Viability and Therapeutic Properties of Probiotic Bacteria

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Abstract

Enumeration of viable probiotic bacteria (*Lactobacillus acidophilus* and bifidobacteria) in yogurt products obtained from 5 major manufacturers was carried out at 3-day interval over a 5-week period. The results showed that most products contained low numbers of *L. acidophilus* and bifidobacteria. The viable counts of probiotic bacteria were lower in the products which had pH ≤4.1. It was necessary to develop reliable methods for selective enumeration of probiotic bacteria as the available selective media were found to be too inhibitory against the target organisms or some media were not selective sufficiently in order for selective enumeration. Hence, several media were evaluated in order to develop media for selective enumeration of *L. acidophilus* and bifidobacteria. NNLP (nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate) agar, bile agar and galactose agar were selective for bifidobacteria. MRS-salicin agar was developed for selective enumeration of *L. acidophilus*. Nine strains of bifidobacteria and 6 strains of *L. acidophilus* were evaluated for their acid, bile and hydrogen peroxide tolerances. Based on this screening, 3 strains of bifidobacteria and 1 strain of *L. acidophilus* were selected as suitable strains for incorporation into yogurt as probiotic dietary adjuncts. The performance of the selected strains of probiotic bacteria (*L. acidophilus* 2409, *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099) was assessed in terms of their viability and sensory attributes of the products manufactured incorporating these strains as compared with commercially available probiotic starter cultures. The selected strains performed better than the commercial strains in terms of their survival in yogurt and the sensory properties of the products were similar to those manufactured using commercial strains of *L. acidophilus* and bifidobacteria.

The levels of α-galactosidase, β-galactosidase, and phospho-β-galactosidase in each strain of probiotic bacteria were determined and the role of β-galactosidase (β-gal) on growth and viability of probiotic bacteria was studied. It

was found that the growth and viability of *L. acidophilus* and bifidobacteria improved in the presence of β-gal enzyme. Freeze dried yogurt starter cultures were prepared using ruptured yogurt bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* 2515 and *Streptococcus thermophilus* 2010). The rupturing process released intracellular β-gal enzyme which hydrolysed lactose into glucose and galactose. The incubation time for making yogurt using ruptured yogurt bacteria was longer and the initial drop in pH was slow which allowed *L. acidophilus* and bifidobacteria to build up their numbers. In separate studies, the results have shown that growth and viability of probiotic bacteria could be improved by two step fermentation and by using neutralised yogurt mix. Both methods extended the incubation period thus allowing probiotic bacteria to increase their numbers.

The potential therapeutic effects of probiotic bacteria was also studied. Antimicrobial activity against several pathogens (*Salmonella typhimurium*, *Escherichia coli*, *Aeromonas hydrophila*, and *Candida albicans*) was studied. The results have shown that the antimicrobial activity of *L. acidophilus* and bifidobacteria against the pathogens were due to short chain organic acids such as lactic, acetic, propionic and pyruvic acids produced during fermentation but not due to bacteriocin activity. The levels and types of organic acids produced by probiotic bacteria were determined using HPLC. It was found that >90% of the acids produced by *L. acidophilus* strains was lactic acid. Bifidobacterial strains produced higher levels of acetic acid as compared with *L. acidophilus* strains.

Antimutagenic properties of *L. acidophilus* and bifidobacteria and of acids produced by these bacteria against 8 mutagens were studied. It was found that physical binding of mutagens and biochemical inactivation of the potency of mutagens by probiotic bacteria and their acids were responsible for their antimutagenic activity. Among the short chain organic acids, butyric acid seemed to have higher antimutagenic activity.

Ability of the selected strains of probiotic bacteria to adhere to the colonic epithelium cell lines was studied using Ht-29 colonic carcinoma cell line. The results have shown that the ability of probiotic bacteria to adhere to the colonic cells varied with strains of the former. Some strains lacked ability to adhere to the colonic cells. The substances involved in adherence were identified as extracellular proteins produced by probiotic bacteria and polysaccharides produced by intestinal cells. The molecular size of proteins involved in adherence was determined for each adhering strain of probiotic bacteria.

Effect of probiotic bacteria on the growth rate of Ht-29 carcinoma cells was also studied. The results have shown that the growth rate of the carcinoma cells reduced in the presence of probiotic bacteria, especially bifidobacteria. The results have also shown that the inhibitory effect on the growth of cancer cells was higher with the probiotic bacterial strains that produced butyric acid.

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List of Publications

Refereed papers

1. Shah, N.P., Lankaputhra, W.E.V., Britz, M.L. and Kyle, W.S.A. 1995. Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yogurt during refrigerated storage. *International Dairy Journal* 5:515-521

2. Lankaputhra, W.E.V. and Shah, N.P. 1995. Survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in the presence of acid and bile salts. *Cultured Dairy Products Journal* 30:2-7.

3. Lankaputhra, W.E.V. and Shah, N.P. 1996. A simple method for selective enumeration of *Lactobacillus acidophilus* in yoghurt supplemented with *L. acidophilus* and *Bifidobacterium* spp. *Milchwissenschaft* 51(8):446-451.

4. Lankaputhra, W.E.V., Shah, N.P. and Britz, M.L. 1996a. Evaluation of media for selective enumeration of *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Food Australia* 48:113-8.

5. Lankaputhra, W.E.V., Shah, N.P. and Britz, M.L. 1996b. Survival of bifidobacteria during refrigerated storage in the presence of acid and hydrogen peroxide. *Milchwissenschaft* 51:65-70.

6. Lankaputhra, W.E.V. and Shah, N.P. 1997a. Improving viability of *Lactobacillus acidophilus* and bifidobacteria in yogurt using two step fermentation and neutralised yogurt mix. *Food Australia* 49(8): 363-366.

7. Lankaputhra, W.E.V. and Shah, N.P. 1997b. Antimutagenic activity of *Lactobacillus acidophilus* and bifidobacteria and organic acids usually produced by these bacteria. *Mutation Research* (in press)

8. Shah, N.P. and Lankaputhra, W.E.V. 1997C. A new approach for improving viability of *Lactobacillus acidophilus* and bifidobacteria in yogurt. *International Dairy Journal* (in press).

9. Lankaputhra, W.E.V. 1997D. Adherence of probiotic bacteria to human colonic cells. *Applied and Environmental Microbiology* (under review).

Conference abstracts

1. Lankaputhra, W.E.V. and Shah, N.P. 1994. Investigation of factors affecting viability of *Lactobacillus acidophilus* and *Bifidobacterium spp* in yogurt. A paper presented at International Dairy Congress, Melboume, Australia (Poster No. Ha3P), 18-22 September, 1994.

2. Shah, N.P., Lankaputhra, W.E.V. and Britz, M. 1995. Selective enumeration of *Lactobacillus acidophilus* and *Bifidobacterium* spp. 9th World Food Congress, Budapest, Hungary, July 31-Aug.

3. Lankaputhra, W.E.V. and Shah, N.P. 1996. Improved viability of *L. acidophilus* and *Bifidobacterium* spp. 29th Annual Convention of AIFST, Conrad Jupiters, Gold Coast, May 5-8, 1996.

4. Lankaputhra, W.E.V. and Shah, N.P. 1997. Production of volatile acids by probiotic bacteria and their antimicrobial properties. 30th Annual Convention of AIFST, Perth, May 4-9, 1997.

5. Lankaputhra, W.E.V. and Shah, N.P. 1997. Use of intracellular β-galactosidase produced by yogurt bacteria in improving viability of probiotic bacteria in yogurt. 30th Annual Convention of AIFST, Perth, May 4-9, 1997.

List of Abbreviations

AFISC	Australian Food Industry Science Centre
AFTB	Aflatoxin-B
AMIQ	2-amino-1-methyl-6-phenyl-imidazo pyridine
BSA	Bovine serum albumin
CSCC	CSIRO culture collection
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
DSM	Deutsche Saamlung Von Mikroorganismen und Zelkulturen GmbH
FCS	Foetal calf serum
HBSS	Hank's balanced salt solution
MNNG	N-methyl, 4-Nitro N nitro, N-nitrosoguanidine
MRS	deMan, Rogosa and Sharpe
NAD	Nicotinamide adenine diphosphate
NDM	Nonfat dry milk
NF	Nitrofluorene
NGYC	Nonfat dry milk supplemented with 2% glucose, 1% yeast extract and 0.05% L-cysteine hydrochloride
NNLP	Neomycin sulphate, nalidixic acid, lithium chloride, paromomycin sulphate
NPD	4-nitro-O-phenylenediamine
NQO	4-nitroquinolin-N-oxide
ONPG	Ortho-nitrophenyl-β-D-galactopyranoside
PBS	Phosphate buffered saline
PNPG	Para-nitrophenyl-β-D-galactopyranoside
PONPG	Phospho-Ortho-nitrophenyl-β-D-galactopyranoside
RCA	Reconstituted Clostridial Agar
TA	Titratable acidity
TS	Total solids

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INTRODUCTION

In spite of the increasing popularity of probiotic yogurt containing *L. acidophilus* and bifidobacteria, there is a growing concern regarding the viability of these organisms in yogurt type products. However, several studies (Gilliland and Speck, 1977; Schioppa *et al.*, 1981; Hull *et al.*, 1984; Anon., 1992; Shah *et al.*, 1995) have shown low viability of these organisms. Inhibitory substances such as acid and hydrogen peroxide produced by yogurt bacteria (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) are claimed to be responsible for poor survival of *L. acidophilus* and bifidobacteria (Conway *et al.*, 1987; Playne, 1993; Shah and Jelen, 1990; Shah *et al.*, 1995; Lankaputhra and Shah, 1995; Lankaputhra *et al.*, 1996b).

Independant reports regarding the low viable counts of probiotic bacteria in commercial yogurts (Anon., 1992) and unavailability of sufficient scientific data regarding beneficial properties of probiotic bacteria prompted us to investigate the viability and therapeutic properties of probiotic bacteria.

There was 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 may be present in the product. Availability of reliable selective enumeration media is a prerequisite for estimating the viability of probiotic bacteria in the presence of yogurt bacteria. This prompted the development, assessment and validation of media for selective enumeration of probiotic bacteria.

Our preliminary studies (Shah *et al.*, 1995; Lankaputhra and Shah, 1994) justified the need for developing new approaches for improving the viability of probiotic bacteria in yogurt. Thus, two new methods were developed for improving the viability of probiotic bacteria in yogurt in this study.

Inclusion of live probiotic organisms in the diet is claimed to provide several therapeutic benefits to the consumers (Goldin *et al.*, 1980; Pochart *et al.*, 1992; Hawkins, 1993; Hammes *et al.*, 1991). It is assumed that probiotic microorganisms should be able to survive in the gastro-intestinal tract and colonise in the intestine in order to provide

beneficial effects to the host. It was necessary to screen probiotic bacteria for their ability to survive during yogurt production and storage, and in the acid and bile normally encountered in the gastrointestinal tract in order to provide therapeutic properties. Selected strains of probiotic bacteria were studied in order to determine their antimicrobial, and antimutagenic properties and their ability colonise in the intestine. The objectives of this research project were as follows:

1. To survey the viability status of probiotic bacteria in 5 major brands of yogurt produced in Australia and to develop methods for improving the viability of *L. acidophilus* and bifidobacteria in yogurt,

2. To assess, develop and validate media for selective enumeration of *L. acidophilus* and bifidobacteria in yogurt,

3. To screen strains of probiotic bacteria against inhibitory substances encountered during production and storage of yogurt, to select the most tolerant strains, and to assess the performance of selected strains of probiotic bacteria under laboratory and pilot scale experiments,

4. To determine the antimicrobial and antimutagenic properties of selected strains of probiotic bacteria and of organic acids usually produced by these bacteria, and

5. To determine the adherence levels of selected probiotic bacteria to colon cells and the major factors affecting adherence.

Chapter 1 contains the Literature Review regarding the general aspects of lactic acid bacteria and probiotic bacteria. Chapter 2 contains the Materials and Methods section. Chapters 3-7 contain experimental details of survival of probiotic bacteria in commercial yogurts (Chapter 3), selective enumeration of probiotic bacteria in yogurt (Chapter 4), survival of probiotic bacteria in the presence of acid, bile and hydrogen peroxide (Chapter 5), methods developed to improve the viability of probiotic bacteria in yogurt (Chapter 6), and the therapeutic properties of probiotic bacteria (Chapter 7). Chapter 8 contains Overall Conclusion of the study. Chapter 9 contains Future Research Directions and Chapter 10 contains List of References.

1.0. LITERATURE REVIEW

1.1. Characteristics of *Lactobacillus acidophilus* and bifidobacteria

Lactobacillus acidophilus and bifidobacteria are normal inhabitants of the intestine of humans and animals (Speck, 1978; Gilliland, 1979; Scardovi, 1986; Gilliland, 1989; Chitow and Trenev, 1990; Hammes and Tichaczeek, 1994). *L. acidophilus* and bifidobacteria belong to lactic acid bacteria (LAB), which are a group of Gram-positive, non sporing and non respiring organisms with rod, cocci, branched or amorphic morphology and common physiological and ecological characteristics (Finegold *et al.*, 1983; Simon and Gorbach, 1986; Fuller, 1989; Gorbach, 1990).

1.1.1. Historical background and growth characteristics

In the beginning of this century the term "lactic acid bacteria" was used synonymously with "milk souring organisms". When similar characteristics between milk souring bacteria and other lactic acid producing bacteria in various habitats were recognised, important progress in classification of these bacteria was made (Henneberg, 1904; Lohnis, 1907). Orla-Jensen (1919) used certain characteristics as the basis for classification of LAB such as cellular morphology, mode of sugar fermentation, growth temperature and the form of lactic acid (D or L) produced.

Tissier (1899) at the Pasteur Institute isolated a bacterium from the stools of infants with an unusual Y-shaped morphology. This was the first recorded observation of bifidobacteria, although at that time the researchers were not aware of the group or genus to which these bacteria would belong to. In 1900, Moro isolated a similar bacterium in faecal matter and reported this organism as belonging to a member of genus *Lactobacillus*. In 1967, De Vries and Stouthamer demonstrated the presence of fructose-6-phosphate phosphoketolase (F6PPK) and the absence of

aldolase and glucose-6-phosphate dehydrogenase in bifidobacteria. Aldolase and glucose-6-phosphate dehydrogenase were found in lactobacilli. Based on these findings, DeVries and Stouthamer (1967) concluded that classification of bifidobacteria in the genus *Lactobacillus* was not justified. However, Rasic and Kurmann (1983) and other researchers including Prevot (1955) preferred to have bifidobacteria within the genus *Lactobacillus*.

In most LAB, lactic acid is a major metabolic by-product produced as a result of carbohydrate metabolism. Some members of LAB group produce acetic and propionic acids in addition to lactic acid (Bisset and Anderson, 1974; Thomas *et al.*, 1980; Kandler, 1983; Kandler and Weise, 1986). The group of LAB can be homofermentative or heterofermentative based on their pattern of carbohydrate fermentation. LAB which produce lactic acid as a major by-product are referred to as homofermentative and those which produce CO_2, ethanol and acetic acid as major by-products in addition to lactic acid are referred to as heterofermentative.

1.1.2. Carbohydrate metabolism

Homofermentative LAB follow Embden-Meyerhof-Parnas (EMP) pathway for glycolysis. However, unlike in animal tissues LAB may either form D or L lactic acid or a racemic mixture of the two isomers. There are three major pathways associated with hexose metabolism in LAB (Wood and Holzapfel, 1995) as shown in Table 1.1. and Fig. 1.1.

Table 1.1. Major metabolic products of hexose metabolism through homo, hetero and bifidus fermentative pathways.

Homo fermentative pathway	$C_6H_{12}O_6$ ---------->	$CH_3.CHOH.COOH$
Hetero fermentative pathway	$C_6H_{12}O_6$ ---------->	$CH_3.CHOH.COOH + CO_2$
Bifidus fermentative pathway	$C_6H_{12}O_6$---------->	$CH_3.CHOH.COOH + CH_3COOH$

Aldolase plays a key role in EMP route of glycolytic homofermentation as compared with the phosphoketolase serving as key enzyme in the other two pathways. The 6-phosphogluconate pathway which yields carbon dioxide, lactate and ethanol is characteristic of some organisms exhibiting the heterolactic type fermentation. However, bifidobacteria utilise sugar via a different pathway known as 'bifidus pathway' (Wood and Holzapfel, 1995).

1.1.3. Hexose metabolism in Genus *Bifidobacterium* by Fructose-6-phosphate pathway (bifidus pathway)

As suggested by the name, 'bifidus pathway' is a major pathway of carbohydrate metabolism occurring in all bifidobacteria. This pathway is also known as 'fructose-6-phosphate shunt'. In the genus *Bifidobacterium*, hexoses are metabolised exclusively and specifically by the fructose-6-phosphate pathway (Scardovi and Trovatelli, 1965). Many authors use bifidus pathway as a marker for the genus *Bifidobacterium*.

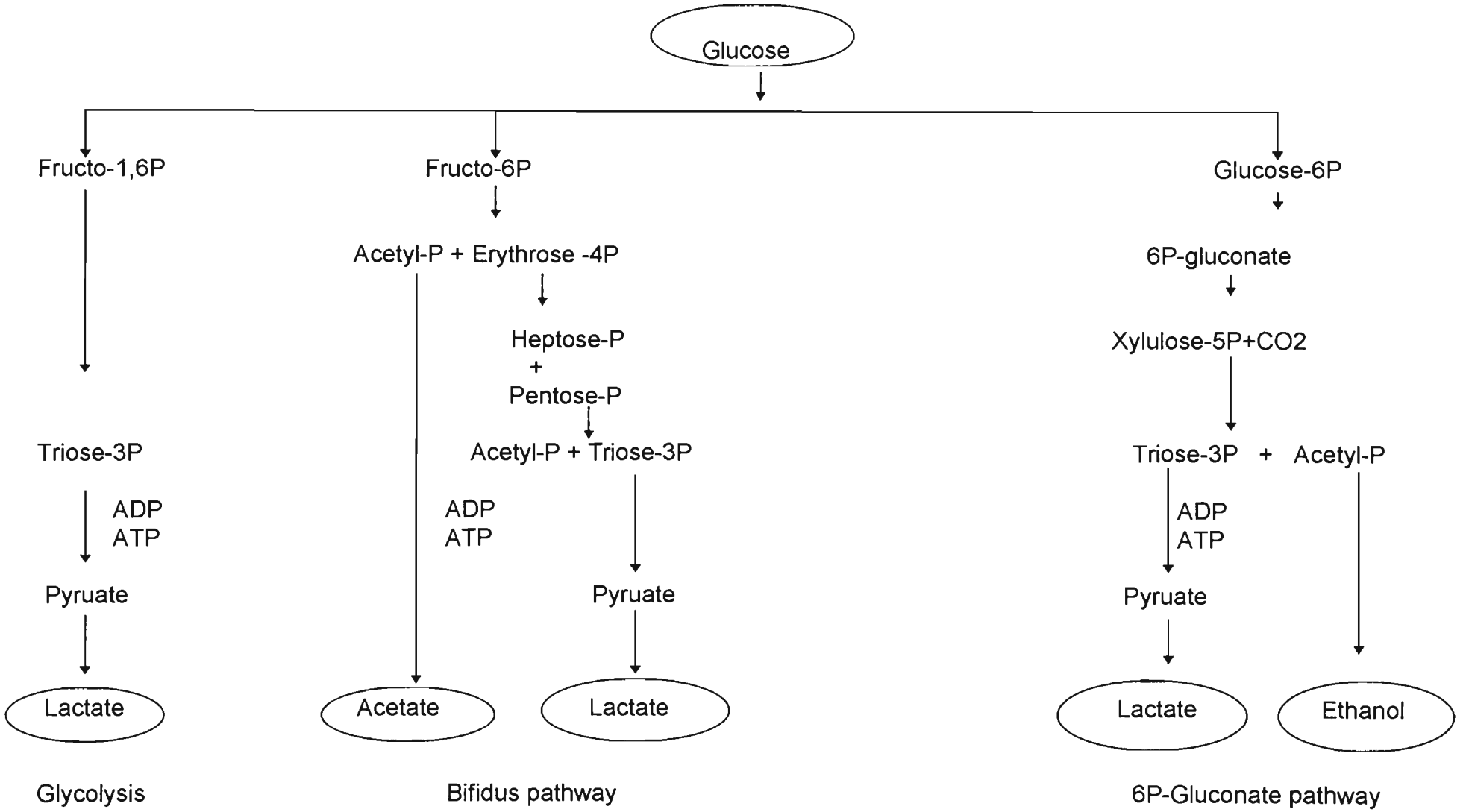

Fig. 1.1. Three pathways associated with hexose metabolism in LAB

The key enzyme for bifidus pathway (Fig. 1.1.) is F6PPK which hydrolyses hexose phosphate to erythrose-4-phosphate and acetyl phosphate. From tetrose and hexose phosphates through subsequent action of transaldolase and transketolase pentose phosphates are formed. The final fermentation products are formed by the action of transaldolase, transketolase, xylulose-5-phosphate phosphoketolase and enzymes belonging to EMP pathway (glyceraldehyde-3-phosphate dehydrogenase, pyruate kinase and lactate dehydrogenase) which act on glyceraldehyde -3- phosphate. In bifidus pathway, fermentation of two moles of glucose leads to three moles of acetate and two moles of lactate. Phosphoraclastic cleavage of pyruate to formic and acetic acids and the reduction of acetate to ethanol can often alter the fermentation balance of end products to a great extent.

Although F6PPK is found in bifidobacteria, aldolase and glucose-6-phosphate dehydrogenase are not present in these oerganisms (DeVries and Stouthhamer, 1967). Absence of F6PPK in other Gram positive anaerobic bacteria such as *Lactobacillus, Arthrobacter, Propionobacterium, Corynebacterium* and *Actinomycetaceae,* which could be morphologically confused with bifidobacteria, is important for identification of the members of the genus *Bifidobacterium.*

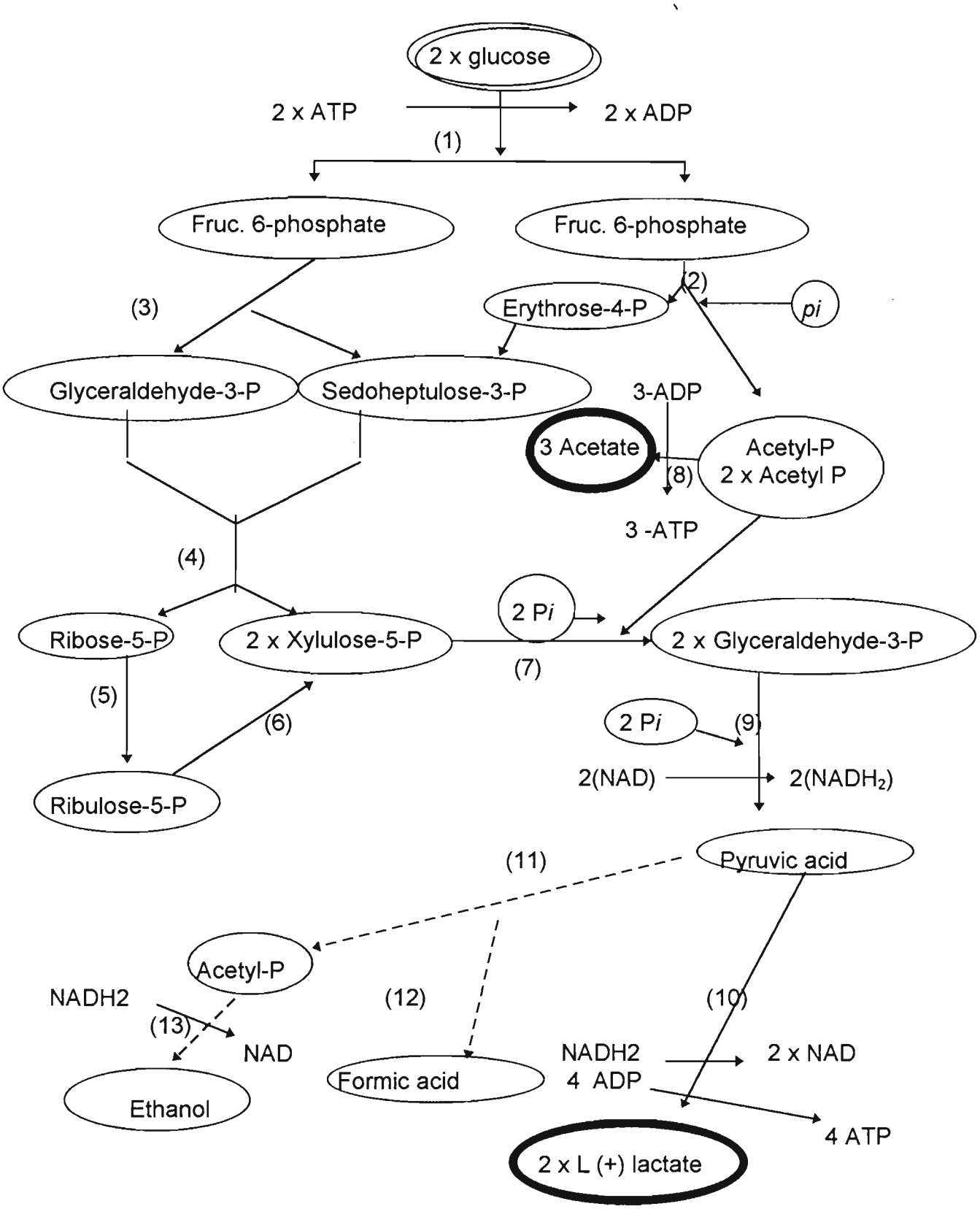

Fig.1.2. The bifidus pathway (fructose-6-phosphate shunt). Refer to Table 1.2. for the enzymes involved.

Table 1.2. Enzymes of the fructose-6-phosphate shunt (reference Fig 1.2.)

Reaction	Enzyme
1	hexokinase and glucose -6-phosphate isomerase
2	fructose-6-phosphate phosphoketolase
3	transaldolase
4	transketolase
5	ribose-5-phosphate isomerase
6	ribulose-5-phosphate epimerase
7	xylulose-5-phosphatecetolase
8	acetate kinase
9	glyceraldehyde -3 - phosphate dehydrogenase, pyruate kinase and lactate dehydrogenase
10	L (+) lactate dehydrogenase
11	phosphoroclastic enzyme
12	formate dehydrogenase
13	alcohol dehydrogenase

1.2. Taxonomic diversity

1.2.1. Lactic acid bacteria in general

Recent taxonomic revisions suggest that LAB group could be comprised of genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus*, and *Vagococcus* (Moss, 1981; Collins *et al.*, 1987; Stackebrandt and Teuber, 1988; Collins *et al.*, 1989, 1990). Originally, bifidobacteria were included in genus *Lactobacillus* and the organism was referred to as *Lactobacillus bifidus* (Breed *et al.*, 1957; Denhert, 1957). Although the classification of LAB into different genera is mainly based on the characteristics used by Orla-Jensen (1919) including morphology and mode of sugar fermentation, for some of the newly described genera of LAB, additional characteristics such as fatty acid composition and motility are used as the basis of classification.

The measurement of true phylogenetic relationships with rRNA sequencing has been used to classify some members of LAB. Most genera in the group of LAB form phylogenetically distinct groups. However, some genera such as *Lactobacillus* and *Leuconostoc* are heterogeneous and phylogenetic grouping does not correlate with the current classification based on phenotypic characters (Garvie, 1986a, b; Yang and Woese, 1989; Fox *et al.*, 1990; Olson, 1990).

Although most authors prefer to include the genus *Bifidobacterium* under the group of LAB, molecular percentage values of G + C contents of DNA show that all members of genus *Bifidobacterium* contain >50 mol percent G + C in DNA. Other LAB contain <50 mol percent G + C in DNA. Based on the mol percent G+C contents, all lactic acid producers have been allocated into two branches called clostridium and actinomycetes. All members of bifidobacteria fall within the actinomycetes branch (Table 1.3.).

L. acidophilus is rod shaped whereas bifidobacteria show variable morphology characterised by branching and pleomorphism. Both organisms are Gram positive. *L. acidophilus* is microaerophilic and bifidobacteria prefer anaerobic conditions for their growth (Sharpe, 1979; Yang and Woese, 1989).

Table 1.3. Phylogenetic relationship of lactic acid bacteria based on the mol percent of G + C content in DNA.

Mol percent of G + C content in DNA	Branch
<50	**Clostridium**
	Lactobacillus
	Lactococcus
	Enterococcus
	Leuconostoc
	Pediococcus
	Streptococcus
	Staphylococcus aureus
	Bacillus subtilis
>50	**Actinomycete**
	Bifidobacterium
	Propionibacterium
	Microbacterium
	Corynebacterium
	Brevibacterium
	Atophobium

Adopted from Salminen and Wright (1993).

1.2.2. Genus *Lactobacillus*

Lactobacilli are found in association with substrates rich in various carbohydrates, and thus, in a variety of habitats such as mucosal membranes of humans and animals, mainly in oral cavity, intestine and vagina, or on plant material and fermenting food (Hammes *et al.*, 1991; Pot *et al.*, 1994). Lactobacilli are strictly fermentative, aero-tolerant to anaerobic, aciduric or acidophilic and have complex nutritional requirements. Lactobacilli do not synthesise porphyroids and therefore are devoid of heme dependent activities. However, some strains of lactobacilli can use porphyroids from the environment and exhibit activities of catalase, nitrite reduction or even cytochromes.

With glucose as a carbon source, lactobacilli could be either homofermentative or heterofermentative. When homofermentative, they could produce more than 85% lactic acid and the heterofermentative strains produce lactic acid, carbon dioxide, ethanol or acetic acid in equimolar quantities. In the presence of oxygen or other oxidants increased amounts of acetate may be produced at the expense of lactate or ethanol.

A total of 56 species of lactobacilli (Table 1.4.) have been divided into 3 fundamental groups, A, B and C and could be briefly described as follows;

(1) Group A: Obligately homofermentative and hexoses are almost exclusively fermented to lactic acid by EMP pathway. The members possess fructose 1,6-biphosphate-aldolase but lack phosphoketolase, and therefore, neither gluconate nor pentose is fermented.

(2) Group B: Facultatively heterofermentative and hexoses are almost exclusively fermented to lactic acid by EMP pathway. The organisms possess both aldolase and phosphoketolase and therefore, they can ferment hexoses, pentoses and gluconates. In the presence of glucose, the enzymes of the phosphogluconate pathway are repressed.

(3) Group C: Obligately heterofermentative lactobacilli. Hexoses are fermented by the phosphogluconate pathway yielding lactate, ethanol (or acetic acid) and CO_2 in equimolar quantities.

L. acidophilus come under Group A and is physiologically related to *Lactobacillus delbrueckii*. The cluster of *L. acidophilus* species have very similar physiological properties. However, they seemed quite heterogeneous in DNA-DNA hybridisation studies. Based on the heterogeneous nature of *L. acidophilus* strains, they were divided into two main genotypic subgroups referred to as A and B which shared less than 25% DNA-DNA homology whereas strains within each subgroup shared a similarity of 75-100%. Recent studies on the systematics of *L.*

acidophilus employing electrophoresis of soluble proteins or lactate dehydrogenase and DNA-DNA reassociation indicated that *L. acidophilus* strains include six genomospecies. This finding was confirmed by the results of highly standardised SDS-PAGE of whole cell proteins (Pot *et al.*, 1994) and rRNA targeted oligonucleotide probes.

Table 1.4. 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. plantarum*
7	*L. gaserri*	35	*L. brevis*
8	*L. helveticus*	36	*L. buchneri*
9	*L. gensenii*	37	*L. collinoides*
10	*L. johnsonii*	38	*L. fermentum*
11	*L. kefiranofaciens*	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*

Adapted from Wood and Holzapfel (1995).

These new techniques allow clear differentiation between *L. acidophilus* strains of the six subgroups; *L. acidophilus, L. crispatus, L. amylovorus, L. gallinarum, L. gasseri* and *L. johnsonii*. The former four subgroups (*L. acidophilus,*

L. crispatus, L. amylovorus and *L. gallinarum*) and the latter two subgroups (*L. gasseri* and *L. johnsonii*) were placed under subgroups "A" and "B", respectively.

1.2.3. Genus *Bifidobacterium*

After bifidobacteria was first discovered in the beginning of the century (Table 1.5.), a number of more important discoveries were made after the advent of chemotaxonomy during 1960s. Sebald *et al.* (1965) showed that the percentage of G + C in the DNA of bifidobacteria differed from that of *Lactobacillus, Corynebacterium* and *Propionibacterium.*

In 1974, the 8th edition of Bergy's Manual of Determinative Bacteriology recognised *Bifidobacterium* as genus in its own right consisting of 11 species (Buchnan and Gibbons, 1974). Scardovi (1986) included 24 species in the genus *Bifidobacterium.* These 24 species were grouped according to their ecological origin and 15 strains were isolated from animals and the rest of the 9 species were found in the natural cavities and surfaces of humans. According to the latest collection of species in the genus of *Bifidobacterium*, additional five species have been described (Table 1.6.) making the total number of species as twenty nine (Biavati and Mattarelli, 1991; Biavati *et al.*, 1991).

Table 1.5. 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. 1-4	1920
5	*Bifidobacterium bifidum*	Holland	1924
6	*Bacterium bifidum*	Orla-Jensen	1927
7	*Tisseria bifida*	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 eds. 5-7.	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	Denhert	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 (Bergy's Manual ed. 8)	1974
24	Inclusion of 24 species to genus *Bifidobacterium*	Scardovi (Bergy's Manual ed. 9)	1986
25	Inclusion of 29 species to genus *Bifidobacterium*	Sgorbati *et al.* (Genera of Lactic acid bacteria)	1995

Adapted from Sgorbati *et al.* (1995)

Table 1.6. Species of genus *Bifidobacterium* and their mol percent G + C contents

	Species	percent G + C
1.	*B. bifidum*	60.8
2	*B. longum*	60.8
3.	*B. infantis*	60.5
4.	*B. breve*	58.4
5.	*B. adolescentis*	58.9
6.	*B. angulatum*	59.0
7.	*B. catenulatum*	54.0
8.	*B. pseudocatenulatum*	57.5
9.	*B. dentium*	61.2
10.	*B. globosum*	63.8
11.	*B. pseudolongum*	59.5
12.	*B. cuniculi*	64.1
13.	*B. choerinum*	66.3
14.	*B. animalis*	60.0
15.	*B. thermophilum*	60.0
16.	*B. boum*	60.0
17.	*B. magnum*	60.0
18.	*B. pullorum*	67.5
19.	*B. gallinarum*	65.7
20.	*B. suis*	62.0
21	*B. minimum*	61.6
22.	*B. subtile*	61.5
23.	*B. coryneformes*	--
24.	*B. asteroides*	59.0
25.	*B. indicum*	60.0
26.	*B. gallicum*	61.0
27.	*B. ruminatium*	57.0
28.	*B. mericicum*	59.0
29.	*B. saeculare*	63.0

Adapted from Sgorbati *et al.* (1995).

All members of genus *Bifidobacterium* show a bacillar form. Some strains develop ramifications giving V, Y, X or other shapes. However, their polymorphism depends mainly on culture medium and the growth conditions. The levels of N-acetylglucosamine, which is involved in the synthesis of peptidoglycan, a component of the cell wall, affect the branching of bifidobacteria. While lower levels of N-glucosamine and amino acids produce more highly branched shapes, rich and favourable growth conditions produce longer and bacillus-form morphology.

Generally, bifidobacteria are considered to be strict anaerobes. However, their ability to tolerate and survive in the presence of oxygen depend on the species or strain and the composition of the culture medium. Upon exposure to aerobic conditions from anaerobic environment, various species of bifidobacteria can produce three different types of responses as follows:

(i) Aerobic growth without hydrogen peroxide accumulation. Some strains form minute quantities of hydrogen peroxide by NADH oxidation. However, hydrogen peroxide may not be present in the growth medium due to the activity of an unknown peroxidase system which could destroy hydrogen peroxide.

(ii) Limited aerobic growth with the accumulation of H_2O_2. Accumulation of H_2O_2 could kill the cells as it is inhibitory to the key enzyme F6PPK. Species without a peroxidase system could soon die as H_2O_2 starts accumulating in the cells.

(iii) No growth without accumulation of H_2O_2 in the presence of O_2 (Fig. 1.3). Such strains always require a strict anaerobic condition and low redox potential for growth and fermentation.

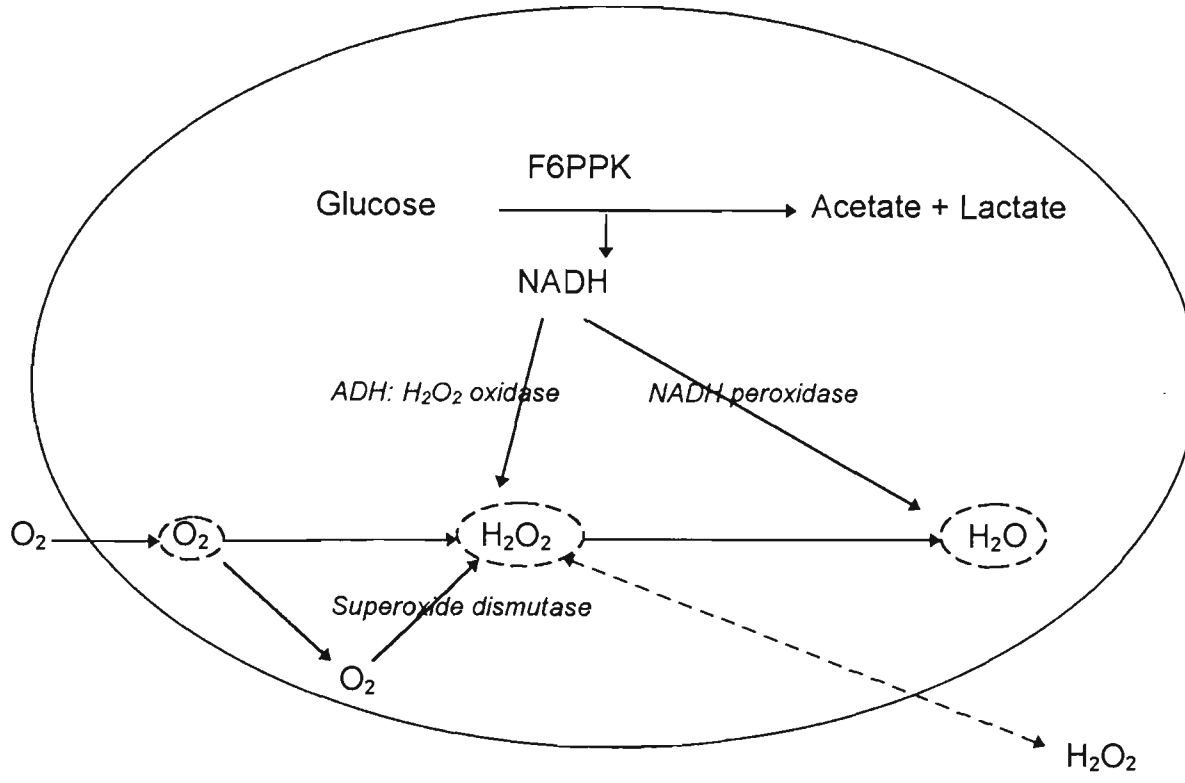

Fig. 1.3. Oxygen dissimilation in bifidobacteria (adapted from Salminen and Wright, 1993)

Optimum growth temperature of the species of human origin is around 37±1°C and that of animal origin is around 42±1°C. Most bifidobacteria die at 60°C (Rasic and Kurman, 1983). Optimum growth pH is between 6.5 to 7.0 and no growth occur below 5.0 or above 8.0 (Scardovi, 1986). Below pH 4.1, most species die within less than a week even at 4°C and below pH 2.5 most species die within less than 3h (Lankaputhra and Shah, 1995; Lankaputhra and Shah, 1996).

Most species of bifidobacteria of human origin produce vitamins such as thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), folic acid (B_9), cobalamin (B_{12}), ascorbic acid (C), nicotinic acid (PP) and biotin (H) (Deguchi *et al.*, 1985). Ability

to synthesise these vitamins could be important to the animal or human hosts as the vitamin supplies for the requirement of the host may not be affected as the demand for the vitamins by these bacteria would be minimum or nil within the gastrointestinal system.

Resistance of bifidobacteria to antibiotics is an important parameter in assessing the possibility of maintaining bifidobacteria in the digestive tract without aggression, particularly during antibiotic treatment to the hosts. Knowledge of resistance to antibiotics also is important due to their applicability as selective agents in selective media for various species of bifidobacteria. Although sufficient research is not available regarding the antibiotic resistance of bifidobacteria (Salminen and Wright, 1993), it has been claimed that bifidobacteria are resistant to antibiotics such as nalidixic acid, gentamycin, kanamycin, metronidazole, neomycin, polymixin B, and streptomycin. Sensitivity of these antibiotics vary from 10-500 μg/mL (La Vergne *et al.*, 1959; Miller and Finegold, 1967). Bifidobacteria could be strongly inhibited by ampicillin, bacitracin, chloramphenicol, clindamycin, erythromycin, lincomycin, nitrofurantoin, oleandomycin, penicillin G, and vancomycin (Scardovi, 1986).

1.3. Gastrointestinal ecology of *L. acidophilus* and bifidobacteria

1.3.1. Natural microflora in gastrointestinal system

Among the genera of LAB, *Lactobacillus, Streptococcus, Enterococcus* and bifidobacteria are found with the intestinal flora of humans and animals. Microflora of the gastro-intestinal system is comprised of about 400 different species. Intestinal contents have a viable microbial count of about 10^{12}cfu/g (Simon and Gorbach, 1986; Fuller, 1989). In addition to *Lactobacillus, Streptococcus, Enterococcus*, and bifidobacteria, there are other genera such as *Bacteroides, Clostridium, Eubacteria, Peptococcus*, and *Fusobacteria*. (Table 1.7.) Stomach

has lowest population of microorganisms and its contents have counts in the range of 10^0- 10^3 cfu/mL.

1.3.2. Equilibrium of gastrointestinal microflora and health of the host

L. acidophilus and bifidobacteria constitute a major part of the natural microflora of the human intestine and when present in sufficient numbers, these organisms create a healthy equilibrium between beneficial and potentially harmful microorganisms in the gut (Kolars *et al.*, 1984; Chitow and Trenev, 1990; Clark and Martin, 1994). Inclusion of live cultures of *L. acidophilus* and bifidobacteria in the diet produces several therapeutic benefits to the host (Kim, 1988; Pochart *et al.*, 1992).

As gastrointestinal system has a complex and varied microflora, various internal and external factors can affect the healthy equilibrium. External factors such as food and food or water-borne infections in the gastrointestinal system, antibiotics and other medications and radio-therapy perturb the microbial flora.

1.3.3. Factors affecting the flora of gastrointestinal tract

Disturbed or destroyed microbial flora could be replaced with opportunistic microorganisms such as the members of *Enterobacteriaceae* family or pathogenic yeast such as *Candida albicans*, which remain in lower numbers under normal circumstances. Various metabolic products secreted by these opportunistic microorganisms can cause short term and long term disease conditions in the host. Levels of pH and the presence of bile can limit the microflora in the gut. Use of broad spectrum antibiotics could produce sudden microbial vacuum in the gut. Dietary pattern and composition of the major diets (high meat or vegetable) can also influence the type of flora establishing in the gut.

Various levels of pH in different parts of the gastrointestinal system determine the diversity of the flora to a greater extent. Depending on the amount

of food available in the stomach and the time of measurements, the pH levels in the stomach can vary from 1.5 to 3.5. Low pH levels in the stomach are inhibitory to most microorganisms. As a result, the numbers of microoganisms reduce and only *Streptococcus* and *Lactobacillus* are found in the stomach (Table 1.7) (Ducluzeau, 1989).

Duodenum and jejunum also contain *Streptococcus* and *Lactobacillus* and their contents have counts of 10^2-10^4cfu/mL of these organisms. Ileal and caecal areas contain *Bacteroides, Clostridium*, *Streptococcus* and *Lactobacillus* up to 10^6-10^8cfu/mL in their contents.

Colon contains the heaviest microbial load, $10^{11.5}$-10^{12}cfu/g, mainly comprised of *Bacteroides, Clostridium, Eubacterium, Peptococcus, Bifidobacterium, Streptococcus,* and *Fusobacterium* (Ducluzeau and Raibaud, 1976; Ducluzeau *et al.*, 1980; Ducluzeau, 1981). All these microorganisms are in a delicate equilibrium between potentially harmful, non harmful and beneficial bacteria in healthy hosts.

Table 1.7. Distribution of human gastrointestinal flora in different segments of the gastrointestinal tract.

	Stomach	Jejunum	Ileum	Colon
Total microbial count[a]	0-10^3	0-10^5	10^3-10^7	10^{10}-10^{12}
Aerobic or facultative anaerobes count[a]				
Enterobacteria	0-10^2	0-10^3	10^2-10^5	10^4-10^{10}
Streptococcus	0-10^3	0-10^4	10^2-10^6	10^5-10^{10}
Staphylococcus	0-10^2	0-10^3	10^2-10^5	10^4-10^7
Lactobacillus	0-10^1	0-10^4	10^2-10^5	10^6-10^1
Anaerobes count[a]				
Bacteroides	rare	0-10^2	10^3-10^6	10^{10}-0^{12}
Bifidobacterium	rare	0-10^3	10^3-10^7	10^8-10^{12}
Peptococcus	rare	0-10^3	10^3-10^4	10^8-10^{12}
Clostridium	rare	rare	10^2-10^4	10^6-10^{11}
Fusobacterium	rare	rare	rare	10^9-10^{10}
Eubacteria	rare	rare	10^3-10^5	10^9-10^{12}
Veilonellae	rare	0-10^2	10^3-10^4	10^3-10^4

[a] Number of organisms per g of intestinal content (Salminen and Wright,1993)

1.4. Therapeutic properties of probiotic organisms

L. acidophilus and bifidobacteria are considered to be probiotic organisms. Probiotic organisms can be defined as "live microorganisms which can produce beneficial effects to the host and contribute to maintain a healthy equilibrium in the microflora of the gut of the host humans or animals".

Inclusion of live probiotic organisms in the diet produces several therapeutic benefits to the hosts (Goldin *et al.*, 1980; Pochart *et al.*, 1992; Hawkins, 1993; Hammes *et al.*, 1991). Probiotic microorganisms should be able to survive the passage through the gastro-intestinal tract and preferably colonise in the intestine in order to provide beneficial effects to the host.

Microoganisms which are considered to be probiotic must have several important characteristics. Firstly, these organisms are required to be normal inhabitants in the gut. They must not produce any harmful effects to the host under any circumstances. They are required to be able to survive under acidic conditions in the gastro-intestinal system. As acidity in the stomach is generally very high, probiotic microorganisms must be able to survive exposure to acidic conditions encountered in the stomach as well as in the duodenum and jejunum. The intestine also contains bile acids which are inhibitory to many microorganisms. However, normal inhabitants of the intestine are tolerant to bile. Probiotic bacteria of human origin are preferred for humans than those of animal origin as it is widely believed that the former bacteria are more compatible with the human intestine, as a result, such strains would have better chances of adhering to the intestinal epithelium of humans.

1.4.1. Antimicrobial properties

One of the important properties of probiotic microorganisms is their ability to produce antimicrobial substances of non-proteinaceous or proteinaceous in nature. Non-proteinaceous antimicrobial substances include organic acids such as lactic, acetic and orotic acids which possess antimicrobial properties against pathogenic microorganisms such as *Escherichia coli, Salmonella typhimurium, Aeromonas hydrophila*, and *Candida albicans*. Hydrogen peroxide is another inhibitory substance produced by probiotic bacteria. Hydrogen peroxide in the presence of organic acids such as lactic acids is more inhibitory against bacteria (Lankaputhra *et al.*, 1996 b). In addition to lactic and other organic acids produced by probiotic bacteria, volatile substances such as acetaldehyde and diacetyl could be effective in inhibiting unwanted bacteria in the intestine.

Some probiotic bacteria produce proteinaceous inhibitory substances, known as bacteriocins against some specific groups of pathogenic bacteria (Klaenhammer, 1988). In 1987, a low molecular weight proteinaceous substance produced by *Lactobacillus* GG was reported. This compound inhibited 54 strains including *Escherichia, Streptococcus, Pseudomonas, Salmonella, Bacillus, Clostridium,* and *Bifidobacterium*. Bhunia *et al.* (1988) and Harris *et al.* (1989) reported about bacteriocins produced by LAB against Listeria.

Although bacteriocins have antimicrobial effect against an array of pathogens and non pathogens, their effect *in vivo* may not be as significant as *in vitro*. Within the intestine, various proteases may hydrolyse the protein structure of bacteriocins. Further, bacteriocins produced by LAB are mainly inhibitory against other closely related strains of LAB. Hence, the antimicrobial activity based on non-protien substances such as organic acids could be more useful for intestinal conditions as compared with the inhibitory effects due to bacteriocins.

1.4.2. Antimutagenic and anticarcinogenic properties

Some strains of *L. acidophilus* and bifidobacteria have been reported to show antimutagenic and anticarcinogenic properties and this has been proven by experiments conducted using mice (Hosono *et al.*, 1986a, b, 1990; Zang *et al.*, 1990; Zang and Ohta, 1991a, b). The evidence of anticancer effects are available in four catagories: *in vitro* studies on the inhibition of mutagen activity; *in vivo* decrease of faecal enzymes involved in conversion of procarcinogens to carcinogens; *in vivo* studies on tumour suppression or incidence in laboratory animals; and epidemiology correlating cancer and certain dietary regimes.

Although there are no direct evidence regarding antimutagenic or anticarcinogenic properties of probiotic organisms directly on human subjects, a few studies conducted using human cell lines have shown that certain strains have positive effects that could lead to prevention of cancer (Nadathur *et al.*, 1995; Orrahage *et al.*, 1995). Recent reports have suggested that short chain fatty acids have effects at DNA level on rectifying genetic misreading (Tanaka *et al.*, 1990). As most of the probiotic organisms produce various short chain fatty acids such as acetic and butyric acids, it can be expected that these acids may be responsible for antimutagenic effect observed in probiotic bacteria.

These organisms also lower the levels of harmful enzymes such as β-glucosidase and β-glucuronidase responsible for catalysing the conversion of harmful amines. Goldin *et al.* (1980) studied the effect of *L. acidophilus* on the presence of four faecal enzymes including β-glucuronidase, nitroreductase, azoreductase and steroid 7-α-dehydroxylase in human omnivores and found that nitroreductase and β-glucuronidase levels were reduced. Goldin *et al.* (1992) showed that consumption of *Lactobacillus* GG can mediate a reduction in faecal β-glucuronidase levels in humans. In this study, eight control subjects were given *S. thermophilus* or *L. delbrueckii* ssp. *bulgaricus* and further eight subjects were given

L. acidophilus GG at a daily dose of microorganisms of 10^{10}. β-glucuronidase levels fell to 80% of baseline values only in subjects consuming *L. acidophilus* GG.

Antimutagenic effect of fermented milks has been detected against a range of mutagens and promutagens in various test systems based on microbial and mammalian cells (Bodana and Rao, 1990; Hosoda *et al.*, 1992*a, b*; Hosono *et al.*, 1986*a, b*; 1990; Renner and Munzner, 1991). Consumption of fermented milk inhibited the growth of certain types of tumours in mice and rats (Ayebo *et al.*, 1981; 1982; Esser and Lund, 1983; Fernandes *et al.*, 1987; Reddy *et al.*, 1973). Oral supplementation of *L. acidophilus* in humans reduced activities of faecal bacterial enzymes such as β-glucuronidase, nitroreductase and azoreductase that are involved in procarcinogen activation and reduced excretion of mutagens in faeces and urine (Lidbeck *et al.*, 1992). In studies with humans and other animals, dietary lactobacilli were found to stimulate the immune system (Gilliland, 1991; Perdigon *et al.*, 1993; Perdigon *et al.*, 1993; Sellars, 1991). Epidemiological evidences indicated negative correlations between the incidence of certain cancers and consumption of fermented milk products (Peters *et al.*, 1992, Van'T Veer *et al.*, 1991). Peters *et al.* (1992) reported that yogurt was found to be protective against colon cancer.

The mechanisms of antimutagenicity of the fermented dairy products, probiotic bacteria or of any specific chemical compounds have not been understood or identified so far (Nadathur *et al.*, 1995). Orrahage *et al.* (1995) suggested that microbial binding of mutagens could be the mechanism of antimutagenicity. Nadathur *et al.* (1995) reported that a crude extract of milk fermented with probiotic bacteria produced substantial antimutagenic effects. Renner and Munzner (1991) suggested the involvement of fibre in reduction of carcinogenicity. Presence of fibre promotes the production of short chain fatty acids (SCFA) by intestinal flora. Acetate, propionate and butyrate are the major

products of microbial fermentation of plant fibre polysaccharides in the human colon (Cummings, 1985; Wohin and Miller, 1983). Butyrate is a major source of energy for colonic epithelial cells (Roediger, 1982) and at low concentration it causes differentiation of mammalian cells and carcinoma cells (Kruth, 1982; Tanaka *et al.*, 1990).

1.4.3. Production of short chain fatty acids by intestinal bacteria and their therapeutic properties

Human colonic bacteria generate a wide range of low molecular weight products as a result of fermentation reactions in the large intestine. Quantitatively, SCFAs are the major end products of carbohydrate and protein breakdown. Acetate, propionate and butyrate are the main SCFAs found in the colonic contents. The caecum and proximal colon generally have high luminal concentrations of organic nutrients which serve to maintain high bacterial growth rates (Moor and Holdeman, 1975; Macfarlane and Cummings, 1991). Antiperistalsis in the proximal colon moves luminal content in a cephalad direction so that faeces are retained and mixed thoroughly. This provides ideal conditions for fermentation and maximal production of SCFA (Cummings *et al.*, 1987). As SCFA absorption is concentration dependant, absorption occurs most readily from the proximal colon. Butyrate production and absorption are also maximum in the proximal colon. Butyrate is a major energy source for human colonocytes, particularly in the distal colon and is an important factor for colonocyte growth and differentiation (Whitehead *et al.*, 1986; Kim *et al.*, 1980; Wilson, 1989). In addition to its potential inhibitory effect on colonic tumorigenesis, critical role of butyrate as energy source for human distal colonocytes has raised the possibility that defective SCFA metabolism, particularly of butyrate may be a causative factor in ulcerative colitis (Vernia *et al.*, 1988; Senagore *et al.* 1992). This idea is supported by a recent trial which has shown that SCFA enemas containing acetate,

propionate and butyrate are effective in the treatment of distal ulcerative colitis (Senagore *et al.*, 1992). Dietary supplementation of rats with wheat bran has recently been shown to increase total faecal SCFA concentrations by three fold and to maintain high faecal butyrate concentrations in distal colon.

In addition to their effect on increasing the concentrations of SCFA, within the colonic lumen, many studies have shown that changes in dietary fibre alter the metabolic activity of colonic bacteria and the faecal concentrations of bacterial β-glucuronidase, azoreductase and nitroreductase which are responsible for the generation of a variety of potential carcinogens (Goldin and Gorbach, 1975). In this regard faecal levels of bacterial 7α-dehydroxylase, which converts primary bile acids such as cholic acid and chenodeoxycholic acid to secondary bile acids (deoxycholic acids and lithocholic acids respectively), have been strongly implicated in the pathogenesis of colon cancer (Hill, 1975). Wheat fibre has been shown to have significant effect in altering the segmental growth patterns of colonic bacteria and thus SCFA and secondary bile acid concentrations in such a way as to protect the distal colon from the effects of inflammation and malignant transformation.

Butyrate induces many alterations when added to cells in culture and also *in vivo*. These alterations result from an effect of butyrate on gene expression in most cases and are summarised as follows:

(a) Alteration of the amount of a limited number of proteins. In many cases, these alterations have been shown to correlate with the level of the specific messenger RNAs.

(B) Inhibition of cell proliferation. In general butyrate stops the progression of the cells in the cell cycle. The addition of butyrate to cultured cancer cells *in vitro* strongly inhibits or suppresses DNA synthesis.

(C) Alteration of cell morphology and ultrastructure. In most cases butyrate suppresses cancer specific properties of the cells which recover normal molecular and cellular characteristics. However, the effect of butyrate is reversible and it disappears shortly after the removal of butyrate from the medium. Other short chain fatty acids are less effective or not effective at all.

1.4.4. Adherence and colonisation of probiotic bacteria

Microorganisms isolated from human intestines are believed to adhere better on the intestinal mucosal surface and therefore the organisms which are able to adhere and multiply are better candidates as probiotics. Adherence to intestinal surface is mediated by proteins and polysaccharides present in intestinal cell surfaces and microbial cell surfaces. Ability to adhere to the intestinal surfaces enables probiotic microorganisms to colonise in the gut and dominate over opportunistic and harmful organisms.

Recent reports concerning human gastrointestinal flora demonstrate that all strains do not possess the ability to adhere to human intestinal cells (Conway *et al.*, 1987; Ducluzeau, 1989; Kleeman and Klaenhammer, 1982: Simon and Gorbach, 1986). Selected strains such as *Lactobacillus acidophilus* BG2FO4 (Coconnier *et al.*, 1992), LB 7 (Chauviere *et al.*, 1992) and *Lactobacillus casei* GG (Elo and Salminen, 1991) exhibited adhesive properties which allow interaction with the brush border of human polarised intestinal epithelial cell lines in culture. Lack of adhesion ability of several bifidobacterial strains, as observed by Elo and Salminen (1991), suggest that adhesive factors are not expressed by all *Bifidobacterium* strains (Bernet *et al.*, 1993). Bernet *et al.* (1993) reported that *B. infantis* and *B. breve* showed adherence properties to HT-29 and Caco-2 cell lines.

Adherence differs from colonisation because the latter implies the ability to adhere and also to multiply. Mechanisms of adherence are diverse and

complicated. However, retention of bacteria in the human intestine can result from specific adherence to epithelial cells, from non-specific adherence to other intestinal bacteria, or from entanglement in mucus.

The intestines of different species vary in chemical composition and nutrient availability (Tannock, 1990). Therefore, the observation of host specificity of adherence can be expected (Tannock *et al.*, 1982). The host specificity can be achieved by specific adhesions and receptors on bacterial and host cells or by nutritional or physiological adaptation to different cell types or gut environments. Conway and Kjelleberg (1989) identified an extracellular protein from *Lactobacillus fermentum* that mediated host-specific *L. fermentum* adhesion and patented a process that uses this protein to promote adhesion (Conway and Kjelleberg, 1989). Sato *et al.* (1982) reported that a *Bifidobacterium* strain required polysaccharide fraction to adhere. However, as they used intestinal cells of pigs for their study, the significance of their findings depends on how well *in vitro* studies using pig intestinal cells represent a human system. Hood and Zottola (1988) showed that *L. acidophilus* strain BG2FO4 needed bacterial polysaccharide layer to adhere to intestinal surfaces. Mukai and Arihara (1994) found the presence of lectin binding glycoproteins on the cell surface of *L. acidophilus* cells. They suggested that the interaction between bacterial cell glycoproteins and intestinal lectins are required for specific adherence of the bacterium to intestinal epithelial surfaces.

1.5. Probiotic organisms in yogurt

1.5.1. Yogurt consumption

Yogurt containing probiotic bacteria has become a very popular product in recent years probably due to increased consumer awareness of the importance of probiotic bacteria. Yogurt production and consumption in Australia have increased

over the past two decades and the increase in production has been steady. Total yogurt production in Australia was reported to be 76,402,000 litres in 1993 while, in 1994, the production was more than 83,799,000 and in 1995, the production was 93,246,000 litres. This shows a 11% increase in yogurt production each year (Table 1.8).

Table 1.8. yogurt production in Australia

Year	Yoghurt (x 1000 L)
1984	35,879
1985	44,919
1986	---
1987	50,968
1988	53,157
1989	60,781
1990	59,424
1991	64,745
1992	72,055
1993	76,402
1994	85,627
1995	93,246
1996	----

Australian Dairy Corporation (1996).

In 1993, 1596 tonnes of yogurt was imported to Australia from New Zealand and other countries and import of yogurt products has increased to 2529 tonnes in 1994. This shows more than 58% increase in yogurt importation in one year (Table 1.9).

Table 1.9. Comparision of yogurt importation to Australia with other dairy products in 1993 and 1994.

	Imports (tonnes)		
Product	1993	1994	% increase
Yogurt	1596	2529	58.4
Butter	1827	1967	7.6
Cheese	25,448	27,392	7.6
Ice cream (000 Lt)	4339	4360	0.5

Australian Dairy Corporation, 1996.

Per capita consumption of yogurt in 1993 was 4.3 kg, which increased to 4.8 kg in 1994 and 4.9 kg in 1996. This shows an increase of 11.6% in consumption of yogurt. Consumption of other products including wholemilk powder, butter, cheese and ice cream either decreased or remained unchanged.

1.5.2. Regulatory aspects of yogurt

Dairy manufactures in many parts of the world incorporate probiotic bacteria such as *L. acidophilus* and bifidobacteria in yogurt. Yogurt containing *L. acidophilus* and bifidobacteria are referred to as "AB" yogurt. Some yogurt manufacturers incorporate *Lactobacillus caseii* in addition to *L. acidophilus* and bifidobacteria, such products are known as "ABC" yogurt. Yogurts are traditionally manufactured using *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* as starter cultures. However, these yogurt organisms are not natural inhabitants of intestine and can not survive under acidic conditions and bile concentrations encountered in the gastrointestinal tract. Therefore, for yogurt to

be considered as a probiotic product, *L. acidophilus* and bifidobacteria must be incorporated as adjuncts.

In order to produce the desired antimicrobial, antimutagenic, and anticarcinogenic effects, to reduce cholesterol levels in blood and to have improved lactose digestion, the probiotic organisms must be available in sufficient numbers. Several researchers have suggested that to have the desired effects, these organisms must be present in food at a minimum level of 10^6cfu/g or daily intake should be about 10^8cfu/g (Robinson, 1987; Ishbashi and Shimamura, 1993). Such high numbers are required to compensate for the possible reduction in numbers of the probiotic organisms during passage through the stomach and the intestine.

According to Australian Food Standards Code (Standard H8), yogurt is required to have a pH $\leq$ 4.5 and could be prepared with *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* or other suitable lactic acid bacteria. However, Australian Food Standards Code does not specify any requirements regarding the numbers of *L. acidophilus* or bifidobacteria in probiotic yogurt. However, in other countries standards have been imposed regarding the requirement of the numbers of the probiotic bacteria in yogurt. In Japan a standard has been developed by the Fermented Milks and Lactic Acid Bacteria Beverages Association which requires a minimum of 10^7 viable bifidobacteria cells/mL to be present in fresh dairy products.

1.6. Selective enumeration of *L. acidophilus* and bifidobacteria

1.6.1. Media based on selective utilisation of sugars by yogurt and probiotic bacteria and use of inhibitory substances for improving selectivity

In order to ensure that yogurt contain sufficient numbers of *L. acidophilus* and bifidobacteria required to confer beneficial effects to the consumer, suitable methods are required for enumeration of these organisms in yogurt. The need to

monitor survival of *L. acidophilus* and *Bifidobacterium* species in yogurt has often been neglected, with the result that a number of products reach the market with few viable bacteria (Anon., 1992; Hull and Roberts 1984; Iwana *et al.*, 1993, Shah *et al.*, 1995). An important parameter in monitoring viable organisms in assessing product quality and performance is the ability to determine the viable count of *L. acidophilus* and *Bifidobacterium* species and differentiate their species readily and rapidly. Differential enumeration of *L. acidophilus* and *Bifidobacterium* species in yogurt is difficult due to the presence of similar microbes which are used traditionally in yogurt manufacturing, including typically, *L. delbrueckii* ssp *bulgaricus* and *S. thermophilus*.

Several media have been developed for differential enumeration of *L. acidophilus* and *Bifidobacterium* species. Scardovi (1986) has reviewed several complex media and media containing a wide variety of antibiotics to enumerate selectively *Bifidobacterium* species and concluded that one selective medium is not appropriate for all species. Munoa and Pares (1988) have developed a selective medium for isolation and enumeration of bifidobacteria from aquatic environments. This medium consisted of reinforced clostridial 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* species. 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* species in the presence of other LAB or from yogurt

which 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 was 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* and bifidobacteria and that counts obtained are not necessarily representative of viable cells present in the product. Therefore it is necessary to have a broader knowledge of applicability of various media and selective ingredients for selective enumeration of *L. acidophilus* and bifidobacteria in yogurt.

1.6.2. Importance of using non-inhibitory media for selective enumeration of probiotic bacteria in yogurt

Most antibiotics and other inhibitory substances used to improve the selective growth of probiotic bacteria can be toxic to the strains of interest as well. As a result, lower viable counts shown in such media could lead to wrong estimation of viability of the strains of interest (Lankaputhra *et al.*, 1996 a; Dave and Shah, 1996 a).

All selective media employing antibiotics or other inhibitory substances should be validated before using them. Pure cultures of the strains should be separately plated in the selective media and control media such as MRS or MRS supplemented with bifidobacterial growth promoters such as L-cystein·HCl (Lankaputhra *et al.*, 1996a; Dave and Shah, 1996a).

1.7. Survival of *L. acidophilus* and bifidobacteria in commercial yogurt during refrigerated storage

In spite of the increasing popularity of yogurt containing *L. acidophilus* and bifidobacteria, there is a growing concern regarding the presence of these organisms in yogurt products in sufficient numbers required to confer expected health benefits at the time of consumption (Gilliland and Speck, 1977; Schioppa *et al.*, 1981; Hull *et al.*, 1984; Anon., 1992; Shah *et al.*, 1995). Inhibitory substances such as acid and hydrogen peroxide produced by yogurt bacteria (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) are claimed to be responsible for poor survival of *L. acidophilus* and bifidobacteria (Conway *et al.*, 1987; Playne, 1993; Shah and Jelen, 1990; Shah *et al.*, 1995; Lankaputhra and Shah, 1995; Lankaputhra *et al.*, 1996b).

Studies have shown that *L. acidophilus* and bifidobacteria are unstable in yogurt (Gilliland and Speck, 1977; Schiopa *et al.*, 1981; Hull *et al.*, 1984). *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 (Reddy 1989). When this organism was added with the yogurt culture organisms during yogurt manufacture, loss of viability still occurred but the organism survived better. Several brands of commercial yogurts from Australian supermarkets were analysed for the presence of *L. acidophilus* and *B. bifidum* (Anon., 1992; Shah *et al.*, 1995). All the products contained very low numbers of *B. bifidum*. *L. acidophilus* count was also very low in some products.

Survival of *L. acidophilus* and bifidobacteria is affected by low pH of the product. Although *L. acidophilus* survives better than yogurt bacteria, a rapid decrease in their numbers has been observed under acidic conditions, both *in vitro* and *in vivo* (Conway *et al.*, 1987; Hood and Zottola, 1988; Shah and Jelen, 1990). Bifidobacteria are not as acid tolerant as *L. acidophilus* and the growth of the latter organism ceases

below pH 4.0 (Playne, 1993). Most strains of bifidobacteria prefer anaerobic conditions or low redox potential for multiplication and some can grow under microaerophillic conditions.

1.8. Inhibitory factors against *L. acidophilus* and bifidobacteria

L. acidophilus and bifidobacteria are affected by several inhibitory substances during production, cold storage and after consumption of yogurt. During production, yogurt bacteria produce lactic acid which reduces the pH to 4.5. Hydrogen peroxide is also produced by yogurt bacteria during yogurt fermentation. Acid and hydrogen peroxide can affect the growth of *L. acidophilus* and bifidobacteria in yogurt. During storage of the product at low temperature, there is further reduction in acidity and the pH can reach about 3.7 (Shah *et al.*, 1995) which can reduce the viability of *L. acidophilus* and bifidobacteria in yogurt.

L. acidophilus and bifidobacteria are exposed to low pH conditions and bile concentrations in the gastrointestinal tract upon consumption of yogurt. Several investigators have studied the survival of *L. acidophilus* and bifidobacteria in the presence of acid and bile salts. Hood and Zotolla (1988) have shown that *L. acidophilus* rapidly lost viable counts at pH 2.0. Clark *et al.* (1993) studied the survival of *B. infantis, B. adolescentis, B. longum* and *B. bifidum* in acidic conditions and reported that *B. longum* survived best.

Clark and Martin (1994) showed that *B. longum* tolerated bile concentrations as high as 4% whereas Ibrahim and Bezkorovainy (1993) found *B. longum* to be least resistant to bile. In the latter study, *B. infantis, B. bifidum, B. breve*, and *B. longum* were exposed to 0.6 to 3.0 g/L (0.06 to 0.30%) of sodium glycolate. *B. infantis* showed the best survival followed by *B. bifidum* and *B. breve*.

In selecting *L. acidophilus* and bifidobacteria as probiotic dietary adjuncts, it is necessary to consider their survival against acid during fermentation, storage and in the gut against hydrogen peroxide during fermentation and storage and the presence of bile acids in the intestine.

2. MATERIALS AND METHODS

2.1. Sources of chemicals, reagents and microbiological media

2.1.1. Chemicals and reagents

All chemicals were obtained from Sigma Chemicals Company (Anella Avenue, Castle Hill, New South Wales, Australia) or from BDH Chemicals Company (Corporate Avenue, Rowville, Victoria, Australia). Reagents were obtained from Boehringer Mannheim Australia Pty. Ltd. (Victoria Avenue, Castle Hill, New south Wales, Australia).

2.1.2. Microbiological media

All microbiological media were obtained from Oxoid (West Heidelberg, Victoria, Australia). Some media including basal growth medium and minimal agar were prepared in the laboratory using basic ingredients.

2.2. Yogurt samples and bacterial strains

2.2.1. Yogurt samples

Yogurt samples for microbiological enumeration and chemical analyses were obtained from five yogurt manufacturers in Australia. The yogurt samples were despatched by the manufacturers under refrigerated conditions on the day of manufacture and the samples were received at the Centre for Bioprocessing and Food Technology, Werribee Campus of Victoria University of Technology. Upon receipt, the samples were stored at 4°C in a walk-in-cooler. A fresh cup of yogurt was opened every three days for microbiological and chemical analyses.

2.2.2. Lactic acid bacteria

Pure cultures of six strains of *Streptococcus thermophilus*, five strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*, six strains of *Lactobacillus acidophilus* and nine strains of *Bifidobacterium* spp. were obtained from the Dairy Research

Laboratory, Division of Food Science and Technology, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Highett, Victoria, Australia. All strains of *S. thermophillus*, *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus*, and the strains of *Bifidobacterium* spp. 1900, 1901, 1912, 1920 and 1941 have CSCC (CSIRO Culture Collection) strain numbers, and *Bifidobacterium* spp. strains 20097, 20099 and 20210 have DSM (Deutsche Saamlung Von Mikroorganismen und Zellkulturen GmbH) strain numbers. Working cultures were maintained in reconstituted 12% nonfat dry milk supplemented with 2% glucose, 1% yeast extract and 0.05% L-cysteine hydrochloride (NGYC). Cultures for long storage were prepared either in milk based medium or in glycerol nutrient broth. For the former, the cultures were grown overnight in NGYC and 10% of these cultures were mixed with fresh sterile NGYC, dispensed in small cryovials and stored in a -20°C freezer. For the latter, cultures were grown in sterile MRS (DeMann, Rogosa and Sharpe) broth for 18h, the cultures were centrifuged at 3000 rpm for 20 min under sterile conditions and the pellets were suspended in a 60:40 mixture of double strength MRS and sterile glycerol, respectively. Aliquots of cultures were dispensed in cryovials, frozen and kept in a freezer at -80°C.

2.2.3. Pathogenic bacteria

Cultures of *Salmonella typhimurium*, *Aeromonas hydrophila*, *Escherichia coli* and *Candida albicans* were obtained from the culture collection of Australian Food Industry Science Centre (AFISC), Sneydes Road, Werribee, Victoria, Australia. Working cultures of these organisms were grown in Oxoid nutrient broth No. 2. Cultures for long storage were stored at -80°C in a nutrient broth and glycerol (in a ratio of 60:40, respectively) mixture.

2.3. Equipment and Instruments

2.3.1. Anaerobic jars

Anaerobic jars with sixteen and fourty eight plate capacities and catalysts were obtained from Oxoid. In order to create anaerobic condition, H_2 and CO_2

generating sachets (Oxoid Australia) or a gas mixture containing H_2, CO_2 and N_2 in ratios of 5:10:85 respectively, were used.

2.3.2. HPLC

HPLC (Varian) was used with an autosampler, solvent delivery system, UV-Vis and refractive index detectors, and Star software (Varian, Mulgrave, Victoria, Australia). For determining organic acids, an Aminex HPX-87H ion exclusion column - 300 x 7.8 mm (Bio-Rad, North Ryde, New South Wales, Australia) was used. For determining the concentrations of organic acids UV-Vis detector was used and for sugars refractive index detector was used.

2.3.3. Centrifuge and microcentrifuge

Beckmann J2-HS centrifuge (Beckmann Instruments Inc., Palo Alto, California, USA) was used for centrifuging large samples of about 10 -1000 mL. For volumes smaller than 2 mL, a microcentrifuge (Beckmann) was used. For samples between 2 - 10 mL and requiring rpm of <4000, a bench top centrifuge (Beckmann) was used.

2.3.4. Sonication of bacterial cultures

Bacterial cultures grown in milk or MRS broth were sonicated using a Branson sonifier (Branson Ultrasonics Corporation, Eagle Road, Danbury, CT, USA). Diluted or undiluted samples were placed in 20 mL glass container and immersed in an ice bath. The sonicator was set for medium output and the culture samples were sonicated for 20 seconds followed by cooling period of one minute. With this arrangement, the temperature in the sonicated samples did not rise above 10°C.

2.3.5. Cell rupturing

Cell rupturing was carried out in a MSK cell homogeniser (B. Braun Melsungen AG, Melsungen, Germany). Centrifuged cell pellets were suspended in

phosphate buffered saline (PBS), chilled in ice for 10 min, and placed in a sterilised stainless steel container which were used in the cell homogeniser. Cell breaking was carried out for 30 seconds. Ruptured cell suspensions were chilled and refrigerated or frozen.

2.3.6. Freeze drying of bacterial cultures for yogurt preparation

S. thermophilus, L. delbrueckii ssp. *bulgaricus, L. acidophilus* and *Bifidobacterium* spp. were grown separately in MRS broth for 18 h at 37°C. For *Bifidobacterium* spp., MRS broth was supplemented with 0.05% L-cysteine hydrochloride. Cells were recovered by centrifuging at 5000 rpm using Beckmann J2-HS refrigerated centrifuge (Beckmann Instruments Inc., Palo Alto, California, USA). The cells were washed with sterile PBS and centrifuged again. The cell pellets were suspended in reconstituted 12% nonfat dry milk (NDM), poured into plastic containers with lids and frozen immediately at -20°C for 6 h. The frozen cultures were placed in a Dynavac FD 300 freeze dryer (Dynavac Engineering Pty. Ltd. Inc., Melbourne, Victoria, Australia) and freeze drying was carried out at -60°C for 12 h. Freeze dried cultures were packed aseptically in sterile containers and kept in a refrigerator at 4°C.

2.4. Physico-chemical analyses

2.4.1. Titratable acidity and pH

Titratable acidity (TA) of yogurt and milk samples was measured by titrating 9 g samples with 0.1 N NaOH using 0.1 mL phenolphthalein. Titration was repeated three times for each sample and average volume of NaOH was used in the following equation for estimating the TA;

$$\%TA = \frac{(\text{mL of NaOH used in titration} \times 0.009)}{\text{g of sample}} \times 100$$

pH of the samples was measured using a HI 8418 pH meter (Hanna Instruments, New South Wales, Australia). Average of three measurements was recorded for each sample.

2.4.2. Acetaldehyde

Acetaldehyde content was measured using the methods of Lindsay and Day (1965) (method 1) and Millies *et al.*, (1989) (method 2).

Acetaldehyde determination using method 1:

A quantity of five grams of yogurt was weighed accurately into a 25 x 250 mm test tube, and 15 g of distilled water was added and mixed thoroughly. A collection trap was prepared using a thin (10 mm) test tube and a collection reagent (2.5 mL distilled water, 2.5 mL of 0.4% aqueous 3-methyl-2-benzothiazolone hydrazone hydrochloride, and 0.5 mL of DMSO) was placed in the tube (Fig. 2.1.). The collection system was assembled and culture samples and a control containing only distilled water were placed in a 65°C water bath and purged with 100 - 125 mL of N_2 per min for 1 h. Collection tubes were removed from the assembly, allowed to stand in room temperature for 25 min, added with 12.5 mL of 0.2% ferric chloride in 0.1 N HCl, and allowed to stand for exactly 25 min. Finally 20 μL of acetone was added, mixed immediately, transferred the reaction mix into a 50 mL volumetric flask and brought to the mark with acetone. Absorbence was measured using a spectrophotometer at 666 nm against a reagent blank (section 2.2.4.). A standard curve was prepared by adding 6 different known concentrations of acetaldehyde into 5 g of milk acidified with 1N phosphoric acid using the procedure described above.

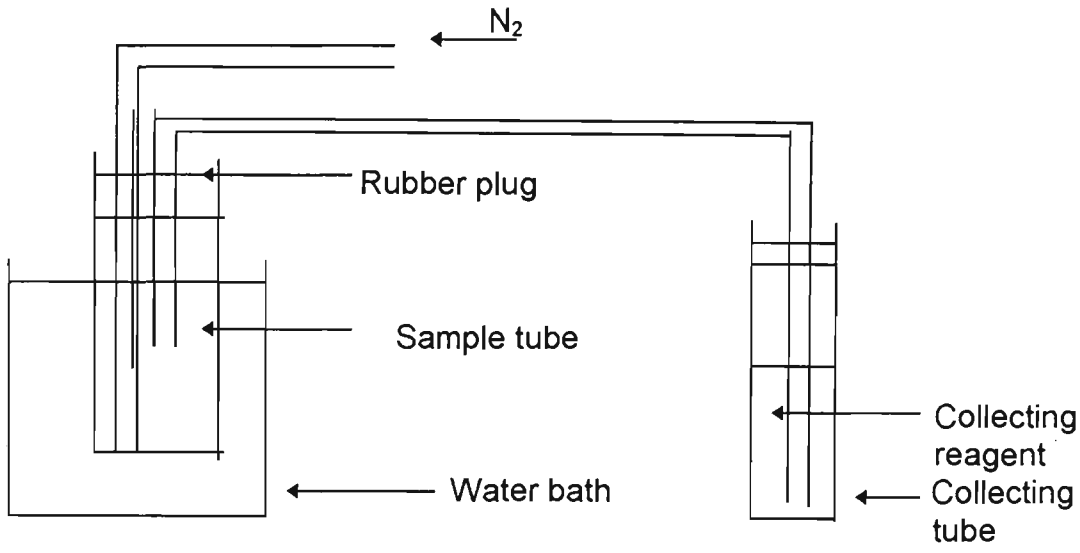

Fig. 2.1. Setup for acetaldehyde determination by the method of Lindsay and Day (1965)

Acetaldehyde determination using method 2: One g of yogurt was weighed accurately and mixed with 4 g of distilled water, mixed thoroughly and filtered through a Whatman No. 42 filter paper. An aliquot of 0.2 mL of the filtrate was added to 3 mL of NAD (0.25 mg in 0.1 M phosphate buffer at pH 9.0), allowed to stand for 3 min, 0.1 mL of acetaldehyde dehydrogenase (4 U/mL in distilled water) was added and incubated for 5 min at 25°C. Absorbance was measured at 340 nm. A standard curve was prepared by adding dilutions of acetaldehyde into 1 g of acidified (with phosphoric acid) milk using the same procedure.

2.4.3. Hydrogen peroxide

Hydrogen peroxide content was measured by the method described by Gilliland (1968). Ten gram of yogurt was mixed with 0.1 **M** acetate buffer (section 2.6.2.). The yogurt-buffer mixture was diluted to 20 mL with distilled water and filtered through a Whatman No. 42 filter paper. Five millilitres of the filtrate, 1 mL of distilled water, and 0.1 mL of o-dionisidine (1% in methanol) were added into a 10 mL test tube (control sample), and 5 mL of the filtrate, 1 mL of peroxidase solution (10 μg/mL) and 0.1 mL o-dionisidine were added to another test tube (test

sample). Both the control and the test samples were incubated at 25°C in a water bath for 10 min and 0.2 mL of 4N HCl was added in control and test samples. After 5 min, optical density was measured using a UV-Vis spectrophotometer (section 2.4.2.) at 400 nm. Average of 3 readings were compared against a standard curve prepared with known concentration of pure H_2O_2.

2.4.4. Determination of the α-D-galactosidase, β-D-galactosidase and phospho-β-D-galactosidase activities

Bacterial cells were prepared as described in section 2.3.5. Ruptured bacterial cells were centrifuged at 5000 rpm for 10 min and the extract was diluted 5 times with 0.1 M phosphate buffer at pH 7.0. Solutions (0.005 M) of o-nitrophenyl-β-D-galactopyranoside (ONPG), p-nitrophenyl-β-D-galactopyranoside (PNPG), and phospho-o-nitrophenyl-β-galactopyranoside (PONPG) were prepared using 0.1 M phosphate buffer. One millilitre of each cell extract was added with 5 mL of ONPG, PNPG, or phospho-ONPG separately, incubated at 37°C for 15 min, and the reaction was stopped by adding 2.5 mL cold Na_2CO_3 solution. The amount of o-notrophenol (ONP) released was measured with a spectrophotometer (section 2.4.2.) at 420 nm. Enzyme activities were determined using a standard curve.

2.4.5. Determination of protein levels in bacterial extracts

A modified version of Lowrey assay was used to determine protein contents in bacterial extracts. An aliquot of 0.5 mL of the sample containing 0-100 μg of protein was added with 0.5 mL of solution A (0.1 mL of 5% $CuSO_4$ (A1); 0.9 mL of 1% potassium tartrate (A2); 10 mL of 10% Na_2CO_3 in 0.5 M NaOH (A3)). After 10 min at 37°C, 1.5. mL of solution B (1 mL Folin-Ciocalteau's reagent and 10 mL of double distilled water) was added and the solutions were mixed immediately using a vortex mixer. Absorbance of samples was recorded at 680 nm after incubation at 52° C for 20 min. Standards containing 0-100 μg of protein were

prepared from 0.1% bovine serum albumin (BSA) solution. Reagents A and B were prepared immediately before use.

Stock solution A = (A1 + A2+ A3)

A1	5% w/v CuSO4	0.1 mL
A2	1% w/v KNaTartarate	0.9 mL
A3	10% w/v Na_2CO_3 in 0.5M NaOH	10.0 mL

2.5. Enumeration of probiotic bacteria

2.5.1. Peptone and water diluent

Peptone and water diluent (0.1%) was prepared by dissolving 1.0 g of peptone medium (Oxoid, Australia) in 1 litre of distilled water followed by autoclaving 9 mL aliquots at 121°C for 15 min. After autoclaving, peptone and water diluent in glass vials was stored for up to 2 weeks at room temperature (~ 20°C).

2.5.2. MRS agar and broth

MRS (deMan Rogosa Sharpe) broth was used for growing *L. acidophilus*, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, and for bifidobacteria MRS broth was supplemented with 0.05% L-cysteine HCl was added to the broth. Ingredients for MRS broth are shown in Table 2.1. To prepare MRS agar, 15 g of bacteriological agar (Oxoid) was added to the broth which was sterilised at 121°C for 15 min.

Table 2.1. Ingredients for deMan, Rogosa and Sharpe (MRS) broth

Ingredients	Amount (g/L)
Proteose peptone # 3	10.0
Beef extract	10.0
Yeast extract	5.0
Glucose	20.0
Tween 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium phosphate	2.0

2.5.3. Preparation of serial dilutions for spread and pour plating

One gram of yogurt culture was added to 9 mL of 0.1% peptone and water diluent, vortexed and 1 mL of the dilution was transferred into 9 mL of peptone and water and a series of ten-fold dilutions were prepared. One millilitre aliquots were placed in an empty petridish, molten agar medium was poured into the plate followed by mixing in a circular motion to left and right hand sides and the agar was allowed to solidify. Spread plating was carried out by placing 0.1 mL aliquot of the diluted samples in a pre-prepared petridish and spreading with a bent glass rod.

2.5.4. MRS-galactose agar, MRS-maltose agar, MRS-dextrose agar and MRS-L-arabinose agar

To prepare MRS-galactose, MRS-maltose, MRS-dextrose and MRS-L-arabinose agars, ingredients of MRS agar (Table 2.8.), with the exception of dextrose, were separately weighed and reconstituted with distilled water. The pH of the media was adjusted to 6.8 and the media were sterilised by autoclaving at 121°C for 15 min. Twenty per cent stock solution of galactose, maltose, dextrose, and L-arabinose were prepared separately and each sugar solution was filter sterilised. Each sugar was

added separately to the autoclaved MRS basal medium held at 45°C to achieve a final sugar concentration of 2% and the media were used immediately for enumeration using the pour plating method.

2.5.5. Modified salicin agar, cellobiose agar, fructose agar, mannitol agar and sorbitol agar

Ten per cent sugar solutions of salicin, cellobiose, fructose, mannitol and sorbitol were filter sterilised and mixed with minimal nutrient agar base (Table 2.9.) at 45°C to achieve a final sugar concentration of 1% in the medium.

2.5.6. NNLP agar

NNLP (nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate) (Sigma Chemicals Company) agar was prepared according to the method described by Laroia and Martin (1991). The composition of MRS basal medium, is given in Table 2.2. Nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate as selective agents were added to the autoclaved MRS base. Filter sterilised L-cysteine hydrochloride (final concentration 0.05%) was added to lower the oxidation-reduction potential of the medium and to enhance the anaerobic growth of bifidobacteria.

Table 2.2. Ingredients for minimal basal agar

Ingredients	Amount (g/L)
Tryptone (pancreatic digest of casein)	20.0
Yeast extract	2.0
Tween 80	1.0
Tri sodium citrate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium phosphate	2.0
Agar	15.0

2.5.7. Reconstituted clostridial agar

Reconstituted clostridial agar (RCA) (Oxoid, Australia) was prepared according to the manufacture's instructions (i.e., 38 g powder was dissolved in 1 litre of distilled water) and the pH was adjusted to 5.5 with 1N HCl. The agar was sterilised by autoclaving at 121°C for 15 min.

2.5.8. Bile agar

Bile agar was prepared by adding 2.0 g of dessicated ox bile extract (Oxoid Australia, W. Heidelberg, Australia) per 100 mL of MRS medium. The pH of the medium was adjusted to 6.8 and the medium was sterilised by autoclaving at 121°C for 15 min.

2.5.9. Use of antibiotics for selective growth of bacteria

Antibiotic discs were prepared by soaking sterile filter paper discs with measured micro volumes of concentrated antibiotic solutions. Antibiotics used were: chloramphenicol (10 μg), cloxacillin (10 μg), carbenicillin (10 μg), penicillin-G (5 units), trimethoprim (2.5 μg), tetracycline (30 μg), ampicillin (10 μg), erythromycin (15 μg), and gentamycin (10 μg). Quantities of each antibiotic used in the discs are the standard quantities recommended by Oxoid. Pure culture suspensions of each of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and *Bifidobacterium* spp. with a viable count of 10^7-10^8 cfu/g were prepared in 0.1% sterile peptone water and 0.1 mL aliquots were separately spread plated on MRS agar. The plates were incubated in an anaerobic chamber for 3 h at room temperature and then antibiotic discs were placed on the agar surfaces. The plates were incubated anaerobically at 37°C for 72 h and zones of inhibition were recorded.

2.6. Assessment of survival of *L. acidophilus* and bifidobacteria in commercial yogurt during refrigerated storage

Samples of five brands of yogurt were obtained directly from the yogurt manufacturers within 24 -72 h of production (as described in section 2.2.1). All

yogurts were full cream, stirred products and were claimed to contain live *L. acidophilus* and *B. bifidum* cultures in addition to yogurt culture bacteria. Samples were received in normal retail packs of 200 or 500 mL consumer packs and stored at 4°C. Serial dilutions were prepared as described in section 2.5.1 and the pour plates were prepared as in section 2.5.2. Enumeration of *L. acidophilus* and *B. bifidum* was carried out with NNLP agar and MRS-maltose agar at pH 5.5 as in sections 2.5.1 and 2.5.5. Duplicate plates were incubated anaerobically in Oxoid anaerobic jars using a gas mixture of 10% CO_2, 5% H_2 and 85% N_2 as in section 2.3.1 at 37°C for 48-72 h. Plates were counted using a colony counter.

2.7. Characterisation of strains of probiotic bacteria and their application in yogurt manufacture

2.7.1. Assessment of survival in the presence of acid and bile

To evaluate the survival of six strains of *L. acidophilus* and nine strains of *Bifidobacterium* spp. under acidic conditions similar to those encountered in the gut, aliquots of active cultures grown in NGYC for 18 h at 37°C were adjusted to pH 3.0, 2.5, 2.0 and 1.5 with 5 **N** HCl and incubated at 37°C for 3 h. Samples were taken in triplicate every hour for 3 h and the viable numbers of *L. acidophilus* and *Bifidobacterium* spp. were enumerated using the pour plate technique (section 2.5.2).

To evaluate the survival of these probiotic organisms in the presence of various concentrations of bile salts as encountered in the intestine, aliquots of active cultures grown in NGYC were adjusted to pH 4.5 using 0.1 **N** HCl or 0.1 **N** NaOH, depending on the final pH of the cultures after 18 h of incubation. Concentrated bile solution was prepared separately using powdered bile extract (Oxoid Australia Pty. Ltd.), filter sterilised and added to two of the cultures to achieve a final concentration of 1.0% or 1.5% and the third culture served as a control. The cultures were incubated at 37°C for 3 h. Samples were taken in

triplicate prior to adding bile and every hour for 3 h and the viable counts of *L. acidophilus* and bifidobacteria were determined by the pour plate count technique.

2.7.2. Assessment of survival of probiotic bacteria in the presence of acid and hydrogen peroxide

To evaluate the survival of *L. acidophilus* and bifidobacteria in the presence of acid encountered during processing and storage conditions, aliquots of active cultures grown in NGYC were adjusted to pH 4.5, 4.3, 4.1, 3.9 and 3.7 using sterile 4N lactic acid and stored at 4°C for 6 weeks. Samples were taken at 6 day intervals and the viable count of *L. acidophilus* and bifidobacteria were determined as described in section 2.5.2.

To evaluate the synergistic effect of acid and hydrogen peroxide on the survival of these probiotic microorganisms, aliquots of active cultures grown in NGYC for 18 h at 37°C were adjusted to pH 4.3, 4.1, 3.9 and 3.7 with sterile 4N lactic acid, and freshly prepared hydrogen peroxide solution (10,000 μg) was added to the cultures to achieve a final concentration of 100 μg/mL. The cultures were mixed and stored at 4°C for 6 weeks. Samples were taken at 6 day intervals and the viable counts of bifidobacteria were determined by pour plate technique.

2.7.3. Viability and organoleptic assessment of yogurt prepared with selected strains of *L. acidophilus* and bifidobacteria and commercial probiotic starter cultures

Commercial freeze dried probiotic starter cultures were obtained from 3 suppliers. Commercial starter cultures from supplier 1 were *S. thermophilus* (St), *L. acidophilus* (La-1) and *B. bifidum* (Bb-1). Commercial starter culture from supplier 2 provided *L. acidophilus* (La-2), *B. longum* (B-I2) and *B. infantis* (Bi-2). Commercial starter culture from supplier 3 provided *L. acidophilus* (La-3), *B. bifidum* (Bb-3) and *B. longum* (Bl-3). From Victoria University culture collection, *B. longum* 1941 (Bl-1941), *B. infantis* 1912 (Bi-1912), *B. pseudolongum* 20099 (Bp-20099), *L. acidophilus* 2409 (La-2409) and *L. delbrueckii* ssp *bulgaricus* 2515 (Lb) were freeze dried (as described in section 2.3.3.) for yogurt preparation. Viable

counts of all freeze dried bacterial cultures were determined as described in section 2.5.2. using MRS agar with added L-cysteine· HCl (0.05%).

Nine experimental batches of yogurt were prepared as shown in Table 2.3. In each batch, there was a combination of *S. thermophilus*, *L. delbrueckii* ssp *bulgaricus*, *L. acidophilus* and *Bifidobacterium* spp. (*B. bifidum*, B. *infantis*, B. *longum* or B. *pseudolongum*) except batch 1 which was made only with *S. thermophilus* supplied by the supplier 1 and *L. delbrueckii* ssp. *bulgaricus* 2515.

Five litres of vacuum concentrated milk with a total solid content of 17% was used for each batch of yogurt. The concentrated milk was heated to 80°C for 30 min, cooled to 40°C, and inoculated with freeze dried yogurt and probiotic bacterial cultures at rates recommended by the starter culture suppliers (50g of freeze dried culture per 1000 L of yogurt mix). After inoculation and mixing, the yogurt mixes were distributed into 100 mL cups, incubated at 42°C until the pH reached 4.5, and stored at 4°C. Samples were drawn immediately after manufacturing and every week for 6 weeks for enumeration of probiotic bacteria. Sensory evaluation was carried out using a panel of 10 members after 3 days, 2 weeks and 5 weeks of manufacture.

Table 2.3. Preparation of yogurt with different probiotic starter culture combinations

Batch No.	Bacterial culture combination for making various batches of yogurt		
1	*S. thermophilus*	------	------
2	*S .thermophilus*	La-1	Bb-1
3	*S. thermophilus*	La-2	Bi-2
4	*S. thermophilus*	La-2	Bl-2
5	*S. thermophilus*	La-3	Bb-3
6	*S. thermophilus*	La-3	Bl-3
7	*S. thermophilus*	La-2409	Bl-1941
8	*S. thermophilus*	La-2409	Bp-20099
9	*S. thermophilus*	La-2409	Bi-1912

2.8. Improving viability of probiotic bacteria: impact of availability of β-galactosidase, higher levels of solids and various fermentation strategies

2.8.1. Preparation of freeze dried starter culture using ruptured yogurt starter bacteria in yogurt manufacture

2.8.1.1. Bacterial strains

L. delbrueckii ssp. *bulgaricus* 2515, *S. thermophilus* 2010, *L. acidophilus* 2409, *Bifidobacterium longum* 1941, *B. pseudolongum* 20099, *B. infantis* 1912 and *B. bifidum* 1900 and 1901 were obtained as described in section 2.2.2. *L. delbrueckii* ssp. *bulgaricus* 2515 and *S. thermophilus* 2010 were selected on the basis of their high extracellular β-galactosidase activity (Lankaputhra & Shah, unpublished data) and *L. acidophilus* 2409 was selected on the basis of acid and bile tolerance as reported earlier (Lankaputhra & Shah, 1995). Cultures were maintained and their identity confirmed as described earlier (Lankaputhra & Shah, 1995).

2.8.1.2. Harvesting, rupturing and freeze drying of yogurt bacteria

L. delbrueckii ssp *bulgaricus* and *S. thermophilus* were grown separately in deMan Rogosa and Sharpe (MRS) broth for 16 h and the cells in their early log phase were recovered by centrifuging at 10,000 rpm for 15 min using a Beckman Model L-70 ultracentrifuge and JA-14 rotor (Beckman Instruments, Palo Alto, CA). The cells were washed by suspending in sterile phosphate buffered saline and re-centrifuged. The cell pellet was suspended in 50 mL sterile saline solution, the suspension cooled to <4°C and 10 mL by volume of glass beads of 0.1 mm size was added. The cell suspension and glass beads were placed in a 70 mL sterile stainless steel adaptor and mechanical vibration was applied using an MSK cell homogeniser (B. Braun Melsungen AG, Melsungen, Germany) for 30, 60 or 90 sec in order to rupture the cells. Samples were taken before and after cell rupture to enumerate viable counts and to measure β-galactosidase activity. The ruptured cell suspension was centrifuged at 3000 rpm for 1 min using Beckman

ultracentrifuge to remove the glass beads. The cell suspension was mixed with 12% (w/v) reconstituted nonfat dry milk (NDM), frozen at -20°C and freeze dried using a Dynavac freeze dryer (Dynavac Engineering, New South Wales, Australia).

2.8.1.3. Harvesting and freeze drying of probiotic cultures

L. acidophilus 2409 and five species of *Bifidobacterium* (*B. longum* 1941, *B. pseudolongum* 20099, *B. infantis* 1912, *B. bifidum* 1900 and 1901) were grown separately in MRS broth for 16 h and cells were harvested and washed as with yogurt bacteria. The washed cells were suspended in NDM, frozen at -20°C and freeze dried as with yogurt bacterial cultures. All freeze dried starter cultures were packed in MaCartney glass bottles with airtight seals and stored at 4°C until used.

2.8.1.4. Preparation of yogurt

Homogenised and pasteurised milk supplemented with 5% nonfat dry milk was heated to 85°C for 30 min, cooled to 42°C and freeze dried starter cultures of yogurt and probiotic bacteria were added to the yogurt mix at the rate of 0.1 g/L. Five different types of yogurt were prepared as shown in Table 2, each containing *L. delbrueckii* ssp. *bulgaricus* 2515, *S. thermophilus* 2010, *L. acidophilus* 2409 and one species of *Bifidobacterium* (*B. longum* 1941, *B. pseudolongum* 20099, *B. infantis* 1912, *B. bifidum* 1900 or *B. bifidum* 1901). After mixing with the starter cultures, the yogurt mix was incubated at 42°C and samples were taken during fermentation at 0 h and then at hourly intervals till the pH reached 4.5 for measurement of pH and titratable acidity, enumeration of yogurt and probiotic bacteria and for determination of β-D-galactosidase activity, hydrogen peroxide and acetaldehyde. The yogurt was then stored for 6 weeks at 4°C and viable counts of probiotic bacteria were determined at weekly intervals.

2.8.1.5. Enumeration of yogurt and probiotic bacteria

L. delbrueckii ssp. *bulgaricus* was enumerated according to the method of Dave and Shah (1996a) using MRS agar (Oxoid, W. Heidelberg, Australia) adjusted to pH 5.2 and anaerobic incubation at 43°C for 72 h. *Streptococcus thermophilus* agar and aerobic incubation at 37°C were used for selective enumeration of *S. thermophilus* as per the method of Dave and Shah (1996a). *L. acidophilus* was enumerated according to the method of Lankaputhra and Shah (1996) using MRS-salicin agar and bifidobacteria were enumerated according to the method of Lankaputhra *et al.* (1996a) using MRS-NNLP agar.

