SURVIVAL OF *BIFIDOBACTERIUM LONGUM* DURING FROZEN (-196 °C) AND ENSUING REFRIGERATED (5 °C) STORAGE AS INFLUENCED BY GROWTH AT VARIOUS PH

By

SIOBHAN SUE REILLY

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University of Florida

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Thesis Approved:

Stanley E. Gilliland
Thesis Advisor

P. Larry Claypool

Barbara J. Stoecker

Thomas C. Collins
Dean of the Graduate College

PREFACE

The effect of pH of growth on the viability and bile tolerance of four strains of *Bifidobacterium longum* during frozen storage at -196 °C and ensuing refrigerated storage in milk at 5 °C was investigated.

I would like to express my most sincere appreciation to Dr. Stanley Gilliland. Thank you for your guidance, leadership, and encouragement throughout this study and, the opportunity to grow both academically and professionally. You have made the difference.

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CHAPTER I

INTRODUCTION

Bifidobacteria have gained considerable attention over the last two decades with *B. longum* emerging as an important candidate as a dietary adjunct. Research efforts have indicated that when *B. longum* is taken as a dietary adjunct it may provide probiotic effects. Possible benefits include increased lactose utilization, control of serum cholesterol, anticarcinogenic activity and protection from food borne pathogens.

Both nonfermented and fermented products are made such that they incorporate bifidobacteria either alone or in conjunction with other lactic acid bacteria. Nonfermented milk products which incorporate *Bifidobacterium* spp into cold, pasteurized milk prior to packaging are gaining popularity in the United States.

Potential probiotic activity of the culture once ingested probably will be maximized if a high number of cells are present during consumption. The number of viable organisms in dairy products containing bifidobacteria can drop over 4 log cycles during the first two weeks of refrigerated storage (65). To ensure high numbers of viable cells, the production of the concentrated cell crop should involve growth under optimal conditions with respect to media, harvest time, and pH.

In addition, the concentrated cell crop should be stable during freezing and frozen storage (-196 °C). Also, the subsequent milk product produced from the concentrated culture should contain sufficient viable cells which survive refrigerated storage conditions.

The objectives of this study were to determine the effect on storage stability of different strains of *B. longum*, when grown at various pH levels, during frozen storage (-196 °C) and subsequent storage in milk (5 °C).

CHAPTER II

REVIEW OF THE LITERATURE

For the past century, bifidobacteria have been examined as potential health promoting bacteria. The first reported encounter with these unique organisms occurred in 1899 when a French scientist named Harry Tissier isolated a culture and named it *Bacillus bifidus* (65). The bacteria was noted as the predominant organism in the feces of breast fed infants and was gram positive, rod shaped with a characteristic "bifid" or "Y" shaped morphology (51,65,81). Later investigations led to its isolation from adults as well as infants, identification of various types of cellular morphology and characteristic bifidogenic factors that were responsible for the proliferation of this organism (65). Based on this information, this microorganism was placed in the genus *Lactobacillus* and was considered related to *L. acidophilus*. In 1934, researchers from Yale University concluded that the differences between bifidobacteria and *L. acidophilus* were so slight that they should be considered a variant of *L. acidophilus* (81). Hence, for the next 30 years this new organism became *Lactobacillus bifidus*. It wasn't until 1974 that these organisms (11 total species) were separated into the genus *Bifidobacterium* (72). Today, there are twenty-four species within the genus listed in *Bergey's Manual for Systematic Bacteriology* (72), five of which are important to the dairy industry for the production of a wide range of products. *B. longum*, *B. bifidum*, *B. breve*, *B. adolescentis* and *B. infantis* are of human origin and are currently considered potential organisms used as probiotic agents in food (43,76). Early on, Tissier regarded this organism as health promoting and subsequently used bifidobacteria to supplement the diets of infants with intestinal distress symptoms (65). Due to host specificity, the strains of human origin are preferred when used as dietary adjuncts (43, 65). These five species of bifidobacteria have some common characteristics but their differences are the basis for selection as a dietary

adjunct. *B. longum* and *B. bifidum*, when compared to the other three species may be better choices for use as a probiotic agent in food. Mitsuoka (58) indicates that only these two species can be found in the intestines of both infants and adults. Five different species were compared by Ishibasi (43), for their effectiveness in yogurt manufacture. *B. longum* performed the best when added to 15 % nonfat dry milk (NFDM) supplemented with yeast extract. It produced a relatively short coagulation time coupled with stable growth whereas the other four species (*B. adolescentis, B. infantis, B. bifidum* and *B. breve*) produced poor coagulation within a reasonable time or failed completely to grow in milk (43). Numerous reports have indicated that strains of *B. longum* perform well with respect to pH tolerance, growth, survivability, and bile salt deconjugation activity (13,42,43,59). Also, *B. longum* was found to be the predominant strain in adults and therefore more likely to establish and proliferate in the adult human intestine (38,58,59,76). According to Molder and coworkers (59), the use of *B. longum* in commercial applications currently is preferred over the other species.

Characterization

In general, bifidobacteria are gram-positive, nonspore-forming, catalase-negative, nonmotile rods having variable morphology. The optimum growth temperature ranges from 37 to 41 °C with a minimum and maximum of 25 °C and 45 °C, respectively. Growth of bifidobacteria does not usually occur below pH 5.0 or above pH 8.0 with an optimum range between 6.5 and 7.0 (72).

Glucose fermentation occurs by the unique metabolic pathway called the fructose-6-phosphate shunt (bifid shunt) and produces both acetic and L(+) lactic acids in a ratio of approximately 3:2 (65,72). These metabolic products are not only responsible for flavors and textures of various dairy products but may play a role in the control of harmful bacteria in both the food product and the human intestine (59,65). Metabolism by

bifidobacteria involves fructose-6-phosphate phosphoketolase (F6PFK), an enzyme which is an essential tool for distinguishing bifidobacteria from other microorganisms (72).

Most strains exhibit minimal tolerance to oxygen and are considered anaerobes. How a particular strain of *B. longum* reacts to the presence of oxygen may be dependent upon the intracellular catalase activity. Those that produce catalase, which is usually very weak, subsequently remove metabolically produced hydrogen peroxide. If not, the presence of H_2O_2 impedes the metabolic functions of bifidobacteria by inactivating F6PFK thus adversely affecting growth and survival (59,64,65,76). Another reaction that may occur in the presence of oxygen results in formation of superoxide and/or hydroxyl free radicals because of the lack of regulatory enzymes such as superoxide dismutase (76). These free radicals are electron scavengers which may destroy the cells of *Bifidobacterium*.

Bifidobacteria produce small quantities of ethanol, formic acid and succinic acid but do not produce CO_2, butyric or propionic acid (59,72). Many important vitamins are also synthesized by bifidobacteria including vitamin K, thiamin, and riboflavin(65,76).

Probiotic Properties

For almost 100 years there have been active investigations into the use of microorganisms to enhance or maintain health. The normal human intestine may contain pathogenic, non-pathogenic, putrefactive and lactic acid bacteria in various numbers depending upon the age, gender, health, and diet of the individual (58). Lactic acid bacteria such as *Streptococcus faecalis*, *S. faecium*, *Lactobacillus* spp., and *Bifidobacterium* spp., make up part of the normal intestinal flora (58) . The majority of previous work has concentrated on the genus *Lactobacillus* for providing potential health benefits when incorporated into the diet. However, research on bifidobacteria has been extensive for the last twenty years, with *Bifidobacterium longum* emerging as a

predominant potential dietary adjunct (58). Some of the possible benefits of consuming such lactic acid bacteria include resistance to intestinal infections, increased lactose utilization, anticarcinogenic activity, and control of serum cholesterol.

Antagonistic Action

Hoover (37) reported the use of bifidobacteria to prevent or minimize enteric infections. Japanese children were treated with large doses of bifidobacteria to reduce or eliminate gastrointestinal infections (37). Harmful bacteria (i.e., coliforms /putrifactive organisms) can easily infect the host and challenge an already compromised biological system when this normal gut flora is altered or destroyed. Some of the most problematic are enteropathogenic *Escherichia coli*, *Staphylococcus* spp., *Pseudomonas aeruginosa*, *Salmonella* spp., *Vibrio* spp., and *Clostriduim* spp. (58). Bifidobacteria may prevent the establishment and/or growth of undesirable bacteria by monopolizing the space and nutrients available in the intestine (37,65). In addition, metabolic products produced in the intestine by *B. longum* include acetic and lactic acid, which, are inhibitory to putrefactive bacteria. Inhibition of growth possibly results from the reduced pH environment created by the presence of either of the organic acids (37,65). Ibrahim (42), implied that the ability of five strains of bifidobacteria (*B. bifidum* ATCC 15696, *B. breve* ATCC 15700, *B. infantis* ATCC 15697, *B. longum* ATCC 15707 and *B. thermophilum* ATCC 25866) to inhibit growth of *E. coli* was due exclusively to the production of acetic and lactic acid. The inhibitory effects of the spent broth of each of the strains of bifidobacteria could be duplicated by using a mixture of these acids (41). Earlier research indicated that in addition to the effect of lactic acid, an inhibitory substance ("Bifidin") produced by a particular strain of *B. bifidum* also could provide some antibacterial activity against seven pathogenic microorganisms (2,3). Additional findings have indicated that changing the oxidation-reduction potential, motivated by growth of bifidobacteria in the

intestine, also is inhibitory toward some harmful bacteria (65). This is likely due to altered metabolism of aerobic and facultative anaerobes due to the change in available oxygen required for biochemical processes (58,73). Furthermore, Bernet, et al. (4), demonstrated the potential of various strains of bifidobacteria to prevent the invasion of human intestinal epithelial cells by enteropathogenic *E. coli*, *Yersinia pseudotuberculosis* and *S. typhimurium* (4).

Bifidobacteria have been implicated in providing the host with an increased immunological response. In particular, cell-mediated immunity was induced in mice exposed to a pathogenic strain of *E. coli*. In a review by Robinson and Samona (69), a protective barrier generated by the bifidobacteria may be responsible for preventing infection by some of the possible harmful bacteria. Germ-free mice to which *B. longum* had been administered either directly into the digestive system or intravenously were able to survive lethal injections of *E. coli* compared to mice without *B. longum*. Oral administration of *B. longum* prevented infection of *E. coli* fed 20 days later, conversely, mice not fed *B. longum* before being exposed to *E. coli* did not survive more than 48 hours (59,69). Overall, the antagonistic action of bifidobacteria may reduce the chances of obtaining an intestinal infection by preventing the growth of pathogenic or putrefactive bacteria or by inducing an increased immunological response therefore affecting the overall health of those consuming dairy products containing *B. longum* (37,43,58,59,65).

Anticarcinogenic Properties

There are numerous reported mechanisms by which bifidobacteria may provide anticarcinogenic effects (20,43,59,65,69,76). Antimicrobial activity of *B. longum* may lead to reduction of carcinogenic/toxic compounds that are generated by some of the putrefactive bacteria (59,65). These compounds may result from the microbial breakdown of various foods and drugs ingested by humans (78). For example, nitrates in the diet may

be reduced to nitrites, which, in conjunction with secondary amines, form nitrosamines in the stomach and intestine (58). Nitrosamines have been established as a causative agent for stomach and colon cancer. Also, some putrefactive bacteria degrade proteins and amino acids forming carcinogenic compounds such as amines, indols, and phenols (43). Mitsuoka (58) reported that the amino acids tyrosine and phenylalanine are changed into phenol by *E. coli* and *Clostridum* species producing a carcinogenic effect in mice. In addition, the enzymes involved in the conversion of procarcinogenic factors into carcinogens may be inactivated by bifidobacteria (20,58,59). Another possible mechanism is the involvement of lactic acid bacteria in the biotransformation of bile salts. Fernandes (20) reported that the presence of lactic acid bacteria either reduced the conversion of primary bile salts into secondary derivatives or adversely affected the population of microoganisms (*Clostridium*, *Bacteroides* and *Eubacterium*) that have been reported to participate in the transformation process of these cancer causing precursors. Using bifidobacteria to exert control of various harmful bacteria which produce these compounds may be an effective way of reducing the occurrence of certain types of cancer.

Serum Cholesterol Control

Lactic acid bacteria have been studied in terms of their ability to control serum cholesterol levels. This subject draws much attention since one of the leading causes of death in the United States results from coronary heart disease which has been linked to dietary fat and cholesterol. The average cholesterol intake has been approximated at 600 mg/d and increasing dietary levels above this has been implicated as possible factors in related diseases (40).

One possible mechanism by which bifidobacteria affect the cholesterol levels in the body is by the direct assimilation of cholesterol. Cholesterol can be taken up by some bacterial cells and incorporated into the membrane (67). This cholesterol thus, could be

removed from the intestines with subsequent microbial excretion. In 1975, the intestinal microflora was noted to have an effect on serum cholesterol levels in infants (35). The authors indicated that when the microflora consisted primarily of lactobacilli, the mean cholesterol value was low (119mg/100ml) whereas infants having more *E. coli* than lactobacilli had higher mean cholesterol values (147 mg/100ml). Grunewald (34) indicated that rats fed fermented skim milk containing *L. acidophilus* had significantly lower ($P < .05$) serum cholesterol (65mg/dl) than rats fed diets without *L. acidophilus* (78-79mg/dl). Gilliland, et al. (28), showed that, *L. acidophilus* RP32 had the ability to prevent increases of serum cholesterol of pigs fed a cholesterol rich diet. In a related study (18), pigs were fed a diet supplemented with 0.5% crystalline cholesterol for 14 days to create hypercholesterolemic conditions. On day 15, the cholesterol was removed from the diet and pigs were fed a regular diet with/without *L. acidophilus* ATCC 43121. The researchers indicated that total serum cholesterol levels of pigs receiving *L. acidophilus* were 11.8% lower than pigs fed a diet without the organism (18). Rasic (66), indicated that two strains of *B. bifidum* used for commercial yogurt production readily assimilated cholesterol. Jaspers et al. (44), showed that the consumption of yogurt containing *L. bulgaricus* and *S. thermophilus* significantly reduced serum cholesterol by 10-12% in adult humans. However, with continued yogurt consumption, cholesterol values equilibrated to the control levels after 14 days of continuous consumption (44).

Klaver et al.(48), studied the mechanism of cholesterol assimilation for both lactobacilli and *Bifidobacterium bifidum*. They concluded that cholesterol assimilation was not responsible for cholesterol removal, instead, cholesterol was coprecipitated with deconjugated bile acids (48). In response, Tahri et al. (75), determined the effects of three strains of bifidobacteria on cholesterol. Cholesterol precipitation in resting cells (pH5.0) of *B. breve* ATCC 15700, *B. longum* BB536 and *B. animalis* ATCC 25527 appeared to be transient since the cholesterol readily redissolved at pH 7.0. However in growing cells, the three strains of bifidobacteria (*B. breve* ATCC 15700, *B. longum* BB536 and *B.*

animalis ATCC 25527) removed 60, 50 and 39% of the cholesterol respectively. Cell extracts were recovered after repeated washings and determined to contained 42, 30 and 22 % respectively, of the missing cholesterol. This research suggested that cholesterol assimilation was dependent on cell growth and the presence of bile salts. Coprecipitation of cholesterol with free bile salts could not account for significant cholesterol removal (75).

Cholesterol removal occurs only in the presence of bile, therefore an organism selected for cholesterol assimilation should be tolerant to bile concentrations similar to that present in the gastrointestinal tract (10,28,33,80). Research by Gilliland et al (26,28), indicated that both assimilation and bile acid deconjugation by the organism are probable mechanisms involved in controlling serum cholesterol levels. The reduction of blood serum cholesterol by bile acid deconjugation can result from two possible routes: 1) if taurocholate and/or glycocolic acid is deconjugated in the intestine, the rate of cholesterol absorbed into the body is reduced. Since deconjugated bile acids do not support cholesterol absorption as well as do conjugated ones (77) and 2) once bile acids are deconjugated, they cannot readily enter back into the enterohepatic circulatory system, consequently, they are excreted in the feces (12). This reduces the body's bile acid pool, which inturn requires cholesterol (the precursor to bile acid synthesis) for replacement. Synthesis of replacement bile acids can lower the amount of cholesterol in the body (26,38). Research with bifidobacteria, has not been as extensive as that on lactobacilli. However, it has been established that certain strains of bifidobacteria do deconjugate bile acids (i.e., bile salt hydrolase activity) and assimilate cholesterol. Both are important criteria for dietary adjuncts used in cultured dairy products to provide potential for control of serum cholesterol. (42,66,75).

Lactose Maldigestion

In order to efficiently digest lactose, a carbohydrate commonly found in dairy products, the enzyme lactase must be present in sufficient amounts in the small intestine. Lactose is normally hydrolyzed into primary units of glucose and galactose, which can be absorbed from the intestines. However, when there is insufficient lactase, the lactose molecule may remain intact until it is broken down microbially in the large intestine leading to abdominal disorders (i.e., bloating, cramps, etc.). Surprisingly, a large percentage of adults throughout the world cannot tolerate lactose, the exceptions include northern Europeans, white Americans, and a few tribes in Africa. This problem is also very prevalent among the elderly since enzyme activity is likely to decrease with age (58). Many people suffer nutritional disorders which might otherwise be eliminated if they could efficiently digest dairy products (40). Reports indicate that the apparent benefit from consuming dairy products containing lactic acid bacteria occur, in part, to 1) a reduced amount of lactose in the cultured product and 2) the presence β-galactosidase in the cultures that can hydrolyze lactose in the small intestine (1,17,31,36,38). The first instance occurs within the dairy product during the fermentation process. Alm (1) reported that a significant amount of the lactose was utilized in fermented milk products such as bifidus and acidophilus milk. In addition, several lactose maldigestors showed no symptoms if they consumed these fermented milk products but had severe abdominal disorders when regular low fat milk was ingested (1). Strains of bifidobacteria may produce sufficient β-galactosidase to ensure efficient digestion of lactose in the gut, thereby allowing lactose maldigestors to consume and digest fermented dairy products (19,38). Desjardins (19) indicated that β-galctosidase activity of four different strains of bifidobacteria varied due to growth and the time in which β-galactosidase was induced. *B. infantis* ATCC 27920 and *B. longum* ATCC 15707 displayed the greatest β-galactosidase activity compared to *B. bifidum* ATCC 15696 and *B. breve* ATCC 15698. Interestingly,

in *B. longum* ATCC 15707, β-galactosidase was induced during the exponential phase (2-8 hrs) and again during the stationary phase (15 hrs) suggesting the presence of two β-galactosidase enzymes while the other three strains only showed signs of one induction period (19). Some research has indicted that results obtained are widely varied with some suggesting that there is no real effect (62,71). However, there is an overwhelming amount of evidence indicating enhanced lactose digestion in persons classified as lactose maldigestors can result from consumption of fermented dairy products (1,17,29,36,38). It is likely that these conflicting reports are due to the variation in the strains of bacterial cultures used, and that variation in β-galctosidase activity is due, in part, to variation among strains (31,54).

Product(s) Characteristics

Products made from bifidobacteria have been produced and promoted as therapeutic supplements for infants and adults for almost fifty years. Some of these include baby food, fermented and nonfermented milks, yogurt, cheese, ice cream, soya milk products, dried milk powder, and pharmaceuticals. In the United States the acceptance of such products is not as extensive as in Japan and Europe (59). However, during the last ten years there has been a dramatic increase in the consumption of these beneficial microorganisms and the associated products. These dairy products may contain only a strain of *Bifidobacterium* or a mixture of lactic acid bacteria such as, *Streptococcus salivarius* subsp. *thermophilus*, *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, or *Pediococcus acidilactici* (76).

The following product characteristics are summarized based on reports in the literature (43,49,65,76).

1) Nonfermented Milk Products: These products contain either bifidobacteria and/or lactobacillus cultures which are added to the milk after pasteurization and cooling (49,65,76). They have none of the characteristics of a typical fermented dairy product (i.e., sour taste) since the added cultures do not grow and produce the metabolic products responsible for the taste and aroma of fermented milk products. This type of product is more likely to be accepted by individuals not accustomed to the unique taste of cultured dairy products. Acidophilus/Bifidus (A/B) milk products available in the United States contain *Lactobacillus acidophilus* and *Bifidobacterium* spp. which are added to pasteurized and cooled milk prior to packaging (49). Bifidus milk is also a common product in Japan and Germany where it is consumed for its therapeutic properties (65,76).
2) Fermented Milk Products: Bifidus yogurt can be found in many countries and may also contain other organisms (i.e., *L. acidophilus*, *S. thermophilus*, etc.). These products are generally made with standardized, homogenized and pasteurized milk which has been cooled prior to inoculation. The mixture is then incubated for a predetermined time sufficient for coagulation and development of desired flavor attributes. Incubation temperature usually ranges from 37 ^{o}C to 42 ^{o}C and the product is cooled prior to packaging (76).

    a) Bifidus milk/yogurt: *Bifidobacterium longum*, *bifidum*, or *infantis* are commonly used to produce these dairy foods by incubating at 37 °C until the desired texture is achieved. The United Kingdom and Germany are currently producing such products (76).

    b) Acidophilus-Bifidus milk/yogurt: Russia, Germany, Denmark and Japan have a variety of products on the market that contain a mixture of *L .acidophilus* and *B.*

longum or *B. bifidum*. Fermentation of these cultures takes approximately 16 hours at 37 ^{o}C depending upon the desired consistency and flavor. "Mil-Mil" is a popular fermented drink produced in Japan which is made in this manner (43,65,76).

c) A variety of products containing bifidobacterium and other types of starter cultures (i.e., *S. thermophilus*, *L. delbruekii* subsp. *bulgaricus*) are produced in Germany. For example, Bifighurt is made with a slime producing strain of *B. longum* in combination with *S. thermophilus* and is fermented for about 4 hours at 42 ^{o}C. Biomild is a lowfat milk product fermented with *L. acidophilus* in addition to bifidobacteria (76). Each combination produces a slightly different taste and texture of fermented dairy food.

