USE OF CRYOPROTECTANTS, MICROENCAPSULATION OF BACTERIAL CELLS AND ACID AND LOW TEMPERATURE ADAPTATIONS IN IMPROVING VIABILITY OF PROBIOTIC BACTERIA IN FERMENTED FROZEN DAIRY DESSERTS

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By

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ABSTRACT

The main aim of this study was to improve the viability of probiotic bacteria (*Lactobacillus acidophilus* and *Bifidobacterium* spp.) in fermented frozen dairy desserts. In order to improve the viability of probiotic bacteria, initial studies were undertaken to determine the existing levels of probiotic bacteria present in fermented frozen dairy desserts. Samples of yogurt frozen on stick (with 18% overrun) and in cups (with 100% overrun) were directly received from a manufacturer and were analysed for the viable populations of *L. acidophilus*, *Bifidobacterium* spp, *S. thermophillus* and *L. delbrueckii ssp. bulgaricus*. The populations of the two probiotic organisms were very low ($<10^3$ cfu/g) even in the freshly frozen products.

Viability of yogurt bacteria (*S. thermophilus* and *L. delbruekii* ssp. *bulgaricus*) and probiotic bacteria (*L. acidophilus* and *Bifidobacterium* spp.) were assessed in several fermented frozen dairy dessert preparations obtained directly from commercial manufacturers. The counts of the four groups of organisms were monitored for 12 weeks storage at -18⁰C. The counts were found to be poor, especially in the case of *L. acidophilus* and bifidobacteria. The counts of *S. thermophilus* were better than those of *L. delbrueckii* ssp. *bulgaricus*.

Forty-one strains of probiotic and yogurt bacteria were screened for their ability to survive under frozen conditions, in presence of sugar (0, 4, 8, 12 and 16%) and under acidic conditions. Five batches (one litre size) of probiotic yogurts were made. Yogurt made without any added sugar was used as a control (batch 1). Yogurts were also made with 4, 8, 12 and 16% sugar (sucrose) levels (batches 2, 3, 4, and 5 respectively). The fermentation process was monitored by measuring

pH, acidity, and the incubation time taken to reach pH 4.5. Initial and final counts of yogurt bacteria and probiotic bacteria in yogurts were also assessed. The water activity decreased as the percentage of sugar increased. Higher levels of sugar (> 8%) prolonged the fermentation time taken to reach the pH of 4.5. There was no significant difference in the initial counts of yogurt and probiotic bacteria in all the five batches of yogurt. Final counts of *S. thermophilus* increased significantly in batches 1, 2, and 3 when compared to batches 4 and 5. The final viable counts of *L. delbrueckii* ssp. *bulgaricus* in batches 1 and 2 increased by about 2 log cycles and decreased in batches 3, 4 and 5. The final viable counts *L. acidophilus* in the batches 1, 2 and 3 were almost similar to initial counts, whereas in batches 4 and 5 the counts decreased by about 2 and 5 log cycles. The final viable counts of bifidobacteria in the batches 1, 2 and 3 increased by about one log cycle and the counts decreased in batches 4 and 5 by 2 to 3 log cycles.

Five one-litre batches of probiotic yogurts were made using reconstituted skim milk supplemented with 0, 4, 8, 12 or 16% sucrose. The incubation time to decrease the pH to 4.5 and changes in organic acids production and counts of yogurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) and probiotic bacteria (*L. acidophilus* and *Bifidobacterium* spp.) in yogurt were assessed for up to 30 days. The organisms produced pyruvic acid, acetic acid, lactic acid and propionic acid at varying levels. There were no major differences in the counts of yogurt and probiotic bacteria in all the five batches. However, the counts of all the four groups of organisms declined during storage, in particular in the batches containing 12 and 16% sugar. Thus sugar addition appeared deleterious to the

growth of yogurt and probiotic bacteria, especially in the products such as fermented frozen dairy desserts, which contain approximately 16% sugar.

Three batches of yogurts were prepared using three selected strains of each of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and *Bifidobacterium* spp. mixed with a base mix and liquid sugar (67% brix) and the mix frozen to prepare fermented frozen dairy desserts. In a separate experiment, three batches of yogurt were made with yogurt bacteria only and probiotic bacteria were added just before freezing. The viability of yogurt and probiotic organisms in the fermented frozen dairy desserts was monitored over a 12-week storage at -18^0C. Further, two 1000 L batches of fermented dairy desserts were made using selected strains of yogurt and probiotic bacteria using a continuous freezer to stimulate industrial setting and the viability of both organisms was monitored over a 12 week storage at -18^0C. Most of the organisms survived freezing, acidic conditions and the presence of high levels of sugar. Thus the survival of these organisms appeared to be strain dependent.

Three batches of fermented frozen dairy desserts were made with milk supplemented with acid casein hydrolysate or cysteine. The control batch was made with milk supplemented with 2% skim milk powder. Viable counts of yogurt bacteria and probiotic bacteria were enumerated and pH, titratable acidity and β-D-galactosidase activity measured at fortnightly intervals over a 12-week storage at -18^0C. *S. thermophilus* was most stable in all the samples of fermented frozen dairy desserts with counts $\geq 10^7$ cfu/g throughout the storage period. The counts of *L. delbrueckii* ssp. *bulgaricus* were higher in products supplemented with acid casein hydrolysate and cysteine. The counts of *L. acidophilus* and

bifidobacteria were >10^5 cfu/g in the batches supplemented with acid casein hydrolysate or cysteine. The β-D-galactosidase activity decreased rapidly in the control sample during 12 weeks storage at -18^0C as compared with those prepared with acid casein hydrolysate and cysteine.

A selective medium (which will be referred to as LC agar) was developed in this study for enumeration of *Lactobacillus casei* populations from commercial yogurts and fermented milk drinks that may contain strains of yogurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*), probiotic bacteria (*L. acidophilus* and bifidobacteria) and *L. casei*. Appropriate dilutions were pour plated in specially formulated LC agar acidified to pH 5.1 and the plates incubated at 27^0C for 72 to 96 h under anaerobic conditions. The growth of *S. thermophilus* was prevented by adjusting the pH to 5.1. *L. delbrueckii* ssp. *bulgaricus* did not ferment ribose as the carbon source, as a result the organisms did not form colonies. *L. acidophilus* formed colonies on MRS-ribose agar; however, this organism did not grow in the specially formulated LC agar containing ribose. Similarly, *Bifidobacterium* spp. did not form colonies in LC agar. *L. casei* formed colonies on LC agar.

Survival of probiotic bacteria was studied in fermented frozen dairy desserts made with or without cryoprotectants using a bench top and a commercial freezer. Cryoprotective agents were added at a rate of 0.2% to yogurt mix at a temperature of 30-35°C before pasteurising at 85°C for 30 min. Various cryoprotective agents were initially evaluated for their effect on textural quality and the best resulting cryoprotectants were used in this study. The cryoprotectants that were used included RS 150 (pectin), glucose and GCF 639

(alginate). These cryoprotectants were of food grade standard and are suitable for the manufacture of dairy products. The final product was made by blending 45% yogurt, 45% of base mix and 10% of 65% syrup (liquid sucrose) followed by freezing through an ice cream freezer. The control batch and the batches containing RS 150, glucose and GCF 639 were made using a bench top ice cream freezer (1 L batch; ~18% overrun). Two batches of fermented frozen dairy desserts were made with cryoprotectant RS 150 and without cryoprotectant (control) using a commercial freezer (20 L batch; ~100% overrun). The product was filled in plastic containers and stored at $-18^{\circ}C$ for 12 weeks.

The initial pH of milk (6.51-6.67 at 0 h) decreased to pH 4.52-4.59 during yogurt making. In general, there was a gradual decrease in the pH in all the products during storage for 12 weeks. The pH by the end of storage period was 4.41-4.46. The titratable acidity of yogurt varied between 0.71 to 0.78% after fermentation and the acidity of fermented frozen dairy desserts varied between 0.79 to 0.86% at the end of the storage period. The counts of *L. acidophilus* and bifidobacteria at the end of 12 weeks storage period was $\geq 10^{5}$ cfu/g in all the products except the product containing glucose as the cryoprotectant which had a viable count of $<10^{4}$ cfu/g. The counts *L. acidophilus* and bifidobacteria decreased rapidly in both products and the viable counts were $<10^{2}$ and $<10^{3}$ cfu/g, respectively by the end of the 12-week storage period. Loss of viability of *L. acidophilus* and bifidobacteria in the products made using the commercial freezer was possibly due to the presence of oxygen. The products containing cryoprotectants performed better in improving viability of probiotic bacteria. Glucose did not help improve their survival. Overall, there was a slight improvement in the viable

counts of *L. acidophilus* and *Bifidobacterium* spp. in the presence of the cryoprotectant RS 150.

Viability of microencapsulated *L. acidophilus* and bifidobacteria during a storage period of 12 week was assessed in fermented frozen dairy desserts. Two batches of fermented frozen dairy desserts were made with or without encapsulated probiotic bacteria. Viable counts of probiotic bacteria were enumerated and pH, titratable acidity and β-D-galactosidase activity measured at fortnightly intervals for 12 weeks. The counts of *L. acidophilus* and bifidobacteria decreased to $<10^3$ cfu/g in the control batch, whereas the counts were $>10^5$ cfu/g in the batches in which microencapsulated probiotic bacteria were used. The β-D-galactosidase activity decreased rapidly in the control during 12 weeks storage at -18^0C as compared with those prepared with microencapsulated cells.

Selected strains of *L. acidophilus* and bifidobacteria were adapted to acid and low temperature conditions. The organisms were stressed at lethal pH (pH 3.5) or sub-lethal pH (pH 5.5) for various times. The organisms survived better in the environment where sub-lethal stress was given. The organisms stressed in acid and at -18^0C for 24 h were used for incorporation into fermented dairy desserts. Viability of stress adapted probiotic bacteria in fermented frozen dairy desserts was assessed during a storage period of 12 week. Six 1 L batches of fermented frozen dairy desserts were made on a bench scale with or without acid and low temperature stress adapted probiotic bacteria. The counts of *L. acidophilus* and bifidobacteria decreased to $<10^3$ cfu/g in the control batch, whereas the counts were $>10^5$ cfu/g in the products made with the bacteria which were adapted to acid and low temperature.

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1.0   INTRODUCTION

Dairy products containing *L. acidophilus* and *Bifidobacterium* spp. are rapidly gaining popularity in Australia. The shelf life of yogurts is approximately 3 weeks. As a result, it is not feasible to ship yogurts to overseas countries. Thus, the sales of such products are limited to local markets. With increasing popularity of frozen dairy desserts containing yogurt cultures, a market could be developed for dairy desserts similar to soft-serve and hard pack frozen yogurts but containing cultures selected for dietary benefits such as *L. acidophilus* and *Bifidobacterium* spp. Frozen dairy desserts have longer shelf life and thus the products could be shipped to overseas countries such as Japan.

It has been suggested that to have therapeutic effects, a minimum of one million viable cells of *L. acidophilus* and *Bifidobacterium* spp. per gram of a product must be present in fermented frozen dairy desserts. However, introducing probiotic organisms into the frozen food chain can be a difficult task as the organisms are unstable in such products.

This study aimed at achieving improved viability of probiotic bacteria in fermented frozen dairy desserts. The specific objectives of this project were:

1.   To assess the viability of yogurt and probiotic bacteria in commercial products such as yogurts and fermented dairy desserts,

2. To evaluate several media in order to selectively enumerate *L. casei* from mixed populations of yogurt bacteria and probiotic bacteria,

3. To select strains of yogurt bacteria and probiotic bacteria that survive acidic conditions, low temperatures, high sugar concentrations and low water activity conditions,

4. To investigate the use of micronutrients and cryoprotectants on improving the survival of the probiotic bacteria,

5. To investigate the feasibility of encapsulating probiotic bacteria and of incorporating them in fermented frozen dairy deserts, and

6. To examine the effects of low temperature and acid adaptations on survival of probiotic bacteria.

Chapter 2 of this thesis contains the review of literature; chapter 3 contains materials and methods and chapter 4 deals with the viability of probiotic bacteria in commercial products. Chapters 5 and 6 studied the viability of the 5 groups of bacteria (*S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus,* bifidobacteria and *L. casei*) in commercial dairy products. Chapters 7, 8, and 9 focus on selection of strains and effects of water activity on fermentation, organic acid production and viability of probiotic bacteria. Chapters 10 and 11 contain viability of probiotic bacteria in products made using a bench top and a

continuous ice cream freezer. Chapters 12 and 13 deal with the effects of micronutrient and cryoprotectants on the viability of probiotic bacteria. Chapters 14 and 15 studied use of microencapsulation technique and acid and low temperature adaptations in improving viability of probiotic bacteria and finally chapters 16, 17, and 18 give overall conclusions, future research directions and references.

2.0 LITERATURE REVIEW

2.1 Yogurt

2.1.1 *Definition*

Yogurt is defined as a product resulting from milk by fermentation with the two organisms, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* growing together symbiotically. Carbon dioxide, acetic acid, di-acetyl, acetaldehyde and several other substances are formed in the conversion process, and these impart to the product its characteristic fresh taste and aroma. Yogurt varies considerably in composition, flavour and texture, according to the nature of fermentation and the type of organisms added, although some controversy still exists regarding the exact definition of yogurt in terms of its chemical composition and types of starter organisms used (Kosikowski, 1977; Tamime and Deeth, 1981).

2.1.2 *Types of yogurt*

Several types of yogurts are manufactured in Australia, however, the main types are: set, stirred and drinking yogurts. The minimum standards including composition and pH are fixed by the Australian standards H8. The set yogurt is prepared by heating the standardised milk to 85°C for 30 min or 95°C for 15 min and adding yogurt bacteria or a combination of yogurt and probiotic bacteria after cooling to 42–45°C. The milk containing culture bacteria are filled in desired containers and incubated at the same temperature until a pH of 4.5 is attained. Similar procedures of set yogurts are used in preparing stirred yogurts; however, the total solids are higher in this case and inoculated milk is set in bulk rather

than in containers. The incubated mix after attaining pH 4.5 is stirred and chilled followed by addition of fruits before packing into containers. Drinking yogurts are prepared using less total solids (5.5 – 11%) and incubating at lower temperatures for long hours (37°C for 16-18h). Fruit puree, fruit pulp and flavours are added before filling into bottles.

The post-incubation processing of yogurt may lead to various types of yogurts such as pasteurised, UHT, concentrated and dried yogurts. Pasteurised yogurt is heated after incubation using a shell tube pasteuriser to about 60-65^{0}C leading to destruction of yogurt. In some cases probiotic bacteria are incorporated in order to avoid antagonism with yogurt bacteria and to improve the survival of probiotic bacteria. The dried yogurt can be produced either by sun drying, spray drying or freeze-drying. The drying process transforms the junket into powder and also causes loss of some flavour compounds and destruction of starter cultures. The dried yogurt is mainly used in the Middle East. The dried yogurt is used in making some traditional cooking. The manufacture of yogurt has been reviewed by several authors (Tamime and Robinson, 1985; Kurmann, 1988; Rasic and Kurmann, 1978).

2.1.3 *Consumption pattern*

Fermented milk products originated from the near east and subsequently gained popularity in eastern and central Europe. The earliest example of cultured milk was presumably produced accidentally by the nomads. This milk "turned sour" and coagulated under the influence of certain infecting microorganisms. As luck

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would have it, the bacteria were of harmless, acidifying type and were not toxin-producing organisms. Among the fermented milks, yogurt is one of the common examples.

Yogurt is one of the most popular and best known in almost all corners of the world, particularly Mediterranean, Asia, and Central Europe, and is becoming more popular in America, Australia and Japan. The origin of yogurt is not very clear. However according to Tamime and Deeth (1980), the word yogurt is derived from the Turkish word 'jugurt'. The Slavs adopted the procedure of yogurt preparation and yogurt became their traditional food. Yogurt is prepared in Bulgaria from goats or boiled cows milk, inoculated at 40-45°C with a portion of previously soured milk. To keep the temperature constant, the containers containing inoculated milk are thoroughly wrapped in furs and placed for 8-10 h in a warm place until a smooth gel was formed. Table 2.1 shows the various types of yogurts and their composition, manufactured in Australia.

Table 2.1   Types of yogurts and their nutritional values that are available in
Australian markets (data shown is for 100 grams).

Type	Protein (g)	Fat (g)	Total Sugars (g)	Energy (kJ)	Calcium (mg)	Sodium (mg)	Cholesterol (mg)
Natural	4.7	3.4	4.7	281	171	55	16
Reduced fat	5.0	1.7	7.5	266	160	63	10
Low fat	5.9	0.2	5.8	200	209	70	5
Acidophilus	5.6	0.2	5.5	191	215	75	6
Vanilla skim	5.9	0.2	12.2	304	185	79	6
Strawberry skim	5.9	0.3	10.9	270	181	67	5
Fruit strawberry	4.9	2.8	12.4	381	171	60	12
Strawberry reduced fat	4.9	0.9	14.5	348	160	50	6
Strawberry low fat	5.2	0.2	12.8	299	179	61	3
Strawberry artificially sweetened	5.0	0.2	11.4	186	170	57	4
Strawberry A B	4.9	3.1	5.9	379	165	58	12

(Source: Australian yogurt and ice cream, Australian Dairy Corporation
nutritional guide, 1998).

2.2 Functional foods

Functional foods are foods that convey nutritional/therapeutic benefits on human health. Functional foods are defined as "the foods similar in appearance to conventional foods and are intended to be consumed as part of a normal diet, but have been modified to subserve physiological roles beyond the provision of simple nutrient requirements" (National Food Authority, 1994). The interest in functional foods continues to increase world wide and with the rapid growth of nutritional and medical sciences, it is hard to imagine that all the 'functional' components have been detected yet in the foods we commonly consume. The first international conference on East-west perspective on functional foods was held in Singapore in 1995. The purpose of the conference was to discuss the many issues surrounding functional foods at an international level. The concept originated in Japan because of two reasons, the highly competitive market and the great interest by consumers in health foods. This has now found a firm basis in the western world and is considered to be the most important development for the future.

Dairy products containing probiotic bacteria are a good example of functional foods. Yogurts and fermented frozen dairy desserts containing probiotic bacteria are popular dairy products. Japan has provided the technology for the introduction of probiotic functional foods and import probiotic functional foods into the country. The Japanese Food Authority has implemented minimum standards on the products before it is marketed to the consumers. Providing an affirmed legal product and specific labelling benefit, functional foods (Known

in English as <u>F</u>oods for <u>S</u>pecified <u>H</u>ealth <u>U</u>se, or FOSHU) are currently marketed

in Japan, although many foods, which would be considered as functional foods,

have not been submitted for formal FOSHU certification. As of June, 1997, 80

FOSHU foods have been approved (Bailey, 1997; Sanders, 1998). In the United

States, although consumer and corporate interest is high, functional foods have no

legal definition. This has not stopped food companies from entering into

functional food market (Sanders, 1998). In Europe the probiotic functional yogurt

market offers considerable opportunities for Europe's dairy industry. Grijspaardt-

Vink (1996) identified health and convenience as the two major trends in the

European food market, citing keen awareness of European consumers to the

relationship between diet and health. A leadership role in approaching the

functional food area is being taken by International Life Sciences Institute

(ILSL), which is managing a project started in 1995 to establish a science-based

approach for targeted modification of food and food constituents. In Australia

probiotic functional foods are popular and the products are exported to Japan and

the middle-east countries. Fermented dairy products such as yogurt and

fermented frozen dairy desserts have been the focus of many studies in terms of

their alleged physiological functionality. In contrast to the biologically active

components contained in the milk itself, the health-promoting effects of the

fermented dairy products may be related to the biological activity of the bacteria

used in the production of these foods and the metabolites generated during the

fermentation process (Jelen and Lutz, 1998).

2.3 Fermented frozen dairy desserts

2.3.1 *Definition*

Frozen yogurt is a food product derived by blending of pasteurised ice-cream base mix with plain yogurt followed by mixing before freezing. The proportion of blending varies from manufacturer to manufacturer. The ice-cream mix may contain whole milk, partially de-fatted milk, skim milk, cream, butter, butter oil, sweeteners, stabilisers and emulsifiers. The yogurt mix may contain, milk, sucrose, cream, butter, butter oil and lactic acid bacteria and/or probiotic bacteria.

2.3.2 *Types of fermented frozen dairy desserts*

There are two types of fermented frozen dairy desserts available in the market: soft serve, hard pack and ice-poll type. Soft serve fermented frozen dairy desserts may contain about 100% overrun and are packed in 200 mL, 500 mL, 1 L, 2 L and 5 L containers. Soft serve fermented frozen dairy desserts are available in different flavours and with fruits and/or fruit pulp, whereas stick ice-poll may have about 18% overrun and is moulded in fancy shapes and packed individually in a box of 12 and 24.

2.3.3 *Consumption pattern*

Yogurt has proven to be an unacceptable vehicle to incorporate bifidobacteria into human diet due to the high acidity of most products. Several commercial products have been evaluated and only one low acid Japanese product was found to contain bifidobacteria. The remaining samples were either negative for

bifidobacteria or lacked fructose-6-phosphate phosphoketolase (F6PPK) activity. F6PPK is indicative of bifidobacteria (Scardovi, 1981; Modler and Garcia, 1993).

The ice cream industry has investigated several means of participating in the ever-growing market for acceptable yogurt products. The industry wanted to produce a desirable flavoured frozen yogurt, which could be manufactured in an ice-cream plant and marketed through existing channels of distribution. Due to competitive reasons the quality of the new product might suffer without a published standard. Therefore it was decided that frozen yogurt was in fact a new frozen dessert and, therefore entitled to its own standard of identity. Frozen yogurt in its own right can be identified by several features.

In early 70s frozen yogurt was established in the market but was unsuccessful by the late 70s due to its sour taste and being gummy, icy and not very creamy. In 80s health consciousness of consumers have spurred the demand for the frozen yogurt. The acceptance and popularity have pushed frozen yogurt into full-service, year-round, any-time-of-day of product demand.

Dairy products containing *L. acidophilus* and *Bifidobacterium* spp. are rapidly gaining popularity in Australia. The shelf life of yogurt is approximately 3 weeks. As a result, it is not feasible to ship yogurt to overseas countries. Thus, the sales of such products are limited to local markets. With increasing popularity of frozen dairy desserts containing yogurt cultures a market could be developed for dairy desserts similar to soft-serve and hard pack frozen yoghurts, but containing

cultures selected for health benefits such as *L. acidophilus* and *Bifidobacterium* spp. Frozen dairy desserts have longer shelf life and thus the products could be shipped to overseas countries such as Japan.

It has been suggested that to have therapeutic effects, a minimum of one million viable cells of *L. acidophilus* and *Bifidobacterium* spp. per gram of a product must be present in fermented frozen dairy desserts. However, introducing probiotic organisms into the frozen food chain can be a difficult task as the organisms are unstable in such products.

2.4   Yogurt bacteria

In 1905, Grigoroff conducted several studies on yogurt bacteria. The classification of the lactic acid bacteria by Orla-Jensen is still recognised as the standard method for distinguishing these organisms, ie the sphere shape was *Streptococcus* and the rod forms were *Thermobacterium*, *Treptobacterium* and *Betabacterium*. According to Orla Jensen the yogurt starter organisms were thermophilic lactic acid bacteria capable of growing at 40-45°C.

According to the Garvie (1986), yogurt bacteria are classified into two separate families, the *Streptococcaceae* and *Lactobacillaceae* (Tamime and Robinson, 1985). The yogurt starter cultures are mixed strains of *S. thermophilus* and *L. delbrueckii* spp. *bulgaricus,* which are normally propagated together at around 42°C. There is a symbiotic relationship between *S. thermophilus* and *L. delbrueckii* spp. *bulgaricus.* Pette and Lolkema (1950) observed a rapid acid

development in mixed cultures, compared to single strain cultures due to increase in numbers of *streptococci*. Dahiya and Speck (1968) also investigated the symbiosis theory, and supported the view that *L. delbrueckii* ssp. *bulgaricus* stimulates *S. thermophilus* by releasing glycine and histidine into the growth medium. They concluded that histidine rather than valine was the most important requirement. Under anaerobic conditions, the former organism produces a stimulating factor for *L. delbrueckii* ssp. *bulgaricus* that is equal to or can be replaced by formic acid.

Faster growth of streptococci at the beginning of fermentation brings about accumulation of moderate amounts of lactic and acetic acids, acetaldehyde, diacetyl and formic acid. Availability of formate and the changes in oxidation-reduction potential in the medium stimulate the growth of *L. delbrueckii* spp. *bulgaricus*. The proteolytic activity is more in *L. delbrueckii* spp. *bulgaricus* than *S. thermophilus*. The metabolic activity of yogurt bacteria results in a considerable increase in cell numbers. The total numbers of yogurt bacteria ranges from 10^7 to 10^8 and may decrease during the storage period (Ravula and Shah, 1998b).

2.5 Prebiotics

The term "prebiotic" has been applied to compounds such as oligosaccharides, which promote the growth of beneficial organisms. Oligosaccharides have been recognised for health benefits in Japan, since the early 1990s many products, especially drinks, have been developed and are being promoted for their

oligosaccharide content. Some oligosaccharides, due to their chemical structure, are resistant to digestive enzymes and therefore pass into large intestines where they become available for fermentation by the saccharolytic bacteria (O'Sullivan, 1996).

2.6   Probiotics

The term probiotic is derived from the Greek word, which means for life. Probiotic is defined as a viable mono-or mixed-culture of microorganisms, which when applied to animal or a man beneficially affects the host by improving the properties of the indigenous microflora. The term probiotic was first used by Lilly and Stillwell (1965) to describe the growth promoting effect of one or more microorganisms against another. Soon afterwards, the term was applied primarily to animal feed supplements. Further changes in use of this term implied a live microbial feed supplement that improved the intestinal microbial balance of the host (Fuller, 1989). The term is now also being used with reference to human consumption of live microorganisms as food additives for nutritional health and well-being (Hull *et al.,* 1992). The organisms that have been considered as probiotics are *L. acidophilus* and *Bifidobacterium* spp., which must be capable of reaching and colonising the intestinal tract of the human. These organisms must be acid and bile tolerant and must provide a nutrient source, which selectively promotes growth of beneficial intestinal microflora (Lyons, 1988).

These bacteria are the most important group in intestinal organisms to be suggested for use as dietary adjuncts in dairy foods. The probiotic organisms are

predominant microflora of breast fed infants (Jao *et al.*, 1978; Gilliland, 1979; Rasic and Kurmann, 1978). As the person ages the number of intestinal bifidobacteria decreases and the number of clostridia, streptococci and coliforms increases (Stark and Lee, 1982).

The basis of the probiotic concept is that these are the gastrointestinal tract microorganisms, which have a beneficial effect on the host. Modern life styles may have adverse affect on the delicate balance that exists between the many different species of microorganisms, which inhabit the gut. Our unnatural diet (most of which is heat sterilised by cooking), antibiotic therapy and stress are all factors which can alter the composition of the gut microflora and nutritional and health consequences. Probiotics can reverse or prevent these consequences by restoring the flora to its natural state (Fuller, 1989)

2.7 *Lactobacillus acidophilus*

In the early 19th century Metchnikoff suggested that man should consume milk fermented with lactobacilli to prolong life (Metchnikoff, 1908). His theory was that the lactobacilli would displace the microorganisms normally occurring in the intestinal tract that produced "toxins", resulting in reduction of life span. Continued worldwide interest in beneficial lactobacilli as dietary adjuncts has resulted in extensive research and has led to the development of foods including various dairy products. Several antibiotic-like substances such as acidolin (Hamdan and Mikolajcik, 1974), acidophilin (Shahani *et al.*, 1977), and lactocidin (Vincent *et al.*, 1959) are produced by various strains of *L.*

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acidophilus. Mehta *et al.* (1984) reported a broad-spectrum inhibitor produced by *L. acidophilus*, which was active against a number of both gram-positive and gram-negative organisms. This inhibitory material was characterised as being a protein. This inhibitory system also was active against both gram-negative and gram-positive organisms. It was characterised as being resistant to proteolytic enzymes, heat resistant, and of low molecular weight. *L. acidophilus* is capable of producing more than one inhibitory system and the antagonistic action they exert towards intestinal pathogens are the likely results from the involvement of more than one particular inhibitor.

Shahani *et al.* (1977) studied the influence of *L. acidophilus* on tumour cells using rats as an animal model. The authors reported that the rats fed with milk containing *L. acidophilus* had less tumour cells than the rats, which did not receive milk containing *L. acidophilus*.

Goldin *et al.* (1980) showed that the consumption of milk containing *L. acidophilus* could cause significant reduction in the activity of the β-glucuronidase, azoreductase, and nitoreductase enzymes. These enzymes can convert procarcinogens into carcinogens in the intestinal tract. Lactose maldigestion refers to the inability of the person to digest lactose adequately. The inability to digest lactose adequately is due to the absence of sufficient amounts of the enzyme β-galactosidase in the small intestines. The microorganisms in the gastrointestinal tract can influence the ability to digest lactose. A total of 56 of lactobacilli are shown in Table 2.2.

Table 2.2 Species of genus *Lactobacillus*

1	Lactobacillus acidophilus	29	L. paracasei
2	L. amylophilus	30	L. rhamnosus
3	L. amylovorus	31	L. sake
4	L. crispatus	32	L. agilis
5	L. delbrueckii	33	L. pentosus
6	L. gallinarum	34	L.plantarium
7	L. gaserri	35	L. brevis
8	L. heliveticus	36	L. buchneri
9	L. gensenii	37	L. collinoides
10	L. johnsonii	38	L. fermentum
11	L. kefiranofacens	39	L. fructivorans
12	L. aviarius	40	L. hilgardii
13	L. farciminis	41	L. kefir
14	L. salivarius	42	L. malofermentans
15	L. mali	43	L. oris
16	L. ruminis	44	L. parabuchnery
17	L. sharpeae	45	L. ruteri
18	L. acetotolerans	46	L. pontis
19	L. hamsteri	47	L. vaginalis
20	L. alimentarius	48	L. suebicus
21	L. bifermentans	49	L. vaccinostercus
22	L. casei	50	L. sanfracisco
23	L. coryneformis	51	L. confusus

24	L. curvatus	52	L. fructosus
25	L. graminis	53	L. halotolerans
26	L. homohiochii	54	L. viridescens
27	L. intestinalis	55	L. kandlery
28	L. murinus	56	L. minor

(Source: Wood and Holzapfel, 1995)

2.8   Bifidobacterium spp.

The isolation of bifidobacteria was made by Tissier in the year 1899 at the Pasteur Institute in France. These organisms were isolated from infant stools and are most predominant component of normal intestinal microflora of infants and adults. Since then these organisms have been treated with a blend of endorsement and skepticism regarding the implied nutritional benefit derived from the ingestion of viable bifidobacteria or bifidogenic substances. The microbiology appears straightforward (Hughes and Hoover, 1991). Bifidobacteria are indigenous gut organisms well adapted for metabolism in the gastrointestinal tract of humans. Acetic, formic and lactic acids are produced in relatively high amount from the fermentation of sugars. The volatile fatty acids, particularly acetic acid is much more inhibitory toward gram-negative bacteria than is lactic acid (Hoover, 1993). Bifidobacteria are one of the first groups of organisms to establish in the intestinal tract and usually are the largest group represented in infants. Bottle fed babies normally have one log count less bifidobacteria present in faecal samples than breast fed babies (Braun, 1981) and bottle fed infants generally have higher levels of enterobacteriaceae, streptococci and anaerobes

other than bifidobacteria (Yuhara *et al.*, 1983). For breast fed babies, levels of 10^{10}-10^{11} bifidobacteria per gram of faeces are common (Modler *et al.*, 1990). The feces from breast-fed infants contained higher levels of acetic acid and acetate than did the faeces from bottle fed infants (Bullen *et al.*, 1977). Enteric infection in children can be prevented or minimised when their intestinal tracts contain high levels of bifidobacteria (Bullen and Willis, 1971). In Japan, successful treatment of diarrhoeal diseases in children is accomplished by feeding dairy products containing large numbers of bifidobacteria (Tojo *et al.*, 1987).

Bifidobacteria can colonise the intestinal tract. Colonisation varies depending upon the specific bacterium and their host affinity. Bacteria are in competition for colonisation space. The original resident has an advantage over transient bacteria, however, the composition of the microflora is rather dynamic. If harmful bacteria proliferate, as in the case of diarrhoeal infections, the equilibrium is disturbed and it becomes difficult even for indigenous bacteria to maintain the necessary territory to colonise the intestine (Homma, 1988; Rasic, 1989).

With new technology available for providing anaerobic conditions, bifidobacteria are now being studied more extensively and are emerging as possibly the most important group of intestinal organisms with regard to human health. Among bifidobacteria species, *B. bifidum* is suggested for use as dietary adjuncts especially in dairy foods. Table 2.3 shows the chronological order of the development of taxonomy of bifidobacteria.

Table 2.3   Chronological order of the development of taxonomy of bifidobacteria

	Stage of development	Author/reference	Year
1	*Bacillus bifidus*	Tissier	1900
2	*Bacteroides bifidus*	Castellani	1919
3	*Bacteroides bifidus*	Chalmers	1923-1934
4	*Lactobacillus bifidus*	Bergey's Manual eds	1920
5	*Bifidobacterium bifidum*	Holland	1924
6	*Bacterium bifidum*	Orla-Jensen	1927
7	*Tisser bifidia*	Lehmann and Neumann	1929
8	*Norcadia bifida*	Pribram	1931
9	*Actynomyces bifidus*	Vuillemin	1934
10	*Actinobacterium bifidum*	Nanni	1937
11	*Lactobacillus acidophilus var. bifidus*	Weiss and Rettger	1938
12	*Lactobacillus parabifidus*	Weiss and Rettger	1938
13	*Bifidobacterium bifidum*	Weiss and Rettger	1938
14	*Lactobacillus bifidus*	Prevot	1939-1957
15	*Cohnistreptothrix bifidus*	Bergey's Manual	1944
16	*Corynebacterium bifidum*	Negrovi and Fisher	1949
17	*Lactobacillus bifidus*	Olsen	1950
18	*Lactobacillus bifidus var. pennsylvanicus*	Norris et al.	1953
19	Description of human species	Dehnert	1963
20	New animal species	Reuter	1969
21	New animal species	Mitsuoka	1969
22	New animal species	Scardovi	1972
23	Creation of genus *Bifidobacterium* constituting 11 species	Holdeman and Moore	1974
24	Inclusion of 24 species to genius *Bifidobacterium*	Scardovi	1986
25	Inclusion of 29 species to genus *Bifidobacterium*	Sgorbati et al.	1995

Adapted from Sgorbati *et al.* (1995)

According to the latest collection of species in the genus of *Bifidobacterium*, additional five species have been described (Table 2.4) making the total number of species as twenty nine (Biavati and Mattarelli, 1991; Biavati *et al.* 1991).

Table 2.4   Species of genus *Bifidobacterium*

1	*B. bifidum*	16	*B. boum*
2	*B. longum*	17	*B. magnum*
3	*B. infantis*	18	*B. pullorum*
3	*B. breve*	19	*B. gallinarum*
5	*B. adolescentis*	20	*B. suis*
6	*B. angulatum*	21	*B. minimum*
7	*B. catenulatum*	22	*B. subtile*
8	*B. pseudocatenulatum*	23	*B. coryneformis*
9	*B. dentium*	24	*B. asteroids*
10	*B. globosum*	25	*B. indicum*
11	*B. pseudolongum*	26	*B. gallicum*
12	*B. cuniculi*	27	*B. ruminatium*
13	*B. choerinum*	28	*B. mericicum*
14	*B. animalis*	29	*B. saeculare*
15	*B. thermopilum*		

Adopted from Sgorbati *et al.* (1995)

2.9   *Lactobacillus casei*

Due to viability problems associated with *L. acidophilus* and bifidobacteria during storage (Anon, 1992; Shah *et al.*, 1995; Dave and Shah, 1997 a, b), the recent trend is to add *Lactobacillus casei* as an adjunct bacteria to yogurt or probiotic cultures. *L. casei* is claimed to be more stable than *L. acidophilus* or bifidobacteria (McCann *et al.*, 1996). Several yogurts and fermented milk drinks currently available on markets already contain *L. casei* in addition to *L. acidophilus* and bifidobacteria. *L. casei*, recently introduced to Australian yogurts, has shown good potential as a probiotic organism (Perdigon *et al.*, 1995; Wagner *et al.*, 1997). Table 2.5 shows various dairy products available in Australia with *L. acidophilus*, bifidobacteria and *L. casei*.

Table 2.5   Dairy products available in Australia with *L. acidophilus*, bifidobacteria and *L. casei*.

Type of culture	Company	Brand name	Type of product
L. acidophilus	Amarina Health Foods Co. Pty. Ltd.	Amarina	Yogurt
L. acidophilus	Casa Cheese	Casa	Yogurt
L. acidophilus	Kenilworth Country Foods Pty. Ltd.	Kenilworth	Yogurt
L. acidophilus	Queensco-unity Dairy Foods	Bornhoffen	Yogurt
L. acidophilus	Mundella Foods Pty. Ltd.	Mundella	Yogurt
L. acidophilus	Yoplait Australia	Yoplait yoplus	Yogurt
L. acidophilus	Attiki Pty. Ltd.	Attiki	Skim yogurt

L. acidophilus	Burra Foods	Gippsland	Organic fruit yogurt
L. acidophilus	Regal Cream Products Pty. Ltd.	Bulla lite	Fruit yogurt Reduced fat
L. acidophilus	Hastings Co-operative Limited	Hastings	Fruit yogurt Reduced fat
L. acidophilus	Nestle Australia Pty. Ltd.	LC1	Yogurt
L. acidophilus	National Dairies Limited	Yo-Split	Flavoured yogurt
L. acidophilus, Bifidum	Fonti Dairy Foods Pty. Ltd.	Fonti farm	Yogurt
L. acidophilus, Bifidum	Jalna Dairy Foods Pty. Ltd.	Jalna	Yogurt
L. acidophilus, Bifidum	Midcoast yogurt	Mid Coast	Yogurt
L. acidophilus, Bifidum	Jalna Dairy Foods Pty. Ltd.	Jalna	Biodynamic
L. acidophilus, Bifidum	Westhaven	Weasthaven	Yogurt
L. acidophilus, Bifidum	Regal Cream Products Pty. Ltd.	Bulla	Drinking yogurt
L. acidophilus, Bifidum	Jalna Dairy Foods Pty. Ltd.	Jalna	Drinking yogurt
L. acidophilus, Bifidum	Peter Farms	Peter	Yogurt
L. acidophilus, Bifidum	Nestle Australia Pty. Ltd.	Nestle	Yogurt
L. acidophilus, Bifidum	Q.U.F. Industries Ltd.	Danone	Yogurt
L. acidophilus,	Regal Cream	Bulla	Frozen yogurt

Bifidum	Products Pty. Ltd.		
L. acidophilus, Bifidum, *L. casei*	Mundella Foods Pty. Ltd.	Mundella	Yogurt
L. acidophilus, Bifidum, *L. casei*	Jalna Dairy Foods Pty. Ltd.	Jalna	Yogurt
I N A	Australian Co-operative Foods Lid	Oak	Frozen yogurt
I N A	Charioto Natural Ice cream	Charioto frozen yogurt	Frozen yogurt
I N A	Dairy Vale	Eve	Frozen yogurt
I N A	Norco Co-operative Limited	Norco	Frozen yogurt
I N A	Royal Copenhagen Ice Cream Cone Co	Royal Copenhagen	Frozen yogurt
	Seendipity ice Cream	Seendipity	Frozen yogurt
I N A	Everest	Colonial	Frozen yogurt
I N A	Dairyfields Co-operative Assoc Ltd.	That's Y	Long Life yogurt
I N A	Amarina Health Food Co Pty Ltd.	Amarina	Gourmet yogurt
I N A	Amarina Health Food Co Pty Ltd.	Amarina	Greek style yogurt
I N A	Delco Foods Products	Delco	Greek style yogurt
I N A	National Dairies Ltd.	Yo-Fresh	Greek style yogurt
L. casei Shirota	Yakult Australia Pty. Ltd.	Yakult	Fermented milk drink

L. casei	K.O.Z. World Wide	Maeil	Fermented milk drink
L. casei GG	Q.U.F Industries	Vaalia	Fermented milk drink

(Source: Australian yogurt and ice cream, Australian Dairy corporation nutritional guide 1998. I N A: information not available)

2.10 Enumeration of *S. thermophilus*, *L. delbrueckii* ssp *bulgaricus*, *L. acidophilus*, *Bifidobacterium* spp. and *L. casei*

Several media have been developed for differential enumeration of *L. acidophilus* and *Bifidobacterium* ssp. Scardovi (1986) reviewed several complex media and media containing a wide variety of antibiotics to enumerate selectively *Bifidobacterium* spp. and concluded that one selective medium is not appropriate for all species. For isolation and enumeration of bifidobacteria from aquatic environments, Munoa and Pares (1988) developed a selective medium, which consisted of reinforced clostridal agar (RCA) containing nalidixic acid, polymyxin B, kanamycin and iodoacetate. Iwana *et al.* (1993) developed galactose agar containing lithium chloride and galactose as selective agents for enumeration of *Bifidobacterium* spp. Several other selective media have been reported, including one containing an oxygen reducing membrane fraction from *Escherichia coli* (Burford, 1989), Rogosa modified selective medium and tryptone phytone yeast extract-S-agar (Samona and Robinson, 1991), X-gal-based medium (Chevalier *et al.*, 1991) and dicloxacillin-based medium (Sozzi *et al.*, 1990). Arroyo *et al.* (1994) evaluated brain heart infusion agar, modified columbia agar, RCA, modified deMan Rogosa Sharpe (MRS) agar and modified bile agar for enumeration of *B. adolescentis*, *B. infantis* and *B. longum* from pure cultures. However, these media may not be suitable for selective enumeration of *Bifidobacterium* spp. in the presence of other lactic acid bacteria or from yogurt which typically contains *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*. M17 agar, developed by Terzaghi and Sandine (1975) from lactose yeast phosphate agar, has been found to support the growth of *S. thermophilus* and suppress the

growth of *L. delbrueckii* ssp. *bulgaricus* when the pH is 6.8 (Shankar and Davies, 1977; Terzaghi and Sandine, 1975). RCA has been found to be selective for *L. delbrueckii* ssp *bulgaricus* by suppressing the growth of *S. thermophilus* when the pH of the agar was 5.5 (Johns *et al.*, 1988). There is a growing concern that some media which contain antibiotics or bile may also restrict the growth of *L. acidophilus* or bifidobacteria and that counts obtained are not necessarily representative of viable cells which are in the product .

Selective enumeration of *L. casei* in yogurt- type fermented milks containing probiotic bacteria based on 15^0C incubation temperature and 14 days incubation time was studied by Champagne *et al.* (1997). However, an incubation time of 14 days may not be practical for the dairy industry if the results are required in a short time.

Table 2.6   Therapeutic properties of probiotic bacteria.

No	Therapeutic properties	Possible causes and mechanisms
1.	Colonisation of gut and inhibition of spoilage type organisms	Survive gastric acid, resist lysozyme, tolerate high bile concentration, adhere to intestinal surface and production of inhibitory compounds i.e. acids, H_2O_2 and bacteriocins.
2.	Improved digestibility and enhanced growth	Partial breakdown of protein, fat, carbohydrates and improved bioavailability of nutrients.
3.	Lactose tolerance	Reduced lactose in the product and further availability of bacterial lactase enzymes.
4.	Hypocholesterolaemic effect	Production of inhibitors of cholesterol synthesis, deconjugation of biles and assimilation of cholesterol.
5.	Anticarcinogenic effect	Inhibition of carcinogens and enzymes involved in converting procarcinogens to carcinogens, inhibition of growth of putrefying organisms and stimulation of host immune system.
6.	Stimulation of the host immunological system	Enhancement of macrophage formation, stimulation of suppressor cells and production of interferon.
7.	Control of vaginal infections	Inhibition of fungi and bacteria responsible for the infection.
8.	Improved vitamin metabolism.	Synthesis of group B vitamins.
9.	Prevention of constipation	Improvement in bowel movement and stabilisation of ecological balance in the intestinal tract.
10.	Recovery of damaged liver, effectiveness against aspects of nausea, liver diseases, acne, etc	Reduction in concentration of metabolites and enterotoxins produced by putrefying organisms.
11.	Prolongation of life	Reduced intestinal putrefaction and auto-intoxication.

(Source: Metchnikoff, 1908; Gilliland *et al.*, 1975; Le *et al.*, 1986; Van't Veer *et al.*, 1989; Modler *et al.* 1990; Kurmann and Rasic, 1991; Huges and Hoover, 1991; Misra and Kuila, 1991; Kanbe, 1992; Mital and Garg, 1992; Ishibashi and Shimamura, 1993; Yamamoto *et al.*, 1994).

2.11 Factors affecting the viability of probiotic bacteria in fermented frozen dairy desserts

Probiotic foods containing live microorganisms with beneficial effects on human health have been produced for many years. *Lactobacillus acidophilus* and *Bifidobacterium* spp. are helpful in improving intestinal problems including bowel movements. These organisms can colonise the intestinal tract and offer antagonism against various pathogenic and spoilage organisms. Lactose intolerant people can exploit the lactase enzyme of the organisms, which could benefit in reducing lactose intolerance problem. Some workers have studied the effect of administering *L. acidophilus* and *B. bifidum* to children suffering from leukaemia and children with obstructive jaundice.

The major factors affecting the healthy intestinal microflora of humans are diet, drugs, stress and ageing. As a result, use of probiotics or feeding of desirable types of bacteria to re-establish or maintain a healthy balance of gut microorganisms has gained considerable interest over the last several years (Hull *et al.*, 1992). *B. bifidum* accounts for about 40% of the faecal flora in the large intestine of adults, therefore, the consumption of the minimal culture plays a beneficial role in maintaining a balanced intestinal flora (Rasic and Kurmann, 1978). *L. acidophilus* and *Bifidobacterium* spp. have shown to correct intestinal disorders, lower serum cholesterol, posses anti-carcinogenic properties and improve lactose utilisation in lactose intolerant people (Gilliland, 1989; Shah and Jelen, 1990; Shah, 1993; Shah, 1994). These organisms are inhibitory towards Gram-negative bacteria (Hekmat and McMahon, 1992).

Poor survival of probiotic organisms in fermented frozen product is possibly due to pH and acidity, freeze injury and due to incorporation of air. *L. acidophilus* and *B. bifidum* prefer to grow at 37°C and at a pH of 6.5 to 7.0 (Scardovi, 1986). Gram positive bacteria are generally less susceptible to freeze injury than Gram negative bacteria (Speck and Ray, 1977). *B. bifidum* is not as acid tolerant as *L. acidophilus* and the growth of this organisms is affected below pH. 5.0. Another factor affecting the viability of *L. acidophilus* and *B. bifidum* is the incorporation of air due to whipping air into the product during freezing. As *B. bifidum* is anaerobic while *L. acidophilus* is microaerophilic, the viability of *B. bifidum* is expected to be reduced more rapidly than that of *L. acidophilus*.

Survival of *L. acidophilus* and *B. bifidum* in ice cream for use as a probiotic food was studied by Hekmat and McMahon (1992). They have demonstrated that the beneficial microorganisms such as *L. acidophilus* and *B. bifidum* can be grown to high numbers in ice cream mix and the organisms remained viable during frozen storage. Studies by Gilliland and Speck (1977), Schioppa *et al.* (1981), and Hull *et al.* (1984) have shown that *L. acidophilus* and bifidobacteria are unstable in fermented dairy products. *L. acidophilus*, when added to set yogurt after its manufacture, rapidly lost viability (90-99%) within three to five days and completely disappeared within seven days. When the organisms were added with yogurt cultures organisms during yogurt manufacture, loss of viability still occurred but the organisms survived better. In several studies (Anon, 1992; Shah *et al.*, 1999) several brands of commercial yogurts and fermented milk drinks from Australian supermarkets were analysed for the presence of *L. acidophilus*

and *B. bifidum*. All the products contained very low numbers of *L. acidophilus* and bifidobacteria.

Several approaches have been taken to improve the viability of probiotic bacteria including selection of various strains on the basis of pH, effect of sugar, and freezing, use of various cryoprotectans, using acid casein hydrolysate and cysteine, encapsulating the bacteria and using acid and low temperature adaptations.

2.11.1 *Effect of sugar*

High concentrations of sugar or total solids added to milk before fermentation with yogurt starters may result in conditions inhibitory to bacterial growth, leading to long fermentation times and poor acidity development (Tramer, 1973; Zourari *et al.*, 1992). This is due to both adverse osmotic effects of the solutes in milk and low a_w (Larsen and Anon, 1989, 1990; Zourari *et al.*, 1992). Influence of water activity on the fermentation of yogurt made with extracellular polysaccharide producing or non-producing starters were studied. The authors reveal that glycerol reduced a_w of the milk considerably, but it was less inhibitory to acid production than sucrose. The ropy strains did not show any advantage over non-ropy ones when they were used to produce yogurt in milk with reduced a_w their performance was similar to non-producer strains in terms of lactic acid production.

Effect of water activity of milk on acid production by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* was studied by Larsen and Anon (1989). The rate of acid production by *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* were measured at temperature between 30^0C and 50^0C in milks, which had been adjusted from 0.992 to 0.943 a_w with glycerol. Optimal temperatures of acid production were unaffected by change of a_w. Minimum a_w for acid production was always lower when a_w of milk was adjusted with glucose or sucrose than with glycerol. Media adjusted with sucrose generally were more inhibitory than those in which glycerol was the humectant. Titratable acidity although not related to the type of humectant, did depend on a_w of the medium and was directly related to the extent of growth. Acid production appeared to be related to the presence and extent of growth at all of a_w levels studied.

2.11.2 *Freeze injury*

Freezing is one of the main end points in manufacturing of fermented frozen dairy desserts. Intracellular ice formation during freezing has been described for many cellular systems, and the rate at which ice is formed determines the type of freezing damage suffered by cells (Mazur, 1970; Toner *et al.*, 1993). Cells can be injured during freezing by physical factors such as ice crystal formation and dehydration. At high freezing rates, intracellular freezing occurs, leading to cell damage mainly by ice crystal formation. However, at low freezing rates, extracellular ice formation predominates, leading to intracellular dehydration (Mazur, 1970).

2.11.3 pH and acidity

As the bacteria ferment lactose to lactic acid, the buffering-capacity of the growth medium is eventually overcome and the pH drops until acidic conditions become unfavourable for the bacteria and acid production stops usually around pH 4.6-4.7 (Thunell *et al.*, 1984). According to Harvey (1960) starter growth in an environment below pH 5.0 results in reduced enzyme activity. If bacteria remain at low pH for extended periods of time, a steady decrease in acid producing activity results and increase amounts of starter must be used to form comparable amounts of lactic acid over a given period of time (Thunell *et al.*, 1984). The survival of microorganisms is effected by low pH of the environment (Shah and Jelen, 1990). The populations of *L. acidophilus* decreased rapidly at pH 2.0; however, there was no decrease in the number of viable cells at pH 4.0 (Hood and Zottola, 1988). *S. thermophilus* is not as acid tolerant as *L. delbruekii* ssp. *bulgaricus*.

The major inhibitory substances produced by yogurt bacteria are claimed to be acid and hydrogen peroxide (Lankaputhra and Shah, 1994; Shah *et al.*, 1995). The pH of yogurt may decrease to as low as 3.7 during storage (Kolars *et al.*, 1984; Shah, 1994; Shah *et al.*, 1995), which may be detrimental to bifidobacteria as the growth of bifidobacteria is significantly retarded below pH 5.0 (Lankaputhra and Shah, 1995). Similarly hydrogen peroxide produced by yogurt organisms, especially by *L. delbruekii* ssp. *bulgaricus*, may affect the viability of bifidobacteria and the presence of hydrogen peroxide in acidic conditions, such

as found in fermented dairy products, may cause synergistic inhibition of bifidobacteria.

Survival of bifidobacteria during refrigerated storage in the presence of acid and hydrogen peroxide was studied by Lankaputra *et al.* (1996 a, b). The authors concluded that the viability of 6 strains of bifidobacteria (*B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 and *B. thermophilum* 20210) decreased with pH. The presence of acid and hydrogen peroxide resulted in further decrease in the viable counts of these 6 strains of bifidobacteria, indicating a synergistic inhibition of bifidobacteria in the presence of acid and hydrogen peroxide. The remaining 3 strains of bifidobacteria (*B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099) survived well in the presence of acid and hydrogen peroxide.

The effect of pH on survival of *L. acidophilus* and *B. bifidum* in frozen fermented dairy desserts was studied by Laroia and Martin (1991). *L. acidophilus* and *B. bifidum* were incorporated into frozen fermented dairy desserts and their survival during frozen storage was determined before and just after freezing and at 2 weeks interval during 8 week storage at -29°C. *L. acidophilus* and *B. bifidum* survived the freezing process and frozen storage in pH 5.6-5.8 in frozen fermented dairy products for 8 weeks. *B. bifidum* did not survive well in the fermented product at pH 3.9-4.6.

Frozen yogurt can have a widely variable level of acidity depending on manufacturers. The soft and hard frozen yogurts were observed to have mean acidities of 0.25–0.32%, respectively. These acidities are considerably higher than ice-cream mix and are about one–quarter of the acidity normally associated with fresh yogurts.

The viability of *L. acidophilus* and *B. bifidum* in soft-serve frozen yogurt was studied by Holcomb *et al.* (1991) to determine if frozen yogurt would be an appropriate vehicle for these two organisms. Bile and acid tolerance of the two organisms, both before and after freezing in yogurt mix was determined. These organisms were able to survive and grow after freezing and both were found to grow in up to 0.45% bile salts before and after freezing. The two organisms were able to survive and grow at pH 5.4 before and after freezing in yogurt mix. However, the authors found only *L. acidophilus* to be able to survive and grow after exposure to 0.01N HCl for 2 h at 37°C.

2.12 Approaches taken to improve the viability

2.12.1 *Use of cryoprotective agents in improving the viability of probiotic bacteria*

Bacteria differ in their susceptibility to freezing and thawing. Cryoprotective agents provide protection against freeze-injury. Cellular damage is most likely due to elevated solute concentration (which causes cell dehydration) and membrane destruction by intracellular ice crystals. The addition of cryoprotectants results in greater survival rate during freezing and frozen storage by binding water and inhibiting either intracellular or extracellular ice crystal formation. The presence of most cryoprotectants results in formation of extracellular amorphous ice glass instead of crystalline ice upon freezing. These agents may include sorbitol, sucrose, sodium tripolyphosphate, glucose, xylitol, glycerol, raffinose and maltodextrin (Thunnell *et al.*, 1984).

The effect of various cryoprotectants on the viability of freeze-dried lactic acid bacteria has been studied by Valdez *et al.* (1985). Adonitol produced the smallest changes in water content at various times during drying and allowed the highest rate of survival. Use of β-glycerophosphate as a cryoprotective agent gave greatest protection to *L. acidophilus*.

Kilara *et al.* (1976) reported that the extent of cryoprotection provided by any given agent varied from culture to culture. Malt extract and milk solids as the cryoprotective agents resulted in the survival ratio of 58 and 80% for *L. delbrueckii* ssp. *bulgaricus*, respectively, while with *L. acidophilus* the same two

cryoprotectants provided 89% and 48% survival. Whey powder improved viability of *L. delbrueckii* ssp. *bulgaricus* and pectin that of *S. thermophilus*. For improved viability of probiotic and yogurt organisms, a combination of cryopotectants may be required.

2.12.2 *Use of microencapsulation*

Microencapsulation is a process where the cells are retained within the encapsulating membrane in order to reduce the cell injury or cell death. Microencapsulation tends to stabilise cells, potentially enhancing viability and stability in the production, storage and handling of lactic cultures (Kim *et al.,* 1988). In some food formulations, immobilised cells are more active than free cells (Kearney *et al.,* 1990). Microencapsulation of lactic starter cultures is of interest to the dairy industry since it improves the control of the fermentation process (Champagne and Cote, 1987; Champagne, 1990).

Two-phase dispersion process for the production of biopolymer gel beads, the effect of various parameters on bead size and their distribution was studied by Audet and Lacroix (1989). The authors concluded that the two-phase dispersion process could be effective in large-scale production of biocatalysts with high mechanical resistance and high productivity when used in fermentation process.

Covalent stabilisation of alginate gel for the entrapment of living whole cells was studied by Staffan *et al.* (1981) employing three methods. In the first method performed, alginate beads containing entrapped cells were treated with

polyethyleneimine followed by glutaraldehyde. In the second method alginate sol was treated with carbodiimide and n-hydroxysuccinimide and mixed with cells and extruded into calcium chloride solution. In the third method the alginate was treated with periodate. The beads were subsequently cross-linked with polyethyleneimine.

Lactococcus lactis released from calcium alginate beads was studied by Champagne *et al.* (1988). The authors concluded that ethanol treatment or heating of beads appears to be one of the most promising methods for maintaining acidification activity while minimising viable cell release due to loosely entrapped cells near the surface of the alginate beads.

Improving survival of culture bacteria in frozen desserts by microentrapment was studied by Sheu *et al.* (1993). The authors concluded that the death of yogurt bacteria in frozen desserts reduces the potential to produce health benefits. Freezing with agitation kills a large portion of added lactobacilli. Loss of viable cells was markedly decreased by microentrapment of cells in 3% calcium alginate beads. Addition of 6% glycerol further enhanced the viability of entrapped lactobacilli and addition of glycerol did not affect overrun, texture, or body characteristics of frozen ice milk. Therefore, addition of similar entrapped yogurt bacteria to frozen dairy desserts would be advantageous to provide desirable marketing and health benefits.

No reduction in cell umber was observed when lactic cultures were immobilised within alginate beads (Champagne and Cote, 1987; Kolot, 1988). Immobilisation prevents the cultures from phage contamination (Champagne *et al.*, 1988; Steenson *et al.*, 1987). Immobilisation improves culture stability (Champagne and Boyaval, 1986). Lactic cultures have been previously immobilised by k-carrageenan and locust bean gum beads. The advantage of immobilisation using natural polymers is that the reagents are non-toxic and the procedures are gentle to the microorganisms (Lacroix *et al.*, 1990).

Lactococcus lactis ssp. *cremoris* was microencapsulated within alginate(alg)/poly-L-lysine (PLL), nylon or crosselinked polyethyleneimine (PEI) membranes. Toxic effects were observed with solvents and reagents used in nylon and PEI membrane formation. Alg/PLL encapsulation resulted in viable and active cell preparations, which acidified milk at a rate proportional to the cell concentration, but at rates less than that of free cell preparations. At 4×10^8 cfu per ml milk, encapsulated cells took 17% longer incubation time than free lactocci to reduce the pH of milk to 5.5. Similar activities of free and microencapsulated cells may be attained at higher cell concentrations. The authors observed the rate of lactic acid production of approximately 2 Mmol/h using an encapsulated cell concentration of 4×10^8 cfu/ ml (Larisch *et al.*, 1994).

Viability and acidification activity of *Lactococcus lactis* ssp. *lactis* was demonstrated by Hyndman *et al.* (1993) by immobilisation within crosslinked gelatine microcapsules. Contact with the cross linking agent, toluene

diisocyanate, was minimised by a reduction in the reaction agent concentration to 34 mol dm^{-3} during capsule production and by minimising the surface to volume ratio of the microcapsules. A low viscosity oil (silicone) used in the dispersion step during microcapsule formation reduced the surface to volume ratio by 45%, yielding a 44% increase in activity over capsules produced in higher viscosity oil. After encapsulation, activity and viability of the immobilised culture was increased by cell growth within the capsules.

2.12.3 *Use of micronutrients*

Probiotic bacteria grow slowly in milk due to lack of proteolytic activity. Dave and Shah (1997a, b; 1998) and Klaver (1993) have shown that milk supplemented with peptides and amino acids such as cysteine improved the survival of bifidobacteria. Cysteine is a sulfur containing amino acid that is incorporated into agar media for the growth of bifidobacteria (Laroia and Martin 1991). Cysteine is also known for lowering redox potential and addition of this amino acid could improve viability of anaerobic bifidobacteria. Similarly acid casein hydrolysate is a product that is made from hydrolysis of casein and is rich in amino acids and peptides. Thus, acid casein hydrolysate and cysteine may provide growth factors essential for the growth of probiotic bacteria and could improve their viability.

2.12.4 *Acid and low temperature adaptations*

Acid and low temperature adaptation is a novel method where the cells are exposed to a particular mild acidic environment followed by exposure to sub-lethal acidic environment and frozen for various time and temperature combinations in order to determine whether any proteins are released during freezing. These proteins help the organisms cope under stressful conditions. While the physiology, biochemical and genetic mechanisms of many of these responses have not been delineated, what is clear is the following. When exposed to a mild dose of stress, microorganisms may adapt to the stress, thus developing tolerance or resistance to stronger doses of stress. Also it is becoming increasingly clear that the exposure and subsequent adaptation to one stress can confer resistance to other different stress. Most microorganisms must accommodate to a variety of changing conditions and stress in their environment in order to survive. Because of the impact of temperature on all reactions, cell adaptations to fluctuations in temperature are possibly the most common. In many organisms specific sets of cold shock proteins are induced upon abrupt shifts to colder temperatures. While this cold shock response has not been fully delineated, it appears to be adaptive and may function to promote the expression of genes involved in translation when cells are displaced to low temperatures. The cold shock response of *Escherichia coli* has been extensively studied and the major cold shock protein CspA appears to be involved in the regulation of response. Upon cold shock, the induction of CspA and its counterparts in most microorganisms studied is prominent, but transitory. Studies of this response in some psychotropic bacteria have reported constitutive synthesis and continued

synthesis during cold temperature growth of CspA homologous (Berry and Foegeding, 1997).

Freezing is one of the main factors in manufacturing of fermented frozen dairy desserts. Intracellular ice formation during freezing has been described for many cellular systems, and the rate at which ice is formed determines the type of freezing damage suffered by cells (Mazur, 1990; Toner *et al.*, 1993). Cells can be injured during freezing by physical factors such as ice crystal formation and dehydration. At high freezing rates, intracellular freezing occurs, leading to cell damage mainly by ice crystal formation. However, at low freezing rates, extracellular ice formation predominates, leading to intracellular dehydration (Mazur, 1990). The pretreatment of cells with a mild stress induces higher stress tolerance and in some cases this confers cross protection to other, different types of stress (Mager and Ferreira, 1993). It has been assumed that different stress conditions act through a variety of effects in cells, such as generation of abnormal or denatured proteins, internal acidification, alterations in the cytoskeleton or modulation of second-messenger levels, to produce signals that can provide coverage and stimulate more general stress-responding system (Chowdhury *et al.* 1992; Coote *et al.*, 1991; Craig and Gross 1991). The freeze-thaw stress response of yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the Ras-cyclic AMP signal transduction pathway as studied by Jong *et al.* (1997). The authors concluded that the ability of cells to survive freezing and thawing is expected to depend on the physiological conditions experienced prior to freezing. They examined factors affecting yeast cell survival

during a freeze thaw stress, including those associated with growth phase, requirement for mitochondrial functions and prior stress treatment. Supercooling occurred without reducing cell survival and was followed by freezing. Loss of viability was proportional to the freezing duration, indicating that freezing was the main determinant of freeze-thaw damage.

The initial discovery of this response was made by Jones *et al.* (1987) in *E. coli* growing at 37^0C followed by a reduction in temperature to 10^0C. The growth was halted for approximately 4 hours before exponential growth was resumed (Jones *et al.* 1987; Ng *et al.*, 1962). During this lag period, there is an induction of some 16 proteins, called the cold shock proteins (Jones *et al.* 1996; La Teana *et al.*, 1991) Lelivelt and Kawula (1995) and Jones *et al.* (1992) further examined the relationship of stringent control and the cold shock response and found that the induction of the cold shock response is negatively regulated by (p) ppGpp (guanosine 5'-triphosphate 3'-diphosphate and guanosine 5'-diphosphate 3'-diphosphate). The induction of the stringent response, and therefore increased (p)ppGpp, in wild-type *E. coli* prior to a temperature downshift resulted in decreased synthesis of many transcriptional and translational proteins decreased induction of many of the cold shock proteins. When a real spot mutant, which cannot synthesize (p)ppGpp, was shifted from 24^0C to 10^0C, increased synthesis of transcriptional and translational proteins and greater induction of the cold shock proteins was observed. In *E. coli* following a shift to 10^0C cold shock proteins are transiently synthesised at rates 2 to 10 fold greater than rates at 37^0C (Jones *et al.*, 1987). However one cold shock protein CspA is more prominently

induced and because of this it has been designated the major cold shock protein (Goldstein *et al.*, 1990). Cold shock responses and cold shock proteins have been demonstrated in many other organisms, including psychrotrophic and psychrophilic microorganisms. Cloutier *et al.* (1992) compared the cold and heat shock protein synthesis of three psychrotrophic and three mesophilic strains of rhizobia. In their examination of cold shock protein synthesis the rhizobia were shocked from their respective optimal temperature to various cold temperatures according to their individual thermo-adaptation characteristics, such that the temperature ranges of the cold shocks were equivalent for different isolates. These workers found that both psychrotrophic and mesophilic rhizobia produced similar numbers of cold shock proteins, thus concluding different thermo-adaption levels were not reflected in the absolute number of cold shock proteins. Julseth and Inniss (1990) examined the cold shock response of the psychrotrophic yeast *Trichosporon pullulans* and found that 26 cold shock proteins were induced upon a shift from 21 to 5^0C. Only 6 of the 26 cold shock proteins were induced upon a shift from 15 to 5^0C; these data are in line with observations in *E. coli* (Jones *et al.*, 1992). The magnitude of the induction is dependent upon the range of the temperature shift ie the greater the range of the temperature shift, the more pronounced the induction. Some cold adapted bacteria have been shown to produce both cold shock proteins and cold acclamation proteins (Berger *et al.*, 1996; Roberts and Inniss, 1992). The results observed in yeast *T. pullulans* showed that the greater number of cold shock proteins were produced by a greater temperature shift. The induction of nine, seven and five cold shock

proteins was observed upon cold shocks from 20 to 0, 5 and 10^0C (Julseth and Inniss, 1990).

Acid tolerance in *Listeria monocytogens* such as the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance was studied by Davis *et al.* (1996). *L. monocytogenes* acquired increased acid tolerance during exponential growth upon exposure to sublethal acid stress. Maximum acid resistance was seen when the organism was exposed to pH 5.0 for 1 hour prior to the challenge at pH 3.0, although intermediate levels of protection were afforded by exposure to pH values ranging from 4.0 to 6.0. One hour adaptive period was required for the development of maximal acid tolerance; during this period the level of acid tolerance increased gradually. Full expression of ATR required de novo protein synthesis. Chloramphenicol, a protein synthesis inhibitor, prevented full induction of acid tolerance. *L. monocytogenes* also developed increased acid resistance upon entry into the stationary phase; this response appeared to be independent of the pH dependent ATR seen during exponential growth.

Acid adapted survival of *Bifidobacterium breve* against environmental stress was studied by Lee *et al.* (1995). The authors concluded that acid adapted cells survived better than non-adapted cells. Acid tolerance in *Leuconostoc oenos* and isolation and characterisation of acid-resistance mutant was studied by Drici *et al.* (1996) and found that the acid adapted cells survived better than non-adapted cells. Acid adaptation of *L. monocytogenes* and their survival in acidic foods and during milk fermentation was studied by Gahn *et al.* (1996). The authors

concluded that the ATR mechanism whether constitutive or induced could greatly influence the survival of *L. monocytogenes* in low pH food environment.

Adaptive acidification tolerance response of *Salmonella typhimurium* was studied by John and Holly (1990). The authors concluded that logarithmically grown cells (pH 7.6) shifted to mild acid (pH 5.8) for one doubling as an adaptive procedure were 100 to 1000 times more resistant to subsequent strong acid challenge (pH 3.3) than were unadapted cells shifted directly from pH 7.6 to 3.3. This acidification tolerance response required protein synthesis and appears to be a specific defence mechanism for acid. No cross protection was observed for hydrogen peroxide, or heat shock. The $Mg2++$-dependent proton-translocating ATPase was also found to play an important role in acid tolerance.

Most of the dairy products in Japan, United States and Europe contain a mixed culture of bifidobacteria and *L. acidophilus*. In spite of all the commercial products available, viability of probiotic microorganisms is still a major problem. Hence, development of fermented frozen dairy dessert with high numbers of viable *L. acidophilus* and bifidobacteria is needed in order to enhance the export potential of the product because of improved viability and shelf life.

3.0 MATERIALS AND METHOD

3.1 Sources of chemical, media and equipment

3.1.1 *Chemicals and media*

All chemicals were obtained from BDH Chemicals Company (Corporate Avenue, Rowville, Australia) or from Sigma Chemicals Company (Castle Hill, New South Wales, Australia).

3.2 EQUIPMENT

3.2.1 *Pasteuriser*

A pasteuriser (APV) was used in pasterurising milk for yogurt manufacture (95^0C for 15 min) and for ice cream base mix (73^0C for 15 sec).

3.2.2 *Ice cream freezer*

A continuous ice cream freezer (Crepaco Freezer, Model KM 240; USA) was used to make pilot scale (1000 L) fermented frozen dairy desserts with 100% overrun.

A bench top ice cream freezer (2 L size) (Kenwood Ice Cream Freezer (Model Im 300 PK 185/w, Kenwood Appliances, Italy) was used to make laboratory scale fermented frozen dairy desserts with 18% overrun.

3.2.3 Laminar flow

A laminar airflow (Gelman Sciences, HWS series Vic. Australia) was used as work-station for plating and inoculating culture bacteria.

3.2.4 Anaerobic jar

Anaerobic jars were used to enumerate *L. delbrueckii* ssp *bulgaricus, L. acidophilus*, bifidobacteria and *L. casei*. Anarobic jars having a capacity of sixteen and forty-eight plates were used. To create anaerobic conditions catalysts and gas generating kit (BR 38) were used according to the manufacturer instructions.

3.2.5 Centrifuge and microcentrifuge

Large volumes (100 to 1000 mL) of samples were centrifuged using Beckmann refrigerated J2-HS (Beckmann Instruments Inc., Palo Alto, CA, USA). For medium volumes (10 to 100 mL) of samples Bekmann refrigerated centrifuge and for small volumes microcentrifuge (Beckmann) were used.

3.2.6 Freeze drier and freeze drying of bacteria

A freeze drier was used for freeze drying microencapsulated probiotic bacteria for incorporation into fermented frozen dairy desserts. Microencapsulated probiotic bacteria was placed in petridishes with lids and frozen at −25°C for 24 h using a freeze drier (Dynavac Engineering Pty. Ltd. Inc., Melbourne, Australia). The freeze dried microencapsulated probiotic bacteria were kept in the same petridishes and sealed with parafilm and stored in a freezer at -18°C until used.

3.2.7 *Compound microscope and examination of beads*

Microencapsulated calcium alginate beads were stained with safranin and placed on a glass slide. Diameters of calcium alginate beads were measured with an eyepiece micrometer using an optical microscope (CHT, Olympus Optical Co. Ltd., Japan) at a magnification of 100. At least 80 randomly selected beads were measured for each sample.

3.2.8 *Scanning electron microscope and scanning electron microscopy*

Scanning electron microscopy was employed to examine the external and internal appearance of calcium alginate beads. The beads were freeze-dried using a freeze drier. For external structure intact beads were attached to the aluminium stubs using conductive carbon paint (CCP) (Probing and Structure, now Pro Science Tech., Melbourne). For internal structure the freeze-dried beads were mechanically broken using fine tweezers and mounted using CCP as described above.

The specimens were coated with 10 μm gold using Edwards Sputter Coater. External and internal structures were observed using scanning electron microscopy (SEM) (Philip 500 at 20 kv, Holland).

3.3 Bacterial culture and maintenance

3.3.1 *Lactic acid bacteria and probiotic bacteria*

Pure strains (yogurt and probiotic organisms) were collected from the Division of Food Science and Technology, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Highett, Victoria, Australia; Mauri Laboratory (now, Gist-brocades) and Chr. Hansen. The organisms were maintained at –20°C in 12% reconstituted skim milk (RSM) supplemented with 1.0% glucose and 0.5% yeast extract. L-cysteine hydrochloride (0.05%) was added for the maintenance of bifidobacteria. Working cultures were propagated using frozen stock cultures. Sterile RSM (10 mL) portions were propagated by inoculating 1% of each culture and incubated at 37°C for 18 h. All cultures were activated by propagating into sterile RSM on a weekly basis for a maximum of twenty propagations. After which new working cultures were propagated using frozen stock culture. This was done to minimize the loss of any plasmid associated inhibitory activities.

3.4 Microbiological analyses

S. thermophilus was enumerated on *S. thermophilus* agar (ST agar; 10.0 g of tryptone, 10.0 g of sucrose, 5.0 g of yeast extract and 2.0 g K_2HPO_4 in 1 L of distilled water) by incubating the plates aerobically at 37^0C for 24 ± 3h (Dave and Shah, 1996). Counts of *L. delbrueckii* ssp. *bulgaricus* were determined on MRS agar (Oxoid, Basingstoke, Hampshire, UK) adjusted to pH 5.2 and incubated anaerobically at 43^0C for 72h. MRS-salicin and MRS-sorbitol agars were used for enumeration of *L. acidophilus* (Dave and Shah, 1996; Lankaputra

et al., 1996a, b). Counts of bifidobacteria were estimated on MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, paromomycin sulfate) agar (Laroia and Martin, 1991). The enumeration was carried out using the pour plate technique. Plates containing 25 to 250 colonies were enumerated and recorded as colony forming (cfu) units per gram of culture. All the experiments and analyses were repeated at least twice. The results presented are averages of two replicates.

