 90 mL of buffered peptone water (pH 7.1) and homogenized for 1 min in a Stomacher Lab-Blender 400. Serial dilutions ranging from $10^{-5}$ to $10^{-8}$ were made in 0.1% peptone water and samples of appropriate dilutions were surface plated on MRS agar and on MRS agar with streptomycin to determine total viable counts and probiotics counts, respectively. Total microbial counts (natural microflora and probiotic bacteria) and control were determined on MRS agar. Probiotic bacteria were determined on modified MRS agar plates with 1500 μg/mL streptomycin. The control cheese was also plated on MRS agar with 1500 μg/mL streptomycin (MRSST) to ensure that growth of background microflora was inhibited. Bacterial counts on MRSST agar were subtracted from bacterial counts on MRS agar to determine actual viability of background flora in the presence of probiotics. Each sample was surface plated in duplicate. *B. breve, B. infantis* and *B. longum* were placed in anaerobic jars with AnaeroGen™ 3.5L gas packs (Oxoid, Inc., Bashingstroke, Hampshire England). *P.*

*acidilactici* and control are oxygen tolerant and were grown aerobically. All inoculated plates were incubated at 37°C for 48 h and bacterial colonies were counted following incubation.

**pH**

pH of all cheese slurries were recorded by using a Corning 430 digital pH meter (Corning, NY). The pH meter was calibrated before use with pH 4 and pH 7 standard buffer solutions.

**Statistical Analysis**

The experiment was designed as a 2-way factorial with bacterial treatment and ripening time as the main factors. Analysis of variance and Fisher's LSD was conducted to determine the effects of the main factors and interactions between main factors of pH and the viability of probiotic bacteia and Swiss cheese natural microflora. Statistical analyses were performed (SYSTAT ver. 9.01; SPSS, Inc.; Chicago, IL) with a significance level of $P \geq 0.05$. Each treatment (bacteria/time) was prepared in duplicate. The experiment was replicated three times.

## Results and Discussion

The ability to distinguish between background microflora of a cheese and an added probiotic bacterium is critical to verifying that the probiotic bacteria are present in the food at therapeutic levels. Streptomycin has been used in previous experiments for probiotic bacteria detection (Lim and others 1993). Although 1500 $\mu$g/mL streptomycin was effective, the resistance threshold can be variable based on the type of probiotic bacteria and a cheeses' natural microflora. A series of concentrations of streptomycin tested against the background cheese microflora and each probiotic bacteria on MRS agar proved to be an effective test for differentiating probiotic bacteria from background cheese microflora (Table 1). A method of differentiating between the different bacteria needed to be used for differenting between the

probiotic bacteria and background microflora. There are different mediums that can be used for distinguishing bifidobacteria from other lactic acid bacteria but they are not always effective. A gradient plate allowed for detection of single colonies at a specific concentration of Streptomycin. At a concentration of 1500$\mu$g/mL all four probiotic bacteria survived and the background microflora were completely inhibited. This streptomycin concentration was used for further studies to enumerate the probiotic bacteria in the cheese slurries. While a higher concentration of Streptomycin could be used, some inhibition of the *Bifidobacterium* species and *Pediococcus acidilactici* may occur.

The rapid method of the cheese slurry model system developed by Kristofferson and others (1967) used a high temperature to accelerate the biochemical reaction of cheese ripening. By using this rapid method, viability of probiotics bacteria in a Swiss cheese environment can be determined to identify the best species or strain for incorporation into the cheese. *Bifidobacterium breve*, *B. infanits*, *B. longum* and *P. acidilactici* maintained viability above 9 $\log_{10}$ cfu/g in Swiss cheese. Figure 1 shows the viability of the probiotic bacteria in a Swiss cheese slurry model system. Based on statistical data a significant difference based on ripening time ($P < 0.05$) but not between bacterial treatments. Also, no significant interactions were seen between ripening time and bacterial treatment. Initially, *B. infantis* had a concentration of 9.11 $\log_{10}$ cfu/g while the other three bacteria had counts to greater than 8 $\log_{10}$ cfu/g after 10 days. *B. breve* had a final concentration of 10.22 $\log_{10}$ cfu/g while the other three bacteria had counts to greater than 9 $\log_{10}$ cfu/g after 10 days. Although *B. infantis* had the highest initial concentration, the bacterial counts decreased over 7 and 10 days. *P. acidilactici* remained stable for the first 7 days of ripening and then increased by day 10. *B. breve* and *B. longum* gradually increased over the 10-day ripening period. This high level is not always seen in all studies and may be attributed to the unique environment of

Swiss cheese model system used in the present study. A near-neutral pH, low salt content and an anaerobic environment produced by propionibacteria may have attributed to the growth and survivability of these bacteria.

Only *B. infantis* and *B. longum* have been incorporated and examined for viability in other cheeses. The viability of *B. infantis* was variable in cottage and Cheddar cheese. Cottage cheese dressing at pH 4.5 reached viable counts of 9.15 $log_{10}$ cfu/g for *B. infantis*. Cottage cheese is an unripened product; therefore, reduction in viability would only occur during storage and not ripening (Blanchette and others 1995, 1996). When *B. infantis* was incorporated into a ripened product, the viability of *B. infantis* decreased during ripening and storage periods resulting in lower final counts. *B. infantis* survived the ripening process of Cheddar and Crescenza cheeses with final bacterial counts of 6.59 and 5.23 $log_{10}$ cfu/g, respectively, (Daigle and others 1999; Gobbetti and others 1998). Similar results were seen in *B. longum* in Crescenza and Canestrato Pugliese cheese. *B. longum* was below therapeutic levels of 6.0 $log_{10}$ cfu/g in Canestrato Pugliese but remained viable at 7.12 $log_{10}$ cfu/g in Crescenza cheese (Gobbetti and others 1998; Corbo and others 2001). The viability of probiotic bacteria in Crescenza cheese was inhibited by the rapid acid production of the starter cultures (Gobbetti and others 1998). Gouda, Canestrato Pugliese and white-brined cheeses showed decrease in viability of probiotic bacteria because of their high salt content (Gomes and others 1995; Ghoddusi and Robinson 1996; Corbo and others 2001). Gardiner and others (1998) reported that all but two their probiotic Cheddar cheeses survived above 6 $log_{10}$ cfu/g due to loss during storage. These results demonstrated that the survivability of a specific probiotic bacterium may be effective at concentrations greater than 6 $log_{10}$ cfu/g in one dairy product and not survive in another.

A concern when producing a probiotic cheese is the effect on the natural cheese microflora. A crucial drop in natural microflora could produce defects in the cheese and off-flavors or not allow for proper ripening. Starter cultures such as *Streptococcus thermophilus* and *Lactobacillus acidophilus* work together to lower the pH and produce sharp flavor characteristics (Davis and others 1971). Figure 2 shows the counts of background microflora (Swiss cheese) in the presence of the four probiotic bacteria. No significant decreases were seen in the four Swiss cheese slurries with incorporated probiotics. Also, there was no significant interaction between bacterial treatments and ripening time for the natural microflora. A significant difference was not seen in the counts of background microflora in the control cheese and the probiotic cheese but there was a significant difference seen in terms of days of ripening. *Bifidobacterium breve* showed the greatest increase in Swiss cheese background microflora after 10 days. The natural microflora of the control sample, *B. breve* and *B. longum* gradually increased during the 10 days of ripening. The background microflora of the cheese containing *B. breve* was greater than the control sample. The background microflora of the cheese containing *P. acidilactici* and *B. infanits* remained stable over the 10 days. Those cheeses that used conventional starter cultures and added probiotic bacteria have shown positive results. Crescenza cheese with added *B. bifidum*, *B. infantis* and *B. longum* were able to coexist with the starter cultures (Gobbetti and others 1998). *Bifidobacterium lactis* and *Lactobacillus acidophilus* strain Ki replaced the conventional starter cultures of Gouda cheese (Gomes and others 1995, 1998). In either case, based on microbiological data, detrimental results have not been show for the coexistence of starter cultures and incorporated probiotic bacteria.

