ENHANCED RECOVERY OF INJURED AND NONINJURED CELLS OF
BIFIDOBACTERIUM SPECIES FROM WATER AND DAIRY PRODUCTS

by

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(ABSTRACT)

Bifidobacterium spp. are anaerobic, Gram-positive, nonmotile bacteria that are a major component of intestinal microbiota. They are potential indicators of human fecal pollution in shellfish harvesting waters. In addition, Bifidobacterium spp. are used as supplements in dairy products. These bacteria are easily injured and it is important to have methods that will recover all cells (injured and uninjured) present in water and food samples. The objectives of this research were to develop a repair detection (RD) procedure to be used with VPI's anaerobic roll tube apparatus for improving the recovery of Bifidobacterium and to compare this method with existing enumeration methods.

Bifidobacterium adolescentis cells were injured by exposure to pond water. Cells were enumerated using a modification of Human Bifid Sorbitol Agar (MHBSA). MHBSA was modified by substituting phenyl red for bromocresol purple and adding methylene blue as an indicator of
oxidation/reduction. *Bifidobacterium bifidum* and *animalis/longum* cells were injured by exposure to frozen yogurt. Cells were enumerated using Modified Human Bifid Glucose Agar (MHGBA), a modification of MHBSA which substituted glucose for sorbitol.

For the RD procedure using roll tubes, the cells were inoculated in 7mL of nonselective medium, allowed to repair, and then overlaid with 10mL of selective medium containing nalidixic acid, kanamycin sulfate and polymyxin B. Recovery of *B. adolescentis* from pond water and *B. bifidum* and *animalis/longum* from frozen yogurt was observed using the roll tube RD procedure and conventional techniques. Results indicated that the RD roll tube procedure was superior to conventional methods for the enumeration of *Bifidobacterium* spp. from water and frozen yogurt. Optimum repair time was between one to two hours before overlaying with the selective medium.
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INTRODUCTION

*Bifidobacterium* spp. are anaerobic bacteria that are a major component of the human intestinal microflora (Scardovi, 1986, Moore and Holdeman, 1974, Holdeman, et al., 1976, Mara and Oragui, 1983). *Bifidobacterium* spp. have a couple of applications in the field of Food Science and Technology. The ingestion of *Bifidobacterium*-containing foods has been linked to various health benefits. These benefits include the maintenance of intestinal balance, an improvement in lactose tolerance, a reduction of serum cholesterol levels, anti-carcinogenic effects and the production of B complex vitamins (Hughes and Hoover, 1991, Hose and Sozzi, 1991). In addition, *Bifidobacterium adolescentis* has been isolated from only human feces and, therefore, would be an ideal indicator of human fecal pollution (Kator and Rhodes, 1988, Hackney, et al., 1991a). An indicator that targets human fecal contaminations is needed since human illnesses associated with sewage frequently have a viral etiology and viruses tend to be species specific.

The purpose of this study was to develop methodology to enumerate injured and noninjured cells of *Bifidobacterium*. This methodology was applied to the enumeration of *Bifidobacterium* cells from water and frozen yogurt.
CHAPTER I: IMPROVED RECOVERY OF BIFIDOBACTERIUM FROM WATER

I. INTRODUCTION

Mossel (1958) suggested that Bifidobacterium would be an ideal alternative indicator of human fecal pollution for shellfish harvesting waters. Mara and Oragui (1983), using Human Bifid Sorbitol Agar (HBSA), isolated the species Bifidobacterium adolescentis from only human feces. Because of its limited survival properties, Bifidobacterium adolescentis, when detected in harvesting waters specifically may indicate recent human fecal pollution (Kator and Rhodes, 1988, Hackney, et al., 1991a).

Stress factors in the harvesting water environment, such as oxygen, pH and temperature variations, can cause injury to the cells of indicator bacteria (Hackney, et al., 1991a). In order to determine the level of pollution that is present in harvesting waters, it is essential to enumerate both injured and noninjured indicator bacteria. When injured, a bacterium does not grow on a selective medium; however, a selective medium is a necessary component for the isolation of Bifidobacterium from harvesting waters. A second problem is that the oxygen in the medium used for the enumeration can cause further injury. Anaerobe jars are commonly used to create anaerobic conditions for the enumeration of anaerobic bacteria; however, the jars can
take up to four hours to turn anaerobic. During this four hour period, the *Bifidobacterium* can acquire further injury. Also, non-pre-reduced media can undergo autooxidation and form hydrogen peroxide. Hydrogen peroxide is lethal to injured, catalase negative *B. adolescentis* (Carlsson, et al., 1978, personal communication with Hackney and Sutton, 1990).

A possible solution to the problems of recovering injured anaerobic cells, is the use of a repair-detection system (Speck, et al., 1975) in combination with VPI's Anaerobic Roll Tube Procedure (Holdeman, et al., 1977). The VPI Anaerobic Roll Tube Procedure uses pre-reduced media and anaerobic procedures as described in the Anaerobe Laboratory Manual (Holdeman, et al., 1977). In the repair-detection system, injured cells are allowed to recover in nonselective media for a set period of time and then are overlaid with selective agents. To date, pre-reduced media and the repair detection technique have not been used together nor used to enumerate *Bifidobacterium adolescentis*.

There were two objectives in this study. The first objective was to develop a medium that would enumerate and select for *Bifidobacterium adolescentis* and that could be used in the anaerobic roll tube procedure. The second objective was to compare the efficiency of this new roll tube repair-detection procedure to anaerobe jars in the
enumeration of *B. adolescentis*.

The first step involved modifying Mara and Oragui's (1983) Human Bifid Sorbitol Agar (HBSA) so that it could be used in the roll tubes. The second step included determining the proper amounts of medium to be placed in the roll tube since during the overlay, the roll tube had to be respun and the first nonselective layer had a tendency to detach from the tube walls. Specifically, this step included finding the amount of medium needed in the first nonselective layer and the amount needed in the second selective overlay. The third step involved finding the amount of repair time that injured *Bifidobacterium* cells needed before they were overlaid with selective agents and whether this amount of time varied with the amount of time that the cells were injured. The last step involved comparing the roll tube repair-detection procedure to a repair-detection procedure using anaerobe jars.

II. LITERATURE REVIEW

A. *Bifidobacterium*

*Bifidobacterium* spp. are obligatory anaerobic, Gram-positive, nonmotile, non-spore-forming rods that possess a characteristic pleomorphic and branching cell morphology (Scardovi, 1986, Levin and Resnick, 1981). A unique metabolic feature of this genus is that glucose is
metabolized exclusively and characteristically through the fructose-6-phosphate shunt (bifid shunt) (Bezkorovainy and Miller-Catchpole, 1989, Scardovi, 1986). The first step is mediated by the enzyme fructose-6-phosphate phosphoenolpyruvate carboxykinase (F6PPK) (Enzyme Commission Serial Number 4.1.2.f.). The detection of this enzyme is a reliable phenotypic characteristic of the genus (Scardovi, 1981, Kator and Rhodes, 1991, Hackney, et al., 1991a, Levin and Resnick, 1981). Bifidobacterium produces both acetic and lactic acid from glucose fermentation and, therefore, gas chromatography tests to detect these products are also useful in identifying the bacterium. A ratio greater than 1:1 of acetic acid to lactic acid is needed to identify Bifidobacterium at the generic level (Levin, 1977, Holdeman, et al., 1977, Levin and Resnick, 1981).

Bifidobacterium can utilize ammonia as a sole source of nitrogen if strictly anaerobic conditions are provided (Tanaka and Mutai, 1980, Kator and Rhodes, 1991, Hackney et al., 1991a). Some strains of the bacterium can tolerate oxygen in the presence of CO₂. Bifidobacterium spp., except for B. indicum and B. asteroides when grown in the presence of air, are catalase negative. Their optimum pH for growth is 6.5 to 7.0 and their optimum temperature for growth is 37-41°C (Scardovi, 1986).
Bifidobacterium spp. occur in the intestines of humans, various animals and honey bees (Scardovi, 1986). In humans, Bifidobacterium is a major component of the intestinal microflora occurring at densities of greater than $1 \times 10^{10}$ cells/g dry feces and may comprise more than 6% of the culturable microbiota (Moore and Holdeman, 1974, Holdeman, et al., 1976).

The first species of Bifidobacterium was discovered in 1900 by Henry Tissier of the Pasteur laboratory who named it Bacillus bifidus communis (Hughes and Hoover, 1991). Bifidobacteria were later grouped with lactobacilli in a single species, Lactobacillus bifidum. This confusion may have been due to the fact that Bifidobacterium and L. acidophilus have an intimate relationship in the intestines and therefore, L. acidophilus may have been carried over as a contaminant in what were thought to be pure cultures of bifidobacteria (Levin and Resnick, 1981). The 1986 edition of Bergey's Manual (Scardovi, 1986) lists twenty-four species of Bifidobacterium. A key difference between bifidobacteria and lactobacilli is the presence of the enzyme F6PPK (Scardovi, 1981). Another difference between Bifidobacterium and L. acidophilus, is the amount of guanine and cytosine in their DNA (Levin and Resnick, 1981, Hughes and Hoover, 1991). L. acidophilus contains 36.7% of G + C in its DNA (Sharpe, 1981) while Bifidobacterium contains 58%
(Scardovi, 1986). Also, while *Bifidobacterium* produces both lactic and acetic acids, *L. acidophilus* only produces lactic acid. Lactobacilli, as facultative anaerobes, can also be distinguished from bifidobacteria by their ability to grow in aerobic environments (Holdeman, et al., 1977, Levin and Resnick, 1981).

B. **Bifidobacterium as an Indicator**

1. **Indicators**

   The enumeration of indicators is the most common method of assessing the safety of shellfish growing waters. It has been recognized that direct detection of pathogens to assess water quality is fundamentally limited in usefulness, because of difficulties associated with detection of multiple pathogens and their unpredictable occurrence. Coliforms and fecal coliforms are the groups of indicators most often used to determine the sanitary quality of water. Their use is considered a reasonable means to detect the presence of fecal contamination in receiving waters. Fecal coliforms are present in high numbers in raw sewage. When they are present in high numbers in shellfish harvesting areas, they are considered "indicators" of fecal pollution, which may signify the presence of bacterial and viral human enteric pathogens. The term "fecal-coliform" has no taxonomic validity and refers to any Gram-negative bacteria that can produce gas in a medium containing lactose at
44.5°C (APHA, 1984) or 45.5°C (USFDA, 1984) for food other than shellfish (Paille, et al., 1987, Hackney et al., 1991a). The fecal coliform standard established to detect raw sewage contamination of shellfish growing waters is 14 most probable number (MPN) fecal coliforms/100mL growing waters (USFDA, 1988).

In general, *Escherichia coli* is considered the major component of the fecal coliform population. In most cases, *E. coli* comprises 95-98% of the total population (Paille, et al., 1987). Some problems exist with the current indicator system. One problem is that current fecal coliform standards do not distinguish between human and animal fecal pollution. Mara and Oragui (1983) reported that *E. coli* is present in the feces of humans, cattle, sheep, pigs, horses, rabbits, rats, mice, hens, cocks and dogs. They also observed a wide variation in numbers of *E. coli* among different animals and even among animals of the same species.