3.5 Commercial products used for enumeration of *L. casei*

Nine samples of commercial yogurts and fermented milk drinks claimed to contain *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, bifidobacteria and/or *L. casei* were analyzed to validate the selective medium (LC agar) in composite culture conditions. The products analyzed were: Mundella (Mundella Foods Private Limited, Western Australia, Australia) claimed to contain *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, bifidobacteria and *L. casei*; Jalna skim milk yogurt and Jalna mild continental yogurt (Jalna Dairy Foods Private Limited, Thomastown, Australia) claimed to contain *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, bifidobacteria and *L. casei*; Yoplait yoplus natural yogurt (National Foods Limited, Morewell, Australia) claimed to contain *S. thermophilus*, *L. acidophilus*, and bifidobacteria; Nestle LC1 (Nestle (Australia) Private Limited, Mulgrave, Australia) claimed to contain *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, and *L. acidophilus*; Yakult (Yakult (Australia) Limited, Dandenong, Australia) claimed to contain *L. casei Shirota* strain; Maeil (imported by KOZ Worldwide, Queensland, Australia) claimed to contain *L. casei*; Bulla drinking yogurt (Regal

Cream Products Private Limited, North Melbourne, Australia) claimed to contain

S. thermophilus, L. delbrueckii ssp. *bulgaricus, L. acidophilus* and bifidobacteria;

Vaalia (QUF Industries Limited, South Brisbane, Australia) claimed to contain *S.*

thermophilus, L. delbrueckii ssp. *bulgaricus, L. acidophilus,* bifidobacteria and *L.*

casei ssp. *GG.* All experiments and analyses were carried out in duplicate. The

results presented are averages of duplicate experiments.

3.6   Screening of strains of yogurt and probiotic bacteria for selecting strains that survive freezing

The organisms were propagated three times before using for freeze tolerance

studies. Ten millilitre of sterile RSM was inoculated with 2% culture followed by

incubation at 37^0C till the pH 4.5 was attained. The samples were transferred to a

refrigerator till the temperature dropped to 4^0C followed by freezing for 4 h at -

25^0C using a glycol bath. The temperature dropped to -25^0C in 15 min. Samples

were thawed using tap water (~20^0C). Bacterial counts before and after freezing

were determined. The organisms resistant to freeze injury were used for making

fermented frozen dairy desserts and the viable counts of yogurt and probiotic

organisms were monitored at weekly intervals for 12 weeks.

3.7   Analysis of samples to determine viability of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus, bifidobacteria and/or L. casei* in commercial fermented dairy products

Twenty-six commercial yogurts and fermented milk drinks claimed to contain *S.*

thermophilus, L. delbrueckii ssp. *bulgaricus, L. acidophilus,* bifidobacteria and/or

L. casei were analysed for their population levels. The products that were

analysed included Mundella containing *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus*, bifidobacteria and *L. casei*. (Mundella Foods Private Limited, Western Australia, Australia); Jalna skim milk yogurt and Jalna mild continental yogurt containing *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus*, bifidobacteria and *L. casei*. (Jalna Dairy Foods Private Limited, Thomastown, Australia); Yoplait yoplus, natural yogurt containing *S. thermophilus, L. acidophilus*, and bifidobacteria (National Foods Limited, Morewell, Australia); Nestle LC1 containing *S. thermophilus, L. delbrueckii* ssp. *bulgaricus,* and *L. acidophilus* (Nestle (Australia) Private Limited, Mulgrave, Australia); Yakult containing *L. casei* Shirota strain (Yakult (Australia) Limited, Dandenong, Australia); Maeil containing *L. casei* (imported by KOZ Worldwide, Queensland, Australia); Bulla drinking yogurt containing *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and bifidobacteria (Regal Cream Products Private Limited, North Melbourne, Australia). Vaalia, containing *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus*, bifidobacteria and *L. casei GG* (QUF Industries Limited, South Brisbane, Australia). All experiments and analyses were carried out in duplicate. The results presented are averages of duplicate experiments.

3.8 Effects of various sugar levels on survival of probiotic bacteria.

Ten millilitre of RSM containing 8 and 16% sucrose were inoculated with 2% each of *L. acidophilus* and bifidobacteria and incubated at 37^0C till the pH of 4.5 was attained. Samples were transferred to a refrigerator till the temperature dropped to 4^0C and then frozen at -25^0C for 4 h using a glycol bath. The

temperature dropped to -25⁰C in 18 min. Samples were thawed using tap water (~20⁰C). Bacterial counts before and after freezing were determined as before.

3.9 Effects of acidic and freezing conditions on survival of yogurt and probiotic bacteria.

Samples of RSM were adjusted to pH 6.2 (control), 5.0, 4.5 and 4.0 with lactic acid (BDH Laboratory Supplies, Poole, England). One millilitre of each culture of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus,* and *Bifidobacterium* spp. was added to 9 mL of the pH adjusted RSM in separate test tubes. The test tubes were kept in a refrigerator at 4⁰C for 5 h followed by freezing at -25⁰C for 4 h using a glycol bath. The temperature dropped to -25⁰C in 15 min. Samples were thawed using tap water (~20⁰C). Bacterial counts before and after freezing were determined as before.

3.10 Influence of water activity on fermentation of probiotic yogurt and on viability of yogurt and probiotic bacteria

Five batches of yogurt (1 L) batch size were made using 12% RSM containing 0, 4, 8, 12 and 16% sugar levels. Yogurt made without added sugar was used as control (batch 1). The mix was heated at 85⁰C for 30 min, cooled to 42⁰C and yogurt bacteria (*S. thermophilus* ST WJ7 and *L. delbrueckii* ssp. *bulgaricus* LB WJ7) and probiotic bacteria (*L. acidophilus* LA 2415 and bifidobacteria BB 1941) were added at the rate 0.5% of each, mixed for 5 min using a magnetic stirrer and filled in 100 mL plastic containers and incubated at 42⁰C till the pH 4.5 was attained. The yogurt was transferred to a walk-in-cooler and kept over night at 4⁰C.

3.11 Survival of yogurt and probiotic bacteria in fermented frozen dairy desserts made using a bench top ice cream freezer

Six different batches (B1-6) of fermented frozen dairy desserts were made as follows:

For batch B1, yogurt was made by using ST 2010, LB 2501, LA 2415 and BB 20099. Similarly, B2 was made with ST 2008, LB 2515, LA 2401, BB 1912 and B3 with ST/LB Wj7, MjLA1, BDBB2. B4, B5 and B6 were made with ST 2010, 2008, ST WJ7 and LB 2501, 2515, LB WJ7 only and probiotic strains LA 2415, 2401, MJLA1, BB 20099, 1912, BDBB2 were incorporated just before freezing. Yogurt and base mix were added at a ratio of 45:45 and 10% of liquid sucrose syrup (65%) was added, followed by freezing the contents using a bench top Kenwood ice chef ice cream freezer (Model Im 300 PK 185/w, Kenwood Appliances, Italy). The pH of the final product ranged between 4.51 and 4.59.

3.12 Survival of yogurt and probiotic bacteria in fermented frozen dairy desserts made using a continuous freezer

Two different batches of fermented frozen dairy desserts each of 1000 L were made on a pilot scale using an ice cream freezer. For batch 1, yogurt was made by using ST 2008, LB 2501, LA 2401, and BB 20099. Similarly, batch 2 was made with ST/LB Wj7, MjLa1, and BDBB2. Yogurt and base mix were added at a ratio of 45:45 and 10% of sucrose syrup (65%) was added, followed by freezing the contents using a continuous ice cream freezer (Crepaco Freezer, Model KM 240, U.S.A). The pH of the final product ranged between 4.51 and 4.59.

3.13 Use of acid casein hydrolysate and cysteine in manufacture of fermented frozen dairy desserts

This study investigated the use of acid casein hydrolysate and cysteine in improving the viability of *L. acidophilus* and *B. bifidum* noting that *Bifidobacterium* spp is a strict anaerobe and *L. acidophilus* is considered to be microaerophilic, although the latter organism grows well under anaerobic conditions. Effect of cysteine as an oxygen scavenging agent was investigated. L-Cysteine has been found to be essential for the growth of bifidobacteria (Laroia and Martin, 1991).

Fermented frozen dairy desserts were made according to the method of Ravula and Shah (1998b). Yogurt and base mix were prepared separately and blended at a rate of 45% of each and 10% sucrose syrup was added. The blend was then frozen using a bench top ice cream freezer (Model Im 300 PK 185/w, Kenwood Appliances, Italy).

Three batches of yogurt (3L) were made with homogenised and pasteurised milk (1% fat) supplemented with 2% skim milk powder. The mix was heated at 85^0C for 30 min, cooled to 42^0C and divided into three lots. Acid casein hydrolysate (0.5g/L) (Sigma Chemical Co. St. Louis, MO) and L-cysteine (0.05g/L) (Sigma) were added to the two batches of heated and cooled yogurt mix to study the effect of these growth factors on viability of probiotic bacteria in fermented frozen dairy desserts. The third batch was made with milk supplemented with 2% skim milk powder only (control). Yogurt bacteria (*S. thermophilus* STWJ7 and *L.*

delbrueckii ssp. *bulgaricus* LBWJ7), and probiotic bacteria (*L. acidophilus* LA MJLA1 and bifidobacteria BB BDBB2) were added at the rate of 0.5% each and the inoculated mix incubated at 42^0C till pH 4.5 was attained. The yogurt was transferred to a cold room till the temperature decreased to 4^0C. The yogurt and base mix were mixed at a rate of 45% of each and 10% of liquid sucrose (65%) was added. All the three batches of fermented frozen dairy desserts were made using a bench top ice cream freezer with an overrun of 18%.

3.14 Assessment of effects of cryoprotectants for improving viability of *L. acidophilus* and *Bifidobacterium* spp.

Effects of cryoprotectants such as fructose, glucose, corn syrup solids, vegetable gum, glycerol and pectin etc. for improving the viability of *L. acidophilus and Bifidobacterium* spp. was assessed. Products were made from fermenting a mix with organisms selected from previous studies, containing variable amounts of cryoprotectants and mixed with a pasteurised base containing 16% sugar and 10% milk solids not fat and whipped air while freezing to achieve 90 to 100% overrun. Viable numbers of probiotic and yogurt organisms were enumerated at weekly intervals for 12 weeks

3.15 Compositional analysis

3.15.1 *Determination of pH and titratable acidity*

The pH values of yogurt, fermented frozen dairy desserts, milk samples, broth samples, and agar samples were measured at 22-25°C using a pH meter (Model 420 A, Orion Research Inc. Boston, U.S.A.). The pH meter was calibrated between pH 7.0 and 4.0 using a standard buffer solution according to the

manufacturer instructions. The titratable acidity was determined after mixing a sample with 10 mL of hot distilled water and titrating with 0.1N NaOH using 0.5% phenolphthalein indicator to the end point of faint pink colour that persisted for at least 30 seconds.

3.15.2 *Determination of sugar*

Two milliliters of sugar solution containing between 10 and 70 µl of sugar is pipetted into a colorimetric tube, and 0.05 ml of 80% phenol is added followed by rapid addition of 5 ml of concentrated sulfuric acid. The stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand for 10 minutes, mixed well and placed for 10 –20 minutes in a water bath at 25 to 30° C and the readings are taken. The absorbance values were measured at 490 nm (Dubois *et al.*, 1956).

3.15.3 *Determination of protein*

Protein content of fermented frozen dairy desserts was determined by the Kjeldahl method using Kjeltec system and 1002 distillation unit (Tecator ab, Hoganas, Sweden). One gram of well mixed fermented frozen dairy desserts was measured carefully (avoiding the sample touching the sides of the tube) into digestion tubes and catalyst tablets were added according to the manufacturer instructions into the digestion tube containing the samples. To the tubes, sulfuric acid (12.5 mL) was carefully added using a dispenser and the content was mixed by swirling the tubes with hand (protected with heavy duty gloves) and the tubes

were placed in the preheated digester, preset at (420°C) with maximum air flow through the exhaust for 3-5 min. The digestion was continued until all samples were digested as indicated by a clear solution and the lack of black residue in the tubes. The digestion time for the most samples ranged between 45 to 55 min. The tubes containing digested samples were removed from the digester and were placed in the cooling stand. After cooling, 50 mL of nitrogen free tap water was added for dilution. The distillation was carried out and the distillates were collected into a receiver flask containing 25 mL boric acid solution. After complete distillation, the content was titrated against 0.05 M H_2SO_4 until the solution in the receiver flask turn to natural grey.

3.15.4 *Determination of total solids*

The total solids contents were determined by standard gravimetric method using an oven at 100°C. One gram of well mixed sample was weighed into moisture dish and dried for one hour. The dish was cooled using a desiccator jar containing silica gel. The cooled dish was measured and dried again for another one hour and weighed and the drying process continued for further one hour. The difference in the weight of the sample before and after drying was calculated and percent total solids were obtained.

$$TS = \frac{\text{initial wt.} - \text{final wt.}}{\text{initial wt.}} \times 100$$

3.15.5 *Determination of water activity*

Water activity was measured using a water activity meter. Three milliliters of the sample were measured and placed in the chamber at 25^0C according to the instructions of the manufacturer and the results were obtained. Three determinations were performed and results were presented as average of three replicates.

3.15.6 *Determination of oxygen content and redox potential*

The dissolved oxygen in parts per million (ppm) was measured using LC82 Oxygen meter (TPS Pty. Ltd., Brisbane, Australia), after calibrating each time before use. The redox potential was measured with a platinum electrode (Model P 14805-SC-DPAS-K85/325, Ingold (Mettler Toledo), Urdorf, Switzerland) connected to pH meter (model H 18418, Hanna Instruments, Padova, Italy).

3.15.7 *Determination of β-D galactosidase*

The β-D-galactosidase activity was determined according to the method of Shah and Jelen (1990). Ten grams of each product were mixed with distilled water, blended for 1 min and the final volume made up to 100 mL in a volumetric flask. One millilitre of the solution was used in the assay. A solution of 0.005M O-nitrophenyl-β-D-galactopyranoside (ONGP) was prepared in 0.1M phosphate buffer (pH 7.0), and 1 mL aliquots of the diluted samples were incubated with 5 mL ONGP solution for 15 min at 37^0C. The reaction was stopped by adding 2.5 mL 1M cold sodium carbonate solution. The amount of O-nitrophenol released was measured with a spectrophotometer at 420 nm. One unit of β-D-

galactosidase activity was defined as the amount of enzyme, which liberated one μmole O-nitrophenol from ONGP per min per gram sample at 37^0C.

3.15.8 *Determination of the growth curve*

To achieve maximum number of viable bacteria, it was felt necessary to study the log phase of the organisms. The growth curve was determined using a spectrophotometer. Probiotic bacteria, *L. acidophilus* and *Bifidobacterium* spp., were propagated three times before inoculating in MRS broth (L-cysteine was added for bifidobacteria). The inoculated MRS broth was incubated at 37°C and hourly readings of absorbance (OD) were measured by weighing the samples in cuvettes, using a spectrometer at 450 nm. The pH of the broth was also measured at hourly basis using a pH meter. A growth curve was plotted to identify the log phase of the organisms to have maximum survival.

3.15.9 *Determination of fat content*

The total fat content of the samples were estimated using Babcock method (Atherton and Newlander, 1977).

3.15.10 *Detection and quantification of organic acids with HPLC*

3.15.10.1   Extraction of organic acids

Four grams of yogurt samples were diluted with 25 mL of 0.01N H_2SO_4 and filtered through 0.02-μm Teflon filters (Schleicher and Schuell, Dassel, Germany). The extraction of organic acids was carried out in duplicates.

3.15.10.2   HPLC of organic acids

The standards of organic acids were pipetted into HPLC vials through 0.02 μm millipore filters (Millipore Australia, Lane Cove, Australia). Similarly, diluted samples of yogurts were pipetted into HPLC vials through 0.02 μm filters. The vials were used in an auto sampler for analysis of organic acids. The concentration of each acid was determined with Varian HPLC (Varian, Mulgrave, Australia) using an UV-Vis detector. An Aminex HPX-87H ion exclusion column (300 x 7.8 mm) (BioRad, North Ryde, Australia) and a mobile phase of 0.0075 M H_2SO_4 were used for the analysis. Quantification of organic acids was carried out by the external standard method. Linear regression curves based on peak heights were calculated for individual organic acids after duplicate injections. Identifications of acids were based on matching retention times of standards.

3.16   Media preparation

3.16.1 *Peptone and water diluent*

Diluent of peptone and water was prepared by dissolving 15 g of peptone and water medium (Oxoide, West Heidelberg, Australia) in 1L of distilled water, the pH adjusted to 7.0 ± 0.2, followed by autoclaving 9 mL portions at 121^0C for 15 min. After autoclaving, peptone and water diluent in McCartney bottles were stored at room temperature for up to 15 days.

3.16.2 *Preparation of serial dilutions for pour plating*

One gram of well-stirred sample was weighed accurately added to 9 mL of 1.5% peptone water diluent. The sample and peptone water diluent were vortexed and 1 mL of the dilution was transferred into 9 ml of peptone water diluent using standard sterile pipettes (1 mL) and a series of ten-fold dilutions were prepared. One millilitre of aliquot was placed in an empty sterile petridish. Melted agar medium (45^0C) was poured into the plates followed by gentle mixing and the agar was allowed to solidify.

3.16.3 *Streptococcus thermophilus* agar (ST agar)

The ST agar was prepared according to the method of Dave and Shah (1996). The ingredients were dissolved in 1 L of distilled water, warmed to 25°C while stirring the medium, using a hot plate and a magnetic stirrer. The pH was adjusted to 6.8 ± 0.1, and 6 mL of 0.5% bromocresol purple was added to the medium. Twelve grams of bacteriological agar was added to 1L of the medium. The medium was autoclaved at 121^0C for 15 min. Plates in duplicate were incubated aerobically at 37^0C for 24 h.

3.16.4 *M17 agar*

The M17 agar (Amyl Media, Dandenong, Australia) was prepared as recommended by the manufacturer. The medium was autoclaved at 121^0C for 15 min. Plates in duplicate were incubated aerobically at 37^0C for 24 h.

3.16.5 *MRS-basal, MRS-salicin, MRS-sorbitol, MRS-ribose, MRS-gluconate agars*

MRS-basal medium was prepared according to Lankaputhra and Shah (1996) without any sugars and sterilised at 121^0C for 15 min. The ingredients were dissolved in 1 L of distilled water, warmed to 25°C while stirring the medium, using a hot plate and a magnetic stirrer. Ten millilitres of membrane-filtered sterile solutions of 10% salicin, sorbitol, ribose or glauconite were added per 90 ml of the basal medium (1% final concentration) just before pouring the agar medium. Plates in duplicate were incubated anaerobically using anaerobic jars (Oxoid) at 37^0C for 72 h (Dave and Shah, 1996).

3.16.6 *MRS-pH modified agar*

MRS broth (Oxoid) was prepared according to the manufacturer instructions. The pH was adjusted to 5.2 using 1.0 M HCl. After the broth was prepared, the agar powder was added at the rate of 1.2%, and the medium was autoclaved at 121^0C for 15 min. Plates in duplicate were incubated anaerobically at 43^0C for 72 h (Dave and Shah, 1996)

3.16.7 *MRS-NNLP agar*

The MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate (all from Sigma Chemical Co., Castle Hill, Australia) agar was prepared according to the method described by Laroia and Martin (1991). The basal medium was MRS agar. Filter-sterilised NNLP was added to the autoclaved MRS basal medium just before pouring. Filter-sterilised L-cysteine

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HCl (0.05% final concentration) was also added at the same time to lower the oxidation-reduction potential of the medium and to enhance the growth of bifidobacteria. Plates in duplicate were incubated anaerobically at 37^0C for 72 h.

3.16.8 *LC agar*

The ingredients of LC agar were dissolved in 1 L of distilled water. The pH was adjusted to 5.1 ± 0.1, and 6 ml of bromocresol green and 12 g of agar were added to the medium. The medium was sterilised at 121^0C for 15 min. Ten millilitre of membrane-filtered sterile solution of 10% ribose was added per 90 ml of the medium (1% final concentration) just before pouring the agar medium. Plates in duplicate were incubated anaerobically at 27^0C for 72 to 96 h.

3.17 Preparation of fermented frozen dairy desserts

3.17.1 *Preparation of yogurt*

Pasteurized skim milk was warmed to 40-45°C in a saucepan using a hot plate and magnetic stirrer and skim milk powder (2%) was added. The milk was heated to 85°C for 30 min and cooled to 43°C. Yogurt bacteria and/or probiotic bacteria were added at 0.5% level and the mix was incubated at 43°C till the pH reached to 4.5 and the yogurt stored overnight before adding into the base mix.

3.17.2 *Preparation of base mix*

Pasteurized full cream milk was warmed to 40-45°C in a saucepan using a hot plate and a magnetic stirrer. Sugar (16%) and gelatin (0.3%) were added to milk slowly while stirring. The mix was heated to 85°C for 15 min and cooled to 4°C. Fresh pasteurised cream was added to make up the fat percentage to 10.5%. The base mix was left overnight for maturing in the cold room before blending with yogurt.

3.18 Preparation of microencapsulation of probiotic bacteria and their incorporation into fermented frozen dairy desserts

Immobilisation of cells were carried out as follows: cultures grown in deMan Rogosa and Sharpe (MRS) broth were harvested by centrifugation in a refrigerated centrifuge, suspended in broth and the cell slurry added to an equal weight of 3% k-carrageenan solution in sterile distilled water. The mixed solution, with the help a syringe, was added drop-wise into a solution of 3M KCl

at 20°C in a 500 mL Buchner flask connected to a supply of nitrogen to help maintain anaerobic atmosphere and develop a positive pressure in the flask. The mixture of k-carrageenan and cell slurry was gelled into small beads on contact with the cold KCl solution, the gelled beads were separated by decantation and lyophilised. The resulting freeze-dried organisms were incorporated into the base mix followed by mixing and freezing.

3.18.1 *Preparation of cell pellet*

Microorganisms were propagated three times in sterile reconstituted skim milk and the fermentation terminated at the log phase. The organisms (2%) from the third propagation were inoculated in MRS broth and incubated for 18 h and the broth was cooled to 4^0C. The cooled broth was centrifuged using a refrigerated centrifuge at 4000 RPM (4^0C) for 25 min. The supernatant was suspended and the cell pellet was collected. The cell pellet was washed and resuspended in saline to give 10^8 cfu/gram.

3.18.2 *Entrapment of the microorganisms in beads*

Various concentrations of sodium alginate were analysed using a viscometer. Sodium alginate (3%) was prepared using a volumetric flask. Cells were microentraped by mixing 50 ml of culture concentrate with 200 ml of 3% (w/v) sodium alginate. The mixture was added dropwise to 5 parts of vegetable oil containing Tween 80 (0.2%), stirring at 200 rpm without any evidence of free aqueous phase. Calcium alginate beads were formed by addition of calcium chloride quickly but gently down the side of the beaker until water/oil emulsion

was broken. The beads were separated and washed by using sterile distilled water by gentle centrifugation (500 rpm).

3.18.3 *Effect of surface tension and emulsifier on bead size*

Effect of surface tension and emulsifier on bead size were studied. Gel strength was studied using a viscometer. The surface tension of 3% sodium alginate was varied by addition of various levels of sodium lauryl sulfate (0, 0.1 and 0.5%) and three different concentrations of Tween 80 (0, 0.1 and 0.2%) were added to the oil phase.

3.18.4 *Effect of freeze-drying on beads and viability of cells*

The entrapped cells were freeze-dried using a freeze dryer. The beads were placed in sterile petri-dishes with lids on and freeze-dried for 24 hrs. The beads were collected and plates were sealed with parafilm and stored for further testing. Viable counts before and after freeze-drying were enumerated. The size of the bead was studied.

3.18.5 *Solubilization of beads*

The entrapped bacteria were released from the gel according to the method of Ohlson et al. (1979). One millilitre of the bead suspension (1g in 59 mL water) was added to 9 mL phosphate solution (concentrations from 0.001 to 0.1M) followed by gentle shaking for 30 min. The pH of the phosphate solutions was varied from 4.8 to 8.5 by adjusting the ratio of monosodium phosphate and

disodium phosphate buffer solutions. The number of released cells was determined by plate count using MRS agar.

3.19 Acid and low temperature adaptations of probiotic bacteria and their incorporation into fermented dairy desserts

Two adaptation conditions were used: one with sublethal pH of 5.5 and the other with lethal pH 3.5. For adaptation studies, the organisms were propagated three time in MRS broth (0.05% cysteine was added for bifidobacteria) at 2% level and incubated at 37^0C for 18 h. The fermentation was slowed down by transferring the samples into a refrigerator and the temperature was decreased to 4^0C. The initial pH of the sample was assessed with a pH meter and the sample plated for enumeration for viable counts. Ten millilitres of the samples were stored in a freezer for gel-electrophoresis. The pH of the broth (100 ml) was increased to 6.5 using 10% NaOH and the broth kept in a refrigerator for one hour. After 1 h of refrigeration (temperature adaptation), the broth was divided into two portions (A & B) of 50 ml each and decreased to lethal pH of 3.5. The sample B was kept at sublethal pH of 5.5 for one hour before dropping the pH to the lethal pH of 3.5. The samples A and B were maintained at this pH (3.5) for 3 h, followed by freeze stressing at -20^0C for 1 hour. The samples were thawed and the pH increased to 6.5 with 35% NaOH and allowed to recover the stress at this pH for one hour and the pH of the samples was decreased to 4.5. to observe any increased survival in the counts.

Similarly bacterial cells were stressed at pH 3.5 as before and freeze stressed for 2h, 4h, 8h, 12h, 16h or 24h. Studies showed that sample A when adapted to sublethal pH survived better. On this basis, 3 strains each of *L. acdophilus* and bifidobacteria were selected and incorporated into fermented frozen dairy desserts. The viable counts were monitored for 12 weeks.

3.19.1 *Preparation of buffers and reagents*

3.19.1.1 Tris-HCl buffer (1.875M and pH 8.8)

Tris–HCL buffer (1.875M and pH 8.8) was prepared by dissolving 56.8 g of Tris in 150 mL distilled water. The solution was made up to 250 mL and adjusted to pH 8.8 with 5N HCl and stored at 4^0C for up to four weeks.

3.19.1.2 Tris-HCl buffer (1.25 M and pH 6.6)

Tris-HCl buffer (1.25 M and pH 6.6) was prepared by dissolving 37.8 g of Tris in 150 mL of distilled water. The pH was adjusted to 6.8 with 5 N HCl and the solution was made up to 250 mL with distilled water.

3.19.1.3 SDS (Sodium dodecyl sulphate) (10%) solution

Ten gram of SDS was dissolved in 85 mL distilled water and the solution was made up to 100 mL with distilled water. SDS was stored at room temperature up to four months.

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3.19.1.3 SDS (Sodium dodecyl sulphate) (10%) solution

Ten gram of SDS was dissolved in 85 mL distilled water and the solution was made up to 100 mL with distilled water. SDS was stored at room temperature up to four months.

3.19.1.4 Poly-acrylamide gel electrophoresis (PAGE), staining of the gels, and preparation of running gel

Aliquots of 3.33 mL of stock (30%) acrylamide, 2.00 mL of 1.875 M tris –HCl (pH 8.8) and 4.70 mL distilled water, 50 µl 10% APS, 150 µl 10% SDS were mixed and degassed for 15 min. Ten microlitre of TEMED was added to the degassed mix quickly. A 3.5 mL quantity was added per gel.

3.19.1.5 Preparation of stacking gel

Aliquots of 0.66 mL of acrylamide (30%) 0.50 mL of 1.25 M tris HCL (pH 6.8) and 3.80 mL distilled water, 10 µl 10% APS and 50 µl 10% SDS were degassed for 15 min under vacuum. Five microlitre of TEMED was added to the degassed mix and mixed quickly. The stacking mix was applied on to the top of the running gel and combs were inserted.

3.19.1.6 Staining the gels with coumassie blue

The protein gels were transferred from the gel plates into a fixing solution prepared by adding 4 parts of ethanol, 1 part of acetic acid and 5 parts of H_2O. The gels were left in the fixing solution for 30 min and transferred into a staining solution prepared by mixing 1.5 g of Coomassie blue, 105 ml of 95% ethanol, 30 mL of acetic acid and 165 mL of distilled water. After staining for 30 min in coomassie blue, the gels were transferred into a de-staining solution, which was similar in composition to the fixing solution (4 parts of ethanol, 1 part of acetic acid, and 5 parts of H_2O) during de-staining, the gels were transferred into fresh

de-staining solutions three times. After de-staining, the gels were transferred into a preserving solution prepared by mixing 50 mL of glycerol, 175 ml of 95% ethanol, 50 mL of acetic acid and the volume made up to 500 mL with distilled water.

3.19.1.7 Staining the gels with silver stain

The protein gels were transferred from the gel plates into fixing solution prepared by adding 4 parts of ethanol, 1 part of acetic acid and 5 parts of dH_2O. The gels were left in the fixing solution for 30 min and transferred into an incubation solution prepared by mixing 75 mL ethanol, 17 g of sodium acetate, 3 mL dH_2O, 1.3 ml glutaraldehyde (25% w/v), 0.5g sodiumthiosulphate (pentahydrate) and the volume made up to 250 mL with distilled water. After incubation, the gels were washed with distilled water 3 times and placed in a silver solution prepared by mixing 0.25 g of silver nitrate and 50 µl of formaldehyde in 250 mL distilled water. The gels were transferred into developing solution (made with 6.25 g Na_2CO_3 and 25µl of formaldehyde in 250 mL of distilled water) after 40 min of silver reaction. The gels were then placed in stop solution prepared by dissolving 3.65 g of EDTA-Na_2 dehydrate in 250 mL distilled water. The gels were then placed in preserving solution prepared with 20 ml of glycerol made up to 200 ml of distilled water.

3.19.1.8 Gel preparation and electrophoresis

The frozen cells extract was thawed at room temperature and the protein contents were determined using Lowrey assay. The extracts were diluted with double

distilled water to achieve a final concentration of 10 μl/mL, and the samples were mixed with loading buffer and boiled for 5 min in Eppendorf tubes. The boiled samples were briefly centrifuged and loaded in to the gel along with the molecular size standards (in the first and the last well). A standard was also loaded alongside the samples into the gel and electrophoresis was carried out at 30 MAmp using a power-pack and the gel stained with Coomassie blue. Stained gels were photographed with a Polaroid camera and dried using a gel drier.

4.0 SELECTIVE ENUMERATION OF *LACTOBACILLUS CASEI* FROM YOGURTS AND FERMENTED MILK DRINKS

4.1 Introduction

Yogurts are made from the symbiotic growth of the two bacteria: *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. These yogurt bacteria do not survive the gastric passage or colonise in the gut (Shah and Jelen, 1990). Dairy products containing probiotic bacteria such as *Lactobacillus acidophilus* and bifidobacteria are becoming popular because of their potential therapeutic benefits. However, due to viability problems associated with *L. acidophilus* and bifidobacteria during storage (Anon, 1992; Shah *et al.*, 1995; Dave and Shah, 1997a, b, c), the recent trend is to add *L. casei* as an adjunct bacteria to yogurt or probiotic cultures. *L. casei* is claimed to be more stable than *L. acidophilus* or bifidobacteria (McCann *et al.*, 1996).

In order to achieve health benefits, a minimum of one million viable cells per gram of a product must be present. It is important that the cells must remain viable throughout the projected shelf life of a product so that when consumed the product contains sufficient viable cells.

Several media have been developed for selective enumeration of *L. acidophilus* and bifidobacteria in yogurts and fermented milks (Kneifel and Pacher, 1993; Int. Dairy Federation, 1997; Dave and Shah, 1996; Lankaputhra and Shah, 1996). However, there is little information on selective enumeration of *L. casei* in yogurt and fermented milk drinks, which may contain yogurt bacteria and probiotic bacteria.

Selective enumeration of *L. casei* in yogurt-type fermented milks containing probiotic bacteria based on $15°C$ incubation temperature and 14 day incubation time was studied by Champagne *et al.* (1997). However, an incubation time of 14 days may not be practical for the dairy industry if the population of *L. casei* is to be known in a short time.

In this study, the ability of *L. casei* to ferment ribose was used as an approach to develop a medium for selective enumeration of this organism in fermented products containing yogurt bacteria, probiotic bacteria and *L. casei*. The efficacy of the medium for selective enumeration of *L. casei* was assessed using nine commercial yogurts and fermented milk drinks obtained from local supermarkets.

4.2   Materials and methods

4.2.1 *Bacteria*

Two strains each of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and bifidobacteria were used as reference for the validation of the medium developed in this study. *S. thermophlius* ST WJ7, *L. delbrueckii* ssp.

bulgaricus LB Wj7, *L. acidophilus* LA MJLA1 and bifidobacteria BB BDBB2 were obtained from Gist-brocades (Moorebank, Australia). *S. thermophlius* ST 2008, *L. delbrueckii* ssp. *bulgaricus* LB 2501, *L. acidophilus* LA 2415, and bifidobacteria BB 20099 (*B. pseudolongum*) were obtained from the Division of Food Science and Technology, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Highett, Australia. *L. casei* was obtained from Chr. Hansen Pty Ltd. (Bayswater, Australia).

4.2.2 *Microbiological analysis*

4.2.2.1 *Streptococcus thermophilus* agar (ST agar*)*

The ST agar was prepared according to the method described by Dave and Shah (1996). Plates in duplicate were incubated aerobically at 37°C for 24 h.

4.2.2.2 M17 agar

The M17 agar (Amyl Media, Dandenong, Australia) was prepared as recommended by the manufacturer. The medium was autoclaved at 121°C for 15 min. Plates in duplicate were incubated aerobically at 37°C for 24 h.

4.2.2.3 MRS-basal, MRS-salicin, MRS-sorbitol, MRS-ribose, MRS-gluconate agars

deMan, Rogosa, and Sharpe (MRS)-basal medium was prepared according to Lankaputhra and Shah (1996) without any sugars and the medium sterilised at 121°C for 15 min. Ten millilitres of membrane-filtered sterile solutions of 10%

salicin, sorbitol, ribose or gluconate were added per 90 ml of the basal medium

(1% final concentration) just before pouring the agar medium. Plates in duplicate

were incubated anaerobically using anaerobic jars (Oxoid) at 37°C for 72 h (Dave

and Shah, 1996).

4.2.2.4 MRS-pH modified agar

MRS broth (Oxoid) was prepared according to the manufacturer instructions. The

pH of the broth was adjusted to 5.2 using 1.0 **M** HCl, agar powder added at the

rate of 1.2%, and the medium was autoclaved at 121°C for 15 min. Plates in

duplicate were incubated anaerobically at 43°C for 72 h (Dave and Shah, 1996).

4.2.2.5 MRS-NNLP agar

The MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and

paromomycin sulfate (all from Sigma Chemical Co., Castle Hill, Australia) agar

was prepared according to the method described by Laroia and Martin (1991).

The basal medium was MRS agar. Filter-sterilised NNLP was added to the

autoclaved MRS basal medium just before pouring. Filter-sterilised L-

cysteine·HCl (0.05% final concentration) was also added. Plates in duplicate were

incubated anaerobically at 37°C for 72 h.

4.2.2.6 LC agar

The ingredients of LC agar (10.0 g bacteriological peptone; 1.0 g yeast extract;

4.0 g Lab Lemco (Oxoid); 2.0 g KH_2PO_4; 3.0 g sodium acetate; 1.0 g tri-

ammonium citrate; 0.2 g MgSO₄; 0.05 g MnSO₄ (BDH Chemicals Pty. Ltd.,
Kilsyth, Australia); 1.0 g acid casein hydrolysate; 1.0 g tween 80 (Sigma
Chemicals Co. St. Louis, USA) were dissolved in 1 L of distilled water. The pH
was adjusted to 5.1 ± 0.1, and 6 ml of bromocresol green and 12 g of
bacteriological agar (Amyl Media, Dandenong, Australia) were added to the
medium. The medium was sterilised at 121°C for 15 min. Ten millilitre of
membrane-filtered sterile solution of 10% D (-)ribose (Sigma) was added per 90
ml of the medium (1% final concentration) just before pouring the agar medium.
Plates in duplicate were incubated anaerobically at 27°C for 72 to 96 h.

4.2.3 Enumeration of bacteria

Pure strains were given three successive transfers before enumeration. One gram
of each culture was 10-fold serially diluted (10^3 to 10^7) in 0.15% sterile peptone
and water diluent. The enumeration was carried out using the pour plate
technique. Anaerobic jars and gas generating kits (Anaerobic System BR 38,
Oxoid Ltd., Hampshire, England) were used for creating anaerobic conditions.
Plates with 25-250 colonies were enumerated and recorded as colony forming
units (cfu) per gram of the product or culture.

4.2.4 Commercial products

Nine commercial yogurts and fermented milk drinks claiming to contain single or
mixed strains of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus,*
bifidobacteria and *L. casei* were analysed to validate the efficacy of the method.
The products analysed were: Product 1- yogurt; Product 2-skim milk yogurt;

Product 3- mild continental yogurt; Product 4-natural yogurt; Product 5-yogurt; Product 6- fermented milk drink; Product 7-fermented milk drink; Product 8-drinking yogurt; and Product 9- dairy fruit drink. The organisms that were claimed in these products are shown in Table 2.

All experiments and analyses were carried out in duplicate. The results presented are averages of duplicate experiments.

4.3   Results and Discussion

Table 4.1 shows the viable counts of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, bifidobacteria and *L. casei* on various bacteriological media. ST agar and M17 agar were selective for *S. thermophilus* and MRS-pH modified agar was selective for *L. delbrueckii* ssp. *bulgaricus*. *L. acidophilus* and *L. casei* formed colonies on MRS-salicin, MRS-sorbitol agar, MRS-ribose agar and MRS-gluconate agar. Thus these media cannot be used for selective enumeration of *L. casei*. MRS-salicin agar was reported to be selective for *L. acidophilus* (Dave and Shah, 1996; Lankaputhara and Shah, 1996). However, as shown in Table 4.1, *L. casei* also grew in this medium. Hence, MRS-salicin medium cannot be used for selective enumeration of *L. casei* in products containing *L. acidophilus* and *L. casei*. MRS-NNLP medium (Laroia and Martin, 1991) was selective for enumeration of bifidobacteria. LC agar inhibited the growth of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and bifidobacteria, and thus the medium was selective for *L. casei*.

The efficacy of the medium was validated using nine commercial yogurts and fermented milk drinks claiming to contain single or mixed strains of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus*, bifidobacteria and *L. casei*. Table 4.2 shows the counts of *L. casei* in various commercial products.

Products 4, 5 and 8 did not contain *L. casei* and this organism was not detected in these products. Products 1, 2, 3 and 9 claimed to contain yogurt bacteria, probiotic bacteria and *L. casei* and *L. casei* was detected in these products. However, the population of *L. casei* in both Products 2 and 3 were low. An earlier report (Anon, 1992) on Australian dairy products also showed low counts of *L. acidophilus* and bifidobacteria in several products. Products 6 and 7 claimed to contain strains of *L. casei* and *L. casei* was detected in these products. Thus, it appears that LC agar was selective for *L. casei*. Figure 4.1 shows colonies formed by *L. casei* on LC agar, whereas no other organisms formed colonies on the LC agar confirming that LC agar can be selective for enumerating *L. casei*

4.4   Conclusion

LC agar developed in this study was found to be selective for *L. casei* from pure cultures. The efficacy of the medium was verified using several commercial products containing single or mixed population of yogurt bacteria, probiotic bacteria and *L. casei*. LC agar could be used for selective enumeration of *L. casei* in yogurts and fermented milk drinks containing mixed population of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and bifidobacteria.

Table 4.1 Counts ($\log_{10}$ cfu/g) of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus,* bifidobacteria, and *L. casei* on various bacteriological media

Strains	ST agar	M17 agar	MRS-basal agar	MRS-salicin agar	MRS-sorbitol agar	MRS-ribose agar	MRS-gluconate agar	MRS-pH-modified agar	MRS-NNLP[1] agar	LC[2] agar
ST[3] 2008	8.56	8.72	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
ST WJ7	8.68	8.59	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
LB[4] 2501	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	7.66	<3.00	<3.00
LB WJ7	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	7.91	<3.00	<3.00
LA[5] 2415	<3.00	<3.00	<3.00	8.62	8.41	8.68	8.85	<3.00	<3.00	<3.00
LA MJLA	<3.00	<3.00	<3.00	8.68	8.46	8.55	8.55	<3.00	<3.00	<3.00
BB[6] 20099	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	8.85	<3.00
BB BDBB2	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00	8.93	<3.00
L. casei	<3.00	<3.00	<3.00	8.89	8.68	8.73	8.50	<3.00	<3.00	9.08

[1]NNLP = Nalidixic acid, neomycin sulfate, lithium chloride and paromomycin sulfate.
[2]LC = *Lactobacillus casei*
[3]ST = *Streptococcus thermophilus*
[4]LB = *Lactobacillus delbrueckii* ssp. *bulgaricus*
[5]LA = *Lactobacillus acidophilus*
[6]BB = Bifidobacteria

Table 4.2 Counts of *L. casei* from different commercial products selectively enumerated on LC agar

Products	Organisms claimed to contained in the product	LC agar
Product 1- yogurt	*S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus*, bifidobacteria and *L. casei*	7.49
Product 2- skim milk yogurt	*S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus*, bifidobacteria and *L. casei*	3.41
Product 3- mild continental yogurt	*S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus*, bifidobacteria and *L. casei*	3.72
Product 4- yogurt	*S. thermophilus, L. acidophilus* and bifidobacteria	<3.00
Product 5- yogurt	*S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus*	<3.00
Product 6- fermented milk drink	*L. casei*	8.22
Product 7- fermented milk drink	*L. casei*	6.98
Product 8- drinking yogurt	*S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus*, and bifidobacteria	<3.00
Product 9- dairy fruit drink	*S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus*, bifidobacteria and *L. casei.*	8.08
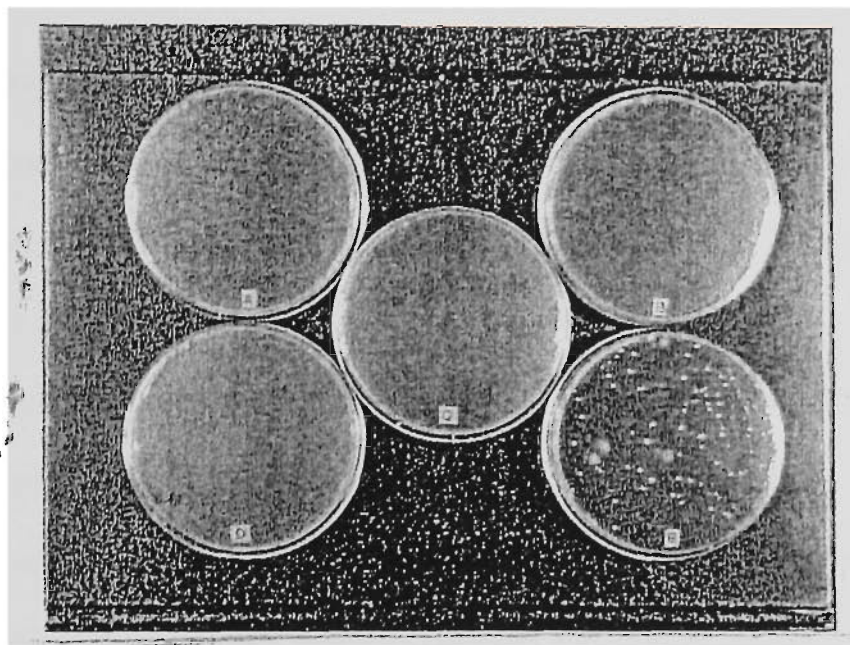
Figure 4.1. Colonies formed by *L. casei* on LC agar

5.0 Populations of *Lactobacillus acidophilus*, *Bifidobacterium* spp., and *Lactobacillus casei* in commercial fermented milk products

5.1 Introduction

Over the past decade, the per capita consumption of yogurt in Australia has increased by 112% (IDF, 1997). Yogurt has been considered a highly nutritious food and a useful therapeutic agent (Tamime and Deeth, 1981). There is a growing consumer demand for 'bio-yogurts' and other milk products that contain live probiotic cultures. Japan is the world leader in probiotics. In Europe, probiotic containing food and beverages remains one of the fastest growing segments of the cultured dairy products (Hughes and Hoover, 1991).

Probiotic bacteria are claimed to prevent the growth of intestinal pathogens such as *Salmonella*, and *Shigella*. Bifidobacteria are useful for the maintenance of human health and the reduction or disappearance of bifidobacteria in the human intestine would imply an 'unhealthy' state (Mitsuoka, 1990; Shah *et al.*, 1995). Probiotic organisms are also claimed to stimulate immune response and to prevent cancer. In addition, these organisms improve digestibility of lactose in lactose intolerant people (Shahani and Chandan, 1979).

In order to obtain health benefits associated with probiotics, it is necessary to ingest a minimum of one million organisms per gram of a product (Sellars, 1991). Studies (Hamann and Marth, 1984; Rohm *et al.*, 1990) have shown that yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckeii* spp. *bulgaricus*) survive well in yogurt until the use-by-date. However, probiotic bacteria (*L. acidophillus* and *Bifidobacterium* spp.) showed a rapid decline after manufacture (Gilliland and Speck, 1977; Medina and Jordano 1994; Shah *et al.*, 1995; Rybka and Fleet, 1997). Thus, it is misleading to describe a probiotic product as having health promoting properties unless the minimum level of viable cells are present at the time of consumption (Kailaspathy and Rybka, 1997).

Lactobacillus casei, recently introduced to Australian yogurts, has shown good potential as a probiotic organism (Perdigon *et al.*, 1995; Hudault *et al.*, 1997; Wagner *et al.*, 1997). Additionally, this organism is claimed to have good viability in yogurt products. However, the population of *L. casei* in Australian yogurt has not been reported.

This paper reports the populations of *L. acidophillus, Bifidobacterium* spp. and *L. casei* in 26 fermented milk products representing 14 companies. Enumeration of bacterial populations was carried out immediately after purchase and at the expiry date.

5.2 Materials and Methods

5.2.1 Commercial products

Twenty-six commercial yogurts and fermented milk drinks representing 14 companies were purchased from local super markets in the Melbourne metropolitan area. The types of product that were analysed included fermented liquid milk drink, set yogurt, drinking yogurt, fruit yogurt and soy yogurt. All products claimed to contain probiotic bacteria. Six products (products A-F) contained *L. acidophilus* only, 12 products (G-R) contained *L. acidophilus* and bifidobacteria, 6 products (S-X) claimed *L. acidophilus*, bifidobacteria and *L. casei*, and 2 products (Y-Z) contained *L. casei* only. Relevant information was recorded from the package including the expiry date, flavour, composition, type of probiotic bacteria, and the address. The products were stored at 5°C during the stated shelf life. To protect reputations of the companies, the details of the manufacturers are not given.

5.2.2 *Measurement of pH*

The pH was measured with a pH meter (Model 420 A; Orion Research Inc., Boston, MA).

5.2.3 *Microbiological analyses*

MRS-salicin agar and MRS-sorbitol agar were used for enumeration of *L. acidophilus* (Dave and Shah 1996; Lankaputhra *et al.*, 1996a, b). Counts of bifidobacteria were enumerated on MRS-NNLP (nalidixic acid, meomycin sulfate, lithium chloride, and paromomycin sulfate) agar (Laroia and Martin 1991; Dave and Shah 1996). Counts of *L. casei* were enumerated on LC agar

(Ravula and Shah 1998). Enumeration was carried out using the pour plate technique and plates were incubated at 27°C for 72 to 96 h under anaerobic conditions. Plates containing 25 to 250 colonies were enumerated and recorded as colony forming units (cfu) per gram of the product.

The determination of pH and microbiological analyses were carried out in duplicate. The results presented are averages of duplicate experiments.

The products that were analysed included: (A) banana flavoured yogurt containing *L. acidophilus*; (B) vanilla flavoured yogurt containing *L. acidophilus*; (C) fruit yogurt containing *L. acidophilus*; (D) vanilla flavoured yogurt containing *L. acidophilus*; (E) black cherry fruit yogurt containing *L. acidophilus*; (F) low fat black cherry yogurt containing *L. acidophilus*; (G) vanilla flavoured yogurt with fruit and nuts containing *L. acidophilus* and bifidobacteria; (H) passion fruit reducted fat yogurt, d'lite containing *L. acidophilus* and bifidobacteria; (I) peach 'n' mango reduced fat and fruit yogurt containing *L. acidophilus*; and bifidobacteria; (J) strawberry organic soy yogurt containing *L. acidophilus* and bifidobacteria; (K) so natural non-dairy soy strawberry yogurt containing *L. acidophilus* and bifidobacteria; (L) non-dairy soy wild-berry yogurt containing *L. acidophilus* and bifidobacteria; (M) reduced fat raspberry drinking yogurt containing *L. acidophilus* and bifidobacteria; (N) reduced fat fruit salad fruit yogurt containing *L. acidophilus* and bifidobacteria; (O) yo-split reduced fat vanilla yogurt with museli 'n' fruit containing *L. acidophilus* and bifidobacteria; (P) diet lite, boysenberry low fat yogurt

containing *L. acidophilus* and bifidobacteria; (Q) fruit salad yogurt with honey

containing *L. acidophilus* and bifidobacteria; (R) apricot yogurt with honey

containing *L acidophilus* and bifidobacteria; (S) apricot mango, reduced-fat

yogurt containing *L. acidophilus*, bifidobacteria and *L. casei*; (T) strawberry

sundae, reduced-fat set yogurt containing *L. acidophilus*, bifidobacteria and *L.*

casei; (U) skim milk berry fruit yogurt containing *L. acidophilus*, bifidobacteria

and *L. casei*; (V) premium vanilla creamy yogurt containing *L. acidophilus*,

bifidobacteria and *L. casei*; (W) banana custard yogurt containing *L. acidophilus*,

bifidobacteria and *L. casei*; (X) Swiss vanilla reduced-fat fruit drinking yogurt

containing *L. acidophilus*, bifidobacteria and *L. casei*; (Y) probiotic fermented

milk drink containing *L. casei*; (Z) fermented milk drink containing *L. casei*

Shirota strain.

5.3   Results and Discussion

The populations of *Lactobacillus acidophilus, Bifidobacterium* spp. and

Lactobacillus casei were determined in 26 commercial dairy products and the

results grouped together on the basis of the type of organisms contained in the

products.

Table 5.1 shows the initial and final pH and viable counts in the products claimed

to contain *L. acidophilus* (products A-F). There was a slight decrease in pH (.04

to .07 units) in all the products. Three of the 6 products showed the initial viable

counts of *L. acidophilus* above 10^6 cfu/g, while the viable counts and at the

expiry date decreased below 10^5 cfu/g. In the product C, the initial counts were 10^4 cfu/g and at the expiry date the counts decreased below 10^2 cfu/g.

Table 5.2 shows the initial and final pH and viable counts of *L. acidophilus* and bifidobacteria in the AB products (products G-R). The initial pH ranged between 3.69 to 4.55 and the final between pH 3.64 to 4.26. The drop in pH ranged between .04 to .32. The initial counts of *L. acidophilus* in only 50% of the products (products I, L, O, P, Q, R) were above 10^6 cfu/g, while only 33% of the products (products O, P, Q, R) showed counts above 10^6 cfu/g. The initial counts of *L. acidophilus* in 25% of the products (products G, H, K) were very low ($<10^2$ cfu/g). The product L showed a decline of 6 log in the count of *L. acidophilus*. Only one product (product P) of 12 products (80%) showed initial and final counts of bifidobacteria of $>10^6$ cfu/g.

Table 5.3 shows the initial and final pH and viable counts of *L. acidophilus*, bifidobacteria and *L. casei*. Overall, there was a slight decrease in pH (.02 to .07) in all the products. The initial counts of *L. acidophilus* in 50% of the products (products S, T, V) were above 10^6 cfu/g. The initial counts of bifidobacteria in only one product (product S) was above 10^6 cfu/g. All the products showed bifidobacteria count of $<10^6$ at the expiry date. Four of 6 products (products S, T, V, X) had initial counts of *L. casei* above 10^6 cfu/g, while 3 of these 4 products maintained viability of above 10^6 at the expiry date. The counts of *L. casei* in the product X dropped below $<10^2$ cfu/g at the expiry date.

Table 5.4 shows the pH and viable counts in the products claimed to contain $L.$ *casei* only. There was a slight decrease in pH in both products. The initial counts in both products were >10^6 cfu/g, however, at the expiry date the counts in the product Y decreased below 10^2 cfu/g.

Initially, $L.$ *acidophilus* was added to fermented product as a probiotic organism. Then AB products containing $L.$ *acidophilus* and bifidobacteria were introduced. The recent trend is to incorporate all the three groups of bacteria ($L.$ *acidophilus*, bifidobacteria and $L.$ *casei*) called ABC products. $L.$ *casei* is claimed to be hardy, as a result, this organism is incorporated in fermented products. However, results suggest that 50% of the ABC products had <10^5 cfu/g viable counts.

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It is recommended that, to have therapeutic benefits, the product must contain viable probiotic population of 10^6 cfu/g. However, the viable populations of probiotic bacteria decline after manufacturing. Several factors have been found to affect the viability of probiotic bacteria including pH of the product, post-acidification due to yogurt bacteria, production of hydrogen peroxide by $L.$ *delbrueckii* ssp. *bulgaricus* (Dave & Shah, 1997 a, b), and antagonism among yogurt and probiotic bacteria (Joseph et al., 1998). Previous reports by Shah *et al.*, (1995) and Rybka and Fleet (1997) have also found variable levels of these organisms in the probiotic products. Fourteen to 24% of the samples in the study of Rybka and Fleet (1997) had viable population of $L.$ *acidophilus* and *Bifidobacterium* spp. above 10^6 cfu/g. The viable population of $L.$ *casei* in commercial products has not been reported.

5.4   Conclusion

Five of the AB and two ABC products contained *L. acidophilus* counts of above 10^6 cfu/g, whereas only one of AB and ABC products contained bifidobacteria counts of above 10^6 cfu/g at the expiry date. Three of the ABC products and only one LC product contained *L. casei* counts of $> 10^6$ cfu/g at the expiry date. To have therapeutic effects a minimum of one million viable cells per gram of the product has been recommended. The products containing low viable counts may not provide any health benefits. Advances in the understanding of the problems associated with the viability and survival of probiotic organisms in fermented milk products are yet to reach the commercial market.

Table 5.1 Initial and final pH and viable counts of *Lactobacillus acidophilus*

Products	Remaining shelf life (days)	pH initial	pH final	LA initial	LA final
A. Banana flavoured yogurt	25	4.17	4.10	3.7×10^6	3.0×10^5
B. Vanilla flavoured yogurt	25	4.18	4.13	9.1×10^5	9.8×10^5
C. Fruit yogurt	26	4.18	4.11	4.0×10^4	$<10^2$
D. Vanilla flavoured yogurt	24	4.15	4.10	1.9×10^6	1.9×10^4
E. Black cherry fruit yogurt	23	4.15	4.11	2.5×10^6	3.7×10^4
F. Low fat black cherry yogurt	28	4.24	4.17	3.0×10^5	3.0×10^4

LA = *Lactobacillus acidophilus*

Table 5.2 Initial and final pH and viable counts of *Lactobacillus acidophilus* and bifidobacteria

Products	Remaining shelf life (days)	pH initial	pH final	LA initial	LA final	BB initial	BB final
G. Vanilla yogurt with fruit and nuts	30	4.55	4.23	$<10^2$	$<10^2$	2.3×10^3	$<10^2$
H. Passion fruit reduced fat yogurt	22	4.22	4.23	$<10^2$	$<10^2$	1.0×10^3	1.1×10^3
I. Peach 'n' mango reduced fat and fruit yogurt	23	4.28	4.19	1.9×10^6	6.3×10^5	$<10^2$	$<10^2$
J. Strawberry organic soy yogurt	22	3.69	3.64	3.1×10^3	$<10^2$	$<10^2$	$<10^2$
K. So natural non dairy soy yogurt strawberry	26	4.06	4.02	$<10^2$	$<10^2$	3.2×10^5	$<10^2$
L. Non dairy soy yogurt wild-berry	29	4.14	4.14	1.2×10^8	$<10^2$	1.2×10^4	1.0×10^4
M. Reduced fat raspberry drinking yogurt	34	4.11	4.05	6.4×10^5	4.1×10^5	$<10^2$	$<10^2$
N. Reduced fat fruit salad fruit yogurt	24	4.03	3.98	5.3×10^5	$<10^2$	$<10^2$	$<10^2$
O. Yo-split reduced fat vanilla yogurt	25	4.33	4.26	2.2×10^8	1.1×10^7	4.2×10^5	8.9×10^4
boysenberry low fat yogurt	27	4.16	4.13	3.3×10^7	1.4×10^6	2.8×10^6	1.5×10^6
Q. Fruit salad yogurt with honey	23	3.74	3.66	1.8×10^6	1.7×10^6	4.0×10^3	$<10^2$
R. Apricot with honey	27	3.79	3.74	1.3×10^6	1.9×10^6	1.7×10^4	3.2×10^3

LA = *Lactobacillus acidophilus*; BB = bifidobacteria.

Table 5.3. Initial and final pH and viable counts of *Lactobacillus acidophilus*, bifidobacteria and *Lactobacillus casei*

Products	Remaining shelf life (days)	PH Initial	pH final	LA initial	LA final	BB initial	BB final	LC initial	LC final
S. Apricot mango, reduced fat yogurt	26	4.18	4.16	1.8×10^8	6.7×10^6	7.3×10^6	8.3×10^5	7.7×10^7	6.1×1
T. Strawberry sundae Reduced fat set yogurt	34	4.27	4.21	1.5×10^8	1.0×10^8	4.7×10^4	1.3×10^4	1.5×10^8	1.2×1
U. Skim milk berry fruit yogurt	34	4.45	4.40	5.4×10^5	$<10^2$	1.4×10^4	$<10^2$	4.5×10^5	3.8×1
V. Premium vanilla creamy yogurt	22	4.49	4.46	7.7×10^6	1.6×10^3	1.6×10^3	$<10^2$	5.5×10^6	3.4×10^6
W. Banana custard yogurt	23	4.42	4.35	7.3×10^5	5.7×10^5	9.0×10^3	5.8×10^3	5.1×10^5	3.1×10^3
X. Swiss vanilla reduced fat fruit drinking yogurt	27	4.23	4.19	7.0×10^3	1.1×10^3	3.8×10^4	6.8×10^3	1.3×10^6	$<10^2$

LA = *Lactobacillus acidophilus*; BB = bifidobacteria; LC = *Lactobacillus casei*

Table 5.4. Initial and final pH and viable counts of *Lactobacillus casei*

Products	Remaining shelf life (days)	pH initial	pH final	LC initial	LC final
Y. Probiotic fermented milk drink	29	3.50	3.38	4.5×10^6	$<10^2$
Z. Fermented milk drink	34	3.53	3.55	2.2×10^8	2.0×10^7

LA = *Lactobacillus casei*

6.0 SELECTION OF STRAINS OF YOGURT AND PROBIOTIC BACTERIA TO INCORPORATE IN FERMENTED FROZEN DAIRY DESSERTS

6.1 Introduction

Probiotic organisms such as *Lactobacillus acidophilus* and *Bifidobacterium* spp. are claimed to correct intestinal disorders, lower serum cholesterol, posses anti-carcinogenic properties and improve lactose utilisation in lactose intolerant people (Gilliland, 1989; Shah and Jelen, 1990; Shah, 1993).

Dairy products containing *L. acidophilus* and *Bifidobacterium* spp. are rapidly gaining popularity in Australia. The shelf life of yogurt is approximately 3 weeks. As a result, it may not be feasible to ship yogurt to overseas countries and the sales of such product are limited to local markets. With an increasing popularity of frozen dairy desserts containing yogurt cultures, a market could develop for dairy desserts similar to soft-serve frozen yogurt but containing *L. acidophilus* and *Bifidobacterium* spp. Frozen dairy desserts have longer shelf life and thus the products could be shipped to overseas countries. However, introducing probiotic organisms into the frozen food chain can be a difficult task as the organisms are unstable in such products. The loss of viability of probiotic organisms in frozen yogurt may be due to acidity of the product, freeze injury, sugar concentration and oxygen toxicity.

Using ice cream as a mechanism to incorporate bifidobacteria and fructooligosaccharides into the human diet was studied by Modler *et al.* (1990). In the presence of neosugars and Jerusalem artichoke flour, 90% survival of bifidobacteria (*Bifidobacterium adolescentis, Bifidobacterium infantis* and *Bifidobacterium longum*) was observed at the end of 70 days. The 10% loss in viable counts occurred at the time of freezing due to incorporation of air to achieve 100% overrun. The initial counts were 7.5, 6.9 and 6.6 $\log_{10}$ cfu/ml and the final counts were 7.3, 6.8 and 6.3 $\log_{10}$ cfu/ml respectively in the presence of neosugars. In the presence of Jerusalem artichoke flour the initial counts were 7.3, 7.4 and 6.9 and the final counts were 7.3, 7.2 and 6.4, respectively. The effect of pH on survival of *L. acidophilus* and *Bifidobacterium bifidum* in frozen fermented dairy desserts was studied by Laroia and Martin (1991). *L. acidophilus* and *B. bifidum* survived the freezing process and frozen storage of 8 weeks in pH 5.6-5.8 frozen fermented dairy desserts. The initial counts of *B. bifidum* were 5.7 cfu/ml and the final counts were 4.0 cfu/ml and the initial counts of *L. acidophilus* were 11.4 cfu/ml and the final counts were 11.4 cfu/ml.

Viability of *L. acidophilus* and *B. bifidum* in soft serve frozen yogurt was studied by Holcomb *et al.* (1991). *L. acidophilus* was able to survive when exposed to 0.01N HCl for 2 h at 37^{0}C. The initial counts of *L. acidophilus* before freezing were 5.90 log cfu and the final counts after freezing were 5.86 log cfu. Survival of *L. acidophilus* and *B. bifidum* in ice cream was also studied by Hekmat and McMahon (1992). Initial counts of the organisms were 1.5 x 10^8 cfu/ml for *L. acidophilus* and

2.5 x 10^8 cfu/ml for *B. bifidum.* After freezing the fermented mix at -29^{0}C and storing for 17 weeks the bacterial counts decreased to 4 x 10^6 and 1 x 10^7 cfu/ml, respectively. Survival of *L. acidophilus* and *Bifidobacterium* spp. in the presence of acid and bile salts was studied by Lankaputra and Shah (1995). Of several strains *L. acidophilus,* of 2401, 2409 and 2415 survived best under acidic condition, while *B. longum* and *B. pseudolongum* showed best survival under acidic conditions.

In this study, several yogurt and probiotic bacteria were screened for their ability to survive under freezing and acidic conditions and in the presence of high levels of sugar. Frozen fermented dairy desserts were made with selected yogurt and probiotic organisms and survival of these organisms was monitored for 12 weeks during frozen storage at -18^{0}C. Further, fermented frozen dairy desserts were made on a pilot scale (1000 L) and survival of yogurt and probiotic organisms were monitored for 12 weeks during frozen storage at -18^{0}C.

6.2   Materials and method

6.2.1 *Propagation of yogurt and probiotic cultures*

Yogurt and probiotic organisms were obtained from the Division of Food Science and Technology, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Highett, Victoria, Australia; Mauri Laboratory (now, Gist-brocades Pty Ltd) and Chr. Hansen Pty. Ltd. A total of 41 strains were used for initial study including 10 strains of *Streptococcus thermophlius* (ST 1, ST 2, ST 2000, ST 2002, ST 2008, ST 2010, ST 2013, ST 2014, Ts1, ST WJ7), 7 strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB 4, LB 2501, LB 2505, LB 2515, LB 2517, LB

2519,LB Wj7), 13 strains of *Lactobacillus acidophilus* (LA 2400, LA 2401, LA 2404, LA 2405, LA 2406, LA 2411, LA 2412, LA 2414, LA 2415, MOLA2, PLA2, BDLA2, MJLA1) and 11 strains of bifidobacteria (BB 1912, BB 1920, BB 1941, BB 1978, BB 5089, BB 5092, BB 5094, BB 5095, BB 20099, BB 20210, BDBB2).

6.2.2 Screening of strains of yogurt and probiotic bacteria for selecting strains that survive freezing

The organisms were propagated three times before using for freeze tolerance studies. Ten millilitre of sterile RSM was taken in Pyrex test tubes and inoculated with 2% culture followed by incubation at 37^0C till a pH 4.5 was attained. The samples were transferred to a refrigerator till the temperature dropped to 4^0C followed by freezing for 4 h at -25^0C using a glycol bath. The temperature dropped to -25^0C in 15 min. Samples were thawed using tap water ($\sim20^0C$). Bacterial counts before and after freezing were determined.

6.2.3 Survival of probiotic bacteria at various sugar levels

Ten millilitre of RSM containing 8 and 16% sucrose were inoculated with 2% each of *L. acidophilus* and bifidobacteria and incubated at 37^0C till a pH of 4.5 was attained. Samples were transferred to a refrigerator till the temperature dropped to 4^0C and then frozen at -25^0C for 4 h using a glycol bath. The temperature dropped to -25^0C in 18 min. Samples were thawed using tap water ($\sim20^0C$). Bacterial counts before and after freezing were determined as before.

6.2.4 Survival of yogurt and probiotic bacteria under acidic and frozen conditions

Samples of RSM were adjusted to pH 6.2 (control), 5.0, 4.5 and 4.0 with lactic acid (BDH Laboratory Supplies, Poole, England). One millilitre of each culture of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus,* and *Bifidobacterium* spp. was added to 9 mL of the pH adjusted RSM in separate test tubes. The test tubes were kept in a cold room (4^0C) for 5 h followed by freezing at -25^0C for 4 h using a glycol bath. The temperature dropped to -25^0C in 15 min. Samples were thawed using tap water (~20^0C). Bacterial counts before and after freezing were determined as before.

6.3   Results and discussion

6.3.1 *Composition*

The protein content of the fermented frozen dairy dessert was ~3.75%. The total solids contents were in the range of 32.5-32.8%. The titratable acidity ranged between 0.74% and 0.76%. The fat content was 10.3%, while the sugar content was 16.8%. The pH ranged between 4.51 and 4.59. The overrun of the product made with a bench top freezer was 18% and that made with Crepaco freezer was 100%.

6.3.2 Effect of freezing on survival of yogurt and probiotic bacteria

The effect of freezing on survival of yogurt and probiotic bacteria before and after freezing is shown in Tables 6.1 and 6.2. There was no major difference in the viable counts of *S. thermophilus* (Table 6.1), *L. delbrueckii* ssp. *bulgaricus* (Table 6.2), *L.*

acidophilus (Table 6.3) and bifidobacteria (Table 6.4) before and after freezing at -25^{0}C for 4 h.

6.3.3 *Effect of sugar levels on survival of probiotic bacteria before and after freezing*

The effect of sucrose levels on survival of yogurt and probiotic bacteria before and after freezing is shown in Tables (6.3 – 6.6). At 8 and 16% sucrose levels, *L. acidophilus* showed minor differences after freezing at -25^{0}C for 4 h. Counts of *L. acidophilus* 2414 were reduced by about 1 log cycle. *L. acidophilus* strains 2404 and 2411 were reduced by about 2 log cycles at both sugar levels. Counts of bifidobacteria 5092 were reduced by about 1 log cycle after freezing both at 8 and 16% sucrose levels. However, other strains did not show any decrease in cell counts.

6.3.4 *Survival of yogurt and probiotic bacteria under acidic and frozen conditions*

The survival of yogurt and probiotic bacteria before and after freezing under acidic and frozen conditions is shown in Tables (6.7 – 6.22). Counts of *S. thermophilus* 2013 were reduced by about 1 log cycle after freezing at pH 4.5 and by about 2 log cycles at pH 4.0. Counts of *L. delbrueckii* ssp. *bulgaricus* 2515 were reduced by about 1 log cycle after freezing at pH 4.0. Counts of *L. acidophilus* MJLA1 were reduced by about 1 log cycle at pH 4 and pH 4.5. Conversely, all five strains of bifidobacteria (1912, 1920, 1978, 20099 and BDBB2) did not show any decrease in counts at various pH levels.