2.8.1.6. Measurement of enzyme activity, hydrogen peroxide and acetaldehyde

β-D-galactosidase activity of the freeze dried cell preparations and that in the yogurt mix during fermentation was determined according to the method of Shah and Jelen (1990, 1991) using ONPG as substrate. The unit of lactase activity was estimated according to the method of Mahoney *et al.* (1975) as the amount of the enzyme which liberated one μmole o-nitrophenol from ONPG. Concentrations of hydrogen peroxide and acetaldehyde were determined according to the methods of Gilliland (1968) and Millies *et al.* (1989), respectively.

2.8.2. Single step and two step fermentation of yogurt

Single step fermentation: Homogenised and pasteurised milk with a total solid (TS) content of 12% was supplemented with 5% nonfat dry milk, heat treated at 85°C for 30 min and cooled to 42°C (Fig. 2. 2.). For the control batch, overnight grown fresh culture of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* was added at the rate of 0.5% and that of *L. acidophilus* 2409 and *B. longum* 1941 at 2%. Inoculated mixes were poured into plastic cups and incubated at 42°C till the pH reached 4.5. Changes in the viable counts of *L. acidophilus* 2409 and *B. longum* 1941, and pH and levels of H_2O_2 and acetaldehyde were monitored at weekly intervals for 6 weeks. Selective enumeration of *L. acidophilus* 2409 and *B. longum* 1941 and the contents of hydrogen peroxide and acetaldehyde in yogurt

were determined by the methods described in sections 2.5.4 , 2.5.2, 2.4.2 and 2.4.3, respectively.

Two step fermentation: Homogenised and pasteurised milk containing 17% TS was heat treated at 85°C for 30 min, cooled to 42°C, and overnight grown fresh culture of *L. acidophilus* 2409 and *B. longum* 1941 was added at the rate of 2.0% followed by incubation at 42°C for 2 h (step 1 fermentation). After the initial fermentation, overnight grown yogurt bacterial culture was added at the rate of 0.5%. The mix was distributed in 100 mL plastic cups and incubated at 42°C (step 2 fermentation) until the pH reached 4.5. A control batch of yogurt was manufactured without carrying out step 1 fermentation (Figur 2.2). Changes in the viable counts of *L. acidophilus* 2409 and *B. longum* 1941, and pH, and levels of H_2O_2 and acetaldehyde were monitored at weekly intervals for 6 weeks (sections 2.5.4, 2.5.2, 2.4.2 and 2.4.3).

2.8.3. Use of neutralised yogurt mix in yogurt making

One litre aliquots of pasteurised homogenised milk containing 17% TS were adjusted to pH 6.7, 6.8, and 6.9 from initial pH of 6.6, using a sterile saturated solution of $Ca(OH)_2$ and the mixes were heated at 85°C for 30 min, cooled to 42°C and overnight grown fresh cultures of the yogurt (*S. thermophilus* 2010 and *L. delbrueckii* ssp. b*ulgaricus* 2515) and probiotic bacteria (*L. acidophilus* 2409 and *B. longum* 1941) were added at the rate of 0.5% and 2.0%, respectively. After proper mixing, the inoculated mix was dispensed into plastic cups and incubated at 42°C till the pH reached 4.5. Samples of yogurt were chilled to 4°C and the initial counts of *L. acidophilus 2409 and B. longum* 1941, and pH, hydrogen peroxide and acetaldehyde were monitored at weekly intervals for 6 weeks (sections 2.5.4, 2.5.2, 2.4.2 and 2.4.3).

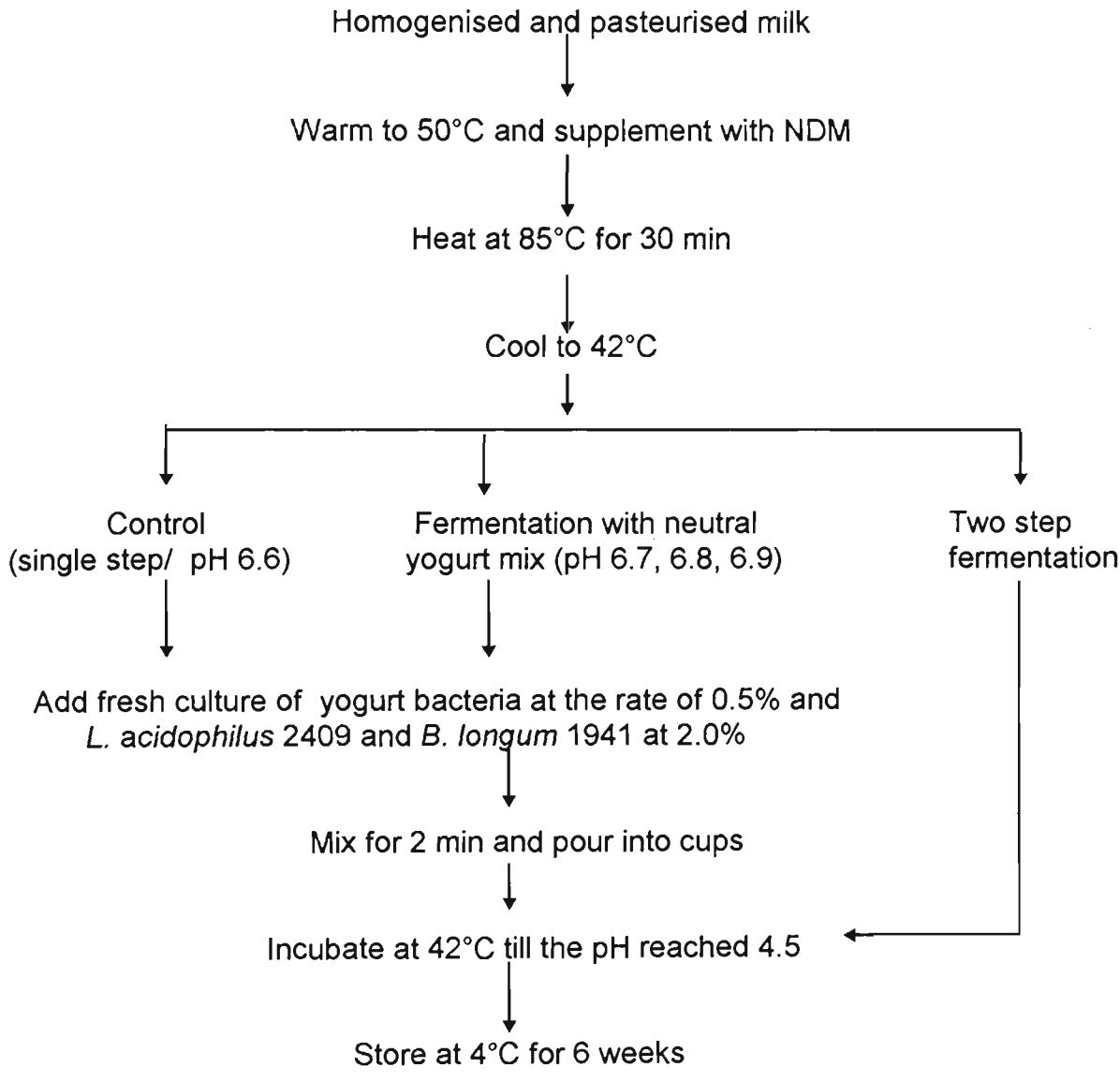

Fig. 2. 2. Flow chart for making yogurt using single or two step fermentation or with neutralised yogurt mix.

2.9. Organic acid production by yogurt and probiotic bacteria due to fermentation of sugars

2.9.1. Preparation of sample extracts from yogurt

An aliquot of 4 g of yogurt or pure cultures grown in milk or MRS broth were weighed accurately into a 25 mL volumetric flask and made up to the volume with 0.005 **M** H_2SO_4. The suspension was mixed using a vortex for 1 min, centrifuged at 10,000 rpm for 5 min, the supernatant filtered through 0.4 μM filter papers, and 1 mL aliquots were pipetted into HPLC vials.

2.9.2. HPLC analysis of organic acids

Levels of organic acids were measured using an Aminex HPX-87H ion exclusion column (section 2.3.2.), a mobile phase of 0.0035 H_2SO_4, and UV-vis detector at 210 nM. Standard curves for lactic, acetic, pyruvic, orotic, butyric, citric, uric and hyppuric was prepared using pure acids. Levels of sugars (glucose, galactose, and lactose) were determined in the same samples using refractive index detector. An Aminex HPX-87H column and a mobile phase of 0.0035 H2SO4 were used. Standard curves were prepared using known quantities of sugars.

2.10. Preparation of buffers and reagents

2.10.1. Phosphate buffered saline (PBS) and phosphate buffer

Phosphate buffered saline (pH 7.4) was prepared by mixing 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g KH_2PO_4 in 1 litre of distilled water. The pH of the buffer solution was adjusted to 7.4 using 1 **N** HCl. Phosphate buffer was prepared (excluding NaCl) by mixing other ingredients in 1 litre of distilled water.

2.10.2. Tris·HCl buffer (1.875 M and pH 8.8)

Tris·HCl buffer (1.875 M 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 5**N** HCl and stored at 4°C for up to four weeks.

2.10.3. Tris·HCl buffer (1.25 M and pH 6.8)

Tris·HCl buffer (1.25 M and pH 6.8) 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.

2.10.4. 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.

2.11. Poly acrylamide gel electrophoresis (PAGE) and staining of the gels

2.11.1. 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 were mixed and degassed for 15 min. The degassed mix was added with 50µl 10% APS, 150 µl 10% SDS and 10 µl of TEMED, and quickly mixed. A quantity of 3.5 mL was added per gel.

2.11.2. 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 were mixed and degassed for 15 min under vacuum. The degassed mix was added with 10µl of 10% APS, 50 µl of 10% SDS and 5 µl of TEMED and mixed quickly. The stacking mix was applied on to the top of the running gel and combs were inserted.

2.11.3. 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_20. 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 destaining solution which was similar in composition to the fixing solution (4 parts of ethanol, 1 part of acetic acid, and 5 parts of H_20). During destaining, the gels were transferred into fresh destaining solutions three times. After destaining, 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.

2.11.4. Staining the gels with silver stain

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_20. 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 H_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 silver solution prepared by mixing 0.25 g of silver nitrate and 50 µl of formaldehyde in 250 mL distilled water. The gels were transfered into developing solution (made with 6.25 g Na_2CO_3 and 25 µl 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 dihydrate in 250 mL of distilled water. The gels were then placed in preserving solution prepared with 25 mL of glycerol made up to 250 mL of distilled water.

2.12. PAGE studies of lab protein profiles

2.12.1. Preparation of cell extracts

Yogurt and probiotic bacteria were grown in MRS broth (section 2.5.3) at 37°C for 18 h and the cells were harvested by centrifuging at 10,000 rpm using a Beckmann J2-HS centrifuge (2.3.6). The harvested cells were suspended in phosphate saline buffer (pH 7.0) and refrigerated at 4°C for 3 h. Cells were ruptured using MSK cell homogeniser (section 2.3.5), and the cell suspensions

were stored in ice and the cell debris was removed by centrifuging in Eppendorf tubes using a micro-centrifuge. The clear supernatant was aliquoted in 200μL quantities and stored in a -20°C freezer until needed for gel electrophoresis.

2.12.2. Gel preparation and electrophoresis

The frozen cell extracts were thawed at room temperature and the protein contents were determined by Lowrey assay (section 2.4.5). The extracts were diluted with double distilled water to achieve a final concentration of 10μg/mL, and the samples were mixed with loading buffer (section 2.9.6.) and boiled for 5 min in Eppendorf tubes placed in boiling water bath. The boiled samples were briefly centrifuged and loaded to the gel along with molecular size standards (in the first and the last well). Pure samples of enzymes obtained from Sigma Chemicals (section 2.1.1) were also loaded alongside the samples into the gel (section 2.11.) and electrophorasis was carried out at using a current of 30 milli Ampere using a powerpack and stained with Coomassie blue or silver stains. Stained gels were photographed with a polaroid camera and dried using a gel dryer.

2.13. Preparation of human cell culture media and reagents

2.13.1. McCoy's 5A Modified Medium and Dulbecco's Modified Eagle's Medium (DMEM)

McCoy's 5A modified medium (Sigma Chemical Company) was prepared by dissolving 16 g contents of a bottle containing dry powder of the medium in 1 L of sterile distilled water. The solution was supplemented with 2.2.g of Na_2HCO_3 and 1.5 g of glucose. The powdered medium contained phenol red as a pH indicator and the pH of the solution was 7.3 ± 0.3 after addition of Na_2HCO_3. The solution was filtered through a 0.2 μ sterile filter paper, stored at 4°C and used within two weeks of preparation. Dulbecco's Modified Eagle's Medium (DMEM) was also obtained from Sigma (Sigma Chemical Company) which was available in the powdered form. This medium also was prepared the same way as the McCoy 5A

medium except that 3.7 g of Na_2HCO_3 and 3.5 g of glucose were dissolved before filtration. The final pH of DMEM medium was 7.3 ± 0.3.

2.13.2. Trypsine-versene

Trypsine-versene solution was prepared by mixing 0.1% trypsin and 0.02% versene solutions. Trypsin solution (2.5%) was prepared by dissolving 0.5 g in 20 mL of sterile distilled water. Versene solution (x10) was prepared by mixing 40 g of NaCl, 1 g of KH_2PO_4, 1 g of KCl, 5.75 g of Na_2HPO_4, 0.335 g of NaOH, 1.0 g of EDTA in 500 mL of sterile distilled water. To prepare trypsin-versene solution (0.1% trypsin and 0.02% versene), 430 mL of distilled water was added with 50 mL of versene and 20 mL of trypsin solution and the pH was adjusted to 7.2 -7.4 with 2 N HCl. The trypsin-versene solution was sterilised by filtering through a 0.2 μ filter paper and stored at -20°C.

2.13.3. Foetal calf serum (FCS)

Foetal calf serum (Sigma Chemicals Company) was received in heat inactivated (56°C for 30 min) and frozen form. The thawed solution of FCS was aliquoted in 10 mL portions and stored at -20°C. FCS (10%) was used in McCoy 5A medium and DMEM before using for cell culture.

2.13.4. Antibiotic preparations for cell culture

Penicillin-streptomycin (Penstrep) solution (Sigma Chemicals Company) containing 10,000 units of penicillin and 10 mg of streptomycin per g was used in cell culture. Penstrep solution was used in Maccoy 5A or in DMEM at a concentration of 1 mL/100 mL of liquid medium. Gentamycin (Sigma Chemicals Company) was used at a concentration of 50 μg per/ mL.

2.13.5. Revival of frozen cell cultures

Frozen cell cultures were thawed by immersing the vials of cells in a water bath at 37°C for about 30 seconds. The thawed cells were transferred immediately

into 50 mL of cell culture medium and centrifuged at 1800 rpm for 2 min. The supernatant was discarded and the remaining cell pellets were suspended in 20 mL of the cell culture medium and transferred to one or more T-flasks and incubated at 37°C.

2.13.6. Subculturing of Ht-29 cell cultures

Cell cultures grown from frozen stocks were periodically subcultured when the monolayers reached confluency. In order to subculture, the monolayers of cells which were bound to each other by intercellular protein bonds were required to be separated to single cells. Culture medium of a confluent culture was removed and 3 mL of Trypsine-versene solution was added to the cell layer. After spreading on the cell layer by slowly slanting the flask sideways, the trypsin-versene solution was quickly removed and a fresh aliquot of 3 mL of the same solution was added to the culture and incubated at 37°C for 10 min. After removing from the incubator, the bottom of the flask was tapped on the palm to loosen the cells and 10 mL of the cell culture medium was squirted in to the flask using a pipette. The cell suspension was sucked through the pipette, re-squirted several times to separate the cells properly, dispensed into 5 new flasks and each flask was added with 5 mL of fresh cell culture medium. The flasks were incubated at 37°C until the cells reached confluency.

2.14. Effect of lactic acid bacteria on Ht-29 cells

2.14.1. Measurement of the effect of LAB in growth of Ht-29 cells

Ht-29 cells were grown in 25 cm^2 T-flasks by adding 5mL of trypsinised cell suspension (1 x 10^6 cells/mL) into each flask followed by 10 mL of McCoy 5-A medium. The flasks were incubated at 37°C for 48 h and the medium was removed gently and fresh medium was added. Each flask was added with penicillin-G at a concentration of 1 IU/mL in order to prevent the growth of bacteria and subsequent acidification which may be harmful to the survival.

Thirty six cell culture T-flasks were used for each bacterial strain. One mL of thawed neutralised bacterial suspensions were added to 18 T-flasks and the other T-flasks (controls) were not added with the bacterial suspension. Every 24 h the cell culture media were changed and bacterial suspension was added freshly. Cells were harvested from 3 flasks each time on day 1, 7, 14, 21, 28 and 35 using trypsin solution in order to segregate the intercellular bonds of Ht-29 cell monolayers. Similarly, 3 control flasks of cells (untreated with probiotic bacteria) were also harvested. Harvested Ht-29 cell suspensions from each flask was separately stained with trypan blue and the count of viable cells determined using a haemocytometer as described in section (2.13). Same procedure was followed after 14, 21, 28 and 35 days too.

2.14.1.1. Use of trypan blue

Trypan blue was used to determine the numbers of dead and live cells in culture. The method was based on the principle that the live cells do not take up the dye whereas the dead cells do (Sigma Biosciences Cell Culture Catalogue, 1996). The cell suspension prepared in Hank's Balanced Salt Solution (HBSS) and 0.5 mL of 0.4% trypan blue (W/V) was transferred to a test tube, 0.3 mL of HBSS and 0.2 mL of the cell suspension (dilution factor = 5) was add and mixed thoroughly, and the mixture was allowed to stand 5 to 15 minutes.

2.14.1.2. Direct counting of dead and viable cells using haemocytometer

Using a Pasteur pipette, a drop of the cell suspension was placed on a haemocytometer with the cover-slip in place and the chamber was allowed to fill with capillary action., All the dead and live cells in the centre square and the four one mm corner squares were counted starting with the chamber 1 of the haemocytometer. The procedure was repeated for chamber two.

Each square of the haemocytometer, represents a total volume of 0.1 mm^3 (10^{-4} cm^3). As 1 cm^3 is equivalent to 1 mL, the cell concentration per mL will be determined as follows:

Cells per mL = average count per square x dilution factor x 10^4
and, Cell viability (%) = total viable cells (unstained) /total cells (stained and unstained) x 100

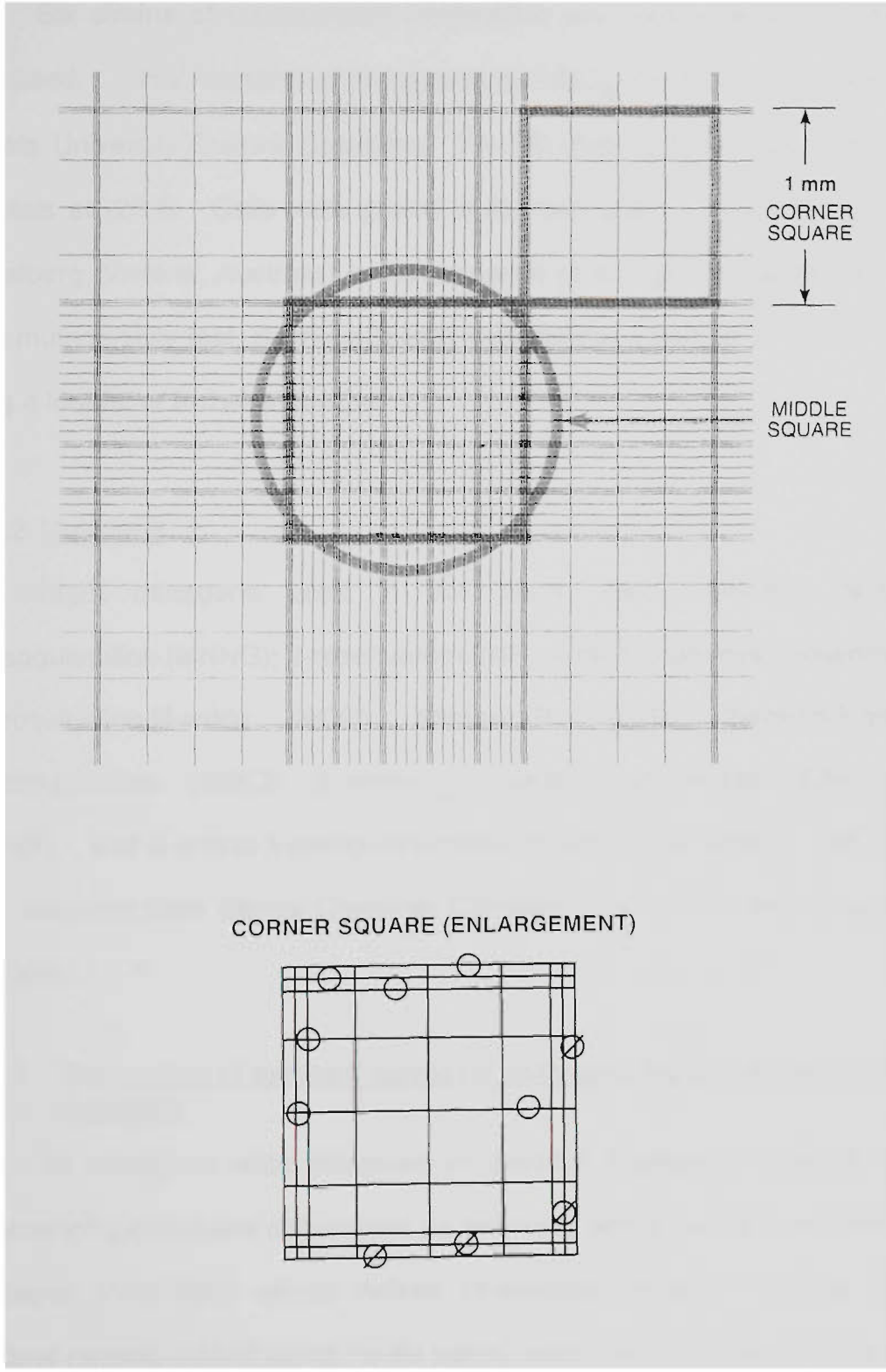

Fig. 2.3. Standard haemocytometer counting chamber

2.15. Determination of antimutagenic activity of probiotic bacteria

2.15.1. Bacterial strains

Six strains of *Lactobacillus acidophilus* and nine strains of bifidobacteria were used. $His^{(-)}$ mutants of *Salmonella typhimurium* TA-100 was obtained from Victoria University Culture Collection. TA-100 stock cultures were kept in 1 mL cryovials at -20°C. Cells were grown in Nutrient Broth II (Oxoid Australia, West Heidelberg, Victoria, Australia) in the presence of 25 μg/mL of ampicillin. Prior to each mutagenicity test, *Salmonella* cells were freshly grown at 37°C for 10 to 12 h using a loopful of frozen inoculum.

2.15.2. Mutagens

Eight mutagens used in this study were: N-methyl, N'-nitro, N-nitrosoguanidine (MNNG); 2-nitrofluorene (NF); 4-nitro-O-phenylenediamine (NPD); 4-nitroquinoline-N-oxide (NQO); Aflatoxin-B (AFTB); 2-amino-3-methyl-3H-imidazoquinoline (AMIQ); 2-amino-1-methyl-6-phenyl-imidazo (4,5-b) pyridine (AMPIP), and 2-amino-3-methyl-9H-pyrido (3,3-6) indole (AMPI). All mutagens were obtained from Sigma Chemical Company (Castle Hill, New South Wales, Australia).

2.15.3. Preparation of standard curves for estimating the concentration of mutagens

All mutagens were dissolved in dimethyl sulphoxide (DMSO) and their absorbence peaks were determined by scanning with a uv-vis spectrophotometer (Ultrospec Plus 4054 uv-vis, Amrad Pharmacia, Boronia, Victoria, Australia). Dilutions ranging from 2 μg/mL to 50 μg/mL were used for preparing the standard curves. For all mutagens, straight line graphs were obtained below 25 μg/mL. Standard curves were prepared according to the method given by Maron and Ames (1983) using TA-100 mutant of *Salmonella typhimurium* (His-) strain.

2.15.4. Ames Salmonella test and mutagenic reaction

S. typhimurium TA-100 mutant strain requiring histidine was used. This strain is resistant to ampicillin at a concentration of 25 ug/mL. Minimal mineral and glucose solution and minimal agar medium were prepared according to Maron and Ames (1983). TA-100 mutant strain of *S. typhimurium* requires histidine for growth and the organism can not form colonies in minimal nutrient agar plates without histidine. However, this mutant can revert to histidine non-requiring state by undergoing mutation in the presence of strong mutagen. Such revertant mutant is able to grow in the absence of histidine in minimal agar plates. The number of revertant in a minimal agar plate could increase when the concentration of mutagens is increased. However, at higher concentration most mutagens can be toxic to the *Salmonella* cells leading to death of the cells, as a result, the number of colonies could decrease in the plates causing a sudden and abnormal change to the standard curve. Therefore, working concentration for a given mutagen was selected within a range of concentration which gave a straight line standard curve.

2.15.5. Preparation of probiotic bacterial cells

Pure strains of *L. acidophilus* and bifidobacteria were grown in MRS broth at 37°C for 12-15 h and the cells were harvested by centrifuging at 5000 rpm at 4°C for 15 min using a refrigerated Beckman J2-HS centrifuge and Beckmann JA-14 rotor (Beckman Instruments Inc., Palo Alto, California, USA) and washed twice with cold sterile phosphate buffered saline (PBS), resuspended in PBS and the absorbence of the cell suspension was adjusted to 1.00 at 600 nm. The standardised bacterial suspensions were stored at $\leq$ 4°C and used within 24 h.

2.15.6. Preparation of killed cell suspensions of probiotic bacteria

The cell suspensions with absorbence value of 1.00 were heat treated by immersing in water bath at 100°C for 15 min. After the heat treatment, the cells were vortexed for about 5 min to break any coagulum formed during heating. The

heat killed cells were plated in MRS agar in order to determine the efficiency of heat treatment.

2.15.7. Binding of mutagens by live or killed probiotic bacteria

Stock solutions of mutagens were prepared by dissolving in dimethyl sulphoxide (DMSO) to give a concentration of 1 mg/mL. One millilitre aliquots of the probiotic bacterial suspensions were placed in small sterile bottles in triplicate and measured quantity of each mutagen stock solution was added to the bacterial suspensions to give a final concentration of 10 μg/mL. Control samples were prepared for each mutagen in PBS without probiotic bacteria. The suspensions of mutagens with or without probiotic bacteria were incubated at 37°C for 3 h in a shaker incubator, cell suspensions centrifuged at 5000 rpm at 4°C using a refrigerated centrifuge, supernatant decanted and filtered using 0.45μ filter papers. The filtrate was divided into two portions and refrigerated, one portion was used to determine the quantity of unbound mutagen concentration by measuring the absorbence values using a UV-VIS spectrophotometer at relevant wave lengths as determined earlier and the other portion was used to determine the remaining mutagenic activity in the bacterial cell-mutagen suspensions using Ames test (Maron and Ames, 1983). For each strain of bacteria and for each mutagen, antimutagenic activity was calculated.

2.15.8. Recovery of bound mutagens from the killed bacterial cells

Incubation was carried out as before. After incubation, the bacterial suspensions were centrifuged and the supernatant were refrigerated for determining the concentrations. Dead bacterial cells pellets were washed with PBS twice, suspended in DMSO, vortexed for 5 min, centrifuged and the supernatant separated for determining the quantity of mutagens recovered from the dead cells.

2.15.9. Antimutagenic activity of probiotic bacteria

Antimutagenic activity of cell free suspensions of mutagens, incubated with or without probiotic bacteria, were used in the Ames test (Maron and Ames, 1983). The number of revertant colonies produced by the supernatant as compared with the controls were determined. The number of spontaneous revertants was determined by preparing triplicate plates as per the Ames test (Maron and Ames, 1983) without any mutagens. At a concentration of $1x10^8$ cfu/mL *Salmonella* cells, 15-20 revertant colonies appeared. The number of $His^{(+)}$ revertant colonies were counted in each plate.

2.15.10. Antimutagenic activity of short chain fatty acids

One percent solutions of lactic, acetic, pyruvic and butyric acids were prepared in Milli Q (double distilled grade) water, the solutions neutralised with 1 N NaOH to a pH of 6.5, filtered through a 0.45μm filter paper using sterile syringes, and 200μL aliquots of each solution were added to the top agar mix along with mutagen and allowed to stand for 30 min at 40°C before pouring the top agar mix on to the minimal agar plates. The top agar mix contained 2 mL of 0.6% agar, 0.4% of NaCl, mutagen, acid and *Salmonella* cells. Controls were prepared without acid solutions. The number of revertant colonies in the plates was determined in comparison with the control which did not contain any acid.

2.15.11. Determination of antimutagenic activity from the plate counts

The number of revertant colonies increased in the agar plates with increasing concentration of mutagens within the range of concentration that produced straight line in the standard curves. Reduction in the number of colonies in the test plates as compared with those in the control indicated a reduction in the mutagenic activity. Percentage reduction in the number of revertant colonies as compared with those produced by the control was expressed as percentage antimutagenicity.

Antimutagenicity of each cell or acid preparation was determined based on the percentage reduction in the number of revertant colonies in the presence of probiotic bacteria or acids. The mean value of revertant colonies appeared in the control plates, which were prepared without probiotic bacterial preparations or acids, was used as the basis for comparison and percentage values of the number of revertant colonies were determined as compared to the base values from the control tests.

2.16. Adherence of probiotic bacteria to Ht-29 cells

2.16.1. Preparation of Ht-29 monolayers for adherence assay

Sterile glass coverslips were placed on the bottom surface of 8 well cell culture plates and an aliquot of freshly trypsinised cells were pipetted into the wells so that the cells settle on the upper surface of the glass coverslips. These culture plates were incubated at 37°C for 2-3 weeks, with daily changes of cell culture medium. When the monolayers became confluent, the culture medium was carefully pipetted out leaving the cell layers attached to the cover slips and the latter were used for adherence assays and microscopical studies.

2.16.2. Light microscopic study of adherence

Two to three weeks old confluent monolayers of Ht-29 cultures were used in this study. Bacterial suspensions were pipetted into the wells which contained the coverslips with monolayers. After incubating the plates at 37°C for a desired period of time, the bacterial suspensions were carefully pipetted out and the wells were rinsed 6 times using PBS in order to remove any loose cells remaining on the surface of monolayers. After rinsing, the monolayers were fixed by pipetting 2 mL of cold (chilled in a -20°C freezer) undiluted methanol for 20 min. After removing methanol, the coverslips were air dried. Immediately before staining, the coverslips were wetted with distilled water and a drop of crystal violet was placed on the coverslip and washed with water after 10 sec. The sample was counter

stained with Gram's iodine, washed with water after 10 sec and with 95% ethanol for 10 seconds followed by a quick rinse with water. The coverslips were mounted on slides and looked under the a microscope after blotting gently on the surface of the monolayers.

2.16.3. Effect of proteins in spent broth on adherence of probiotic bacteria

Probiotic bacteria were grown in MRS broth for 18 h at 37 °C and the cells were separated by centrifuging in 10 mL tubes at 5000 rpm for 10 min. The supernatant was decanted and stored in a refrigerator. The cell pellets were washed with PBS, centrifuged and resuspended in 10 mL of PBS and stored in a refrigerator. Four sets of experiments were carried out as shown below:

(1). Cells in the original broth without centrifuging (control)
(2). Washed pellets suspended in fresh MRS medium
(3). Washed pellets suspended in PBS
(4). Washed pellets suspended in supernatant treated with trypsin (section 2.15.3.1)
(5). Washed pellets treated with trypsin (section 2.15.3.2)

All samples were added with 1 mL of bacterial cell suspension and mixed by vortexing for 1 min. One millilitre aliquots of the cell preparations were added to the Ht-29 monolayers prepared as described in section 2.15.1. After incubating for 2 h at 37°C, bacterial suspensions were pipetted out and the monolayers were rinsed, fixed, and stained as described in 2.51.2. The bacterial cells adhering on to Ht-29 cells were clearly visible under a light microscope (x 1000) and the numbers of adhering bacteria were counted in 10 random microscopic fields.

2.16.3.1. Treatment of supernatant with trypsin

An aliquot of 10 mL of supernatant was added in a tube with 0.1 mL of (100 mg/mL) trypsin and incubated at 37°C for 1 h, the contents of the tube was mixed by vortexing for 1 min, 0.5 mL of foetal calf serum added to (FCS) to inactivate trypsin and incubated at 37°C for 1 h.

2.16.3.2. Treatment of bacterial cells with trypsin

Bacterial cell pellets were suspended in 10 mL of PBS in a tube and 0.5 mL of trypsin (100 mg/mL) was added and incubated at 37 °C for 1 h, the tube was vortexed for 1 min, 0.5 mL of foetal calf serum (FCS) added to inactivate trypsin and incubated at 37°C for 1 h. The cell suspension was centrifuged at 5000 rpm for 10 min and the pellet was resuspended in fresh PBS.

2.16.4. Study of effect of polysaccharides on adherence of probiotic bacteria

One of the cell pellets was suspended in 8 mL of PBS instead of 10 mL. Two millilitre aliquots of 0.25 **M** sodium periodate was added to the cell suspension and incubated at 37 °C for 1 h. The cells were centrifuged, washed with PBS three times and resuspended in fresh PBS. One millilitre of the cell suspension was added to the monolayer and incubated for 2 h at 37°C. The bacterial suspensions were pipetted out and the monolayers were rinsed, fixed, and stained as described in section 2.51.2. The bacterial cells adhering on to Ht-29 cells were clearly visible under a light microscope (x 1000) and the numbers of adhering bacteria were counted in 10 random microscopic fields.

2.16.5. Electron microscopic study of adherence of probiotic bacteria

Ht-29 monolayers grown on coverslips (as described in section 2.15.1) were allowed to adhere with the bacteria (as described in section 2.15.2). The coverslips were then fixed by immersing in 3% gluteraldehyde for 20 min, washed with PBS and processed for electron microscopy (section 7.2).

2.17. Antimicrobial properties of probiotic bacteria

2.17.1. Antimicrobial activity of the spent broths of probiotic bacteria

Six strains of *L. acidophilus* and nine strains of bifidobacteria were grown at 37°C overnight in MRS broth, centrifuged and the supernatant was sterilised using a 0.45μm filter paper. Pathogenic bacteria (*Aeromonas hydrophila, Candida*

albicans, Escherichia coli and Salmonella typhimurium) were grown in nutrient agar pour plates. Wells were bored in the agar layers using a cork borer with 8 mm diameter. Four wells were made in each inoculated agar plate and 0.1 mL of filter sterilised supernatant was added to well number 1. Well number 2 was added with 0.1 mL of the supernatant (pH to 6.5) neutralised by adding 1 N NaOH. Well number 3 was filled with a similar volume of fresh MRS broth and well number 4 was filled with trypsin treated supernatent. The plates were incubated for 18 h and inhibition zones were observed around the wells.

2.17.2. Antimicrobial activity of acids usually produced by probiotic bacteria

Acetic, butyric, lactic, orotic and pyruvic acids were diluted with distilled water to prepare 20 mL dilutions of 100 μg/mL, 1 mg/mL, 10 mg/mL, and 100 mg/mL. Ten millilitre aliquot of each dilution was neutralised with 1 N NaOH to a pH of 6.5, and aliquots of 0.1 mL from each preparation were added to agar wells with pathogens prepared as described in section 2.16.1. After incubating at 37°C for 18 h, zone of inhibition was measured.

2.17.3. Suppression of growth of pathogens in the presence of probiotic bacteria

Fifty millilitre aliquots of nutrient broth (Oxoid) was prepared and sterilised in 100 mL Schott bottles at 121°C for 15 min. The bottles of nutrient broth were inoculated with the active cultures of pathogens (section 2.17.1), and/or probiotic bacteria.

Table 2.4. Volumes of pathogens and probiotic bacteria added in a co-culture to study the growth pattern of pathogens in the presence of different concentrations of pathogenic bacteria

Test	Pathogen	Probiotic strain
1	1 mL	-----
2	-----	1 mL
3	1 mL	1 mL
4	-----	2 mL
5	1 mL	2 mL
6	-----	3 mL
7	1 mL	3 mL
8	-----	4 mL
9	1 mL	4 mL
10	-----	5 mL
11	1 mL	5 mL

After inoculating according to the volumes shown in Table 2.4, the bottles of nutrient broth were incubated at 37°C in a shaker incubator. Samples were drawn every 30 min for 12 h and absorbency at 600 nm was recorded. Growth curves were prepared based on absorbency values.

3.0. SURVIVAL OF *LACTOBACILLUS ACIDOPHILUS* AND *BIFIDOBACTERIUM BIFIDUM* IN COMMERCIAL YOGURT DURING REFRIGERATED STORAGE

3.1. Introduction

It is important to maintain the viability of probiotic bacteria, until the products are consumed in order to ensure maximum delivery of organisms. Beneficial effects of *L. acidophilus* and bifidobacteria may be greater if the ingested viable cells can colonise in the intestine. The most commonly used species in commercial products are: *L. acidophilus, L. caseii, Lactobacillus GG* (closely related to *L. casei* subgroup *rhamnosus*), *B. bifidum, B. longum, B. breve, B. infantis* (Rasic and Kurmann, 1983; Ishibashi & Shimamura, 1993). *L. acidophilus* and *B. bifidum* are unstable in yogurt when added after its manufacture and these organisms lost their viability rapidly (90-99%) within three to five days and completely disappeared within seven days (Gilliland and Speck, 1977; Schioppa *et al.*, 1981; Hull *et al.*, 1984). In an independent study (Anon, 1992) several brands of commercial yoghurts from Australian supermarkets were analysed for the presence of *L. acidophilus and B. bifidum.* All products contained very low numbers of *B. bifidum.* The *L. acidophilus* counts were also very low in some products. Survival of *L. acidophilus* and bifidobacteria is affected by low pH of the environment. Although *L. acidophilus* survives better than yogurt culture organisms (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*), a rapid decrease in their numbers has been observed under acidic conditions, both *in vitro* and *in vivo* (Conway *et al.*, 1987; Hood and Zotolla, 1988; Shah and Jelen, 1990). Bifidobacteria are not as acid tolerant as *L. acidophilus* and the growth of the former organism is significantly retarded below pH 5.0. Growth of *L. acidophilus* ceases below pH 4.0 (Playne, 1993).

In order to investigate the viability status of *L. acidophilus* and *B. bifidum* in yogurt a survey was carried out using yogurt products from leading yogurt

manufacturers in Australia. Five yogurt products were used in this study and the viability of the two probiotic bacteria were investigated during 5 weeks of storage. Changes in pH and titratable acidity and protein and total solids content of the five brands of yogurt were also studied.

3.2. Materials and methods

Yogurt samples were obtained as described in section 2.2.1. Enumeration of *L. acidophilus* and *B. bifidum* was carried out at 3 day intervals for a period of 5 weeks as described in section 2.5.3 using MRS-maltose agar (section 2.5.4.) and NNLP agar (section 2.5.6.) in order to selectively enumerate *L. acidophilus* and *B. bifidum*, respectively. Titratable acidity (TA) and pH were also measured at 3 day intervals as in section 2.4.1. Total solids (TS) (section 2.4.6.) and protein content (section 2.4.6.2.) were determined for all the products.

3.3. Results and discussion

Changes in pH and TA of yogurt during five weeks of storage at 4°C are shown in Figs. 3.1. and 3.2. All products showed a decrease in pH (0.07 - 0.42). Traditionally most yogurts are manufactured by addition of yogurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*). These organisms seem to be active even at low temperatures and produce small amount of lactic acid by fermentation of lactose resulting in gradual pH decrease. Initial pH was lowest in product 1 (pH 4.06). Products 2 and 3 had a higher pH value at day 0 (pH 4.36 and 4.33) as compared with the other products and after 5 weeks of storage, pH of these two strains remained highest at 4.25 and 4.13. Product 1 and 2 showed smallest drop in pH (< 0.1), during five weeks of storage followed by product 3 which reduced pH by < 0.2 . Product 4 showed the highest reduction in pH during refrigerated storage. At day 0, the pH of product 4 was 4.22 and within 9 days pH

dropped by about 0.35 followed by further drop by about 0.1 during the rest of the storage period. Product 5 showed a decrease in pH of 0.2 - 0.3.

Figures 3.1. and 3.2. show the change in TA of the products during 5 weeks of storage at 4°C. As shown, the products 1 and 4 had higher TA levels as compared with the other products. High TA levels in these products were complementary with low pH levels of the products. After five weeks of storage, product 1 and 4 reached 1.56 and 1.60%, respectively. Products 2, 3, and 5 reached TA levels of 1.37, 1.41, and 1.48%, respectively. The products which had low TA levels showed high pH levels during storage.

The pH of yogurt can affect the viability of *L. acidophilus* and bifidobacteria (Laroia and Martin, 1991). It has been found that acid production ability of yogurt bacteria, especially post incubation (post acidification) affected the viability of *L. acidophilus* and bifidobacteria (Ishibashi and Shimamura, 1993).

Changes in the viable counts of *L. acidophilus* and *B. bifidum* in five brands of yogurt are given in Figures 3.3 and 3.4 Products 1 - 3 contained 10^7 to 10^8/g viable cells of *L. acidophilus* when fresh. In these products, the number of viable cells of *L. acidophilus* remained high until 30 days from the date of production and then declined (Fig. 3.3). Products 4 and 5 contained $\leq 10^5$/g viable cells of *L. acidophilus* when fresh and their number declined rapidly (Fig. 3.4). Products 2 and 4 contained 10^6 to 10^7/g viable cells of *B. bifidum* initially. In the other products (1, 3 and 5), the viable number of *B. bifidum* in fresh sample was $\leq 10^3$/g. All the products showed a constant decline in the numbers of *B. bifidum* after production. The decline was more rapid for *B. bifidum* as compared *to L. acidophilus* in all the yogurts during storage. At the end of five weeks storage, very few viable cells of *B. bifidum* were found in the products.

It has been suggested that to have therapeutic effects, the minimum number of these organisms in a product should be > 10^5/g and that one should

aim to consume 100 million (10^8) live cells of these bacteria per day (Anon, 1992). A serving of 100 g yogurt containing 10^6 cells/g would supply this amount. Some fresh products examined in this study, especially products 2 and 4, would be suitable for this purpose. However, other yogurts had low levels of these organisms initially and contained very few or none at the end of five weeks of storage, as the probiotic bacteria had died off in the product during the refrigerated storage, most likely due to post-acidification by yogurt culture bacteria. Several other factors may be responsible for the reduced viability of these organisms, such as hydrogen peroxide produced by the yogurt culture bacteria, oxygen level in the product or oxygen permeation through the package.

Fig. 3.5. illustrates the relationship among initial and final pH, protein content, loss of viability of *L. acidophilus* and *b. bifidum* and total solid content in yogurt. Products 1, 2, and 3 had high TS levels (16-18%) and high protein contents ranging from 4.9 to 5.9% whereas products 4, and 5 had low total solid levels (14%) and a protein content of 4.2%. The results showed that increased solid levels also increased the protein contents in yogurt. As shown, loss of viable counts of *L. acidophilus* in the products was low in products 1, 2 and 3 and that in product 4 and 5 were high. Although bifidobacteria did not show a clear relationship, products 1 and 3 showed a lower reduction in viability of *B. bifidum* and products 4 and 5 had high reduction in viable counts. Although product 2 also had high protein content, the loss of viable count of *B. bifidum* was high. The results suggest that the higher contents of proteins may have effect in reducing the loss of viable counts of *L. acidophilus* and *B. bifidum*. This could be possible as proteins act as buffers against pH changes. However, as different products may have different strains of *L. acidophilus* and *B. bifidum*, it may be difficult to draw conclusions on the effect of change of pH or high solid content on the viability of probiotic bacteria. In order to confirm that high levels of milk solids and

proteins have effect on improved viability, it may be necessary to design experiments using yogurt manufactured with various levels of milk solids and proteins and known strains of *L. acidophilus* and bifidobacteria.

3.4. Conclusion

Enumeration of viable *L. acidophilus* and *B. bifidum* in five commercial yogurts showed variable levels of these organisms in the products surveyed. All the products showed a constant decline in the numbers of viable *B. bifidum* during 6 weeks storage, while viability of *L. acidophilus* remained high in three of five products. The decrease in pH values of between 0.07 and 0.42 pH units during the storage period may have affected the viability of the organisms. As the results suggested that higher solid levels seemed to have beneficial effect on the viability of the probiotic bacteria, especially *L. acidophilus*, the contribution of increased solid levels in improving the viability of *L. acidophilus* and bifidobacteria in yogurt should be further investigsted.

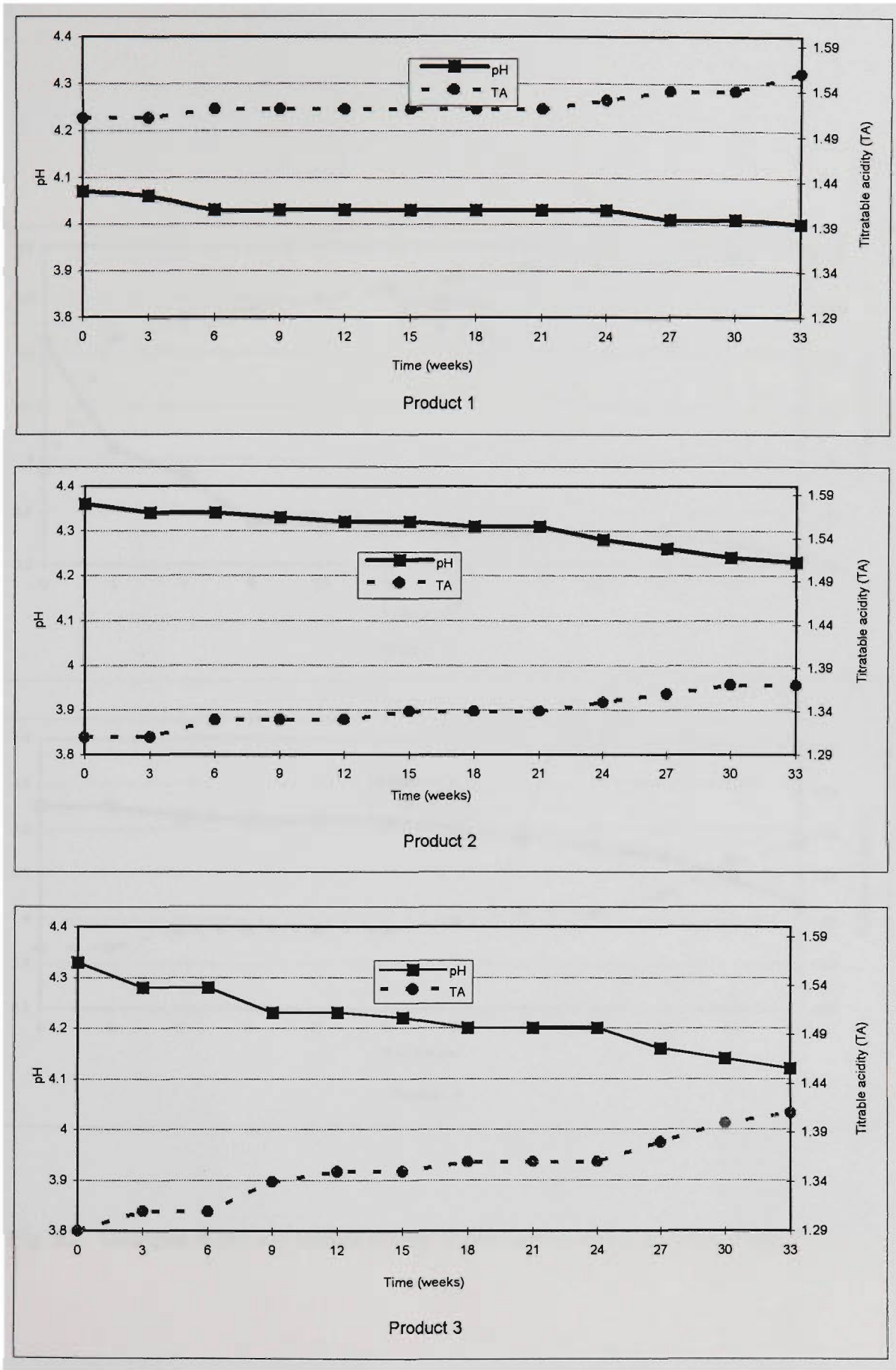

Fig. 3.1. Changes in pH and titrable acidity of products 1, 2, and 3 during storage at 4°C

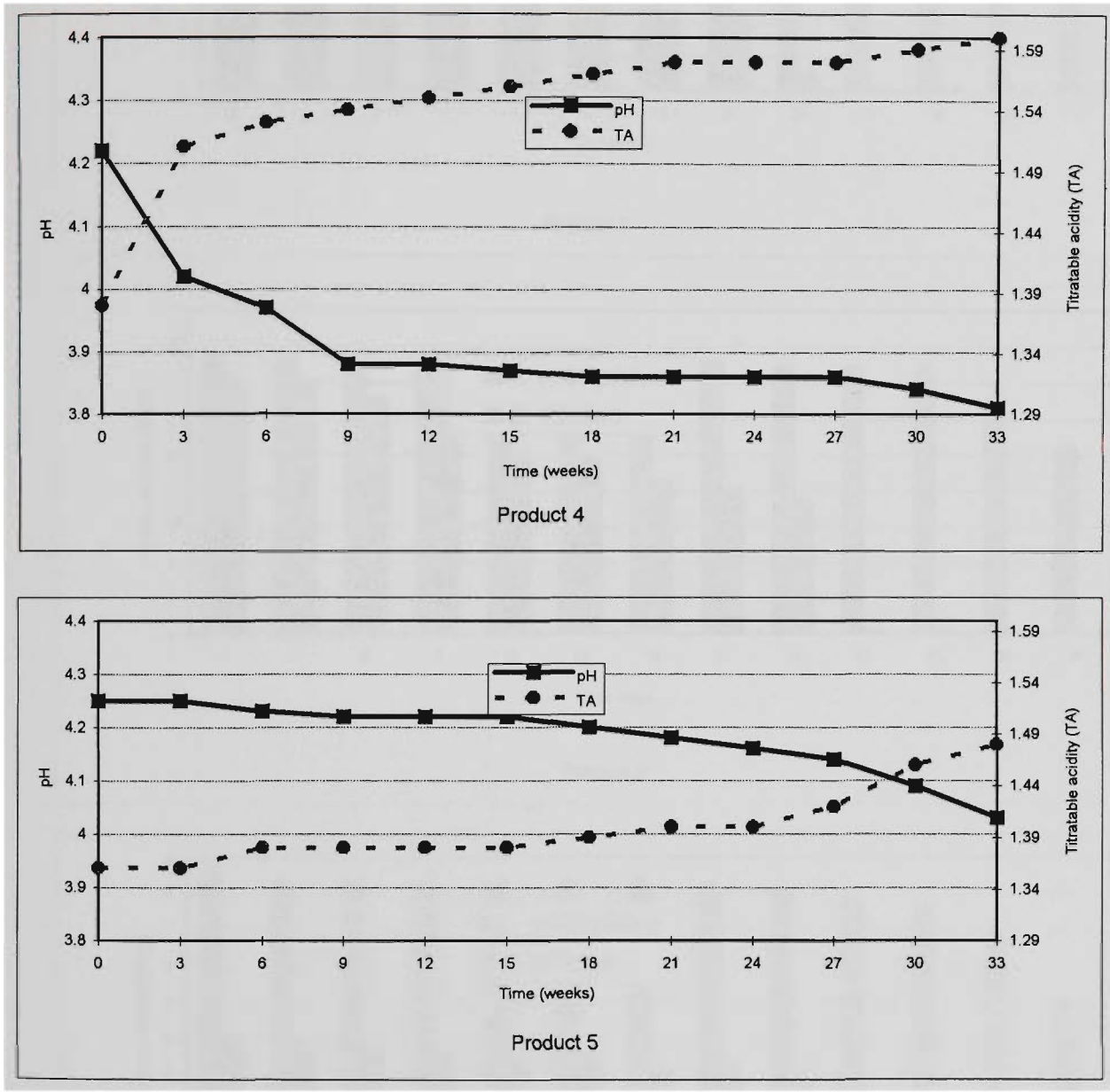

Fig. 3.2. Changes in pH and titrable acidity of products 4, and 5 during storage at 4°C

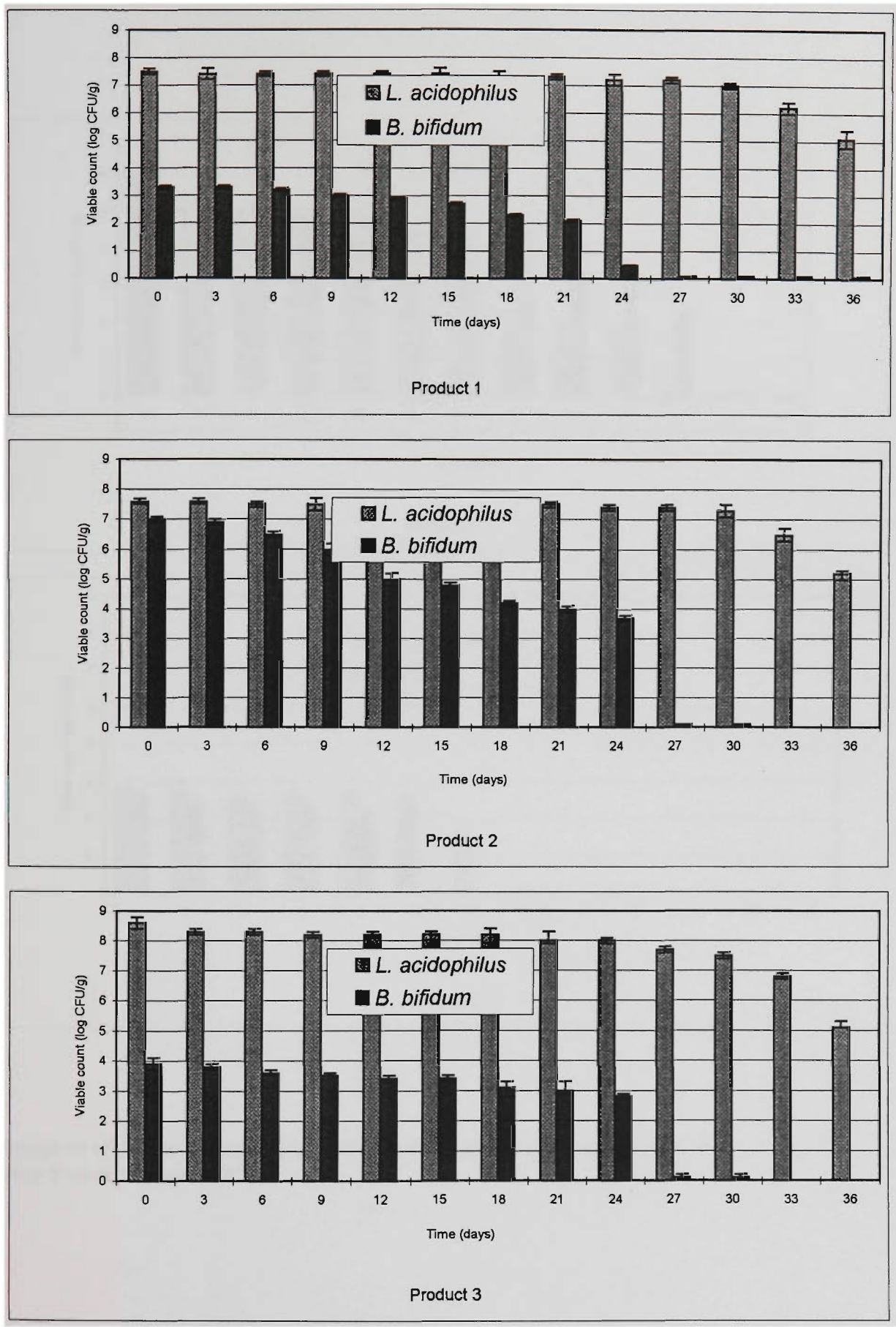

Fig 3.3. Changes in viable counts of *L. acidophilus* and *B. bifidum* in yogurt 1, 2, and 3 during 5 week storage at 4°C

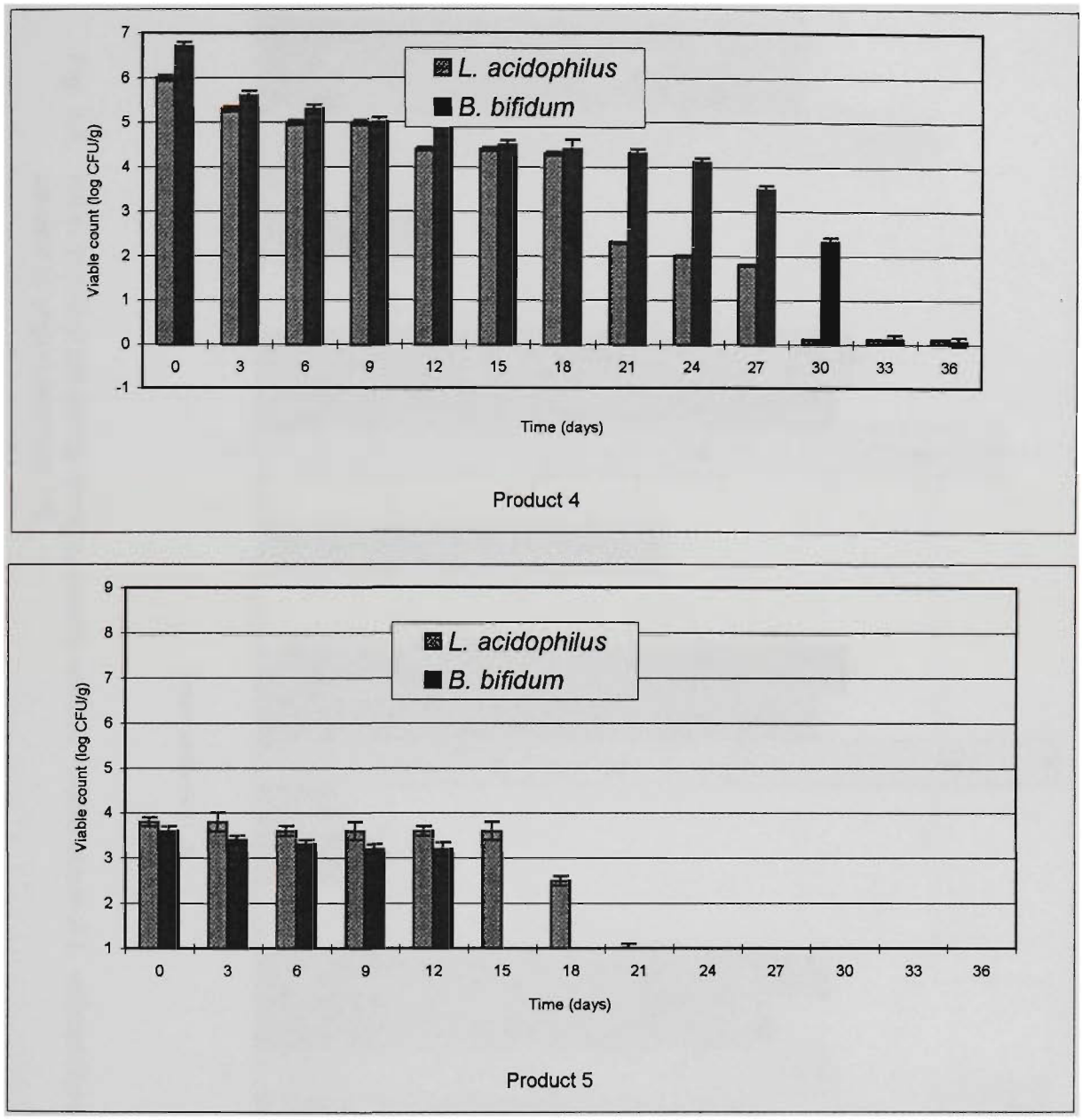

Fig 3.4. Change of viable counts of *L. acidophilus* and *B. bifidum* in yogurt *4, and 5* during 5 week storage at 4°C

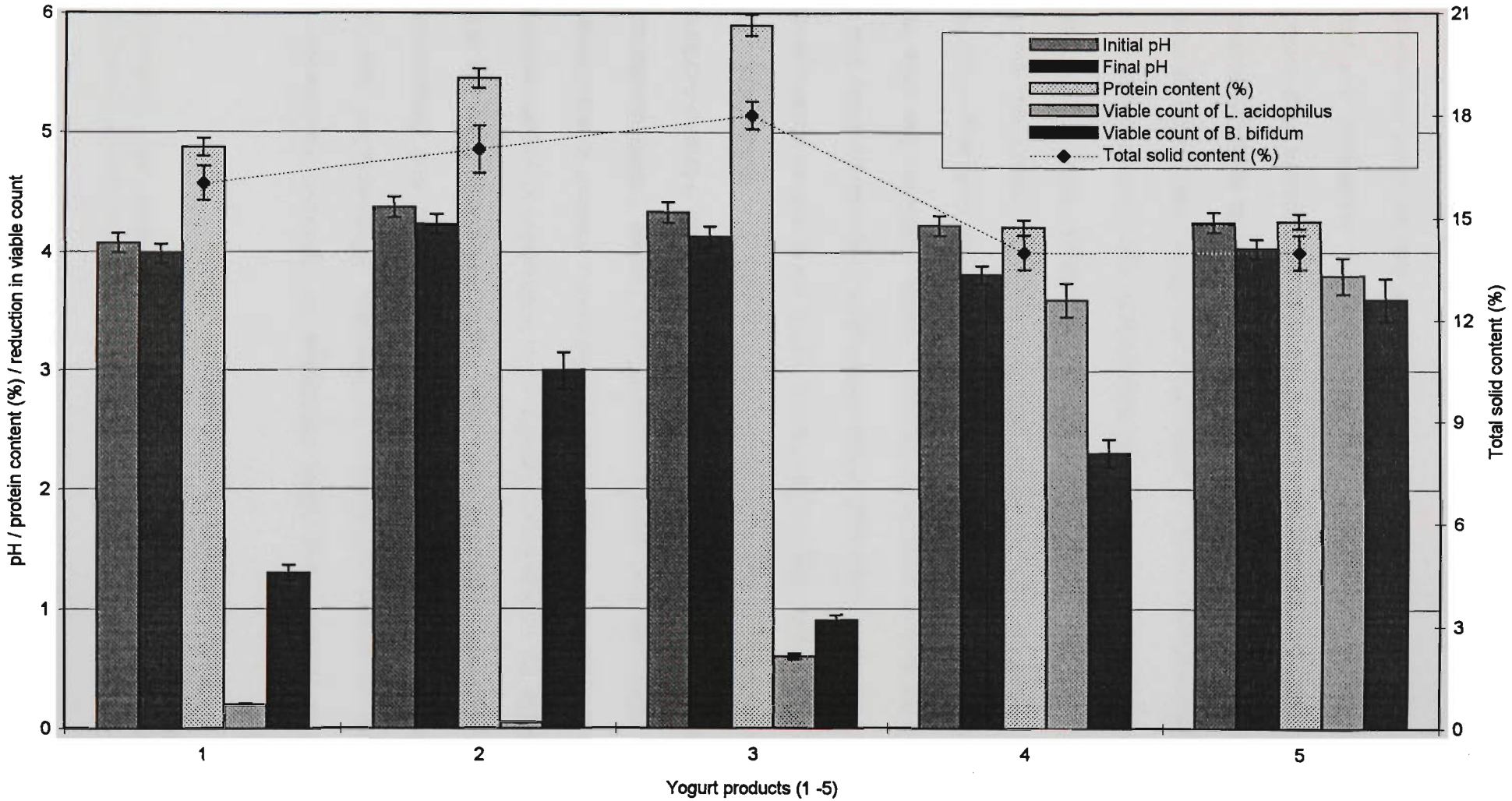

Fig. 3.5. Initial and final pH during storage, protein content, viable count of *L. acidophilus* and *B. bifidum* and total solid content of yogurt products 1-5

4.0. ENUMERATION OF PROBIOTIC BACTERIA

4.1. Selective enumeration of *Lactobacillus acidophilus* in yogurt containing *L. acidophilus*, bifidobacteria, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*

4.1.1. Introduction

A number of media have been developed for selective enumeration of *Bifidobacterium* spp. in yogurt containing *L. acidophilus*, *Bifidobacterium* spp., and yogurt culture bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) (Arroyo *et al.*, 1994; Chevalier *et al.*, 1991). However, there are only a few methods available for selective enumeration of *L. acidophilus* in yogurt containing these four groups of organisms. Hunger (1989) developed a medium to enumerate *L. acidophilus* using cellobiose and esculin. Kneifel and Pacher (1993) developed a medium designated X-Glu agar containing Rogosa medium with 5-bromo-4-chloro-3-indole-β-D-glucopyranoside (X-Glu) for selective enumeration of *L. acidophilus*. Both these methods (Hunger, 1989; Kneifel and Pacher, 1993) are based on chromogenicity of esculin or X-glu. However, high cost of the chromogenic compounds could limit the use of such media for routine analysis of samples. Media containing bile salts have been used to enumerate *L. acidophilus* in sweet acidophilus milk or in yogurt (Gilliland and Speck, 1977). However, the use of bile salts as a selective inhibitor was found to reduce the recovery of viable *L. acidophilus* (Gilliland and Speck, 1977; Speck, 1978). Bile salts allow the growth of several *Bifidobacterium* spp. mainly used in AB (acidophilus and bifidobacteria) yogurt (Lankaputhra and Shah, 1995; Lankaputhra *et al.*, 1996a) thus, limiting the selectivity of the medium. MRS-maltose agar, developed by Hull and Roberts (1984) by replacing glucose of MRS agar with maltose, is suitable for selective enumeration of *L. acidophilus* in yogurt containing *L. acidophilus* and yogurt culture bacteria. However, MRS-maltose medium could not be used for selective enumeration of *L. acidophilus* in yogurt containing *L. acidophilus* and *Bifidobacterium* spp. as the latter organisms form colonies in this medium.

The objective of this study was to develop a simple medium for selective enumeration of a broad range of *L. acidophilus* in yogurt containing yogurt bacteria, *L. acidophilus* and *Bifidobacterium* spp.

4.1.2. Materials and methods

4.1.2.1. Bacterial cultures

Pure cultures of six strains of *S. thermophilus*, five strains of *L. delbrueckii* ssp. *bulgaricus*, six strains of *L. acidophilus* and nine strains of *Bifidobacterium* spp. were obtained as described in 2.2.2.

4.1.2.2. Utilisation of various sugars as carbon source by bacteria

To determine the ability of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and *Bifidobacterium* spp. to utilise various sugars, these bacteria were grown in minimal nutrient agar (MNA) supplemented with salicin, cellobiose, fructose, mannitol and sorbitol (as described in 2.5.5). Glucose was used as the control. One gram of each culture was ten-fold serially diluted (10^2 to 10^6) in 0.1% sterile peptone water, and one mL of each dilution was pour plated in duplicate (as described in 2.5.3). The pour plates were then incubated anaerobically for 72 h at 37°C (12, 20) and after incubation, the colony size was measured randomly.

4.1.2.3. Optimisation of salicin concentration in the media

To determine the optimum concentration of salicin, calculated quantities of 10% salicin solution were added to MNA base to achieve final concentrations of 0.1%, 0.25%, 0.5%, and 1.0% of salicin. One millilitre aliquots of pure culture (10^4-10^6) were plated and incubated anaerobically for 72 h at 37°C. The size of the colonies formed in each concentration of salicin in the medium was measured.

All the experiments and analyses were repeated at least twice. The results presented are means of two replicates.

4.1.3. Results and discussion

4.1.3.1. Utilisation of various sugars as carbon source by S. thermophilus, L. delbrueckii ssp. bulgaricus, L. acidophilus, and Bifidobacterium spp.

Tables 4.1.1 and 4.1.2 show the sugar utilisation patterns of all the four groups of organisms. Glucose and fructose were utilised by all the bacteria studied. MNA medium containing salicin (salicin medium) suppressed the growth of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and all the strains of *Bifidobacterium* spp. studied and supported the growth of *L. acidophilus*. All the strains of *L. acidophilus* formed well developed colonies in salicin agar. As shown in Fig. 4.1.1, *L. acidophilus* strains 2400, 2401, 2404, and 2409 produced sharp and discernible colonies both in MRS and salicin media. Fig. 4.1.2 shows the growth inhibition of *B. bifidum* 1901, *B. longum* 1941 and 20097, and *B. thermophilum* 20210 in salicin medium. These results are in agreement with the data on sugar utilisation of *Bifidobacterium* spp. in Bergey's Manual of Systematic Bacteriology.

L. acidophilus formed large colonies (~1.5 mm) in cellobiose medium; however, this medium suppressed the growth of other groups of bacteria, except that of *B. bifidum* 1900 and *B. pseudolongum* 20099, while *B. longum* 1941 grew poorly. MNA containing mannitol and sorbitol supported the growth of *L. acidophilus*, but the colony size was very small as compared with that formed in salicin and cellobiose media (Table 4.1.2). Further, *B. longum* 1941 formed pin point colonies and *B. pseudolongum* 20099 grew poorly in mannitol and sorbitol media. Growth on MNA containing glucose is shown as a positive control. To validate the efficacy of the salicin medium in selective enumeration of *L. acidophilus*, yogurt was made using the four groups of organisms in a separate experiment. Fig. 4.1.3. shows the growth inhibition of pure cultures of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, and *B. longum* 1941, which along with *L. acidophilus* were used in yogurt manufacture. Pictures X and Y of Fig. 4.1.3. show the selective growth of *L. acidophilus* strains 2401 and 2409, respectively.

Salicin medium was easy to prepare and it involved only a single step. Incorporation of salicin in the MNA medium followed by sterilisation by autoclaving at 121°C produced the same results as those with filter sterilised salicin solution added to sterile MNA base.

4.1.3.2 Optimisation of salicin concentration for selective enumeration of L. acidophilus

It was important to determine the optimum concentration of salicin required to produce sufficiently large colonies. Table 4.1.3 shows the colony size of *L. acidophilus* formed in MNA medium containing various concentrations of salicin. As shown, MNA containing 0.10 or 0.25% salicin formed small colonies, whereas at 0.5 or 1.0% concentration, large colonies were formed which were easy to count even without the aid of a magnifying colony counter. Therefore, a final concentration of 0.5% salicin in MNA base was used for selective enumeration of *L. acidophilus* in yogurt containing *B. longum* 1941 (Fig. 4.1.3). In a separate study, salicin medium was successfully used for selective enumeration of *L. acidophilus* from yogurts manufactured with starter cultures containing yogurt bacteria, *L. acidophilus* and *Bifidobacterium* spp. supplied by Chr. Hansen, Mauri Laboratories, and Rhone Poulenc (section 5.3.2.3).

4.1.4. Conclusion

Minimal nutrient agar base containing salicin was suitable for selective enumeration of *L. acidophilus* from pure cultures and yogurt containing *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* spp. A salicin concentration of 0.5% was appropriate for producing optimum size colonies. Salicin could be filter sterilised and then added to sterilised minimal nutrient base or could be autoclaved along with the minimal nutrient base.

Table 4.1.1. Utilisation of various sugars as carbon source by *S. thermophilus*, and *L. delbrueckii* ssp. *bulgaricus*

Strains	Sugars					
	Sali	Cello	Fruc	Man	Sor	Gluc
S. thermophilus 2000	-	-	++	-	-	++
S. thermophilus 2002	-	-	++	-	-	++
S. thermophilus 2008	-	-	++	-	-	++
S. thermophilus 2010	-	-	++	-	-	++
S. thermophilus 2013	-	-	++	-	-	++
S. thermophilus 2014	-	-	++	-	-	++
L. del. ssp. *bulgaricus* 2501	-	-	++	-	-	++
L. del. ssp. *bulgaricus* 2505	-	-	++	-	-	++
L. del. ssp. *bulgaricus* 2515	-	-	++	-	-	++
L. del. ssp. *bulgaricus* 2517	-	-	++	-	-	++
L. del. ssp. *bulgaricus* 2519	-	-	++	-	-	++

Note: (-) no growth, (±) Pin point colonies, (+) colony size 0.1-0.5mm, (++) Colony size 0.6mm-1.5mm, (+++) colony size >1.5mm
Sali=salicin, Cello=cellobiose, Fruc=fructose, Man=mannitol, Sor=sorbitol, Gluc=glucose

Table 4.1.2. Utilisation of various sugars as carbon source by *L. acidophilus* and *Bifidobacterium* spp.

Strains	Sugars					
	Sali	Cello	Fruc	Man	Sor	Gluc
L. acidophilus 2400	+++	++	++	+	+	++
L. acidophilus 2401	+++	++	++	+	+	++
L. acidophilus 2404	+++	++	++	+	+	++
L. acidophilus 2405	+++	+++	++	+	+	++
L. acidophilus 2409	+++	+++	++	+	+	++
L. acidophilus 2415	+++	+++	++	+	+	++
B. bifidum 1900	-	+	++	-	-	++
B. bifidum 1901	-	-	++	-	-	++
B. infantis 1912	-	-	++	-	-	++
B. adolescentis 1920	-	-	++	-	-	++
B. breve 1930	-	-	++	-	-	++
B. longum 1941	-	+	++	±	±	++
B. longum 20097	-	-	++	-	-	++
B. pseudolongum 20099	-	++	++	+	+	++
B. thermophilum 20210	-	-	++	-	-	++

Note: (-) no growth, (±) Pin point colonies, (+) colony size 0.1-0.5mm, (++) Colony size 0.6mm-1.5mm, (+++) colony size >1.5mm
Sali=salicin, Cello=cellobiose, Fruc=fructose, Man=mannitol, Sor=sorbitol, Gluc=glucose

Table 4.1.3. Colony size of *L. acidophilus* at various concentrations of salicin in minimal nutrient agar base

Strains	% Salicin			
	0.1	0.25	0.50	1.00
L. acidophilus 2400	±	+	++	+++
L. acidophilus 2401	±	+	+++	+++
L. acidophilus 2404	±	+	++	+++
L. acidophilus 2405	±	+	++	+++
L. acidophilus 2409	±	+	+++	+++
L. acidophilus 2415	±	+	++	+++

Note: (±) Pin point colonies, size >1.5mm (+) colony size 0.2-0.5mm, (++) colony size 0.6-1.5mm, (+++) colony

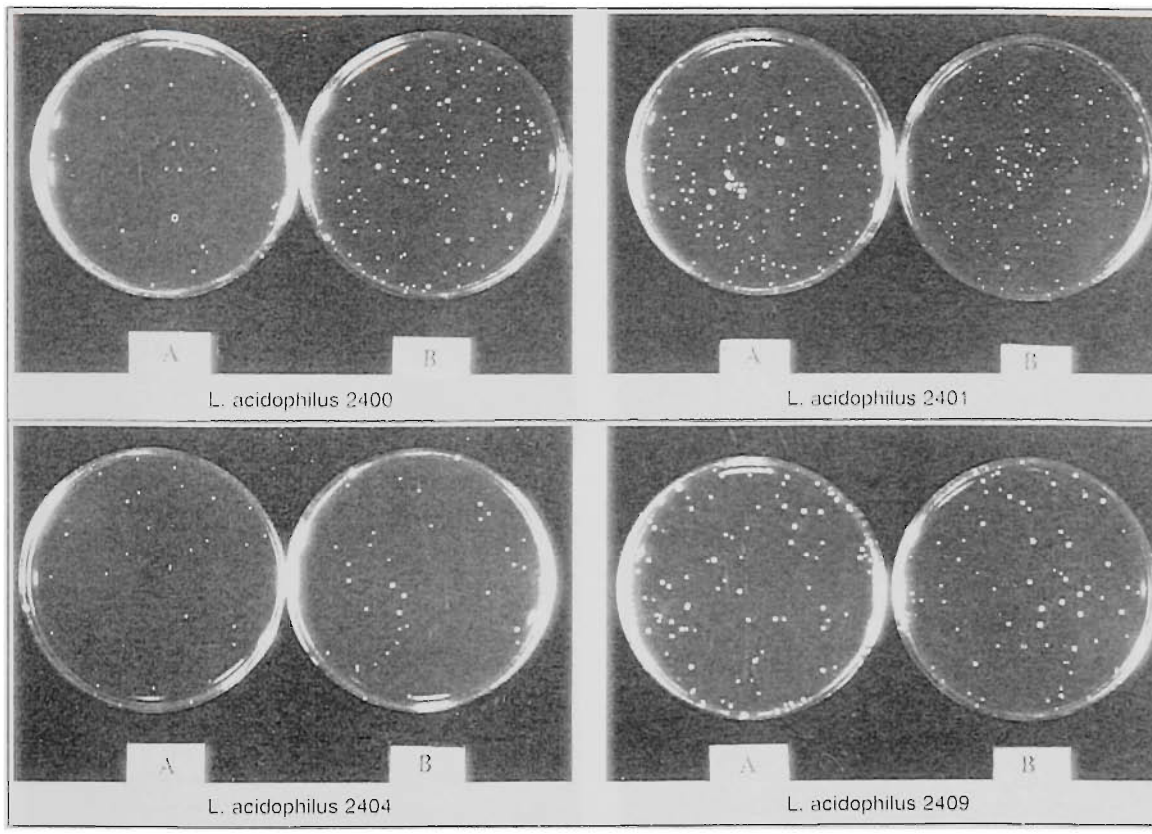

Fig. 4.1.1. *L. acidophilus* strains 2400, 2401, 2404 and 2409 grown in (A) MRS medium and (B) MNA (minimal nutrient agar) containing salicin. Colony formation in the latter medium is similar in shape and size with that formed in MRS medium.

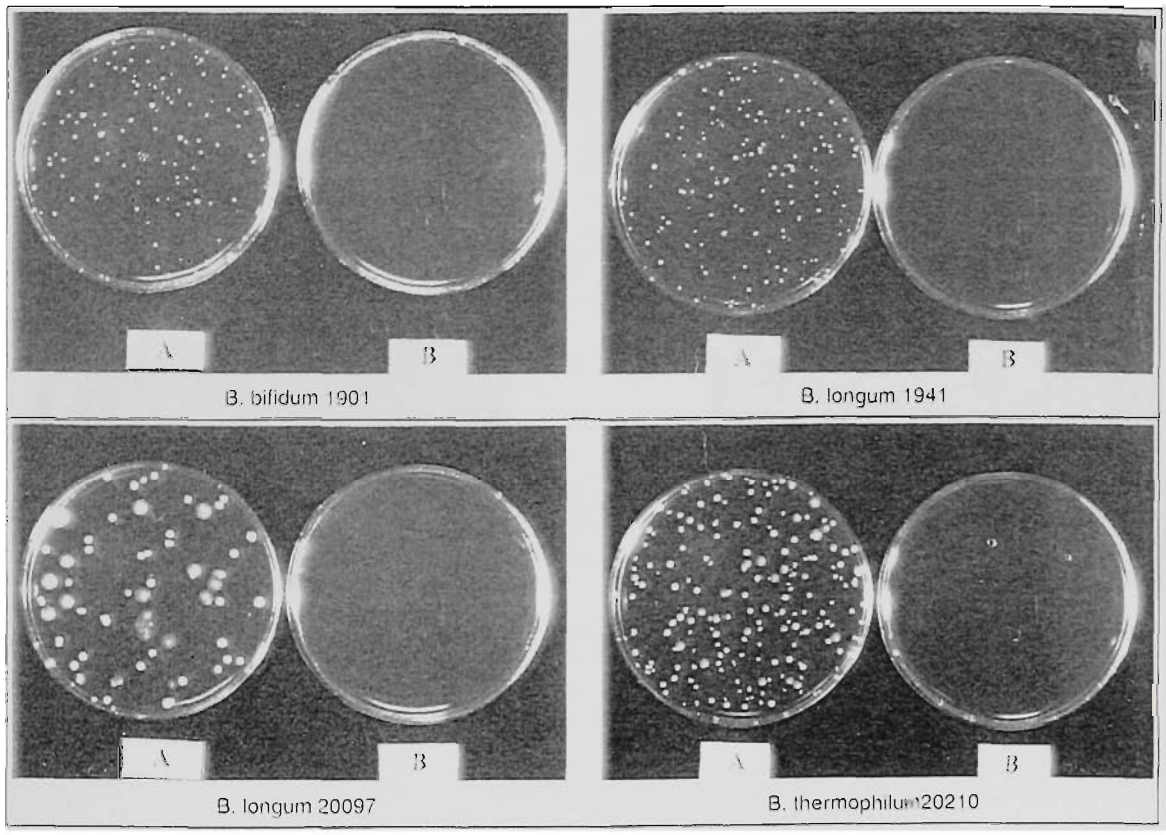

Fig. 4.1.2. *B. bifidum* 1901, *B. longum* 1941 and 20097, and *B. thermophilum* 20210 grown in (A) MRS agar, and (B) MNA (minimal nutrient agar) containing salicin. In the latter medium, *Bifidobacterium* spp. did not form colonies.

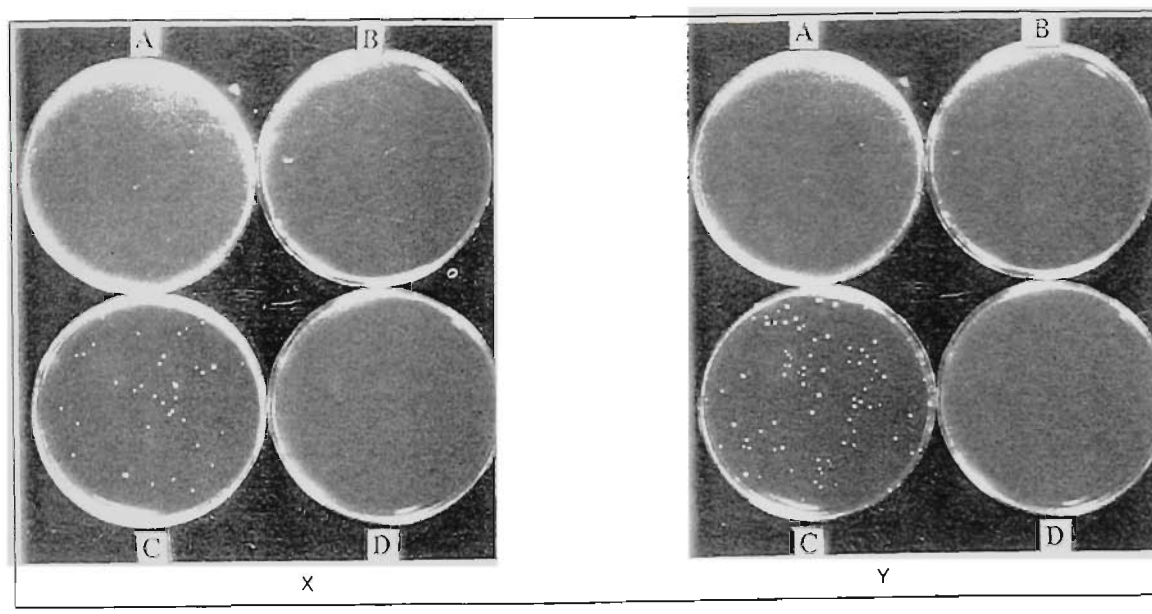

Fig. 4.1.3. Plates containing MNA with salicin were inoculated with *S. thermophilus* (A), *L. delbrueckii* ssp. *bulgaricus* (B), *L. acidophilus* (C), and *B. longum* 1941 (D). Colonies formed by *L. acidophilus* strains 2401 (in picture X), and 2409 (in picture Y).

4.2. Selective enumeration of bifidobacteria in yogurt containing *Lactobacillus acidophilus*, bifidobacteria, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*.

4.2.1. Introduction

Several media have been developed for differential enumeration of *L. acidophilus* and *Bifidobacterium* species. Scardovi (1986) has reviewed several complex media and media containing a wide variety of antibiotics to selectively enumerate *Bifidobacterium* species and concluded that one selective medium is not appropriate for all species. Munoa and Pares (1988) have developed a selective medium for isolation and enumeration of bifidobacteria from aquatic environments. This medium consisted of reinforced clostridial 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* species. 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* species 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. The aim of this study was to examine a range of media which could possibly be used in selective enumeration of *L. acidophilus* and *Bifidobacterium* species in the presence of yogurt culture organisms (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*).

4.2.2. Materials and methods

4.2.2.1. Bacterial cultures

Pure cultures of six strains of *S. thermophilus*, five strains of *L. delbrueckii* ssp. *bulgaricus*, six strains of *L. acidophilus* and nine strains of *Bifidobacterium* spp. were obtained as described in 2.2.2.

4.2.2.2. Media preparation

NNLP (nalidixic acid, neomycin sulphate, lithium chloride and paromomycin sulphate) agar was prepared according to the method described in 2.5.6. Filter sterilised L-cysteine hydrochloride (final concentration 0.05%) was added to lower the oxidation-reduction potential of the medium and to enhance the anaerobic growth of bifidobacteria. Bile agar was prepared by the method described in 2.5.8. The pH of the medium was adjusted to 6.8. MRS-galactose, MRS-maltose, MRS-dextrose and MRS-L-arabinose agars were prepared as described in 2.5.4. Each sugar was added separately to the autoclaved MRS basal medium held at 45°C to achieve a final sugar concentration of 2% and the media were used immediately for enumeration using the pour plating method. RCA agar (Oxoid, Australia) was prepared as described in 2.5.7. Peptone and water diluent (0.1%) was prepared as described in 2.5.1.

4.2.2.3. Enumeration

One gram of each culture was 10-fold serially diluted (10^2 to 10^8) in 0.1% sterile peptone and water diluent. Preparation of dilutions and pour plating was carried out as described in 2.5.3. Plates with colonies between 25-250 were enumerated and recorded as colony forming units (cfu) per gram of culture.

All experiments and analyses were duplicated. The results presented are averages of duplicate experiments.

4.2.3. Results and discussion

Our results showed that NNLP agar inhibited the growth of *S. thermophilus* (Table 4.2.1), *L. delbrueckii* ssp. *bulgaricus* (Table 4.2.2) and *L. acidophilus* (Table 4.2.3). As shown in Table 4.2.4, NNLP agar was found to be selective for *B. bifidum* strains 1900 and 1901, *B. longum* strains 1941 and 20097, *B. pseudolongum* 20099 and *B. thermophilum* 20210. However, this medium suppressed the growth of *B. infantis* 1912, *B. adolescentis* 1920, and *B. breve* 1930, to a certain extent. Four types of antibiotics are used as selective agents in NNLP medium and one or more of these may have affected the growth of *B. infantis* 1912, *B. adolescentis* 1920 and *B. breve* 1930 (Wijman *et al.* 1989). NNLP medium developed by Teraguchi *et al.* (1978) is a selective medium for *Bifidobacterium* species. In a study by Lim *et al.* (1993), *Bifidobacterium* strains were shown to be resistant to kanamycin, neomycin, paromomycin sulphate, nalidixic acid and polymyxin B sulphate, validating the use of these antimicrobial agents as selective agents in NNLP agar. Several studies (Clark *et al.,* 1993; Arroyo *et al.,* 1994; Lankaputhra and Shah, 1994) have shown that *B. longum* would be the species of choice for use as a dietary adjunct in cultured dairy products. However, most of the probiotic organisms containing yogurt manufactured in Australia claim to contain *B. bifidum* (Shah *et al.,*1995). Therefore, NNLP agar can be used for selective enumeration of *B. bifidum* and other bifidobacteria studied (except *B. infantis* 1912, *B. adolescentis* 1920 and *B. breve* 1930) from yogurt. However, NNLP medium is

composed of several components and requires laborious preparation which may make this unattractive for routine analysis of yogurt.

Bile is used as a selective agent in bile agar. *L. acidophilus* and *Bifidobacterium* species have been reported to be bile tolerant when compared to other lactic acid bacteria (Clark *et al.,* 1993; Lankaputhra and Shah, 1994) and this finding was used to assist in developing this selective medium. Bile agar inhibited the growth of *S. salivarius* ssp. *thermophilus* (Table 4.2.1.), and *L. delbrueckii* ssp. *bulgaricus*, except *L. delbrueckii* ssp. *bulgaricus* strain 2501 which produced tiny colonies (Table 4.2.2). All the species of *L. acidophilus* studied grew on bile agar plates (Table 4.2.3). As shown in Table 4.2.4, bile agar supported the growth of *B. infantis* 1912, *B. longum* 1941, *B. pseudolongum* 20099 and *B. thermophilum* 20210. Other *Bifidobacterium* species such as *B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930 and *B. longum* 20097 were inhibited in bile agar. Since strains of *L. acidophilus* and *Bifidobacterium* species were able to grow in bile agar, it may not be possible to selectively enumerate all *Bifidobacterium* species selectively using this medium from a product containing *L. acidophilus* and bifidobacteria. However, bile agar can be used as a selective medium for enumeration of *L. acidophilus* from product containing *B. bifidum*, *B. adolescentis*, *B. breve* or *B. longum* 20097 as the latter organisms did not grow in bile agar. Most Australian yogurt manufacturers use *B. bifidum* as an adjunct (Shah *et al.,* 1995); the strains that were used in this study did not grow in bile agar, thus this medium could be used for selective enumeration of *L. acidophilus* in yogurt. However, if viable count of *L. acidophilus* in a product is to be determined, it may be necessary to validate the selective medium for growth or inhibition of the strains used, as the product may contain strains of *B. bifidum* which may grow in bile agar.

Trials using MRS agar as a basal medium and substituting dextrose with other sugars such as galactose, maltose, and arabinose were carried out in order to examine the suitability of these media for selective enumeration of *L. acidophilus* and *Bifidobacterium* species. MRS-galactose agar inhibited the growth of *S. thermophilus*

strains 2002 and 2013 while allowing the growth of *S. thermophilus* strains 2000, 2008, 2010 and 2014 (Table 4.2.1). The growth of *L. delbrueckii* ssp *bulgaricus* did not occur in MRS-galactose agar (Table 4.2.2) and the growth of *L. acidophilus* was also inhibited (Table 4.2.3). All the species of *Bifidobacterium*, except *B. adolescentis* 1920, were able to grow in MRS-galactose medium. Iwana *et al.* (1993) developed a galactose-based medium (GL) which contains galactose and lithium chloride (0.4 g/L) as selective and inhibitory agents. They reported that GL agar can be used for selective enumeration of bifidobacteria in yogurt. According to Hardie (1986), galactose is weakly fermented by *S. thermophilus*. Our results have shown that *S. thermophilus* strains 2000, 2008, 2010, and 2014 were able to grow on MRS-galactose agar plates. Therefore, it seems that this medium may be unsuitable for selective enumeration of *Bifidobacterium* species in yogurt as *S. thermophilus* is an inherent part of yogurt culture.

MRS medium modified by replacing glucose with maltose (MRS-maltose agar) was found to be suitable for enumeration of *L. acidophilus* in yogurt in presence of *L. delbrueckii* ssp *bulgaricus* and *S. thermophilus* (Hull and Roberts, 1984). *S. thermophilus* (Table 4.2.1) and *L. delbrueckii* ssp *bulgaricus* did not grow on MRS-maltose agar plates except *L. delbrueckii* ssp *bulgaricus* 2501 which formed very small colonies (Table 4.2.2). Similarly, all the bifidobacteria species except *B. adolescentis* 1920 formed colonies on MRS-maltose agar plates and *B. bifidum* 1900, *B. breve* 1930 and *B. longum* 20097 were partially inhibited (Table 4.2.5). Because there is an increasing tendency to incorporate *Bifidobacterium* species in yogurt along with *L. acidophilus*, MRS-maltose medium may not be suitable for differential enumeration of *L. acidophilus* from bifidobacteria in yogurt. *S. thermophilus* and most of the *L. delbrueckii* ssp. *bulgaricus* studied did not grow on the plates, which suggest that this medium can be used for selective enumeration of *L. acidophilus* provided bifidobacteria are not present in yogurt.

All the strains of *S. salivarius* ssp *thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and bifidobacteria studied (Tables 4.2.1-4.2.4) grew on MRS-dextrose agar.

Dextrose is fermented by most of the microorganisms and therefore MRS-dextrose medium may not be suitable for differential enumeration of *L. acidophilus* or *Bifidobacterium* species in presence of other lactic acid bacteria.

According to Bergey's Manual of Determinative Bacteriology (Scardovi, 1986), L-arabinose is not fermented by most dairy lactobacilli and streptococci and by *B. bifidum*, *B. infantis*, and *B. breve*. However, other `human' type strains, *B. longum*, *B. adolescentis*, *B. angulatum*, *B. catenulatum* are reported to ferment L-arabinose. Our results have shown that all the *S. thermophilus* (Table 4.2.1) and *L. delbrueckii* ssp. *bulgaricus* (Table 4.2.2) strains studied did not grow on MRS-L-arabinose agar plates. Similarly, *L. acidophilus* 2404 did not grow (Table 4.2.3), while other strains of *L. acidophilus* formed pin point colonies on the plates and can easily be differentiated from *Bifidobacterium* species such as *B. longum* 1941 and 20097 and *B. pseudolongum* 20099, which formed large colonies on the plates. However, MRS-L-arabinose agar did not support the growth of *B. bifidum*, *B. infantis*, *B. adolescentis*, *B. breve* and *B. thermophilum*. Thus, this medium can only be used for selective enumeration of *B. longum* 1941 and 20097 and *B. pseudolongum* 20099. Our study (Lankaputhra and Shah, 1994) has shown that *B. longum* and *B. pseudolongum* are tolerant to acid and bile generally encountered in the gastrointestinal tract and would be the species of choice for use as a dietary adjunct in yogurt.

Ideally, a medium for use in routine laboratory testing of yogurt containing *L. acidophilus* and *Bifidobacterium* species should enumerate *L. acidophilus* and *Bifidobacterium* species selectively and differentiate these from yogurt culture organisms (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*). Most of the selective medium described to date in the literature for use in selective enumeration of *L. acidophilus* and *Bifidobacterium* species have been evaluated using pure cultures. In this study, a similar approach has been used for both these and yogurt culture strains, followed by selective enumeration of one of the strains of bifidobacteria from yogurt containing all the four strains.

Figures 4.2.1 and 4.2.2 show plates inoculated with serially diluted cultures of *S. salivarius* ssp *thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* 2409 and *B. longum* 1941 and incubated anaerobically at 37°C for 72 h. As shown in the Figures, both MRS-L arabinose agar (Figure 4.2.1) and NNLP agar (Figure 4.2.2.) selectively supported the growth of *B. longum* 1941 and suppressed the growth of *S. salivarius* ssp *thermophilus* (a), *L. delbrueckii* ssp. *bulgaricus* (b), while *L. acidophilus* 2409 (c) produced tiny colonies, easily distinguishable from those of *B. longum* 1941 (d) or *B. pseudolongum* 20099 (data not shown). To confirm these findings, yogurt was prepared with fresh cultures of *S. salivarius* ssp *thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* 2409, and *B. longum* 1941 in a separate experiment. Enumeration was carried out using MRS-L-arabinose agar and NNLP agar. As shown in Figure 4.2.3, only *B. longum* formed well developed colonies in MRS-L-arabinose agar (a) and NNLP agar (b), and *L. acidophilus* formed tiny colonies while other organisms did not grow.

RCA agar, best known for its use in isolation of clostridial species, has been reported to detect *L. delbrueckii* ssp *bulgaricus* selectively by suppressing the growth of *S. salivarius* ssp *thermophilus* when the pH of the agar is 5.5 (Johns *et al.,* 1988). In RCA medium, *S. thermophilus* strains 2000, 2002, 2010, and 2013 did not grow on agar plates, however, *S. thermophilus* strains 2008 and 2014 were partially inhibited (Table 4.2.1). *L. delbrueckii* ssp. *bulgaricus* formed cloudy and faded colonies (Table 4.2.2), which can be easily differentiated from those of *S. thermophilus*. *L. acidophilus* strains either formed tiny colonies (*L. acidophilus* strains 2400, 2405, 2409) or did not grow (*L. acidophilus* strains 2401, 2404, and 2415) (Table 4.2.3.3). However, all bifidobacteria species studied were able to grow on RCA plates (Table 4.2.3.4). Therefore, this medium may not be suitable for selective enumeration of *L. delbrueckii* ssp. *bulgaricus* from *S. thermophilus*, if a product contains *Bifidobacterium* species. However, this medium can be used to differentiate *S. salivarius* ssp *thermophilus* from *L. delbrueckii* ssp. *bulgaricus* when *L. acidophilus* and *Bifidobacterium* species are not added to yogurt.

4.2.4. Conclusions

Of seven media that were evaluated, NNLP agar can be used for selective enumeration of *B. bifidum* 1900 and 1901, *B. longum* 1941 and 20097, *B. pseudolongum* 20099 and *B. thermophilum* 20210. However, this medium does not support the growth of *B. infantis* 1912, *B. adolescentis* 1920 and *B. breve* 1930 and therefore cannot be used for enumeration of these organisms. NNLP medium requires considerable time in preparation and uses a number of ingredients.

Bile agar can be used for selective enumeration of *L. acidophilus* from yogurt supplemented with *L. acidophilus* along with *B. bifidum*, *B. adolescentis* or *B. breve*. All the strains of *L. acidophilus* used in this study grew well in bile agar while the strains of *S. salivarius* ssp *thermophilus* and *L. delbrueckii* ssp. *bulgaricus* did not form colonies. Maltose agar can be used to differentiate *L. acidophilus* from *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp *bulgaricus* if a product does not contain bifidobacteria. Our results have shown that MRS-L-arabinose agar can be used for selective enumeration of *B. longum* strains 1941 and 20097 and *B. pseudolongum* 20099 from *L. acidophilus*. Strains of *L. acidophilus* formed pin point colonies in MRS-L-arabinose agar and can be easily differentiated from that of bifidobacteria. *B. bifidum*, *B. infantis*, *B. adolescentis*, *B. breve* and *B. thermophilum* did not ferment MRS-L-arabinose and as a result did not form colonies on the plates. Bifidobacteria have the ability to metabolise complex carbohydrates and these carbohydrates may form the basis for the development of differentially selective media.

However, presently there are taxonomical uncertainties regarding classification of bifidobacteria and *L. acidophilus* (Salminen and Wright, 1993). Therefore, some strains which have been classified under the same species may not actually belong to those species. Therefore, it may be necessary to validate the suitability of these selective media to determine the growth and viability of each particular combination of strains in a product before applying such media for selective enumeration of *L. acidophilus* and *Bifidobacterium* spp. in yogurt.