Domestic and feral animals have been found to be important sources of fecal contamination in urban and rural areas. Diffuse or nonpoint pollution is now recognized as a major source of fecal contamination to all types of receiving waters. The most productive shellfish growing estuaries are often those most subject to rainfall runoff from animal non-point sources. Closures due to high fecal
coliform indicator counts from non-point animal sources is one of the major concerns of state regulatory agencies and industry members throughout the country. Human illnesses associated with sewage frequently have a viral etiology and viruses tend to be species specific; therefore, closures of shellfish harvesting areas should be based on contamination from human sources and not animal sources. This problem will worsen as efforts to preserve wetlands succeed and animal populations increase. A specific indicator of human fecal contamination is needed (Kator and Rhodes, 1991, Hackney, et al., 1991a).

Another problem with current fecal coliform indicators is that, as defined, "fecal coliforms" are not necessarily of fecal origin. In some cases, the presence of non-\textit{E. coli} fecal coliforms may predominate in the fecal coliform population analyzed by APHA approved methods. These non-\textit{E. coli} fecal coliforms can be found in shellfish, sediments and the water column, especially in the warm summer temperatures. Characterization of the non-\textit{E. coli} fecal coliform population in Louisiana oysters shows that in the warm months, they often outnumber \textit{E. coli} by 1000 to 1. These non-\textit{E. coli} fecal coliforms, mostly \textit{Klebsiella} and \textit{Enterobacter} spp., are not indicative of sewage pollution (Paille, et al., 1987, Hackney et al., 1991a).
E. coli, in most cases, is not the numerically dominant component of the microbiota of the gastrointestinal tracts of the humans and warm-blooded animals that it represents. The bacterial composition of mammalian feces, which can vary with host species, age, diet, and geographic location, is dominated by obligate anaerobic bacteria belonging to major taxa that include Bacteroides, Bifidobacterium, Clostridium and Eubacterium (Kator and Rhodes, 1991, Hackney, et al., 1991a). Mara and Oragui (1983) reported that the total number of bifidobacteria isolated from human fecal samples is greater by a factor of about 3-4 than the total number of E. coli.

2. Bifidobacterium

Resnick and Levin (1981) credit Mossel (1958) as being the first to propose using Bifidobacterium as an indicator. Some strains of Bifidobacterium have been found to be human specific and are therefore ideal candidates as alternative indicators of human fecal contamination. Tanaka and Mutai (1980) reported that B. adolescentis accounts for 51% of the bifidobacterial strains isolated from human feces. They also reported that the inclusion of the antibiotic nalidixic acid in a medium sufficiently suppresses almost all non-Bifidobacterium microorganisms. Resnick and Levin (1981) developed YN-6, a quantitative, selective and differential medium which includes nalidixic acid. Using this medium,
they were able to identify colonies by their typical morphology with 90% accuracy. Levin and Resnick (1981) reported that the Bifidobacterium species isolated with YN-6 are absent in a variety of domestic and wild animals except swine. Mara and Oragui (1983), using YN-17, a more selective form of Resnick and Levin's YN-6 medium, confirmed Levin and Resnick's finding that the Bifidobacterium species they had isolated with their medium are present in only humans and swine. They also modified YN-17 so that it would select for the sorbitol fermentative Bifidobacterium adolescentis. They reported that Mannitol-fermenting strains are isolated from both human and animal feces, but sorbitol-fermenting strains are only obtained from human fecal samples. They identified the sorbitol fermenting strains as B. adolescentis and B. breve. They were able to isolate Bifidobacterium adolescentis from only the feces of humans with their new medium, Human Bifid Sorbitol Agar (HBSA), which contained sorbitol as the main carbohydrate source. One advantage of their new media, YN-17 and HBSA, is that the addition of kanamycin sulfate substantially reduces the proportion of contaminating fecal streptococci that had been a problem with the use of the medium YN-6.

The value of an indicator organism is based on certain criteria (Banwart, 1989). First, the indicator must be associated with feces or the pathogen in question. As
stated earlier, *Bifidobacterium* is found in densities of $1 \times 10^{10}$ cells/g dry feces (Holdemann et al. 1976). Mara and Oraguí (1983) found that sorbitol fermenting bifidobacteria are consistently isolated from sewage and fresh water polluted with sewage but no bifidobacteria are detected from waters which are not subject to pollution with human feces. Second, the indicator should not be naturally present in the waters being tested. Sorbitol fermentative *Bifidobacterium adolescentis*, which is only isolated from the intestines of humans (Scardovi, 1986), meets this criterion (Resnick and Levin, 1981). Third, the indicator should be easy to grow and differentiate. Mara and Oraguí (1983) developed Human Bifid Sorbitol Agar (HBSA) which effectively grows and selects *B. adolescentis*. Fourth, the indicator should have survival characteristics which are similar to the pathogens of concern. Unfortunately, *Bifidobacterium* may not meet this last criterion.

Various studies have been done to compare the survival characteristics of *Bifidobacterium* to the survival of *E. coli*. *Bifidobacterium* is considered aeroduric: while it cannot reproduce in an aerobic environment, it is capable of surviving in aerobic environments for long periods of time relative to other anaerobic genera (Levin and Resnick, 1981). Gyllenberg et al. (1960) reported that *Bifidobacterium* has survival characteristics similar to
those of *E. coli*, which is currently used as an indicator organism. Gyllenberg et al. reported that the initial ratio of bifidobacteria to coliform bacteria is 23.6. This ratio drops to 5.8 after 2 days but then remains consistent for the next 12 days. In 1981, Levin and Resnick, reported that *Bifidobacterium* exposed in vitro to fresh and marine water samples is less persistent than *E. coli*. This finding contrasted with the report of Gyllenberg et al. (1960) which stated that bifidobacteria, after an initial drop in number, survive as well as *E. coli* in freshwater. Levin and Resnick (1981) suggested that Gyllenberg may have enumerated false-positive colonies of lactobacilli. Resnick (1978) and Evison and James (1973) reported that the *Bifidobacterium/E. coli* ratio decreases dramatically from the source of the pollution to a more downstream location (Levin and Resnick, 1981). Mara and Oragui (1983) reported that the number of bifidobacteria in feces is much higher than the number of bifidobacteria in fresh water samples. They attributed this decline to quick dieaway of bifidobacteria once discharged into the environment. Carillo et al. (1985) observed that *B. adolescentis* populations decline considerably after 48 hours of in situ exposure in tropical freshwaters but under these same conditions *E. coli* survives indefinitely.

Kator and Rhodes (1988) reported that in an in vitro experiment using samples of Chesapeake Bay water, *B.
adolescentis persisted as well as *E. coli* at 6°C but at higher temperatures (25°C) *E. coli* persisted better. This is in contrast with Levin and Resnick's (1981) observation that the survival of bifidobacteria in seawater is independent of temperature (at temperatures of 6°C, 12°C, and 20°C). In their study, approximately 15% of the initial bifidobacterial density remained after about 6 hours with individual species and strains varying in survival capability. However, they did find that in the case of freshwater, temperature has a major effect on the survival of bifidobacteria. After 12 hours, 35% of the bifidobacteria stored at 4°C remain viable, while only 10% of the bacteria exposed to 12°C remain viable and none of the bacteria exposed to 20°C survive. Their finding is in agreement with Evison and James (1973) and Gyllenberg et al. (1960) who reported that temperature affects the survival of bifidobacteria in freshwater. Gyllenberg et al. (1960) observed that bifidobacteria tend to be more persistent at colder temperatures.

As the above studies indicate, *Bifidobacterium*, in contrast to *E. coli*, seems to have limited survival properties. This characteristic of *Bifidobacterium* does not have to be a disadvantage. On the contrary, because of its low survivability in water, its presence in these waters could suggest a recent fecal contamination and perhaps aid
in finding where the contamination originated. The presence of *Bifidobacterium* would also indicate human fecal contamination (Kator and Rhodes, 1991, Hackney, et al., 1991a).

As mentioned earlier, *Bifidobacterium* utilizes the enzyme F6PPK to cleave fructose-6-phosphate to acetyl phosphate and erythrose-4-phosphate. In 1971, Scardovi et al. found that the F6PPK from different species varies in electrophoretic mobility in starch-gel electrophoresis. The distance migrated by the enzyme seems related to the habitat from which the species came from. This characteristic of the bacterium may be useful in cases where it is important to identify the source of a particular fecal pollution (Levin and Resnick, 1981).

Another useful characteristic of *Bifidobacterium*, is that it is very sensitive to chlorination. The presence of bifidobacteria, therefore, not only indicates human fecal pollution, but it also indicates that this pollution comes from a nearby and unchlorinated source (Levin and Resnick, 1981).

3. Injury

Once *Bifidobacterium* leaves the human body through the feces and enters the harvesting waters, the organism is exposed to various stressful factors. When microorganisms are exposed to stress, they may become injured. These
stresses in the marine environment include fluctuations in temperature, changes in pH or ionic conditions, exposure to light (ultra violet or visible), exposure to chemical residues (chlorine, ozone, etc.), exposure to oxygen and a combination of these stresses. In addition, starvation or nutrient depletion can cause cells to be stressed and injured (Hackney et al., 1991a).

a. When Is a Cell Injured?

A cell is considered injured when it grows in a nonselective medium but fails to grow in a medium selective for that organism or it requires more preformed growth nutrients than normal (Ray, 1989, Przybylski and Witter, 1979, Bissonnette, et al., 1975, Hurst, 1980). There are two types of injury: structural and metabolic. Structural injury has taken place when the organism can grow in a nonselective medium but cannot grow in a selective medium. Structural injury involves physical damage to the cell membrane and other components. Metabolic injury has taken place when the organism can grow on a nutritionally complete medium but cannot grow on a minimal medium (Ray, 1989, Hackney, et al., 1991a).

The two types of injury, structural and metabolic, rarely occur separately. Almost all injured cells have damage to the cell structure (i.e. the cell membrane) as well as an extended lag phase and a sensitivity to selective
agents. When the stress becomes more extensive, metabolic systems become damaged (Hackney et al., 1991a, Ray, 1989).