Acid and Bile Tolerance

Another requirement of *B. longum*, to be used as a dietary adjunct to cause benefits resulting from its growth in the intestines, is the ability to tolerate acid and bile after ingestion. In order for bifidobacteria to remain beneficial to the consumer, it must first survive passage through the stomach and survive the influx of bile from the gall bladder as it enters the small intestine. Resistance to gastric acidity by various strains of bifidobacteria has been well documented (5,13,63). An *in vivo* study by Berrada (5) indicated that survival during gastric transport was strain dependent. Two strains of bifidobacteria contained in fermented milk were ingested by twelve healthy volunteers. Samples were taken (via gastric tube) at 0, 30, 60, and 90 minutes following ingestion and total numbers of bifidobacteria enumerated. The transit time for the fermented milk product to be delivered to the small intestine was approximately 90 minutes. The population of strain #1 was reduced less than 2 log cycles during the 90 minute transit time. The decline for strain #2 was approximately 4 log cycles during gastric transit. The author concluded that the product containing strain #1 would deliver 1 X10^{9} total

bifidobacteria in 100g of product (5). The resistance to acidity would be a primary criteria for bifidobacteria used in the manufacture of dairy products containing these dietary adjuncts (5).

Biliary salts can impose bacteristatic and/or bactericidal effects on strains of bifidobacterium. Ibrahim and Bezkorovainy (42) reported the ability of selected strains of *Bifidobacterium* to survive a concentration of bile equal to or higher than that present the gastrointestinal system. Four strains of bifidobacteria (*B. bifidum* ATCC 15696, *B. breve* ATCC 15707, *B. infantis* ATCC 15697 and *B. longum* ATCC 15707) were tested for survival in the presence of bile salt (sodium glycocholate) at concentrations ranging from 0 to 0.3%. All four strains exhibited limited growth in the presence of increasing bile concentrations. The cells remained viable for 48 hours in the presence of 0.3% sodium glycocholate indicating the potential for survival through the intestines where the transit time is much shorter and the normal concentration of bile salts may range from 0.2 to 0.5% (42). Bouhnik (8), indicated that various strains of *Bifidobacterium* did remain viable following ingestion in fermented milk and subsequent fecal recovery. However, once the test subjects stopped consuming the milk product, bifidobacteria were not detectable after 8 days following the final ingestion of the milk (8). This is important since ultimate benefits remain dependent upon survival of bifidobacteria after ingestion and suggests the need to ingest the organism on a regular basis if benefits are to be realized.

Growth Requirements of a Dietary Adjunct

With continued research efforts, a criteria for selecting strains to use as dietary adjuncts has been developed. Steps must be taken to ensure the organism has the greatest chance of surviving processing and storage. Without this, there is uncertainty that the culture will be present in the food product at the time of consumption. During processing of the cell crop, maximum growth and cell numbers is necessary to ensure substantial

metabolic activity (23,25). Increased metabolic activity may promote optimal flavor and texture development and/or associated potential health/nutritional benefits (i.e., cholesterol reduction, lactose hydrolysis). If the commercial product is to be effective as a probiotic agent then the number of viable organisms consumed should be greater than $1X10^6$ CFU/ml (76).

Maintenance of an anaerobic environment at an optimum pH level for the growth of bifidobacteria is the key to the production of the organism for use as a dietary adjunct (65,79). It was also suggested that cultures able to grow at lower pH levels may tolerate higher levels of acidity in fermented milk products (25). Gilliland and Rich (32), tested two different strains of *L. acidophilus* grown at pH 5.0, 5.5, 6.0, 6.5 and 7.0 for stability during frozen and subsequent refrigerated storage in milk. They observed higher storage stability for both strains of *L. acidophilus* in milk held for 21 days at 5°C when the cells had been grown at pH 5.0 compared to those grown at any of the other four pH levels (32). The growth of bifidobacteria at certain pH levels may produce extracellular polysaccharide material that may add protection to the cells during frozen storage (68). Production of the polysaccharide was reported as being pH dependent with optimal production occurring at pH 7.0-7.5 for specific organisms (6,64). Optimal pH levels for maximum growth in cultures of *Bifidobacterium* have been reported to be around 6.5-7.0 but this may vary from strain to strain (72).

The ingredients that make up the growth medium should not restrict the production of the cell crop (25,57). In general, bifidobacteria rely upon an abundance of nutrients to grow and require a relatively expensive medium. It is common to use media enriched with large quantities of blood and tissue extracts which tend to be tedious to prepare and use (76). Ideally, a milk based medium should be used in order to facilitate addition to dairy products (79), however, this may result in problems associated with harvesting and concentrating the cell crop. Hughes and Hoover (39) found that *B. breve* 2258, *B. bifidum* 2715 and *B. longum* 15707 grew better in MRS broth supplemented

with lactose than in milk. In addition, Collins and Hall (14) suggested that the addition of 2% modified MRS broth to milk enhanced the growth of *B. adolescentis*, *B. infantis*, *B. longum* and *B. bifidum* strains.

To produce an optimum growth environment the medium should have a low oxidation-reduction potential to ensure the growth of oxygen sensitive bifidobacteria. This can be accomplished by adding a reducing agent (i.e., cysteine, thioglycolate) directly to the media, purging with nitrogen gas or a combination of both. Care must be taken when using reducing agents with bifidobacteria, since, if used in excess they may be detrimental to growth and subsequent survival(65).

Harvest time plays a vital role in the overall behavior of a cell crop. In general, the most desirable time to harvest cells occurs at the onset of the stationary phase of growth when cell growth and activity are optimum (15,16,74). Collecting cells too soon (during the early logarithmic growth phase) leads to lower total numbers of organisms and perhaps to lower metabolic activities. When testing three different strains of *L. acidophilus*, Brashears et al. (9), found there were no significant ($P>.05$) differences for two of the strains when harvesting cells in either the early or late stationary phase of growth with respect to storage stability (at either -196 °C or 7 °C), bile tolerance, β-galactosidase activity, or cholesterol assimilation (9). The third strain, remained significantly ($P<.05$) more stable during storage at 7 °C when harvested during the late stationary phase than when harvested in the early stationary phase. This stability may be due to the production of capsular material that the researchers observed during this phase of growth (9).

Preservation of Cell Crops

Strains of *Bifidobacterium* intended for use as dietary adjuncts may be prepared as concentrated cell suspensions (i.e., concentrated cultures) in order to be added directly to

the food product. For example, non-fermented milk products containing *L. acidophilus* and/or *Bifidobacterium* spp., are prepared by adding a concentrated culture to the milk just prior to packaging (76). In this case, the initial population of organisms should be known as well as their ability to survive shipping and storage until consumption. Methods used to efficiently preserve a concentrated cell crop are freezing and/or drying.

Frozen Storage

Extensive research has been done in order to determine which conditions are best for the preservation of concentrated cultures and maximum retention of viability and biochemical activities. Even though numbers of viable cells remain high, biochemical/probiotic activities may be lowered or destroyed due to cell damage during freezing and subsequent frozen storage. The loss of acid production activity due to injury and death is greater for cultures stored at -20 °C than for those stored in liquid nitrogen (-196 °C) thus, the optimum temperature for long storage periods is -196 °C (16). Gilliland and Lara (30), evaluated β-galactosidase activity of three strains of *L. acidophilus* as influenced by storage at -196 °C. There were no significant differences ($P>.05$) in viability of the three concentrated cultures of *L. acidophilus* during storage for 28 days at -196 °C. However, one strain of *L. acidophilus* was significantly affected ($P<.05$) after only 14 days of frozen storage with respect to β-galactosidase activity (30).

Concentrated cultures can be stored at temperatures higher than -196 °C without significant loss of activity if they are suspended in a cryoprotective medium, however, minimal loss of viability is attained if the temperature is -110 °C or lower (53). The survivability and activity of concentrated cultures resuspended in skim milk at -110 °C were 100% whereas survival rates at -40 °C and -20 °C averaged 94% and 42% respectively (53). Relationships reportedly occur between cellular composition, growth media, growth pH and freezing menstruum with respect to survival of lactic acid bacteria

at frozen storage temperatures (22,24,60). Moss and Speck indicated that the freezing menstruum was critical to the survival of *Lactococcus lactis* subsp. *lactis* during the early stages of freezing at -20 °C. They indicated that freezing in 10% nonfat milk solids resulted in less injury and death than cells frozen in only water (60). Gibson et al. (22), indicated that L-malic acid, when added to the media aided in survival of lactic streptococci at -23.3 °C. In a study by Gilliland and Speck (24), several cellular components were related to survival of lactic streptococci at -17 °C. They observed that cultures were more resistant to freezing if they were grown at pH 6.0 rather than statically (no pH control). They also observed a negative correlation between the cellular content of octadecanoic acid (18:1) and resistance of the lactic streptococci to freezing (24). Tween 80 (polyethylene sorbitan monooleate) is a surfactant consisting of about 70% oleic acid (octadecanoic acid) which has been shown to provide protection to cells during freezing (24,74). Smittle et al. (74), indicated that if *L. bulgaricus* cells were grown in the presence of Tween 80, storage stability in liquid nitrogen of the concentrated cell suspension was greater than observed for cells grown without Tween 80. It was determined that Tween 80, or specifically octadecanoic acid, was converted by *L. bulgaricus* into C_{19} cyclopropane fatty acids and incorporated into the phospholipid portion of the cell membrane lending to increased stability at freezing temperatures (74). Gilliland and Speck (24) also pointed out that the amount of capsular material surrounding the cells of lactic streptococci was related to their resistance to freezing. Specifically, as capsular material increased, loss of viability during freezing and subsequent storage at -17 °C was reduced (24). Sanders et al. (70), measured the stability of five strains of bifidobacteria commercially prepared as frozen concentrates. The initial population for four of the strains was approximately $1X10^{11}$ cfu/ml while one culture concentrate contained only $4X10^{8}$ cfu/ml. Three of the cultures remained relatively stable for 6 weeks at -20 °C whereas the other 2 cultures exhibited >1 log cycle reduction in numbers of viable cells (70).

Differences between storage at -20 °C and -196 °C may be attributed to the rate of freezing (7,55). Bacterial cells undergoing a transition from liquid to solid at higher temperatures exhibited elevated loss of cellular integrity and intensified dehydration. Intracellular ice crystals may form at either temperature and rupture the cell membrane, however, the accelerated rate of freezing associated with liquid nitrogen (-196 °C) generally results in smaller ice crystal formation (52,55,82). Subsequent thawing of the frozen concentrated culture may also affect the survival rates. Mazur (55) indicated that an increased rate of warming can prevent recrystallization of any intracellular ice crystals before the melting point is reached. Cryoprotective agents such as glycerol do not necessarily protect from intracellular ice formation but influence the rate of cooling (55), and intracellular solute concentrations (61). With increasing concentrations of glycerol, Mazur (55) suggested that the rate of cooling was substantially decreased resulting in an increase in survival of cells. In addition, increasing the viscosity of the freezing menstruum (i.e., increasing glycerol concentrations) may prevent intracellular water from leaving the cell during a slower cooling process (-20 °C) and subsequently affect survivability (55). Cell death may be related to the rapid exosmosis of water from the cell, associated with slower cooling rates, and may lead to lethal concentrations of intracellular solutes (55).

Frozen concentrated cultures have been scrutinized since liquid nitrogen storage is not always practical and the subsequent cultures require special handling and shipping. Freeze drying of culture concentrates involves freezing, sublimation and drying (11). Wright and Klaenhammer (82) pointed out the effects of freezing (-20 °C) and freeze drying on the survival of *Lactobacillus bulgaricus* grown in the presence of calcium. Beneficial effects were observed for two different strains of *L. bulgaricus* grown in the presence of calcium and subsequently frozen to -20 °C. The strains of *L. bulgaricus*, grown statically without calcium fortification, declined by over 2 log cycles after 24 hours at -20 °C. In comparison, the strains exhibited substantial improvement in stability (<1 log cycle reduction) when grown in the presence of $5.4X10^{-3}$ M calcium chloride. This effect

was also prevalent in freeze-dried cells. Both strains, grown in the absence of calcium, exhibited nearly a 3 log cycle reduction immediately after freeze drying. Declines for both strains continued by approximately 1 log cycle when stored for 5 days at -20 °C. When calcium was included during growth of these strains, the loss of viability immediately after freeze drying was not as great (approximately 1 log cycle). In addition, the inclusion of calcium seemed to offer some stability during storage for five days at -20 °C, but for only one of the two strains (82).

Spray drying generally involves pumping a concentrated cell suspension through an atomizer into circulating hot air (21). After the cells are dried the powder is collected and stored. Johnson and Etzel (45) compared the effects of spray drying, freeze-drying or freezing (-20 °C) on *Lactobacillus acidophilus* strain CNRZ-32. The overall survivability was highest for frozen and freeze dried concentrates at 54% and 48% respectively. Spray dried cells (dried at 82 °C or 120 °C outlet air temperature) had drastically lower survival at 15% and .08% respectively. The authors suggested that even though the spray dried cells had the lowest viability, acid production rates were similar to that of the frozen and freeze dried cell crops. Spray dried (low temperature) cells were reported to have higher L-lysyl aminopetidase and β-galactosidase activity compared to the frozen cells. Cells spray dried at the high temperature and the freeze dried cells were almost entirely destroyed with respect to enzyme activity (45).

Refrigerated Storage

In addition to ensuring optimal conditions during prolonged storage of the bulk cell crop, subsequent survival of bifidobacteria in the food product should be evaluated. This requires refrigerated storage of the product. Potential health attributes related to the use of probiotics can be attained only if the culture used is present in sufficient numbers and remains viable in the product prior to ingestion (27).

Fermented Dairy Products

The incorporation of *Bifidobacterium* spp., into fermented dairy products such as milk and yogurt has been increasing over the past 10 years. Several studies have examined the growth and survival of bifidobacteria in such fermented dairy products in relation to the associative cultures, acid stability, and storage at refrigerated temperatures.

Khedkar (46), prepared fermented milk with *Bifidobacterium adolescentis* alone or in associative culture with *L. acidophilus* or *S. thermophilus* at inoculation rates of 1:1, 1:0.5 and 1:0.1 respectively. Initial plating of *Bifidobacterium* yielded counts of $5.4X10^8$ cfu/ml (bifidobacteria alone), $2.0X10^8$ cfu/ml (bifidobacteria + *L. acidophilus*) and $1.1X10^8$ cfu/ml (bifidobacteria + *S. thermophilus*). After 10 days of refrigerated storage, numbers of bifidobacteria remained stable at $1.1X10^8$, $1.4X10^8$ and $1.7X10^8$ cfu/ml, respectively, in associative culture (46). Similarly, Medina (56), conducted a study on the survival of bifidobacteria in commercially fermented milk during refrigerated storage in the presence of *S. thermophilus* and *L. bulgaricus*. In contrast to the results of Khedkar (46), numbers of bifidobacteria declined 92.6 % after 24 days of storage at 7.5 °C. *L. bulgaricus* and bifidobacteria spp. declined at a much faster rate than *S. thermophilus*. It was concluded that the production of acid by *S. thermophilus* was detrimental to the survival of bifidobacteria (56). Further studies by Klaver (47), et al, indicated the growth and survival of 17 strains of bifidobacteria in milk alone or supplemented with a casein hydrolysate. Out of 17 strains, 15 did not grow in milk without the addition of casein hydrolysate. Klaver (47) indicated that these strains lacked sufficient proteolytic activity and either needed to be supplemented with additional nitrogen sources or grown in the presence of an associative culture that did break down existing milk proteins. The survival of the 17 strains also were monitored in yogurt during storage from 5-7 °C. Only three strains remained viable in yogurt for 30 days of refrigerated storage (47). In addition, Klaver (47) looked at the effect of pH on survival of 10 strains of *Bifidobacterium* during

storage at 5-7 °C in milk acidified with lactic acid to pH 4.0, 4.5, 5.5, and 6.5. While there was variation among strains, only one strain remained stable at all four pH levels for 28 days storage (47).

Additional research was conducted by Lankaputhra et al.(50), to determine the storage stability of various strains of bifidobacteria in the presence of hydrogen peroxide and lactic acid, simulating possible environments created when growing bifidobacteria in association with other starter culture bacteria. Only three of the cultures of bifidobacteria were able to tolerate the addition of both lactic acid (pH 3.7, 3.9, 4.1 and 4.3) and hydrogen peroxide (100μg/ml) to milk and were stable during refrigeration (50).

Nonfermented Milk Products

The use of nonfermented milk products containing added cells of *L. acidophilus* and/or *Bifidobacterium* in the United States has become popular. The most common is acidophilus milk, which is made by adding the desired strain of *L. acidophilus* just prior to packaging and storing at 5-7 °C (32,76). Recently, bifidobacteria either has been incorporated into milk already containing *L. acidophilus* (nonfermented Acidophilus/Bifidus milk) or added exclusively (nonfermented Bifidus Milk). This type of product may be more acceptable in the United States since there is no change in texture or flavor commonly associated with traditional fermented milk products.

Sanders et al.(70), investigated the stability of three strains of lactic acid bacteria (currently used commercially) stored at refrigeration temperatures in pasteurized skim milk. The milk was formulated to contain $1X10^7$ cfu/ml of *L. acidophilus* LH1, $1X10^7$ cfu/ml of *Bifidobacterium* BG9 and $5X10^7$ cfu/ml of *S. thermophilus*. Differential counts and pH were determined periodically during 21 days at 4 and 10 °C for the sample and uninoculated control. The authors indicated that all three cultures remained viable at 4 °C with only slight (<1 log cycle) losses at 10 °C after 21 days. A slight change in pH from

6.8 to 6.5 occurred for both the control and inoculated sample whereas, the 10 °C samples dropped to pH 5.5. This increase in acidity was, in part, due to the growth of psychrotropic bacteria and not the co-cultured lactic acid bacteria (70).

In another study (39), four strains of bifidobacteria and one strain of *L. acidophilus* were tested for the influence of refrigerated storage conditions (4 °C) in sterile milk on viability, acidity, β-galactosidase and α-galactosidase activity. β-galactosidase activity was intended to demonstrate ability of the organisms to break down lactose while α-galactosidase may indicate the utilization of carbohydrate indicating the potential for establishment and colonization on the small intestine. Prior to refrigerated storage nonfermented samples were prepared such that the final concentration of cells was approximately $1X10^6$ cfu/ml. Fermented samples were prepared using a 1% inocula of the appropriate organism followed by incubation for 16 hrs at 37 °C. All samples were plated at 3 day intervals during the 15 day storage period. Bifidobacteria in nonfermented milk remained stable at 4 °C for 15 days. The milk samples had a mean pH of 6.3 and titratable acidity of 0.18%. The author suggested that the higher pH may have contributed to survival of the cultures of bifidobacteria. The fermented milk exhibited substantial losses of numbers of bifidobacterium with a pH of 5.0 and titratable acidity of .50%. β-galactosidase and a-galactosidase activity remained stable for all strains during refrigerated storage conditions for both fermented and nonfermented milk (39).

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CHAPTER III

SURVIVAL OF *BIFIDOBACTERIUM LONGUM* DURING FROZEN (-196 °C) AND ENSUING REFRIGERATED (5 °C) STORAGE AS INFLUENCED BY GROWTH AT VARIOUS PH

Siobhan S. Reilly and Stanley E. Gilliland

Department of Animal Science, Oklahoma State University

Stillwater, Oklahoma 74078

ABSTRACT

Four strains of *Bifidobacterium longum* were grown at pH 5.5, 6.0, 6.5 and 7.0 and evaluated for survival and bile tolerance during frozen and subsequent refrigerated storage in milk. Concentrated cultures of *B. longum* were prepared from cells grown at the various pH levels, frozen and analyzed on day 0, 7, 14, 21 and 28 of storage in liquid nitrogen (-196 °C). Nonfermented milk, prepared from each concentrated culture on day 28 of frozen storage, was stored at 5 °C and analyzed on day 0, 7, 14 and 21 of storage at 5 °C. There were no significant reductions in cell numbers following initial freezing, regardless of the pH at which the cultures had been grown, for any of the four strains of *B. longum*. There were significant effects for strain, pH of growth and storage for some of the four strains *of B. longum* stored at 5 °C in milk. *B. longum* S9 was more stable than the other strains in that, no significant losses occurred, regardless of pH of growth. *B. longum* ATCC 15707 was more stable when grown at higher pH levels (6.5 or 7.0) than when grown at pH 5.5 or 6.0. Significant declines in viability occurred *B. longum* III when grown at all pH levels, except pH 5.5, during 21 days of refrigerated storage. *B. longum* II was more stable when grown at pH 6.0 than when grown at any of the other pH levels. *B. longum* S9 did not exhibit loss of bile resistance during refrigerated storage (5 °C) while the other three strains did.

INTRODUCTION

The potential health/nutritional benefits associated with lactic acid bacteria have been well documented (4). Bifidobacteria have emerged as a leading candidate for use as a dietary adjunct. Potential benefits for the consumer from consumption of *B. longum* include: antagonistic action toward intestinal pathogens, improved lactose utilization, anticarcinogenic action and control of serum cholesterol levels.