Table 4.2.1. Viable counts of *S. thermophilus* on different bacteriological media (cfu/g)

Strain	NNLP agar	Bile agar	MRS-galactose agar	MRS-maltose agar	MRS-dextrose agar	MRS-arabinose agar	RCA agar
S. thermophilus 2000	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	5.8×10^4	$< 1.0 \times 10^1$	4.1×10^8	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
S. thermophilus 2002	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	4.8×10^8	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
S. thermophilus 2008	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	5.8×10^6	$< 1.0 \times 10^1$	1.1×10^8	$< 1.0 \times 10^1$	9.4×10^5
S. thermophilus 2010	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	1.1×10^8	$< 1.0 \times 10^1$	8.8×10^7	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
S. thermophilus 2013	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	5.0×10^7	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
S. thermophilus 2014	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	6.0×10^7	$< 1.0 \times 10^1$	5.5×10^7	$< 1.0 \times 10^1$	3.9×10^5

Table 4.2.2. Viable counts of *L. delbrueckii* ssp. *bulgaricus* on different bacteriological media (cfu/g)

Strain	NNLP agar	Bile agar	MRS-galactose agar	MRS-maltose agar	MRS-dextrose agar	MRS-arabinose agar	RCA agar
L. bulgaricus 2501	$< 1.0 \times 10^1$	$9.0 \times 10^{4*}$	$< 1.0 \times 10^1$	6.5×10^4	1.0×10^7	$< 1.0 \times 10^1$	6.0×10^{8} !
L. bulgaricus 2505	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	8.4×10^7	$< 1.0 \times 10^1$	3.5×10^{8} !
L. bulgaricus 2515	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	2.0×10^8	$< 1.0 \times 10^1$	1.0×10^{9} !
L. bulgaricus 2517	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	2.8×10^8	$< 1.0 \times 10^1$	8.6×10^{8} !
L. bulgaricus 2519	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	2.5×10^8	$< 1.0 \times 10^1$	1.2×10^{9} !

Note: (*) very tiny colonies, (!) cloudy and faded colonies

Table 4.2.3. Viable counts of *L. acidophilus* on different bacteriological media (cfu/g)

Strain	NNLP agar	Bile agar	MRS-galactose agar	MRS-maltose agar	MRS-dextrose agar	MRS-L-arabinose agar	RCA agar
L. acidophilus 2400	$< 1.0 \times 10^1$	4.1×10^7	$< 1.0 \times 10^1$	3.6×10^8	3.7×10^8	$8.3 \times 10^{5*}$	7.9×10^{4O}
L. acidophilus 2401	$< 1.0 \times 10^1$	4.9×10^8	$< 1.0 \times 10^1$	4.8×10^8	4.8×10^8	$7.1 \times 10^{8*}$	$< 1.0 \times 10^1$
L. acidophilus 2404	$< 1.0 \times 10^1$	1.1×10^8	$< 1.0 \times 10^1$	1.0×10^8	1.0×10^8	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$
L. acidophilus 2405	$< 1.0 \times 10^1$	4.8×10^8	$< 1.0 \times 10^1$	1.1×10^8	3.1×10^8	$7.2 \times 10^{8*}$	1.0×10^{9O}
L. acidophilus 2409	$< 1.0 \times 10^1$	7.9×10^7	$< 1.0 \times 10^1$	3.2×10^8	3.6×10^8	$1.0 \times 10^{9*}$	8.0×10^{8O}
L. acidophilus 2415	$< 1.0 \times 10^1$	4.9×10^8	$< 1.0 \times 10^1$	4.6×10^8	4.5×10^8	$2.5 \times 10^{9*}$	$< 1.0 \times 10^1$

Note: (*) very tiny colonies , (O) tiny colonies

Table 4.2.4. Viable counts of *Bifidobacterium* spp. on different bacteriological media (cfu/g)

Strain	NNLP agar	Bile agar	MRS-galactose agar	MRS-maltose agar	MRS-dextrose agar	MRS-arabinose agar	RCA agar
B. bifidum 1900	2.0×10^9	$< 1.0 \times 10^1$	2.1×10^9	1.1×10^5	2.4×10^9	$< 1.0 \times 10^1$	4.1×10^8
B. bifidum 1901	1.0×10^9	$< 1.0 \times 10^1$	4.7×10^9	1.0×10^9	4.9×10^9	$< 1.0 \times 10^1$	4.8×10^8
B. infantis 1912	$< 1.0 \times 10^1$	2.5×10^7	4.9×10^7	5.4×10^7	6.1×10^7	$< 1.0 \times 10^1$	1.1×10^8
B. adolescentis 1920	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	1.0×10^8	$< 1.0 \times 10^1$	8.8×10^7
B. breve 1930	3.4×10^5	$< 1.0 \times 10^1$	1.0×10^9	2.0×10^5	9.7×10^9	$< 1.0 \times 10^1$	5.0×10^7
B. longum 1941	7.4×10^8	7.9×10^8	7.6×10^8	7.5×10^8	7.0×10^8	2.5×10^8	5.0×10^7
B. longum 20097	5.4×10^8	$< 1.0 \times 10^1$	7.8×10^7	6.2×10^5	6.0×10^4	8.5×10^7	5.5×10^7
B. pseudolongum 20099	6.3×10^8	4.7×10^9	3.4×10^8	3.2×10^8	3.4×10^8	1.0×10^9	5.5×10^7
B. thermophilum 20210	2.5×10^9	1.7×10^9	2.5×10^9	2.7×10^9	2.5×10^9	$< 1.0 \times 10^1$	5.5×10^7

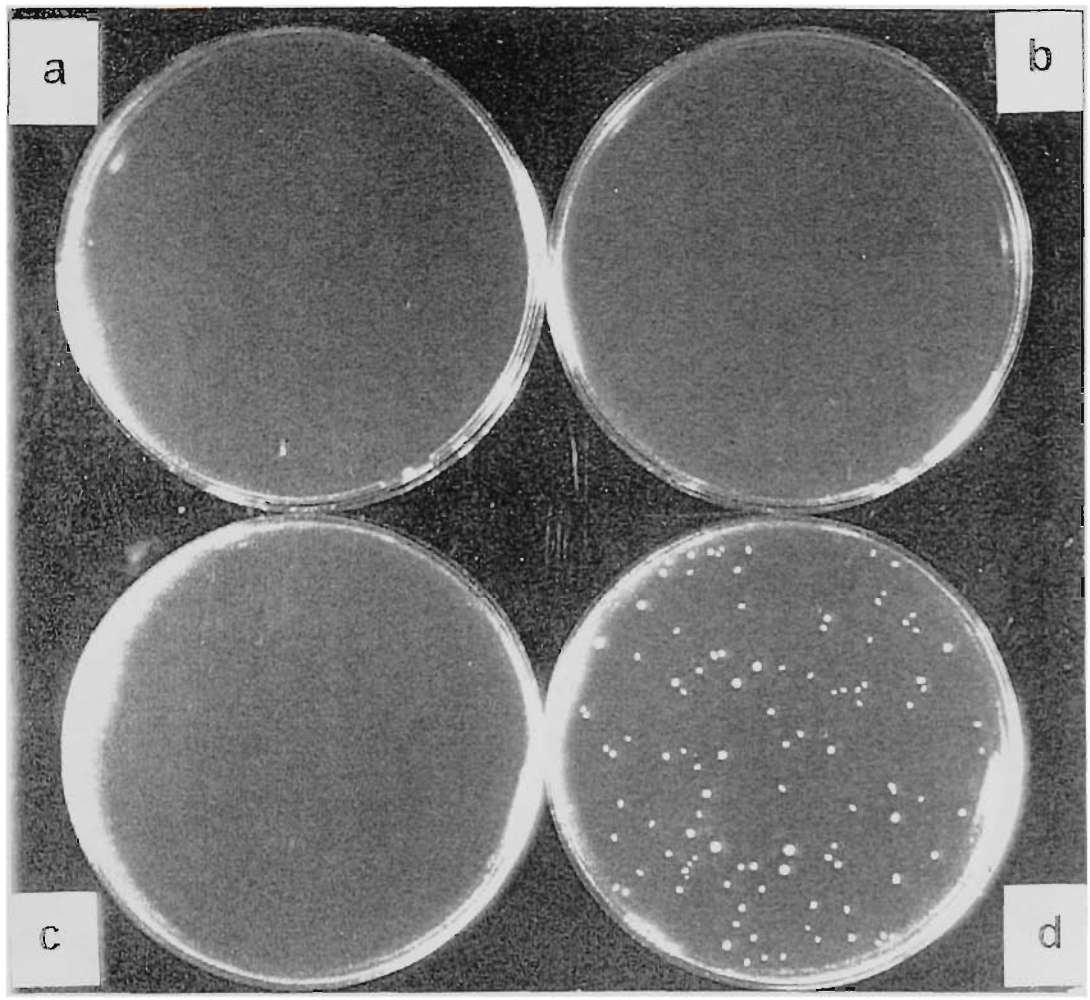

Figure 4.2.1. MRS-L-arabinose agar plates inoculated with pure cultures of (a) *S. thermophilus*, (b) *L. delbrueckii* ssp. *bulgaricus*, (c) *L. acidophilus* and (d) *B. longum* 1941, incubated at 37°C for 72h.

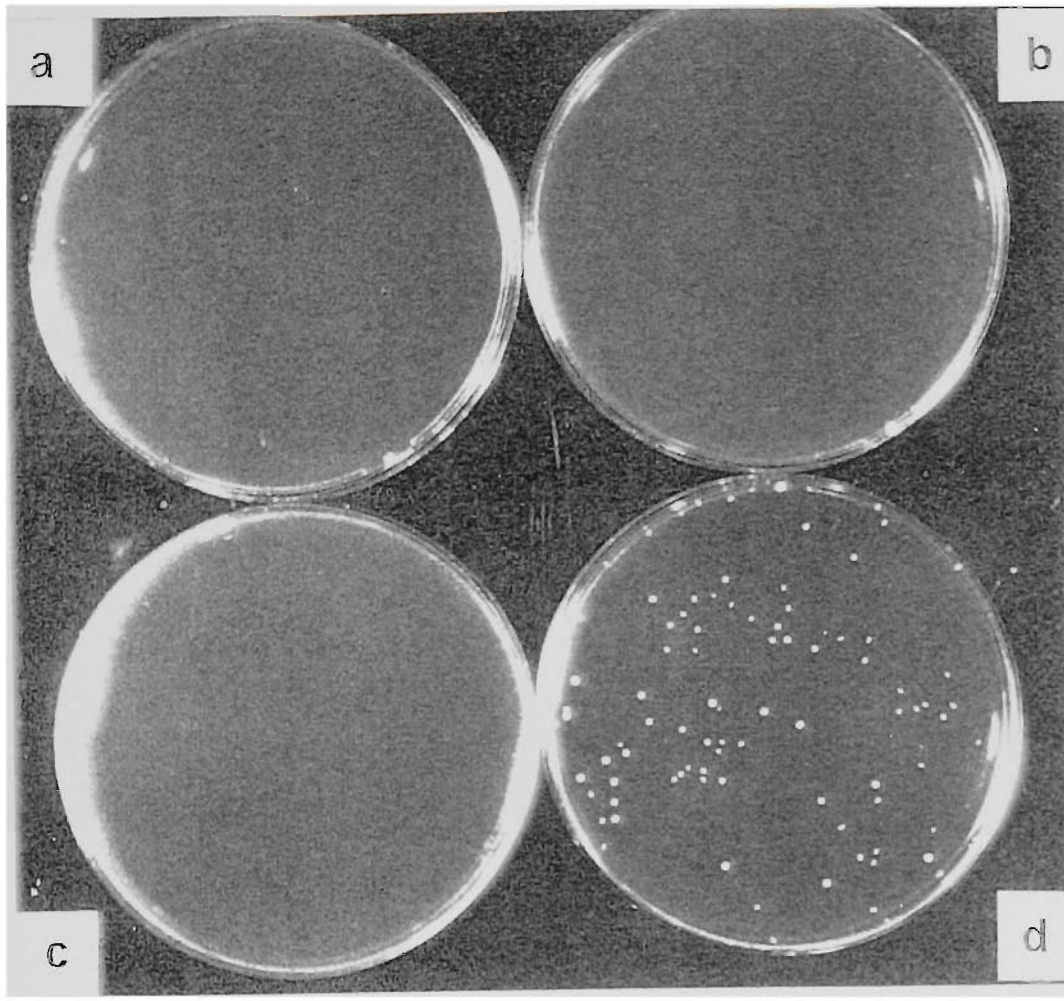

Figure 4.2.2. NNLP agar plates inoculated with pure cultures of (a) *S. thermophilus*, (b) *L. delbrueckii* ssp. *bulgaricus*, (c) *L. acidophilus* and (d) *B. longum* 1941 incubated at 37°C or 72 h.

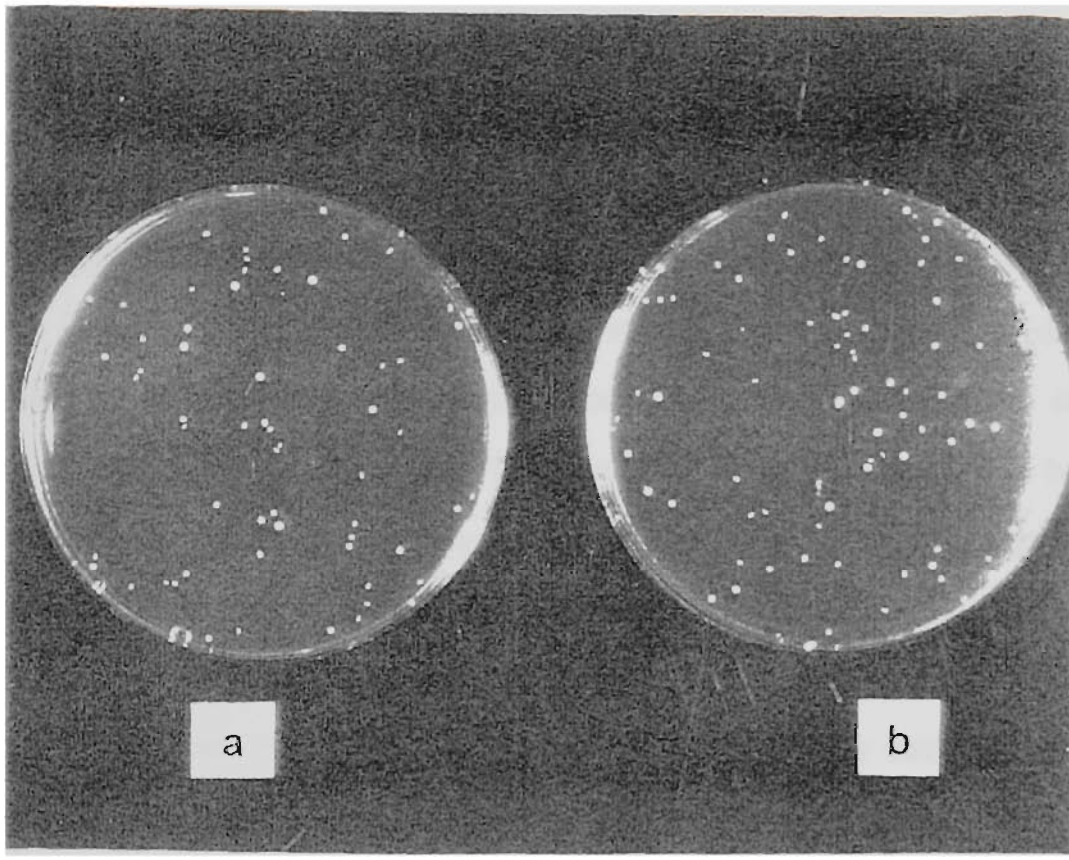

Figure 4.2.3. Plates showing colonies of *B. longum* 1941 from yogurt sample in (a) MRS-L-arabinose agar and, (b) NNLP agar. The plates were incubated anaerobically at 37°C for 72 h.

5.0. SCREENING OF PROBIOTIC BACTERIA AGAINST ANTIMICROBIAL SUBSTANCES AND APPLICATION OF SELECTED STRAINS IN YOGURT MANUFACTURE

5.1. Survival of *Lactobacillus acidophilus* and bifidobacteria in the presence of acid and bile to simulate their survival in gastrointestinal tract[1]

5.1.1 Introduction

One of the important characteristics of the probiotic microorganisms is their ability to survive through acidic conditions in the human stomach and bile concentrations in the intestine in order to colonise in the gut. Strains of *L. acidophilus* and *Bifidobacterium* spp. that lack the ability to survive the harsh conditions in gastrointestinal tract may be unsuitable for use as dietary adjuncts in fermented foods. In order to determine the suitability of the strains of *L. acidophilus* and bifidobacteria for use as dietary adjuncts in fermented dairy products, survival of 6 strains of *L. acidophilus* and 9 strains of bifidobacteria under acidic conditions and bile concentrations commonly encountered in the stomach and the intestine was evaluated.

5.1.2. Materials and Methods

5.1.2.1. Bacterial cultures

Six strains of *L. acidophilus* and 9 strains of bifidobacteria were used in this study. Bacterial cultures were grown and maintained as described in section 2.2.2.

5.1.2.2. Survival of L. acidophilus and bifidobacteria in acidic conditions

To evaluate the survival of 6 strains *L. acidophilus* and 9 strains of bifidobacteria, aliquots of active cultures grown in NGYC for 18 h at 37°C were

[1] a paper titled "Survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in the presence of acid and bile salts" was published in *Cult. Dairy Prod. J.* 30:2-7.

adjusted to pH 3.0, 2.5, 2.0, and 1.5 with 5 N HCl and incubated at 37°C for 3 h. Samples were taken initially and every hour for 3 h and the viable numbers of *L. acidophilus* and bifidobacteria were enumerated by the pour plate techniques using 10-fold serial dilutions prepared in 0.1% peptone and water diluent (as described in sections 2.5.1 to 2.5.3).

5.1.2.3. Survival of L. acidophilus and bifidobacteria in the presence of bile

Aliquots of active cultures grown in NGYC for 18 h at 37°C were adjusted to pH 4.5 with sterile 0.1 N HCl or 0.1 N NaOH depending on the final pH of the culture after 18 h of incubation. Concentrated bile solution was prepared separately by dissolving powdered bile extract (Oxoid, Australia) filter sterilised and was added to the bacterial cultures to achieve final concentrations of 1.0 and 1.5%. The control samples did contain bile extract. The cultures were incubated at 37°C for 3 h. Samples were taken initially and every hour for 3 h and the viable counts of *L. acidophilus* and bifidobacteria were determined by pour plate counts of all the samples using 10-fold serial dilutions prepared in 0.1% peptone and water diluent.

5.1.2.4. Enumeration of L. acidophilus and bifidobacteria

MRS agar supplemented with 0.05% L-cysteine· HCl was used for enumeration of *L. acidophilus* and bifidobacteria. Cysteine·HCl was used to lower the oxidation and reduction potential of the medium and to enhance the anaerobic growth of probiotic bacteria, in particular, bifidobacteria. Enumeration was carried out as described in section 2.2.2.

5.1.3. Results and discussion

5.1.3.1. Survival of L. acidophilus and bifidobacteria under acidic conditions

Acidity in different regions of the gastrointestinal tract varies. Stomach and the regions immediately following stomach have highest acidity and the pH in these areas may fall as low as 1.5.

Survival of *L. acidophilus* strains 2400, 2401, 2404, 2405, 2409 and 2415 in acidic conditions is illustrated in Figs. 5.1.1 and 5.1.2. In general, the viable counts decreased during 3 h incubation at all pH conditions; the decrease was substantial, especially at pH 2.5 or lower. *L. acidophilus* 2409 showed highest survival in acidic conditions followed by strains 2415 and 2401. As shown in Fig. 5.1.1, viable count of *L. acidophilus* 2400 reduced from 10^8 to 10^4 CFU/g in 1 h of incubation and to < 10^1 CFU/g after 2 h of incubation at pH 1.5 the same pH, while at pH 2.5 and 3.0, the viable counts remained at 10^6 and 10^7 CFU/g, respectively. *L. acidophilus* 2401 was found to be slightly more acid tolerant than *L. acidophilus* 2400; there were >10^2 survivors after 3 h of incubation at pH 1.5. The viability of *L. acidophilus* 2404 was low at pH 2.5 or below; the count was 10^1 CFU/g after 1 h of incubation at pH 1.5. For *L. acidophilus* 2405, the results were similar to those of *L. acidophilus* 2404. As shown in Fig. 5.1.2, the viability of *L. acidophilus* 2409 was least affected and even after 3 h of incubation at pH 1.5, the count remained ~10^4 CFU/g while at pH 3.0 the counts remained > 10^8 CFU/g. The count of *L. acidophilus* 2415 was similar to that of *L. acidophilus* 2401. However, *L. acidophilus* 2409 showed better survival than *L. acidophilus* 2401 or 2415.

Hood and Zotolla (1988) studied the survival of *L. acidophilus* in a pH range of 2.0 to 4.0 and observed a rapid decline in their numbers at pH 2.0. However, there was no decrease in the number of viable cells at pH 4.0. Our results are comparable to the findings of Hood and Zottola (1988). All the 6 strains of *L.*

acidophilus studied survived well at pH 3.0 or above and the viable counts remained > 10^7 CFU/g after 3 h incubation.

Survival of 9 strains of *Bifidobacterium* spp. in acidic conditions is shown in Figures 5.1.3, 5.1.4, and 5.1.5. Among the 9 strains of bifidobacteria, *B. longum* 1941 and *B. pseudolongum* 20099 showed the highest survival. As shown in Figures 5.1.3, 5.1.4, counts of *B. bifidum* 1900 and 1901, *B. adolescentis* 1920 and *B. breve* 1930 showed a rapid decline at pH 3.0 after 3 h of incubation. *B. adolescentis* 1920 and *B. breve* 1930 survived poorly at all pH levels studied. The counts of *B. infantis* 1912 reduced to < 10^1 CFU/g from the original level of 10^9 at pH 2.5 or below. However, at pH 3.0, the count of *B. infantis* 1912 remained at 10^7 CFU/g even after 3 h of incubation. Pochart *et al.* (1992) also observed a rapid decline in the counts of bifidobacteria at pH 1.0 with no survivors after 1 h of incubation; however, there was no considerable decrease in the counts at pH 3.0 after 3 h of incubation. *B. thermophilum* 20210 also survived poorly at all the pH levels; even at pH 3.0, the viable count dropped to 10^3 CFU/g after 3 h of incubation.

Figure 5.1.5 illustrates survival of *B. longum* 1941 and 20097 and *B. pseudolongum* 20099 under acidic conditions. As shown in Fig 5.1.3, *B. longum* 1941 *B. pseudolongum* 20099 survived better than other strains studied under similar conditions. As shown, *B. longum* 1941 and *B. pseudolongum* 20099 survived better than other strains studied under similar conditions. Clark *et al.* (1993) also observed better survival of *B. longum* among four strains of bifidobacteria studied. *B. longum* 20097 survived poorly at all the pH levels studied and the results for *B. longum* 20097 were much similar to those of *B. thermophilum* 20210. It is interesting to note that *B. longum* 1941 survived better than *B. longum* 20097 under similar conditions. *B. pseudolongum* 20099 was found to be the most acid tolerant among the bifidobacteria strains studied.

5.1.3.2. Survival of L. acidophilus and bifidobacteria in the presence of bile

Secretion of bile and its concentration in different regions of the intestine varies, depending on the type of food consumed and it may not be possible to predict bile concentration in the intestine at any given moment. While bile concentrations in the intestine can range between 0.5 to 2.0% during the first hour of digestion, its levels may decrease during the second hour. Bile concentrations ranging from 0.5 to 2.0% have been used in several microbiological media for selective isolation of bile tolerant bacteria from mixed cultures. Figures 5.1.6 and 5.1.7 show survival of *L. acidophilus* strains 2400, 2401, 2404, 2405, 2409 and 2415 in bile concentrations of 0, 1.0 and 1.5%. *L. acidophilus* strains 2404 and 2415 survived best in bile followed by strains 2401 and 2409. *L. acidophilus* 2400 counts decreased to 10^5 and 10^3 at 1.0 and 1.5% bile concentrations, respectively in 3 h of incubation at 37°C. *L. acidophilus* 2401 was found to be tolerant to both levels of bile; the viable counts declined only to 10^7 and 10^6 CFU/g from an initial level of 10^8 CFU/g after 3 h of incubation in 1.0 and 1.5% bile concentrations, respectively.

L. acidophilus 2404 survived well under both bile levels; the viable count remained between 10^8 and 10^7 CFU/g at 1.0 and 1.5% bile after 3 h of incubation. The survival of *L. acidophilus* 2409 was slightly better than that of *L. acidophilus* 2405 at 1.0 and 1.5% bile concentrations. *L. acidophilus* 2415 showed tolerance to bile salts and the counts remained > 10^7 CFU/g even after 3 h of incubation in 1.0 or 1.5% bile concentrations. It is interesting to note that *L. acidophilus* 2401 and 2415 were also found to be acid tolerant.

Figures 5.1.8 to 5.1.10 show the survival of 9 strains of bifidobacteria in 1.0 and 1.5% bile concentrations. Count of *B. bifidum* 1900 declined from 10^8 to 10^7 CFU/g after 3 h of incubation in 1.0% bile concentration, while the viable count reduced to 10^5 CFU/g in 1.5% bile concentration. *B. bifidum* 1901 count was also

affected in bile; the count decreased to 10^6 and 10^4 CFU/g from an initial level of 10^9 after 3 h of incubation in 1.0 and 1.5 bile concentrations, respectively. *B. infantis* 1912 was found to be tolerant to bile; the count was unaffected even after 3 h of incubation at both bile concentrations. The viable counts of *B. adolescentis* 1920, *B. breve* 1930 and *B. thermophilum* 20210 were also reduced in the presence of bile.

As shown in Fig. 5.1.10, *B. pseudolongum* 20099 showed tolerance to bile and the counts remained close to the initial count of 10^9 CFU/g in bile concentrations of 1.0 or 1.5% after 3 h incubation at 37°C. The counts *B. longum* 20097 decreased to 10^5 and 10^4 after 3 h of incubation in 1.0 and 1.5% bile, respectively. *B. longum* 1941 exhibited tolerance to bile; the viable count remained between 10^9 and 10^8 CFU/g after 3 h of incubation in the bile concentration of 1.0 to 1.5%. It is interesting to note that *B. longum* 1941 was also found to be acid tolerant.

Clark and Martin (1994) studied the effect of bile on *B. longum* and reported that the organism survived bile concentrations of as high as 4%. Our results have also shown that *B. longum* 1941 survived best in bile concentrations studied. It is interesting to note that *B. longum* 20097 is not as bile tolerant as *B. longum* 1941. This may have been due to the lower concentrations of bile used in their study. *B. longum* displayed the least tolerance to bile in the study of Ibrahim and Bezkorovainy (1993). However, our study has shown that while one strain of *B. longum* (1941) was tolerant to bile the other strain of *B. longum* (20097) did not survive as well in bile.

5.1.4. Conclusions

Results showed that among 6 strains of lactobacilli, *L. acidophilus* strains 2401, 2409 and 2415 survived best under acidic conditions. *L. acidophilus* strains

2404 and 2415 showed the best tolerance to bile followed by strains 2401 and 2409. However, as *L. acidophilus* 2404 showed poor tolerance to acid conditions, this organism may not be suitable for use as dietary adjuncts. Among the nine strains of bifidobacteria, *B. longum* 1941 and *B. pseudolongum* 20099 survived best under acidic conditions. *B. longum* 1941, *B. pseudolongum* 20099 and *B. infantis* 1912 showed the best tolerance to bile. Thus, *L. acidophilus* strains 2401, 2409 and 2415 and *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 strains can be used as dietary adjuncts in fermented dairy products.

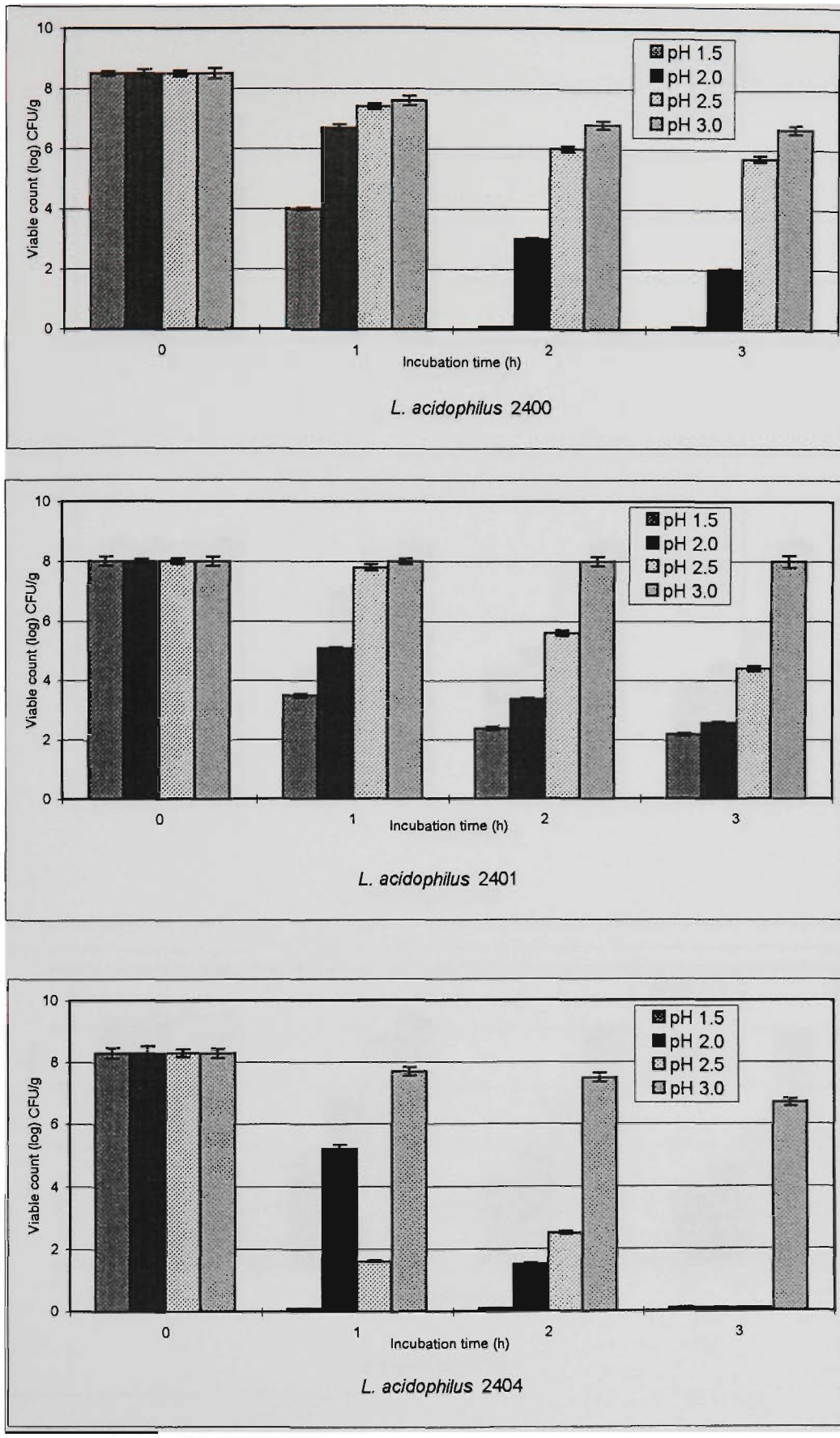

Fig. 5.1.1. Survival of *L. acidophilus* strains 2400, 2401 and 2404 during 3 h incubation in HCl solutions

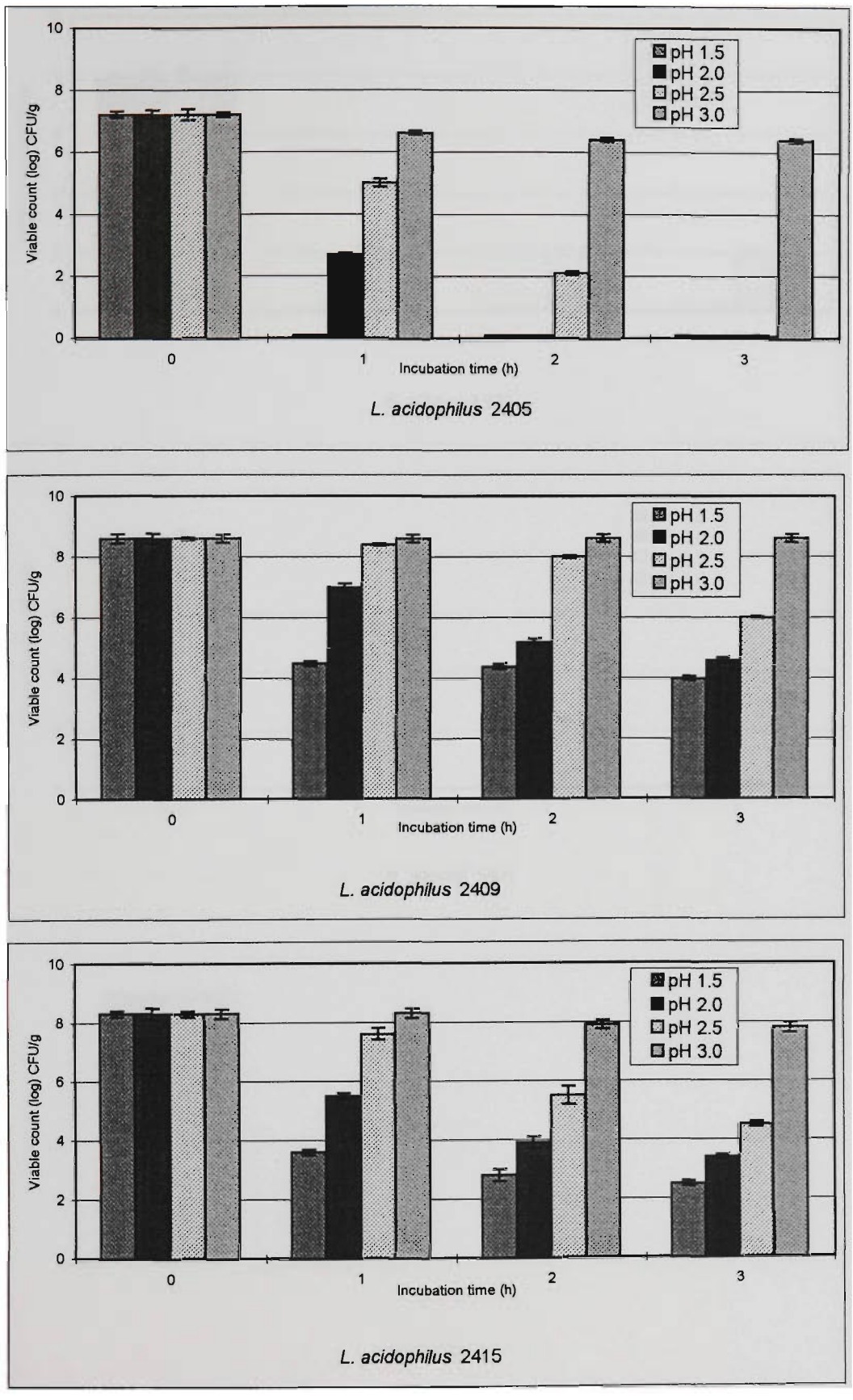

Fig. 5.1.2. Survival of *L. acidophilus* strains 2405, 2409 and 2415 during 3 h incubation in HCl solutions

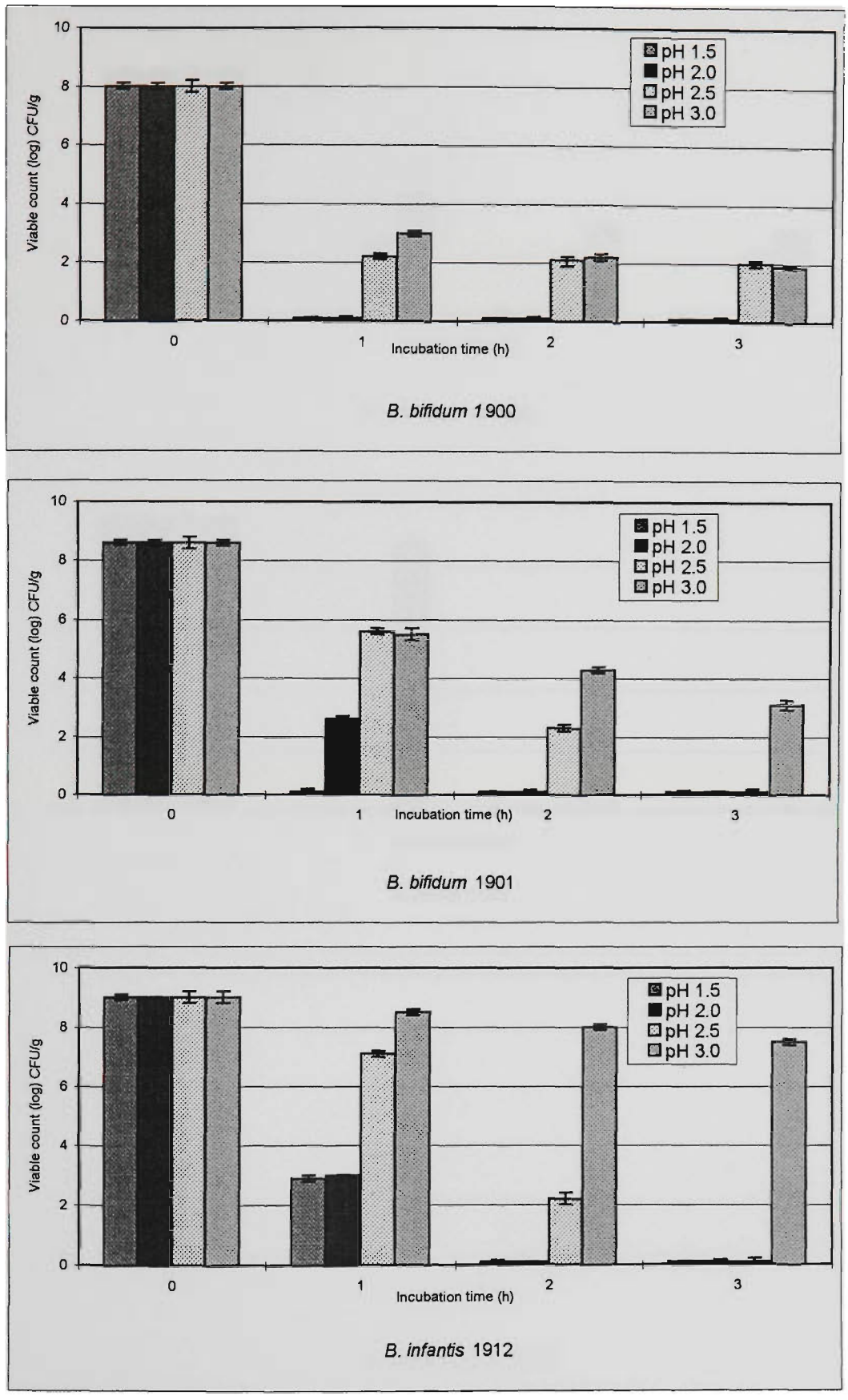

Fig. 5.1.3. Survival of *B. bifidum 1900 and 1901 and B. infantis 1912* during 3 h incubation in HCl solutions

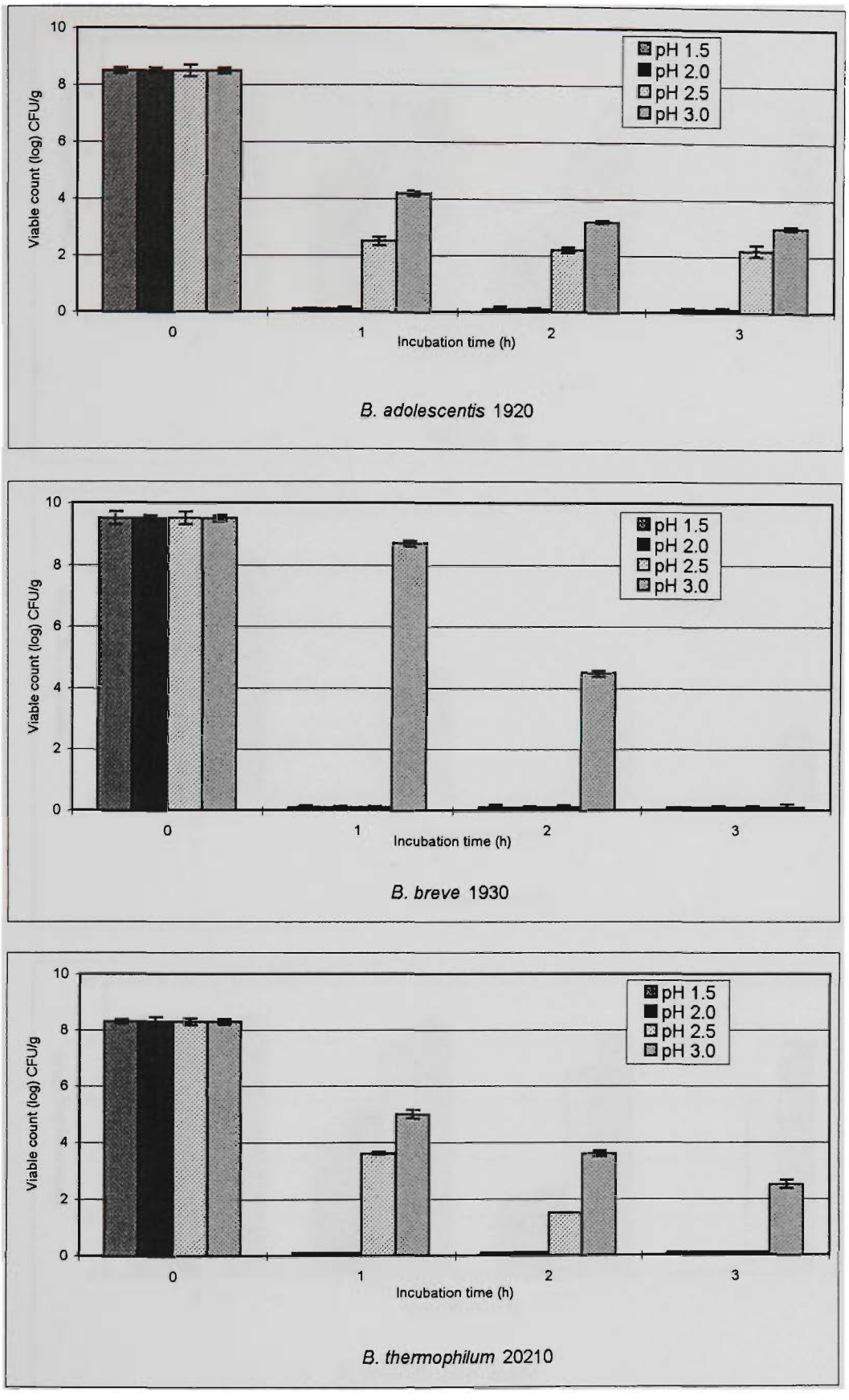

Fig. 5.1.4. Survival of *B. adolescentis* 1920, *B. breve* 1930 and *B. thermophilum* 20210 during 3 h incubation in HCl solutions

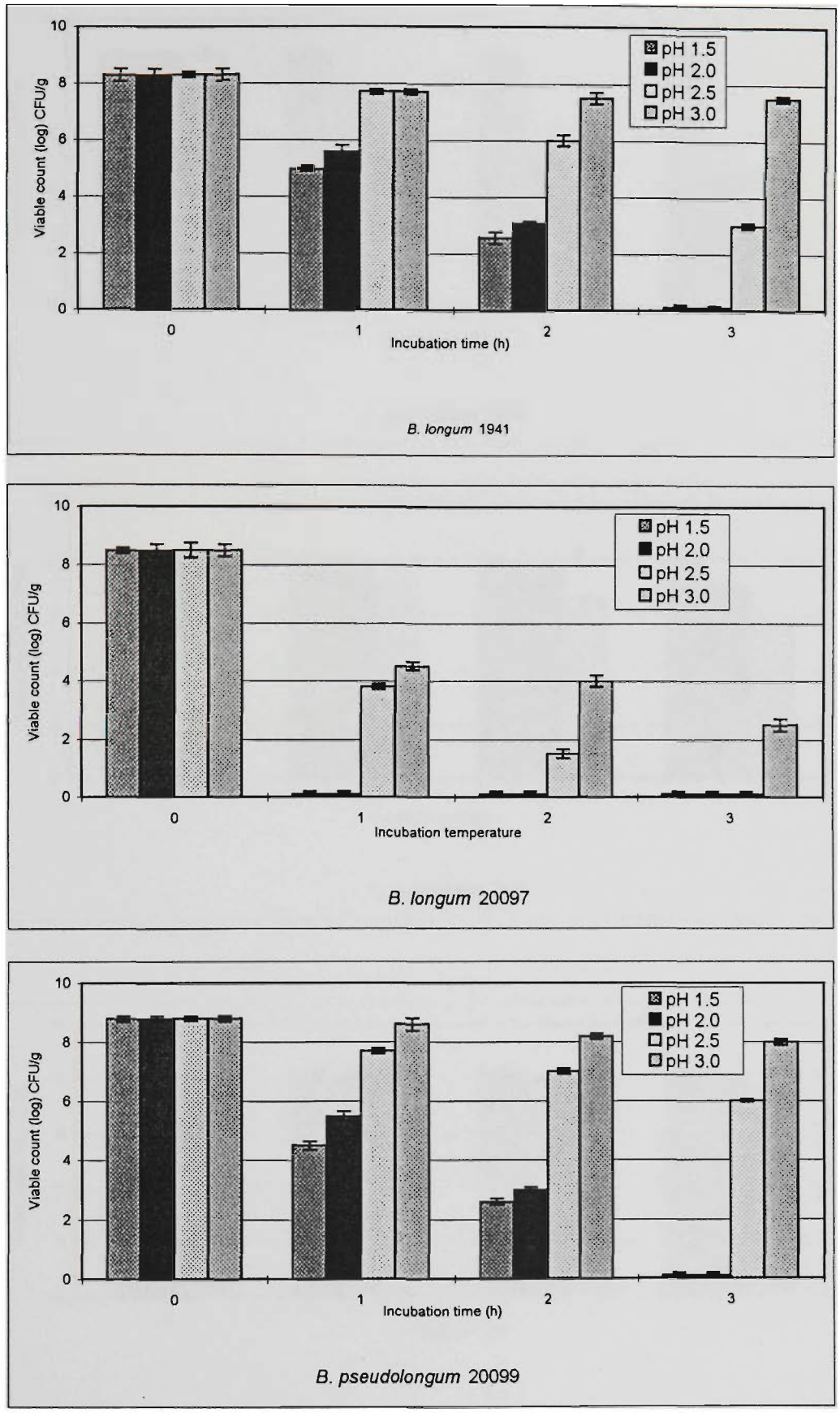

Fig. 5.1.5. Survival of *B. longum1941*, *B. longum 20097* and *B. pseudolongum* 20099 during 3 h incubation in HCl solutions

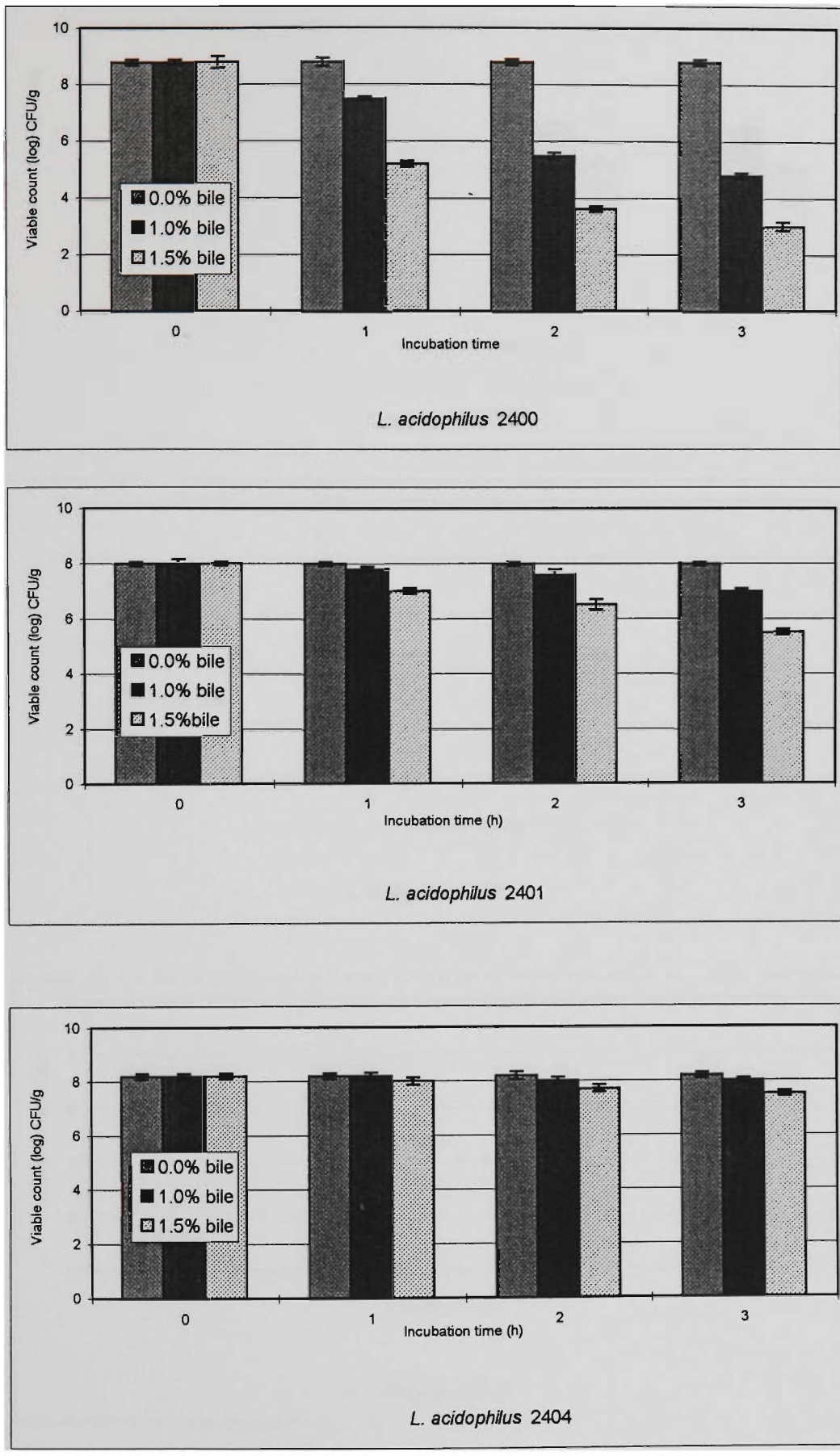

Fig. 5.1.6. Survival of *L.acidophilus* 2400, 2401 and 2404 during 3 h incubation in bile

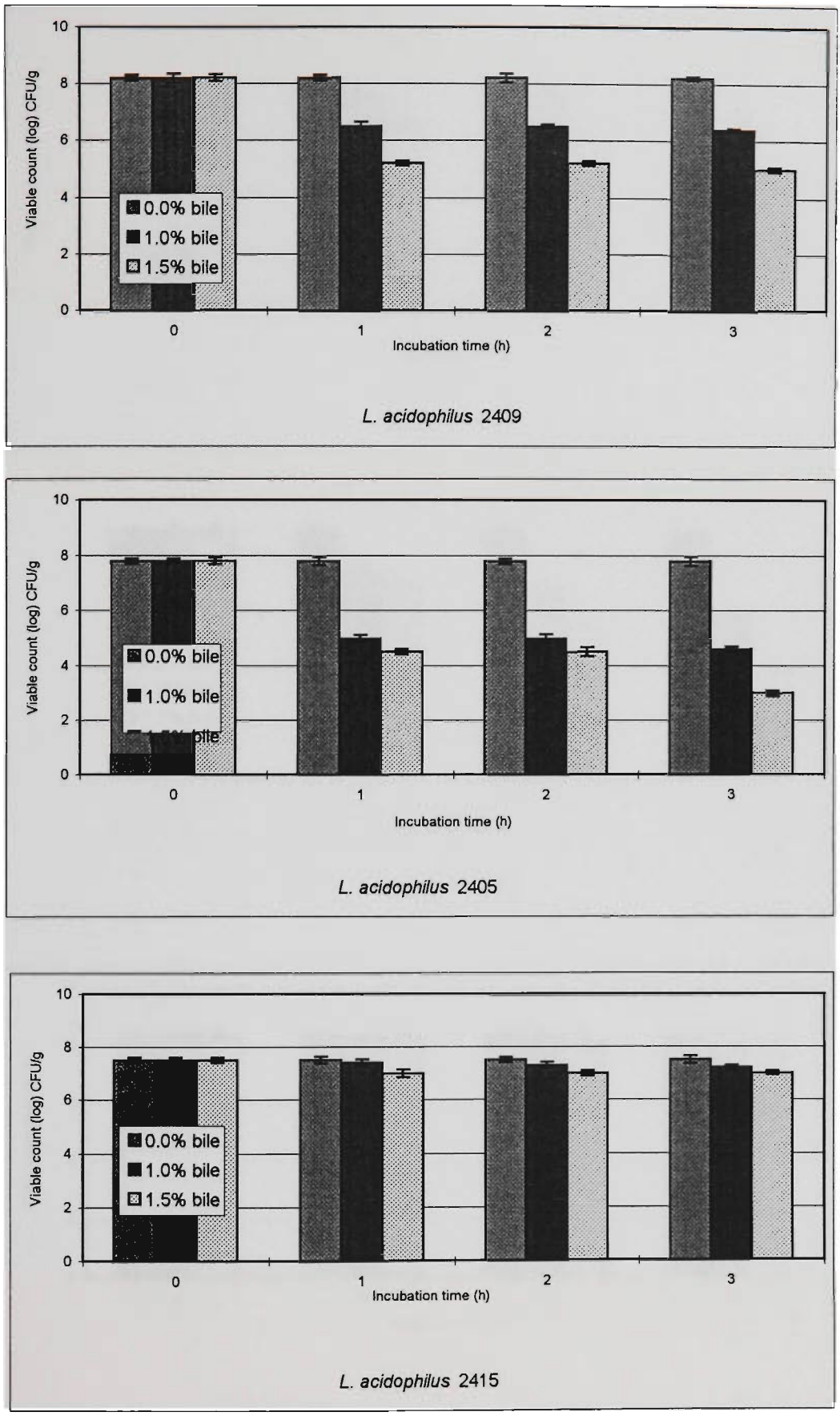

Fig. 5.1.7. Survival of *L.acidophilus 2405, 2409 and 2415* during 3 h incubation in bile

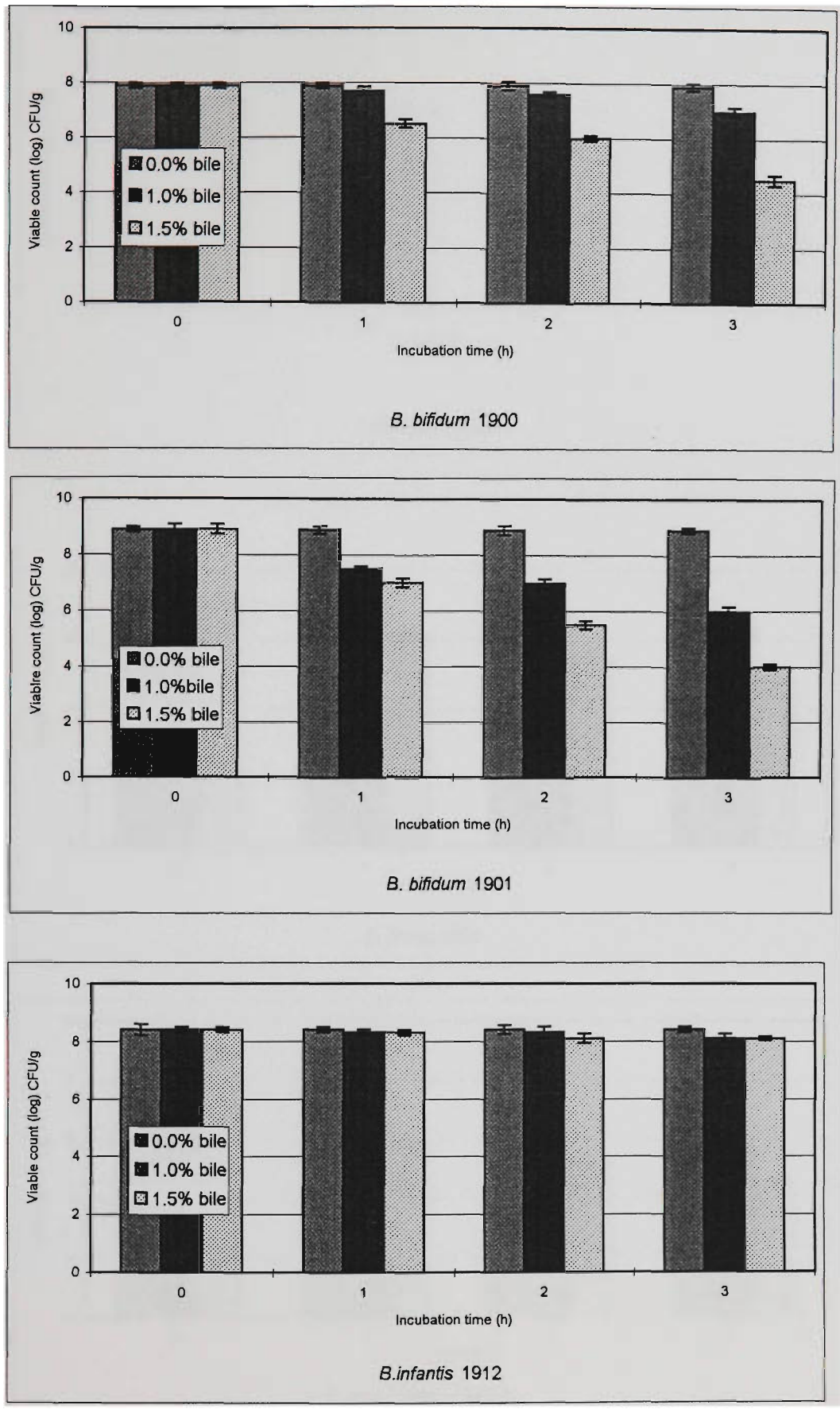

Fig. 5.1.8. Survival of *B. bifidum 1900 and 1901 and B. infantis* 1912 during 3 h incubation in bile

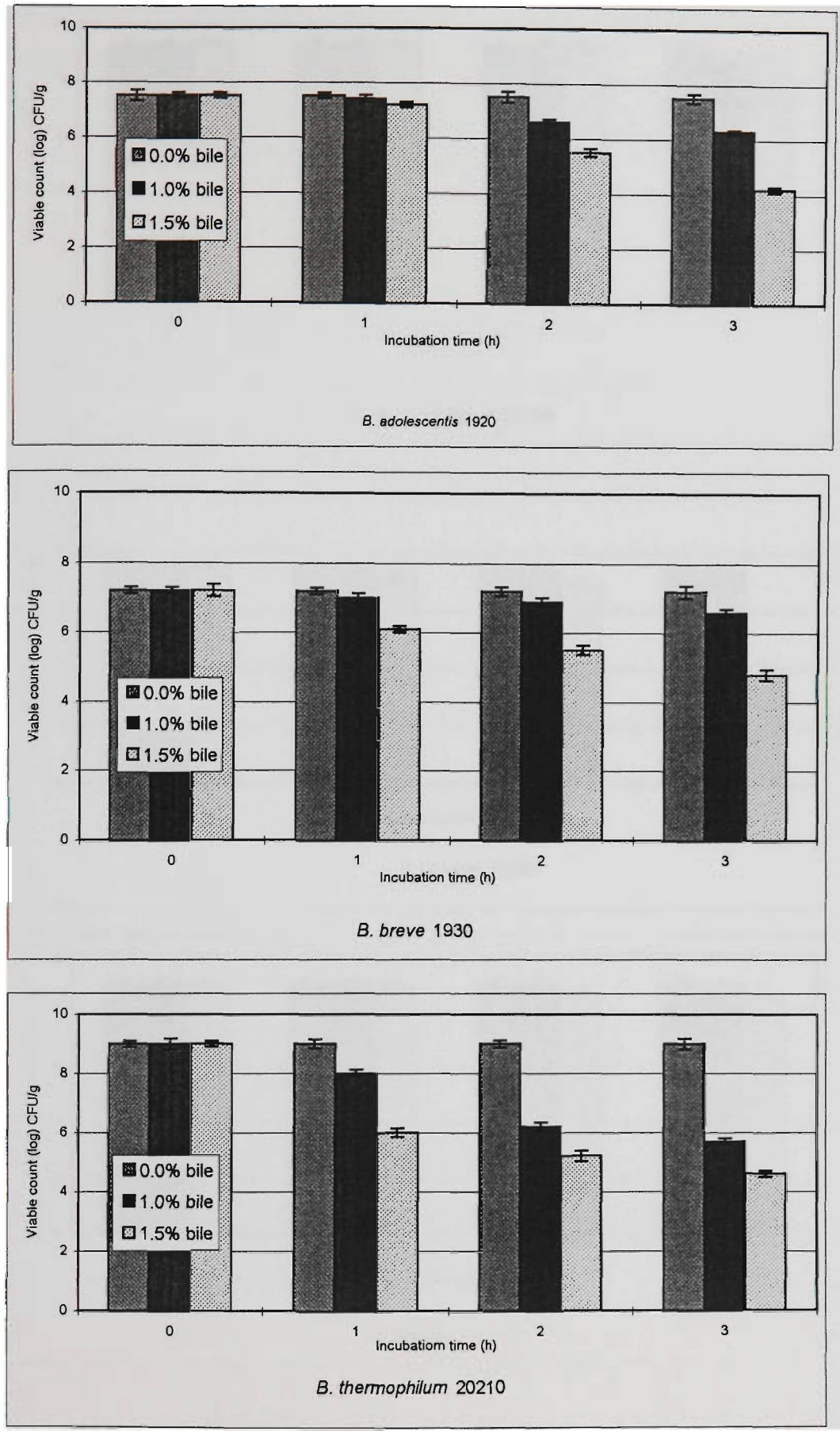

Fig. 5.1.9. Survival of *B. adolescentis 1920, B. breve 1930 and B. thermophilum* 20210 during 3 h incubation in bile

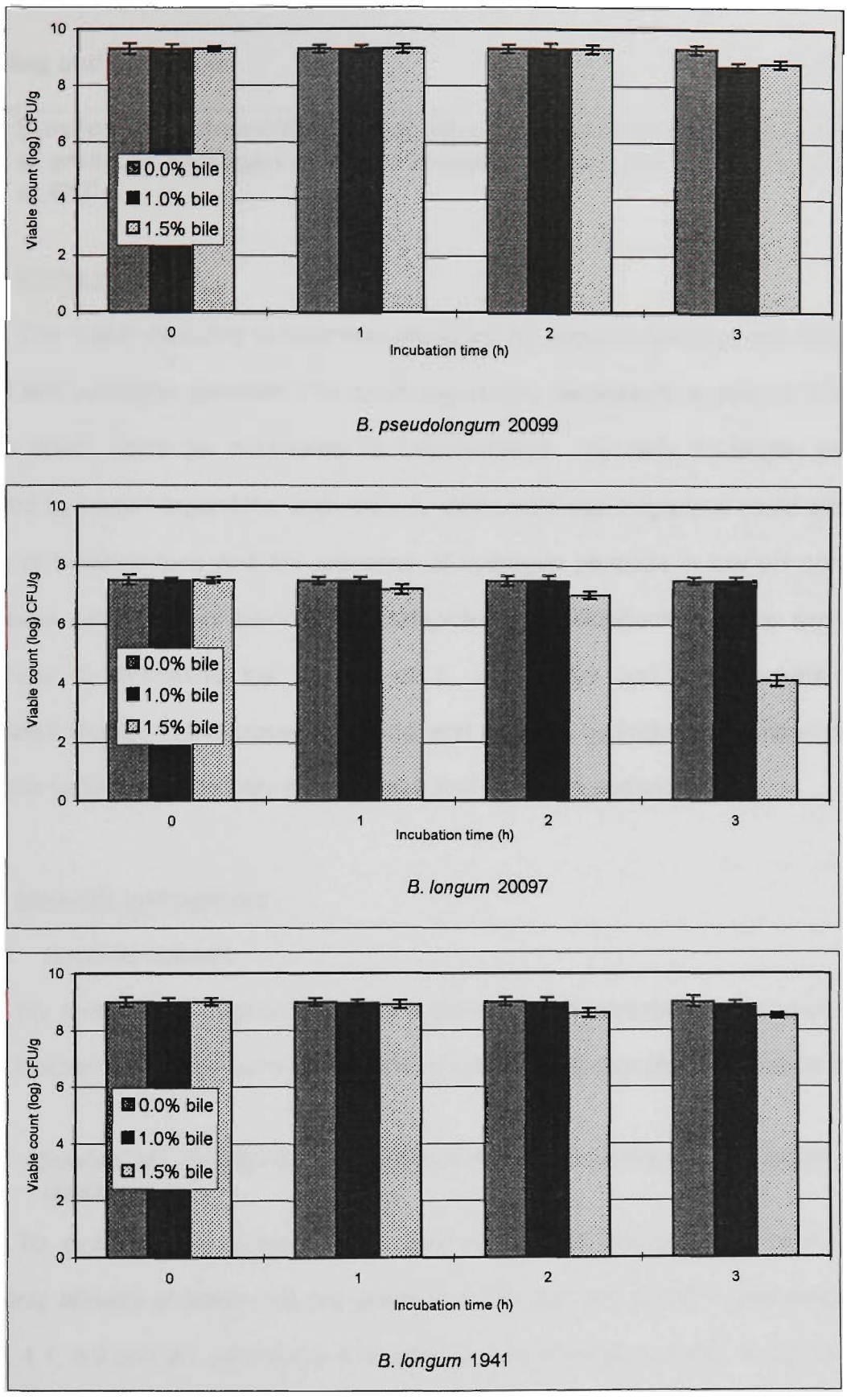

Fig. 5.1.10. Survival of *B. pseudolongum* 20099, *B. longum* 20097 and *B. longum* 1941 during 3 h incubation in bile

5.2. Survival of *Lactobacillus acidophilus* and bifidobacteria in the presence of acid and hydrogen peroxide to simulate their survival during storage at 4°C[1]

5.2.1. Introduction

The major inhibitory substances produced by yogurt organisms are claimed to be acid and hydrogen peroxide. The pH of yogurt may decrease to as low as 3.7 during storage which could be detrimental to bifidobacteria. Similarly, hydrogen peroxide produced by yogurt organisms, especially *L. delbrueckii* ssp *bulgaricus* could affect the viability of bifidobacteria and the presence of hydrogen peroxide in low pH conditions may cause synergistic inhibition of *L. acidophilus* and bifidobacteria. The aim of this study was to determine the survival of *L. acidophilus* and bifidobacteria during refrigerated storage in the presence of acid, and acid and hydrogen peroxide in order to determine synergistic inhibitory effect of acid and hydrogen peroxide.

5.2.2. Materials and methods

5.2.2.1. Bacterial cultures

Six strains of *L. acidophilus* and 9 strains of bifidobacteria were used in this study. Bacterial cultures were grown and maintained as described in section 2.2.2.

5.2.2.2. Survival of L. acidophilus and bifidobacteria during storage under acidic conditions

To evaluate the survival of *L. acidophilus* and bifidobacteria under acidic conditions, aliquots of active cultures grown in NGYC for 18 h at 37°C were adjusted to pH 4.3, 4.1, 3.9 and 3.7 with sterile 4 N lactic acid and stored in a walk-in-cooler at 4°C for 6 weeks. Samples were taken at 6-day intervals and the counts of bifidobacteria were determined by the pour plate technique using 10-fold serial dilutions prepared in sterile 0.1% peptone and water diluent as described in section 2.2.2.

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[1] A paper titled "Survival of bifidobacteria during refrigerated storage in the presence of acid and hydrogen peroxide" was published in *Milchwissenschaft* 51:65-70

5.2.2.3. Survival of L. acidophilus and bifidobacteria during storage in the presence of acid and hydrogen peroxide

To evaluate the synergistic effect of acid and hydrogen peroxide on the survival of *L. acidophilus* and bifidobacteria, aliquots of active cultures grown in NGYC for 18 h at 37°C were adjusted to pH 4.3, 4.1, 3.9 and 3.7 with sterile 4 **N** lactic acid and freshly prepared hydrogen peroxide solution (10 mg/mL) was added to the cultures to achieve a final concentration of 100µg/mL. The cultures were stored in a walk-in-cooler at 4°C for 6 weeks. Samples were taken at 6-day intervals and the counts were determined by the pour plate technique using 10-fold serial dilutions prepared in sterile 0.1% peptone and water.

5.2.2. 4. Enumeration of L. acidophilus and bifidobacetria

MRS agar supplemented with 0.05% L-cystein·HCl was used for enumeration of *L. acidophilus* and bifidobacteria. L-cystein·HCl was used to lower the oxidation and reduction potential of the medium and to enhance the anaerobic growth of probiotic bacteria, particularly bifidobacteria. Enumeration was carried out as described in section 2.2.2.

5.2.3. Results and discussion

This study was carried out in order to determine the survival of bifidobacteria and *L. acidophilus* in the presence of antimicrobial substances such as acid and hydrogen peroxide produced during yogurt manufacture and storage. As lactic acid and hydrogen peroxide are the major inhibitory substances produced by yogurt culture bacteria, it was desirable to study their synergistic effect on the survival of bifidobacteria and *L. acidophilus*.

The survival of nine strains of bifidobacteria during 6 weeks of refrigerated storage at 4°C at pH 3.7 to 4.3 with or without H_2O_2 is illustrated in Figs. 5.2.1. - 5.2.9.

Section (A) of each figure shows the survival of bifidobacteria under acidic conditions without H_2O_2 and section (B) of the figures shows the survival in acidic conditions and in the presence of 100 μg/mL of H_2O_2.

In general, as shown in the section (A) of the figures, 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) declined as the pH decreased from 4.3 to 3.7. The remaining 3 strains of bifidobacteria (*B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099) survived well for 6 weeks under all pH levels studied.

The viable count of *B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 and *B. thermophilum* 20210 decreased to 10^2 to 10^4cfu/g from an initial count of 10^6 to 10^7 cfu/g in 30 days at pH 4.3 and these strains lost viability more rapidly at pH 4.1 or lower. The viable counts of *B. bifidum* 1900, *B. adolescentis* 1920, and *B. longum* 20097 reduced to 10^1 to $<10^1$ cfu/g in 12 days at pH 4.1 or lower while *B. bifidum* 1901 and *B. thermophilum* 20210 survived slightly better under acidic conditions. *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 survived well under acidic conditions. The counts of these 3 strains reduced by <2 log cycles after 6 weeks storage even at pH 3.7, while the viable counts of *B. longum* 1941 remained similar to the original count of 10^8 cfu/g. Our previous studies (Lankaputhra *et al.*, 1996b) showed that *B. infantis 1912*, *B. longum 1941* and *B. pseudolongum* were tolerant to high acid conditions (pH 1.5 to 3.0) for 2 to 3 h. Shah *et al.* (1995) reported that pH of some yogurts reduced as low as pH 3.7 during refrigerated storage, hence the pH levels used in this study (pH 3.7 to 4.3) were selected based on the findings of this study (Shah *et al.*, 1995).

Section B of the figures shows the survival of *B. bifidum* 1900 and 1901, *B. infantis* 1912, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 1941 and 20097, *B. pseudolongum* 20099 and *B. thermophilum* 20210 in the presence of hydrogen peroxide at various pH levels (pH 3.7 to 4.3). The presence of acid and hydrogen

peroxide seemed to have adverse effect on the viability of *B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 and *B. thermophilum* 20210. The counts of *B. bifidum* 1901 reduced to 10^1 cfu/g in 12 days at pH 4.3 in the presence of acid and hydrogen peroxide, whereas in the absence of hydrogen peroxide (Fig. 5.2.2. A), the viable count of *B. bifidum* 1901 remained $>10^6$ cfu/g after 12 days. Similarly, the viable counts of *B. adolescentis* 1920 and *B. breve* 1930 decreased to $<10^1$ cfu/g in 6 days storage at 4°C under all pH levels studied (section B of the figures). However, in the absence of hydrogen peroxide the viable count of these 2 strains were 10^2 and 10^5 cfu/g respectively after 12 days storage at pH 4.3 (section A of the figures). The viable counts of *B. longum* 20097 reduced to $<10^1$ cfu/g within 6 days storage in the presence of hydrogen peroxide at all pH levels studied, whereas the viable count of *B. longum* 20097 remained at $>10^6$cfu/g after 6 days storage at pH 4.3 in the absence of hydrogen peroxide (section A of the figures). A similar pattern was observed for *B. themophilum* 20210; the viable count reduced to 10^1 cfu/g after 24 days storage at 4°C in the presence of hydrogen peroxide at pH 4.3 (section B of the figures), whereas in the absence of hydrogen peroxide at pH 4.3, the viable count was 10^5 cfu/g after 24 days storage at 4°C (section B of the figures). The viability of *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 were not substantially affected by the presence of hydrogen peroxide at all pH levels studied; even after 6 weeks of storage at 4°C, these 3 strains had a count of 10^6 to 10^8cfu/g.

Thus, it appears that there may be a synergistic effect of acid and hydrogen peroxide in reducing the viable counts of *B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 and *B. thermophilum* 20210. However, *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 appeared to be resistant to acid and hydrogen peroxide; thus, *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 can be used as dietary adjuncts in fermented dairy products

whereas, *B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 and *B. thermophilum* 20210 are not suitable for inclusion as dietary adjuncts.

Figures 5.2.10 - 5.2.15. show the survival of *L. acidophilus* strains 2400, 2401, 2404, 2405, 2409 and 2415 under acidic conditions with or without H_2O_2. Section A of each figure shows the survival of *L. acidophilus* under acidic conditions without H_2O_2 and section B of the figures show the survival of the former bacteria in acidic conditions with 100 μg/mL of H_2O_2 added to the medium.

All strains of *L. acidophilus* showed more reduction in viable counts at lower pH levels during storage in the absence or presence of H_2O_2. However, as shown in bifidobacterial strains, no synergistic effect was shown against *L. acidophilus*. *L. acidophilus* 2400, 2401, 2404, 2405 and 2409 showed slight improvement in viability in the presence of H_2O_2. This may be due to the ability of H_2O_2 to increase O_2 concentration in the medium by reducing to H_2O and O_2 in the presence of catalase. Generally, bifidobacteria are anaerobic and presence of O_2 is inhibitory to these bacteria. However, *L. acidophilus* is microaerophillic and therefore they may prefer slightly oxygenated condition. Our previous study (Shah *et al.*, 1995) showed that *L. acidophilus* survived better than bifidobacteria in commercial yogurts.

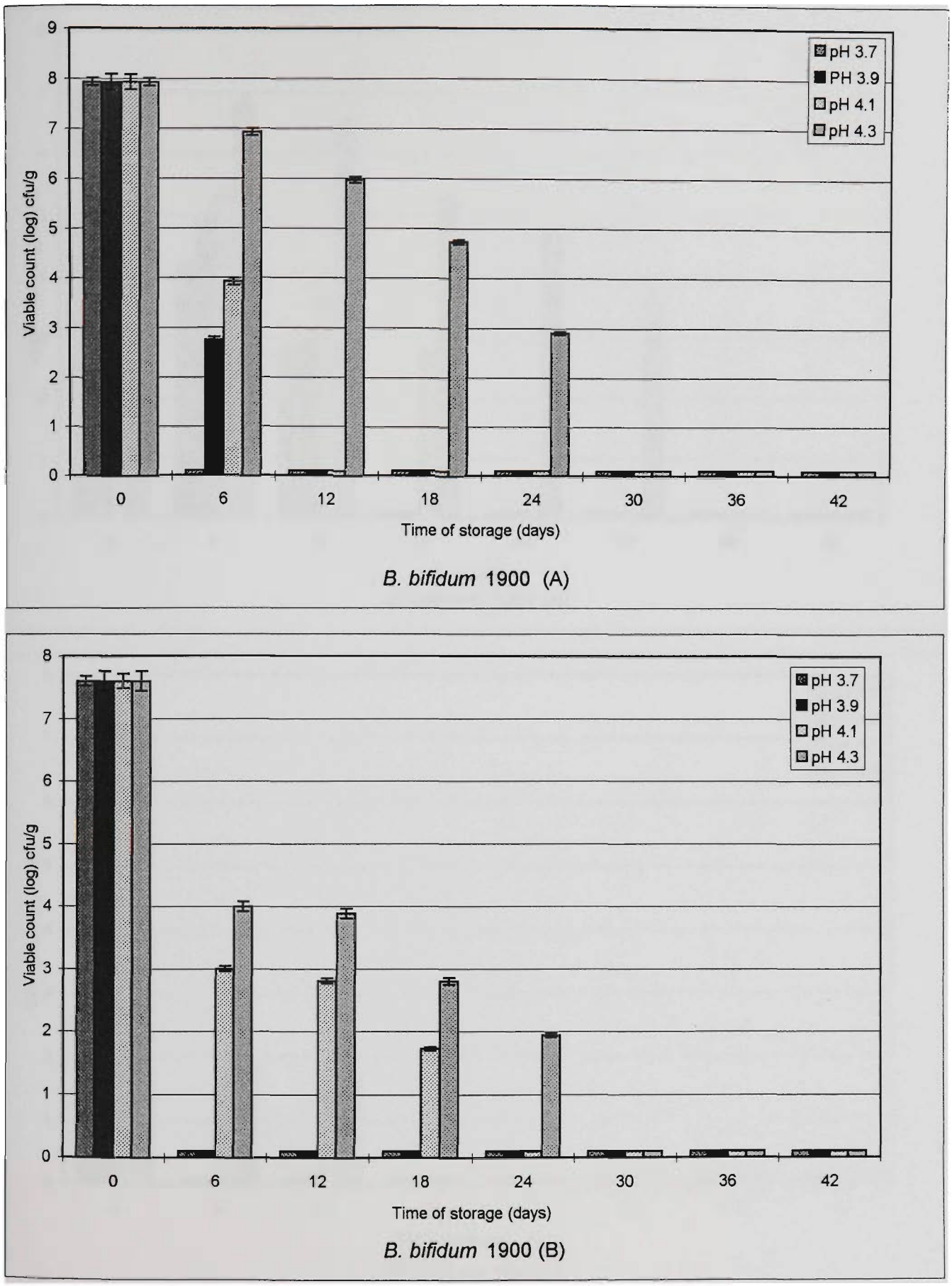


Fig. 5.2.1. Survival of *B. bifidum 1900* in acidic conditions (A) and in the presence of acid and H_2O_2 (B) during storage at 4°C.

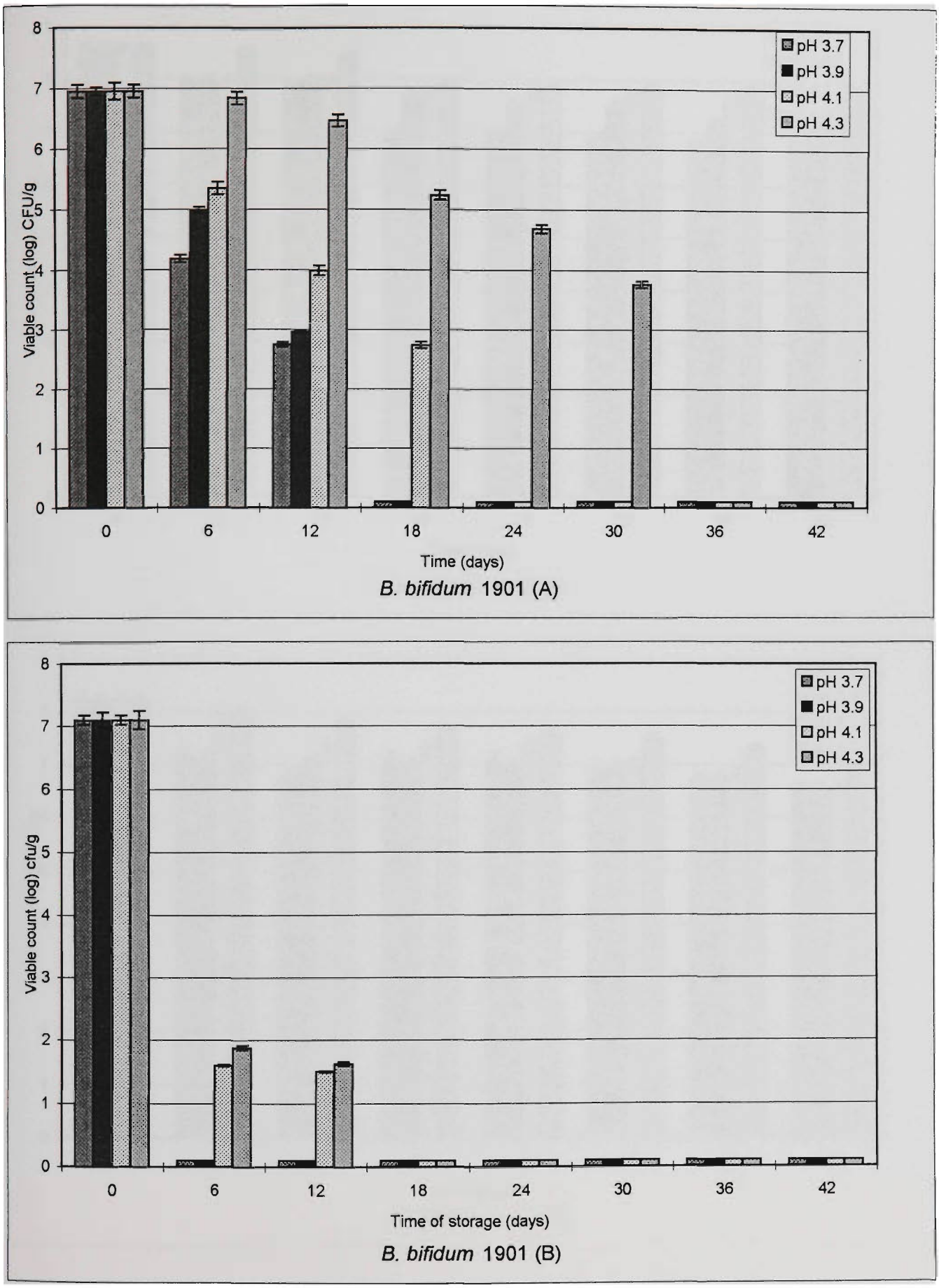


Fig. 5.2.2. Survival of *B. bifidum 1901* in acidic conditions (A) and in the presence of acid and H_2O_2 (B).

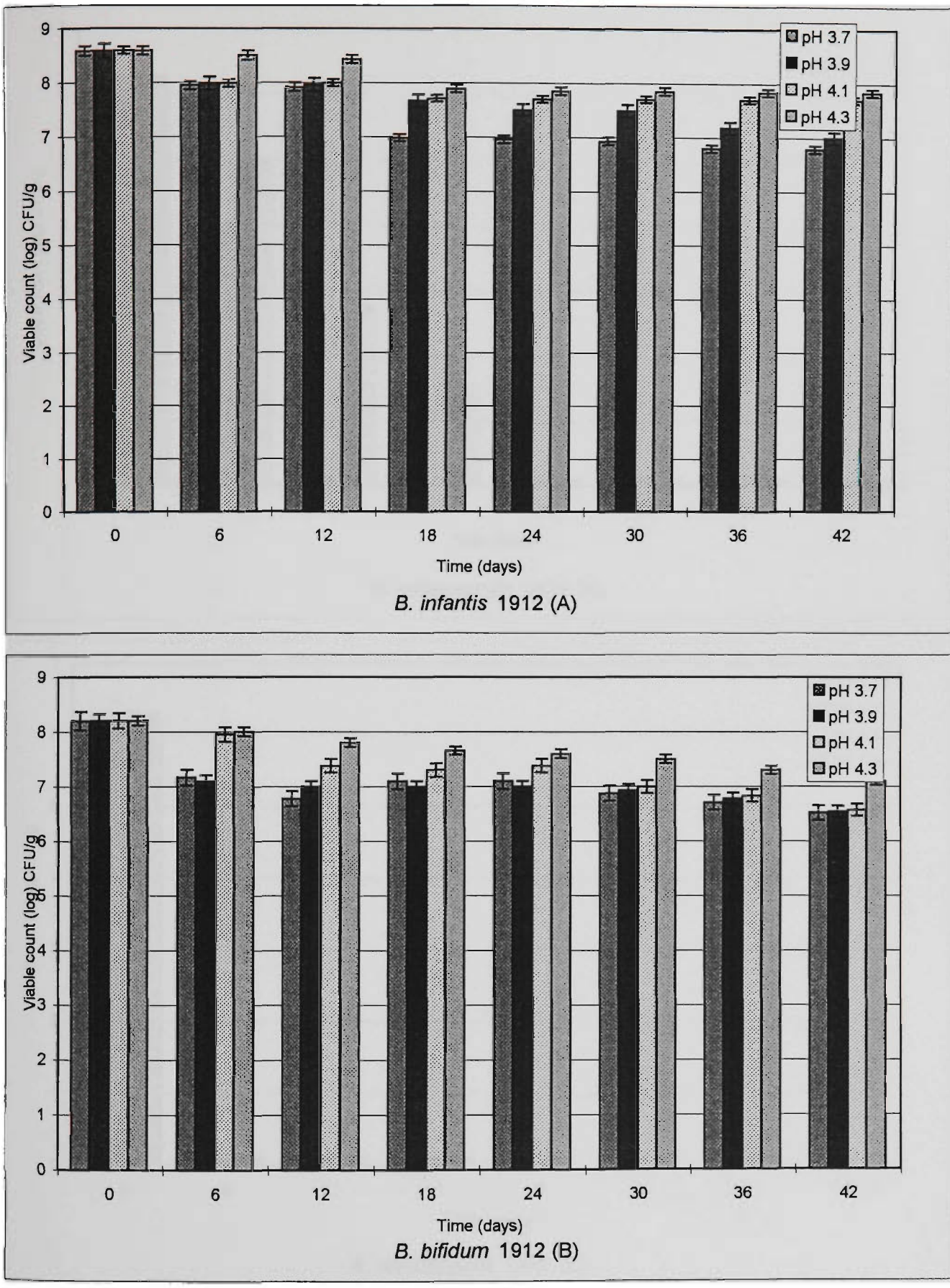


Fig. 5.2.3. Survival of *B. bifidum 1912* in acidic conditions (A) and in the presence of acid and H_2O_2 (B).

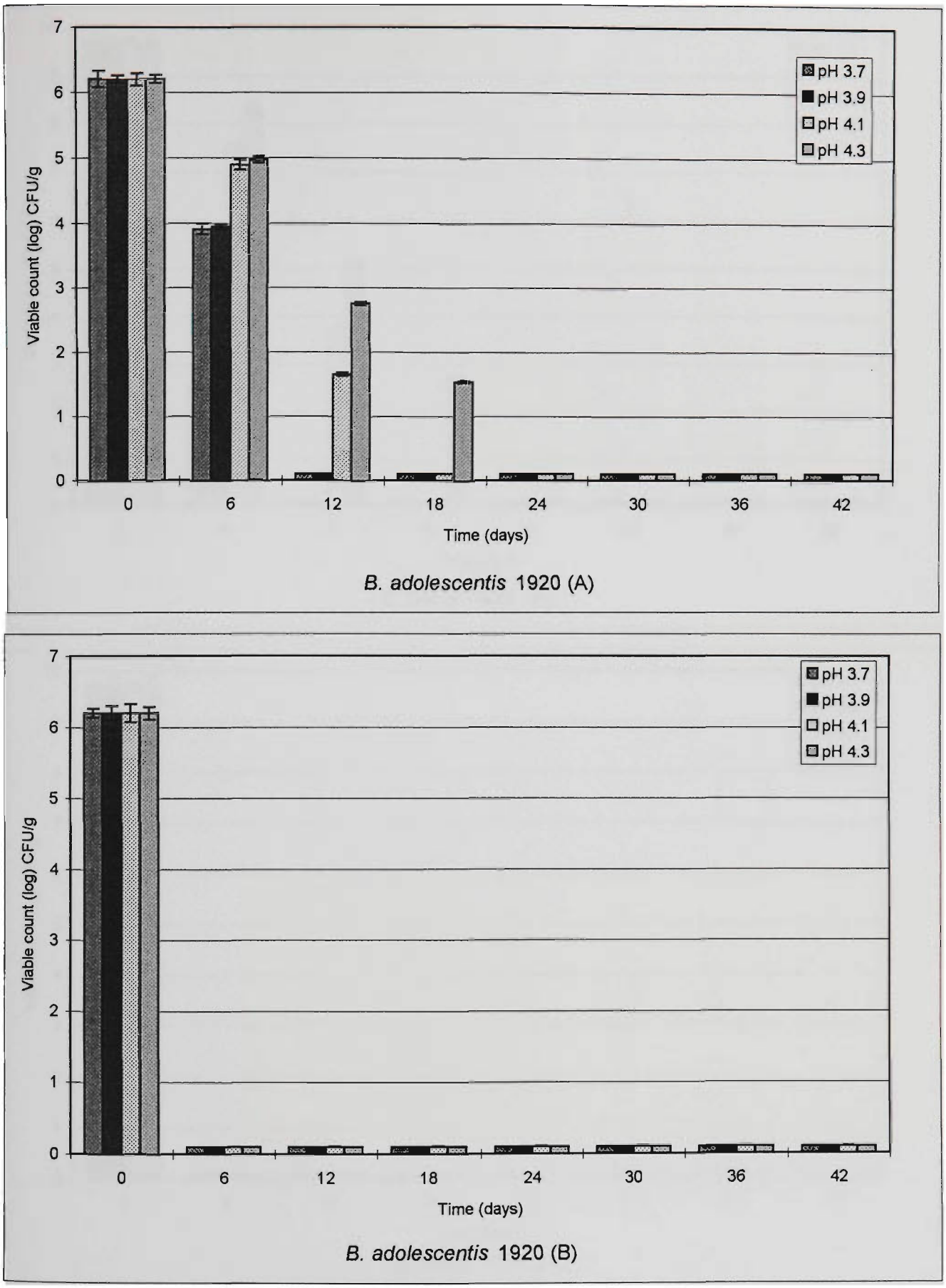


Fig. 5.2.4. Survival of *B. adolescentis 1920* in acidic conditions (A) and in the presence of acid and H_2O_2 (B).

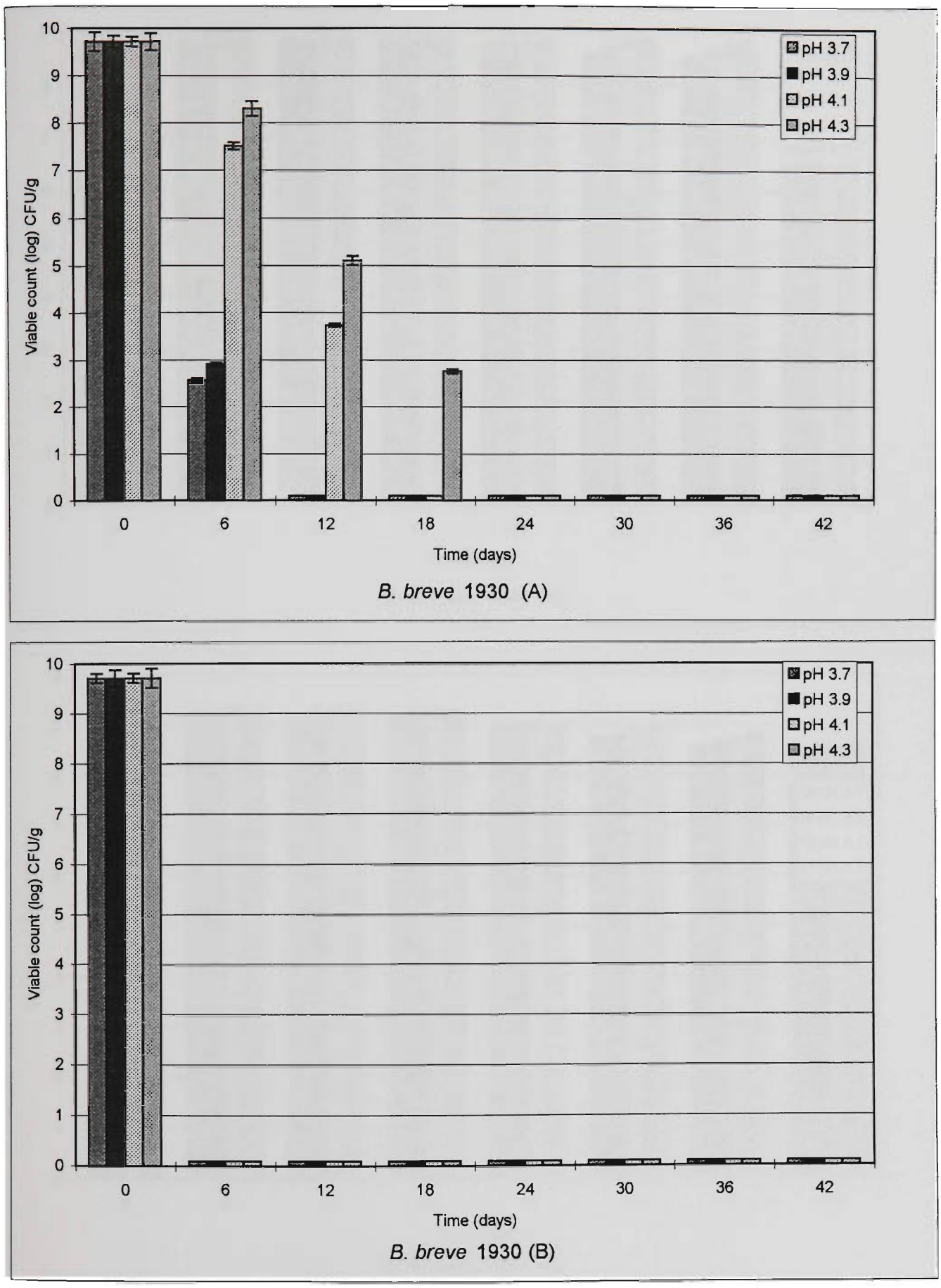


Fig. 5.2.5. Survival of *B. breve 1930* in acidic conditions (A) and in the presence of acid and H_2O_2 (B).

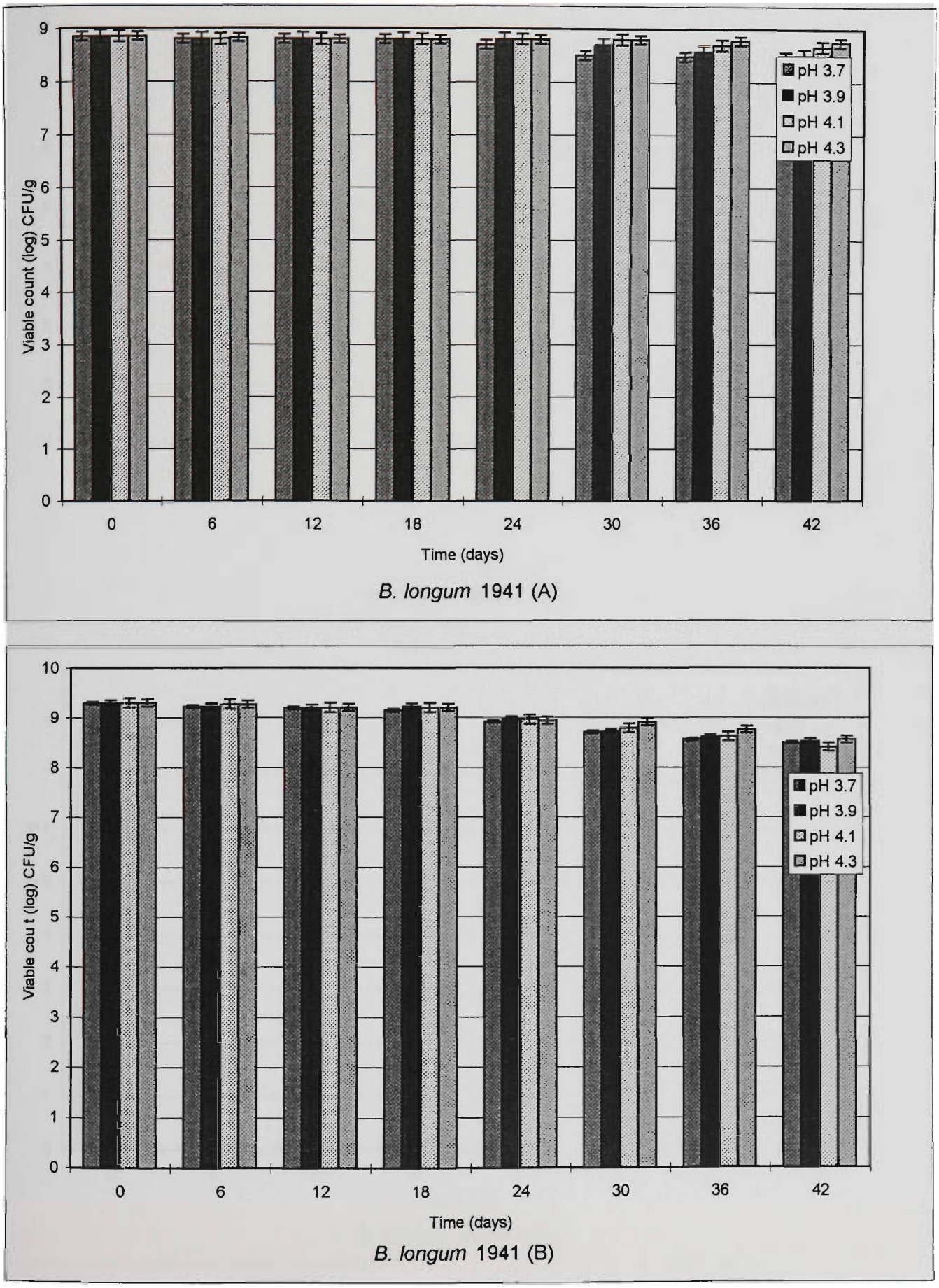


Fig. 5.2.6. Survival of *B. longum 1941* in acidic conditions (A) and in the presence of acid and H_2O_2 (B).