Injured cells can be detected indirectly by observing the difference in counts on selective and non-selective media. Specifically, the number of injured cells is determined by subtraction:

\[
\text{\# injured cells} = \text{CFU nonselective} - \text{CFU selective}
\]

The formula used to determine the percentage of injury in the entire bacterial population is:

\[
\text{% injury} = \frac{\text{CFU nonselective} - \text{CFU selective}}{\text{CFU nonselective}} \times 100
\]

In a stressed population, three types of cells may exist: uninjured cells, injured cells or dead cells. A nonselective medium will pick up all living cells, whether injured or not, and therefore represents the entire population. A selective medium will grow only uninjured cells (Hurst, 1980, Przybylski and Witter, 1979, Hackney, et al., 1991a)

b. Problems with the Enumeration of Injured Cells

It is important to note that several factors can affect the outcome when enumerating a bacterial population. These
factors include the type of selective agents present, and the composition of the medium. In addition, under certain conditions a nonselective medium may not recover all injured cells; such as when an anaerobic medium is exposed to oxygen. When this occurs, autoxidation can take place and may lead to the formation of hydrogen peroxide, which can be lethal to injured cells and catalase-negative bacteria (Carlsson et al., 1978). Harmon and Kauter (1976) and Flowers and Ordal (1979) reported that the presence of hydrogen peroxide can be a strong factor against the enumeration of stressed cells. They stated that the addition of degraders of peroxides can greatly improve the enumeration of injured cells on selective media. McDonald et al. (1983) showed that the addition of compounds such as sodium pyruvate and 3,3'-thiodopropionic acid that degrade or block the formation of hydrogen peroxide in media will greatly increase the recovery in freeze and heat injured cells. Hackney and Sutton (1990) observed that while pyruvate improves counts in non-pre-reduced media, it did not affect counts in pre-reduced media, suggesting that pre-reduced media are sufficient barriers against autoxidation (personal communication). Pierson, et al. (1974) and Shoemaker and Pierson (1976) observed that injured cells of Clostridium botulinum and Clostridium perfringens were able to repair in pre-reduced media.
Another factor that may affect the outcome when enumerating a bacterial population involves the length of media storage. In general, the longer the time between the preparation of the medium and its use, the greater the inhibition of injured cells. Storage conditions of prepared media will also affect the recovery of injured cells. Prepared media should be stored in the dark (Hackney, et al., 1991a).

c. Solutions to the Enumeration of Injured Cells

When enumerating indicator organisms, an accurate estimate of their numbers is desired. An accurate count is not possible, however, when the injured cells do not grow on the selective medium used in the enumeration. Bissonnette, et al. (1975) found that injured indicator cells make up a large proportion of the total viable population. The possibility exists, therefore, of underestimating the number of indicators present (Ray, 1989, Hackney, et al., 1991a).

A method has been developed which allows injured cells to repair their damage before they are placed in the selective medium (Speck et al., 1975). This method is called the repair-detection system. This system is based on the concept that injured cells retain their capacity to repair their damage when they are placed in a suitable environment such as a nonselective medium (Hurst, 1980, Hurst and Hughes, 1981, Przybylski and Witter, 1979, Ray,
Repair occurs during the lag phase of growth. The lag phase of growth of injured cells, therefore, is usually greatly extended (Bissonnette, et al., 1975). During this lag, the cells do not divide but remain physiologically active which allows the microorganisms to adjust to the new environment. Cell size increases and protoplasm and enzymes are synthesized. Cell division occurs at the end of the lag phase. The newly repaired cells can then respond to selective media in the same manner as uninjured cells (Bissonnette, et al., 1975, Busta, 1976, Flowers and Ordal, 1979, Hackney, et al., 1991a).

Fecal bacteria used as indicators are never present in pure populations; therefore, it is necessary to use selective agents in order to isolate and enumerate them. The repair-detection system mentioned earlier is therefore very useful in the enumeration of injured indicator bacteria. In this system, the bacteria are first placed in nonselective medium and are given time to repair. The nonselective medium is then overlaid with selective medium. At this point, as mentioned previously, the repaired cells will respond to the selective medium in the same manner as the noninjured cells. Inhibitors in the selective medium will diffuse into the nonselective medium, creating the desired selective environment needed for recovery of a particular bacterium. Speck et al. (1975) developed this
procedure to recover injured coliforms. Hackney et al. (1979) adapted this method for enumeration of fecal coliforms and enterococci.

d. The Repair of *Bifidobacterium*

*Bifidobacterium* is generally found in low densities in receiving waters. This low recoverability may be attributed to the stresses encountered by the bacterium in the waters and the subsequent injuries it suffers. Munoz and Pares (1988) demonstrated that the inability of *Bifidobacterium* spp. cells to produce colonies on a selective medium is caused by sublethal injury following exposure to seawater. To minimize the effect of sublethal stress on enumeration, they developed a two-layer recovery procedure, similar to the repair-detection technique, that incorporates plating and incubating the sample on resuscitative medium (reinforced clostridial agar). This is followed with an overlay of BIM-25, a selective medium. BIM-25 was developed to improve the poor selectivity of YN-6 medium with environmental samples (Levin and Resnick, 1981). Mara and Oragu (1983) had reported that YN-6 is not selective enough and that it allows contaminating fecal streptococci to grow. Unfortunately, *B. adolescentis* does not grow as well on BIM-25 as other *Bifidobacterium* spp. (Munoz and Pares 1988). To date, the only successful selective medium for *B. adolescentis* is Mara Oragu's (1983)
HBSA medium but it has not been applied to recovering injured cells. An objective of future research efforts should be to improve recovery methods for indicator microorganisms, especially human-specific species.

Hackney and Sutton (1990) observed that prerduced medium can prevent autoxidation from occurring (personal communication). Levin and Resnick (1981) suggested that oxygen toxicity is not a concern with recovery of bifidobacteria but sorbitol fermenting strains such as B. adolescentis are catalase negative and therefore cannot degrade hydrogen peroxide if it is formed by autoxidation. Munro and Pares (1988) and Kator and Rhodes (1988) suggest that factors, such as oxygen, that could exacerbate sublethal stress should be minimized. Despite the recognition that bifidobacteria are sensitive to oxygen, use of a prerduced medium or an oxygen-free environment such as VPI's Anaerobic Roll Tube Procedure (Holdeman, et al., 1977) for recovering Bifidobacterium adolescentis has not been applied (Hackney, et al., 1991a).

The purpose of this study was to develop a prerduced medium with the VPI Anaerobic Roll Tube Procedure (Holdeman, et al., 1977) that would enumerate and select Bifidobacterium from water samples. In addition, Speck et al.'s (1975) repair-detection system was applied to the roll tube procedure so that both injured and noninjured cells of
Bifidobacterium could be recovered. Lastly, the efficiency of this new roll tube repair-detection procedure was compared to plates and anaerobe jars.
III. MATERIALS AND METHODS

A. Source of Bacteria

A culture of *Bifidobacterium adolescentis* (ATCC #7501) was acquired from the VPI&SU Anaerobic Laboratory (Blacksburg, Virginia). The organism was stored at 40°C in an 18 X 142 mm anaerobic culture tube (Bellco, Inc., Vineland, New Jersey) containing Peptone Yeast Extract Glucose (PYG) broth (Holdeman, et al., 1977). Throughout this study, prior to use, the organism was streaked on PYG agar and incubated at 37°C for 48 hours to isolate a pure colony. The isolated colony was grown in PYG broth and was used for inoculations while it was in late log growth.

B. Growth Curve

Late log growth of *B. adolescentis* was determined with a Bausch and Lomb Spectronic 70 spectrometer set at 650nm. Anaerobe culture tubes (18 X 42 mm) containing 7mL of PYG broth were placed in the spectrometer and the absorbance setting was adjusted to 0. The tube was then inoculated with 0.1mL of *B. adolescentis* and placed in a 37°C water bath. The tube's increase in turbidity was observed hourly (beginning with 0 hour) and recorded. A noninoculated anaerobe culture tube containing 7mL of PYG broth was used as the control.
C. Media

A modification of Mara and Oragui's HBSA medium (1983) was used for this study. The original medium contained (g/l distilled water): sorbitol 10, polypeptone 10, yeast extract 20, casamino acids 8, sodium chloride 3.2 bromocresol purple 0.1 and cysteine hydrochloride 0.4. The medium also contained the following amounts of selective agents: nalidixic acid (30mg/L), kanamycin sulfate (50ug/mL) and polymyxin B (10IU/mL). The pH of the medium was adjusted to 6.9 +/- 0.1. Mara and Oragui's (1983) HBSA was modified so that it could be used in the roll tube procedure and in the repair-detection technique. Various combinations of oxidation-reduction and acid-base indicators were tested.

Peptone blanks were prepared in 18 X 142 mm anaerobe culture tubes following the guidelines of the Anaerobe Laboratory Manual (Holdeman, et al., 1977). Each tube contained 9mL of a 0.1% peptone solution which included 1.0g of peptone and 3.0mL of methylene blue stock solution per 1L of distilled water.

All media were stored in the dark at room temperature and were used within two weeks of preparation.

D. Nonselective/selective Media Combinations

The amount of medium in the nonselective layer and the selective layer were varied to determine which combination worked best with the roll tube procedure (Table 1).
Table 1: Combinations of medium amounts in the selective and nonselective layers were varied and observed during the roll tube repair-detection procedure for breakage of medium from the tube walls.

<table>
<thead>
<tr>
<th>Nonselective Layer</th>
<th>Selective Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5mL</td>
<td>5mL</td>
</tr>
<tr>
<td>10mL</td>
<td>10mL</td>
</tr>
<tr>
<td>5mL</td>
<td>10mL</td>
</tr>
<tr>
<td>10mL</td>
<td>5mL</td>
</tr>
<tr>
<td>7mL</td>
<td>10mL</td>
</tr>
</tbody>
</table>
E. Selective Agents

A stock solution of the three selective agents used in HBSA was prepared so that each 0.1mL of this solution would be 17x the strength recommended per mL of medium, since each tube, once overlaid, would contain 17mL of medium (as shown in the results section). Based on Mara and Oragui's (1983) recommendations the stock solution contained (g/10mL distilled water): nalidixic acid 0.056, kanamycin sulphate 0.085 and polymyxin B 0.00215. The agents were mixed into 10mL of distilled water and filter sterilized into a sterile test tube. The stock solution was frozen for long periods of storage, and refrigerated 24 hours prior to its use.

F. Repair-detection Procedure Using Roll Tubes

All streaks and inoculations which involved anaerobic roll tubes were performed with the Anaerobic Roll Tube Apparatus (Bellco, Inc., Vineland, New Jersey) and followed the procedures described in the Anaerobe Laboratory Manual (Holdeman, et al., 1977). The repair-detection technique was applied to anaerobic roll tubes and a procedure was developed. The procedure used in this study was as follows: first, the modified HBSA (MHBSA) medium without selective agents was prepared according to the VPI Anaerobic Manual (Holdeman, et al., 1977) and inoculated into 25 X 142mm isolation roll tubes (Bellco, Inc., Vineland, New Jersey) in both 7mL and 10mL quantities; the tubes were autoclaved and
stored for up to two weeks; prior to use, the tubes were autoclaved for 3 minutes and tempered in a 49°C water bath. A nonselective (7mL) tube was opened and placed under a canula, inoculated according to the specific study being performed, stoppered, spun until the medium hardened and then incubated at 37°C for up to five hours to allow injured cells to repair. Once the incubation time was achieved, the tube was reopened and again placed under a canula. A 10mL tube of tempered MHBSA was opened and quickly poured into the 7mL tube. The tube was then inoculated with 0.1mL of the stock solution of selective agents, stoppered and respun. This was considered the selective overlay. The tube was then returned to the incubator.

G. Repair-detection Procedure Using Plates

MHBSA was also used for the plates. The medium was prepared by mixing the ingredients in a flask (selective agents excluded), adjusting the pH, boiling 1-2 minutes and autoclaving. The repair-detection procedure was similar to the tubes (see: Repair detection procedure for roll tubes). Pour plates with 7mLs of non selective MHBSA were prepared, allowed to solidify and placed upside down in an anaerobe jar prepared with a gas pack™ and an indicator strip. The jar was then incubated at 37°C for a set period of time. As each plate had to be overlaid, the jar was opened and the plate removed. The plate was then overlaid with 10mL of
MHBSA with 0.1mL of the selective stock solution. Once solidified, the plate was returned to the jar which was prepared with a new gas pack™ and indicator strip.