The changes in pH show metabolic activity during the ripening process. pH readings of the slurry model system can not be compared with a pilot scale model system of cheese

making. During the cheesemaking process, the pH gradually drops throughout the process. Metabolic and biochemical activity during ripening and storage will increase the pH slightly. In Swiss cheese the typical final pH would be 5.2. In the slurry model system, the cheese remained at a pH close to that of cheese curd before press. Table 2 shows the average pH for each Swiss cheese slurry based on day and treatment. No significant difference was seen based on the interaction between ripening time and bacterial treatment for pH. Any differences found for pH would mean that a change in pH might have an effect on the final product of Swiss cheese. A significant difference ($P < 0.05$) is seen between day 0 and days 7 and 10.

## Conclusions

The testing methods used in this study proved to be effective for detection of probiotic bacteria in the presence of background Swiss cheese microflora. Modified MRS with 1500 $\mu$g/mL streptomycin is a possible alternative to other more expensive media used for probiotic detection in cheeses. The use of a simple nitrogen purging system proved to be an effective alternative to an anaerobic chamber. *Bifidobacterium breve*, *B. infantis*, *B. longum* and *Pediococcus acidilactici* were successfully incorporated and remained viable in Swiss cheese slurry. The four bacteria remained viable at $10^9$ to $10^{10}$ cfu/g. The natural microflora was not inhibited by the incorporation of probiotic bacteria. Even though the final pH readings were not similar to traditional processed Swiss cheese, there were no significant differences seen between the control sample and those with added probiotics. The environment of Swiss cheese provides viability of probiotic bacteria at concentrations that give a therapeutic effect.

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**Table 1 – Growth of background microflora (Swiss cheese) and probiotics cultures in MRS with added streptomycin**

| | Microbial Growth | | | | |
|---|---|---|---|---|---|
| Streptomycin conc (μg/ml) | Natural Microflora | *B. breve* | *B. infantis* | *B. longum* | *P. acidilactici* |
| 0 | Growth | Growth | Growth | Growth | Growth |
| 100 | Growth | Growth | Growth | Growth | Growth |
| 500 | Growth | Growth | Growth | Growth | Growth |
| 1000 | Small Growth | Growth | Growth | Growth | Growth |
| 1500 | No Growth | Growth | Growth | Growth | Growth |

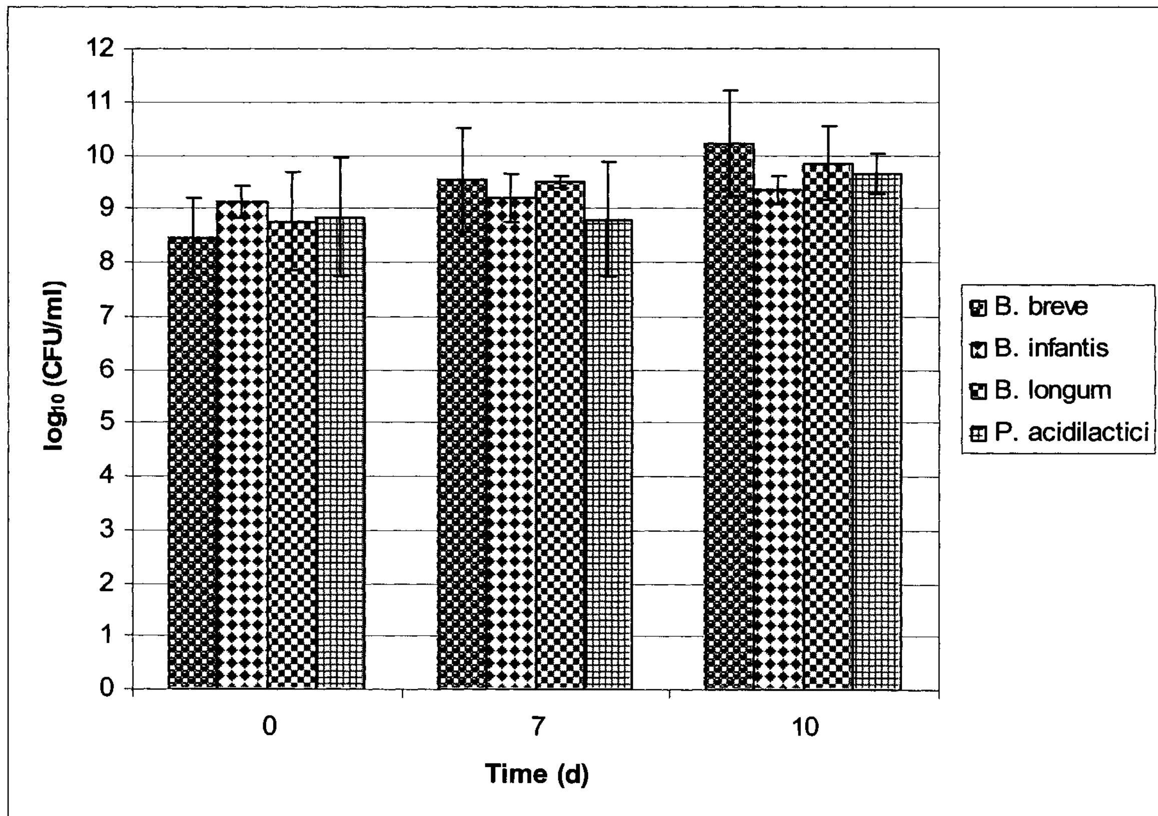


**Figure 1 – Viability of probiotic bacteria in Swiss cheese on MRS agar + 1500 ug/ml Streptomycin @ 37°C. Each bar is a mean of duplicate analyses of three replications.**