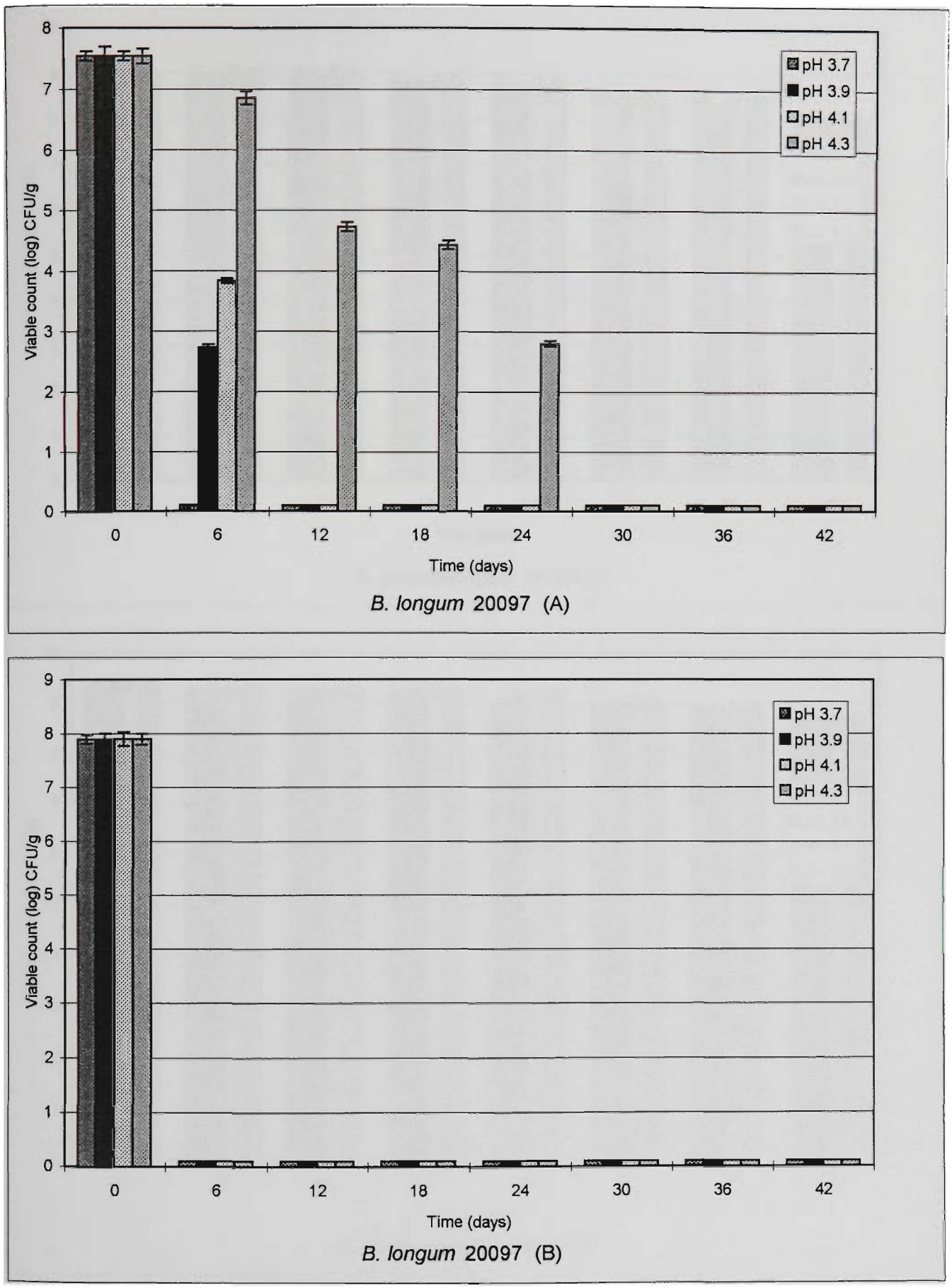


Fig. 5.2.7. Survival of *B. longum 20097* in acidic conditions (A) and in the presence of acid and H_2O_2 (B).

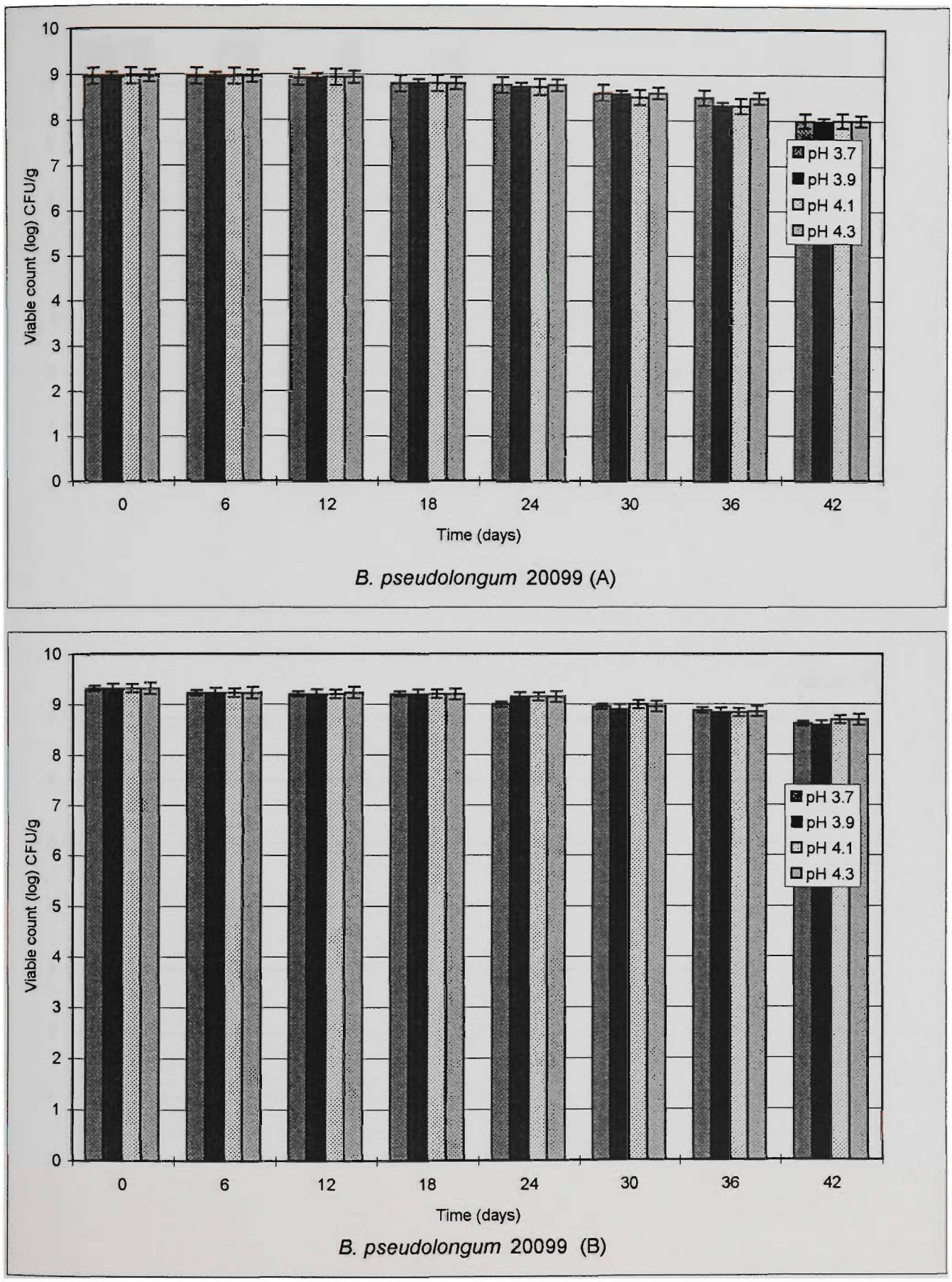


Fig. 5.2.8. Survival of *B. pseudolongum 20099* in acidic conditions (A) and in the presence of acid and H_2O_2 (B).

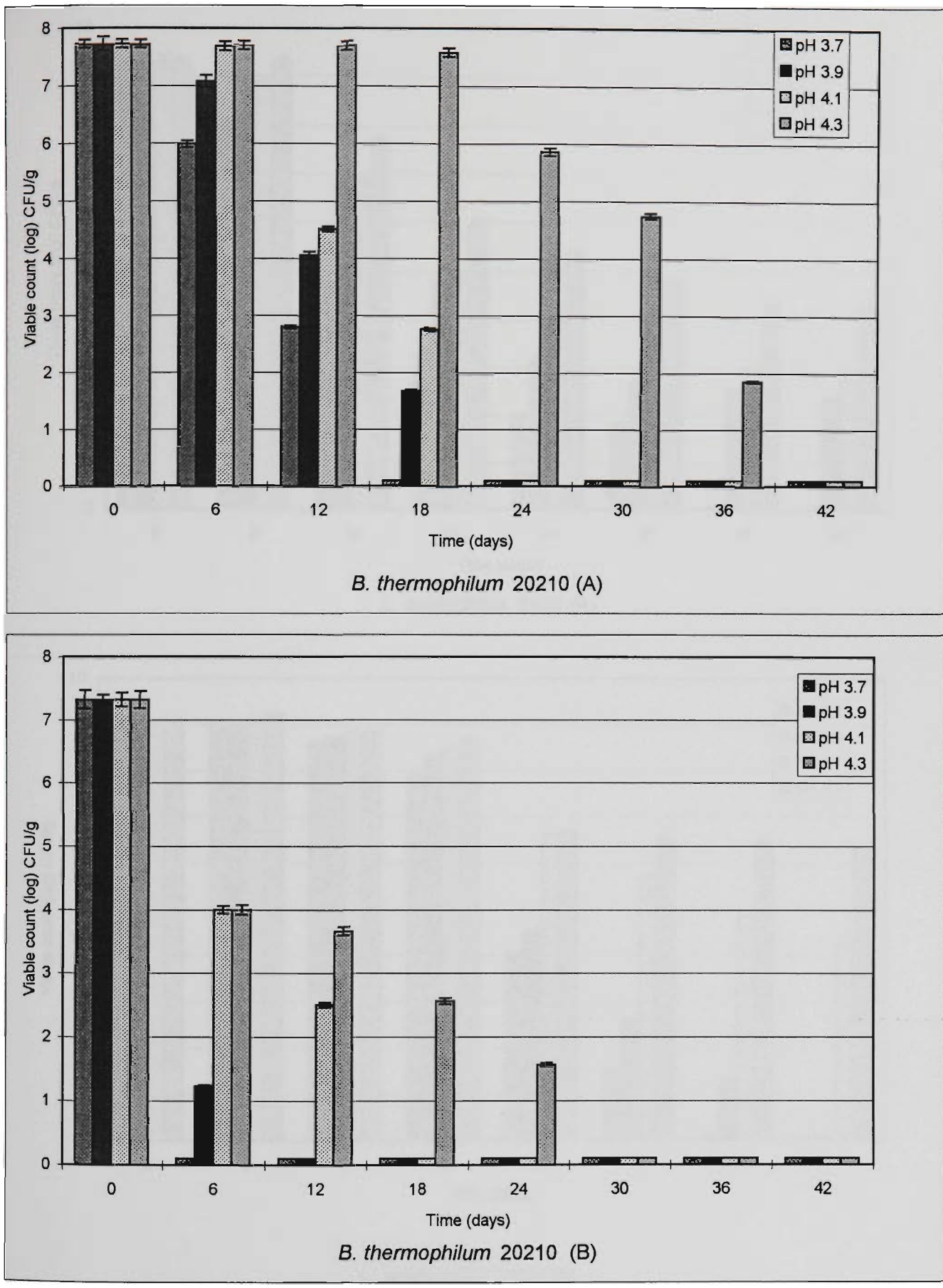
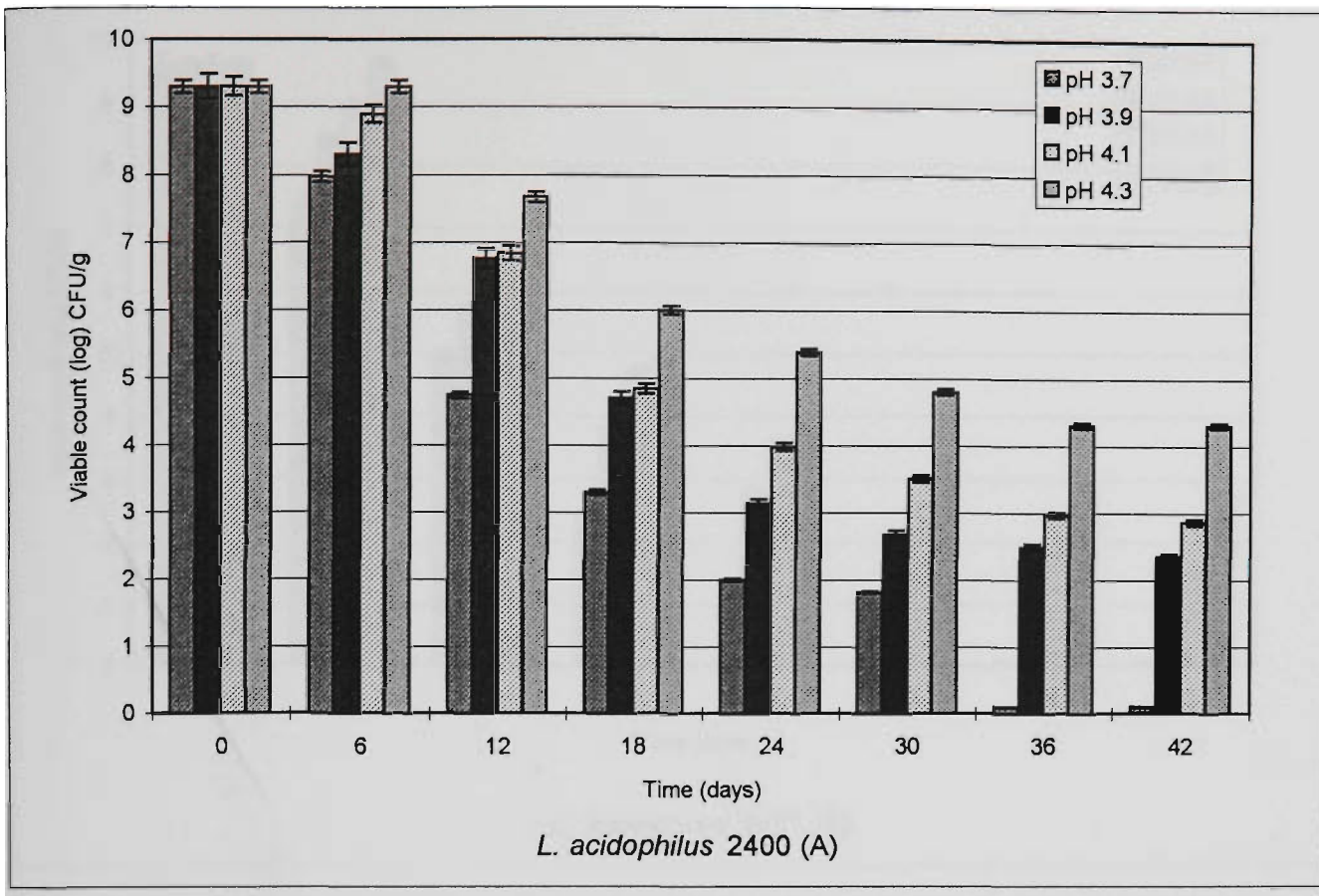


Fig. 5.2.9. Survival of *B.thermophilum 20210* in acidic conditions (A) and in the presence of acid and H_2O_2 (B).



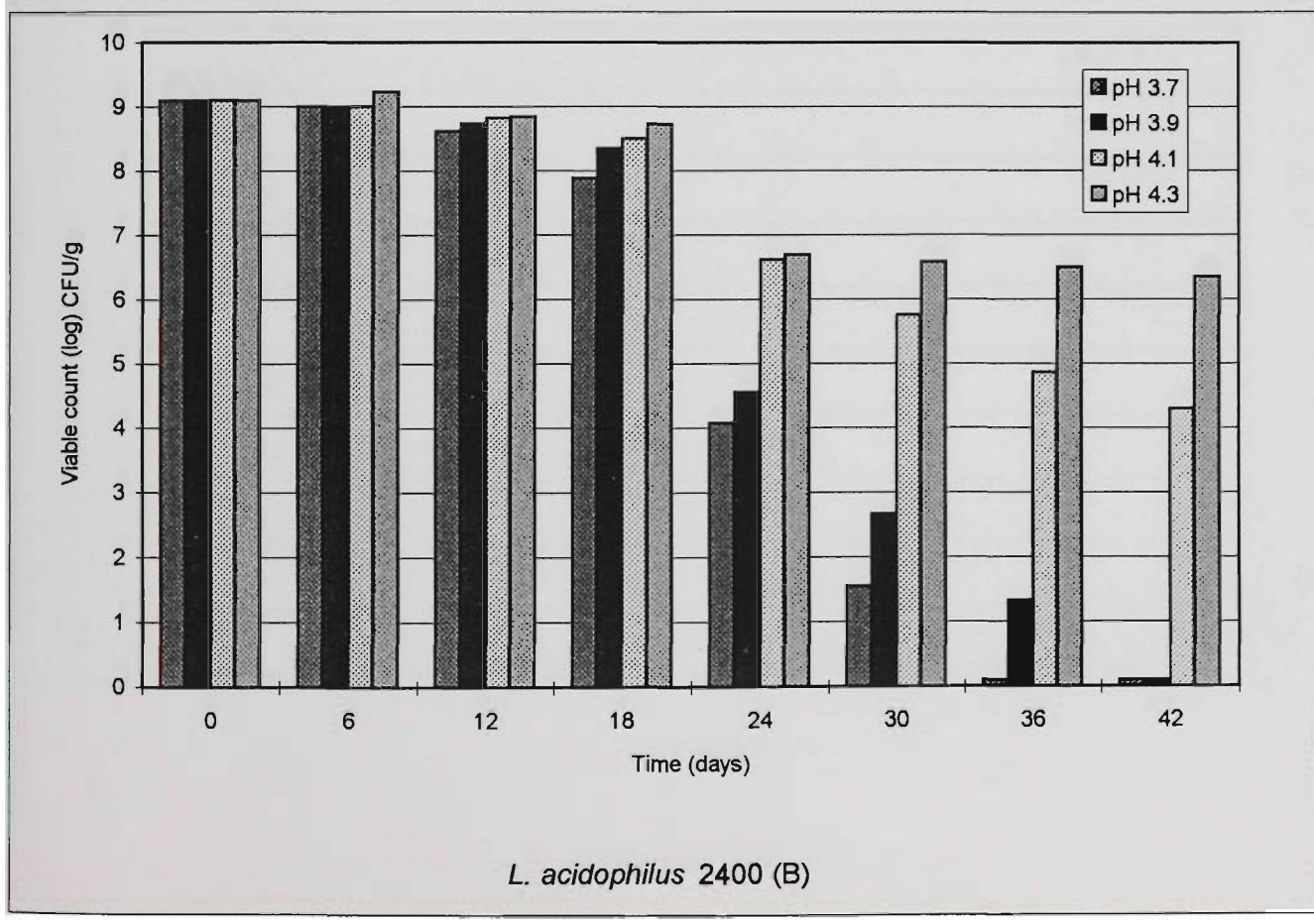
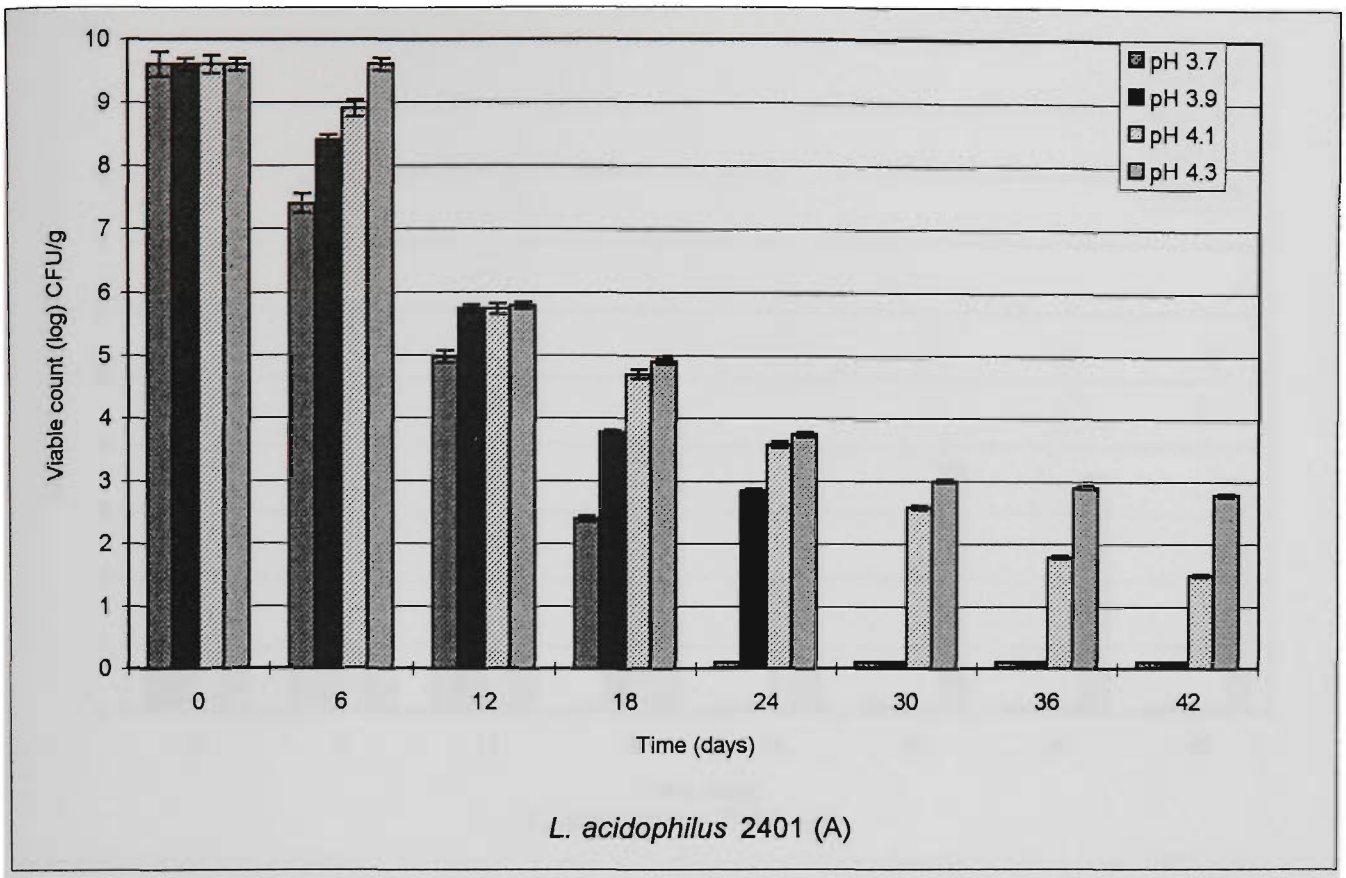


Fig. 5.2.10. Survival of *L. acidophilus* 2400 in acidic conditions (A) and in the presence of acid and H_2O_2 (B).



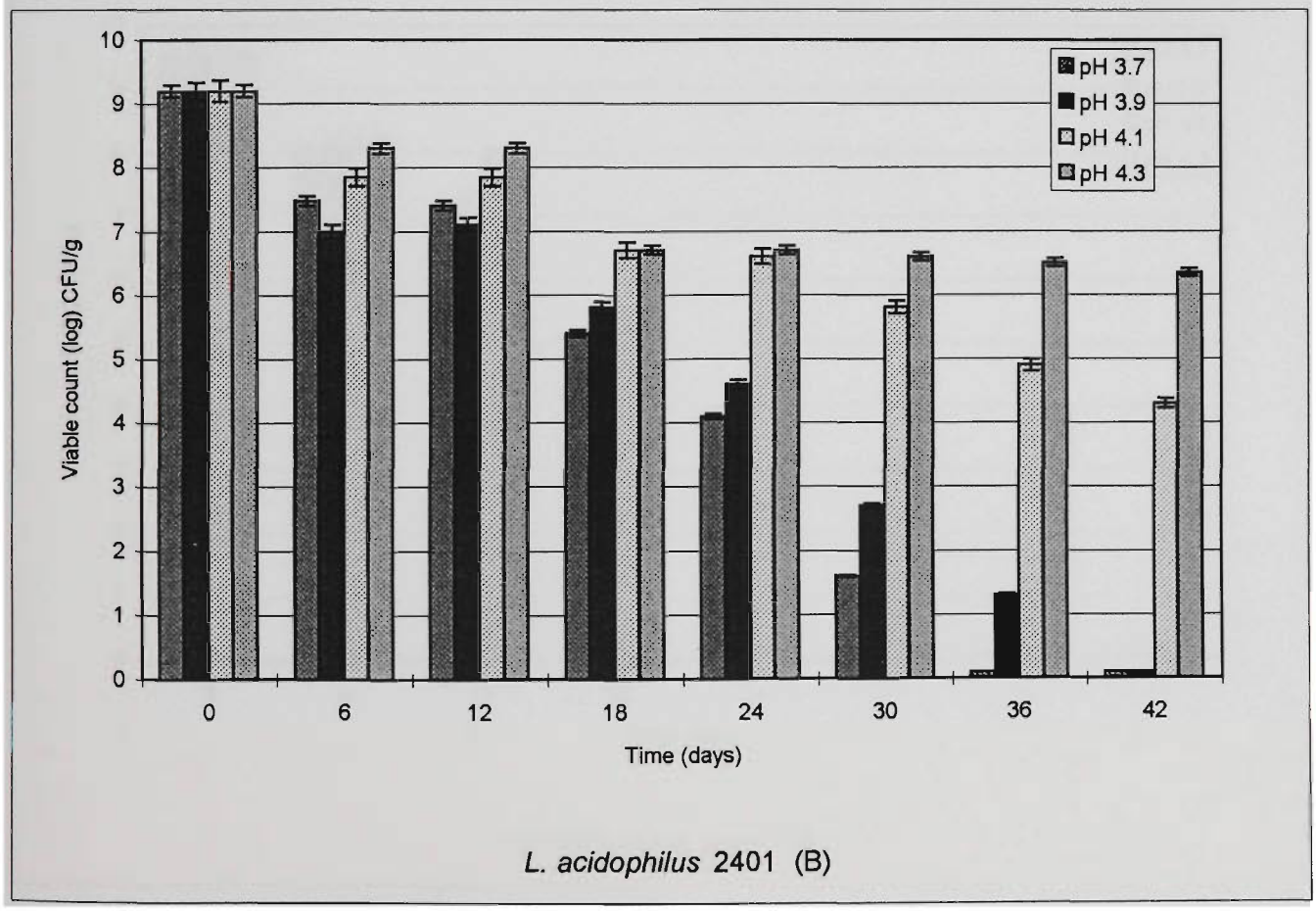


Fig. 5.2.11. Survival of *L. acidophilus* 2401 in acidic conditions (A) an in the presence of acid and H_2O_2 (B).

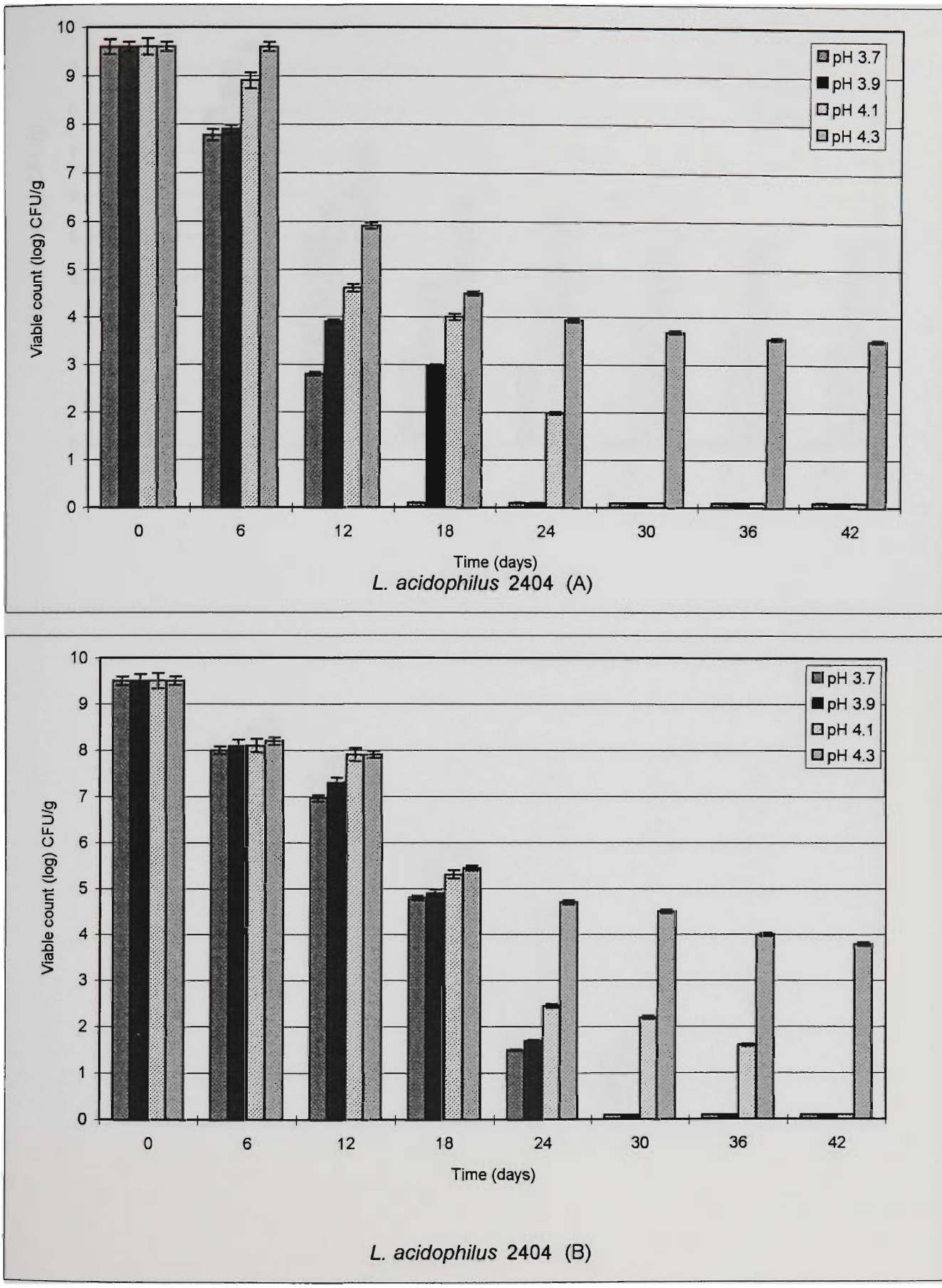
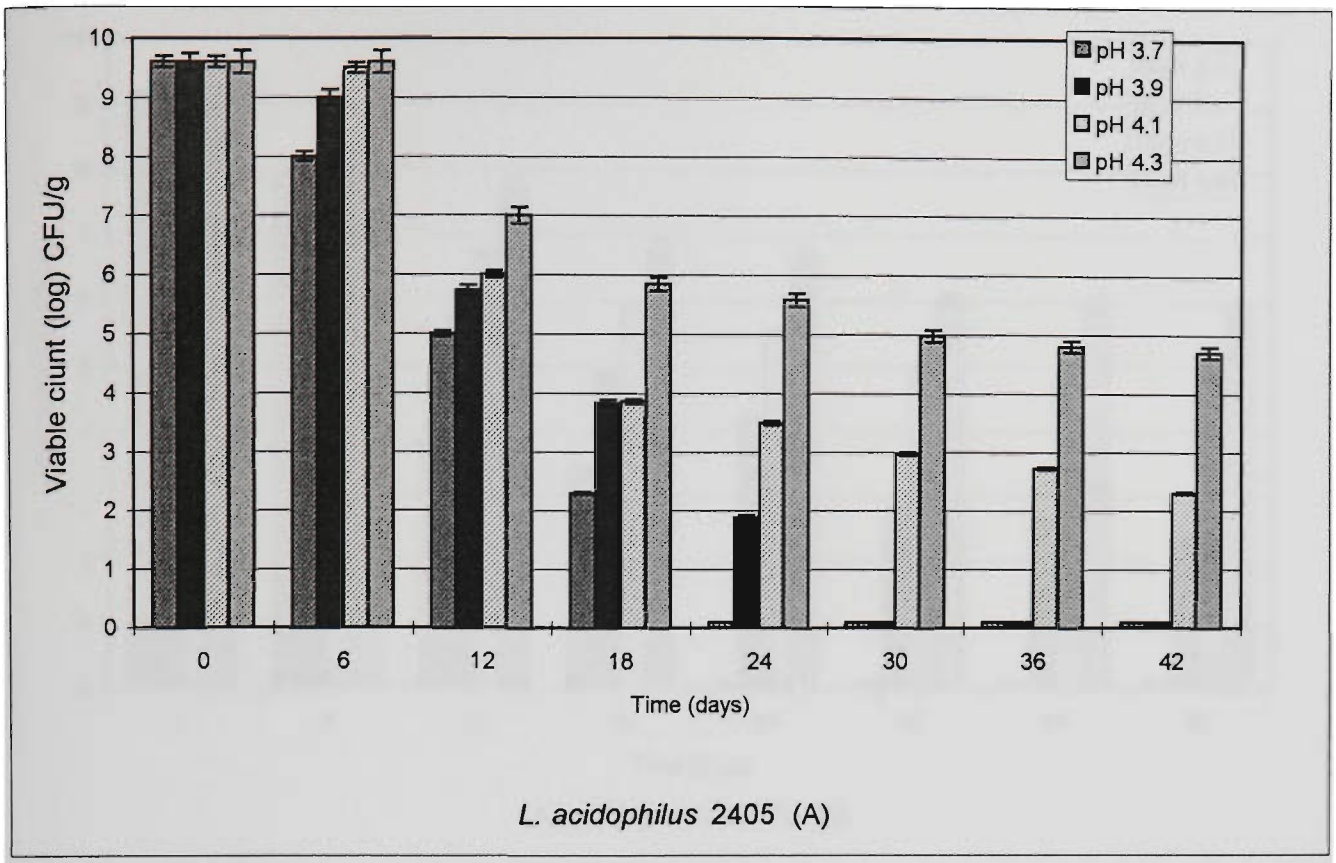


Fig. 5.2.12. Survival of *L. acidophilus* 2404 in acidic conditions (A) and in the presence of acid and H_2O_2 (B).



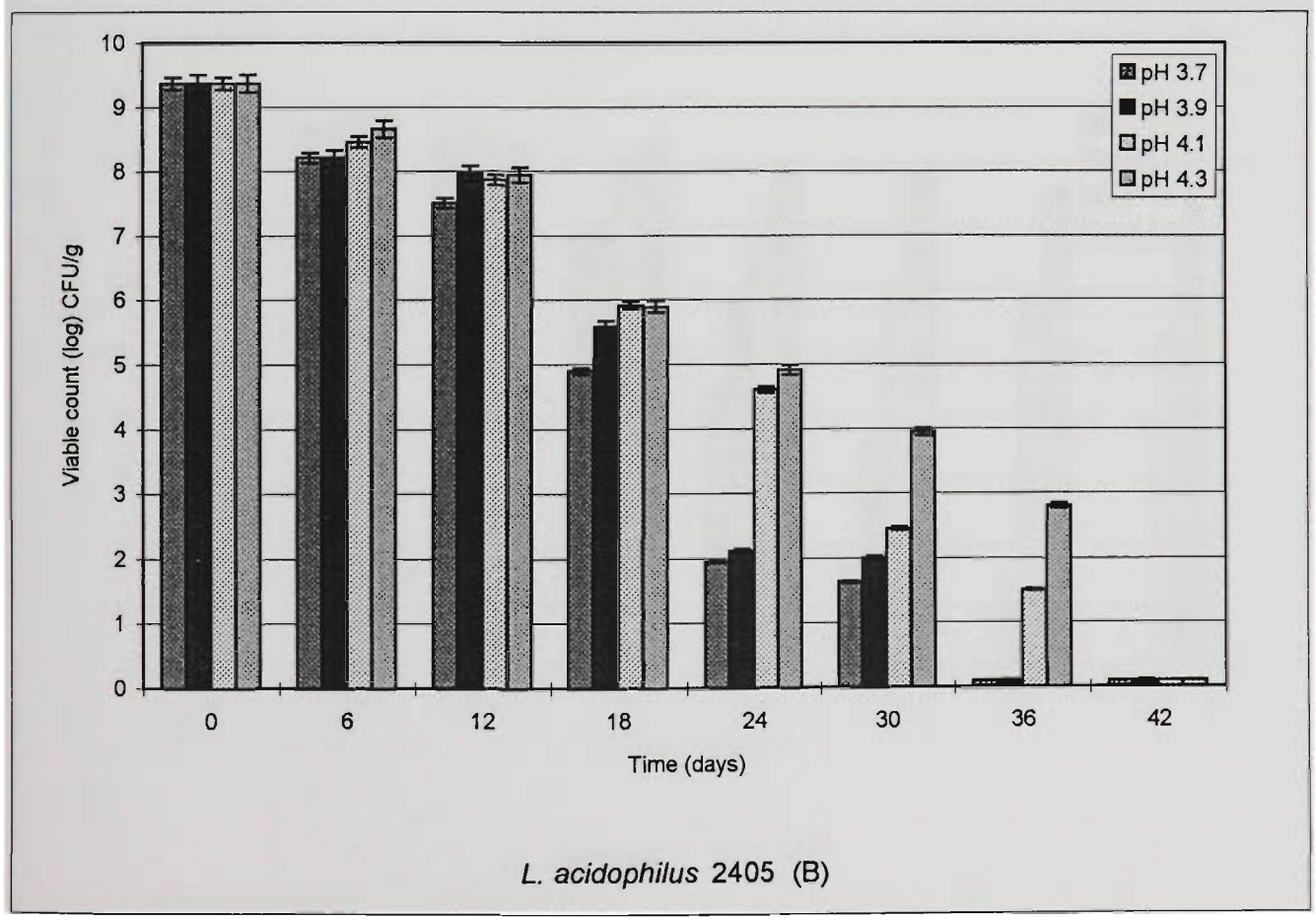


Fig. 5.2.13. Survival of *L. acidophilus* 2405 in acidic conditions (A) and in the presence of acid and H_2O_2 (B).

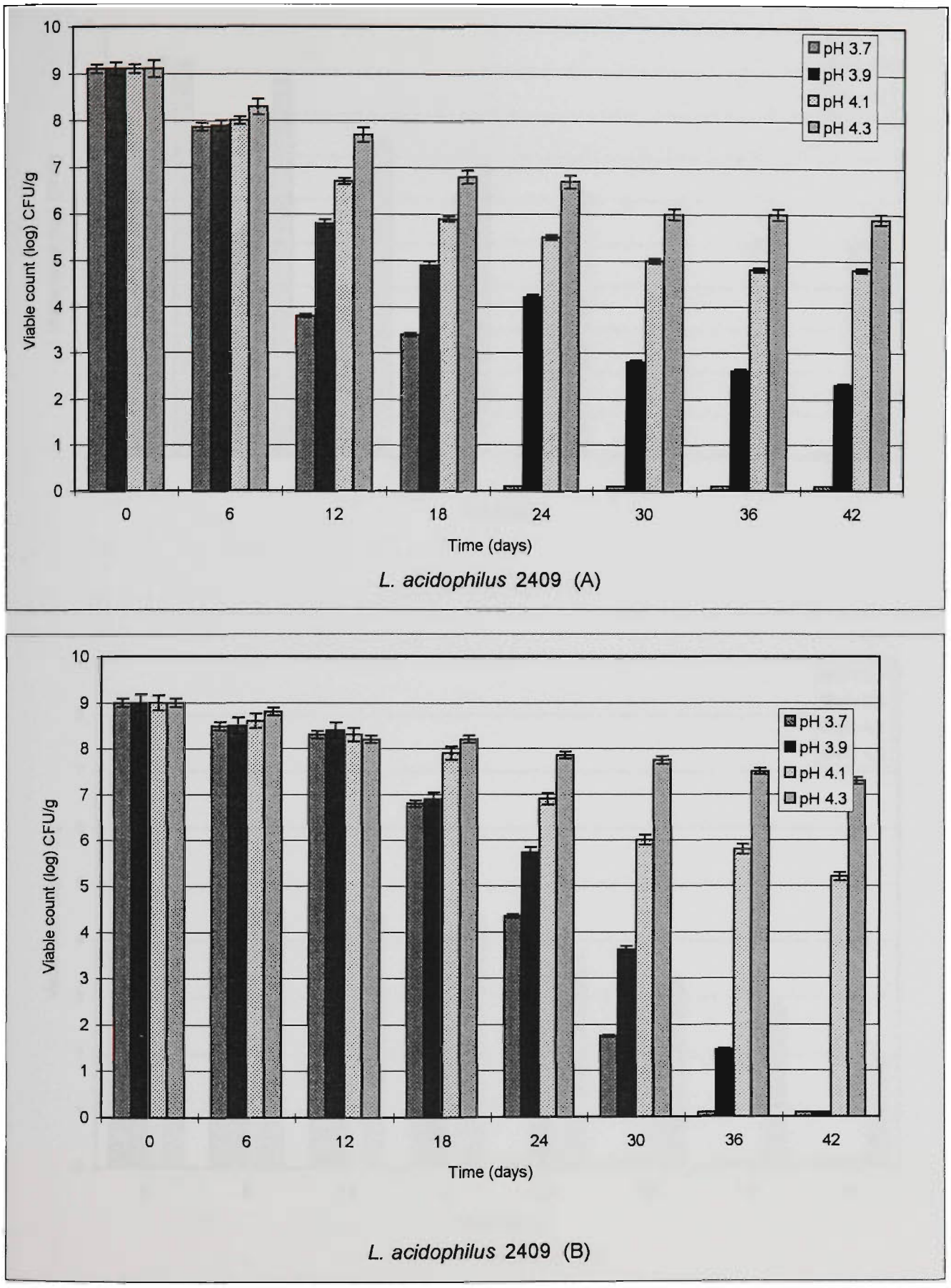


Fig. 5.2.14. Survival of *L. acidophilus* 2409 in acidic conditions (A) and in the presence of acid and H_2O_2 (B).

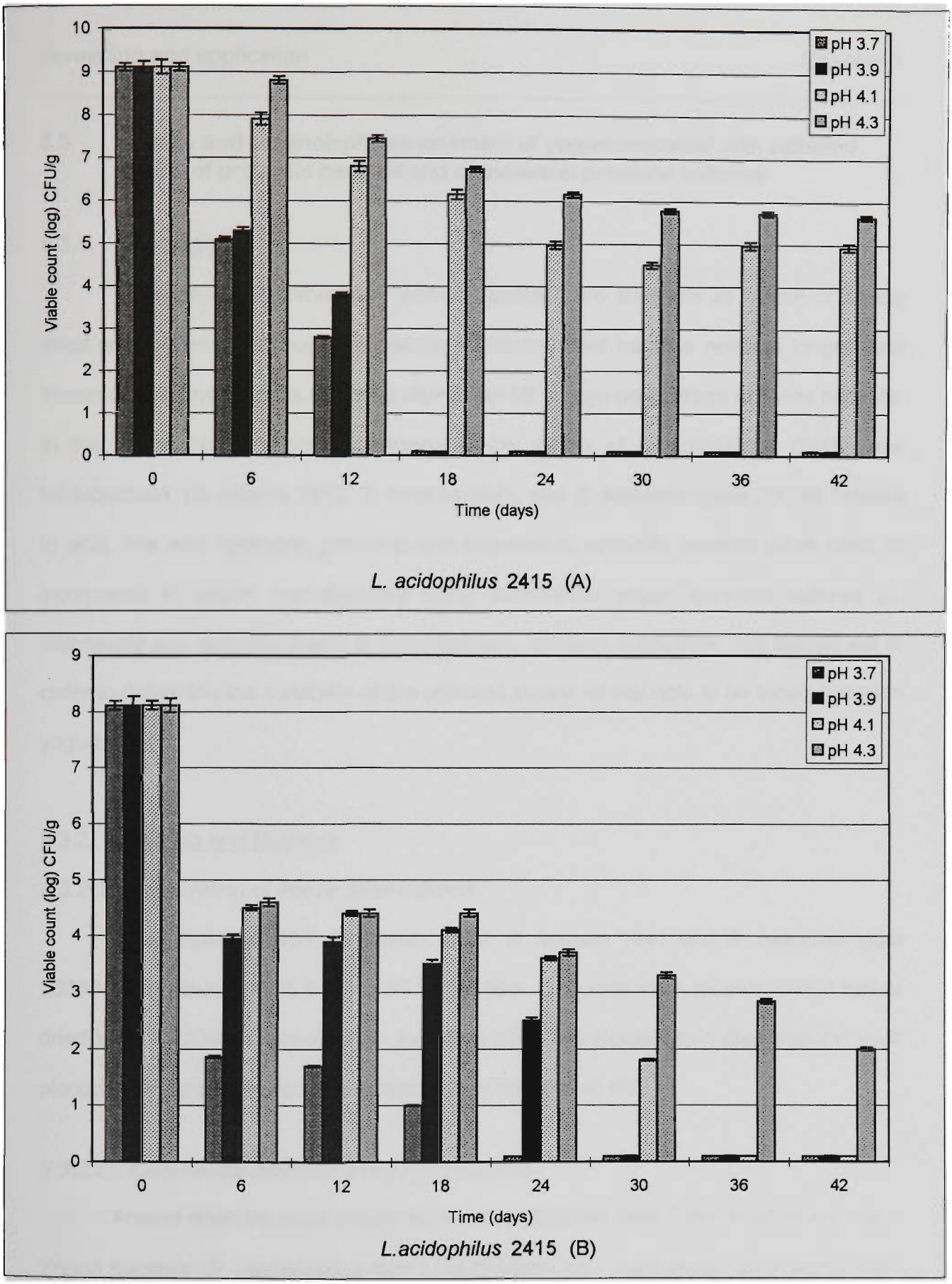


Fig. 5.2.15. Survival of *L. acidophilus* 2415 in acidic conditions (A) and in the presence of acid and H_2O_2 (B).

5.3. Viability and organoleptic assessment of yogurt prepared with selected strains of probiotic bacteria and commercial probiotic cultures

5.3.1. Introduction

Generally most commercial probiotic bacteria are available as frozen or freeze dried preparations. Although the viability of freeze dried bacteria remains longer than frozen culture, the process of freeze drying can kill a large percentage of these bacteria. In this study, freeze dried preparations of the strains of *L. acidophilus* (2409) and bifidobacteria (*B. infantis* 1912, *B. longum* 1941, and *B. pseudolongum* 20099) tolerant to acid, bile and hydrogen peroxide and commercial probiotic bacteria were used to incorporate in yogurt manufactured using commercial yogurt bacterial cultures (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*). Sensory evaluation was carried out in order to determine the suitability of the selected strains as adjuncts to be incorporated in yogurt.

5.3.2. Materials and Methods

5.3.2.1. Preparation of freeze dried cultures

L. acidophilus 2409, *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 were grown in MRS broth at 37 °C for 18 h. The cells were recovered and freeze dried in 12% NDM as described in section 2.3.6. The freeze dried preparations were placed in air tight containers and stored in a refrigerator at 4°C.

5.3.2.2. Commercial probiotic and yogurt bacteria

Freeze dried bacterial preparations were obtained from 3 commercial suppliers. Yogurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*), and freeze dried probiotic preparations were obtained as described in section 2.7.3. All preparations contained viable counts of bacteria ranging from 3.0×10^{10} to 3.0×10^{11} CFU/g. Nine

batches of yogurt were manufactured with the culture combinations as shown in Table 5.3.1.

Table 5.3.1. Probiotic and yogurt bacterial combinations for preparation of yogurt.

Batch number	Culture combination
1	Yogurt bacteria only (control)
2	Yogurt bacteria, *L. acidophilus* (CH), and *B. bifidum* (CH)
3	Yogurt bacteria, *L. acidophilus* (ML), and *B. bifidum* (ML)
4	Yogurt bacteria, *L. acidophilus* (ML), and *B. bifidum* (ML)
5	Yogurt bacteria, *L. acidophilus* (RPH), and *B. infantis* (RPH)
6	Yogurt bacteria, *L. acidophilus* (RPH), and *B. longum* (RPH)
7	Yogurt bacteria, *L. acidophilus* (2409), and *B. longum* (1941)
8	Yogurt bacteria, *L. acidophilus* (2409), and *B. pseudolongum* (20099)
9	Yogurt bacteria, *L. acidophilus* (2409), and *B. infantis* (1912)

5.3.2.3. Preparation of yogurt

Preparation of yogurt was carried out as described in section 2.7.3. Yogurt and probiotic bacteria were added as shown in the Table 2.7.3.2 in section 2. 7.3. After manufacturing, yogurt was stored at 4°C for 6 weeks and samples were taken weekly for microbiological assessment. Organoleptic assessment was carried out at day 1, 21, and 35.

5.3.2.4. Organoleptic evaluation

Organoleptic evaluation was carried out using the samples from 9 batches of yogurt. Twelve members were included in the panel of evaluation. The members of the panel were provided with an sensory evaluation score card (Table 5.3.2) requested to carry out the evaluation based on odour, taste and mouth feel and general appearance of yogurt such as viscosity and syneresis.

Table 5.3.2. Sensory evaluation score card for the panellists

SENSORY EVALUATION OF YOGURT										
		<----------Samples---------->								
		1	2	3	4	5	6	7	8	9
FLAVOUR - SCORE--------->										
CRITICISMS										
	Bitter									
If no	*Cooked*									
criticism	*Foreign*									
score 10	*Lacks fine flavor*									
	Lacks freshness									
	Lacks sweetness									
	Low acid									
Range 1-10	*Oxidised*									
	Rancid									
	Too sweetness									
	Unnatural flavor									
	Unclean									
	Yeasty									
BODY & TEXTURE - SCORE--------->										
CRITICISMS										
If no	*Gel like*									
criticism	*Grainy*									
score 5	*Ropy*									
	Too Firm									
Range 1-5	*Weak*									
APPEARANCE - SCORE--------->										
CRITICISMS										
If no	*Atypical colour*									
criticism	*Entrapped gases*									
score 5	*Free whey*									
	Lumpy									
Range 1-5	*Shrunken*									

5.3.3. Results and discussion

Changes in viable counts of *L. acidophilus* and bifidobacteria in yogurt prepared using commercial or selected strains of probiotic bacteria is illustrated in Figs. 5.3.1 and 5.3.2. Product 1 was manufactured using the probiotic strains from culture supplier 1. Products 2 and 3 were manufactured using the probiotic strains from culture supplier 2 and products 4 and 5 were manufactured using the probiotic strains from culture supplier 3. Names of the culture suppliers are not mentioned due to confidentiality reasons. Products 6, 7 and 8 were manufactured using selected strains (based on acid tolerance) of probiotic bacteria from the collection of Victoria University of Technology, Werribee Campus, Victoria Australia.

As shown in Fig. 5.3.1, *L. acidophilus* strain used in product 1 showed poor survival and the counts declined by about 3 log cycles during 6 weeks of storage as compared to the *L. acidophilus* strains in products 2, 3, and 4. Although the products 2 and 3 were manufactured using the same commercial strain of *L. acidophilus*, the viability of the former strain in product 3 reduced more as compared to the product 2. This may be due to incompatibility between *L. acidophilus* and bifidobacterial strains used in the product 3. *L. acidophilus* in products 2 and 4 lost < 1 log cycle of viable count during storage for 6 weeks at 4°C.

Fig. 5.3.2 shows the survival of *L. acidophilus* and bifidobacteria in products 5, 6, 7, and 8. In products 5, both *L. acidophilus* and *B. longum* survived well despite the initial low levels of viable cells of the latter. *L. acidophilus* 2409 survived well in products 6, 7, and 8. *B. longum* 1941, *B. pseudolongum* 20099 and *B. infantis* 1912 in products 6, 7 and 8 respectively, survived well.

Fig. 5.3.3 illustrates the change in pH of the products after manufacture. All products showed a reduction in pH (in a range of 4.45 to 4.20) after 24 h in storage although all products were shifted from the incubator to cold store at pH 4.5 $\pm$ 0.1. The

pH of all products showed gradual reduction during 6 weeks of storage and after storage pH of all products ranged between 4.0 and 4.2.

The average score of sensory assessment of all the products are shown in Fig. 5.3.4. The score for all products remained between 8 - 9 after 1 day of storage and 7.7 - 8.7 after 35 days of manufacture. Sensory score of all products remained above 8 out of 10 during storage except product 4 which indicated lowest sensory score (7.6) after 35 days.

5.3.4. Conclusions

Selected strains of *L. acidophilus* 2409, *B. longum* 1941, *B. pseudolongum* 20099 and *B. infantis* 1912 showed better survival in yogurt as compared to the commercial strains. Among commercial strains, all *L. acidophilus* strains showed better survival than bifidobacteria. Reduction in the viable counts of *L. acidophilus* strain in the presence of *B. longum* from supplier 1 suggested the possibility of antagonism or incompatibility between those two organisms. Organoleptic score remained 8 ± 1 out of 10 for all products suggesting that the selected strains of probiotic bacteria also could be successfully used to substitute commercial cultures of probiotic bacteria.

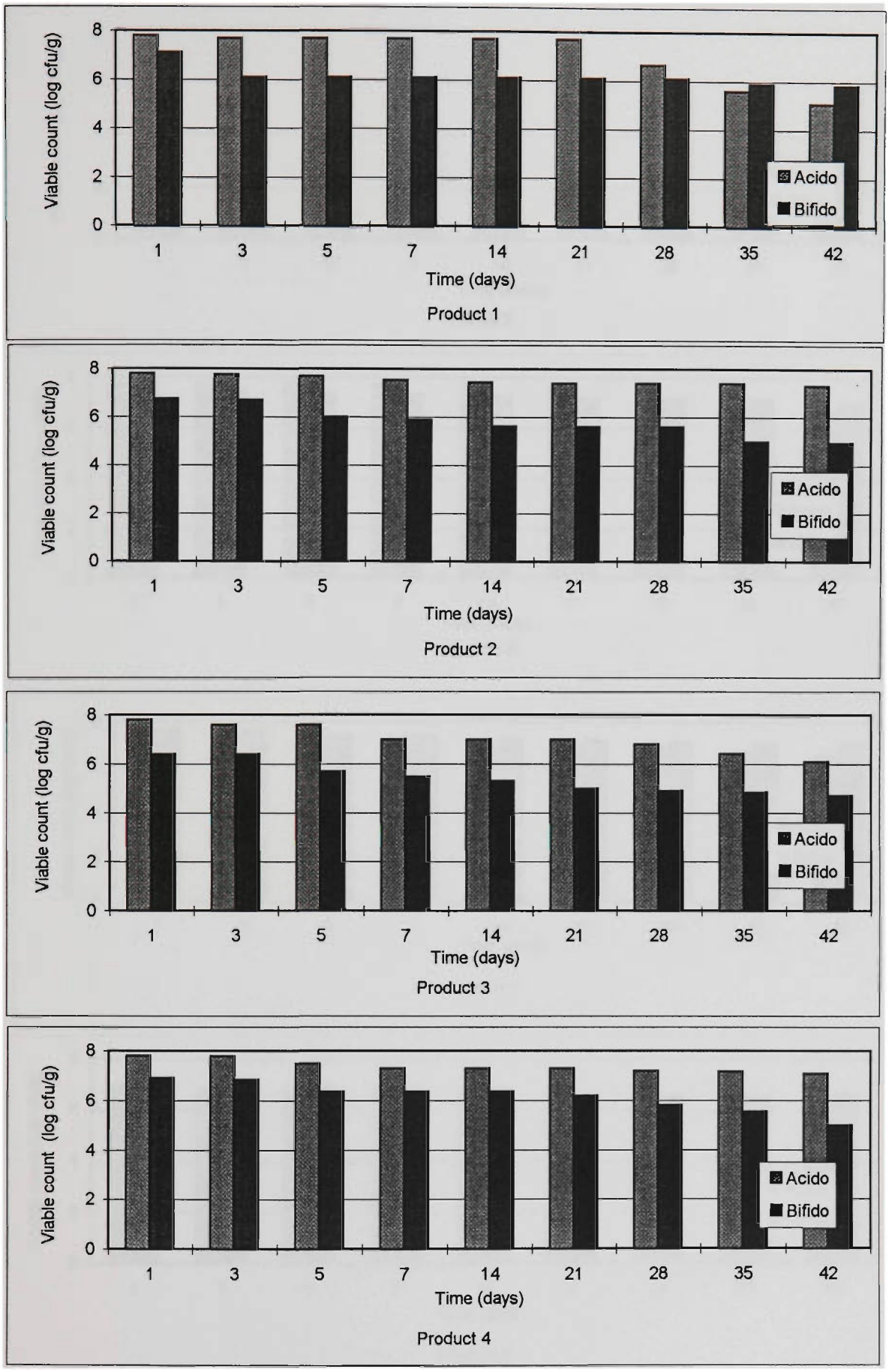


Fig. 5.3.1. Survival of *L. acidophilus* (Acido)and bifidobacteria (Bifido) in products 1, 2, 3, and 4.

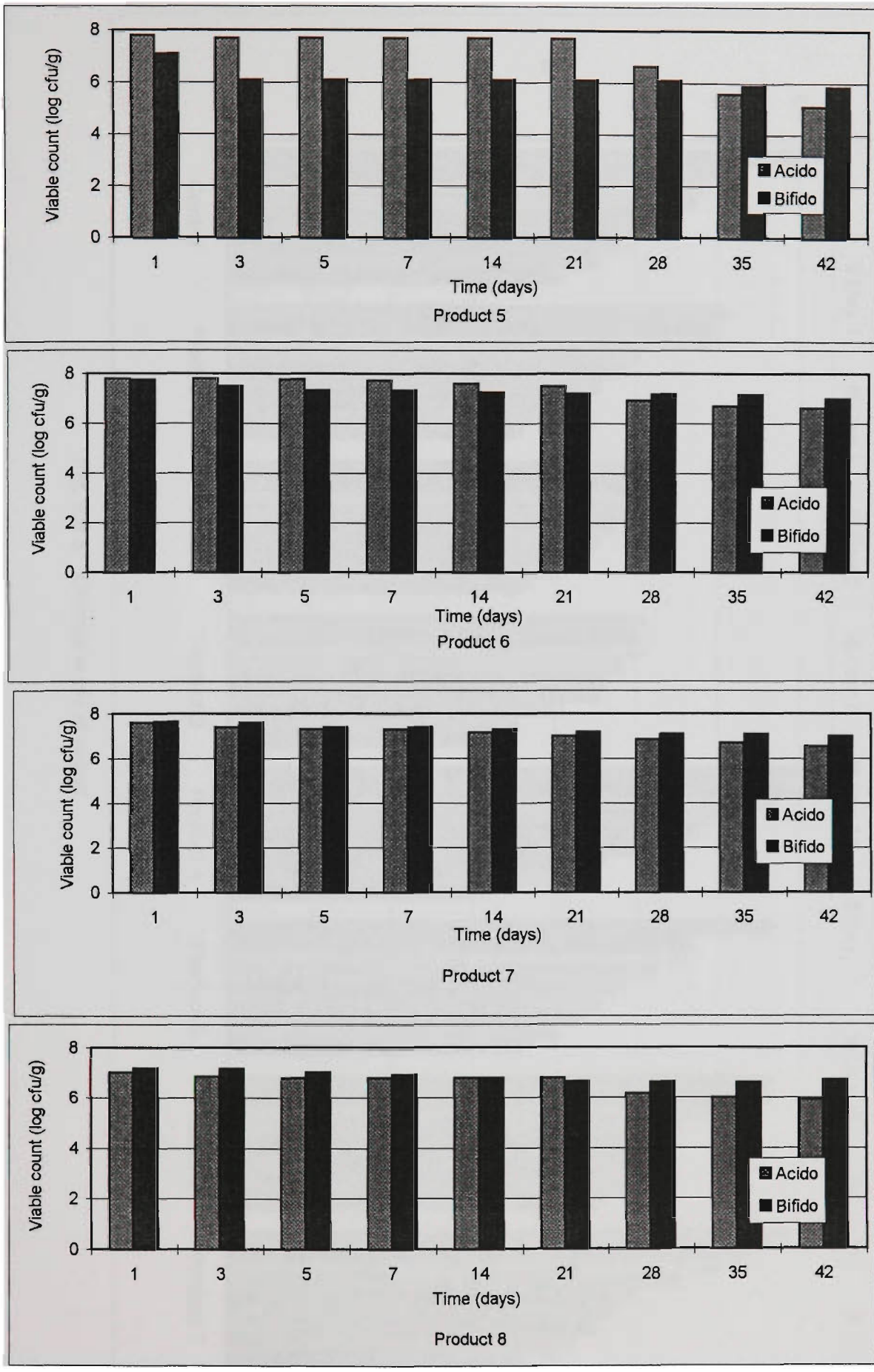


Fig. 5.3.2. Survival of *L. acidophilus* (Acido) and bifodobacteria (Bifido) in products 5, 6, 7, and 8

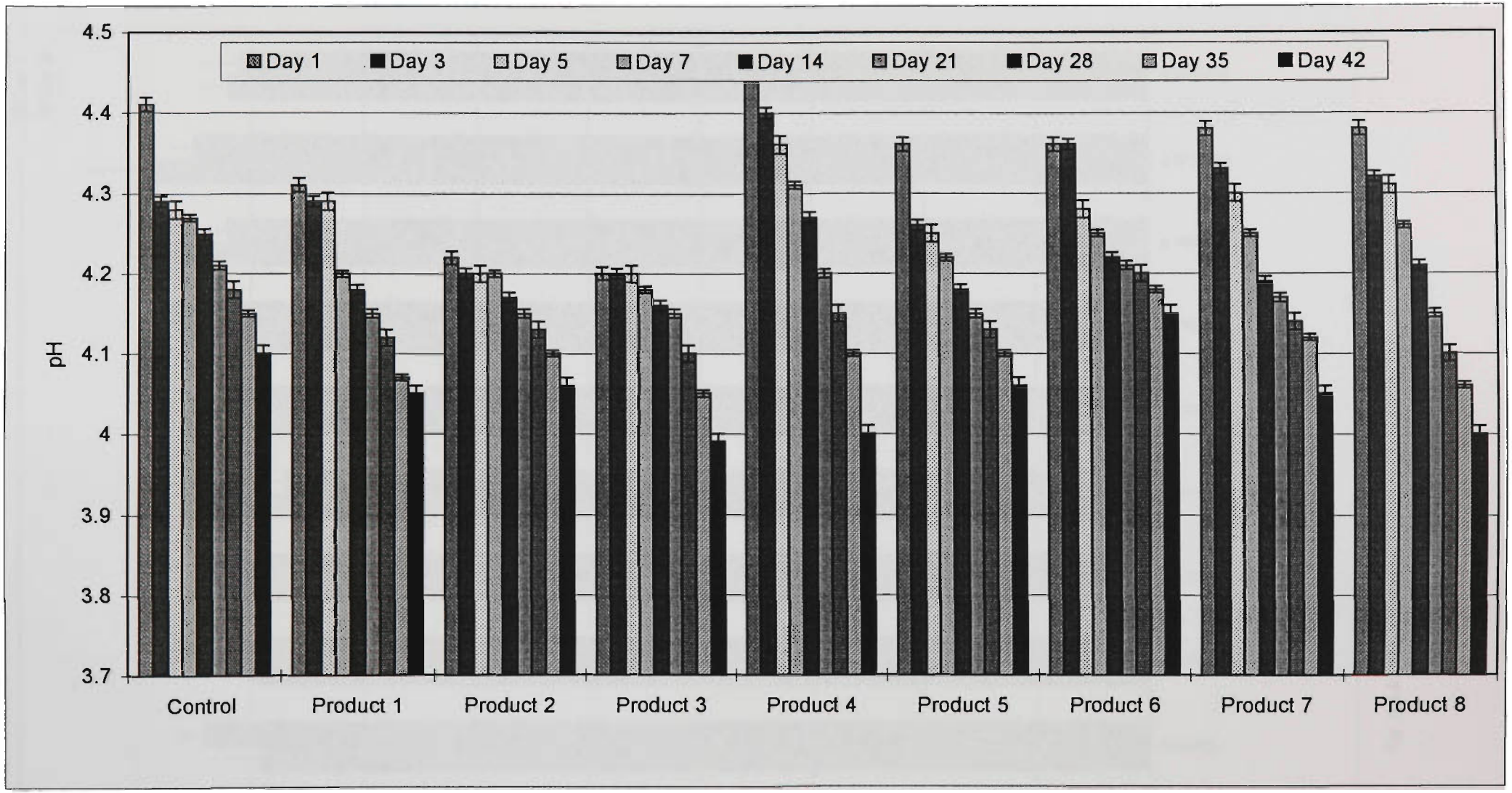


Fig. 5.3.3. Change in pH of yogurt

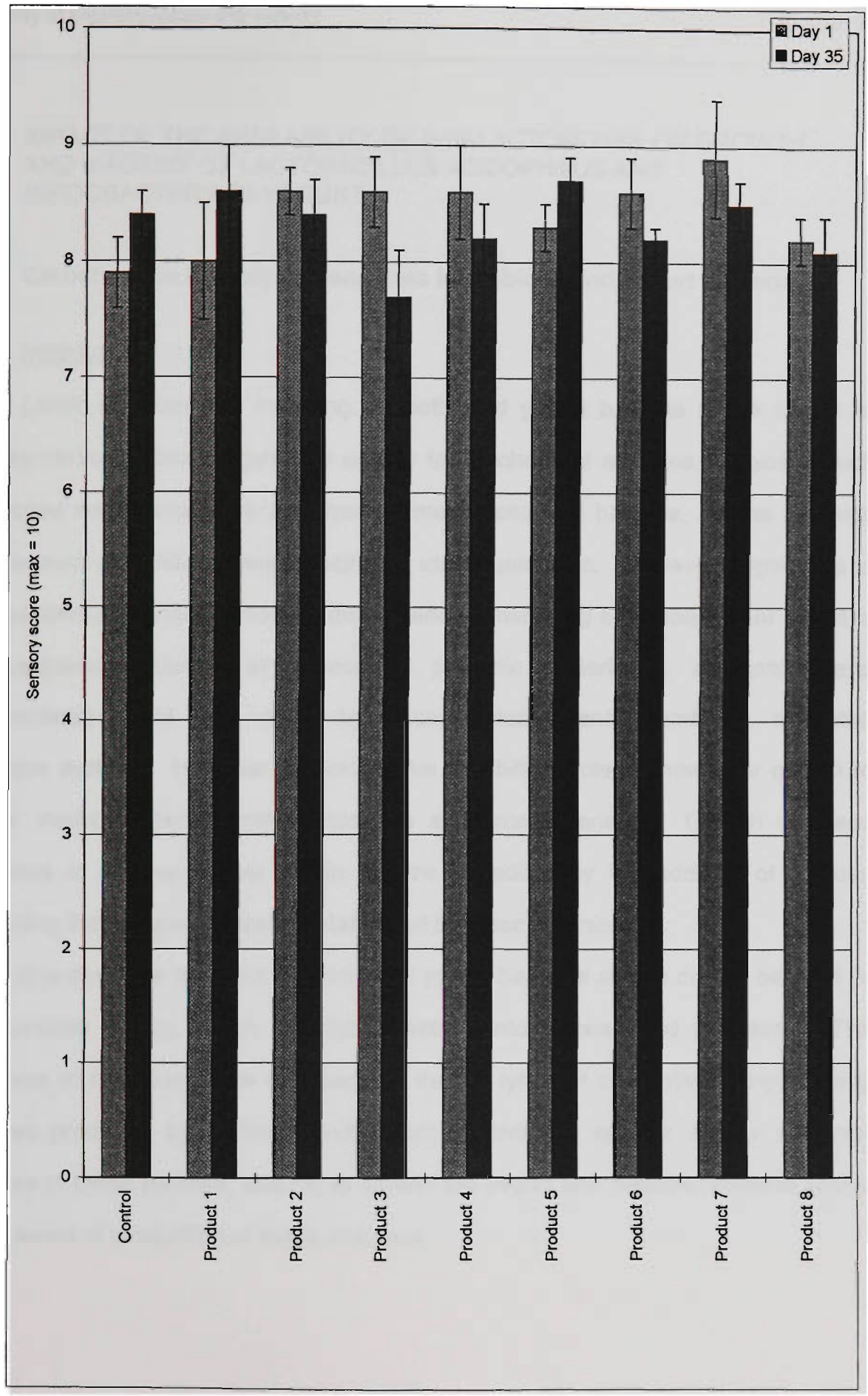


Fig. 5.3.4. Flavour component of sensory evaluation score of yogurt

6.0. IMPACT OF THE AVAILABILITY OF β-GALACTOSIDASE ON GROWTH AND VIABILITY OF LACTOBACILLUS ACIDOPHILUS AND BIFIDOBACTERIA IN YOGURT

6.1. Carbohydrate hydrolysing enzymes in probiotic and yogurt bacteria

6.1.1. Introduction

Lactic acid bacteria including probiotic and yogurt bacteria utilise simple to complex carbohydrates to generate energy for biochemical activities. Hexoses such as glucose and fructose are preferred by most lactic acid bacteria. Some probiotic bacteria such as bifidobacteria specifically utilise galactose. However, organisms in cultured dairy milk utilise lactose, a disaccharide constituting of a molecule of glucose and galactose. Uptake of lactose by probiotic bacteria (*L. acidophilus* and bifidobacteria) could take place by phosphorylation and membrane mediated permease systems. However, *L. acidophilus* and bifidobacteria show poor growth in milk or media containing only lactose as a source of energy. Growth of these organisms in milk and other media can be expedited by the addition of glucose suggesting that glucose is easily metabolised by these organisms.

It is desirable to select probiotic and yogurt bacterial strains on the basis of β-galactosidase activity, which hydrolyses lactose into glucose and galactose. The objectives of this study were to determine the (1) types of carbohydrate hydrolysing enzymes produced by probiotic and yogurt bacteria (2) specific activity of these enzymes in these bacteria, and (3) to screen the yogurt and probiotic bacteria based on the levels of production of these enzymes.

6.1.2. Materials and methods

6.1.2.1. Probiotic and yogurt bacterial cultures

Six strains of *L. acidophilus* and 9 strains of bifidobacteria were obtained as described in section 2.2.2. Five strains of *L. delbrueckii* ssp. *bulgaricus* and 6 strains of *S. thermophilus* were also obtained from CSIRO, Highett, Victoria, Australia.

6.1.2.2. Determination of enzyme activity

Cells of *L. acidophilus*, bifidobacteria, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* were grown in MRS broth for 18 h at 37°C, centrifuged and ruptured as described in section 2.3.5. Activities of α-D-galactosidase (α-gal), β-D-galactosidase (β-gal) and phospho-β-D-galactosidase (p-β-gal) were determined by the methods described in section 2.4.4. Total protein levels in cell extracts were determined using modified Lowrey assay described in section 2.4.5. Specific activities of the enzymes were calculated as a factor of the total protein level (enzyme activity per gram of protein).

6.1.2.3. Gel electrophoresis of probiotic and yogurt bacterial cell extracts

Poly acrylamide gel electrophoresis was carried out in order to determine the presence of carbohydrate hydrolysing enzymes as described in section 2:11. The gels were stained with Coumassie blue.

6.1.3. Results and discussion

Table 6.1.1. shows the levels of activity of α-gal, β-gal and P-β-gal levels in the cell free extracts of probiotic and yogurt bacteria. P-β-gal was available in small quantities in most strains of *L. acidophilus* and bifidobacteria. *L. acidophilus* 2409 and 2415, *B. infantis* 1912 and *B. longum* 20097 showed the highest activities (0.077, 0.062, 0.194 and 0.110, respectively) of this enzyme. However, yogurt bacterial strains, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* did not possess P-β-gal. This suggests that these two types of bacteria do not rely on phosphorilation. All

strains of *L. acidophilus* except strain 2401 had higher activity of β-gal as compared with the levels of P-β-gal in *L. acidophilus*. *L. acidophilus* 2409 and 2415 showed the highest activities (1.027 and 0.590, respectively). All bifidobacterial strains except, *B. infantis* 1912 and *B. thermophilum* 20210 showed high β-gal activity (1.870-2.869). α-gal was present in all *L. acidophilus* strains studied; *L. acidophilus* 2409 exhibited the highest activity. All strains of bifidobacteria except *B. infantis 1912* showed high levels of activitity (1.245 - 2.785). However, *B. infantis* 1912 which showed low activity of α-gal and β-gal showed high activity for P-β-gal.

Table 6.1.2. shows β-gal and α-gal activities elaborated into phosphate buffered saline (PBS) solution by freshly grown unbroken cells of probiotic and yogurt bacteria. Both enzymes were not detected in *L. acidophilus* strains except in *L. acidophilus* 2409. Among the bifidobacterial strains, *B. breve* and *B. longum* showed β-gal and α-gal activities. Strains of bifidobacteria showed various levels of enzyme activity, while *B. longum* 1941 showed highest activity. Yogurt bacteria (*L. delbrueckii* ssp. *bulgaricus* and *S. thrmophilus*) also showed β-gal activity.

Fig. 6.1.1. shows the enzyme profiles of probiotic and yogurt bacterial strains. As illustrated, *L. acidophilus* 2409 (lane 12) showed the presence of α-gal (as compared with lane 4 which contains standard α-gal). Lanes 14-20 show the presence of this enzyme in bifidobacteria. *B. longum* 1941 (lane 17) seemed to have high concentration of this enzyme. Yogurt bacterial strains did not show α-gal. Lane 5 shows standard β-gal; all bacteria showed the presence of this enzyme.

Table 6.1.1. Enzyme activities in the intracellular extracts of probiotic and yogurt bacteria.

Bacterial strain	Phospho-β-D-galactosidase	β-D-galactosidase	α-D-galactosidase
L. acidophilus		.....units[1]....	
2400	0.045	0.463	0.571
2401	0.048	0.015	0.382
2404	0.025	0.098	0.165
2405	0.008	0.076	0.145
2409	0.077	1.027	2.762
2415	0.062	0.590	0.336
Bifidobacterium spp.			
1900	0.035	1.870	1.245
1901	0.017	2.747	1.802
1912	0.194	0.121	0.000
1920	0.000	2.773	2.727
1930	0.028	2.773	2.725
1941	0.003	2.809	2.785
20097	0.110	2.869	1.886
20099	0.019	2.809	2.777
20210	0.021	0.276	2.641
L. delbrueckii ssp. *bulgaricus*			
2505	0.004	1.620	0.000
2515	0.005	2.790	0.000
S. thermophilus			
2002	0.009	2.820	0.000
2010	0.005	2.818	0.000

[1] μ moles of ortho-nitrophenyl from ortho-nitrophenyl β-D-galactopyranoside per gram culture per minute at 37°C.

Table 6.1.2. Activities of β-D-gal and α-D-gal in whole cells of probiotic and yogurt bacteria.

Bacterial strain	β-D-galactosidase	α-D-galactosidase
L. acidophilus		.....units[1].....
2400	0.015	0.012
2401	0.000	0.000
2404	0.000	0.000
2405	0.000	0.000
2409	0.089	0.045
2415	0.000	0.000
Bifidobacterium spp.		
1900	0.000	0.020
1901	0.000	0.022
1912	0.000	0.045
1920	0.000	0.032
1930	0.477	0.078
1941	2.834	1.997
20097	0.000	0.023
20099	0.000	0.102
20210	0.000	0.052
L. delbrueckii ssp. *bulgaricus*		
2505	0.055	0.005
2515	0.065	0.000
S. thermophilus		
2002	0.152	0.015
2010	0.310	0.020

[1]μ moles of ortho-nitrophenyl from ortho-nitrophenyl β-D-galactopyranoside per gram culture per minute at 37°C.

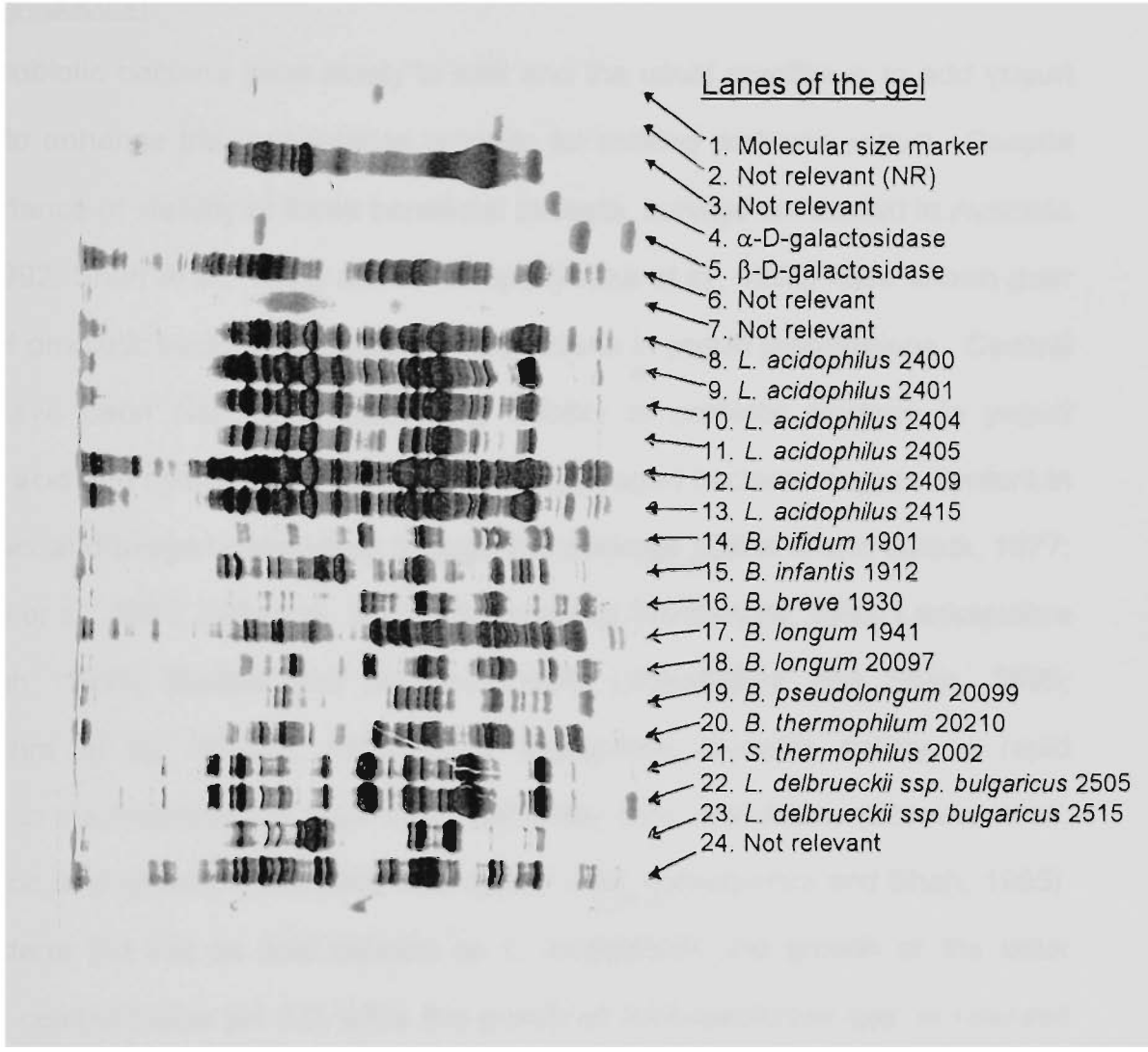


Fig. 6.1.1. Poly acrylamide gel electrophoresis of probiotic and yogurt bacterial extracts showing presence of α- and β-D-galactosidases.

6.2. Survival of probiotic bacteria in yogurt prepared with freeze dried preparations of ruptured yogurt bacteria[3]

6.2.1. Introduction

Probiotic bacteria grow slowly in milk and the usual practice is to add yogurt bacteria to enhance the fermentation process for making probiotic yogurt. Despite the importance of viability of these beneficial bacteria, surveys conducted in Australia (Anon, 1992; Shah *et al.*, 1995) and in Europe (Iwana *et al.*, 1993) have shown poor viability of probiotic bacteria, especially bifidobacteria in yogurt preparations. Several factors have been claimed to affect the viability of probiotic bacteria in yogurt including acid and hydrogen peroxide produced by yogurt bacteria, oxygen content in the product and oxygen permeation through the package (Gilliland and Speck, 1977; Schioppa *et al.*, 1981; Hull *et al.*, 1984; Ishibashi and Shimamura, 1993; Lankaputhra and Shah, 1994; Medina and Jordono, 1994; Lankaputhra and Shah, 1995; Lankaputhra *et al.*, 1996b). Although *L. acidophilus* tolerates acidity, a rapid decrease in their number has been observed under acidic conditions (Conway *et al.*, 1987; Hood and Zottola, 1988; Shah and Jelen, 1990; Lankaputhra and Shah, 1995). Bifidobacteria are not as acid tolerant as *L. acidophilus*; the growth of the latter organism ceases below pH 4.0, while the growth of *Bifidobacterium* spp. is retarded below pH 5.0 (Shah, 1997).

Among lactic acid bacteria, yogurt bacteria contain the highest lactase activity (Shah and Jelen, 1990, 1991). Lactase or β-D-galactosidase (β-gal) is an endoenzyme and whole microbial cells exhibit very little exogenous lactase activity (Kilara and Shahani, 1976; Shah and Jelen, 1990). Activity of the β-gal can be increased several times by cell lysis induced by sonication or Eaton press.

β-gal released after rupturing of the yogurt bacterial cells could be used to hydrolyse a portion of lactose in milk and the products of lactose hydrolysis, glucose

[3]A paper based on the findings of this section is in press in *International Dairy Journal* under the title "A new approach for improving viability of *Lactobacillus acidophilus* and bifidobacterium spp. in yogurt".

and galactose, could be used by organisms such as *L. acidophilus* and *Bifidobacterium* spp. Rupturing of yogurt bacteria using high velocity glass beads could also reduce the viable count of yogurt bacteria and thus the amount of hydrogen peroxide produced by these bacteria. Studies (Lankaputhra *et al.*, 1996b; Dave and Shah, 1996b) has shown that hydrogen peroxide produced by *L. delbrueckii* ssp. *bulgaricus* affected the growth of probiotic bacteria. The objective of this study was to determine whether viability of probiotic bacteria could be improved by rupturing yogurt bacterial cells in order to release their intracellular β-gal and to reduce their initial viable counts.

6.2.2. Materials and methods

6.2.2.1. Bacterial strains

L. delbrueckii ssp. *bulgaricus* 2515, *S. thermophilus* 2010, *L. acidophilus* 2409, *Bifidobacterium longum* 1941, *B. pseudolongum* 20099, *B. infantis* 1912 and *B. bifidum* 1900 and 1901 were obtained as described in section 2.2.2. *L. delbrueckii* ssp. *bulgaricus* 2515 and *S. thermophilus* 2010 were selected on the basis of high extracellular β-gal activity (section 6.1.3.) and *L. acidophilus* 2409 was selected on the basis of acid and bile tolerance as reported earlier (Lankaputhra and Shah, 1995).

6.2.2.2. Rupturing and freeze drying of yogurt bacteria

The two yogurt bacteria were grown separately in 1 litre of deMan Rogosa and Sharpe (MRS) broth for 16 h at 37°C and the cells in their early log phase were recovered by centrifuging at 10,000 rpm for 15 min at 4°C using a Beckman Model L-70 ultracentrifuge and JA-14 rotor (Beckman Instruments, Palo Alto, CA, USA). The cells were washed in sterile phosphate buffered saline, centrifuged and the supernatant was decanted. The cell pellet was suspended in 50 mL (concentration

factor 20) of sterile saline solution, the suspension cooled to <4°C and 10 mL by volume of glass beads of 0.1 mm size were added. The cell suspension and glass beads were placed in a 70 mL sterile stainless steel adaptor and mechanical vibration was applied using an MSK cell homogeniser (B. Braun Melsungen AG, Melsungen, Germany) for 30, 60 or 90 sec in order to rupture the cells. Samples were taken before and after cell rupture to enumerate viable counts and to measure β-galactosidase activity. The ruptured cell suspension was centrifuged at 3000 rpm for 1 min using Beckman ultracentrifuge to remove the glass beads. The cell suspension was mixed with 12% (w/v) reconstituted nonfat dry milk (NDM) at 1:1 ratio, frozen at -20°C and freeze dried at -60°C for 10 h using a Dynavac FD 300 freeze drier (Dynavac Engineering Pty. Ltd. Inc, Melbourne, Victoria, Australia).

6.2.2.3. Harvesting and freeze drying of probiotic cultures

L. acidophilus 2409 and 5 strains of bifidobacteria representing 4 species of *Bifidobacterium* (*B. longum* 1941, *B. pseudolongum* 20099, *B. infantis* 1912, *B. bifidum* 1900 and 1901) were grown separately in MRS broth for 16 h and the cells were harvested and washed as described for yogurt bacteria in section 6.2.2.2. The washed cells were suspended in 50 mL of sterile NDM, frozen at -20°C and freeze dried as with yogurt bacterial cultures. All freeze dried starter cultures were packed in MacCartney glass bottles with airtight seals and stored at 4°C until used.

6.2.2.4. Preparation of yogurt

Homogenised and pasteurised milk supplemented with 5% nonfat dry milk was heated to 85°C for 30 min, cooled to 42°C and 60 sec-ruptured freeze dried starter cultures of *L. delbruckii* ssp. *bulgaricus* 2515, *S. thermophilus* 2010, and *L. acidophilus* 2409 and *Bifidobacterium* spp. were added to the yogurt mix at the rate of 0.1%. Five different types of yogurt were prepared each containing *L. delbruckii*

ssp. *bulgaricus* 2515, *S. thermophilus* 2010, *L. acidophilus* 2409 and one species of *Bifidobacterium* (*B. longum* 1941, *B. pseudolongum* 20099, *B. infantis* 1912, *B. bifidum* 1900 or *B. bifidum* 1901). The yogurt mix was incubated at 42°C and samples were taken during fermentation at 0 h and then at hourly intervals till the pH reached 4.5 for measurement of pH and titratable acidity, determination of β-D-galactosidase activity, level of hydrolysis of lactose and utilisation of glucose and galactose, hydrogen peroxide and acetaldehyde, and for enumeration of yogurt and probiotic bacteria. The yogurt was then stored for 6 weeks at 4°C and viable counts of probiotic bacteria were determined at weekly intervals.

6.2.2.5. Enumeration of bacteria

L. delbrueckii ssp. *bulgaricus* was enumerated according to the method of Dave and Shah (1996a) using MRS agar (Oxoid, W. Heidelberg, Australia) adjusted to pH 5.2 and anaerobic incubation at 43°C for 72 h. *Streptococcus thermophilus* agar and aerobic incubation at 37°C were used for selective enumeration of *S. thermophilus* as per the method of Dave and Shah (1996a). *L. acidophilus* was enumerated according to the method of Lankaputhra and Shah (1996) using modified MRS-salicin agar and bifidobacteria were enumerated according to the method of Lankaputhra *et al.* (1996a) using MRS-NNLP agar.

6.2.2.6. Measurement of enzyme activity

β-D-galactosidase activity of the freeze dried cell preparations and of yogurt mix during fermentation was determined according to the method of Shah and Jelen (1990, 1991) using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate as described in section 2.4.4. The unit of lactase activity was estimated according to the method of Mahoney *et al.* (1975) as the amount of enzyme which liberated one μmole o-nitrophenol from ONPG.

6.2.2.7. Measurement of hydrogen peroxide and acetaldehyde

Concentrations of hydrogen peroxide and acetaldehyde were determined according to the methods of Gilliland (1968) and Millies *et al.* (1989), respectively as described in sections 2.4.3. and 2.4.2., respectively. All experiments and analyses were replicated three times. The results presented are averages of all the replicates.

6.2.2.8. HPLC analysis of the rates of lactose hydrolysis and uptake of glucose and galactose by yogurt and probiotic bacteria

Hourly samples were taken during fermentation of yogurt. Samples were snap frozen by dipping the sample tubes in dry ice. Extraction of samples for HPLC analysis was carried out as described in section 2.9.1. and the levels of glucose, galactose and lactose were determined as described in section 2.9.2.

6.2.3. Results and discussion

Viable counts and β-gal activity of *L. delbrueckii* ssp. *bulgaricus* 2515 and *S. thermophilus* 2010 before and after cell rupture are shown in Table 6.2.1. After 60 sec cell rupture treatment, the viable counts of *L. delbrueckii* ssp. *bulgaricus* 2515 were reduced from 4.2×10^{10} to 1.0×10^{5} and those of *S. thermophilus* 2010 from 3.6×10^{11} to 4.2×10^{6}. The cell rupture time of 60 sec was used throughout the study. The β-gal activity increased from 38 to 310 unit per gram of cell suspension for *L. delbrueckii* ssp. *bulgaricus* 2515 and from 25 to 286 for that of *S. thermophilus* 2010 culture. An increase in the β-gal activity of bacterial cells after sonication has been observed by Shah and Jelen (1990). In their study, a 5-fold increase in the lactase activity upon sonication of a *L. delbrueckii* ssp. *bulgaricus* culture was observed, while a *S. thermophilus* culture produced more enzyme activity per gram of dry cell weight in glucose and lactose containing APT (All Purpose Tween) broths than that of *L. delbrueckii* ssp. *bulgaricus*. This could be due to differences in strain or due to variation in growth medium.

Five different batches of yogurt were made. The strains of *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus* and *L. acidophilus* were kept the same in all the five batches, however, the species of *Bifidobacterium* varied in each batch of yogurt. Several studies report varying viability of various *Bifidobacterium* spp. (Anon, 1992; Shah *et al.*, 1995; Dave and Shah, 1996b). *B. longum 1941* and *B. pseudolongum* 20099 have been found to survive well in the presence of acid and bile salts (Lankaputhra and Shah, 1995).

Figure 6.2.1. shows the changes in the viable counts of yogurt and probiotic bacteria during yogurt fermentation using ruptured (9 h) or whole (7 h) cells of yogurt bacteria with whole cells of probiotic bacteria. As shown, the viable count of *L. delbrueckii* ssp. *bulgaricus* 2515 and *S. thermophilus* 2010 gradually increased from 10^2 - 10^3 to 10^5 - 10^6 at the end of 9 h of fermentation (Fig 6.2.1.a) using ruptured yogurt bacteria and whole cells of probiotic bacteria, while the numbers of *L. acidophilus* 2409 and *B. longum* 1941 increased to 10^7 to 10^8 from initial counts of 10^5 cfu/g. Similar trends were observed during fermentation with yogurt bacteria, *L. acidophilus* 2409 and other species of *Bifidobacterium* such as *B. pseudolongum* 20099, *B. infantis* 2912, *B. bifidum* 1900 and *B. bifidum* (data not included).

Viable counts of *L. delbrueckii* ssp. *bulgaricus* 2515 and *S. thermophilus* 2010 increased from 10^5-10^6 to 10^7-10^8 in 7 h of fermentation (Fig 6.2.1.b) when whole cells of the two yogurt bacteria were used. The fermentation time was 2 h shorter and the final counts of the two yogurt bacteria were approximately 2 log cycles higher in yogurt made with whole cells as compared with that made with ruptured cells. Counts of *L. acidophilus* 2409 and *B. longum* 1941 were the same at 0 h in yogurts made with ruptured or whole cells, however, the final counts of *L. acidophilus* 2409 and *B. longum* 1941 after 9 h of fermentation with ruptured cells were 1-2 log cycles higher. Viable counts of yogurt bacteria in yogurt made using whole or ruptured cells of *L. delbrueckii* ssp. *bulgaricus* 2515 and, *S. thermophilus* 2010, and *L. acidophilus* 2409

and other 4 strains of *Bifidobacterium* spp. during 7 h fermentations showed similar trends (data not included) as shown in Fig. 6.2.1.(a) and (b).

Rupturing yogurt bacteria decreased the viable counts of yogurt bacteria by about 7 to 8 log cycles and increased β-gal activity about 15 fold (Table 6.2.1). The initial counts of yogurt bacteria at 0 h were approximately 3 log cycles lower as a result of cell rupture. Reduced viable counts of yogurt bacteria may have helped the probiotic bacteria to build up their numbers. When yogurt bacteria are present in high numbers, *L. acidophilus* and *Bifidobacterium* spp. are easily dominated and outgrown by the former bacteria. Higher counts of probiotic bacteria in yogurt prepared using ruptured yogurt bacterial cells could also be due to increased levels of β-gal produced by yogurt bacteria as compared with yogurt prepared with whole cells of both groups of organisms.

The final counts of the two yogurt bacteria, *L. acidophilus* 2409 and *Bifidobacterium spp.* in five batches of yogurt after 9 or 7 h of fermentation are summarised in Table 6.2.2. In all the five batches, bacterial counts of yogurt bacteria were 2-3 log cycles higher in yogurt made from whole cells as compared with that made using ruptured cells, while the probiotic bacterial counts were about 1-2 log cycles lower. Among the *Bifidobacterium* spp., *B. longum* 1941 and *B. pseudolongum* 20099 showed the highest counts in yogurt made using either whole or ruptured cells and *B. bifidum* 1901 the lowest. However, *B. pseudolongum* is claimed to be of animal origin and may not provide therapeutic benefits and thus could not be considered as a good candidate for incorporation into fermented dairy foods such as yogurt. *B. bifidum* is commonly used by the Australian yogurt manufacturers, however, this organism has been found to be poorly tolerant to acid, bile and hydrogen peroxide (Lankaputhra and Shah, 1995; Shah, 1996).

These results are consistent with previous findings. Reuter (1990) conducted a survey of fermented milk products containing bifidobacteria in Germany, France

and Japan and found that *B. longum* was widely used in Germany. Clark *et al.* (1993) studied the survival *of B. infantis*, *B. adolescentis*, *B. longum* and *B. bifidum* in acidic conditions and reported that *B. longum* survived the best. Clark and Martin (1994) and Lankaputhra and Shah (1996) reported that *B. longum* tolerated bile concentration as high as 4%. Thus, it appears that *B. longum* could be the best candidate for use as dietary adjunct in fermented dairy products such as yogurt.

Changes in viable counts of probiotic bacteria in yogurt made with ruptured or whole cells of yogurt bacteria during 6 weeks storage are shown in Fig 6.2.2. (a, b). In general, the viability of *L. acidophilus* 2409 and *B. longum* 1941 decreased during storage. Viability of probiotic bacteria in yogurts made with the two yogurt bacteria, *L. acidophilus* 2409 and 4 other species of *Bifidobacterium* showed similar trend (data not included). Counts of *L. acidophilus* 2409 and *B. longum* 1941 in yogurt made from ruptured yogurt bacteria and whole cells of probiotic bacteria decreased by about 58 and 3.4 folds respectively, whereas the decrease of these bacteria in yogurt made from whole cells of yogurt and probiotic bacteria was 11.6 and 8.7 folds, respectively, after 6 weeks of storage. In the case of *B. longum*, a 2.6 fold faster death rate was observed as compared with 0.2 fold for *L. acidophilus* in the whole versus ruptured cell fermentations. However, the counts of probiotic bacteria remained above the recommended level of 1 million viable cells in yogurt prepared with ruptured cells; the improved viable counts of probiotic bacteria could be due to higher initial counts of these organisms.

Table 6.2.3 shows changes in titratable acidity (TA), β-gal activity, hydrogen peroxide and acetaldehyde during yogurt manufacture containing *L. acidophilus* 2409 and *B. longum* 1941. Changes in these parameters in other batches of yogurt made using the other 4 species of *Bifidobacterium* are not included in Table 6.2.3. The TA increased to 1.4% in yogurt after 9 or 7 h of fermentation. There was a slight increase in the TA in yogurt during 6 weeks storage (data not shown). As expected,

β-gal activity was higher in the yogurt mix containing ruptured cells. The enzyme activity reached about 2.0 unit per gram of mix during fermentation then declined as the pH decreased. Acidification of sonicated culture has been found to result in loss of enzyme activity (Shah and Jelen, 1990). The enzyme activity increased to 1.31 unit per gram of mix during fermentation and the loss in activity was minimal in yogurt made with whole cells of yogurt and probiotic bacteria. Microbial cell membrane, cell wall, or both may have aided in protecting the β-gal from acid denaturation in yogurt made with whole cells of yogurt and probiotic bacteria.

Production of hydrogen peroxide was higher in yogurt made with whole cells of yogurt and probiotic bacteria as compared with that made using ruptured cells. This may be due to reduction in the initial viable count of yogurt bacteria as a result of cell rupture. No hydrogen peroxide was detected after one week of storage of the product (data not shown).

Yogurt made with ruptured or whole cells of yogurt bacteria showed almost the same level of acetaldehyde after 9 or 7 h of fermentation, respectively. The levels of acetaldehyde should be sufficient to produce desired flavour as the threshold level of acetaldehyde for development of characteristic yogurt flavour is about 0.4 ppm. The production of acetaldehyde was slow in yogurt made with ruptured yogurt bacteria possibly due to lower levels of live yogurt bacteria.

Figures 6.2.3 to 6.2.7 show the pattern of lactose hydrolysis and utilisation of hydrolysed products (glucose and galactose) during yogurt fermentation with ruptured or whole yogurt bacteria. Each batch of yogurt (Figs. 6.2.3 to 6.2.7) was supplemented with *L. acidophilus* 2409 and *B. bifidum* 1900 and 1901, *B. infantis* 1912, *B. longum* 1941 or *B. pseudolongum* 20099, respectively. As shown in the figures, more lactose (15 -18 mg/mL) was hydrolysed in yogurt manufactured with ruptured yogurt bacteria as compared with yogurt prepared with whole (unruptured) yogurt bacteria. In yogurt made with ruptured yogurt bacteria, hydrolysis of lactose

was rapid after 3 h of fermentation during which time the probiotic bacterial counts also increased. In yogurt made with whole (unruptured) yogurt bacteria, hydrolysis of lactose was rapid and it took place after 4 h of fermentation. The level of glucose during fermentation in all batches of yogurt remained < 1.5 mg/mL. This shows that glucose was regularly used up by the organisms. After storage at 4°C for 24 h, there was no detectable levels of glucose in any of the products. In yogurt made with ruptured yogurt bacteria, the galactose concentrations remained higher than glucose (7-8.5 mg/mL) showing that both yogurt and probiotic and bacteria preferred to utilize glucose. In yogurt manufactured with unruptured yogurt bacteria, the galactose content was about 5 mg/mL indicating that less quantity of lactose had been hydrolysed. As shown in Figures 6.2.3-6.2.7, in yogurt manufactured with ruptured yogurt bacteria about 16% of the initial level of lactose was hydrolysed as compared to 10% hydrolysis of lactose in yogurt manufactured with ruptured yogurt bacteria.