6.4   Conclusion

All strains screened for freeze tolerance remained viable before and after freezing and at 8 and 16% sugar levels. Of the various pH levels studied, pH 4.5 and 4.0 were found to be detrimental for *S. thermophilus* 2013, *L. delbrueckii ssp. bulgaricus* 2515 and *L. acidophilus* MJLA1, while rest of the organisms were not affected by pH.
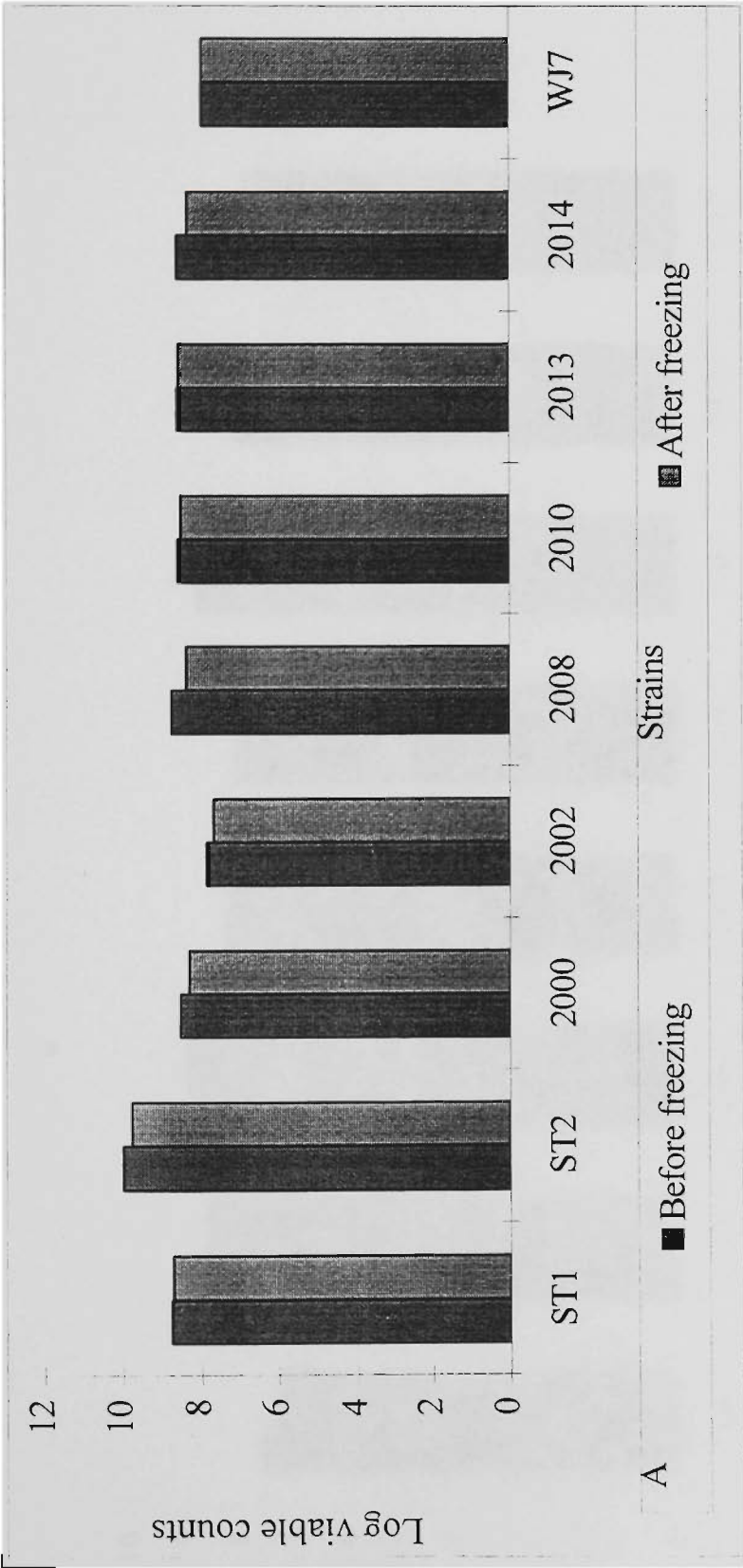
Figure 6.1 Effects of freezing on survival of *S. thermophilus* before and after freezing at -25^{0}C for 4h.
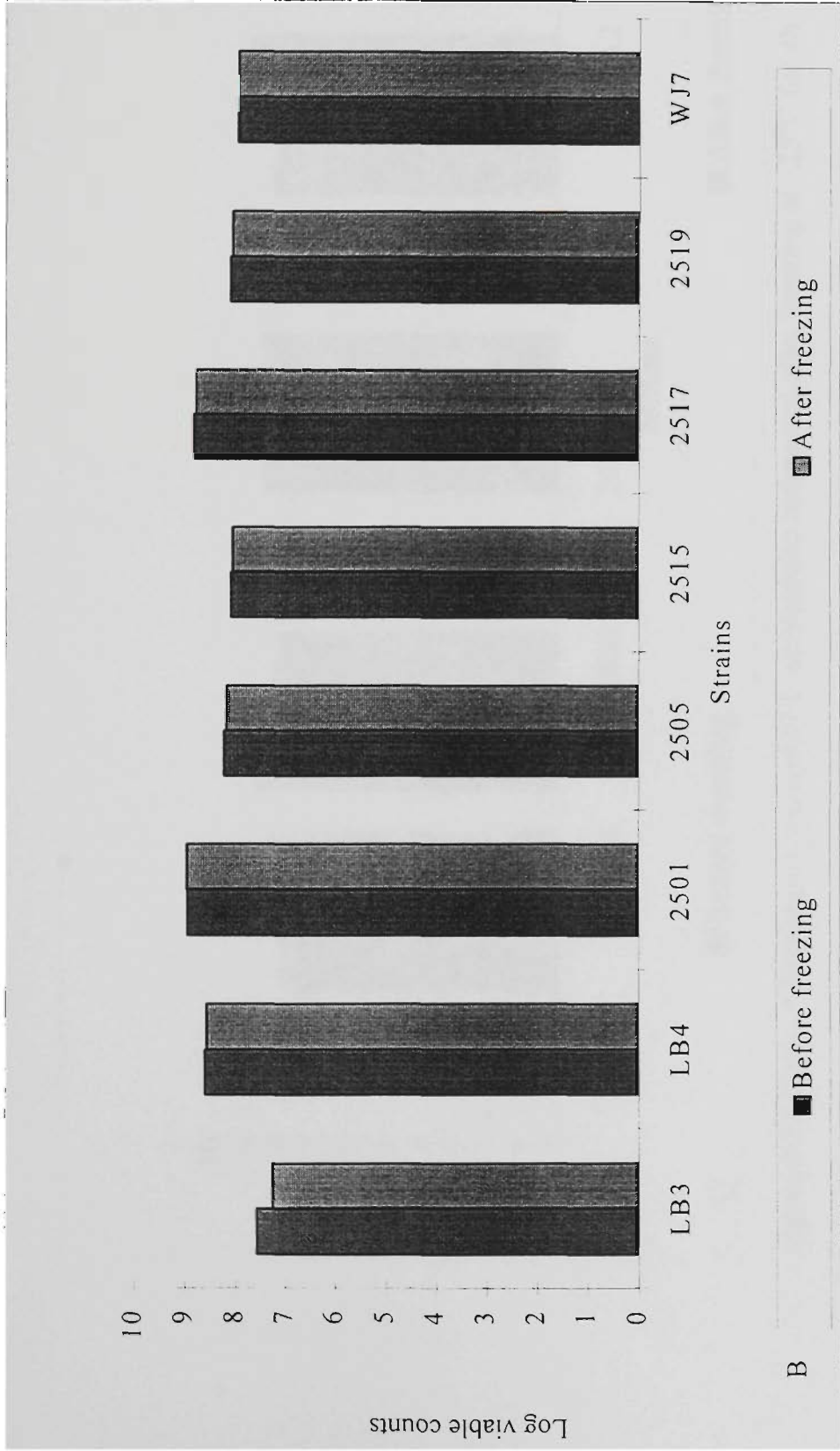
Figure 6.2 Effects of freezing on survival of *L. delbrueckii* ssp. *bulgaricus* before and after freezing at -25^0C for 4h.
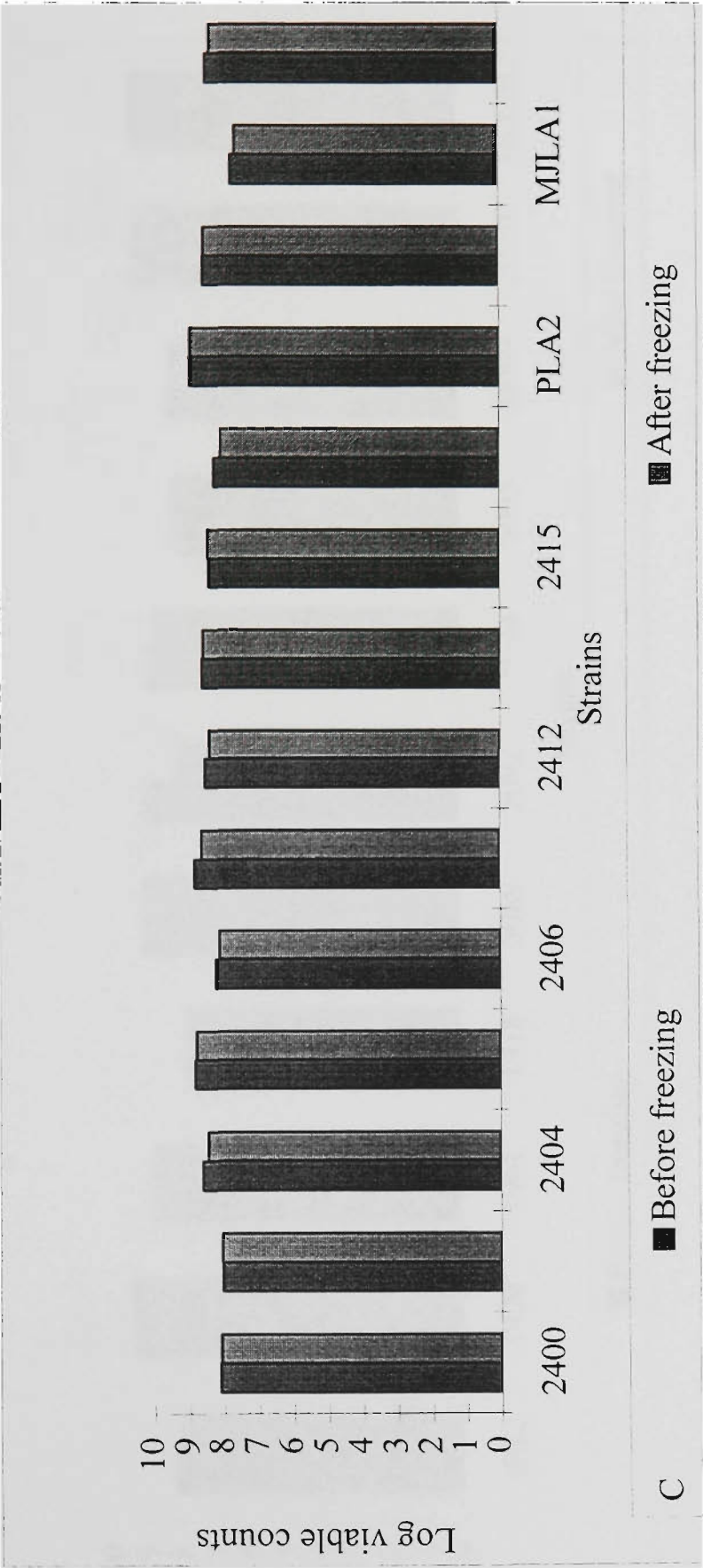
Figure 6.3 Effects of freezing on survival of *L. acidophilus* before and after freezing at -25^0C for 4h.

106
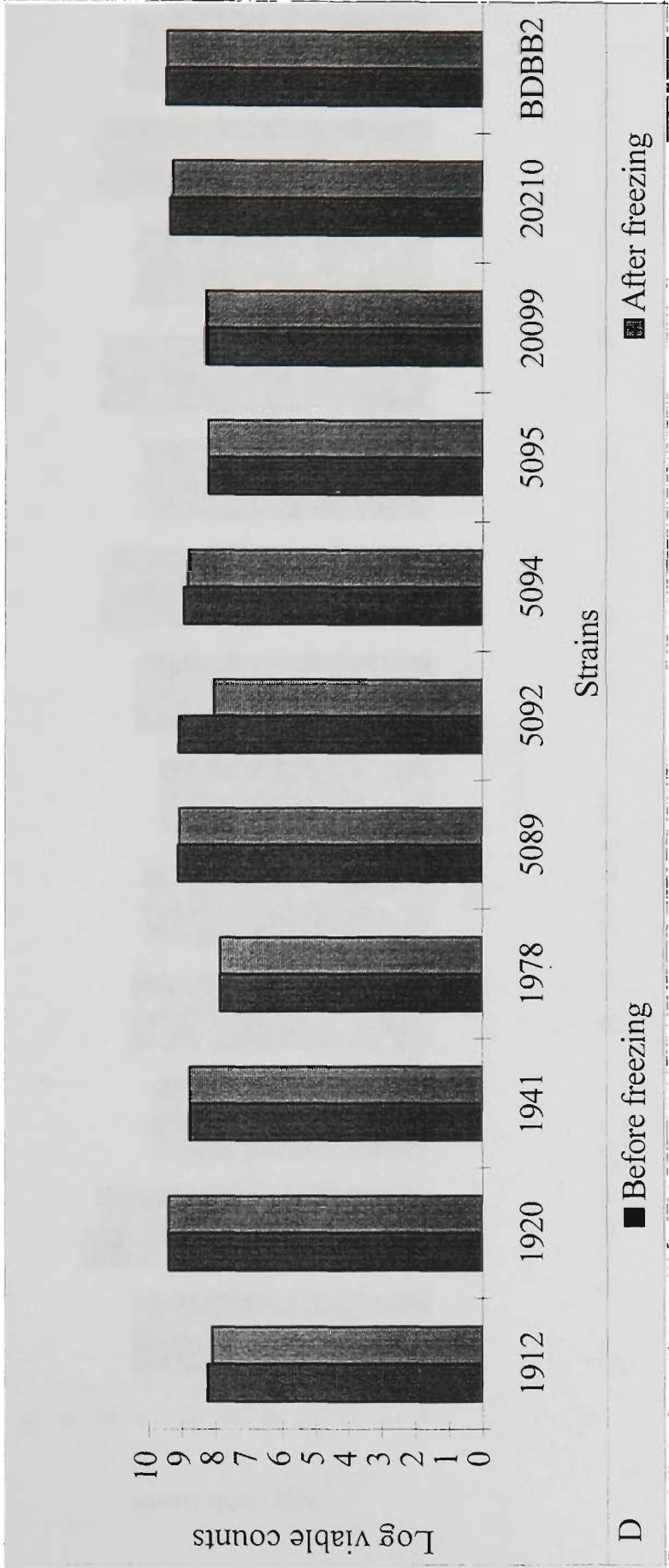
Figure 6.4 Effects of freezing on survival of bifidobateria before and after freezing at -25^{0}C for 4h.

Figure 6.5 Effects of freezing on survival of *L. acidophilus* before and after freezing at -25^0C for 4h at 8 and 16 % sugar levels.

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Fig.6 6 Effects of freezing on survival of bifidobacteria before and after freezing at –25ºC for 4h at 8 and 16% sugar levels.

109
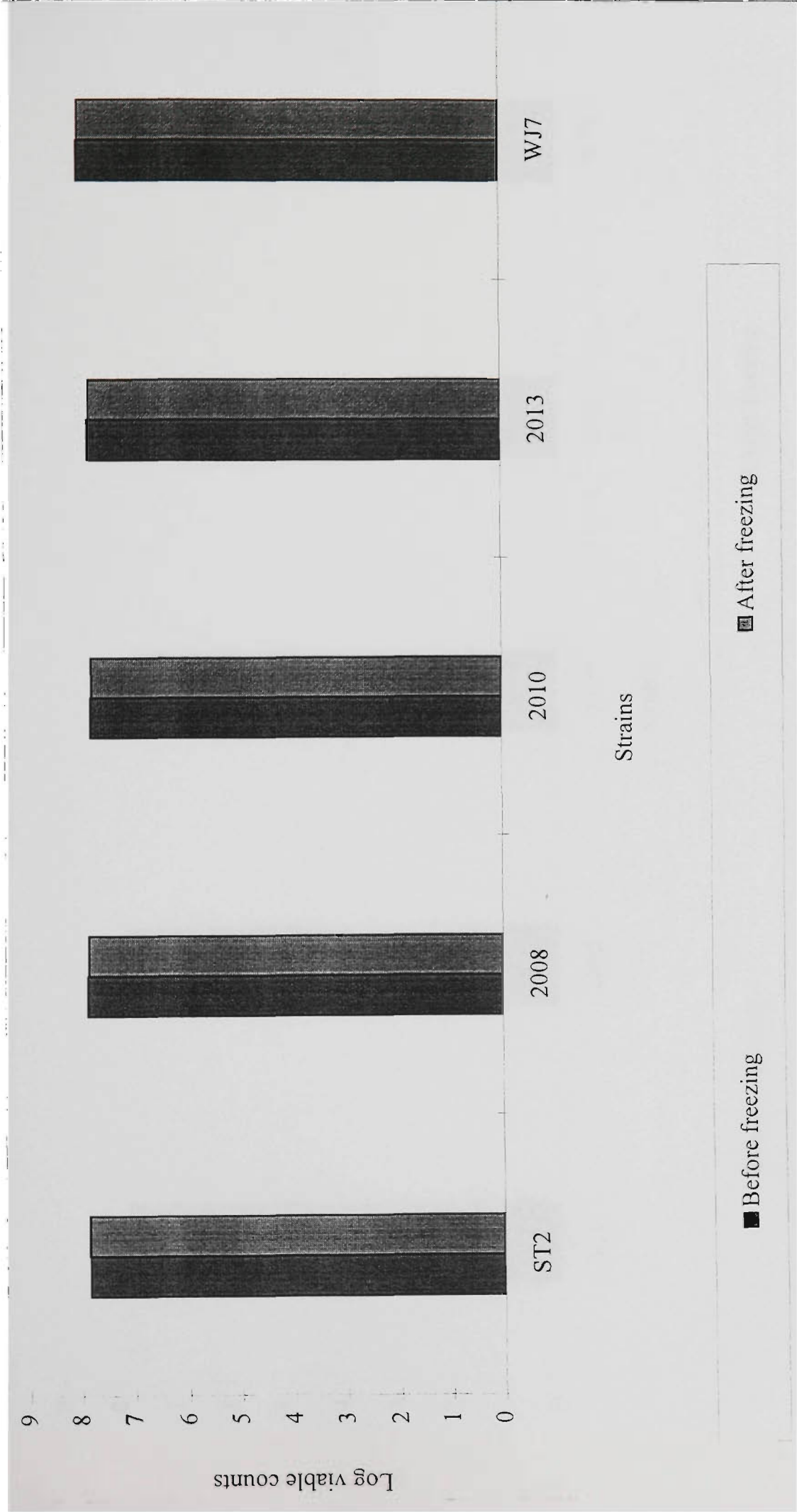
Fig 6.7 : Viability of *S. thermophilus* at pH 6.2 before and after freezing at -25 deg C.
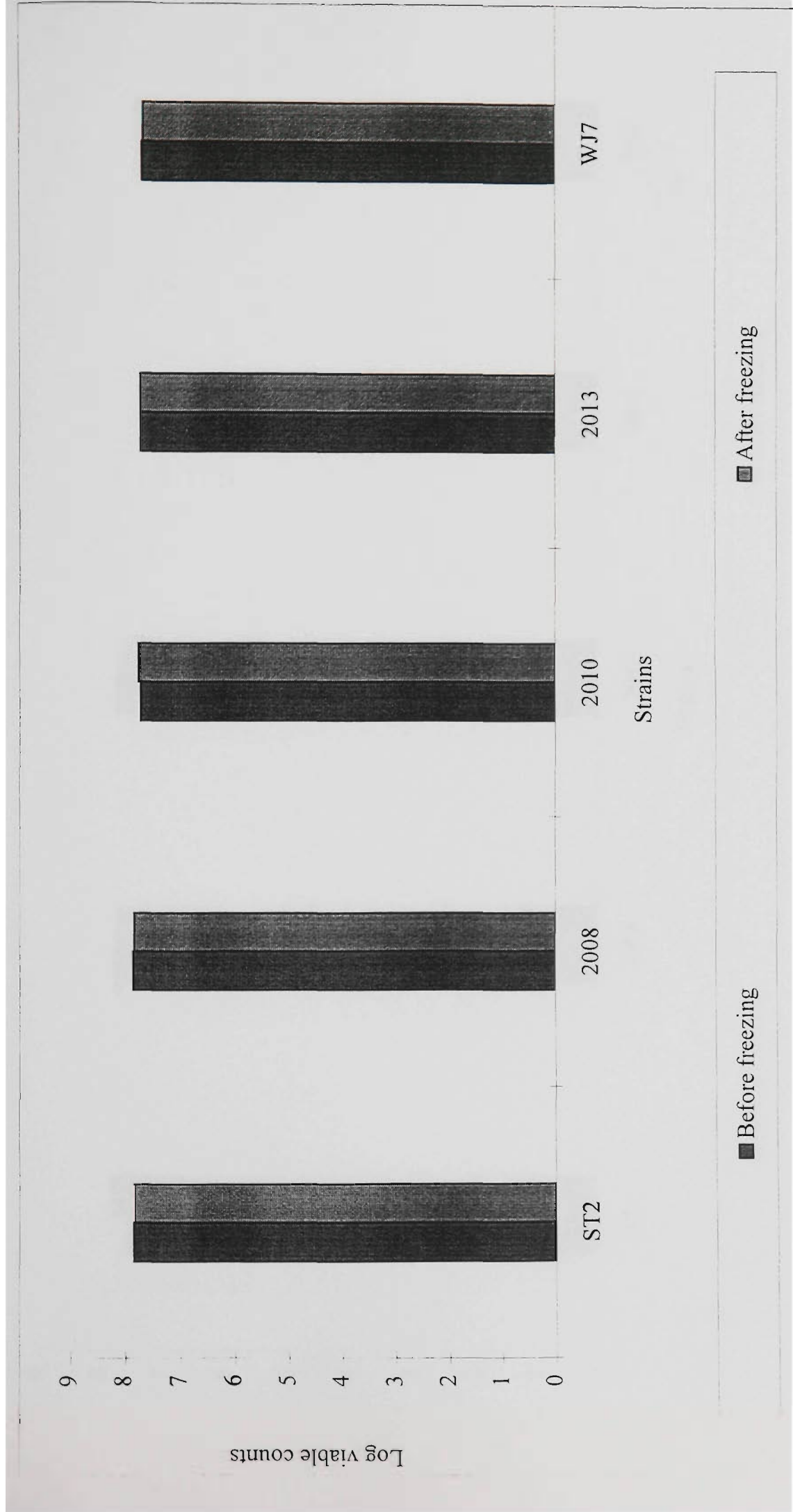
Fig 6.8 : Viability of *S. thermophilus* at pH 5.0 before and after freezing at -25 deg C.
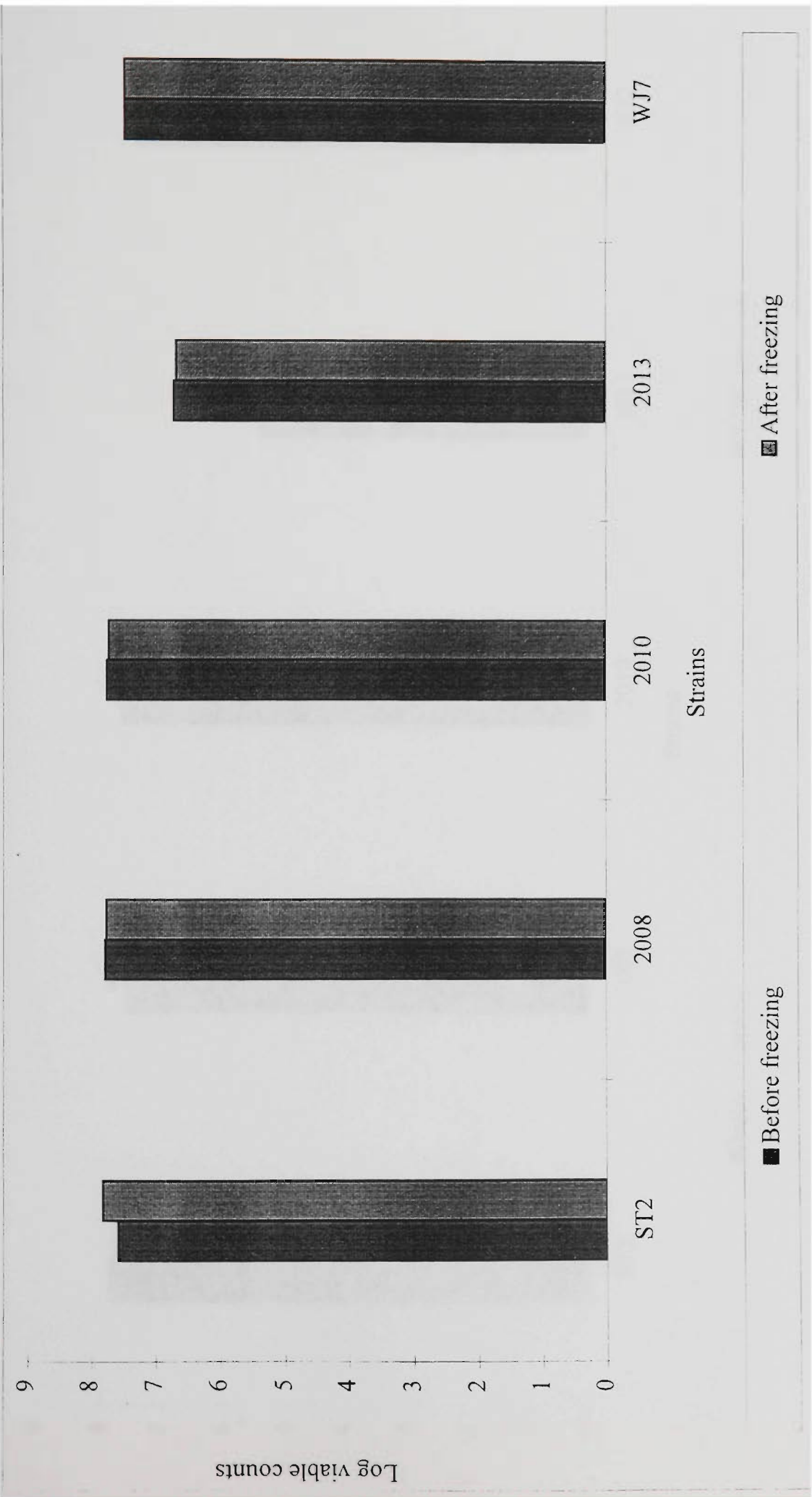
C   Fig 6.9 : Viability of *S. thermophilus* at pH 4.5 before and after freezing at -25 deg C.
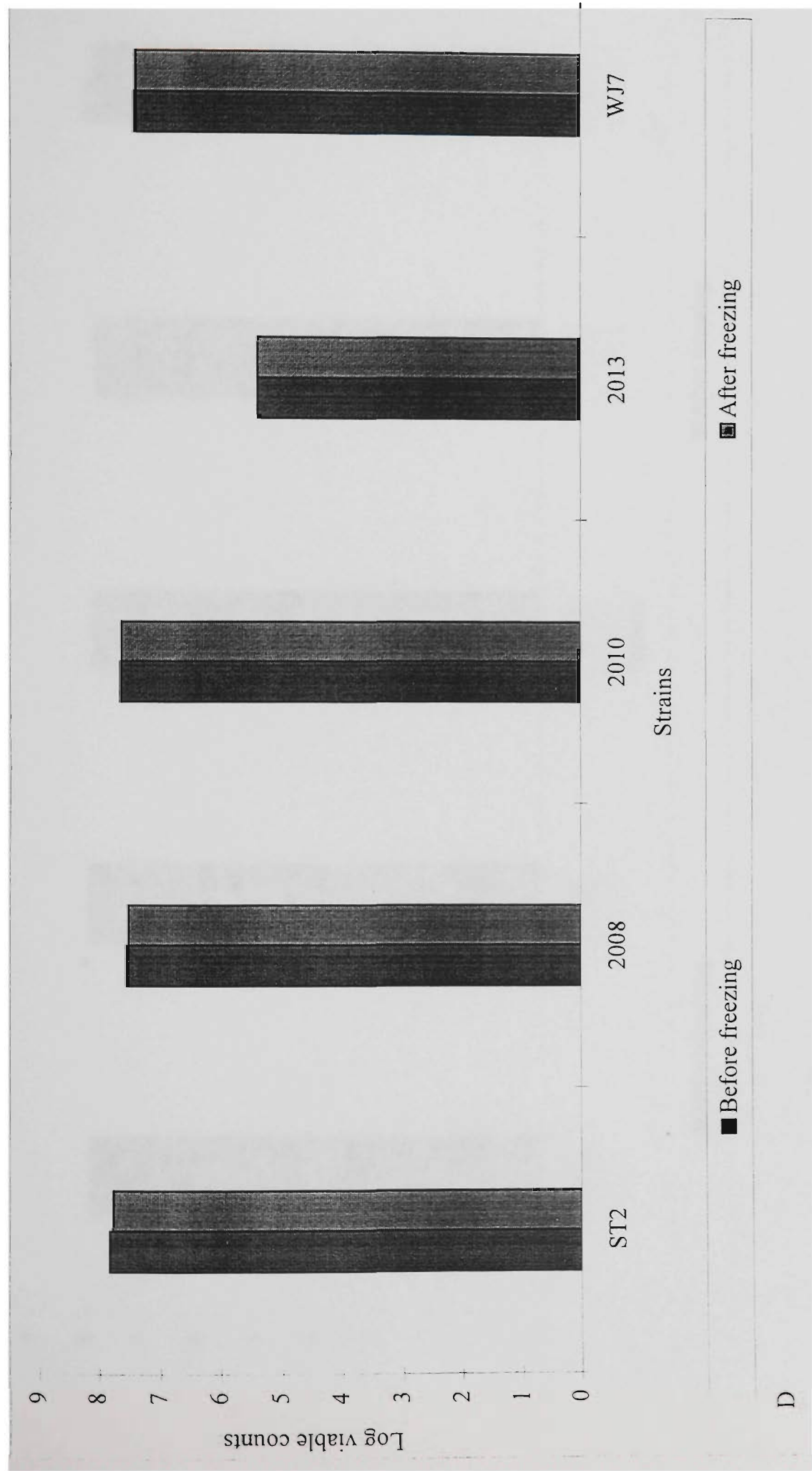
Fig 6.10 : Viability of *S. thermophilus* at pH 4.0 before and after freezing at -25 deg C.
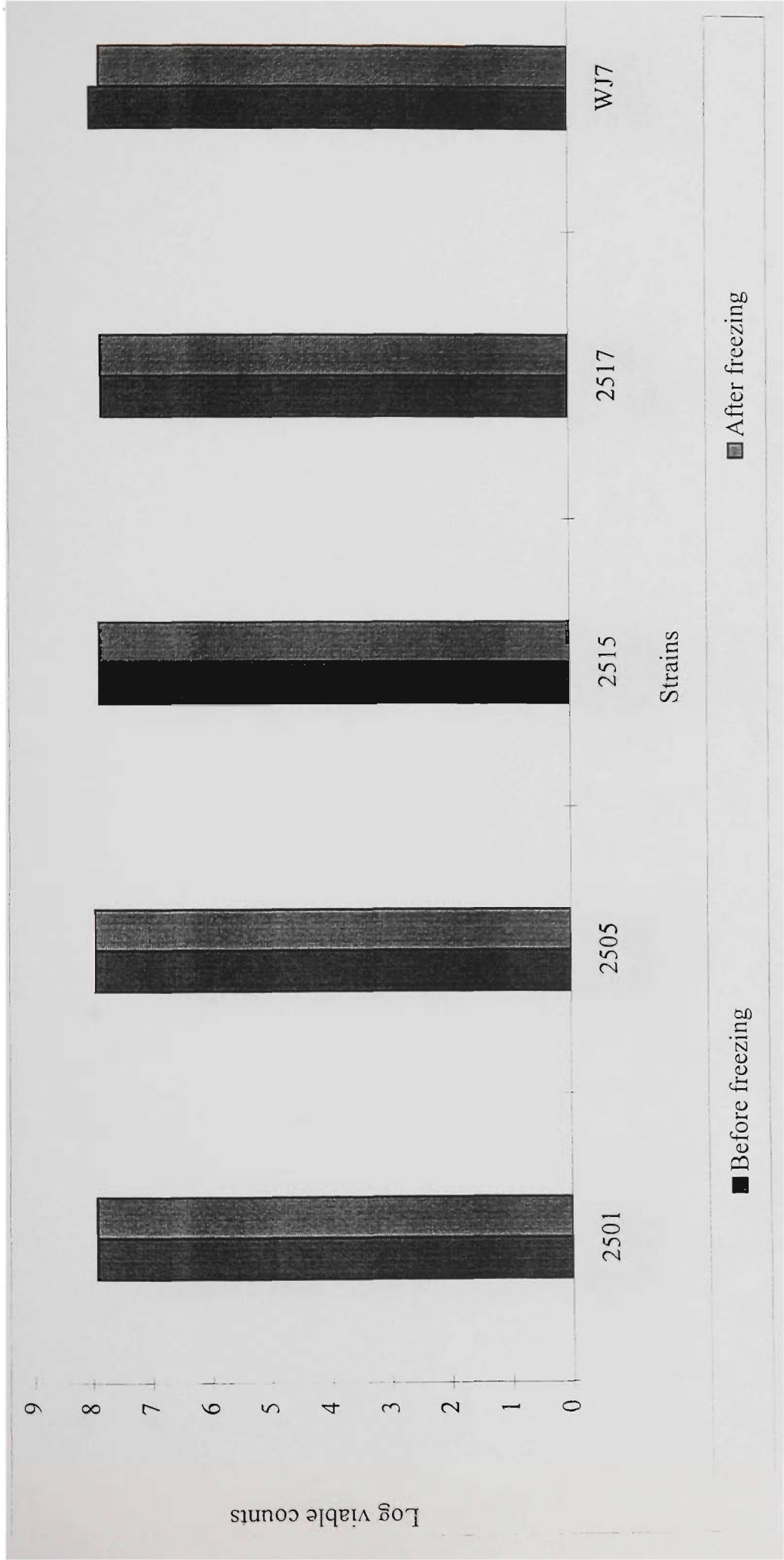
Fig 6.11 : Viability of *L. delbrueckii* ssp. *bulgaricus* at pH 6.2 before and after freezing at -25 deg C.
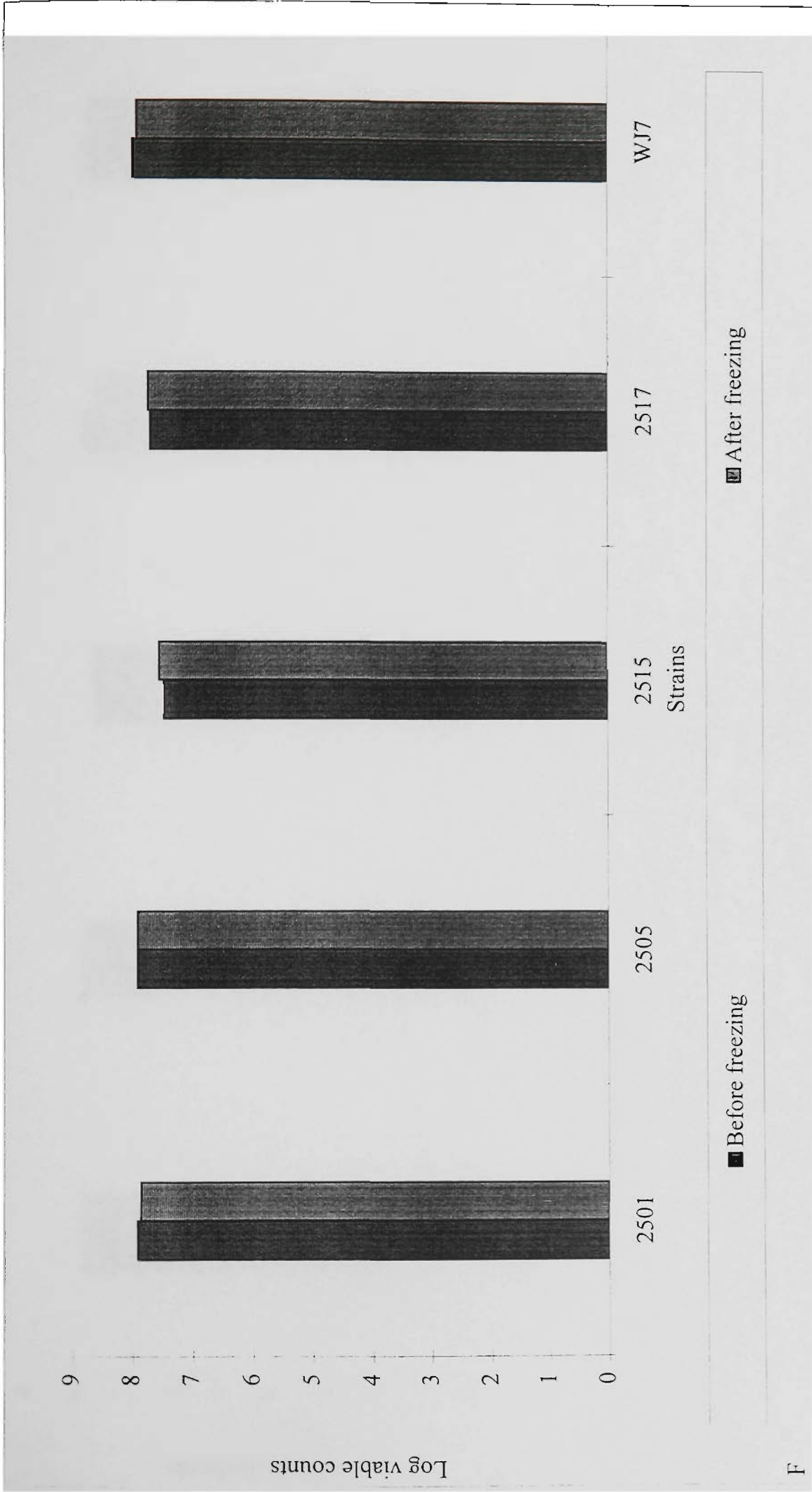
Fig 6.12 : Viability of *L. delbrueckii* ssp. *bulgaricus* at pH 5.0 before and after freezing at -25 deg C.
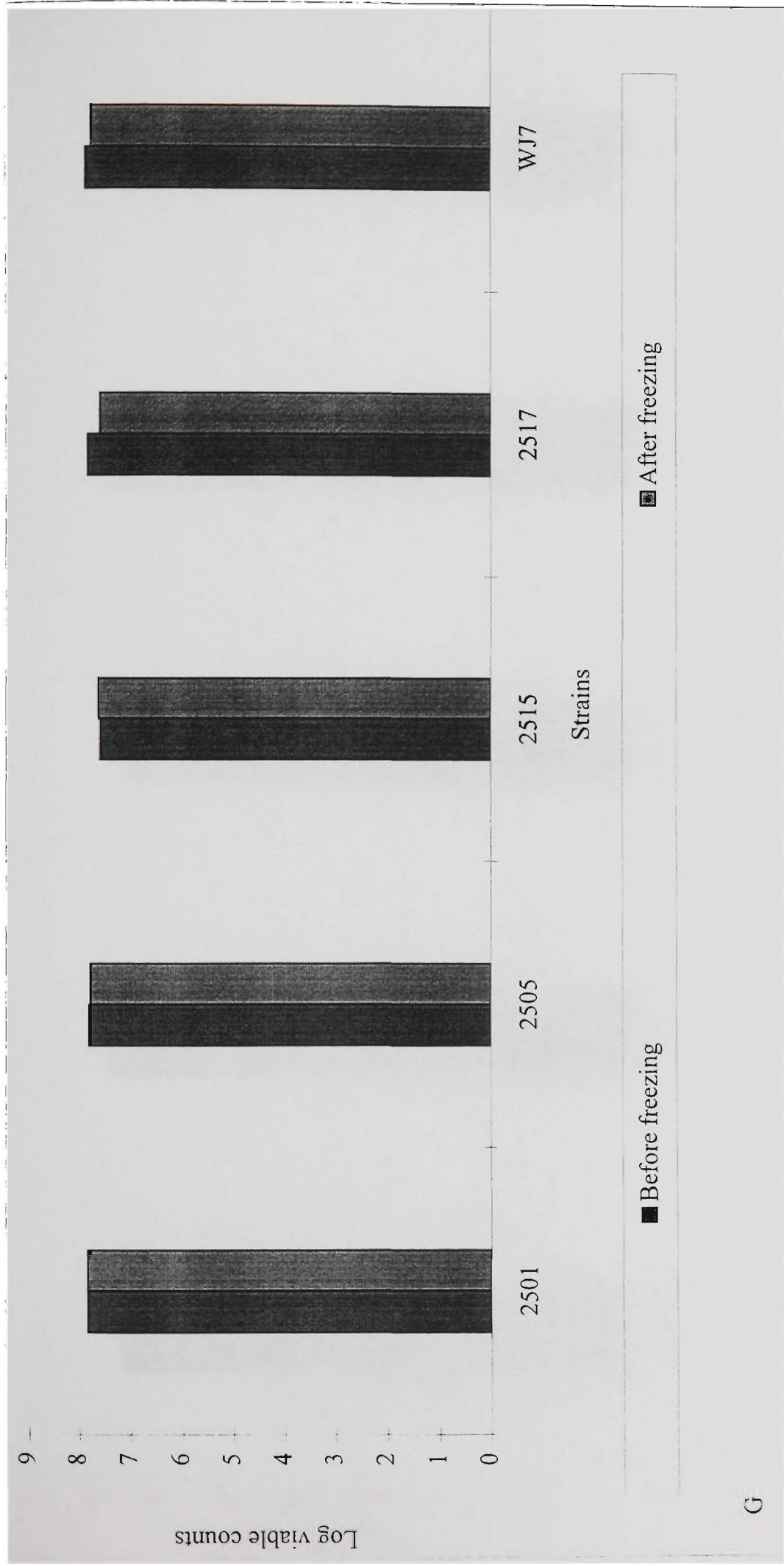
Fig 6.13 : Viability of *L. delbrueckii* ssp. *bulgaricus* at pH 4.5 before and after freezing at -25 deg C.
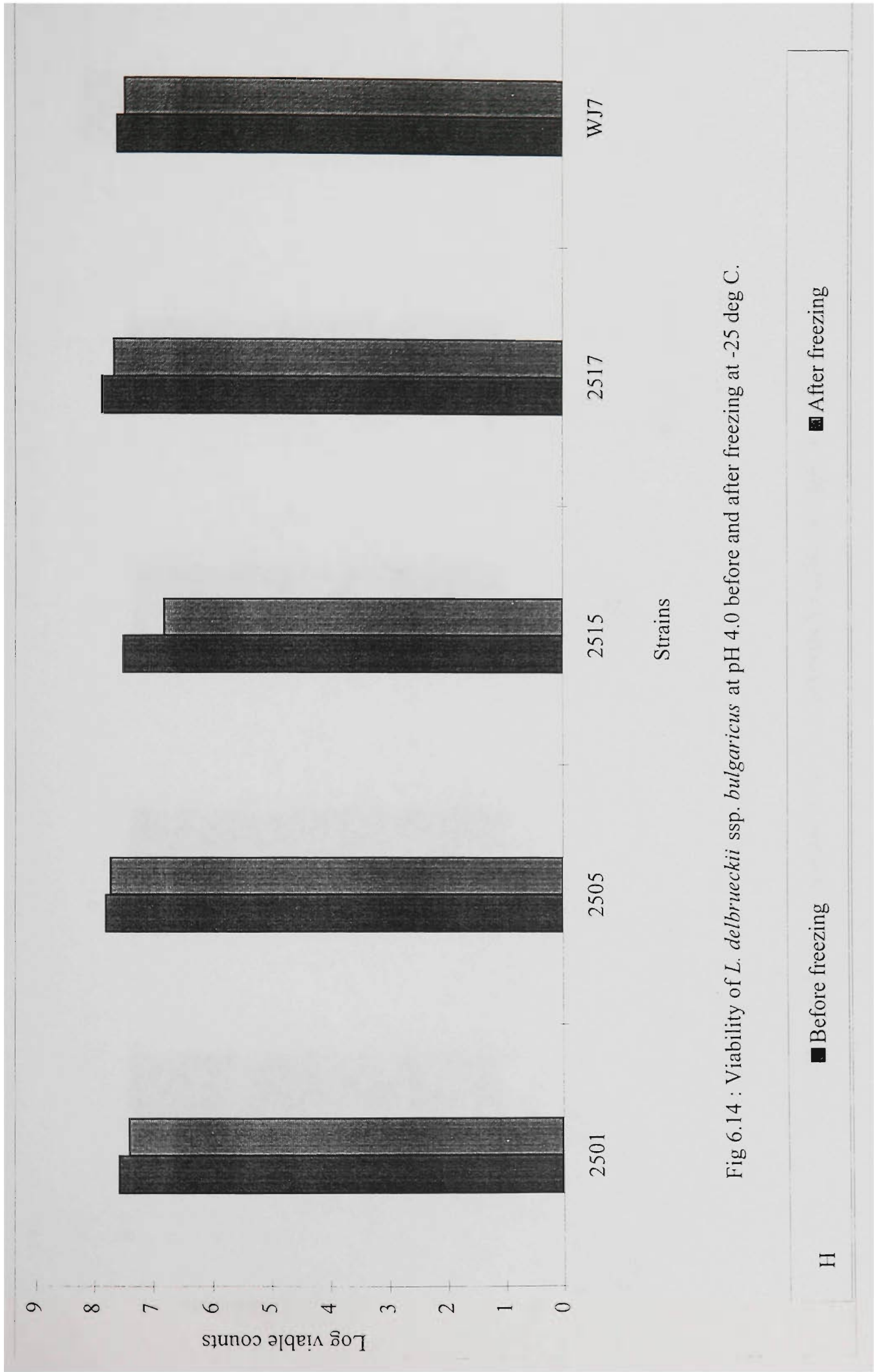
Fig 6.14 : Viability of *L. delbrueckii* ssp. *bulgaricus* at pH 4.0 before and after freezing at -25 deg C.

■ Before freezing   ■ After freezing
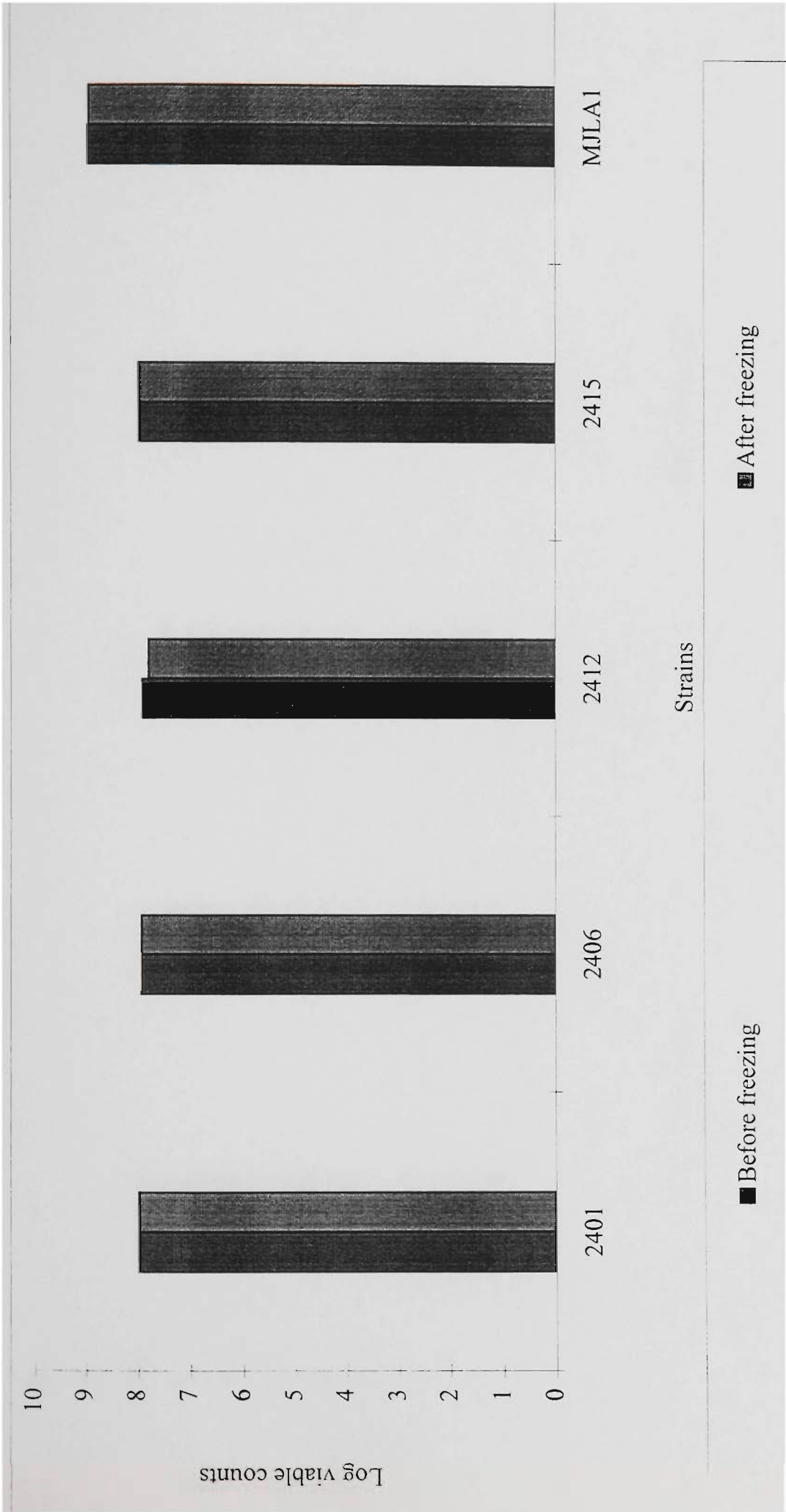
Fig 6.15 : Viability of *L. acidophilus* at pH 6.20 before and after freezing at -25 deg C.

A
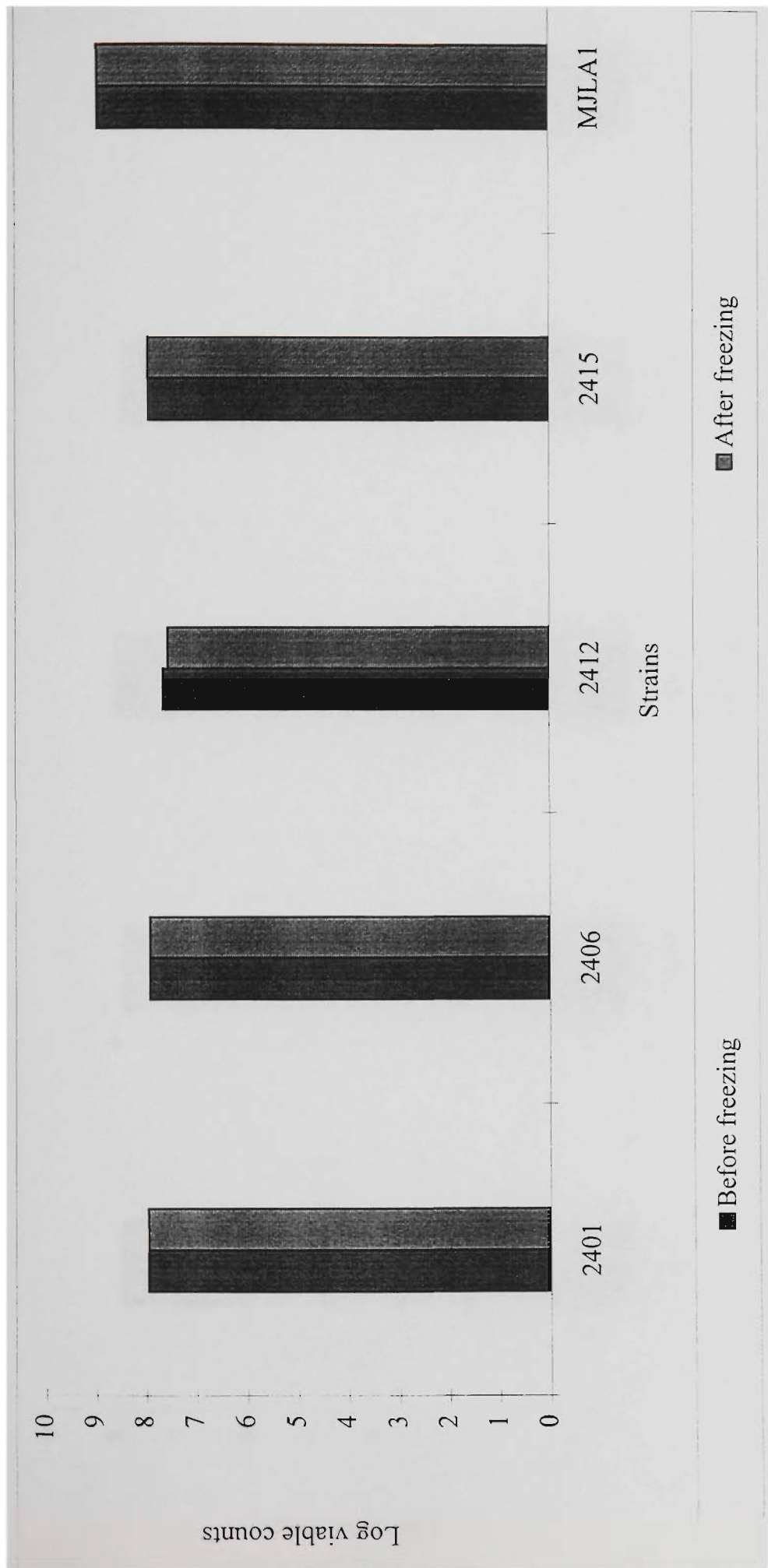
Fig 6.16 : Viability of *L. acidophilus* at pH 5.0 before and after freezing at -25 deg C.

B
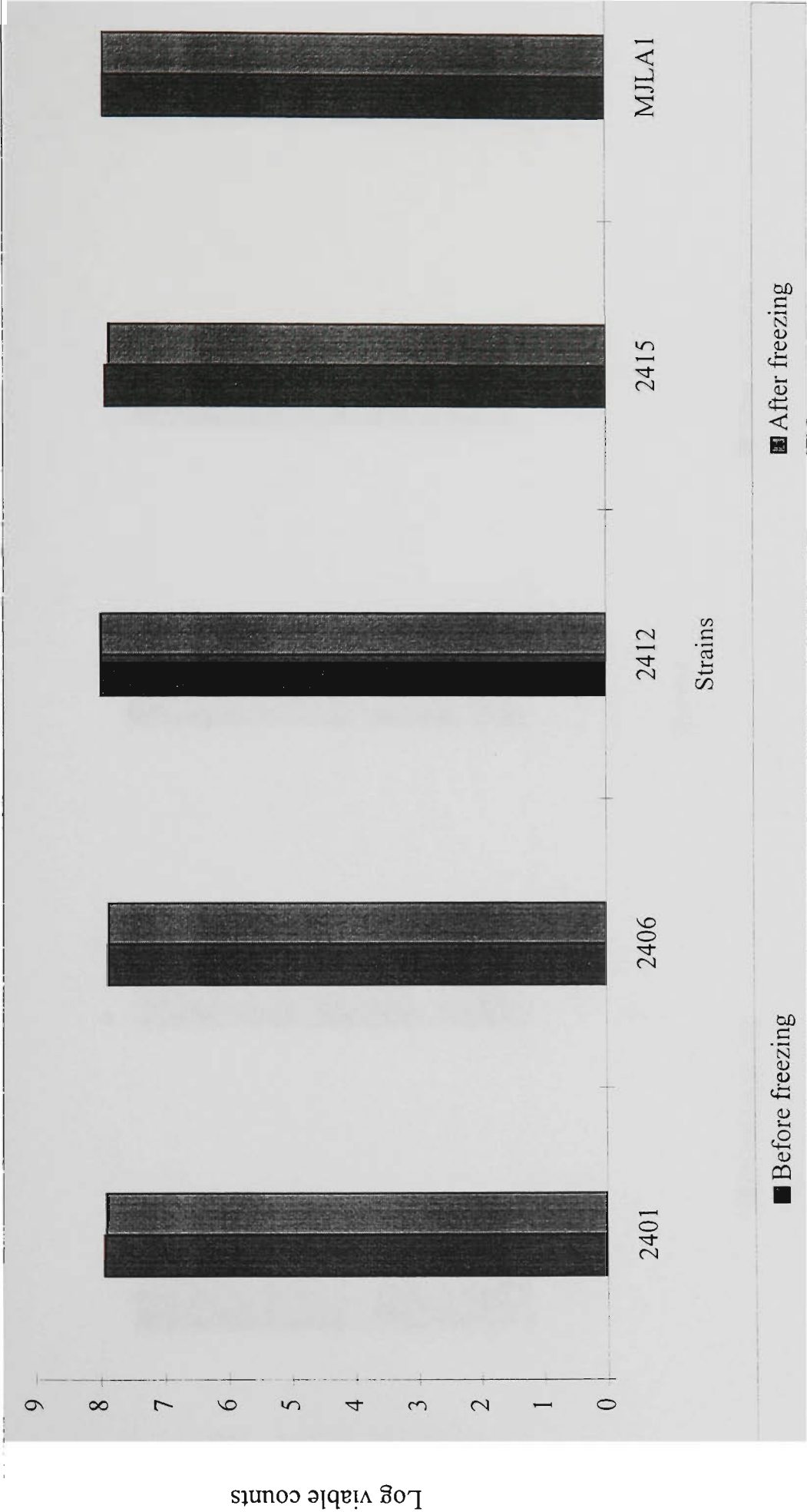
Fig 6.17 : Viability of *L. acidophilus* at pH 4.50 before and after freezing at -25 deg C.

C
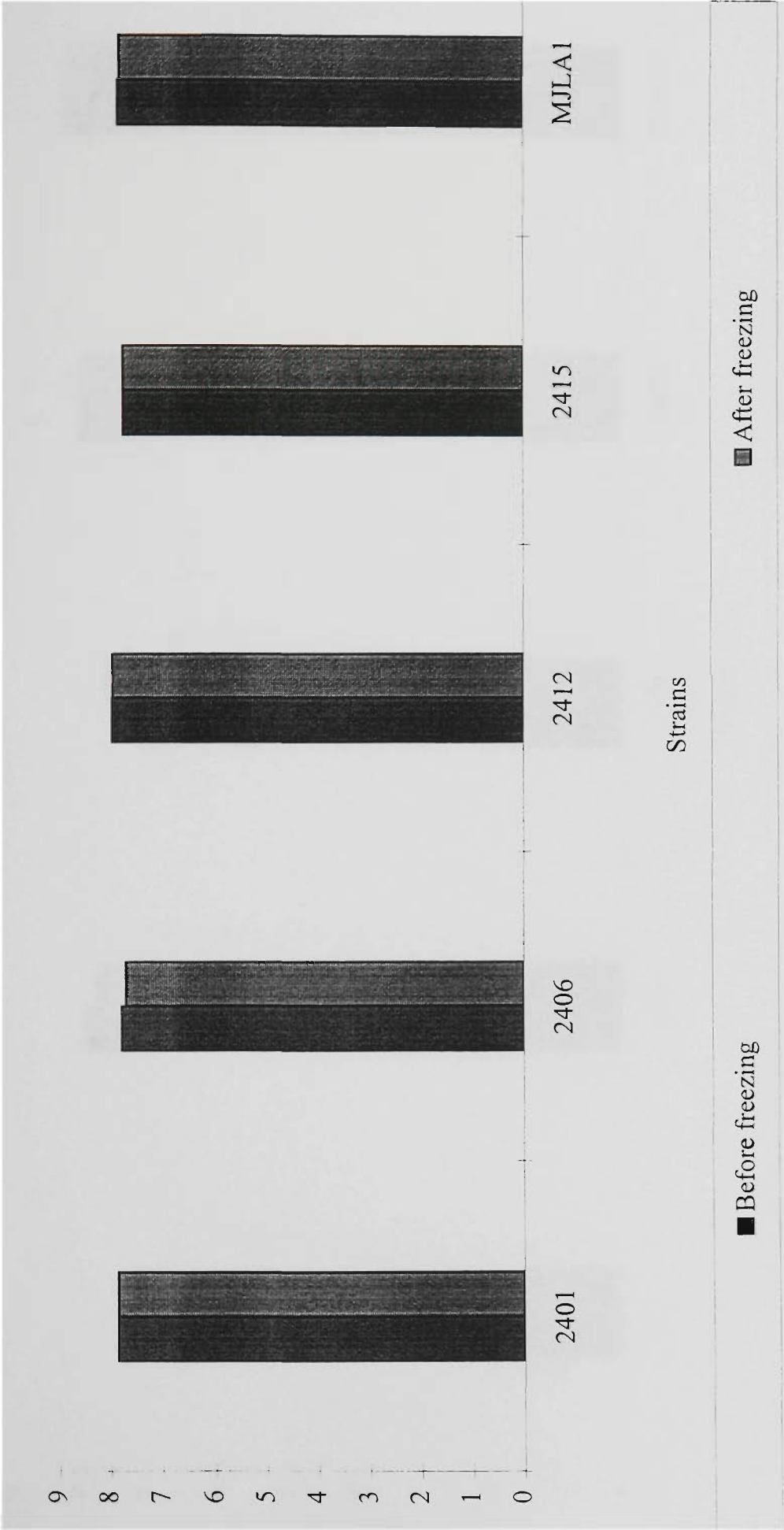
Fig 6.18 : Viability of *L.acidophilus* at pH 4.0 before and after freezing at -25 deg C.

D
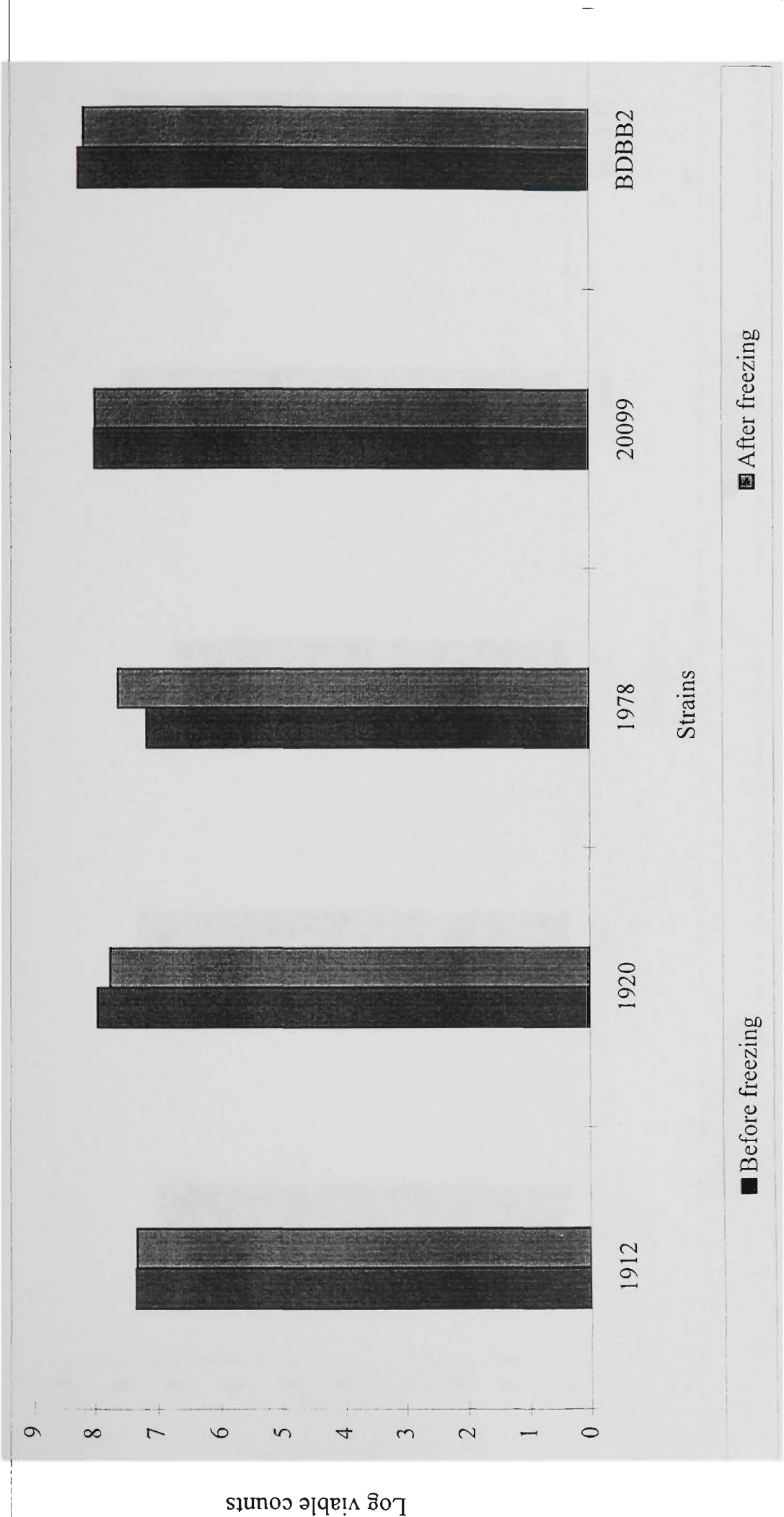
Fig 6.19 : Viability of bifidobacteria at pH 6.20 before and after freezing at -25 deg C.
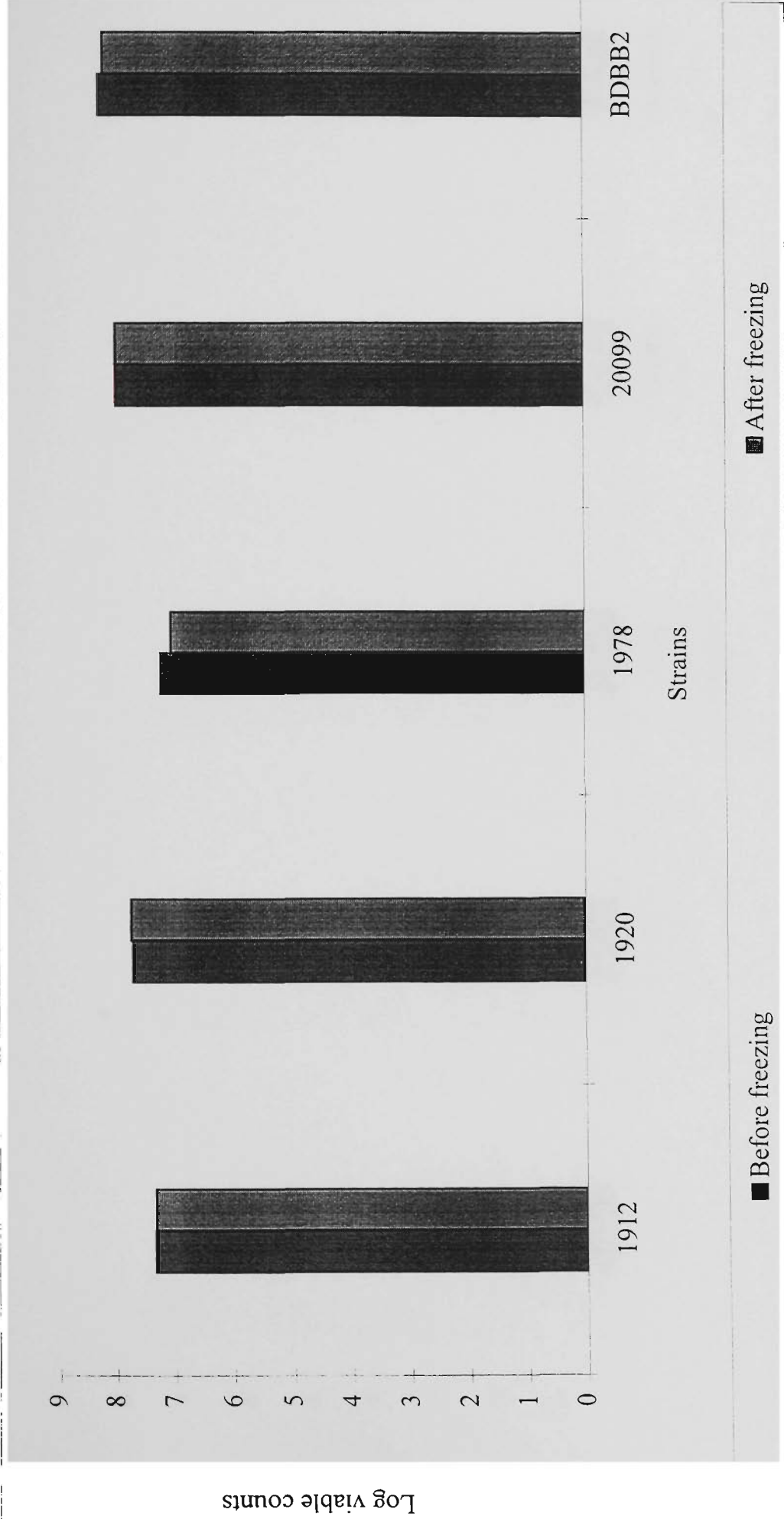
Fig 6.20 : Viability of bifidobacteria at pH 5.0 before and after freezing at -25 deg C.
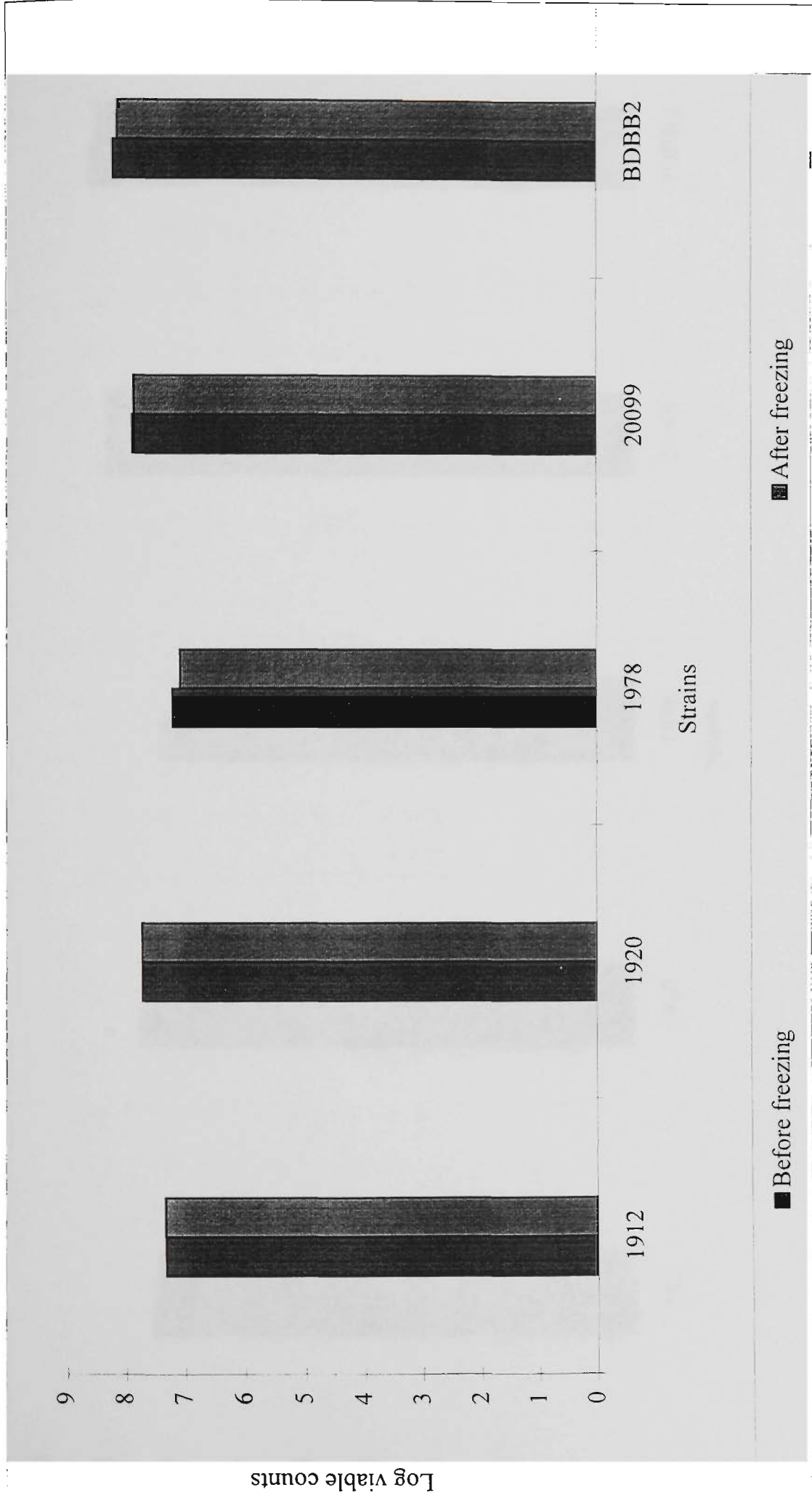
Fig 6.21 : Viability of bifidobacteria at pH 4.50 before and after freezing at -25 deg C.

G
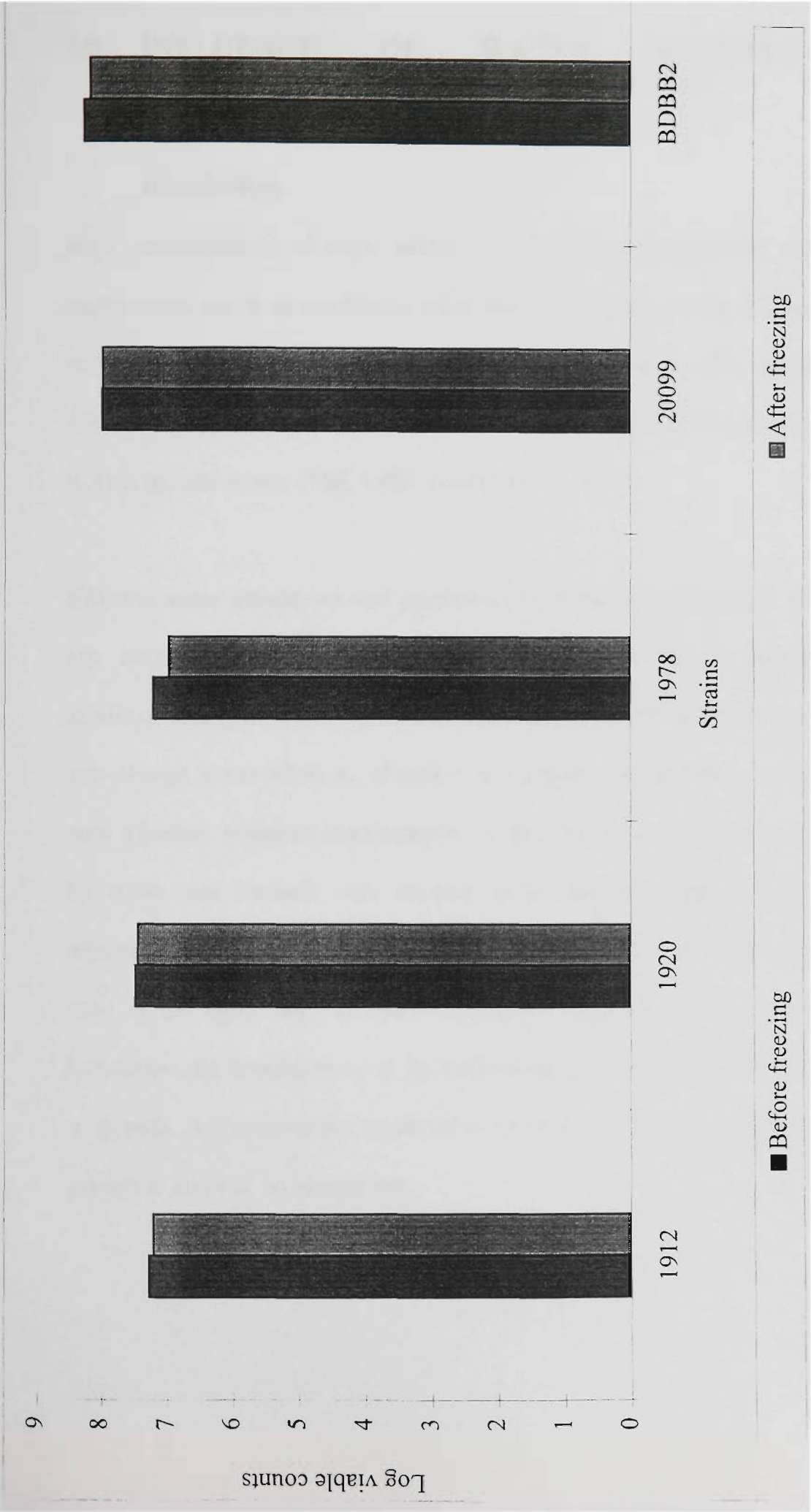
Fig 6.22 : Viability of bifidobacteria at pH 4.0 before and after freezing at -25 deg C.

7.0 INFLUENCE OF WATER ACTIVITY ON PRODUCTION OF PROBIOTIC YOGURT

7.1 Introduction

High concentrations of sugar added to milk before fermentation with yogurt starters may result in conditions inhibitory to bacterial growth, leading to long fermentation times and poor acidity development (Tramer, 1973; Zourari *et al.*, 1992). This is due to both adverse osmotic effects of the solutes in milk and low a_w (Larsen and Anon, 1989, 1990; Zourari *et al.*, 1992).

Effect of water activity on acid production by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* was studied by Larsen and Anon (1989. A decrease in a_w inhibited acid production by both organisms. Minimum a_w for acid production was always lower when a_w of milk was adjusted with glucose or sucrose than with glycerol. Moisture requirements for the growth and metabolite production by lactic acid bacteria were studied by Troller and Stinson (1981). Media adjusted with sucrose generally were inhibitory than those in which glycerol was used as the humectant. Titratable acidity although not related to the type of humectant, did depend on a_w of the medium and was directly related to the extent of growth. Acid production appeared to be related to the presence and extent of growth at all of a_w levels studied.

7.2   MATERIALS AND METHODS

7.2.1   *Yogurt bacteria and probiotic bacteria*

7.2.2   *Manufacture of yogurt*

Yogurt was manufactured as outlined in Fig 7.1. Five batches of yogurt (1 L size) were made using 12% reconstituted skim milk (RSM).