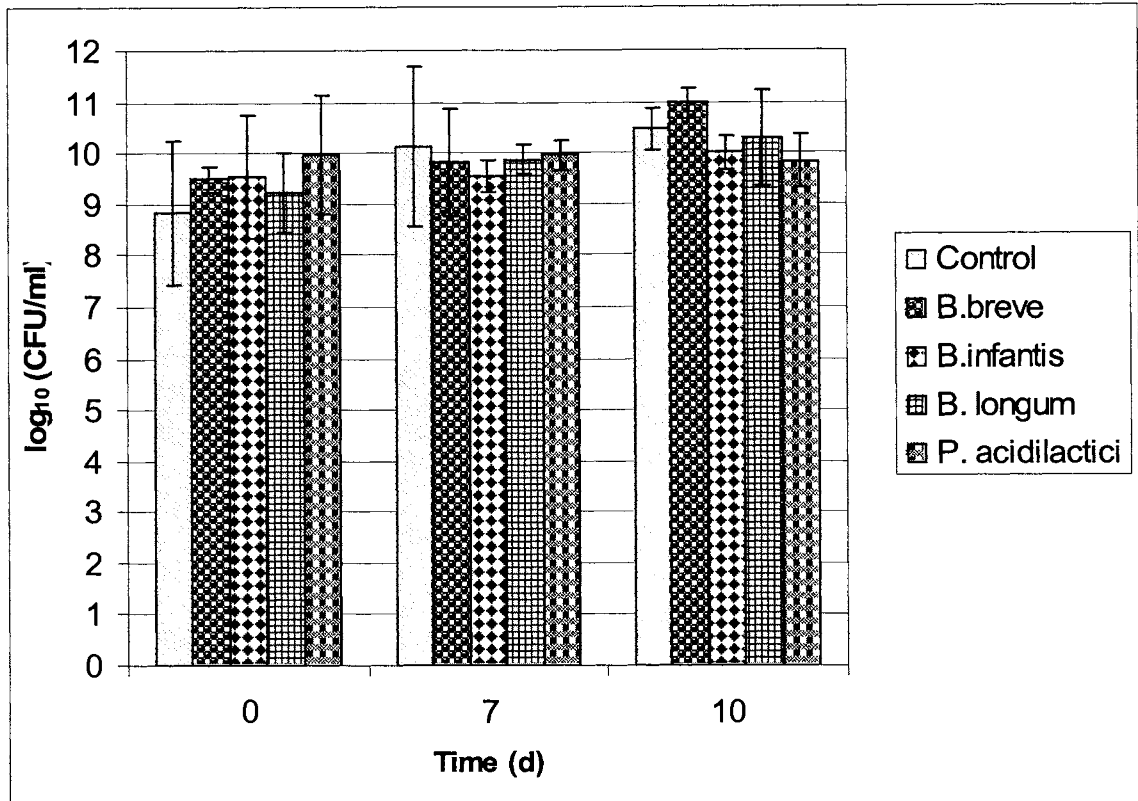


**Figure 2 – Viability of background microflora in Swiss cheese slurry model system in the presence of probiotic bacteria. Each bar represents a mean of duplicate analyses of three replications.**

**Table 2 – The effect of probiotic bacteria and incubation time on pH of Swiss cheese slurry[a]**

| | Day 0 | Day 7 | Day 10 |
|---|---|---|---|
| Control | 6.23a | 6.22a | 6.41b |
| B. breve | 6.23a | 6.39b | 6.51c |
| B. infantis | 6.23a | 6.32b | 6.54b |
| B. longum | 6.23a | 6.34b | 6.57b |
| P. acidilactici | 6.23a | 6.11b | 6.22b |

[a]Means are duplicate analysis of 3 replications.

Means followed by different letters within a column are significantly different ($P<0.05$) based on day of ripening.

# FLAVOR CHARACTERISTICS OF A SWISS CHEESE SLURRY MODEL SYSTEM WITH ADDED BIFIDOBACTERIUM SPP. AND *PEDIOCOCCUS ACIDILACTICI*

A paper to be submitted for publication in the Food Chemistry and Toxicology section of the Journal of Food Science

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## Abstract

The effects of the addition of *Bifidobacterium breve*, *B. infantis*, *B. longum* and *Pediococcus acidilactici* on the flavor characteristics of Swiss cheese were determined using a slurry model system. Volatile flavor compounds, free amino acids, titratable acidity (TA) and pH were analyzed as contributors to flavors. Ripening time but not bacterial treatment had a significant effect for all analyses except for free amino acids. Propionic acid, dimethyl disulfide and 2-heptanone were among the flavor compounds detected in all cheese slurries. The detection of free amino acids was an indication of proteolytic activity in the slurry samples. Proline and methionine have been associated with flavor formation in Swiss cheese. Bifidobacteria samples showed increased amounts alloisoleucine, thiaproline and serine when compared with the control and *P. acidilactici* samples. Titratable acidity and pH showed little difference between bacterial treatments of slurry samples. The result of this

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study showed the addition of Bifidobacterium spp. or *P. acidilactici* did not significantly alter the flavor profile of a Swiss cheese slurry model system.

## Introduction

Health claims have driven an increase in the market of functional foods in recent years (Stanton and others 1998). The application of probiotics in functional foods has provided a carrier system for the delivery of probiotic bacteria to the human gut. Numerous dairy products, such as fermented milk, various cheeses, yogurt and other dairy desserts, have functioned as vehicles for incorporation of probiotic bacteria (Lourens-Hattingh and Vilijoen 2001).

There are concerns when developing a cheese containing probiotic bacteria. Flavor and texture attributes of cheeses are critical to what the consumer buys. Any changes in flavor that occur in a cheese may be undesirable and discourage the consumer from future purchase of that product. The incorporation of probiotic bacteria allows for the possible metabolic and biochemical changes to occur which could produce undesireable results. Another concern is developing a cheese that will survive the environment of the human gut and without altering flavor or texture attributes to ensure survival of the probiotic bacteria (Mattila-Sandholm and others 1999).

In a review of probiotic cheeses, probiotic bacteria have been incorporated into cottage cheese, Gouda, Fresco, Crescenza, Cheddar, Canestrato Pugliese, and White-brined cheeses (Boylston and others 2003). Each study has examined similar results of cheese composition based on pH, moisture content and salt content when looking for quality attributes of probiotic cheeses. All of the above mentioned studies on probiotic cheeses were examined for cheese composition based on pH, moisture content and salt content. Proteolytic and lipolytic activity was examined through total N and pH 4.6-soluble nitrogen and free

fatty acids, respectively. The probiotic bacteria were also monitored for lactic acid and acetic acid production and $\beta$-galactosidase activity. Many of these tests are crucial for detecting changes within the cheese that occur through the addition of probiotics. The data produced can help to identify and quantify compounds found in cheese.

Swiss cheese like many cheeses has characteristic flavor and off-flavors. The detection of increased percentages of ethanol in Swiss cheese is an indication of ripening. Some flavor compounds are distinctive of cheeses. Sulfur compounds and methyl thioacetate are found in Swiss cheese but not in Cheddar cheese. Compounds detected at larger than normal amounts can be an indication of defects in a cheese (Yang and Min 1994). Langsrud and others (1977) reported the effect the amino acid proline on the flavor of Swiss cheese. The production proline from propionibacterium was said to be a possible pathway. It was also proposed that proline is associated with the sweet flavor of Swiss cheese (Hintz and other 1956). Griffth and Hammond (1989) also reported the importance of amino acids on Swiss cheese flavor. Methionine, proline, lysine and cysteine play key roles in the flavor formation for Swiss cheese.

Previous research on the viability of probiotic bacteria incorporated into Swiss cheese curd slurry model system showed viable results *Bifidobacterium breve*, *B. infantis*, *B. longum* and *Pediococcus acidlactici*. With all four bacteria remaining viable after a 10-d ripening time, their impacts on the flavor in comparison the control sample will designate which bacteria not only remain viable to provide beneficial health effect but also have little or no adverse effects on flavor or biochemical activity. The objective of this study is to analyze flavor compounds of Swiss cheese with and without added probiotics through gas chromatography (GC) and GC/MS. Also, the biochemical activity of the Swiss cheese will be analyzed for pH, titratable acidity and free amino acids.

## Methods and Materials

### Bacterial Culture Preparation

Freeze-dried cultures of *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum* and *Pediococcus acidilactici* were obtained from by Institut Rosell/Lallemand (Montreal, Canada).