6.2.4. Conclusion

Viable counts of yogurt bacteria were 2 log cycles lower and of probiotic bacteria 1-2 log cycles higher in yogurt made with ruptured yogurt bacteria cells and whole cells of probiotic bacteria. Higher counts of probiotic bacteria are possibly due to higher activity of β-gal released as a result of cell rupture of yogurt bacteria and/or lower level of hydrogen peroxide produced during fermentation. In general, the counts of probiotic bacteria decreased during storage but was better in yogurt prepared using ruptured cells of yogurt bacteria and whole cells of probiotic bacteria and the level of probiotic bacteria remained above 10^6 cfu per gram. Among the bifidobacteria used in this study, the viability of *B. longum* 1941 and *B. pseudolongum* was the highest and that of *B. bifidum* the lowest. However, *B. pseudolongum* is of animal origin and thus may not provide any health benefits to humans. Production of hydrogen peroxide was higher in yogurt made with whole

cells as compared with that made with ruptured cells. Although the production of acetaldehyde was slow with ruptured cells of yogurt bacteria, final amount of acetaldehyde produced was similar in yogurts made with either ruptured or whole cells of yogurt bacteria and whole cells of probiotic bacteria. Higher level of lactose was hydrolysed in yogurt made using ruptured yogurt bacteria as compared with those made using whole yogurt bacteria. During the period of fermentation, glucose was utilised rapidly by the bacteria whereas most of galactose was accumulated unused.

Table 6.2.1. Viable counts and β-D-galactosidase activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* 2515 and *Streptococcus thermophilus* 2010 before and after cell rupture.

Organisms	Before cell rupture	After cell rupture for 30 sec	60 sec	90 sec
		——Viable counts (cfu/g)——		
L. delbrueckii ssp. bulgaricus 2515	4.2×10^{10}	3.2×10^{7}	1.0×10^{5}	1.2×10^{2}
S. thermophilus 2010	3.6×10^{11}	1.0×10^{8}	4.2×10^{6}	1.8×10^{4}
		——β-D-galactosidase activity[1]——		
L. delbrueckii ssp. bulgaricus 2515	38	202	310	572
S. thermophilus 2010	25	242	286	371

[1]μmole o-nitrophenol per min per gram culture

Table 6.2.2. Counts of yogurt bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* 2515 and *Streptococcus thermophilus* 2010) and probiotic bacteria (*Lactobacillus acidophilus* 2409 and *Bifidobacterium* spp.) in five batches of yogurt made using ruptured or whole cells of yogurt bacteria and whole cells of probiotic bacteria (counts are taken after 9 or 7 h of fermentation of milk at 42°C till the pH reached 4.5).

	–Batches of yogurt made with yogurt bacteria and various strains of probiotic organisms–				
Organisms	1	2	3	4	5
Viable counts (cfu/g) after 9 h of fermentation of milk with ruptured yogurt bacteria and whole cells of probiotic bacteria					
L. delbrueckii ssp. bulgaricus 2515	4.2×10^5	4.7×10^5	4.1×10^5	4.6×10^5	1.0×10^5
S. thermophilus 2010	2.6×10^6	3.1×10^6	2.6×10^6	7.2×10^6	7.2×10^6
L. acidophilus 2409	7.8×10^7	7.4×10^7	5.8×10^7	3.1×10^7	1.5×10^7
Bifidobacterium spp.[1]	1.2×10^8	1.6×10^8	4.7×10^7	8.5×10^7	8.5×10^6
Viable counts (cfu/g) after 7 h of fermentation with whole cells of yogurt and probiotic bacteria					
L. delbrueckii ssp. *bulgaricus* 2515	9.8×10^7	8.6×10^7	8.6×10^7	2.8×10^8	1.9×10^8
S. thermophilus 2010	8.2×10^8	9.2×10^8	6.8×10^8	1.6×10^9	1.1×10^9
L. acidophilus 2409	1.4×10^6	1.6×10^6	1.2×10^6	4.1×10^6	1.9×10^6
Bifidobacterium spp.[1]	6.7×10^6	1.0×10^7	9.0×10^6	1.5×10^6	9.7×10^4

[1]*B. longum* 1941, *B. pseudolongum* 20099, *B. infantis* 1912, *B. bifidum* 1900 and *B. bifidum* 1901 were used for manufacturing batches of yogurts 1 to 5, respectively.

Table 6.2.3. Changes in titratable acidity, β-galactosidase activity, hydrogen peroxide and acetaldehyde concentrations during fermentation of milk with ruptured or whole cells of yogurt bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* 2515 and *Streptococcus thermophilus* 2010) and probiotic bacteria (*Lactobacillus acidophilus* 2409 and *Bifidobacterium longum* 1941).

Time of incubation (h)	Titratable acidity (%)	β-gal activity[1]	Hydrogen peroxide (μg/g)	Acetaldehyde (μg/g)
Yogurt made with ruptured cells of yogurt bacteria and whole cells of probiotic bacteria				
0	0.32 ± .02	1.81 ± .13	ND	ND
1	0.33 ± .02	1.82 ± .10	ND	ND
2	0.40 ± .03	1.84 ± .14	ND	ND
3	0.51 ± .02	1.90 ± .12	1.1 ± .1	0.6 ± .1
4	0.75 ± .02	2.01 ± .11	2.2 ± .2	1.2 ± .2
5	0.81 ± .02	2.02 ± .12	2.7 ± .1	1.9 ± .1
6	0.90 ± .01	1.76 ± .08	3.2 ± .2	2.6 ± .1
7	1.21 ± .02	1.68 ± .09	2.8 ± .2	3.3 ± .2
8	1.30 ± .01	1.53 ± .12	2.5 ± .1	3.3 ± .1
9	1.40 ± .03	1.31 ± .10	1.8 ± .1	3.3 ± .1
Yogurt made with whole cells of yogurt and probiotic bacteria				
0	0.32 ± .02	0.12 ± .04	ND	ND
1	0.33 ± .02	0.12 ± .03	1.3 ± .2	0.5 ± .1
2	0.53 ± .01	0.14 ± .06	2.6 ± .1	1.0 ± .1
3	0.74 ± .01	0.58 ± .09	2.8 ± .1	1.6 ± .2
4	0.91 ± .03	0.86 ± .08	3.1 ± .3	2.2 ± .2
5	1.08 ± .03	1.31 ± .10	3.8 ± .2	2.6 ± .1
6	1.27 ± .01	1.30 ± .08	3.6 ± .3	3.1 ± .1
7	1.40 ± .02	1.30 ± .07	3.2 ± .2	3.9 ± .2

[1]μmole o-nitrophenol per min per g mix.
ND= not detected.

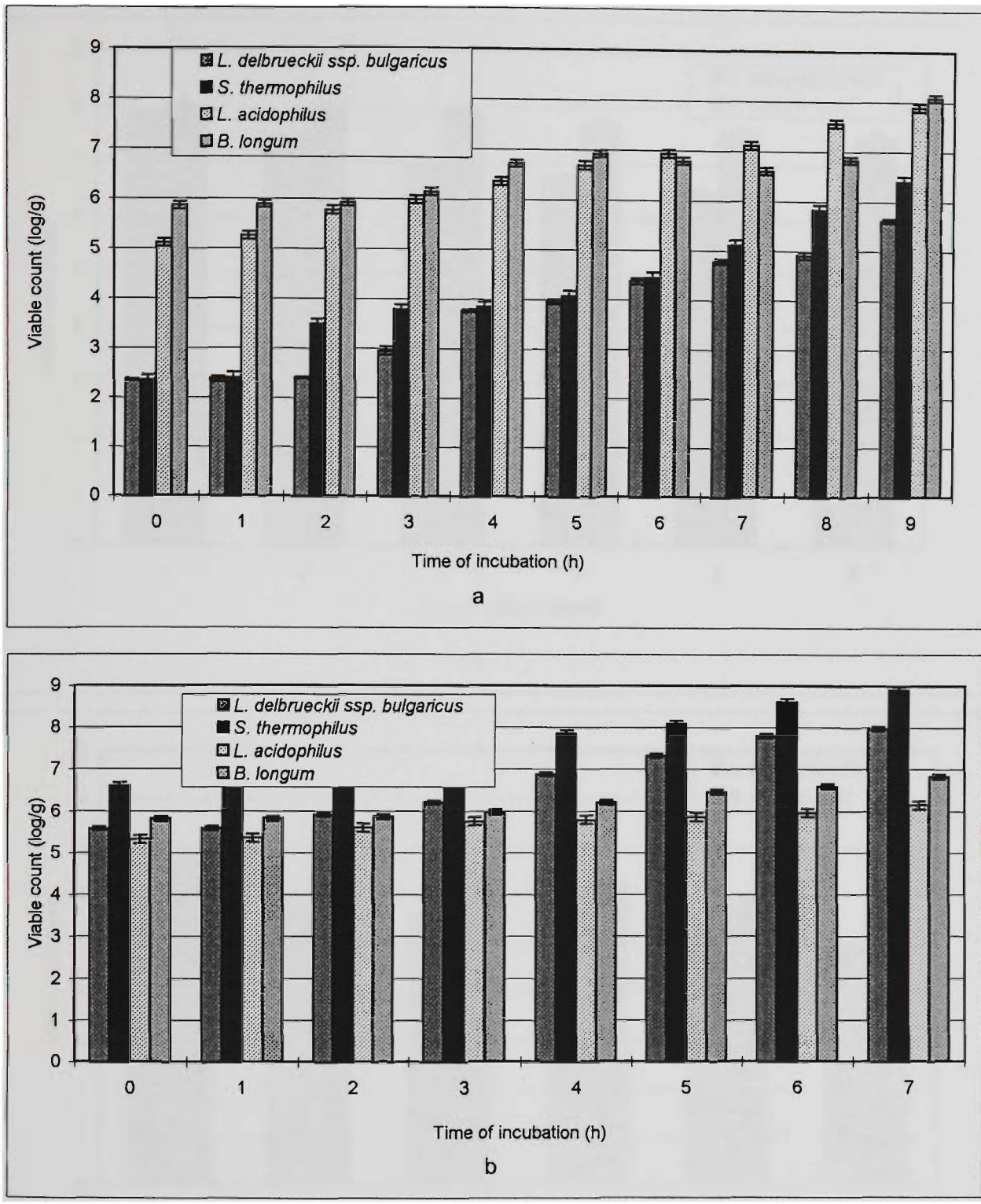


Figure 6.2.1. Changes in viable counts of two yogurt bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* 2515 and *Streptococcus thermophilus* 2010) and two probiotic bacteria (*Lactobacillus acidophilus* 2409 and *Bifidobacterium longum* 1941) during fermentation of milk with (a) ruptured cells of yogurt bacteria and whole cells of probiotic bacteria, and (b) whole cells of yogurt and probiotic bacteria.

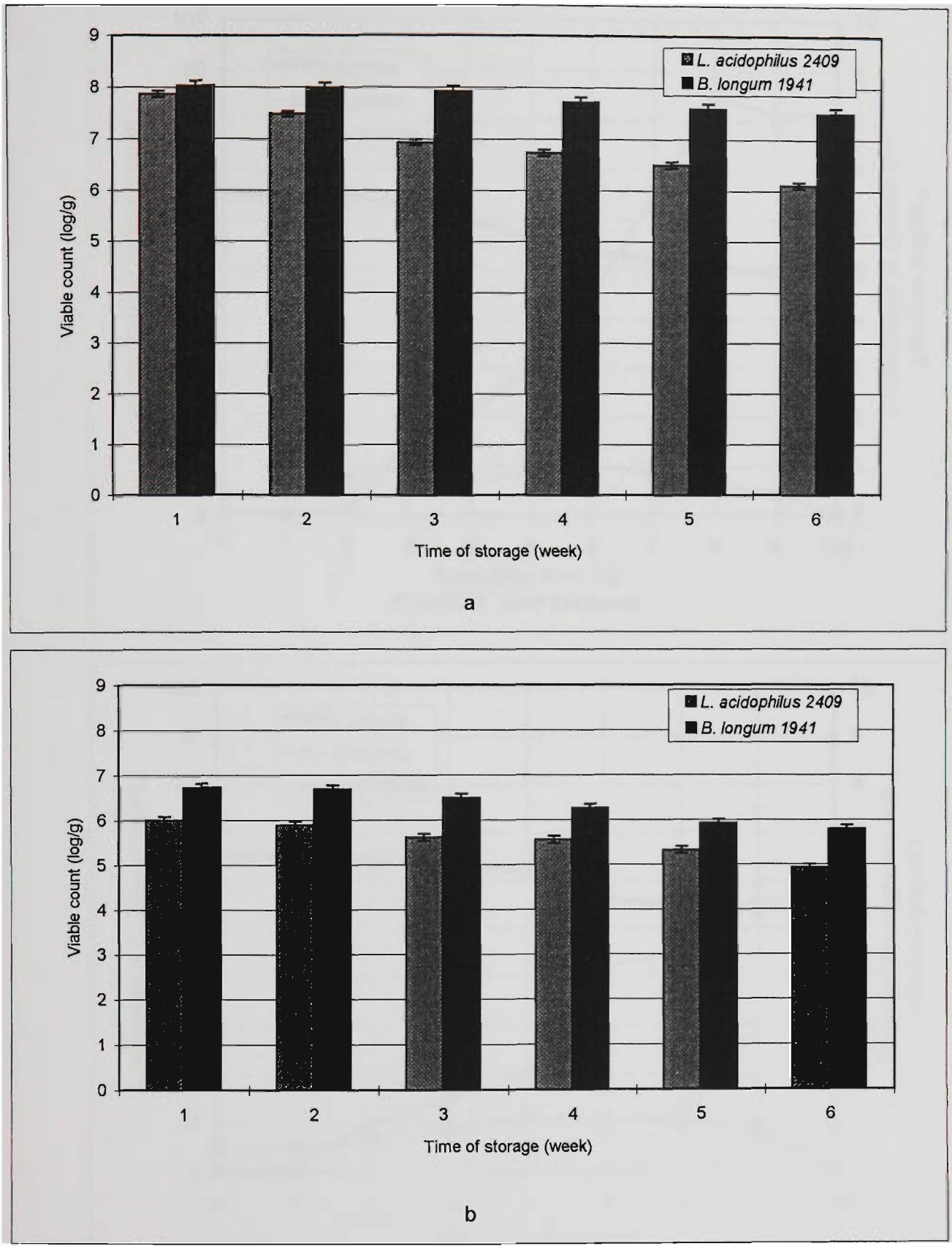


Figure 6.2.2. Changes in viable counts of two probiotic bacteria (*Lactobacillus acidophilus* 2409 and *Bifidobacterium longum* 1941) during storage of yogurt made with (a) ruptured cells yogurt bacteria and whole cells of probiotic bacteria, and (b) whole cells of yogurt and probiotic bacteria.

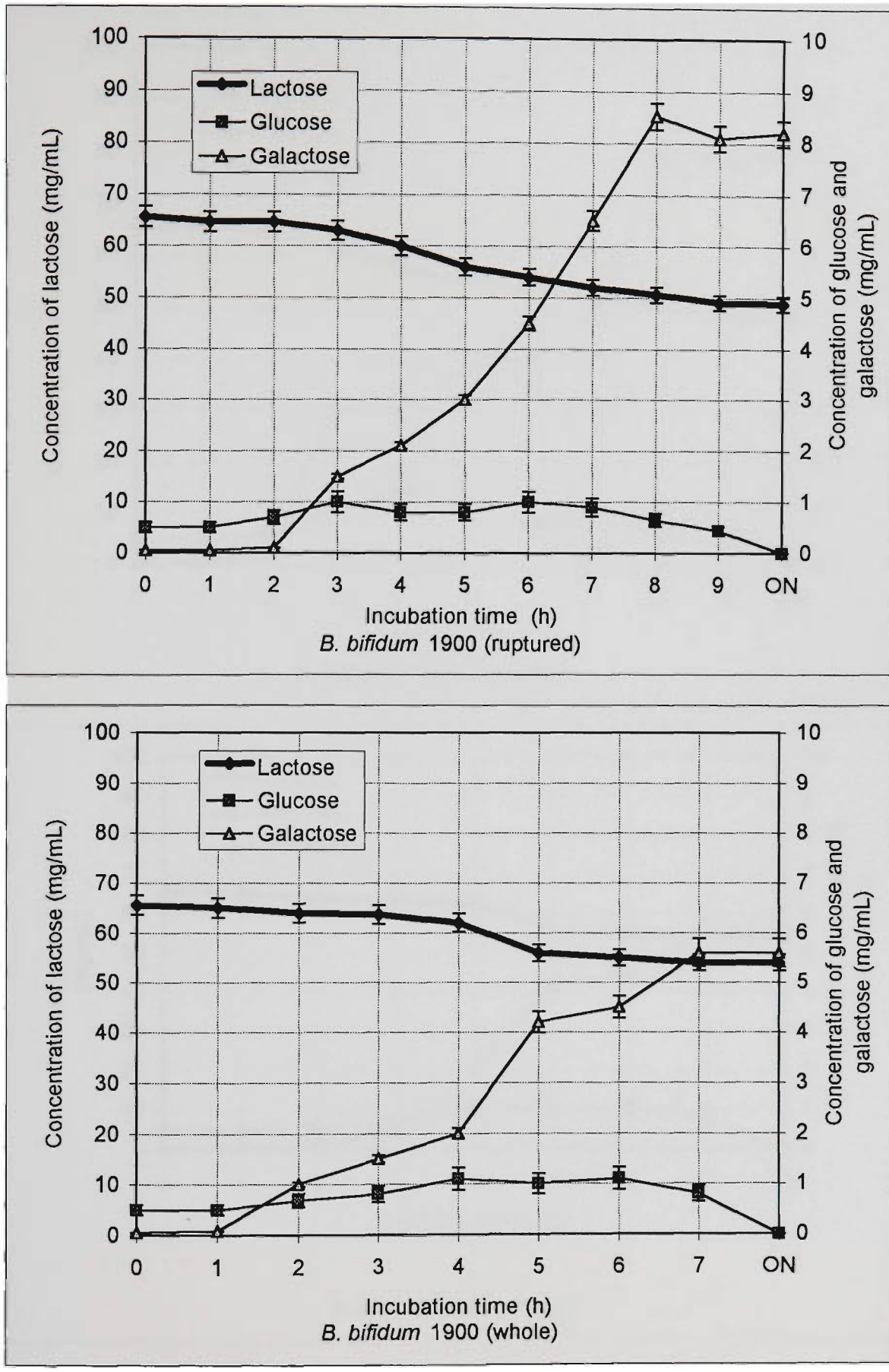


Fig. 6.2.3. Hydrolysis of lactose and use of glucose and galactose in yogurt mix containing ruptured or whole yogurt bacteria, *L. acidophilus* 2409, and *B. bifidum* 1900 during yogurt fermentation at 42°C and during overnight (24 h) storage at 4°C (ON=overnight)

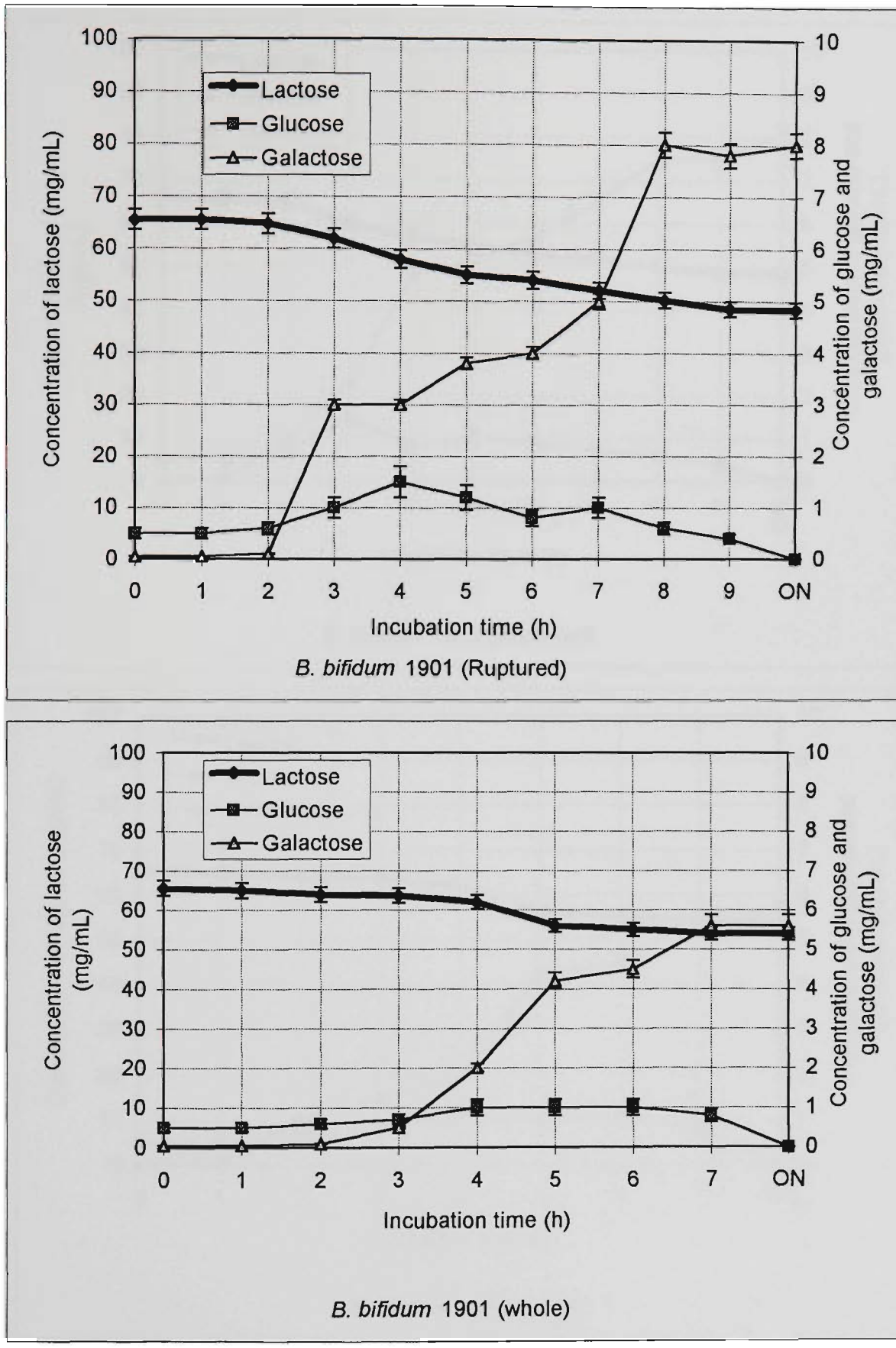


Fig. 6.2.4. Hydrolysis of lactose and use of glucose and galactose in yogurt mix containing ruptured or whole yogurt bacteria, *L. acidophilus* 2409, and *B. bifidum* 1901 during yogurt fermentation at 42°C and during overnight (24 h) storage at 4°C) (ON=overnight)

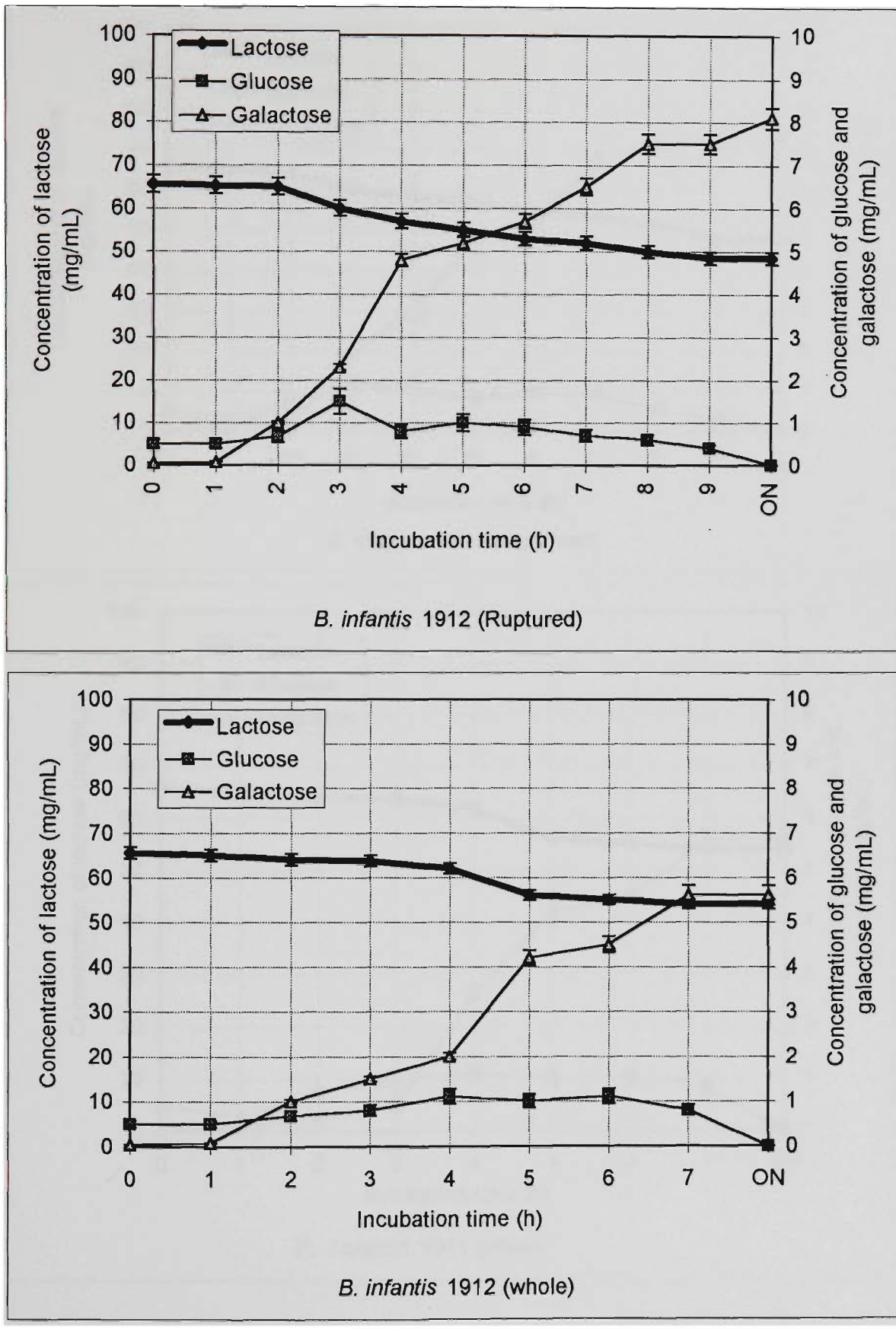


Fig. 6.2.5. Hydrolysis of lactose and use of glucose and galactose in yogurt mix containing ruptured or whole yogurt bacteria, *L. acidophilus* 2409, and *B. infantis* 1912 during yogurt fermentation at 42°C and during overnight (24 h) storage at 4°C)
(note:-ON=overnight)

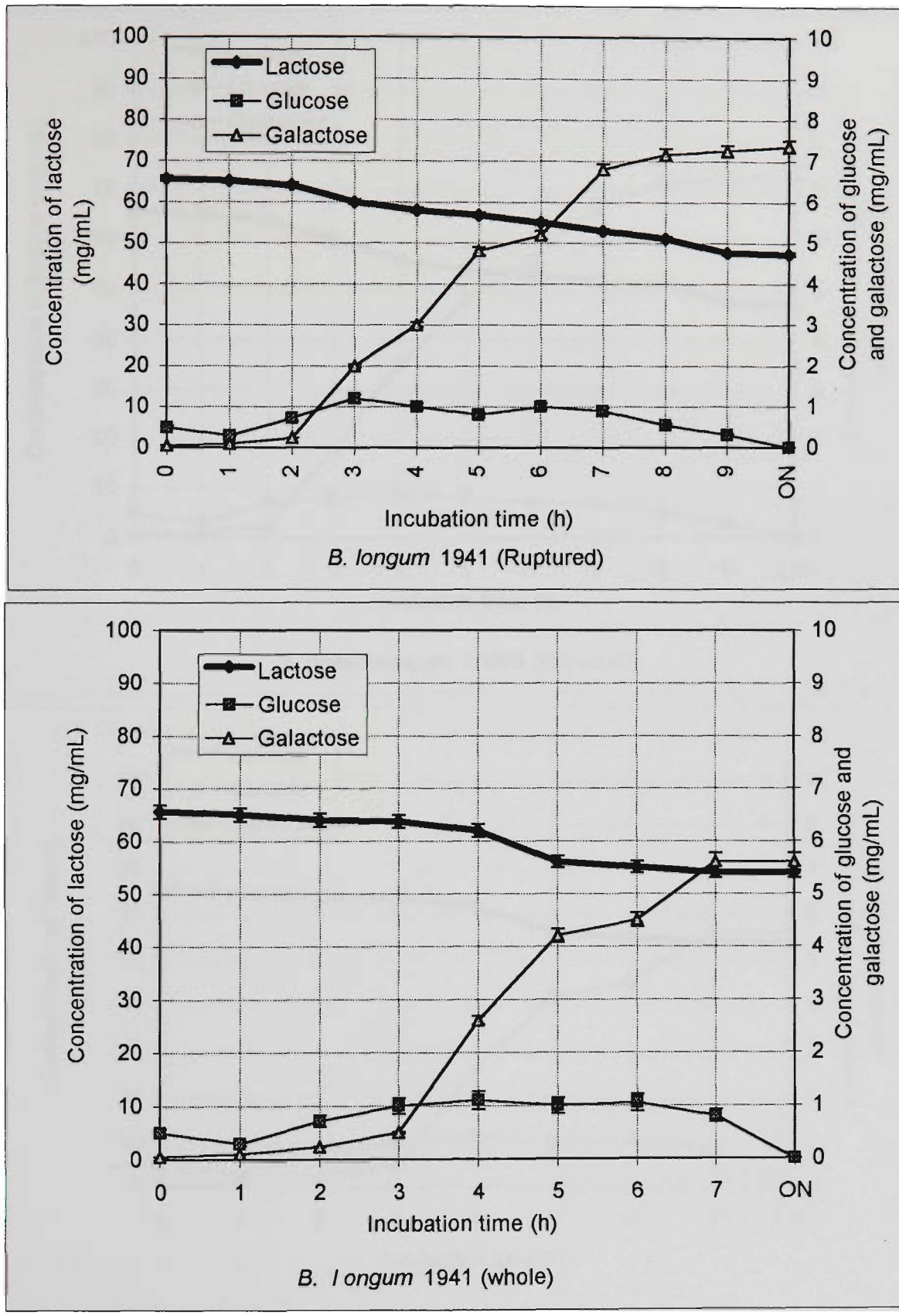


Fig. 6.2.6. Hydrolysis of lactose and use of glucose and galactose in yogurt mix containing ruptured or whole yogurt bacteria, *L. acidophilus* 2409, and *B. longum* 1941 during yogurt fermentation at 42°C and during overnight (24 h) storage at 4°C) (note:-ON=overnight

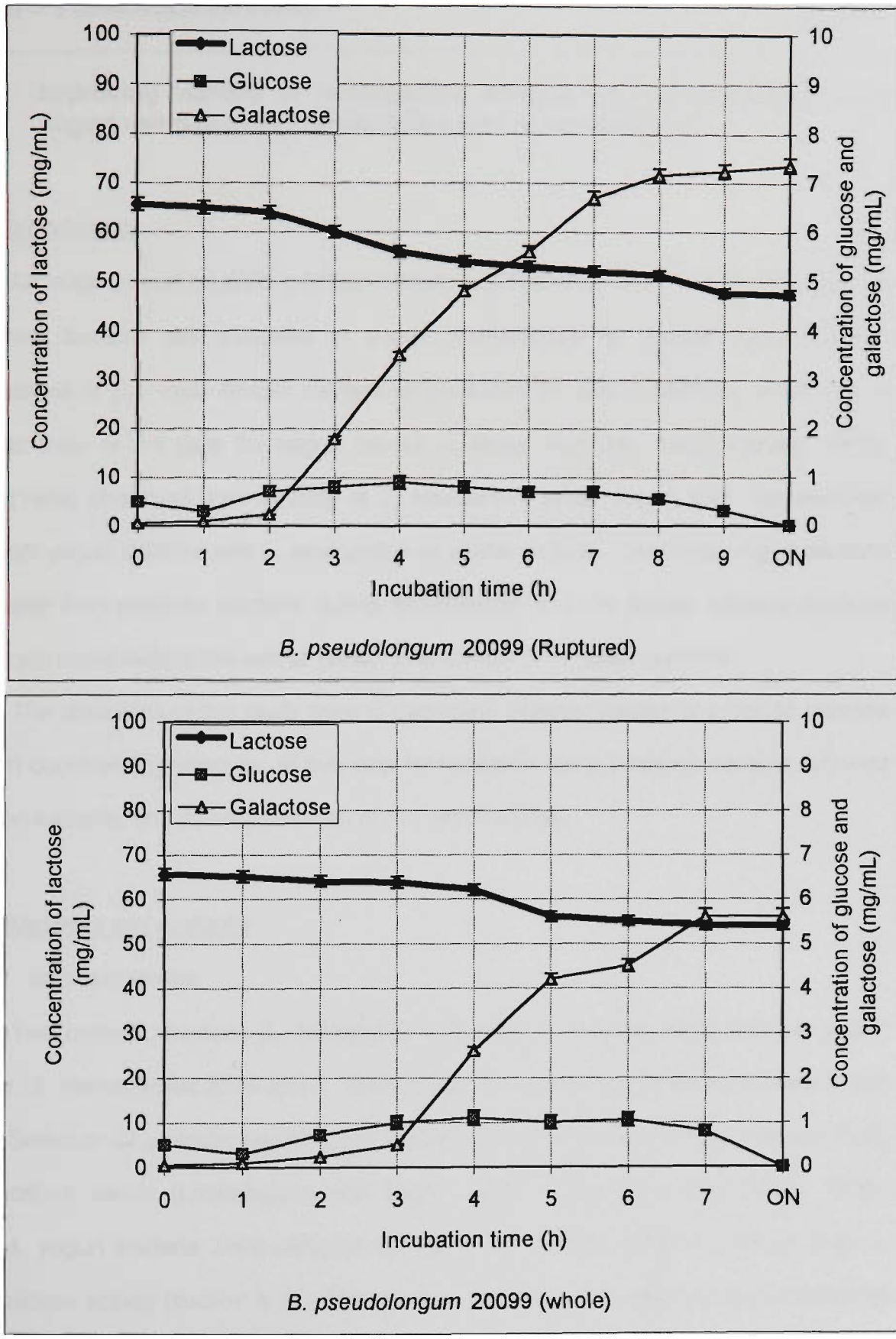


Fig. 6.2.7. Hydrolysis of lactose and use of glucose and galactose in yogurt mix containing ruptured or whole yogurt bacteria, *L. acidophilus* 2409, and *B. pseudolongum* 20099 during yogurt fermentation at 42°C and during overnight (24 h) storage at 4°C (ON=overnight)

6.3. Improving viability of *lactobacillus acidophilus* and bifidobacteria in yogurt using two step fermentation and neutralised mix[1]

6.3.1. Introduction

Although yogurt bacteria produce inhibitory substances against probiotic bacteria, the former bacteria are essential in yogurt manufacture for typical yogurt flavour. Acetaldehyde is the main flavour compound produced by yogurt bacteria, which has a threshold level of 0.4 ppm for yogurt flavour (Lindsay and Day, 1965; Harvey, 1960). Reddy (1989) observed poor viability of *L. acidophilus* when yogurt was manufactured using both yogurt bacteria and *L. acidophilus* as starter culture. Generally, yogurt bacteria grow faster than probiotic bacteria during fermentation and the former bacteria produce acids which could reduce the rate of growth and viability of probiotic bacteria.

The objectives of this study were to determine whether viability of probiotic bacteria in yogurt could be improved by: (i) two step fermentation using probiotic bacteria followed by yogurt bacteria, and (ii) neutralisation of mix with $Ca(OH)_2$.

6.3.2. Materials and methods

6.3.2.1. Bacterial strains

Two probiotic bacteria (*L. acidophilus* 2409 and *B. longum* 1941) and two yogurt bacteria (*S. thermophilus* 2010 and *L. delbrueckii* ssp. *bulgaricus* 2515) were used in this study. Selection of probiotic bacteria was based on their tolerance to acid, bile and H_2O_2 as described earlier (Lankaputhra and Shah, 1995; Lankaputhra and Shah, 1996); whereas, yogurt bacteria were selected on the basis of their ability to produce high β-galactosidase activity (section 6.1). The bacterial cultures were obtained and maintained as described in section 2.2.2.

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[1] A refereed paper based on this section is in press in Food Australia under the title "Improving viability of *Lactobacillus acidophilus* and bifidobacteria in yogurt using two step fermentation and neutralised yogurt mix".

6.3.2.2. Yogurt making using single step fermentation

Homogenised pasteurised milk with a total solid (TS) content of 12% was supplemented with 5% nonfat dry milk (NDM), heat treated at 85°C for 30 min and cooled to 42°C (Fig. 2.2). For the control batch, separately overnight grown fresh culture of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* was added at the rate of 0.5% of each and that of *L. acidophilus* 2409 and *B. longum* 1941 each at 2%.

Inoculated mixes were poured into plastic cups and incubated at 42°C till the pH reached 4.5. Changes in viable counts of *L. acidophilus* 2409 and *B. longum* 1941, pH, and levels of H_2O_2 and acetaldehyde were monitored at weekly intervals for 6 weeks.

6.3.2.3. Yogurt making using two step fermentation

Homogenised and pasteurised milk with a total solid content of 12% was supplemented with 5% NDM, heat treated at 85°C for 30 min, cooled to 42°C, and separately overnight grown fresh culture of *L. acidophilus* 2409 and *B. longum* 1941 was added at the rate of 2.0% of each followed by incubation at 42°C for 2 h (step 1 fermentation). After the initial fermentation, separately overnight grown *L. delbrueckii* ssp. *bulgaricus* 2515 and *S. thermophilus* 2010 were added at the rate of 0.5% of each. The mix was filled into 100 mL plastic cups and incubated at 42°C (step 2 fermentation) until the pH reached 4.5. A control batch of yogurt was manufactured without carrying out step 1 fermentation. Changes in viable counts of *L. acidophilus* 2409 and *B. longum* 1941, pH, and levels of H_2O_2 and acetaldehyde were monitored at weekly intervals for 6 weeks.

6.3.2.4. Yogurt making with neutralised mix

One litre aliquots of homogenised and pasteurised milk containing 17% TS prepared as in previous section were adjusted to pH 6.7, 6.8, and 6.9 from initial pH of

6.6 using a sterile saturated solution of $Ca(OH)_2$ and the mixes were heated at 85°C for 30 min, cooled to 42°C and overnight grown fresh cultures of the yogurt (*S. thermophilus* 2010 and *L. delbrueckii* ssp. b*ulgaricus* 2515) and probiotic bacteria (*L. acidophilus* 2409 and *B. longum* 1941) were added at the rate of 0.5% and 2.0%, respectively. After proper mixing, the inoculated mix was poured into plastic cups and incubated at 42°C till the pH reached 4.5. Samples of yogurt were chilled to 4°C and the changes in counts of probiotic bacteria, and pH, hydrogen peroxide and acetaldehyde contents were monitored at weekly intervals for 6 weeks.

6.3.2.5. Enumeration and chemical analyses

Selective enumeration of *L. acidophilus* 2409 was carried out using MRS-salicin agar (Lankaputhra and Shah, 1996). *B. longum* 1941 was selectively enumerated using neomycin sulphate, nalidixic acid, lithium chloride and paromomycine sulphate (NNLP) agar (Lankaputhra *et al.*, 1996b). The plates were incubated anaerobically using anaerobic jars HP 11 (Oxoid Australia, W. Heidelberg, Australia). Hydrogen peroxide and acetaldehyde contents in yogurt were determined by the methods described by Gilliland (1969) and Lindsay and Day (1965), respectively.

All experiments and analyses were replicated three times. The results presented are averages of all the available replicates.

6.3.3. Results and discussion

Figures 6.3.1. and 6.3.2. illustrate effect of single versus two step fermentation on viability of *L. acidophilus* 2409 and *B. longum* 1941 in yogurt, respectively. As shown, yogurt prepared using two step fermentation process showed counts of 7.94 and 9.00 log cfu/g of *L. acidophilus* 2409 and *B. longum* 1941 as compared with counts of 7.48 and 8.63 cfu/g in that prepared using single step fermentation process. Initial counts of probiotic bacteria increased by about 4-5 times in the product made using two step fermentation

process. Final counts of *L. acidophilus* 2409 and *B. longum* 1941 after 6 weeks storage were 6.85 and 7.93 log cfu/g, and 7.60 and 8.84 cfu/g for yogurt prepared using single and two step processes, respectively.

When yogurt bacteria are introduced after the initial fermentation with *L. acidophilus* 2409 and *B. longum* 1941, the probiotic bacteria would be in their final stage of lag phase or initial stage of log phase and thus could dominate the flora resulting in higher counts. Reddy (1989) reported improved viability of *L. acidophilus* in yogurt made using two step fermentation.

Figures 6.3.3. and 6.3.4. show changes in counts of *L. acidophilus* 2409 and *B. longum* 1941 in yogurt made with neutralised mix. At pH 6.6 (control), the incubation time required to reach the pH of 4.5 was 5.0 h, whereas, those at pH 6.7, 6.8 and 6.9 were 5 h 20 min, 5 h 40 min and 6 h, respectively (Table 6.3.1). In general, the initial counts of the two bacteria increased with increasing pH of the mix. The initial counts of *L. acidophilus* 2409 and *B. longum* 1941 in the control yogurt after fermentation was 8.00 and 8.20 log cfu/g and those in the product made with the mix at pH 6.9 had counts of 8.81 and 8.95 log cfu/g, respectively. The final counts of *L. acidophilus* 2409 and *B. longum* 1941 in the product prepared with the mix at pH 6.9 were 7.61 and 7.95 log cfu/g, respectively, as compared with 6.91 and 7.18 log cfu/g in the control sample. Improved survival of the probiotic bacteria may be due to the presence of $Ca(OH)_2$ which could enhance the buffering effect or due to longer incubation time. Our results are in accordance with the findings of Reddy (1989) who reported improved survival of *L. acidophilus* in yogurt made with added $Ca(OH)_2$.

Table 6.3.1. shows the levels of acetaldehyde in yogurt prepared using single step and two step fermentation processes or using neutralised mix. Yogurt made using single step fermentation produced slightly higher level of acetaldehyde (6.46 ppm) as compared with the product made using two step process (6.12 ppm). In both types of products, the

levels of acetaldehyde during storage were close to the initial levels. The products made with mix at pH 6.7, 6.8 and 6.9 showed similar levels of acetaldehyde.

Table 6.3.2. shows the changes in pH of yogurt prepared using single or two step fermentation process and with mix at pH 6.6, 6.7, 6.8, and 6.9. The pH of yogurt made using single step fermentation process was lower as compared with that made using two step process. This may be due to higher numbers of probiotic bacteria in the latter product which are claimed to be slow acid producers. Yogurt prepared with mix at pH 6.6 and 6.7 showed final pH of 4.0 whereas that prepared with mix at pH 6.8 and 6.9 had final pH of 4.1 and 4.2, respectively. This could be due to the presence of higher $Ca(OH)_2$ levels in the mix at higher pH.

All the products prepared using single and two step fermentation processes and with neutralised mix had initial hydrogen peroxide levels ranging from 3.8 to 4.2 ppm (data not shown). However, hydrogen peroxide was undetectable after 7 days of storage. Similar results have been observed previously (Lankaputhra and Shah, 1996).

6.3.4. Conclusions

Counts of probiotic bacteria were about 5 times higher in yogurt made using two step fermentation process as compared with those made using single step fermentation process. In general, the counts of probiotic bacteria reduced in all the products during storage, however, yogurt made using two step process showed higher counts than that made using single step process. Neutralisation of the mix before fermentation also increased the initial and final counts of the two probiotic bacteria by about 4-6 times. All the products showed similar levels of acetaldehyde.

Table 6.3.1. Acetaldehyde content in yogurt manufactured using single or two step fermentation process or with neutralised yogurt mix during refrigerated storage at 4°C.

-----Storage period (days)-----								
Type of yogurt	0	7	14	21	28	35	42	Incubation time
-----Acetaldehyde (ppm)-----								
Yogurt made using single or two step fermentation								
Single step	6.46	6.50	6.51	6.51	6.50	6.48	6.48	5 h
Two step	6.12	6.18	6.21	6.21	6.20	6.20	6.18	7 h
Yogurt made with mix at								
pH 6.6 (control)	6.51	6.54	6.55	6.54	6.53	6.53	6.53	5 h
pH 6.7	6.55	6.57	6.58	6.57	6.57	6.57	6.55	5 h 20 min
pH 6.8	6.61	6.62	6.63	6.63	6.62	6.62	6.60	5 h 40 min
pH 6.9	6.68	6.68	6.71	6.70	6.70	6.68	6.68	6 h

Table 6.3.2. Change in pH of yogurt prepared using single or two step fermentation and using neutralised mix during six weeks of storage at 4°C.

-----Storage period (days)-----							
Type of yogurt	0	7	14	21	28	35	42
----- pH -----							
Yogurt made using single or two step fermentation							
Single step	4.50	4.42	4.37	4.30	4.21	4.15	4.04
Two step	4.50	4.44	4.39	4.32	4.25	4.19	4.15
Yogurt made with mix at							
pH 6.6 (control)	4.50	4.42	4.36	4.29	4.21	4.13	4.04
pH 6.7	4.50	4.43	4.36	4.30	4.21	4.13	4.04
pH 6.8	4.50	4.45	4.38	4.34	4.30	4.25	4.14
pH 6.9	4.50	4.45	4.38	4.35	4.32	4.28	4.25

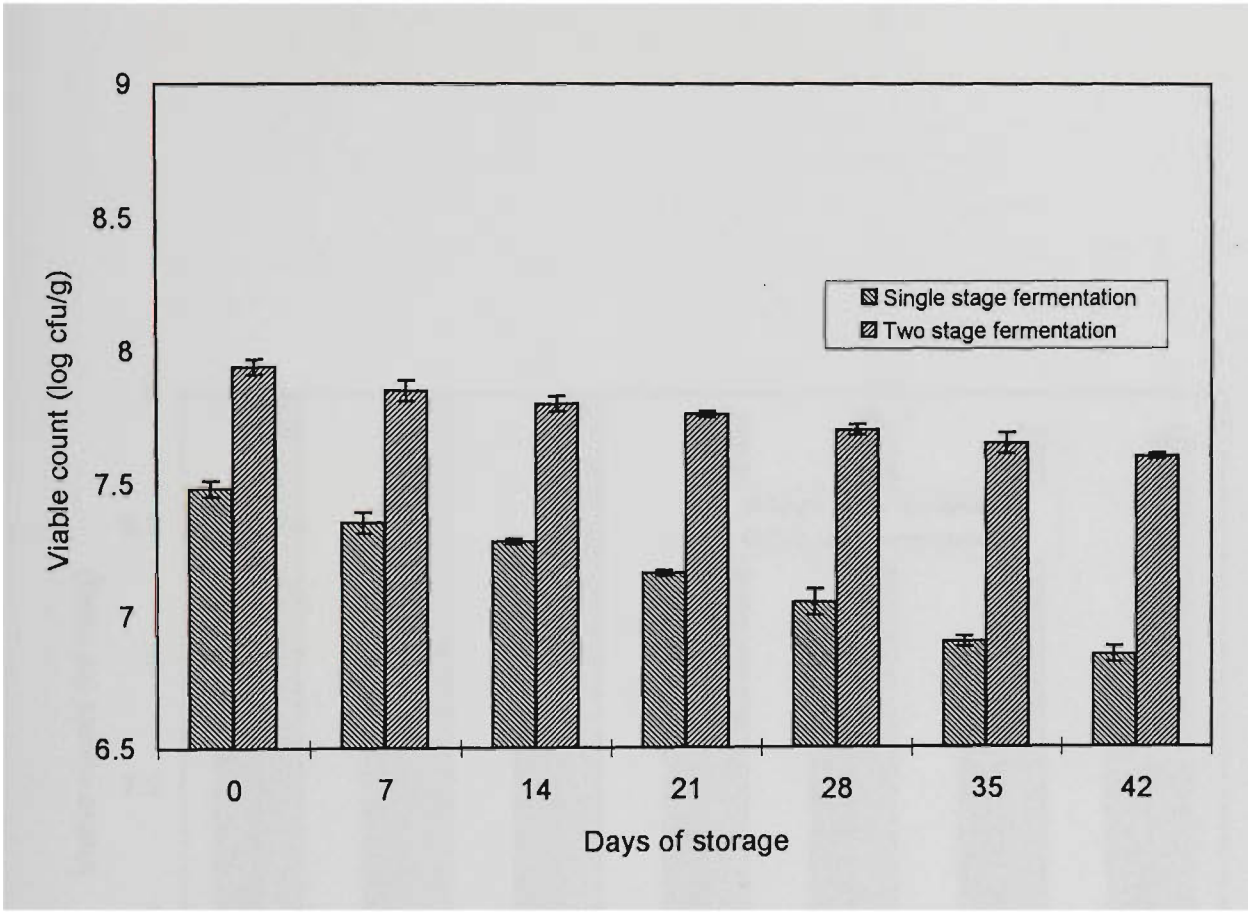


Fig 6.3.1. Changes in viable counts of *L. acidophilus* 2409 in yogurt prepared using single or two stage fermentation process.

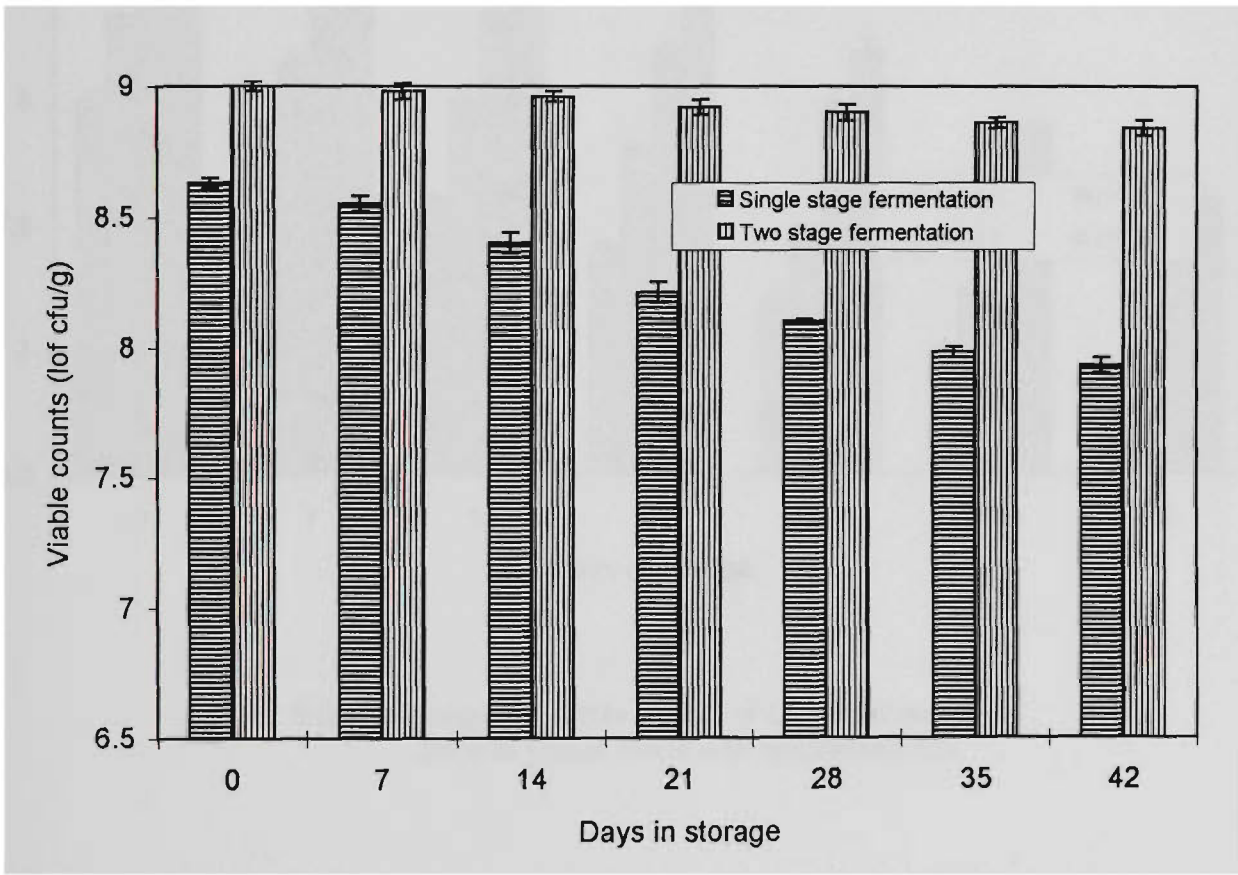


Fig 6.3.2. Changes in viable counts of *B. longum* 1941 in yogurt prepared using single or two stage fermentation process.

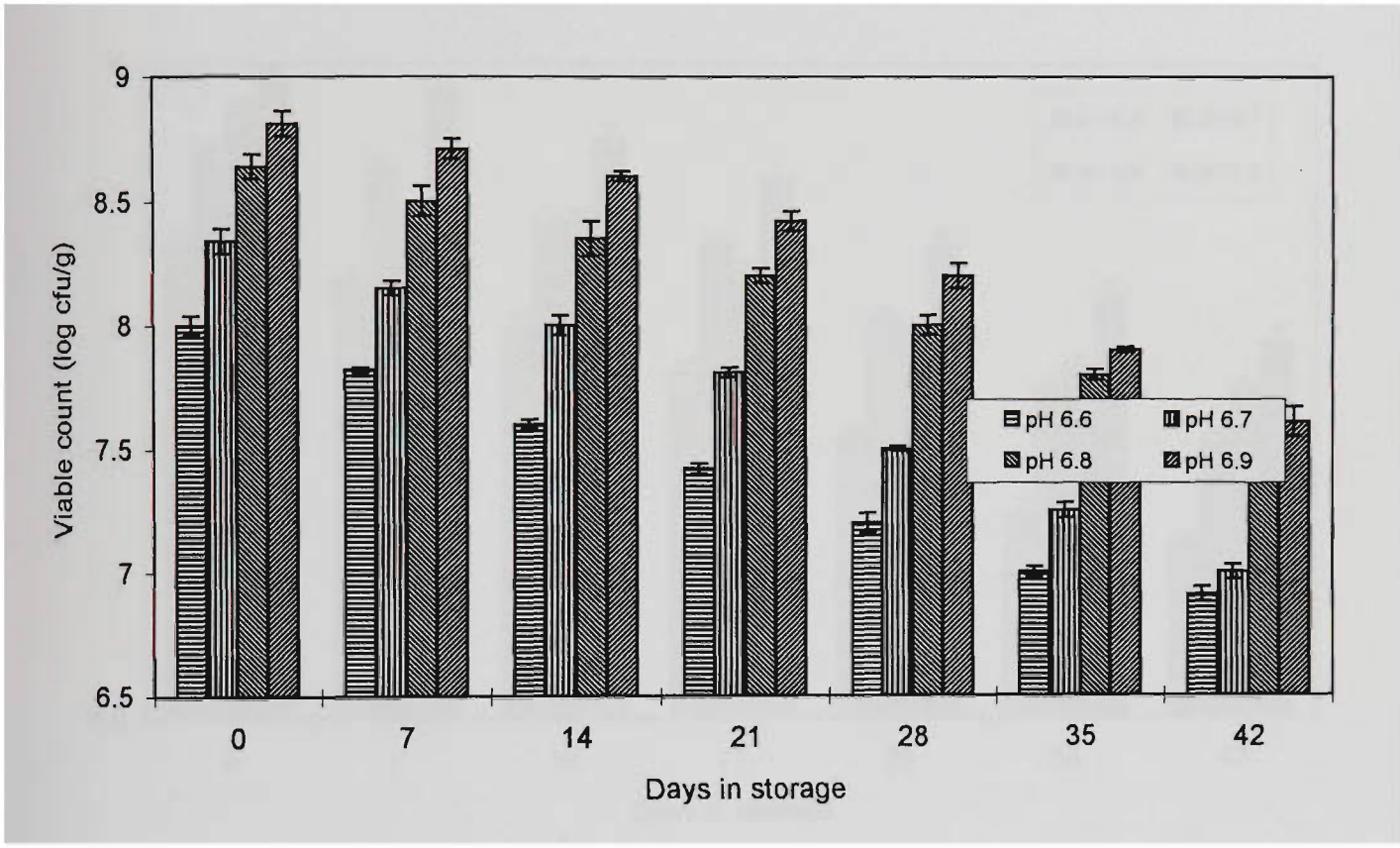


Fig 6.3.3. Changes in viable counts of *L. acidophilus* 2409 in yogurt made with neutralised mix.

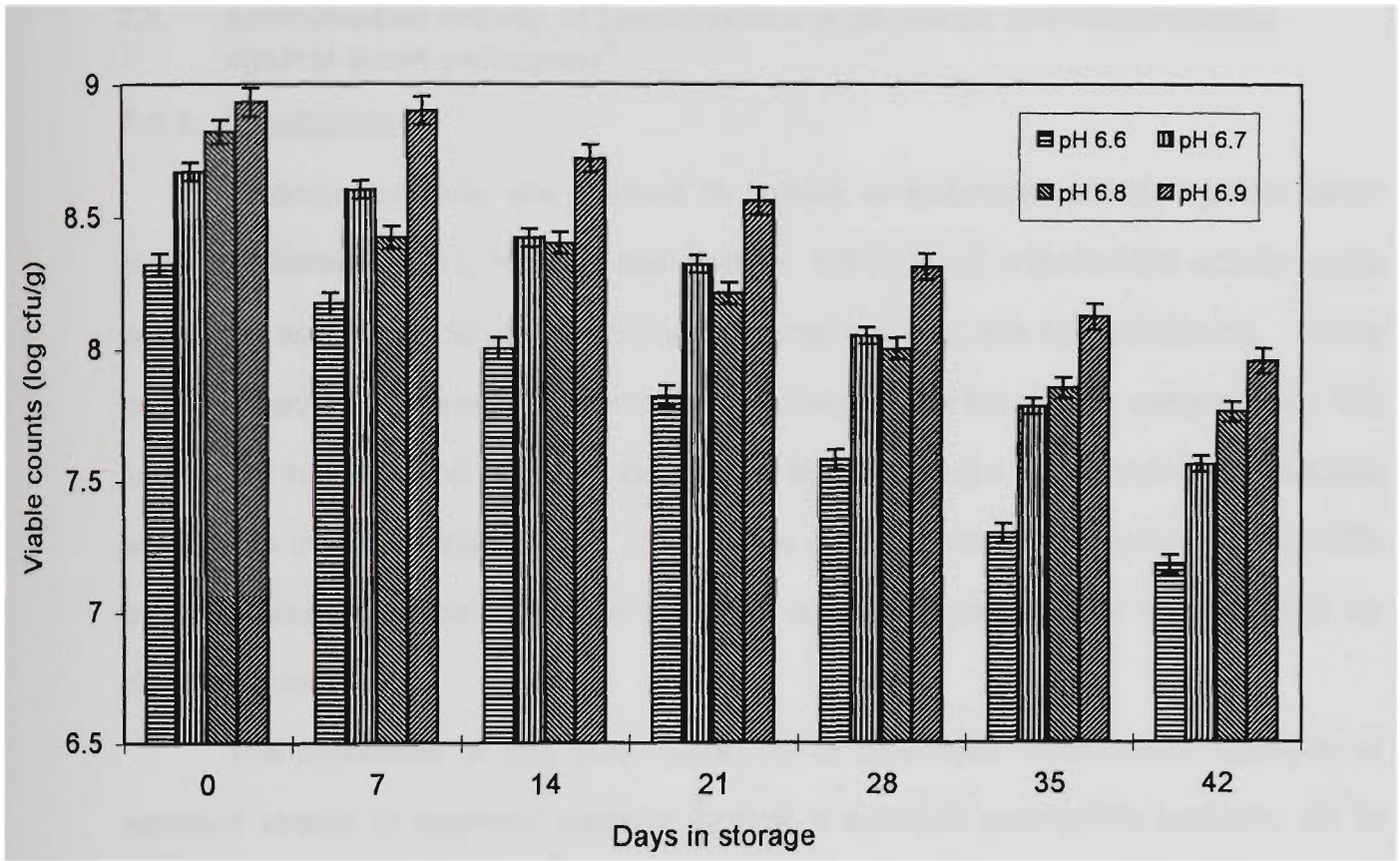


Fig 6.3.4. Changes in viable counts of *B. longum* 1941 in yogurt prepared with neutralised mix.

7.0. POTENTIAL THERAPEUTIC BENEFITS OF SELECTED STRAINS OF *LACTOBACILLUS ACIDOPHILUS* AND *BIFIDOBACTERIUM* SPECIES AND OF ORGANIC ACID PRODUCTION BY THESE BACTERIA

7.1. Antimicrobial activity of *Lactobacillus acidophilus* and bifidobacteria against some pathogens[1]

7.1.1. Introduction

Probiotic bacteria are claimed to exhibit antimicrobial activity against other bacteria (Gilliland, 1991; Hughes and Hoover, 1991). Such antimicrobial activity could be due to organic acids produced during fermentation or due to bacteriocins. Those probiotic bacteria possessing antimicrobial activity would be able to compete with the 'unfriendly' bacteria and establish dominance in the intestine, thus achieving beneficial equilibrium in the intestinal flora. Information of the antimicrobial activity of probiotic bacteria would also be helpful in selecting suitable organisms for incorporation as dietary adjuncts.

The objectives of this study were: (1) to determine antimicrobial activities of selected strains of probiotic bacteria against 4 selected pathogenic bacteria, (2) to identify nature of antimicrobial substances produced by probiotic bacteria, and (3) to study antimicrobial effect of main organic acids produced by probiotic bacteria.

7.1.2. Materials and Methods

7.1.2.1. Antimicrobial activities of spent broths of probiotic bacteria

Antimicrobial activities of 6 strains of *L. acidophilus* and 9 strains of bifidobacteria against 4 pathogens (*Aeromonas hydrophila*, *Candida albicans*, *Escherichia coli* and *Salmonella typhimurium*) were determined. *L. acidophilus* and bifidobacteria were grown in MRS broth at 37°C for 15 h, the broth centrifuged and the supernatant filter sterilised using 0.45μm filter papers. The pathogenic bacteria were grown separately in nutrient broth and aliquots (5%) of these cultures were inoculated

[1] A paper titled "Production of volatile acids by probiotic bacteria and their antimicrobial properties" was presented at 30th Annual Convention of AIFST, Perth, Australia, May 4-9, 1997.

into sterile molten nutrient agar. Inoculated molten agar was poured into Petri plates, allowed to solidify for 1 h at room temperature, and 4 wells were bored in the solidified agar using a cork borer of 7 mm diameter. The wells were filled with aliquots of 0.1 mL of filter sterilised supernatant, neutralised supernatant (pH 6.5), fresh MRS broth and supernatant treated with 0.1% trypsin. The plates were incubated for 18 h and zones of inhibition around the wells were observed.

7.1.2.2. HPLC analysis of organic acids produced by probiotic bacteria

Probiotic bacteria were grown in MRS broth for 18 h at 37°C and supernatants were separated by centrifuging at 10,000 rpm for 10 min using a Beckman J2-HS centrifuge. Four millilitre aliquot of each supernatant was pipetted into a 25 mL volumetric flask and the volume made up with 0.005 **M** H_2SO_4. Each solution was filter sterilised with a 0.45 μM millipore filter (Millipore Australia, Lane Cove, NSW, Australia). One millilitre aliquot of each filtrate was pipetted into HPLC vials which were used in an auto sampler for analysis of organic acids. Concentrations of the acids were determined with a Varian HPLC (Varian, Mulgrave, Australia) using a UV-Vis detector. An Aminex HPX-87H ion exclusion column (300 x 7.8 mm) (Bio-Rad, North Ryde, NSW, Australia) and a mobile phase of 0.00375 **M** H_2SO_4 were used for the analysis.

7.1.2.3. Effects of organic acids usually produced by probiotic bacteria

Effects of organic acids against 4 pathogens (*Aeromonas hydrophila*, *Candida albicans*, *Escherichia coli* and *Salmonella typhimurium*) were determined. Dilution (1 mg/mL) of each of acetic, butyric, lactic, orotic and pyruvic acid was prepared. Ten millilitre aliquot of each dilution was neutralised with 1 **N** NaOH to a pH of 6.5 and 0.1 mL from each was added to agar wells. After incubating at 37 °C for 18 h, the zones of inhibition were measured.

7.1.2.4. Effect of the presence of probiotic bacteria on the growth of pathogenic bacteria

Effect of selected probiotic bacteria on the growth of *Aeromonas hydrophila*, *Candida albicans*, *Escherichia coli* and *Salmonella typhimurium* was studied. Fifty millilitre aliquots of nutrient broth ii (Oxoid) was prepared and sterilised in 100 mL Schott bottles and 4 such bottles were used for each pathogen. The control sample was inoculated with 1 mL of overnight grown each pathogenic bacteria and the remaining 3 bottles were inoculated with 1, 2 or 5 mL of freshly grown probiotic bacterial cultures along with each pathogen. A second set of control sample was prepared with probiotic bacterial cultures only. After inoculation, the bottles were incubated at 37°C in a shaker incubator. Samples were drawn every 2 h for 12 h and absorbency at 600 nm was measured to determine effect of various levels of probiotic bacteria on the growth of pathogenic bacteria.

7.1.3. Results and discussion

Antimicrobial activity of 15 probiotic strains against *Aeromonas hydrophila*, *Candida albicans*, *Escherichia coli* and *Salmonella typhimurium* is shown in Tables 7.1.1-7.1.4 In general, supernatants of each probiotic strains studied showed varying levels of inhibition against the 4 pathogens. However, neutralised supernatants did not show any inhibition. Supernatants treated with trypsin also showed inhibition similar to those that were untreated.

As shown in Table 7.1.1, supernatants of all probiotic strains produced zones of inhibition against *A. hydrophila*. *L. acidophilus* strains produced stronger inhibition against *A. hydrophila* than the strains of *Bifidobacterium* spp. *Bifidobacterium* spp. showed larger zones of inhibition against *C. albicans* as compared with *L. acidophilus* (Table 7.1.2). As shown in Tables 7.1.3 and 7.1.4, all probiotic strains showed smaller zones of inhibition against *E. coli* and *S. typhimurium*.

Non-inhibitory nature of the neutralised supernatant confirmed that the inhibition of the probiotic bacterial supernatant was due to acids. Unaltered inhibitory activity of the trypsinised supernatant suggested that the inhibition caused by the probiotic strains was not due to bacteriocins. Stronger inhibition against *A. hydrophilla* by *L. acidophilus* strains could be due to higher levels of lactic acids in the supernatants as compared with those produced by *Bifidobacterium* spp. On the other hand, stronger level of inhibition against *C. albicans* by *Bifidobacterium* spp. strains could be due to higher levels of acetic acid in the supernatants as compared with those produced by *L. acidophilus* (Table 7.1.5). Table 7.1.5 shows the level of organic acids produced by probiotic bacteria. The major acids produced by these organisms were lactic and acetic acids. *L. acidophilus* produced higher levels of lactic acid than other acids. Bifidobacteria produced more acetic than other acids.

All organic acids produced by probiotic bacteria had an antimicrobial effect (Table 7.1.6). Inhibitions by these acids varied with organisms. Lactic, propionic and pyruvic acids showed stronger inhibition against *A. hydrophila* and acetic acid exhibited stronger inhibition against *C. albicans*.

Growth of *A. hydrophila* in the presence of various inoculum levels of *L. acidophilus* 2409, *B. infantis* 1912 and *B. longum* 1941 are illustrated in Figures 7.1.1-7.1.3. These 3 strains were selected for this study based on their tolerance to acid and bile concentrations (Lankaputhra and Shah, 1995). Presence of probiotic bacteria seemed to retard the growth of *A. hydrophila*; the effect was higher with higher levels of probiotic bacteria. As shown in Figures 7.1.4-7.1.6, *Candida albicans* also showed similar pattern of inhibition. However, *E. coli* and *S. typhimurium* did not show such inhibition (data not shown).

7. 1. 4. Conclusions

L. acidophilus and bifidobacteria showed antimicrobial activity against *A. hydrphila*, *C. albicans*. *E. coli* and *S. typhimurium*. However, this antimicrobial activity

was due to acidity ony. The probiotic bacteria did not show bacteriocin activity against the pathogens studied. In general *L. acidophilus* produced more lactic acid, whereas bifidobacteria produced more acetic acid. When *A. hydrophila* and *C. albicans* were grown in a co-culture, presence of probiotic bacteria inhibited the growth of the former pathogens. Therefore, the selected probiotic strains (*L. acidophilus* 2409, *B. infantis* 1912 and *B. longum* 1941) could be useful as probiotic dietary adjuncts.

Table 7.1.1. Antimicrobial activity of probiotic bacteria against *Aeromonas hydrophila*.

Probiotic strains	Supernatant	Neutralised supernatant (pH 6.5)	Supernatant treated with trypsin
L. acidophilus	----------Size of zones of inhibition ----------		
2400	++	-	++
2401	++	-	++
2404	+++	-	+++
2405	+++	-	+++
2409	++	-	++
2415	++	-	++
Bifidobacterium spp.			
1900	+	-	+
1901	+	-	+
1920	+	-	+
1930	+	-	+
1941	+	-	+
20097	+	-	+
20099	+	-	+
20210	+	-	+

(-) = no inhibition zone; (+) = inhibition zones of 0.7-0.8 cm; (++) = inhibition zones of 0.8-1.2 cm; (+++) = inhibition zones of 1.2-1.5 cm; (++++) = inhibition zones of 1.5-2.0 cm.

Table 7.1.2. Antimicrobial activity of probiotic bacteria against *Candida albicans*.

Probiotic strains	Supernatant	Neutralised supernatant (pH 6.5)	Supernatant treated with trypsin
L. acidophilus	----------Size of zones of inhibition ----------		
2400	+	-	+
2401	+	-	+
2404	+	-	+
2405	+	-	+
2409	+	-	+
2415	+	-	+
Bifidobacterium spp.			
1900	+++	-	+++
1901	+++	-	+++
1920	+++	-	+++
1930	++	-	++
1941	++	-	++
20097	++	-	++
20099	++	-	++
20210	++++	-	++++

(-) = no inhibition zone; (+) = inhibition zones of 0.7-0.8 cm; (++) = inhibition zones of 0.8-1.2 cm; (+++) = inhibition zones of 1.2-1.5 cm; (++++) = inhibition zones of 1.5-2.0 cm.

Table 7.1.3. Antimicrobial activity of probiotic bacteria against *Escherichia coli*.

Probiotic strains	Supernatant	Neutralised supernatant (pH 6.5)	Supernatant treated with trypsin
L. acidophilus	----------Size of zones of inhibition ----------		
2400	+	-	+
2401	+	-	+
2404	+	-	+
2405	+	-	+
2409	+	-	+
2415	+	-	+
Bifidobacterium spp.			
1900	+	-	+
1901	+	-	+
1920	+	-	+
1930	+	-	+
1941	+	-	+
20097	+	-	+
20099	+	-	+
20210	+	-	+

(-) = no inhibition zone; (+) = inhibition zones of 0.7-0.8 cm; (++) = inhibition zones of 0.8-1.2 cm; (+++) = inhibition zones of 1.2-1.5 cm; (++++) = inhibition zones of 1.5-2.0 cm.

Table. 7.1.4. Antimicrobial activity of probiotic bacteria against *Salmonella typhimurium*.

Probiotic strains	Supernatant	Neutralised supernatant (pH 6.5)	Supernatant treated with trypsin
L. acidophilus	----------Size of zones of inhibition ----------		
2400	+	-	+
2401	+	-	+
2404	+	-	+
2405	+	-	+
2409	+	-	+
2415	+	-	+
Bifidobacterium spp.			
1900	+	-	+
1901	+	-	+
1920	+	-	+
1930	+	-	+
1941	+	-	+
20097	+	-	+
20099	+	-	+
20210	+	-	+

(-) = no inhibition zone; (+) = inhibition zones of 0.7-0.8 cm; (++) = inhibition zones of 0.8-1.2 cm; (+++) = inhibition zones of 1.2-1.5 cm; (++++) = inhibition zones of 1.5-2.0 cm.

Table 7.1.5. Levels of organic acids produced by probiotic bacteria in cultures grown in MRS broth for 18 h at 37°C as determined by HPLC.

	Organic acids			
Bacterial strains	Acetic	Butyric	Lactic	Pyruvic
			µg/mL	
L. acidophilus				
2400	65	50	1850	13
2401	80	50	2100	22
2404	175	52	2670	16
2405	67	8	1480	7
2409	170	26	3276	20
2415	58	--	780	6
Bifidobacterium spp.				
1900	60	85	420	3
1901	137	50	256	3
1912	96	147	567	3
1920	240	151	234	4
1930	680	--	1576	3
1941	600	18	887	5
20097	98	75	436	4
20099	620	--	1843	5
20210	65	8	265	3

Table 7.1.6. Antimicrobial activity of organic acid (0.1%) solutions.

	Organic acids						
Pathogens	Acetic	Butyric	Hippuric	Lactic	Orotic	Propionic	Pyruvic
A. hydrophila	++	++	++	++++	++	++++	++++
C. albicans	++++	++	++	++	-	++	++
E. coli	++	++	-	++	-	++	++
S. typhimurium	++	++	-	++	-	++	++

(-) = no inhibition zone; (+) = inhibition zones of 0.7-0.8 cm; (++) = inhibition zones of 0.8-1.2 cm; (+++) = inhibition zones of 1.2-1.5 cm; (++++) = inhibition zones of 1.5-2.0 cm.

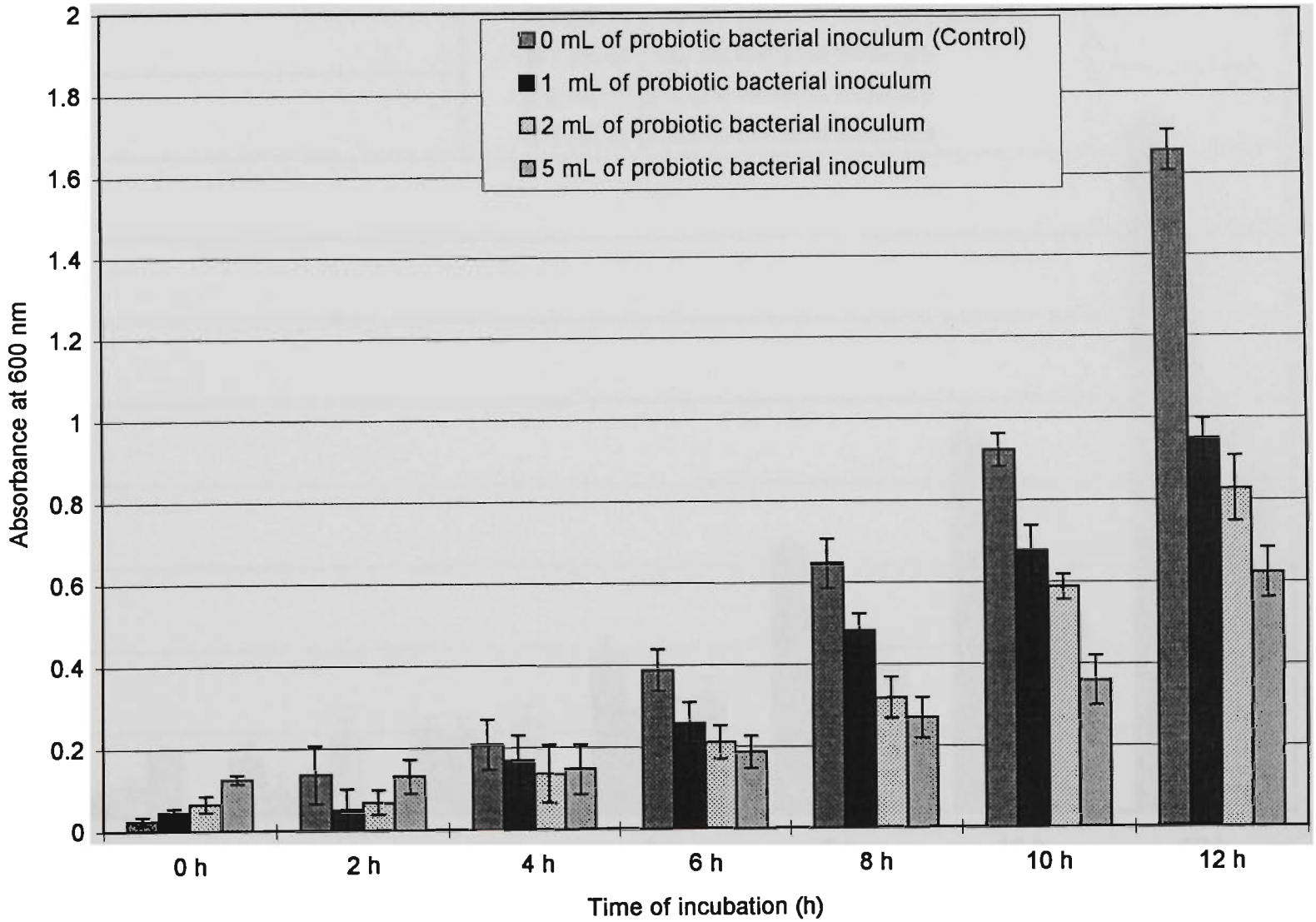


Fig. 7.1.1. Growth of *Aromonas hydrophila* in the presence of various lavels of inoculum of *L. acidophilus* 2409

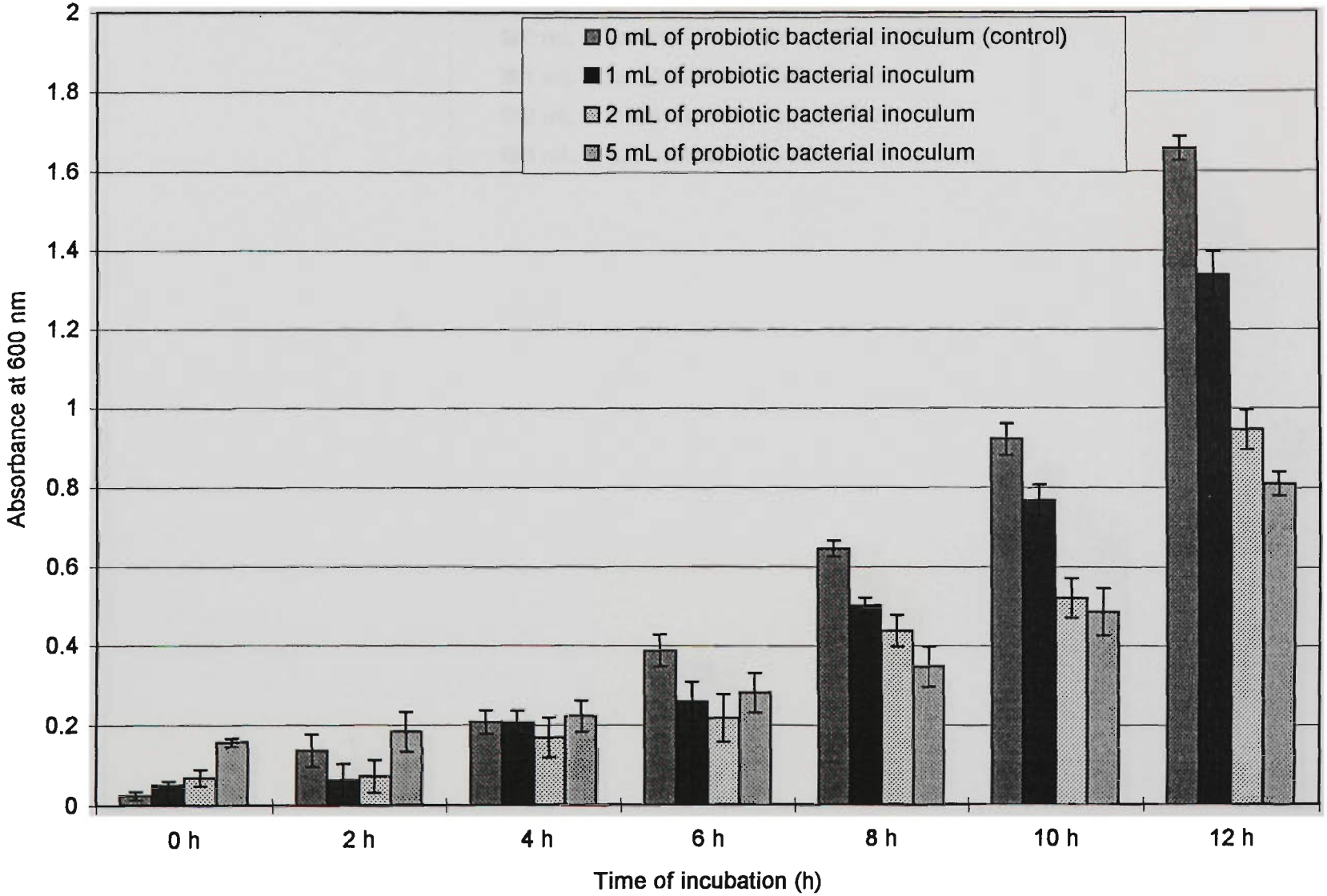


Fig. 7.1.2. Growth of *Aromonas hydrophila* in the presence of various lavels of inoculum of *B. infantis* 1912

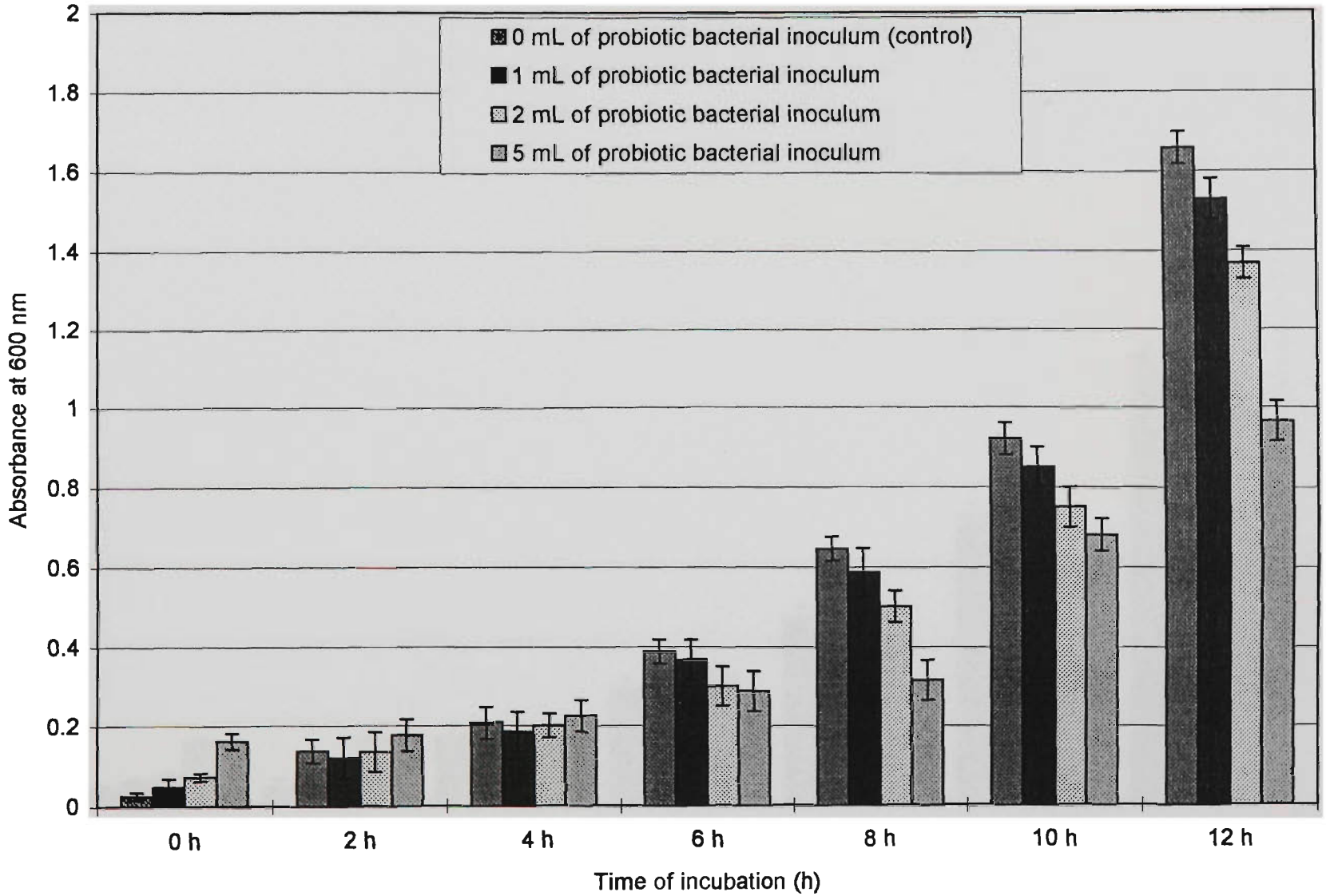


Fig. 7.1.3. Growth of *Aromonas hydrophila* in the presence of various lavels of inoculum of *B. longum* 1941

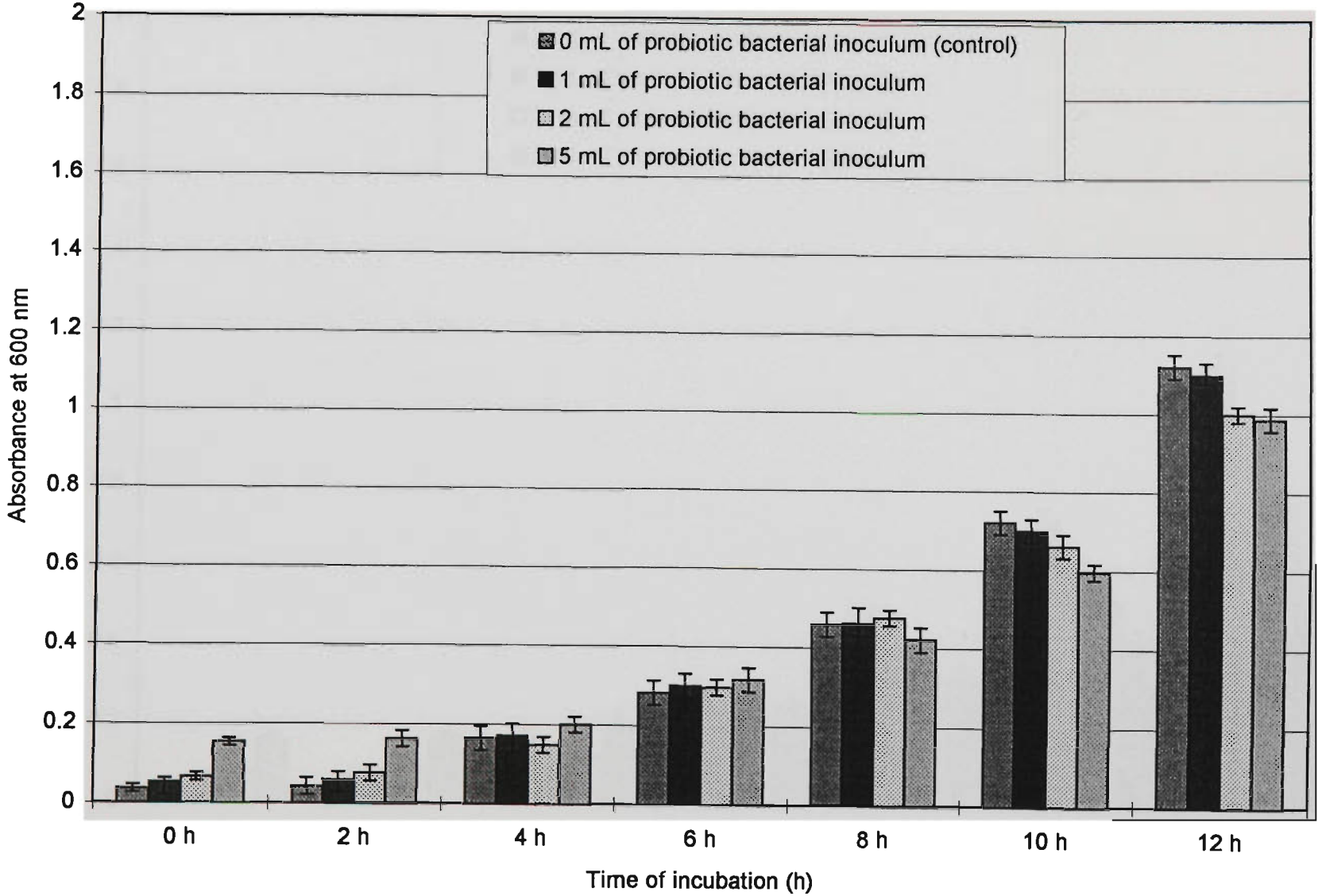


Fig. 7.1.4. Growth of *Candida albicans* in the presence of various lavels of inoculum of *L. acidophilus 2409.*

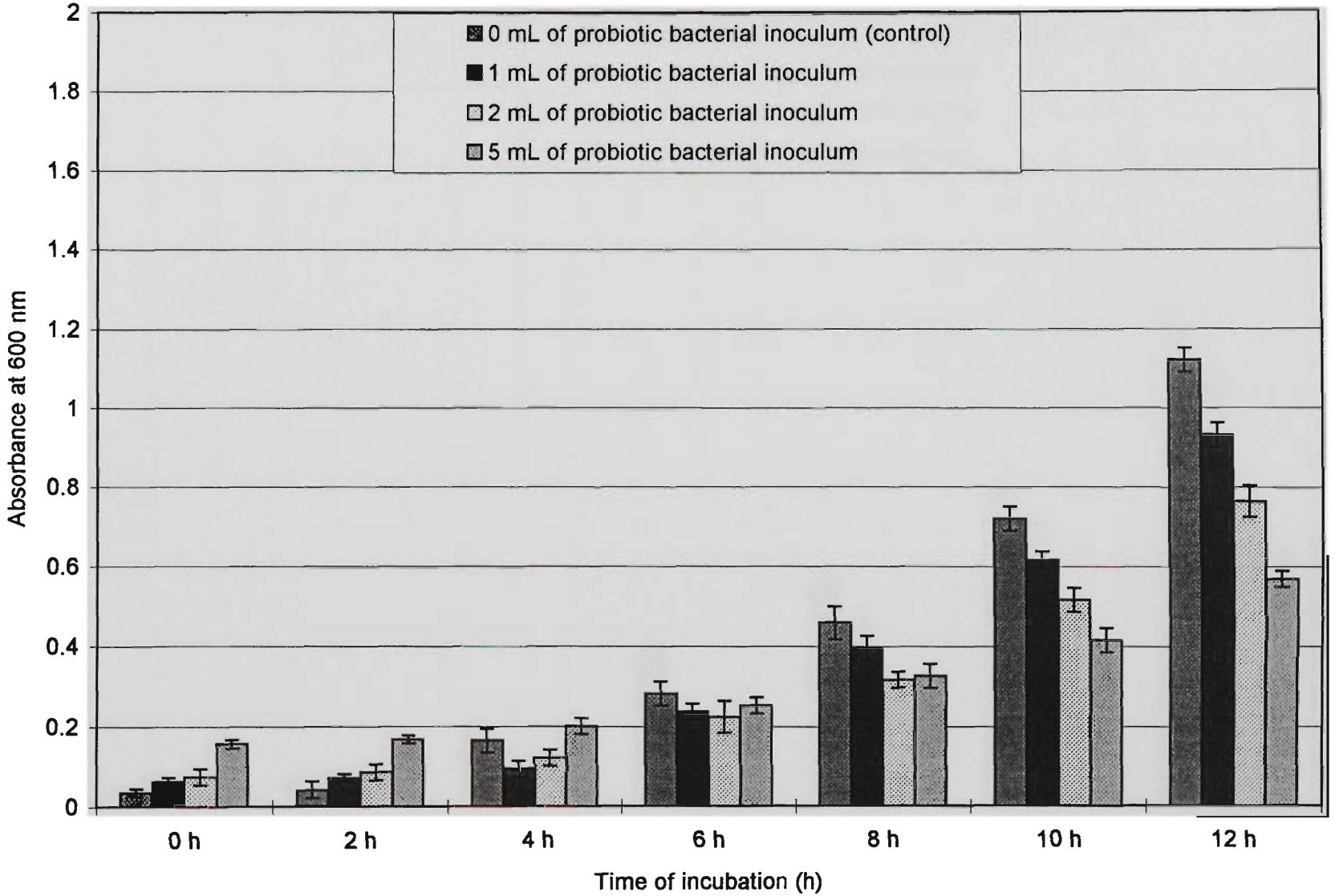


Fig. 7.1.5. Growth of *Candida albicans* in the presence of various lavels of inoculum of *B. infantis* 1912.

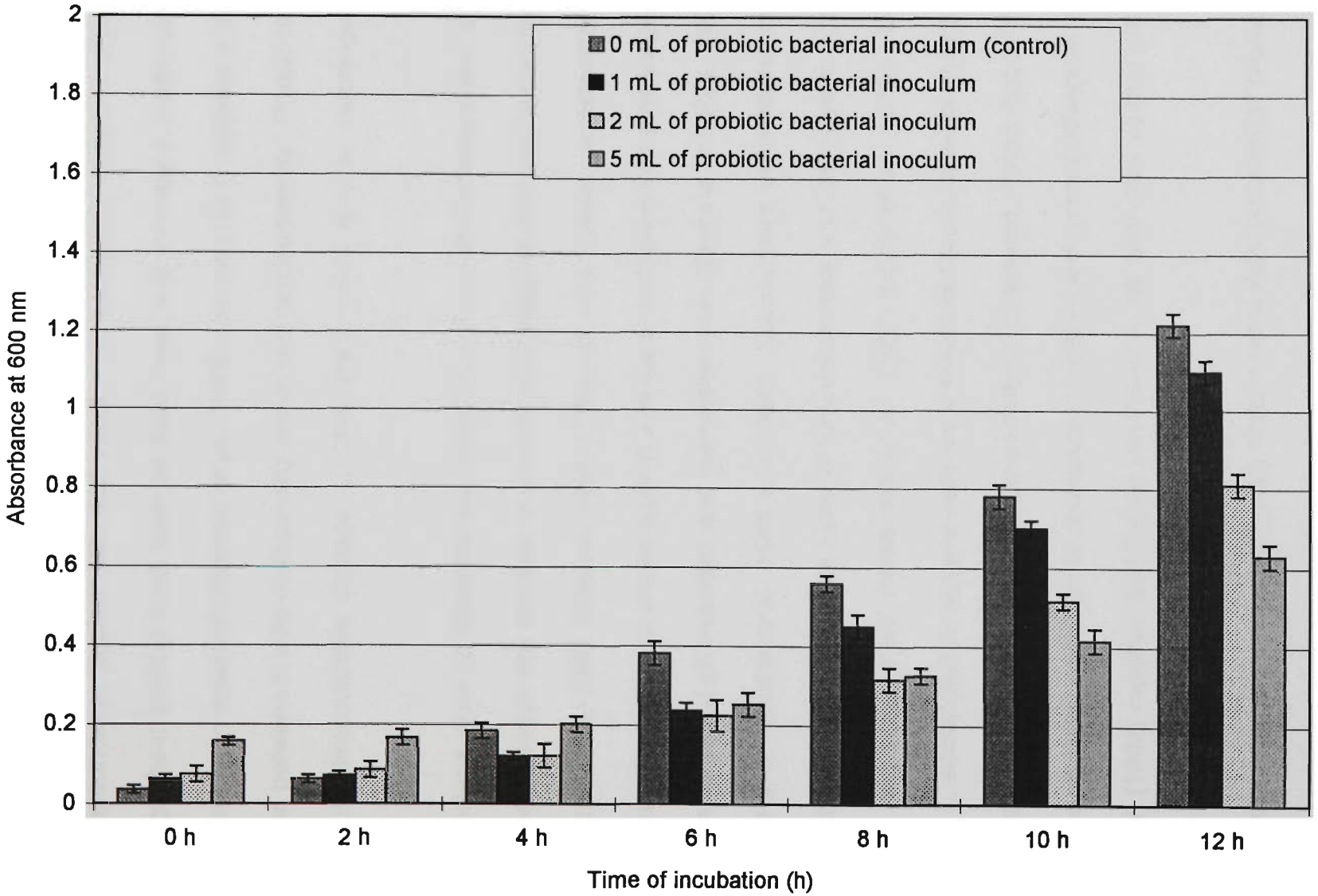


Fig. 7.1.6. Growth of *Candida albicans* in the presence of various lavels of inoculum of *B. longum* 1941.

7. 2. Antimutagenic properties of probiotic bacteria and of organic acids

7.2.1. Introduction

Studies (Sujimura *et al.*, 1977; Commoner *et al.*, 1978) have reported the presence of mutagenic compounds in broiled fish and meat. A correlation between regular consumption of high heat cooked food such as fried meat and increased incidence of colorectal cancer has been reported (Steineck *et al.*, 1993). Zeiger (1987) reported a high degree of correlation between *in vitro* mutagenicity and *in vivo* carcinogenicity. Presence of mutagens can cause irrecoverable damage to DNA which could lead to carcinogenic conditions.

Probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium* spp. and their products of fermentation are claimed to provide antimutagenic and anticarcinogenic properties (Fernandes *et al.*, 1987; Gilliland, 1991; O'Sullivan *et al.*, 1992). Antimutagenic activity of fermented milks has been shown against a range of mutagens and promutagens in various test systems based on microbial and mammalian cells (Bodana and Rao, 1990; Hosoda *et al.*, 1992 *a*, *b*; Hosono *et al.*, 1986 *a, b*; 1990). Consumption of fermented milk is reported to inhibit the growth of certain types of tumours in mice and rats (Ayebo *et al.*, 1981; 1982; Esser and Lund, 1983; Fernandes *et al.*, 1987; Reddy *et al.*, 1973). Oral supplementation of *L. acidophilus* in humans reduced activities of faecal bacterial enzymes such as β-glucuronidase, nitroreductase and azoreductase that activate procarcinogen into carcinogen and also reduced excretion of mutagens in faeces and urine (Lidbeck *et al.*, 1992). Peters *et al.* (1992) reported that yogurt was found to be protective against colon cancer.