Experiments were carried each using four levels of sugar (4, 8, 12 and 16%) added to RSM. Yogurt made without added sugar was used as a control (batch 1). The mix was heated at 85^0C for 30 min, cooled to 42^0C, and yogurt bacteria (*S. thermophilus* ST WJ7 and *L. delbrueckii* ssp. *bulgaricus* LB WJ7) and probiotic bacteria (*L. acidophilus* LA 2415 and bifidobacteria BB 1941) were added at the rate of 0.5% each. The organisms were allowed to mix for 5 min using a sterile magnetic stirrer. The inoculated mix was filled in 100 millilitre plastic containers and incubated at 42^0C till pH 4.5 was attained. The yogurts were transferred to a cold room and kept overnight at 4^0C. Microbiological and chemical analysis were monitored for 24 hrs and then for 30 days at 10-day intervals.

7.3   RESULTS AND DISCUSSION

7.3.1 *pH and titratable acidity*

The initial pH of reconstituted skim milk ranged between 6.25 to 6.36 and the final pH after fermentation ranged from 4.45 to 4.58. The fermentation time to decrease pH to 4.5 ranged between 3.25 h to 22.5 h. As the sugar concentration increased, the fermentation time increased. For the batch containing 16% sugar,

the fermentation time was 22.5h. The final pH by the end of storage period was 4.50-4.44 (Figure 7.4).

High concentrations of sugar added to milk before fermentation with yogurt starters may result in conditions inhibitory to bacterial growth, leading to long fermentation times and poor acidity development (Tramer, 1973; Zourari *et al.*, 1992). This is due to both adverse osmotic effects of the solutes in milk and low a_w (Larsen and Anon, 1989, 1990; Zourari *et al.*, 1992).

Figure 7.3 shows the water activity of all the five batches of yogurt. The water activity decreased as the percentage of sugar increased. Fajardo-Lira *et al.* (1997) studied the effect of water activity on exopolysaccharide producing or non-producing starters and the authors concluded that sucrose had an inhibitory effect on fermentation process. Effect of water activity of milk on acid production by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* was studied by Larsen and Anon (1989). Decrease of a_w inhibited acid production by both organisms. Minimum a_w for acid production was always lower when a_w of milk that was adjusted with glucose or sucrose than with glycerol.

Figure 7.2 shows the total solids content in all the five batches of yogurt. The increase in total solid showed increased fermentation time. The total solids contents ranged between 12.53 to 27.56%.

7.3.2 Viable counts of yogurt and probiotic bacteria

Figure 7.5 shows the viable counts of yogurt bacteria and probiotic bacteria in the five batches of yogurt. In all the five batches, the initial counts (before incubation) of yogurt bacteria and probiotic bacteria were almost similar. The final counts (at pH 4.5) of *S. thermophilus* in batches containing 0, 4 or 8% sugar increased approximately by 3 log cycles, whereas in the batches with 12 and 16% sugar, the counts increased by 2 and 1 log cycle, respectively. There was approximately 2 log cycle increase in the counts of *L. delbrueckii* ssp. *bulgaricus* in the batches containing 0 and 4% sugar. The viable counts decreased in the other batches. The viable counts of *L. acidophilus* in the batches containing 0, 4 and 8% sugar were almost similar as the initial counts. The viable counts in the batches containing 12 and 16% sugar decreased by 2 and 5 log cycles. The viable counts of bifidobacteria in batches made with 0, 4 and 8% sugar increased by almost 1 log cycle, however, the counts decreased by 2 and 3 log cycles in the batches made with 12 and 16% sugar. The decrease in the counts of yogurt and probiotic bacteria clearly indicates the role of low water activity caused by addition of sugar.

Lankaputhra and Shah (1996) studied the survival of probiotic bacteria at various pH levels. Of the various strains studied, over 50% of the strains lost viability after 6 days of storage at pH <4.3. The minimum pH for survival of *L. acidophilus* and bifidobacteria is 5.5 (Holt, 1986). Thus a decline in their counts is expected to occur. However, the authors concluded that the strain La 2415 and BB 1941 survived pH 4.5. However our study revels that the strains, which

survive low pH and high acidity does not necessarily survive high levels of sugar and total solids.

High concentrations of sugar added to milk before fermentation with yogurt starters may result in conditions inhibitory to bacterial growth, leading to long fermentation times and poor acidity development (Tramer, 1973; Zourari *et al.*, 1992). This is due to both adverse osmotic effects of the solutes in milk and low a_w (Larsen and Anon, 1989, 1990; Zourari *et al.*, 1992).

7.4   Conclusion

Influence of water activity on fermentation of functional probiotic yogurt used in the manufacture of fermented frozen dairy desserts was studied. Higher levels of sugars (sucrose) lead to longer fermentation time and poor survival of yogurt and probiotic bacteria. Fermented frozen dairy desserts require incorporation of high levels of sugar, thus it may not be feasible to make such products.

Reconstituted skim milk (12%)

$\downarrow$

Tempering (40-45^{0}C)

$\downarrow$

Addition of sugar (sucrose)

(0, 4, 8, 12 or 16%)

$\downarrow$

Heat treatment (85^{0}C/30 min)

$\downarrow$

Cooling to incubation temperature (42^{0}C)

$\downarrow$

Addition of yogurt bacteria and probiotic bacteria, mixing

$\downarrow$

Filling in containers

$\downarrow$

Incubation at 42^{0}C till the pH reached 4.5

$\downarrow$

Storage at 4^{0}C
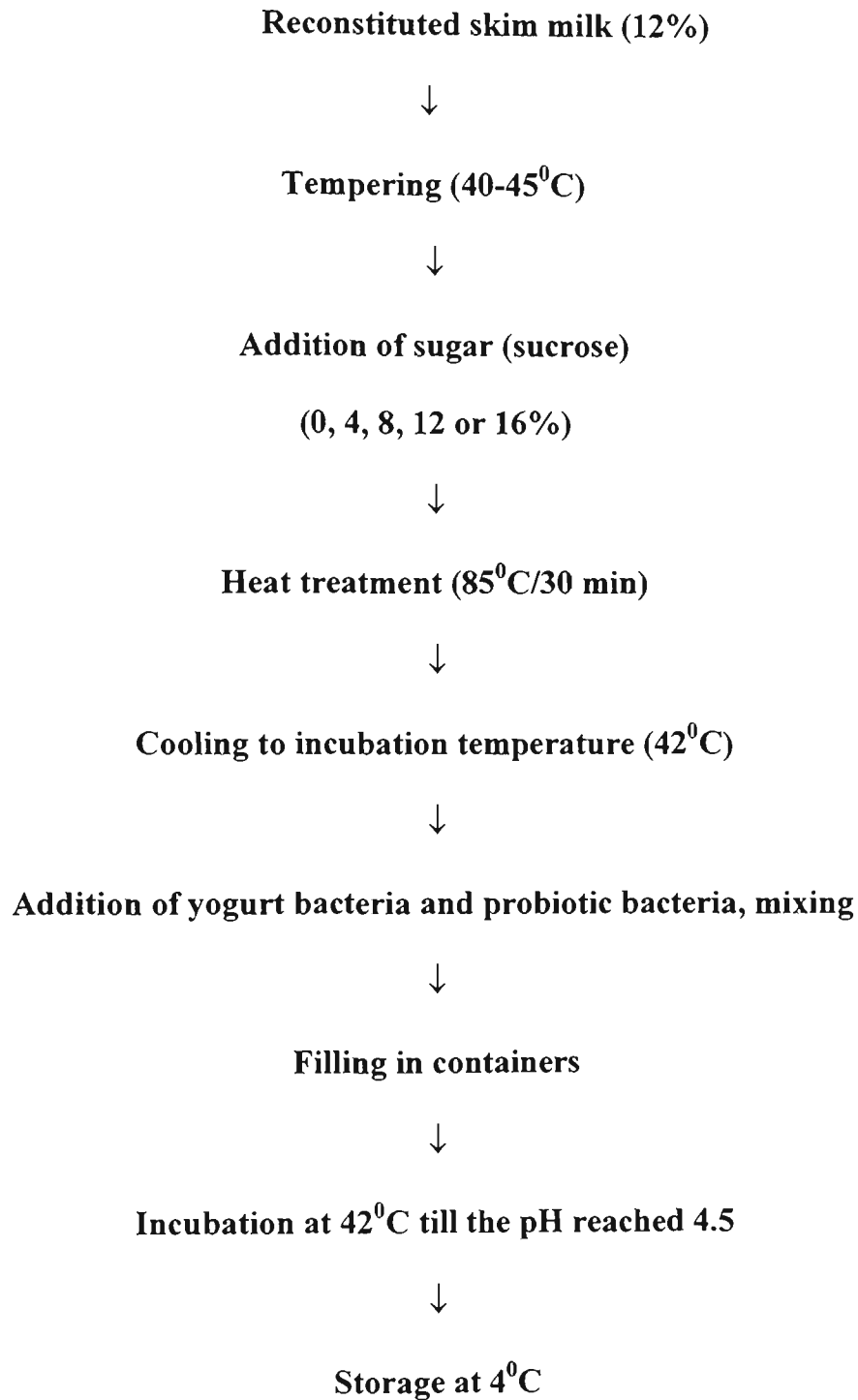
Fig 7.1 A Flow diagram for making yogurt.
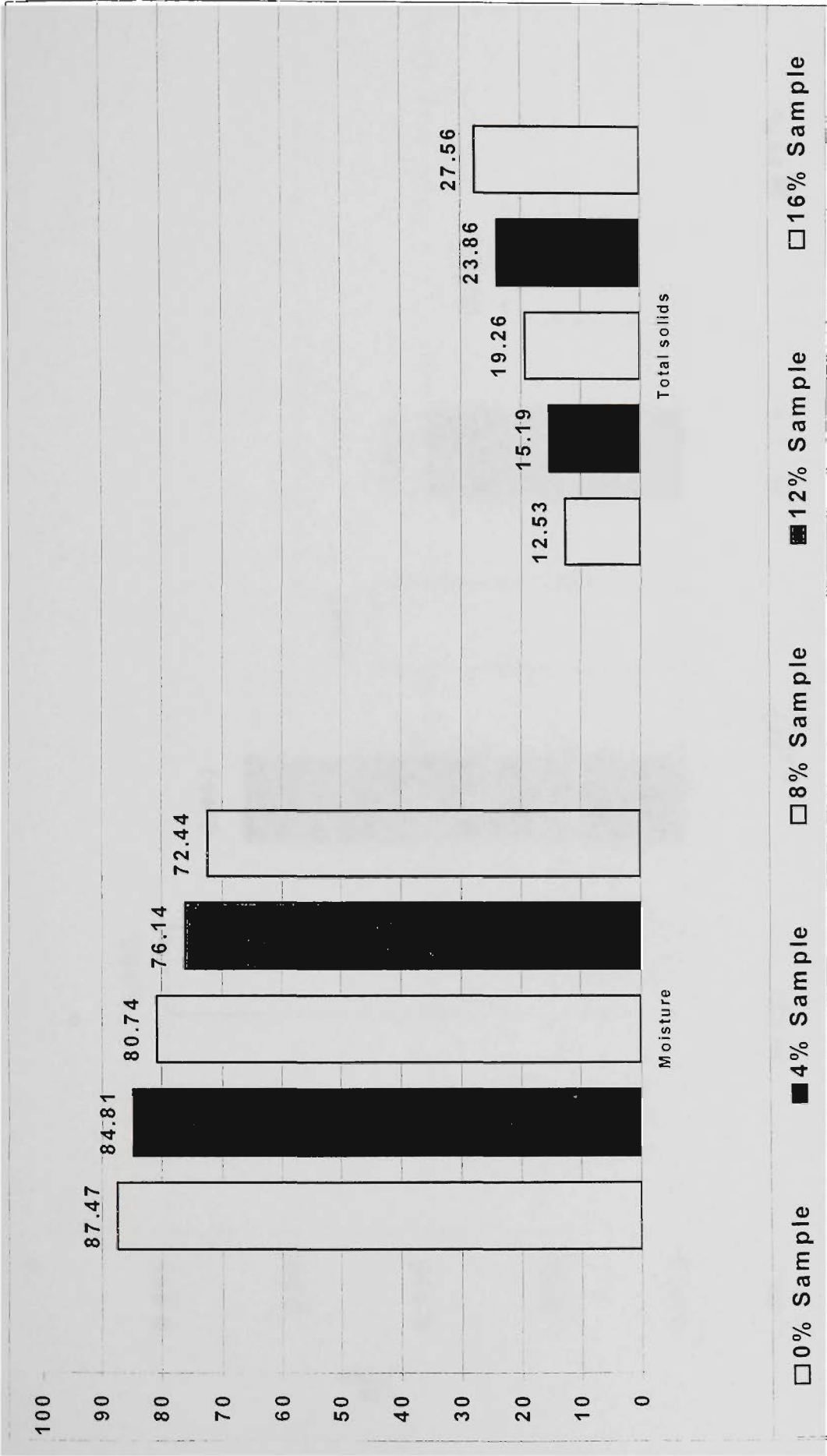
Fig 7.2 Total solids and moisture contents of samples after incorporation with various levels of sugar

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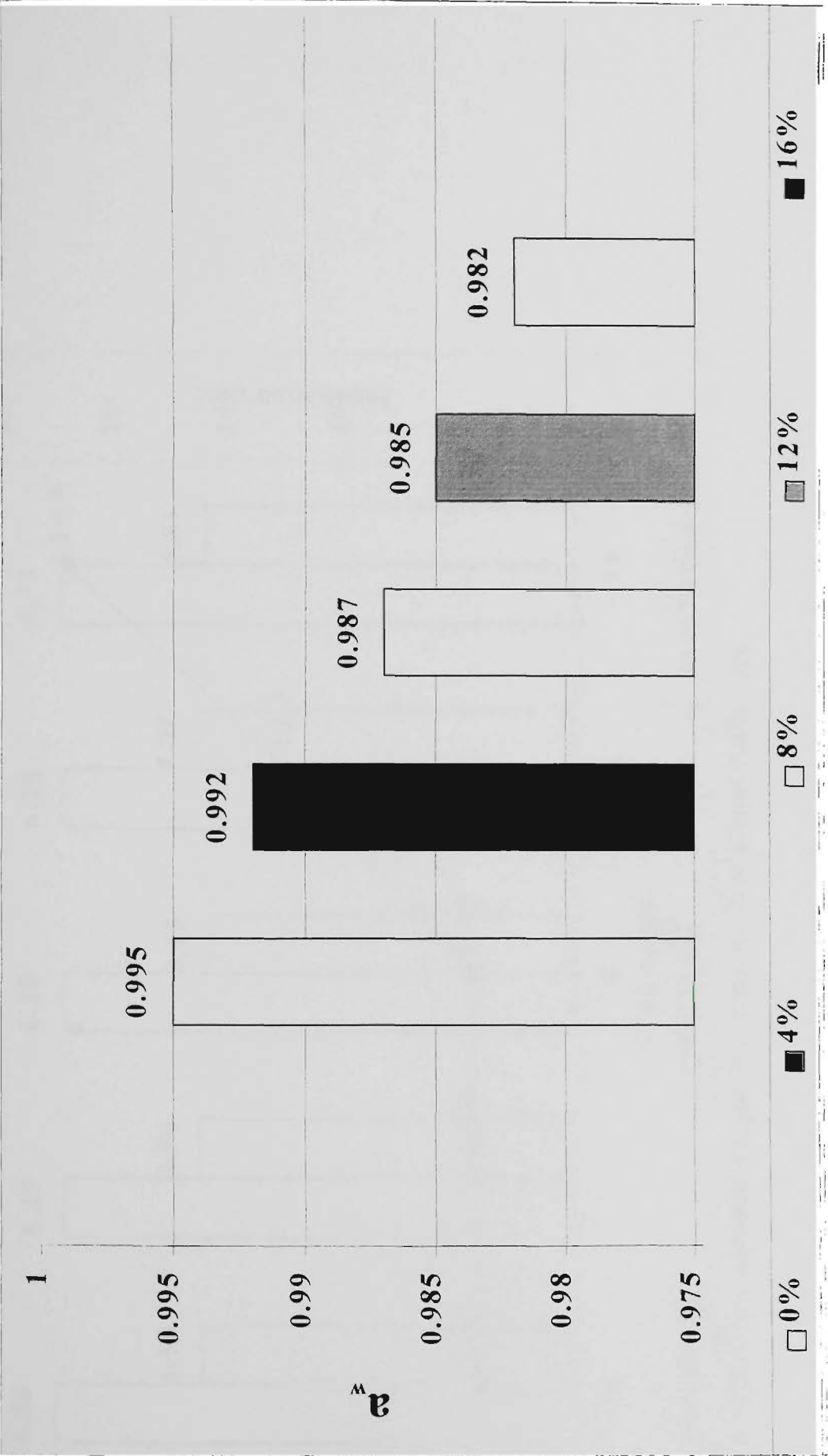
Fig 7.3   Effects of various concentrations of sugars on water activity

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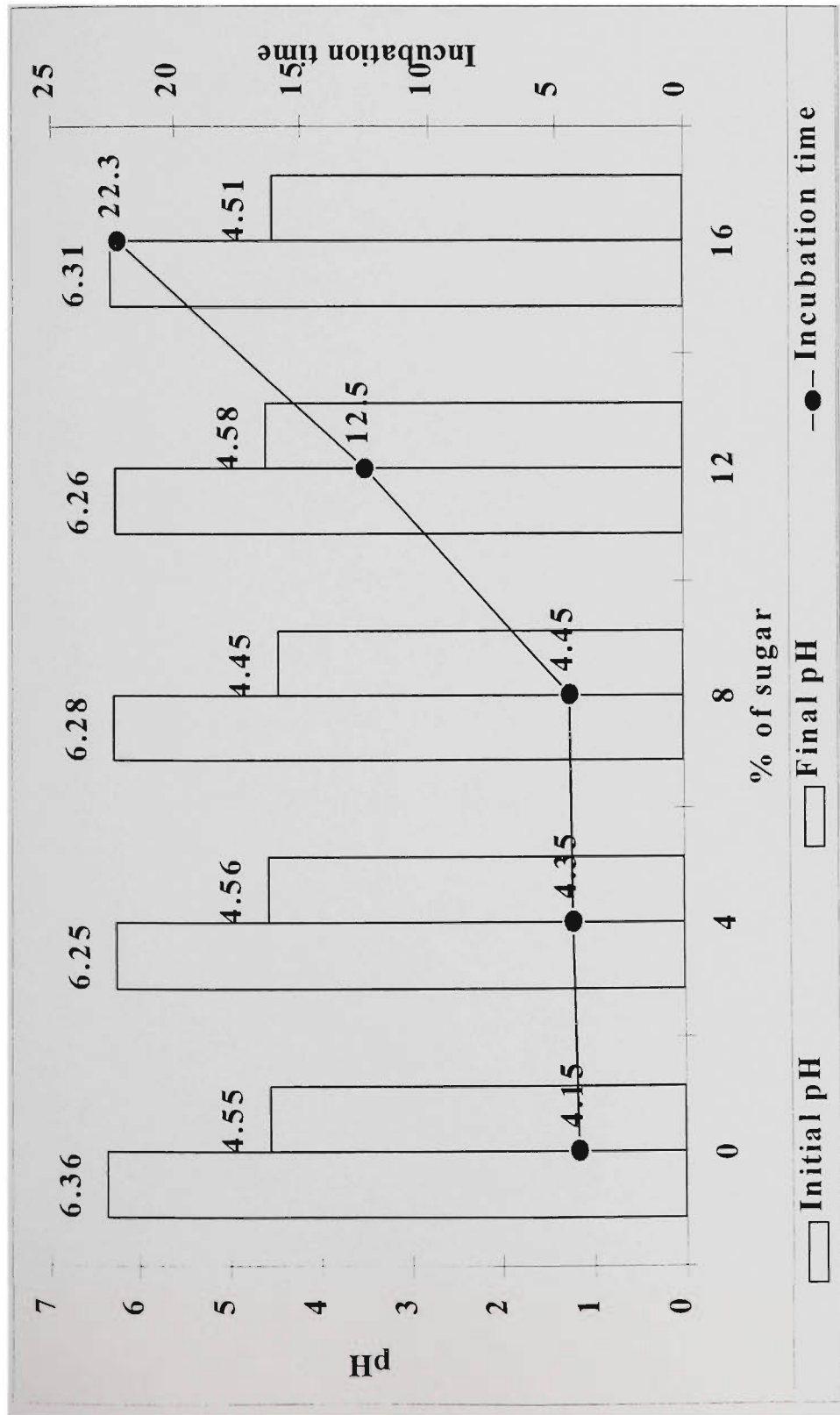
Fig 7.4 Effect of addition of sugar on changes in pH and fermentation time

Fig 7.5 Initial and final viable counts of *S. thermophilus L. delbrueckii* ssp. *bulgaricus L. acidophilus* and bifidobacteria in the products made with various levels of sugar. (ST = *Streptococcus thermophilus*; LA = *Lactobacillus*

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8.0 INFLUENCE OF WATER ACTIVITY ON FERMENTATION, ORGANIC ACIDS PRODUCTION AND VIABILITY OF YOGURT AND PROBIOTIC BACTERIA

8.1 Introduction

A number of health benefits associated with the consumption of probiotic organisms such as *Lactobacillus acidophilus* and *Bifidobacterium* spp. have been claimed (Hughes and Hoover, 1991; Kanbe, 1992; Mital and Garg, 1992). It has been suggested that, to produce health benefits, a minimum level of probiotic bacteria in fermented dairy products should be 10^6 viable cells per gram of a product. However, several studies (Anon, 1992; Shah *et al.*, 1995, 1999) report that the probiotic organisms do not survive well in fermented products.

Several factors have been claimed to affect the viability of probiotic bacteria in fermented milk products. According to the Australian Food Standards H8, the pH of yogurt must be ≤ 4.5, which affects the viability of probiotic bacteria. Similarly, oxygen is incorporated in fermented frozen dairy desserts and has been identified to affect their viability during manufacture and storage (Lankaputhra and Shah, 1994, 1995; Lankaputhra and Shah 1996; Ravula and Shah, 1998b).

Fermented frozen dairy desserts typically contain 16% sugar (Ravula and Shah, 1998b) High concentrations of sugar added to milk before fermentation may result in conditions inhibitory to yogurt starter growth, leading to long fermentation times and poor acidity development (Tramer, 1973; Zourari *et al.*, 1992). This is due to both adverse osmotic effects of the solutes in milk and low a_w (Larsen and Anon, 1989, 1990; Zourari *et al.*, 1992).

Organic acids are particularly important for the final properties of fermented dairy products such as yogurt. These acids help preserve the sensory characteristics of the product. Lactic acid has been found to inhibit the growth of certain pathogenic bacteria in yogurt (Zourari *et al.*, 1992)

Effects of water activity on acid production by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were studied by Larsen and Anon (1989). A decrease in a_w inhibited acid production by both organisms. The minimum a_w for acid production was always lower when a_w of milk was adjusted with glucose or sucrose than with glycerol.

The final pH of yogurt can affect the growth and viability of *L. acidophilus* and *Bifidobacterium* spp., most notably of the latter organism (Laroia and Martin 1991; Hekmat and McMahon, 1992). Acid production by lactic acid bacteria, especially post-acidification, affected cell viability of *L. acidophilus* and *B. bifidum* (Ishibashi and Shimamura, 1993). This may be due to residual activity of lactic bacteria that produce lactic or acetic acid.

The aims of this study were: (i) to investigate the influence of water activity due to addition of sucrose on incubation time and viability of yogurt and probiotic bacteria, and (ii) to assess changes in organic acids during a 30-day storage period.

8.2 Materials and Methods

8.2.1 *Yogurt bacteria and probiotic bacteria*

Yogurt bacteria (*Streptococcus thermophilus* STWJ7 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LBWJ7) and probiotic bacteria (*Lactobacillus acidophilus* MJLA1 and *Bifidobacterium* BDBB2) were obtained from Gist-brocades (formerly Mauri Laboratories Pty. Ltd., Moorebank, Australia). All the strains were tested for purity by Gram stain and propagated every week in sterile 12% reconstituted skim milk (RSM) supplemented with 2% glucose and 1% yeast extract. All the cultures were grown for 18 h at 37^0C using 1% inoculum. For propagation of *Bifidobacterium* spp., sterile RSM was supplemented with 0.05% L-cysteine hydrochloride in order to provide anaerobic condition and to stimulate their growth. The cultures were maintained in the same medium at 4^0C. Before yogurt manufacture, the cultures were transferred successively three times for activation.

8.2.2 *Manufacture of yogurt*

Five batches of yogurt (1 litre size) were made using 12% RSM with four levels of sugar (4, 8, 12 and 16%). A control batch was made without added sucrose. The mix was heated at 85^0C for 30 min, cooled to 42^0C and yogurt bacteria (*S. thermophilus*

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STWJ7 and *L. delbrueckii* ssp. *bulgaricus* LBWJ7) and probiotic bacteria (*L. acidophilus* MJLA1 and *Bifidobacterium* spp. BDBB2) were added at the rate of 0.5% of each. The inoculated mix was filled in 100 millilitre plastic containers and incubated at 42^0C until the pH of 4.5 ± 1 was attained. The yogurts were stored in a cold room (4^0C) and microbiological and chemical analyses were monitored at 24 h and at 10-day intervals for 30 days.

8.2.3 *Detection and quantification of organic acids with HPLC*

8.2.3.1 Extraction of organic acids

Four grams of yogurt samples were diluted with 25 mL of 0.01N H_2SO_4, and filtered through 0.02-μm Teflon filters (Schleicher and Schuell, Kassel, Germany). The extraction of organic acids was carried out in duplicates.

8.2.3.2 HPLC of organic acids

The standards of organic acids were pipetted into HPLC vials through 0.02 μm millipore filters (Millipore Australia, Lane Cove, Australia). Similarly, diluted samples of yogurts were pipetted into HPLC vials through 0.02 μm filters. The vials were used in an auto sampler for analysis of organic acids. The concentration of each acid was determined with Varian HPLC (Varian, Mulgrave, Australia) using an UV-Vis detector. An Aminex HPX-87H ion exclusion column (300 x 7.8 mm) (BioRad, North Ryde, Australia) and a mobile phase of 0.0075 M H_2SO_4 were used for the analysis. Quantification of organic acids was carried out by the external standard method. Linear regression curves based on peak heights were calculated for

individual organic acids after duplicate injections. Identifications of acids were based on matching retention times of standards.

8.2.4 *Microbiological analysis*

8.2.4.1 Peptone and water diluent

Peptone and water diluent were prepared by dissolving 15 g of peptone and water medium (Oxoid, West Heidelberg, Australia) in 1L of distilled water, the pH adjusted to 7.0 ± 0.2, followed by autoclaving 9 mL portions at 121^{0}C for 15 min.

8.2.4.2 Enumeration of bacteria

S. thermophilus was enumerated on ST agar (composition: 10.0g tryptone; 10.0g sucrose; 5.0g yeast extract; 2.0g K_2HPO_4 and 6 mL bromo-cresol purple (0.05%) per litre of water, pH adjusted to 6.8) and by incubating plates aerobically at 37^{0}C for 24 ± 3 h (Dave and Shah, 1996; Lankaputhra and Shah, 1996b). MRS agar (Oxoid) adjusted to pH 5.2 and anaerobic incubation at 43^{0}C for 72 h were used for selective enumeration of *L. delbrueckii* ssp. *bulgaricus*. MRS-salicin and MRS-sorbitol agars were used for the selective enumeration of *L. acidophilus* (Dave and Shah, 1996). The populations of bifidobacteria were enumerated on MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate) agar (Laroia and Martin, 1991). The initial counts (counts after inoculation but before incubation) and those after reaching pH of 4.5 (final counts) of both yogurt and probiotic bacteria were enumerated. The populations of the four types of organisms were monitored at 24 hour and then at 10-day intervals for 30 days.

8.2.5 Analyses

The water activity (a_w) was measured at 25^0C with a water activity meter (Deacon CX-1; Decagon Devices Inc., Pullman, WA). The pH was measured with a pH meter (Model 420A, Orion Research Inc., Boston, MA). All the measurements were carried out in triplicates. The results presented are averages of the three replicates.

8.3. Results and Discussion

8.3.1   pH, sugar concentration and incubation time

Initial pH of RSM ranged between 6.25 to 6.36 and the final pH 4.45 to 4.58 (Table 8.1). The incubation time to decrease the pH of yogurt mix to 4.5 ranged between 4 h to 24 h (Figure 8.7). In general, the incubation time increased as the sugar concentration increased, in particular in batches containing 12 or 16% sugar.

8.3.2   Water activity

Figure 8.8 shows the water activity of all the five batches of yogurt. The water activity decreased as the percentage of sugar increased. Fajardo-Lira *et al.* (1997) studied the effects of water activity on exopolysaccharide producing or non-producing starters and found that sucrose had an inhibitory effect on fermentation process. Effects of water activity of milk on acid production by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were studied by Larsen and Anon (1989). A decrease in a_w inhibited acid production by both organisms. The minimum a_w for acid production was always lower when a_w of milk was adjusted with glucose or sucrose than with glycerol.

8.3.3 *Production of organic acids*

Figures (8.1 – 8.5) show the percent organic acids present in the inoculated milk, after fermentation and during the storage period of 30 days. Pyruvic acid, acetic acid, lactic acid, propionic acid, and hippuric acid were identified and quantified in inoculated yogurt mix, after fermentation and during storage. The levels of pyruvic acid after fermentation increased with the level of sugar, but decreased during storage.

In general, the level of acetic acid increased during fermentation. There was a slight increase in the level of acetic acid during storage. The decrease was inversely proportional to the level of sugar. The production of lactic acid followed a similar pattern except that there was no production of lactic acid in inoculated but unfermented products.

Low levels of propionic acid were detected in inoculated but unfermented batches (Figure 8.4). The propionic acid level at 16% sugar level was lower than at 12%, but similar to the 4% and 8% sugar levels and higher than the zero sugar control. Low levels of hippuric acid were detected in inoculated milk not after fermentation or storage.

8.3.4 *Viable counts of yogurt and probiotic bacteria*

Figures (8.6 – 8.9) show the viable counts of yogurt bacteria and probiotic bacteria in the five batches of yogurt. In general, the initial counts after inoculation but

before incubation of yogurt bacteria and probiotic bacteria were similar. The counts of *S. thermophilus* at 16% sugar level was lower than at 12%, but similar to the 0%, 4% and 8% sugar levels after 24 hour storage in batches containing 0, 4 or 8% sugar increased by approximately 2 log cycles (Figure 8.6). There was approximately 1 log cycle increase in the counts of *L. delbrueckii* ssp. *bulgaricus* in the batches containing 0, 4 and 8% sugar (Figure 8.7). The viable counts decreased as with *S. thermophilus* in the two batches containing 12 and 16% sugar. The initial viable counts of *L. acidophilus* in the batches containing 0, 4 and 8% sugar were similar (Figure 8.8). There was a sharp decline in the viable counts during storage in the batches containing 12 and 16% sugar. The viable counts of bifidobacteria showed a similar trend (Figure 8.9). The decrease in the counts of yogurt and probiotic bacteria indicates the adverse effects of lowering water activity due to addition of sugar. The viability of *L. acidophilus* and *Bifidobacterium* spp. was most adversely affected followed by that of yogurt bacteria, *L. delbrueckii.* ssp. *bulgaricus,* and *S. thermophilus.*

Lankaputhra and Shah (1996) studied the survival of probiotic bacteria at low pH conditions. Of the various strains studied, over 50% of the strains lost viability after 6 days of storage at pH 4.3. The minimum pH for survival of *L. acidophilus* and bifidobacteria is 5.5 (Holt, 1986). Thus a decline in their counts at pH 4.3 is expected to occur.

Frozen fermented dairy products typically contain 16% sugar. Thus such a high concentration of sugar is likely to inhibit the growth of probiotic bacteria and production of organic acids. In this study, only one set of four bacterial strains was tested. However, results from these experiments are indicative of issues that might need to be considered in understanding the effects of sugar in yogurt, but other strains must be evaluated on a case-by-case basis.

8.4 Conclusions

The influence of water activity due to addition of sucrose on fermentation of probiotic yogurt and viable counts of yogurt and probiotic bacteria were studied. High levels of sugar, especially 12% and 16% led to longer fermentation time, decreased levels of organic acids and poor survival of yogurt and probiotic bacteria. The growth of *L. acidophilus* and *Bifidobacterium* spp. was most adversely affected at sugar concentrations of 16% followed by that of yogurt bacteria, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*. Fermented frozen dairy desserts typically contain 16% sugar, thus it may not be feasible to achieve a satisfactory count of probiotic bacteria in such products if sugar is added prior to fermentation.

Table 8.1. Effect of various concentrations of sugar on water activity, pH, and fermentation time.

Sugar concentration (%)	Water activity	Initial pH	pH after fermentation	Incubation time (h)
0	0.998	6.36	4.55	4.00
4	0.994	6.25	4.56	4.25
8	0.987	6.28	4.45	4.40
12	0.984	6.25	4.58	13.25
16	0.982	6.31	4.51	23.45
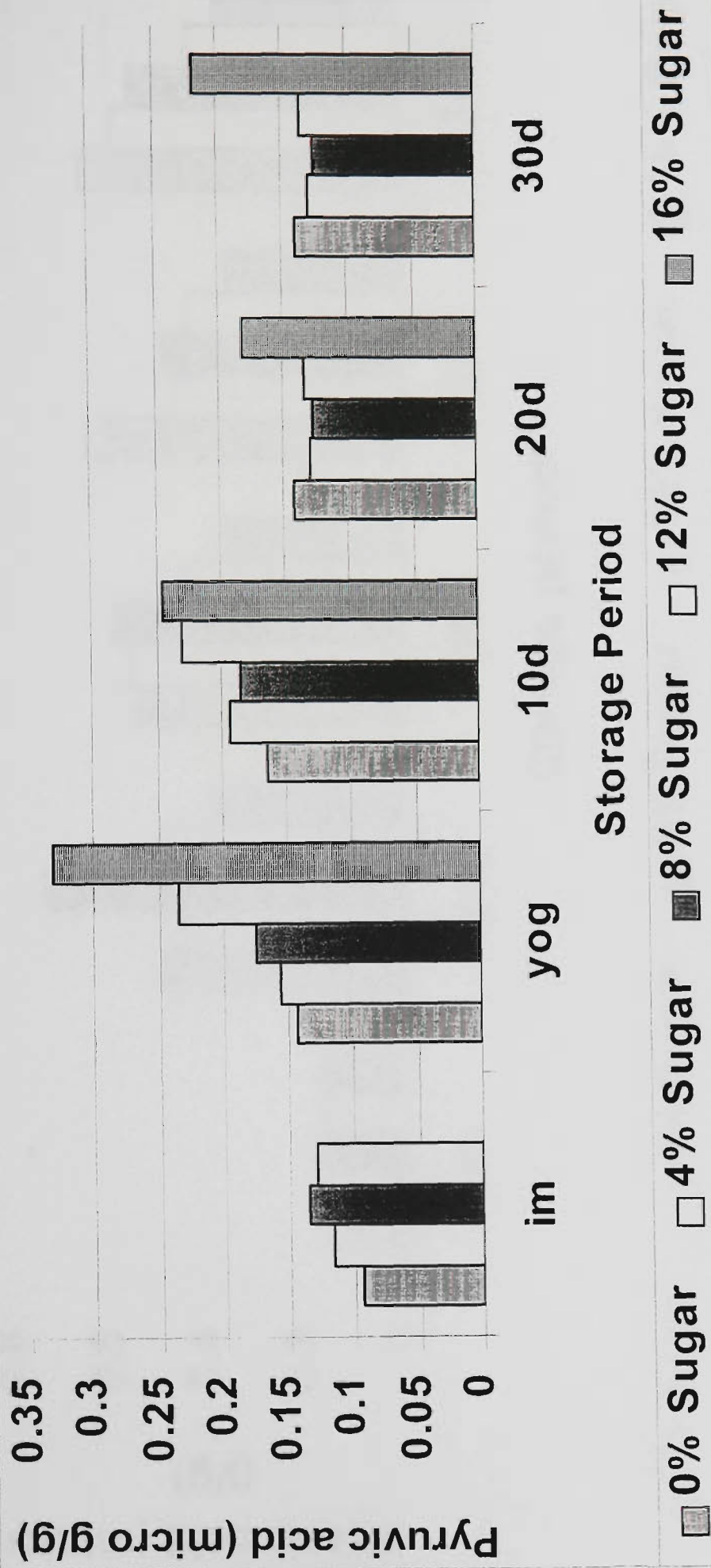
Fig 8.1 Levels of pyruvic acid in inoculated milk, after fermentation, and during 30-day storage at various concentrations of sugar in measurement of organic acids (Im = inoculated milk; Yog = yogurt).

146
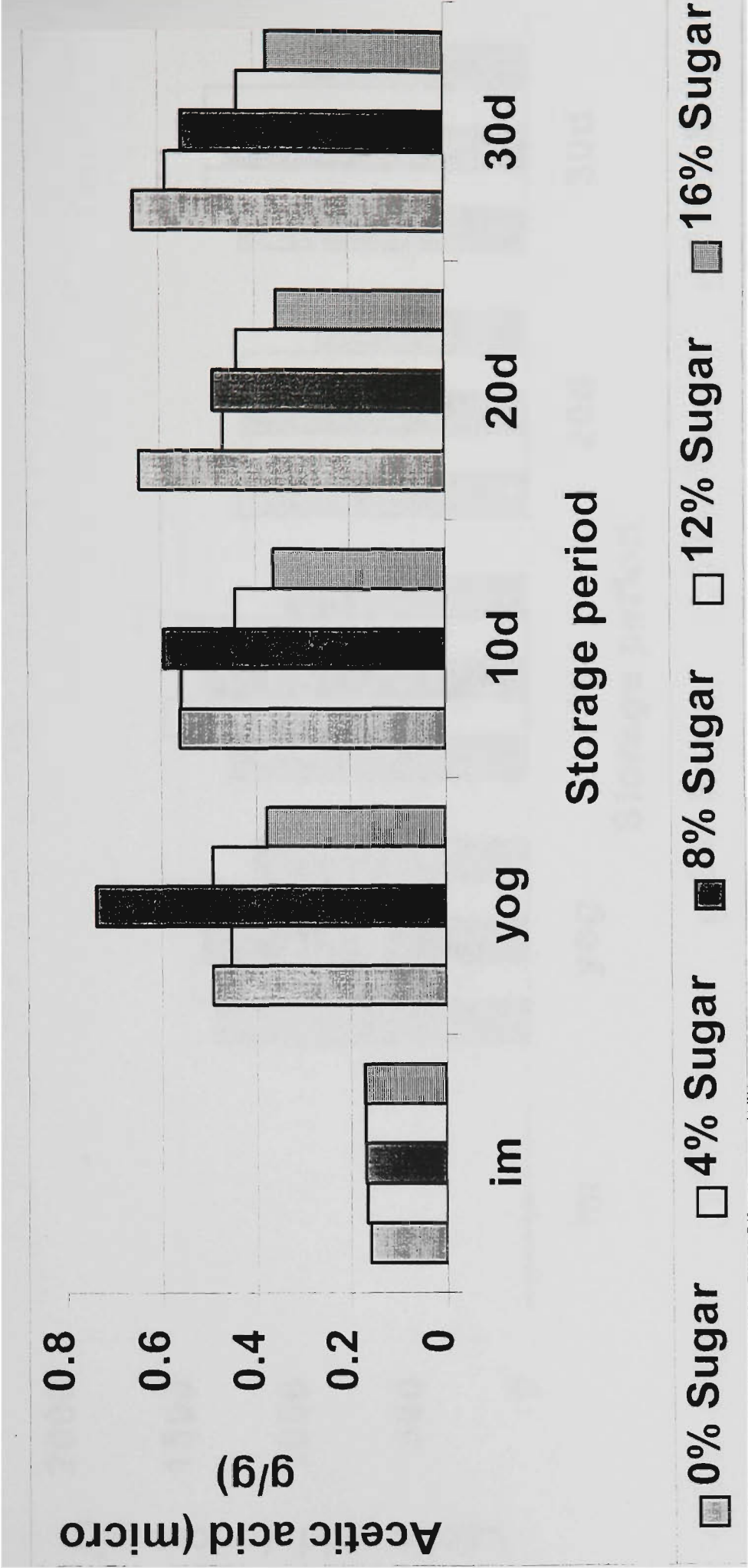
Fig 8.2 Levels of acetic acid in inoculated milk, after fermentation, and during 30-day storage at various concentrations of sugar in measurement of organic acids (Im = inoculated milk; Yog = yogurt)

147
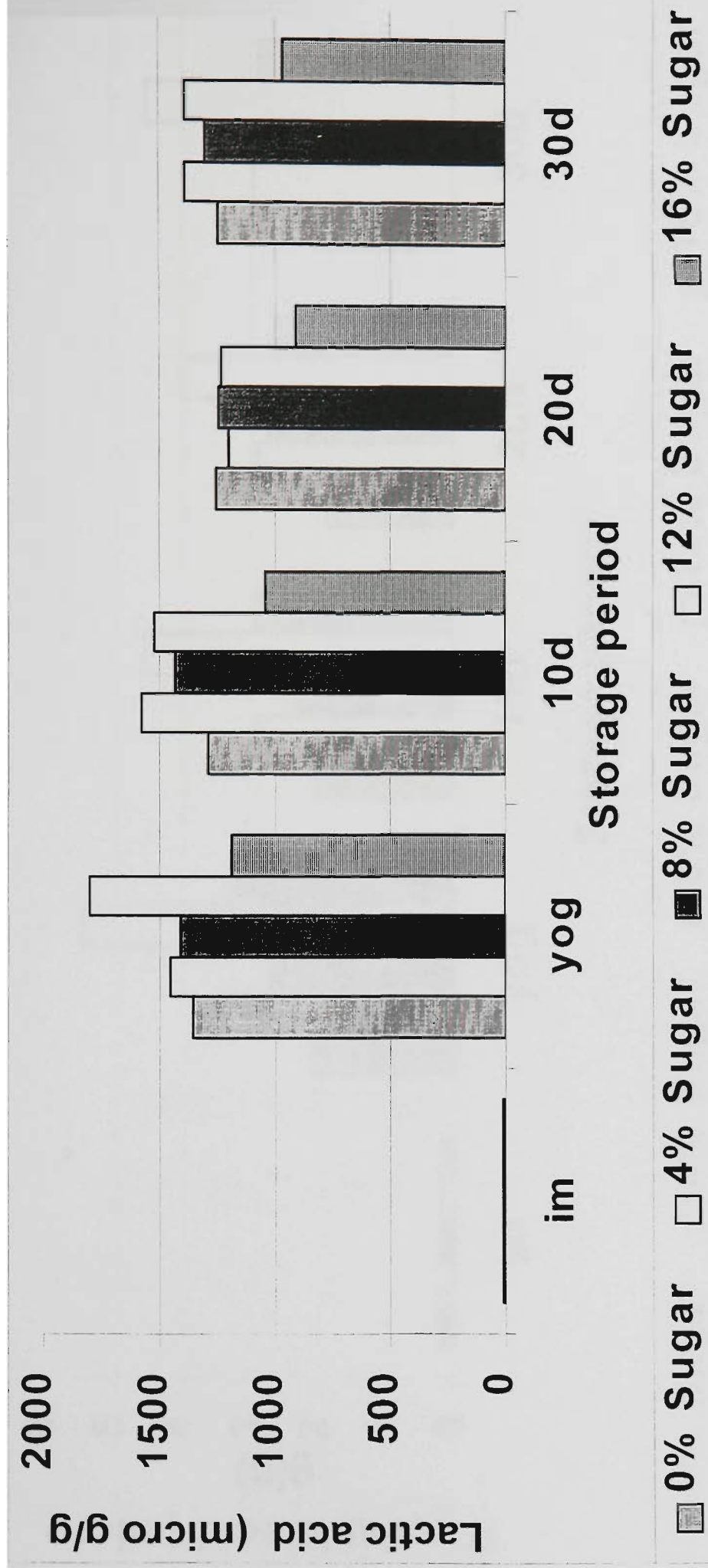
Fig 8.3 Levels of lactic acid in inoculated milk, after fermentation, and during 30-day storage at various concentrations of sugar in measurement of organic acids (Im = inoculated milk; Yog = yogurt).

148
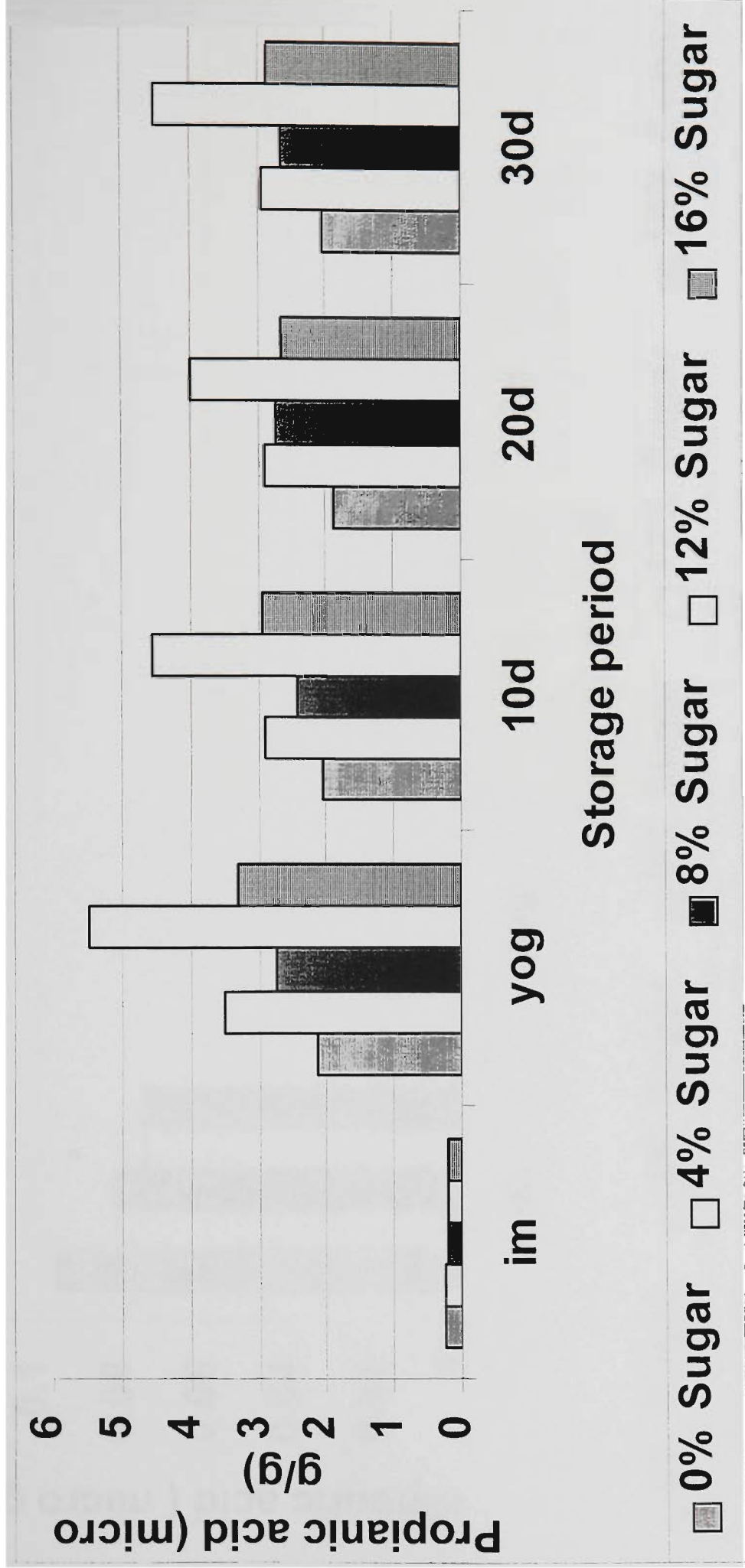
Fig 8.4 Levels of propianic acid in inoculated milk, after fermentation, and during 30-day storage at various concentrations of sugar in measurement of organic acids (Im = inoculated milk; Yog = yogurt).

149
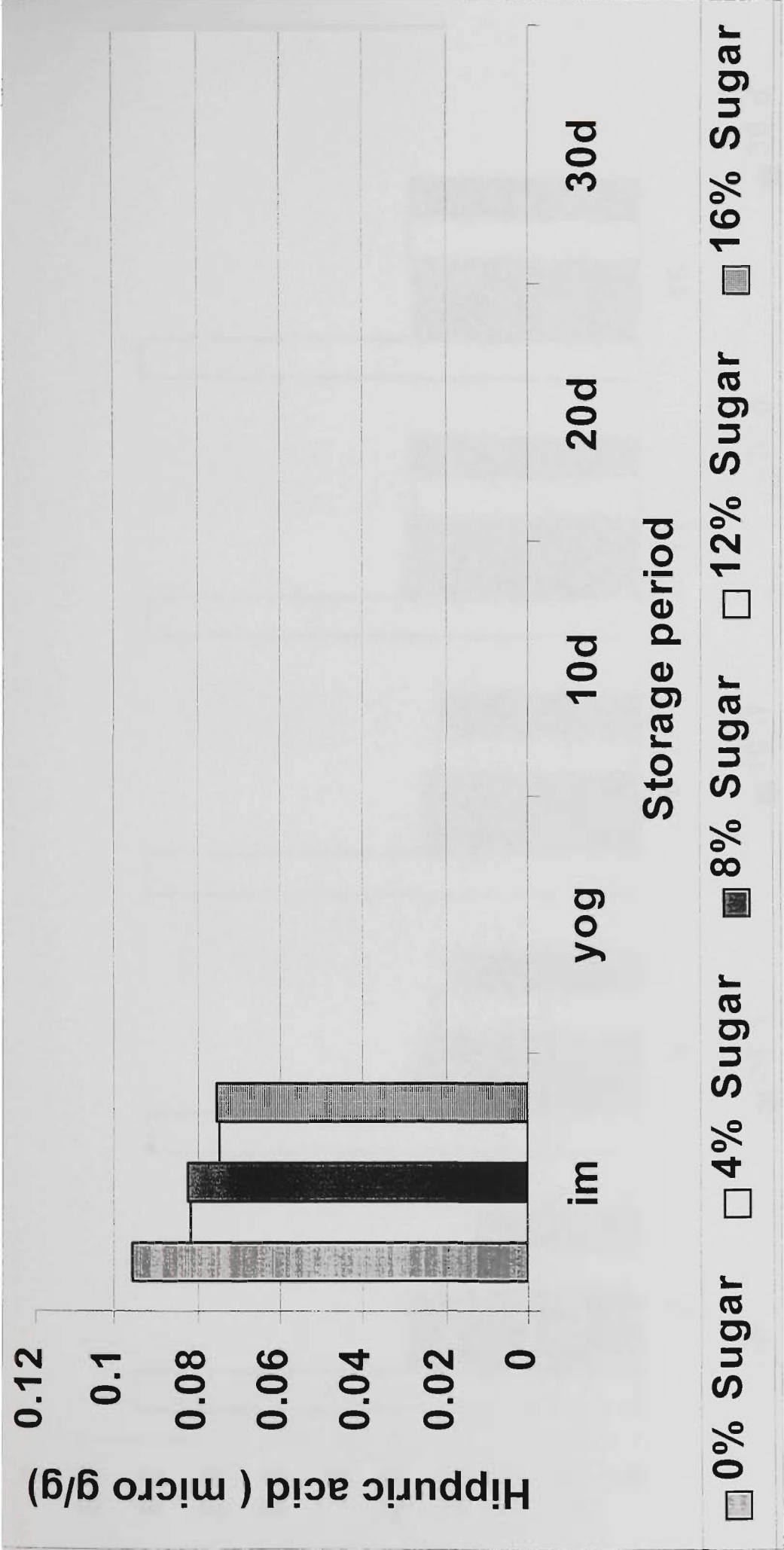
Fig 8.5 Levels of Hippuric acid in inoculated milk, after fermentation, and during 30-day storage at various concentrations of sugar in measurement of organic acids (Im = inoculated milk; Yog = yogurt)
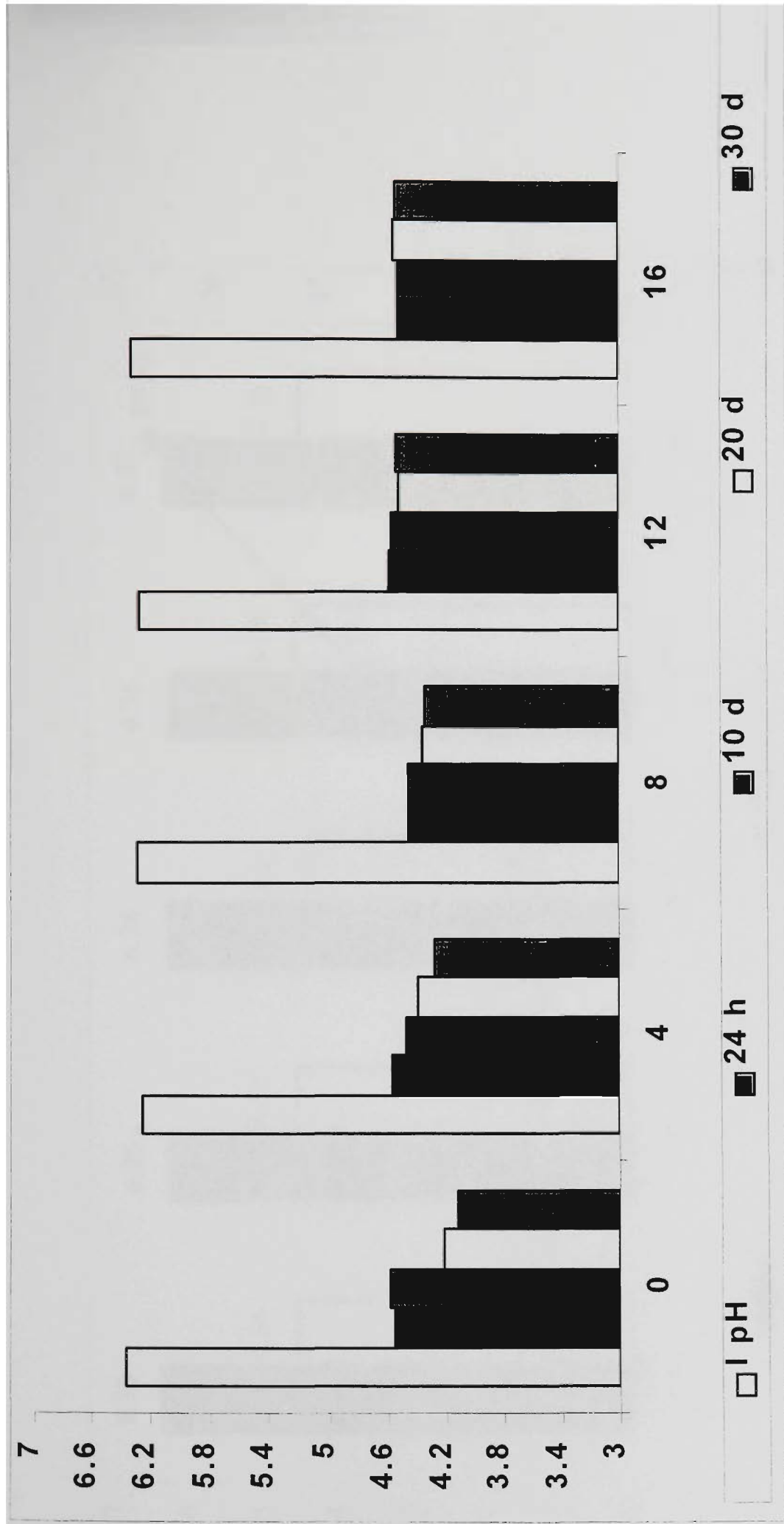
Figure 8.6 Effects of sugar concentration on pH over 30 day storage period

151
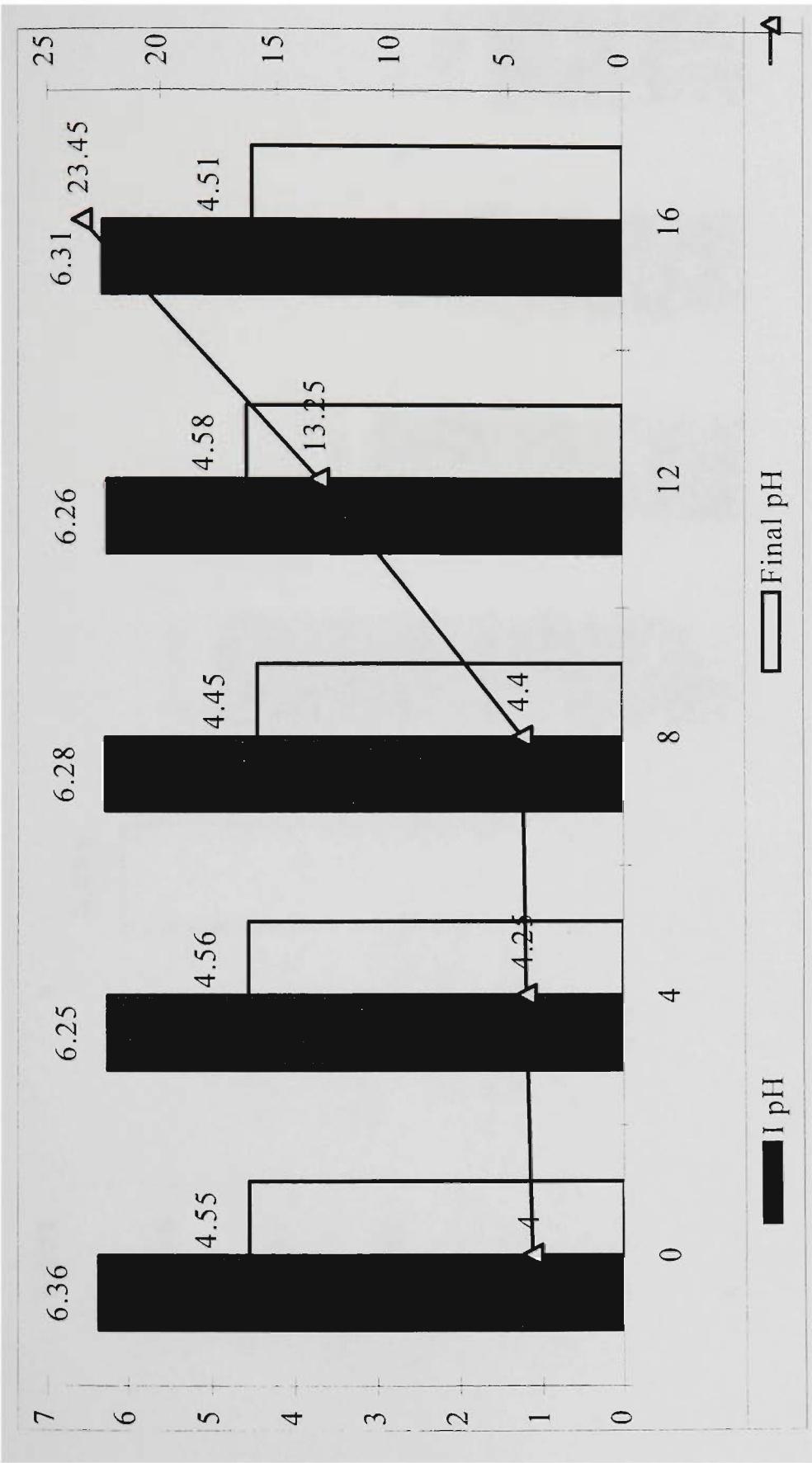
Figure 8.7 Effects of sugars on pH and fermentation time
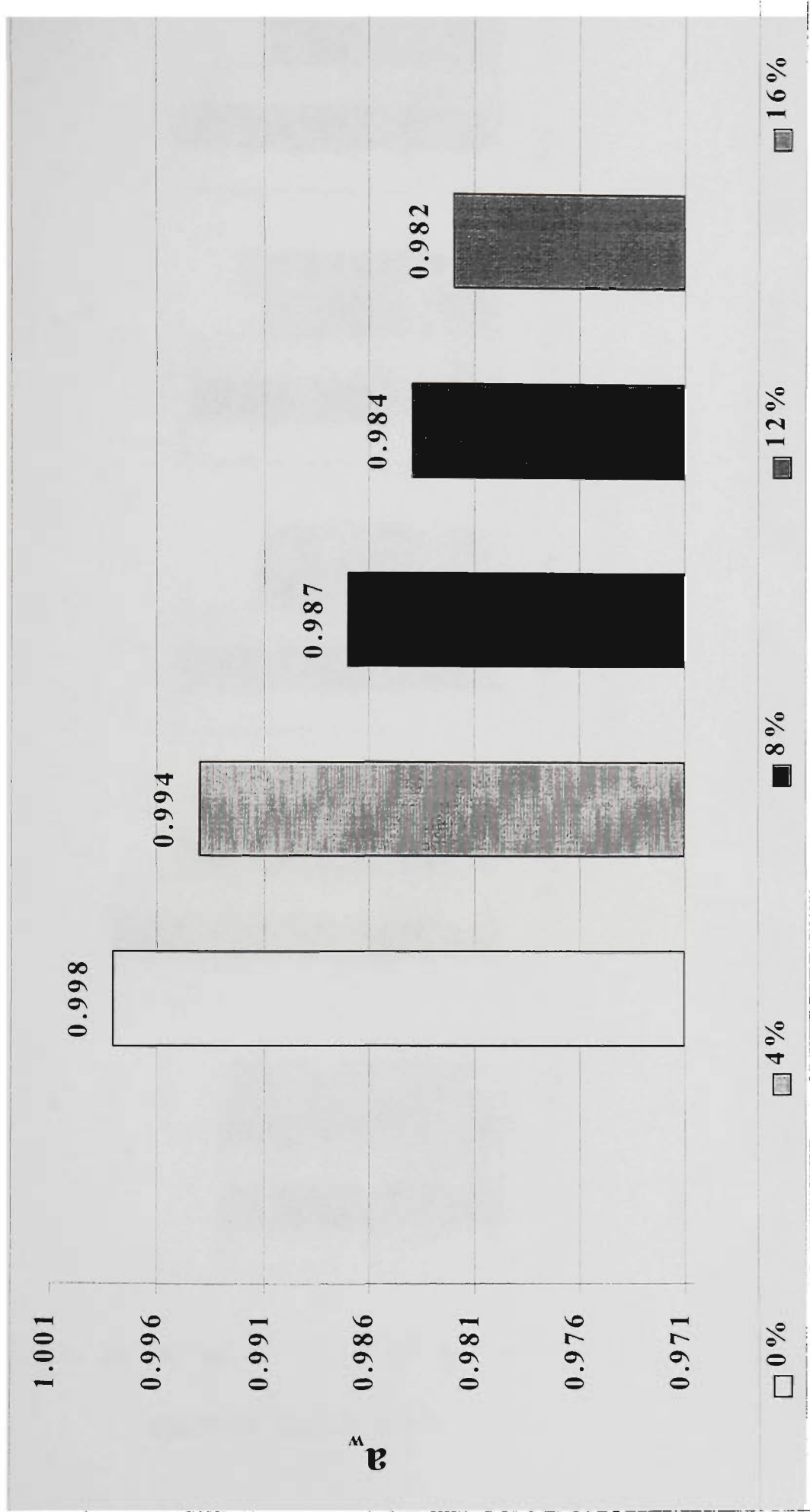
Figure 8.8 Water activity at various levels of sugar

153
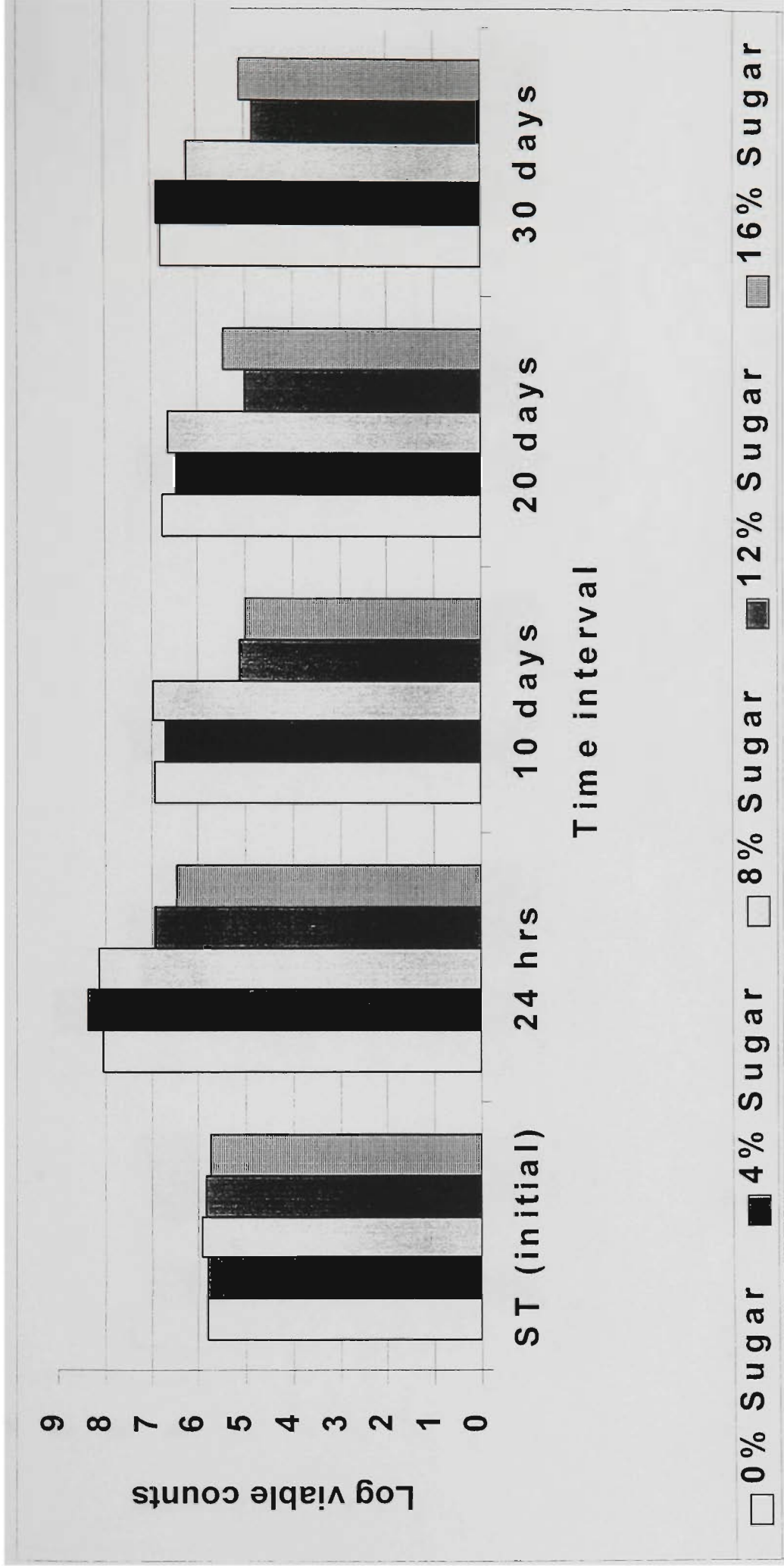
Figure 8.9 Counts of *S. thermophilus* after inoculation, at 24 h and during storage in the products made with various levels of sugar.

154
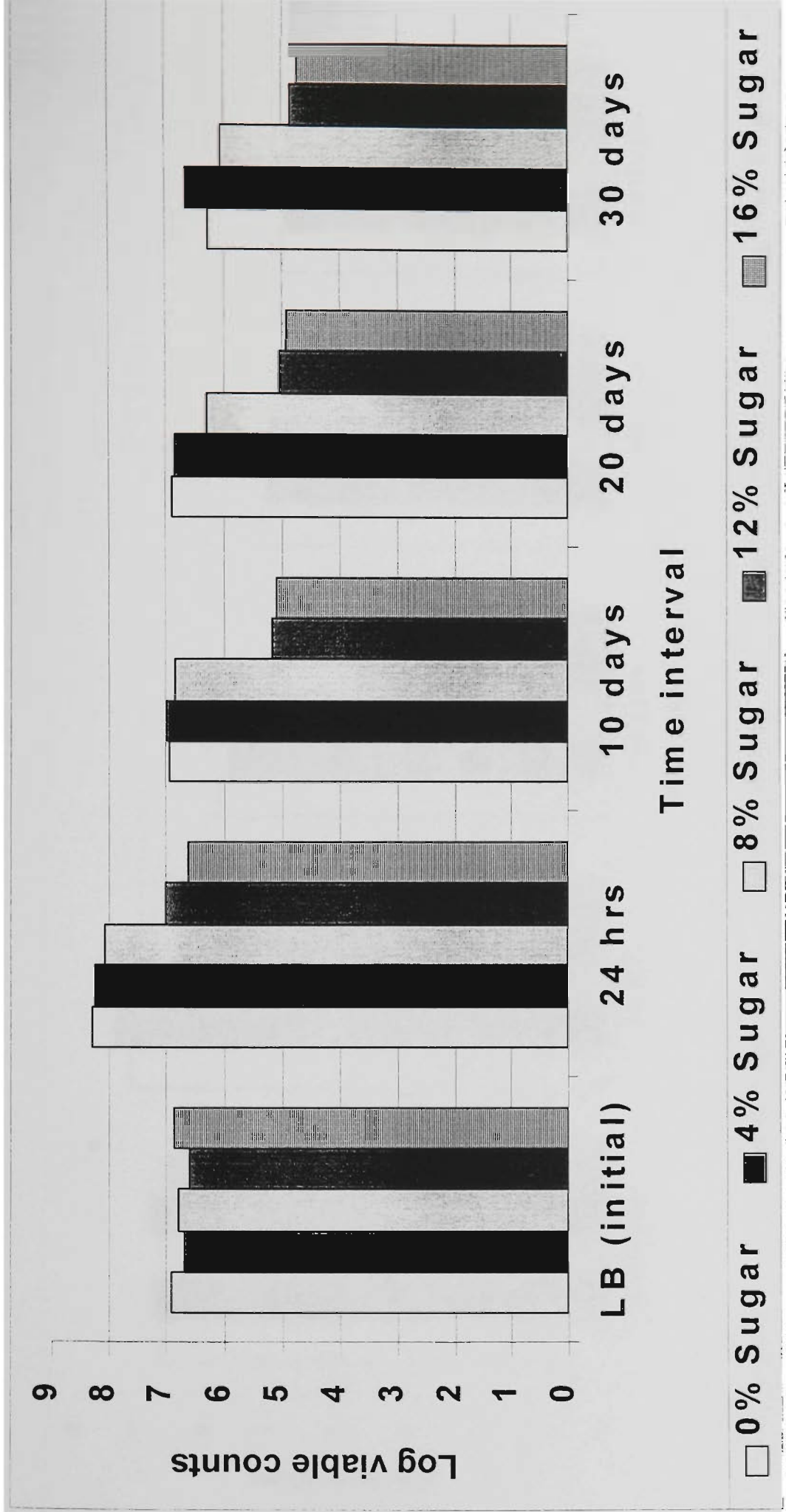
Figure 8.10 Counts of *L. debrueckii* ssp. *bulgaricus* after inoculation, at 24 h and during storage in the products made with various levels of sugar.

LB = *L. debrueckii* ssp. *bulgaricus*

155
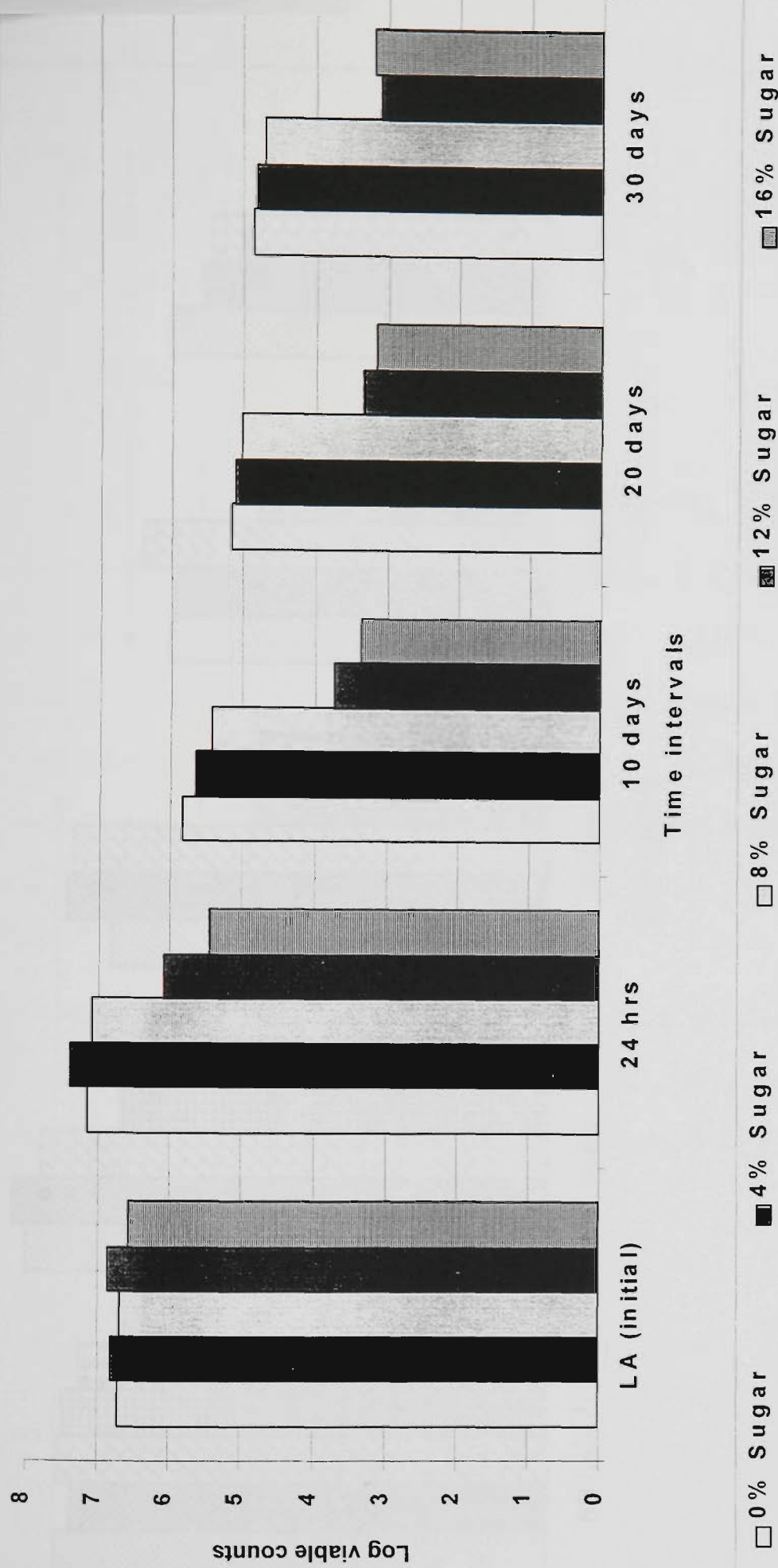
Fig 8.11 Counts of *Lactobacillus acidophilus* after inoculation, at 24 h and during storage in the products made with various levels of sugar. LA = *Lactobacillus acidophilus*.