Since bifidobacterium are strict anaerobes, an oxygen-free environment is needed for optimal growth. A nitrogen purging apparatus used to create an oxygen-free environment for the probiotic bacteria which consisted of a nitrogen tank (Linweld, Des Moines, IA), rubber tubing, glass wool and a canula (Becton-Dickinson, San Jose, CA). The probiotics were nitrogen purged and grown in a 50 ml roll tube containing modified MRS broth (Difco Laboratories, Detroit, MI) with 0.05% L-cysteine·HCl (Sigma Chemical Co., St. Louis, MO) and 0.02% resazurin (Sigma Chemical Co.). The bacteria were grown in a 37°C incubator and were transferred into fresh modified MRS broth for homogeneity. After the 48-h period of growth, each bacterium was centrifuged, supernatant was decanted and the pellets were resuspended in 0.1% buffered peptone water (Difco Laboratories). The final concentrations of Bifidobacteria species and *Pediococcus acidilactici* were 9.0 $\log_{10}$ cfu/ml.

### Swiss Cheese Curd Slurry Preparation

The cheese curd slurries prepared for microbial viability of bifidobacterium spp. and *P. acidilactici* were also used for volatile flavor analysis, free amino acids, titratable acidity, and pH. Unsalted Swiss cheese curd obtained from Swiss Valley Farms (Luana, IA) was used for the cheese slurry model system. The prepared cheese slurry contained Swiss cheese curd (100 g), sterile deionized water (40 mL) and NaCl (0.5 g) which were placed in sterile Stomacher bags. The duplicate samples inoculated with individual treatments of probiotic bacteria for 0, 7 and 10 d.

**Volatile Flavor Analysis**

Volatile flavor compounds of the cheese curd slurries were isolated using solid-phase microextraction (SPME). Aliquots of 40 g of Swiss cheese curd slurry was transferred to 100-mL headspace bottles and sealed with a Teflon septum. Samples were equilibrated under constant stirring in a 40 °C water bath with stirring for 45 min and absorbed onto the SPME fiber (2 cm-50/30 μm divinylbenzene /carboxen/ polydimethylsiloxane; Supelco, Inc., Bellefonte, PA).

Volatile compounds were separated on a Hewlett-Packard gas chromatograph (Model 6890; Hewlett-Packard, Inc., Wilmington, DE) equipped with a splitless injection port and flame ionization detector (FID). Volatile flavor compounds were thermally desorbed (220 °C) from the SPME fiber for 3 min via the GC injection port onto a fused-silica capillary column (SPB-1000, 30 m x 0.25 mm x 0.25 μm film thickness; Supelco, Inc.). An initial column temperature of 30 °C was held for 3 min, then increased to 80 °C at 5 °C/min, to 95 °C at 4 °C/min, to 115 °C at 5 °C/min, and to 190 °C at 10 °C/min. The detector temperature was set at 220 °C, and the column pressure was set at 124.0 kPa with a helium flow rate of 1.9 mL/min. Flow rates of detector gases consisted of air at 400 mL/min, hydrogen at 30 mL/min and nitrogen (make-up gas) at 25 mL/min. Volatile flavor standards were identified using authentic standards (Sigma-Aldrich, Milwaukee, WI; AccuStandard, Inc., New Haven, CT). Analyses were conducted in duplicate and averaged for further statistical analysis.

Identification of volatile flavor compounds was performed using a gas chromatograph-mass spectrometer (GC-MS) (Micromass GCT, Waters Corp., Milford, MA). Swiss cheese slurry volatiles were thermally desorbed into the GC injection port with a split injector onto a fused-silica capillary column (SPB-5, 30 m x 0.25 mm x 0.25 μm film thickness, Supelco, Inc.) with a 100:1 split ratio. The GC has an initial temperature of 38°C

with a 1 min hold, then increased to 150°C at a rate of 4°C/min and a final temperature increase to 280°C at a rate of 50°C/min. The mass spectrometer conditions were set as the following: electron ionization positive (EI+) polarity, source electron energy at 70 eV, source electron current at 200 μA, ion source temperature at 180°C, source ion repeller at 0.8 V, electron multiplier voltage at 2700 V, scan range from 41 to 400 m/z, at a frequency of scanning cycle every 0.75 seconds. Volatile flavor compounds from GC-MS were analyzed using MassLynx version 4.0 (Waters Corp., Milford, MA) for identification and were compared to a spectral library (Wiley Library).

**Free Amino Acid Analysis**

Free amino acids from Swiss cheese slurry samples were extracted according to method reported by Standara and others (2000). Cheese slurry (10 g) was mixed with 90 mL trichloroacetic acid (TCA) and homogenized. The top lay of fat was skimmed off, and the remaining sample was held at 3°C for further cream separation. After the cream was separated, the mixture was centrifuged (Sorvall Super T21, Newon, CT) at 3°C for 10 min at 8000 x g to separate of any remaining cream. A clear liquid portion was then vacuum filtered using a No. 1 Whatman paper (Whatman International Ltd; Maidstone, England). The isolated free amino acids were derivatized according to protocol of EZ:faast™ kit (Phenomenex; Torrance, CA). The derivatized amino acids were analyzed using a gas chromatography (Model 6890; Hewlett-Packard, Inc., Wilmington, DE) with a split injection port and flame ionization detector (FID) for separation of amino acids. Amino acid samples were injected using a Hewlett Packard 7683 series autosampler at 2 μL each with a 1:15 split ratio injection at 250°C onto a Zebron ZB-PAAC column (10m X 0.25mm; Phenomenex). Helium was used as a carrier gas at 60 kPa. The detector temperature was set at 320°C. The oven temperature increased from 110° to 320°C with a ramp time of 35°C/min ramp time

with a 1 min hold at 320°C. Amino acid standards included with EZ: faast™ kit were used for identification of amino acids in the samples.

**Titratable acidity**

Titratable acidity was performed using AOAC Official Method 920.124 for cheese slurry samples. Results were calculated and expressed in terms of g lactic acid/100 g of cheese.

**pH**

The pH of all cheese samples were recorded using a Corning 430 digital pH meter (Corning, NY). The pH meter was calibrated before use with pH 4 and pH 7 standard buffer solutions.

**Statistical Analysis**

The experiment was designed as a 2-way factorial with bacterial treatment and ripening time as the main factors. Analysis of variance was conducted to determine the effects of the main factors and interactions between main factors on the contents of volatile flavor compounds, free amino acids, and titratable acidity and pH. Statistical analyses were performed with Fisher's LSD using SYSTAT ver. 9.01 (SPSS, Inc.; Chicago, IL) with a significance level of $P \geq 0.05$. Each treatment (bacteria/time) was prepared in duplicate. The experiment was replicated three times.

## Results and Discussion

**Volatile Flavor Compounds**

Volatile flavor compounds identified in the Swiss cheese slurry included ketones, alcohols, aldehydes, esters, fatty acids and sulfur compounds which are characteristic of Swiss cheese. Fifteen volatile compounds from the control and probiotics Swiss cheese slurries were found in Swiss cheese flavor analysis (table 1 and table 2). Table 1 and 2 show

the volatile flavor compounds that were statistically significant ($P<0.05$). In table 1, the volatile flavor compounds were significantly different for each ripening time. Sulfide compounds increased in peak area intensity with the increasing ripening time. The acid compound increased in peak area intensity with increased ripening time except for propionic acid. Table 2 indicates that there was significant difference seen in bacterial treatments ($P<0.05$). Butyl propionate, dimethyl disulfide, dimethyl trisulfide and 2-heptanone were among common compounds with Swiss cheese (Yang and Min 1994). Dimethyl sulfide and trimethyl sulfide are among the sulfur compounds formed through oxidation of methanethiol (Griffth and Hammond 1989). A number of acid compounds were detected in all cheese slurries that were not reported in previous research. Propionibacterium used for eye hole formation in Swiss cheese also produces with acetic and propionic acids (Langsrud and others 1977). While both acids were present at high concentration in the Swiss cheese curd slurries, ripening time had a significant effect only on acetic acid content.