The mechanism of antimutagenic activities of fermented dairy products or probiotic bacteria has not been clearly understood (Nadathur *et al.*, 1995). Binding of mutagens to

microbial cells has been suggested to be a possible mechanism of antimutagenicity (Orrhage *et al.*, 1995).

Acetate, propionate and butyrate are the major products of microbial fermentation in the human colon of dietary fibre and other polysaccharides such as amylose and amylopectin (Cummings, 1985; Wohin and Miller, 1983). Butyrate, a major source of energy for colonic epithelial cells (Cummings, 1981; Roediger, 1982; Cummings *et al.*, 1987) caused differentiation of mammalian and carcinoma cells at low concentration (Kruth, 1982; Tanaka *et al.*, 1990).

The objectives of this study were: (i) to evaluate antimutagenic activity of 6 strains of *L. acidophilus* and 9 strains of *Bifidobacterium* spp. against 8 potent mutagens, (ii) to determine the mechanism of antimutagenic activity of probiotic bacteria, (iii) to evaluate antimutagenic potential of killed cells of probiotic bacteria, and (iv) to determine the antimutagenic activity of organic acids usually produced by probiotic bacteria.

7.2.2. Materials and methods

7.2.2.1. Bacterial strains

Six strains of *Lactobacillus acidophilus* and 9 strains of bifidobacteria were obtained as described in section 2.2.2. Working cultures were grown in 12% reconstituted skim milk (RSM) supplemented with 1% glucose, 1% yeast extract and 0.05% L-cysteine hydrochloride.

$His^{(-)}$ mutants of *Salmonella typhimurium* TA-100 was obtained from the Victoria University Culture Collection. Stock culture of *S. typhimurium* was kept in 1 mL cryovials at -20°C. Cells were grown in Nutrient Broth II (Oxoid Australia, West Heidelberg, Australia) in the presence of 25 μg/mL of ampicillin. Prior to each mutagenicity test, *Salmonella* cells were freshly grown at 37°C for 10 to 12 h using a loopful of frozen inoculum.

7.2.2.2. Mutagens

Eight mutagens used in this study were: N-methyl, N'-nitro, N-nitrosoguanidine (MNNG); 2-nitroflourene (NF); 4-nitro-O-phenylenediamine (NPD); 4-nitroquinoline-N-oxide (NQO); Aflatoxin-B (AFTB); 2-amino-3-methyl-3H-imidazoquinoline (AMIQ); 2-amino-1-methyl-6-phenyl-imidazo (4,5-b) pyridine (AMPIP), and 2-amino-3-methyl-9H-pyrido (3,3-6) indole (AMPI). All mutagens were obtained from Sigma Chemical Company (Castle Hill, New South Wales, Australia).

7.2.2.3. Standard curves for estimating the concentration of mutagens

All mutagens were dissolved in dimethyl sulphoxide (DMSO) and their absorbence peaks were determined by scanning with a Ultrospec Plus Model 4054 uv-vis spectrophotometer (Amrad Pharmacia, Boronia, Australia). Dilutions ranging from 2 μg/mL to 50 μg/mL were used for preparing the standard curves. For all mutagens, straight line curves were obtained below 25 μg/mL. Standard curves were prepared according to the method of Maron and Ames (1983) using TA-100 mutant of *S. typhimurium* (His-) strain.

7.2.2.4. Mutagen assay

Mutagen assay was carried out using Ames Salmonella Test (Maron and Ames, 1983). *S. typhimurium* TA-100 mutant strain requiring histidine and resistant to ampicillin at a concentration of 25 μg/mL was used in the assay. Minimal mineral mix, glucose solution, and minimal agar medium for the assay were prepared according to Maron and Ames (1983).

7.2.2.5. Preparation of probiotic bacterial cells for mutagenic studies

Six strains of *L. acidophilus* and 9 strains of bifidobacteria were grown in MRS broth at 37°C for 12-15 h, and the cells were harvested by centrifuging at 5000 rpm at 4°C for 15

min using a Beckman J2-HS refrigerated centrifuge (Beckman Instruments Inc., Palo Alto, California, USA). The cell pellets were washed twice with cold sterile phosphate buffered saline (PBS), resuspended in the PBS buffer and the absorbence of the cell suspension was adjusted to 1.00 at 600 nm. The standardised bacterial cell suspensions were stored at 4°C and used within 24 h.

7.2.2.6. Preparation of killed probiotic bacterial cell suspension for mutagenic studies

The cell suspensions with absorbence value of 1.00 were heat treated in test tubes by immersing in a water bath at 100°C for 15 min. After the heat treatment, the cells were vortexed for 5 min to break any coagulum formed during heating and the cells were plated in MRS agar in order to determine the efficacy of heat treatment.

7.2.2.7. Binding of mutagens by live or killed cells of probiotic bacteria

Stock solutions of each mutagen were dissolved in DMSO to obtain a concentration of 1 mg/mL. One millilitre aliquots of the probiotic bacterial suspensions were placed in small sterile bottles in triplicate and measured quantity of each mutagen solution was added to give a final concentration of 10 μg/mL. Control samples were prepared for each mutagen in PBS without probiotic bacteria. Suspensions of each mutagen with or without probiotic bacteria were incubated at 37°C for 3 h in a shaker incubator, the suspensions centrifuged at 5000 rpm at 4°C using a refrigerated centrifuge (Beckman J2-HS) and supernatant decanted and filtered with a 0.45μ filter paper (Millipore, Australia). The filtrate was divided into 2 portions and refrigerated; one portion was used to determine the quantity of unbound mutagen by measuring the absorbence values using a UV-Vis spectrophotometer at relevant wave lengths and the other portion was used to determine the remaining mutagenic activity in the bacterial cell-mutagen suspensions using Ames test

(Maron and Ames, 1983). Antimutagenic activity of each probiotic strain against each mutagen was calculated as a percentage as compared with the control.

7.2.2.8. Recovery of mutagens from killed bacterial cells

Incubation was carried out as before. After incubation, each bacterial cell-mutagen suspension was centrifuged and the supernatant refrigerated until the concentration of each mutagen was determined. Killed bacterial cell pellets were washed twice with PBS, suspended in DMSO, vortexed for 5 min, centrifuged and the supernatants separated for determining the quantity of mutagens recovered from the killed bacterial cells.

7.2.2.7. Antimutagenicity of probiotic bacteria

Mutagenic activity of each mutagen solution, incubated with or without probiotic bacteria, was determined using Ames test (Maron and Ames, 1983). Mutagen solution incubated with live or killed probiotic bacteria was centrifuged to obtain bacterial cell free supernatant. The number of revertant colonies produced by plating the supernatant and the controls (without probiotic bacteria) was determined. The number of spontaneous revertant was determined by preparing triplicate plates as per the Ames test (Maron and Ames, 1983) without any mutagen. Fifteen to 20 spontaneous revertant colonies appeared at a concentration of $1x10^8$ CFU/mL *Salmonella* cells. The number of $His^{(+)}$ revertant colonies was counted in each plate.

7.2.2.8. Ames Salmonella test and mutagenic reaction

S. typhimurium TA-100 mutant requiring histidine was used in this study. This organism cannot form colonies in minimal nutrient agar plates without histidine. However, this mutant can revert to histidine non-requiring state by undergoing mutation in the

presence of strong mutagen. Such revertant mutant is able to grow in the absence of histidine in minimal agar plates. The number of revertant colonies in minimal agar plates increased when the concentration of mutagens is increased. However, at higher concentrations, most mutagens can be toxic to *Salmonella* cells leading to death of the cells. As a result, the number of colonies can decrease in the plates causing a sudden and abnormal change to the standard curve. Therefore, working concentration for each mutagen was selected within a range of concentration which gave a straight line standard curve.

7.2.2.9. HPLC analysis of acetic, butyric, lactic and pyruvic acids produced by probiotic bacteria

Each probiotic bacteria was grown in MRS broth for 18 h at 37°C and supernatant was separated by centrifuging at 10,000 rpm for 10 min using a Beckman J2-HS centrifuge. Four millilitre aliquots of each supernatant were pipetted into a 25 mL volumetric flask and the volume made up with 0.005 **M** H_2SO_4. Each solution was filtered through a 0.45 μM millipore filter (Millipore Australia, Lane Cove, NSW, Australia). One millilitre aliquot of the filtrate was pipetted into HPLC vials and used in the auto sampler for estimation of organic acids. Concentration of each acid was determined with a Varian HPLC (Varian, Mulgrave, Australia) using a UV-Vis detector.

7.2.2.10. Antimutagenicity of organic acids usually produced by probiotic bacteria

One percent solution each of acetic, butyric, lactic, and pyruvic acids was prepared in Milli Q (double distilled grade) water, the solution neutralised with 1 **N** NaOH to a pH of 6.5, filtered through a 0.45μm filter paper using a sterile syringe. A 200 μL aliquot of each acid solution was added to a glass sample test tube with top agar mix (containing 2 mL of 0.6% agar, 0.4% of NaCl) followed by 200 μL of 300 μg/mL solultion of each mutagen, and

100 μL aliquot of a suspension of *Salmonella* cells and allowed to stand for 30 min at 40°C before pouring the top agar mix on to minimal agar plates. A control test sample was prepared with all the ingredients except the addition of 200 μL of sterile distilled water instead of an acid solution and incubated for 30 min at 40°C before pouring the top agar mix on to minimal agar plate. The number of revertant colonies in the plates was enumerated in comparison with the control which did not contain any acid.

7.2.2.11. Determination of antimutagenicity from the bacterial counts

The number of revertant colonies increased in the agar plates with increasing concentration of mutagen within the range of concentration that produced straight line section of the standard curve. Reduction in the number of colonies on the test plates as compared with the control indicated a reduction in mutagenic activity. Percentage reduction in the number of revertant colonies in the test sample as compared with that produced by the control sample was expressed as percentage antimutagenicity.

Antimutagenicity of each bacterial cell or acid preparation was determined based on the percentage reduction in the number of revertant colonies in the presence of probiotic bacteria or acid as compared with reduction in the number of colonies in the absence of probiotic bacteria or acid preparation (control sample). Both control and test sample contained equal concentration of the mutagen and *Salmonella* cells, whereas the control sample did not contain any bacteria or acid preparation. Thus, a reduction in the number of revertant colonies indicated a decrease in mutagenicity.

7.2.3. Results and discussion

7.2.3.1. Antimutagenicity of live and killed bacterial cells

Antimutagenic activity of live or killed cells of *L. acidophilus* strains 2400, 2401, 2404, 2405, 2409 and 2415 against mutagens MNNG, NF, NPD, NQO, AFTB, AMIQ, AMPIP and AMPI is shown in Fig. 7.2.1. In general, live bacterial cells always showed higher antimutagenicity against the mutagens studied. Live cells of *L. acidophilus* 2400 showed >50% antimutagenicity against 6 of the 8 mutagens. *L. acidophilus* strains 2401, 2404, 2405 and 2409 showed common patterns of antimutagenic activity against all the mutagens. These four strains exhibited low antimutagenicity against NQO (<10%) and MNNG (<40%). In general, all *L. acidophilus* strains showed high antimutagenicity against NF and NPD, in particular *L. acidophilus* 2409 manifested high antimutagenicity (> 70%) against NF and NPD. However, *L. acidophilus* 2415 exhibited low level of antimutagenic activity against all the mutagens studied.

Figures 7.2.2 and 7.2.3 show antimutagenic activity of 9 strains of bifidobacteria against 8 mutagens. In general, live cells of bifidobacteria showed higher antimutagenic activity than killed cells as observed with *L. acidophilus*. As shown, strains of bifidobacteria exhibited different levels of antimutagenicity against the 8 mutagens. All live bifidobacteria strains exhibited high activity against NF, in particular cells of *B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. longum* 1941 (Fig. 7.2.2) and 20097 and *B. pseudolongum* 20099 (Fig. 7.2.3) showed > 90% antimutagenicity against NF. Live cells of bifidobacteria strains 1901, 1912, 1920, 1941 (Fig 7.2.2) and 20097 and 20210 (Fig 7.2.3) showed > 40% antimutagenic activities against MNNG, while *B. bifidum* 1900 and *B. breve* 1930 (Fig. 7.2.2) exhibited the lowest levels of antimutagenicity (<20%). Similarly, live cells of *B. pseudolongum* 20099 and *B. thermophilum* 20210 (Fig. 7.2.3) showed highest

antimutagenic activity (> 60%) against AFTB, whereas killed cells of these 2 strains showed very low levels (<18%) of antimutagenicity.

In the study of Nadathur *et al.* (1995) a strain of *L. acidophilus* showed 59-95% inhibition of MNNG when extracts of fermented milk was used. Cassand *et al.* (1994) reported that cultured milk containing *L. acidophilus* and bifidobacteria reduced mutagenic activity of NQO and NF by 20-60%.

7.2.3.2. Binding of the mutagens by live and killed probiotic bacteria

Figures 7.2.4-7.2.6 show binding levels of the 8 mutagens to live or killed probiotic bacterial cells and the recoverability of the bound mutagens. In general, live cells of all the strains studied showed higher levels of binding than killed cells. When the bound mutagens were extracted with DMSO, live cells showed less recovery indicating permanent binding to cells, while killed cells were unable to bind the mutagens permanently.

As shown in Fig. 7.2.4, live cells of 6 strains of *L. acidophilus* bound higher level of all the 8 mutagens as compared with the killed cells and recoverability of the mutagens from live cells was very low (< 5%). However, 80-95% of the bound mutagens were recovered from killed cells. All strains of live *L. acidophilus* except 2400 and 2405 bound MNNG at lower levels. All strains of *L. acidophilus* except 2415 bound > 70% of NF. Binding of NPD by all strains of live *L. acidophilus* ranged from 40 to 85%. AFTB was bound at high concentration (> 50%) by all strains except 2415.

In general, live cells of bifidobacteria bound higher levels of mutagens than the killed cells as with *L. acidophilus*. Higher level of mutagen was recovered from the killed cells as compared with the live cells. Live cells of *B. bifidum* 1901, *B. infantis* 1912, *B. adolescentis* 1920 and *B. longum* 1941 bound 30-50% of MNNG. All live strains bound 68-80% of NF while binding of NPD ranged from 48-70 % by all strains except *B. adolescentis*

1920. Live cells of *B. bifidum* 1900, *B. adolescentis* 1920 and *B. breve* 1930 bound 70-75% of NQO. All 6 strains of bifidobacteria (Fig. 7.2.5) bound AFTB poorly. All live strains bound high levels (48-72%) of AMIQ. Binding of AMPIP and AMPI was low for all 6 strains of bifidobacteria. *B. longum* 20097, *B. pseudolongum* 20099, and *B. thermophilum* 20210 bound 50-55% MNNG and 55-80% NF (Fig. 7.2.6). *B. longum* 20097 bound 86% and other 2 strains bound 45% of NPD. *B. longum* 20097 bound 65% of NQO. *B. thermophilum* 20210 and *B. pseudolongum* 20099 bound highest levels (45-77%) of AFTB (Figures 7.2.5 and 7.2.6). All 3 strains bound 40-60 % of AMIQ. AMPIP and AMPI were bound poorly by all strains.

Orrhage *et al.* (1995) reported that *L. acidophilus* and bifidobacteria bound AMPIP up to 50% and that binding efficiency of bacteria correlated with their antimutagenic activity. Our results also showed that the strains showing higher binding abilities to mutagens also exhibited high antimutagenic activity.

7.2.3.3. Antimutagenic activities of organic acids usually produced by probiotic bacteria

Table 7.2.1. shows the level of acetic, butyric, lactic, and pyruvic acids produced by each of the probiotic bacteria as determined by HPLC. As shown in the table, all strains produced acetic, lactic and pyruvic acids. Butyric acid was produced by all strains except *L. acidophilus* 2415, *B. breve* 1930 and *B. pseudolongum* 20099. The major products of fermentation were lactic and acetic acids which accounted for > 90% of organic acids produced. Other acids produced in small quantity were citric, hippuric, orotic and uric acid (data not reported). It was desired to study the antimutagenic activity of the major organic acids produced in order to determine the mechanism of antimutagenic activity.

Fig. 7.2.7. shows antimutagenic activity of acetic, butyric, lactic, and pyruvic acids against the 8 mutagens. Acetic acid showed higher antimutagenic activity against NQO,

NF and NPD, whereas butyric acid showed highest antimutagenic activity against all the 8 mutagens studied. Lactic and pyruvic acids showed lower antimutagenic activities against all the mutagens studied except NQO. Thus it appears that lactic acid produced by lactic acid bacteria play a minor role in antimutagenic activity.

Butyric acid is claimed to prevent carcinogenic effects at molecular (DNA) level (Smith, 1995). It has been hypothesised that the initial effect of butyrate occures on histone deacetylase, which results in a hyperacetylation of histone. The consequence of this could be a release of the bonds between DNA and histones, which results in an increase of the accessibility of DNA not only to nucleases but also to various factors involved in the control of gene expression. Yangi *et al.* (1993) reported that addition of butyric acid to a diet containing 20% margarine prevented mammary tumour formation by 7, 12-dimethylbenz(a)anthracene in rats. Our results also showed that butyric acid was a strong inhibitor of mutagenic activity of chemical mutagens studied. Thus, it appears that antimutagenic effects of probiotic bacteria may be due to both binding by bacterial cells and production of organic acids, especially butyric acid.

7.2.4. Conclusions

Strains of probiotic bacteria showed different levels of antimutagenic activity and binding of mutagens. Generally, most strains of *L. acidophilus* and bifidobacteria were effective in inhibiting NF (nitrofluorene), NDP (4-nitro-O-phenylenediamine), and AFTB (aflatoxin-B). Similarly, most strains of bifidobacteria showed antimutagenic activity against AMIQ (2-amino-3-methyl-3H-imidazoquinoline).

Live probiotic bacteria exhibited higher antimutagenic activity and greater binding of mutagens as compared with killed cells of probiotic bacteria. Binding of mutagens to probiotic bacteria appeared to be permanent for live cells and temporary for killed cells. Killed cells released bound mutagens when extracted with DMSO. The results emphasised

the importance of consuming live probiotic bacteria and of maintaining viability of these bacteria in the intestine so that efficient inhibition of mutagens can be acheived in order to provide benefit to consumers.

Acetic and butyric acids reduced mutagenicity of the mutagens studied. Butyric acid inhibited effect of all mutagens, while acetic acid showed antimutagenic effect against 3 of 8 mutagens studied. Thus, it appears that organic acids, especially butyric and acetic acids produced by probiotic bacteria contributed to the antimutagenic activity.

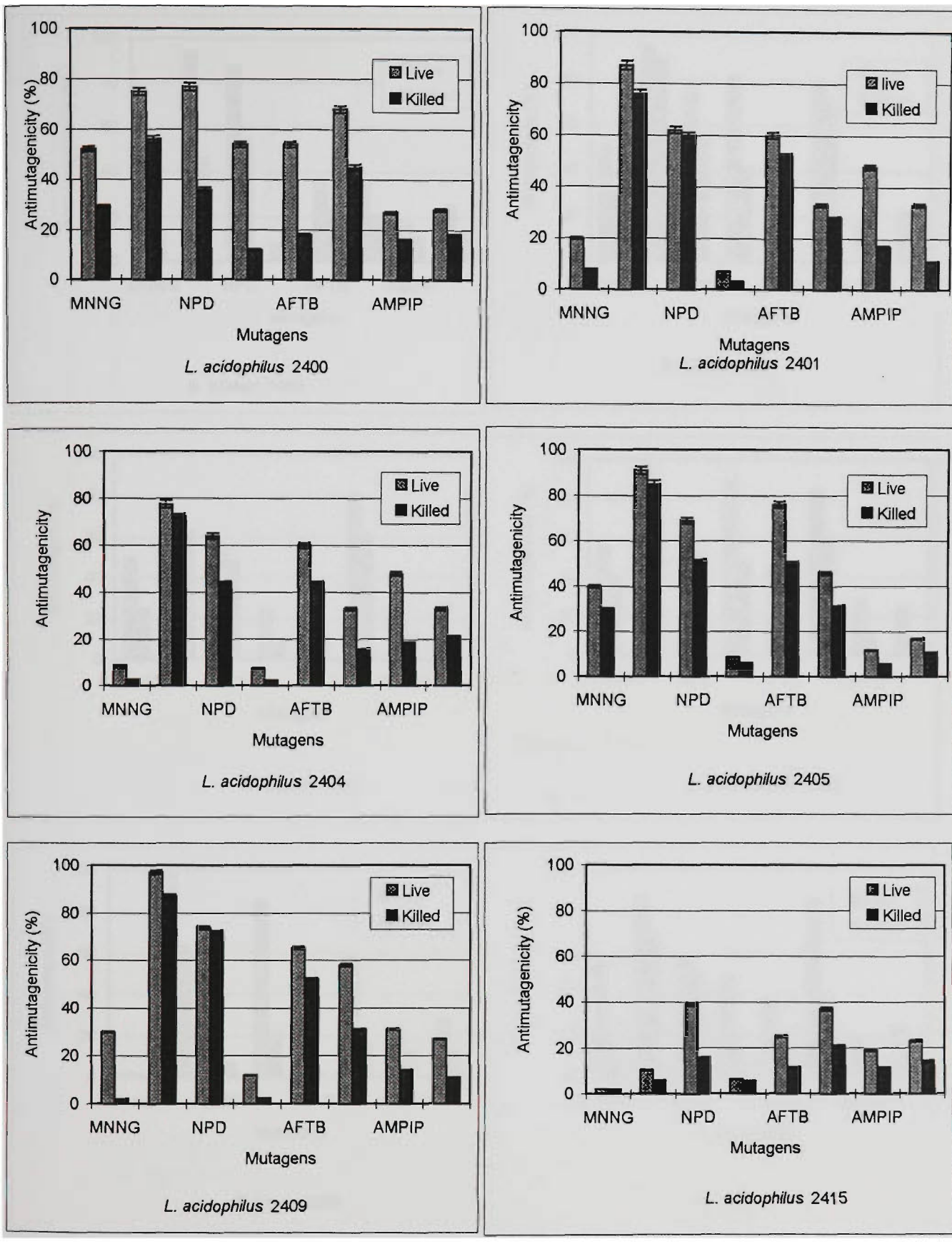


Fig. 7.2.1. Antimutagenic activity of live and killed cells of 6 strains of *L. acidophilus* as determined using Ames mutagenicity assay against 8 mutagens

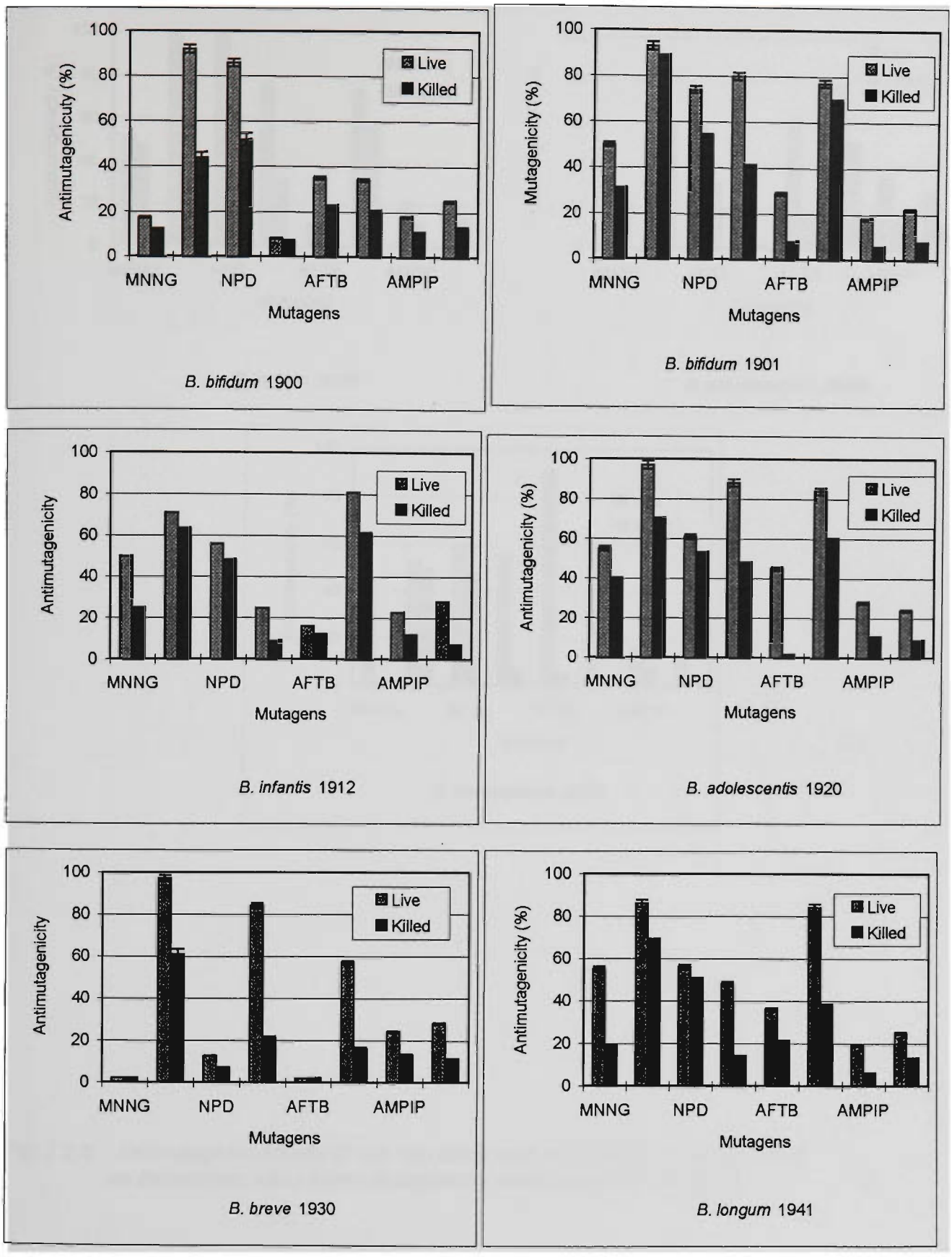


Fig. 7.2.2. Antimutagenic activity of live and killed cells of 6 strains of *bifidobacteria* as determined using Ames mutagenicity assay against 8 mutagens

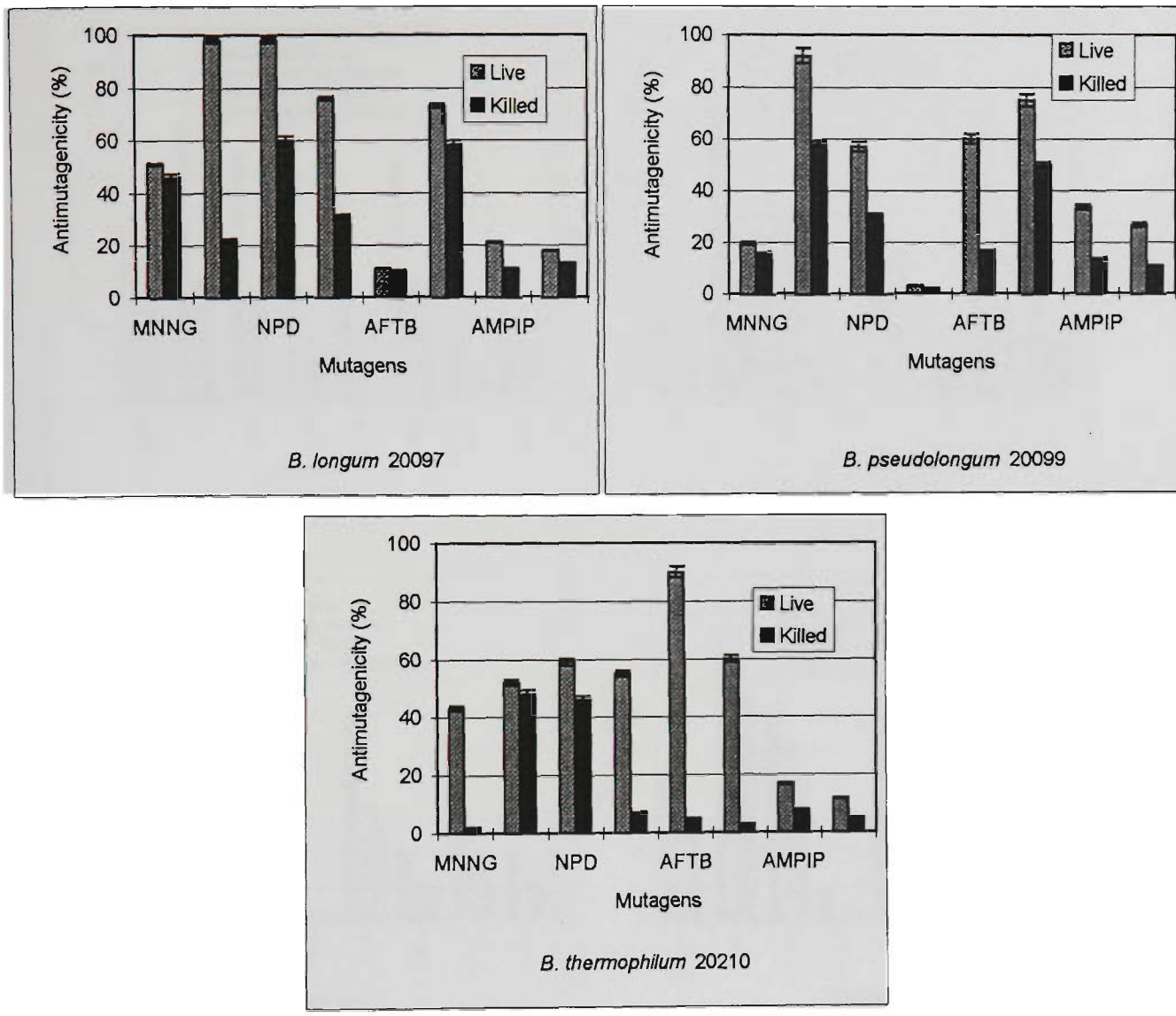


Fig. 7.2.3. Antimutagenic activity of live and killed cells of 3 strains of *bifidobacteria* as determined using Ames mutagenicity assay against 8 mutagens

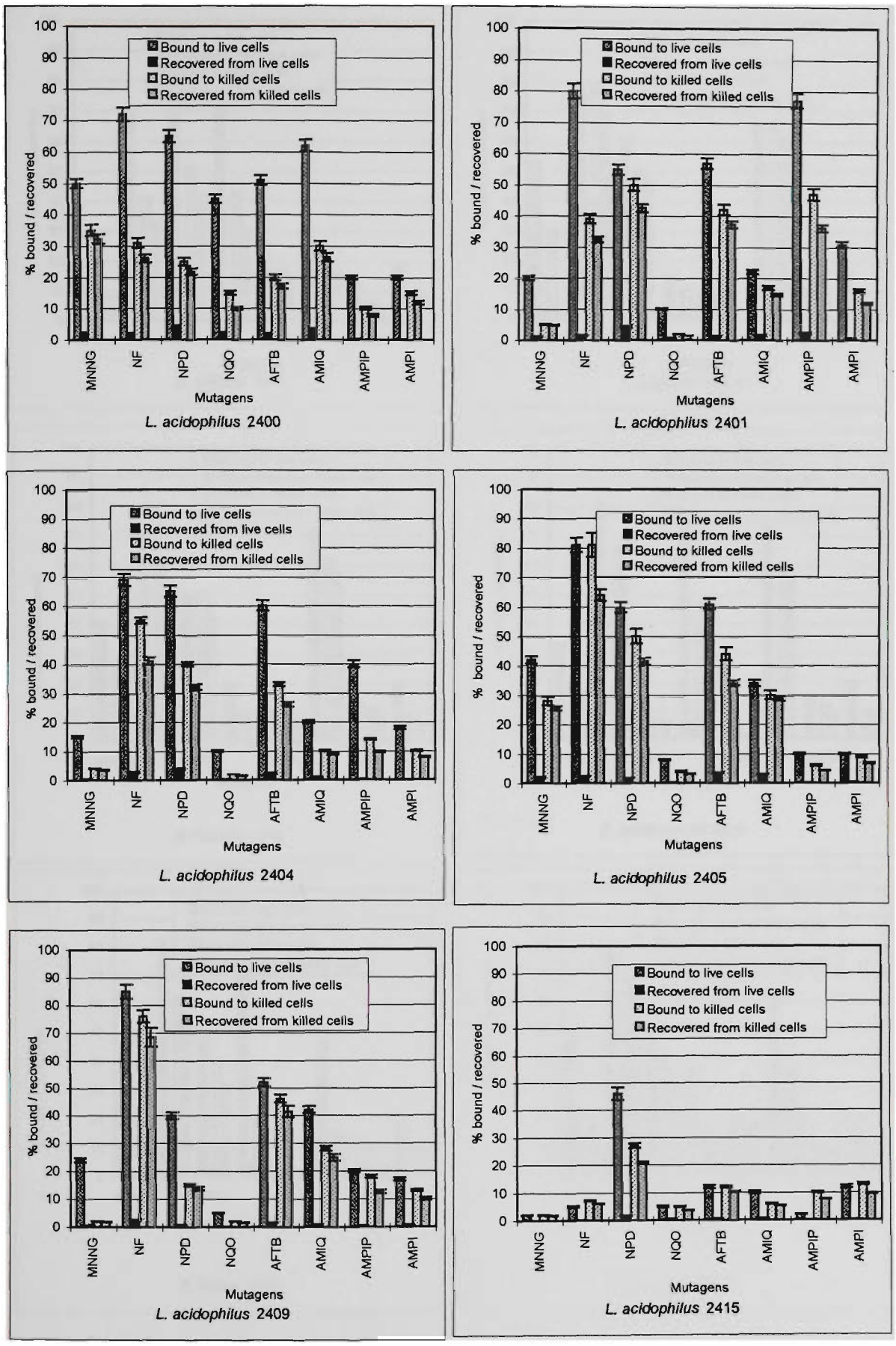


Fig. 7.2.4. Binding of mutagens to live and killed cells of 6 strains of *L. acidophilus* and subsequent recovery of the mutagens from live and killed cells

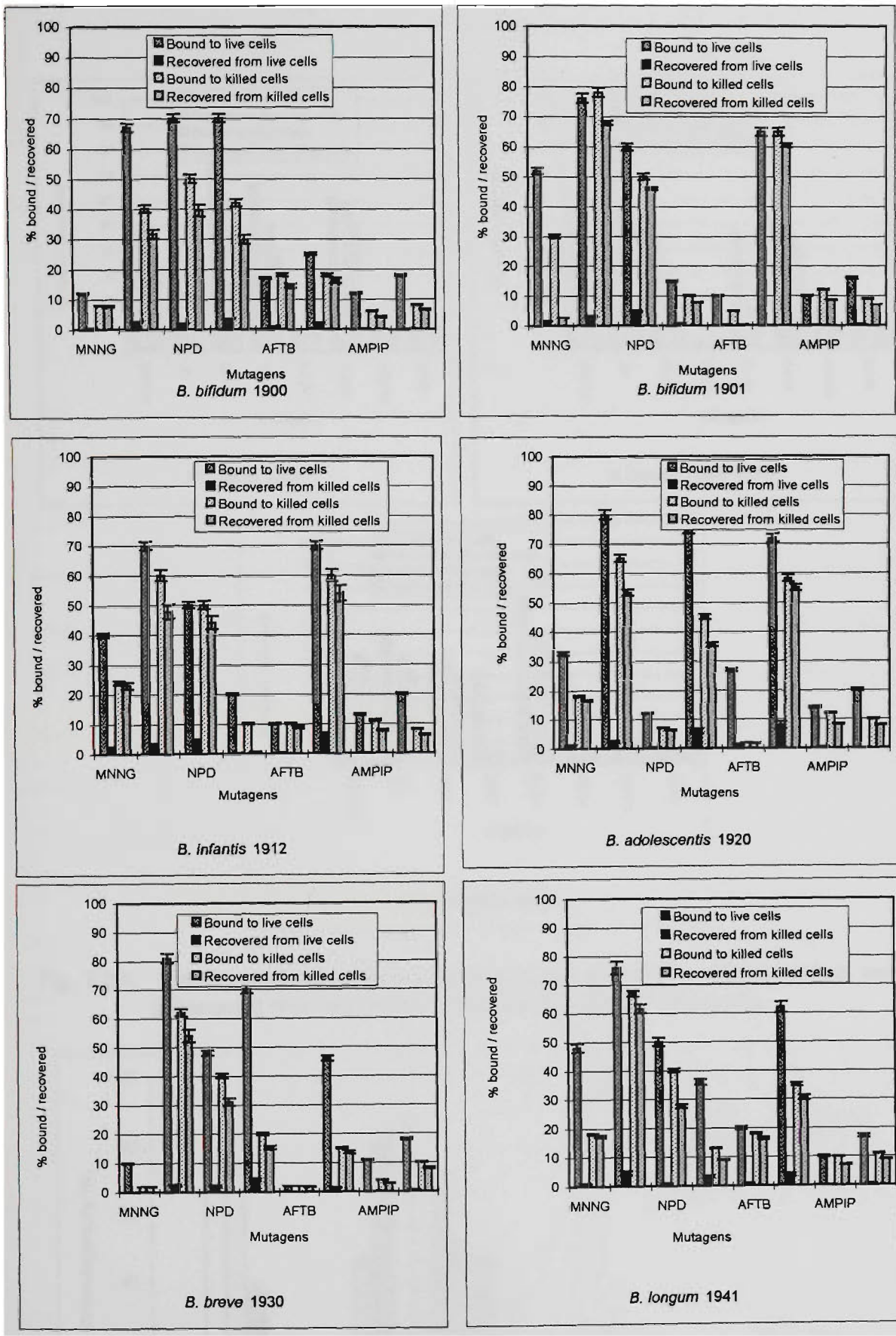


Fig. 7.2.5. Binding of mutagens to live and killed cells of 6 strains of *bifidobacteria* and subsequent recovery of the mutagens from live and killed cells

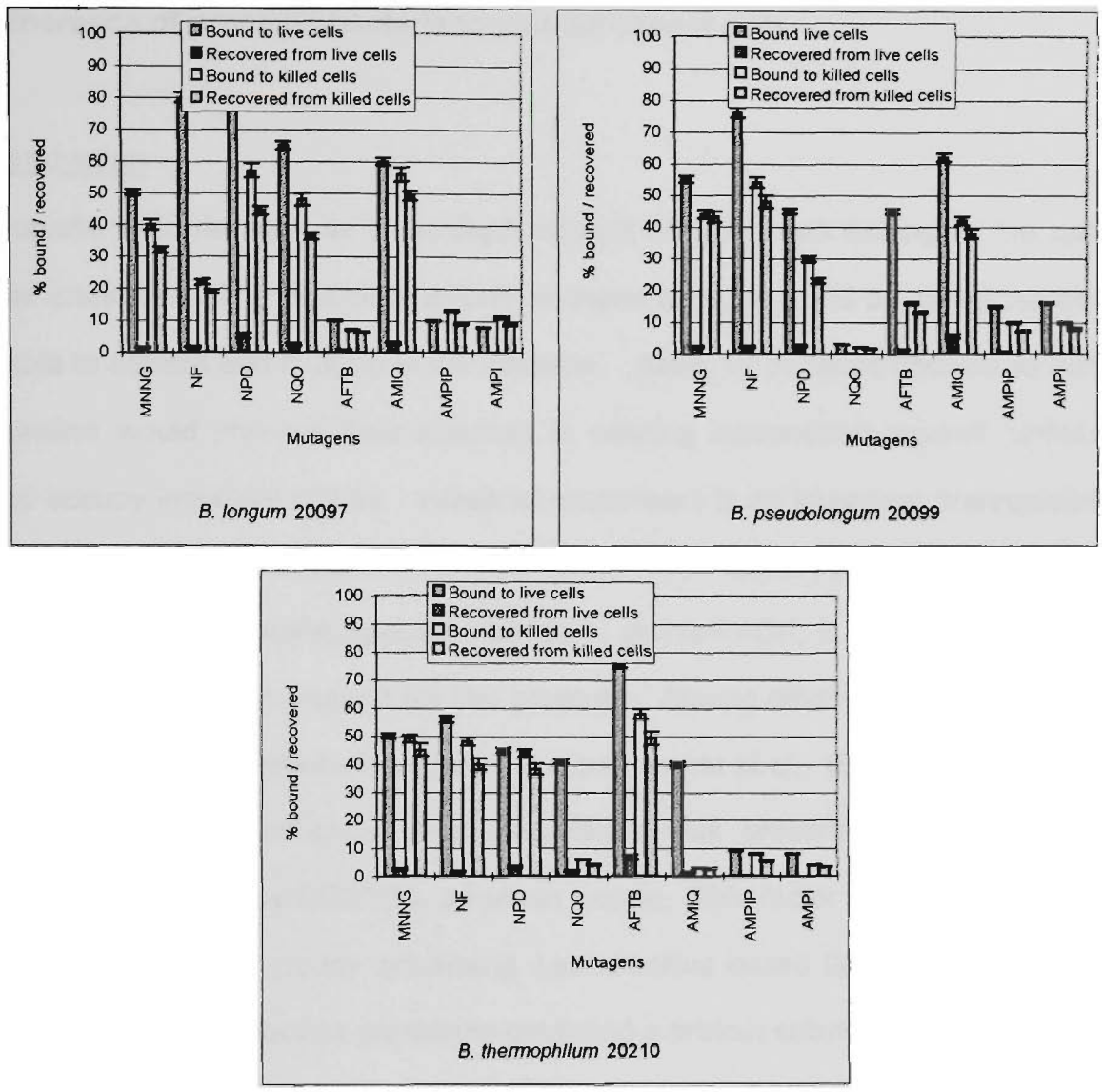


Fig. 7.2.6. Binding of mutagens to live and killed cells of 3 strains of *bifidobacteria* and subsequent recovery of the mutagens from live and killed cells

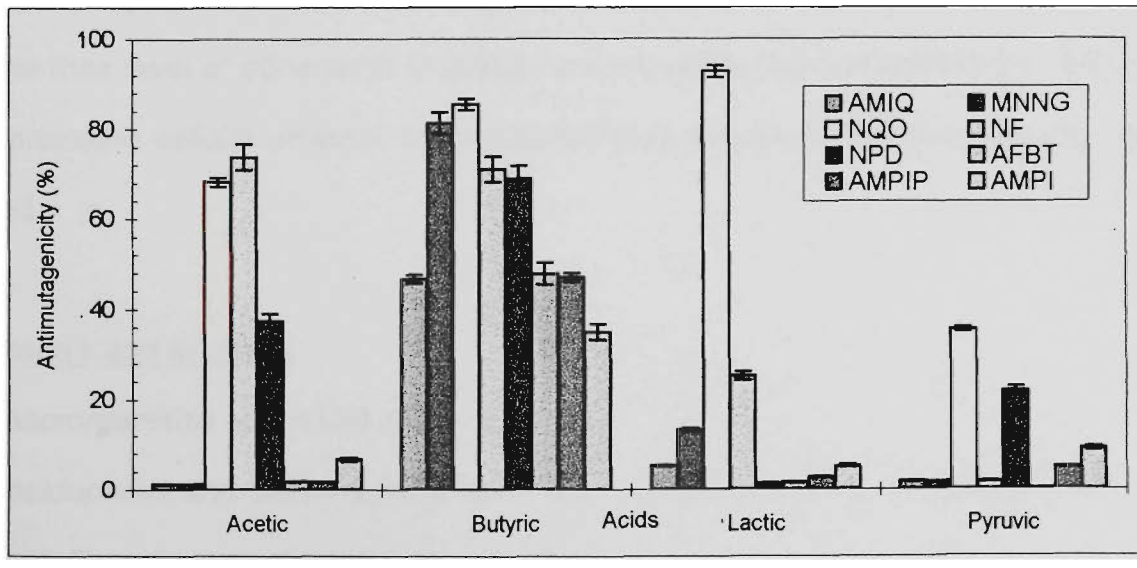


Fig. 7.2.7. Antimutagenicity of organic acids usually produced by probiotic bacteria

7.3. Adherence of probiotic bacteria to colonic cancer cells

7.3.1. Introduction

Probiotic bacteria such as *L. acidophilus* and bifidobacteria belong to the natural flora of the intestine. The desirable effects of these organisms will be produced only if they are able to adhere and multiply in the intestine. Ability of probiotic bacteria to adhere to the intestine would improve their chances in winning competition against 'unfriendly bacteria' to occupy intestinal niches. Intestinal attachment is an important prerequisite for colonisation of gastrointestinal tract (Coconnier *et al.*, 1992; Bernet *et al.*, 1993). However, thus far only a few *Lactobacillus* species such as *L. gasseri* ADH, *L. acidophilus* BG2FO4 and *L. casei* GG have been studied for this property. Among bifidobacteria, *B. breve*, *B. longum, B. bifidum* and *B. infantis* have been studied (Bernet *et al.*, 1993). Coconnier *et al.* (1992) reported that an adhesion promoting factor was present in the spent broth supernatant of *L. acidophilus* BG2FO4, a human isolate. This factor was reported to have promoted the adhesion of poorly adhereing *Lactobacillus caseii* GG. Coconnier *et al.* (1992) reported that *Lactobacillus plantarum* produced a protein substance which promoted adherence to Ht-29 cells. Mukai and Arihara (1994) reported the presence of lectin binding glycoproteins on the cell surface of *L. acidophilus.*

In this study, 9 strains of bifidobacteria and 6 strains of *L. acidophilus* were studied to determine their level of adherence to human colonic carcinoma cell line HT-29. Effect of bacterial/carcinoma cellular proteins and carbohydrates in supporting the adherence were also studied.

7.3.2. Materials and Methods

7.3.2.1. Microrganisms and Ht-29 cells

L. acidophilus and bifidobacteria were obtained as described in section 2.2.2. *C. albicans* and *E. coli* were obtained as described in section 2.2.3. Ht-29 cell line was

obtained from American Type Culture Collection (ATCC) (12301 Parklawn Drive, Rockville, Maryland 20852, USA).

7.3.2.2. Preparation of Ht-29 cells for adherence assay

Ht-29 cells were propagated in McCoy-5A medium and passaged 2 times. Monolayer cells of Ht-29 were prepared on sterile glass cover slips placed in 8 well cell culture plates as described in section 2.16.1.

7.3.2.3. Preparation of probiotic bacteria for adherence assay

Probiotic bacteria were grown in MRS broth for 18 h at 37°C. Cells were separated as described in section 2.16.3.

7.3.2.4. Light microscopic study of adherence of probiotic bacteria to Ht-29 cells

Probiotic cells were allowed to contact with Ht-29 monolayer cells and incubated for 2 h as described in section 2.16.2. After incubation the cells were washed, fixed, stained and observed microscopically as described in section 2.16.2.

7.3.2.5. Effect of bacterial and Ht-29 cellular proteins on adherence

Spent broths of bacterial cultures grown in MRS broth, bacterial pellets and Ht-29 cell monolayer cells were treated with trypsin as described in section 2.16.3. Bacterial adherence levels of trypsin treated samples were compared against the controls which were not treated with trypsin.

7.3.2.6. Effect of polysaccharides on adherence

Bacterial cells were treated with sodium periodate to remove polysaccharides from bacterial cell surfaces. Periodated treated bacterial cells and Ht-29 monolayer cells were incubated for 2 h and the level of adherence was compared with a control which was not treated with sodium periodate.

7.3.2.7. Preparation of adherence specimens for electron microscopy

Ht-29 monolayer cells grown in plastic coverslips were allowed to adhere to probiotic bacterial cultures for 2 h and washed with phosphate buffered saline (pH 7.00) 6 times. Specimens were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffered saline for 30 min at room temperature. After washing 4 times in buffer, samples were postfixed in 2% osmium tetroxide in water for 30 min at room temperature. The samples were washed in distilled water twice and dehydrated in a series of graded acetone solutions and embeded in araldite-epon resin. Blocks were polymerised at 60°C for 48 h. Semithin sections (1μ) and ultrathin sections showing gold and silver interference colours were cut using a Reichter Om U2 ultramicrotome. Semithin sections were mounted on glass microscope slides and stained with a solution of 1% methylene blue and 1% sodium tetraborate. Ultrathin sections were collected on acetone cleaned uncoated 200 mesh copper grids and stained with 5% aqueous solution of uranyl acetate for 10 min and Reynold's lead citrate for 10 min. Sections were examined with a Philips 300 transmission electron microscope at 60 kv, and at 33, 000 and 55,000 magnification.

7.3.3. Results and discussion

7.3.3.1. Level of adherence of probiotic bacteria

Table 7.3.1 shows the level of adherence of 15 strains of probiotic bacteria. Among bifidobacteria, *B. infantis* 1912, *B. adolescentis* 1920, *B. longum* 1941 and *B. thermophilum* 20210 showed high levels of adherence as compared to other strains of bifidobacteria (Table 7.3.1). Among the strains of *L. acidophilus,* 2400 and 2415 showed high level of adherence. Thus, these strains may have potential for successful colonisation in the intestine if they are able to survive the conditions encountered during passage through the GI tract. Fig. 7.3.1 shows light microscopic appearance of Ht-29 monolayer cells at confluent stage, unstained, under 10x10 magnification. Figures 7.3.2-7.3.7 show the levels of adherence of *L. acidophilus* 2400, 2409 and 2415 *B. infantis* 1912, *B. adolescentis* 1920, and *B. longum* 1941, respectively. B. infantis 1900 showed a patchy adherence,

whereas *B. infantis* 1912 and *B. longum* 1941 showed more evenly distributed adherence throughout the Ht-29 monolayer. *L. acidophilus* 2400 showed a higher levels of adherence as compared to 2415. However, as shown *L. acidophilus* 2409 showed nil or very poor adherence.

Table 7.3.1. Adherence of probiotic bacteria to Ht-29 cells as observed using 10x100 magnification. All strains of bacteria were used at a concentration of 1x 10^6/mL.

Strain/species of bacteria	No. of bacterial cells[1] adhered to Ht-29 cells
Lactobacillus acidophilus	
2400	105 ± 13
2401	12 ± 4
2404	5 ± 4
2405	4 ± 3
2409	6 ± 4
2415	380 ± 17
Bifidobacterium spp.	
1900	185 ± 5
1901	170 ± 8
1912	665 ± 15
1920	180 ± 12
1930	32 ± 7
1941	546 ± 13
20097	73 ± 14
20099	8 ± 5
20210	195 ± 17

[1]No. of cells adhered to the Ht-29 monolayer cells as observed per field area of microscope at 10 x 100 magnification.

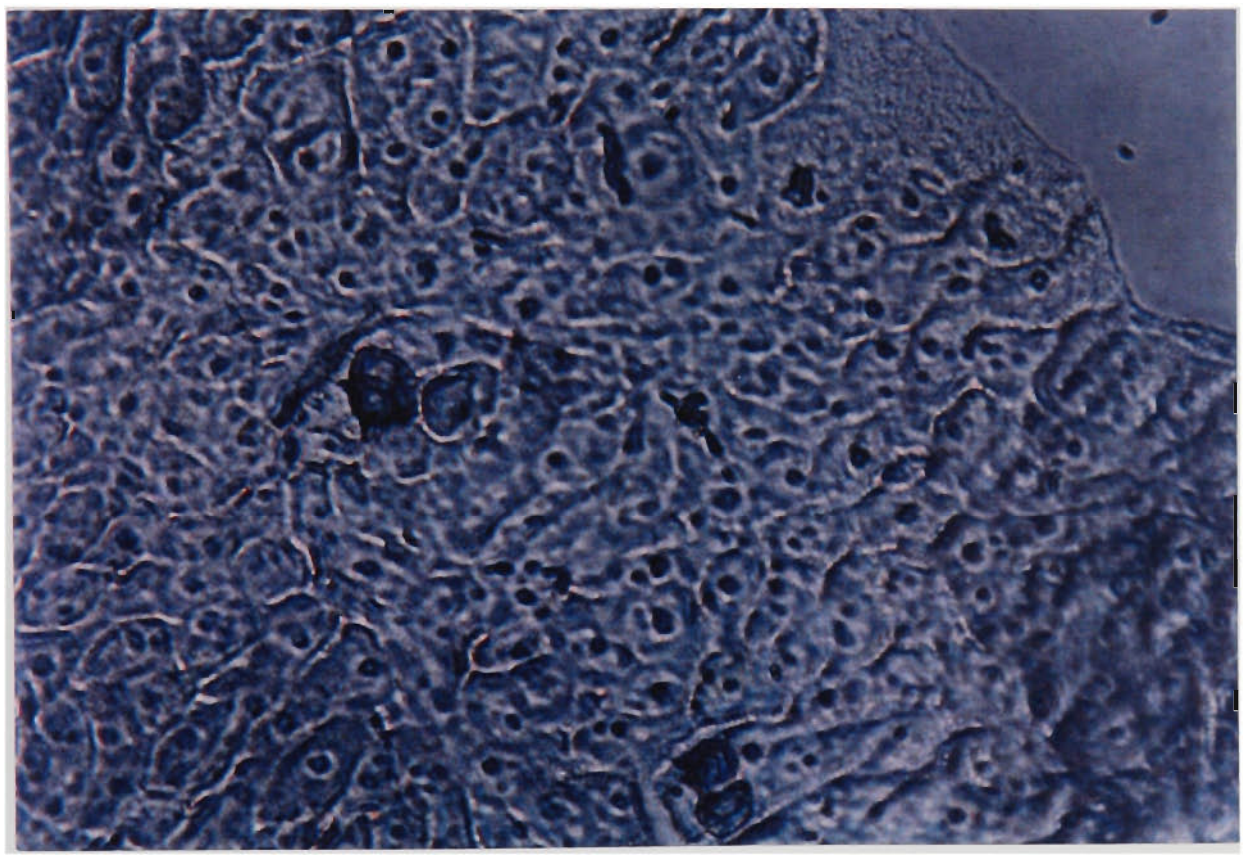


Fig 7.3.1. Unstained monolayer cells of Ht-29 cells as observed with the aid of a light microscope (magnification x 1000).

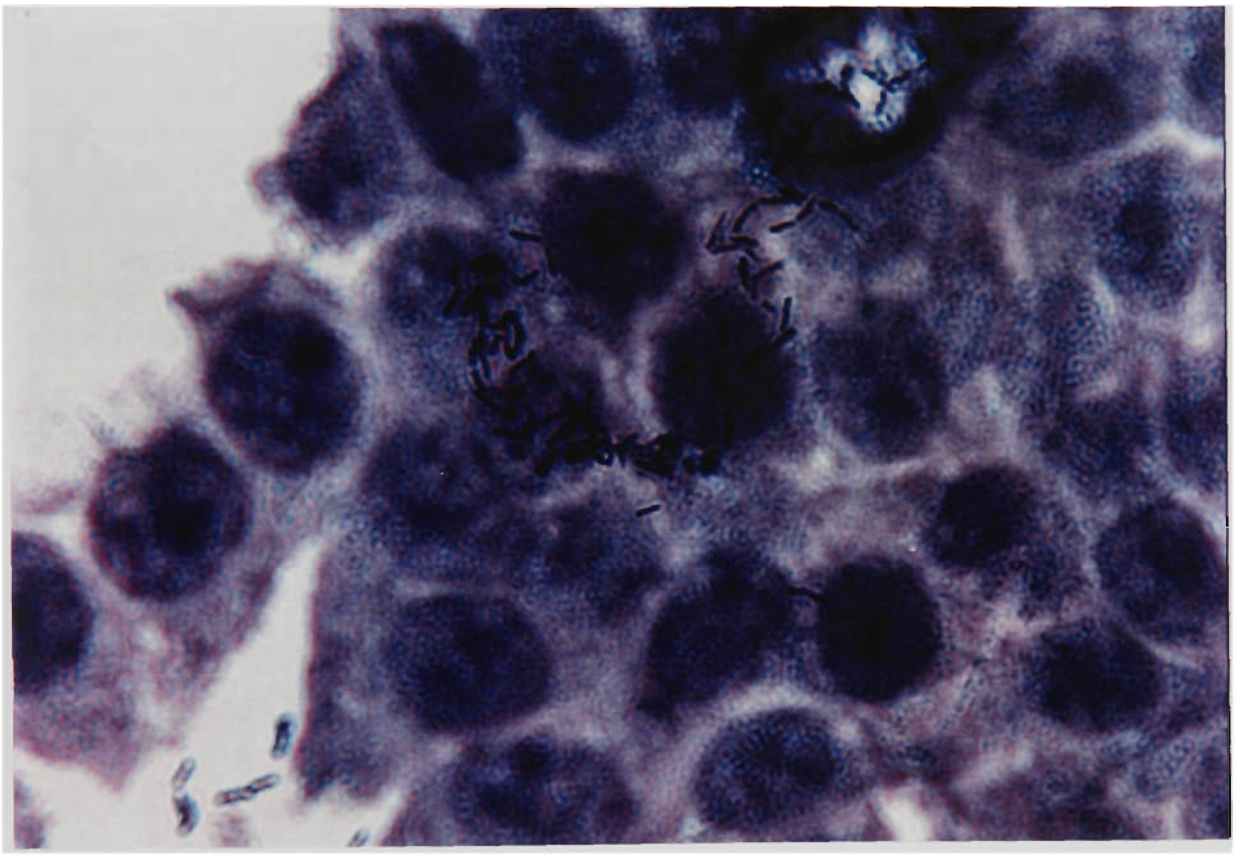


Fig 7.3.2. Adherence of *B. bifidum* 1900 to Ht-29 monolayer cells (magnification x 1000).

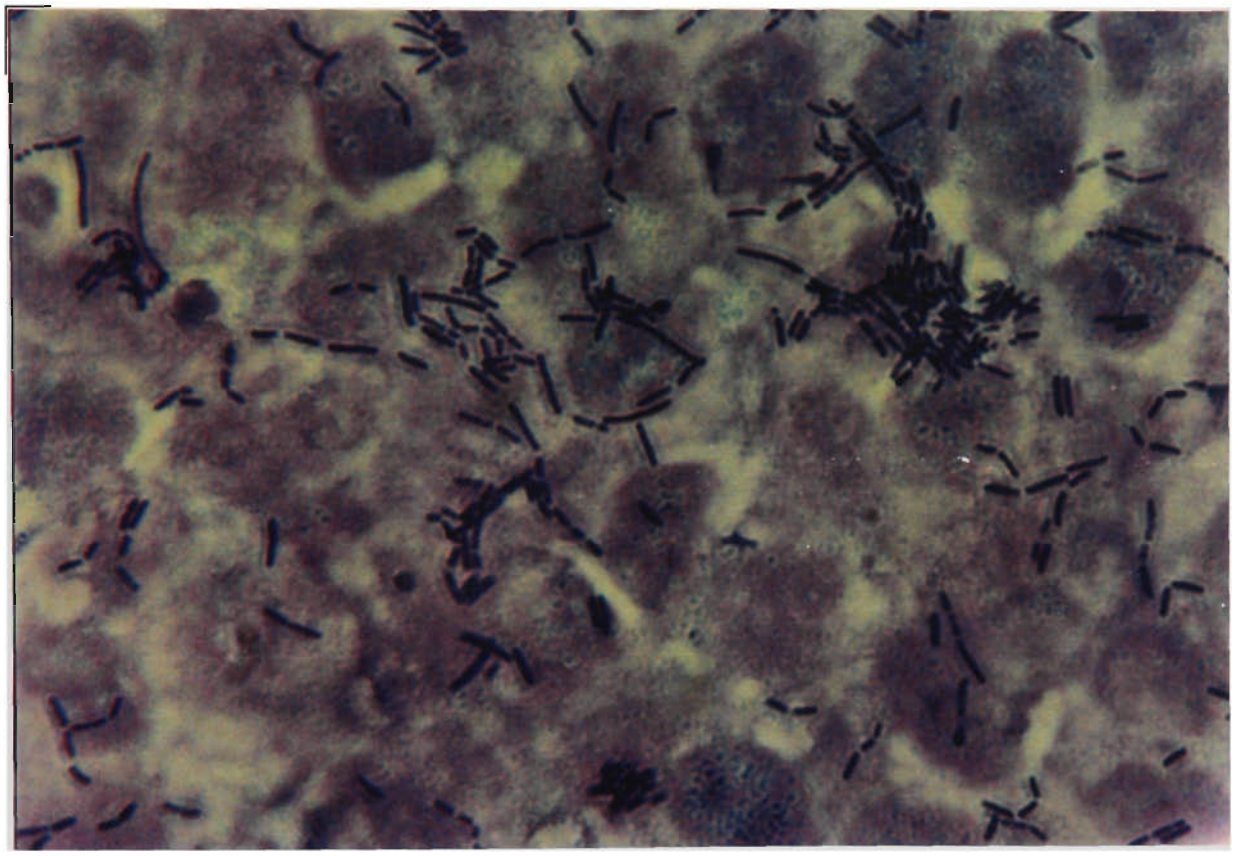


Fig. 7.3.3. Adherence of *B. infantis* 1912 to Ht-29 monolayer cells (magnification x 1000).

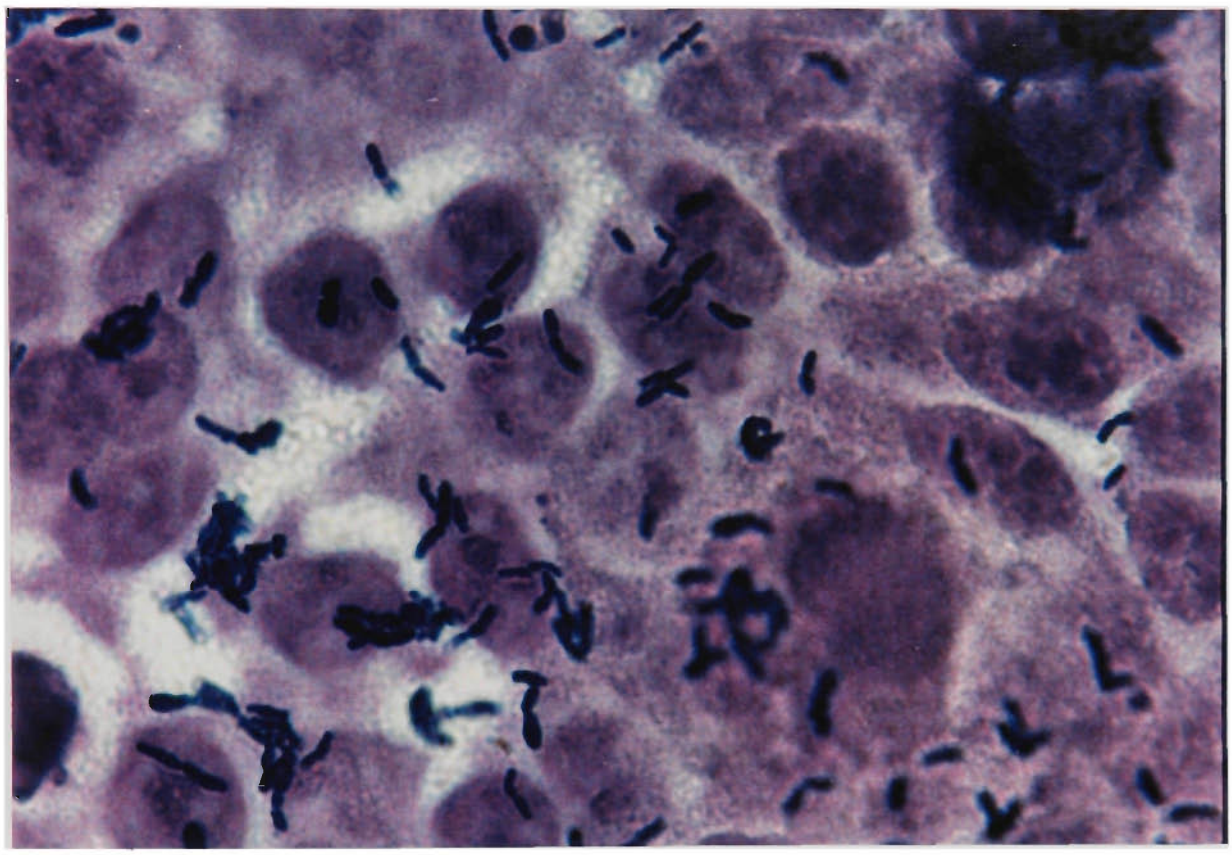


Fig 7.3.4. Adherence of *B. longim* 1941 to Ht-29 monolayer cells (magnification x 1000).

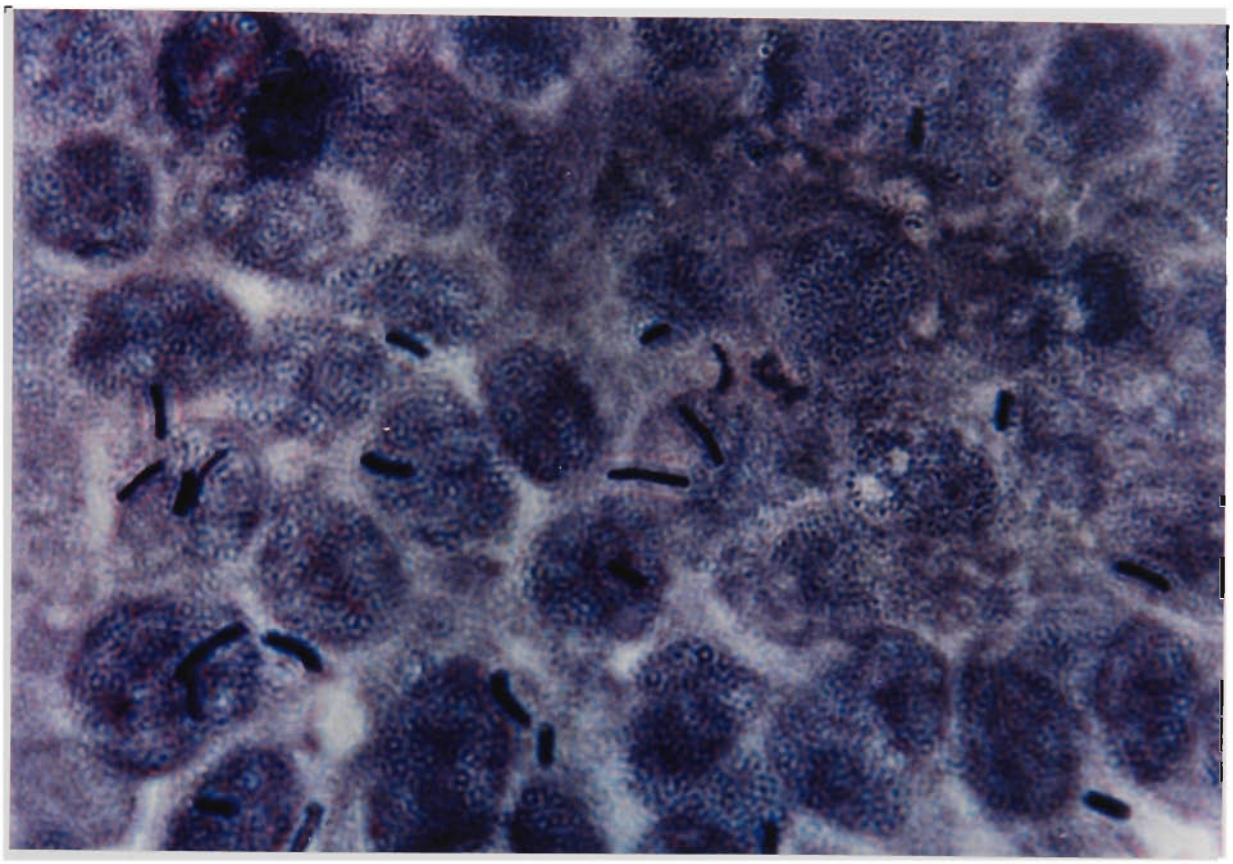


Fig. 7.3.5. Adherence of *L. acidophilus* 2400 to Ht-29 monolayer cells (magnification x 1000).

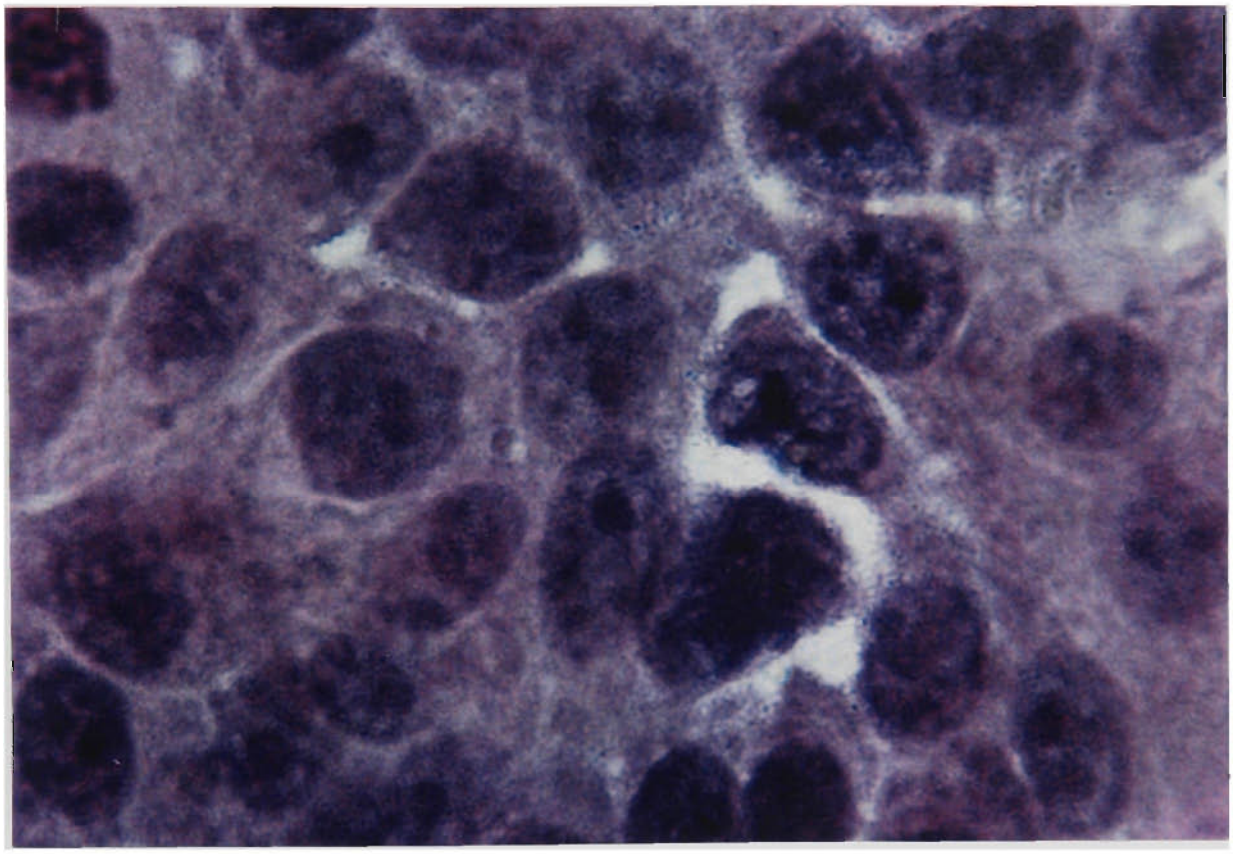


Fig. 7.3.6. Adherence of *L. acidophilus* 2409 to Ht-29 monolayer cells (magnification x 1000).

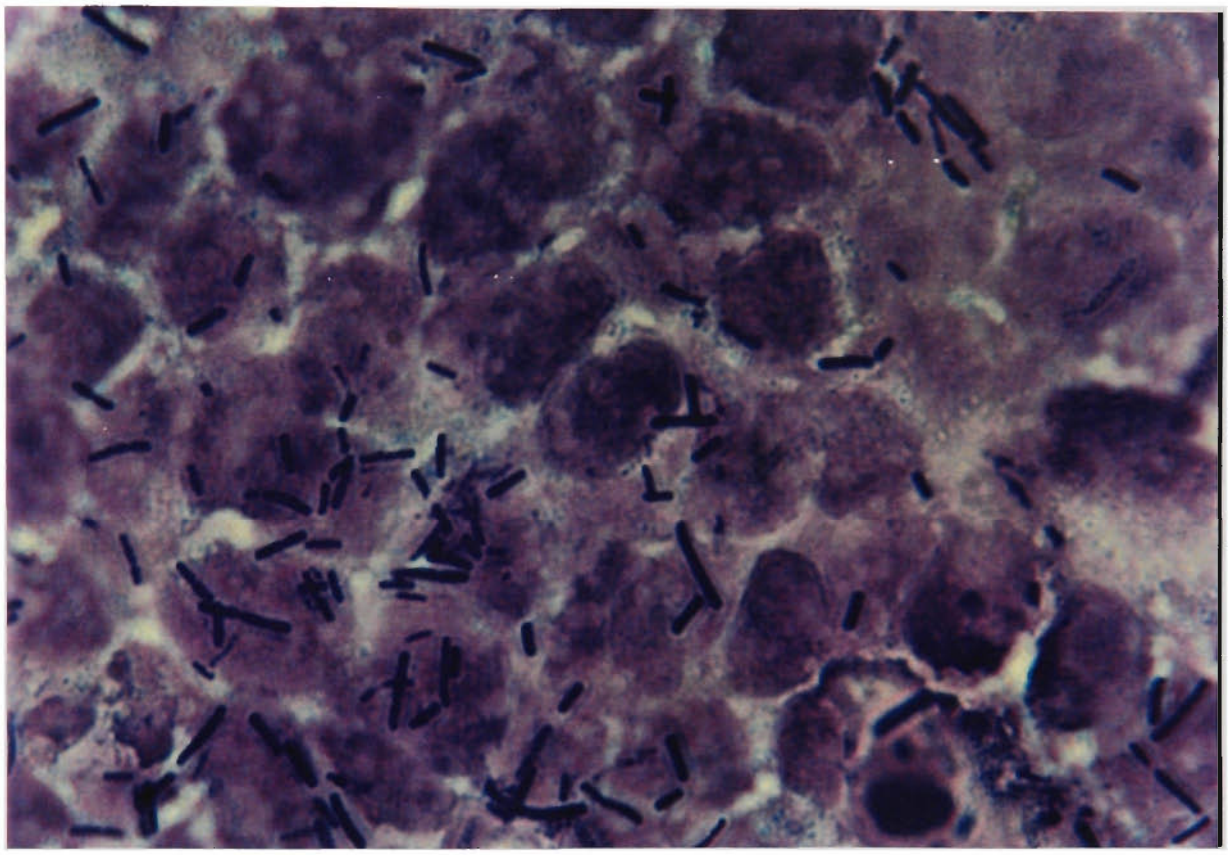


Fig. 7.3.7. Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells (magnification x 1000).

7.3.3.2. Effect of proteins and carbohydrates on adherence of probitic bacteria to Ht-29 monolayer cells

Figures 7.3.8-7.3.16 show the effect of proteins and polysaccarides in adherence of probiotic bacteria to Ht-29 cells. In this experiment, each bacterial suspension was used at a concentration of 10^8 cfu/mL. Figures 7.3.8-7.3.16 show the level of adherence of probiotic bacteria suspended in spent MRS broth (control) or probiotic bacteria treated otherwise (test samples), which were added to Ht-29 cells.

Figures 7.3.8 A-E show the adherence of *B. bifidum* 1900 after treatments[1] (a), (b), (c), (d), and (e), respectively. Sample (b) showed similar level of patchy adherence as compared with the control (a). This suggests that any component liberated into the spent broth during growth of *B. bifidum* 1900 culture would not have been responsible for adherence because sample (b) prepared with bacterial cells suspended in fresh MRS broth also showed similar level of adherence. When the spent broth and bacterial cells were treated with trypsin (Fig. 7.3.8 C, sample c), bacteria showed very little adherence. This could be due to the destruction of protein or polypeptide substances in bacterial cell walls responsible for adherence. When bacterial cells were treated with periodate to remove polysaccharides (Fig. 7.3.8 D, sample d), the pattern of adherence or the number of adhereing cells did not reduce as compared to control (sample a). This suggests that polysaccharide type substances of bacterial origin may not be involved in adherence. When Ht-29 monolayer cells were treated with periodate to remove polysaccharides from cell surfaces, the number of adhereing cells of *B. bifidum* 1900 reduced by 50% or more, which suggests that polysaccharide substances of Ht-29 monolayer cells play an important role in adherence of *B. bifidum* 1900.

[1] Samples for each bacterial strain is described as follows: (a) adherence after adding bacterial cells (suspended in untreated spent broth in which the bacterial cultures were grown for 18 h) to Ht-29 cells (control sample); (b) adherence after adding bacterial cells (suspended in fresh sterile MRS broth) to Ht-29 cells; (c) adherence after adding bacterial cells (suspended in trypsin treated spent broth in which the bacterial cultures were grown for 18 h added to Ht-29 cells broth) to Ht-29 cells; (d) adherence after adding bacterial cells (suspended in periodate treated spent broth in which the bacterial cultures were grown for 18 h) to Ht-29 cells; (e) adherence after adding bacterial cells (suspended in untreated spent broth in which the bacterial cultures were grown for 18 h) to Ht-29 cells treated with periodate.

B. bifidum 1901 also showed a similar pattern of response to the treatments (Figures 7.3.9 A-E). Fig. 7.3.9 C (sample c) showed the involvement of bacterial cell wall protein in adherence because trypsin treated bacterial cells showed nil adherence.

Figures 7.3.10 A-E show the response in adherence of *B. infantis* 1912 to various treatments. As shown in Fig. 7.3.10 A (control), *B. infantis* 1912 showed evenly spreaded distribution of adhereing cells all over the monolayer cells of Ht-29 cells. However, when bacterial cells were suspended in fresh MRS broth and applied on to the monolayer cells, there was >50% reduction in the number of adhereing cells, while higher numbers of cells were limited to patches of the monolayer cells as compared to evenly distributed bacterial cells in sample a (Fig. 7.3.10 A). Fig. 7.3.10 C shows the reduction of adherence of *B. infantis* 1912 when treated with trypsin. When bacterial cell suspension in spent broth was treated with periodate, the number of adhering bacteria reduced similar to sample b. More reduction in adhereing bacteria was observed when Ht-29 monolayer cells were treated with periodate (Fig. 7.3.10 E). These observations suggest that a protein or polypeptide substance present in cell walls of bacteria as well as in the spent broth involved in adhesion of these bacteria. In addition to the protein or polypeptide type substances of bacterial cell walls and the spent broth, polysaccharides in bacteria as well as Ht-29 monolayer cells seemed to involve in adherence. Such multi-factor involvement in adherence of *B. infantis* 1912 can be substantiated by high level of adherence of these bacteria to Ht-29 monolayer cells as compared with the other probiotic strains studied.

B. longum 1941 (Figures 7.3.11 A-E) also exhibited observations similar to *B. infantis* 1912. These observations suggest that a protein or polypeptide substance present in cell wall of bacteria as well as in the spent broth involved in the adhering of these bacteria. In addition to the protein or polypeptide substances of bacterial cell walls and the spent broth, polysaccharides in bacteria as well as in Ht-29 monolayer cells seemed to involve in adherence. *B. longum* 1941 also showed high frequency of adherence to Ht-29 monolayer cells similar to *B. infantis* 1912.

B. thermophilum 20210 (Figures 7.3.12 A-E) showed adhereing patterns similar to *B. bifidum* 1900. However, one specific observation of these bacteria was concentration of adhering bacteria to intracellular gap areas of Ht-29 monolayer cells.

L. acidophilus 2400 (Figures 7.3.13 A-E) also showed a pattern of adherence similar to *B. thermophilum* 20210. Adherence of *L. acidophilus* 2415 is illustrated in Figures 7.3.14 A-E. *L. acidophilus* 2415 cells suspended in untreated spent broth (control) showed higher adherence as compared to *L. acidophilus* 2400 cells suspended in untreated spent broth. When cells were suspended in fresh MRS broth, adherence of bacteria to Ht-29 monolayer cells reduced by about 90% (Fig. 7.3.14 B). Trypsin treatment also gave nil adherence (Fig. 7.3.14 C). Treatment of bacterial cells with periodate did not seem to affect the level of adherence (Fig. 7.3.14 D). However, treatment of Ht-29 monolayer cells with periodate seemed to reduce the number of adhereing cells by about 80-90% (Fig 7.3.14 E). These observations prove that a protein or polypeptide substance in spent broth was directly involved in adherence of *L. acidophilus* 2415. Further, involvement of bacterial polysaccharides was not shown in adherence. However, polysaccharides of Ht-29 monolayer cells seemed to be important in the adherence of *L. acidophilus* 2415..

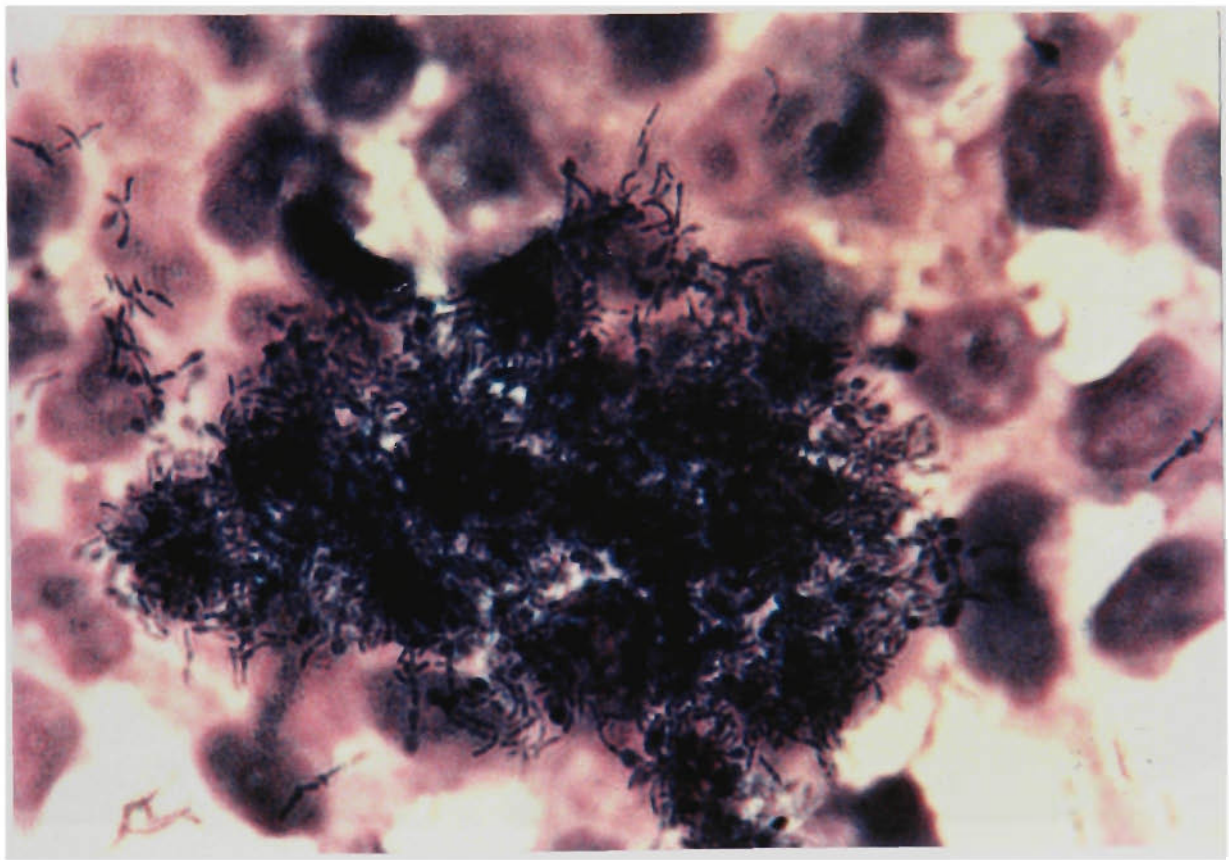


Fig. 7.3.8. A Adherence of *B. bifidum* 1900 to Ht-29 monolayer cells when added with untreated spent broth (magnification x 1000).

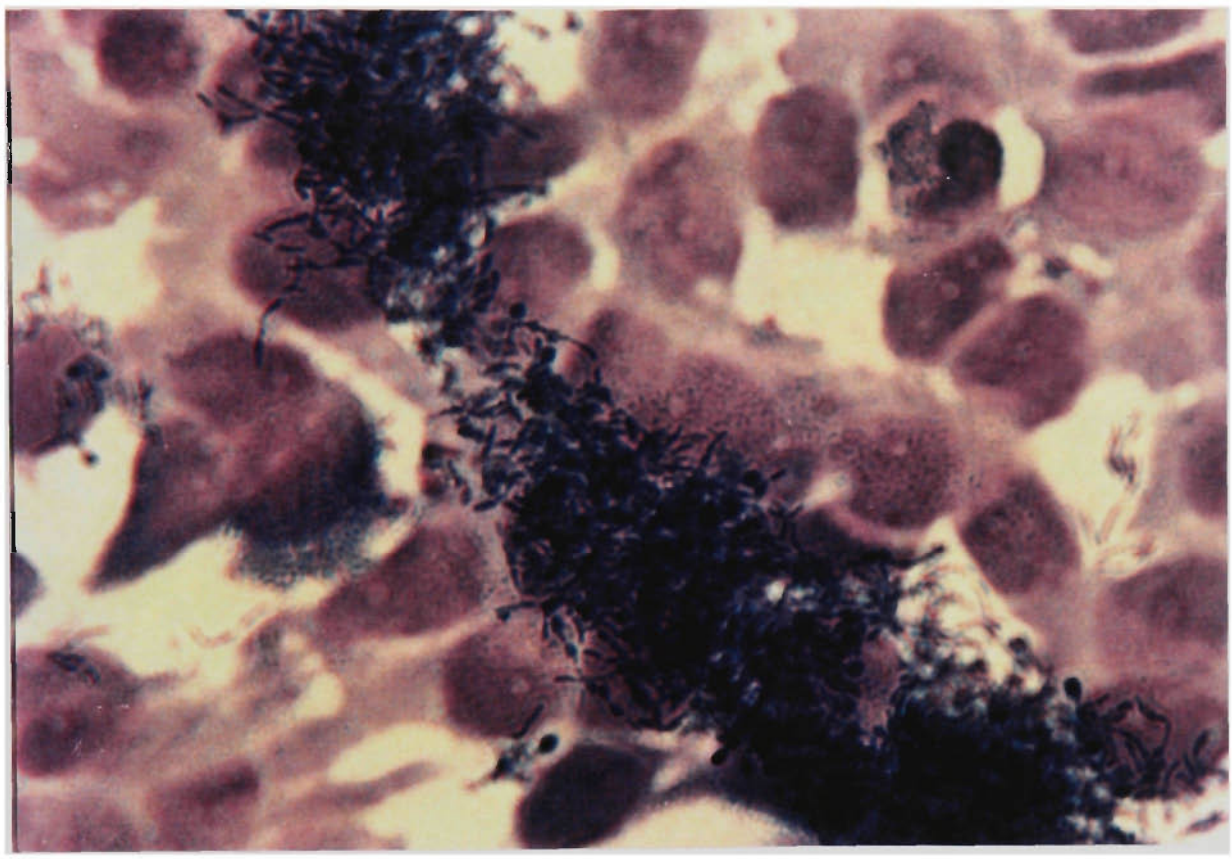


Fig. 7.3.8. B Adherence of *B. bifidum* 1900 to Ht-29 monolayer cells when added with fresh MRS broth (magnification x 1000).

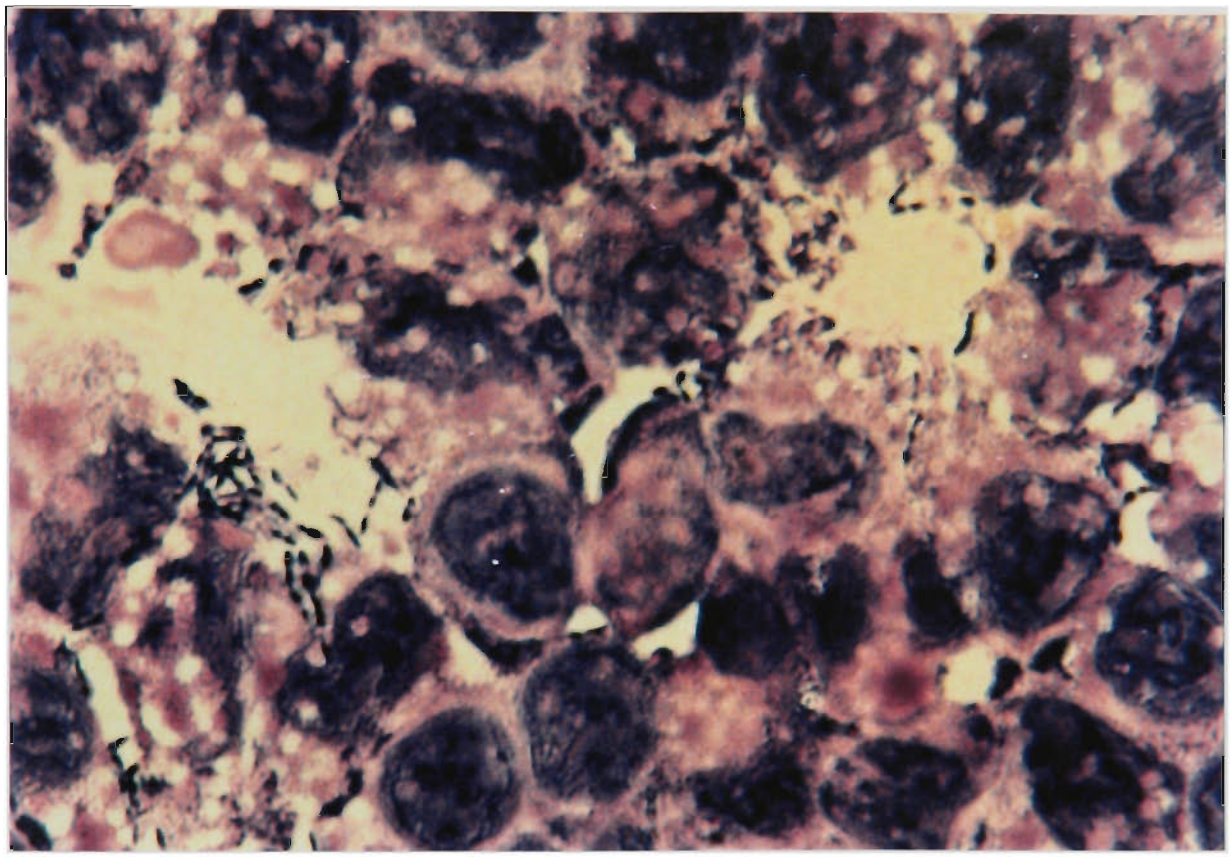


Fig. 7.3.8. C Adherence of *B. bifidum* 1900 to Ht-29 monolayer cells when added with trypsin treated spent broth (magnification x 1000).

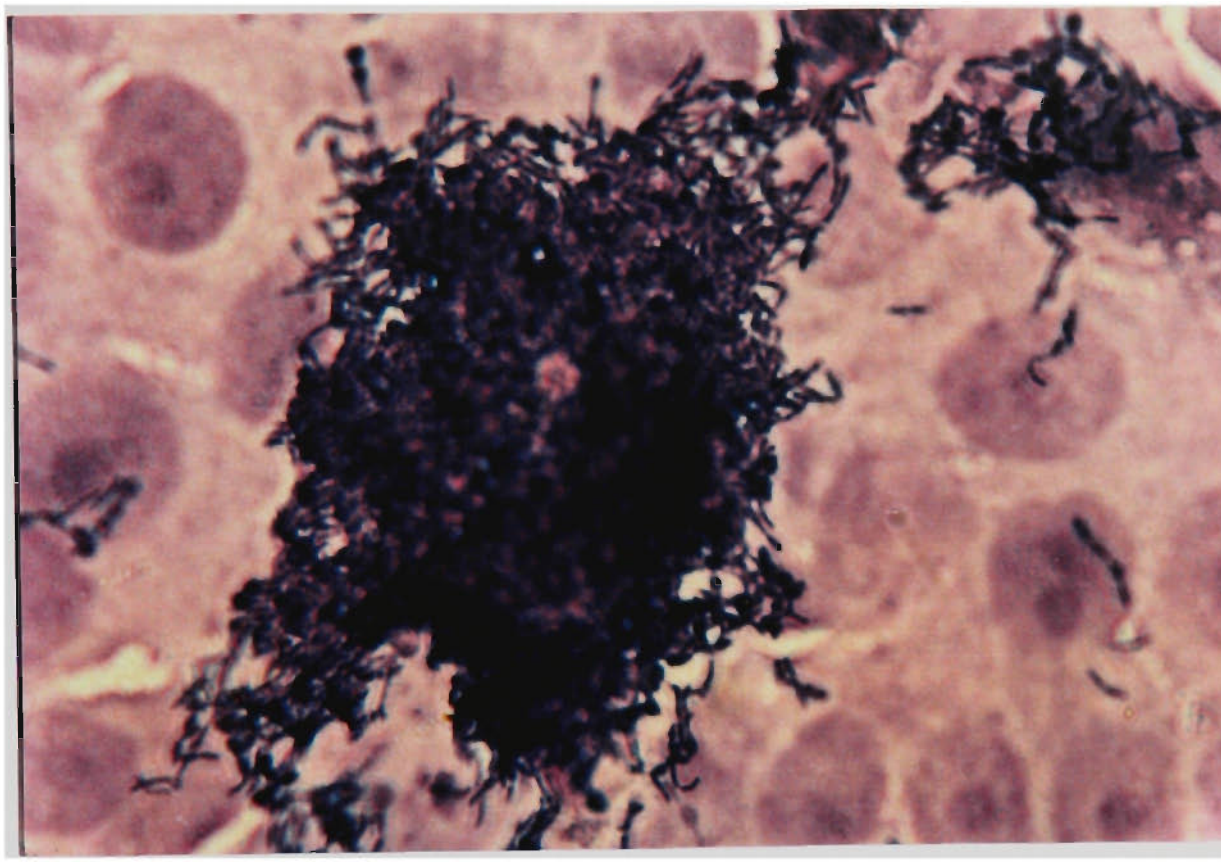


Fig 7.3.8. D Adherence of *B. bifidum* 1900 to Ht-29 monolayer cells when added with spent broth treated with periodate (magnification x 1000).

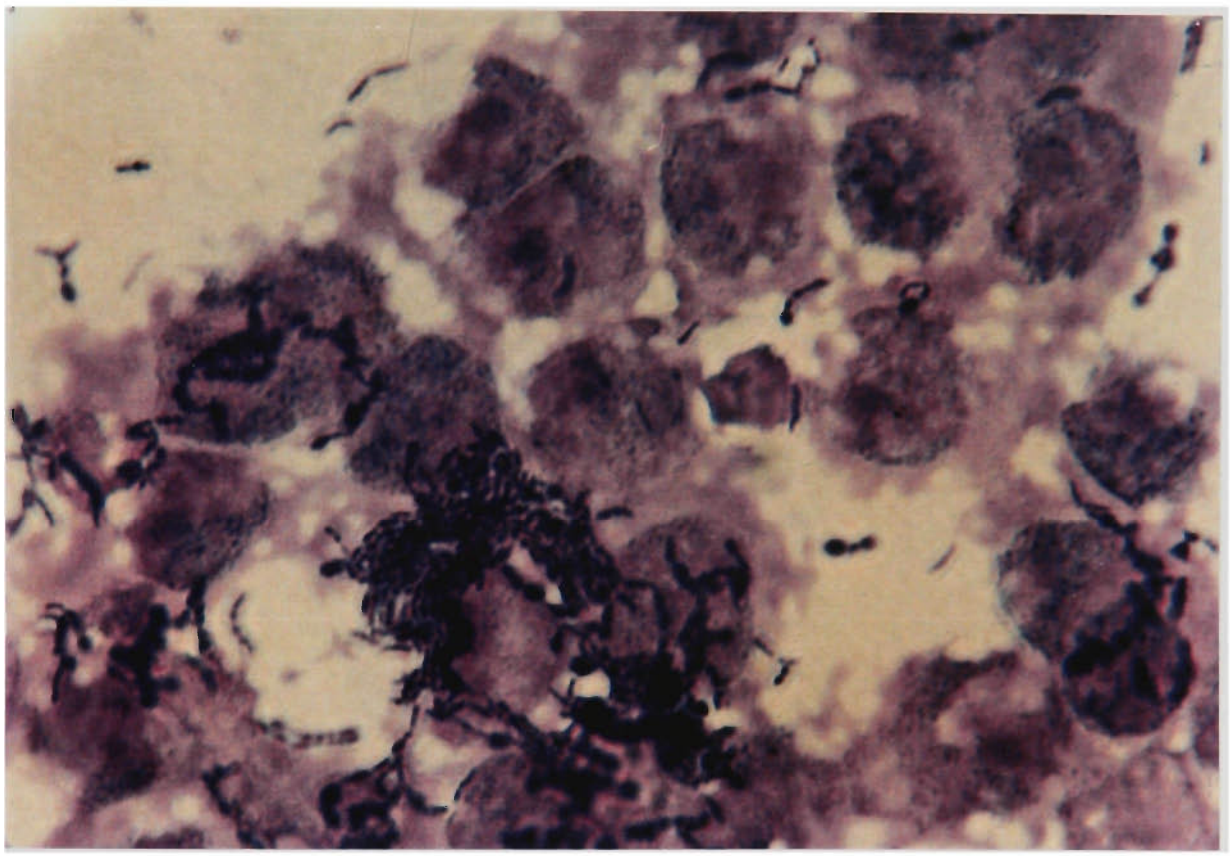


Fig. 7.3.8. E Adherence of *B. bifidum* 1900 to Ht-29 monolayer cells when added with untreated spent broth Ht-29 monolayer cells treated with periodate (magnification x 1000).