In the United States, milk is the main product which has been used as a vehicle for these probiotic cultures. The addition of *B. longum* to pasteurized lowfat milk prior to packaging could provide the consumer with a source of viable cells of this organism in a nonfermented product.

If *B. longum* is to be added to nonfermented milk as a dietary adjunct, the cells likely would be produced and stored as concentrated cultures. The production of frozen concentrated cultures of lactic acid bacteria and storage in liquid nitrogen (-196 °C) is preferred due to the relative low loss of cell viability that is sometimes associated with higher temperatures (-20 °C) or other methods of storage (i.e., freeze drying). However, because of expected variations among strains some may not survive as well as others during frozen and subsequent storage at refrigerated temperatures. It is essential that the cells survive during frozen storage and ensuing storage in the refrigerated milk product in order to provide the consumer with adequate numbers of viable bifidobacteria. Growth conditions used to produce the cells for concentrated cultures can be crucial to the production of cultures that will survive storage. The pH at which cultures of *L. acidophilus* were grown influenced their survival during refrigerated storage in milk (2).

If concentrated cultures of strains of *Bifidobacterium longum* are to be used as dietary adjuncts in milk they must be stable during freezing, frozen storage (-196 °C), as well as, refrigerated storage (5 °C) in milk. The objectives of this study were to determine

the influence of pH during growth of cells of *B. longum* on frozen (-196 °C) and subsequent refrigerated (5 °C) storage in milk.

MATERIALS AND METHODS

Source, Maintenance and Identity of Cultures

Four strains of *Bifidobacterium longum* were evaluated in this study. Two of the cultures, *B. longum* II and *B. longum* III, were commercially available cultures used for the production of cultured dairy products. *B. longum* ATCC 15707 was purchased from the American Type Culture Collection (Rockville, MD., USA) and *B. longum S9* was isolated in our laboratory during a previous study (1). These cultures are kept on reserve in the Dairy Food Microbiology stock culture collection at Oklahoma State University.

For each of the three replications, a new culture was removed from the stock culture collection and grown in MRS-Thio. The broth was MRS broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% sodium thioglycollate (Sigma Chemical Co., St. Louis, MO.). The cultures were maintained by routine subculturing into the MRS-Thio broth (1% inocula and 15 hr incubation at 37 °C) . Immediately before each experiment, the test strain was subcultured at least two times.

Identification of each culture was based on results obtained from API 50 CH test kits (bioMerieux, Inc.), fructose-6-phosphate phosphoketolase assay (12), and gram stain/morphology. Those gram positive rod shaped bacteria, positive for fructose-6-phosphate phosphoketolase were assumed to be species of *Bifidobacterium*. Modifications of the procedure described by the manufacturer for use of the API 50 CH kits for the identification of microorganisms involved incubation of the test kits in an anaerobic chamber (GasPak, BBL) and the elimination of mineral oil overlay prior to incubation at 37 °C. Preliminary tests in our laboratory indicated that a more distinct fermentation pattern was obtained when the mineral oil overlay was omitted from the procedure. Identification of the cultures was based upon their ability to ferment arabinose, ribose, xylose, galactose, fructose, mannose, mannitol, sorbitol, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, and

gluconate. Reaction patterns were compared to those listed for *Bifidobacterium* spp *in Bergy's Manual for Systematic Bacteriology* (12) and used as the basis for confirmation of identity.

Growth of Cell Crops of *B. longum*

Cell crops of *B. longum* were grown in a 7.5 L fermentor (New Brunswick Scientific Co., Inc., Edison, NJ) equipped with an inoculation/filling port, neutralizer port, autoclaveable pH electrode (Ingold, Andover, MA), mechanically coupled impeller, gas sparging line, and a sampling line. The impeller was driven by a mechanical stirrer (Talboys Engineering Corp., Montrose, PA) and maintained at approximately 45 rpm. The entire vessel was autoclaved for 45 min at 121 °C, allowed to cool and placed in a 37 °C waterbath heated by a constant temperature circulator (Fisher Scientific, Pittsburgh, PA). Three liters of sterile MRS-Thio broth were aseptically added through the filling port and slowly agitated until the temperature equilibrated to 37 °C. The pH was maintained at the desired level by using an automatic pH controller (Horizon Ecology Co.) in conjunction with a MasterFlex Pump drive (Cole Parmer, Chicago, Ill) designed to add neutralizer solution as needed. Neutralizer solution consisted of 10% sodium carbonate (Na_2CO_3) in 10% ammonium hydroxide (NH_4OH) prepared according to Gilliland and Rich (5). MRS-Thio broth was adjusted to either pH 5.0, 5.5, 6.0, 6.5, or 7.0 using the neutralizer solution or 20% sterile lactic acid. Nitrogen gas (HPLC grade) was sparged (7cc/min) through a sterile cotton filter and through the medium prior to the addition of a 1% inocula of a freshly prepared culture of *B. longum* and throughout the incubation period. The nitrogen flow rate was monitored using a nitrogen gas flowmeter (Cole Parmer, Chicago, Ill).

Harvesting and concentration of cell crops was done as described by Gilliland and Rich (5). The concentrated cell suspension (2 g portions) was then aseptically placed in

sterile cryogenic vials (2 ml volume) and submerged in liquid nitrogen for storage (-196 ^{o}C). A small (2 g) sample was reserved for immediate plating on MRS agar to determine the initial (Day 0) population of *B. longum*.

Enumeration Procedures

Enumeration of the bifidobacteria was accomplished by the pour plate method (including an overlay) using MRS-Thio agar or MRSO-Thio agar. MRS-Thio agar was prepared by adding 1.5% agar to MRS-thio broth. MRSO-Thio agar was prepared by adding 0.3% Oxgall (Difco Laboratories, Detroit, Mich.) to MRS-Thio agar. Appropriate dilutions were prepared using distilled water containing 5 % non fat dry milk (NFDM) and were plated in duplicate with the desired agar media. The 5% NFDM diluent was prepared by mixing the appropriate quantity of instantized nonfat dry milk powder with distilled water and 0.1% antifoam solution (Sigma Chemical), dispensing in 90 or 99 ml volumes followed by autoclaving for 15 minutes at 121 °C. A 1 ml portion of culture or milk was serially diluted using 5% NFDM dilution blanks. Preliminary studies indicated substantial losses of viable bacteria when using peptone diluent, whereas, no loss was observed when 5% NFDM was used. The plates were incubated at 37 °C for 48 hours in a GasPak (BBL) anaerobic chamber. All colonies visible with the aid of a Quebec colony counter were counted and recorded.

Determination of Harvest Time

Preliminary experiments, consisting of at least two replications, were performed to determine the harvest time for each culture at each pH level. Each of the four strains of *B. longum* were grown at each pH level according to methodology described above. Samples (10 ml) were aseptically removed hourly from the fermentor in order to

determine the population based on plate counts. The $\log_{10}$ of bifidobacteria per ml were plotted against incubation time to construct growth curves for each culture at each pH level. From the growth curves, the time required to reach early stationary phase for each was determined.

Survival During Frozen and Subsequent Refrigerated Storage

Survival of each of the four strains of *B. longum* was monitored during 28 days of frozen storage (-196 ^{o}C). Samples were plated on MRS-Thio agar prior to freezing (day 0) and on days 7, 14, 21 and 28. On the designated day, one vial was removed from the liquid nitrogen tank and placed in a 45 ^{o}C water bath for 5 minutes to thaw. Prior to opening, the outer portion of the sample vial was sanitized by dipping in a 70% ethanol solution. On day 28, samples were tested for numbers of bile tolerant bifidobacteria by plating on MRSO-Thio agar. The total and bile tolerant numbers of viable bifidobacteria were determined by the procedure described above.

Nonfermented milk containing *B. longum* was prepared using the concentrated cultures after 28 days of frozen storage. Following thawing, the appropriate amount of the concentrated cell suspension of *B. longum* to yield populations of approximately 1 X 10^7 to 1 X 10^8 CFU/ml was added to each of three bottles of sterile 10% NFDM. The bottles were then stored at 5 ^{o}C . Numbers of total and bile tolerant bifidobacteria were determined in each sample on days 0, 7, 14 and 21 days of storage at 5 ^{o}C. Total numbers were determined by plating on MRS-Thio agar as described in a previous section. Numbers of bile tolerant bifidobacteria were determined by plating appropriate dilution on MRSO-Thio agar.

Statistical Methods

The experimental design was a split plot in a randomized block design with a 4 X 4 factorial arrangement of treatments (main unit treatment factors = strain and pH, subunit treatment factor = storage time). Analysis of numbers of total and bile tolerant bifidobacteria was performed separately. The randomization of treatment combinations was done by the random numbers generator function (RANUNI) in SAS. PROC GLM and PROC MIXED in SAS was used to determine if differences exist between treatments and combinations of treatments.

RESULTS

Confirmation of Identity of Cultures

The identity of the four strains was confirmed to *B. longum* when fermentation patterns obtained from the API system were compared to those listed in Bergey's Manual of Systematic Bacteriology (12) for *B. longum* (Table 1). All four strains were gram +, rods and tested positive for fructose-6-phosphate phosphoketolase activity. Positive reactions for the fermentations of arabinose, ribose, xylose, galactose, fructose, maltose, lactose, melibiose, sucrose, melezitose, and raffinose were consistent for all strains with the exception of *B. longum* II (negative for ribose) and *B. longum* S9 (negative for melizitose). In addition, mannitol, sorbitol, salicin, cellobiose, trehalose, inulin, starch, and gluconate were consistently negative for all strains except for *B. longum* S9, which was able to ferment salicin.

Effect of pH During Growth on Total Numbers

During the preliminary determination of harvest times for each culture at the five different pH levels it was observed that *Bifidobacterium longum* strains ATCC 15707, II, and III did not grow at pH 5.0. Thus, planned experiments for these three strains at pH 5.0 were excluded from the study. *Bifidobacterium longum* S9, which did grow at pH 5.0, was evaluated at all five pH levels.

The overall model comparing strains and pH of growth was not significant ($P>.05$) indicating no differences for any of the treatment combinations. There was no significant interaction ($P>.05$) among the four strains of bifidobacteria and pH of growth. Trends in the data suggest that, when averaged over strains, the greatest growth occurred at pH 6.0 (9.95 Log_{10} CFU/ml) whereas pH 7.0 resulted in the lowest amount of growth (9.10 Log_{10}

CFU/ml). When averaged over pH levels, *B. longum* S9 and *B. longum* ATCC 15707 grew better (9.77 and 9.64 Log_{10} CFU/ml, respectively), compared to the other two strains, *B. longum* II and *B. longum* III (9.42 and 9.31 Log_{10} CFU/ml., respectively) (Table 2).

Effect of Storage at -196 °C

Frozen storage had little effect on the survival of four strains of *Bifidobacterium* grown at various pH levels. There were no significant ($P>.05$) two or three way interactions between any combination of strains, pH level or storage time. The data obtained from three replications did, however, indicate differences ($P<.05$) within strain, pH and storage time as individual treatments (Table 3). *B. longum* S9 and *B. longum* III exhibited overall greater numbers ($P<.05$) of total cells when averaged over pH level and storage time (10.86 and 10.81 Log_{10} CFU/ml, respectively) than observed for strains II and 15707. *B. longum* ATCC 15707 exhibited significantly ($P<.05$) higher counts than did *B. longum* II.

Significant differences in means ($P<.05$) with respect to total numbers of cells were found for the cultures grown at the various pH levels. When averaged over strain and storage time, growth at pH 6.0 provided higher ($P<.05$) number of cells (10.74 Log_{10} CFU/ml) than did pH 6.5 and 7.0 but not greater than pH 5.5 (Table 3).

In addition, when averaged over pH and strain, total counts for *B. longum* during frozen storage (-196 °C) declined significantly ($P<.05$) only after the initial freezing of the concentrated cell crops (Day 0 to Day 7) as indicated in Table 3. No significant ($P>.05$) declines in population occurred throughout the remaining storage period (through day 28).

Effect of Storage at 5 °C in Milk on Total Numbers of *Bifidobacterium*

Storage at 5 °C of nonfermented milk containing cells of *B. longum* influenced numbers of viable bifidobacteria. A significant (P<.05) three way interaction representing (strain x pH x day) was observed (Table 4).

Less death of *B. longum* ATCC 15707 occurred during 21 days of refrigerated storage when grown at pH 6.5 or pH 7.0 compared to the lower levels of pH. Total numbers of *B. longum* ATCC 15707 grown at pH 5.5 and 6.0 exhibited declines throughout the 21 days of storage however, the declines were significant (P<.05) only at day 21.

Viability of cells of *B. longum* II during storage at 5 °C was greatly affected by growth at different pH levels. This strain was more stable when grown at pH 6.0 than at pH 5.5, 6.5, or 7.0. However, over 21 days of refrigerated storage, significant (P<.05) declines, 7.35 (day 0) to 6.87 (day 21) did occur for the culture which had been grown at pH 6.0. Progressive declines in viable numbers for the 21 days of refrigerated (5 °C) storage for the remaining pH levels was evident. Cells of *B. longum* II grown at pH 5.5 declined significantly (P<.05) from 7.06 to 6.21 Log10 CFU/ml over 21 days. Even greater declines occurred when cells were grown at pH 6.5 ranging from 7.38 (day 0) to 6.34 (day 21) Log_{10} CFU/ml. Over a 2 log cycle reduction (7.31 to 4.68 Log_{10} CFU/ml) in numbers occurred during 21 days of storage when *B. longum* II had been grown at pH 7.0.

Declines (P<.05) in total numbers of *B. longum* III also were seen over 21 days of storage in milk at 5 °C with the exception of those grown at pH 5.5. There was no difference (P>.05) between 0, 7, 14, and 21 days of refrigerated storage when the cells were grown at pH 5.5. However, declines became increasingly greater with increases in pH. The largest reduction occurred at pH 7.0, where counts declined 0.73 log cycles over 21 days of storage.

B. longum S9 showed no significant (P<.05) declines in viable counts over 21 days of refrigerated storage for any pH level.

Effect of Storage at 5 °C in Milk on Numbers of Bile Tolerant *Bifidobacterium*

Storage at 5 °C of nonfermented milk containing cells of *B. longum* influenced numbers of viable bile tolerant bifidobacteria. A significant (P<.05) three way interaction representing (strain x pH x day) was observed (Table 5).

There was relatively little effect on numbers of bile tolerant *B. longum* ATCC 15707 when grown at pH 6.0, 6.5 or 7.0. Slight declines did occur over 21 days of refrigerated storage, however, none were significant (P>.05). A significant (P<.05) decline did occur between day 0 (7.39 Log_{10} CFU/ml) and day 21 (6.61 Log_{10} CFU/ml) when this strain had been grown at pH 5.5.

The decline in numbers of bile tolerant colony forming units for *B. longum* II was similar when the culture had been grown at either pH 5.5, pH 6.0 or pH 6.5. Counts were statistically different (P<.05) between day 0 and day 21 for all three pH levels. The greatest losses were observed for cells which had been grown at pH 7.0. Significant declines (P<.05) occurred on days 7 (6.66 Log_{10} CFU/ml), 14 (5.59 Log_{10} CFU/ml), and 21 (2.87 Log_{10} CFU/ml).

B. longum III showed similar trends with respect to the influence of increasing pH of growth. The greatest declines in bile tolerant numbers occurred when grown at pH 7.0. Initial counts (7.21 Log_{10} CFU/ml) dropped significantly (P<.05) after 21 days of storage (5.99 Log_{10} CFU/ml). The declines observed for cells grown at pH 6.0 and 6.5 were similar over the 21 day period. No significant (P<.05) declines occurred during refrigerated (5 °C) storage in milk for *B. longum* III when it had been grown at pH 5.5.

No significant (P<.05) declines in bile tolerant population occurred for *B. longum* S9 over 21 days of refrigerated (5 °C) storage in milk at any of the four pH levels.

Comparison of Numbers of Total and Bile Tolerant *B. longum* During Storage at 5 °C

Data to compare numbers of total and bile tolerant bifidobacteria were not statistically analyzed, however, graphical comparisons revealed some apparent differences due to strain variation, pH and storage time in nonfermented milk (Figures 1-4).

When grown at pH 5.5, *B. longum* ATCC 15707 appeared to become less bile tolerant during storage at 5 °C than did the other three strains. Figure 1A indicates an increasingly greater loss of bile tolerant numbers (MRSO) than total numbers (MRS) from day 0 to day 21. On day 0, the counts for ATCC 15707 were equal on MRS and MRSO agars however, as storage increased the numbers on MRSO agar declined progressively greater compared to the counts on MRS agar. Comparison of total and bile tolerant numbers the other three strains revealed about the same degree of decline for both media types (Figure 1B, 1C and 1D).

Comparison of total and bile tolerant counts for the four strains of *B. longum* grown at pH 6.0 revealed comparatively little loss of bile tolerance over the 21 days of storage at refrigeration temperatures (5°C) in milk in that the differences between counts on MRS and MRSO at each storage day were about equal (Figure 2A). *B. longum* S9 appeared to be the most stable of the four cultures in that no differences between counts on MRS and MRSO agar were observed during 21 days of refrigerated storage in milk (Figure 2D). The counts on neither media for this culture exhibited declines during storage.

Bile tolerance of the four strains of *B. longum* grown at pH 6.5 revealed different behavior among them. *B. longum* ATCC 15707 remained stable for both total and bile tolerant counts until day 14, after which slight differences can be seen (Figure 3A). Substantial differences occurred for *B. longum* II during 21 days of refrigerated storage in milk in that the counts on MRSO agar became progressively lower than on MRS agar as

storage time increased (Figure 3B). Both *B. longum* III and *B. longum* S9 remained stable in terms of bile tolerance when grown at pH 6.5 (Figure 3C and 3D).

Growth at pH 7.0 revealed considerable differences among the four strains of *B. longum* (Figure 4). Again, *B. longum* ATCC 15707 lost some ability to tolerate bile after 14 days of storage in milk. Total numbers of *B. longum* ATCC 15707 did not decline during this period, however bile tolerant numbers declined on day 21(Figure 4A). Total numbers of *B. longum* II declined 2.63 log cycles compared to a 4.45 log cycle reduction for bile tolerant numbers during 21 days of refrigerated storage in milk (Figure 4B). Bile tolerance was affected primarily during the first 7 days of refrigerated storage in milk for *B. longum* III. A 0.23 log cycle reduction in total numbers compared to a 0.61 log cycle reduction in bile tolerant numbers occurred between day 0 and 7 (Figure 4C). After day 7 the apparent rates of decline for both total and bile tolerant numbers were similar. *B. longum* S9 remained stable with respect to total and bile tolerant numbers over the 21 day storage period (Figure 4D).

DISCUSSION

Frozen Storage of *B. longum*

Frozen storage of the strains of *Bifidobacterium longum* at -196 °C resulted in relatively small losses in viability. The combined effect of different strains, storage time and growth pH did not influence survival during frozen storage. Storage time by itself, had an effect but only during initial freezing and storage of the concentrated cell crop. Since no loss in viability occurred beyond the first 7 days of frozen storage, it is likely that the observed loss was due to damage to the cells during the freezing process and not storage. Losses due to cell injury or death of lactic acid bacteria have been attributed to disrupted cellular integrity, dehydration, intracellular ice formation, intracellular solute concentration, and rate of freezing (7,8). Moss and Speck (10) indicated that the greatest injury of cells subjected to freezing occurred during the early stages of frozen storage and declined with increasing storage time. Similar results were obtained by Mitchell and Gilliland (9) with respect to survival of *L. acidophilus* in concentrated cultures during storage in liquid nitrogen. In their study, the greatest decline in viability occurred during the first 24 hours of storage in liquid nitrogen, with no additional losses during subsequent storage for 28 days.

Statistical analysis comparing the effect of pH and strain differences on overall growth of the cultures indicated no differences ($P>.05$) for any individual treatments or combination of treatments. This suggests that any loss of viability that occurred for the four strains of *B. longum* during frozen storage was not related to differences in growth of the four strains. While there were minimal differences among maximum populations of the four strains of *B. longum* grown at different pH levels, there were considerable differences in the numbers of bifidobacteria present in the freshly prepared concentrated cultures. Observed differences in the initial total counts of the concentrated cultures of the four

strains of *Bifidobacterium* were not necessarily due to higher cell populations during growth. *B. longum* S9 and *B. longum* III generally produced firm cell pellets after centrifugation which required the addition of 2X the pellet weight of 10% NFDM for resuspension. *B. longum* ATCC 15707 and *B. longum* II produced viscous cell pellets and thus were resuspended in 1X the pellet weight of 10% NFDM. The production of a firm or soft pellet by the bifidobacteria might have ramifications during commercial production processes. The production of a soft pellet is indicative of difficulty in recovering the cells by centrifugation. Furthermore, more of the spent medium would likely be contained in the concentrated cultures prepared from such cultures. This could impart undesirable flavor into the nonfermented milk containing the bifidobacteria. The viscous nature of the pellet suggests the production of exopolysaccharide material characteristic of some strains of bifidobacteria (11). Such exopolysaccharide material has been shown to provide protection during freezing of some lactic acid bacteria (10). Since all four strains of bifidobacteria in the present study survived similarly during 28 days of frozen storage it seems that the protective effect of any exopolysaccharide did not have a significant impact.