MHBSA which was used for the plates was prepared in small quantities in Erlenmeyer flasks. The medium was not allowed to temper in the 49°C bath for more than two hours to minimize the oxidation of the medium.

H. Water: The Injury Medium

Water for this study was collected from the VPI&SU Pond. One liter samples were collected in an Erlenmeyer flask containing a magnetic stirrer. The sample was covered with foil, autoclaved and left to cool overnight at room temperature. While cooling, the water was constantly aerated using a magnetic stirrer.

The water sample was inoculated with 1.0mL of PYG broth containing Bifidobacterium adolescentis in late log phase. B. adolescentis's late log phase was determined by using the growth curve which had been previously determined (Figure 1). Late log was considered around 0.18 (log of the absorbance).

I. Comparison of Roll Tubes and Anaerobe Jars

The roll-tube repair-detection procedure was compared to the repair-detection procedure using anaerobe jars. B. adolescentis was left in the water sample for 24 hours. Its recovery was examined in the roll tubes and the plates. In
both the plates and the tubes the following factors were observed: nonselective Modified HBSA (MHBSA) only, no repair time allowed between the 7mL nonselective MHBSA and 10mL selective MHBSA overlays, one hour of repair allowed (in a 37°C incubator), 2 hours, 3 hours, 4 hours and 5 hours. All counts were done in duplicates.

J. Determination of Incubation Time

A study was performed with the roll tubes to determine how long of a repair time cells of B. adolescentis needed after 2 days and 4 days in the water. Again, duplicate counts were performed.

The pond water was prepared as previously described. It was inoculated with 1.0mL of B. adolescentis in late log and immediately a sample was removed from the water and enumerated. This sample represented a few minutes of injury. A second sample was removed after 48 hours and a third after 96 hours in the aerated water flask.

All three samples were enumerated following these conditions: nonselective MHBSA only, no repair time allowed between the 7 and 10mL layers, 1 hour of repair between layers, 2 hours, 3 hours and 4 hours.

K. Enumeration

In this study, all tubes and plates were incubated at 35-37°C for 72 hours. Colonies were counted using a Quebec colony counter. Large round yellow colonies were counted as
B. adolescentis. At first, the identity of the colonies was confirmed by gas chromatography (Holdeman, et al., 1977). Each colony was streaked, inoculated into a PYG broth tube and incubated at 37°C for 48 hours. The tube was brought to a technician at the VPI&SU Anaerobic Laboratory who performed the confirmatory tests. Confirmatory tests showed the presence of lactic and acetic acids, the two major fermentation products of Bifidobacterium. As it was confirmed that all round yellow colonies were Bifidobacterium, cell morphology was used as a sole method of enumeration. All tubes and plates that were purple and had no growth were considered as oxidized and were not included in the results.

IV. RESULTS AND DISCUSSION

A. Growth Curve

A curve, plotting log of absorbance versus time, was constructed based on the data acquired from the spectrometer readings (Figure 1).

B. Modified HBSA

The first modification to Mara and Oragui's HBSA involved finding an efficient oxidation-reduction indicator and pairing it to an acid-base indicator (table 2). In the first combination, the efficiency of resazurin as an indicator of oxidation-reduction for HBSA was observed.
Figure 1: Growth curve of *Bifidobacterium* in PYG broth at 37°C. The curve was determined by observing the increase in turbidity in an anaerobe culture tube containing PYG broth and inoculated with *Bifidobacterium*. Absorbance was observed with a Bausch and Lomb Spectronic 70 spectrometer.
Table 2: Combinations of oxidation-reduction indicators and acid-base indicators were varied in HBSA to determine which combination most efficiently separated the oxidation-reduction and acid-base reactions.

<table>
<thead>
<tr>
<th>Oxidation-reduction indicator</th>
<th>Acid-base indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resazurin</td>
<td>Bromocresol purple</td>
</tr>
<tr>
<td>Resazurin</td>
<td>Bromocresol green</td>
</tr>
<tr>
<td>Resazurin</td>
<td>Phenol red</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>Phenol red</td>
</tr>
</tbody>
</table>
Resazurin and bromocresol purple were found to be incompatible in the roll tubes. Proper and necessary detection of oxidation in the roll tube was impossible. The purple color of the medium masked the pink color that resazurin acquires when oxidized. Likewise, when bromocresol green was substituted for bromocresol purple, resazurin's pink reaction upon oxidation was masked. Also, when phenol red and resazurin were tested in combination, the oxidation of the medium could not be detected. However, the combination of phenol red as the acid-base indicator and methylene blue as the oxidation-reduction indicator successfully separated the oxidation-reduction and acid-base reactions and was therefore chosen for this study. The resulting colors of the media were as shown in table 3. Other modifications of the medium included setting the pH of the medium at 7.1 and doubling the amount of phenol red. The effect of these two changes was to deepen the red color of the neutral reduced medium and differentiate it further from the purple color of the oxidized medium. Table 4 shows the final ingredients of MHBSA and the stock solutions used.

C. Nonselective/selective Media Combinations

The amount of medium in the nonselective and the selective layers were tested in different combinations (Table 1). The most successful combination was 7mL of nonselective medium overlaid with 10mL on selective medium.
Table 3: The color of reduced and oxidized HBSA under acid and basic conditions was observed with various combinations of oxidation-reduction and acid-base indicators.

<table>
<thead>
<tr>
<th>oxid/red indic.</th>
<th>acid/base indic.</th>
<th>reduced acid</th>
<th>reduced basic</th>
<th>oxidized acid</th>
<th>oxidized basic</th>
</tr>
</thead>
<tbody>
<tr>
<td>resaz.</td>
<td>bromo. purple</td>
<td>purple</td>
<td>purple</td>
<td>purple</td>
<td>purple</td>
</tr>
<tr>
<td>resaz.</td>
<td>bromo. green</td>
<td>green</td>
<td>green</td>
<td>green</td>
<td>green</td>
</tr>
<tr>
<td>resaz.</td>
<td>phenol red</td>
<td>yellow</td>
<td>red</td>
<td>red</td>
<td>red</td>
</tr>
<tr>
<td>methy. blue</td>
<td>phenol red</td>
<td>yellow</td>
<td>red</td>
<td>purple</td>
<td>purple</td>
</tr>
</tbody>
</table>

oxid./red. = oxidation-reduction
indic. = indicator
resaz. = resazurin
bromo. = bromocresol
methy. = methylene
Table 4: The final ingredients and their amount in MHBSA and the stock solutions.

<table>
<thead>
<tr>
<th>MHBSA:</th>
<th>Selective SS:</th>
<th>Phenol Red SS:</th>
<th>Methylene Blue SS:</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/L of dw</td>
<td>g/10mL of dw</td>
<td>g/L of dw</td>
<td>g/100mL of dw</td>
</tr>
<tr>
<td>Sorbitol 10</td>
<td>Nalidixic</td>
<td>Phenol Red 0.675</td>
<td>Methylene Blue 0.2</td>
</tr>
<tr>
<td>Polyp. 10</td>
<td>Acid 0.056</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast Ex. 20</td>
<td>Kanamycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casamamino</td>
<td>Sulphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acids 8</td>
<td>0.085</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl 3.2</td>
<td>Polymyxin B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl red 40mL</td>
<td>0.00215</td>
<td></td>
<td></td>
</tr>
<tr>
<td>of SS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meth. Blue 3mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of SS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar 25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine 0.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SS = Stock Solution

Meth. Blue = Methylene Blue

dw = distilled water

Yeast Ex. = Yeast Extract

polyp. = polypeptone
This combination completely covered the roll tube with a thin layer of medium which did not break off from the tube walls during the second spin.

D. Comparison of Roll Tubes and Anaerobe Jars

A study was performed to compare the ability of the roll tube technique and the anaerobe jar technique to recover *Bifidobacterium adolescentis* cells (exposed to pond water for 24 hours). Results are shown in Figure 2. The nonselective medium enumerated all living (injured and noninjured) cells whereas the selective medium enumerated noninjured cells only. As can be seen from Figure 2, more cell death occurred when the anaerobe jar technique was performed than when the roll tube technique was performed. One possible explanation as to why the roll tube technique was superior to the anaerobe jar technique is that the medium in roll tubes was pre-reduced while the medium in plates was not. Pre-reduced medium has been shown to prevent autooxidation and the production of hydrogen peroxide which is toxic to *Bifidobacterium* (Sutton and Hackney, 1990, personal communication). In addition, once the cells were placed in MHBSA in the roll tubes, they were no longer exposed to aerobic conditions; whereas cells growing in MHBSA in plates were exposed to aerobic conditions for as long as the gas pack in the anaerobic jars took to produce an anaerobic environment; and the cells were
The repair-detection curves of *Bifidobacterium adolescentis* in MHBSA in both the roll tubes and the plates. The study included a repair in nonselective MHBSA; a repair for 0 hours in nonselective MHBSA at 37°C before an overlay with selective MHBSA; a repair for 1 hour before the overlay; a repair for 2 hours; 3 hours; 4 hours; and 5 hours.
re-exposed to an aerobic environment whenever overlays were performed.

In both the plate and tube techniques, most repair of injury occurred within the first hour.

E. Determination of Incubation Time

A study was performed to determine if the length of time to which B. adolescentis cells were exposed to water would affect the amount of repair time they needed in nonselective medium. Figure 3 shows that, as in Figure 2, after 48 hours in water, the most repair occurred in the first hour. When cells were exposed to water for 96 hours, the difference between the selective and nonselective curves was negligible which indicated that the decrease in cell count from 0 hours was not due to injury but due to cell death. This observation was consistent with earlier reports that Bifidobacterium has poor survival characteristics (Levin and Resnick, 1981, Resnick, 1978, Evison and James, 1973, Mara and Orugui, 1983 and Carillo et al., 1985).

V. CONCLUSION

In this study, a procedure was developed to enumerate injured and noninjured cells of B. adolescentis from water samples. This roll tube repair-detection procedure which included VPI's roll tube procedure and the repair-detection technique is summarized in Table 5.
Figure 3: The repair-detection curves of *B. adolescentis* in MHBSA in roll tubes after 0 hour, 48 hours and 96 hours of injury in an Erlenmeyer flask containing pond water. The study included a repair in nonselective MHBSA; a repair for 0 hours at 37°C in nonselective MHBSA before an overlay with selective MHBSA; a repair for 1 hour before the overlay; 2 hours; 3 hours; and 4 hours.
Table 5: Summary of the roll tube repair-detection procedure for the recovery of *Bifidobacterium adolescentis* in MHBSA.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description of Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. perform dilutions as needed</td>
<td>use 18 X 142 mm roll tubes with 0.1% peptone</td>
</tr>
<tr>
<td>2. inoculate samples in nonselective roll tube</td>
<td>use 25 X 142 mm roll tubes with 7mL nonselective MHBSA</td>
</tr>
<tr>
<td>3. incubate</td>
<td>at 37°C for one hour</td>
</tr>
<tr>
<td>4. overlay with selective layer</td>
<td>use 25 X 142mm roll tubes with 10mL selective MHBSA and 0.1mL of selective stock solution</td>
</tr>
<tr>
<td>5. incubate and enumerate</td>
<td>at 37°C for 72 hours enumerate all round yellow colonies</td>
</tr>
</tbody>
</table>
The repair-detection roll tube technique was shown to recover *Bifidobacterium* cells better than the plates and anaerobe jar repair-detection technique. Most repair in the roll tubes with nonselective MHBSA by the injured cells of *B. adolescentis* occurred in the first hour (Figures 2 and 3).