156
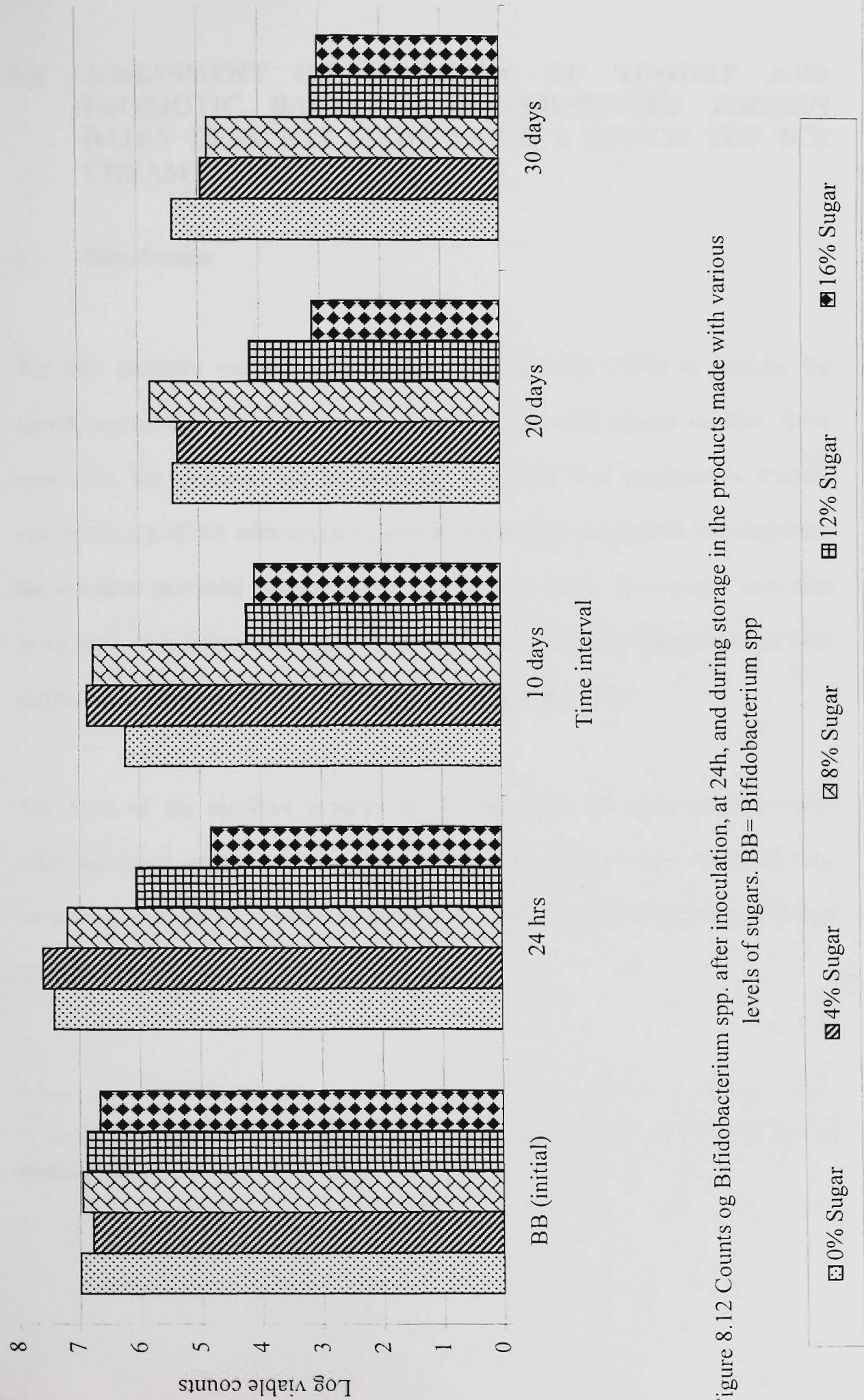
Figure 8.12 Counts og Bifidobacterium spp. after inoculation, at 24h, and during storage in the products made with various levels of sugars. BB= Bifidobacterium spp

9.0 ASSESSMENT OF VIABILITY OF YOGURT AND PROBIOTIC BACTERIA IN FERMENTED FROZEN DAIRY DESSERTS MADE USING A BENCH TOP ICE CREAM FREEZER

9.1 Introduction

The term probiotic was first used by Lilly and Stillwell (1965) to describe the growth promoting effect of one or more microorganisms against another. Soon afterwards, the term was applied primarily to animal feed supplements. Further changes in use of this term implied a live microbial feed supplement that improved the intestinal microbial balance of the host (Fuller, 1989). The term is now also being used with reference to human consumption of live microorganisms as food additives for nutritional health and well-being (Hull et al., 1992).

The basis of the probiotic concept is that these are the gastrointestinal tract microorganisms, which have a beneficial effect on the host. Modern life styles may have adverse effect on the delicate balance that exists between the many different species of microorganisms, which inhabit the gut.

Our unnatural diet (most of which is heat sterilised by cooking), antibiotic therapy and stress are all factors which can alter the composition of the gut microflora and have nutritional and health consequences. Probiotics can reverse or prevent these consequences by restoring the flora to its natural state (Fuller, 1989)

However, introducing probiotic organisms into the frozen food chain can be a difficult task as the organisms are unstable in such products. The loss of viability of probiotic organisms in frozen yogurt may be due to acidity of the product, freeze injury, sugar concentration and oxygen toxicity.

In this study, fermented frozen dairy desserts were made with selected yogurt and probiotic organisms and survival of these organisms was monitored for 12 weeks during frozen storage at -18^{0}C.

9.2   Materials and Method

9.2.1   *Yogurt and probiotic bacteria in fermented frozen dairy desserts made using a bench top ice cream Freezer*

Six different batches (B1-6) of fermented frozen dairy desserts were made as follows:

For batch B1, yogurt was made by using ST 2010, LB 2501, LA 2415 and BB 20099. Similarly, B2 was made with ST 2008, LB 2515, LA 2401, BB 1912 and B3 with ST/LB Wj7, MjLA1, BDBB2. B4, B5 and B6 were made with ST 2010, 2008, ST WJ7 and LB 2501, 2515, LB WJ7 only and probiotic strains LA 2415, 2401,

MJLA1, BB 20099, 1912, BDBB2 were incorporated just before freezing. Yogurt and base mix were added at a ratio of 45:45 and 10% of liquid sucrose syrup (65%) was added, followed by freezing the contents using a bench top Kenwood ice chef ice cream freezer (Model Im 300 PK 185/w, Kenwood Appliances, Italy). The pH of the final product ranged between 4.51 and 4.59.

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9.3 Results and discussion

9.3.1 Viability of yogurt and probiotic bacteria in fermented frozen dairy dessert made using a bench top freezer during frozen storage for 12 weeks

Counts of S. thermophilus (2010, 2008 and STWJ7) were reduced by about 1 log cycle in the first 3 to 5 weeks of storage and there was no further decrease during the rest of the storage period (Figures 9.1-9.3). There was no marked difference in the viable counts when the organisms were added just before freezing (Figures 9.4-9.6). Similar trends were observed with L. delbrueckii ssp. bulgaricus (2501, 2515 and LBWJ7) (Figures 9.1-9.6).

Counts of L. acidophilus 2415 were reduced by about 6 log cycles when added along with S. thermophilus, L. delbrueckii ssp. bulgaricus and bifidobacteria (Figures 9.1-9.3). The viable count at the end of the storage period was 10^2 cfu/g. Counts of L. acidophilus MJLA1 were reduced by about 2 log cycles when the organisms were added along with other bacteria, whereas when the organisms were added just before freezing the counts were reduced by about 4 log cycles by the end of storage period (Figures 9.4- 9.6).

Counts of bifidobacteria 20099 reduced by about 1 log cycle when added along with other bacteria, or added just before freezing. Counts of strain 1912 decreased by 5 log cycles at the end of the storage period when the organisms were added along with other bacteria and also just before freezing (Figures 9.1-9.6). Counts of bifidobacteria BDBB2 were reduced by about 1 log cycles when added along with other bacteria, whereas by about 2 log cycles when added just before freezing.

9.4   Conclusion

All strains screened for freeze tolerance remained viable before and after freezing and at 8 and 16% sugar levels. Of the various pH levels studied, pH 4.5 and 4.0 were found to be detrimental for *S. thermophilus* 2013, *L. delbrueckii ssp. bulgaricus* 2515 and *L. acidophilus* MJLA1, while rest of the organisms were not affected by pH.

Yogurt bacteria reduced by about 1 log cycle in the products made using a bench top freezer during 12 weeks storage. *L. acidophilus* 2415 was adversely affected and the counts declined by 6 log cycles within 8 weeks of storage at -18^0C. Amongst bifidobacteria strain 1912 decreased by 5 log cycles at the end of 12 weeks storage. *L. acidophilus* MJLA1 and bifidobacteria BDBB2 reduced by about 2 log cycles in the products made on a pilot scale after 12 weeks storage.
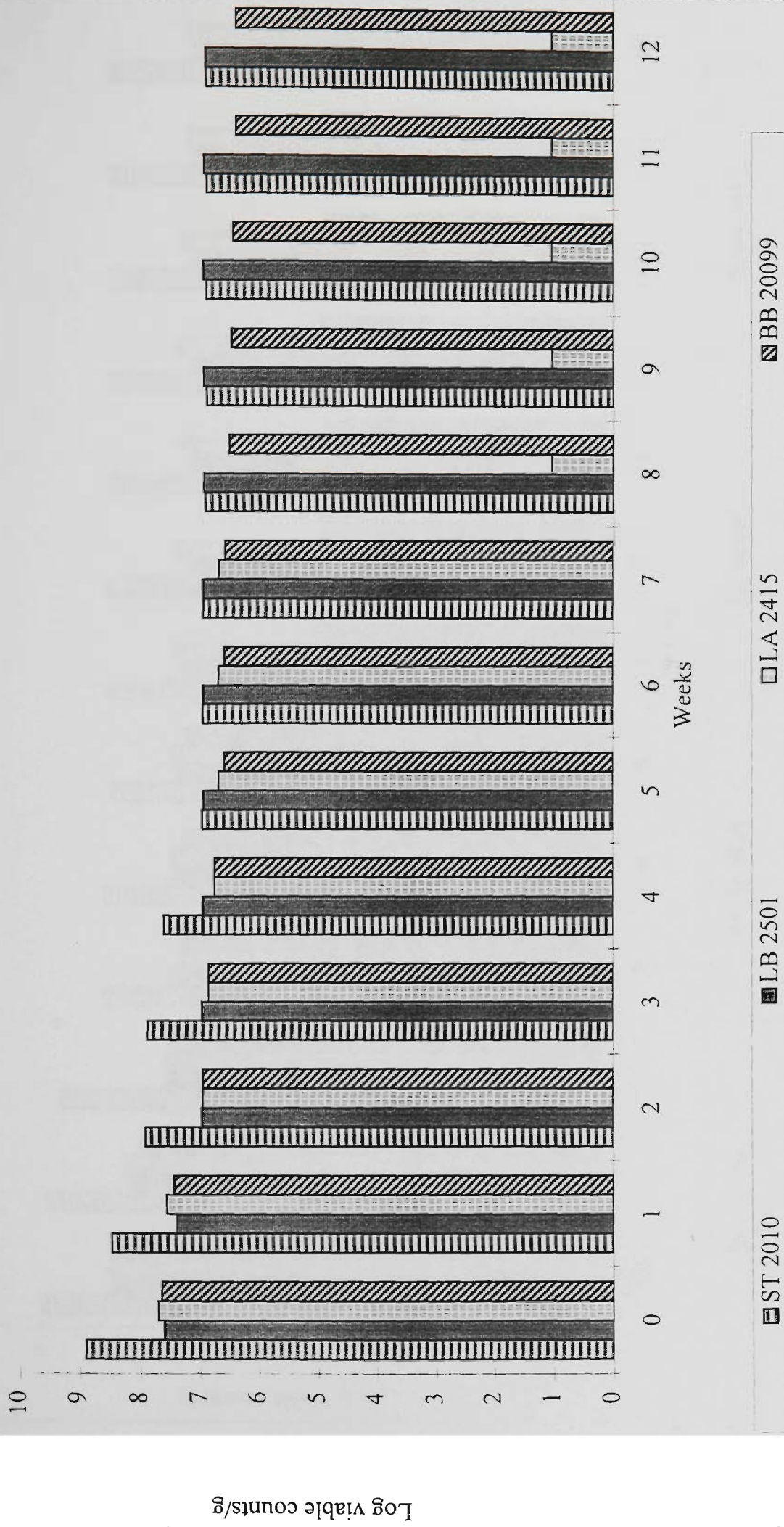
Figure 9.1 Viable counts of yogurt and probiotic bacteria during 12 weeks storage at -18 deg. C. probiotic bacteria were added along with yogurt bacteria

ST 2010   LB 2501   LA 2415   BB 20099

A
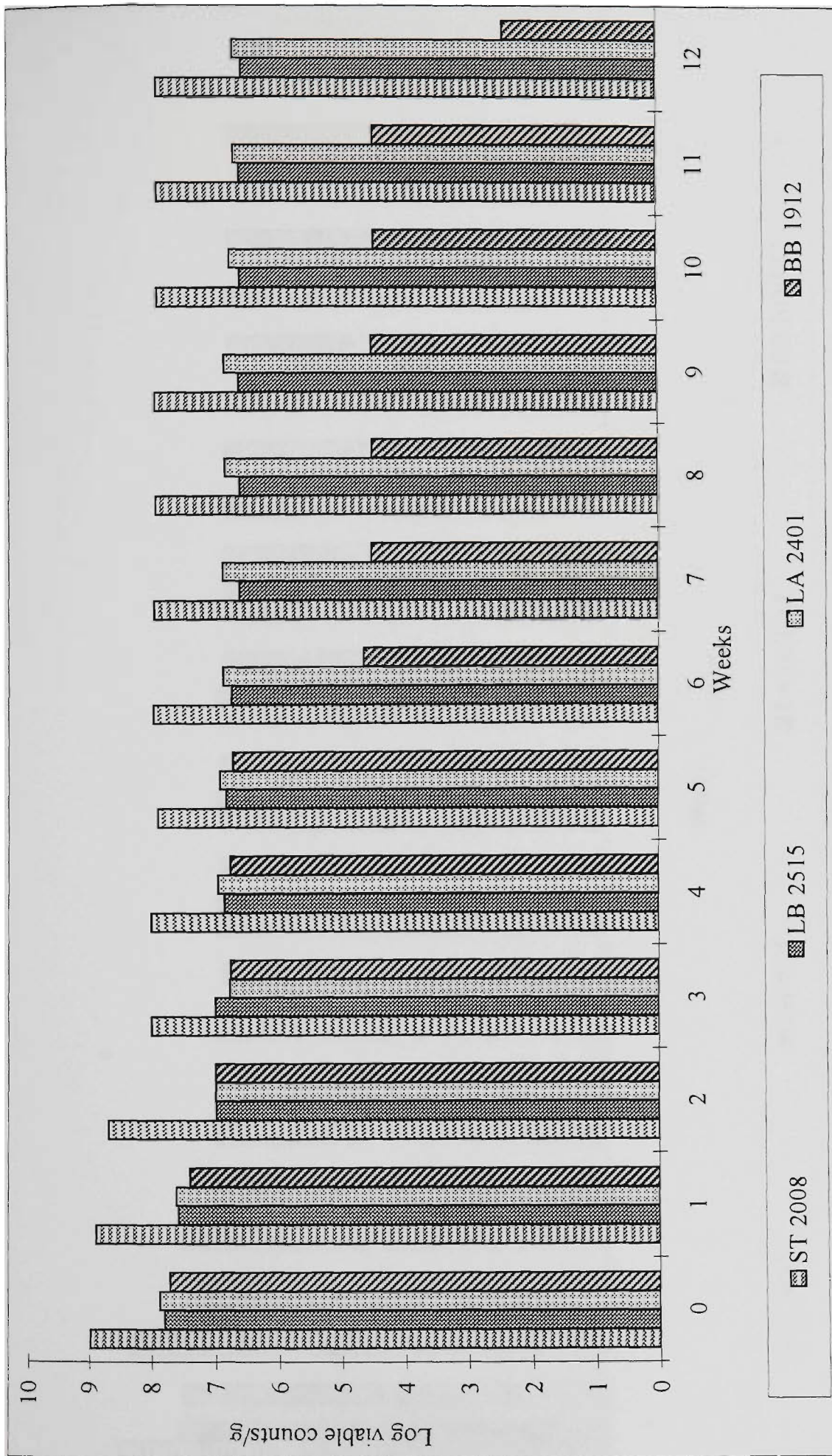
Figure 9.2 Viable counts of yogurt and probiotic bacteria during 12 weeks storage at -18 deg. C. probiotic bacteria were added along with yogurt bacteria

ST 2008   LB 2515   LA 2401   BB 1912

B
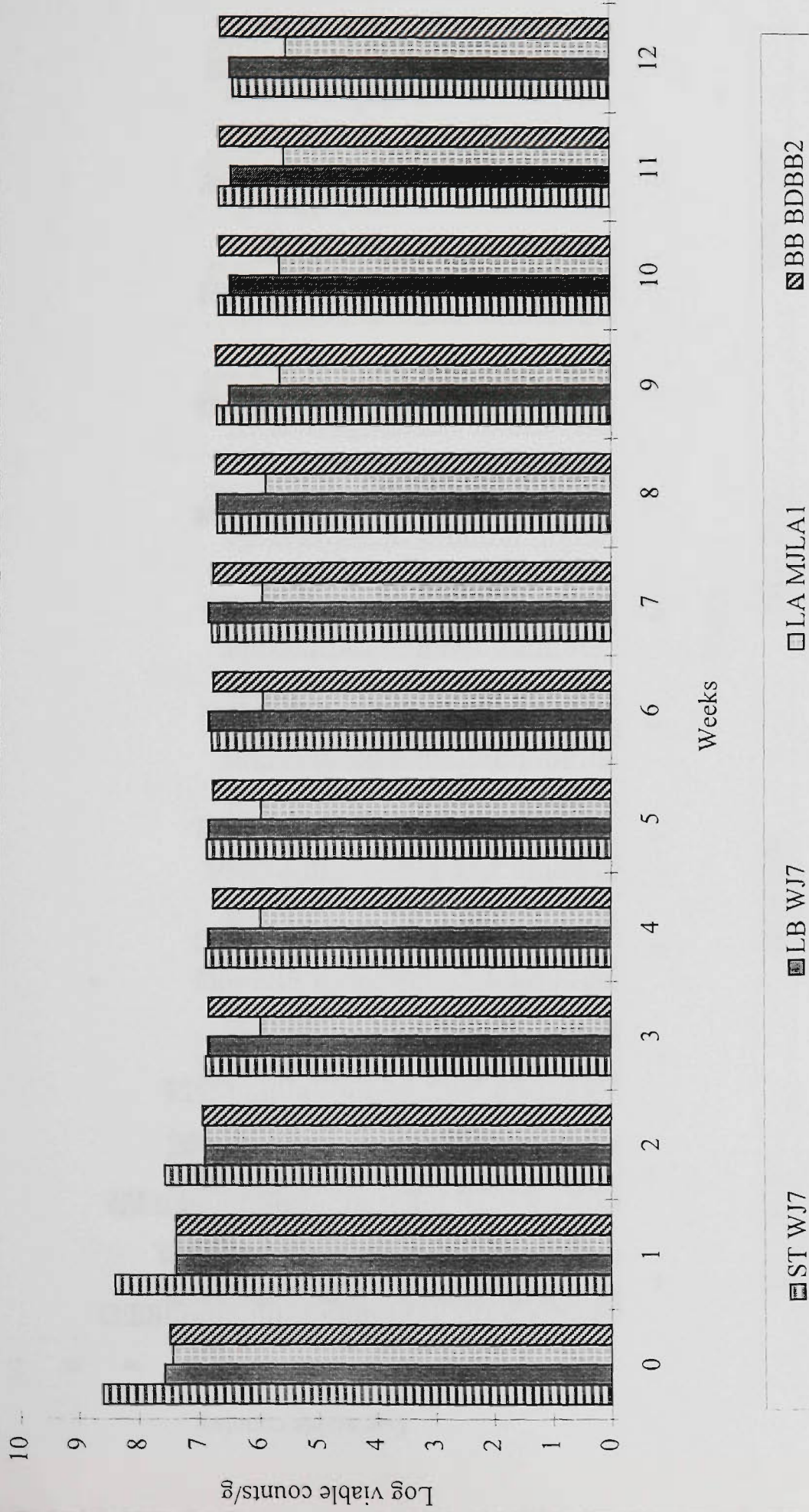
Figure 9.3 Viable counts of yogurt and probiotic bacteria during 12 weeks storage at -18 deg. C. probiotic bacteria were added along with yogurt bacteria

C
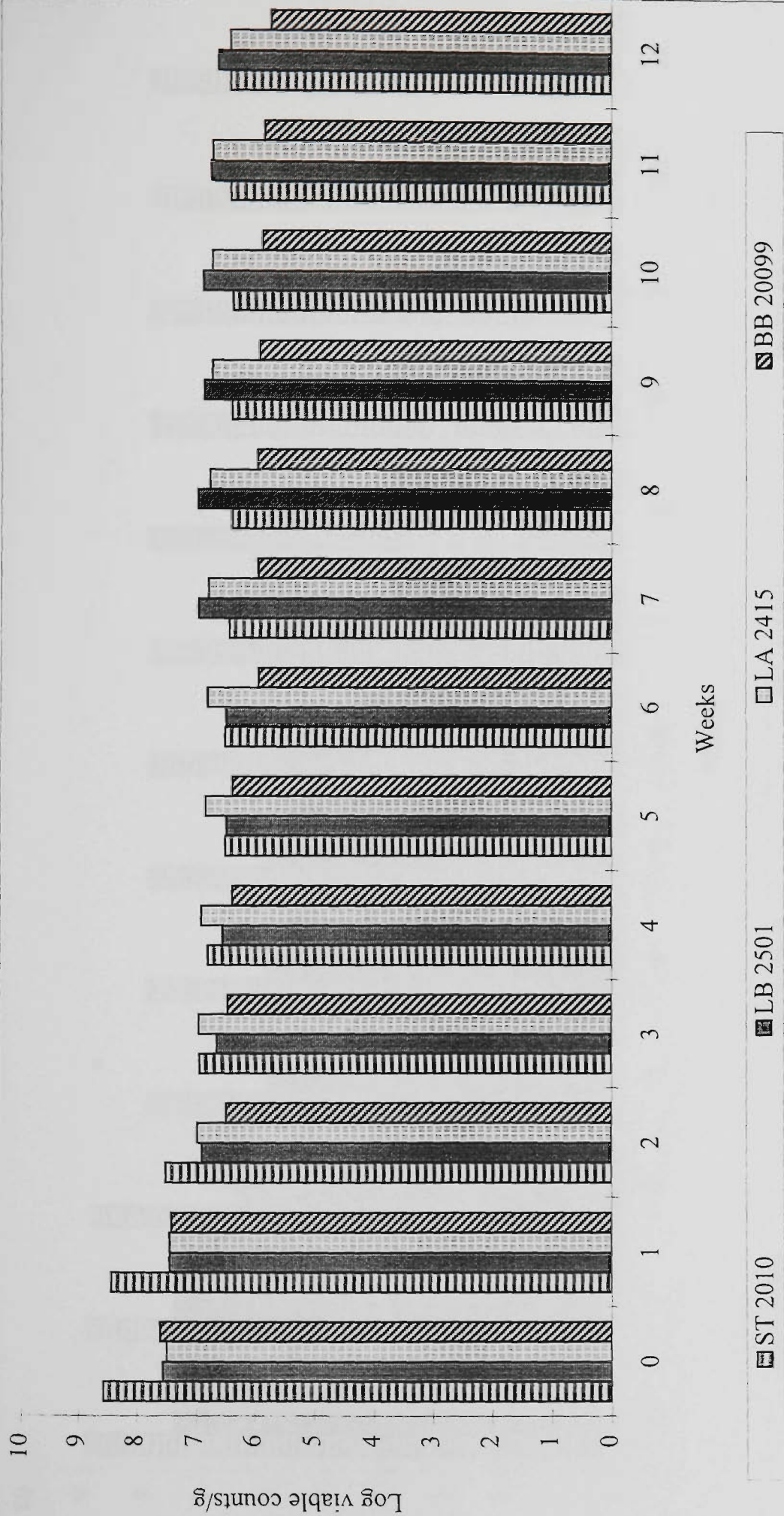
Figure 9.4 Viable counts of yogurt and probiotic bacteria during 12 weeks storage at -18 deg. C. probiotic bacteria were added just before freezing

Figure 9.5 Viable counts of yogurt and probiotic bacteria during 12 weeks storage at -18 deg. C. probiotic bacteria were added just before freezing

E
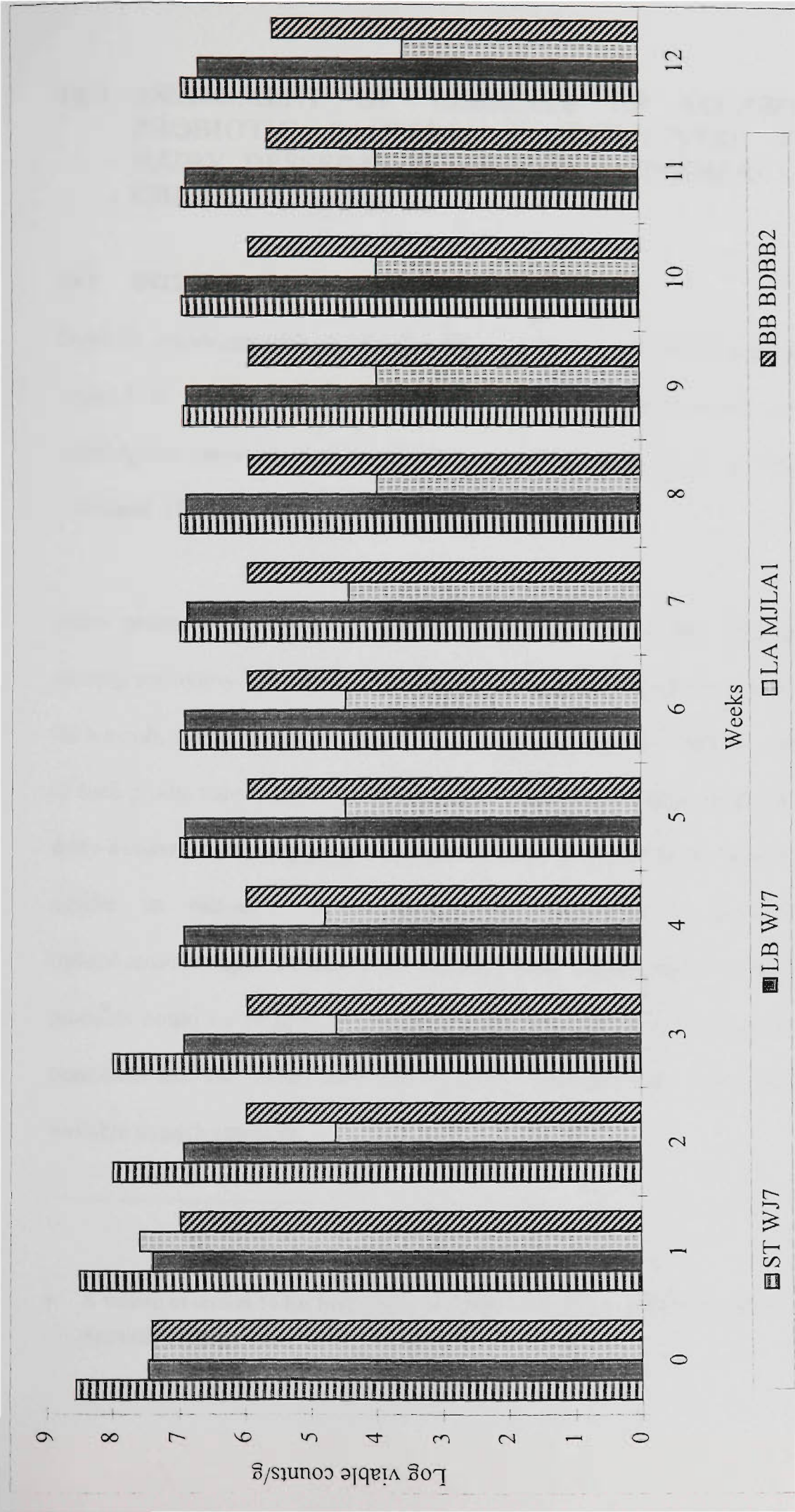
Figure 9.6 Viable counts of yogurt and probiotic bacteria during 12 weeks storage at -18 deg. C. probiotic bacteria were added just before freezing

10.0 ASSESSMENT OF VIABILITY OF YOGURT AND PROBIOTIC BACTERIA IN FERMENTED FROZEN DAIRY DESSERTS MADE USING COMMERCIAL ICE CREAM FREEZER

10.1 INTRODUCTION

Probiotic organisms such as *Lactobacillus acidophilus and Bifidobacterium* spp. are claimed to correct intestinal disorders, lower serum cholesterol, posses anti-carcinogenic properties and improve lactose utilisation in lactose intolerant people (Gilliland, 1989; Shah and Jelen, 1990; Shah, 1993).

Dairy products containing *L. acidophilus* and *Bifidobacterium* spp. are rapidly gaining popularity in Australia. The shelf life of yogurt is approximately 3 weeks. As a result, it may not be feasible to ship yogurt to overseas countries and the sales of such product are limited to local markets. With an increasing popularity of frozen dairy desserts containing yogurt cultures, a market could develop for dairy desserts similar to soft-serve frozen yogurt but containing *L. acidophilus* and *Bifidobacterium* spp. Frozen dairy desserts have longer shelf life and thus the products could be shipped to overseas countries. However, introducing probiotic organisms into the frozen food chain can be a difficult task as the organisms are unstable in such products.

The loss of viability of probiotic organisms in frozen yogurt may be due to acidity of the product, freeze injury, sugar concentration and oxygen toxicity. Using ice cream as a mechanism to incorporate bifidobacteria and fructooligosaccharides into the human diet was studied by Modler *et al.* (1990). In the presence of neosugars and Jerusalem artichoke flour, 90% survival of bifidobacteria (*Bifidobacterium adolescentis, Bifidobacterium infantis* and *Bifidobacterium longum*) was observed at the end of 70 days. The 10% loss in viable counts occurred at the time of freezing due to incorporation of air to achieve 100% overrun. The initial counts were 7.5, 6.9 and 6.6 $\log_{10}$ cfu/ml and the final counts were 7.3, 6.8 and 6.3 $\log_{10}$ cfu/ml respectively in the presence of neosugars. In the presence of Jerusalem artichoke flour the initial counts were 7.3, 7.4 and 6.9 and the final counts were 7.3, 7.2 and 6.4, respectively. The effect of pH on survival of *L. acidophilus* and *Bifidobacterium bifidum* in frozen fermented dairy desserts was studied by Laroia and Martin (1991). *L. acidophilus* and *B. bifidum* survived the freezing process and frozen storage of 8 weeks in pH 5.6-5.8 frozen fermented dairy desserts. The initial counts of *B. bifidum* were 5.7 cfu/ml and the final counts were 4.0 cfu/ml and the initial counts of *L. acidophilus* were 11.4 cfu/ml and the final counts were 11.4 cfu/ml.

Viability of *L. acidophilus* and *B. bifidum* in soft serve frozen yogurt was studied by Holcomb *et al.* (1991). *L. acidophilus* was able to survive when exposed to 0.01N HCl for 2 h at 37^0C. The initial counts of *L. acidophilus* before freezing were 5.90 log cfu and the final counts after freezing were 5.86 log cfu. Survival of *L. acidophilus* and *B. bifidum* in ice cream was also studied by Hekmat and McMahon

(1992). Initial counts of the organisms were 1.5×10^8 cfu/ml for *L. acidophilus* and 2.5×10^8 cfu/ml for *B. bifidum*. After freezing the fermented mix at -29^0C and storing for 17 weeks the bacterial counts were decreased to 4×10^6 and 1×10^7 cfu/ml, respectively. Survival of *L. acidophilus* and *Bifidobacterium* spp. in the presence of acid and bile salts was studied by Lankaputra and Shah (1995). Of several strains *L. acidophilus,* of 2401, 2409 and 2415 survived best under acidic condition, while *B. longum* and *B. pseudolongum* showed best survival under acidic conditions.

In this study, several yogurt and probiotic bacteria were screened for their ability to survive under freezing and acidic conditions and in the presence of high levels of sugar. Frozen fermented dairy desserts were made with selected yogurt and probiotic organisms and survival of these organisms was monitored for 12 weeks during frozen storage at -18^0C. Further, fermented frozen dairy desserts were made on a pilot scale (1000 L) and survival of yogurt and probiotic organisms were monitored for 12 weeks during frozen storage at -18^0C.

10.2   Materials and Method

10.2.1 *Survival of yogurt and probiotic bacteria in fermented frozen dairy desserts made on a pilot scale*

Two different batches of fermented frozen dairy desserts each of 1000 L were made on a pilot scale using an ice cream freezer. For batch 1, yogurt was made by using ST 2008, LB 2501, LA 2401, and BB 20099. Similarly, batch 2 was made with

ST/LB Wj7, MjLa1, and BDBB2. Yogurt and base mix were added at a ratio of 45:45 and 10% of sucrose syrup (65%) was added, followed by freezing the contents using a continuous ice cream freezer (Crepaco Freezer, Model KM 240, U.S.A). The pH of the final product ranged between 4.51 and 4.59.

10.2.2 *Microbiological analyses*

Counts of *S. thermophilus* were enumerated on *Streptococcus thermophilus* agar. Counts of *L. delbrueckii* ssp. *bulgaricus* were enumerated on MRS agar. MRS-salicin and MRS-sorbitol agars were used for enumeration of *L. acidophilus*. Counts of bifidobacteria were enumerated on MRS-NNLP. Enumeration was carried out using the pour plate technique. Plates containing 25 to 250 colonies were enumerated and recorded as colony forming (cfu) units per gram of culture. All the experiments and analyses were repeated at least twice. The results presented are averages of two replicates.

10.3   Results and Discussion

10.3.1 *Composition*

The protein content of the fermented frozen dairy dessert was ~3.75%. The total solids contents were in the range of 32.5-32.8%. The titratable acidity ranged between 0.74% and 0.76%. The fat content was 10.3%, while the sugar content was 16.8%. The pH ranged between 4.51 and 4.59. The overrun of the product made with a bench top freezer was 18% and that made with Crepaco freezer was 100%.

10.3.2 Viability of yogurt and probiotic bacteria in fermented frozen dairy dessert made using a pilot scale ice cream freezer during frozen storage for 12 weeks

Figs (10.1-10.4) shows the viability of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and bifidobacteria in fermented frozen dairy dessert made using a continuous ice cream freezer. The counts of *S. thermophilus* WJ7 decreased by about 1 log cycle after 6 weeks and those of *L. delbrueckii* ssp. *bulgaricus* 2501 and WJ7 reduced by about 1 log cycle after 3 and 8 weeks. The counts of *L. acidophilus* MJLA1 were reduced by about 1 log cycles after 1 week and 2 log cycles after 8 and 9 weeks. There was no decrease in the population of bifidobacteria 20099 for up to 8 weeks of storage; however, there was 1 log cycle decrease during the rest the of storage period. At the end of 12 weeks storage period, the counts of *L. acidophilus* and bifidobacteria were ~ 10^5 cfu/g.

Several factors affect the viability of probiotic bacteria in frozen fermented dairy products. Survival of these organisms is affected by low pH of the environment. The standard requires the pH of the product to be < 4.5, which affects their viability. In the manufacture of fermented frozen dairy desserts air is incorporated to achieve 100% overrun. Bifidobacteria are anaerobic and *L acidophilus* is microaerphilic and these organisms prefer a low oxidation-reduction potential for growth. Addition of sugar increases the osmotic pressure which may have effect on the growth and viability of probiotic bacteria. The optimum temperature for the growth of *L. acidophilus* and bifidobacteria is 37^0C. Thus, when the product is frozen to -18^0C or below, low temperature affects the viability of these organisms. The survival of

these organisms appeared to be strain dependent, thus selection of strains will be an important factor for the survival.

10.4   Conclusion

All strains screened for freeze tolerance remained viable before and after freezing and at 8 and 16% sugar levels. Of the various pH levels studied, pH 4.5 and 4.0 were found to be detrimental for *S. thermophilus* 2013, *L. delbrueckii ssp. bulgaricus* 2515 and *L. acidophilus* MJLA1, while rest of the organisms were not affected by pH.

Counts of yogurt bacteria were reduced by about 1 log cycle in the products made using a bench top freezer during 12 weeks storage. *L. acidophilus* 2415 was adversely affected and the counts declined by 6 log cycles within 8 weeks of storage at -18^0C. Amongst bifidobacteria strain 1912 decreased by 5 log cycles at the end of 12 weeks storage. *L. acidophilus* MJLA1 and bifidobacteria BDBB2 reduced by about 2 log cycles in the products made on a pilot scale after 12 weeks storage.
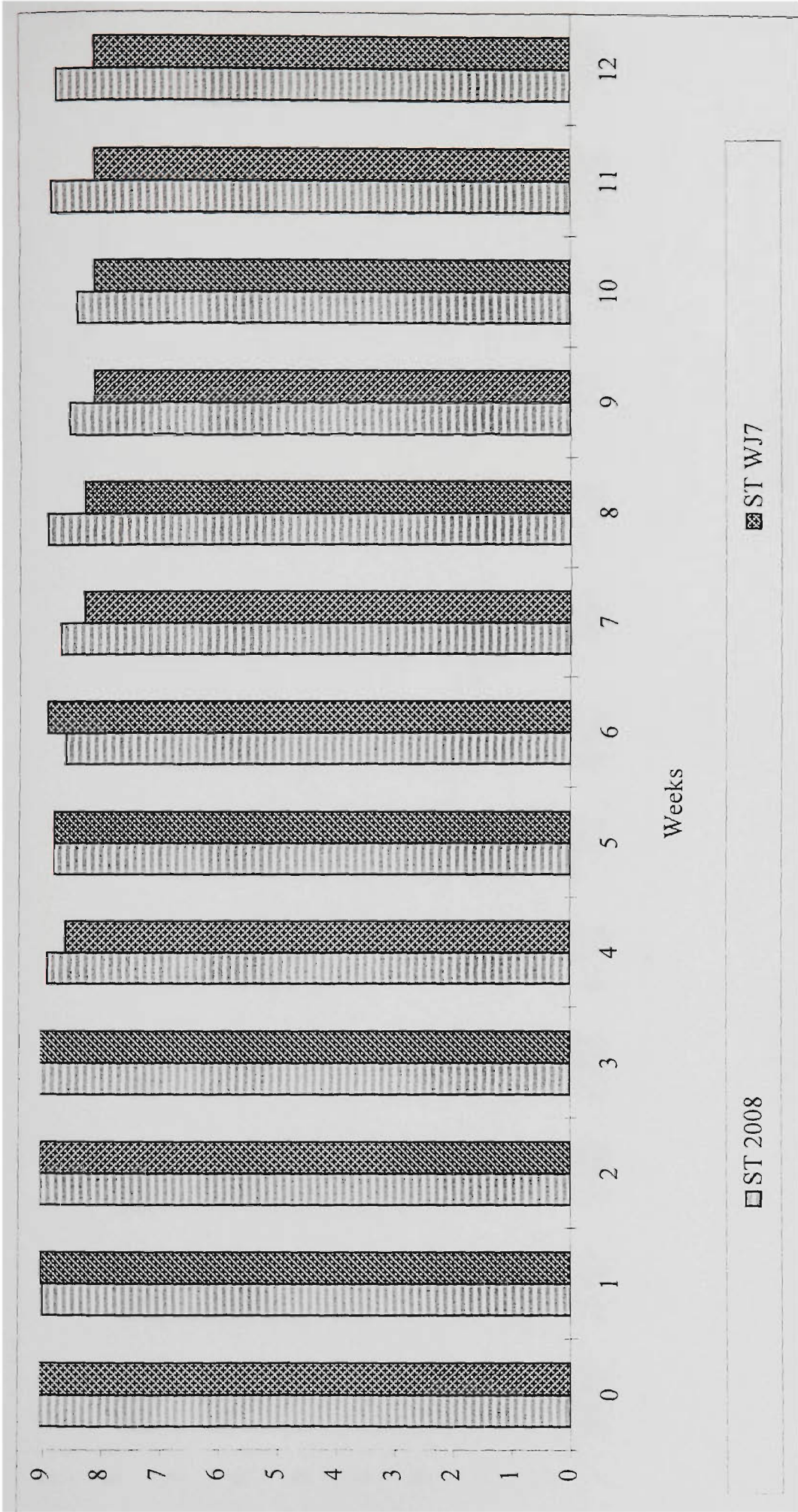
Figure 10.1 Viable counts of *S. thermophilus* during 12 weeks storage at -18 deg. C.

□ ST 2008   ⊠ ST WJ7

A
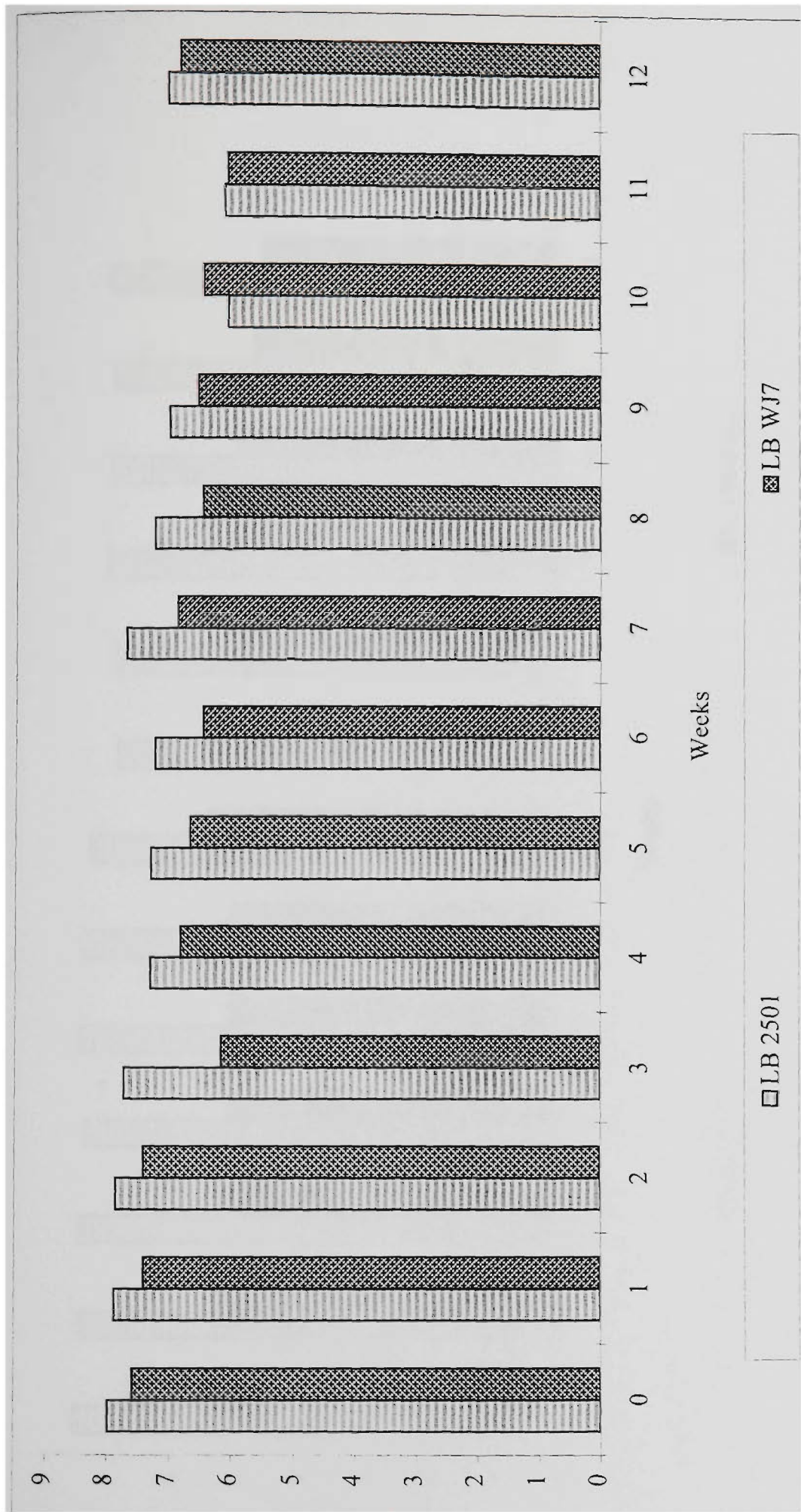
Figure 10.2 Viable counts of *L. delbruekii* ssp. *bulgaricus* during 12 weeks storage at -18 deg. C

B
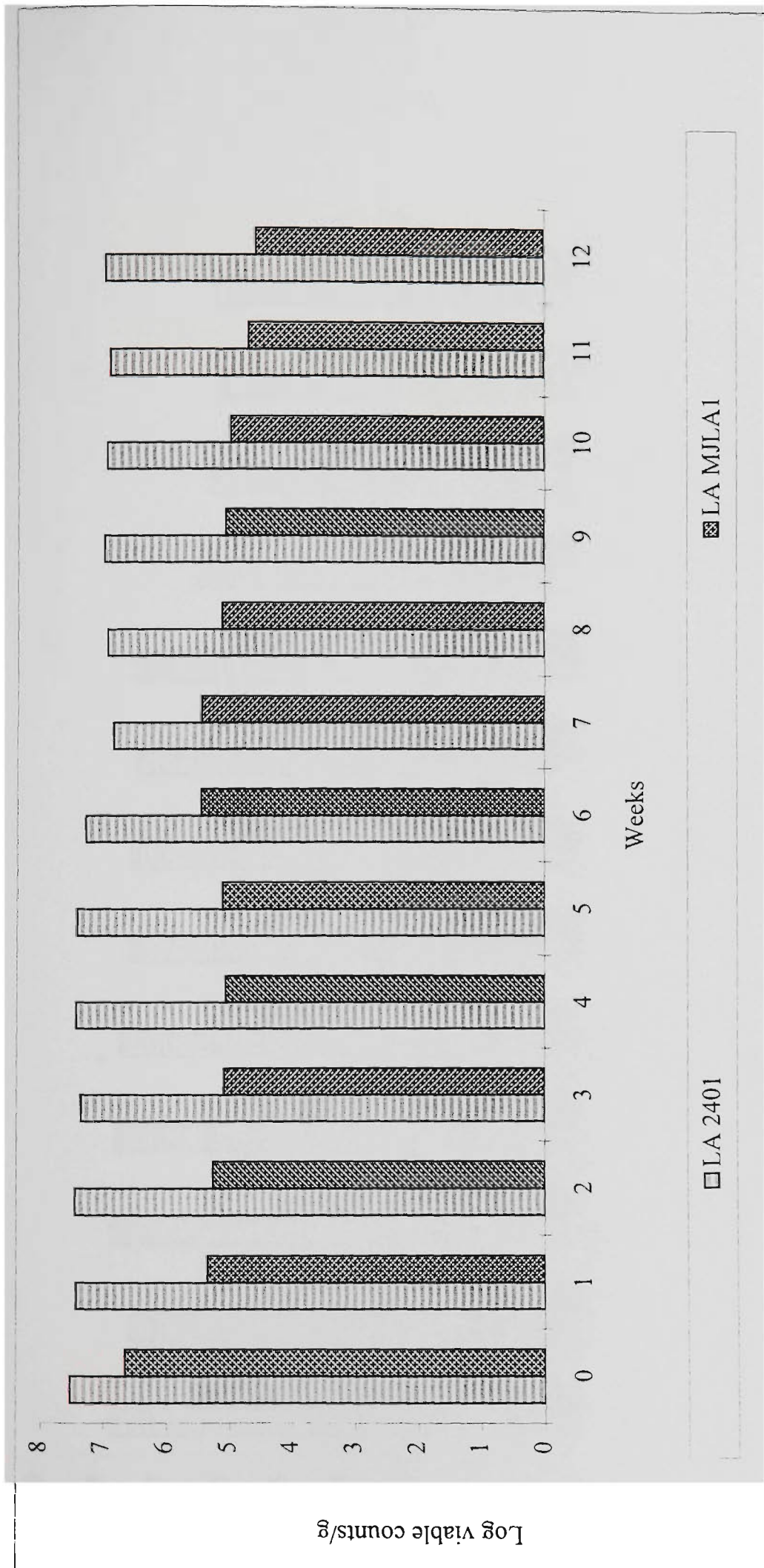
Figure 10.3 Viable counts of *L. acidophilus* during 12 weeks storage at -18 deg. C

C
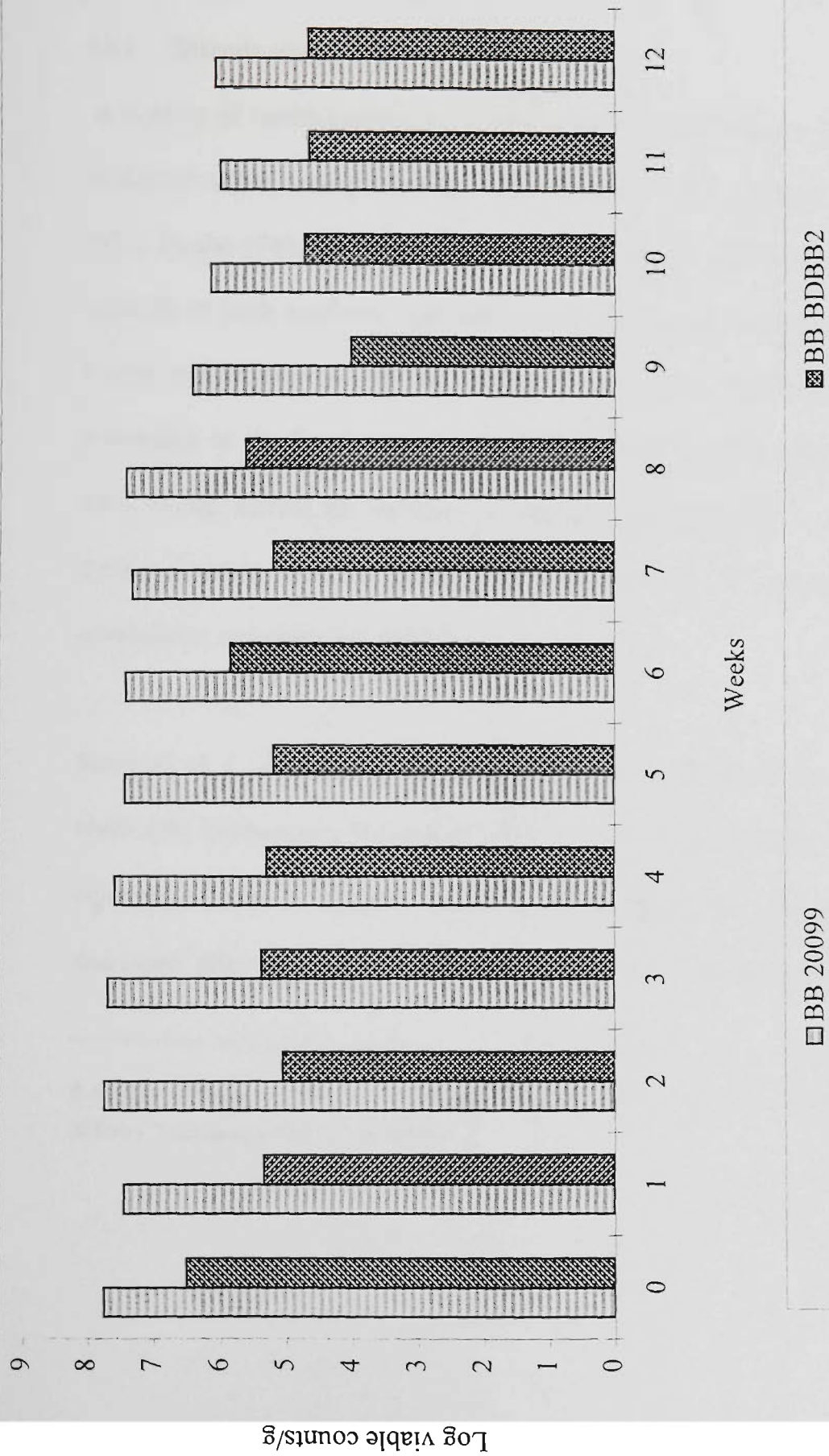
Figure 10.1 Viable counts of bifidobacteria during 12 weeks storage at -18 deg. C

D

11.0 EFFECT OF ACID CASEIN HYDROLYSATE AND CYSTEINE ON VIABILITY OF YOGURT AND PROBIOTIC BACTERIA IN FERMENTED FROZEN DAIRY DESSERTS

11.1 Introduction

A number of health benefits have been claimed for probiotic organisms such as *Lactobacillus acidophilus* and *Bifidobacterium* spp. (Hughes and Hoover 1991; Kanbe 1992; Mital and Garg 1992). However, probiotic organisms are unstable in such products. The loss of viability of probiotic organisms in a frozen yogurt may be due to acidity, freeze injury and oxygen toxicity. According to the Food Standards Code H8, the pH of the product must be ≤4.5, which affects the viability of probiotic bacteria. Air is incorporated during freezing process which affects the growth of microaerophlic *L. acidophilus* and anaerobic bifidobacteria.

Survival of *L. acidophilus* and *Bifidobacterium bifidum* in ice cream was studied by Hekmat and McMahon (1992). They reported that the counts of *L. acidophilus* and *B. bifidum* and their β-D-galactosidase (lactase) activity decreased after freezing at -29^0C and during frozen storage for 17 weeks.

The survival of *L. acidophilus* cells during freezing at -196^{0}C and subsequent refrigerated storage was studied by Brashears and Gilliland (1995). Organisms that assimilated cholesterol were also affected due to freezing (Brashears and Gilliland, 1995).The authors concluded that the number of lactobacilli declined over time as did the β-D-galactosidase activity when the organisms were suspended in milk and stored at 7^{0}C. Using ice cream as a medium to incorporate bifidobacteria and fructo-oligosaccharides into human diet was studied by Modler *et al.* (1990). In the presence of neosugars and Jerusalem artichoke flour, 90% survival of bifidobacteria was observed at the end of 70 days of frozen storage. A 10% loss in viable counts occurred at the time of freezing due to incorporation of air to achieve 100% overrun.

The effect of pH on survival of *L. acidophilus* and *B. bifidum* in frozen fermented dairy desserts was studied by Laroia and Martin (1991). *L. acidophilus* and *B. bifidum* survived the freezing process and frozen storage of 8 weeks in fermented frozen dairy desserts having pH of 5.6-5.8.

Probiotic bacteria grow slowly in milk due to lack of proteolytic activity. Dave and Shah (1997b, 1998) and Klaver (1993) have shown that milk supplemented with peptides and amino acids such as cysteine improved the survival of bifidobacteria. Cysteine is a sulfur containing amino acid that is incorporated into agar media for the growth of bifidobacteria (Laroia and Martin, 1991). Cysteine is also known for lowering redox potential and addition of this amino acid could improve viability of anaerobic

bifidobacteria. Similarly acid casein hydrolysate is a product that is made from hydrolysis of casein and is rich in amino acids and peptides. Thus, acid casein hydrolysate and cysteine may provide growth factors essential for the growth of probiotic bacteria and could improve their viability.

The aim of this study was to investigate the effect of acid casein hydrolysate and cysteine on the viability of *L. acidophilus* and bifidobacteria in fermented frozen dairy desserts.

11.2 Materials and Methods

11.2.1 *Propagation of yogurt bacteria and probiotic bacteria*

Yogurt bacteria (*Streptococcus salivarius* ssp. *thermophilus* ST WJ7 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB Wj7) and probiotic bacteria (*Lactobacillus acidophilus* LA MJLA1 and bifidobacteria BB BDBB2) were obtained from Gist-brocades (formerly Mauri Laboratories Pty. Ltd., Moorebank, Sydney). All the strains were tested for purity by Gram stain and were propagated every week in sterile 12% reconstituted skim milk (RSM) supplemented with 2% glucose and 1% yeast extract. All the cultures were grown for 18 h at 37^0C using 1% inoculum. For propagation of bifidobacteria, sterile RSM was supplemented with 0.05% L-cysteine. hydrochloride in order to provide anaerobic condition and to stimulate their growth. The cultures were maintained in the same medium at 4^0C. Before enumeration, the cultures were transferred successively three times for activation.

11.2.2 *Manufacture of fermented frozen dairy desserts*

Fermented frozen dairy desserts were made according to the method of Ravula and Shah (1998b). Yogurt and base mix were prepared separately and blended at a rate of 45% of each and 10% sucrose syrup was added. The blend was then frozen using a bench top ice cream freezer (Model Im 300 PK 185/w, Kenwood Appliances, Italy).

Three batches of yogurt (3L) were made with homogenised and pasteurised milk (1% fat) supplemented with 2% skim milk powder. The mix was heated at 85^0C for 30 min, cooled to 42^0C and divided into three lots. Acid casein hydrolysate (0.5g/L) (Sigma Chemical Co. St. Louis, MO) and cysteine (0.05g/L) (Sigma) were added to the two batches of heated and cooled yogurt mix to study the effect of these growth factors on viability of probiotic bacteria in fermented frozen dairy desserts. The third batch was made with milk supplemented with 2% skim milk powder only (control). Yogurt bacteria (*S. salivarius* ssp. *thermophilus* STWJ7 and *L. delbrueckii* ssp. *bulgaricus* LBWJ7), and probiotic bacteria (*L. acidophilus* LA MJLA1 and bifidobacteria BB BDBB2) were added at the rate of 0.5% each and the inoculated mix incubated at 42^0C till pH 4.5 was attained. The yogurt was transferred to a cold room till the temperature decreased to 4^0C. The yogurt and base mix were mixed at a rate of 45% of each and 10% of liquid sucrose (65%) was added. All the three batches of fermented frozen dairy desserts were made using a bench top ice cream freezer with an overrun of 18%.

11.2.3 *Microbiological analysis-*

Microbiological analysis described as per material and method section 3.4

11.2.4 *β-D-galactosidase activity*

The β-D-galactosidase activity was determined according to the method of Shah and Jelen (1990). Ten grams of each product were mixed with distilled water, blended for 1 min and the final volume made up to 100 mL in a volumetric flask. One millilitre of the solution was used in the assay. A solution of 0.005M O-nitrophenyl-β-D-galactopyranoside (ONGP) was prepared in 0.1M phosphate buffer (pH 7.0), and 1 mL aliquots of the diluted samples were incubated with 5 mL ONGP solution for 15 min at 37^0C. The reaction was stopped by adding 2.5 mL 1M cold sodium carbonate. The amount of O-nitrophenol released was measured with a spectrophotometer at 420 nm. One unit of β-D-galactosidase activity was defined as the amount of enzyme which liberated one μmole O-nitrophenol from ONGP per min per gram sample at 37^0C.

11.2.4 *Compositional analyses*

The protein content of the product was determined by the Kjeldahl's method using the Kjeltec digestion system and Tecator Kjeltec distillation unit. The total solids contents were determined by drying samples at 101^0C for 2 h using an air oven (Model 500, Memmert, GmbH+ Co., Schwabach) (Atherton and Newlander, 1977). The titratable acidity was measured by titrating 9 g of sample with 0.01N NaOH using phenolphthalein indicator (Atherton and Newlander, 1977). The fat content was determined by the Mojonnier method

(Atherton and Newlander, 1977). The pH was measured with a pH meter (Model 420A, Orion Research Inc., Boston, U.S.A). The sugar content was determined by the colorimetric method (Dubois *et al.*, 1956).

11.3   Results and Discussion

11.3.1 *Composition*

The average protein content of the product was 3.82%. The total solids contents were in the range of 31.4-32.3%. The titratable acidity ranged between 0.77% and 0.79% . The average fat content was 10.3%, while the average sugar content was 16.9%. The pH ranged between 4.51 and 4.53. The overrun of the product was 18%.

The overrun of the commercial frozen desserts is usually 100%. However, an overrun of 100% cannot be achieved with a bench top freezer. Incorporation of air may influence the viability of microaerophlic *L. acidophilus* and anaerobic bifidobacteria.

11.3.2 *pH and titratable acidity*

The initial pH of milk (6.56-6.64 at 0 h) decreased to pH 4.51-4.53 during yogurt making. In general, there was a gradual decrease in the pH in all 3 products during storage for 12 weeks. The final pH by the end of storage period was 4.45 for control, 4.48 for the product supplemented with acid casein hydrolysate and 4.51 for that supplemented with cysteine (Figure 11.1).

The final pH of yogurt or of frozen fermented dairy desserts can affect the growth and viability of *L. acidophilus* and *B. bifidum*, most notably that of bifidobacteria (Laroia and Martin, 1991; Hekmat and McMahon, 1992). It has been found that acid production by lactic acid bacteria, especially post-acidification, affected cell viability of *L. acidophilus* and *B. bifidum* (Ishibashi and Shimamura, 1993). This may be due to residual activity of lactic bacteria that produce lactic or acetic acid that decreases the pH. It is interesting to note that the products containing acid casein hydrolysate and cysteine had higher pH than the control and the two batches also showed better survival of yogurt bacteria and probiotic bacteria. Acid casein hydrolysate and cysteine may have increased buffering capacity of the product (Kailasapathy *et al.*, 1996) and resulted in less decrease in pH.

Lankaputhra and Shah (1995) studied the survival of probiotic bacteria at various pH levels. Of the various strains studied, over 50% of the strains lost viability after 6 days of storage at pH <4.3. The minimum pH for survival of *L. acidophilus* and bifidobacteria is 5.5 (Holt, 1986). Thus a decline in their counts is expected to occur.

The changes in titratable acidity (TA) in fermented frozen dairy desserts is illustrated in Figure 11.2. The initial TA of milk 0.14 increased to 0.77-0.79% after fermentation and the final acidity at the end of storage period further

increased to 0.82-0.89%. The increase in TA of the control sample was the highest (0.89%).

11.3.3 *Enzyme activity*

The β-D-galactosidase activity in the three samples is shown in Figure 11.3. The β-D-galactosidase activity declined by 34% in the control sample, whereas in the sample supplemented with acid casein hydrolysate and cysteine the enzyme activity declined only by 10% and 5.5%, respectively. This may be due to buffering action of acid casein hydrolysate and of cysteine, which may have provided protection to organisms resulting in increased enzyme activity.

Hekmat and McMahon (1992) observed that β-D-galactosidase activity was lost more rapidly in a refrigerated yogurt than in a frozen yogurt. Mashayekh and Brown (1992) observed that 20% of β-D-galactosidase activity in the yogurt was lost after 30 days of refrigerated storage, whereas in a frozen ice cream only 11% activity was lost during the same storage period. Speck and Geoffrion (1980) also found about 50% reduction in the β-D-galactosidase activity of unfrozen yogurt during a 20 days storage period, but there was no further decrease in the β-D-galactosidase activity of the frozen yogurt.

11.3.4 *Viable counts of yogurt bacteria and probiotic bacteria*

Figure 11.4 shows the viable counts of yogurt bacteria in the three batches of fermented frozen dairy desserts. In all the three batches, the counts of *S. thermophilus* remained high until the end of the storage period. There was approximately 2 log cycle decrease in the counts of *L. delbrueckii* ssp. *bulgaricus* and the counts decreased to 10^6 cfu/g by the end of 12 week storage period. However, for the product supplemented with acid casein hydrolysate and cysteine, the counts of *L. delbrueckii* ssp. *bulgaricus* decreased by 1 log cycle only. The viable counts of probiotic bacteria are shown in Figure 11.5. The counts of *L. acidophilus* and bifidobacteria decreased rapidly in the control sample and the viable counts were $<10^2$ cfu/g by the end of 12 weeks storage period. The decrease in the counts of *L. acidophilus* and bifidobacteria in samples supplemented with acid casein hydrolysate or cysteine was less than those with the control sample and the viable counts at the end of 12 weeks storage period was $>10^5$ cfu/g. Improved survival and viability of *L. acidophilus* and bifidobacteria are possibly due to nutrients such as peptides and amino acids supplied through acid casein hydrolysate and cysteine and due to decreased redox potential. Improved viability could have also been due to buffering action of acid casein hydrolysate and cysteine, which resulted in less drop in pH (Figure 1A). Cysteine is a redox-potential reducing agent and is an essential amino acid required for the growth of bifidobacteria. Thus improved survival of probiotic bacteria, in particular bifidobacteria in fermented frozen dairy desserts could also due to redox potential reducing property of cysteine.

Effect of cysteine, whey protein concentrate, acid casein hydrolysate and tryptone on viability of *Streptococcus thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus acidophilus* and bifidobacteria was studied by Dave and Shah (1998). They reported that the addition of these ingredients improved viability of *L. acidophilus* and bifidobacteria by providing growth factors as these probiotic bacteria lack proteolytic activity.

Air is incorporated in fermented frozen dairy desserts and air may affect survival of microaerophilic *L. acidophilus* and anaerobic bifidobacteria. This study was carried out using a bench top ice cream freezer and the level of overrun was only 18%, whereas the level of overrun could be 100% for commercial ice cream freezers. Therefore, in commercial practice, the level of acid casein hydrolysate or cysteine may need to be increased in order to provide sufficient peptides and amino acids, and to decrease redox potential for improved viability of probiotic bacteria.