The pH at which the cultures of *B. longum* were grown influenced survival during storage at -196 °C. When comparing growth behavior at each pH level (averaging over strains), growth at pH 6.0 was only slightly higher than at the other pH levels. Gilliland and Rich (5) observed a similar influence of pH at which cultures of *L. acidophilus* were grown in relation to survival at refrigeration temperatures. The pH during growth could influence some component(s) in the cells making them tolerant to the stress of low temperature.

Although statistical analysis of the frozen storage data indicated variation among strains, growth pH, and storage time, this variation was considerably small. The data reflect substantial precision and subsequently small differences were significant at the P=.05 level. The data clearly show that frozen storage had, in the practical sense, little

effect on the viability of the four strains of *B. longum* regardless of pH during growth and storage (standard error terms support this conclusion).

Refrigerated Storage of *B. longum* in Milk

Storage of the four strains of *Bifidobacterium* at 5 °C nonfermented in milk indicated the potential for survival of these organisms in a refrigerated product on the supermarket shelf was dependent upon strain, pH of growth and storage time at 5 °C. Differences between numbers of total bifidobacteria and bile tolerant bifidobacteria did occur and were dependent upon the strain, growth pH and storage time. Differences observed during refrigerated storage of *L. acidophilus* have been shown to be independent of prior storage in liquid nitrogen (3) and therefore frozen storage is not considered as a source of variation.

The storage time (up to 21 days) at 5 °C is intended to represent how a nonfermented milk product might be handled following manufacture. If these products are to have any potential health/nutritional effect, it is essential to ensure that adequate numbers of *Bifidobacterium* are present at the time of consumption. It is clear that the survival of *B. longum* cells was strain dependent. *B. longum* S9 exhibited no loss of viability over the 21 day storage period regardless of growth conditions whereas, *B. longum* II and *B. longum* III exhibited progressive declines over the same storage time. In addition, with the exception of *B. longum* S9, the pH of the growth medium prior to concentration significantly influenced storage stability once introduced to the milk product. The superior storage stability of *B. longum* S9 in that it exhibited no loss of viability regardless of storage time and growth pH make it an excellent candidate for use as dietary adjunct to prepare a nonfermented milk product.

In addition to survival, based on counts on MRS agar, *B. longum* S9 remained stable for all parameters tested with respect to bile tolerant counts. No significant loss of

counts due to the presence of bile in the enumeration medium were observed during the 21 day storage period in milk regardless of which pH this culture had been grown. Bile resistance *of L. acidophilus* is considered to be a beneficial characteristic (5,6) however, research regarding bile tolerance of bifidobacteria has been limited. Previous research has not addressed the issue of obtaining a bile tolerant strain of bifidobacteria and its incorporation into a milk product. Nonfermented milk products such as this are currently on the market, unfortunately, the level of bile resistant bifidobacteria contained in the refrigerated milk is unknown. Compared to the other three strains tested, strain *B. longum* S9 possesses a clear advantage with respect to potential survival and growth in the gastrointestinal tract where tolerance to bile is a necessity if potential probiotic effects are to occur.

The use of bifidobacteria as a dietary adjunct having potential in control of serum cholesterol, improved lactose utilization, etc., relies upon the ingestion of adequate numbers of viable cells if these effects are to be realized. It is important to select a strain of bifidobacteria capable of survival during production and storage of the product in which the culture is to incorporated. As indicated by this research, of the four strains tested, only one, *B. longum* S9, was suitable with respect to freezing, frozen storage, refrigerated storage in milk, and bile tolerance. The losses in survival and bile tolerance during refrigerated storage may indicate that the other three strains would not be good choices to prepare nonfermented milk products to provide bifidobacteria in adequate numbers when consumed.

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Table 1. Identity characteristics of strains of *Bifidobacterium longum* as compared to those listed in *Bergey's Manual of Systematic Bacteriology* for *B. longum*.

	Bifidobacterium longum reaction response				
Test[1]	Bergey's[2]	ATCC 15707	II	III	S9
Arabinose	+	+	+	+	+
Ribose	+	+	-	+	+
Xylose	+/-	+	+	+	+
Galactose	+	+	+	+	+
Fructose	+	+	+	+	+
Mannose	+/-	-	-	+	+/-
Mannitol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Salicin	-	-	-	-	+
Cellobiose	-	-	-	-	-
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Melibiose	+	+	+	+	+
Sucrose	+	+	+	+	+
Trehalose	-	-	-	-	-
Inulin	-	-	-	-	-
Melezitose	+	+	+	+	-
Raffinose	+	+	+	+	+
Starch	-	-	-	-	-
Gluconate	-	-	-	-	-
Gram Reaction	+	+	+	+	+
Morphology	rods	rods	rods	rods	rods
F6PFK[3]	+	+	+	+	+

[1]API50CH (bioMerieux) test reactions after growth for 48 hours at 37 °C in a GasPak (BBL) anaerobic chamber system, as well as gram stain reaction and morphology.
[2]Responses for *B. longum* reported in Bergey's Manual of Systematic Bacteriology (12).
[3]fructose-6-phosphate phosphoketolase (F6PFK) assay.

Table 2. Growth[1] of four strains of *Bifidobacterium longum* at various pH levels[2].

	Bifidobacterium longum Log_{10} CFU/ml[3](Pellet Type[4])				
pH	ATCC 15707[5]	II[5]	III[5]	S9	Average over strain
5.0	-	-	-	8.70[6] (F)	
5.5	9.77 (S)	9.26 (S)	9.67 (F)	9.37 (F)	9.52
6.0	10.01 (S)	10.12 (S)	9.62 (F)	10.07 (F)	9.95
6.5	9.87 (S)	9.46 (S)	9.16 (F)	9.78 (F)	9.57
7.0	8.92 (S)	8.85 (S)	8.79 (F)	9.86 (F)	9.10
Average over pH	9.64	9.42	9.31	9.77	

[1]Cells were grown in MRS-Thio broth at 37 °C and harvested at early stationary phase (maximum growth).

[2]Overall model was not significant (P>.05), SE=.26.

[3]Each value represents the mean of three replications.

[4]Pellet type consists of either a firm(F) or soft(S) obtained upon centrifugation of the culture. Soft pellet and firm pellet suspensions were resuspended in 1X or 2X their weight in 10% NFDM respectively.

[5]No data presented for pH 5.0 since culture did not grow well at this pH.

[6]Not included in averages.

Table 3. Effect of storage at -196 °C on total numbers of four strains of *Bifidobacterium longum* grown[1] at various pH levels.

Bifidobacterium		Storage Time (Days) Log_{10} CFU/ml[2]				
longum	pH	0	7	14	21	28
ATCC 15707[3]	5.0	-	-	-	-	-
	5.5	10.44	10.29	10.32	10.33	10.32
	6.0	10.62	10.45	10.50	10.53	10.53
	6.5	10.51	10.45	10.39	10.31	10.31
	7.0	9.80	9.90	9.84	9.95	9.93
II[3]	5.0	-	-	-	-	-
	5.5	10.32	9.73	9.77	9.77	9.76
	6.0	10.27	10.22	10.34	10.12	10.06
	6.5	10.02	9.87	9.93	9.97	9.99
	7.0	9.67	9.63	9.49	9.53	9.64
III[3]	5.0	-	-	-	-	-
	5.5	11.08	11.10	11.11	11.14	11.09
	6.0	11.09	11.13	11.08	11.09	11.29
	6.5	10.64	10.63	10.73	10.69	10.67
	7.0	10.43	10.29	10.36	10.31	10.31
S9	5.0	10.42	10.44	10.45	10.43	10.48
	5.5	10.86	10.95	10.87	10.94	10.85
	6.0	11.13	11.17	11.16	10.99	11.03
	6.5	11.04	10.82	10.85	10.86	10.64
	7.0	10.76	10.80	10.40	10.51	10.52

Summary of Independent Treatment Effects (Log_{10} CFU/ml)[1]	Strain[4]		pH[4]		Day[4]	
			5.0	-	0	10.54^{a}
	15707	10.28^{b}	5.5	10.55^{ab}	7	10.46^{b}
	II	9.91^{c}	6.0	10.74^{a}	14	10.45^{b}
	III	10.81^{a}	6.5	10.47^{b}	21	10.44^{b}
	S9	10.86^{a}	7.0	10.10^{c}	28	10.43^{b}
S.E.		.08		.08		.03

[1]Cells were grown in MRS-Thio broth at 37 °C and at early stationary phase (max growth).

[2]Each value represents the mean of three replications.

[3]No data presented for pH 5.0 since culture did not grow well at this pH.

[4]Strain=(average over pH and Day), pH=(average over Strain and Day), Day=(average over Strain and pH)

[abc]Means within columns with the same superscripts do not differ (P>.05).

Table 4. Effect of storage in milk at 5 °C on total numbers of four strains of *Bifidobacterium longum* which had been grown at various pH levels.

Bifidobacterium		Storage Time (Days) Log_{10} CFU/ml[1]			
longum	pH	0	7	14	21
ATCC 15707[2]	5.0	-	-	-	-
	5.5	7.40^{a}	7.25^{a}	7.08^{ab}	6.96^{b}
	6.0	7.55^{a}	7.36^{a}	7.28^{ab}	7.01^{b}
	6.5	7.38^{a}	7.37^{a}	7.26^{a}	7.16^{a}
	7.0	7.24^{a}	7.19^{a}	7.14^{a}	7.14^{a}
II[2]	5.0	-	-	-	-
	5.5	7.06^{a}	6.69^{b}	6.39^{bc}	6.21^{c}
	6.0	7.35^{a}	7.18^{a}	7.14^{ab}	6.87^{b}
	6.5	7.38^{a}	7.01^{b}	6.68^{c}	6.34^{d}
	7.0	7.31^{a}	6.94^{b}	6.03^{c}	4.68^{d}
III[2]	5.0	-	-	-	-
	5.5	7.33^{a}	7.30^{a}	7.25^{a}	7.19^{a}
	6.0	7.96^{a}	7.67^{ab}	7.60^{b}	7.48^{b}
	6.5	7.66^{a}	7.62^{a}	7.33^{ab}	7.12^{b}
	7.0	7.32^{a}	7.09^{ab}	6.93^{b}	6.56^{c}
S9	5.0	7.45	7.41	7.37	7.13
	5.5	7.90^{a}	7.84^{a}	7.74^{a}	7.76^{a}
	6.0	7.78^{a}	7.81^{a}	7.79^{a}	7.81^{a}
	6.5	7.70^{a}	7.98^{a}	7.82^{a}	7.95^{a}
	7.0	7.65^{a}	7.82^{a}	7.76^{a}	7.77^{a}

[1]Each value represents the mean of three replications.

[2]No data presented for pH 5.0 for *B. longum* 15707, II and III since they did not grow at this pH; the data for strain S9 grown at pH 5.0 was not statistically analyzed since it was the only strain that grew at this pH.

^{abcd}Means within rows with the same superscripts do not differ (P>.05). SEM = 0.17.

Table 5. Effect of storage in milk at 5 °C on bile tolerant numbers of four strains of *Bifidobacterium longum* grown at various pH levels.

Bifidobacterium		Storage Time (Days) Log_{10} CFU/ml[1]			
longum	pH	0	7	14	21
ATCC 15707[2]	5.0	-	-	-	-
	5.5	7.39^{a}	7.06^{ab}	6.83^{ab}	6.61^{b}
	6.0	7.39^{a}	7.27^{a}	7.12^{a}	6.79^{a}
	6.5	7.29^{a}	7.32^{a}	7.23^{a}	6.93^{a}
	7.0	7.22^{a}	7.24^{a}	7.16^{a}	6.85^{a}
II[2]	5.0	-	-	-	-
	5.5	6.73^{a}	6.44^{ab}	6.22^{ab}	6.03^{b}
	6.0	7.05^{a}	6.92^{ab}	6.77^{ab}	6.44^{b}
	6.5	7.28^{a}	6.74^{a}	6.08^{b}	5.36^{b}
	7.0	7.32^{a}	6.66^{b}	5.59^{c}	2.87^{d}
III[2]	5.0	-	-	-	-
	5.5	7.33^{a}	7.32^{a}	7.10^{a}	7.11^{a}
	6.0	7.93^{a}	7.64^{ab}	7.56^{ab}	7.32^{b}
	6.5	7.48^{ab}	7.52^{a}	7.10^{ab}	6.90^{b}
	7.0	7.21^{a}	6.60^{ab}	6.45^{bc}	5.99^{c}
S9	5.0	7.35	7.32	7.25	7.09
	5.5	7.90^{a}	7.81^{a}	7.79^{a}	7.81^{a}
	6.0	7.77^{a}	7.81^{a}	7.80^{a}	7.80^{a}
	6.5	7.64^{a}	7.96^{a}	7.75^{a}	7.93^{a}
	7.0	7.63^{a}	7.79^{a}	7.71^{a}	7.73^{a}

[1]Each value represents the mean of three replications.

[2]No data presented for pH 5.0 for *B. longum* 15707, II and III since they did not grow at this pH; the data for strain S9 grown at pH 5.0 was not statistically analyzed since it was the only strain that grew at this pH

^{abcd}Means within rows with the same superscripts do not differ (P>.05). SEM = 0.31.

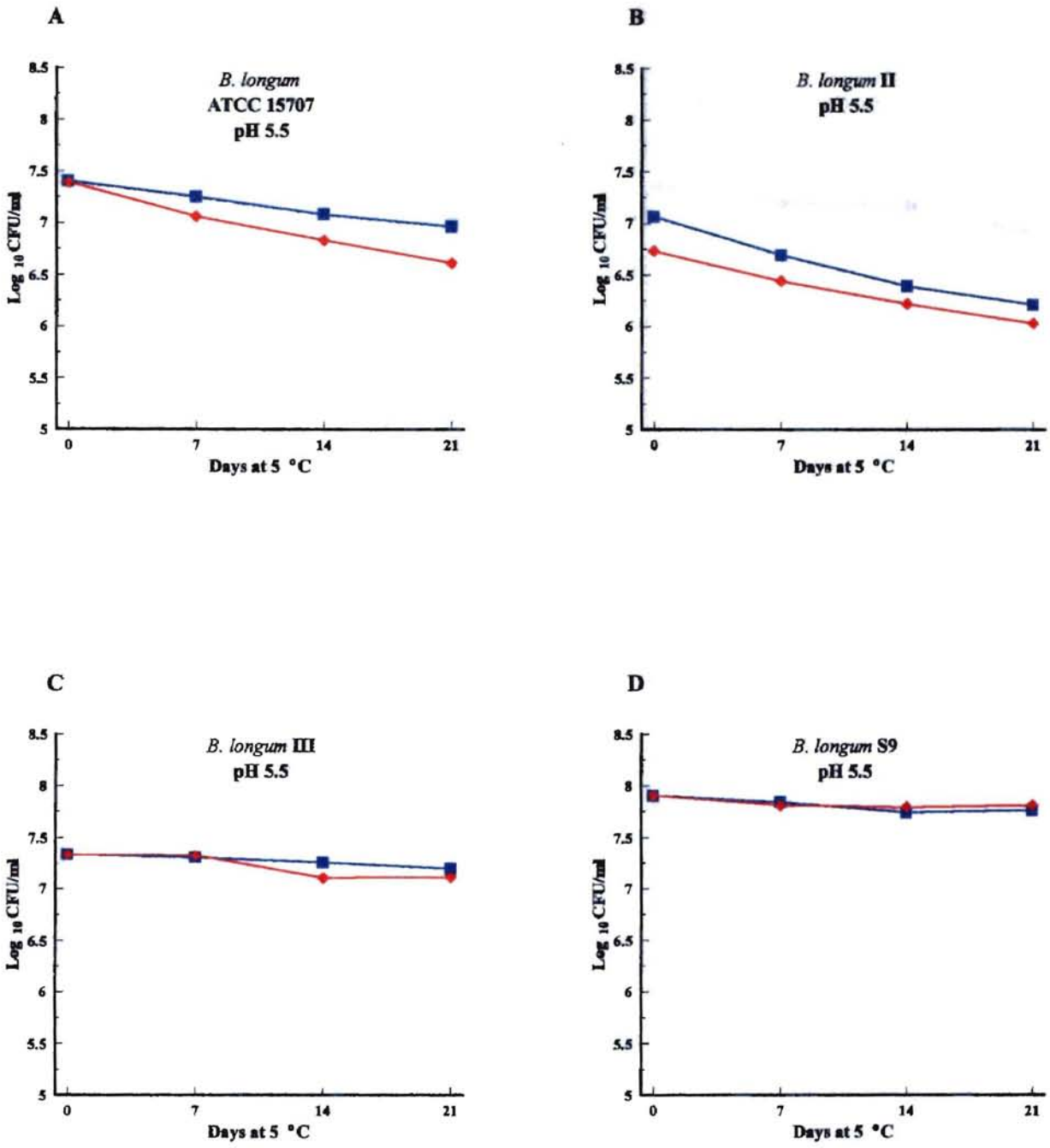

Figure 1. Comparison of total (MRS ■) and bile tolerant (MRSO ◆) numbers of four strains of *Bifidobacterium longum* grown at pH 5.5 and stored at 5 °C in milk.

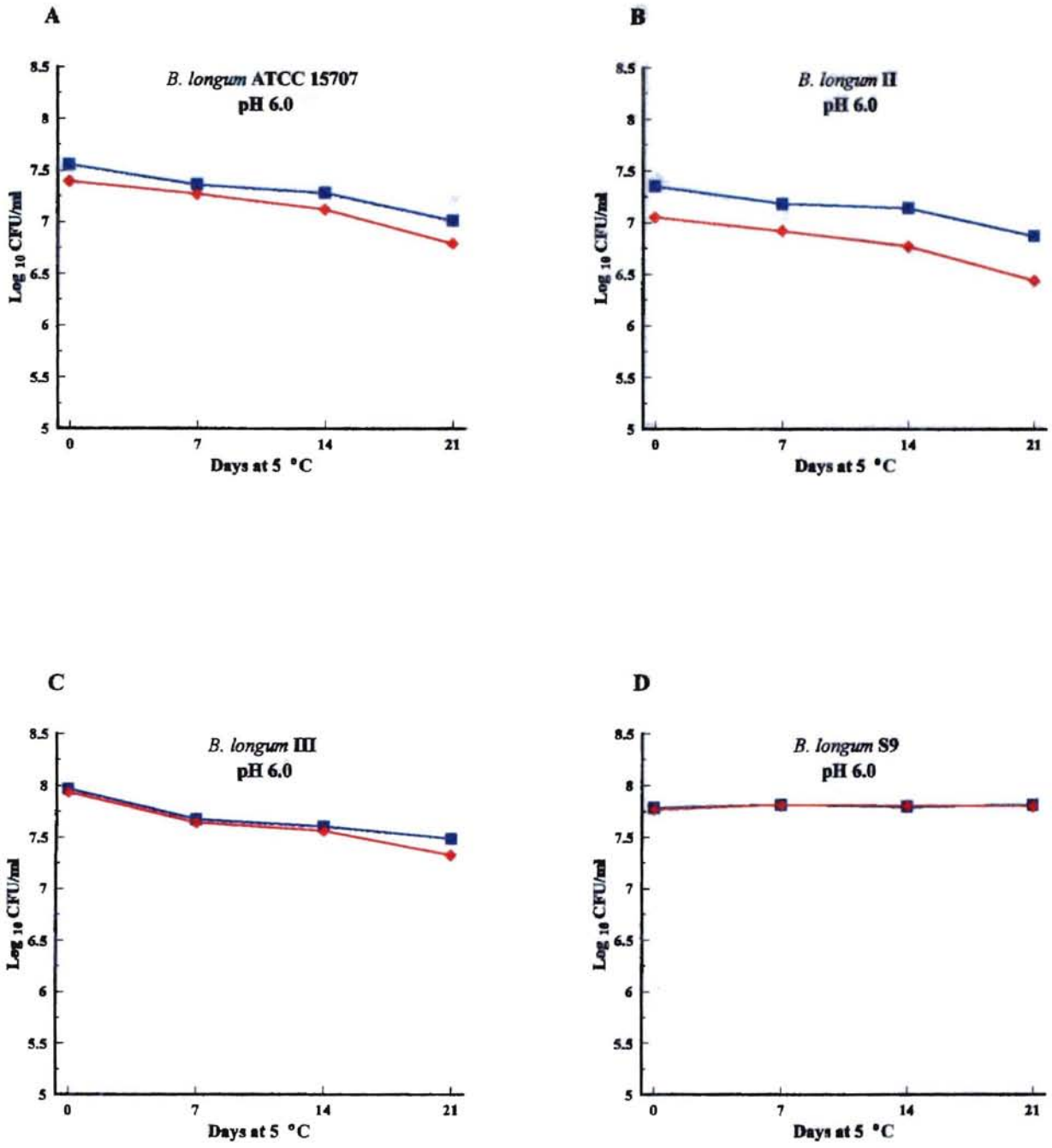

Figure 2. Comparison of total (MRS ■) and bile tolerant (MRSO ◆) numbers of four strains of *Bifidobacterium longum* grown at pH 6.0 and stored at 5 °C in milk.