*Bifidobacterium adolescentis* had a poor survival rate after 96 hours in water. This was consistent with previous studies which have found that *Bifidobacterium* has poor survival characteristics in waters (Levin and Resnick, 1981, Resnick, 1978, Evison and James, 1973, Mara and Oragui, 1983 and Carillo et al., 1985). Therefore, the presence of living cells of *Bifidobacterium adolescentis* in harvesting waters could be indicative of a recent human fecal contamination.

The purpose of this study was to develop a procedure to enumerate injured and noninjured cells of *B. adolescentis*. Studies must follow to determine this procedure's ability to recover *Bifidobacterium* cells from in vivo samples. Further research needs to be performed to study the specific in vivo survival characteristics of *B. adolescentis*, including fresh and marine harvesting waters. The time and entry point of a pollution could be determined by knowing the survival rate of *B. adolescentis* in that particular harvesting area.
CHAPTER II: IMPROVED RECOVERY OF BIFIDOBACTERIUM FROM YOGURT

I. INTRODUCTION

Frozen yogurt has become a popular American snack food probably because of its reputation as a healthy food; however, American consumers may be misguided. There are no Federal Standards on frozen yogurt products and they do not necessarily contain enough viable numbers of the beneficial bacteria associated with yogurt (Tieszen and Baer, 1989). Researchers are currently developing frozen yogurt products that contain viable numbers of *L. acidophilus* and *Bifidobacterium* (Holcomb, et al., 1991).

At the beginning of this century, Elie Metchnikoff suggested that longevity and good health are related to the consumption of fermented products, such as yogurt, which contain bacteria that colonize the intestines. He stated that the intestinal microflora controls aging and mortality (Metchnikoff, 1910). The work of Metchnikoff is currently a popular topic. While it is accepted that *Bifidobacterium* is predominant in the intestines of infants and then becomes the third or fourth largest group in the intestines of adults (Rao et al., 1989, Modler et al., 1990, Marshall, 1988, Mitsuoka, 1978), it is debated as to whether ingesting *Bifidobacterium*-containing food products has health benefits such as maintaining intestinal balance, improving lactose
tolerance, reducing serum cholesterol levels, having anti-
carcinogenic effects and synthesizing B complex vitamins
(Hughes and Hoover, 1991, Hose and Sozzi, 1991). The
beneficial effects of Bifidobacterium-containing food
products are disputed because many authors have reported
that the bacterium is not able to survive the gastric
environment of the stomach and therefore never reaches the

Authors have reported examples of injured bacteria that
are able to recover in the intestines and regain their
pathogenicity (Colwell, et al., 1990, Singh, et al., 1986,
Ray, 1989). It is possible that Bifidobacterium cells may
not die during their passage through the stomach but may
become injured. Therefore, it is important to use
methodology to enumerate all Bifidobacterium cells, injured
and noninjured. Such methodology is also important because
Bifidobacterium cells acquire injury from long-term storage
in frozen yogurt (Hekmat and McMahon, 1992). Another
problem with the enumeration of Bifidobacterium is that it
is difficult to select the bacterium from L. acidophilus,
which is commonly used in food products along with
Bifidobacterium. A repair-detection technique (Speck, et
al., 1975) combined with the roll tube procedure (Holdeman,
et al., 1977) may be appropriate methodology for enumerating
and selecting Bifidobacterium in dairy products.

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The objectives of this study included: modifying Modified Human Bifid Sorbitol Agar (MHBSA) (see Chapter One) to enumerate \textit{B. bifidum} and \textit{B. longum/animalis}; determining if the medium could differentiate \textit{Bifidobacterium} from \textit{L. acidophilus}; applying this medium for use in the repair-detection technique developed in Chapter One to enumerate and select injured and noninjured \textit{Bifidobacterium} cells from frozen yogurt; and comparing the new roll tube repair-detection technique to enumeration with MRSO and anaerobe jars.

\section*{II. LITERATURE REVIEW}
\subsection*{A. FROZEN YOGURT}

Frozen yogurt was introduced into the food market in the early 1970's and quickly gained popularity. It was available in hard pack, soft serve, or as a frozen novelty. The frozen yogurt, usually plain or vanilla flavored, was characterized as having a low pH, a high acid flavor and a body and texture that resembled ice-cream. As the product gained popularity, more flavors became available to the consumer (Tieszen and Baer, 1989).

In the early 1980's the interest in frozen yogurt dwindled and the consumer turned to non-frozen yogurt. The frozen yogurt manufacturers responded by producing a sweeter frozen yogurt with a higher pH. This new product was more
appealing to most American consumers and could still be considered frozen yogurt since there are no Federal Standards of Identity for frozen yogurt (Tieszen and Baer, 1989).

Today, the American population has become more health conscious and is consuming large quantities of frozen yogurt. A problem with frozen yogurt is that it does not necessarily have the health benefits of yogurt. No standards have been set on the amount of yogurt in frozen yogurt and, as a result, frozen yogurt is not required to have any specific amounts of the beneficial bacteria found in yogurt. Most frozen yogurts contain only between 10-25% yogurt and the rest is frozen ice-milk mix (Hackney, et al., 1991b). In 1989, Tieszen and Baer studied the composition of frozen yogurt and found that 14 out of 19 samples tested are well below the advised limits of $5 \times 10^6$ to $7 \times 10^6$ beneficial bacteria per g of food for possible health benefits.

At the beginning of this century, Elie Metchnikoff of the Pasteur Institute, a Nobel Prize laureate biologist, suggested that health and longevity are related to bacteria present in foods such as yogurt, kefir and sour milk, that when ingested could colonize the gut. He developed a theory that the intestinal microflora controls the outcome of infection by enteric pathogens and regulates the natural
chronic toxemia related with aging and mortality. His work has been widely studied and has sparked many debates. Most of the studies on Metchnikoff's theory have been done on yogurt cultures or Lactobacillus spp., which prior to 1974 included Bifidobacterium spp. The benefits of these cultures is widely debated since their ability to survive passage through the stomach, their extent of intestinal colonization and their resistance to bile salts are factors that are still being investigated (Metchnikoff, 1910, Bibel, 1988, Mitsuoka, 1978, Daly, 1991, Hughes and Hoover, 1991).

B. Bifidobacterium

1. Bifidobacterium as a Dietary Adjunct

Most commercial frozen yogurt available is made up by diluting yogurt in ice milk mix. The yogurt is made from the starter cultures of yogurt: Lactococcus thermophilus and Lactobacillus bulgaricus. Recently, two other cultures, Bifidobacterium and Lactobacillus acidophilus, have also been added to frozen yogurt (Holcomb, et al., 1991).

Bifidobacterium is a natural inhabitant of the gut of warm-blooded animals. In 1900, Henry Tissier was the first to detect Bifidobacterium and observed that it predominates in the intestinal lumen of breast-fed infants (Hughes and Hoover, 1991). The most common species in human infants are Bifidobacterium infantis, B. breve and B. longum and B. bifidum. As infants mature, the first two species are
replaced with *B. adolescentis*. Bifidobacteria are the largest group of bacteria in breast fed infants, comprising >99% of the microflora, but the percentage of bifidobacteria declines with adulthood descending to the third or fourth largest group (<15%) and further declines with aging (Rao, et al., 1989, Modler, et al., 1990, Marshall, 1988, Mitsuoka, 1978).

The presence of *Bifidobacterium* in the intestinal tract has various nutritional and health benefits associated with it. Hoover and Hughes (1991) generalized these benefits under the following categories: a) maintenance of normal intestinal balance, especially in the elderly and infants; b) improvement of lactose-tolerance and digestibility of milk products; c) anti-tumorigenic activity; d) reduction of serum cholesterol levels; and e) synthesis of "B-complex" vitamins and absorption of calcium.

**a) Maintenance of Normal Intestinal Balance**

As early as 1906, Tissier had suggested that ingesting *Bifidobacterium* culture could have therapeutic effects for intestinal disorders. He hypothesized that *Bifidobacterium* would replace putrefactive bacteria that caused the intestinal upset and would become the dominant species in the gastrointestinal tract (Hughes and Hoover, 1991). *Bifidobacterium* has been reported to have antagonistic effects towards undesirable organisms such as
enteropathogenic *E. coli*, *Shigella dysenteriae*, *Salmonella typhi*, *Staphylococcus aureus* and *Proteus* spp. (Kim, 1988, Gilliland, 1979, Homma, 1988, Daly, 1991). One way in which *Bifidobacterium* helps inhibit pathogens is with the production of acids. Small amounts of formic acid and ethanol are often produced by bifidobacterial intestinal fermentation (Hughes and Hoover, 1991, de Vries and Stouthamer, 1968). *Bifidobacterium* also produces acetic and lactic acids in a 3:2 ratio (Scardovi and Trovatelli, 1965, Hughes and Hoover, 1991). By releasing these acids, *Bifidobacterium* plays an important part in the control of the intestinal pH. Both acids formed cause the host's intestinal pH to drop and aid the host in restricting the growth of putrefactive and pathogenic bacteria and their metabolic end products (Hughes and Hoover, 1991). It has also been shown that bifidobacteria can cause a substance to be emitted by tissue cells which works on small lymphocytes which in turn leads to an increased production of an antibody against *E. coli* (Daly, 1991, Homma, 1988).

Studies indicate that bifidobacteria may be responsible for the maintenance of normal bowel function. In 1982, Tanaka and Shimosaka reported that elderly patients with bowel obstruction respond well to treatment with bifidus yogurt. Lizko (1987) reported that 34 Soviet cosmonauts suffering from intestinal imbalance were treated
successfully with bifidus tablets and that the use of these tablets is efficient as a method of preventing intestinal imbalance (Hughes and Hoover, 1991, Hackney, et al., 1991b). Tojo, et al. (1987) observed that during campylobacter enteritis, the intestinal flora of patients changes with a decrease in the number of bifidobacteria and an increase in the number of clostridia and enterobacteriaceae. They observed that administration of Bifidobacterium breve is useful in restoring the normal intestinal flora. Hotta, et al. (1987) reported that the intestinal flora of patients with intractable diarrhea is dominated by facultative anaerobes such as E. coli and Enterococcus. After the administration of bifid products the diarrhea is cured and the normal flora, consisting mostly of Bifidobacterium, is restored. Bifidobacterium can also help restore intestinal balance after treatment with antibiotics (Mitsuoka, 1978, Hotta, 1987). Antibiotic therapy often alters the intestinal flora which can have serious consequences to the host since this barrier against invading bacteria is removed (Colombel, et al., 1987, Mitsuoka, 1978).