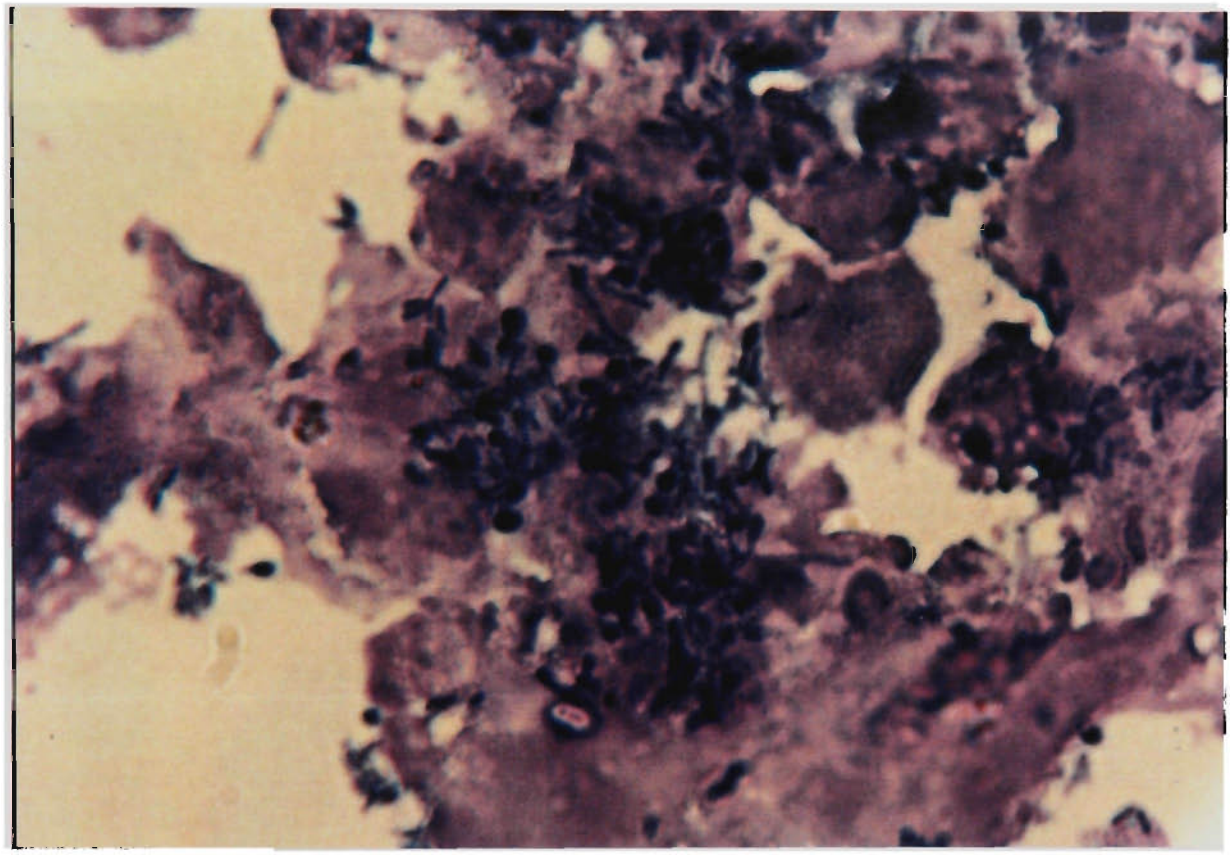


Fig. 7.3.9. A Adherence of *B. bifidum* 1901 to Ht-29 monolayer cells when added with untreated spent broth (magnification x 1000).

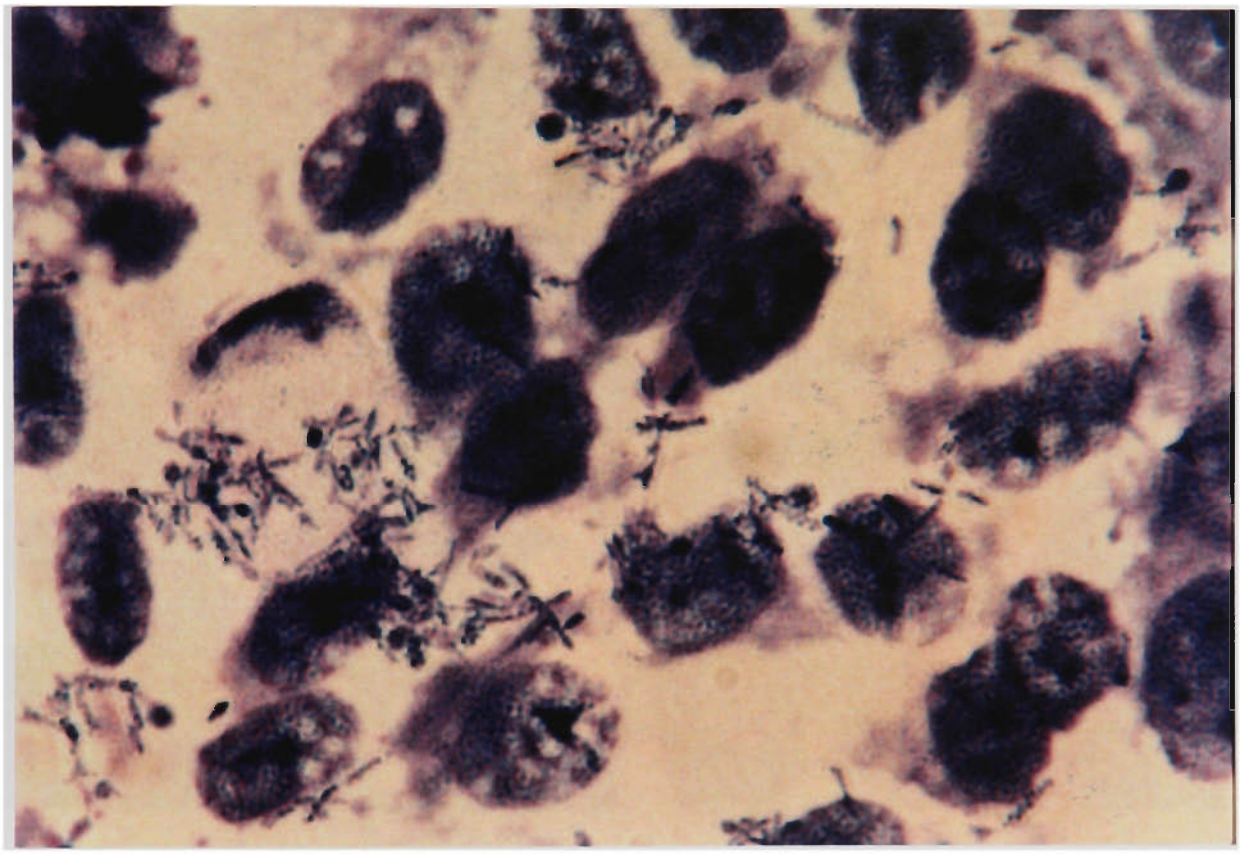


Fig. 7.3.9. B Adherence of *B. bifidum* 1901 to Ht-29 monolayer cells when added with fresh MRS broth (magnification x 1000).

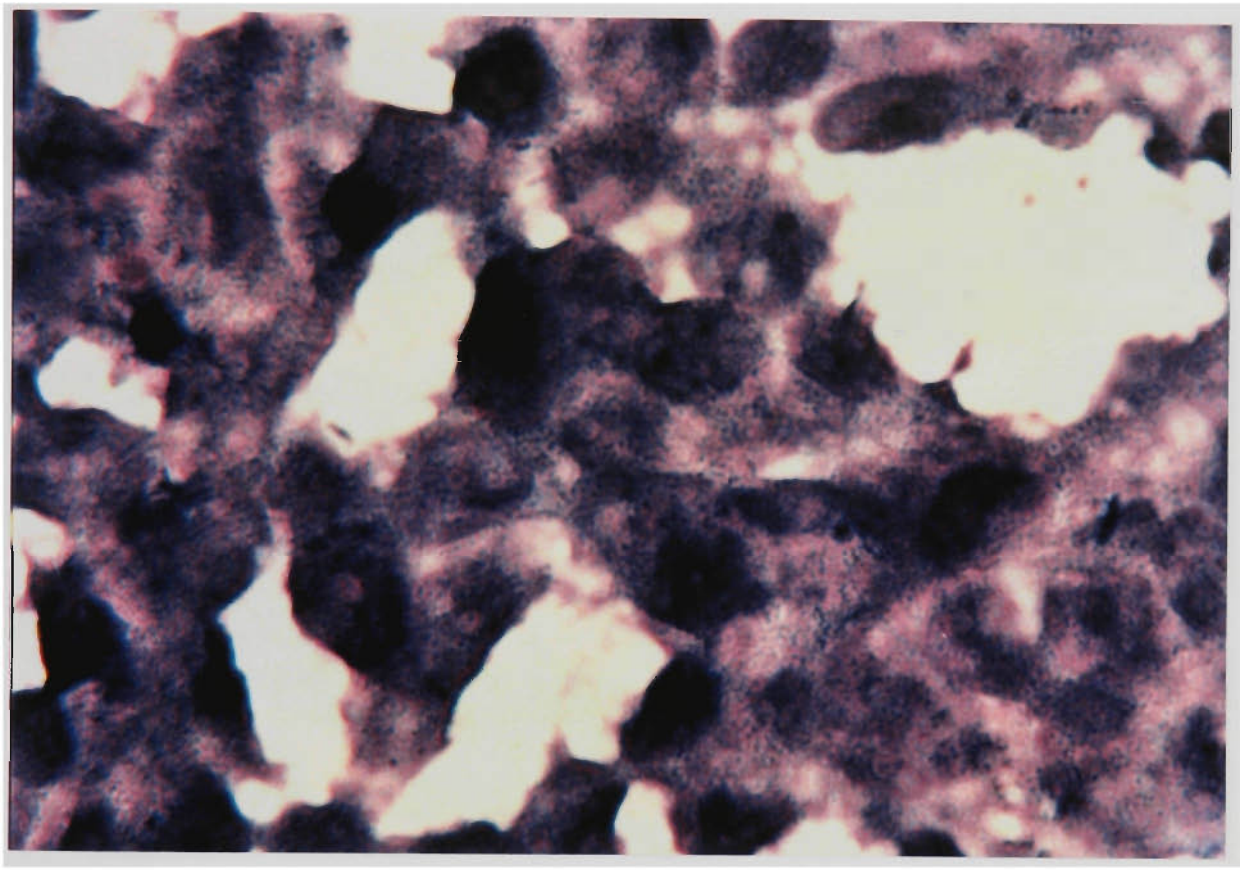


Fig. 7.3.9. C Adherence of *B. bifidum* 1901 to Ht-29 monolayer cells when added with trypsin treated spent broth (magnification x 1000).

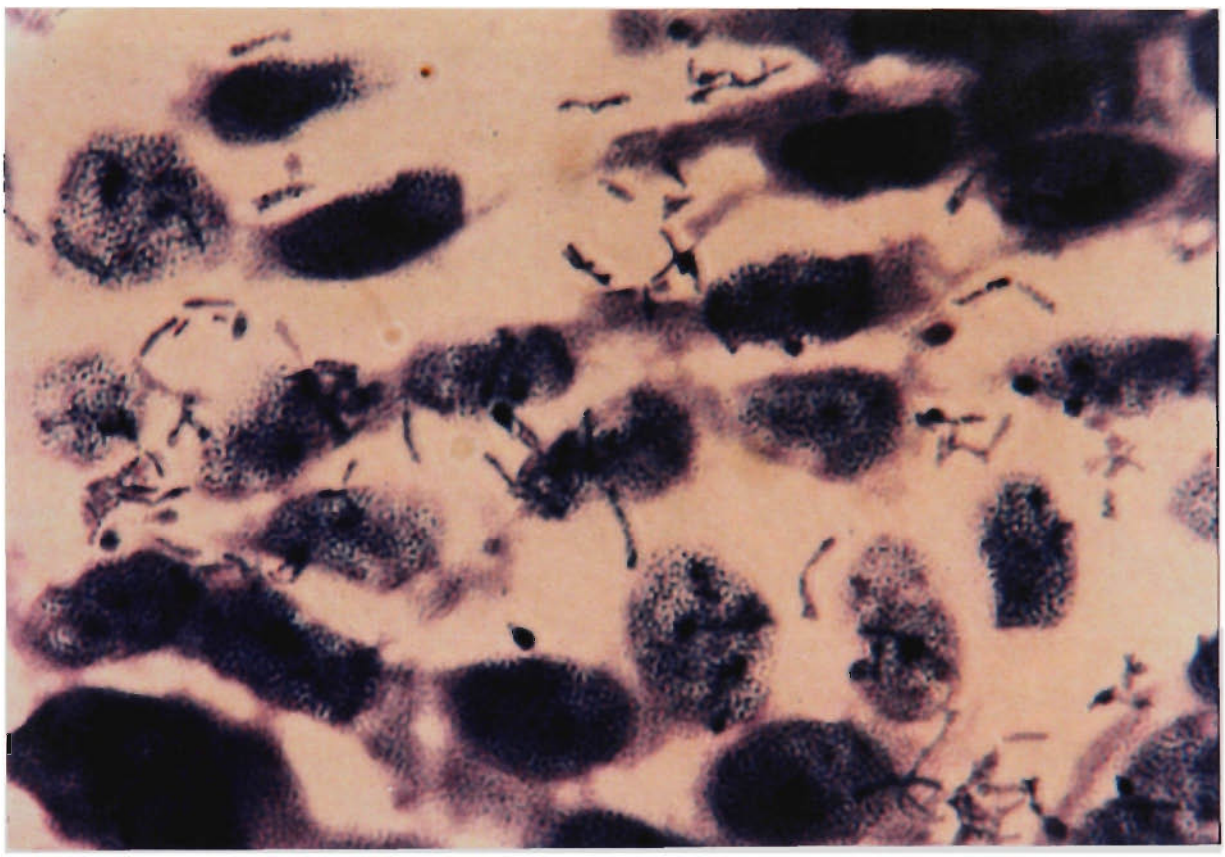


Fig. 7.3.9. D Adherence of *B. bifidum* 1901 to Ht-29 monolayer cells when added with spent broth treated with periodate (magnification x 1000).

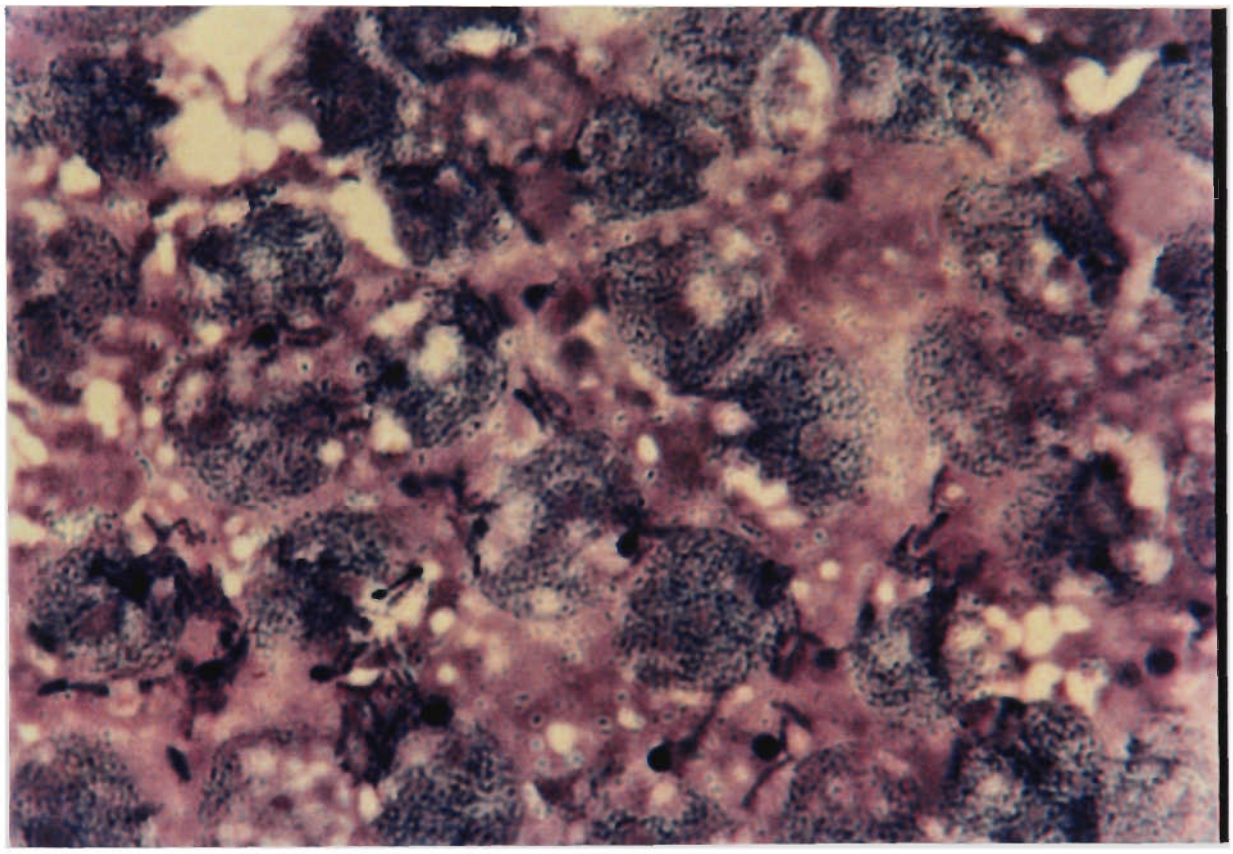


Fig. 7.3.9. E Adherence of *B. bifidum* 1901 to Ht-29 monolayer cells when added with spent broth to Ht-29 monolayer cells treated with periodate (Magnification x 1000).

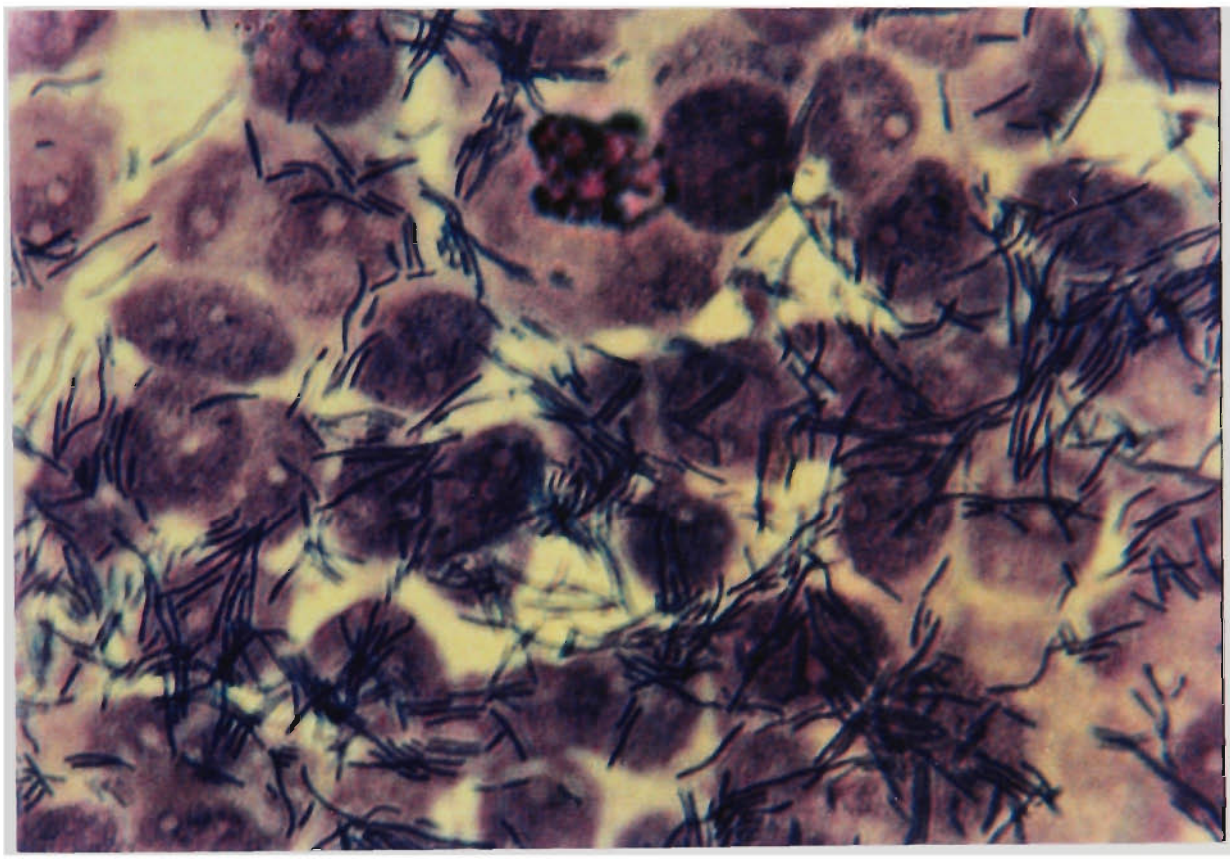


Fig. 7.3.10. A Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with untreated spent broth (magnification x 1000).

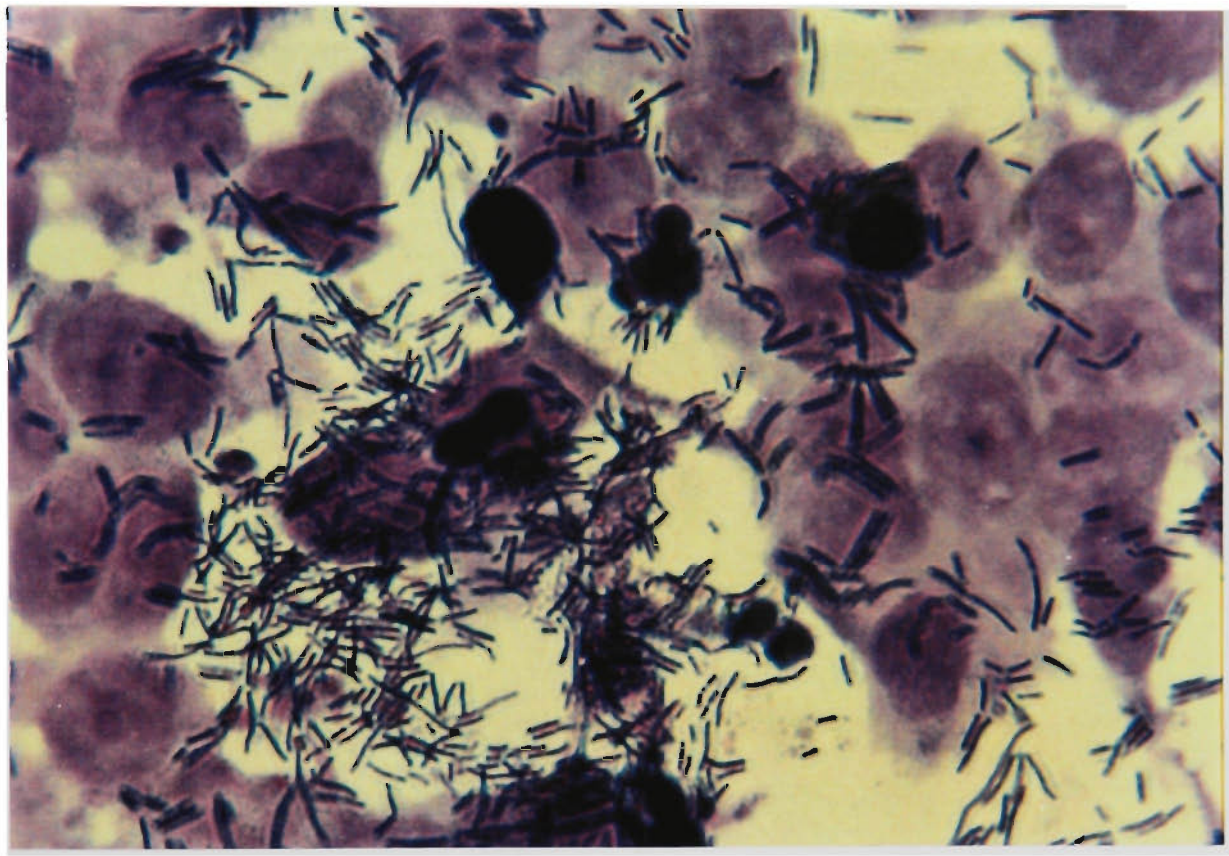


Fig. 7.3.10. B Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with fresh MRS broth (magnification x 1000).

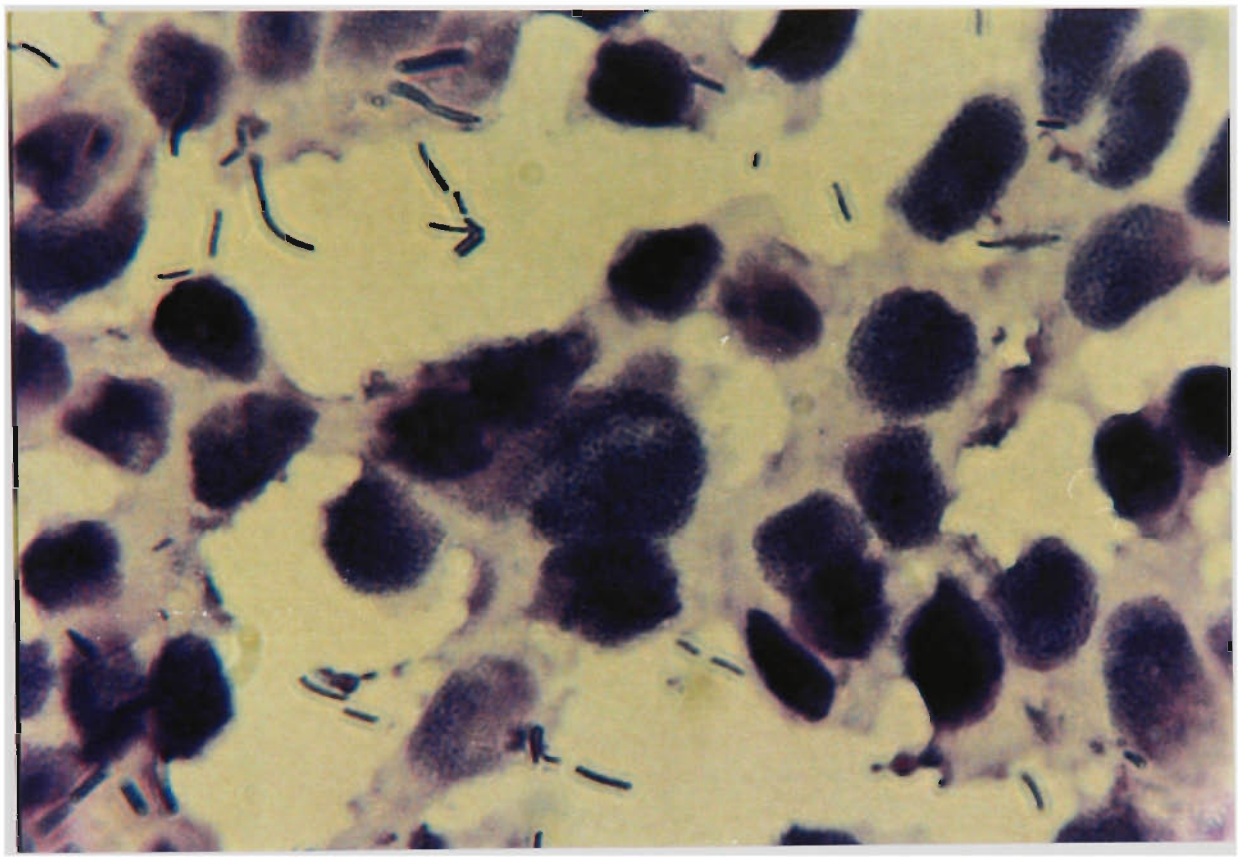


Fig.7.3.10. C Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with trypsin treated spent broth (magnification x 1000).

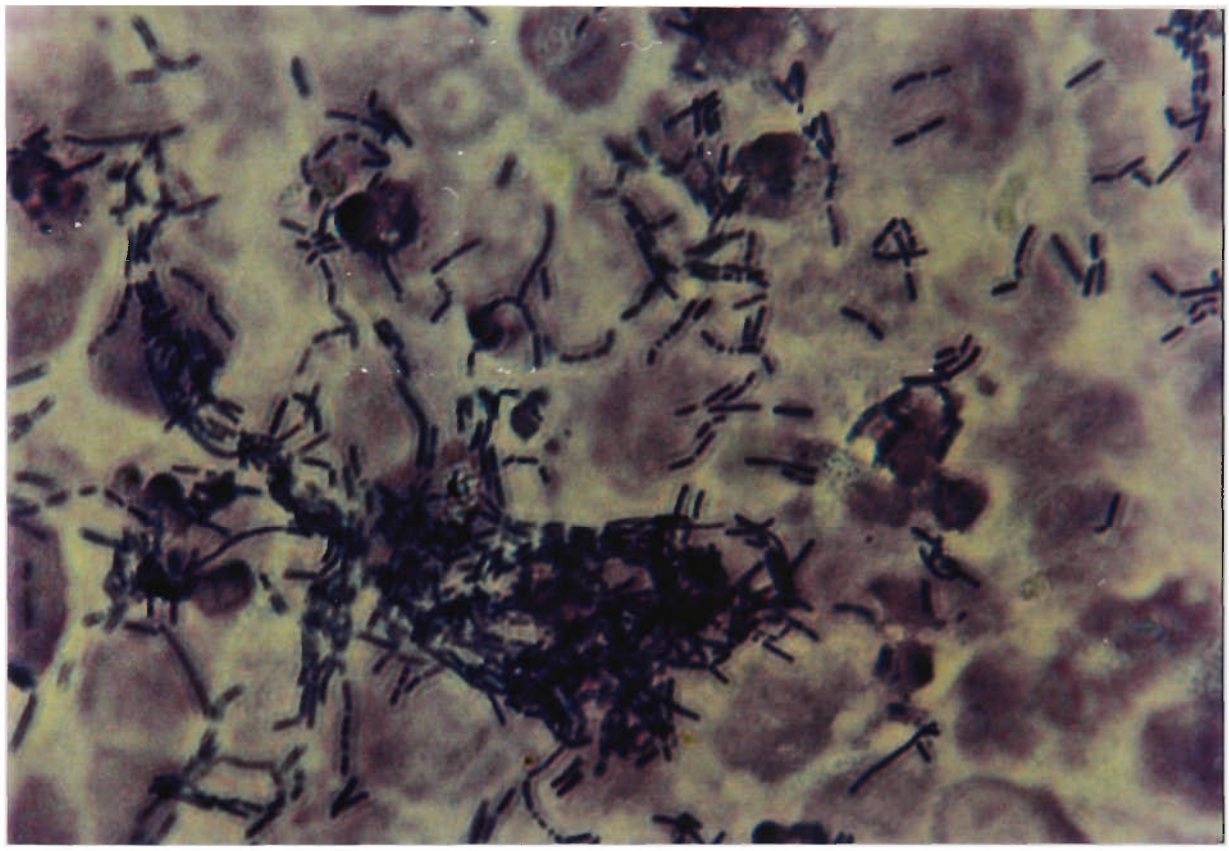


Fig. 7.3.10. D Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with spent broth treated with periodate (magnification x 1000).

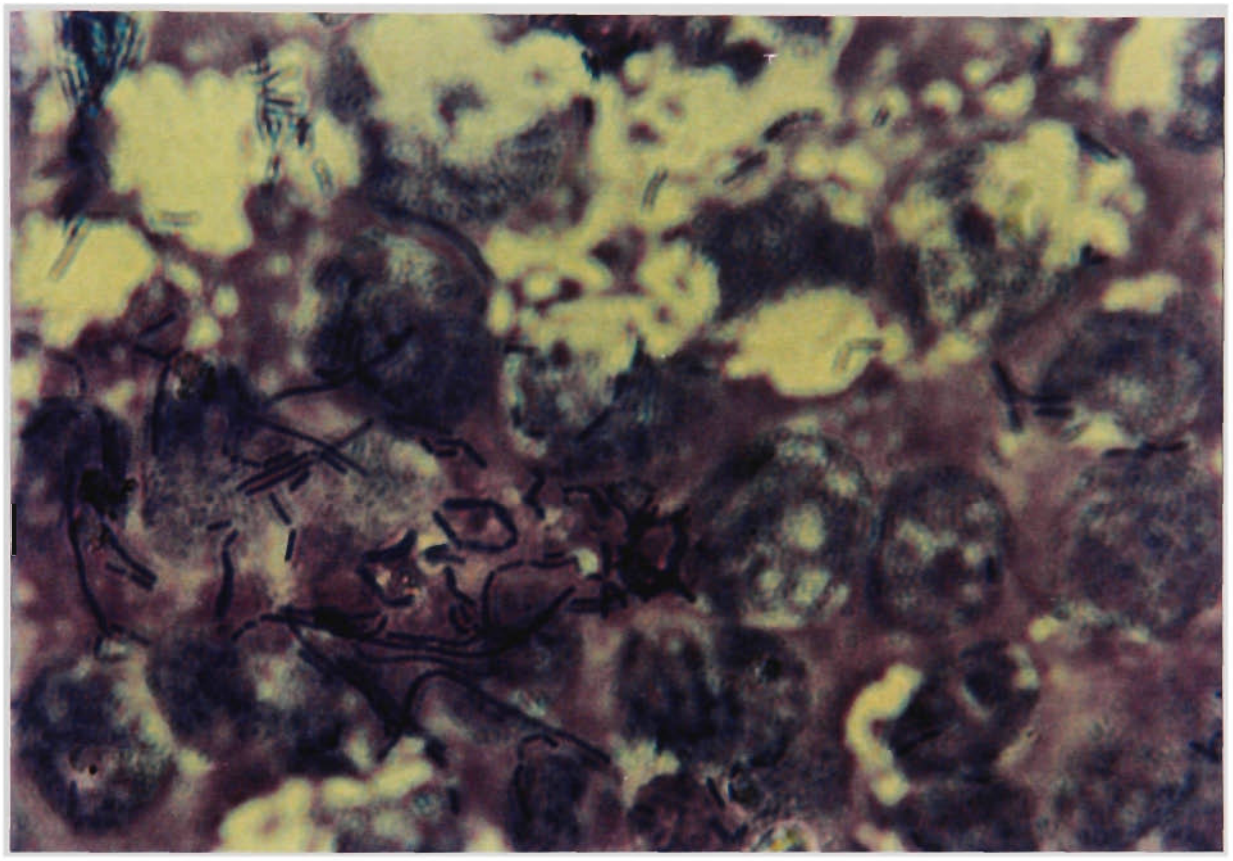


Fig. 7.3.10. E Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with spent broth to Ht-29 monolayer cells treated with periodate (magnification x 1000).

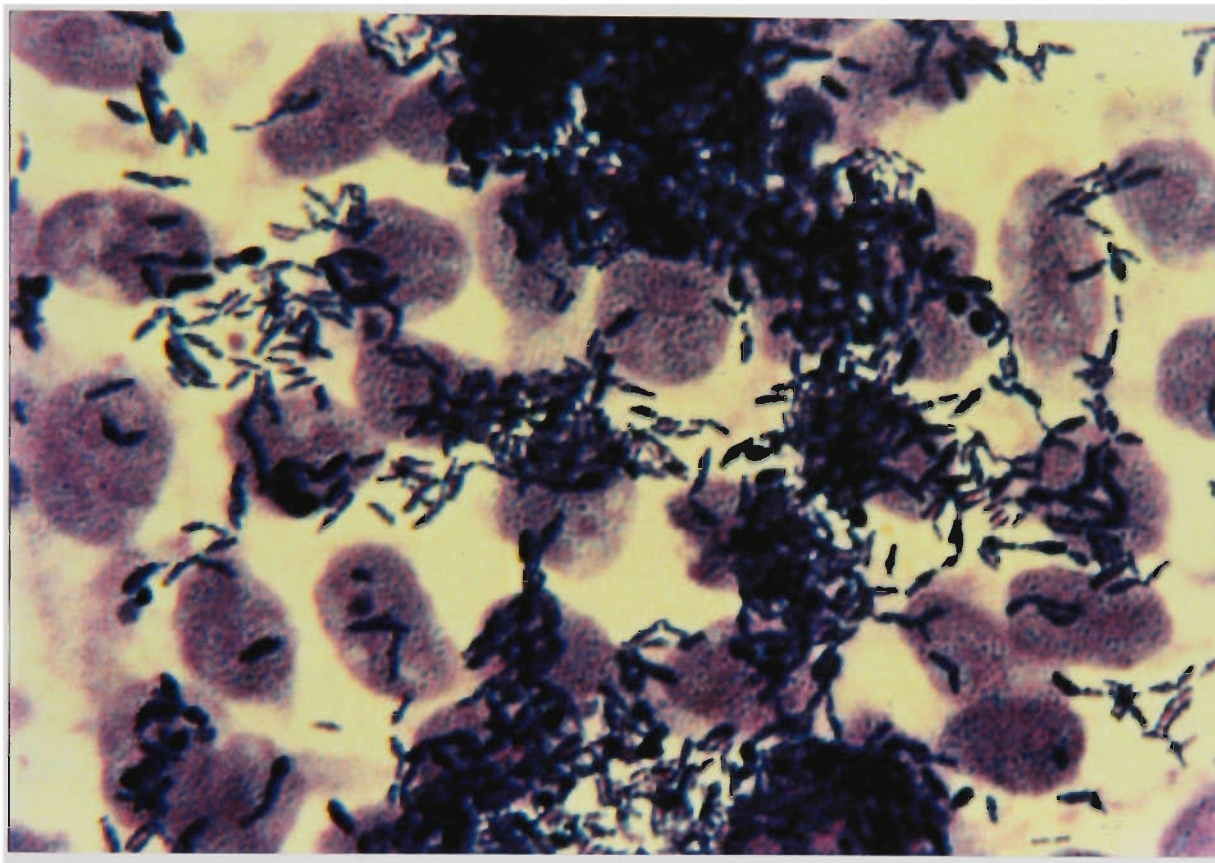


Fig. 7.3.11. (A) Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with untreated spent broth (magnification x 1000).

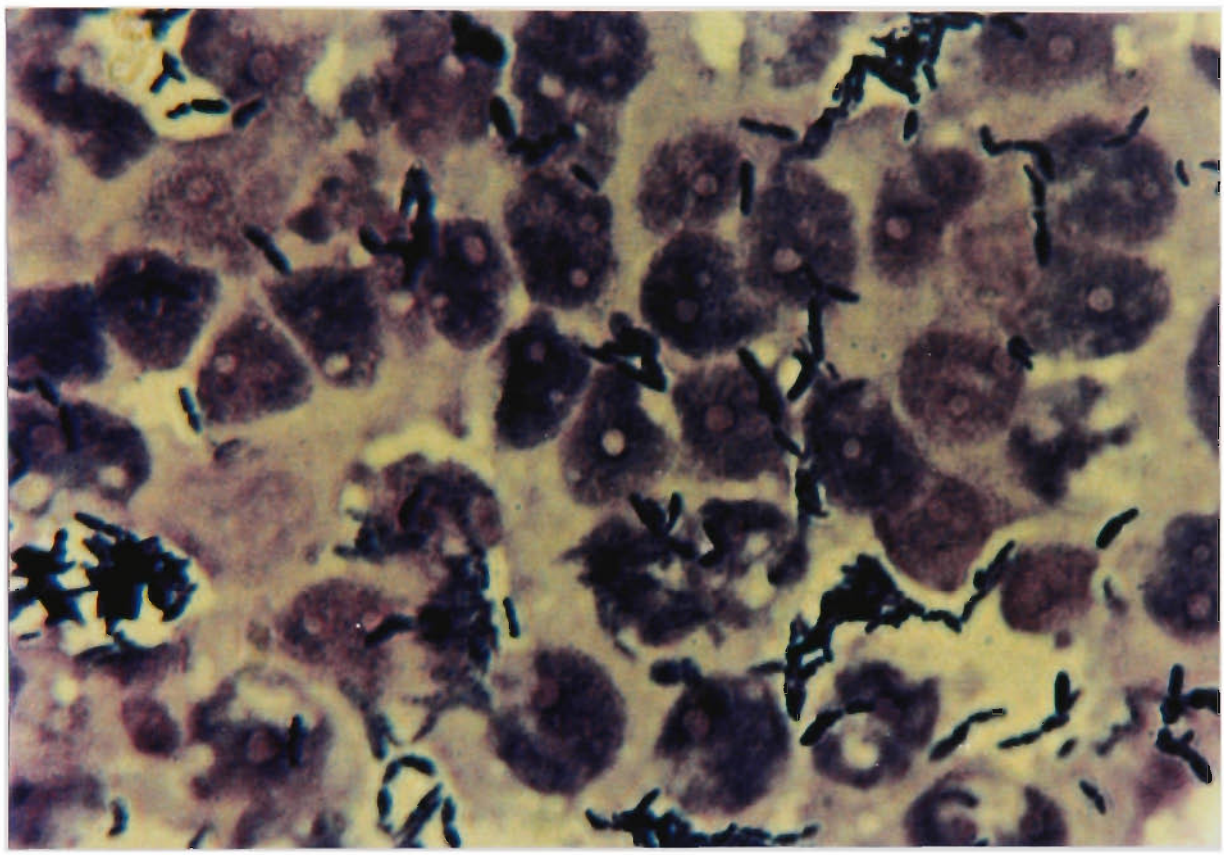


Fig. 7.3.11. B Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with fresh MRS broth (magnification x 1000).

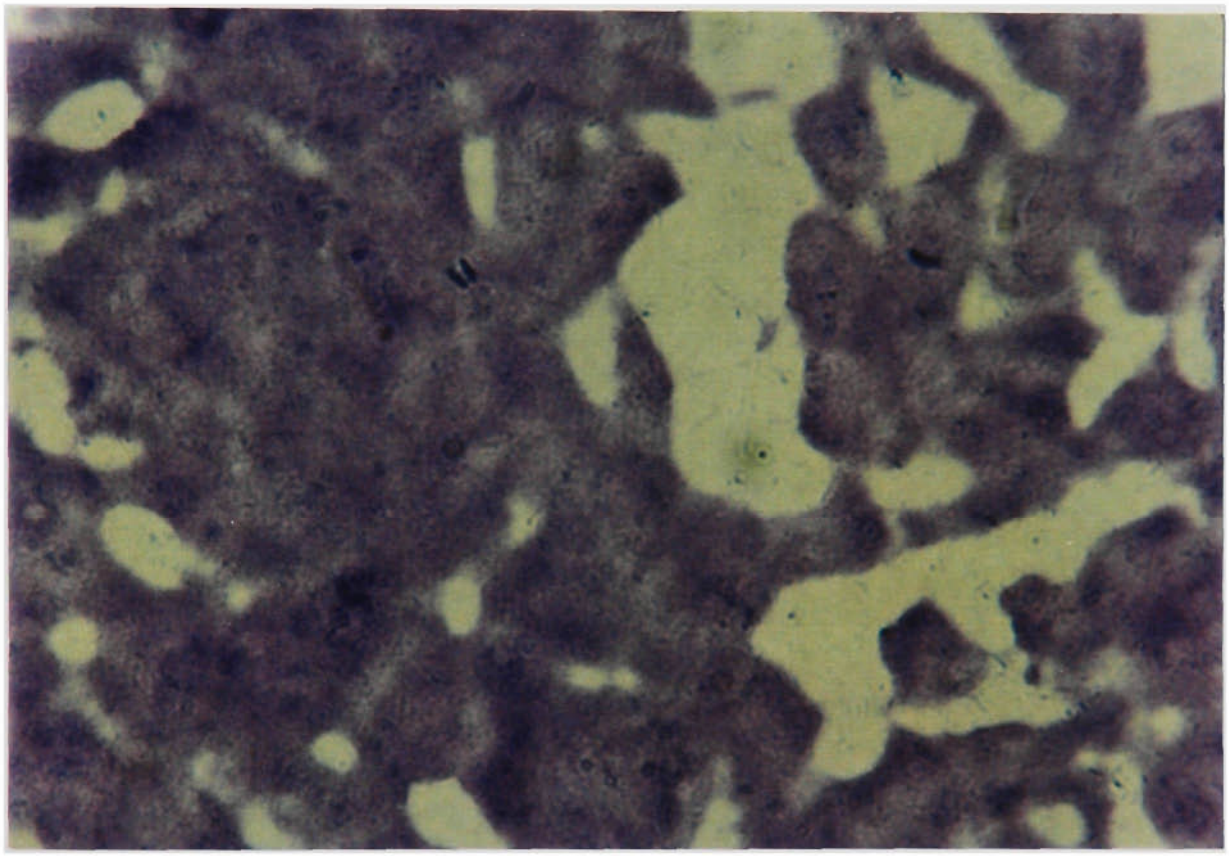


Fig. 7.3.11. C Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with trypsin treated spent broth (magnification x 1000).

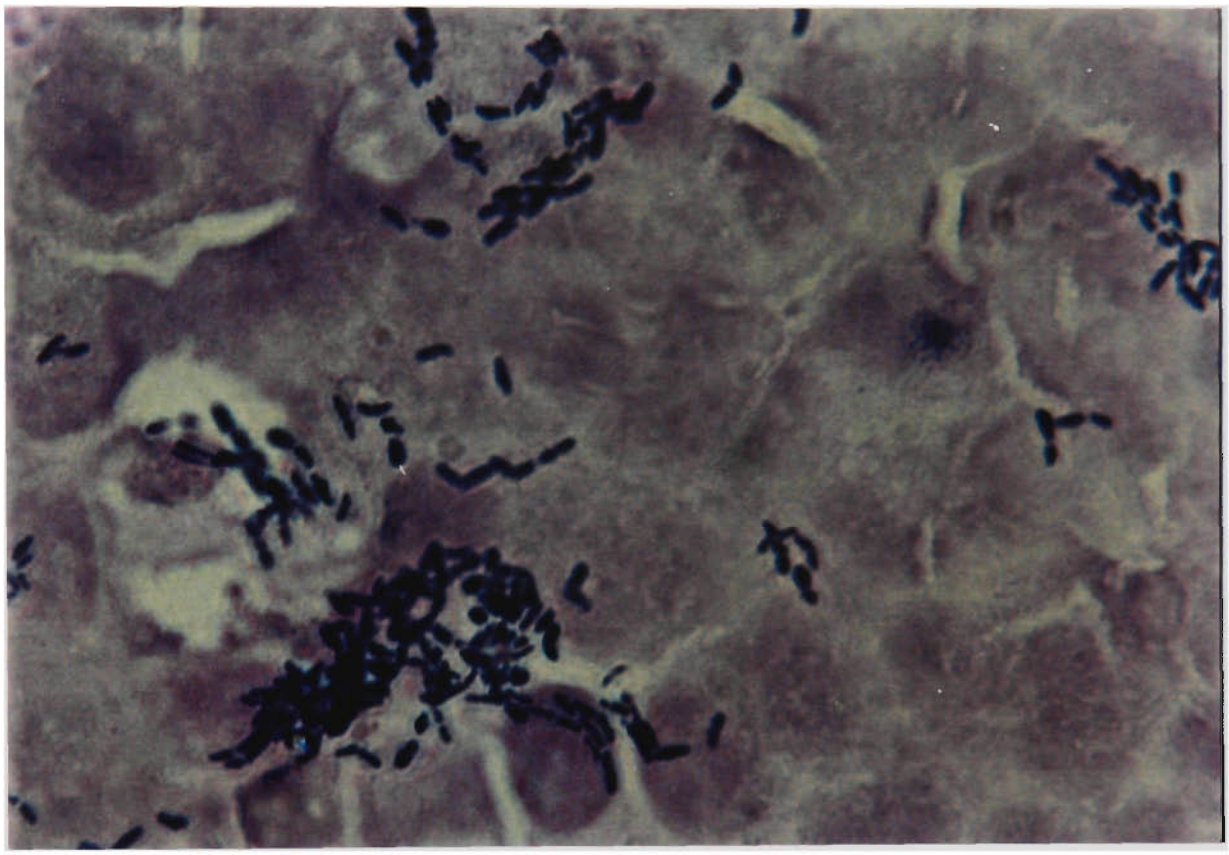


Fig. 7.3.11. D Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with spent broth treated with periodate (magnification x 1000).

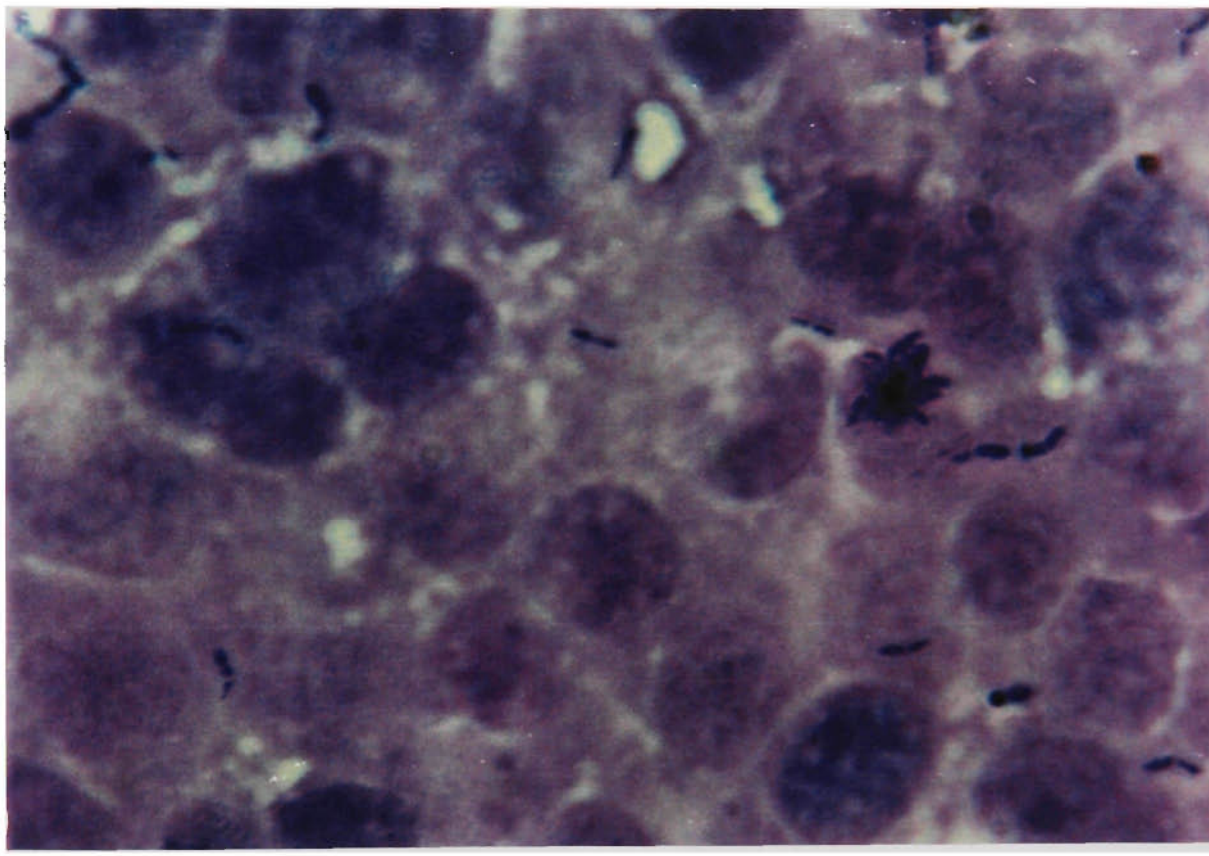


Fig. 7.3.11. E Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with spent broth.to Ht-29 monolayer cells treated with periodate (magnification x 1000).

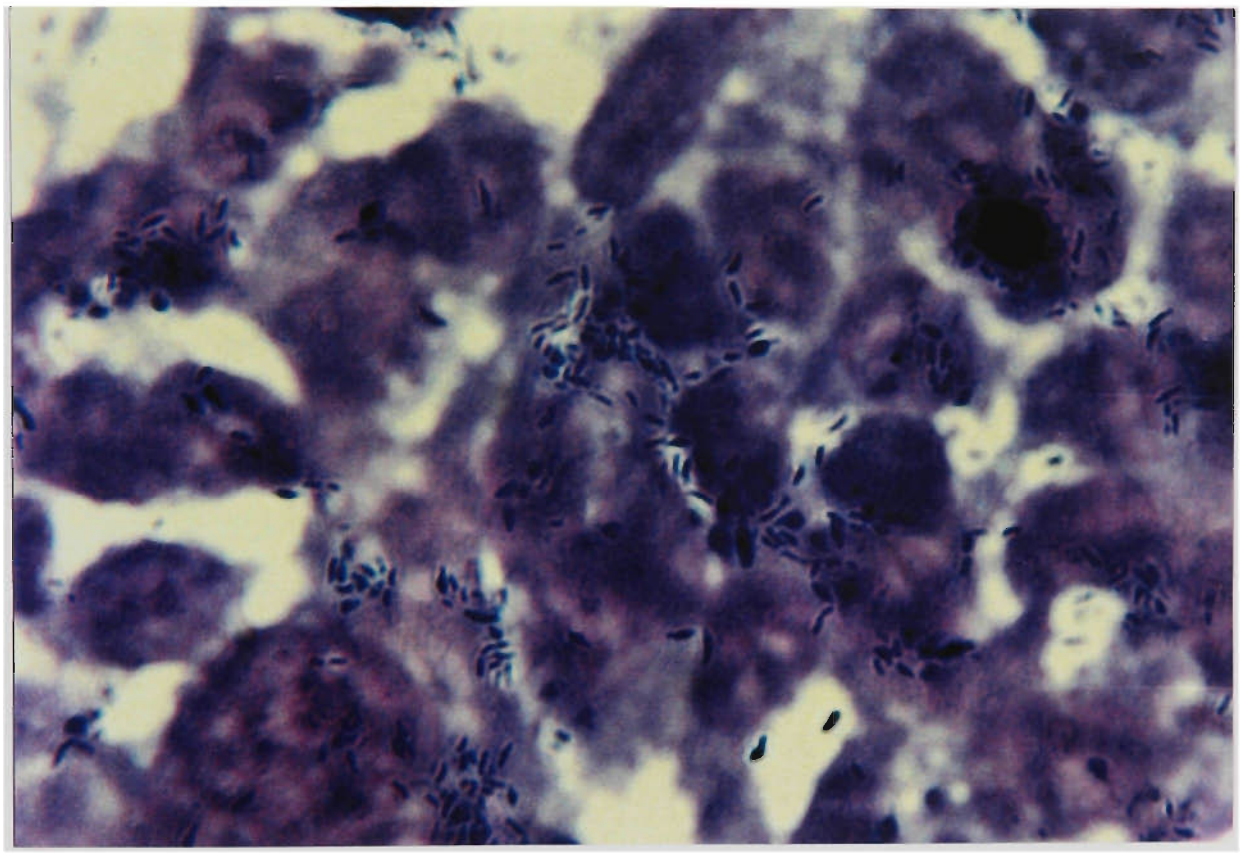


Fig. 7.3.12. A Adherence of *B. thermophilum* 20210 to Ht-29 monolayer cells when added with untreated spent broth (magnification x 1000).

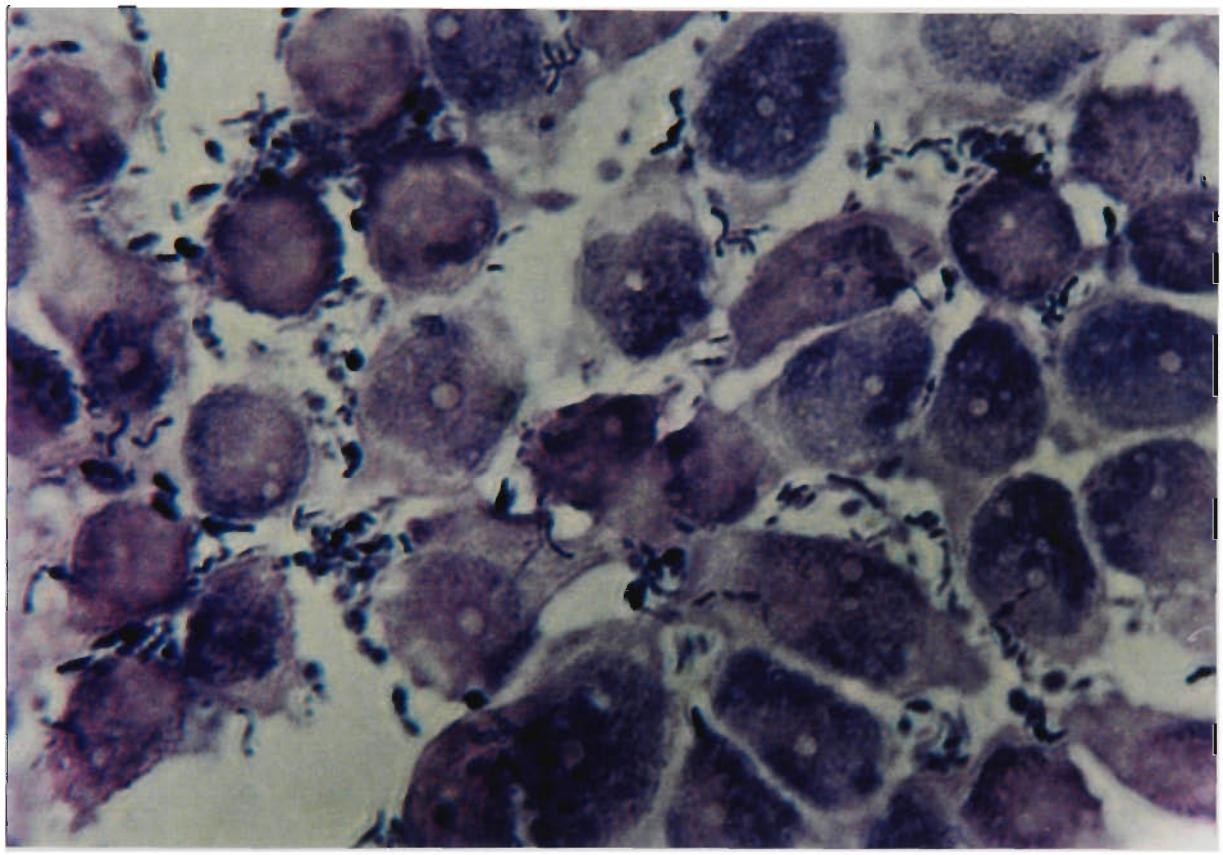


Fig 7.3.12. B. Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with fresh MRS broth (magnification x 1000).

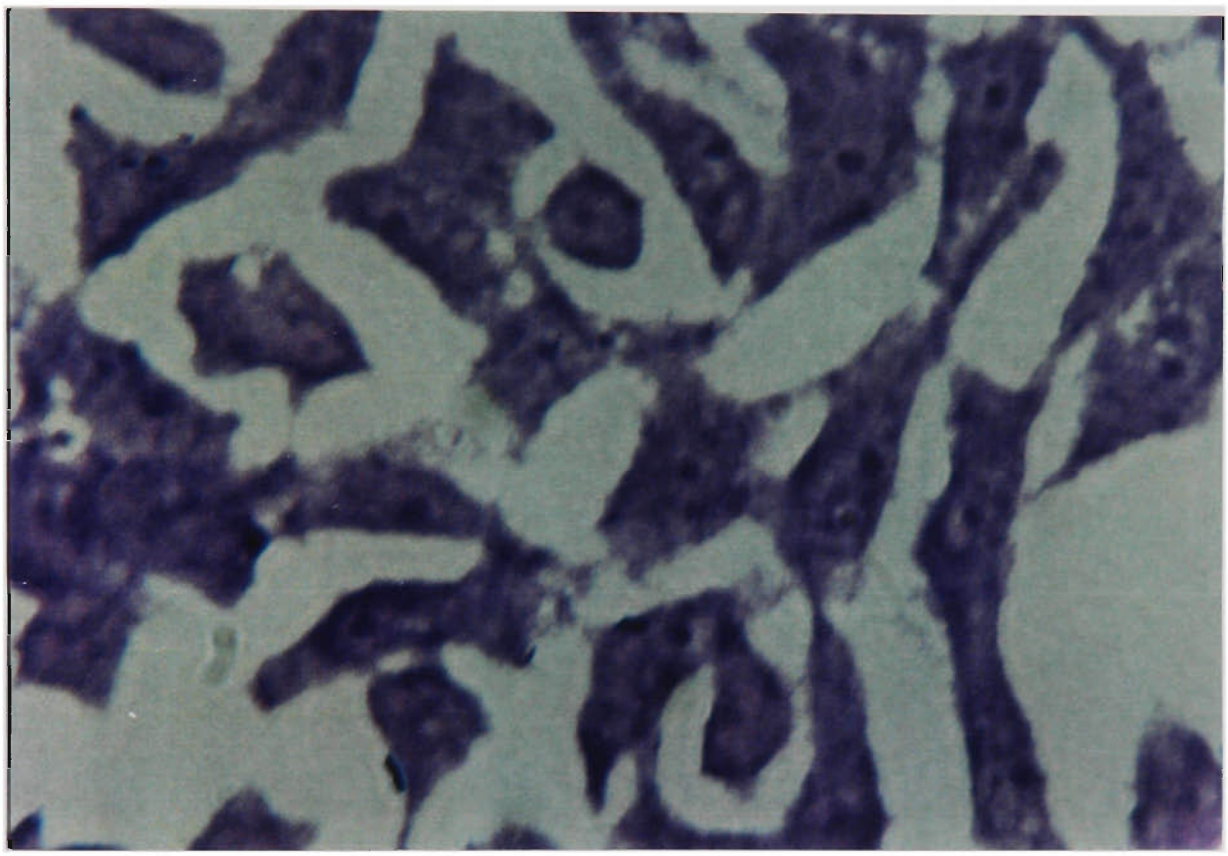


Fig. 7.3.12. C Adherence of *B. thermophilum* 20210 to Ht-29 monolayer cells when added with trypsin treated spent broth (magnification x 1000).

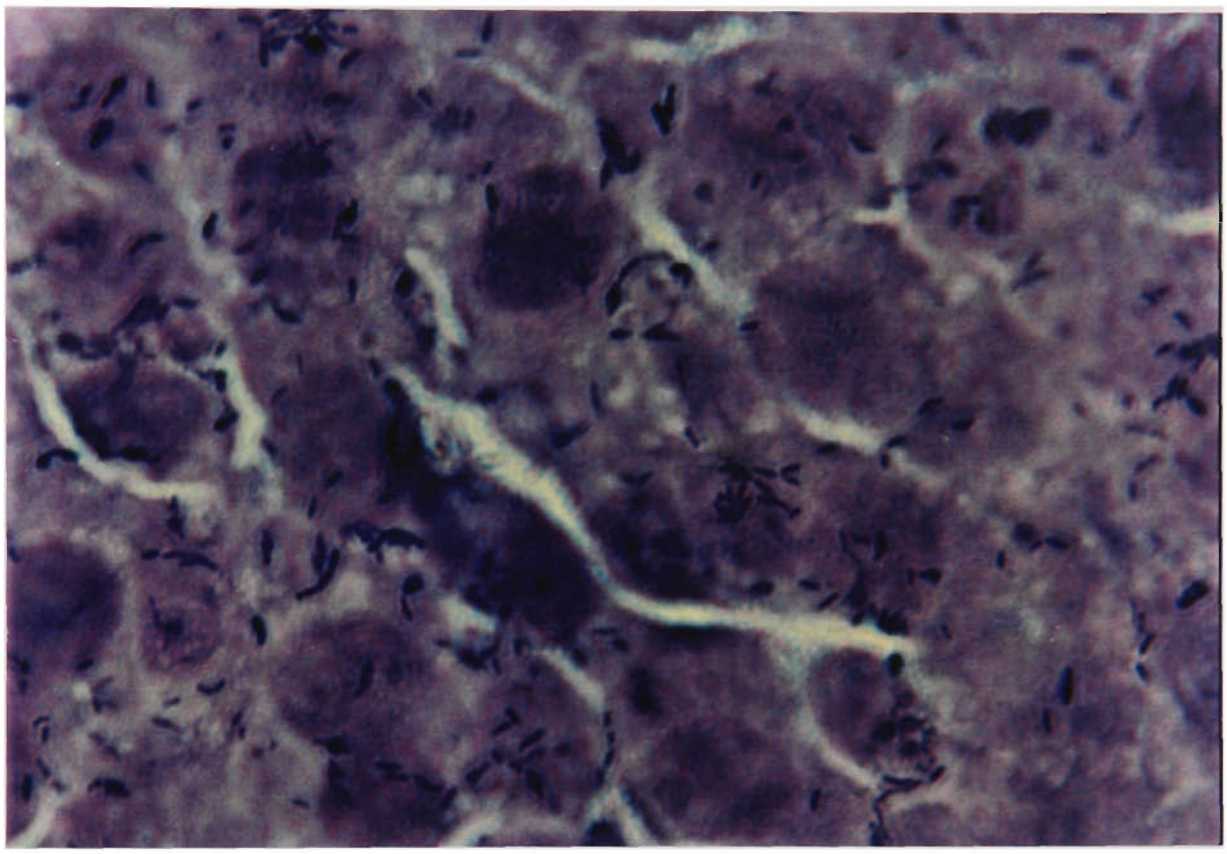


Fig. 7.3.12. D Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with spent broth.treated with periodate (magnification x 1000).

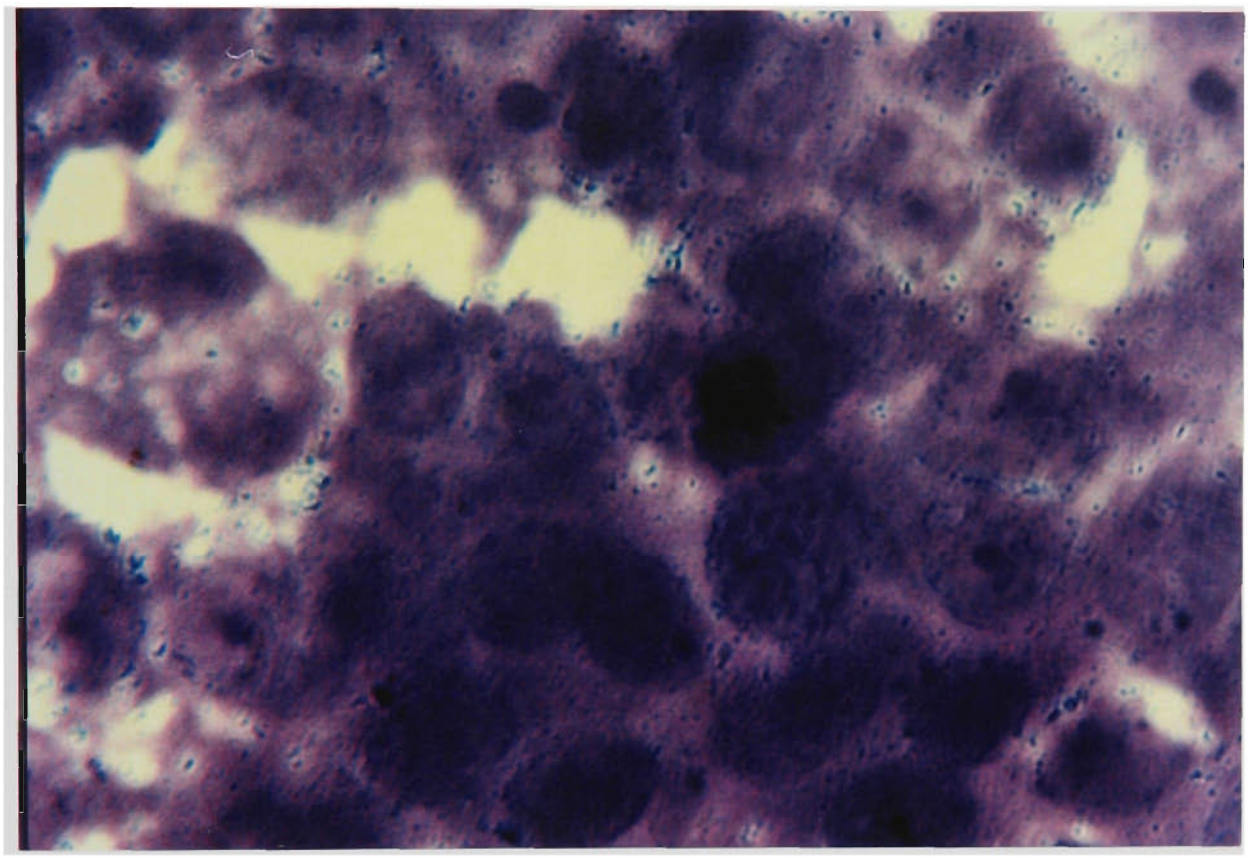


Fig. 7.3.12. E Adherence of *B. thermophilum* 20210 to Ht-29 monolayer cells when added with spent broth.to Ht-29 monolayer cells treated with periodate (magnification x 1000).

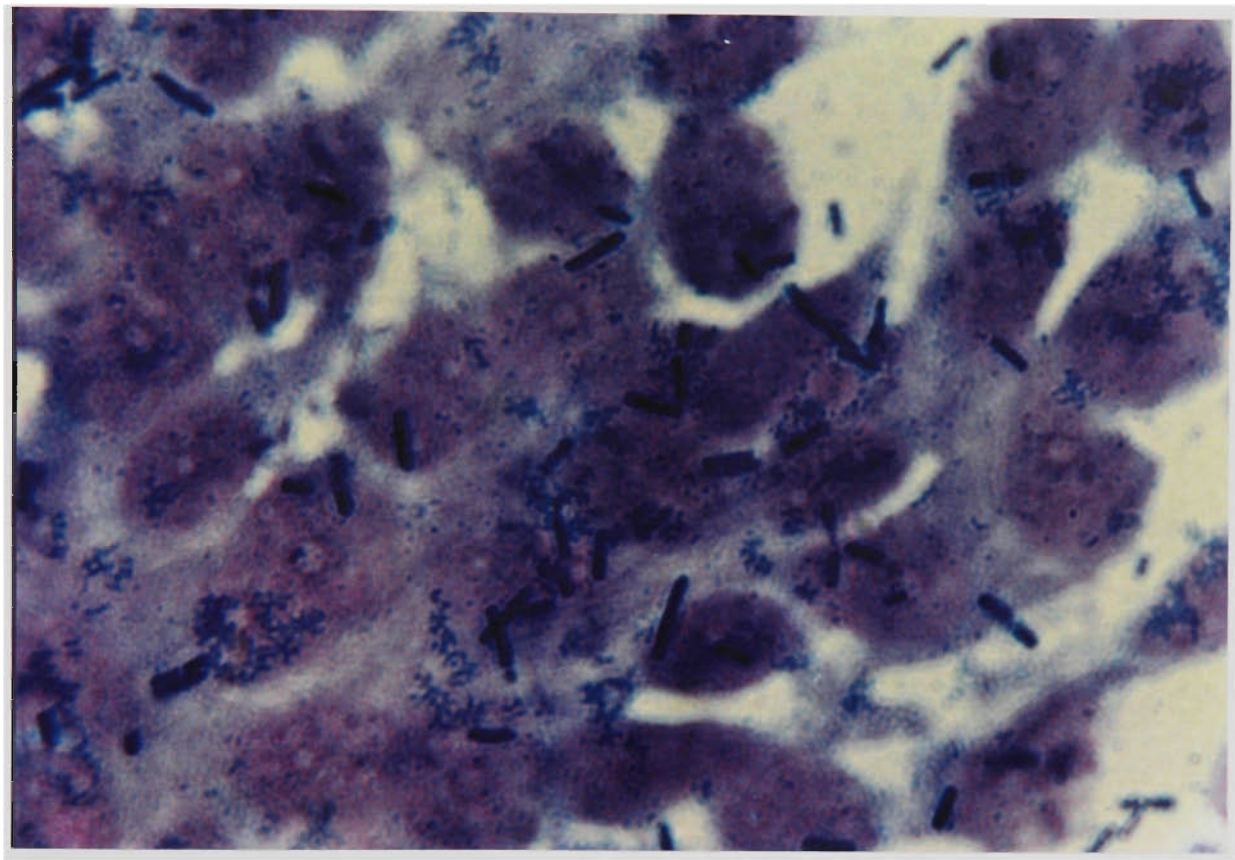


Fig. 7.3.13. A Adherence of *L. acidophilus* 2400 to Ht-29 monolayer cells when added with untreated spent broth (magnification x 1000).

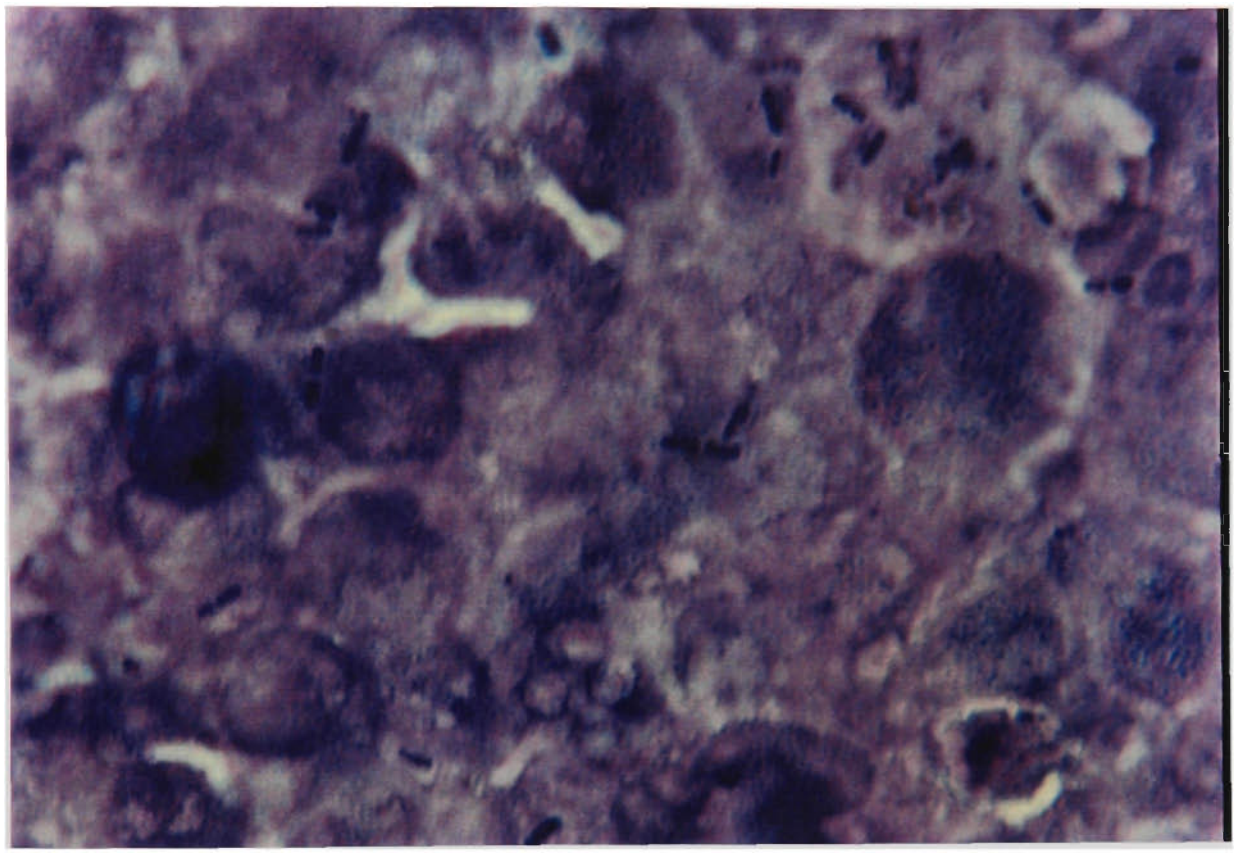


Fig. 7.3.13. B Adherence of *L. acidophilus* 2400 to Ht-29 monolayer cells when added with fresh MRS broth (magnification x 1000).

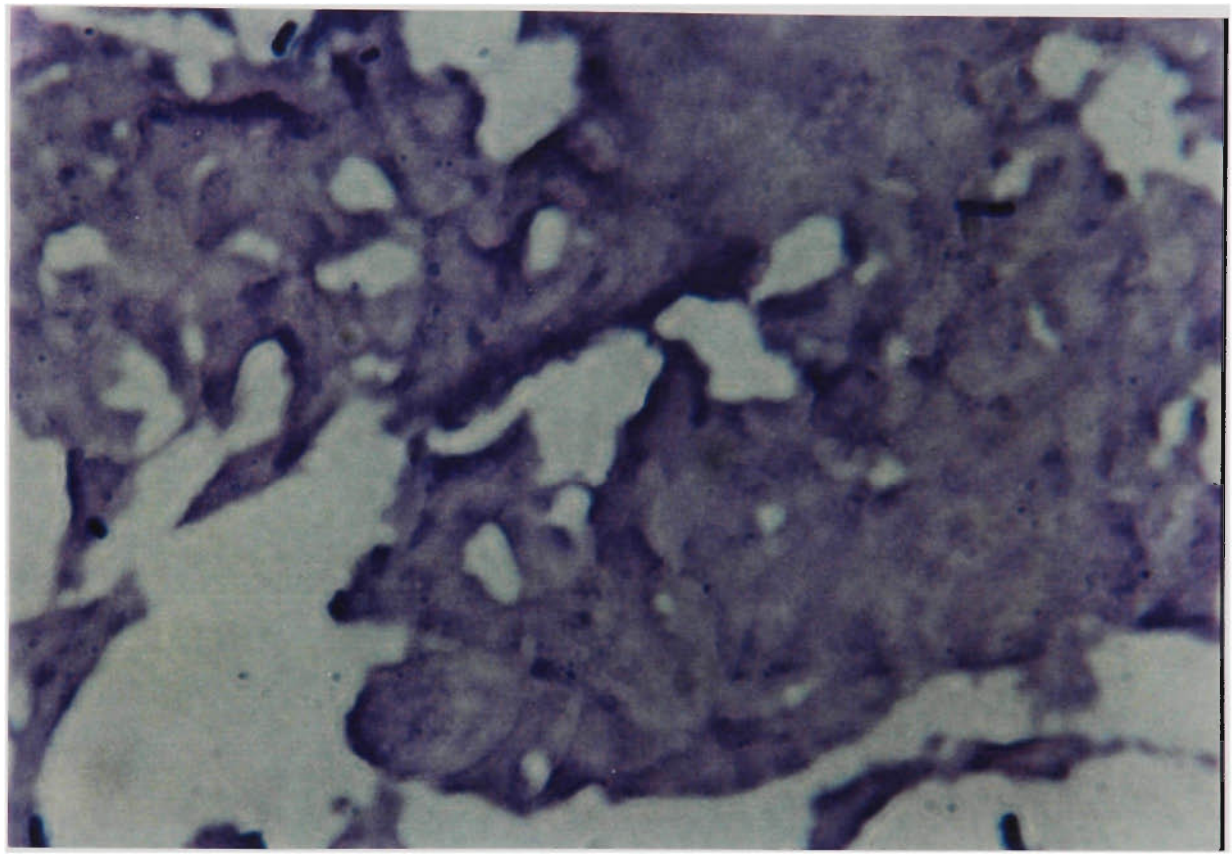


Fig. 7.3.13. C Adherence of *L. acidophilus* 2400 to Ht-29 monolayer cells when added with trypsin treated spent broth (magnification x 1000).

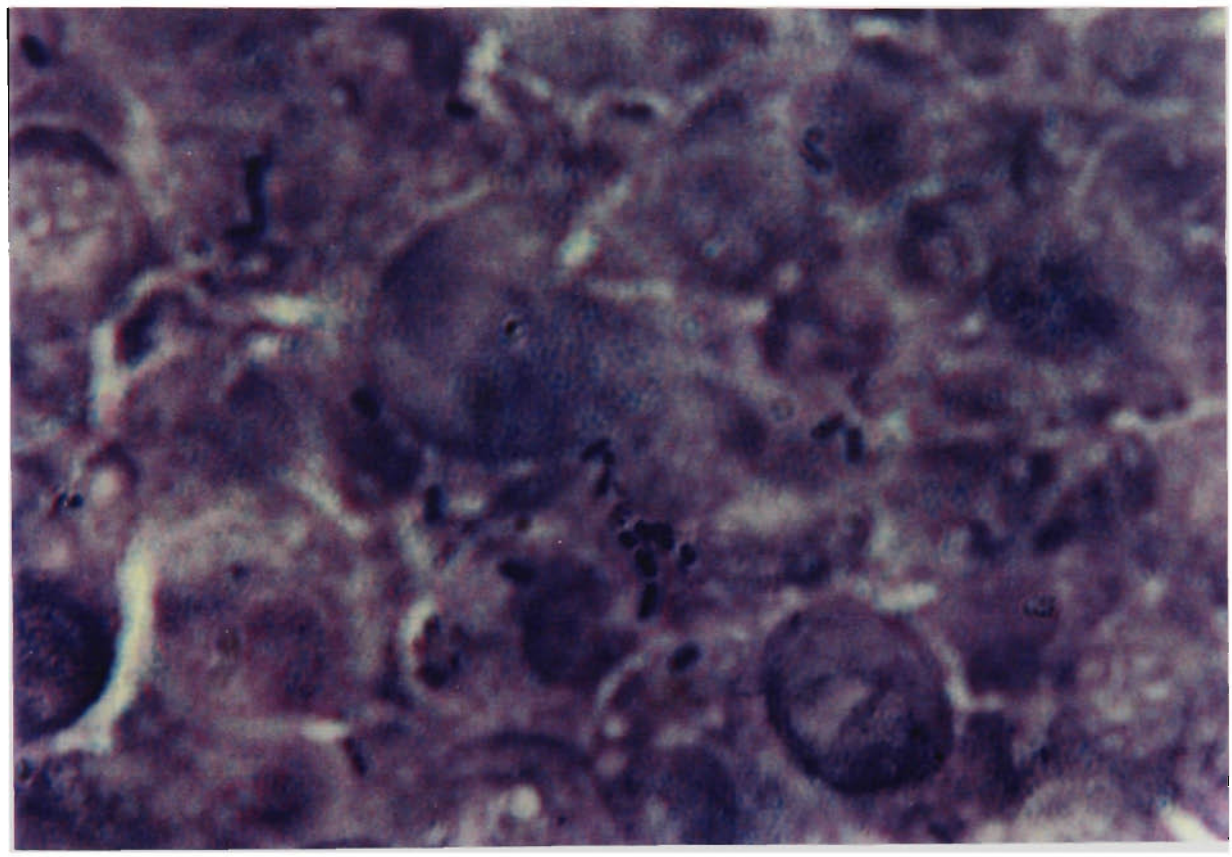


Fig. 7.3.13. D Adherence of *L. acidophilus* 2400 to Ht-29 monolayer cells when added with spent broth.treated with periodate (magnification x 1000).

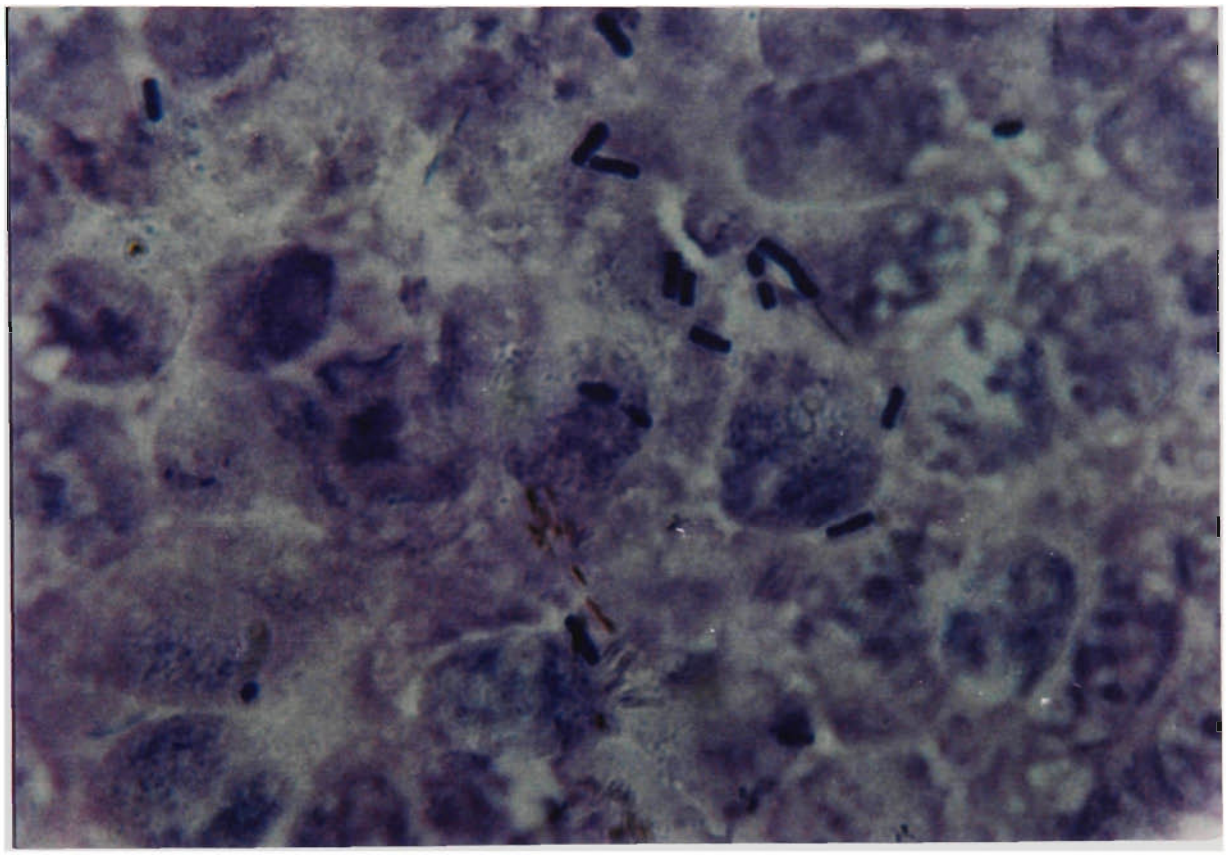


Fig. 7.3.13. E Adherence of *L. acidophilus* 2400 to Ht-29 monolayer cells when added with spent broth.to Ht-29 monolayer cells treated with periodate (magnification x 1000).

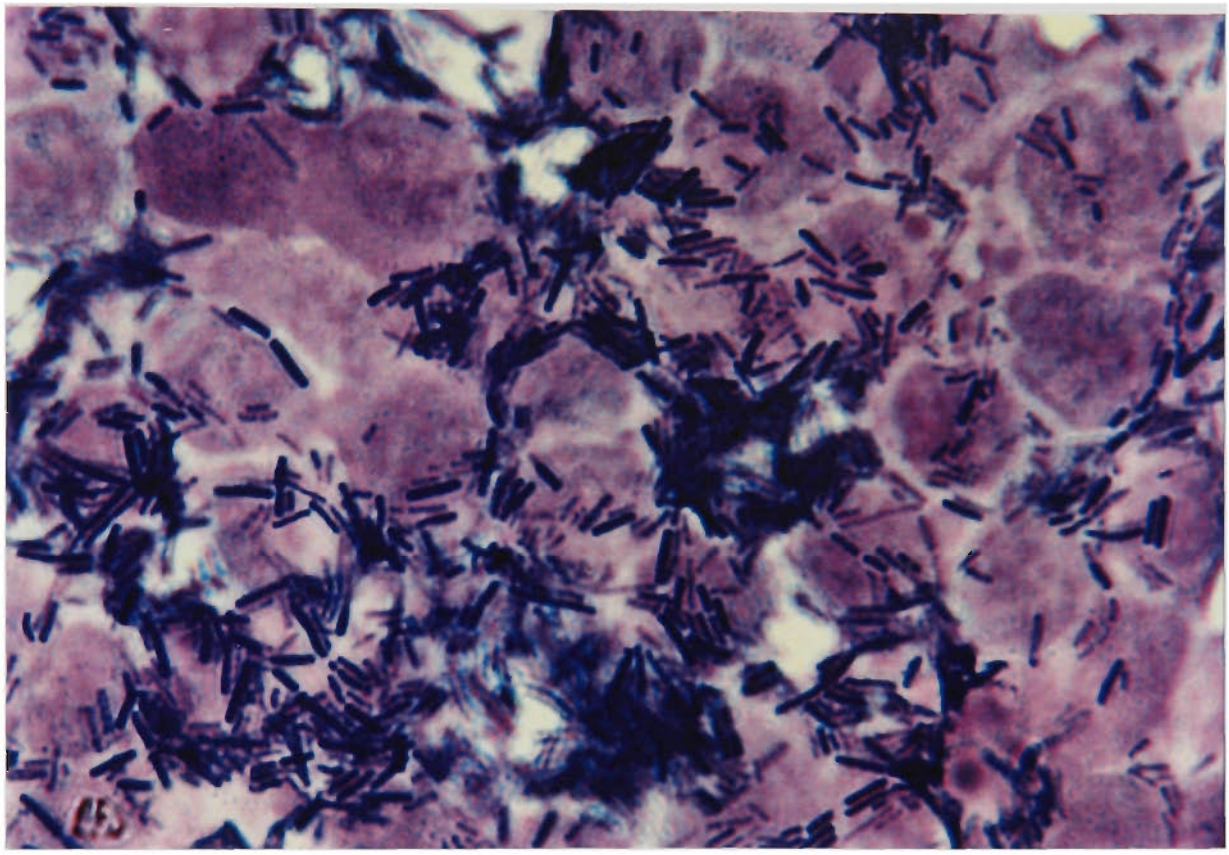


Fig. 7.3.14. A Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells when added with untreated spent broth (magnification x 1000).

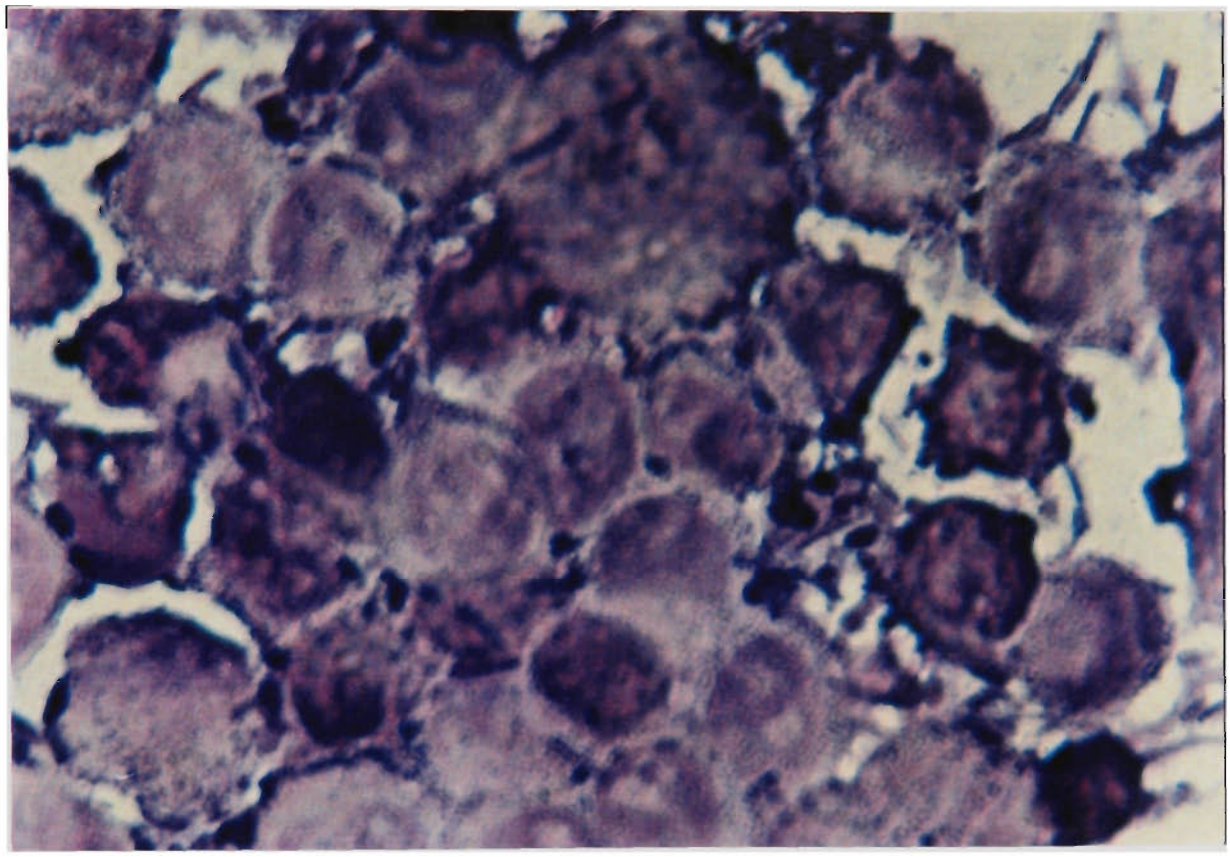


Fig. 7.3.14. B Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells when added with fresh MRS broth (magnification x 1000).

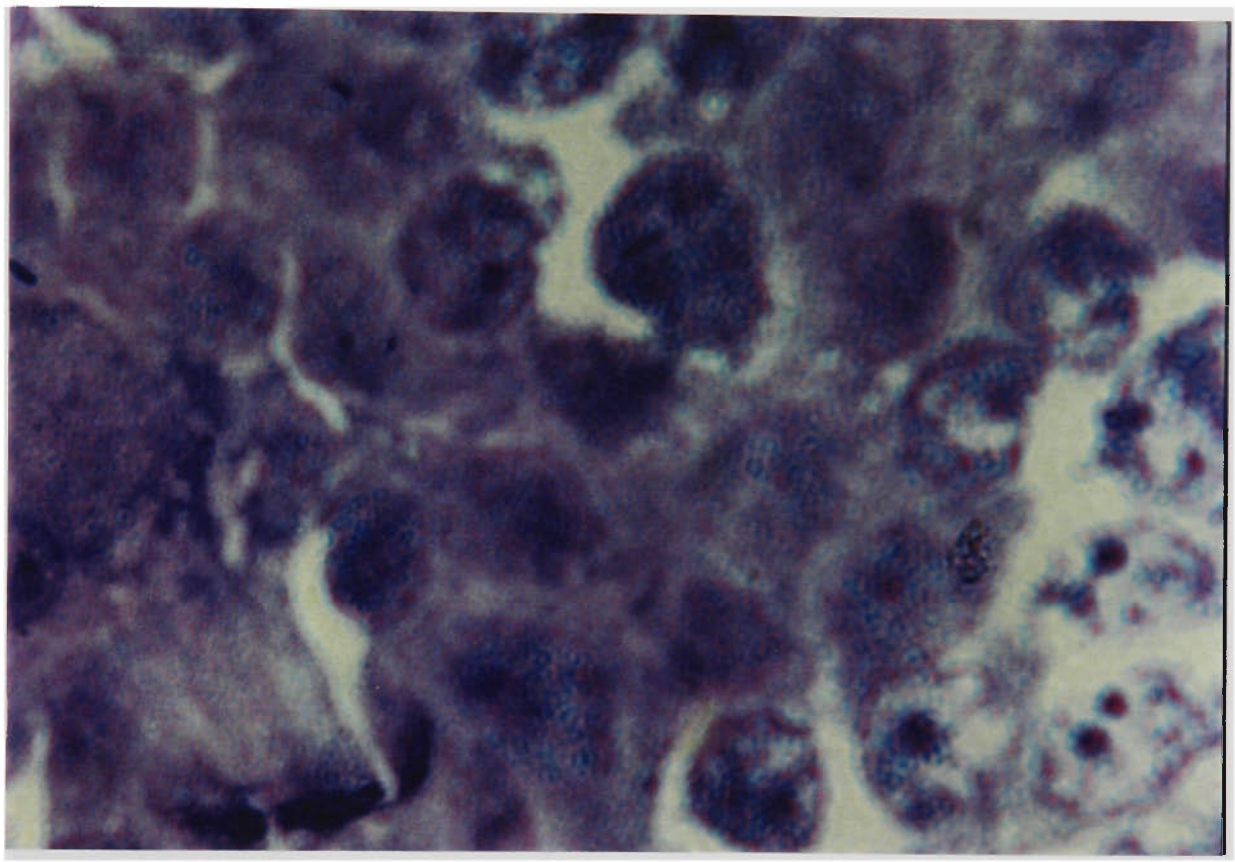


Fig. 7.3.14. C Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells when added with trypsin treated spent broth (magnification x 1000).

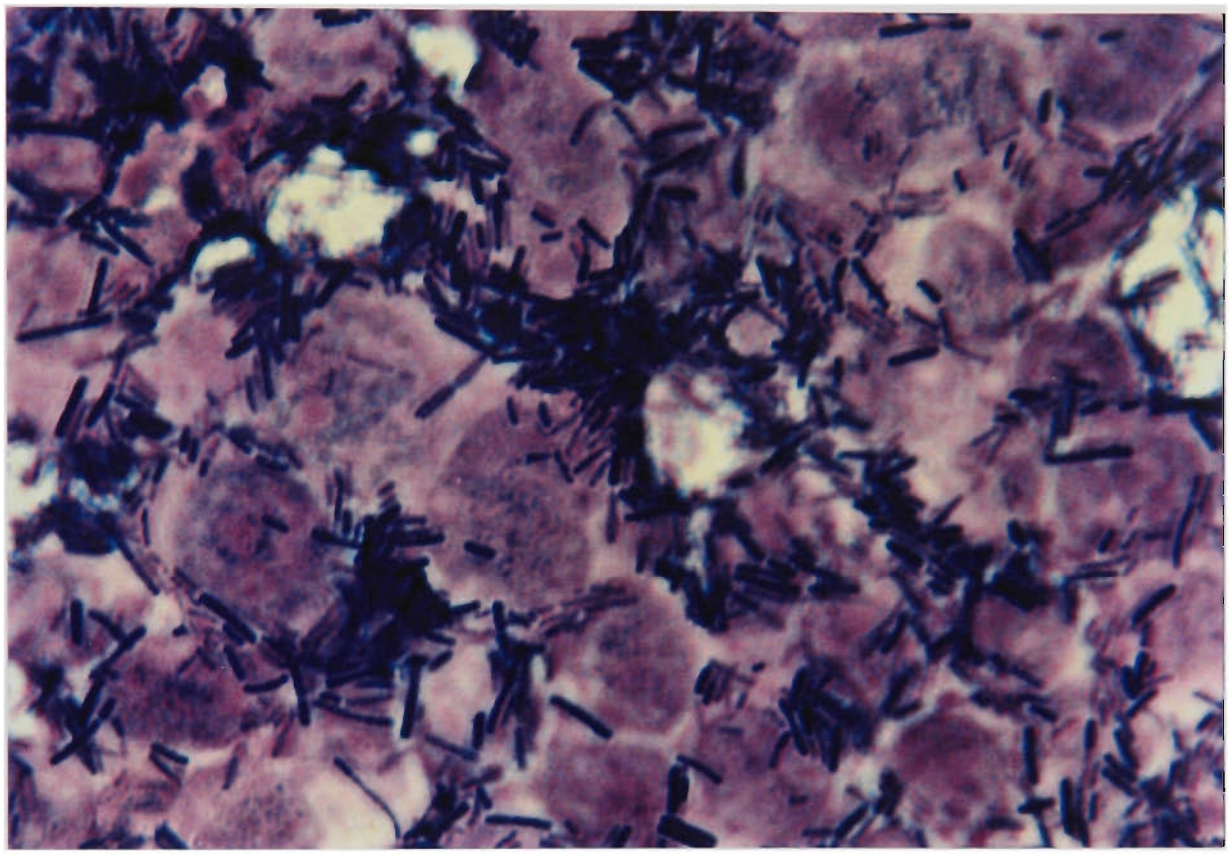


Fig. 7.3.14. D Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells when added with spent broth treated with periodate (magnification x 1000).