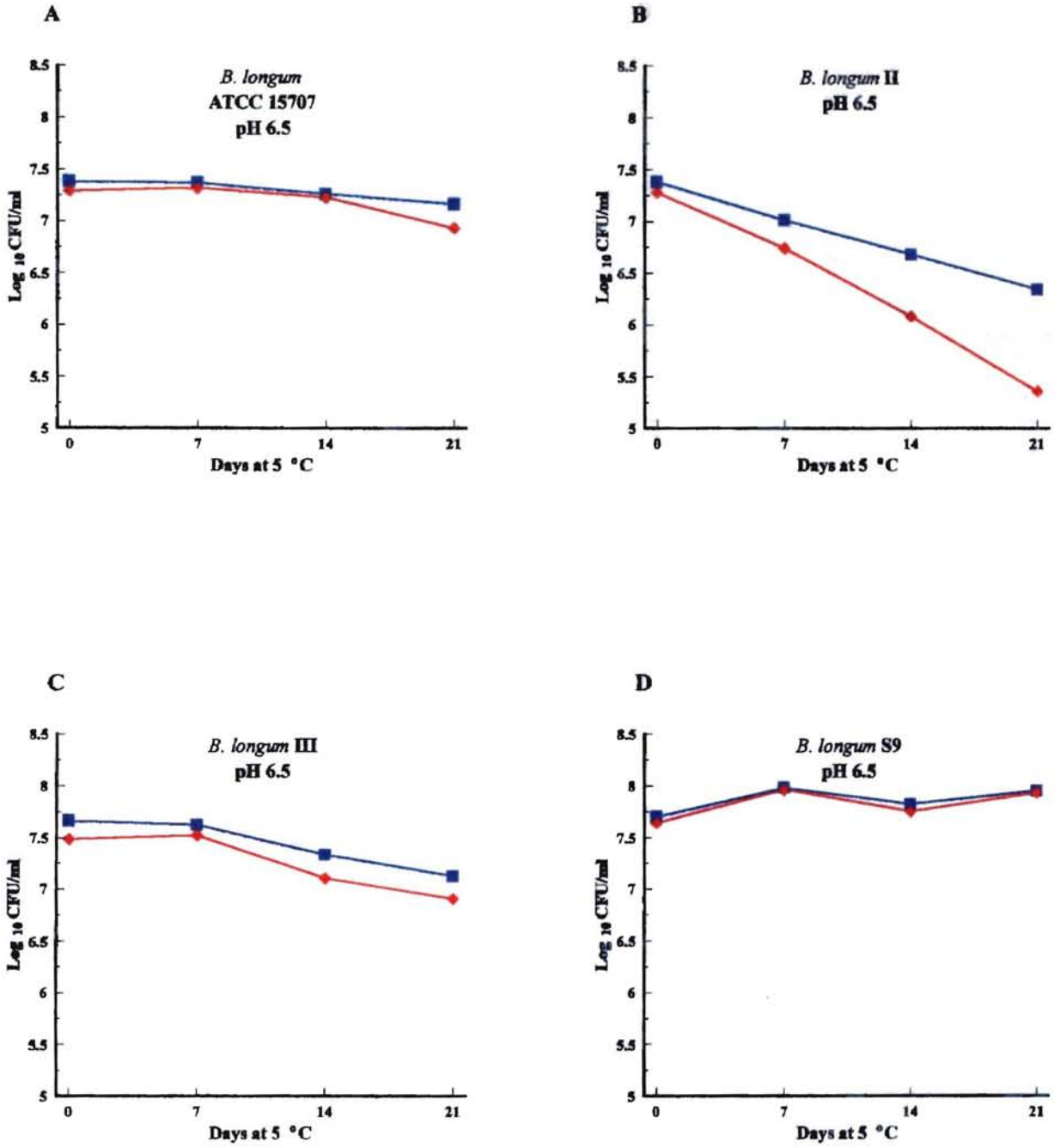

Figure 3. Comparison of total (MRS ■) and bile tolerant (MRSO ◆) numbers of four strains of *Bifidobacterium longum* grown at pH 6.5 and stored at 5 °C in milk.

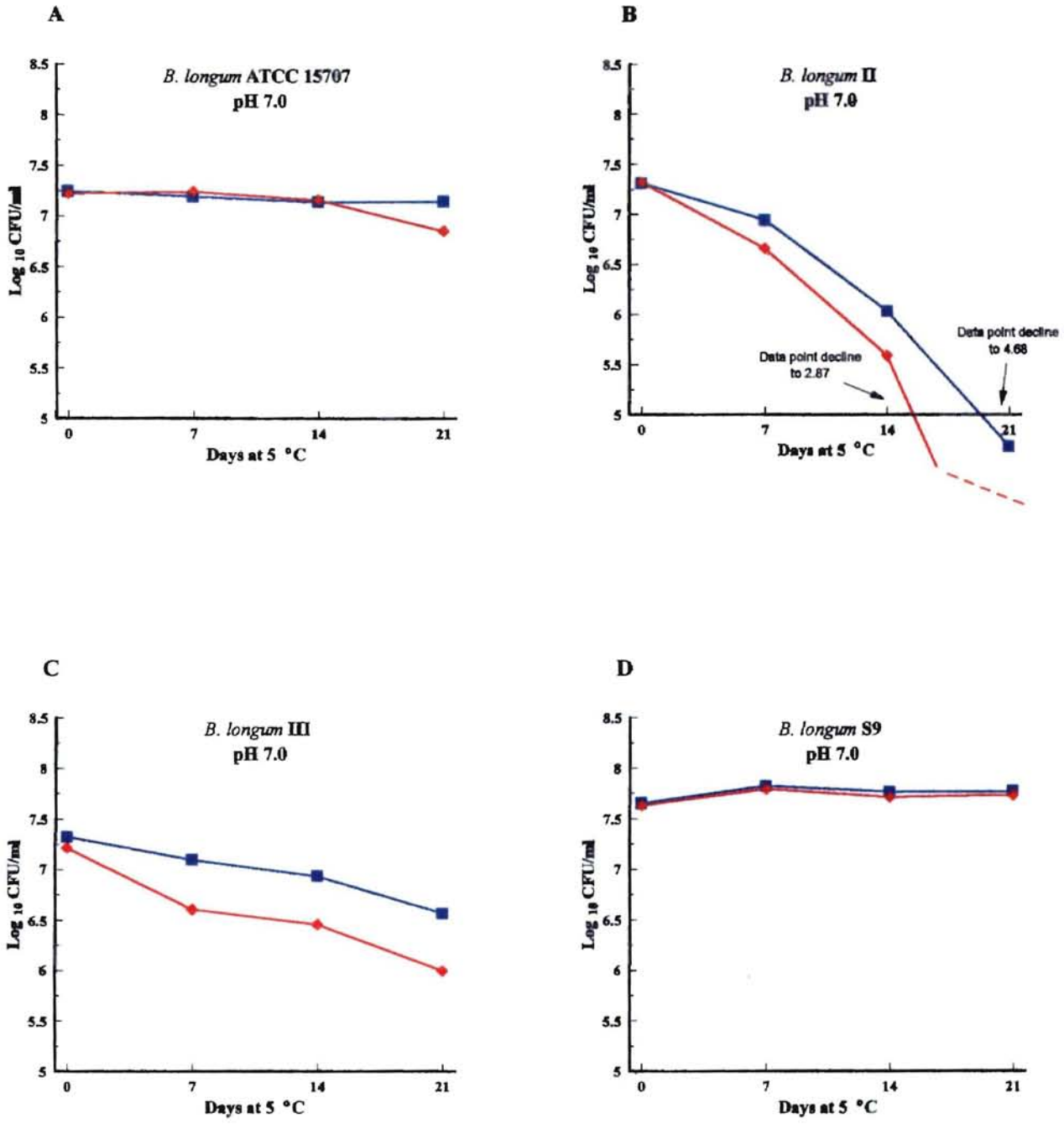

Figure 4. Comparison of total (MRS ■) and bile tolerant (MRSO ◆) numbers of four strains of *Bifidobacterium longum* grown at pH 7.0 and stored at 5 °C in milk.

APPENDIX 1

TOTAL AND BILE TOLERANT NUMBERS OF *BIFIDOBACTERIUM LONGUM* DURING FROZEN STORAGE AT -196 °C

TABLE 6

COUNTS OF *BIFIDOBACTERIUM LONGUM* ATCC 15707 MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 5.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.34	
	7	-	
	14	10.32	
	21	10.34	
	28	10.34	10.28
2	0	10.59	
	7	10.26	
	14	10.30	
	21	10.32	
	28	10.28	10.30
3	0	10.38	
	7	10.34	
	14	10.32	
	21	10.32	
	28	10.34	10.30

TABLE 7

COUNTS OF *BIFIDOBACTERIUM LONGUM* ATCC 15707 MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 6.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.73	
	7	10.53	
	14	10.52	
	21	10.61	
	28	10.66	10.38
2	0	10.58	
	7	10.34	
	14	10.45	
	21	10.38	
	28	10.42	10.34
3	0	10.53	
	7	10.46	
	14	10.54	
	21	10.59	
	28	10.51	10.48

TABLE 8

COUNTS OF *BIFIDOBACTERIUM LONGUM* ATCC 15707 MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 ^{o}C (GROWTH AT pH 6.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.53	
	7	10.57	
	14	10.51	
	21	10.30	
	28	10.36	10.30
2	0	10.51	
	7	10.28	
	14	10.34	
	21	10.20	
	28	10.23	10.18
3	0	10.51	
	7	10.51	
	14	10.32	
	21	10.42	
	28	10.32	10.30

TABLE 9

COUNTS OF *BIFIDOBACTERIUM LONGUM* ATCC 15707 MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 7.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.36	
	7	10.43	
	14	10.30	
	21	10.45	
	28	10.36	10.43
2	0	9.42	
	7	9.18	
	14	9.11	
	21	9.26	
	28	9.23	9.23
3	0	9.61	
	7	10.07	
	14	10.11	
	21	10.15	
	28	10.20	10.20

TABLE 10

COUNTS OF *BIFIDOBACTERIUM LONGUM* S9 MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 5.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.26	
	7	10.20	
	14	10.28	
	21	10.38	
	28	10.30	10.04
2	0	10.45	
	7	10.62	
	14	10.52	
	21	10.38	
	28	10.36	10.34
3	0	10.57	
	7	10.48	
	14	10.56	
	21	10.52	
	28	10.79	10.78

TABLE 11

COUNTS OF *BIFIDOBACTERIUM LONGUM* S9 MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 5.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.85	
	7	11.04	
	14	11.08	
	21	10.94	
	28	11.00	11.04
2	0	10.96	
	7	10.84	
	14	10.86	
	21	10.77	
	28	10.81	10.79
3	0	10.76	
	7	10.98	
	14	10.68	
	21	11.04	
	28	10.74	10.74

TABLE 12

COUNTS OF *BIFIDOBACTERIUM LONGUM* S9 MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 6.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	11.08	
	7	11.18	
	14	10.97	
	21	10.94	
	28	11.04	11.04
2	0	11.23	
	7	11.20	
	14	11.15	
	21	10.85	
	28	10.81	10.89
3	0	11.08	
	7	11.11	
	14	11.36	
	21	11.18	
	28	11.23	11.15

TABLE 13

COUNTS OF *BIFIDOBACTERIUM LONGUM* S9 MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 6.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	11.20	
	7	11.04	
	14	10.90	
	21	10.90	
	28	10.64	10.86
2	0	11.04	
	7	10.49	
	14	10.89	
	21	10.79	
	28	10.63	10.55
3	0	10.88	
	7	10.94	
	14	10.76	
	21	10.90	
	28	10.64	10.78

TABLE 14

COUNTS OF *BIFIDOBACTERIUM LONGUM* S9 MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 7.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	11.04	
	7	10.91	
	14	10.57	
	21	10.59	
	28	10.70	10.85
2	0	10.65	
	7	10.62	
	14	10.20	
	21	10.28	
	28	10.23	10.36
3	0	10.59	
	7	10.86	
	14	10.43	
	21	10.66	
	28	10.62	10.60

TABLE 15

COUNTS OF *BIFIDOBACTERIUM LONGUM* II MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 5.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.11	
	7	10.23	
	14	10.23	
	21	10.18	
	28	10.15	10.08
2	0	10.18	
	7	10.04	
	14	10.04	
	21	10.08	
	28	10.04	9.87
3	0	10.66	
	7	8.93	
	14	9.04	
	21	9.04	
	28	9.08	8.72

TABLE 16

COUNTS OF *BIFIDOBACTERIUM LONGUM* II MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 6.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.28	
	7	9.69	
	14	10.11	
	21	9.99	
	28	9.40	9.08
2	0	10.42	
	7	10.77	
	14	9.92	
	21	10.26	
	28	10.70	10.62
3	0	10.11	
	7	10.20	
	14	10.98	
	21	10.11	
	28	10.08	10.04

TABLE 17

COUNTS OF *BIFIDOBACTERIUM LONGUM* II MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 6.5)

Replication	Storage Time (Days)	Log_{10} CFU/ml MRS AGAR	MRSO AGAR
1	0	10.15	
	7	9.95	
	14	9.99	
	21	9.92	
	28	9.98	9.69
2	0	9.86	
	7	9.70	
	14	9.76	
	21	9.88	
	28	9.97	9.66
3	0	10.04	
	7	-	
	14	10.04	
	21	10.11	
	28	10.04	10.00

TABLE 18

COUNTS OF *BIFIDOBACTERIUM LONGUM* II MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 7.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	9.94	
	7	9.87	
	14	9.69	
	21	9.74	
	28	9.91	9.95
2	0	9.08	
	7	9.11	
	14	8.86	
	21	8.95	
	28	9.04	9.18
3	0	10.00	
	7	9.91	
	14	9.91	
	21	9.91	
	28	9.96	9.81

TABLE 19

COUNTS OF *BIFIDOBACTERIUM LONGUM* III MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 5.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	11.11	
	7	11.23	
	14	11.18	
	21	11.20	
	28	11.18	-
2	0	11.23	
	7	11.15	
	14	11.26	
	21	11.20	
	28	11.18	11.15
3	0	10.91	
	7	10.92	
	14	10.90	
	21	11.00	
	28	10.93	10.80

TABLE 20

COUNTS OF *BIFIDOBACTERIUM LONGUM* III MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 6.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.85	
	7	10.91	
	14	10.96	
	21	10.94	
	28	10.96	10.82
2	0	11.11	
	7	11.26	
	14	11.08	
	21	11.04	
	28	11.11	11.04
3	0	11.32	
	7	11.23	
	14	11.20	
	21	11.30	
	28	11.81	11.82

TABLE 21

COUNTS OF *BIFIDOBACTERIUM LONGUM* III MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 6.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.72	
	7	10.81	
	14	10.95	
	21	10.91	
	28	10.97	10.48
2	0	10.86	
	7	10.73	
	14	11.00	
	21	10.86	
	28	10.81	10.77
3	0	10.32	
	7	10.36	
	14	10.23	
	21	10.30	
	28	10.23	10.26

TABLE 22

COUNTS OF *BIFIDOBACTERIUM LONGUM* III MRS AND MRSO AGAR DURING FROZEN STORAGE AT -196 °C (GROWTH AT pH 7.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	10.28	
	7	10.11	
	14	10.32	
	21	10.30	
	28	10.18	10.04
2	0	10.65	
	7	10.38	
	14	10.42	
	21	10.28	
	28	10.40	10.32
3	0	10.34	
	7	10.38	
	14	10.34	
	21	10.34	
	28	10.36	10.34

APPENDIX 2

TOTAL AND BILE TOLERANT NUMBERS OF *BIFIDOBACTERIUM LONGUM* DURING REFRIGERATED STORAGE IN MILK AT 5 °C

TABLE 23

COUNTS OF *BIFIDOBACTERIUM LONGUM* ATCC 15707 MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C (GROWTH AT pH 5.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.43	7.36
	7	7.30	7.04
	14	7.04	6.71
	21	6.97	6.41
2	0	7.40	7.38
	7	7.26	7.15
	14	7.04	6.93
	21	6.91	6.69
3	0	7.36	7.42
	7	7.20	6.98
	14	7.15	6.86
	21	7.00	6.72

TABLE 24

COUNTS OF *BIFIDOBACTERIUM LONGUM* ATCC 15707 MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C (GROWTH AT pH 6.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.51	7.32
	7	7.40	7.32
	14	7.38	7.18
	21	7.30	7.00
2	0	7.51	7.30
	7	7.30	7.26
	14	7.23	7.18
	21	6.91	6.85
3	0	7.63	7.55
	7	7.38	7.23
	14	7.23	7.00
	21	6.83	6.52

TABLE 25

COUNTS OF *BIFIDOBACTERIUM LONGUM* ATCC 15707 MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C (GROWTH AT pH 6.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.38	7.38
	7	7.49	7.38
	14	7.23	7.15
	21	7.15	6.72
2	0	7.18	7.15
	7	7.30	7.30
	14	7.28	7.26
	21	7.18	6.94
3	0	7.57	7.34
	7	7.30	7.28
	14	7.26	7.28
	21	7.15	7.11

TABLE 26

COUNTS OF *BIFIDOBACTERIUM LONGUM* ATCC 15707 MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C (GROWTH AT pH 7.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.23	7.26
	7	7.28	7.36
	14	7.11	7.15
	21	7.23	6.65
2	0	7.26	7.20
	7	7.11	7.11
	14	7.15	7.11
	21	7.04	6.94
3	0	7.23	7.20
	7	7.18	7.26
	14	7.15	7.20
	21	7.15	6.97

TABLE 27

COUNTS OF *BIFIDOBACTERIUM LONGUM* S9 MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C (GROWTH AT pH 5.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.32	7.08
	7	7.20	7.11
	14	7.20	6.91
	21	6.67	6.54
2	0	7.40	7.38
	7	7.38	7.32
	14	7.40	7.38
	21	7.26	7.23
3	0	7.63	7.59
	7	7.63	7.53
	14	7.51	7.45
	21	7.45	7.51

TABLE 28

COUNTS OF *BIFIDOBACTERIUM LONGUM* S9 MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C
(GROWTH AT pH 5.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	8.00	7.89
	7	7.96	7.92
	14	7.80	7.82
	21	7.80	7.93
2	0	7.95	7.95
	7	7.79	7.75
	14	7.83	7.75
	21	7.81	7.85
3	0	7.75	7.86
	7	7.77	7.75
	14	7.59	7.81
	21	7.68	7.63

TABLE 29

COUNTS OF *BIFIDOBACTERIUM LONGUM* S9 MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C
(GROWTH AT pH 6.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	8.08	8.23
	7	8.08	8.08
	14	8.11	8.15
	21	8.08	8.15
2	0	8.15	8.08
	7	8.18	8.18
	14	8.15	8.15
	21	8.20	8.11
3	0	7.11	7.00
	7	7.18	7.18
	14	7.11	7.11
	21	7.15	7.15

TABLE 30

COUNTS OF *BIFIDOBACTERIUM LONGUM* S9 MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C (GROWTH AT pH 6.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.66	7.74
	7	7.98	7.96
	14	7.98	7.96
	21	7.95	7.91
2	0	7.49	7.45
	7	7.97	8.00
	14	7.51	7.32
	21	7.99	7.93
3	0	7.93	7.74
	7	8.00	7.92
	14	7.98	7.97
	21	7.91	7.96

TABLE 31

COUNTS OF *BIFIDOBACTERIUM LONGUM* S9 MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C
(GROWTH AT pH 7.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.81	7.85
	7	8.08	8.00
	14	8.00	7.99
	21	7.99	7.98
2	0	7.49	7.42
	7	7.61	7.71
	14	7.68	7.59
	21	7.72	7.68
3	0	7.64	7.63
	7	7.76	7.66
	14	7.60	7.56
	21	7.59	7.54

TABLE 32

COUNTS OF *BIFIDOBACTERIUM LONGUM* II MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C (GROWTH AT pH 5.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.26	7.08
	7	7.18	7.11
	14	7.08	6.86
	21	6.89	6.81
2	0	7.04	6.85
	7	6.81	6.40
	14	6.61	6.45
	21	6.45	6.30
3	0	6.89	6.28
	7	6.08	5.81
	14	5.46	5.34
	21	5.30	4.98

TABLE 33

COUNTS OF *BIFIDOBACTERIUM LONGUM* II MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C
(GROWTH AT pH 6.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	6.69	6.52
	7	6.57	6.28
	14	6.46	6.15
	21	6.18	6.00
2	0	8.20	7.60
	7	7.89	7.83
	14	7.96	7.87
	21	7.96	7.85
3	0	7.15	7.04
	7	7.08	6.64
	14	7.00	6.28
	21	6.46	5.46

TABLE 34

COUNTS OF *BIFIDOBACTERIUM LONGUM* II MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C
(GROWTH AT pH 6.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.00	6.97
	7	6.63	6.48
	14	5.93	4.81
	21	5.60	4.32
2	0	8.00	7.80
	7	7.46	7.34
	14	7.26	6.87
	21	6.63	6.30
3	0	7.15	7.08
	7	6.92	6.40
	14	6.84	6.54
	21	6.78	6.28

TABLE 35

COUNTS OF *BIFIDOBACTERIUM LONGUM* II MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C
(GROWTH AT pH 7.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.89	7.86
	7	7.39	7.04
	14	6.68	5.54
	21	5.82	4.48
2	0	7.04	7.18
	7	6.64	6.57
	14	5.20	4.90
	21	3.18	0
3	0	6.99	6.90
	7	6.80	6.38
	14	6.20	6.32
	21	5.04	4.15