**Free amino acids**

Table 3 shows the differences between 30 amino acids during the various ripening times in terms of nmoles per gram of cheese. Most free amino acid that were significant ($P<0.05$) increased in content with ripening time. Proline increased in content by day 7 and then decreased by day 10. Glycine, serine, isoleucine remained stable after day 7. When determining the significant effect of bacterial treatment (Table 4) on free amino acid content, acids only alloisoleucine, thiaproline and serine were significant ($P<0.05$). When examining the five different treatments (Table 4), the three cheese curd slurries containing bifidobacteria showed an increase in free amino acid content during the 10 day ripening period in comparison to *Pediococcous acidilactici* and the control sample. The increase in free amino acid content of bifidobacteria spp. indicates increase in proteolytic activity in those cheese

slurries. Methionine, proline, lysine and cysteine were responsible for key flavors formation in Swiss cheese, which explains the changes in free amino acid content based on ripening time (Griffth and Hammond 1989). Proline was significant based on ripening time in the Swiss cheese curd slurries. The free proline content increased by 7 d and then decreased by 10 d. Langsrud and others (1977) attributed the free proline with propionibacterium. The production of free proline has been associated with Swiss cheese flavor. The decrease in proline is seen based on days of ripening but when analyzing the effects of treatment there is not significant difference in proline. The decrease in proline content could be a natural occurrence for Swiss cheese flavor formation. Compounds like valine and isoleucine were found in Swiss cheese but do not significantly contribute to the overall flavor (Biede and Hammond 1979; Griffth and Hammond 1989). An intense sulfur smell could be detected when each vacuum sealed bag was opened. The increasing free methionine or a metabolite of methionine seen over the 10-d ripening period could have attributed to the detected odor.

**Titratable acidity**

The titratable acidity is expressed in terms of g of lactic acid per 100 g of cheese. The results are based in terms of both lactic acid and free fatty acids found in the Swiss cheese curd slurries. Large amounts of TA were not seen overall during ripening periods or various treatments. A significant difference in the amount of TA was seen in the days of ripening for the curd slurry (Table 5). Although individual free fatty acids were not determined, the flavor indicates that there are acetic and propionic acid in the Swiss cheese curd slurry. Vangtal and Hammond (1986) reported the correlation that propionibacterium had on the free fatty acids, acetic and propionic acid, in Swiss cheese. The amount of lactic acid in the cheese slurries could not be compared with results of previous research with Swiss cheese. Biede and

Hammond (1979) reported that their testing technique yielded higher than normal concentrations of lactic acid in Swiss cheese.

**pH**

The changes in pH show metabolic activity during the ripening process. pH readings of the slurry model system cannot be compared with a pilot scale model system of cheese making. During the cheesemaking process, the pH gradually drops throughout the process because of the acid production by *Lactobacillus bulgaricus*. Metabolic and biochemical activity during ripening and storage will increase the pH slightly. In Swiss cheese, the typical final pH would be 5.2. In the slurry model system, the cheese remains at a pH close to that of cheese curd before press. Table 5 shows the average pH for each Swiss cheese slurry based on day and treatment. A significant difference ($p > 0.05$) is seen between day 0 and days 7 and 10.

## Conclusions

The flavor of the Swiss cheese curd slurry was not affected by the addition of probiotic bacteria. The increased ripening time and not bacterial treatment influenced the volatile flavor compounds in Swiss cheese. Significant flavor compounds found in the Swiss cheese curd slurry were similar to those found in Swiss cheese. There were significant differences found in free amino acid content based on ripening time and treatment. The influence of ripening time affected the content of those free amino acids that were significant. This change in free amino acid might have influence on the flavor and ripening of Swiss cheese. The significant difference seen between the bifidobacteria spp. and the other cheese slurry sample indicates increased proteolytic activity in those curd slurries containing bifidobacterium. The titratable acidity was significantly different in ripening times of the cheese curd slurry and not between bacterial treatments. Even though the final pH readings

were not similar to traditional processed Swiss cheese, there were no significant differences seen between the control sample and those with added probiotics. The high microbial viability and similar quality attributes to a control Swiss cheese could lead to a new functional food.

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**Table 1 – The effect ripening time on content of volatile flavor compounds in Swiss cheese curd slurries[a]**

| | Ripening Time (days)[b] | | | | | |
|---|---|---|---|---|---|---|
| Flavor Compound | 0 | | 7 | | 10 | |
| Dimethyl disulfide | 6.9 | B | 198.8 | a | 208.1 | a |
| Dimethyl trisulfide | - | C | 137.6 | b | 246.3 | a |
| 4-Me-2-pentanol | 19.5 | | 19.1 | | 9.7 | |
| 2-Hexanone | 29.9 | C | 615.0 | b | 1052.9 | a |
| 2-Heptanone | 6.7 | c | 180.2 | a | 104.3 | b |
| 1-Butanol | - | | 26.8 | | 11.3 | |
| 1-pentanol | - | | 7.9 | | 6.0 | |
| 1-Octanol | 32.9 | | 1040.8 | | 867.7 | |
| 2-Me-1-butanol | - | c | 74.6 | | 53.5 | |
| Butyl propionate | 3.5 | | 13.7 | | 7.4 | |
| Acetic acid | - | c | 192.7 | b | 317.2 | a |
| Propionoic acid | 3.5 | | 8190.9 | | 4904.5 | |
| 2-Methylbutyric acid | - | | 967.9 | b | 1484.8 | a |
| Heptanoic acid | 18.0 | c | 76.4 | b | 150.6 | a |
| Octanoic acid | 126.8 | | 70.5 | | 63.9 | |

[a]Means are duplicate analyses of 3 replications with data for bacterial treatment pooled. Means followed by different letters within the same row are significantly different from each other ($P<0.05$).
[b]Peak area/40 g
"--" not detected

**Table 2 – The effect of bacterial treatment on content of volatile flavor compounds[a] in Swiss cheese curd slurries**

| | BacterialTreatments[b] | | | | |
|---|---|---|---|---|---|
| Flavor Compound | Control | B. breve | B. infantis | B. longum | P. acidilactici |
| Dimethyl disulfide | 116.8 a | 117.3 a | 279.7 a | 214.7 a | 233.8 a |
| Dimethyl trisulfide | 80.3 a | 192.1 a | 234.4 a | 262.2 a | 150.6 a |
| 4-Me-2-pentanol | 19.7 | 9.9 | 11.2 | 16.5 | 14.4 |
| 2-Hexanone | 465.6 a | 927.5 a | 978.0 a | 920.1 a | 660.8 a |
| 2-Heptanone | 148.3 a | 97.8 a | 148.5 a | 95.0 a | 151.0 a |
| 1-Butanol | 28.7 | 15.8 | 11.0 | 12.0 | 13.2 |
| 1-pentanol | 5.0 | 6.6 | 11.2 | 8.2 | 1.3 |
| 1-Octanol | 658.0 | 803.6 | 738.5 | 851.8 | 1406.7 |
| 2-Me-1-butanol | 33.2 | 52.2 | 82.1 | 74.2 | 61.9 |
| Butyl propionate | 9.8 | 9.1 | 13.7 | 6.0 | 11.3 |
| Acetic acid | 122.2 a | 356.0 a | 240.8 a | 319.8 a | 175.0 a |
| Propionoic acid | 5544.7 | 4854.1 | 4686.8 | 5342.2 | 9540.1 |
| 2-Methylbutyric acid | 649.0 a | 1438.8 a | 1117.7 a | 1188.0 a | 1413.8 a |
| Heptanoic acid | 63.2 a | 87.0 a | 115.9 a | 115.5 a | 163.3 a |
| Octanoic acid | 56.3 a | 120.1 a | 98.3 a | 73.4 a | 23.2 a |