11.4 Conclusion

Survival of yogurt and probiotic bacteria was studied in fermented frozen dairy desserts, made from milk with or without supplementary acid casein hydrolysate and cysteine, at fortnightly intervals for 12 weeks. Initial counts of the bacteria and their subsequent survival were better in the supplemented products due to nutrients such as peptides and amino acids and due to reduced redox potential. The β-D-galactosidase activity declined more rapidly in the

control sample than in the samples supplemented with acid casein hydrolysate or cysteine. The results suggested that the acid casein hydrolysate and cysteine stimulated the growth of *L. acidophilus* and bifidobacteria which resulted in improved viability of these organisms.
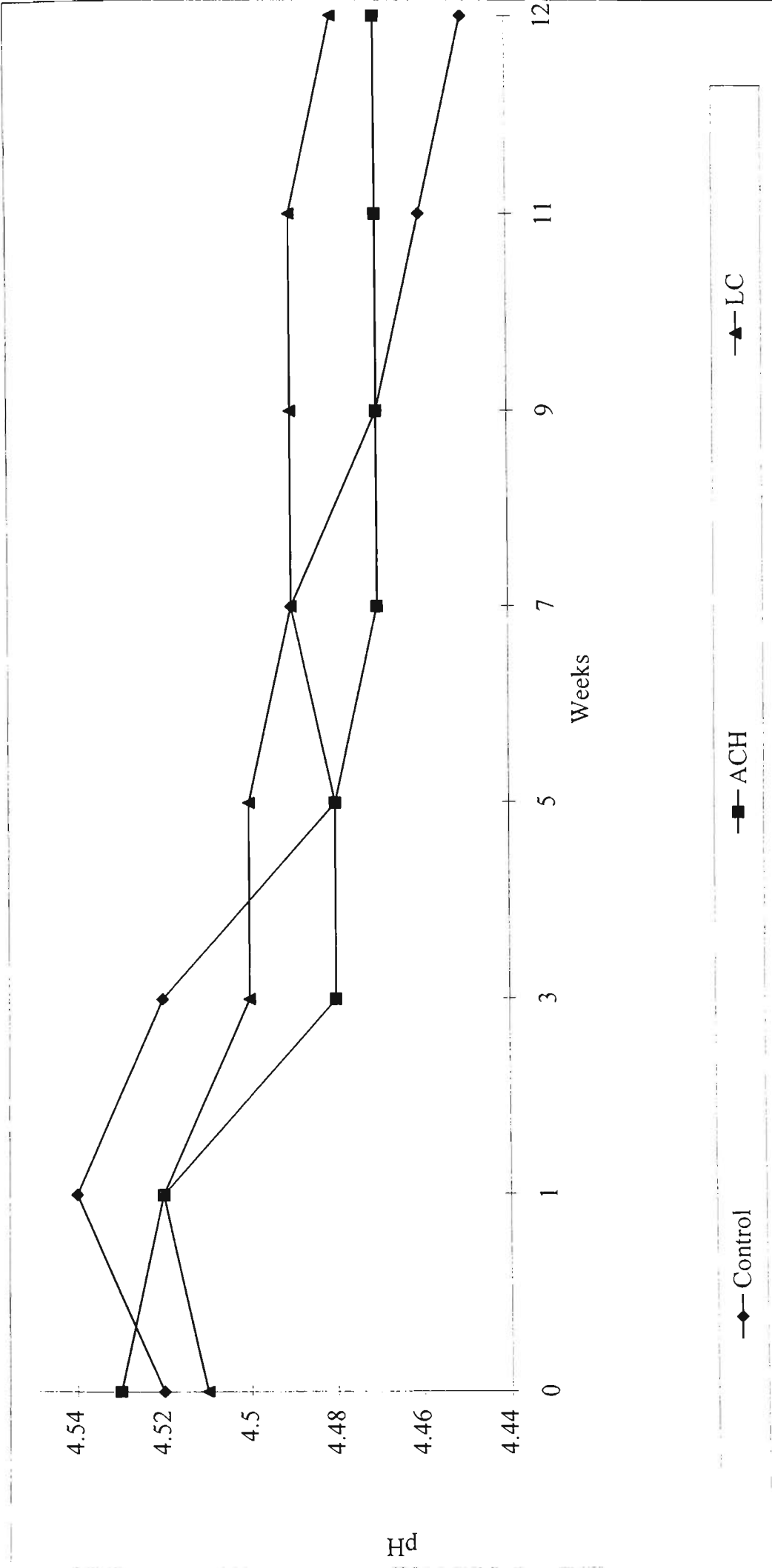
Figure 11.1 Changes in pH in fermented frozen dairy desserts during storage for 12 weeks at -18 deg C
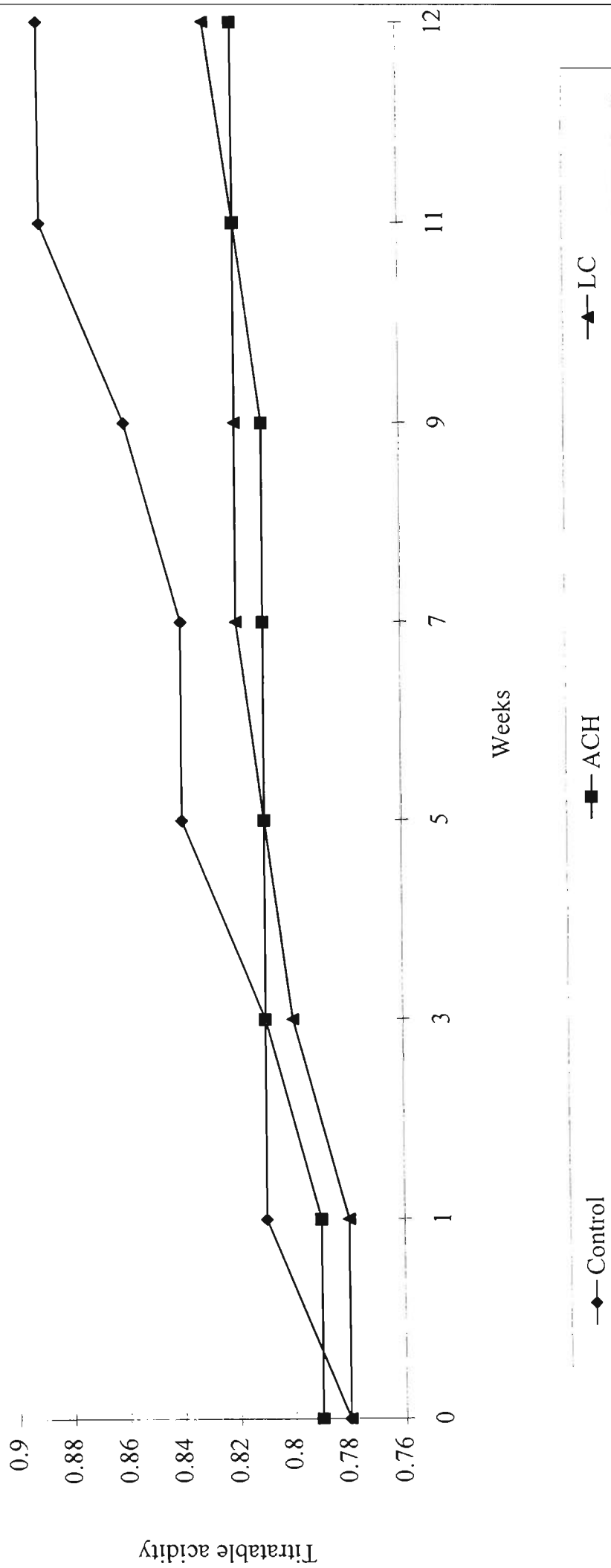
Figure 11.2 Changes in titratable acidity in fermented frozen dairy desserts during storage for 12 weeks at -18 deg C

B
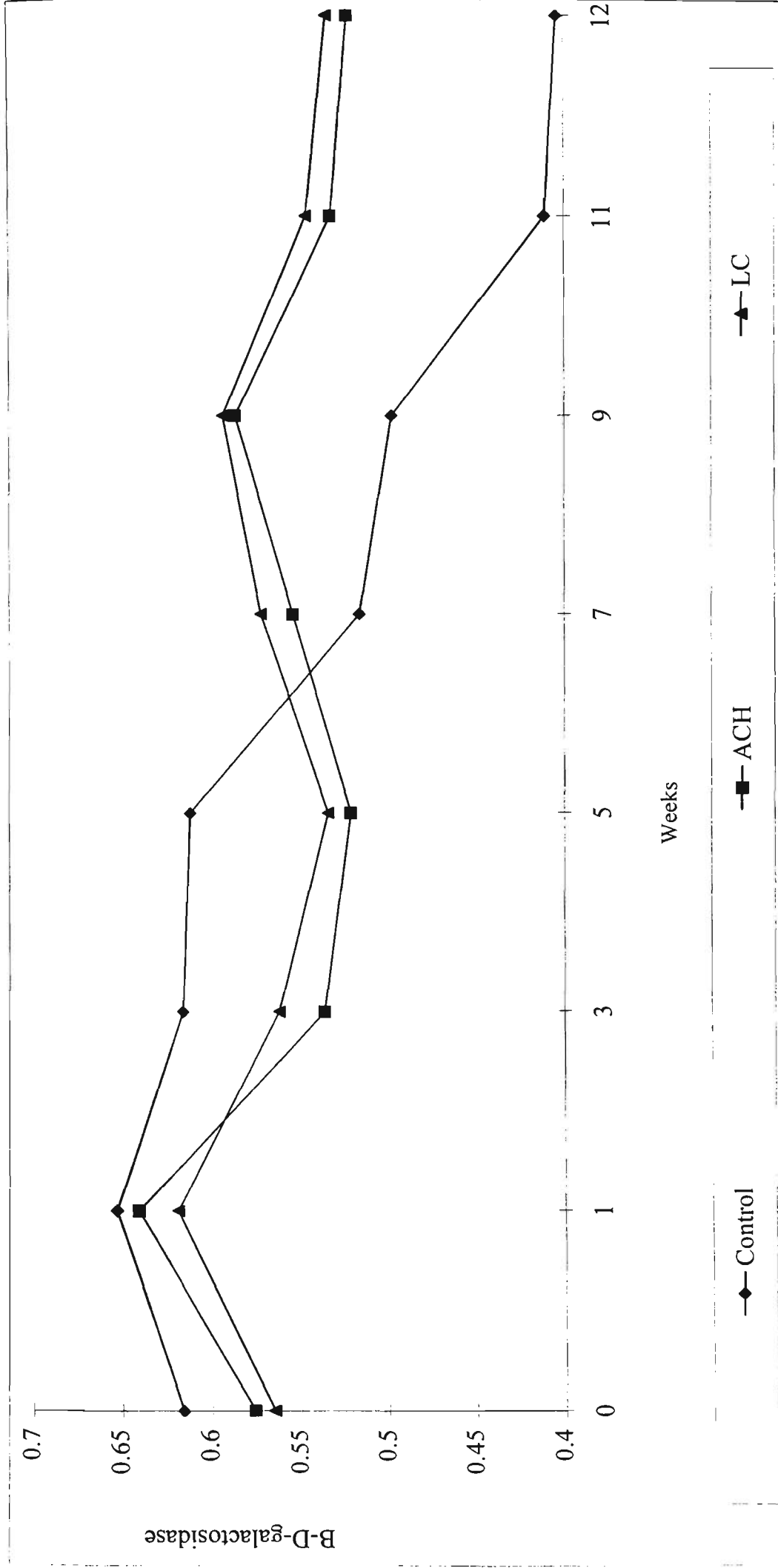
Figure 11.3 Changes in B-D galactosidase in fermented frozen dairy desserts during storage for 12 weeks at -18 deg C
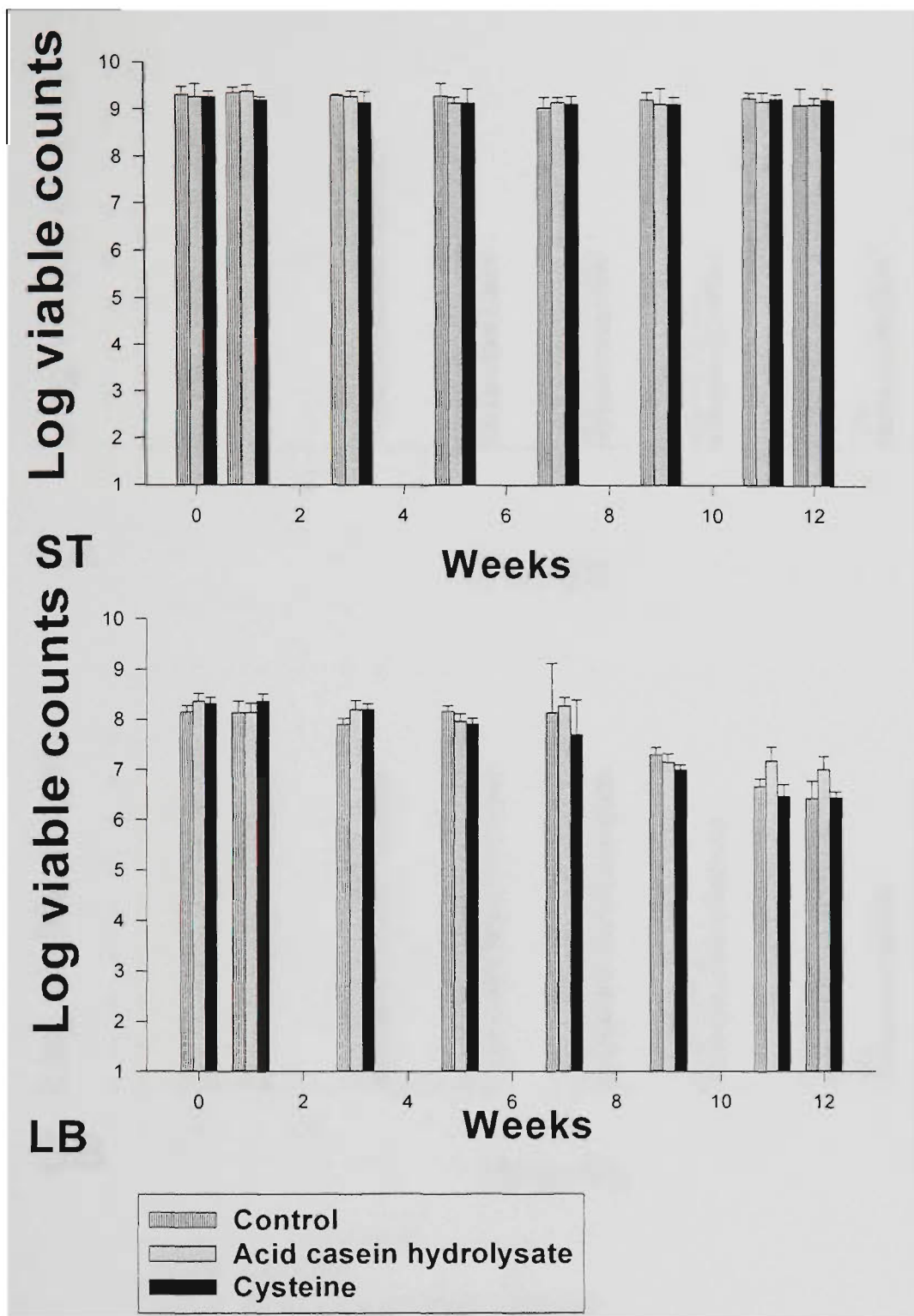
Figure 11.4 Viable counts of yogurt bacteria in the products made with or without acid casein hydrolysate and cysteine during 12 weeks storage at -18^0C
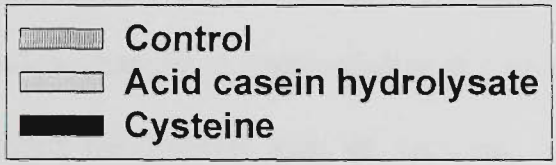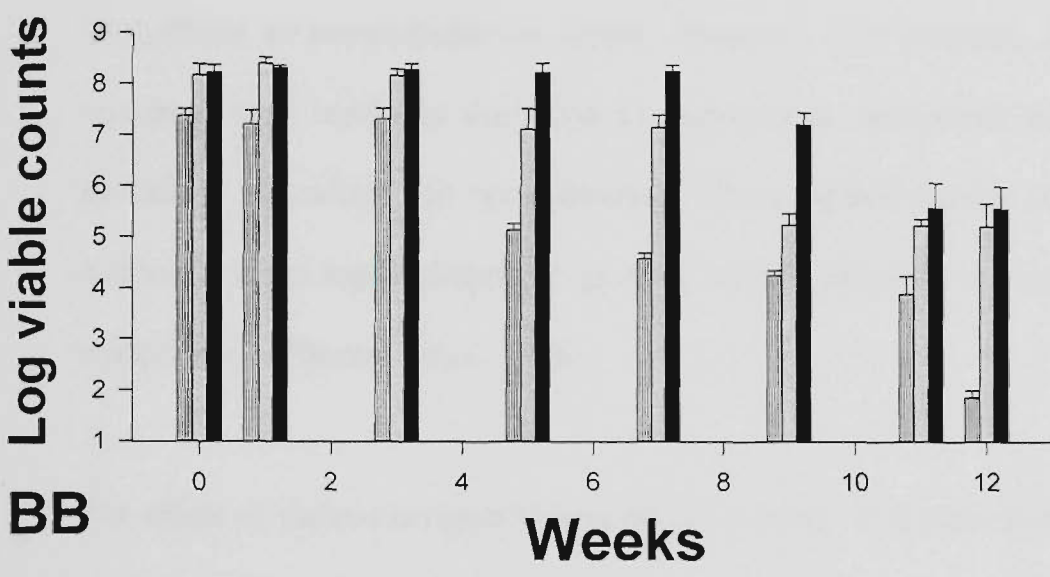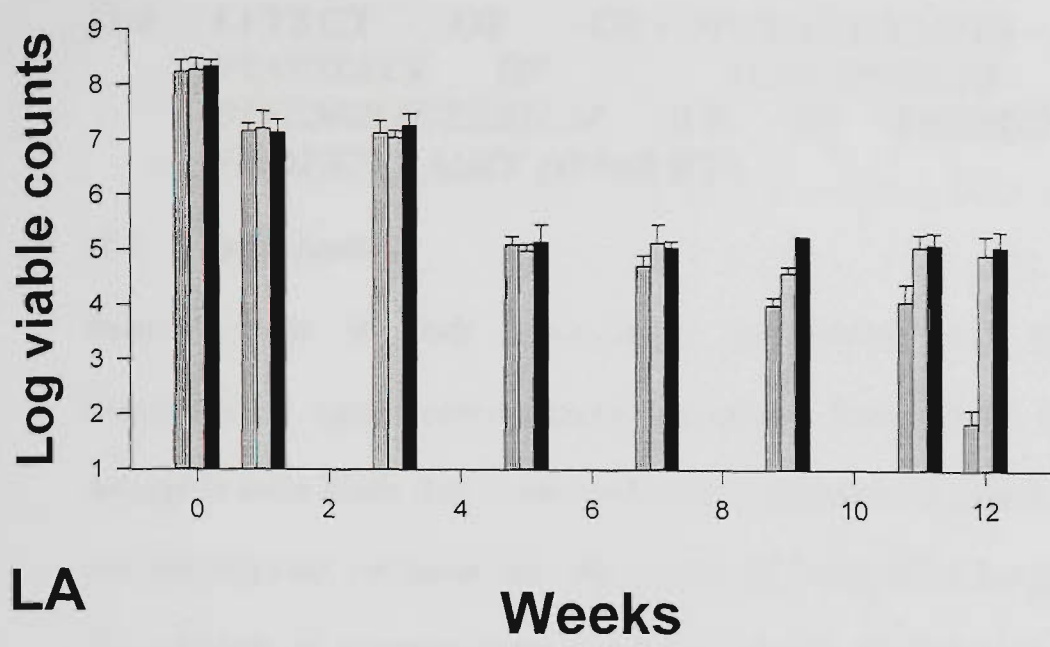
Figure 11.5 Viable counts of probiotic bacteria bacteria in the products made with or without acid casein hydrolysate and cysteine during 12 weeks storage at $-18^{\circ}C$

12.0 EFFECT OF CRYOPROTECTANTS ON VIABILITY OF *L. ACIDOPHILUS* AND *BIFIDOBACTERIUM* SPP. IN FERMENTED FROZEN DAIRY DESSERTS

12.1 Introduction

Bacteria differ in their susceptibility to freezing and thawing. Cryoprotective agents provide protection against freeze-injury. Cellular damage is most likely due to elevated solute concentration (which causes cell dehydration) and membrane destruction by intracellular ice crystals. The addition of cryoprotectants results in greater survival rate during freezing and frozen storage by binding water and inhibiting either intracellular or extracellular ice crystal formation. The presence of most cryoprotectants results in formation of extracellular amorphous ice glass instead of crystalline ice upon freezing. These agents include sorbitol, sucrose, sodium tripolyphosphate, glucose, xylitol, glycerol, raffinose and maltodextrin (Thunnell *et al.*, 1984).

The effect of various cryoprotectants on the viability of freeze-dried lactic acid bacteria has been studied by Valdez *et al.* (1985). Adonitol produced smallest changes in water content at various times during freeze drying and allowed the highest rate of survival. Use of β-glycerophosphate as a cryoprotective agent gave greatest protection to *L. acidophilus.*

Kilara *et al.* (1976) reported that the extent of cryoprotection afforded by any given agent varied from culture to culture. Malt extract and milk solids

as the cryoprotective agents resulted in the survival ratio of 58 and 80% for

L. delbrueckii ssp. *bulgaricus*, respectively, while with *L. acidophilus* the

same two cryoprotectants provided 89 and 48% survival. Whey powder

improved viability of *L. delbrueckii* ssp. *bulgaricus* and pectin that of *S.*

thermophilus. For improved viability of probiotic and yogurt organisms, a

combination of cryopotectants may be required.

The objective of this study was to determine the effect of cryoprotective

agents on improving the viability of probiotic bacteria in fermented frozen

dairy desserts.

12.2   Materials and Methods

13.2.1   *Propagation of yogurt bacteria and probiotic bacteria*

Yogurt bacteria *(Streptococcus thermophilus* STWJ7 and *Lactobacillus*

delbreuckii ssp. *bulgaricus* LBWJ7) and probiotic bacteria (*Lactobacillus*

acidophilus LA MJLA1 and bifidobacteria BB BDBB2) were selected and

propogated as described previously (Ravula and Shah, 1998a).

13.2.2   *Preparation of cryoprotected probiotic bacteria*

The organisms were transferred three times successively in MRS broth and

added to sterile MRS broth. L-cysteine was added to the broth for

bifidobacteria. The fermentation was terminated after 16h. The broth was

cooled to 4°C and transferred to centrifuge tubes and centrifuged at 4000

rpm for 25 min. The supernatant was discarded and the cell pellets were

washed with cold 0.1M phosphate buffer (pH 6.8). The washed cell pellet (10%) was re-suspended into 50 ml of 0.1M phosphate buffer containing 2.0% level (w/v) of cryoprotectants (pectin, sodium alginate, xanthan gum, guar gum, glucose, locust bean gum, fructose, glucose and glycerol). The cell pellet was allowed to mix uniformly in the phosphate buffer and the mixture was transferred into petri-plates. The organisms were frozen before freeze drying. Viable counts were monitored after inoculation, in the MRS broth, after 18 hrs of fermentation, and in cell pellets before and after freeze-drying. The freeze dried cryoprotected probiotic bacteria was added to blend mix of yogurt and base mix and fermented frozen dairy desserts were made using an ice cream freezer. Viable counts were monitored at fortnightly interval for 12 weeks. A similar procedure was used to prepare a control batch except that no the cryoprotective agent was used. All experiments were carried out in duplicate.

12.2.3 *Fermented frozen dairy desserts*

Fermented frozen dairy dessert were made according to the method of Ravula and Shah (1998b). Cryoprotective agents were added at a rate of 0.5% to yogurt mix at a temperature of 30-35°C before pasteurising at 85°C for 30 min. Various cryoprotective agents were initially evaluated for their effect on texture and the best resulting cryoprotectants were used in this study. The cryoprotectants that were used includ: RS 150 (pectin), Glucose and GCF 639 (alginate). These cryoprotectants were of food grade standard and are suitable for the manufacture of dairy products.

The final product was made by blending 45% yogurt, 45% of base mix and 10% of 65% syrup (liquid sucrose) followed by passing through an ice cream freezer. The control batch and the batches containing RS 150, Glucose and GCF 639 were made using a bench top ice cream freezer (1 L batch; 18% overrun). Two batches of fermented frozen dairy desserts were made with cryoprotectant RS 150 and without cryoprotectant (control) using a commercial freezer (20 L batch; 100% overrun). The fermented frozen dairy dessert was filled in plastic containers and stored at $-18^{\circ}C$ for 12 weeks

12.2.4 *Enumeration of probiotic bacteria*

MRS-salicin and MRS-sorbitol agars were used for the selective enumeration of *L. acidophilus*. The population of bifidobacteria was enumerated on MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, paromomycin sulfate) agar.

12.3 Results

12.3.1 *pH and titratable acidity*

The initial pH of milk (6.51-6.67 at 0 h) decreased to pH 4.52-4.59 during yogurt making. In general, there was a gradual decrease in the pH in all the products during storage for 12 weeks. The pH by the end of storage period was 4.41-4.46. The TA of yogurt varied between 0.71 to 0.78% after fermentation and the acidity varied between 0.79 to 0.86% at the end of the storage period.

12.3.2 *Viability*

Figures 12.1 –12.4 show the viable counts of probiotic bacteria in the 4 batches of fermented dairy desserts made using a bench top freezer. The counts of *L. acidophilus* (Figure 12.1) and bifidobacteria (Figure 12.2) at the end of 12 weeks storage period was $\geq 10^5$ cfu/g in all the products except the product containing glucose as the cryoprotectants, which had a viable count of $<10^4$ cfu/g.

Figures 12.3 and 12.4 shows the viable counts of probiotic bacteria in the two batches of fermented frozen dairy desserts made using a continuous freezer on a pilot scale. The counts of *L. acidophilus* (Figure 12.3) and bifidobacteria (Figure 12.4) decreased rapidly in both products and the viable counts were $<10^2$ and $<10^3$ cfu/g, respectively by the end of the 12 week storage period. Loss of viability of *L. acidophilus* and bifidobacteria in the products made using the commercial freezer was possibly due to presence of air. Air is incorporated during the manufacture of fermented frozen dairy desserts to achieve 100% overrun, which may affect the growth of microaerophilic *L. acidophilus* and anaerobic bifidobacteria. The products containing cryoprotectants performed better than without cryoprotectants for improving viability of probiotic bacteria. Glucose did not help improve their survival. Overall, there was a slight improvement in the viable counts of *L. acidophilus* and *Bifidobacterium* spp. in the presence of the cryoprotectant RS 150.

12.4   Conclusions

Survival of probiotic bacteria was studied in fermented frozen dairy desserts made with or without cryoprotectants using a bench top and a commercial freezer. The organisms lost their viability faster in the products made using a commercial freezer. Overall, the viability improved with cryoprotectants RS 150 (pectin) and GCF 639 (alginate) The results suggest that use of RS 150 and GCF 639 cryoprotectants in fermented frozen dairy desserts might improve the viability of probiotic bacteria.

Figure 12.1 Viable counts of *L. acidophilus* (LA MJLA1) in fermented frozen dairy desserts made using a bench top freezer
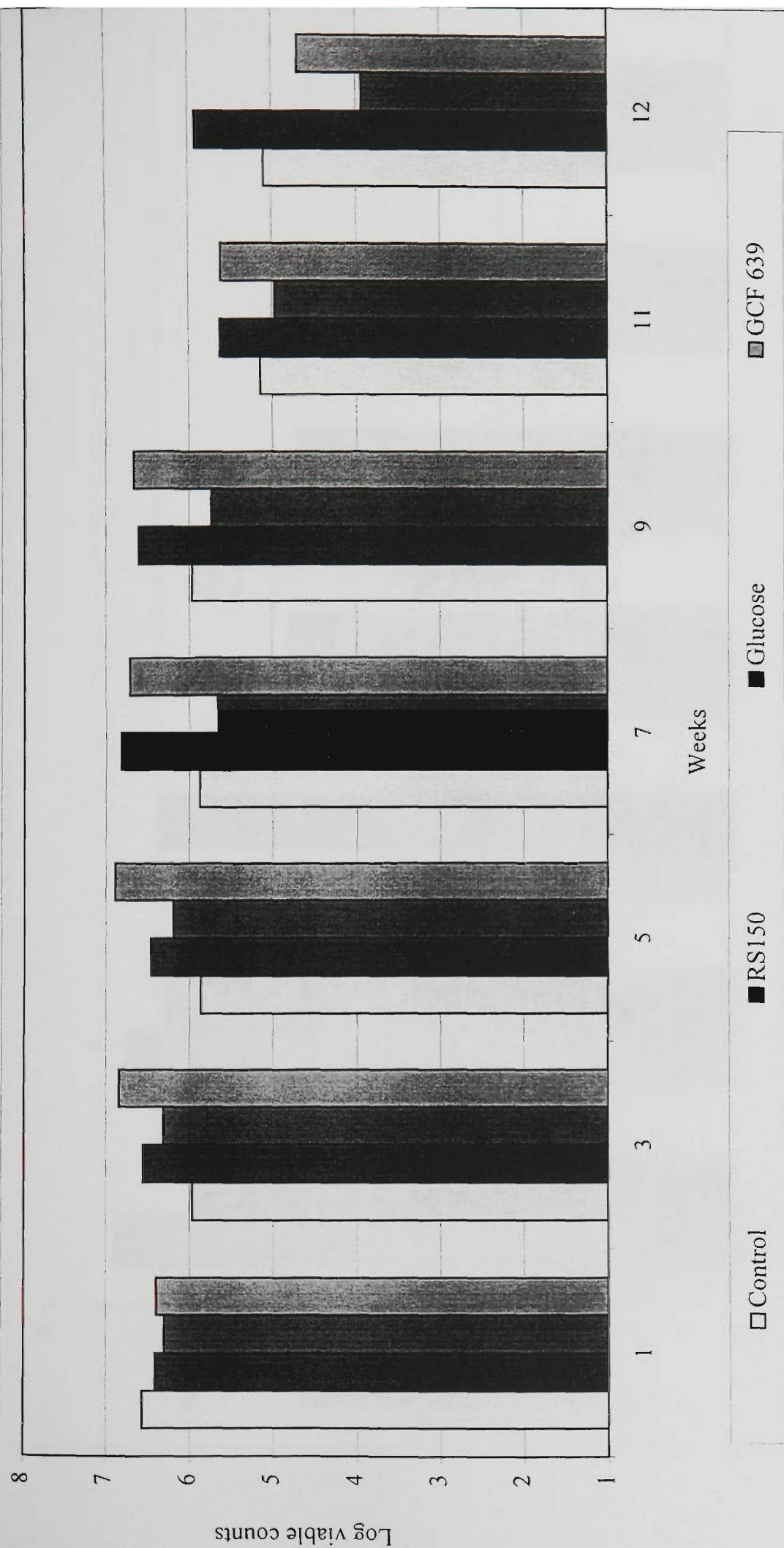
Figure 12.2 Viable counts of bifidobacteria (BDBB2) in fermented frozen dairy desserts made using a bench top freezer

B
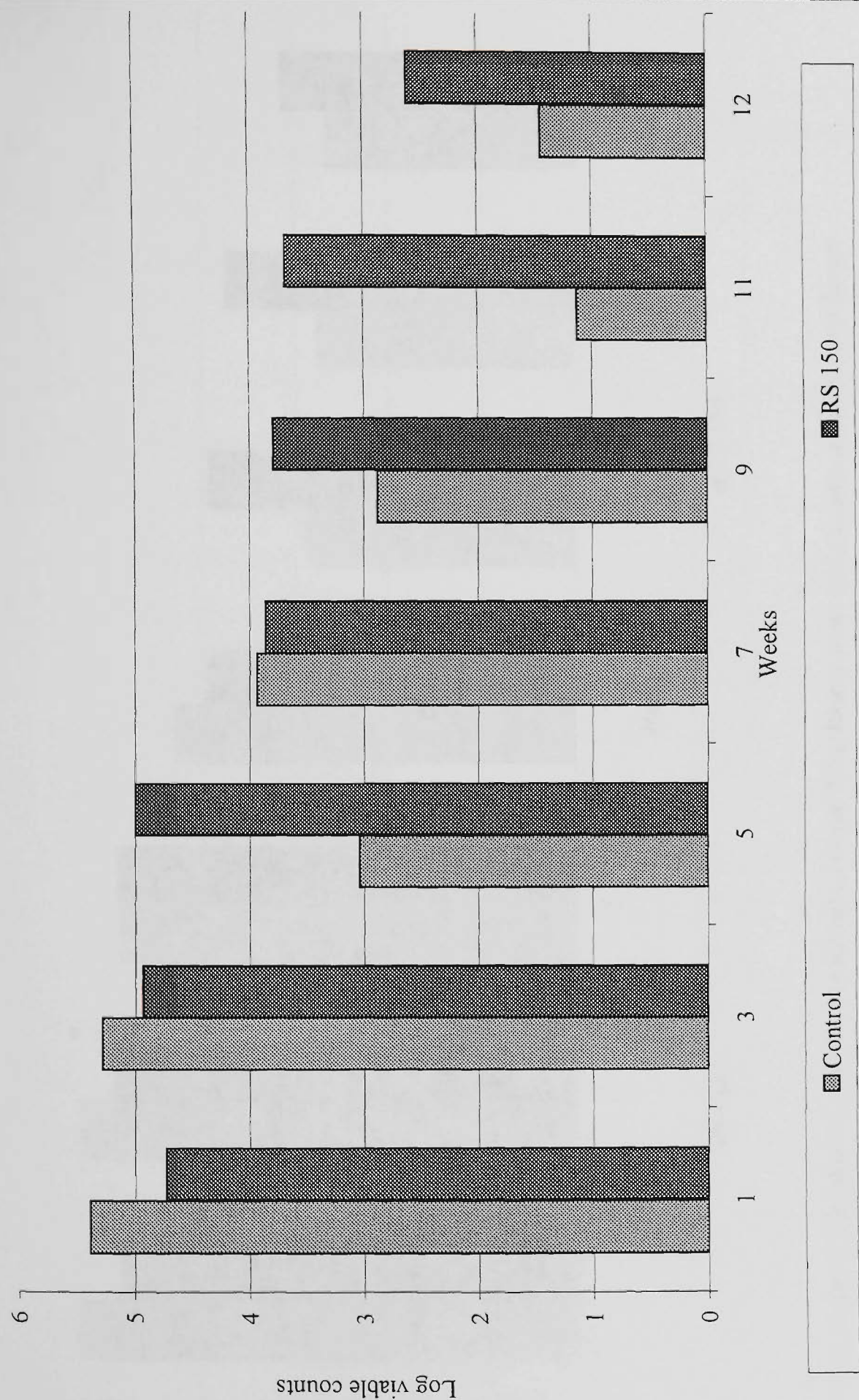
Figure 12.3 Viable counts of *L. acidophilus* (LA MJLA1) in fermented frozen dairy desserts made using a continuous ice cream freezer
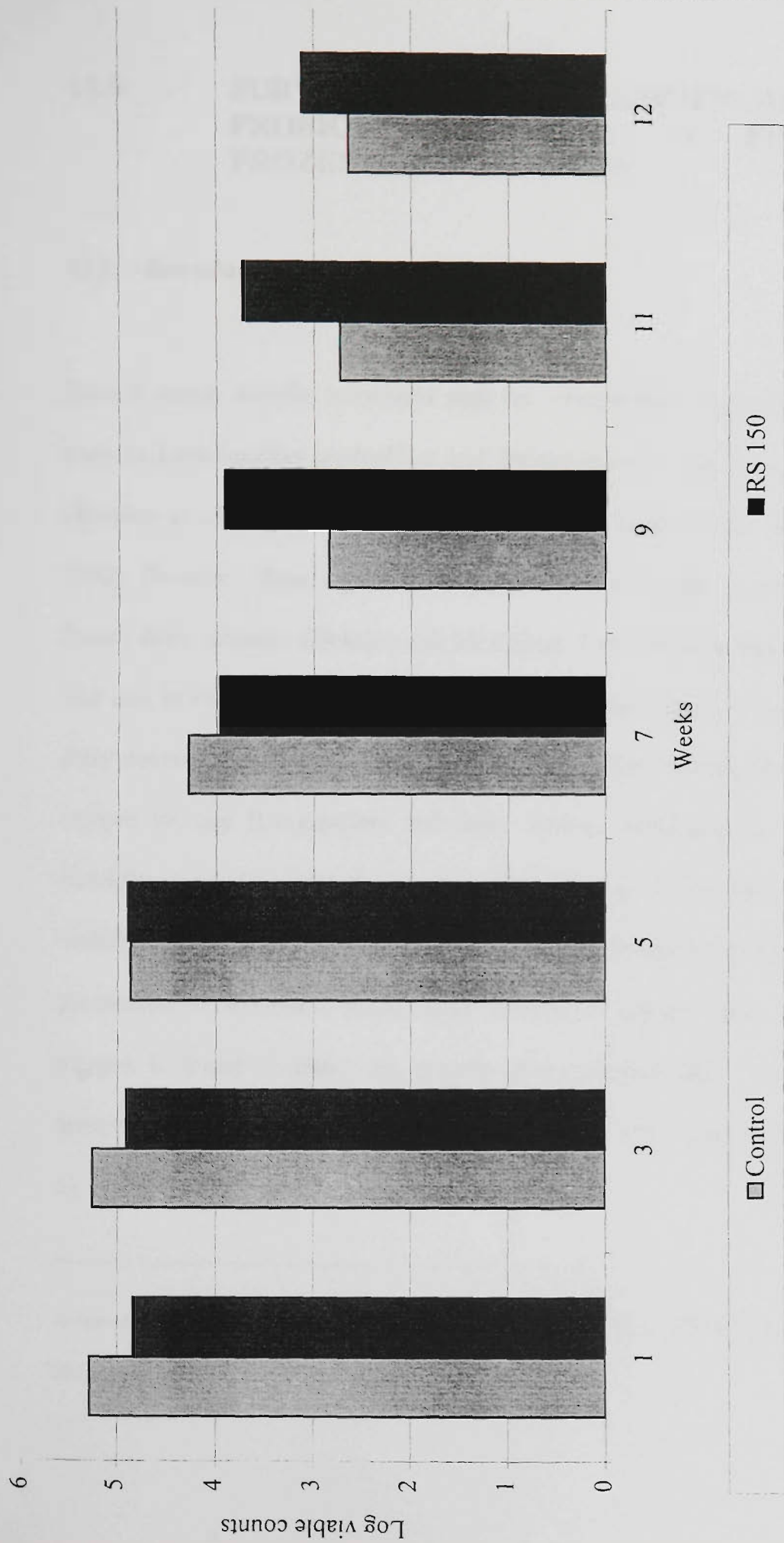
Figure 12.4 Viable counts of bifidobacteria (BDBB2) in fermented frozen dairy desserts made using a continuous ice cream freezer

13.0 SURVIVAL OF MICROENCAPSULATED PROBIOTIC BACTERIA IN FERMENTED FROZEN DAIRY DESSERTS

13.1 Introduction

Several health benefits associated with the consumption of probiotic organisms such as *Lactobacillus acidophilus* and *Bifidobacterium* spp. have been claimed (Kearney *et al.*, 1990; Hughes and Hoover 1991; Kanbe 1992; Mital and Garg, 1992). However, these organisms do not survive in frozen yogurt or fermented frozen dairy desserts (Hekmat and McMahon, 1992; Ravula and Shah, 1998b). The loss in viability of probiotic organisms in frozen yogurt or fermented frozen dairy desserts was claimed to be due to acidity of the product, freeze injury and oxygen toxicity (Lankaputhra and Shah, 1996a). According to the Australian Food Standards Code H8, the pH of yogurt must be ≤ 4.5; such low pH affects the viability of probiotic bacteria. Air is incorporated during the freezing process in production of fermented frozen dairy desserts to achieve desired overrun and oxygen is found to affect the growth of microaerophilic *L. acidophilus* and anaerobic bifidobacteria (Ishibashi and Shimamura, 1993; Dave and Shah, 1997a, b).

Methods to preserve probiotic bacteria in ice cream and frozen fermented dairy desserts have been reported by Laroia and Martin (1991) and Hekmat and McMahon (1992). The survival of probiotic bacteria was reported to be unsatisfactory.

Microencapsulation is a process where the cells are retained within the encapsulating material in order to reduce the cell injury or cell loss. Encapsulation of lactic starter cultures is of interest to the dairy industry since it improves the control of the fermentation process (Champagne and Cote, 1987; Champagne, 1990).

In some food formulations, encapsulated cells are found to be more active than free cells (Kearney *et al.*, 1990). Encapsulation of bacterial cells in calcium alginate beads is simple. The advantage of encapsulation using natural polymers is that the reagents are non-toxic and the matrices formed are gentle to the microorganisms (Lacroix *et al.*, 1990). The other advantage is that calcium alginate can be solubilized by sequestering calcium ions thus releasing the entrapped cells.

Sheu and Marshall (1993) studied the survival of entrapped culture bacteria in frozen ice milk. The viability of bacterial cells was markedly improved by micro-entrapment of cells in calcium alginate gels and 40% more lactobacilli survived freezing of ice milk. Thus addition of entrapped probiotic bacteria to frozen dairy

desserts could be advantageous to provide desirable marketing and health benefits.

Viability and acid production activity of immobilised *Lactococcus lactis* ssp. *lactis* within cross-linked gelatin micro-capsules have been reported by Hyndman et al. (1993). After encapsulation, activity and viability of the immobilised culture was increased by cell growth within the capsules.

This study was focused on factors necessary for producing desirable bead size as well as on the effects of acid, bile, pH of phosphate buffer and stirring time on the release of the organisms from the beads. The influence of micro-entrapment on survival of probiotic organisms in fermented frozen dairy desserts was confirmed in an industrial setting.

13.2   Materials and Methods

13.2.1 *Propagation of probiotic bacteria*

Probiotic bacteria (*Lactobacillus acidophilus* LA MJLA1, LA 2415, LA 2400, LA 2401 and *Bifidobacterium* spp. BB BDBB2, BB 20099, BB 1900, BB 1941) were selected based on our previous study (Ravula and Shah, 1998b). All the strains were tested for purity by Gram stain and sugar fermentation tests. The organisms were propagated every week in sterile 12% reconstituted skim milk (RSM) supplemented with 2% glucose and 1% yeast extract. All the cultures were grown for 18 h at 37^0C using 1% inoculum. For propagation of bifidobacteria, sterile RSM was supplemented with 0.05% L-cysteine

hydrochloride in order to provide anaerobic condition and to stimulate their growth. The cultures were maintained in the same medium at 4^0C. Before enumeration, the cultures were transferred successively three times for activation.

13.2.2 *Preparation of encapsulated beads*

Both probiotic bacteria were microencapsulated separately according to the flow diagram shown in Figure 13.1. The organisms were grown in sterile RSM (12%) containing glucose (1%) and yeast extract (0.5%). Each organism was then transferred twice successively in deMan, Rogosa, and Sharpe (MRS) broth for 18 h at 37°C. The organisms were harvested by centrifuging (3838 x g, 25 min, 4°C), washed, and suspended in saline to obtain 10^{10} cells mL^{-1}. The cell suspension was mixed with equal amount of 3% sodium alginate. The mixture was added drop-wise to 5 parts of vegetable oil containing Tween 80. A turbid emulsion was obtained. Calcium chloride (0.05%) was gently added to the bottom of the flask with the help of a pipette until the emulsion was broken. The calcium alginate encapsulated beads formed were collected by decantation, freeze dried, and incorporated into a mix prior to freezing for manufacturing of fermented frozen dairy desserts as detailed under the manufacture of fermented frozen dairy desserts section.

13.2.3 *Effects of surfactant and emulsifier on bead size*

Effects of a surface tension reducing agent (sodium lauryl sulfate) and an emulsifier (Tween 80) on bead size were investigated. Several concentrations (1 to 4%) of sodium alginate were examined for their effect on bead size, and a 3%

concentration of sodium alginate was found to be optimal. Surface tensions of 3% sodium alginate solution were varied by adding 4 levels of sodium lauryl sulfate (0, 0.1, 0.5 and 1.0%), while four concentrations of Tween 80 (0, 0.1, 0.2 and 0.3%) were added to the oil phase.

The diameters of beads were measured with an eyepiece micrometer using an optical microscope at a magnification of 400x or 1000x after staining with safranin according to the method of Sheu and Marshall (1993). At least 80 randomly selected beads were measured for each sample.

13.2.4 *Measurement of diameter of calcium alginate beads*

Diameters of encapsulated beads were measured with an eyepiece micrometer on an optical microscope at a magnification of 400x or 1000x. At least 80 randomly selected beads were measured for each sample.

13.2.5 *Scanning electron microscopy*

A scanning electron microscope was used to examine the external and internal appearances of freeze-dried beads. For external structure, intact beads were attached to aluminium stubs using conductive carbon paint (CCP) (Probing and Structure, now Pro Sci Tech., Thuringowa, Australia). For internal structure, the freeze-dried beads were mechanically broken using a fine tweezer and mounted on aluminium stubs using CCP.

The specimens were coated with a thin layer of gold using an Edwards Sputter Coater. The external and internal structures were observed using a Philips SEM515 scanning electron microscope at 20 kV.

13.2.6 *Release of probiotic organisms from microencapsulated beads*

One gram of encapsulated beads was added to 9 ml of phosphate buffer at various pH (4.5, 5.5, 6.5 and 7.5). One gram of organisms were further diluted with 59 mL of sterile distilled water. One gram of the mixture containing beads and distilled water were added to 9 mL of peptone water and counts were enumerated by using appropriate peptone water dilutions.

13.2.7 *Release of entrapped bacteria from microencapsulated gel beads*

The effects of pH of phosphate buffer, stirring time and of acid and bile on release of probiotic bacteria from freeze dried encapsulated beads were carried out. One gram of encapsulated beads was added to 59 mL of sterile distilled water and mixed. One millilitre aliquot from this mix was added to 9 mL of a phosphate buffer in test tubes adjusted to various pH levels (4.5, 5.5, 6.5, 7.5, and 8.5) with mono- or di-sodium phosphate followed by stirring at 200 rpm for 0, 5, 10 or 15 min. The released bacterial populations were enumerated using the pour plate technique as outlined above.

13.2.8 *Effect of acid on release of entrapped bacterial cells from encapsulated beads.*

The effects of lactic acid on the release of probiotic bacterial cells from calcium alginate beads were studied by adding 0.1% of beads to sterile distilled H_2O adjusted to pH 2.5, 3.5 and 4.5 using lactic acid, followed by incubation of the mixture for 3 h at 4°C. Samples were taken at hourly intervals for 3 h for enumeration of viable counts. The beads incubated in acid were then added to phosphate buffer (pH 7.5) and plated for enumeration after stirring for 5 minutes at 200 rpm in order to verify the levels of cells released.

13.2.9 *Effect of bile on bacterial cells from encapsulated beads in (bile)*

The effects of bile on the release of probiotic bacterial cells from calcium alginate beads were investigated by adding 0.1% of beads to solutions of bile salts (0.1, 0.5 and 1%) prepared in sterile distilled water, followed by incubation of the mixture for 3 h at 4°C. Samples were taken at hourly intervals for 3 hours for enumeration of bacterial counts. The beads incubated in bile salts were also incubated in phosphate buffer (pH 7.5) and plated for enumeration to verify the levels of cells released.

13.2.10 *Manufacture of fermented frozen dairy desserts*

The industrial procedure for manufacture of fermented frozen dairy desserts typically involves mixing of yogurt (45%), base mix (45%) and liquid sugar (10%) (Ravula and Shah, 1998b). The blend mix is then frozen while

incorporating air to achieve up to 100% overrun, similar to the manufacture of ice

cream. Unlike ice cream, the pH of fermented frozen dairy desserts must typically

be ≤4.5.

Two batches of yogurt (10L) were made with homogenised and pasteurised milk

supplemented with 2% skim milk powder. The mix was heated at 85^0C for 30

min, cooled to 42^0C and commercial yogurt starter bacteria (*Streptococcus*

thermophilus STWJ7 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LBWJ7)

were added at the rate 0.5% of each and the inoculated mix incubated at 42^0C.

The fermentation was terminated at pH 4.5 and the yogurt was stored in a cold

room (4^0C) for 18h (Figure 1). Similarly the base mix was prepared as shown in

the flow diagram in Figure 1. The yogurt, base mix and liquid sugar were mixed

and two batches of fermented frozen dairy desserts were prepared using a

continuous ice cream freezer. One batch of the blend contained micro-entrapped

probiotic bacteria (*L. acidophilus* MJLA1 and *Bifidobacterium* spp. BDBB2), and

for the other batch non-encapsulated cells were added; in both cases the rate of

addition was 0.5 g L^{-1}. The enumeration of *L. acidophilus* and *Bifidobacterium*

spp. was carried out at fortnightly intervals for 12 weeks.

13.2.11   *Enumeration of bacteria*

MRS-salicin agar and MRS-sorbitol agar were used for the selective enumeration

of *L. acidophilus* (Dave and Shah, 1996; Lankaputhra and Shah, 1996). The

population of bifidobacteria was enumerated on MRS-NNLP (nalidixic acid,

neomycin sulfate, lithium chloride, and paromomycin sulfate) agar as reported by Dave and Shah (1996).

All analyses were carried out in duplicate, and all the experiments were repeated at least twice. The results presented are the average of all data.

13.3   Results and Discussion

The use of calcium alginate to microencapsulate cells of lactobacilli was reported by several workers (Nilsson *et al.*, 1983; Sheu and Marshall, 1993; Kim, 1988). Rao *et al.* (1989) reported the survival of microencapsulated *Bifidobacterium pseudolongum* in simulated gastric and intestinal juices. However, details of gel structure and survival of *L. acidophilus* and *Bifidobacterium* spp. in fermented frozen dairy desserts were not reported. Similarly, the effects of acid and bile on release of *L. acidophilus* and *Bifidobacterium* spp. have not been previously studied. To provide any health benefits, probiotic bacteria must survive in acidic conditions encountered in the stomach and bile concentrations in the intestine (Lankaputhra and Shah, 1995; Rao *et al.*, 1989).

13.3.1 *Composition*

The protein content of the product was 3.82%. The total solids contents were in the range of 31.6-33.5%. The titratable acidity ranged between 0.76% and 0.79%. The fat content was 10.3%, while the sugar content was 16.7%. The pH ranged between 4.51 and 4.53

13.3.2 pH and titratable acidity

The initial (at 0 h) pH of milk (6.56-6.64) decreased to pH 4.56-4.58 during yogurt making. In general, there was a gradual decrease in pH in all the products during storage for 12 weeks. The final pH by the end of storage period was 4.50-4.44.

The initial TA of milk 0.14 increased to 0.72-0.76% after fermentation and the final acidity further increased to 0.75-0.82% at the end of storage period. The increase in TA of the control sample was the highest (0.79%).

13.3.4 Effect of surfactant and emulsifier on bead size

Emulsifier is necessary to effect a water/oil emulsion. Tween 80 was used as an emulsifier because it associated with the oil phase. With high association in the oil phase (having high hydrophilic and lipophilic balance value), the Tween 80 was easily removed from the surface of the spheres causing disruption of water/oil emulsion (Govin and Leeder, 1971; Nawar, 1985; Sheu and Marshall, 1993). Tween 80 was essential to prevent spheres from coalescing before the breaking of the emulsion.

For frozen fermented dairy desserts, the diameter of calcium alginate beads is an important aspect as too large a diameter may be detected in the mouth. The mean diameter of the beads without any surfactant or emulsifier was 1.17 mm (Figure 13.2). The diameter of the beads ranged from 0.82mm to 1.44mm.

A solution having a low surface tension resulted in small spheres (Sheu and Marshall, 1993). Sodium lauryl sulfate, a surfactant, was used to lower the surface tension in the alginate mix in order to reduce the size of the spheres. The solution having lower surface tension resulted in smaller beads (Figure 13.2). As the concentration of the sodium lauryl sulfate increased the viscosity decreased. As the concentration of sodium lauryl sulfate and Tween 80 increased, the diameter of calcium alginate beads decreased. At 0.3% Tween 80 and 1% sodium lauryl sulfate, the mean diameter was 0.064 mm. The bead size in the absence of any emulsifier or surfactant of 1.17 mm decreased to 0.064 mm in the presence of 0.3% of Tween 80 and 1% of sodium lauryl sulfate. Thus, there was approximately 18 times reduction in the size of beads due to addition of Tween 80 and sodium lauryl sulfate.

Figures 13.4–13.21 show electron micrographs of a calcium alginate beads showing the external appearance and cross section of beads. The beads appear to be spherical and 1.3 to 1.4 mm in diameter. The microorganisms appear to be located in the matrices formed within the beads.

13.3.5 *Effect of concentration of sodium alginate on bead size*

Several concentrations (1 to 4%) of sodium alginate were examined for their effect on bead size. At a stirring speed of 200 rpm, the apparent viscosity increased with an increase in concentration of sodium alginate from 1 to 4%, making it difficult to form a smooth gel, especially at 4% concentration. Higher concentrations of sodium alginate resulted in larger size bead formation, with the mean diameters approaching a plateau when the concentration of sodium alginate was 3%. Thus, a 3% concentration of sodium alginate was used for the rest of the experiments, unless otherwise indicated.

13.3.6 *Release of entrapped bacteria from microencapsulated gel beads*

In order to estimate the survival of probiotic bacteria in fermented frozen dairy desserts and to investigate the effects of freezing of encapsulated bacteria, it was important to determine optimum conditions including pH of the phosphate buffer and stirring time in the buffer for complete release of the entrapped bacteria. Releasing was carried out after freeze drying of the encapsulated beads. The pH of the phosphate buffer, which ranged from 4.5 to 8.5, did not have any effect on the release of the organisms from the beads. A 5-minute stirring at 200 rpm resulted in complete release of bacterial cells from the encapsulated beads (Figure 13.3).

13.4.7 *Release of entrapped bacterial cells from encapsulated beads acidic conditions*

The encapsulated beads were inoculated for 3 h in lactic acid at various pH (2.5, 3.5 and 4.5) and the viable counts showed that lactic acid had no effect on the release of probiotic bacteria. This was confirmed by adding the acid treated beads to phosphate buffer (pH 7.5) and plating. The viable counts as determined in MRS-salicin agar (for *L. acidophilus*) and MRS-NNLP agar (for bifidobacteria) varied from 6.62 to 6.72 log cfu g^{-1}. The counts after release from beads in phosphate buffer were similar. This may suggest that the organisms could survive in acidic conditions generally encountered in the stomach (Figure 13.22).

13.3.8 *Release of entrapped bacterial cells from encapsulated beads in bile*

The encapsulated beads were incubated for 3 h in bile salts (0.1, 0.5 and 1%) alone and bile and phosphate buffer. It was possible that the organisms may not have been completely released from the beads in the presence of bile. Therefore, the organisms from the beads were released using phosphate buffer to verify the results. The levels of organisms released in the presence of bile were similar to those released in the presence of bile and phosphate buffer (Figure 13.23), suggesting that the organisms could be completely released in the intestine in the presence of bile.

13.3.9 *Effects of addition of beads on product manufacturing*

The products made with encapsulated and non-encapsulated cells were analysed for overrun and mouth feel. It was observed that there were no much difference in overrun and in the taste of the both samples.

13.3.10 *Viable counts of probiotic bacteria in fermented frozen dairy desserts made using continuous ice cream freezer on a pilot scale*

Figures 13.24-13.31 show the viable counts of probiotic bacteria in eight batches of fermented frozen dairy desserts. The counts *L. acidophilus* and *Bifidobacterium* spp decreased in the control samples and the viable counts ranged between $10^2.10^3$ cfu/mL by the end of 12 weeks storage period. The decrease in the counts of *L. acidophilus* and *Bifidobacterium* spp. in samples where encapsulated bacterial cells were used were less than those with the control sample and the viable counts at the end of 12 weeks storage period were $>10^5$ cfu/g. Improved survival and viability of *L. acidophilus* and bifidobacteria were possibly due to the protection given by entrapment in calcium alginate gels. Studies have shown that bifidobacteria did not survive well in fermented products at pH 4.6 and below (Laroia and Martin, 1991), whereas the studies by Rao *et al* (1989) shown that micro-encapsulated bifidobacteria survived in large numbers than non-encapsulated bacteria in gastric and intestinal juices. Reduction of culture bacteria in frozen yogurt was observed after freezing (Mashayekh and Brown, 1992). About 40% more lactobacilli survived freezing of ice milk when they were entrapped in calcium alginate beads (Sheu and Marshall, 1993).

13.4 Conclusion

Fermented frozen dairy desserts typically have pH of 4.5. Such low pH affects the survival of probiotic organisms. Encapsulation of bacterial cells in sodium alginate provided protection to probiotic organisms in fermented frozen dairy desserts. The viable population decreased during storage, however, there was 2-3 log cycle improvement in the viable counts due to encapsulation. The encapsulated probiotic bacteria also survived in acidic conditions (at pH 2.5) in calcium alginate beads; however, the organisms were released in the presence of bile. The study suggests that encapsulated probiotic bacteria incorporated in fermented frozen dairy deserts could survive in low pH of the product and in acidic conditions such as encountered in the human stomach and could be delivered in the intestine.

Legends to figures (electron micrographs)

Figure 13.4 – Scanning electron micrograph of LA 2415 encapsulated in calcium alginate bead

Figure 13.5 – Scanning electron micrograph of LA 2415 encapsulated in calcium alginate bead (cross section).

Figure 13.6 – Scanning electron micrograph of LA 2415 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).

Figure 13.7 – Scanning electron micrograph of LA 2401 encapsulated in calcium alginate bead.

Figure 13.8 – Scanning electron micrograph of LA 2401 encapsulated in calcium alginate bead (cross section).

Figure 13.9 – Scanning electron micrograph of LA 2401 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).

Figure 13.10 – Scanning electron micrograph of LA MJLA1 encapsulated in calcium alginate bead.

Figure 13.11 – Scanning electron micrograph of LA MJLA1 encapsulated in calcium alginate bead (cross section).

Figure 13.12 – Scanning electron micrograph of LA MJLA1 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).

Figure 13.13 – Scanning electron micrograph of BB1900 encapsulated in calcium alginate bead.

Figure 13.14 – Scanning electron micrograph of BB1900 encapsulated in calcium alginate bead (cross section).

Figure 13.15 – Scanning electron micrograph of BB1900 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).

Figure 13.16 – Scanning electron micrograph of BB BDBB2 encapsulated in calcium alginate bead.

Figure 13.17 – Scanning electron micrograph of BB BDBB2 encapsulated in calcium alginate bead (cross section).

Figure 13.18 – Scanning electron micrograph of BB BDBB2 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).

Figure 13.19 – Scanning electron micrograph of BB 1941 encapsulated in calcium alginate bead.

Figure 13.20 – Scanning electron micrograph of BB 1941 encapsulated in calcium alginate bead (cross section).

Figure 13.21 – Scanning electron micrograph of BB 1941 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).

Method of **_yogurt_** **_base mix_**
microencapsulation of
probiotic bacteria

Probiotic bacteria (_L. acidophilus_ or bifidobacteria)	Homogenised and pasteurised milk	Homogenised and pasteurised milk
↓	↓	↓
Incubation 37⁰C/18h	Tempering (40-45⁰C)	Tempering (40-45⁰C)

Incubation 37^{0}C/18h · Tempering (40-45^{0}C) · Tempering (40-45^{0}C)

Centrifugation (4000 rpm 25 min.) · Addition of skim milk powder 2% · Addition of ingredients (skim milk powder, cream, sugar and stabiliser)

Cell pellet · Heat treatment (85^{0}C/30 min) · Heat treatment (85^{0}C/30 min)

Mixing with sodium alginate (3%) · Cooling to incubation temperature (42^{0}C) · Cooling($\sim$4^{0}C)

Addition of mix into veg. oil · Addition of selected strains of yogurt bacteria · Ageing

Addition of calcium chloride · Filling in container

Collecting the beads · Incubation at 42^{0}C till the pH reaches to 4.5

Freeze drying · Storage at 4-6^{0}C for 12h

Blending yogurt and base mix
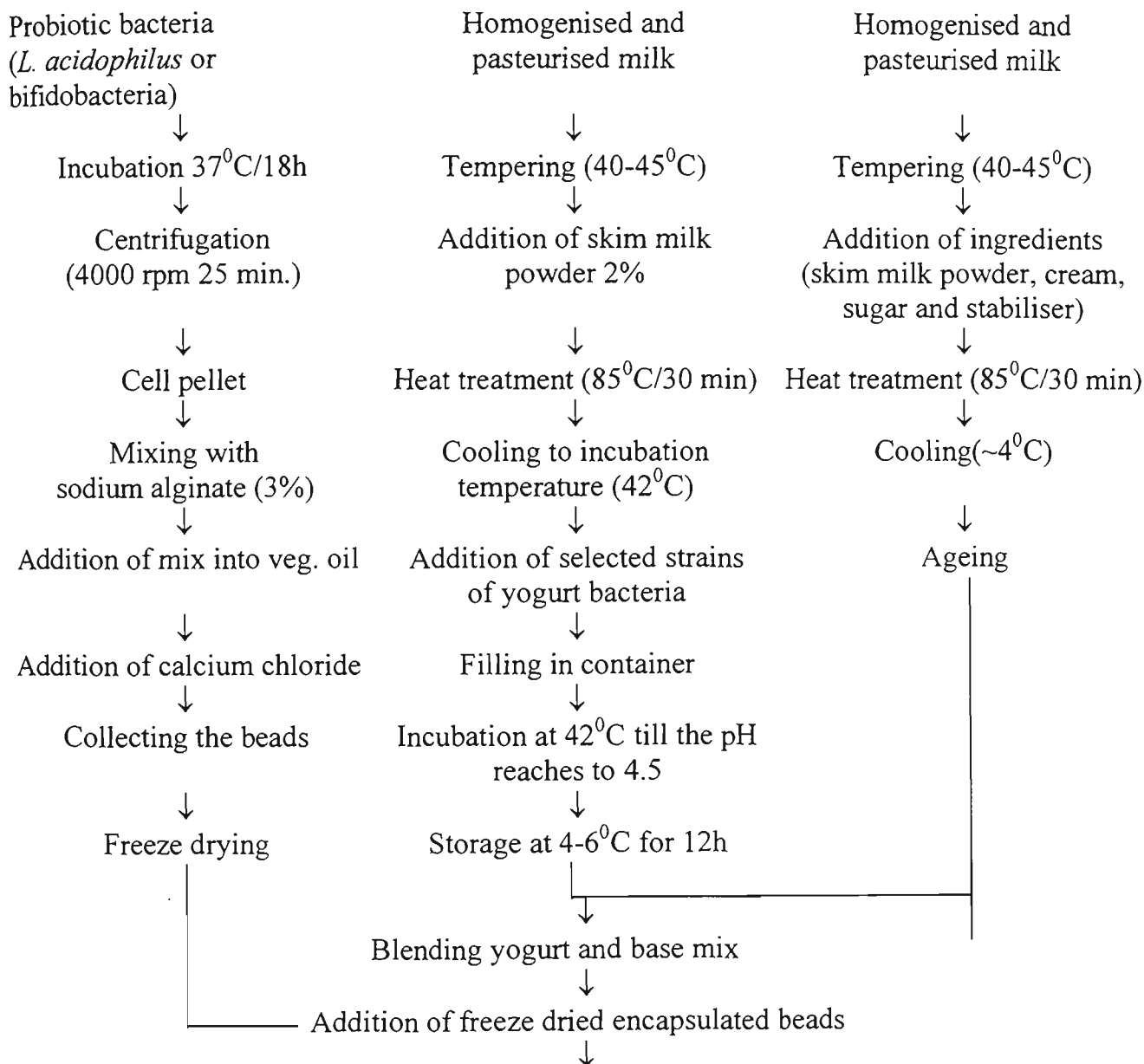
Addition of freeze dried encapsulated beads

Fig 13.1: Preparation of fermented frozen dairy dessert (45% of yogurt, 45% of base mix and 10% of 65% syrup (liquid sucrose)
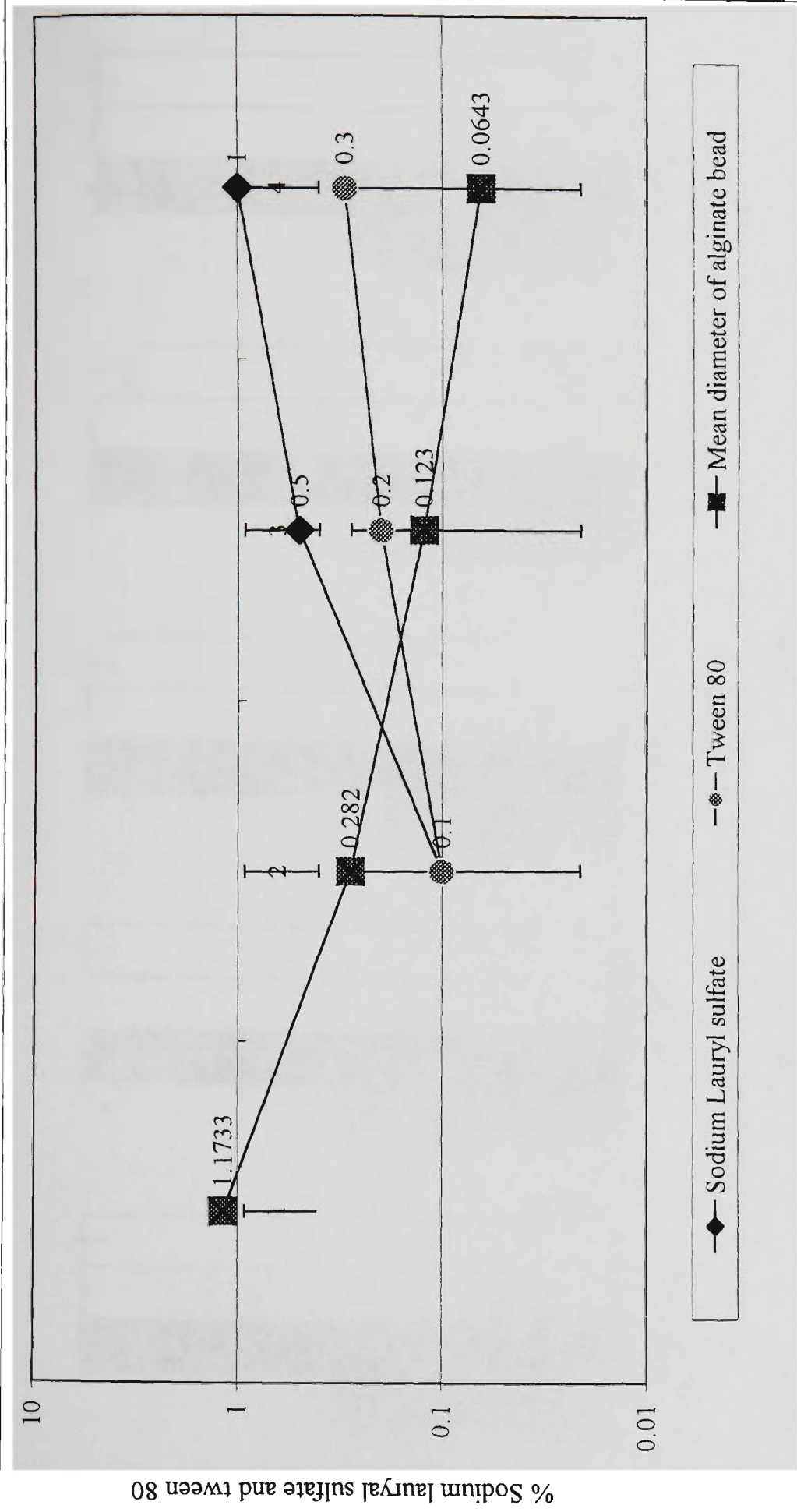
Figure 13.2 Relationship between mean diameter of calcium alginate beads and viscosity of sodium alginate. Error bar indicates standard deviation
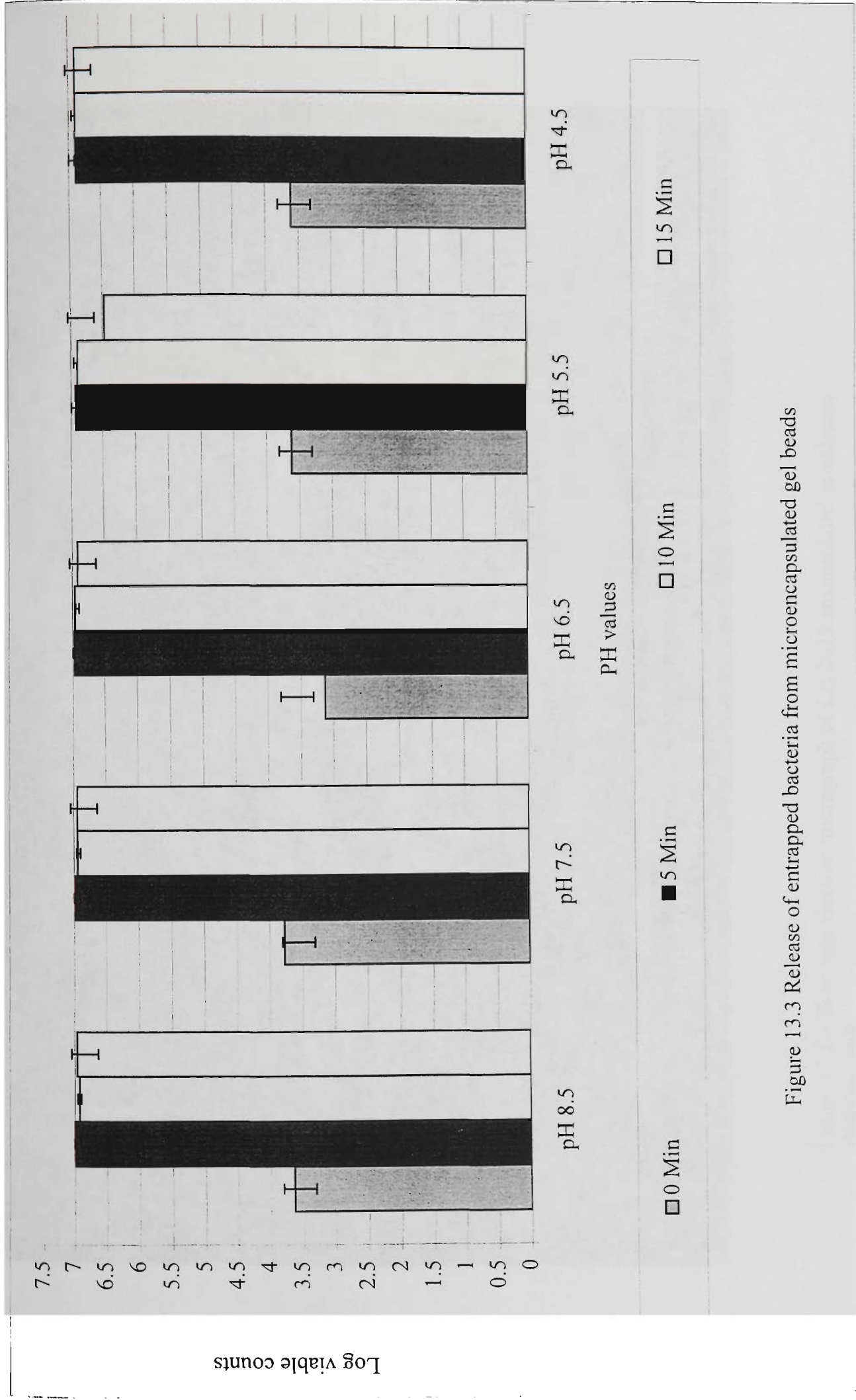
Figure 13.3 Release of entrapped bacteria from microencapsulated gel beads
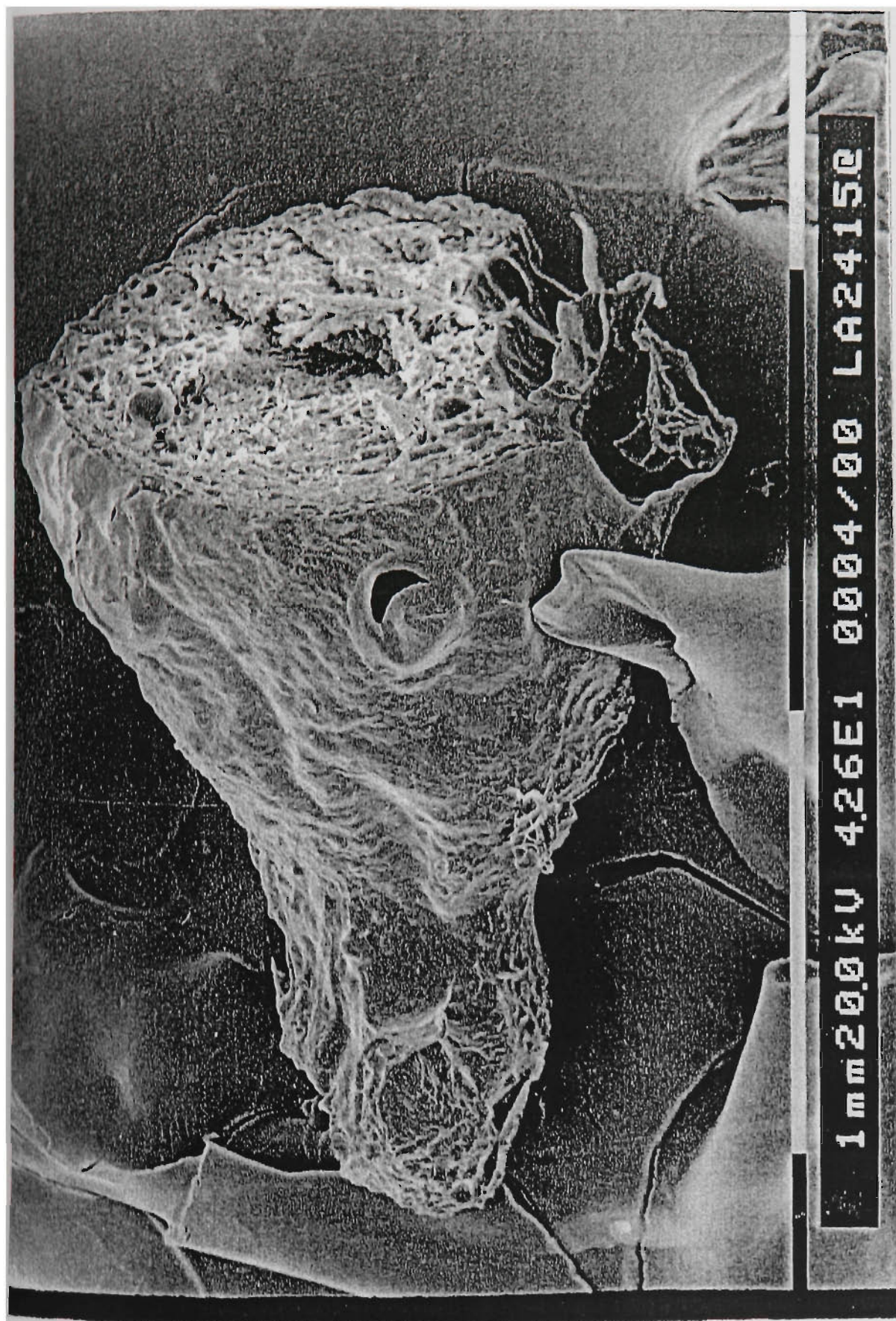
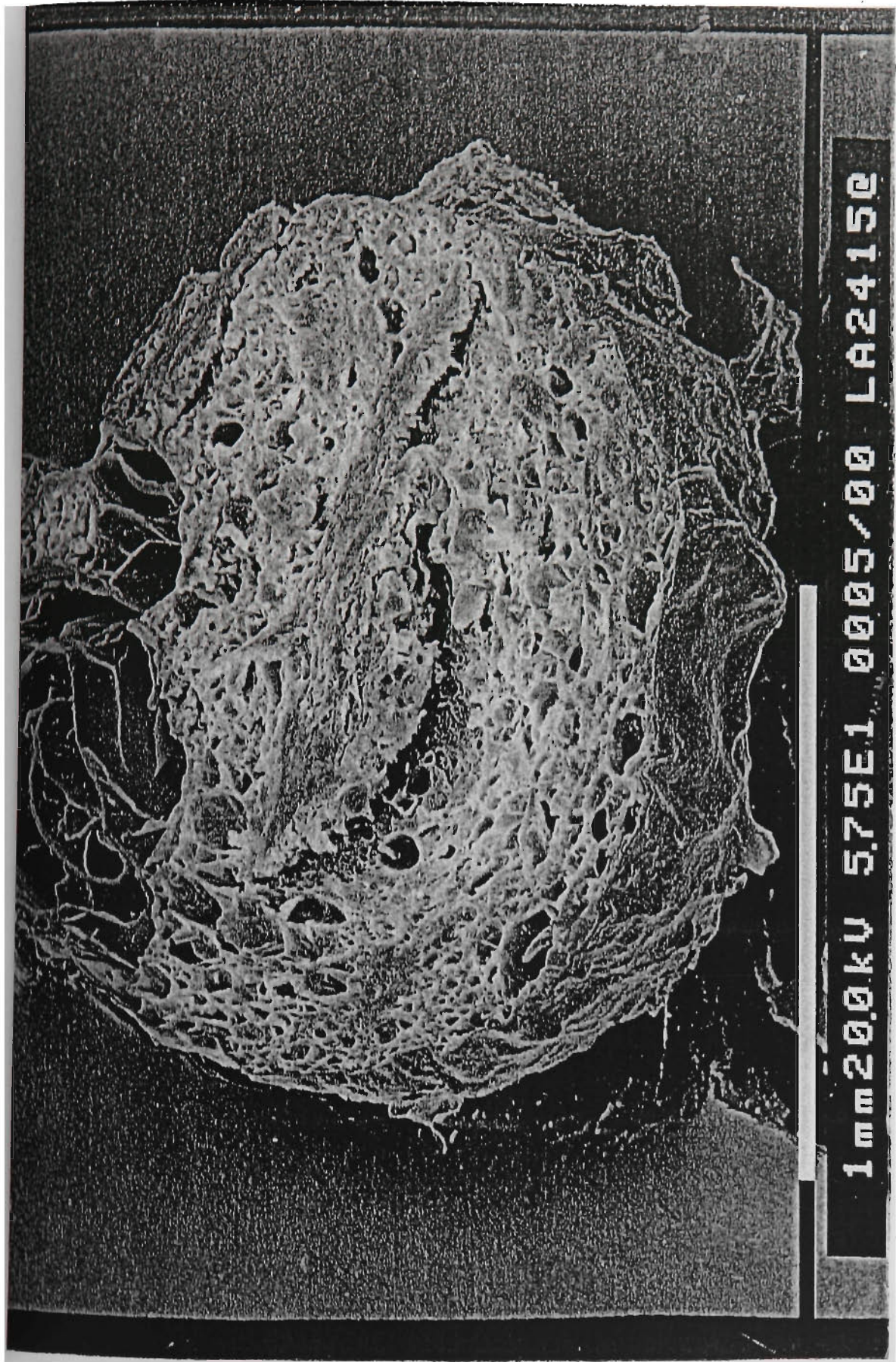Figure 13.4 – Scanning electron micrograph of LA 2415 encapsulated in calcium alginate bead

Figure 13.5 – Scanning electron micrograph of LA 2415 encapsulated in calcium alginate bead (cross section).