TABLE 36

COUNTS OF *BIFIDOBACTERIUM LONGUM* III MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C
(GROWTH AT pH 5.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.00	6.98
	7	7.04	6.90
	14	6.97	6.96
	21	7.08	6.89
2	0	7.11	7.08
	7	7.18	7.23
	14	6.95	6.58
	21	6.89	6.89
3	0	7.88	7.92
	7	7.68	7.82
	14	7.81	7.75
	21	7.59	7.57

TABLE 37

COUNTS OF *BIFIDOBACTERIUM LONGUM* III MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C
(GROWTH AT pH 6.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.92	7.99
	7	7.85	7.90
	14	7.56	7.61
	21	7.48	7.42
2	0	8.04	7.97
	7	8.04	7.94
	14	8.08	7.90
	21	7.80	7.42
3	0	7.92	7.83
	7	7.11	7.08
	14	7.18	7.18
	21	7.18	7.11

TABLE 38

COUNTS OF *BIFIDOBACTERIUM LONGUM* III MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C
(GROWTH AT pH 6.5)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.96	7.40
	7	7.77	7.57
	14	7.30	6.99
	21	6.90	6.56
2	0	7.66	7.72
	7	7.88	7.85
	14	7.61	7.51
	21	7.49	7.65
3	0	7.34	7.32
	7	7.20	7.15
	14	7.08	6.80
	21	6.95	6.48

TABLE 39

COUNTS OF *BIFIDOBACTERIUM LONGUM* III MRS AND MRSO AGAR DURING REFRIGERATED STORAGE IN MILK AT 5 °C
(GROWTH AT pH 7.0)

Replication	Storage Time (Days)	Log $_{10}$ CFU/ml MRS AGAR	MRSO AGAR
1	0	7.15	6.96
	7	7.08	6.40
	14	7.04	6.49
	21	6.72	6.20
2	0	7.42	7.32
	7	7.00	6.30
	14	6.53	5.97
	21	6.15	5.45
3	0	7.40	7.34
	7	7.20	7.11
	14	7.20	6.89
	21	6.82	6.32

APPENDIX 3

HARVEST TIMES FOR *BIFIDOBACTERIUM LONGUM* GROWN AT VARIOUS PH

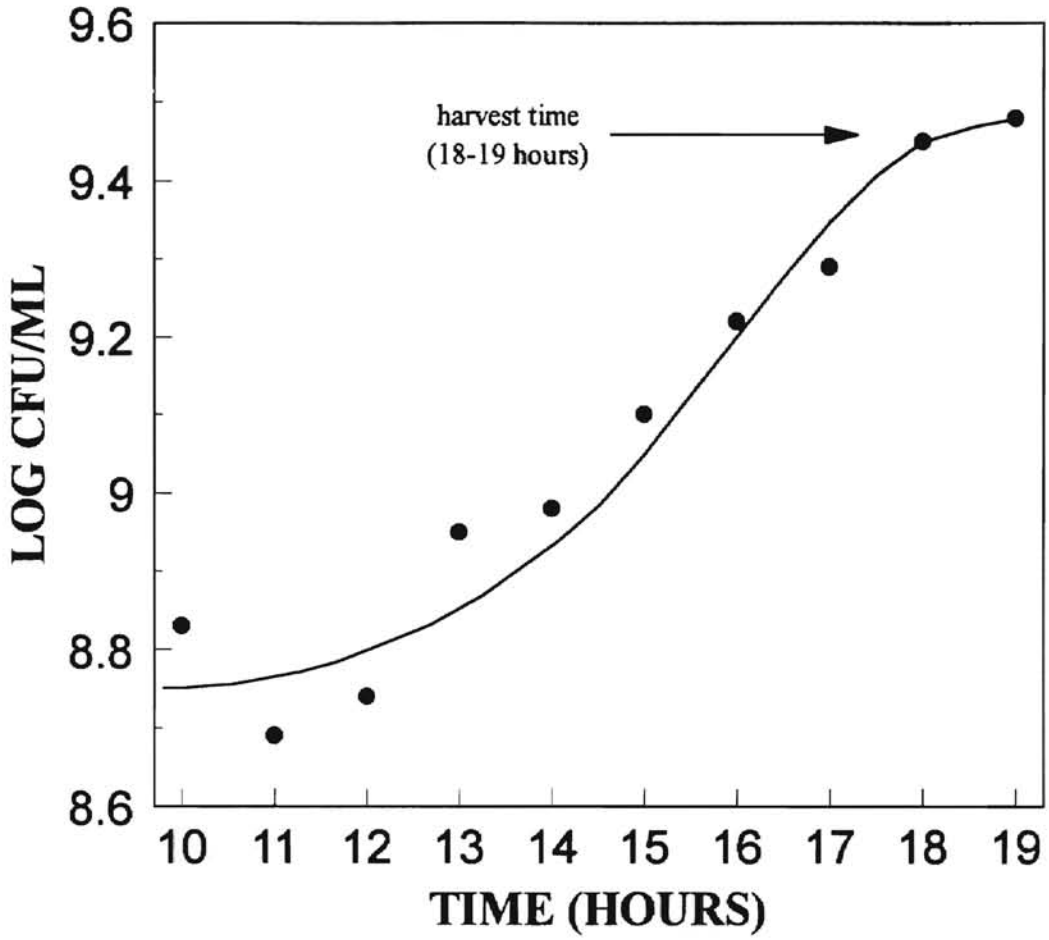

Figure 5. Harvest time (hours) for *Bifidobacterium longum* ATCC 15707 grown at pH 5.5.

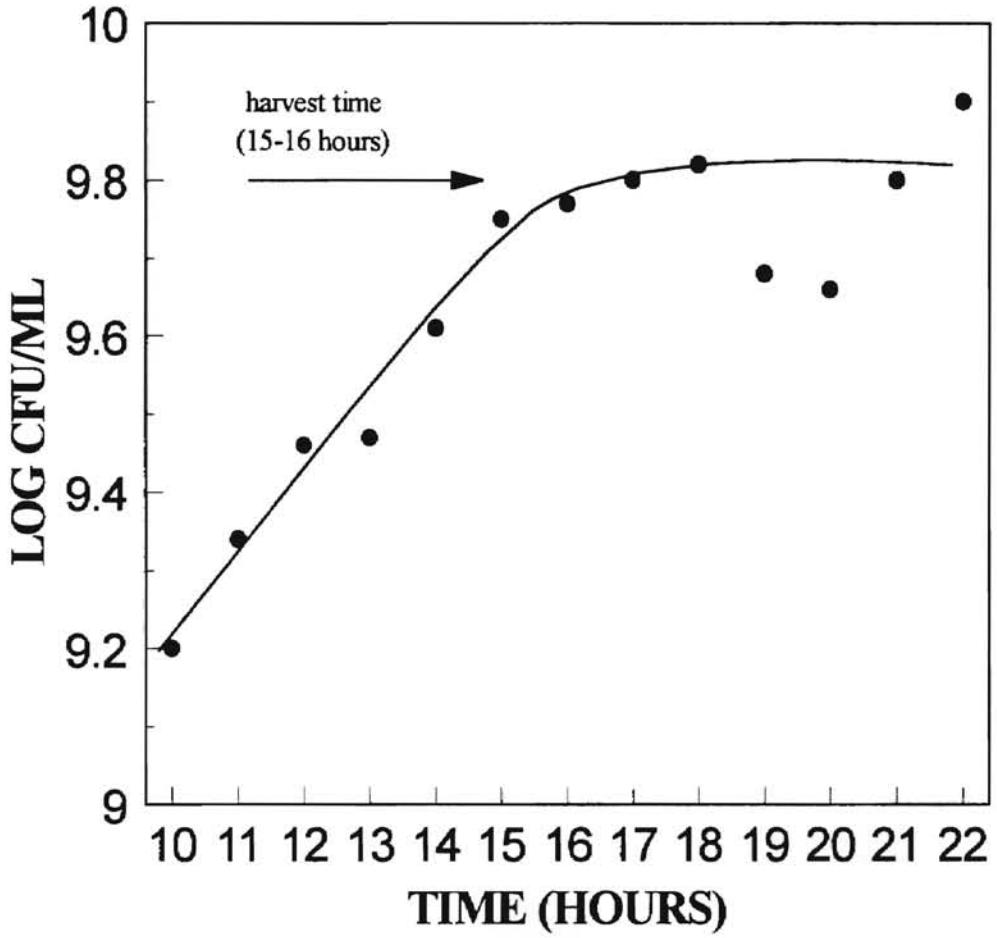

Figure 6. Harvest time (hours) for *Bifidobacterium longum* ATCC 15707 grown at pH 6.0.

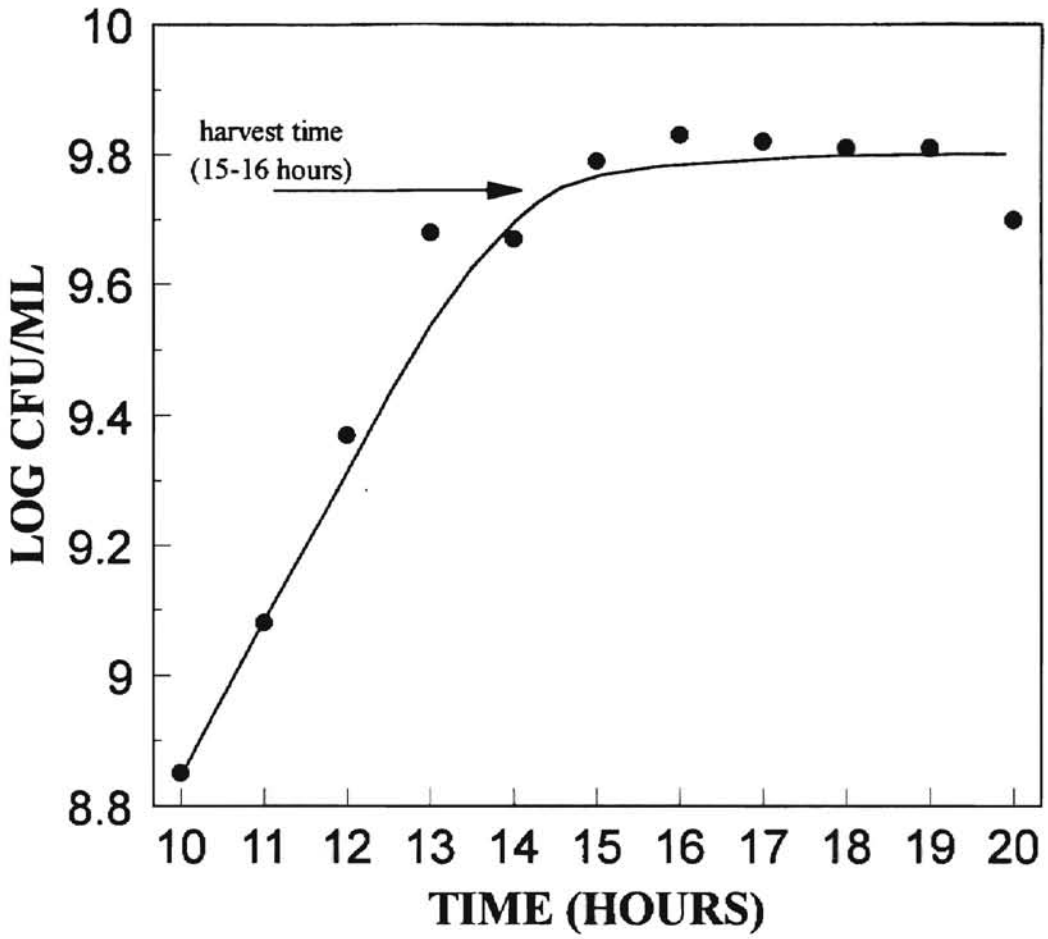

Figure 7. Harvest time (hours) for *Bifidobacterium longum* ATCC 15707 grown at pH 6.5.

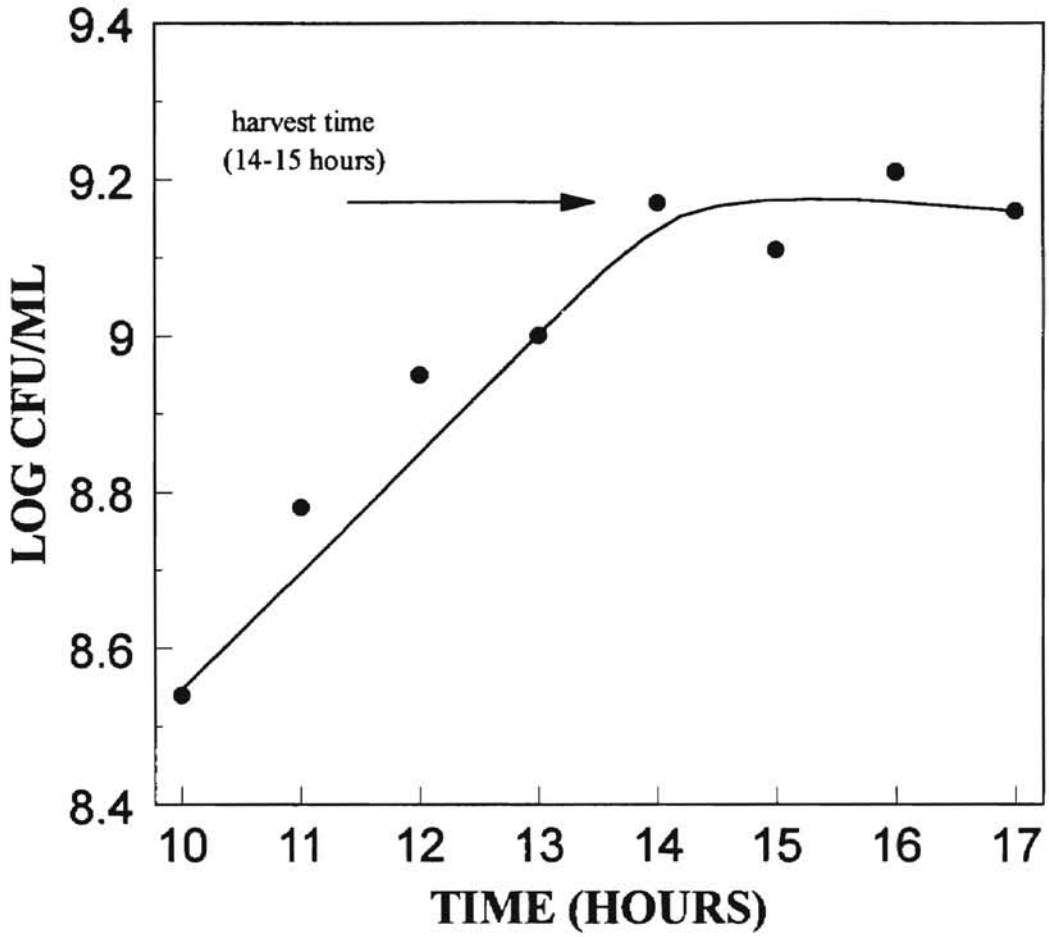

Figure 8. Harvest time (hours) for *Bifidobacterium longum* ATCC 15707 grown at pH 7.0.

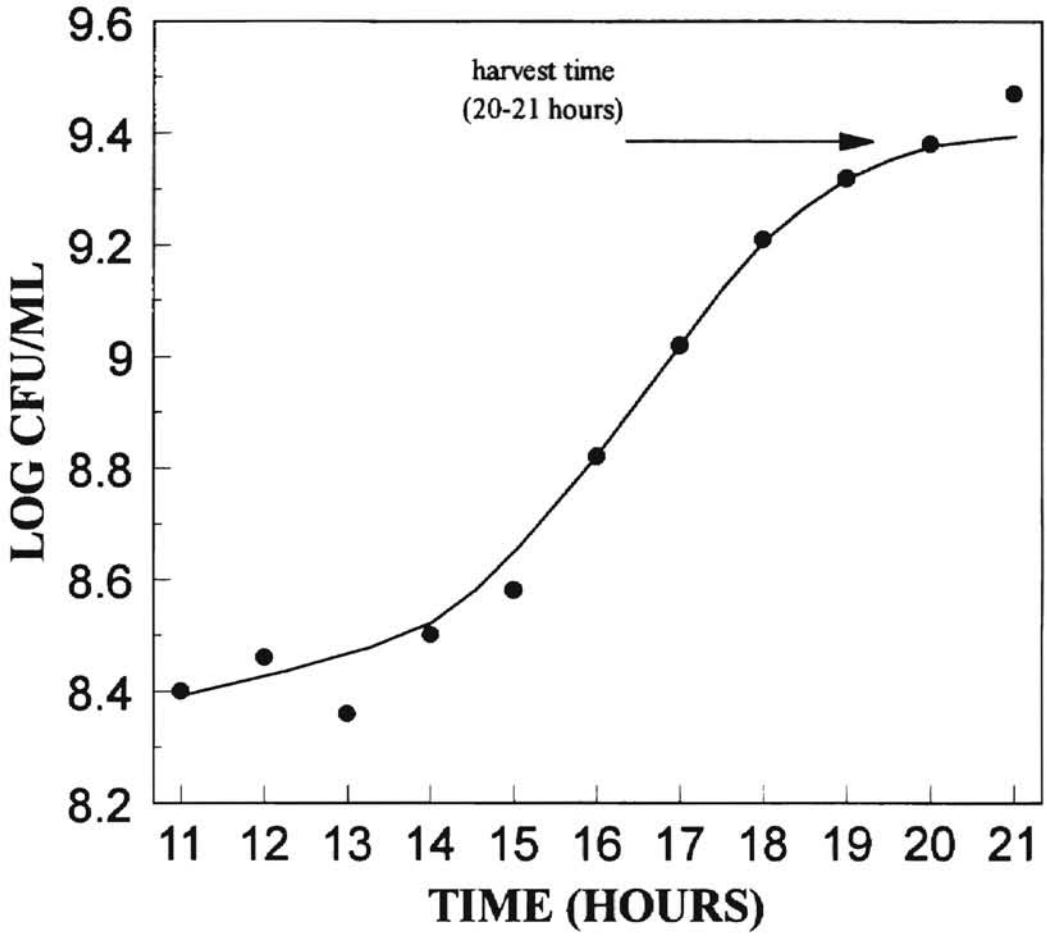

Figure 9. Harvest time (hours) for *Bifidobacterium longum* III grown at pH 5.5.

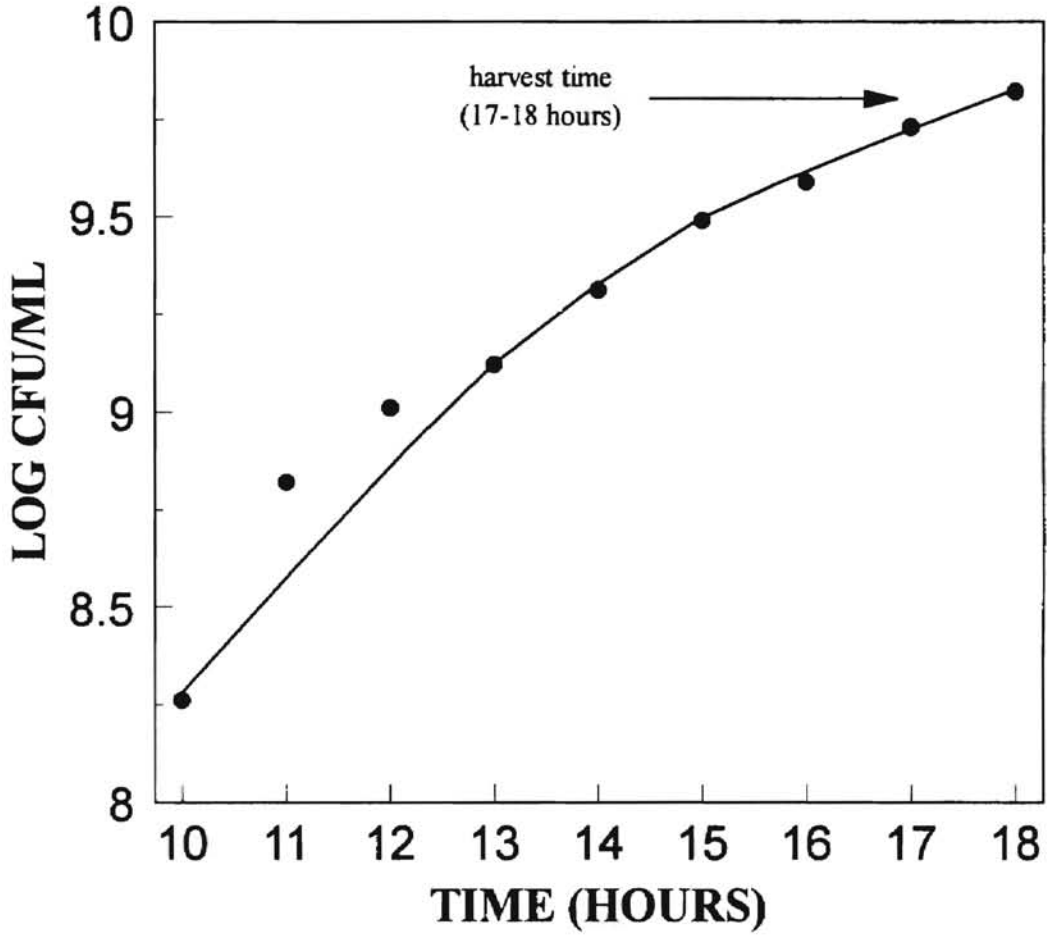

Figure 10. Harvest time (hours) for *Bifidobacterium longum* III grown at pH 6.0.