[a]Means within each column are duplicate analyses of 3 replications. Means followed by different letters are significantly different ($P<0.05$).
[b]Peak area/40g

**Table 3 – The effect of ripening time on content[a] of free amino acids in Swiss cheese curd slurries[b]**

| | Ripening Period (days) | | |
|---|---|---|---|
| | 0 | 7 | 10 |
| Glycine | 246.7 b | 894.9 a | 1358.6 a |
| Alanine | 360.7 c | 2017.0 b | 3575.1 a |
| Valine | 195.0 c | 10620.2 b | 16860.4 a |
| Alloisoleucine | 246.2 c | 18635.2 b | 31038.7 a |
| Isoleucine | 80.9 b | 4441.2 a | 6190.5 a |
| Methionine | 175.1 c | 4855.1 b | 8473.8 a |
| Proline | 225.3 c | 1257.4 a | 795.7 b |
| Proline-hydroxyproline | 250.0 b | 267.6 b | 457.3 a |
| 4-Hydroxyproline | 295.5 a | 2287.2 b | 1194.2 a |
| Thiaproline | 120.6 | 96.3 | 134.6 |
| Phenylalanine | 159.1 c | 1392.9 b | 3941.0 a |
| Tyrosine | 203.6 c | 160.4 b | 1056.2 a |
| Tryptophan | 213.0 c | 975.9 b | 1950.5 a |
| Serine | 1004.9 b | 18084.2 a | 19383.6 a |
| Threonine | 133.7 | 197.9 | 339.8 |
| Cystine | 1822.7 | 2999.9 | 992.2 |
| Lysine | 502.6 c | 3720.0 b | 7269.3 a |
| Histidine | 366.9 c | 1561.2 b | 2945.2 a |
| Aspartic acid | 90.6 c | 1396.6 b | 2402.8 a |
| Glutamic acid | 328.1 c | 4953.7 b | 9935.3 a |
| Asparagine | 373.7 b | 4650.3 a | 5665.2 a |
| Glutamine | 1873.7 c | 12472.6 b | 22147.6 a |
| $\alpha$-aminobutyric acid | 63.1 c | 4356.2 b | 8350.6 a |
| $\beta$-aminobutyric acid | 103.5 | 240.7 | 503.4 |
| Sarcosine | 0.0 | 188.8 | 40.3 |
| Cystoathionine | 538.0 | 826.7 | 848.0 |
| $\alpha$-aminoadipic acid | 204.4 c | 1285.6 a | 1482.3 a |
| $\alpha$-aminopimelic acid | 222.8 | 789.4 | 592.8 |
| Ornithine | 73.3 b | 199.8 a | 231.3 a |
| Glycine-proline dipeptide | 249.4 b | 1064.8 a | 1482.5 a |
| Hydroxylysine | 295.7 b | 590.6 b | 963.2 a |

[a]nmoles/g cheese slurry
[b]Means are duplicate analyses of 3 replications with data for bacterial treatment pooled
Means followed by different letters within the same row are significantly different from each other ($P<0.05$).

**Table 4 – The effect of bacterial treatment on content[a] of free amino acids in Swiss cheese curd slurries[b]**

| | Treatments | | | | |
|---|---|---|---|---|---|
| | Control | B. breve | B. infantis | B. longum | P. acidilactici |
| Glycine | 886.7 | 877.2 | 853.3 | 1004.7 | 545.0 |
| Alanine | 2103.5 | 1869.4 | 1917.6 | 2381.9 | 1648.9 |
| Valine | 7569.5 | 9706.8 | 10237.1 | 11589.7 | 7022.9 |
| Alloisoleucine | 12544.5 bc | 17255.1 abc | 18471.1 ab | 20602.2 a | 12752.0 c |
| Isoleucine | 3313.1 | 4294.2 | 4430.7 | 2520.1 | 3296.1 |
| Methionine | 3857.2 | 4403.5 | 4853.6 | 5970.2 | 3422.3 |
| Proline | 729.1 | 735.8 | 751.1 | 996.9 | 584.3 |
| Proline-hydroxyproline | 278.3 | 366.6 | 332.6 | 407.5 | 239.8 |
| 4-Hydroxyproline | 954.7 | 1401.5 | 1697.4 | 1434.0 | 807.5 |
| Thiaproline | 143.0 a | 128.8 a | 123.6 a | 144.7 a | 45.8 b |
| Phenylalanine | 1621.4 | 1993.4 | 2115.0 | 2277.0 | 1148.3 |
| Tyrosine | 287.8 | 515.7 | 662.7 | 672.3 | 228.3 |
| Tryptophan | 940.9 | 1086.7 | 1146.5 | 1223.0 | 835.1 |
| Serine | 9494.1 c | 15436.6 ab | 12445.5 bc | 19119.8 a | 7625.2 c |
| Threonine | 573.7 | 225.4 | 44.6 | 230.7 | 44.6 |
| Cystine | 3678.7 | 1995.7 | 1151.2 | 1598.5 | 1267.2 |
| Lysine | 2750.4 | 4295.6 | 4410.7 | 4712.0 | 2984.3 |
| Histidine | 1583.3 | 1344.8 | 1648.9 | 1972.1 | 1573.1 |
| Aspartic acid | 1075.4 | 1386.0 | 1340.3 | 1489.3 | 1192.4 |
| Glutamic acid | 4348.9 | 4926.8 | 6173.9 | 5324.8 | 4587.4 |
| Asparagine | 3083.3 | 3912.6 | 3911.4 | 4059.5 | 2848.5 |
| Glutamine | 8489.8 | 14264.3 | 13977.2 | 15183.8 | 8908.0 |
| α-aminobutyric acid | 3553.3 | 4734.6 | 4880.3 | 5318.2 | 2796.8 |
| β-aminobutyric acid | 553.0 | 258.0 | 253.3 | 275.7 | 72.6 |
| Sarcosine | 125.3 | 31.6 | 36.9 | 57.5 | 130.3 |
| Cystoathionine | 474.4 | 991.5 | 576.1 | 1057.1 | 588.7 |
| α-aminoadipic acid | 702.5 | 1127.5 | 1123.2 | 1203.7 | 796.9 |
| α-aminopimelic acid | 516.8 | 423.3 | 501.1 | 883.3 | 350.5 |
| Ornithine | 183.0 | 160.9 | 212.6 | 186.8 | 97.2 |
| Glycine-proline dipeptide | 548.8 | 1072.0 | 787.6 | 1401.5 | 851.3 |
| Hydroxylysine | 588.0 | 684.5 | 615.2 | 692.2 | 502.7 |

[a]nmoles/g cheese slurry
[b]Means within each column are duplicate analyses of 3 replications
Means followed by different letters are significantly different ($P<0.05$) with data for ripening time pooled.