Figure 13.6 – Scanning electron micrograph of LA 2415 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).
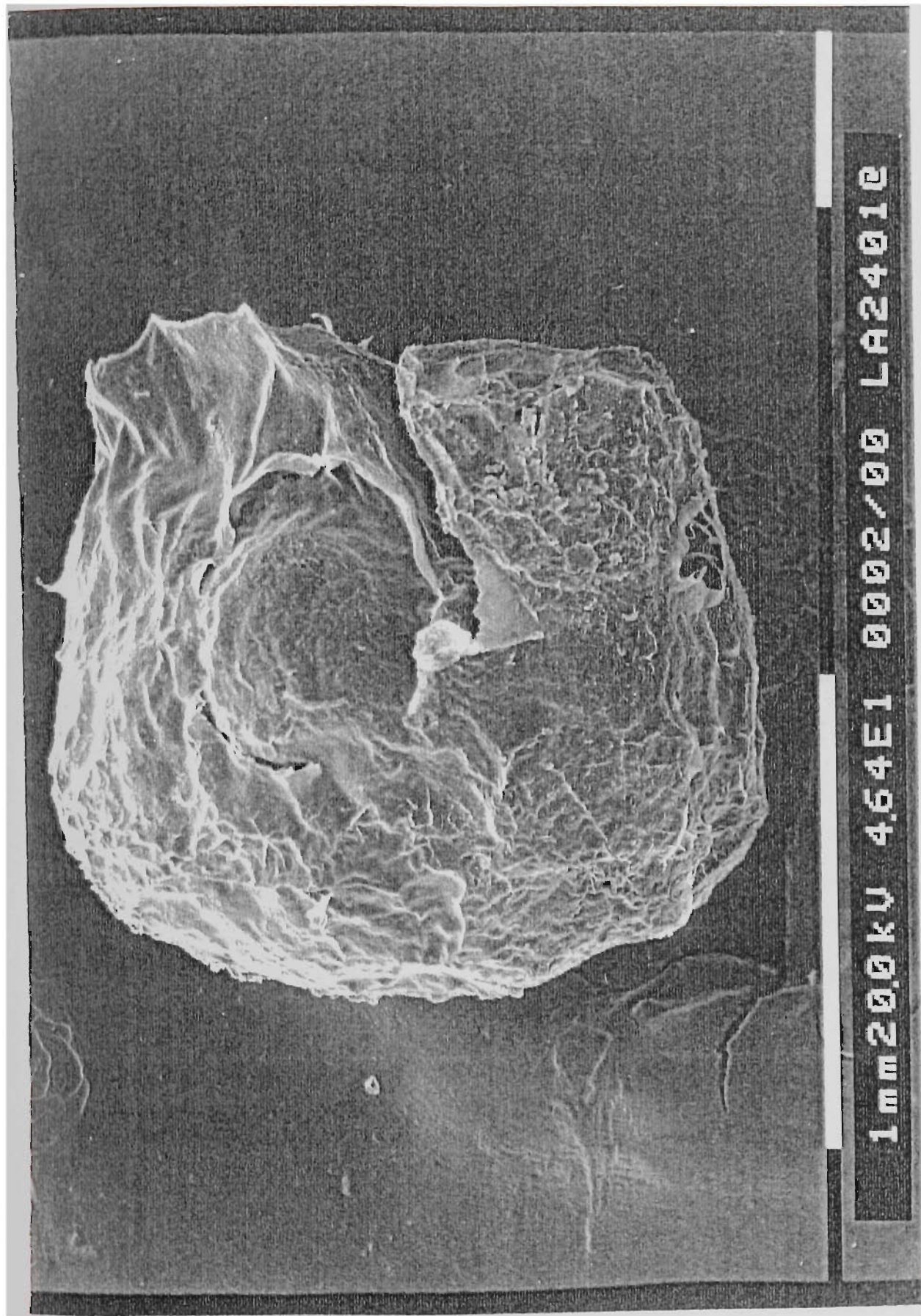
Figure 13.7 – Scanning electron micrograph of LA 2401 encapsulated in calcium alginate bead.
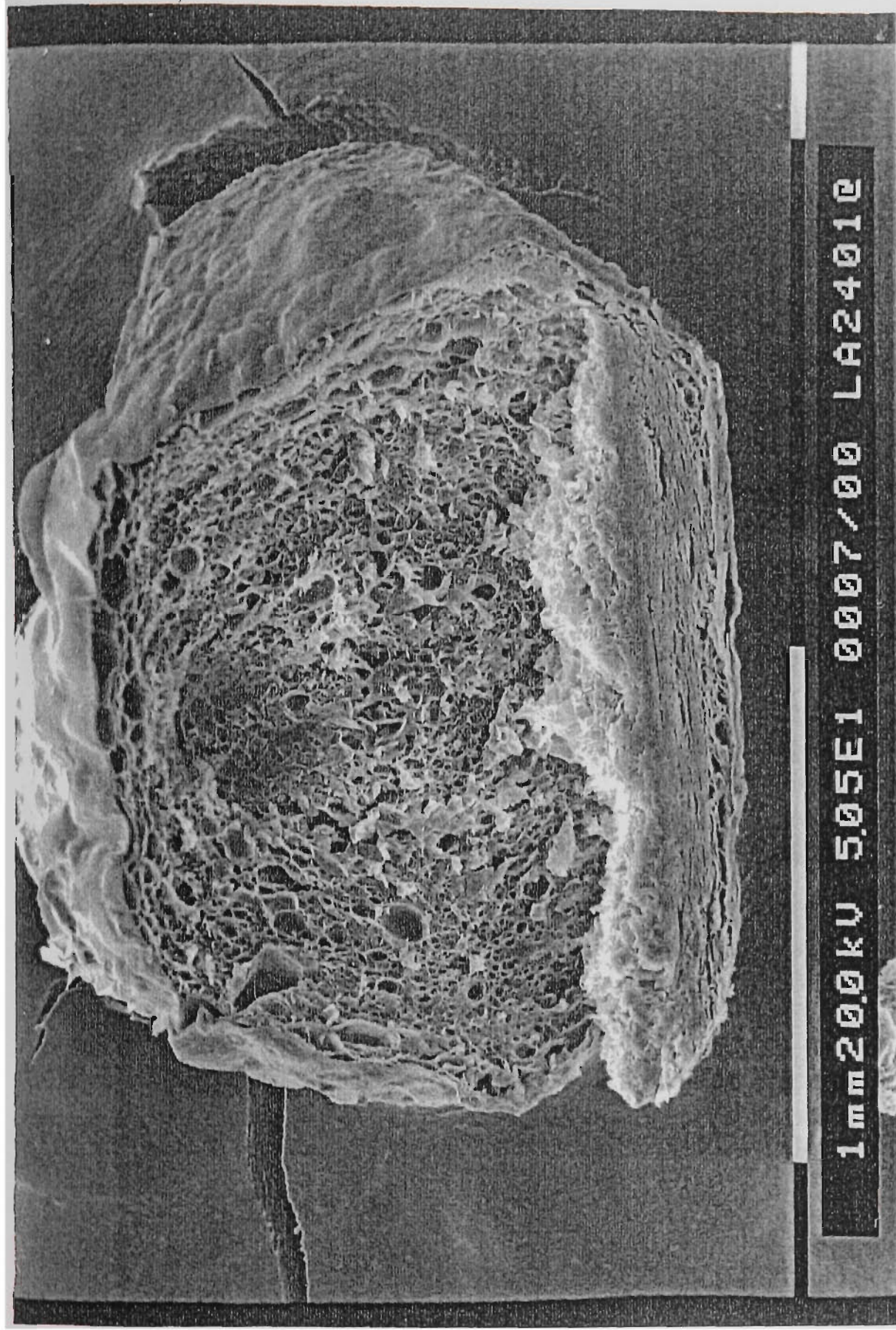
Figure 13.8 – Scanning electron micrograph of LA 2401 encapsulated in calcium alginate bead (cross section).

Figure 13.9 – Scanning electron micrograph of LA 2401 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).
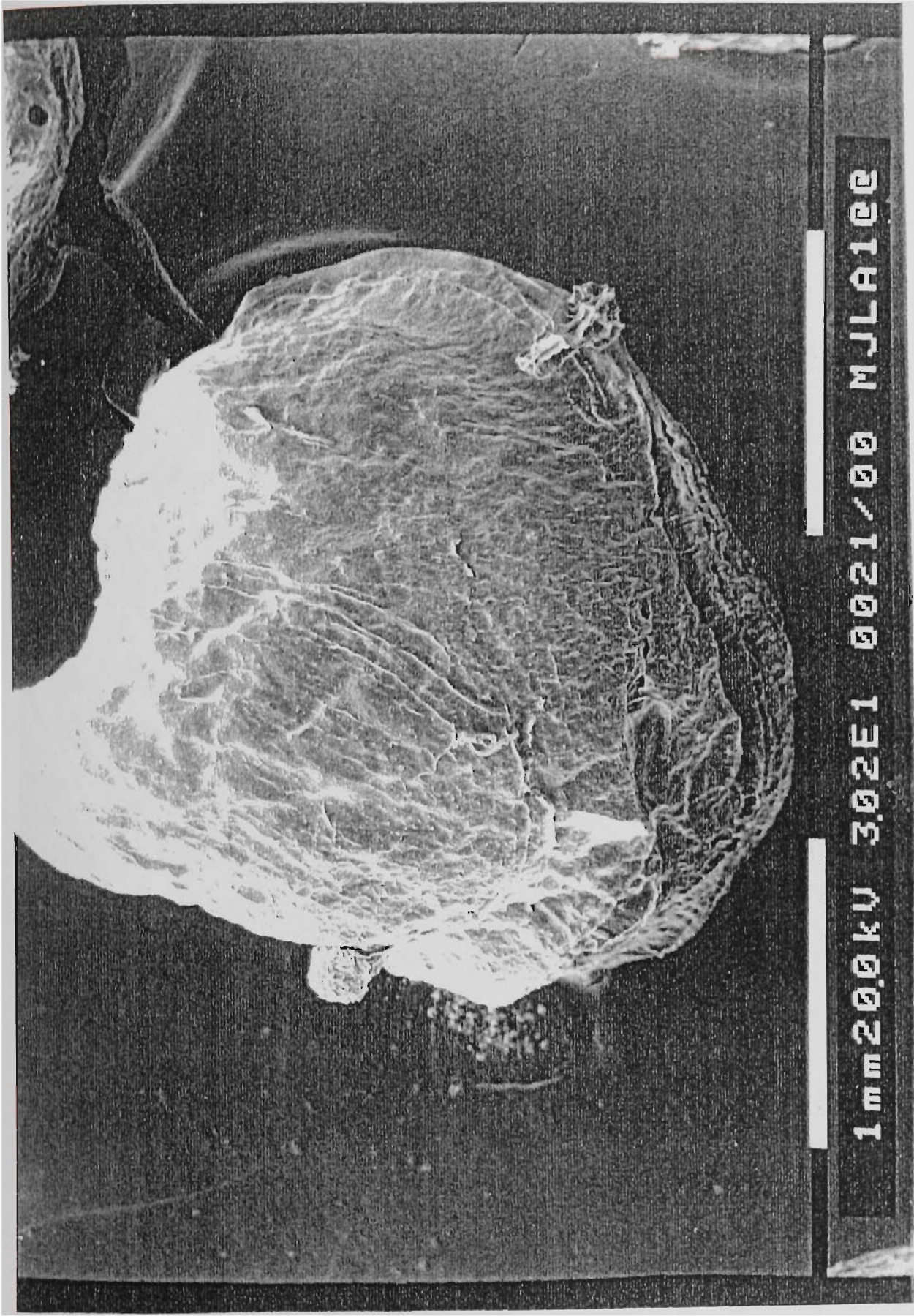
Figure 13.10 – Scanning electron micrograph of LA MJLA1 encapsulated in calcium alginate bead.
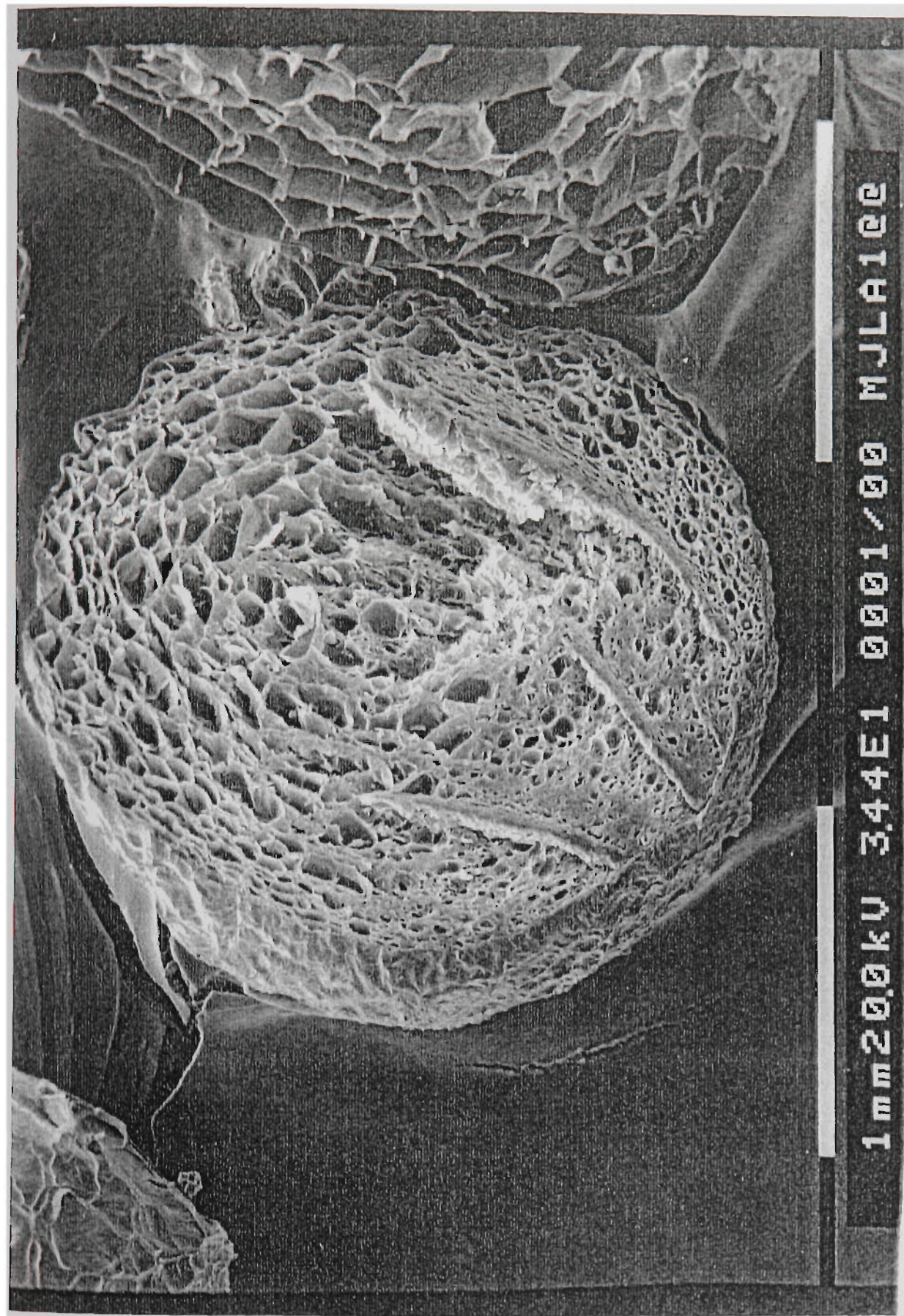
Figure 13.11 – Scanning electron micrograph of LA MJLA1 encapsulated in calcium alginate bead (cross section).
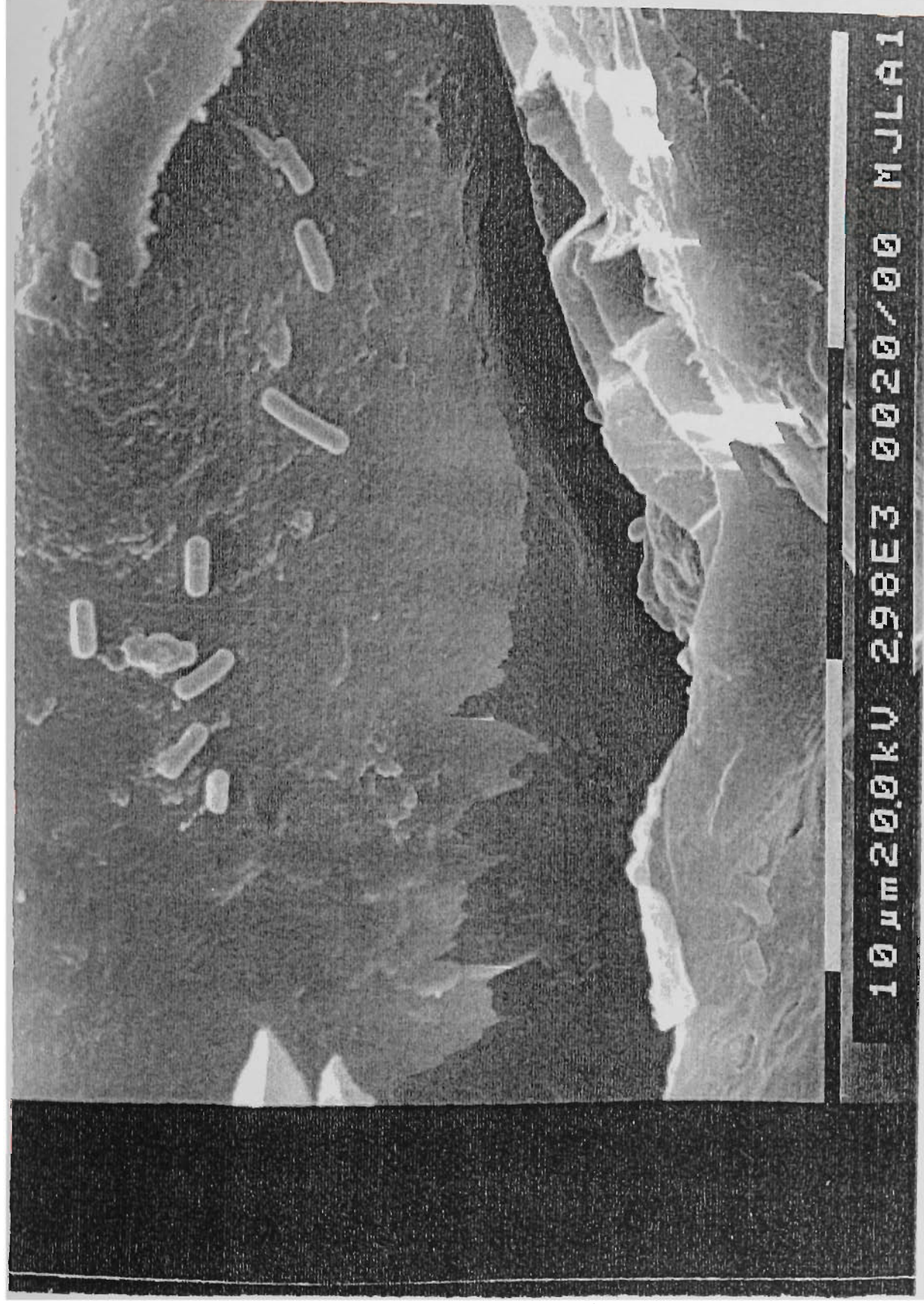
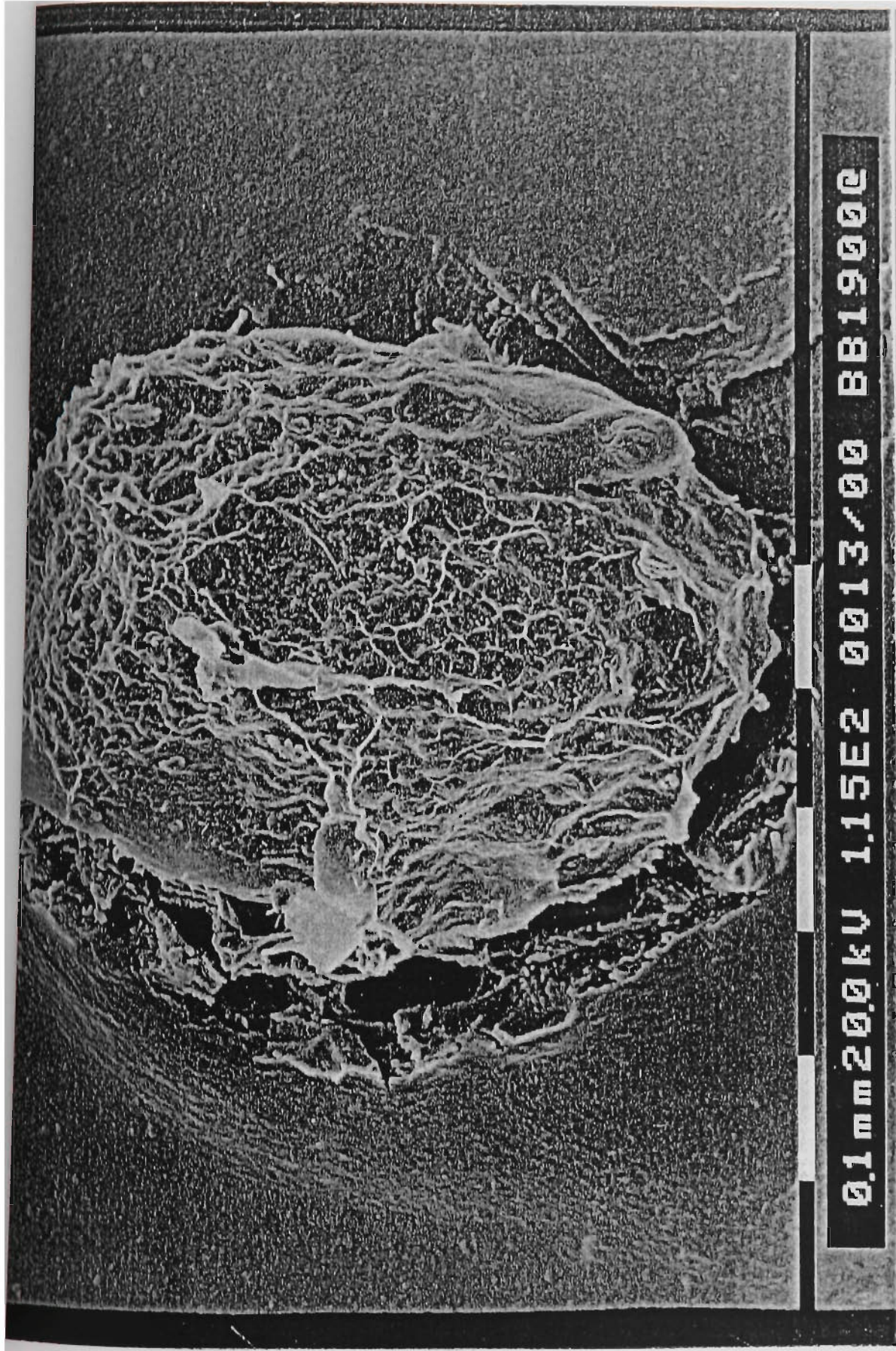Figure 13.12 – Scanning electron micrograph of LA MJLA1 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).

Figure 13.13 — Scanning electron micrograph of BB1900 encapsulated in calcium alginate bead.

0.1mm 20.0kV 1.15E2 0013/00 BB1900

Figure 13.14 – Scanning electron micrograph of BB1900 encapsulated in calcium alginate bead (cross section).

Figure 13.15 – Scanning electron micrograph of BB1900 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).

Figure 13.16 – Scanning electron micrograph of BB BDBB2 encapsulated in calcium alginate bead.

Figure 13.17 – Scanning electron micrograph of BB BDBB2 encapsulated in calcium alginate bead (cross section).

Figure 13.18 – Scanning electron micrograph of BB BDBB2 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).

Figure 13.19 – Scanning electron micrograph of BB 1941 encapsulated in calcium alginate bead.
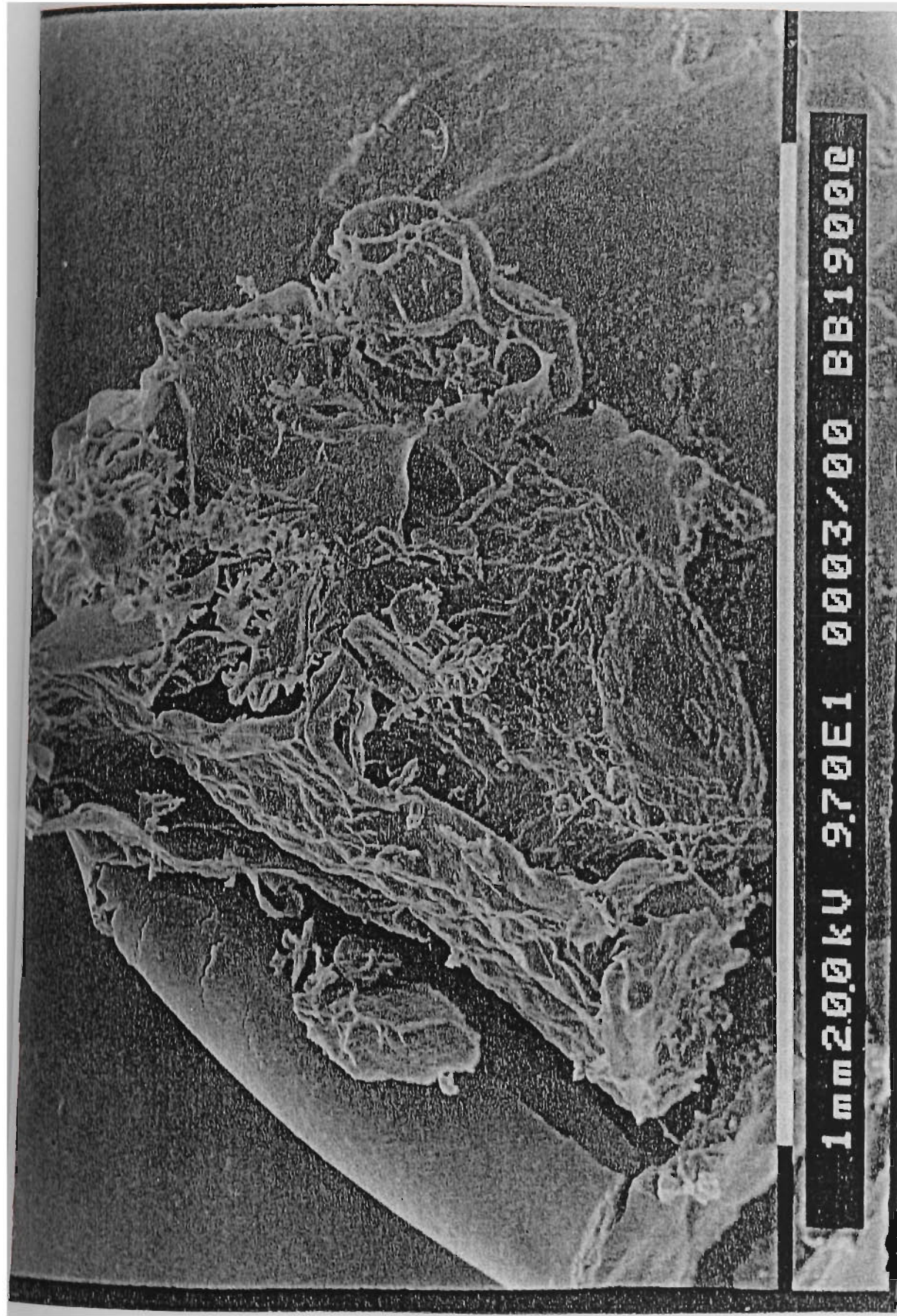
Figure 13.20 – Scanning electron micrograph of BB 1941 encapsulated in calcium alginate bead (cross section).

Figure 13.21 – Scanning electron micrograph of BB 1941 encapsulated in calcium alginate bead (showing the location of the organism in the matrix of the bead).
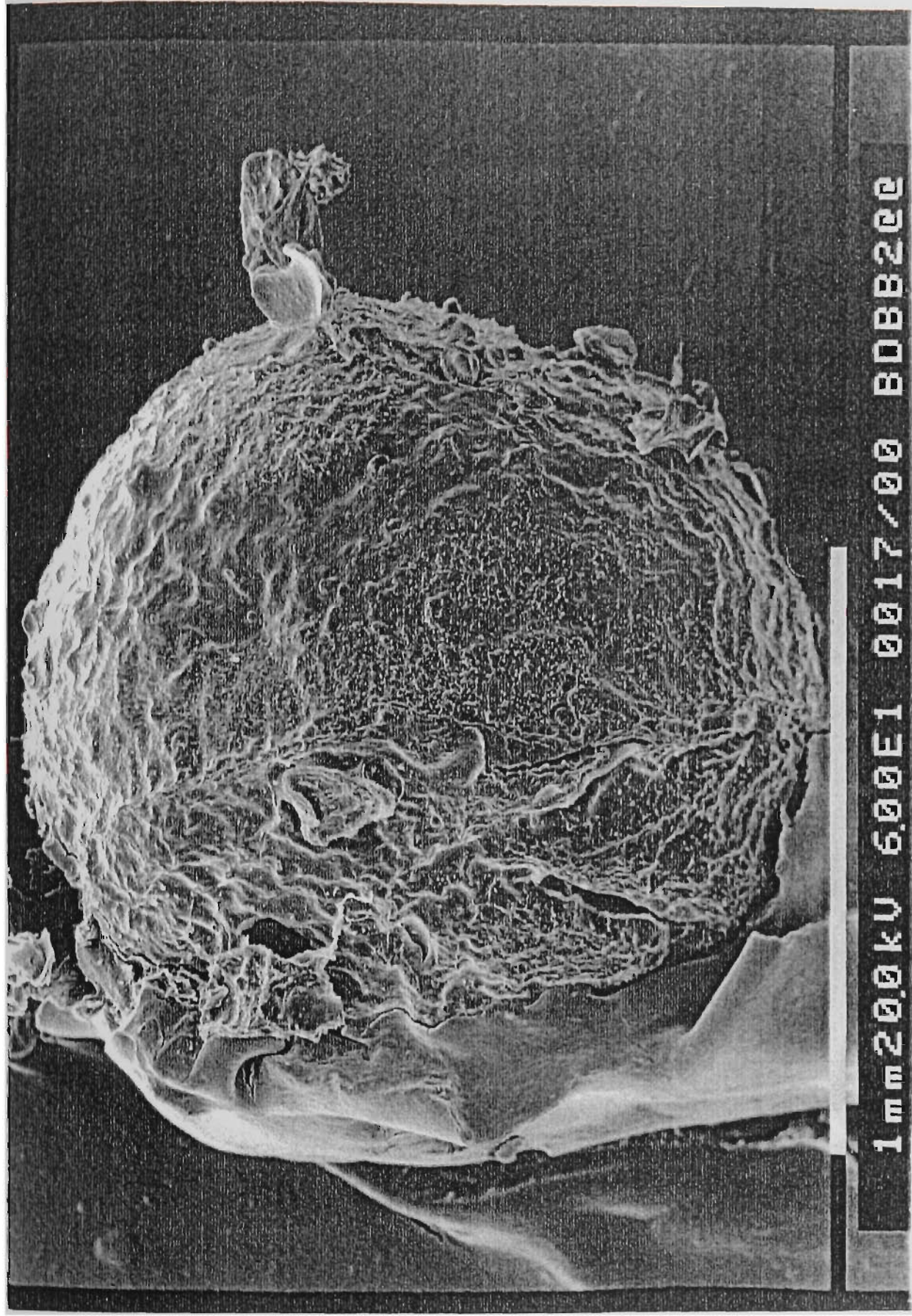
Figure 13.22 Release of beads in various pH levels with lactic acid with or without phosphate buffer
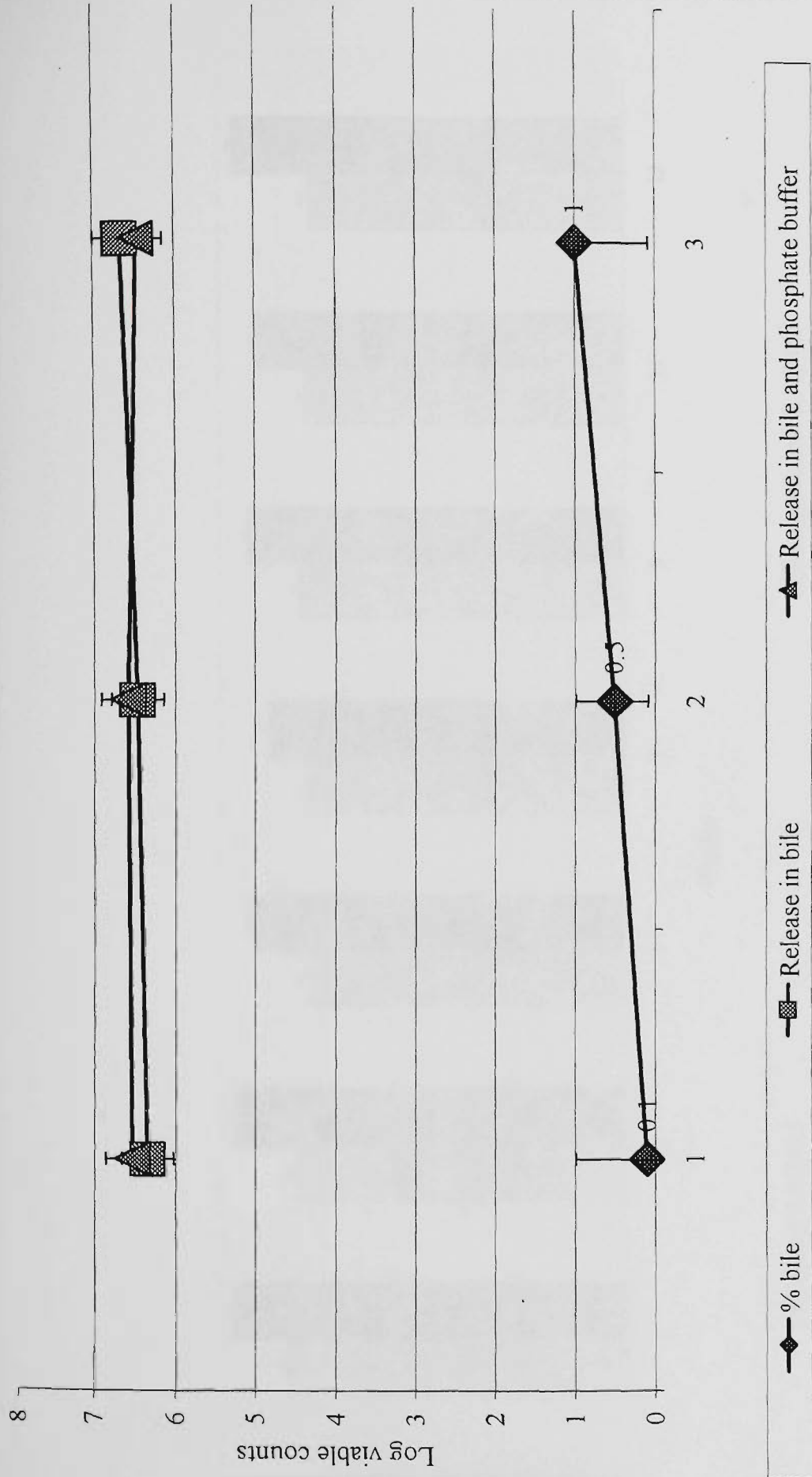
Figure 13.23 Release of beads in various bile concentrations with or without phosphate buffer

Figure 13.24 Viability of *L. acidophilus* with or without encapsulation in fermented frozen dairy desserts
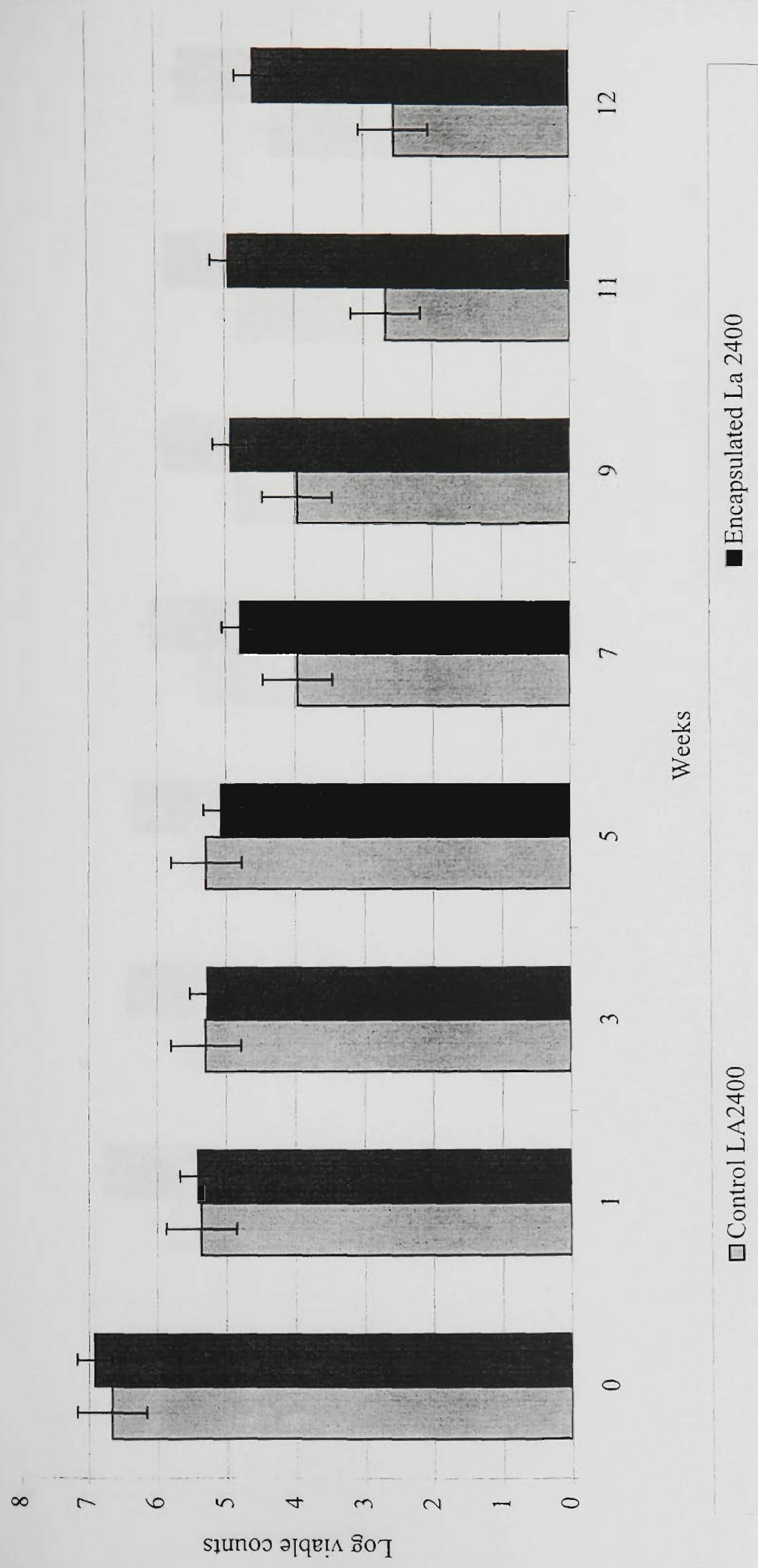
Figure 13.25 Viability of *L. acidophilus* with or without encapsulation in fermented frozen dairy desserts

B
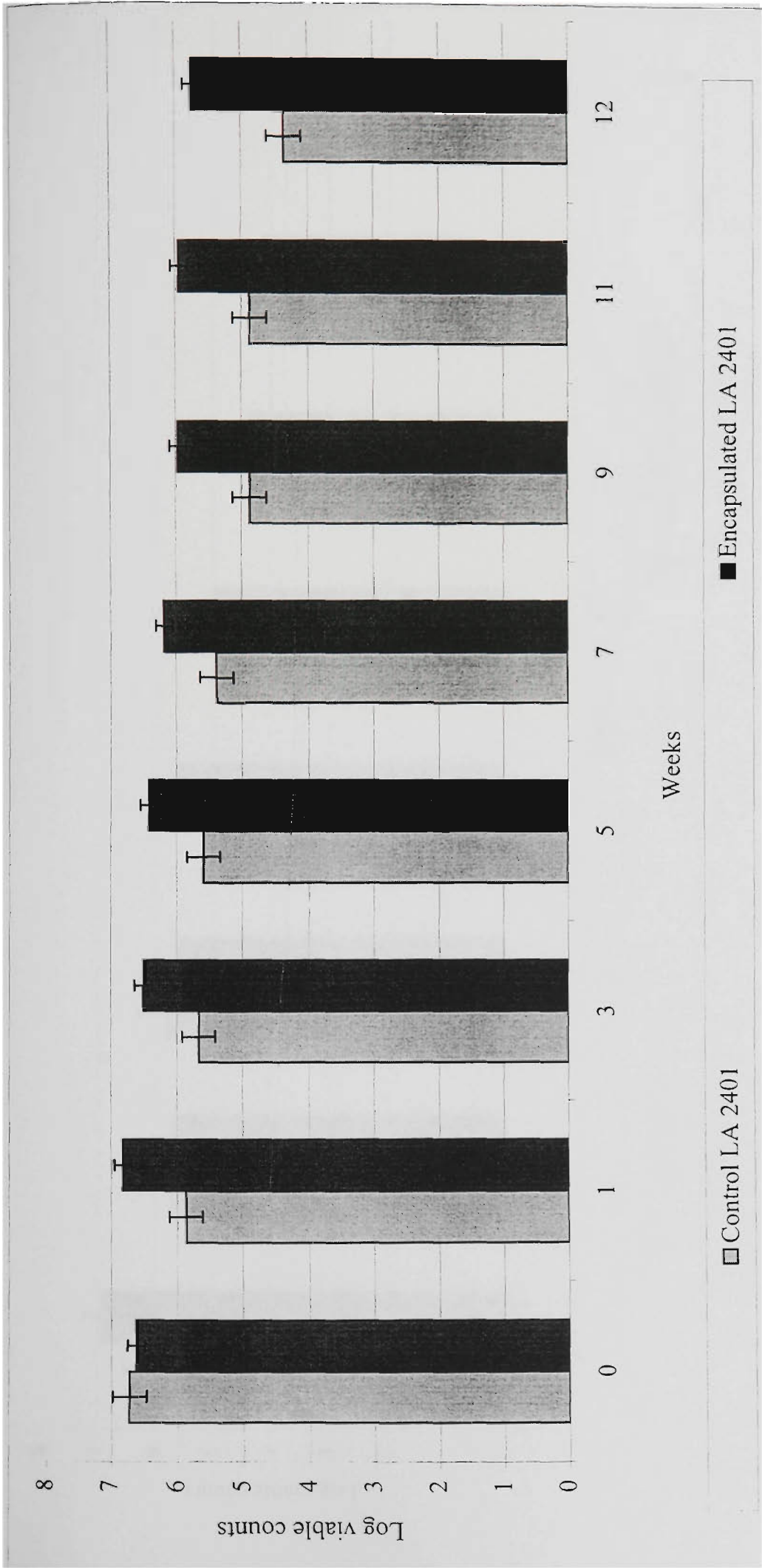
Figure 13.26 Viability of *L. acidophilus* with or without encapsulation in fermented frozen dairy desserts

C
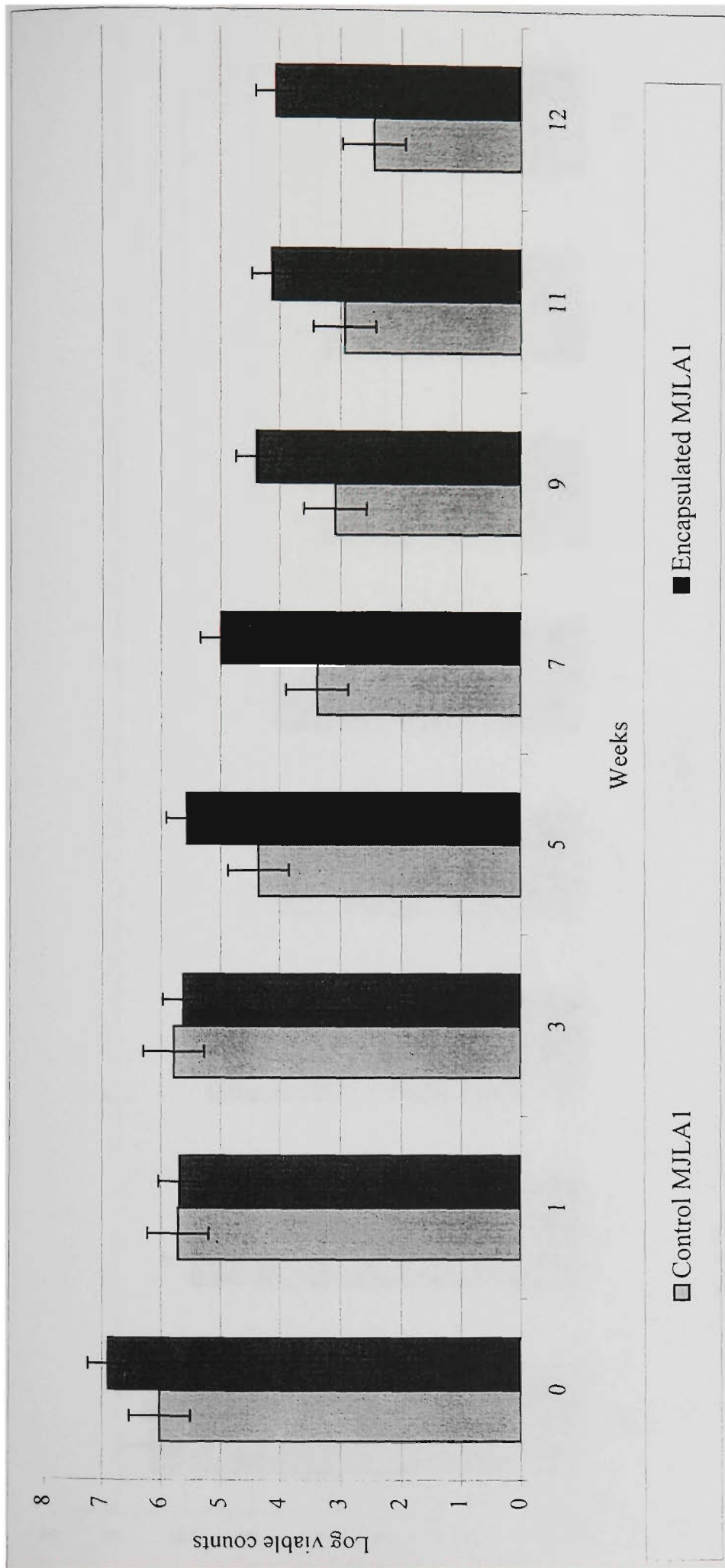
Figure 13.27 Viability of *L. acidophilus* with or without encapsulation in fermented frozen dairy desserts
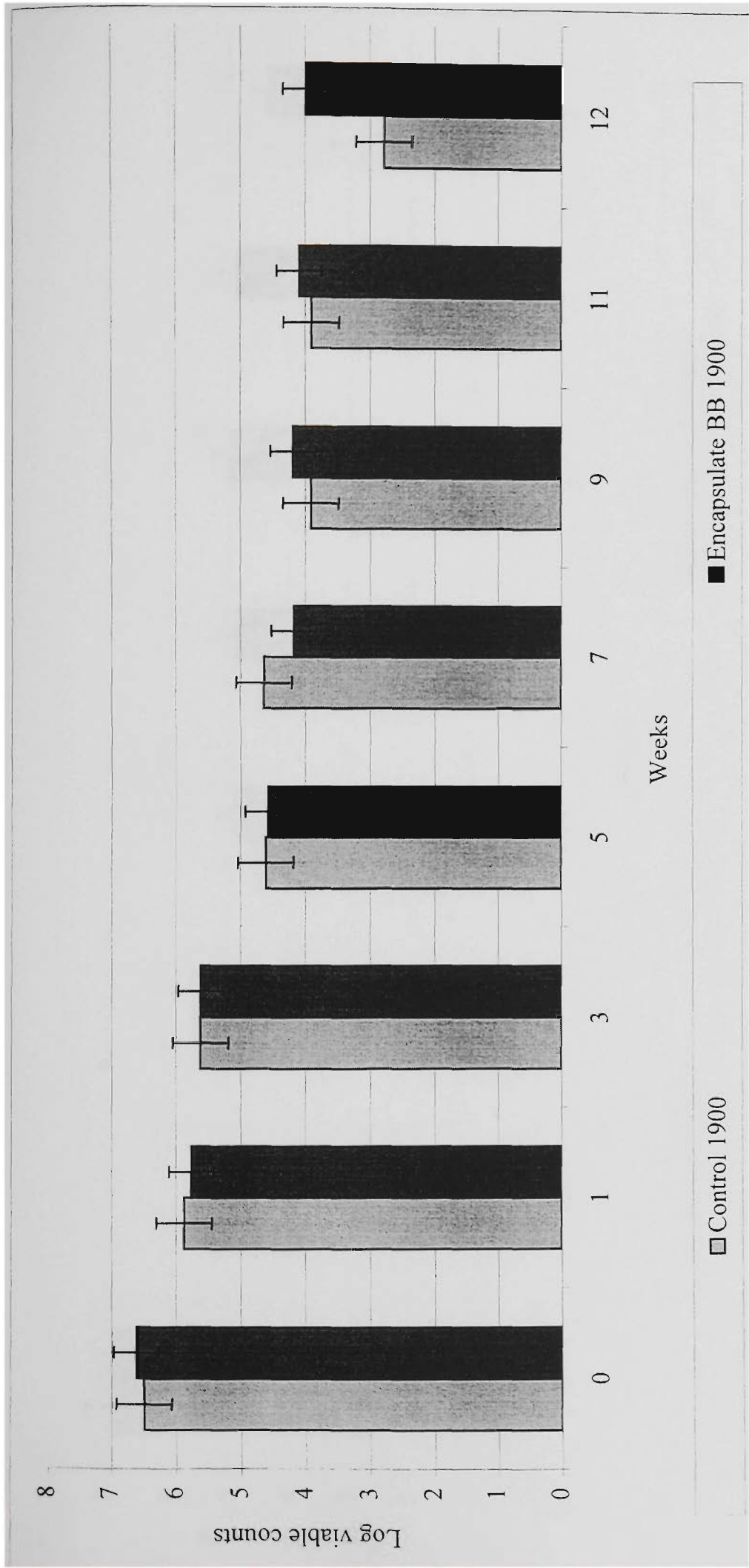
Figure 13.28 Viability of bifidobacteria with or without encapsulation in fermented frozen dairy desserts
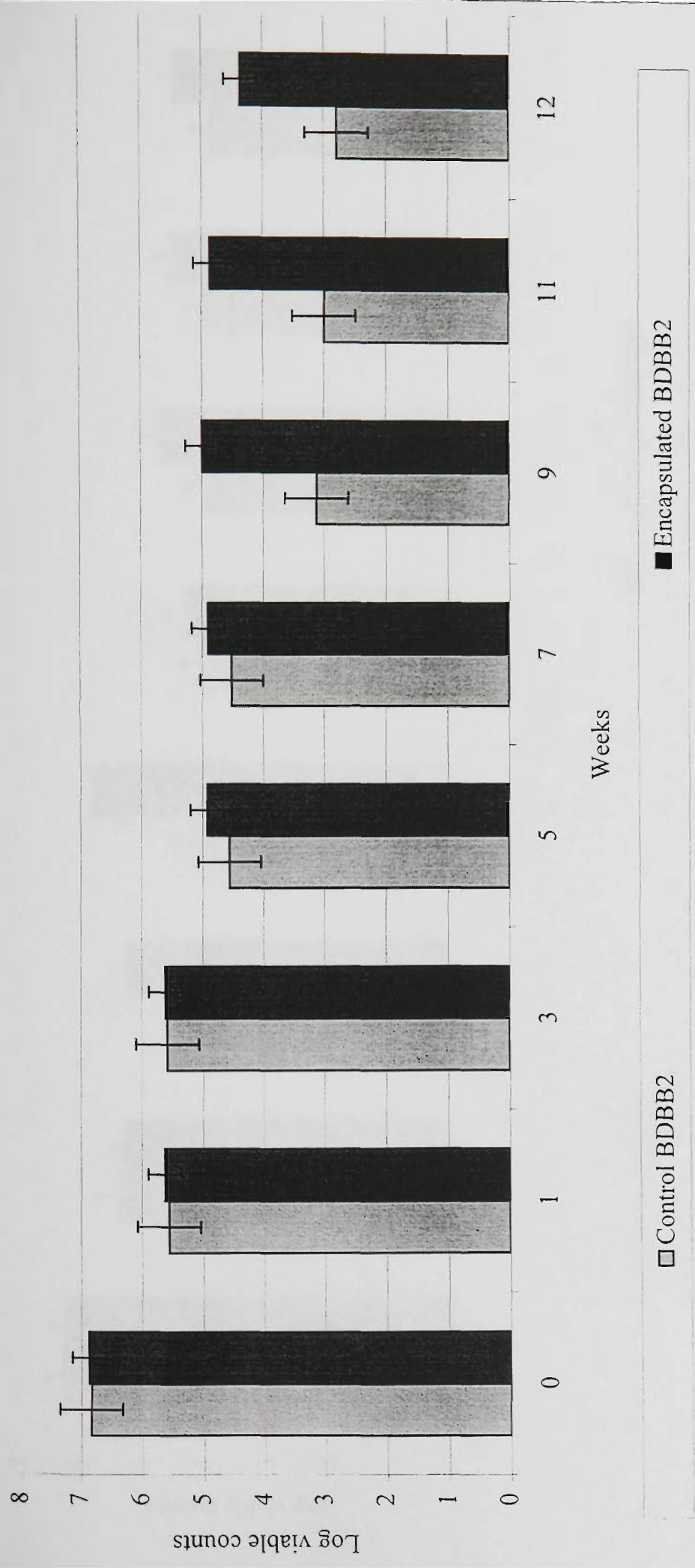
Figure 13.29 Viability of bifidobacteria with or without encapsulation in fermented frozen dairy desserts

☐ Control BDBB2   ■ Encapsulated BDBB2

F
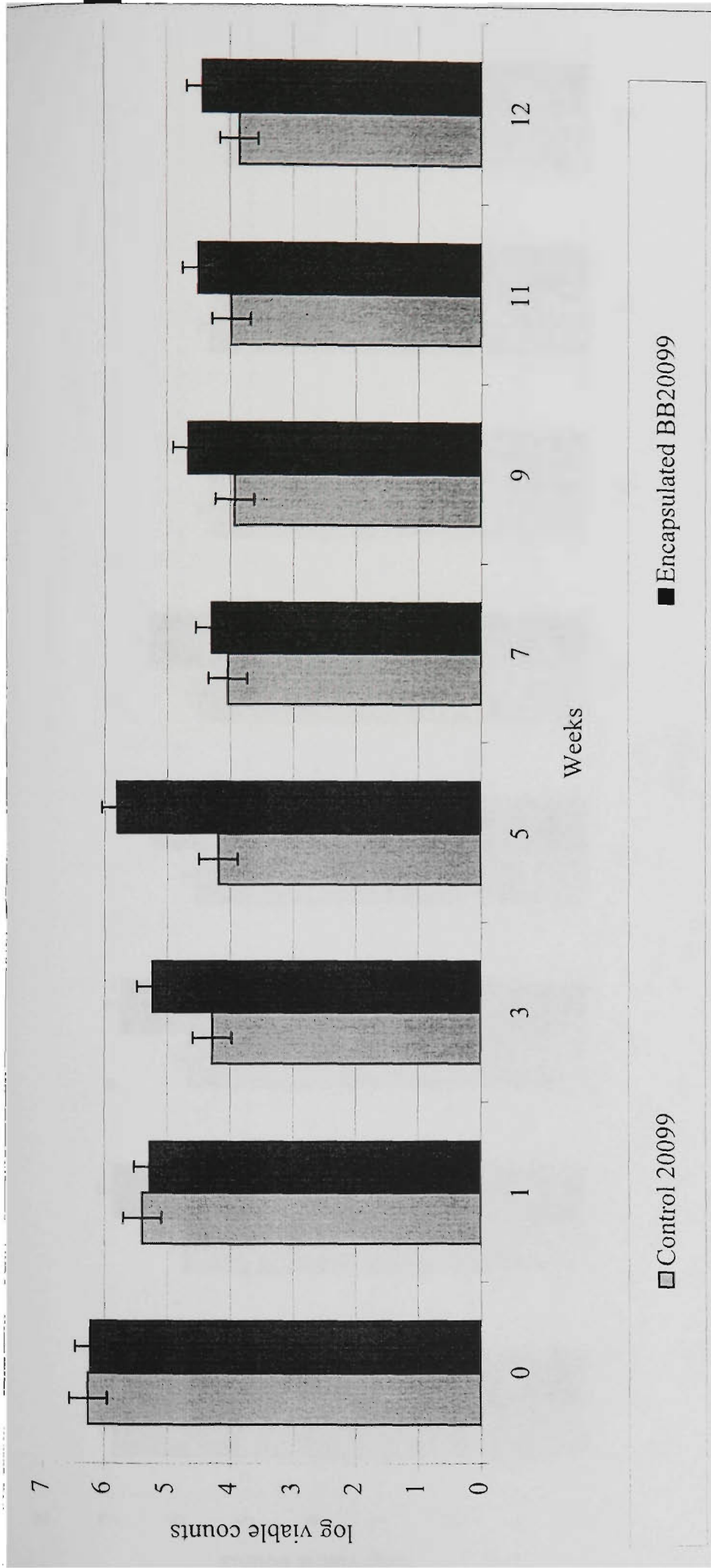
Figure 13.30 Viability of bifidobacteria with or without encapsulation in fermented frozen dairy desserts
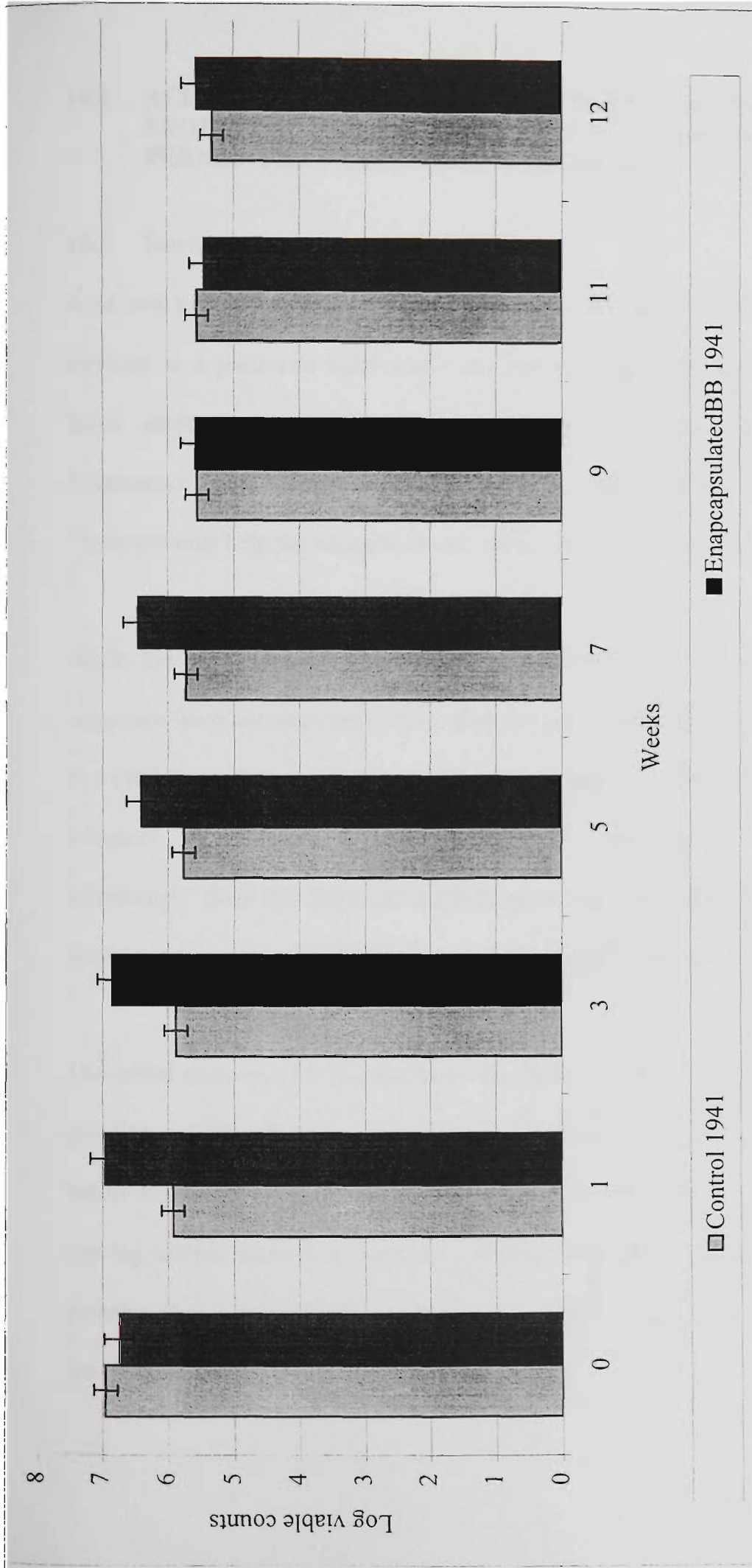
Figure 13.31 Viability of bifidobacteria with or without encapsulation in fermented frozen dairy desserts

14.0 ACID AND LOW TEMPERATURE ADAPTATION OF PROBIOTIC BACTERIA FOR INCORPORATION IN FERMENTED FROZEN DAIRY DESSERTS

14.1 Introduction

Acid and low temperature adaptations are a novel method where the cells are exposed to a particular mild acidic environment and followed by exposure to lethal acidic environment and the organism frozen for various time and temperature combinations in order to see any proteins released during freezing. These proteins help the organisms cope under stressful conditions.

While the physiology, biochemical and genetic mechanisms of many of these responses have not been delineated, what is clear is the following: When exposed to a mild dose of stress, microorganisms may adapt to the stress, thus developing tolerance or resistance to stronger doses of stress. Also it is becoming increasingly clear that the exposure and subsequent adaptation to one stress can confer resistance to other different stress (Berry and Foegeding, 1997).

The initial discovery of this response was made by Jones *et al.* (1987) in *E. coli* growing at 37^0C and shifted to 10^0C. The growth halts for approximately 4 hours before exponential growth is resumed (Jones *et al.* 1987; Ng *et al.*, 1962). During this lag period, there is an induction of some 16 proteins, called the cold shock proteins (Jones *et al.* 1996; La Teana *et al.*, 1991; Lelivelt and Kawula, 1995; Jones *et al.*, 1992)

Most microorganisms must accommodate to a variety of changing conditions and stress in their environment in order to survive. Because of impact of temperature on all reactions of the cell, adaptations to fluctuations in temperature are possibly the most common. In many organisms specific sets of cold shock proteins are induced upon abrupt shifts to colder temperatures. While this cold shock response has not been fully delineated, it appears to be adaptive and may function to promote the expression of genes involved in translation when cells are displaced to low temperatures. The cold shock response of *Escherichia coli* has been extensively studied and the major cold shock protein CspA appears to be involved in the regulation of response. Upon cold shock, the induction of CspA and its counterparts in most microorganisms studied is prominent, but transit. Studies of this response in some psychrotrophic bacteria have reported constitutive synthesis and continued synthesis during cold temperature growth of CspA homologues (Berry and Foegeding, 1997).

Freezing is one of the main factors in manufacturing of fermented frozen dairy desserts. Intracellular ice formation during freezing has been described for many cellular systems, and the rate at which ice is formed determines the type of freezing damage suffered by cells (Mazur, 1970; Toner *et al.* 1993). Cells can be injured during freezing by physical factors such as ice crystal formation and dehydration. At high freezing rates, intracellular freezing occurs, leading to cell damage mainly by ice crystal formation. However, at low freezing rates, extracellular ice formation predominates, leading to intracellular dehydration (Mazur, 1970). The pretreatment of cells with a mild stress induces higher stress

tolerance and in some cases this confers cross protection to other, different types of stress (Mager and Ferreira, 1993). It has been assumed that different stress conditions act through a variety of effects in cells, such as generation of abnormal or denatured proteins, internal acidification, alterations in the cytoskeleton or modulation of second-messenger levels, to produce signals that can provide coverage and stimulate more general stress-responding system (Chowdhury *et al.*, 1993; Coote *et al.*, 1994; Craig and Gross, 1994).

The freeze-thaw stress response of yeast *Saccharomyces cerevisiae* is growth phase specific and is controlled by nutritional state via the Ras-cyclic AMP signal transduction pathway as studied by Jong and Ahn (1997). The authors concluded that the ability of cells to survive freezing and thawing is expected to depend on the physiological conditions experienced prior to freezing. They examined factors affecting yeast cell survival during freeze thaw stress, including those associated with growth phase, requirement for mitochondrial functions and prior stress treatment. Supercooling occurred without reducing cell survival and was followed by freezing. Loss of viability was proportional to the freezing duration, indicating that freezing was the main determinant of freeze-thaw damage.

The aim of this study was to adapt *L. acidophilus* and bifidobacteria to acidic and low temperature conditions so that they can survive in fermented frozen dairy desserts.

14.2 Materials and Methods

14.2.1 *Ice cream freezer*

A bench top ice cream freezer (2 L size) was used to make laboratory scale fermented frozen dairy desserts as described in section 3.2.2.

14.2.2 *Centrifuge and microcentrifuge*

Large volumes (100 to 1000 mL) of samples were centrifuged using Beckmann refrigerated J2-HS and for medium volumes (10 to 100 mL) of samples Beckmann refrigerated centrifuge and for small volumes microcentrifuge (Beckmann) were used as described in section 3.2.5

14.2.3 *Bacterial cultures and maintenance*

The bacterial cultures and maintenance are described in section 3.3.

14.2.4 *Lactic acid bacteria and probiotic bacteria*

Yogurt bacteria and probiotic bacteria were described in section 3.3.1

14.2.5 *Microbiological analyses*

Microbiological analyses were described in section 3.3.3

14.2.6 *Determination of the growth curve*

Probiotic bacteria, *L. acidophilus* and *Bifidobacterium* spp., were propagated three times before inoculating in MRS broth (L-cysteine was added for bifidobacteria). The inoculated MRS broth was incubated at 37°C and hourly absorbance readings were taken from cuvettes, using a spectrometer at 450 nm. The pH of the broth was also measured at hourly intervals using a pH meter. A growth curve was plotted to identify the log phase of the organisms.

14.2.9 *Peptone and water diluent*

As described in section 3.16.1

14.2.10 *Preparation of fermented frozen dairy desserts*

As described in section 3.17

14.2.11 *Acid and low temperature adaptations of probiotic bacteria and their incorporation into fermented dairy desserts*

Two adaptation conditions were used: one with sublethal pH of 5.5 and the other with lethal pH of 3.5. For adaptation studies, the organisms were propagated three time in MRS broth (0.05% cysteine was added for bifidobacteria) at 2% level and incubated at 37^0C for 18 h. The fermentation was slowed down by transferring the samples into a refrigerator and the temperature was decreased to 4^0C. The initial pH of the sample was assessed with a pH meter and the sample plated for enumeration for viable counts. Ten millilitres of the samples were stored in a freezer for gel-electrophoresis. The pH of the broth (100 ml) was increased to 6.5 using 10% NaOH and the broth kept in a refrigerator for one hour. After 1 h of refrigeration (temperature adaptation), the broth was divided into two portions (A & B) of 50 ml each and the pH in the sample A decreased to lethal pH of 3.5. The sample B was kept at sublethal pH of 5.5 for one hour before decreasing the pH to the lethal pH of 3.5. The samples A and B were maintained at this pH (3.5) for 3 h, followed by freeze stressing at -20^0C for 1 hour. The samples were thawed and the pH increased to 6.5 with 35% NaOH and allowed to recover the stress at this pH for one hour and the pH of the samples was decreased to 4.5. to observe any increased survival in the counts.

Similarly bacterial cells were stressed at pH 3.5 as before and freeze-stressed for 2h, 4h, 8h, 12h, 16h or 24h. Studies showed that sample A when adapted to sublethal pH survived better. On this basis, 3 strains each of *L. acidophilus* and bifidobacteria were selected and incorporated into fermented frozen dairy desserts. The viable counts were monitored for 12 weeks.

14.2.12 *Preparation of buffers and reagents*

As described in section 3.19.1 to 3.19.1.8

14.3 Results and Discussion

Most microorganisms must accommodate to a variety of changing conditions and stress in their environment in order to survive and multiply (Berry and Foegeding, 1997).

Figure 14.1 shows the growth curve of 3 strains each of *L. acidophilus* and *Bifidobacterium* ssp. The organisms used in adaptation experiments were grown to log phase before they were used in the experiment. Figures 14.2 – 14.8 shows the results of the adaptation of probiotic bacteria where sub-lethal pH conditions were used. Figure 14.2 shows that when the probiotic bacteria stressed at low temperature for one hour there was no effect on the organisms. The results suggest that the organisms can survive acid and low temperature for one hour. Figures 14.3 show similar trend as with one hour exposure suggesting that the

organisms can survive 2 h of freeze stress. However, exposure time of 4 h affected the viability of the organisms (Figure 14.4)

Figure 14.5 shows that after stress at -18^0C for 6 hour, there was a decrease in one log cycle. As shown in figure 14.6 as the exposure time increased, the viability decreased. The viability of the organisms was lost by about 3 log cycles after stressing for 12 hours.

Figures 14.7 and 14.8 shows more adverse effects on exposure for 18 and 24 hours. Thee organisms lost the viability by 5 log cycles after exposure to 24 hours. The viable counts of the organisms did not improve by increasing the pH suggesting that the effect on the organisms was lethal.

Figures 14.9 – 14.15 shows the results on the probiotic bacteria where the organisms were not exposed to sublethal pH. The organisms were exposed to lethal pH (3.5) only.

The organisms lost the viability by about 1 log cycle even after stressing only for one hour (Figure 14.9). As the exposure time increased, the loss in viability became more obvious (Figure 14.10-14.14). There were no survivors after 24 h of exposure (Figure 14.15).

Gel electrophoresis was used to detect any new protein that could be identified. The results reveal that there was no new protein except that the protein concentration was higher in the band at pH 3.5 at -18^0C.

14.3.1 Survival of acid and low temperature probiotic bacteria in frozen fermented dairy desserts

Figure 14.16 shows the survival of *L. acidophilus* MJLA1 and bifidobacteria BDBB2 in fermented frozen dairy desserts. The counts of *L. acidophilus* MJLA1 in the products incorporated with acid and stress adapted cells were higher at the end of 12 weeks storage period than those made with non-stress adapted cells. The drop in the counts of *L. acidophilus* during storage was by almost 1 log cycle after 7 weeks and remained the same by the end of 12 week storage period, Whereas the control *L. acidophilus* Mjla1 lost the viability by one log cycle after one week and further decreased by one more log cycle after 7 weeks and by the end of 12 week storage period remained 4 log cycles. In the same experiment bifidobacteria lost viability by 1 log cycle after 1 week in both the control and stress adopted organisms and by the end of 12 week storage period control bifidobacteria remained viable by 4 log cycles and stressed by 5 log cycles. However stress adapted *L. acidophilus* showed better response than the other counterparts.

Figure 14.17 shows the survival of *L. acidophilus* La 2409 and bifidobacteria BB 20210 incorporated into fermented frozen dairy desserts The *L. acidophilus* La 2409 lost viability by one log cycle after third week in the control sample and by

the end of 12 week storage period remained at 2 log cycles. A similar trend was observed in the control bifidobacteria. The stressed adapted *L. acidophilus* and bifidobacteria lost the viability after 5 weeks by one log cycle and remained at 5 log cycles at the end of storage period. These strains have been claimed to be poorly survival in acidic and freezing conditions. From the results it can be observed that stress adaptation improves the viability of the bacteria.

In the experiment (Figure 14.18) shows no much effect of acid and low temperature adaptations. There is a minimal decrease in the viable counts of *L. acidophilus* LA 2401 and bifidobacteria BB 1941when compared to the control organisms. This might be the reason that the organisms are resistant to acid and freezing conditions.

14.4   Conclusion

Survival of probiotic bacteria was studied in fermented frozen dairy desserts made with or without acid and low temperature adapted cells at fortnightly intervals for 12 weeks. The counts were higher in the products made with stress adapted organisms. The results suggested that the use of acid and low temperature adapted probiotic bacteria in fermented frozen dairy desserts survive under low pH, and freezing conditions.