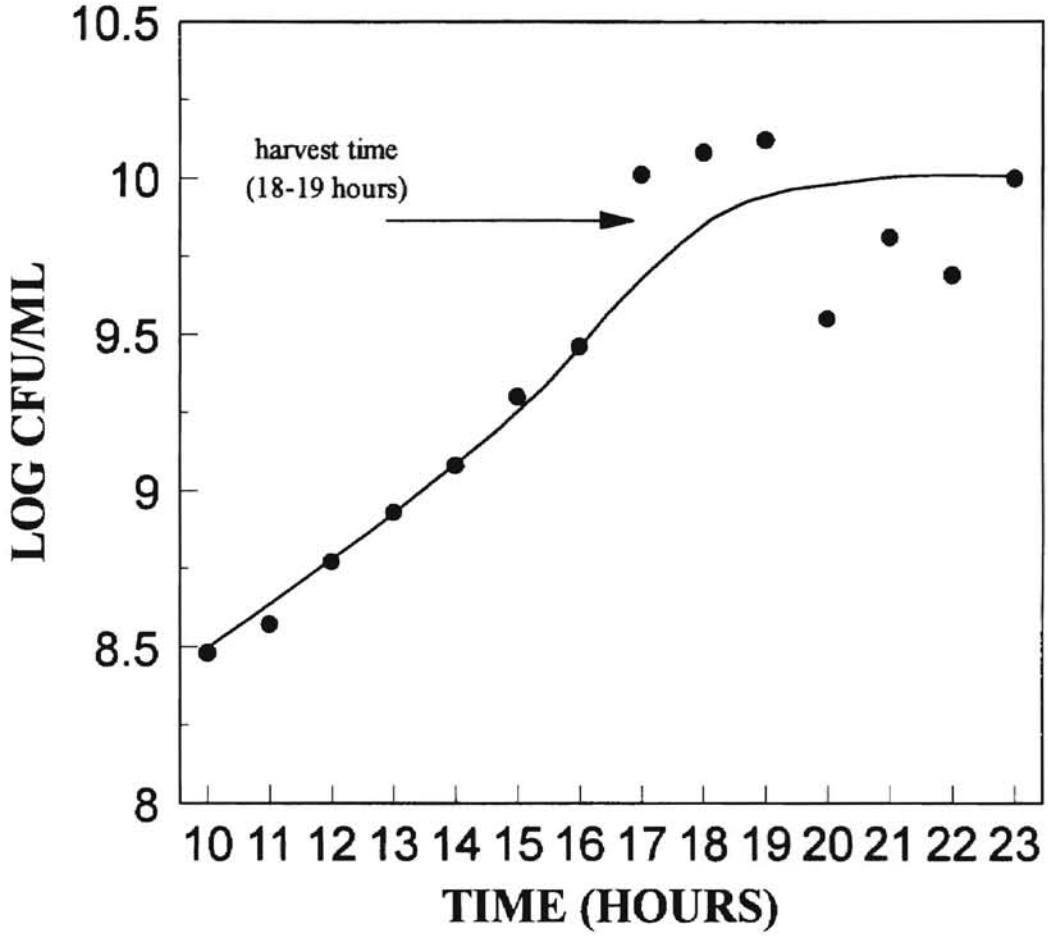

Figure 11. Harvest time (hours) for *Bifidobacterium longum* III grown at pH 6.5.

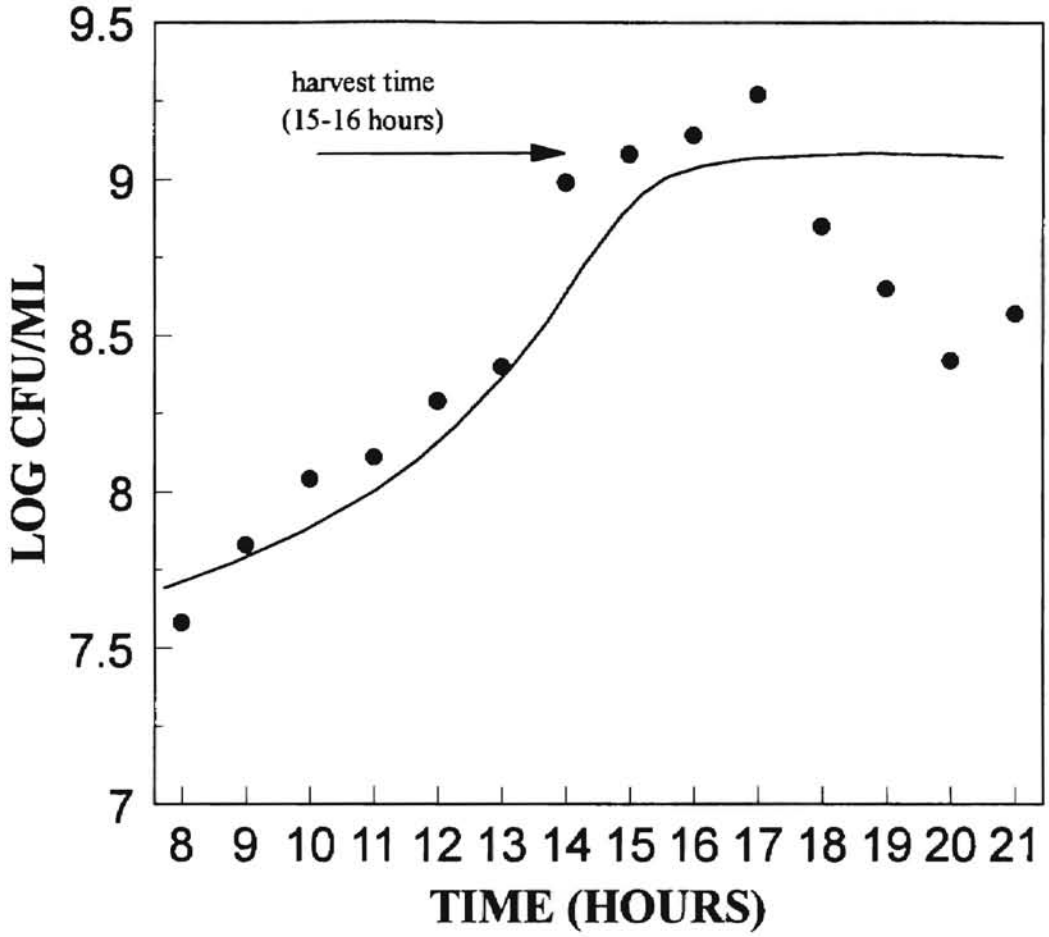

Figure 12. Harvest time (hours) for *Bifidobacterium longum* III grown at pH 7.0.

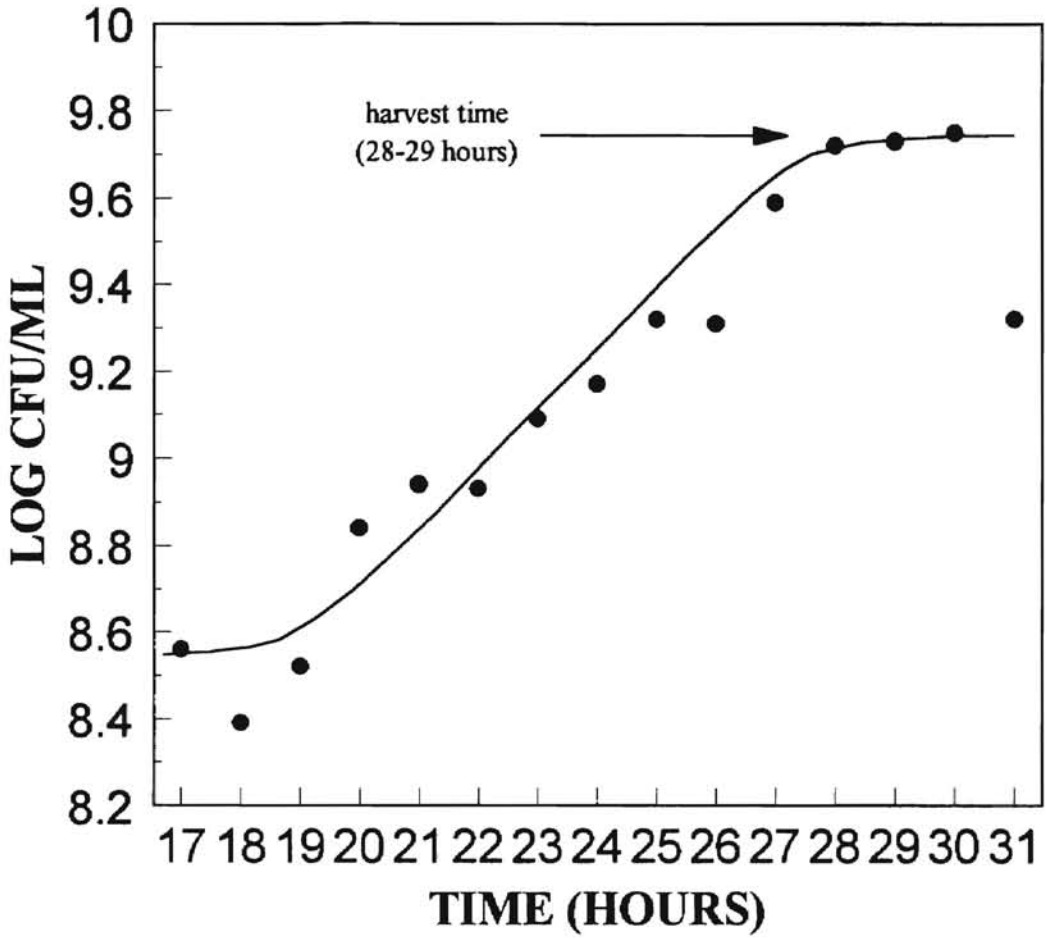

Figure 13. Harvest time (hours) for *Bifidobacterium longum* II grown at pH 7.0.

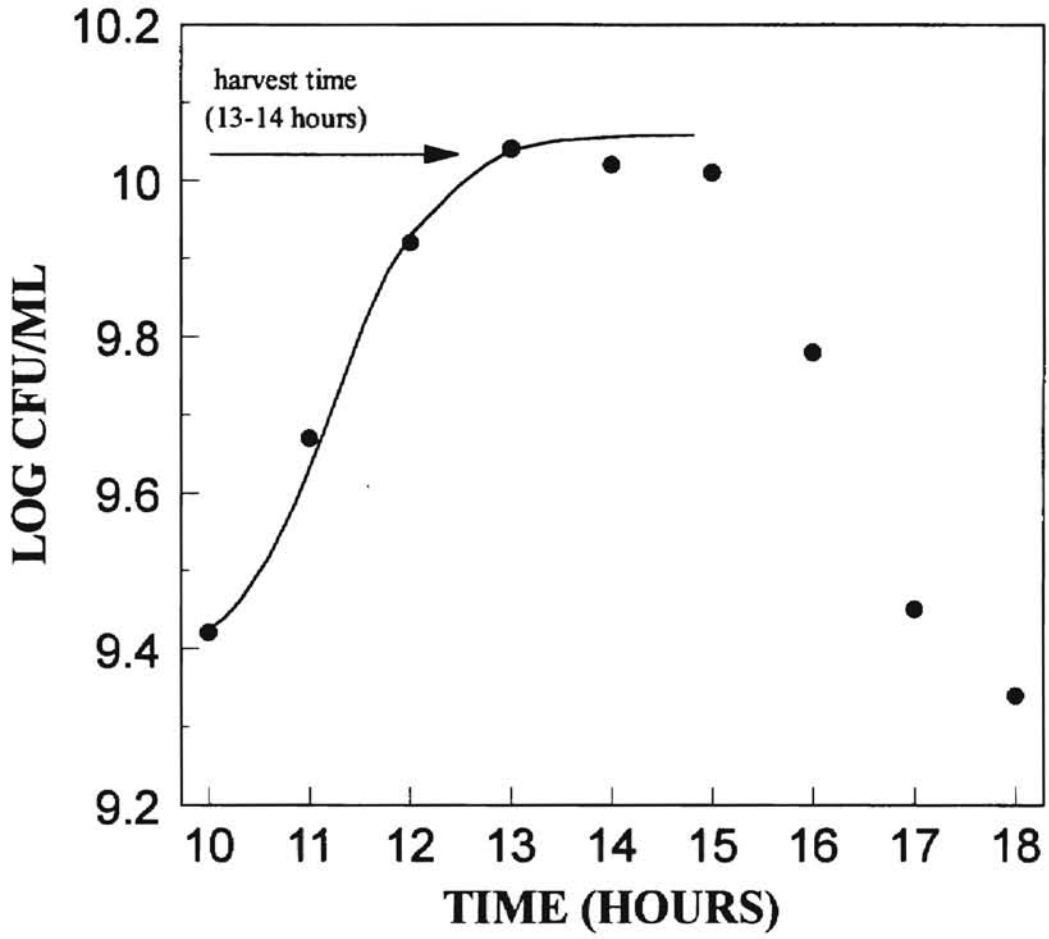

Figure 14. Harvest time (hours) for *Bifidobacterium longum* II grown at pH 5.5.

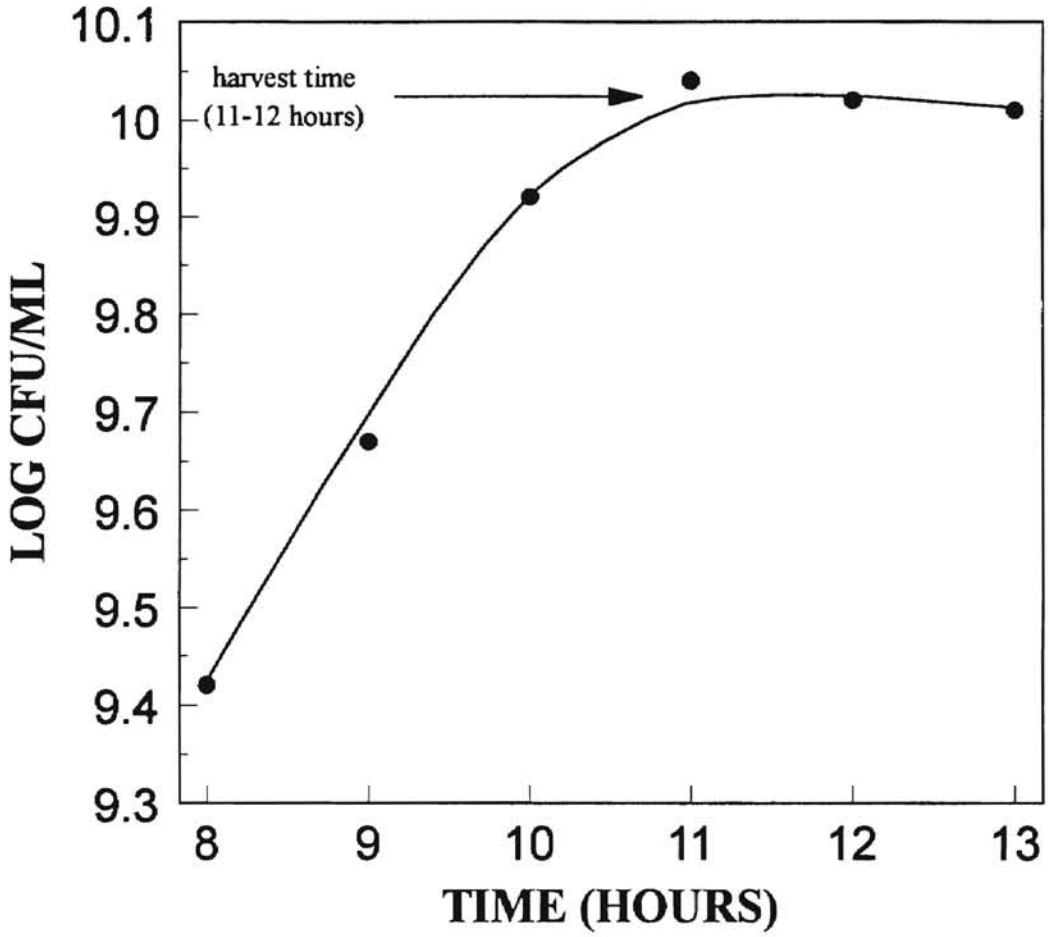

Figure 15. Harvest time (hours) for *Bifidobacterium longum* II grown at pH 6.5.

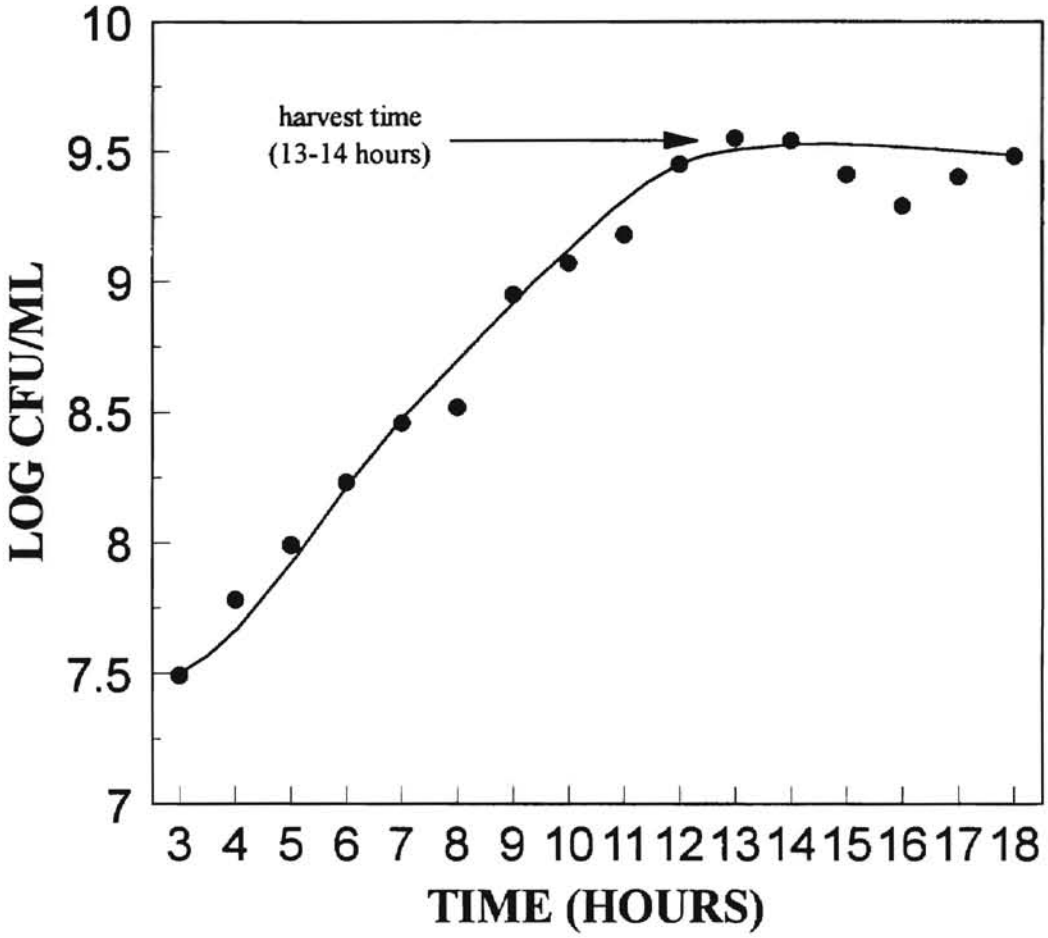

Figure 16. Harvest time (hours) for *Bifidobacterium longum* II grown at pH 7.0.

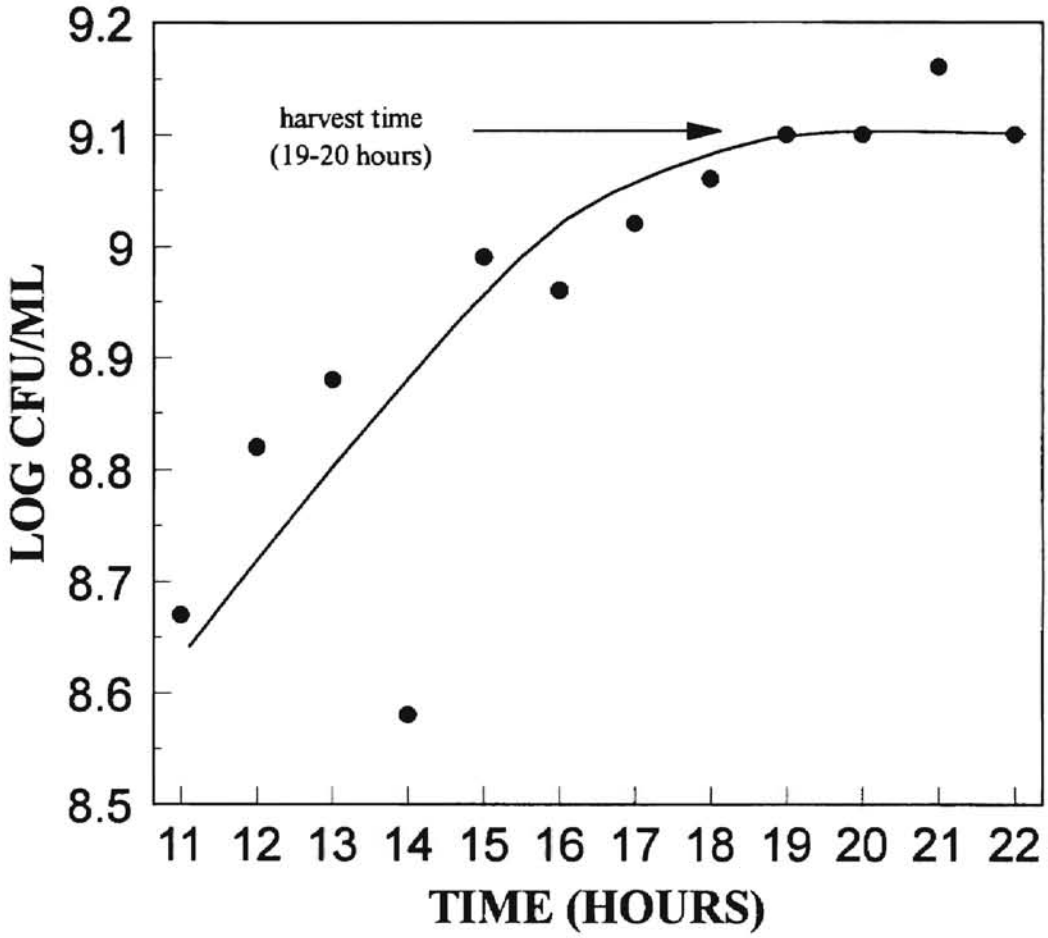

Figure 17. Harvest time (hours) for *Bifidobacterium longum* S9 grown at pH 5.0.