**Table 5 – The effect of ripening time on pH and titratable acidity (TA)[a] of Swiss cheese slurries[b]**

| | pH | TA |
|---|---|---|
| Day 0 | 6.23b | 0.24c |
| Day 7 | 6.28b | 0.74b |
| Day 10 | 6.45a | 1.07a |

[a]g lactic acid/100g cheese
[b]Means are duplicate analysis of 3 replications.
Means followed by different letters are significantly different ($P<0.05$) based on ripening time.

# CONCLUSIONS

The four probiotic bacteria were able to survive in the cheese curd slurry throughout the 10 day ripening period at microbial concentrations above 6 $log_{10}$ cfu/g. The natural microflora of Swiss cheese was not affected by the incorporation of the probiotic bacteria. The Swiss cheese containing *B. breve* showed an increase in natural microflora of Swiss cheese when compared to the control cheese.

The compounds detected in the Swiss cheese slurry were similar to those found in Swiss cheese. Acetic acid, propionic acid, dimethyl disulfide and 2-heptanone were among the compounds identified in the curd slurries. These compounds are characteristic of Swiss cheese. A significant difference was seen between the ripening times of the cheese slurry, but there was not significant difference seen between bacterial treatments. The addition of *B. breve*, *B. infantis*, *B. longum* and *P. acidilactici* had no adverse effects on the flavor Swiss cheese.

Ripening time had a significant effect on titratable acidity (TA) and pH. The bacterial treatments and control did not have a significant effect on TA and pH. The pH increased in all cheese slurries except for *P. acidilactici*. There was a drop in pH during the $7^{th}$ day of ripening. The unusual increase happened because the Swiss cheese curd we used was obtained before pressing. The use of the slurry model did not allow the pH to drop as during production and ripening of Swiss cheese. The acids produced during the cheese making process were stopped during the freezing process of transporting the cheese. The pH was similar to cheese curd before being pressed. The bacterial treatments may have produced some acid but it did not lower the pH of the cheese.

Free amino acid contents were significantly different because of ripening time and bacterial treatment. Alloisoleucine, thiaproline, and serine were free amino acid contents that

were significantly different in all bacterial treatments. In most cases, there was an increase in free amino acid content as the ripening time increased except for proline. The increase in free amino acids with ripening was an indication that proteolysis was occurring in the slurries. The three cheese slurries with the bifidobacteria spp. had increased proteolysis in comparison to the control slurry and slurry with *P. acidilactici*.

The incorporation of *Bifidobacterium breve*, *B. infantis*, *B. longum* and *Pediococcus acidilactici* were viable in a Swiss cheese environment and flavor attributes were not adversely affected. The four probiotic bacteria did not interfere with the growth of the starter cultures for Swiss cheese. In comparison with the control sample, the background microflora in the presence of *B. breve* was increased but was not significant. The other three bacteria showed similar bacterial count to the control slurry. *B. breve* and *B. longum* showed the greatest overall survivability and therefore would be good candidates for incorporation in Swiss cheese.

Future research for probiotic bacteria incorporated into Swiss cheese should be performed on a pilot scale. The cheese should be tested for microbial viability and quality at various stages of the cheesemaking process and during ripening. A concurrent sensory study should be conducted to ensure that consumer panelist could not detect differences between traditional Swiss cheese and cheese added with probiotics. Flavor, free amino acids, fat, moisture, pH and titratable acidity should be monitored to determine differences between the probiotic Swiss cheese and a control Swiss cheese. Any differences detected in sensory evaluation can be correlated through differences seen in these quality tests.

# APPENDIX A. TEMPERATURE STABILITY OF PROBIOTIC BACTERIA AT 48°C

*Bifidobacterium breve*, *B. infantis*, *B. longum* and *Pediococcus acidilactici* were examined to see if they could survive the temperature of the milk vat during the first step of production. The cheese slurry model system was used to determine the effect of temperature on microbial viability. The probiotic bacteria were grown under anaerobic conditions, spun down to form a pellet and resuspended in 0.1% buffered peptone water (Difco) for inoculation into the cheese slurry. The cheese slurry was made with 25 g cheese curd, 10 mL deionized water, and 0.125 g salt. Homogenization and inoculation were performed in the same fashion as our previous study. Each vacuum package contained an individual bacterial treatment for 0, 1.5 and 3 h of incubation. Each bacterial treatment was prepared in duplicate. Samples were vacuum sealed and placed in a 48°C water bath for their designated time period. After each sample reached its designated time period, a series of dilutions was made for each sample and plated in duplicate on MRS agar with 1500 $\mu$g streptomycin/mL to monitor viable counts of the probiotic bacteria. MRS plates were placed in anaerobic jars with gas packs and incubated at 37°C for 48 hrs. Best plate counts were recorded and used to determine viability of each bacterium.

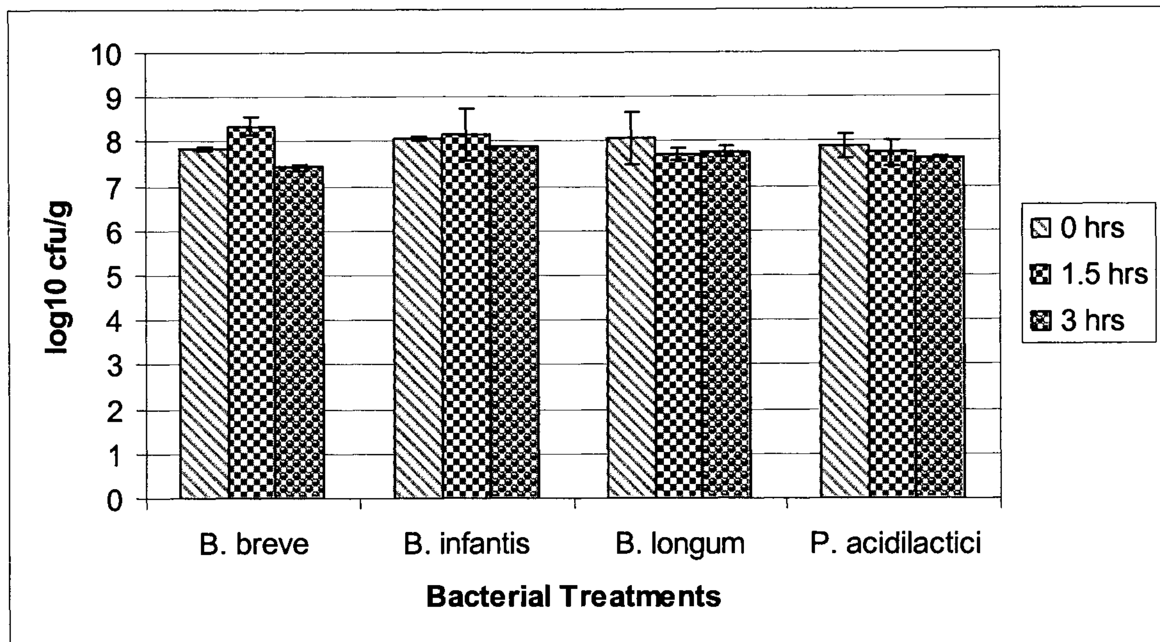


**Figure 1 – Viability of probiotic bacteria at 48°C over a 3-h period. Each bar represents a mean of duplicate analyses.**

*Bifidobacterium breve, B. infantis, B. longum* and *Pediococcus acidilactici* remained viable after 3 h at 48°C. There was no significant interaction seen between bacterial treatment and time in waterbath. There also was no significant difference seen between bacterial treatments or time at 48°C. There were no significant effects seen based on bacterial treatment and heating time. All bacterial treatments decreased slightly in bacterial population after the 3 h at 48°C. *B. longum*'s viability remained stable after 1.5 h. *B. infantis* and *P. acidilactici* have bacterial populations that remain stable throughout the whole 3 h. *B. breve* has an increase in bacterial population before it decreases by the third hour.