It was also observed that the probiotic bacteria survived better in the samples where sub-lethal pH was used when compared to the bacteria where lethal stress was directly used.
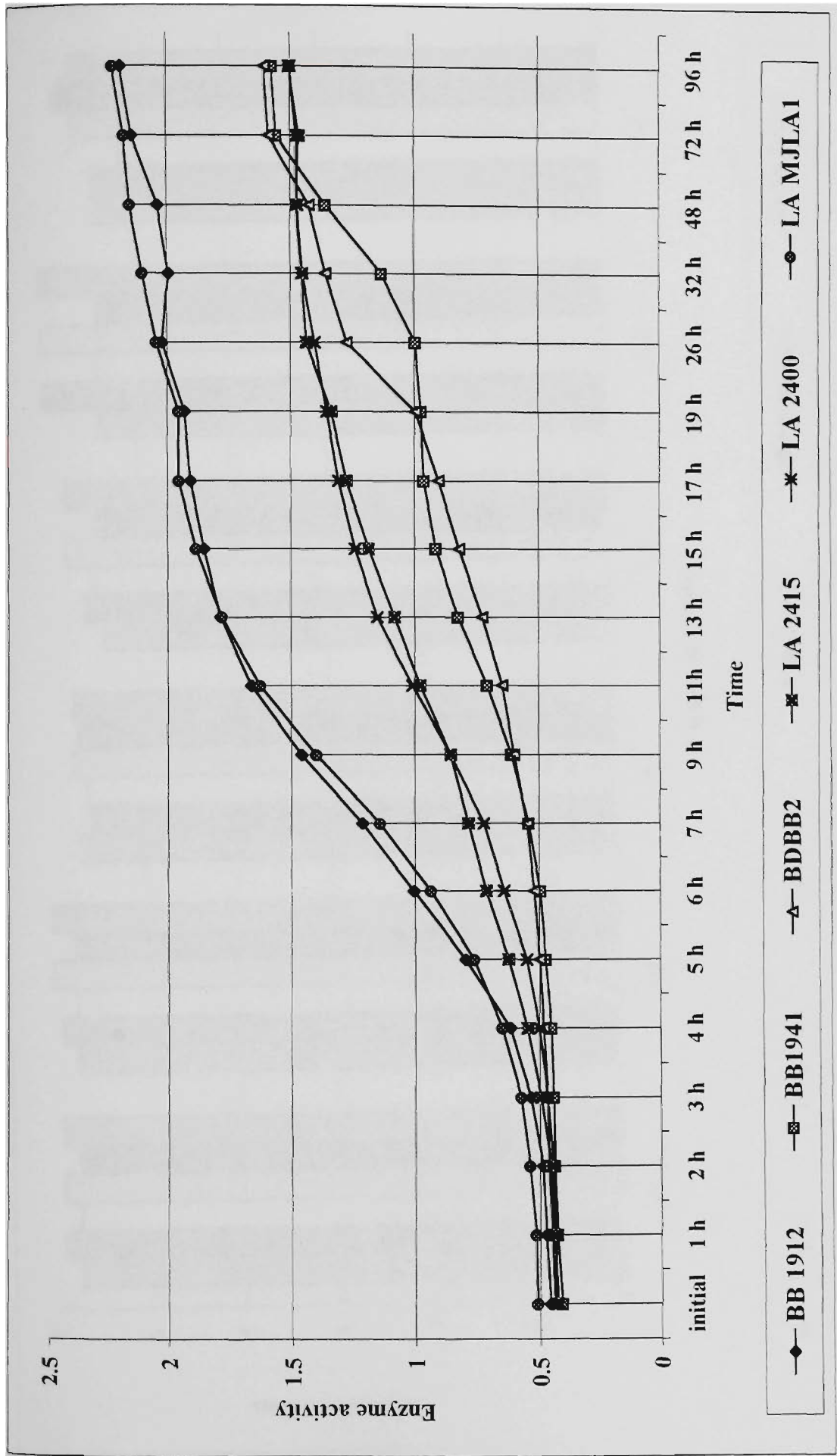
Figure 14.1 Growth curves of probiotic bactera

Figure 14.2 Effects of acid (3.5pH) and freezing at -18 deg C for one hour on the viability of probiotic bacteria
(LA: *L. acidophilus*; BB: Bifidobacteria)

Figure 14.3 Effects of acid (3.5pH) and freezing at -18 deg C for 2 hour on the viability of probiotic bacteria
(LA: *L. acidophilus*; BB: Bifidobacteria)
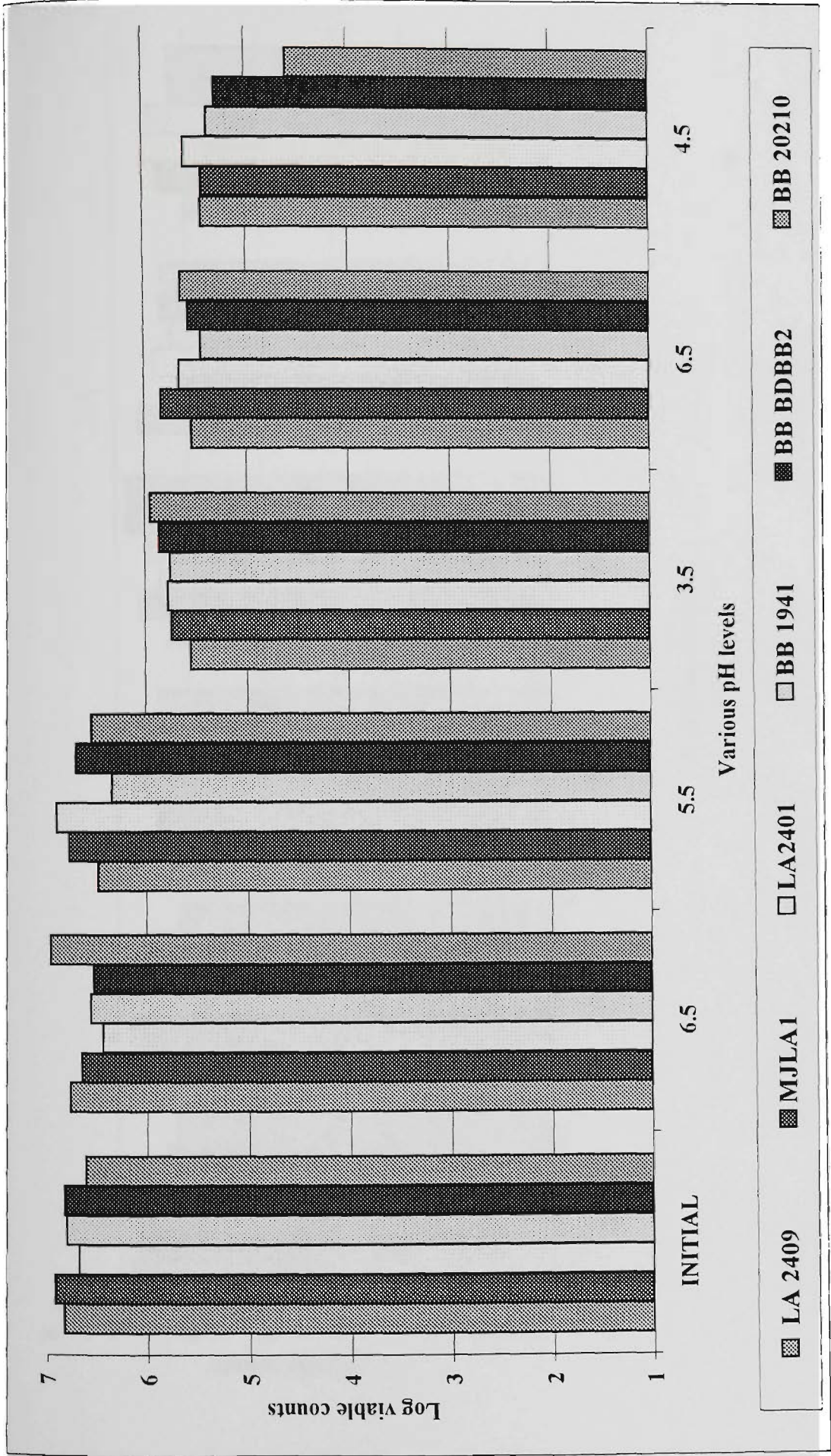
Figure 14.4 Effects of acid (3.5 pH) and freezing at -18 deg C for 4 hour on the viability of probiotic bacteria
(LA: *L. acidophilus*; BB: Bifidobacteria)
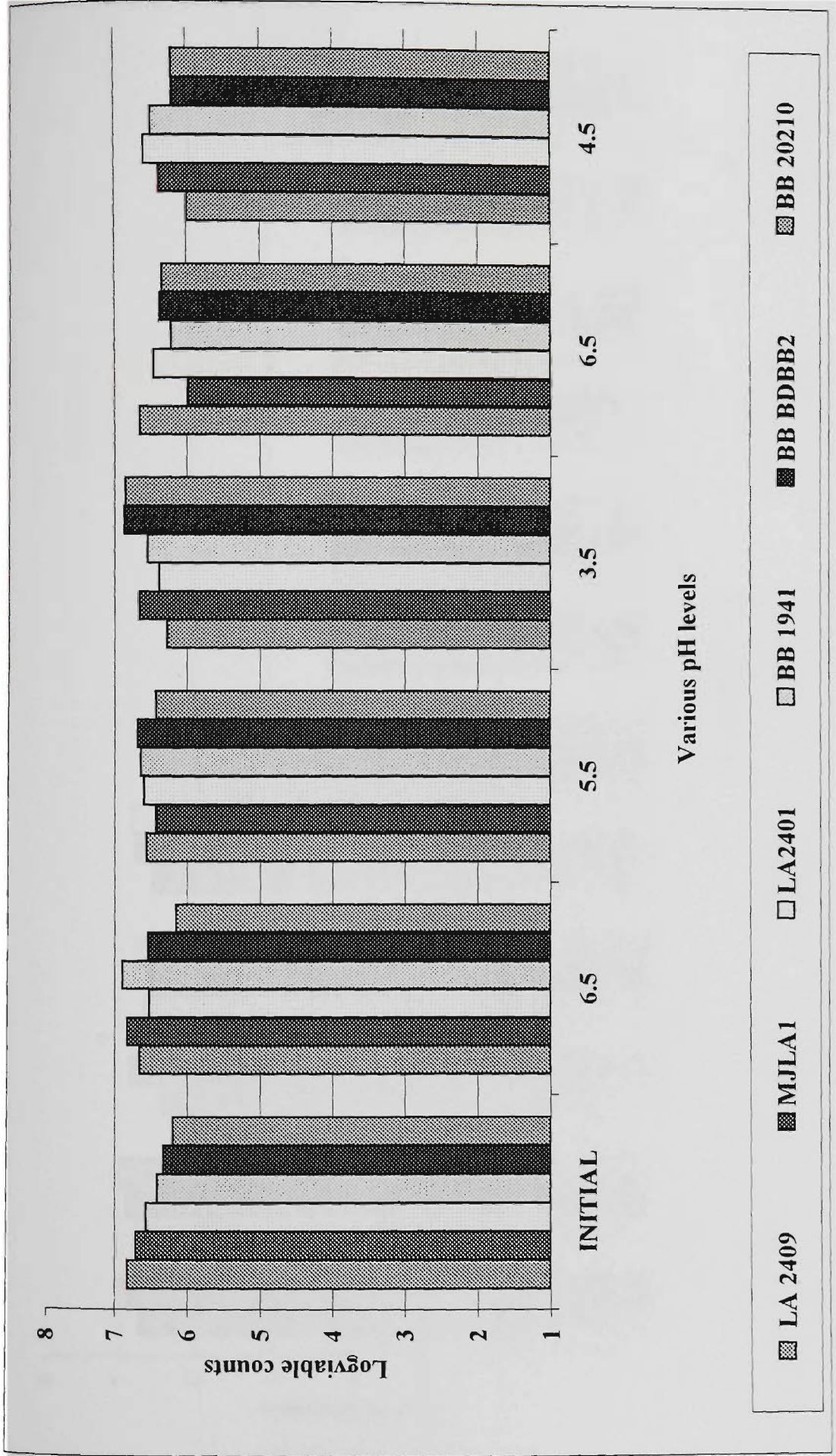
Figure 14.5 Effects of acid (3.5pH) and freezing at -18 deg C for 6 hour on the viability of probiotic bacteria
(LA: *L. acidophilus*; BB: Bifidobacteria)

Figure 14.6 Effects of acid (3.5 pH) and freezing at -18 deg C for 12 hour on the viability of probiotic bacteria
(LA: *L. acidophilus*; BB: Bifidobacteria)
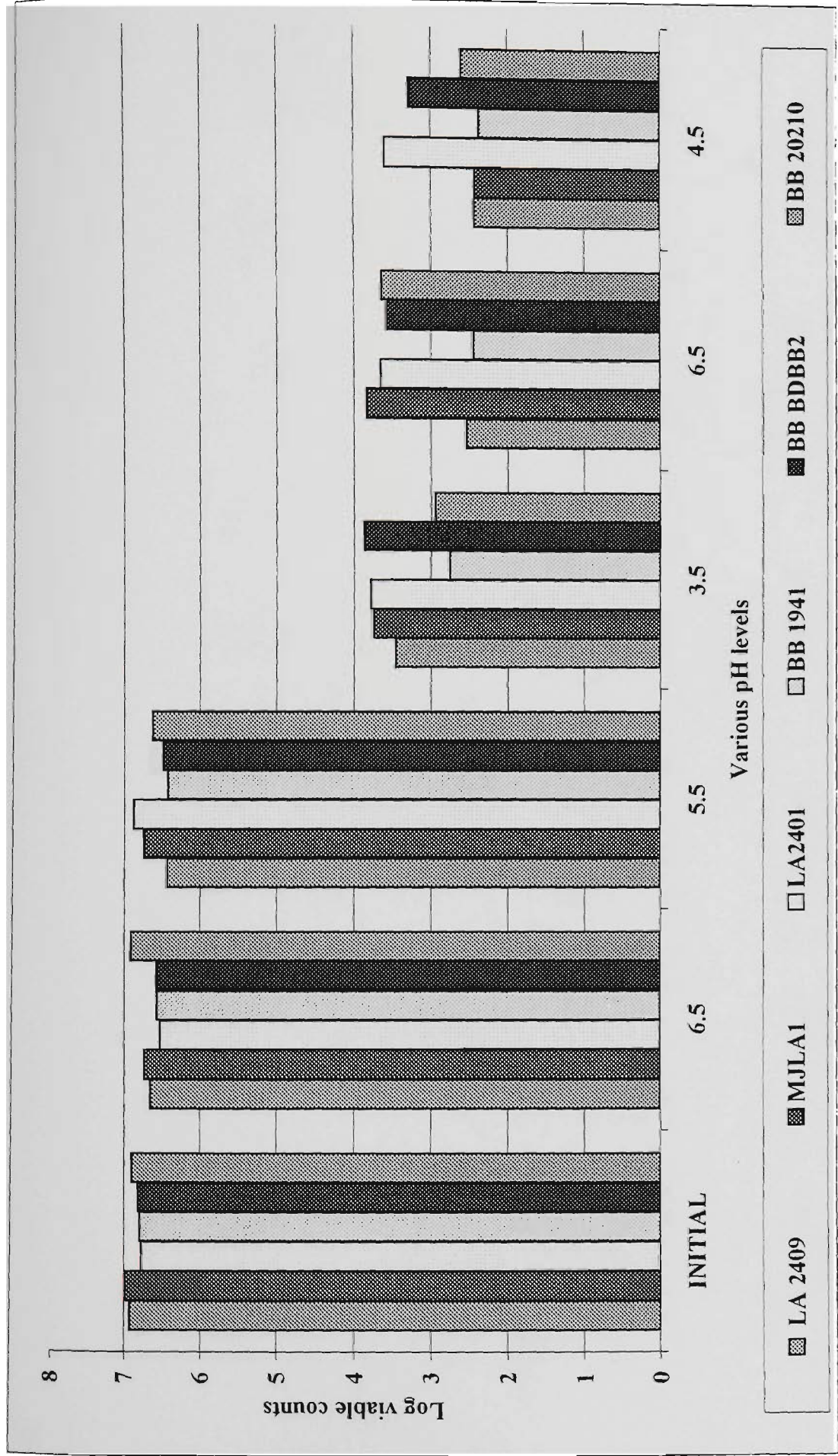
Figure 14.7 Effects of acid (3.5 pH) and freezing at -18 deg C for 18 hour on the viability of probiotic bacteria
(LA: *L. acidophilus*; BB: Bifidobacteria)

Figure 14.8 Effects of acid (3.5pH) and freezing at -18 deg C for 24 hour on the viability of probiotic bacteria
(LA: *L. acidophilus*; BB: Bifidobacteria)
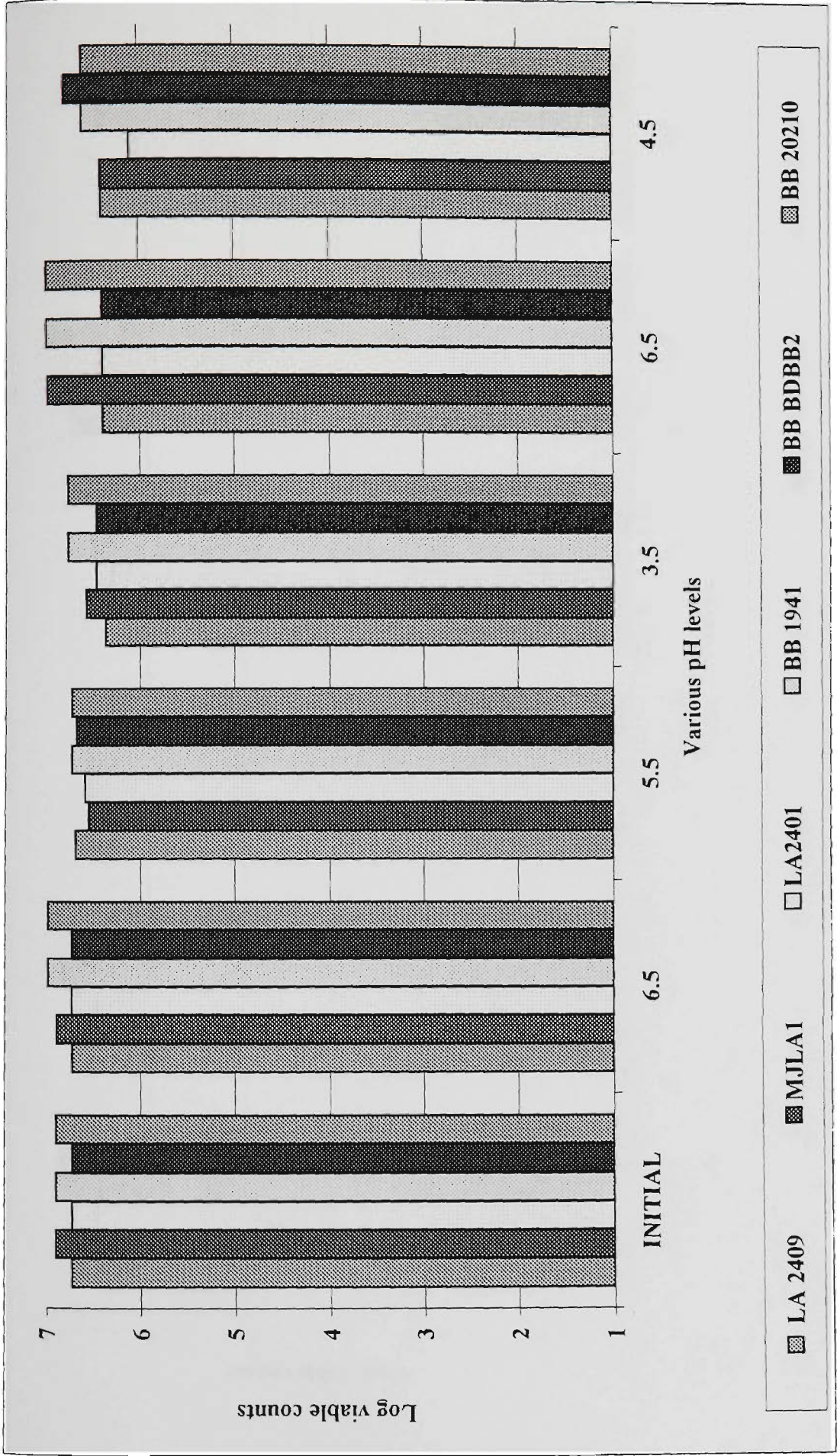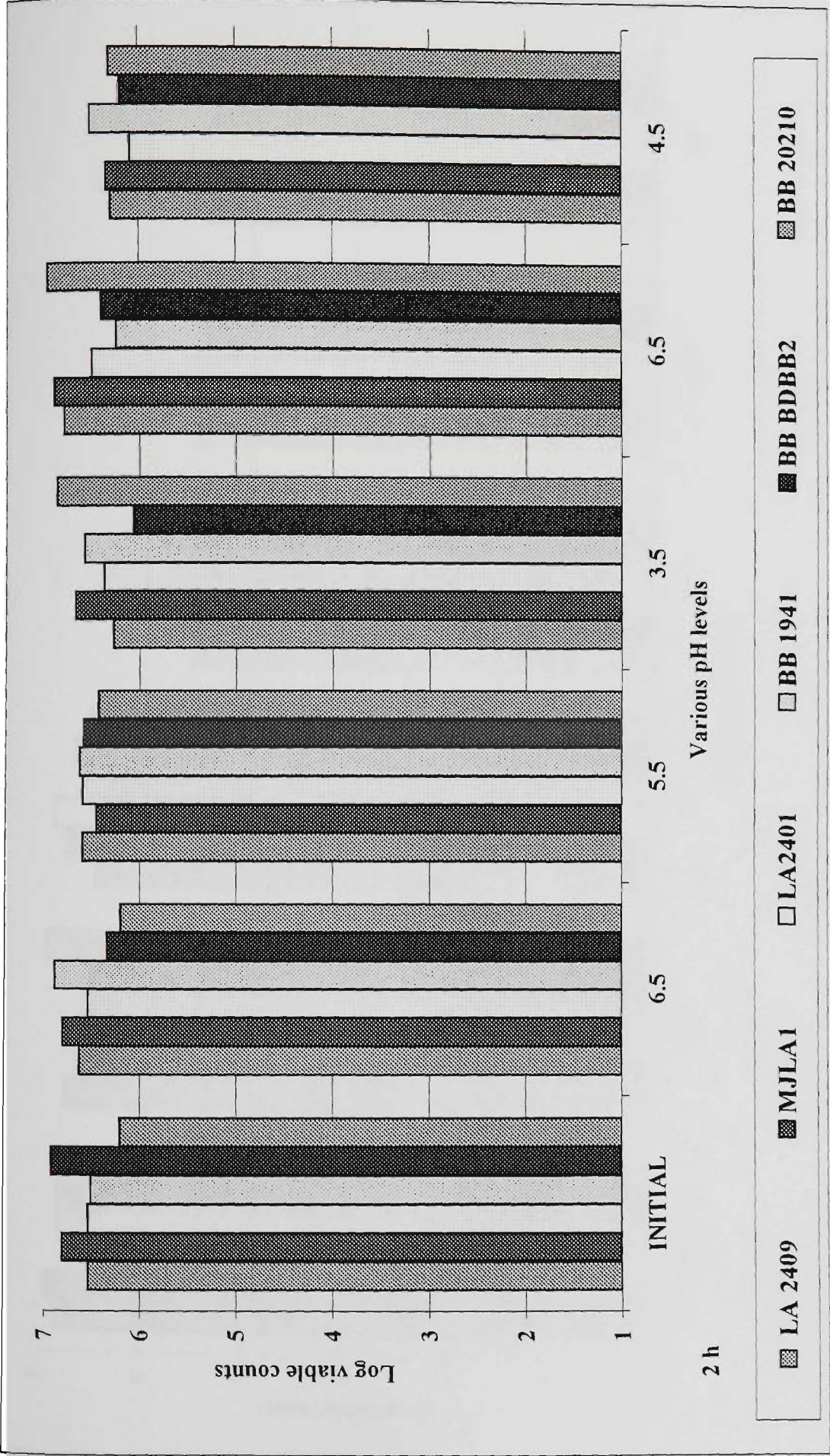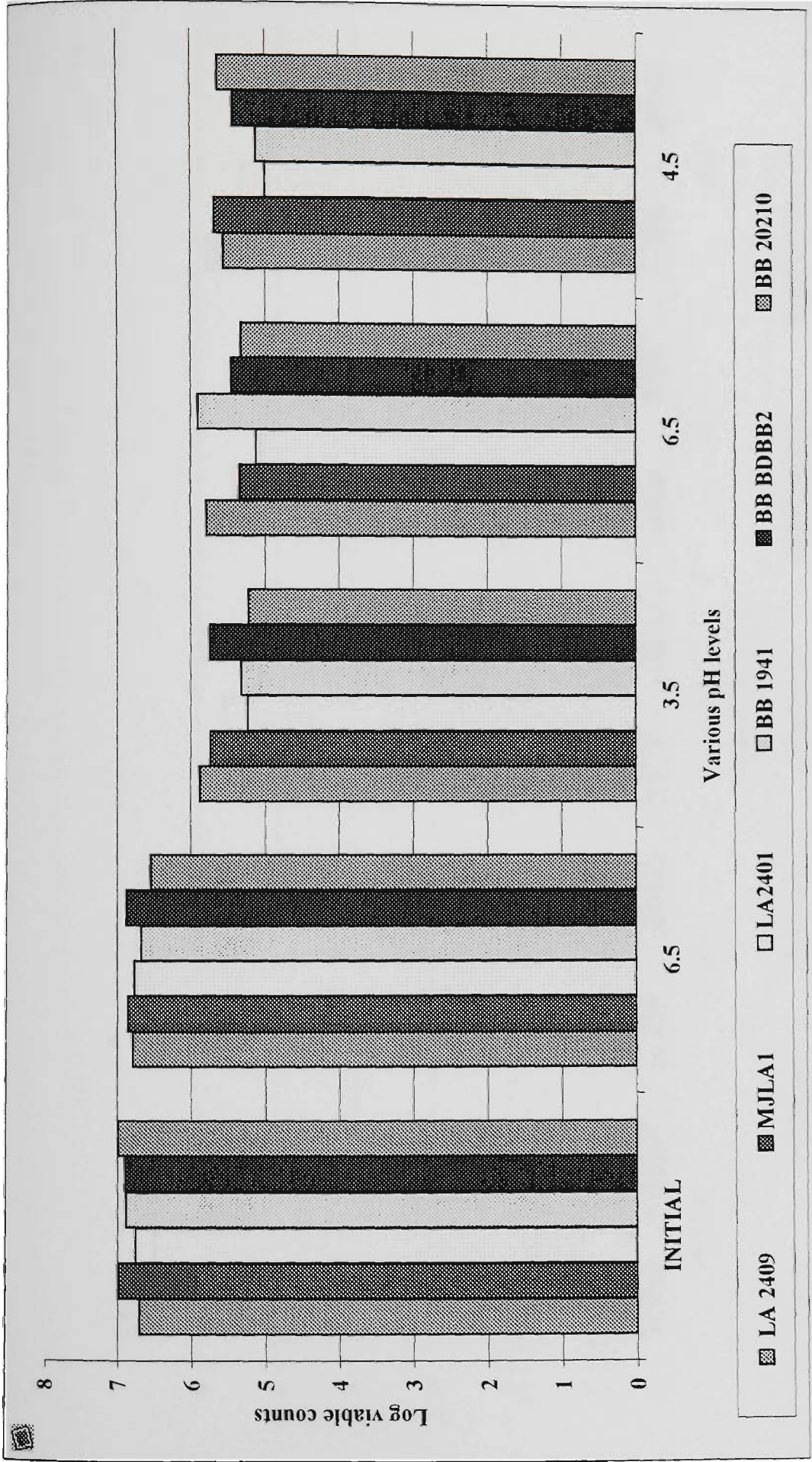
Figure 14.9 Effects of acid (3.5pH) and freezing at -18 deg C for 1 hour on the viability of probiotic bacteria
(The organisms were directly stressed at lethal pH without using sublethal pH)
(LA: *L. acidophilus*; BB: Bifidobacteria)
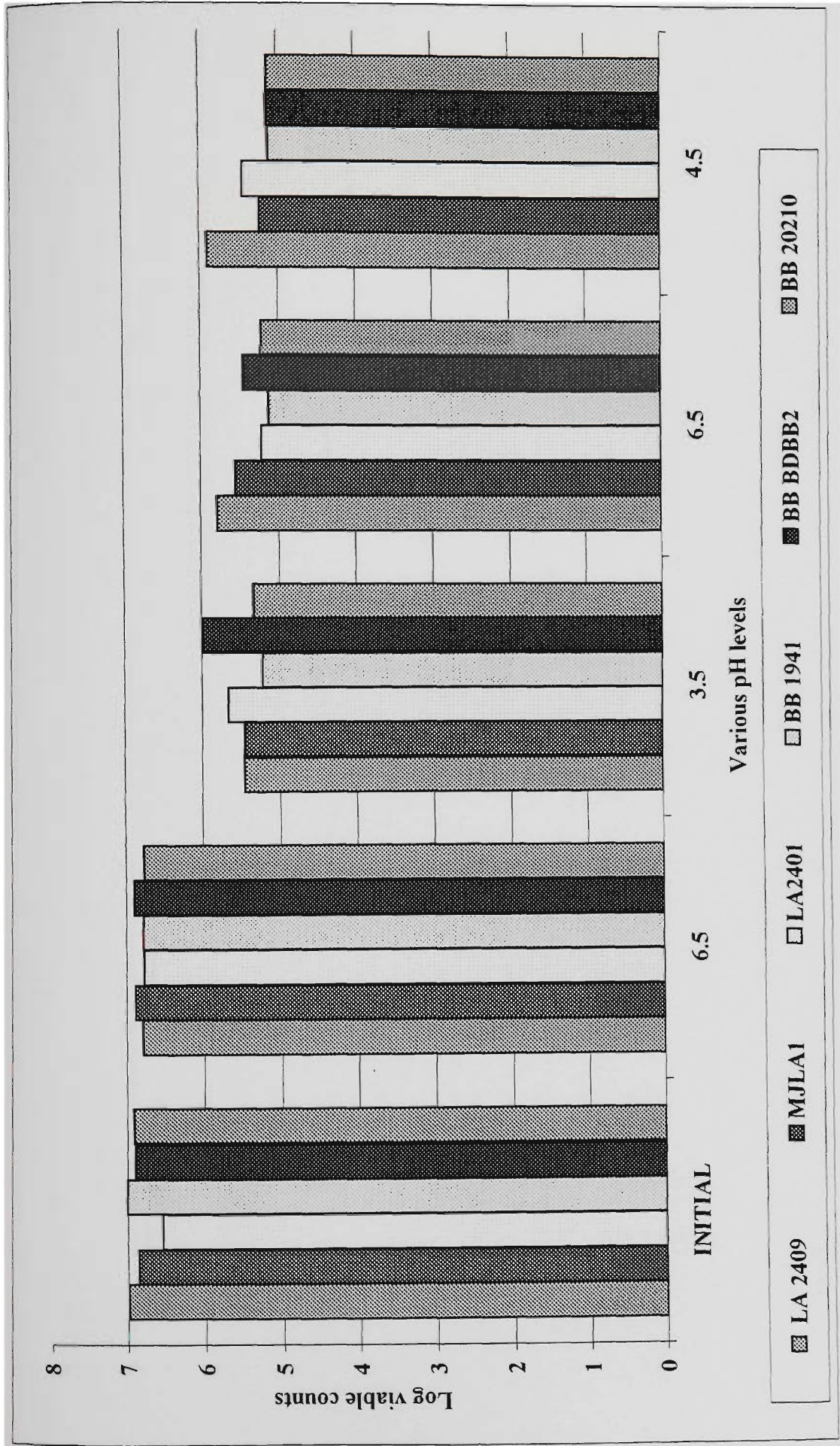
Figure 14.10 Effects of acid (3.5pH) and freezing at -18 deg C for 2 hour on the viability of probiotic bacteria
(The organisms were directly stressed at lethal pH without using sublethal pH)
(LA: *L. acidophilus*; BB: Bifidobacteria)
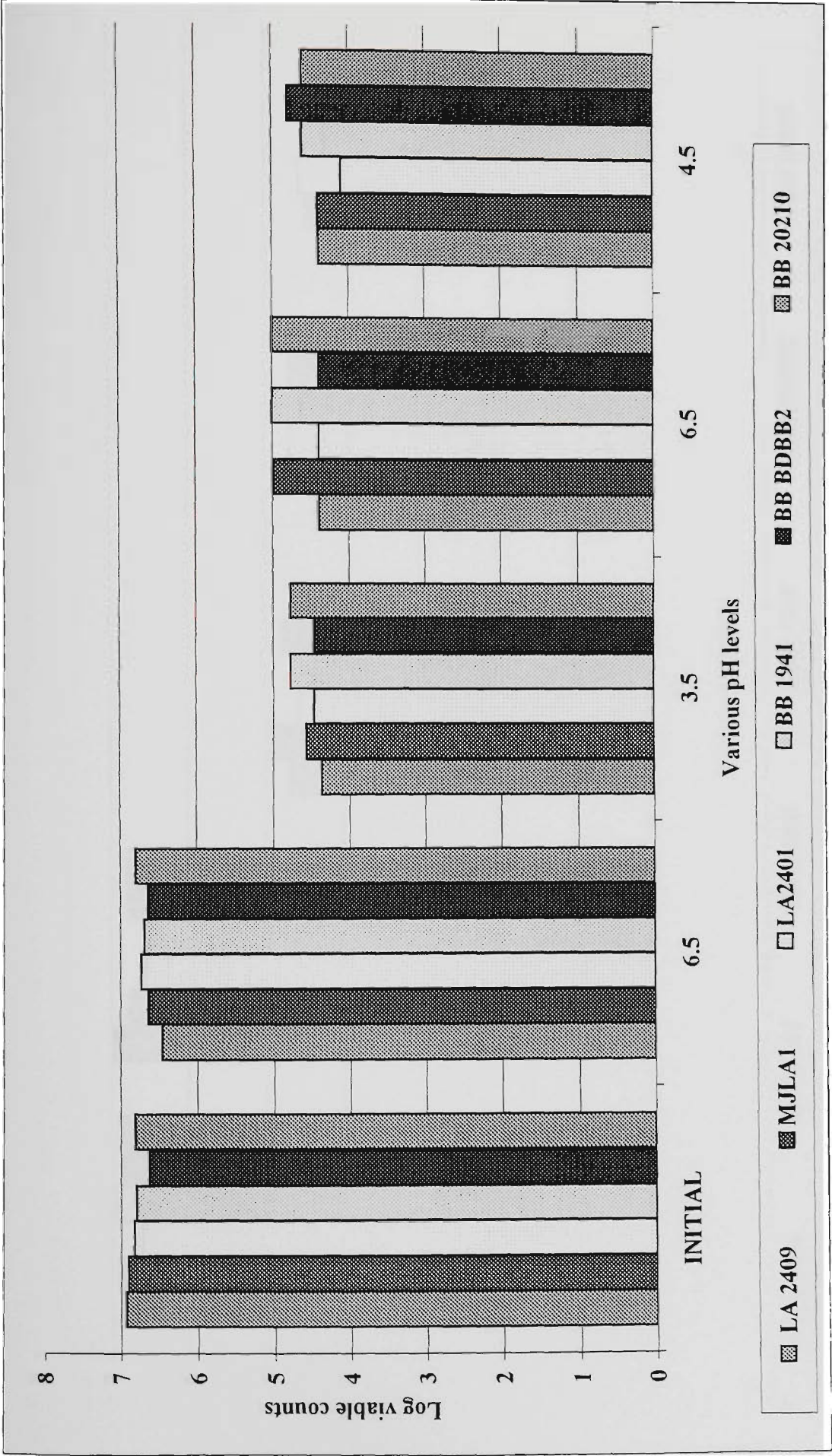
Figure 14.11 Effects of acid (3.5pH) and freezing at -18 deg C for 4 hour on the viability of probiotic bacteria
(The organisms were directly stressed at lethal pH without using sublethal pH)
(LA: *L. acidophillus*; BB: Bifidobacteria)
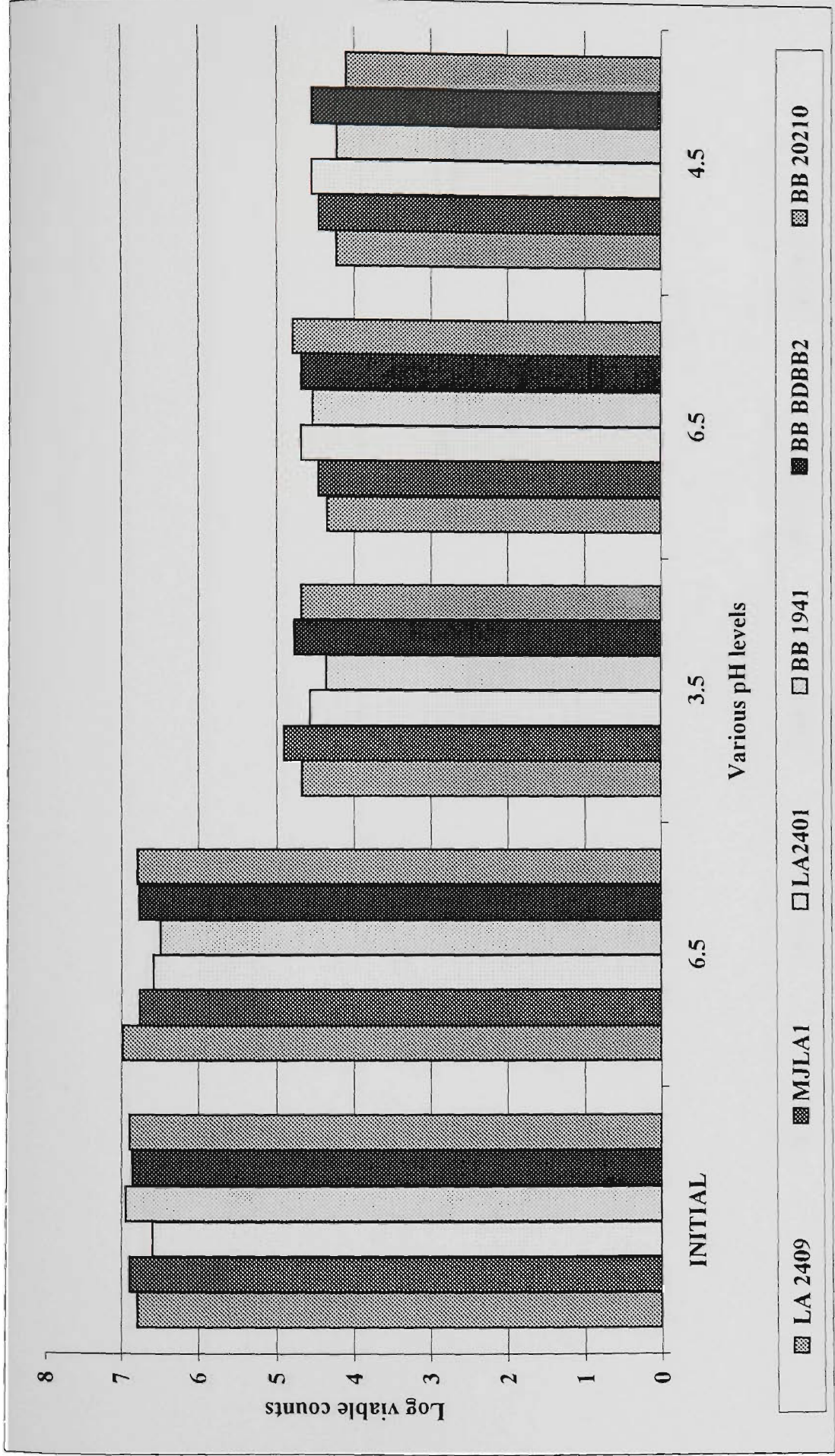
Figure 14.12 Effects of acid (3.5pH) and freezing at -18 deg C for 6 hour on the viability of probiotic bacteria
(The organisms were directly stressed at lethal pH without using sublethal pH)
(LA: *L. acidophilus*; BB: Bifidobacteria)
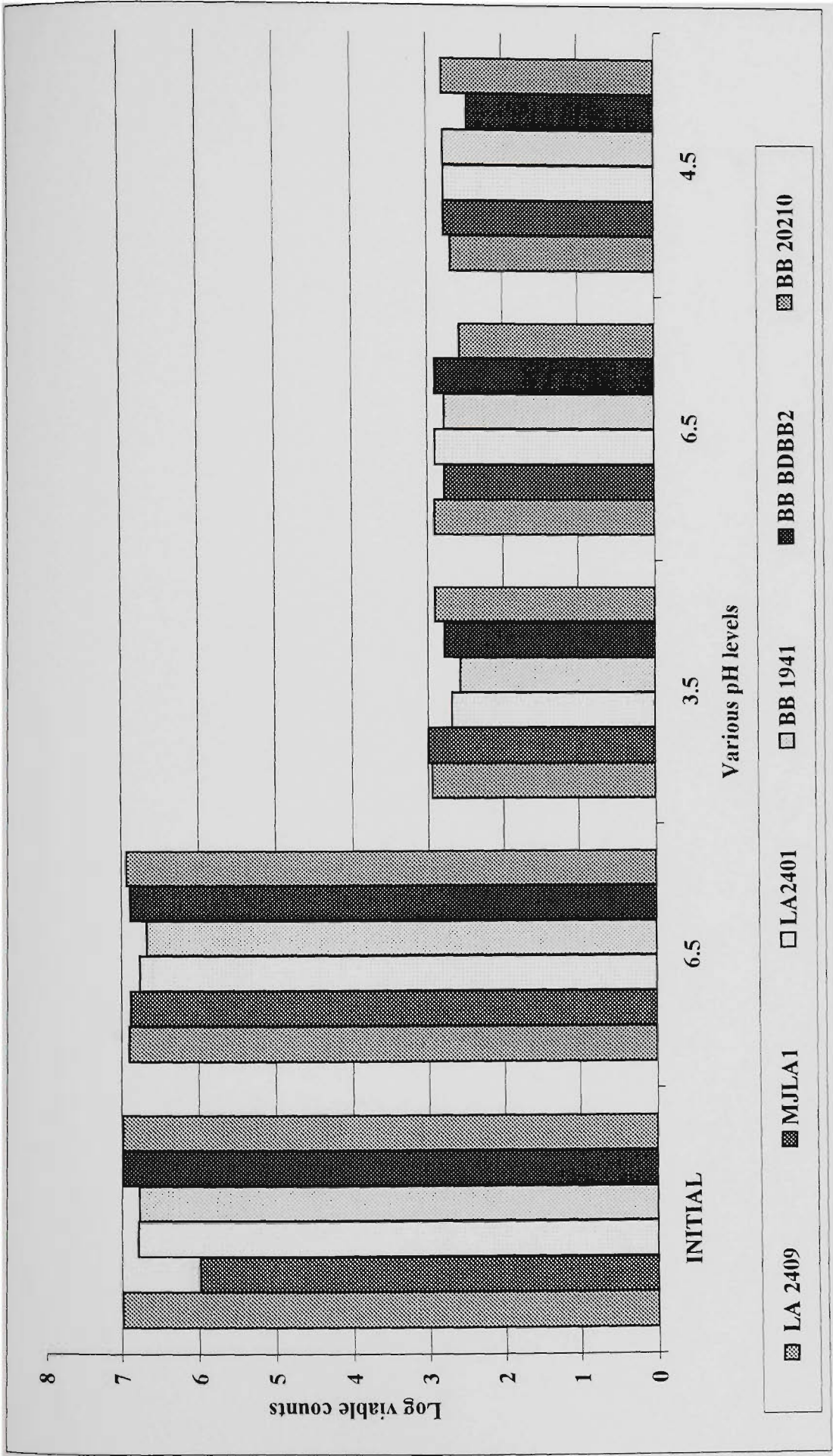
Figure 14.13 Effects of acid (3.5pH) and freezing at -18 deg C for 12 hour on the viability of probiotic bacteria (The organisms were directly stressed at lethal pH without using sublethal pH) (LA: *L. acidophilus*; BB: Bifidobacteria)
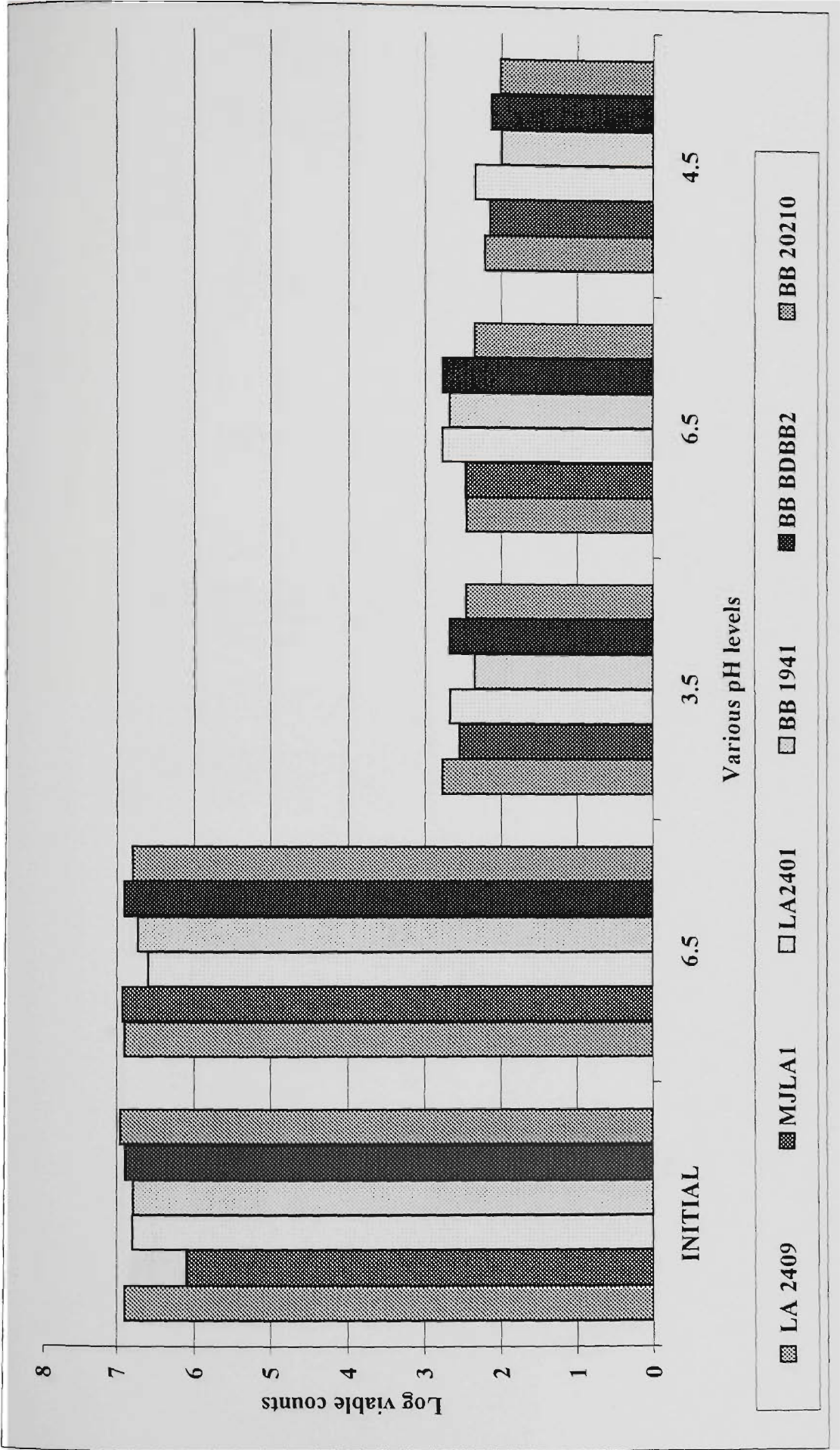
Figure 14.14 Effects of acid (3.5pH) and freezing at -18 deg C for 18 hour on the viability of probiotic bacteria (The organisms were directly stressed at lethal pH without using sublethal pH) (LA: *L. acidophilus*; BB: Bifidobacteria)
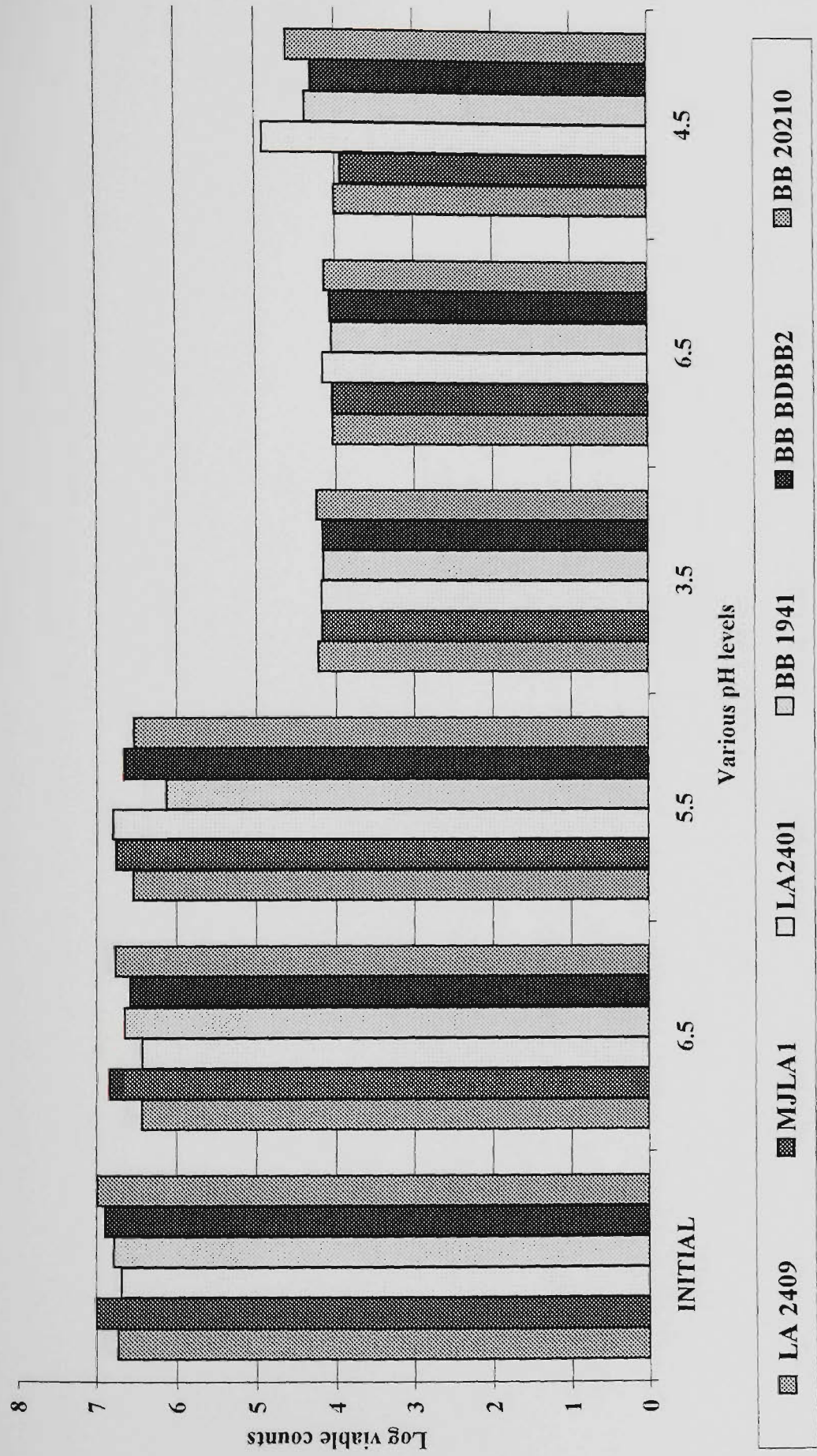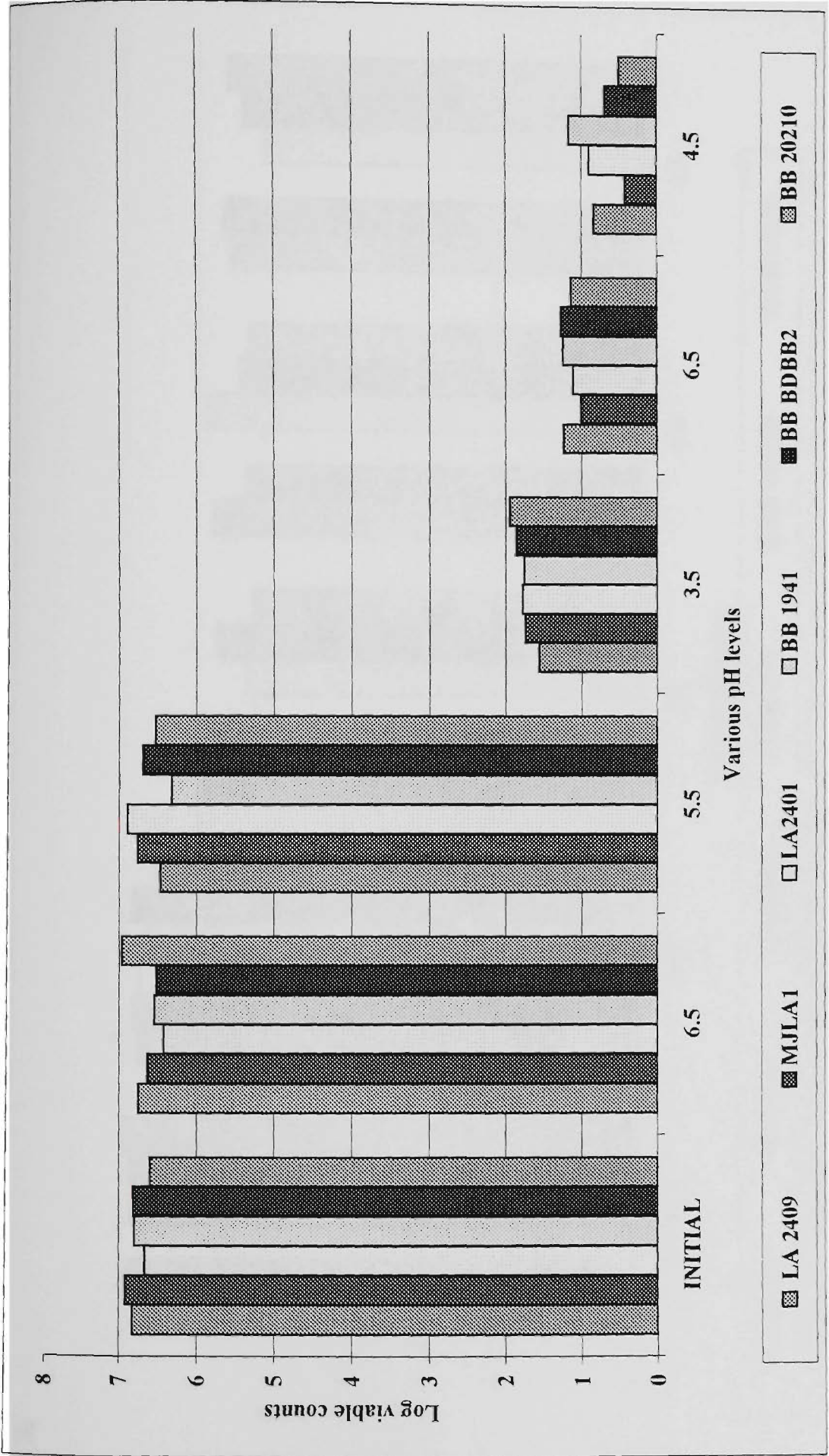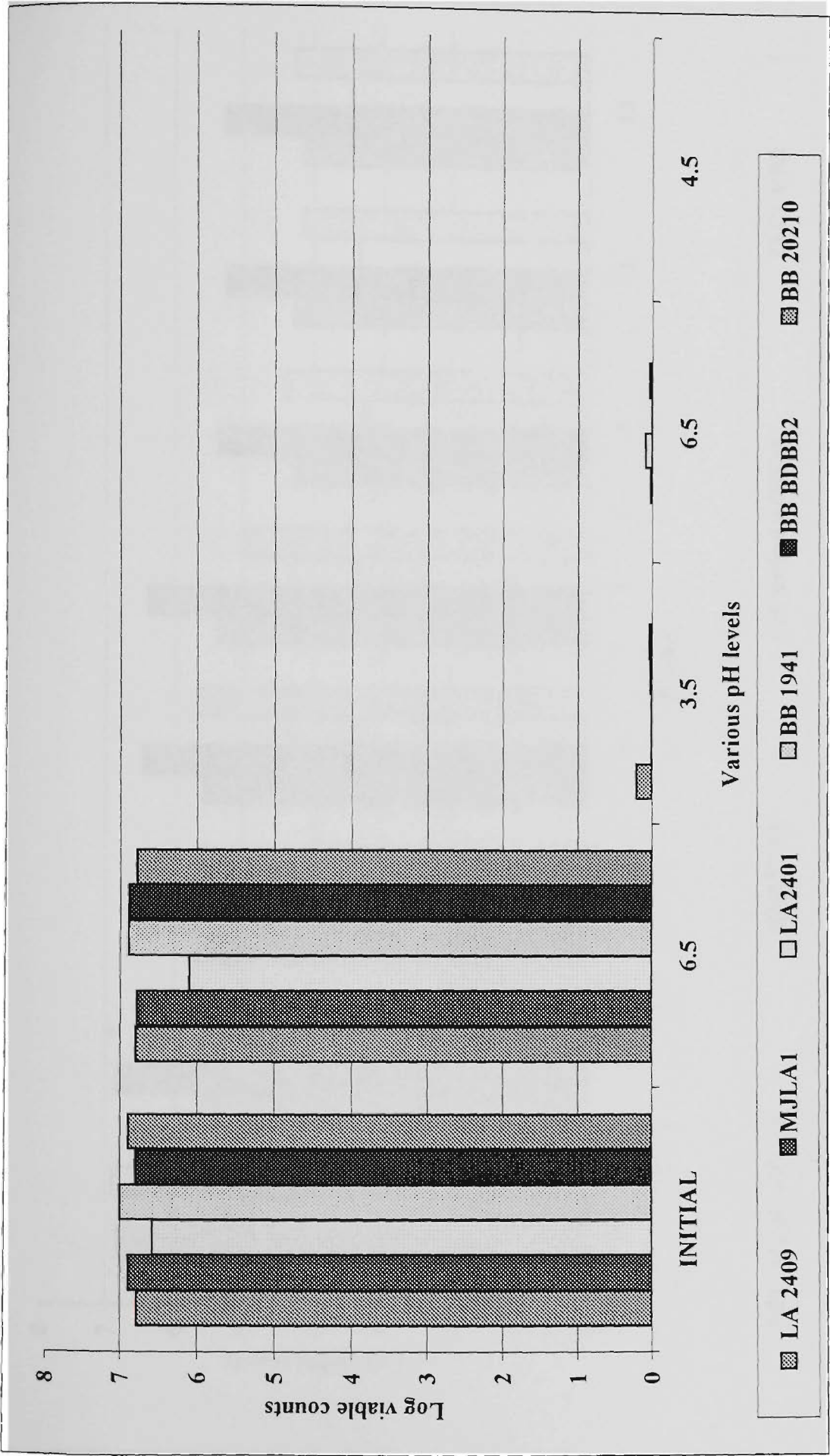
Figure 14.15 Effects of acid (3.5pH) and freezing at -18 deg C for 24 hour on the viability of probiotic bacteria (The organisms were directly stressed at lethal pH without using sublethal pH) (LA: *L. acidophilus*; BB: Bifidobacteria)
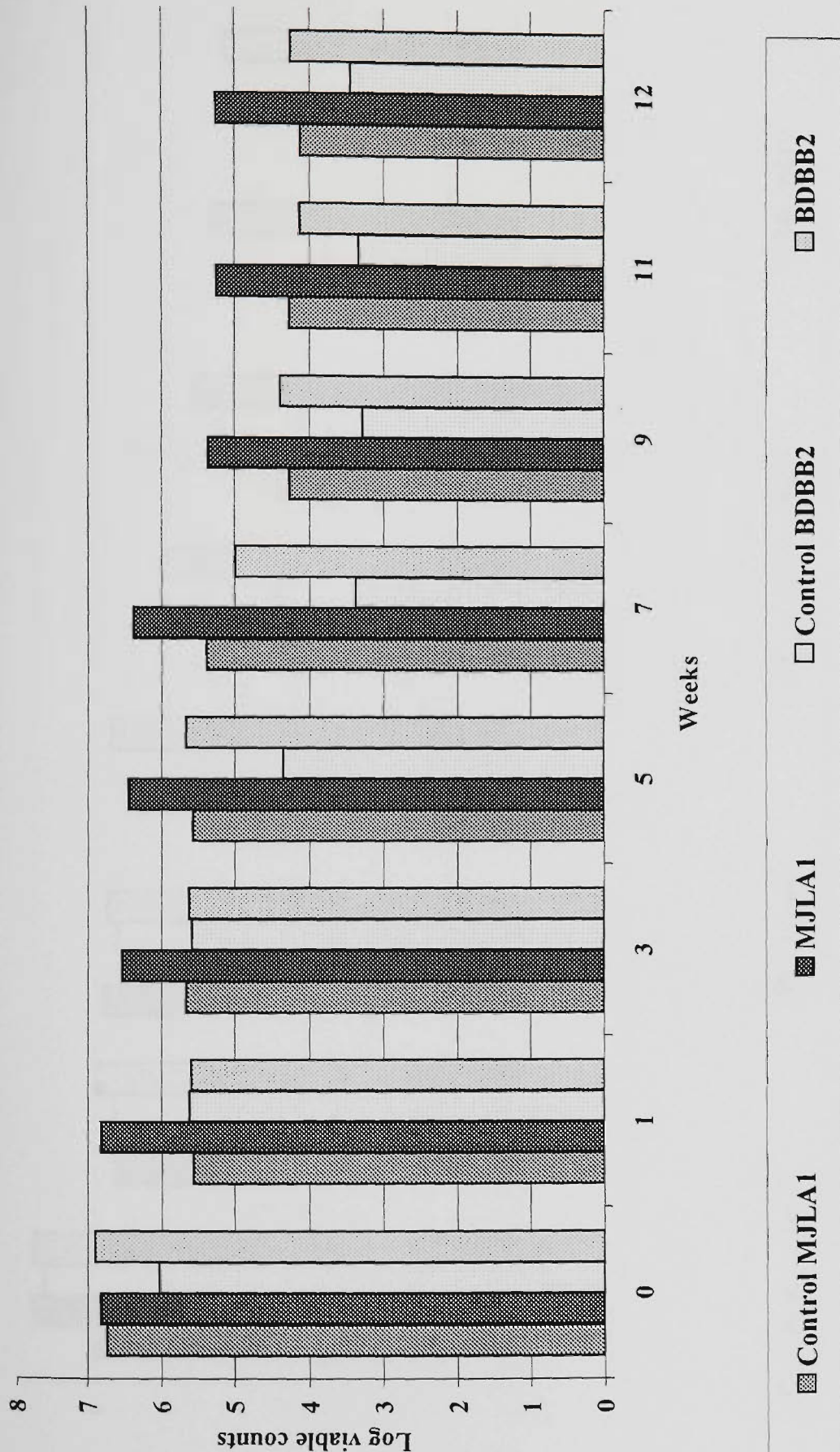
Figure 14.16 Viable couts of stressed probiotic bacteria incorporated into fermented dairy desserts monitored for 12 weeks staorage at -18deg.C.
(LA: Lactobacillus acidophilus; BB Bifidobacteria Spp).

Figure 14.17 Viable counts of stressed probiotic bacteria incorporated into fermented dairy desserts monitored for 12 weeks staorage at -18deg.C.
(LA: Lactobacillus acidophilus; BB Bifidobacteria Spp).
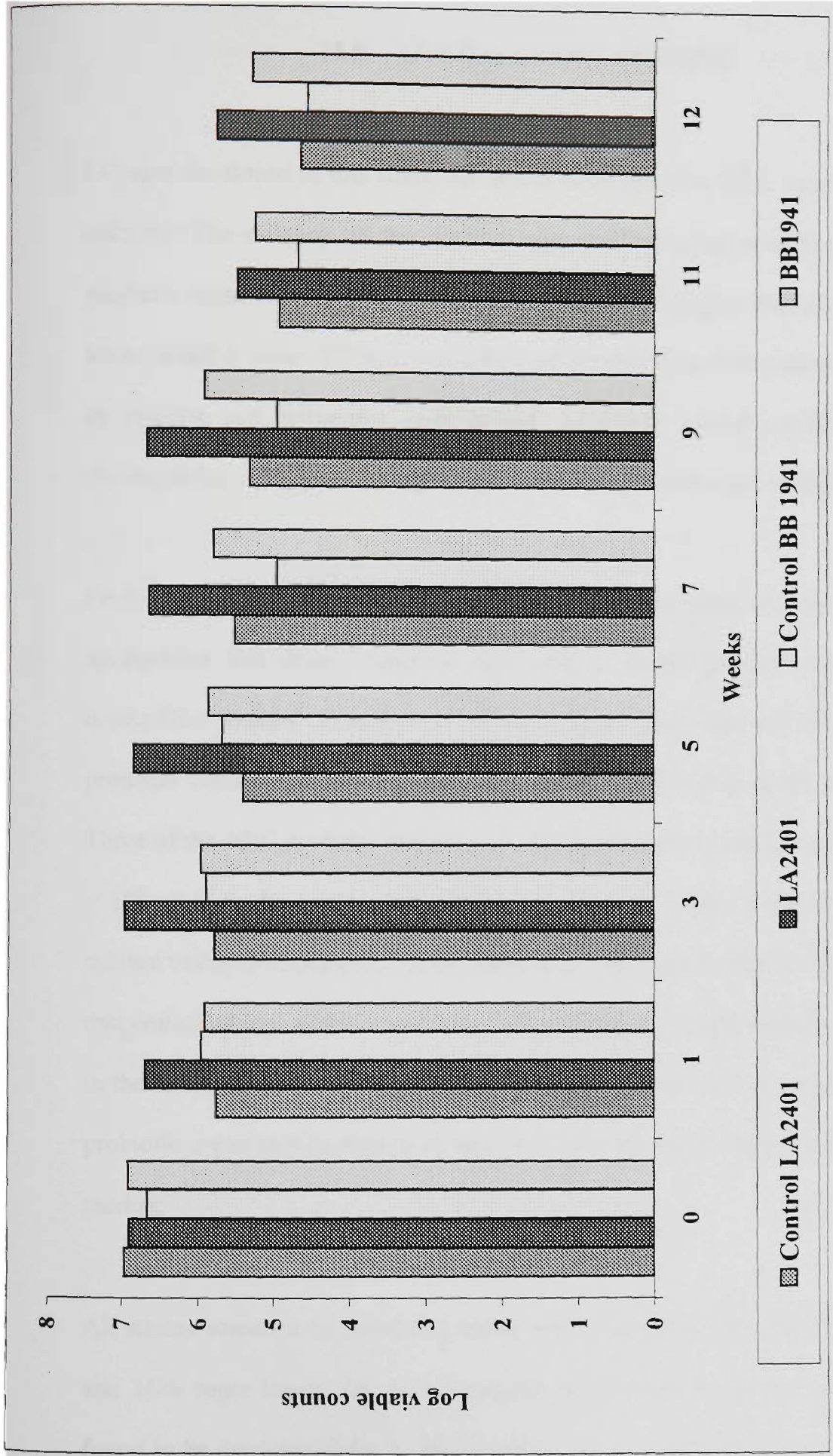
Figure 14.18 Viable couts of stressed probiotic bacteria incorporated into fermented dairy desserts monitored for 12 weeks staorage at -18deg.C.

(LA: Lactobacillus acidophilus; BB Bifidobacteria Spp).

15.0   OVERALL CONCLUSIONS

LC agar developed in this study was found to be selective for *L. casei* from pure cultures. The efficacy of the medium was verified using several commercial products containing single or mixed population of yogurt bacteria, probiotic bacteria and *L. casei*. LC agar could be used for selective enumeration of *L. casei* in yogurts and fermented milk drinks containing mixed population of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and bifidobacteria.

Five of the AB (*L. acidophilus* and *Bifidobacterium* spp.) and two ABC (*L. acidophilus* and *Bifidobacterium* spp. and *L. casei*) products contained *L. acidophilus* counts of above 10^6 cfu/g, whereas only one of AB and ABC products contained bifidobacteria counts of above 10^6 cfu/g at the expiry date. Three of the ABC products and only one LC product contained *L. casei* counts of $> 10^6$ cfu/g at the expiry date. To have therapeutic effects a minimum of one million viable cells per gram of the product has been recommended. The products that contained low viable counts may not provide any health benefits. Advances in the understanding of the problems associated with the viability and survival of probiotic organisms in fermented milk products are yet to reach the commercial market.

All strains screened for freeze tolerance remained viable after freezing and at 8 and 16% sugar levels. Of the various pH levels studied, pH 4.5 and 4.0 were found to be detrimental for *S. thermophilus* 2013, *L. delbrueckii ssp. bulgaricus*

2515 and *L. acidophilus* MJLA1, while rest of the organisms were not affected by pH.

Influence of water activity on fermentation of functional probiotic yogurt used in the manufacture of fermented frozen dairy desserts was studied. High levels of sugars (>12%) lead to longer fermentation time and poor survival of yogurt and probiotic bacteria.

The growth of *L. acidophilus* was most adversely affected at sugar concentrations of 16% followed by that of bifidobacteria. The counts of both yogurt bacteria were also affected at 12 and 16% sugar levels. Fermented frozen dairy desserts typically contain 16% sugar, thus it may not be feasible to achieve a satisfactory count of probiotic bacteria in such products if sugar is added prior to fermentation.

The percent organic acids present in the inoculated milk, after fermentation and during the storage period of 30 days was determined. Pyruvic acid, acetic acid, lactic acid, propionic acid, and hippuric acid were identified and quantified in inoculated yogurt mix, after fermentation and during storage. The levels of pyruvic acid after fermentation increased with the level of sugar, but decreased during storage.

In general, the level of acetic acid increased during fermentation. There was a slight increase in the level of acetic acid during storage. The decrease was inversely proportional to the level of sugar. The production of lactic acid followed a similar trend except that there was no production of lactic acid in inoculated but unfermented products.

Low levels of propionic acid were detected in inoculated but unfermented batches. The propionic acid level at 16% sugar level was lower than at 12%, but similar to the 4% and 8% sugar levels and higher than the zero sugar control. Low levels of hippuric acid were detected in inoculated milk not after fermentation or storage.

All strains screened for freeze tolerance remained viable after freezing and at 8 and 16% sugar levels. Of the various pH levels studied, pH 4.5 and 4.0 were found to be detrimental to *S. thermophilus* 2013, *L. delbrueckii* ssp. *bulgaricus* 2515 and *L. acidophilus* MJLA1, while rest of the organisms were not affected by pH.

Yogurt bacteria counts reduced by about 1 log cycle in fermented frozen dairy desserts made using a bench top freezer during 12 weeks storage. *L. acidophilus* 2415 were adversely affected and the counts declined by 6 log cycles within 8 weeks of storage at -18^0C. For the bifidobacteria strains 1912 decreased by 5 log cycles at the end of 12 weeks storage. *L. acidophilus* MJLA1 and bifidobacteria

BDBB2 counts reduced by about 2 log cycles in the products made on a pilot scale using a commercial freezer after 12 weeks storage.

Survival of yogurt and probiotic bacteria was studied in fermented frozen dairy desserts made from milk with or without supplementation with acid casein hydrolysate and cysteine. Samples were withdrawn at fortnightly intervals for 12 weeks to determine viable counts. Initial counts of the bacteria and their subsequent survival were better in the products supplemented due to nutrients such as peptides and amino acids and with acid casein hydrolysates or cysteine due to reduced redox potential. The β-D-galactosidase activity declined more rapidly in the control sample than in the samples supplemented with acid casein hydrolysate or cysteine. The results suggested that the acid casein hydrolysate and cysteine stimulated the growth of *L. acidophilus* and bifidobacteria, which resulted in improved viability of these organisms.

Survival of probiotic bacteria was studied for 12 weeks in fermented frozen dairy desserts made with or without encapsulated bacteria. The counts were better in the products made with encapsulated cells using a pilot scale and a continuous ice cream freezer. The results suggested that the use of encapsulation of the bacterial cells in fermented frozen dairy desserts protected the organisms against acid stress. The beads did not release the bacterial cell in the presence of acid but released in the presence of bile suggesting that the probiotic organisms can survive in acidic conditions such as found in the stomach and can be delivered into the intestine. Thus incorporating probiotic bacteria into fermented frozen

dairy desserts would be advantageous to provide improved health benefits, and a more marketable product.

16.0 FUTURE RESEARCH DIRECTIONS

Several types of dairy products are manufactured in Australia and fermented frozen dairy desserts are also gaining popularity in the market. The dairy manufacturers would benefit from longer shelf life of fermented frozen dairy desserts for exporting the products to overseas market. Due to an increase in shelf life and increased demand from local and overseas markets increasing number of dairy products manufacturers have started incorporating probiotic bacteria (*L. acidophilus* and bifidobacteria) into dairy products. The more recent trend is to incorporate *L. casei* in addition to *L. acidophilus* and bifidobacteria. At present, there are several brands of fermented frozen dairy desserts available in the super market with yogurt bacteria and/or probiotic bacteria. Different dairy product manufacturers use various strains of probiotic bacteria and due to complexity of these mixed bacteria, there is reason to standardize the species of bacteria which would provide health benefits. There is a need to incorporate prebiotics into the dairy products to increase the viability of probiotic bacteria, especially in the colon. A combination of probiotics and prebiotics will give a symbiotic product.

Our studies demonstrated that incorporation of micronutrients, encapsulation and adapting bacteria to low acid and low temperature conditions improve the viability of the bacteria. However there is a need for extended studies especially in acid and low temperature stress adaptations.

The techniques developed in the encapsulation process provide both the consumers as well as starter culture manufacturers a means of enhancing the

viability of probiotic bacteria by protecting them from acid and low temperature exposures. If this is done then consumer will be more ensured that they are taking the recommended levels of live bacteria for improving their health. Further, human studies can be undertaken in human volunteers to confirm *in vitro* results.

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