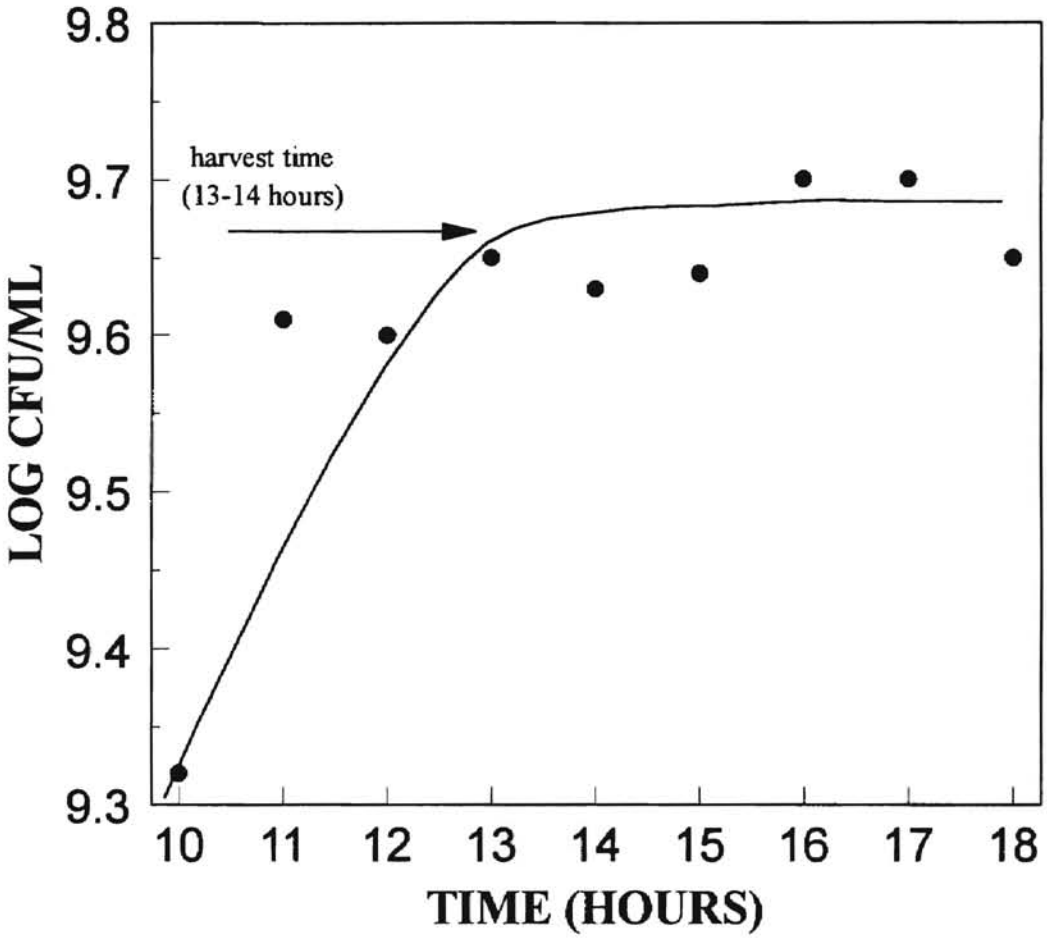

Figure 18. Harvest time (hours) for *Bifidobacterium longum* S9 grown at pH 5.5.

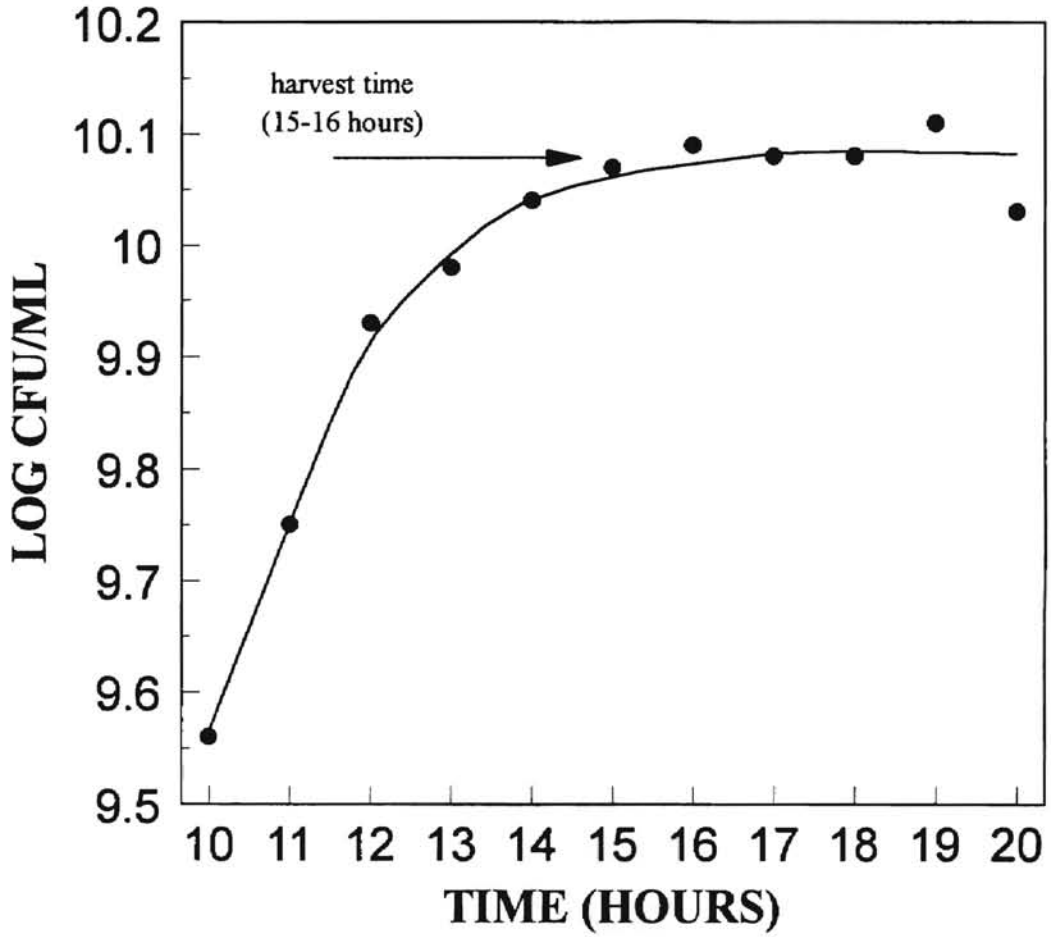

Figure 19. Harvest time (hours) for *Bifidobacterium longum* S9 grown at pH 6.0.

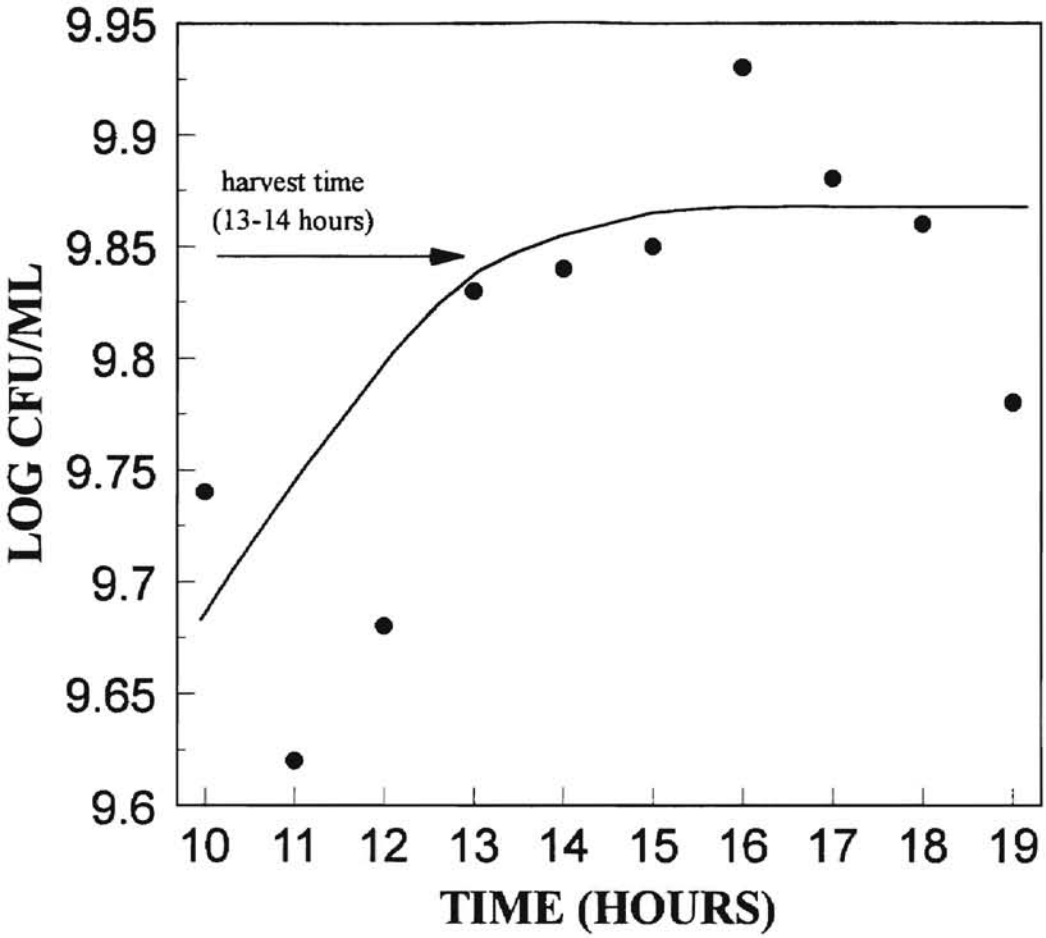

Figure 20. Harvest time (hours) for *Bifidobacterium longum* S9 grown at pH 6.5.

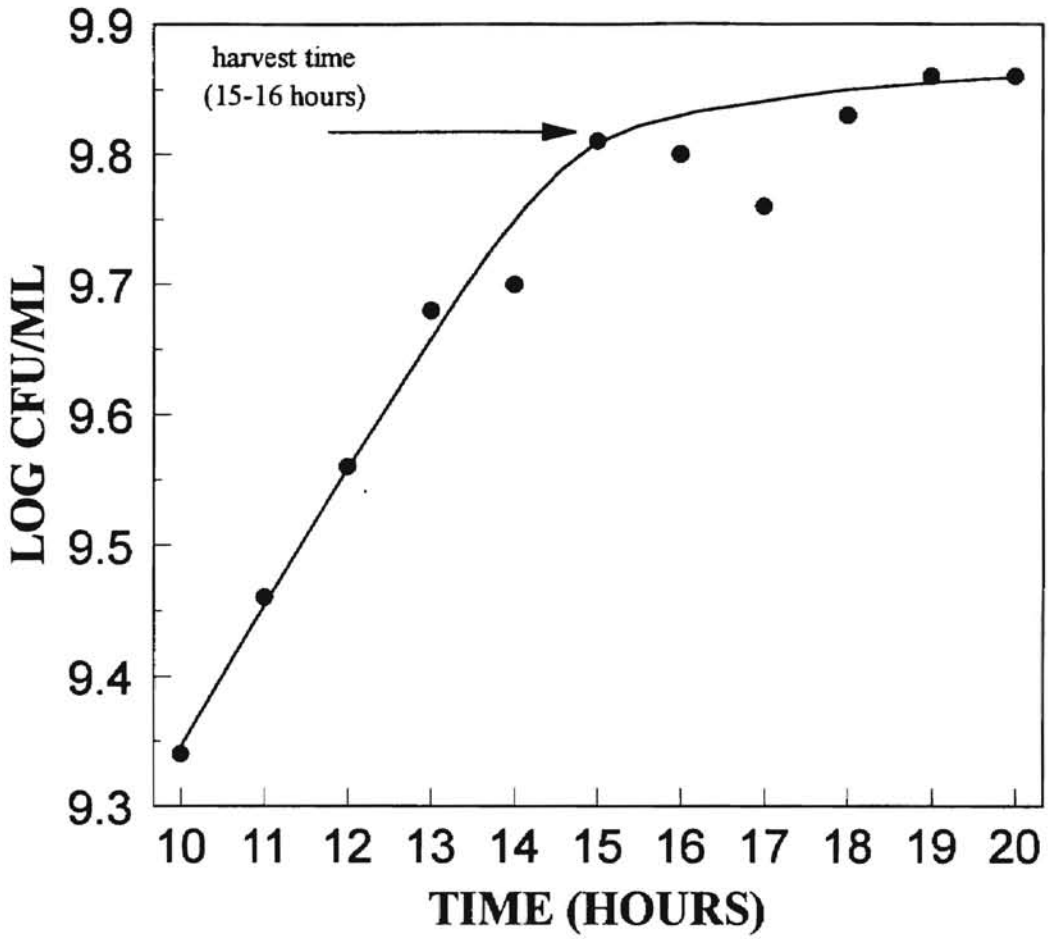

Figure 21. Harvest time (hours) for *Bifidobacterium longum* S9 grown at pH 7.0.

APPENDIX 4

ANALYISIS OF VARIANCE

TABLE 40

ANALYSIS OF VARIANCE
EFFECT ON TOTAL NUMBERS OF *BIFIDOBACTERIUM LONGUM* STRAINS
WHEN GROWN AT VARIOUS PH LEVELS

Source	df	SS	MS	F Value	Pr>F
Model	17	8.53	.5017	1.92	.0605
Error	28	7.30	.2608		
Corrected Total	45	15.83			
Blk	2	.5712	.2856	1.10	.3484
Strain	3	1.63	.5431	2.08	.1251
pH	3	3.74	1.25	4.79	.0082
Strain*pH	9	2.59	.2873	1.10	.3932
Blk	2	.5090	.2545	.98	.3893
Strain	3	1.50	.5005	1.92	.1493
pH	3	3.89	1.30	4.97	.0069
Strain*pH	9	2.59	.2873	1.10	.3932

TABLE 41

ANALYSIS OF VARIANCE
EFFECT ON TOTAL NUMBERS OF *BIFIDOBACTERIUM LONGUM* STRAINS DURING FROZEN STORAGE (-196 °C) WHEN GROWN AT VARIOUS PH LEVELS

Source	df	SS	MS	F Value	Pr>F
Model	111	65.38	.5890	17.69	.0001
Error[b]	126	4.20	.0333		
Corrected Total	237	69.58			
		Type I			
Block	2	.8497	.4249	12.76	.0001
Strain	3	36.96	12.32	370.08	.0001
pH	3	12.83	4.28	128.48	.0001
Strain*pH	9	1.91	.2124	6.38	.0001
Block*Strain*pH[a]	30	10.71	.3571	10.73	.0001
Day	4	.3788	.0947	2.84	.0268
Strain*Day	12	.4082	.0340	1.02	.4332
pH*Day	12	.2276	.0190	.57	.8631
Strain*pH*Day	36	1.096	.0305	.91	.6106
		Type III			
Block	2	.8089	.4044	12.15	.0001
Strain	3	36.65	12.22	366.99	.0001
pH	3	12.80	4.27	128.12	.0001
Strain*pH	9	1.92	.2129	6.40	.0001
Block*Strain*pH[a]	30	10.70	.3566	10.71	.0001
Day	4	.3784	.0946	2.84	.0269
Strain*Day	12	.4135	.0345	1.04	.4212
pH*Day	12	.2287	.0191	0.57	.8609
Strain*pH*Day	36	1.096	.0305	0.91	.6106

[a]Main Unit Treatment Error Term
[b]Sub Unit Treatment Error Term

TABLE 42

ANALYSIS OF VARIANCE
EFFECT ON TOTAL NUMBERS OF *BIFIDOBACTERIUM LONGUM* STRAINS DURING REFRIGERATED STORAGE (5 °C) IN MILK WHEN GROWN AT VARIOUS PH LEVELS

Source	df	SS	MS	F Value	Pr>F
Model	95	77.19	.8126	19.17	.0001
Error[b]	96	4.07	.0424		
Corrected Total	191	81.26			
		Type I			
Block	2	.8688	.4343	.67	.5189
Strain	3	29.49	9.83	15.17	.0001
pH	3	5.28	1.76	2.71	.0624
Strain*pH	9	3.43	.3715	.57	.8079
Block*Strain*pH[a]	30	19.43	.6478	15.28	.0001
Day	3	6.80	2.27	53.47	.0001
Strain*Day	9	5.94	.6598	15.56	.0001
pH*Day	9	1.38	.1538	3.63	.0006
Strain*pH*Day	27	4.66	.1725	4.07	.0001
		Type III			
Block	2	.8688	.4343	.67	.5189
Strain	3	29.49	9.83	15.17	.0001
pH	3	5.28	1.76	2.71	.0624
Strain*pH	9	3.43	.3715	.57	.8079
Block*Strain*pH[a]	30	19.43	.6478	15.28	.0001
Day	3	6.80	2.27	53.47	.0001
Strain*Day	9	5.94	.6598	15.56	.0001
pH*Day	9	1.38	.1538	3.63	.0006
Strain*pH*Day	27	4.66	.1725	4.07	.0001

[a]Main Unit Treatment Error Term
[b]Sub Unit Treatment Error Term

TABLE 43

ANALYSIS OF VARIANCE
EFFECT ON BILE TOLERANT NUMBERS OF *BIFIDOBACTERIUM LONGUM* STRAINS DURING REFRIGERATED STORAGE (5 °C) IN MILK WHEN GROWN AT VARIOUS PH LEVELS

Source	df	SS	MS	F Value	Pr>F
Model	95	144.53	1.52	10.82	.0001
Error[b]	96	13.49	.1405		
Corrected Total	191	158.02			
		Type I			
Block	2	.5858	.2929	.33	.7244
Strain	3	53.87	17.96	19.98	.0001
pH	3	8.70	2.90	3.23	.0363
Strain*pH	9	7.42	.8245	.92	.5237
Block(Strain*pH)[a]	30	26.96	.8987	6.39	.0001
Day	3	14.92	4.97	35.38	.0001
Strain*Day	9	13.00	1.44	10.28	.0001
pH*Day	9	5.52	.6127	4.36	.0001
Strain*pH*Day	27	13.57	.5024	3.57	.0001
		Type III			
Block	2	.5858	.2929	.33	.7244
Strain	3	53.87	17.96	19.98	.0001
pH	3	8.70	2.90	3.23	.0363
Strain*pH	9	7.42	.8245	.92	.5237
Block(Strain*pH)[a]	30	26.96	.8987	6.39	.0001
Day	3	14.92	4.97	35.38	.0001
Strain*Day	9	13.00	1.44	10.28	.0001
pH*Day	9	5.52	.6127	4.36	.0001
Strain*pH*Day	27	13.57	.5024	3.57	.0001

[a]Main Unit Treatment Error Term
[b]Sub Unit Treatment Error Term

VITA

Siobhan Sue Reilly

Candidate for the Degree of

Master of Science

Thesis: SURVIVAL OF *BIFIDOBACTERIUM LONGUM* DURING FROZEN (-196 °C) AND ENSUING REFIGERATED (5 °C) STORAGE AS INFLUENCED BY GROWTH AT VARIOUS PH

Major Field: Food Science

Biographical:

Personal Data:

Born in Madison, Wisconsin, May 20, 1966, the daughter of Joseph and Mildred Reilly.

Education:

Graduated from Clearwater High School, Clearwater, Florida in May 1984. Received Bachelor of Science degree in Food Science from the University of Florida, Gainesville, Florida in May, 1991. Completed the requirements for the Master of Science in Food Science at Oklahoma State University in May, 1997.

Experience:

Began working at age 13 in the restaurant industry until 1985. Employed by the University of Florida, Department of Animal Science as a research assistant from 1986-1990. Employed by Oklahoma State University from 1990-1992 as Laboratory Technician II for the Department of Animal Science in the area of Meat Science. Employed by Oklahoma State University from 1992-1997 as Laboratory Manager II in the Dairy Foods Microbiology Laboratory in the Department of Animal Science. As of April 1, 1997, employed by the Oklahoma Food and Agricultural Products Research and Technology Center as Food Microbiology Specialist.

Organizations: Institute of Food Technologists