# APPENDIX B. pH STABILITY OF PROBIOTIC BACTERIA AFTER 21 H

*Bifidobacterium breve*, *B. infantis*, *B. longum* and *Pediococcus acidilactici* were examined to see if they could survive the pH drop that occurs during the cheese making process. The amount of lactic acid needed to decrease the cheese slurry to the designated pH was performed before the experiment started to ensure a gradual pH change over the 21 h. The probiotic bacteria were grown under anaerobic conditions, spun down to form a pellet and resuspended in 0.1% buffered peptone water (Difco) for inoculation into the cheese slurry. The cheese slurry was made with 25 g cheese curd, 10 mL deionized water, and 0.125 g salt. Homogenization and inoculation were performed in the same fashion as our previous study. Each vacuum package contained an individual bacterial treatment for 0 and 21 h of incubation. Figure 1 shows the gradual pH and temperature adjustments made to simulate that pH changes that occur during a traditional Swiss cheese process. Each bacterial treatment/time was conducted in duplicate. Samples were vacuum sealed and placed in a 48°C water bath for 3h.

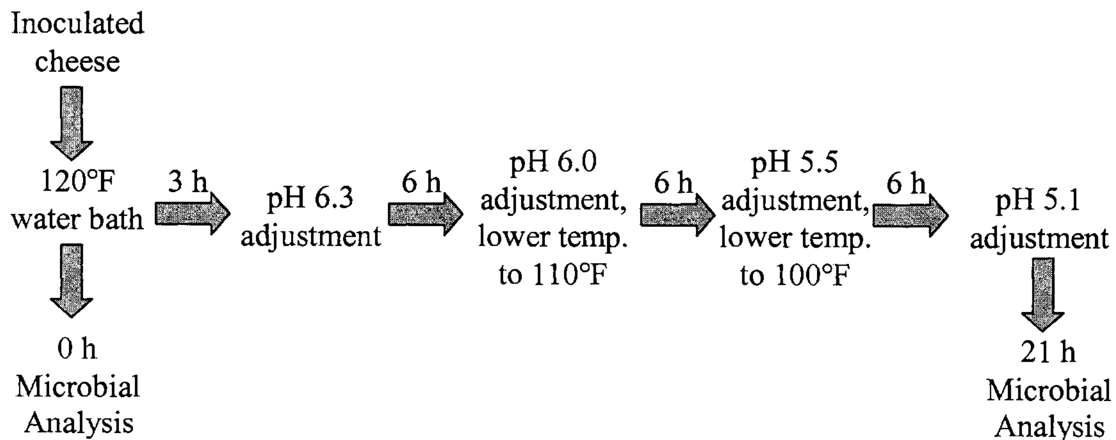


**Figure 1 – The pH adjustments over a 21-h period with gradual temperature drop. The arrowed pathway demonstrates the times and pH changes that occurred in the Swiss cheese curd slurries.**

The sample was allowed hold at this pH for 3 h before plating. The cheese slurries were diluted and plated in duplicate on MRS agar with 1500 $\mu$g streptomycin/mL to monitor viable counts of the probiotic bacteria. MRS plates were placed in anaerobic jars with gas packs and incubated at 37°C for 48 hrs. Best plate counts were recorded and used to determine viability of each bacterium.

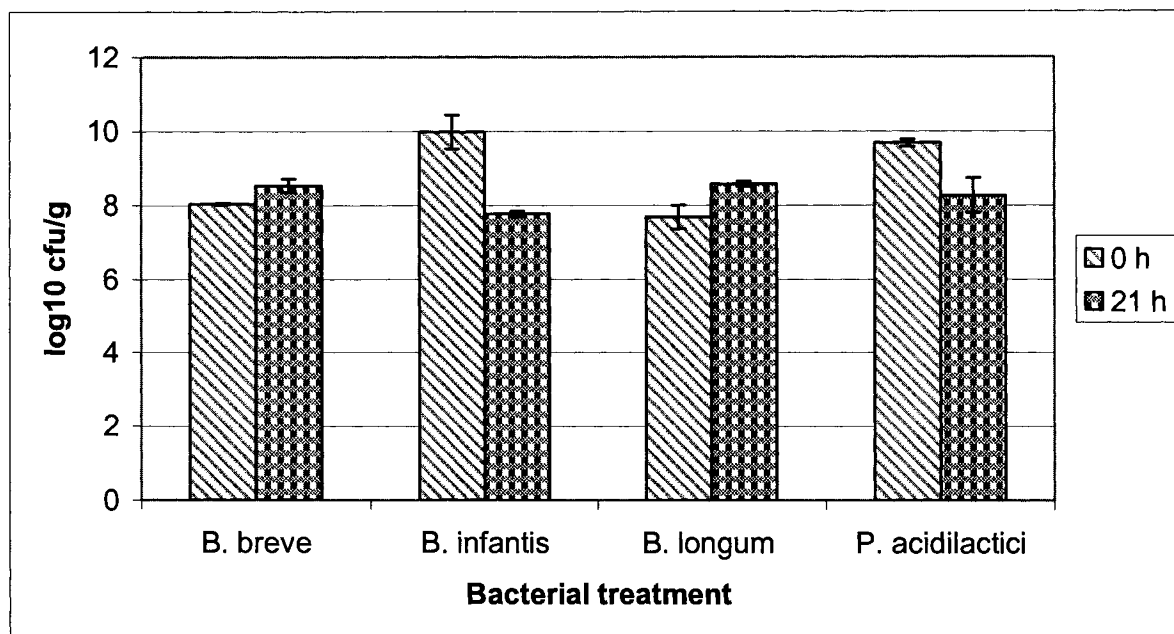


**Figure 2 – Viability of probiotic bacteria during pH changes over a 21 h period Each bar represents a mean of duplicate analyses.**

*Bifidobacterium breve*, *B. infantis*, *B. longum* and *Pediococcus acidilactici* remained viable with a final pH of 5.1 after 21 h. Bacterial treatment and time had a significant effect on viability of probiotics, but there were no significant interactions between the two factors. The 18 h time with the decreased in pH represents the time and pH changes that would normally occur when Swiss cheese is pressed. *P. acidilactici* and *B. infantis* initially had the highest bacterial counts of 9.7 and 9.9 $\log_{10}$ cfu/g, respectively, but they decrease to approximately 8 $\log_{10}$ cfu/g. *B. breve* and *B. longum* increase after 21 h to above 8 $\log_{10}$

cfu/g, which demonstrates some bacterial growth during the 21 h. *B. breve* and *B. longum* would be the better candidates for surviving the Swiss cheese process. The conditions simulating the high temperature in the milk vat and the decreasing pH that occurs while pressing did not cause decreases in viability in the two probiotics bacteria.

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