In 1983, Hori reported that bifidobacteria make up 92% of the intestinal microflora of breast-fed infants, but only 20% of bottle-fed or weaned infants (Hughes and Hoover, 1991). In breast-fed infants, Bifidobacterium is found at a level of $10^{10}$ to $10^{11}$ per gram feces while aerobic bacteria,
such as coliform bacteria and enterococci, are at a level of $10^8$ per gram feces (Marshall, 1988, Mitsuoka, 1978). The number of aerobic bacteria in bottle-fed infants is 10 times that found in the feces of breast-fed infants so that the numbers of bifidobacteria and aerobic bacteria is nearly the same in bottle-fed infants (Mitsuoka, 1978). It has been suggested that breast-fed infants thrive better (Faber and Sutton, 1930) and have lower incidences of colic and other digestive disturbances in part because of a predominant bifid gut flora (Hughes and Hoover, 1991, Shahani and Ayebo, 1980, Mitsuoka, 1978, Haenel, 1970).

The bifidobacteria in the gut of infants prevent the growth of pathogenic bacteria by controlling the pH. The intestinal microflora in infants is not very stable, however, as it is very susceptible to fluctuations caused by changes in the diet or childhood illnesses (Rasic, 1989). Therefore, it is common practice in Japan to supplement infant's diets with bifidobacteria-containing dairy products to treat enteric infections (Tojo, et al., 1987). Bifidobacteria supplements are also used to maintain normal intestinal conditions and are used to correct abnormal conditions such as pediatric intractable diarrhea, that may have been caused by antibiotic therapy (Hughes and Hoover, 1991, Hotta et al., 1987).
Infant diets that include fermented milk products supplemented with bifidobacteria have another advantage over diets with other fermented milk products. Bifidobacteria produce only L-(+)-lactic acid which infants less than one year of age can easily metabolize. Fermented milk products prepared by fermentation with L. bulgaricus and L. acidophilus produce D-(--)-lactic acid which infants cannot easily metabolize (Hughes and Hoover, 1991).

As aging occurs there is a drop in the number of bifidobacteria in the intestines (Mitsuoka, 1978). Bifidobacteria are replaced with C. perfringens, which produces undesirable substances such as toxins and volatile amines. Tanaka et al. (1980) reported that adults who are fed product containing high levels of viable bifidobacteria over a five week period have an increase in their Bifidobacterium count and a decrease in their C. perfringens count (Hughes and Hoover, 1991).

b) Improvement of Lactose-Tolerance

Many individuals, in particular those with oriental or African ancestry, have a deficiency in the enzyme β-galactosidase (Daly, 1991, Savaiano and Levitt, 1987). β-galactosidase is responsible for the cleavage of the milk sugar lactose into glucose and galactose. Those individuals with this deficiency experience gastrointestinal distress whenever they consume unfermented dairy products. This
distress occurs due to the formation of hydrogen gas, which is produced by microbial action on undigested lactose in the large intestine (Savaiano and Levitt, 1987, Gilliland, 1989, Hughes and Hoover, 1991).

Speck (1983) reported that when cultures grow in milk to produce yogurt, they utilize approximately 20% of the lactose. Despite the 80% lactose remaining, people with a B-galactosidase deficiency can still consume fermented milk products. The reason is that the cultures in these fermented dairy products produce the enzyme B-galactosidase which is released into the dairy product. As the product is consumed, more of the enzyme is released during digestion (Speck and Geoffrion, 1980, Speck, 1983, Savaiano and Levitt, 1987). Savaiano and Levitt (1987) observed that the B-galactosidase activity of L. bulgaricus and L. thermophilus is increased when they are incubated in an in vitro gastric digestion system. When yogurt is pasteurized, the starter cultures are killed and the enzyme is inactivated (Speck and Geoffrion, 1980). Savaiano and Levitt (1987) reported that B-galactosidase deficient individuals can easily consume non-pasteurized yogurt, while they have difficulty digesting the pasteurized product. Gilliland (1989) confirmed their finding.

The yogurt starter cultures L. bulgaricus and L. thermophilus contain a substantial amount of the important
enzyme B-galactosidase. These cultures, however, are not resistant to bile (Gilliland, 1989). *Bifidobacterium* is resistant to bile (Rao et al., 1989). *Bifidobacterium*, therefore, may have a better chance of colonizing the gut and delivering the enzyme (Hughes and Hoover, 1991).

c) Anti-Tumorigenic Activity

Kim (1988) reported that *Bifidobacterium* can prevent the formation of carcinogenic material. It does so indirectly by removing the source. Mitsuoka (1989) observed that mice with an intestinal flora that include *E. coli*, *Enterococcus faecalis*, and *Clostridium paraputrificum*, have a decreased proliferation of tumors when *B. longum* is present. The mice with *B. longum* have even fewer tumors than the controls (Hughes and Hoover, 1991).

*Bifidobacterium* also has direct anticarcinogenic effects. There is a correlation between cancer and diet in which bifidobacteria play an important role. Amines, which are contained in food, are transformed to nitrosamines in the stomach and intestines when combined with nitrates. Nitrosamines, which are strongly carcinogenic, can be decomposed by *Bifidobacterium* (Mitsuoka, 1978, Hosono, et al., 1990, Hughes and Hoover, 1991).

Tomoda, et al. (1988) reported that *Bifidobacterium* also plays a role in cancer treatment. The therapy administered to leukemia patients can often lead to
opportunistic infections which is one of the main causes of death in leukemia patients. Tomoda et al. (1988) found that the number of Candida in the intestinal tract is higher in patients receiving antileukemic treatments and that this number is correlated with the incidence of Candida infections. Specifically, they observed that patients with $10^5$ Candida per gram feces have an increase in respiratory and urinary infections. Bifidobacterium administered orally to these patients aids in the prevention of respiratory and urinary infections by inhibiting the growth of intestinal Candida.

**d) Reduction of Serum Cholesterol Levels**

The intestinal microflora may influence serum cholesterol levels. Eyssen (1973) observed that germ free animals have more difficulty excreting cholesterol than conventional animals which suggests that the intestinal microflora may interfere with cholesterol absorption from intestines (Gilliland, 1989). It is not yet understood how bifidobacteria play a role in lowering serum cholesterol, but it has been shown that in rat models, bifidobacteria feedings results in a lowered cholesterol level (Homm, 1988, Hughes and Hoover, 1991).

In 1984, the Lipid Research Clinics Program reported that a reduction in plasma cholesterol level in hypercholesterolemic patients is associated with a
significant reduction in risks of heart attacks. (Gilliland, 1989).

e) Synthesis of B-complex Vitamins and Absorption of Calcium

Bifidobacterium can synthesize B group vitamins in the intestine (Kim, 1988, Shahani and Ayebo, 1980) and increase the amount of calcium absorbed by the body (Rasic, 1989). Hughes and Hoover (1991) reported that adding B. bifidum to the intestinal tract results in a 400% increase of vitamin B₆ content in stools. Bifidobacteria are also known to produce thiamine, riboflavin and vitamins K. The impact of these vitamins on human nutrition, however, is still unknown (Hughes and Hoover, 1991).

2. Viability of Cultures

There is still a lot of controversy surrounding the health claims of Bifidobacterium (Hose and Sozzi, 1991). The debate centers around whether the bacterium stays viable until the product is consumed, whether it is able to survive the low pH and proteases of the stomach before it reaches the intestines, and whether it is successful in colonizing the intestines.

Bifid-containing products, if they are to be effective, need a dose of 10⁶ viable organisms/mL or greater (Hoover and Hughes, 1991) and therefore it is important for the cultures to remain viable until the product is consumed. It
has been reported that *Bifidobacterium* remains viable in yogurt and milk for long periods of time (Daly, 1991). Soft-serve frozen yogurt, however, may be more detrimental to the bacteria since air is pumped into the product as it is frozen. Holcomb et al. (1991) reported that frozen storage for 6 hours at -5°C in soft-serve yogurt mix has no effect on *Bifidobacterium*’s resistance to selective agents, which indicates that the cells survive and are not injured. Soft-serve frozen yogurt is rarely kept for more than a few days. Hard-serve frozen yogurt, however, may be stored for months and therefore *Bifidobacterium* is exposed to a longer period of stress before it is consumed. Laroia and Martin (1991) studied the effects of an eight week storage of 5.5 X 10⁹ CFU of *B. bifidum*/mL in high pH (5.6-5.8) frozen yogurt at -20°C and observed that a significant number (4.0 X 10⁶) of bifidobacteria survived. Hekmat and McMahon (1992) observed that *B. bifidum* stored at -29°C for 17 weeks in ice cream (pH of 4.9) decreased only from 2.5 X 10⁸ CFU/mL to 1 X 10⁷ CFU/mL. They concluded that probiotic ice cream is a suitable vehicle for delivering *Bifidobacterium* to consumers. Marth (1973) reported that the casein, sucrose and lactose in the frozen yogurt mix have cryoprotective properties (Holcomb et al., 1991).

Packaging material is also a factor in the survival of *Bifidobacterium* (Kim, 1988, Yuguchi, 1984). Mil-Mil, a
Japanese product, is filled and sealed into cartons in such a manner that no air is retained (Anon, 1978). It has been reported that Bifidobacterium packaged in glass or aluminum maintains the highest percent survival during a 10 day storage period (Kim, 1988).

Once Bifidobacterium is consumed, it must survive the stressful environment of the stomach. A prerequisite for any effect of ingested bacteria on the intestinal flora is that approximately $10^9$ bacteria pass into the intestines (Berrad, et al., 1990). Rao, et al. (1989) observed that when Bifidobacterium is exposed to a simulated gastric environment for one hour none of the organisms survive. This was confirmed by Holcomb et al. (1991) who observed that when B. bifidum is exposed to 0.01N HCl for 2 hours, a simulation of the conditions and time for the passage through the stomach, it does not survive. In 1990, Berrad et al., observed that the ability to survive a gastric environment is strain specific, although the specific strains were unknown to them. They observed that in an in vitro environment with a pH of 3, one strain decreases by one logarithm unit, while a second strain has a four-logarithm unit decrease. They were able to confirm this in vivo. Sakai et al. (1987), after preparing gastric juices buffered at different pH's, observed that the effect of gastric juice on bifidobacteria is species and strain
specific and is strongly dependent on the pH of the solution. They reported that B. breve 203 is stable at low pH values and therefore could be an ideal bacteria for use in bifid products. Their findings suggest that while Bifidobacterium spp., in general, seem unable to survive the stomach environment, specific species and strains vary in their resistance. In addition, yogurt is an excellent buffer (Savaiano and Levitt, 1987) and can protect cultures from stomach acid and proteases. Because of its high pH, the ice milk mix which comprises up to 90% of the frozen yogurt, would also make an excellent buffer.

Once Bifidobacterium reaches the intestines, it must colonize them in order to be beneficial to the host. The primary barrier to bifidobacteria in the intestines is the presence of bile salts, which are released at the duodenum, the entrance point of the small intestine (Hill, 1986, Mitsuoka, 1978). Bifidobacterium is resistant to bile salts, which are strong surface-active agents (Rao, et al., 1989, Mitsuoka, 1978). Gilliland (1979) reported that Bifidobacterium grows well in the intestines and exhibits resistance to low surface tensions. Homma (1988) reported that Bifidobacterium can adhere easily to the intestinal epithelial cells and far excels E. coli and L. acidophilus in adhesion.
Another topic of debate is which species and strains of bifidobacteria are best suited for frozen yogurt cultures. Different species of *Bifidobacterium* may vary in their ability to colonize the human gut, especially since some species are host-specific. It has been reported that a non-host specific species will induce an immune response from the host (Gilliland, 1979). Indigenous intestinal bacteria, on the other hand, possess antigens that resemble those of the host and are recognized as "self" (Mitsuoka, 1978). Strains and species may also differ in their resistance to bile (Gilliland, 1989). Berrad et al. (1991) observed a large difference between two strains of *Bifidobacterium* in their resistance to a gastric environment. Sakai et al. (1987) reported that *B. breve* shows higher resistance to gastric juice than *B. longum* and that the strain *B. breve* 203 shows more stability at low pH's than other strains. *B. breve*, however, is mostly found in infants and is not a major component of the adult microflora and, therefore, its use may be limited.

Japan uses different cultures than some European countries. Japanese manufacturers use *B. longum* while European manufacturers prefer *B. bifidum*. In Japan it is accepted that *B. longum* performs useful functions to the host. New strains have been produced from selective culturing of parent *B. longum* strain which have been shown
to have increased antibiotic and acid resistance and are antagonists of pathogenic organisms (Kim, 1988). Also, there is extensive research currently being performed in Japan to select for an oxygen resistant strain of bifidobacteria. Mutai, et al. (1980) were successful in selecting an oxygen resistant mutant from B. bifidum (Kim, 1988).

Some authors have suggested using microencapsulation to aid the bacterium in reaching the intestines (Rao, et al., 1989, Kim, et al., 1988). Rao, et al. (1989) developed a procedure for the microencapsulation of bifidobacteria which would aid the bacterium in its passage through the stomach. The coating consists of cellulose acetate phthalate (CAP), a polymer which is insoluble at a pH of 5 and lower. They conducted in vitro studies and observed that microencapsulated bifidobacteria survive a simulated gastric environment in larger numbers than non-encapsulated bifidobacteria. Microencapsulation is a new topic that is showing success and warrants future studies to investigate the in vivo survival of microencapsulated bifidobacteria.

3. Injury and Enumeration of Bifidobacterium

L. acidophilus and Bifidobacterium spp. are often used in combination when used as dietary adjuncts (personal communication with Chris Hansen's Laboratory). Since L. acidophilus is more active in the small intestine and
Bifidobacterium is more active in the large intestine (Laroia and Martin, 1992), a combination of these two cultures in a product renders it more effective then a product with only one culture. The problem with inoculating a product with a combination of \textit{L. acidophilus} and \textit{Bifidobacterium} occurs during the enumeration. Because of the similarities of the two genera, separating one from the other is difficult. Chris Hansen's Laboratory takes advantage of \textit{L. acidophilus}'s ability to grow in both aerobic and anaerobic conditions. They enumerate the cultures from a product by plating duplicate samples on MRS agar and incubating one sample aerobically and the other anaerobically. The difference between the anaerobic count and the aerobic count is the number of bifidobacteria (personal communication). Other authors that have studied \textit{L. acidophilus} in conjunction with \textit{Bifidobacterium} acquired separate cultures of the two bacteria to facilitate enumeration (Holcomb et al., 1991, Hekmat and McMahon, 1992). To date no medium is used by the dairy industry that separates \textit{Bifidobacterium} from \textit{L. acidophilus} without complicated confirmational steps. There exists a need for a medium that would select for \textit{Bifidobacterium}.

A second problem with current enumeration methods, is that they do not take into account the injury that some cells may have acquired. The pH, temperature, % solids,
osmotic pressure and oxygen content of the frozen yogurt may all contribute in stressing and injuring *Bifidobacterium* cells (Sakai, et al., 1987). Also, the passage of *Bifidobacterium* through the stomach and the duodenum can be detrimental to the organism. It is important to enumerate all *Bifidobacterium* cells in frozen yogurt, injured and non-injured, since it has been reported that injured cells, when in the proper environment, can fully repair their damage and function once again as normal undamaged cells (Flowers and Ordal, 1979, Ray, 1989, Hackney, et al., 1991a). To date, no research has been done on whether injured *Bifidobacterium* cells can repair in the intestines and regain their beneficial characteristics but there have been reports on injured organisms which were able to recover their pathogenicity in the host and caused clinical symptoms. Sorrells et al. (1970) reported that freeze injured cells of *Salmonella gallinarum* are pathogenic to White Plymouth Rock chicks. Colwell et al. (1990) observed that when human volunteers ingest nonculturable cells of *Vibrio cholerae* they acquire clinical symptoms of cholera infection and *V. cholerae* is recovered from their stools. Singh et al. (1985) observed that copper- and chlorine-injured *Versinia enterocolitica* cells fed to mice are further injured by the gastric pH but when they reached the small intestines, they are able to resuscitate and produce virulent effects. Singh
et al. (1986) reported that both enteroinvasive and enterotoxigenic strains of *E. coli*, injured with sublethal doses of chlorine and copper, are able to resuscitate in the small intestines of mice and produce enterotoxigenic activity. These studies suggest that injured pathogens can survive the stomach and resuscitate in the small intestines, where they multiply and regain their virulence (Ray, 1989). It is probable that non-pathogens such as *Bifidobacterium* can also repair in the intestines.

Methodologies have been introduced which allow for the enumeration and selection of injured anaerobic cells. They include VPI's Anaerobic Roll Tube Procedure (Holdeman, et al., 1977) and Speck, et al.'s (1975) repair detection system (see Chapter One). The purpose of this study was to modify Modified Human Bifid Sorbitol Agar (see Chapter One) so that it could be used with a roll tube repair-detection procedure to enumerate both injured and noninjured cells of *B. bifidum* and *B. animalis/longum* from frozen yogurt. In addition, the medium was modified so that it would separate *Bifidobacterium* from *L. acidophilus*.

4. *Bifidobacterium* Products

*Bifidobacteria* products can be made one of two ways. *Bifidobacterium* can be added during fermentation of the dairy product or it can be added to the finished fermented product just before it is shipped. Products that have been
fermented with *Bifidobacterium* tend to have a mild acidic flavor. Products to which culture was added prior to shipment, when proper refrigeration temperatures are followed in order to prevent fermentation activity, are organoleptically indistinguishable from the unfortified versions (Hughes and Hoover, 1991).

In 1976, Germany sold 400 million units of Biogarde, a bifid product (Hughes and Hoover, 1991). Biokys, a cultured milk beverage which includes *Bifidobacterium bifidum*, was introduced in Czechoslovakia in 1977 (Lang and Lang, 1978). In 1978, Mil-Mil, a product based on milk cultured with bifidobacteria was introduced in Japan. It is manufactured by using a special packaging system that fills and seals cartons with Mil-Mil without any air being retained (Anon, 1978). Mil-Mil is designed to deliver $10^6$ bifidobacteria per gram (Hughes and Hoover, 1991). In 1981, Lang reported seeing Bifid products at a food exhibition in Munich with new flavors which included mango, blood orange, kiwi gooseberry and Williams pear. The products contained the fruit in a layer at the bottom and the yogurt above. "Nutrish a/B", a two-percent milkfat milk which incorporates both *Bifidobacterium* and *L. acidophilus* was introduced to Scandinavia in the early 1980's by Chris Hansen's Laboratory (Milwaukee) (Morris, 1991). Cultura™, containing $>10^6$ *Bifidobacterium* cells/cm$^3$, is manufactured and sold in
Denmark (Daly, 1991). Yoplait and Dannon, well known yogurt manufacturers, also have introduced bifid products into the European Market (Hughes and Hoover, 1991).

Bifid dairy products have gained wide popularity in Europe and Japan. In Japan, bifid-containing products account for more than one third of the total yogurt sales. In France, bifid-containing products are 4% of total fresh milk sales and 11% of yogurt sales, a 300% rise from when they were first introduced. Bifid products are also sold in Germany, Czechoslovakia, Denmark, Italy, Poland, England, Canada and Brazil (Hughes and Hoover, 1991).

The United States is slowly following the bifid-products trend which is so popular in Europe and Japan. Morris (1991) reported seeing a variety of bifid products at the 1990 International Dairy Show in Annaheim including Nu-trish a/B' and Sbifidus. Nu-trish a/B', already introduced to Scandinavia in the 80's, was added to the American market in 1987. Chris Hansen's Laboratory reports that 31 licensed U.S. dairy processors had produced nearly 6.5 million gallons of Nu-trish a/B' by mid-1990, up from one dairy and 6,600 gallons in 1987 (Morris, 1991). Lieb (1988) reported that Mayfield Dairy Farms of Athens, the first dairy in the country to introduce Nu-trish a/B', quadrupled their sales within 7 months of when they first placed it on the shelf. Mayfield Farms reports that it was contacted by many
satisfied customers who could not drink milk but were able to consume Nu-trish a/B' (Hughes and Hoover, 1991). Many consumers of the new milk claimed to see a vast change in their metabolism. One woman in particular claimed that it cured her infant's diarrhea which had plagued the baby for months (Lieb, 1988). At the International Dairy show in Annaheim, Morris (1991) also reported seeing "Sbifidus", a lowfat frozen yogurt containing Bifidobacterium and L. acidophilus, from Sanobi Bio-Industries (Waukesha, WI).
III. MATERIALS AND METHODS

A. Source of Bacteria

Cultures of B. bifidum, B. animalis/longum and L. acidophilus were provided by the Chris Hansen Laboratory (Milwaukee, Wisconsin). All cultures were received in the form of pellets. Chris Hansen Laboratories prepared these pelletized cultures by first growing the cultures in broth or milk until they reached late log/early stationary phase. Cultures were concentrated with a centrifuge and pelletized by instantly freezing them using liquid Nitrogen. To reactivate the cultures, Chris Hansen Laboratories recommended placing the pellets in a lukewarm mix and mixing 3 to 4 minutes (personal communication). Pellets were kept in a -20°C for up to 4 weeks.

B. Medium

Modified Human Bifid Sorbitol Agar (MHBSA) (see Chapter One) was further modified and was used to enumerate B. bifidum and B. animalis/longum. The further modification to the HBSA involved substituting glucose for sorbitol because B. bifidum and animalis/longum species do not ferment sorbitol. The new medium, Modified Human Bifid Glucose Agar (MHBGA), contained (g/L distilled water): Glucose 10, polypeptone 10, yeast extract 20, casamino acids 8, sodium chloride 3.2, phenyl red 40mL (of stock solution containing 0.675 g phenyl red/1000mL distilled water), methylene blue
3mL (of stock solution containing 0.02g methylene blue/100mL distilled water), cysteine 0.4 and agar 25. The pH of the medium was adjusted to 7.1 +/- 0.1.

Anaerobic dilution blanks were prepared as described in Chapter One.

C. Selective agents

A stock solution containing three selective agents was prepared. It contained (g/10mL distilled water): nalidixic acid 0.056, kanamycin sulfate 0.085 and polymyxin B 0.00215. This stock solution was prepared so that 0.1 mL would be 17X the strength recommended per mL of medium since after the overlay, each roll tube would contain 17mL of medium.

D. Inoculation and Repair-detection Procedures

Strawberry Frozen yogurt from "I Can't Believe It's Yogurt"™ was used in this study. The yogurt had a pH ranging from 6.0 to 6.5. The inoculation procedure involved allowing the yogurt to soften, adding enough pellets to arrive at a count of 10^6 cells/g of frozen yogurt and mixing 3 to 4 minutes with a sterile glass rod. Samples were enumerated with MHBGA in the repair-detection roll tube procedure (as described in Chapter One). All counts, whether in a roll tube or a plate, were done in duplicates.

E. Enumeration of Pellets

Chris Hansen Laboratories claimed that each gram of pellets they provided (containing a mix of B. bifidum, B.
animalis/longum and L. acidophilus) contained 10^{11} CFU. This number was verified by doing a direct pellet count into MHBGA. Pellets were placed directly into anaerobic blanks and mixed 3 to 4 minutes. The appropriate dilutions were made and enumeration was performed using selective roll tubes consisting of 17mL of MHBGA and 0.1mL of the selective stock solution. Incubation was at 37°C for 72 hours.

**F. Bifidobacterium: MHBGA vs MR SO**

The ability of MHBGA to enumerate *B. bifidum* and *B. animalis/longum* in the roll tubes was compared to Chris Hansen Laboratories's enumeration procedure with MR SO in pour plates and anaerobe jars. MR SO plates contained MRS (BBL) medium with the addition of .15% Oxgall. MR SO medium was prepared and tempered in a 49°C water bath for no more than two hours prior to its use.

Pellets containing only the *Bifidobacterium* species were placed directly in an anaerobic dilution blank and mixed for 3 to 4 minutes. This blank was used to inoculate MHBGA roll tubes. The tubes contained 17mL of the medium and 0.1mL of the selective stock solution. The same blank was used to inoculate MR SO pour plates. For each dilution two sets of duplicate plates were poured: one set was incubated aerobically and the other anaerobically (anaerobic plates were stored in anaerobic jars). This followed the Chris Hansen Laboratory technique used to separate
Bifidobacterium from L. Acidophilus which subtracted the anaerobic plate count from the aerobic plate count to determine the number of Bifidobacterium. The time and temperature of incubation were 37°C and 72 hours. After 72 hours, the colony morphology in the MHBGA agar and the MRSO was observed and recorded. Identification was confirmed by gas chromatography (see Chapter One). The presence of L. acidophilus was confirmed by the presence of lactic acid as the main fermentation product.

G. Lactobacillus Acidophilus: MHBGA vs. MRSO

Pellets containing only L. acidophilus cultures were grown in the MHBGA roll tubes and MRSO aerobic and anaerobic plates following the same procedure described above. Colony morphology in MHBGA and MRSO was observed and recorded after 72 hours. Colony identification was confirmed by gas chromatography as described in Chapter One.

H. Lactobacillus Acidophilus and Bifidobacterium:
MHBGA vs. MRSO

Pellets containing a mix of Bifidobacterium bifidum, B. animalis/longum and L. acidophilus were grown in MHBGA with the roll tube repair detection procedure and in MRSO with plates and anaerobe jars following the same procedure described above. Identification of the two species was detected by their morphologies and was confirmed by gas chromatography.
I. Direct Inoculation Vs. Saline-Vehicle Inoculation

A procedure was developed to compare methods of inoculating *Bifidobacterium* into the frozen yogurt. Chris Hansen Laboratories' recommendation of placing the pellets in a lukewarm mix and mixing for 3-4 minutes before adding to the frozen yogurt was compared to a direct inoculation. Pellets containing a mix of *Bifidobacterium* and *L. acidophilus* cultures were inoculated directly into frozen yogurt and stored 24 hours at -12°C. A second inoculation was performed by inoculating the pellets into a sterile saline solution (0.85% NaCl) at room temperature, mixing 3-4 minutes and using the solution as a vehicle to inoculate the frozen yogurt (calculated for $10^6$ cells per g of frozen yogurt) which was also stored at -12°C for 24 hours. Enumerations with the roll tube repair-detection procedure of both the direct and the saline samples followed. Selective overlays were performed after 0, 2 and 4 hours. These counts were compared to the calculated count of $10^6$ cells per g of frozen yogurt.

J. Time Needed for Repair of Injury

A study was performed to detect the amount of repair time that the *Bifidobacterium* cultures would need in the nonselective MHBGA. The saline solution was used as a vehicle to inoculate the frozen yogurt. Duplicate samples were enumerated in MHBGA after 0 hours, 24 hours and 48
hours. For each time period, overlays were performed after 0 hours, 2 hours and 4 hours. The pH of the frozen yogurt was checked at each time period.

IV. RESULTS AND DISCUSSION

A. Enumeration of Pellets

The results of the pellet count confirmed Chris Hansen Laboratories' claim of $10^{11}$ CFU/g of pellets. Specifically, $2.9 \times 10^{11}$ CFU/g of pellets were detected. All inoculations in frozen yogurt performed in this study were based on this count.

B. Bifidobacterium: MHBGA vs MRSO

Chris Hansen Laboratories's method of enumerating Bifidobacterium was confirmed. No colonies were observed on aerobic MRSO plates. Bifidobacterium colonies were detected on anaerobic MRSO plates. The colonies ranged from 1/4 to 1/2 mm in size, varied in color from yellow to white and upon close examination appeared round.

Large (2-3mm) round yellow colonies of Bifidobacterium were observed on the MHBGA roll tubes.

The identity of Bifidobacterium spp. was confirmed with gas chromatography. The presence of the fermentation products, lactic and acetic acids, indicated Bifidobacterium species.
C. *Lactobacillus acidophilus*: MHBGA vs. MRSO

Chris Hansen Laboratories's methodology was again confirmed. *L. acidophilus* grew on both the aerobic and anaerobic plates in each with a CFU of $2.3 \times 10^{10}$. The colonies were 1mm or less in size and upon close examination appeared as white stars.

*L. acidophilus* colonies were difficult to enumerate on the MBHGA roll tubes. All colonies were less than 1/4 mm in size.

The identity of the colonies on the plates and the roll tubes was confirmed to be *L. acidophilus* with gas chromatography. The presence of lactic acid as the major fermentation product indicated *L. acidophilus*.

D. *Lactobacillus acidophilus* and Bifidobacterium: MHBGA vs. MRSO

Results on MRSO plates once again verified Chris Hansens' Laboratories' technique. Aerobic plates contained only *L. acidophilus* colonies, while the anaerobic plates contained both *Bifidobacterium* and *L. acidophilus*. However, morphology could not be used to distinguish the two species.

The MHBGA in the roll tubes was successfully separated the bifidobacteria cultures from the *L. acidophilus* cultures. Bifidobacteria cultures were round, yellow and 1mm or greater. *L. acidophilus* colonies were too small to enumerate with a size of 1/4mm or less.
E. Direct Inoculation Vs Saline-Vehicle Inoculation

Figure 4 shows the counts observed from frozen yogurt inoculated directly with the pellets and the counts observed when using saline as an inoculation vehicle. The inoculation procedure, which involved the saline vehicle, was more effective. As can be seen from Figure 4, the saline vehicle count is more than one log greater that the direct-inoculation count. In both cases, however, the count was below the calculated count of 10^6 cells/g of frozen yogurt. This observation indicated that upon the initial inoculation of the pellets into the yogurt, cell death occurred. In addition, in both the direct- and the saline-inoculations no injury was observed.

F. Time Needed for Repair of Injury

Results shown on Figure 5 indicated that two hours was sufficient time for Bifidobacterium cells to repair their injury. The amount of injury shown on Figure 5, however, was negligible. Results indicated that cell death predominated over cell injury. Again, it was observed that cell death occurred during the inoculation of pellets into the frozen yogurt. The pH of the strawberry frozen yogurt remained the same from 0 hours to 48 and 96 hours.
Figure 4: The repair-detection curve of *B. bifidum* and *B. animalis/longum* in MHBGA after a direct inoculation into an "I Can't Believe It's Yogurt" strawberry frozen yogurt sample and after an inoculation into strawberry frozen yogurt in which .85% saline was used as an inoculation vehicle.
The repair-detection curves of *B. bifidum* and *B. animalis/longum* in MHBGA in anaerobic roll tubes after 0 hour, 24 hours and 48 hours of injury in "I Can't Believe It's Yogurt"™ strawberry frozen yogurt. The study included a repair of 0 hours in nonselective MHBGA at 37°C before an overlay with selective MHBGA; a repair of 1 hour; 2 hours; 3 hours; and 4 hours. The inoculation of cultures was done with a .85% saline solution as an intermediate step.
V. CONCLUSION

A roll tube repair-detection procedure was developed to enumerate injured and noninjured Bifidobacterium cells from frozen yogurt and to separate them from L. acidophilus cells. The final procedure was the same as shown in Table 5 (Chapter One) except that in step 3, the tubes were incubated for two hours.

The MHBGA was able to separate Bifidobacterium from L. acidophilus by cell morphology. Bifidobacterium cells were 1mm or larger, round and yellow, while the L. acidophilus cells were so small (<1/4mm) on the MHBGA that their detection and enumeration was difficult. MHBGA, compared to Chris Hansen Laboratories' MRSO technique seemed more efficient since the MRSO technique does not separate the two genera by cell morphology.

It was observed that when inoculating the pellets of Bifidobacterium and L. acidophilus into frozen yogurt, it was more effective to use a saline solution as an intermediate step. This observation is consistent with Chris Hansen Laboratories' recommendation to place the pellets in a mix and mixing 3-4 minutes before adding to the frozen yogurt (personal communication).

Cell death predominated over cell injury in the frozen yogurt. As can be seen in Figure 5, after 24 and 48 hours there was a decrease in the CFU count from 0 hour, which
could not be recovered with the roll tube repair-detection technique. Figure 4 shows that the inoculation step was lethal to a large number of cells.

There are no Federal Standards for frozen yogurt and therefore one manufacturer's product can be very different from another manufacturer. Future research should focus on using the roll tube repair-detection technique on different frozen yogurts, varying in ingredients, pH and preparation procedures. For example, in this study, the pellets were added to softened frozen yogurt which had already been frozen. Chris Hansen Laboratories recommend adding the pellets to the ice-milk mix just before it is frozen which could be more detrimental to the cells since air is pumped into the mix during the freezing step. In the frozen yogurt used in this study, cell death was predominant over cell injury. Other frozen yogurt products may show different results. In addition, research should continue in the area of Bifidobacterium's reaction to gastric juices. The roll tube repair-detection procedure could prove useful in studying whether Bifidobacterium dies in the stomach or whether it is injured.

Rao, et al. (1989) has suggested using microencapsulation of Bifidobacterium to ensure its survival through the stomach. The roll tube repair-detection procedure could be a useful tool in studying the effects of
microencapsulation. Future research should also follow in using different species and strains of *Bifidobacterium*. Species and strains may differ in their ability to survive and their susceptibility to injury in the stomach.
LITERATURE CITED


VITA

Catherine Beatrice Arany was born December 27, 1968, in Ixelles, Belgium, where she lived for seven years before moving to Madrid, Spain. In 1979, she moved to the United States and became a citizen in 1989. She graduated from Franklin Regional High School, Murrysville, Pennsylvania in 1986. She completed her Bachelor of Science degree in Biology, Summa Cum Laude, from Virginia Polytechnic Institute and State University, Blacksburg, Virginia, in 1990. She is currently pursuing a Master of Science degree in Food Science and Technology from Virginia Polytechnic Institute and State University, Blacksburg, Virginia. She will begin her Ph.D program in September of 1992 at the University of Minnesota.