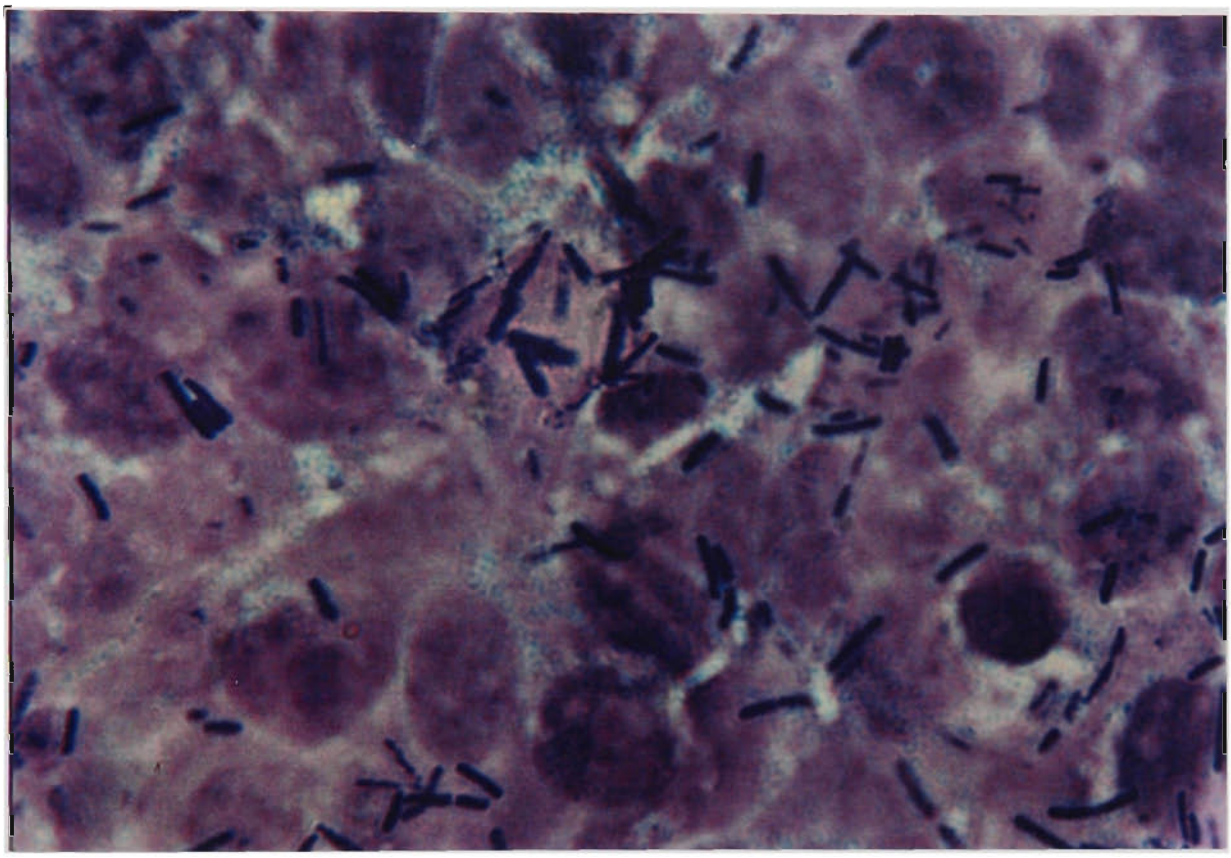


Fig. 7.3.14. E Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells when added with spent broth to Ht-29 monolayer cells treated with periodate (magnification x 1000).

7.3.3.3. Effect of various molecular size fractions of spent broth supernatants in adherence of selected strains of probiotic bacteria

Figures 7.3.15-7.3.17 A-E show the effect of various fractions[2] of spent broth supernatants separated based on molecular size on the adherence of *B. longum* 1941, *B. infantis* 1912 and *L. acidophilus* 2415.

Figures 7.3.15 A-E show the levels of adherence of *B. longum* 1941 when applied on Ht-29 monolayer cells along with whole spent broth or various fractions. As shown in Fig. 7.3.15 A, when applied with whole spent broth supernatant (fraction a), *B. longum* 1941 showed higher level of adherence as compared with the fraction b which contained molecules >50,000 kD. When the fraction c (<50,000 kD) was applied, the bacterial cells showed adherence similar to that was shown with fraction a. Fraction d (30,000-50,000 kD) also produced a similar level of adherence to fractions a and c. When bacterial cells were applied with fraction e (<30,000 kD), the level of adherence reduced substantially (Fig. 7.3.15 E). These observations prove that the spent broth protein responsible for adherence is a protein with a molecular size between 30,000 and 50,000 kD.

Figures 7.3.16 A-E show the levels of adherence of *B. infantis* 1912 when applied on Ht-29 monolayer cells along with whole spent broth or various fractions. As shown, non-fractionated spent broth supernatant, and fraction b and c showed similar level of adherence. Fraction d showed highest level of adherence and as shown in Fig. 7.3.16 D, almost all Ht-29 cells in the monolayer cells were covered by the bacterial cells. Fraction e showed lower levels of adherence as compared to fraction d. Adherence caused by fraction e was substantially low as compared with that caused by all other fractions. These observations show that several proteins >30,000 kD would support the adherence while best adherence promoter had a molecular size of 30,000-50,000 kD.

Figures 7.3.17 A-E show the levels of adherence of *L. acidophilus* 2415 when applied on Ht-29 monolayer cells alongwith whole spent broth or various fractions. As

[2]The fractions were marked a, b, c, d, and e and were described as follows: Bacterial cells suspended in, untreated or non-fractionated spent broth supernatant (a); >50,000 kD fraction of the supernatant (b); <50,000 kD fraction of the supernatant (c); 30,000-50,000 kD fraction of the supernatant (d); <30,000 kD fraction of the supernatant (e).

compared with the unfractionated spent broth (fraction a), fraction b with a molecular size >50,000 kD did not produce any adherence. Fraction c with a molecular size of <50,000 kD showed high level of adherence. However, fraction d (30,000-50,000 kD) did not produce any adherence. However, fraction <30,000 showed higher level of adherence as compared with those produced by fractions a and c. This shows that the adherence of *L. acidophilus* 2415 is supported by a proteine of <30,000 kD. Further, unlike in *B. infantis* 1912, proteins of larger size did not contribute to the adherence at all.

Overall, it can be concluded that different probiotic bacteria adhere to intestinal surfaces at different levels. Various proteins of bacterial origin as well as polysaccarides mainly produced by Ht-29 cells support the adherence of probiotic bacteria. Among the 15 strains of probiotic bacteria *B. longum* 1941, *B. infantis* 1912 and *L. acidophilus* 2415 showed best levels of adherence.

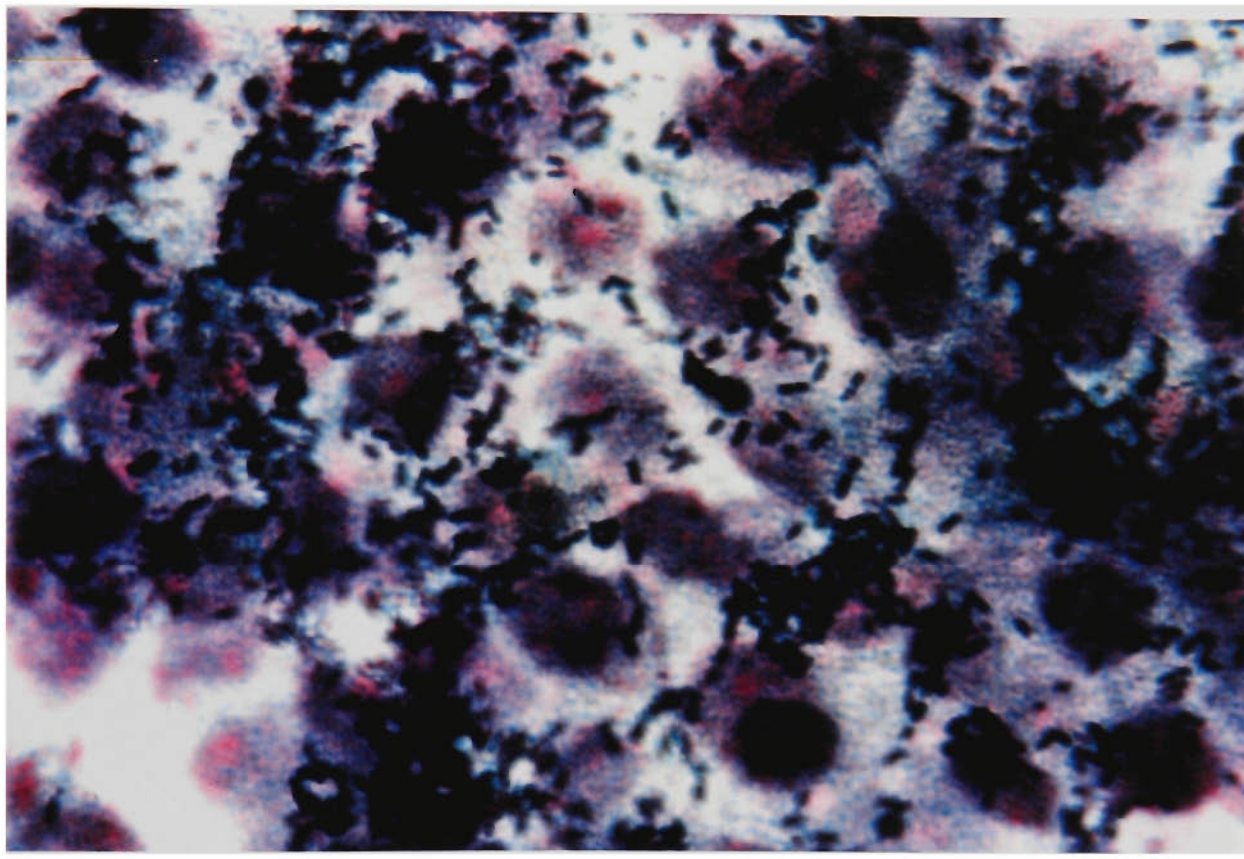


Fig 7.3.15. A Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with non-fractionated spent broth supernatant (magnification x 1000).

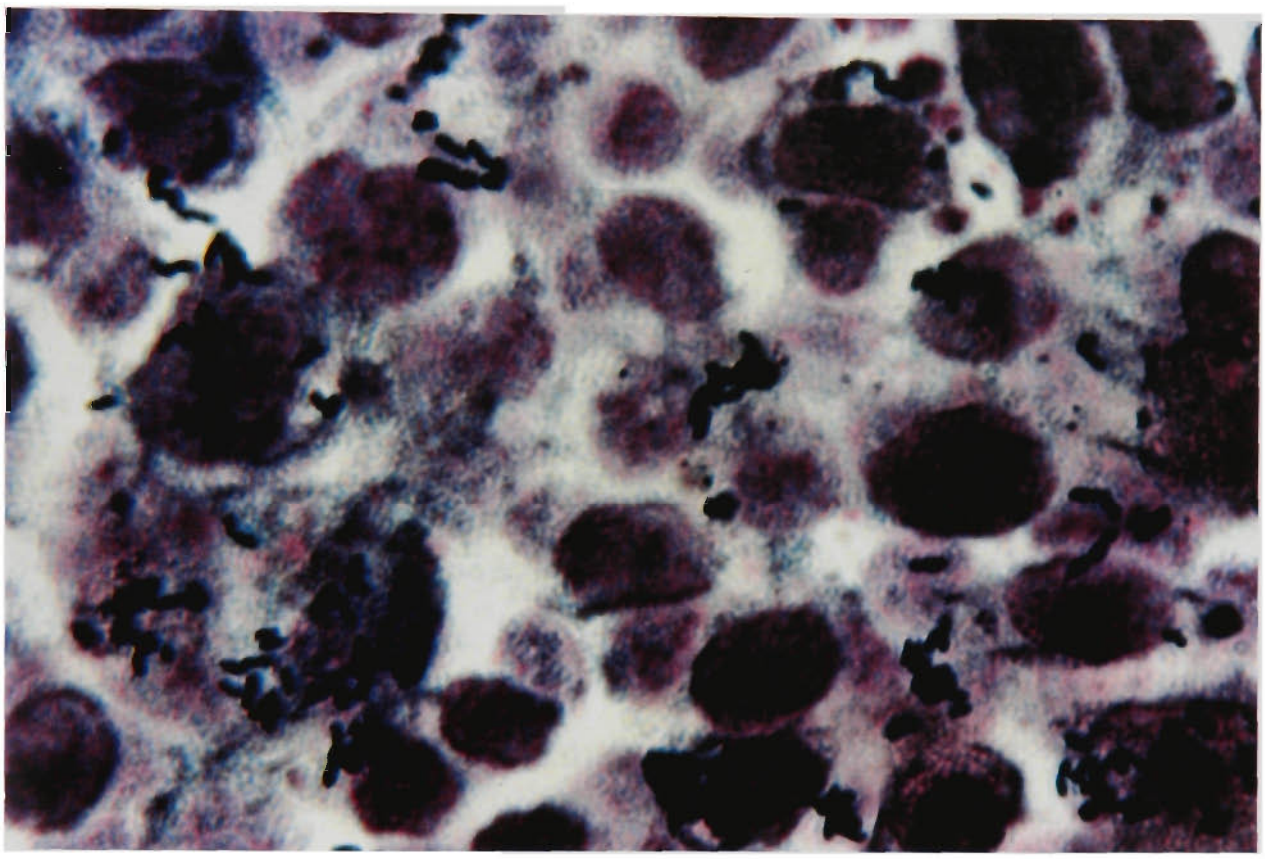


Fig. 7.3.15. B Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with >50,000 kD fraction of the spent broth supernatant (magnification x 1000).

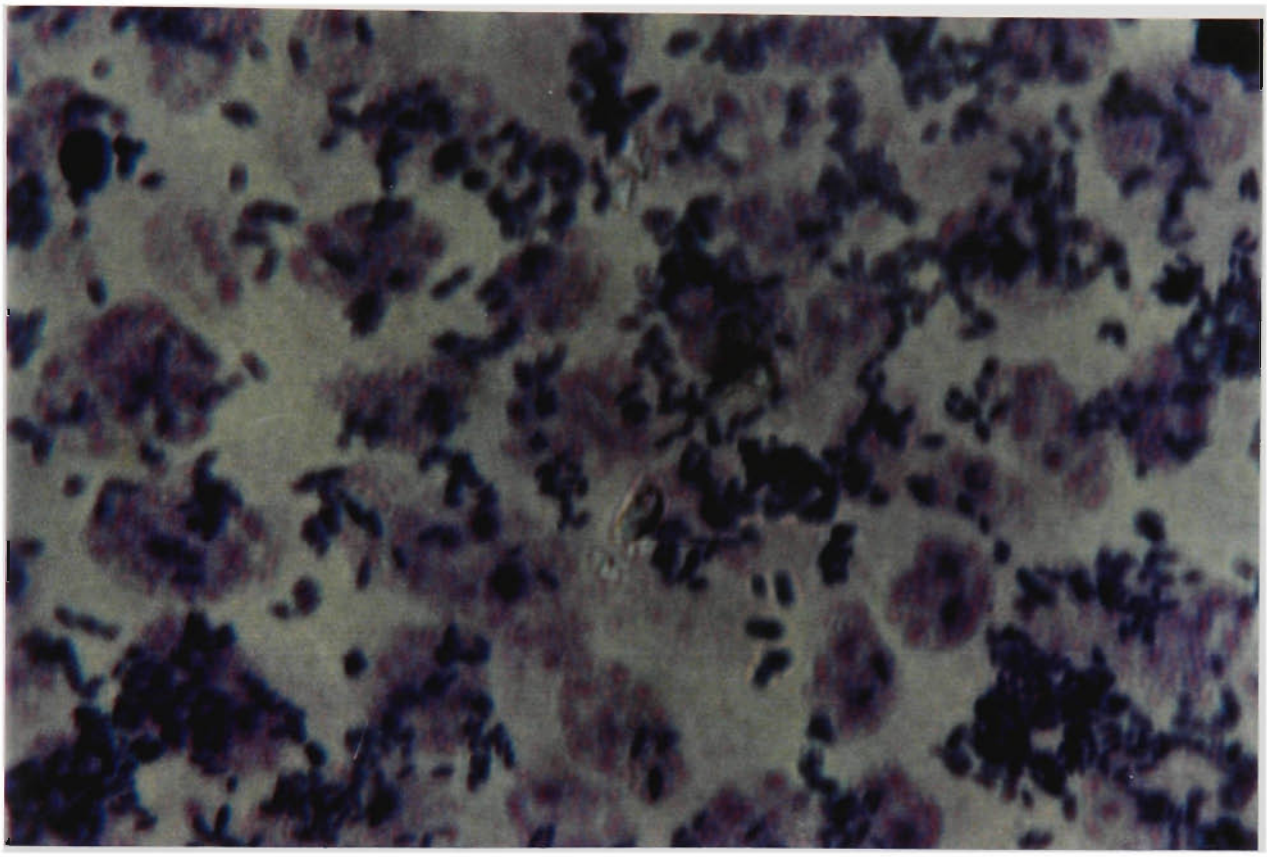


Fig. 7.3.15. C Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with <50,000 kD fraction of the spent broth supernatant (magnification x 1000).

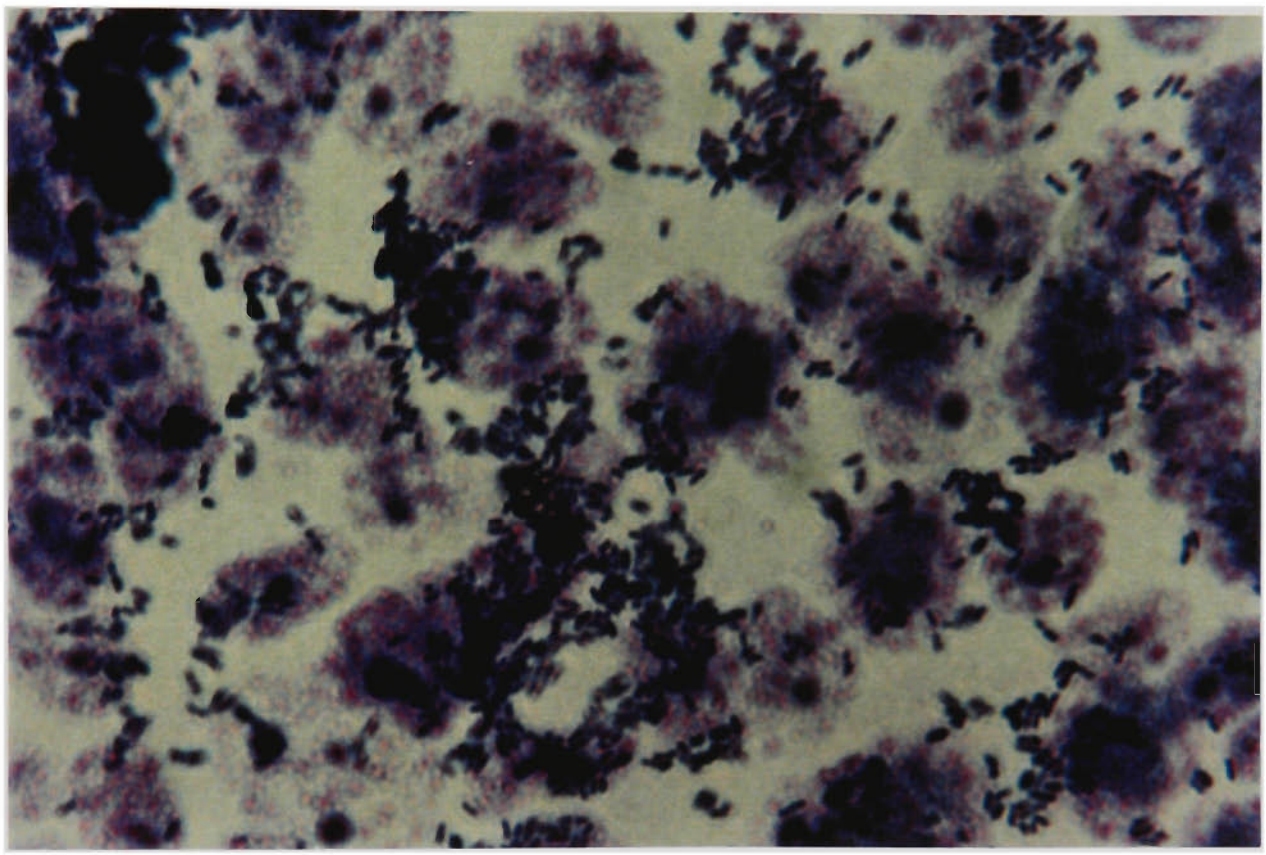


Fig 7.3.15. D Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with 30,000-50,000 kD fraction of the spent broth supernatant (magnification x 1000).

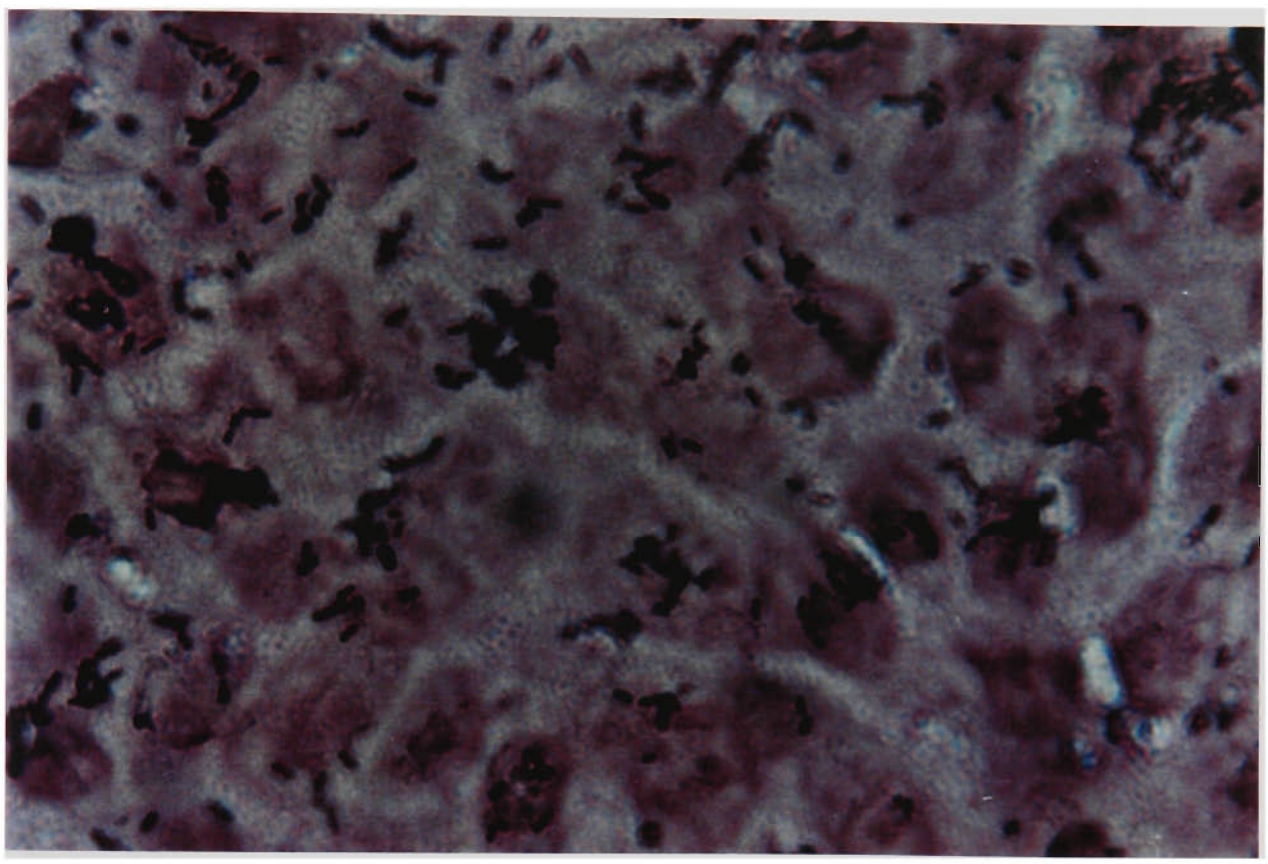


Fig.7.3.15. E Adherence of *B. longum* 1941 to Ht-29 monolayer cells when added with <30,000 kD fraction of the spent broth supernatant (magnification x 1000).

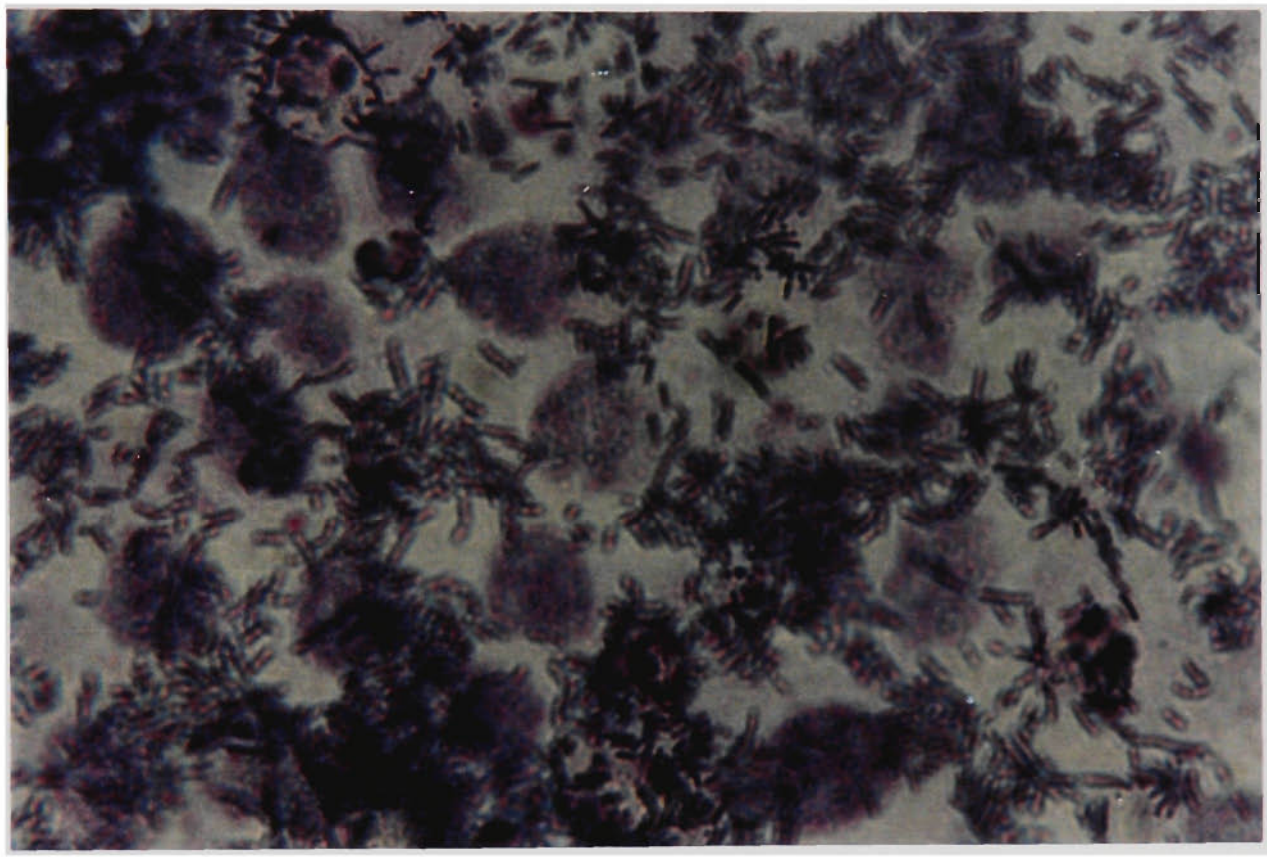


Fig. 7.3.16. A Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with non-fractionated spent broth supernatant (magnification x 1000).

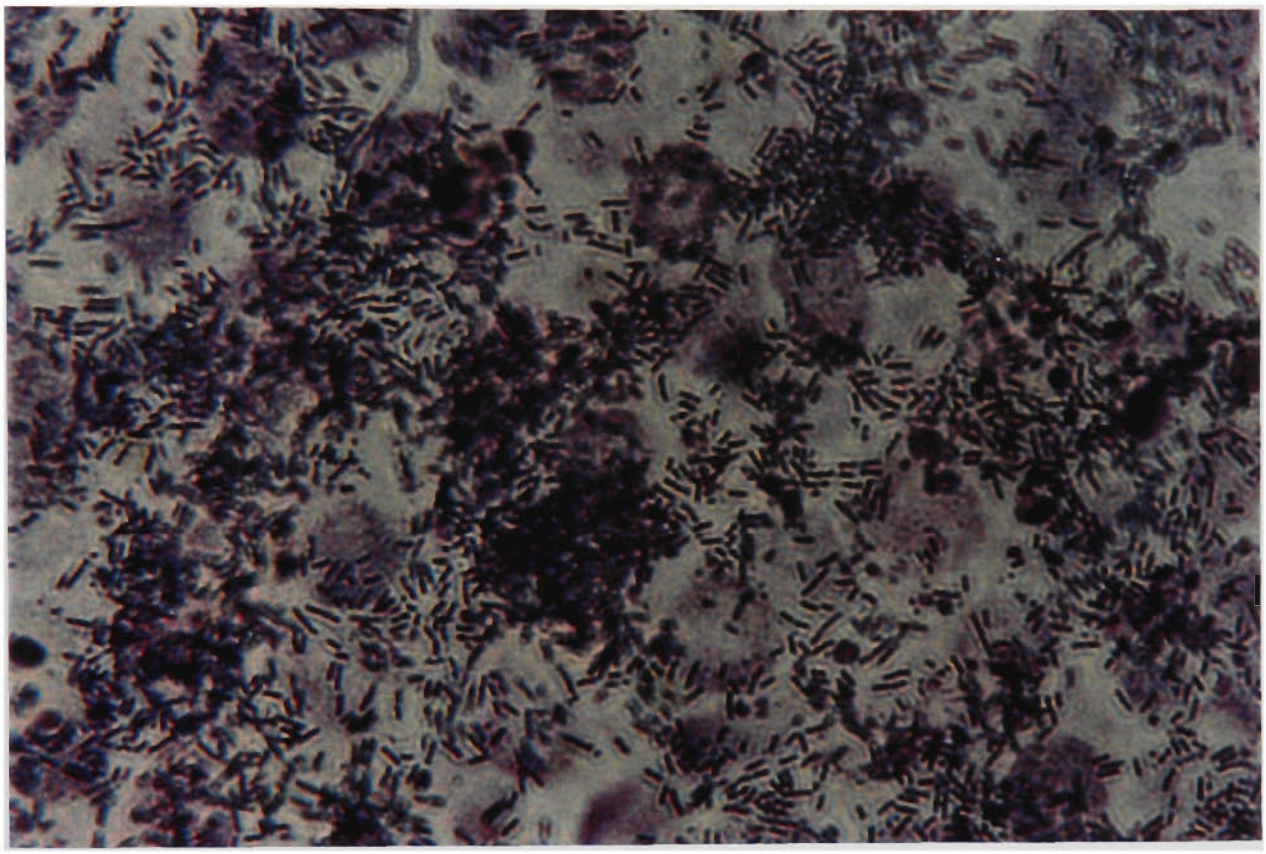


Fig.7.3.16. B Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with >50,000 kD fraction of the spent broth supernatant (magnification x 1000).

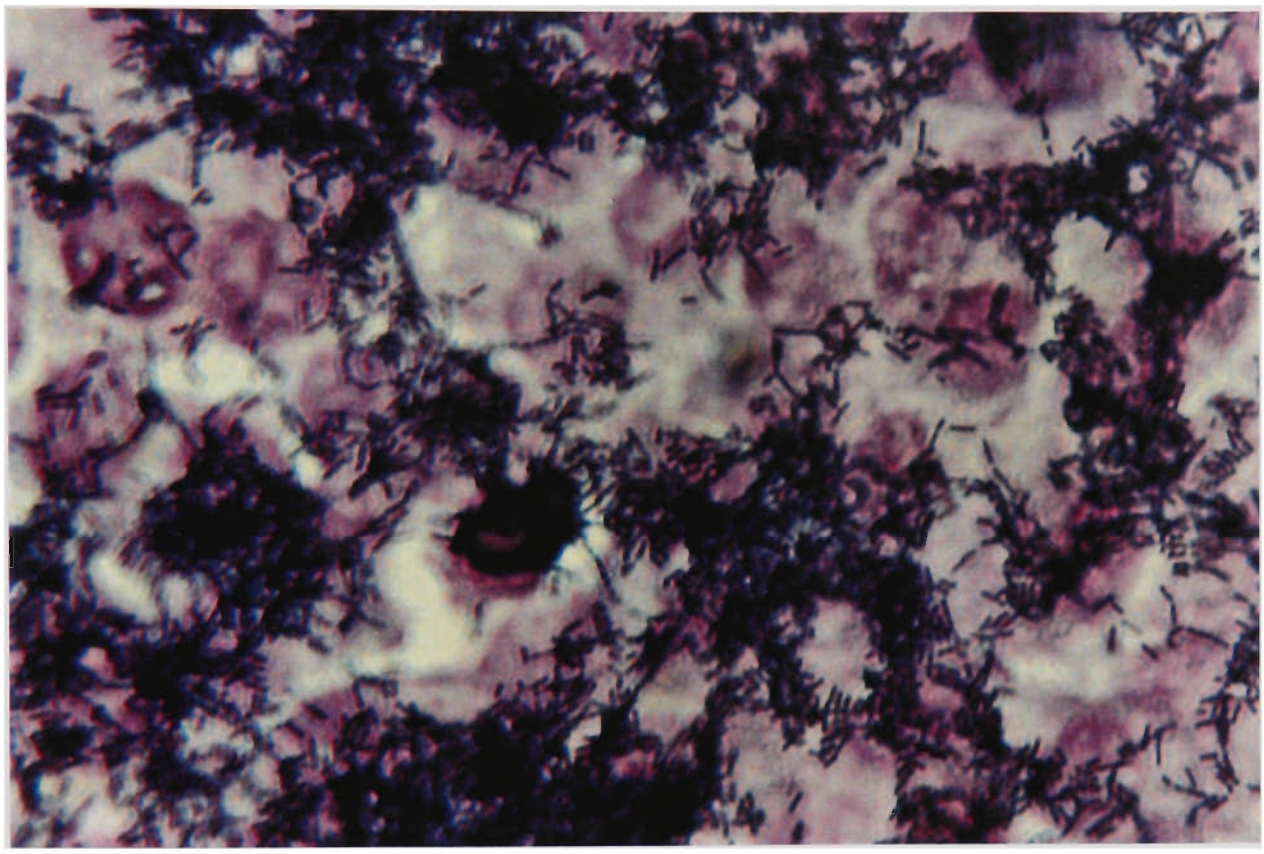


Fig.7.3.16. C Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with <50,000 kD fraction of the spent broth supernatant (magnification x 1000).

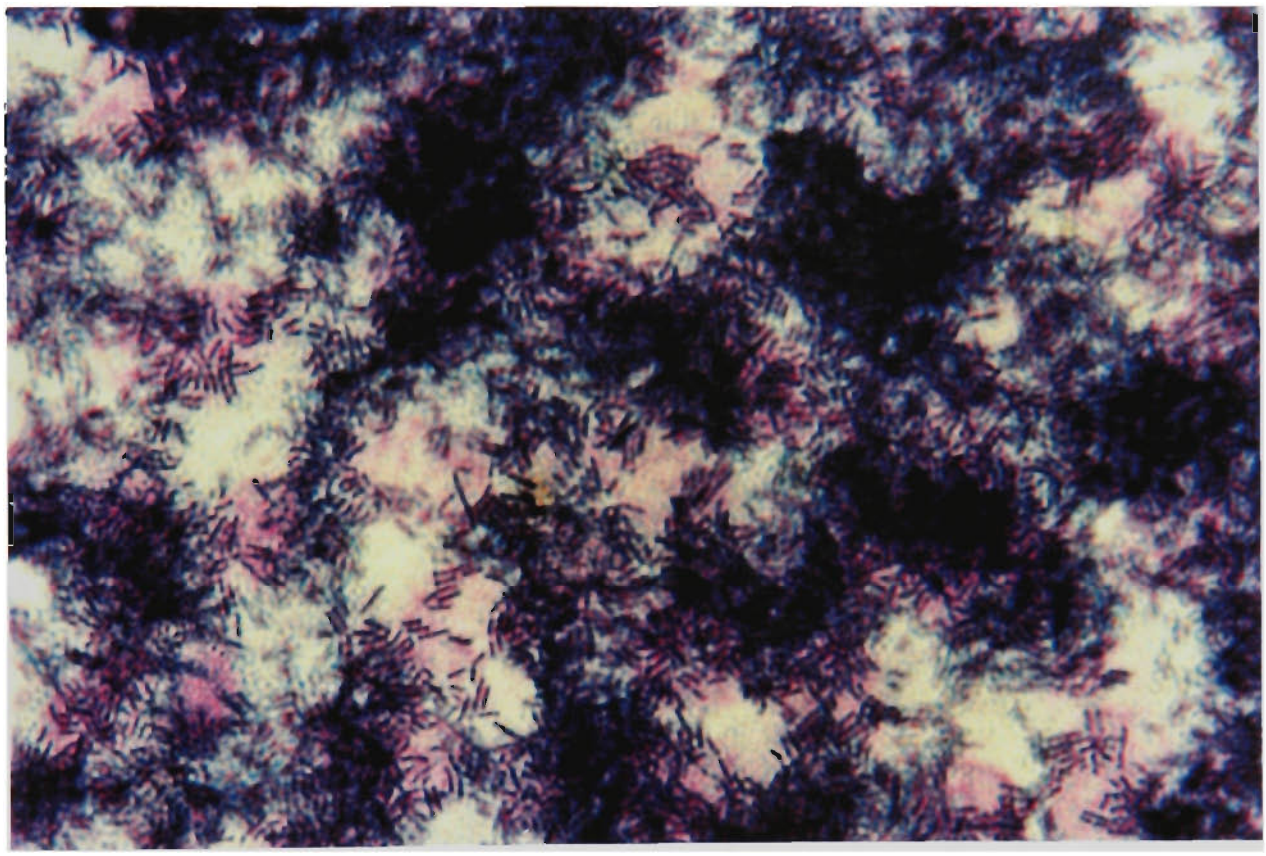


Fig.7.3.16 D Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with 30,000-50,000 kD fraction of the spent broth supernatant (magnification x 1000).

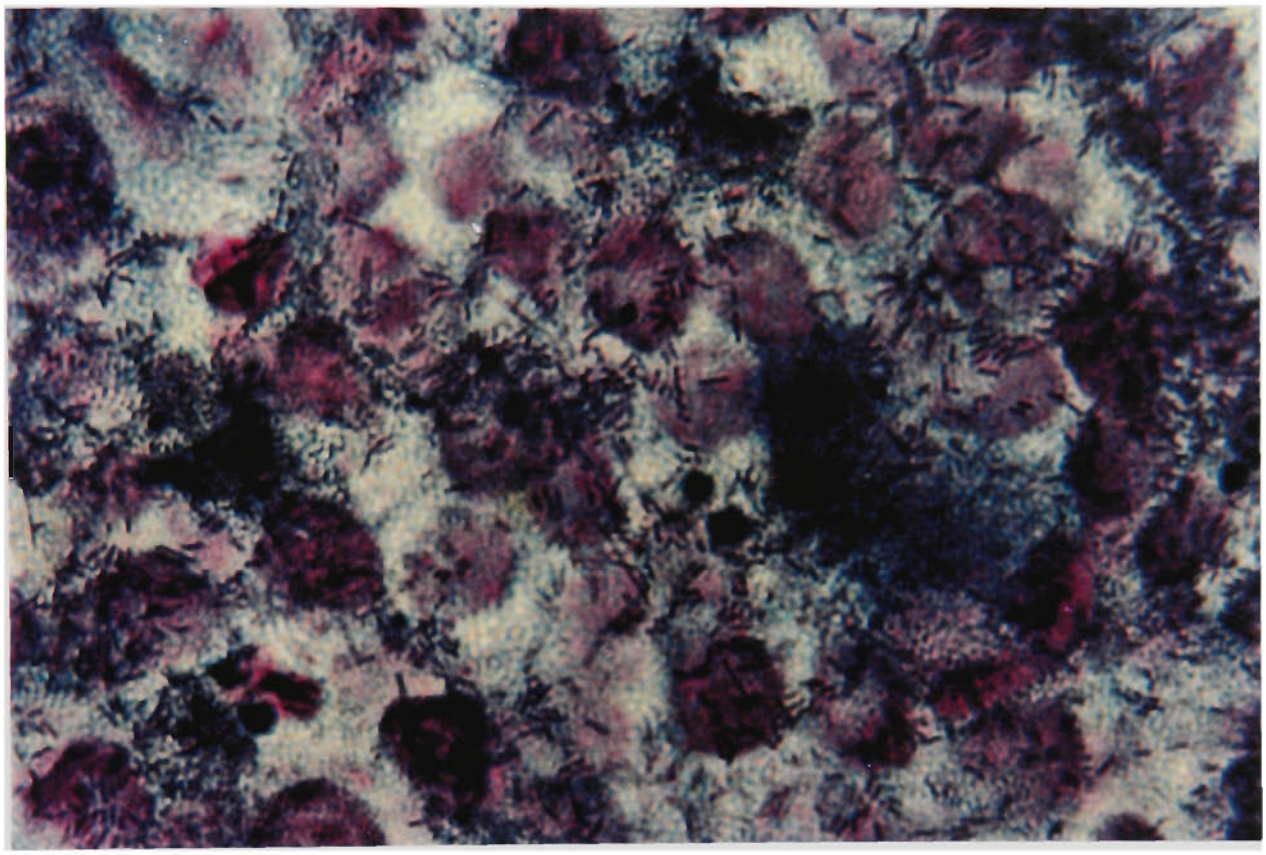


Fig. 7.3.16. E Adherence of *B. infantis* 1912 to Ht-29 monolayer cells when added with <30,000 kD fraction of the spent broth supernatant (magnification x 1000).

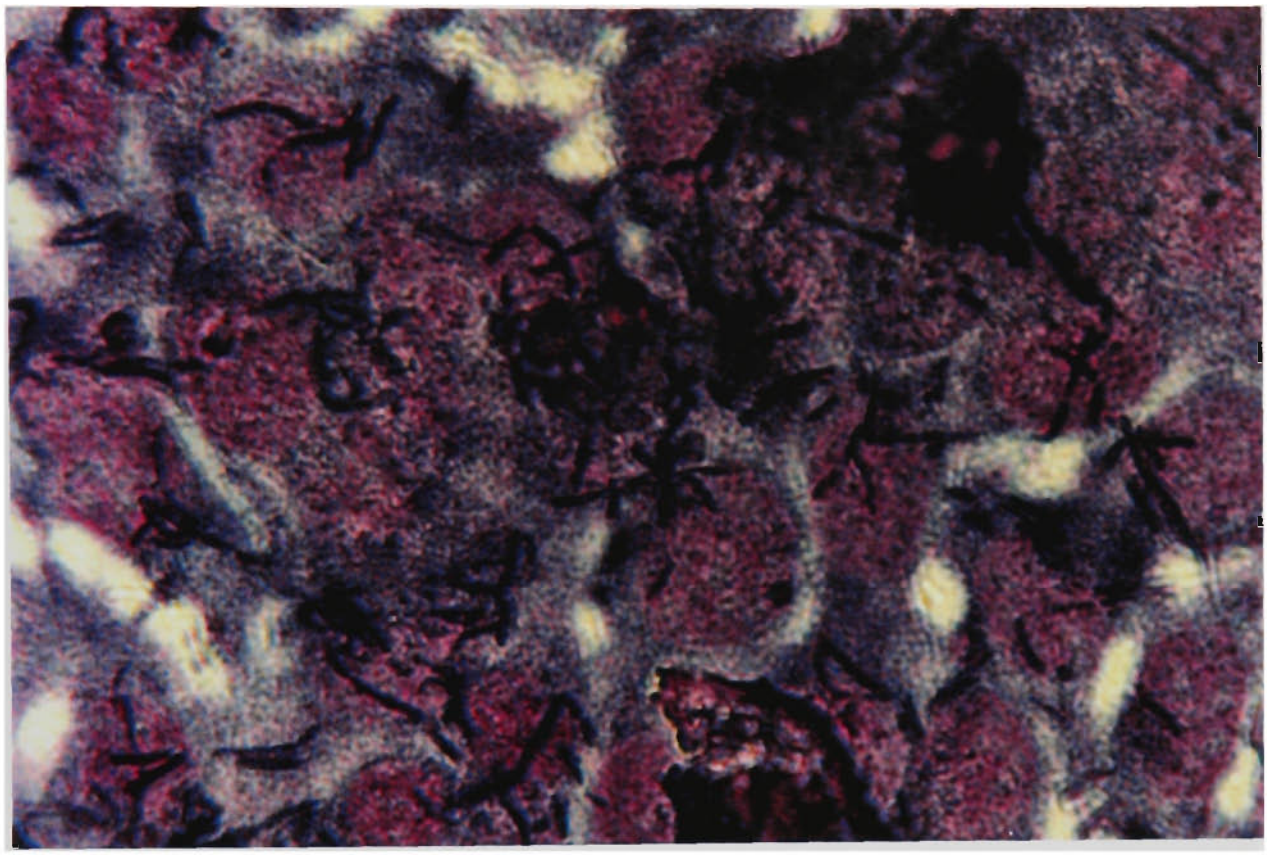


Fig 7.3.17. A Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells when added with non-fractionated spent broth supernatant (magnification x 1000).

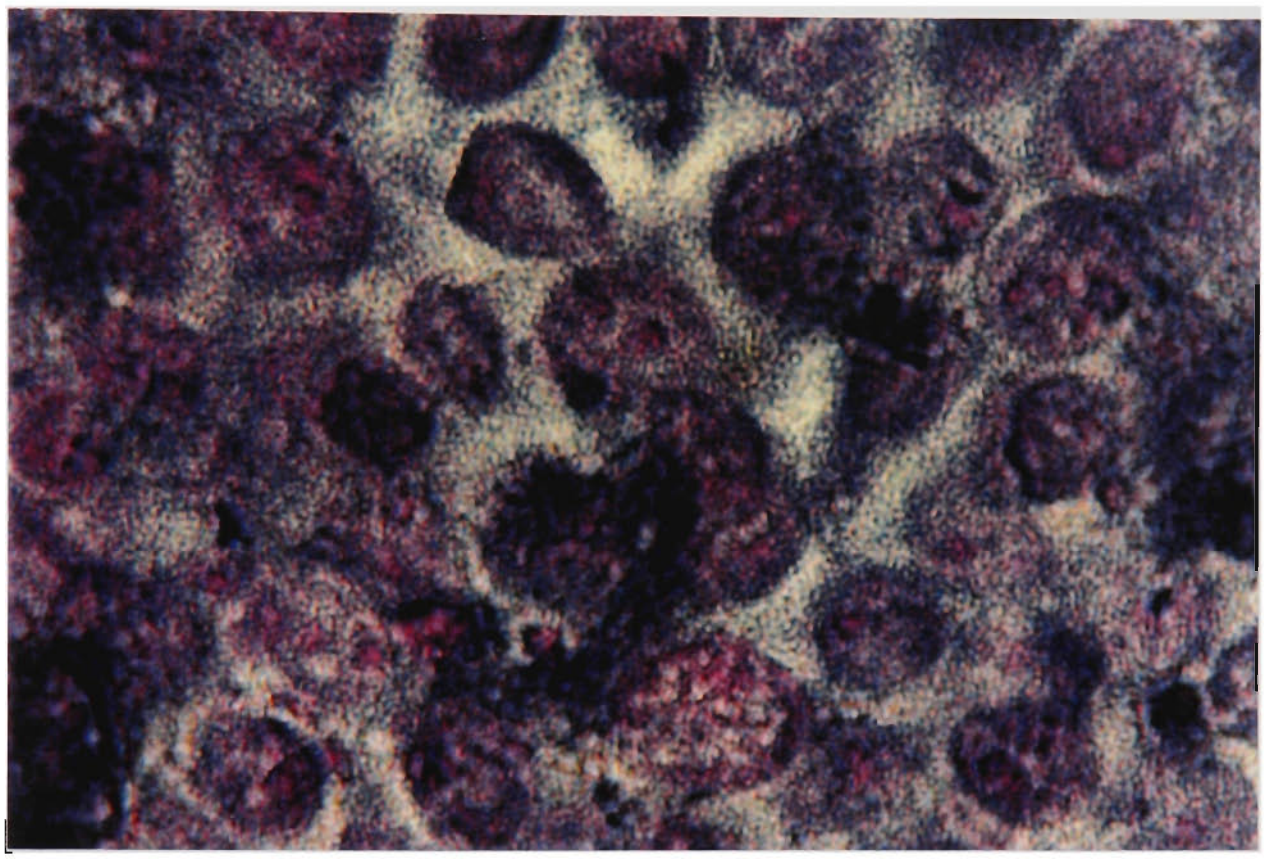


Fig. 7.3.17. B Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells when added with >50,000 kD fraction of spent broth supernatant (magnification x 1000).

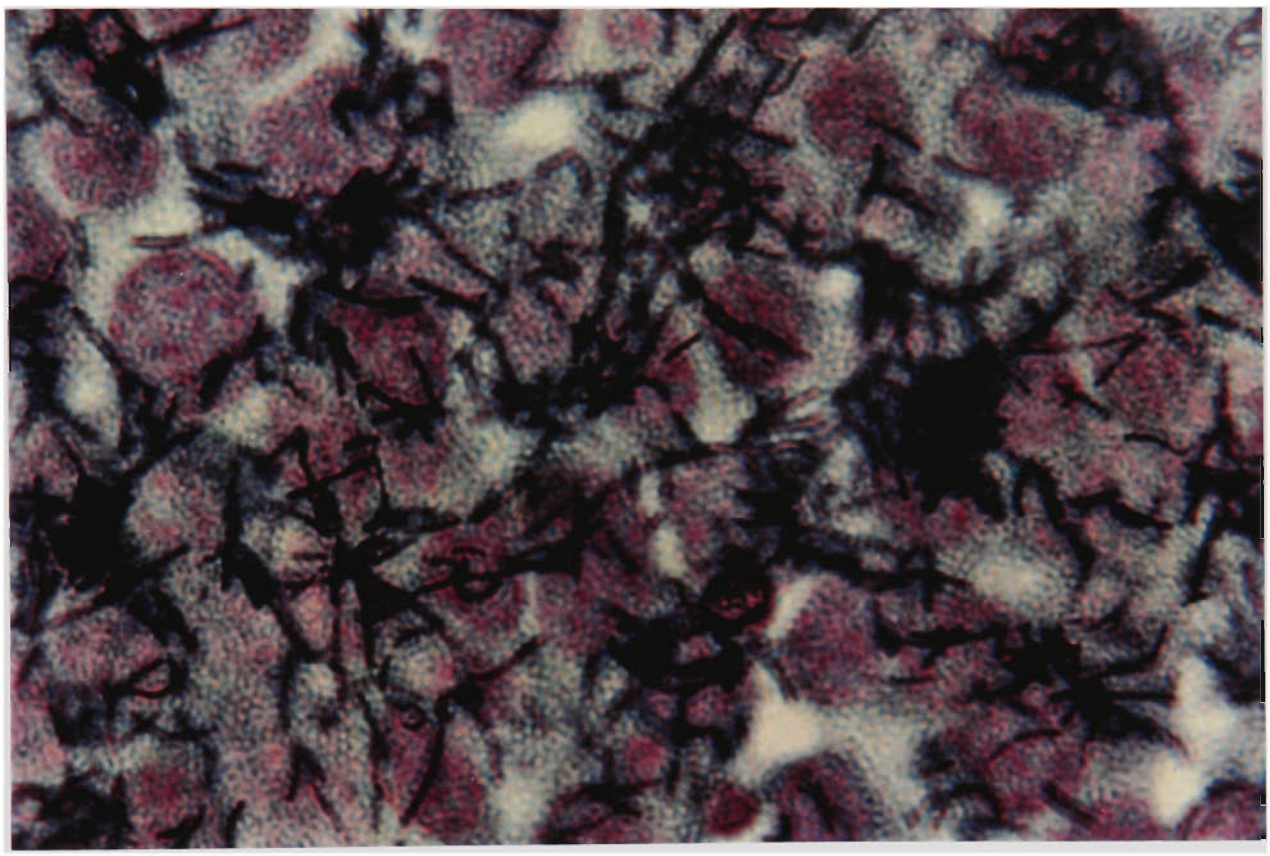


Fig. 7.3.17. C Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells when added with <50,000 kD fraction of the spent broth supernatant (magnification x 1000).

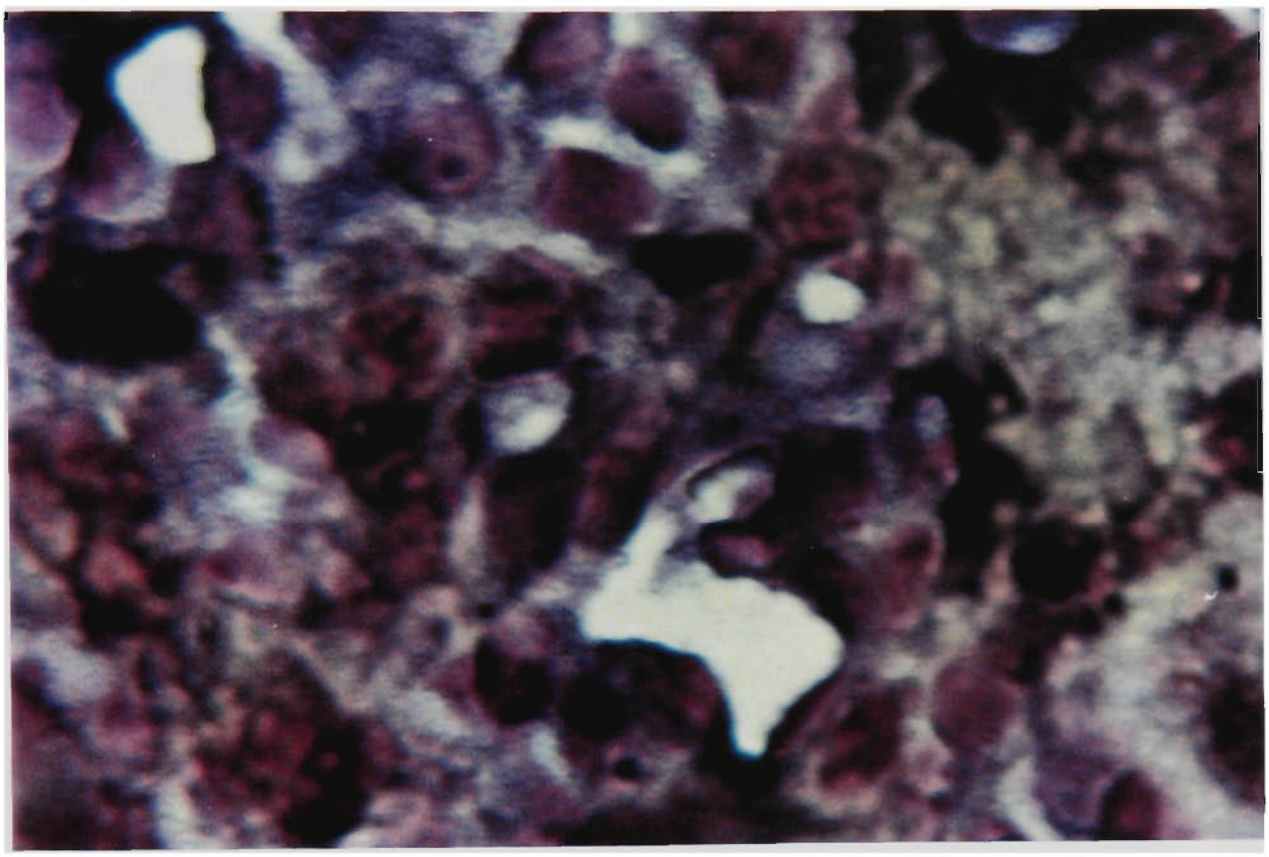


Fig. 7.3.17 D Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells when added with 30,000-50,000 kD fraction of the spent broth supernatan (magnification x 1000).

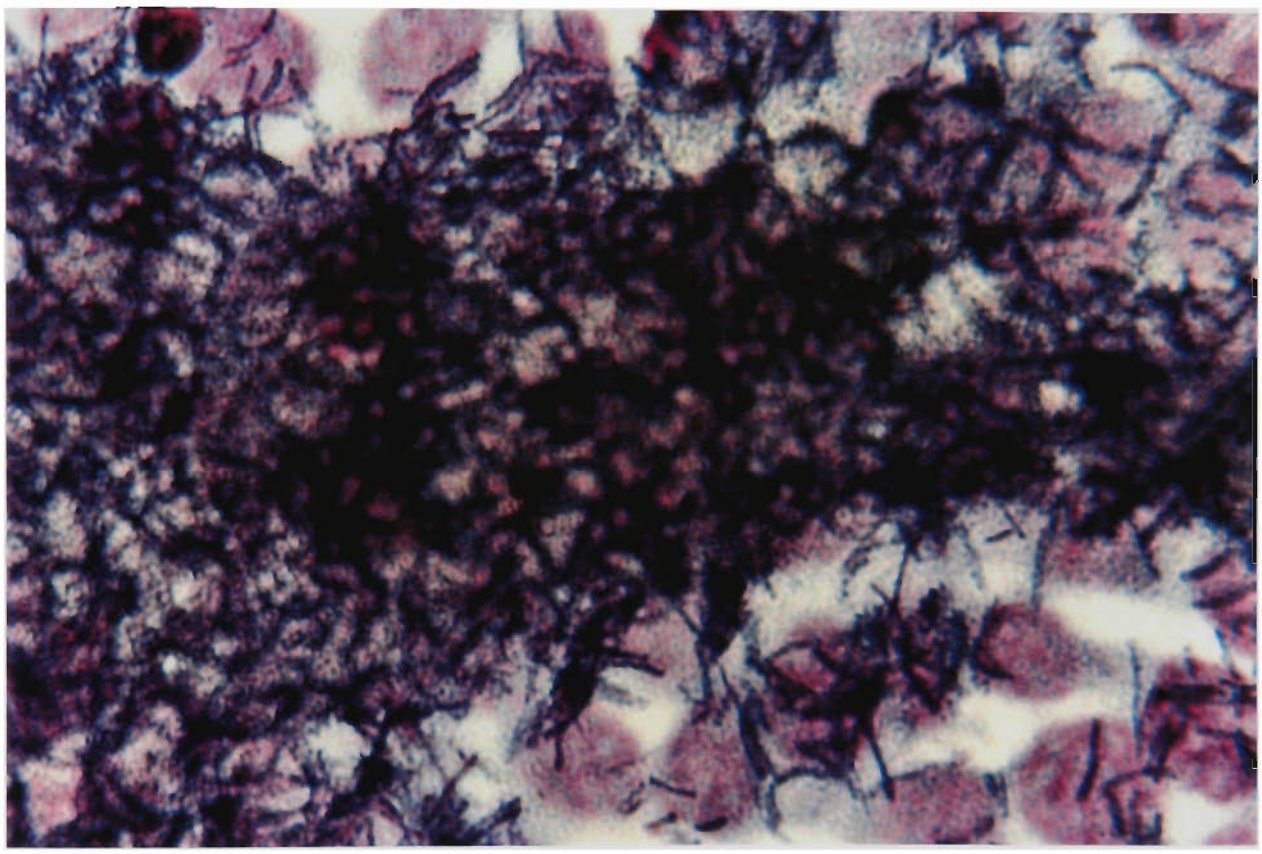


Fig. 7.3.17. E Adherence of *L. acidophilus* 2415 to Ht-29 monolayer cells when added with <30,000 kD fraction of the spent broth supernatant (magnification x 1000).

7.3.3.4. Electron microscopic study of adherence of selected strains of probiotiic bacteria

Pattern of adherence of selected strains of probiotic bacteria to Ht-29 cell monolayer cells was studied using a electron microscope. Figures 18-22 illustrate the adherence of these bacteria to the Ht-29 cells. Specimens were sliced vertically and the electron micrographs were taken using a transmission electron microscope.

Fig 18 shows a vertical cross section of an adhering probiotic bacterial cell (*B. infantis* 1912) to an Ht-29 monolayer cell. As shown, adherence seems be caused by deep embedding of the bacterial cell on the surface of Ht-29 cell. It is also evident that there is a thick layer between the two types of cells which could be a polysaccahride-protein structure which is known to give strong binding between two cells. Fig. 19 shows the formation of an inter-cellular bridge between a probiotic cell (*B. adolescentis* 1920) and an Ht-29 cell. The bridge seems to be extended from the Ht-29 cells. Fig. 20 shows the attachment of a *B. bifidum* 1900 cell to an Ht-29 cell. The formation of a wide bridge between the two cells is visible. Fig. 21 shows the attachment of *B. longum* 1941 cells to Ht-29 monolayer cells. Fig. 22 shows the attachment of *L. acidophilus* 2415 to Ht-29 monolayer cells. The bacterial cell seems to be embedded deep into the Ht-29 cell.

The electron micrographs confirm that intercellular bridging material is formed when probiotic cells are attached to the Ht-29 monolayer cells. This material could be a combination of 2 or more proteins or a protein-polysaccaride substance originating from bacterial cells and from Ht-29 cells as shown in section 7.3.3.3.

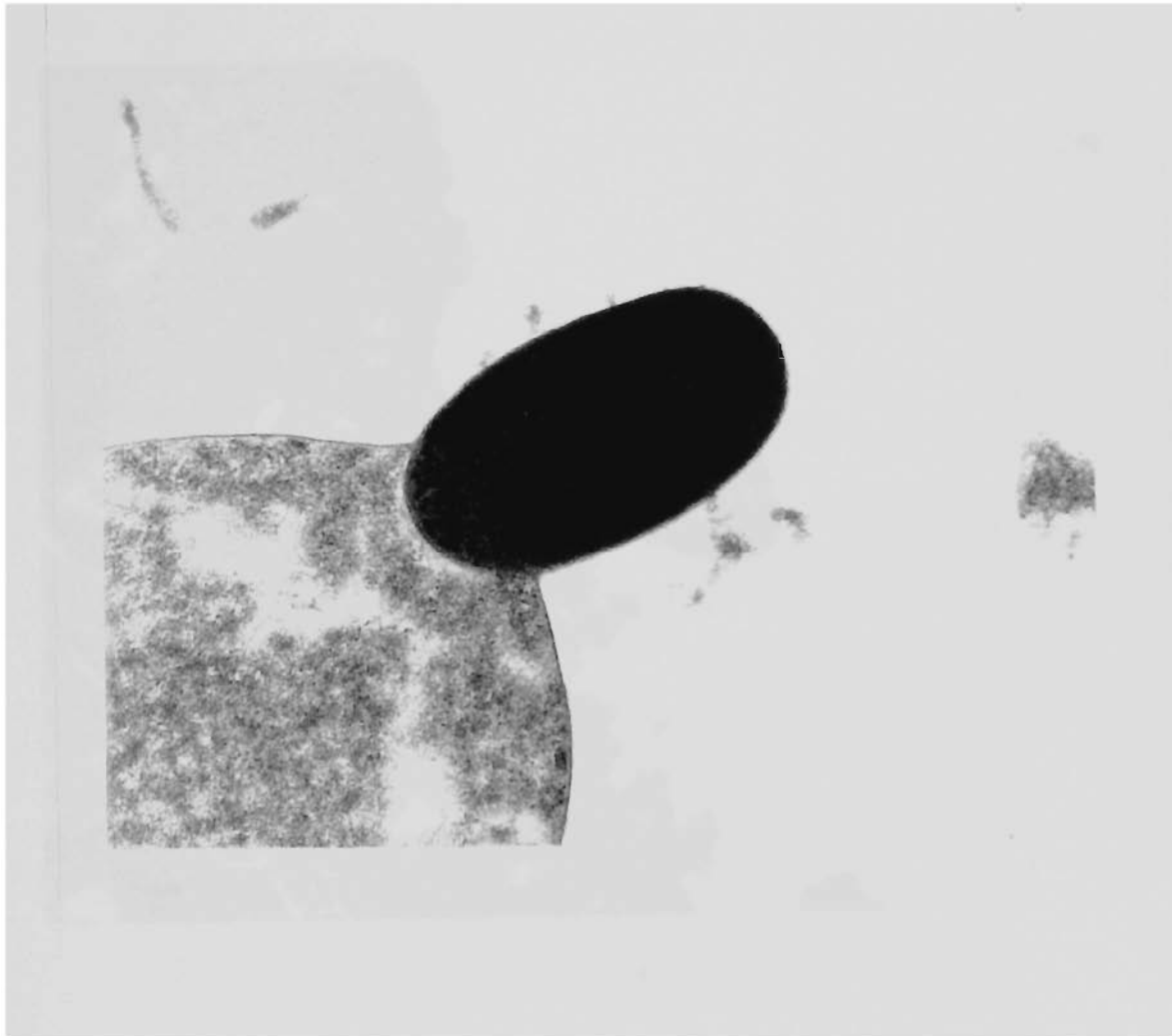


Fig. 7.3.18 Transmission electron micrograph of a vertical cross section of an adhering probiotic bacterial cell (*B. infantis* 1912) to an Ht-29 monolayer cell (magnification x 55,000).

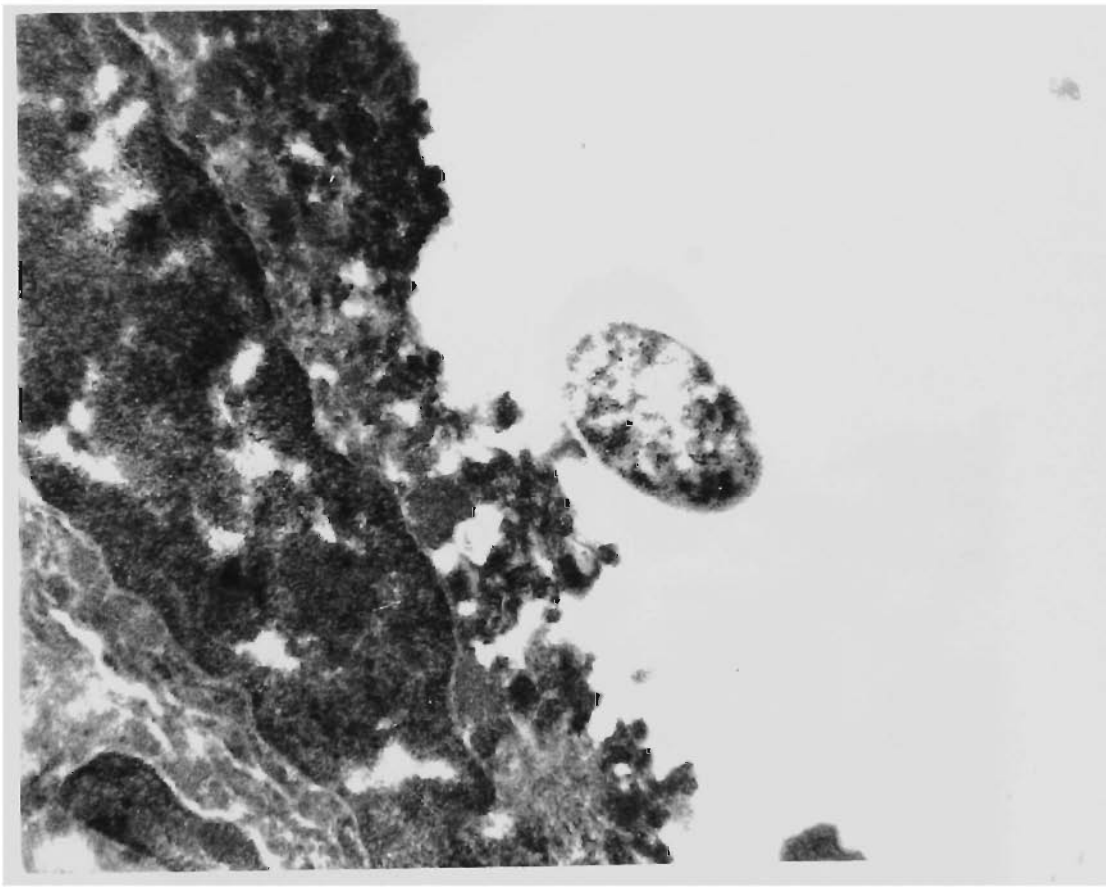


Fig. 7.3.19. Transmission electron micrograph of a vertical cross section illustrating formation of intracellular bridge between *B. adolescentis* 1920 and an Ht-29 cell (magnification x 33,000).

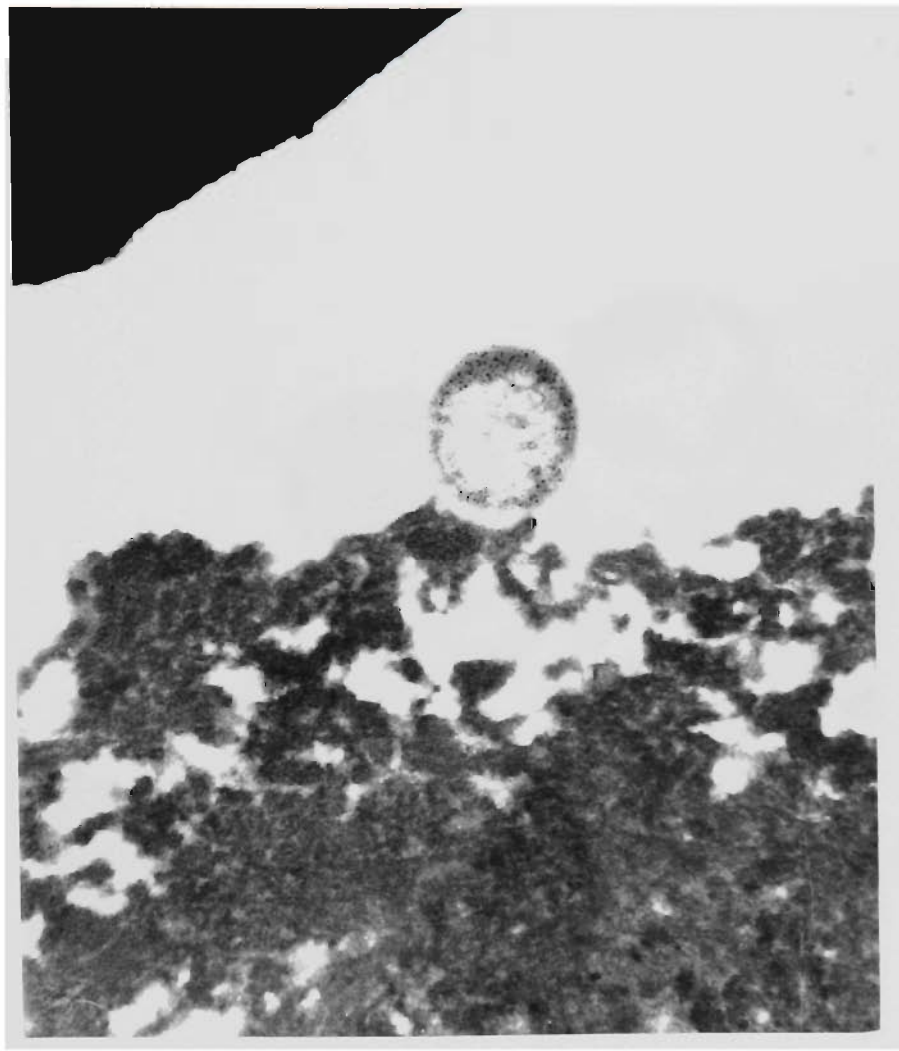


Fig. 7.3.20. Transmission electron micrograph of a vertical section showing adherecence of *B. bifidum* 1900 to an Ht-29 monolayer cell (magnification x 33,000).

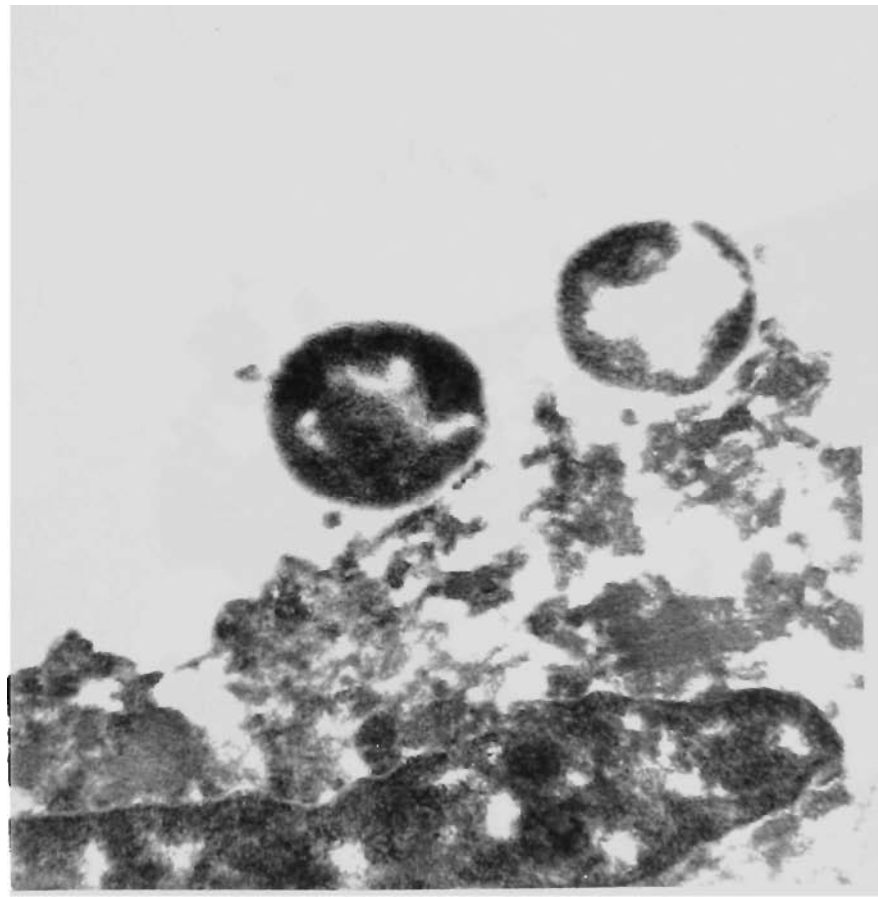


Fig. 7.3.21. Transmission electron micrograph of a vertical cross section showing adherence of *B. longum* 1941 to an Ht-29 monolayer cell (magnification x 33, 000).

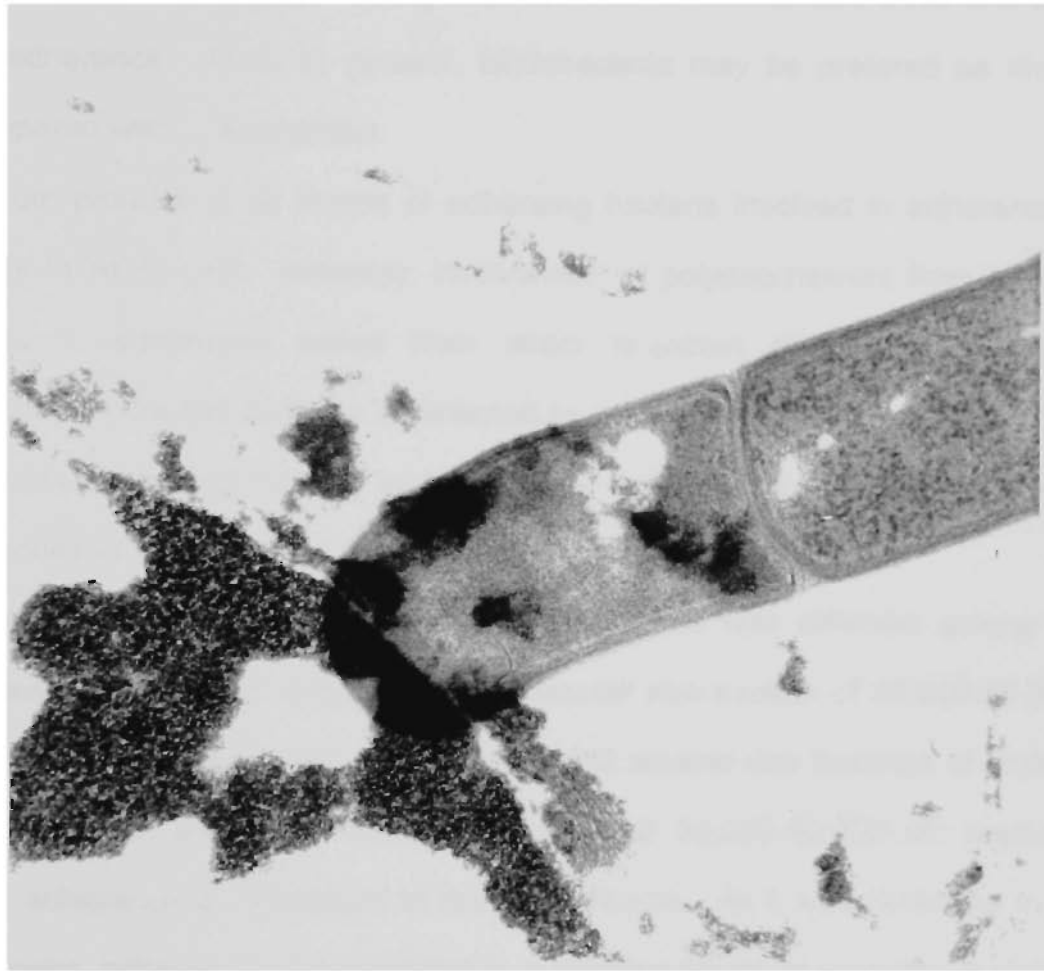


Fig. 7.3.22. Transmission electron micrograph of a vertical cross section showing adherence of *L. acidophilus* 2415 to an Ht-29 cell from a monolayer (magnification x 55,000).

7. 3. 4. Conclusions

Bifidobacteria showed better adherence to Ht-29 colonic cancer monolayer cells as compared with *L. acidophilus*. Among bifidobacteria, *B. infantis* 1912 and *B. longum* 1941 showed highest level of adherence. Among the strains of *L. acidophilus*, 2400 and 2415 showed better adherence. Thus, in general, bifidobacteria may be prefered as dietary adjuncts as compared with *L. acidophilus*.

Spent broth proteins of all strains of adhereing bacteria involved in adherence of probiotic bacteria to Ht-29 cells. However, involvement of polysaccharides from bacteria and Ht-29 cells in adherence varied from strain to strain of probiotic bacteria. Polysaccarides from Ht-29 cell surfaces contributed to adherence more as compared with the polysaccharides originating from the bacterial cells. In *B. infantis* 1912 and *B. longum* 1941, polysaccarides of both bacterial and Ht-29 origin involved in adherence.

The molecular size of proteins involved in adherence was different among the strains of probiotic bacteria. In *B. longum* 1941, molecular size fraction of 30,000-50,0000 kD was responsible for adherence and in *B. infantis* 1912 several size fractions of proteins > 30,000 kD involved in adherence while the fraction of 30,000-50,000 kD produced highest effect in adherence as compared to the other strains. As it was illustrated in the electron micrographs, adherence was mediated by a bridging structure (possibly a protein-polysaccharide structure) formed between bacterial and Ht-29 cells.

7.4. Effect of probiotic bacteria on growth of cultured human colon cancer cell line, Ht-29.

7.4.1. Introduction

Studies have suggested that milk fermented with probiotic bacteria may affect the development of colon cancer (Hosono *et al.*, 1990). Effects of these organisms in differentiation of cancer cell lines have been studied recently. HT-29 human colon cell line has been used by a few workers to study the anticarcinogenic trends of probiotic bacteria.

Under normal culture conditions Ht-29 cells are undifferentiated: morphologically they grow as a multilayer of unpolarized cells and functionally they do not express any particular characteristic of epithelial intestinal cells. Treatment with sodium butyrate resulted in the occurrence of various differentiated phenotypes. When Ht-29 cells were permanently passaged in the presence of sodium butyrate, they differentiated showing marked reduction in glucose utilisation and increase in glycogen accumulation (Zweibaum, 1991). After exposing to sodium butyrate for 3 weeks, Augeron and Laboisse (1984) showed that Ht-29 cells differentiated permanently to functional enterocytes. When these cells undergo differentiation, they get polarised and secrete large amounts of mucin. If the cancerous characteristics of these cells reduce when they differetiate, it may also be expected that the rate of growth of these cells also would reduce during differentiation. The objective of this study was to determine whether the selected strains of probiotic bacteria would affect the rate of growth of Ht-29 cells.

7.4.2. Materials and Methods

7.4.2.1. Culture of Ht-29 cells

Ht-29 cells were obtained and cultured as described in section 2.13. Cell line was passaged as described in section 2.13.6.

7.4.2.2. Preparation of probiotic cultures

Selected strains of probiotic bacteria (*B. bifidum* 1900, *B. infantis* 1912, *B. adolescentis* 1920 and *B. longum* 1941 and *L. acidophilus* 2415) were grown for 18 h in 12% reconstituted skim milk. The pH of the cultures were adjusted to 7.0 by adding 1N NaOH. The neutralised cultures in milk were distributed in ependorph tubes and stored in a -20^{0}C freezer.

7.4.2.3. Treatment of Ht-29 cells with probiotic cultures

Ht-29 cells were grown in 25 cm^2 T-flasks by adding 5mL of trypsinised cell suspension (1 x 10^6 cells/mL) into each flask followed by 10 mL of McCoy 5-A medium. The flasks were incubated at 37°C for 48 h and the medium was removed gently and fresh medium was added. Each flask was added with penicillin-G at a concentration of 1 IU/mL in order to prevent the growth of bacteria and subsequent acidification which may be harmful to the survival.

Thirty six cell culture T-flasks were used for each bacterial strain. One mL of thawed neutralised bacterial suspensions were added to 18 T-flasks and the other T-flasks (controls) were not added with the bacterial suspension. Every 24 h the cell culture media were changed and bacterial suspension was added newly. Cells were harvested from 3 flasks each time on day 1, 7, 14, 21, 28 and 35 using trypsin solution in order to segregate the intercellular bonds of Ht-29 cell monolayers. Similarly, 3 control flasks of cells (untreated with probiotic bacteria) were also harvested. Harvested Ht-29 cell suspensions from each flask was separately stained with trypan blue and the count of viable cells determined using a haemocytometer as described in section (2.13). Same procedure was followed after 14, 21, 28 and 35 days too.

7.4.3. Results and discussion

Growth of Ht-29 cells in the presence or absence of probiotic bacteria is illustrated in Figures 7.4.1. and 7.4.2. As shown in Fig. 7.4.1, Ht-29 cells showed about 10 times (1 log cycle) reduction in the number of cells in the presence of *B. infantis* 1900 as compared with the control. In the presence of *B. infantis* 1912 and *B. adolescentis* 1920 showed about 50 times (1.5 log cycles) reduction in the number of growing Ht-29 cells after 35 days of incubation at 37°C. As shown in Fig. 7.4.2., B. longum 1941 also showed about 10 times reduction in the growth of Ht-29 cells. However, in the presence of *L. acidophilus* 2415, the reduction in the growth of Ht-29 cells was < 5 time after 35 days of incubation at 37°C.

Reduction in the growth of Ht-29 cells in the presence of probiotic bacteria could be due to several reasons such as organic acids produced by probiotic bacteria, especially butyric acid, and competition for nutrients, especially for glucose. However, the effect of organic acids in reducing the growth of these cells is more justifiable as compared to the latter reason. As shown in Table 7.1.5. (in section 7.1.), *B. infantis* 1912 and *B. adolescentis* produced highest levels of butyric acids followed by *B. bifidum* 1900 and *B. longum* 1941. However, *L. acidophilus* 2415 did not produce butyric acid.

Several researchers have shown that when Ht-29 cells are treated with butyrate, they differentiate to absorptive or mucus secreting cells after an initial phase of mortality (Augeron and Laboisse, 1984; and Lesuffleur *et al.*, 1990). Differentiation of Ht-29 cell lines is evident by reduction of cell multiplication, polarisation, morphological changes such as formation of villi and increased production of metabolic enzymes (Zweibaum *et al.*, 1991).

7.4.4. Conclusions

In the presence of probiotic bacteria growth rate of Ht-29 cells reduced. This could mainly be due to differentiation of cells and losing their fast growing characteristic. This could be attributed to the butyric acid produced by the probiotic bacteria. This also could

be due to the competition for nutrients between bacterial cells and Ht-29 cells. However, *L. acidophilus* 2415 which did not produce butyric acid could not reduce the growth rate of Ht-29 cells as efficiently as the other strains which produced butyric acid. This can substantiate the fact that the presence of butyric acid in probiotic bacteria was responsible for reducing the growth rate of Ht-29 cells.

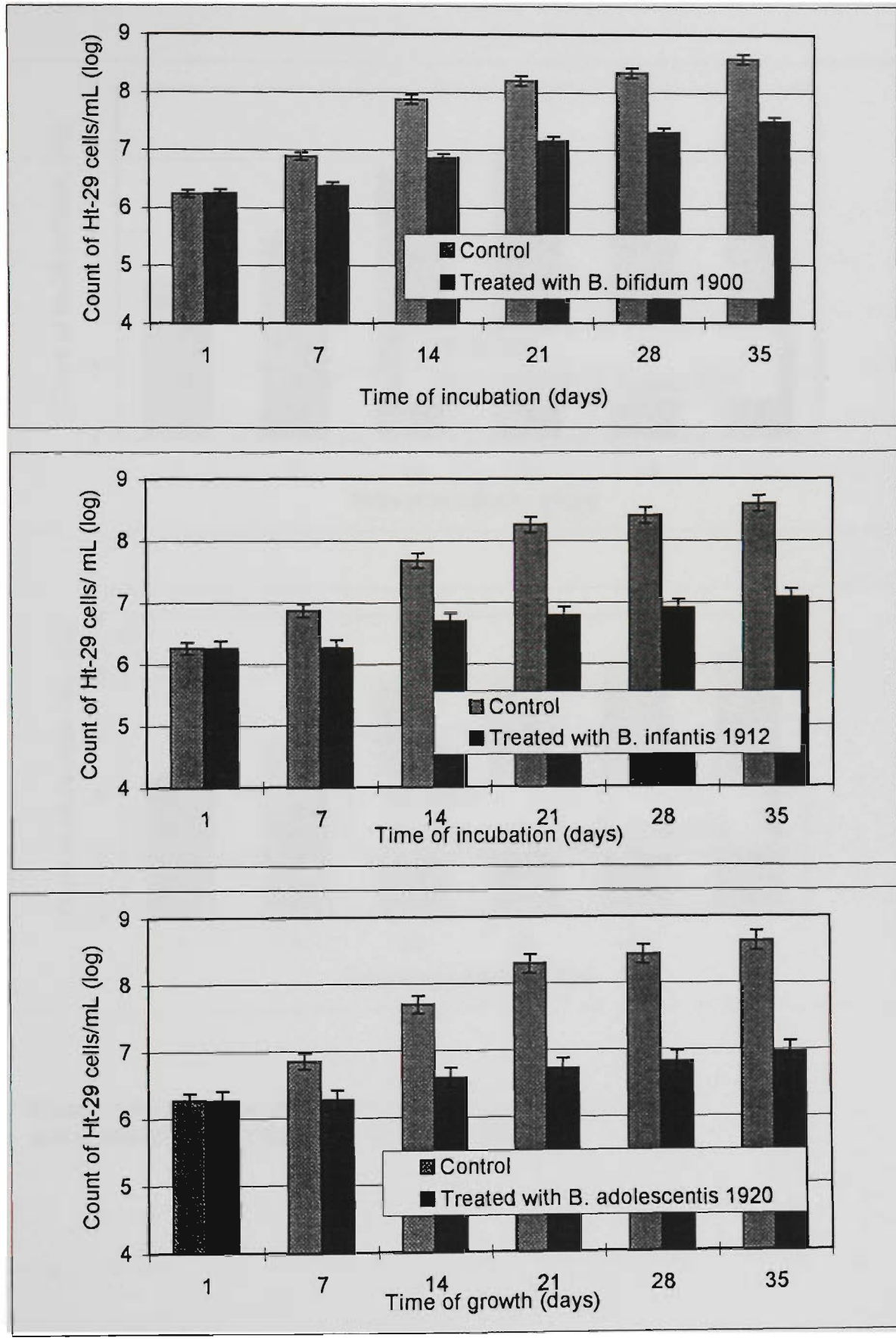
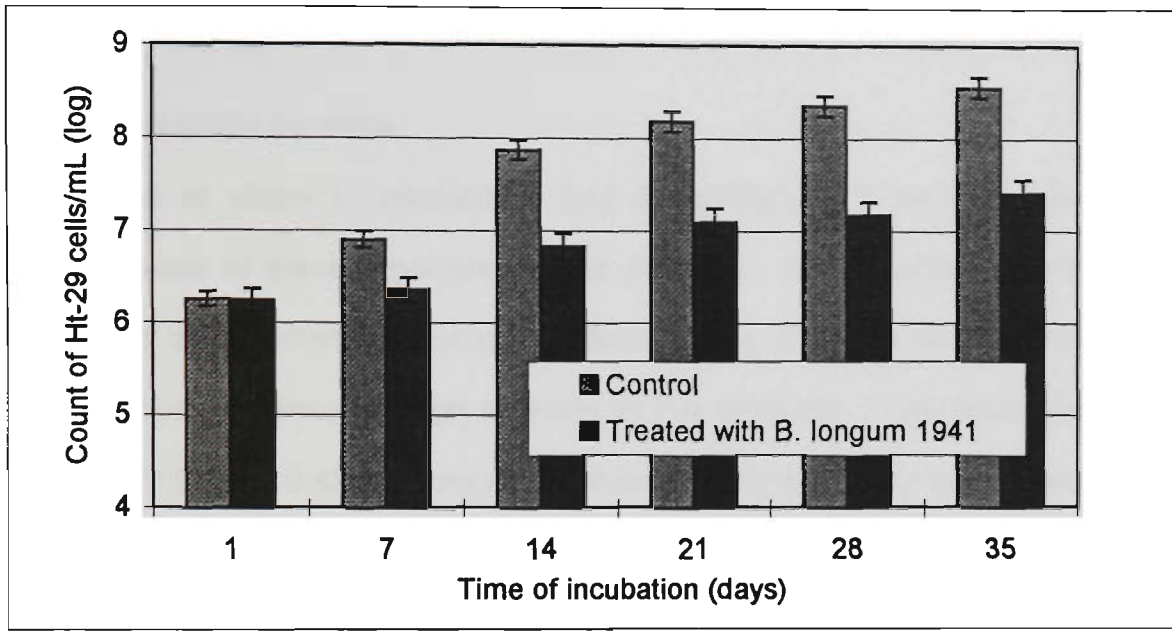


Fig. 7.4.1. Growth of Ht-29 cells in presence or absence of *B. bifidum* 1900, *B. infantis* 1912 and *B. adolescentis* 1920 in McCoy 5A growth medium



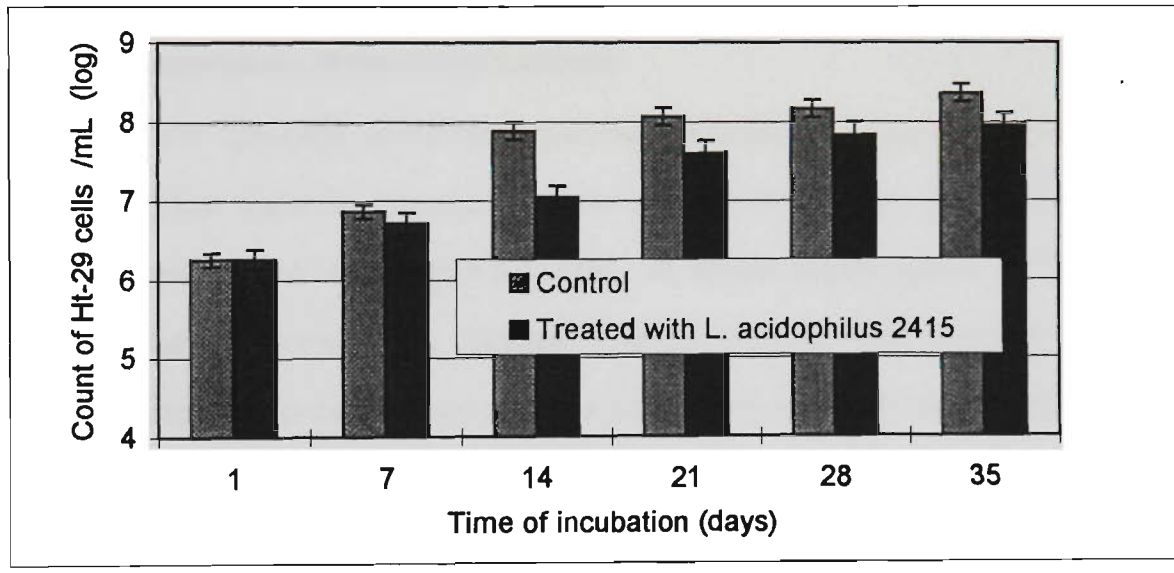


Fig. 7.4.2. Growth of Ht-29 cells in presence or absence of *B. longum* 1941, and *L. acidophilus* 2415in McCoy 5A growth medium

8.0. OVERALL CONCLUSIONS

8.1. Viability of probiotic bacteria

Enumeration of viable *L. acidophilus* and *B. bifidum* in 5 commercial yogurts showed variable levels of these organisms in the products. All the products showed a constant decline in the numbers of viable *B. bifidum* during 6 weeks storage, while the viability of *L. acidophilus* remained high in three of five products. The decrease in pH values of between 0.07 and 0.42 pH units during the storage period may have affected the viability of the organisms. The results suggested that higher solid levels seemed to have beneficial effect on the viability of the probiotic bacteria, especially *L. acidophilus*.

8.2. Selective enumeration of probiotic bacteria

Minimal nutrient agar base containing salicin was suitable for selective enumeration of *L. acidophilus* from pure cultures and yogurt containing *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Bifidobacterium* spp. A salicin concentration of 0.5% was appropriate for producing optimum size colonies. Salicin could be filter sterilised and then added to sterilised minimal nutrient base or could be autoclaved along with the minimal nutrient base.

8.3. Evaluation of media for enumeration of probiotic bacteria

Of seven media that were evaluated, NNLP agar can be used for selective enumeration of *B. bifidum* 1900 and 1901, *B. longum* 1941 and 20097, *B. pseudolongum* 20099 and *B. thermophilum* 20210. However, this medium does not support the growth of *B. infantis* 1912, *B. adolescentis* 1920 and *B. breve* 1930 and therefore cannot be used for enumeration of these organisms. NNLP medium requires considerable time in preparation and uses a number of ingredients.

Bile agar can be used for selective enumeration of *L. acidophilus* from yogurt supplemented with *L. acidophilus* along with *B. bifidum*, *B. adolescentis* or *B. breve*. All the

strains of *L. acidophilus* used in this study grew well in bile agar while the strains of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* did not form colonies. Maltose agar can be used to differentiate *L. acidophilus* from *S. thermophilus* and *L. delbrueckii* ssp *bulgaricus* if a product does not contain bifidobacteria. The results have shown that MRS-L-arabinose agar can be used for selective enumeration of *B. longum* strains 1941 and 20097 and *B. pseudolongum* 20099 from *L. acidophilus*. Strains of *L. acidophilus* formed pin point colonies in MRS-L-arabinose agar and can be easily differentiated from that of bifidobacteria. *B. bifidum*, *B. infantis*, *B. adolescentis*, *B. breve* and *B. thermophilum* did not ferment MRS-L-arabinose and as a result did not form colonies on the plates. Bifidobacteria have the ability to metabolise complex carbohydrates and these carbohydrates may form the basis for the development of differentially selective media.

However, presently there are taxonomical uncertainties regarding classification of bifidobacteria and *L. acidophilus* (Salminen and Wright, 1993). Therefore, some strains which have been classified under the same species may not actually belong to those species. Thus, it may be necessary to validate the suitability of these selective media to determine the growth and viability of each particular combination of strains in a product before applying such media for selective enumeration of *L. acidophilus* and *Bifidobacterium* spp. in yogurt.

8.4. Survival of probiotic bacteria in acid and bile

Results showed that among 6 strains of lactobacilli, *L. acidophilus* strains 2401, 2409 and 2415 survived best under acidic conditions. *L. acidophilus* strains 2404 and 2415 showed the best tolerance to bile followed by strains 2401 and 2409. However, as *L. acidophilus* 2404 showed poor tolerance to acid conditions, this organism may not be suitable for use as dietary adjuncts. Among the nine strains of bifidobacteria, *B. longum* 1941 and *B. pseudolongum* 20099 survived best under acidic conditions. *B. longum* 1941, *B. pseudolongum* 20099 and *B. infantis* 1912 showed the best tolerance to bile. Thus, *L. acidophilus* strains 2401, 2409 and 2415 and *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 strains can be used as dietary adjuncts in fermented dairy products.

8.5. Survival of probiotic bacteria in acid and hydrogen peroxide

Thus, it appears that there may be a synergistic effect of acid and hydrogen peroxide in reducing the viable counts of *B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 and *B. thermophilum* 20210. However, *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 appeared to be resistant to acid and hydrogen peroxide. Thus, *B. infantis* 1912, *B. longum* 1941 and *B. pseudolongum* 20099 can be used as dietary adjuncts in fermented dairy products whereas, *B. bifidum* 1900 and 1901, *B. adolescentis* 1920, *B. breve* 1930, *B. longum* 20097 and *B. thermophilum* 20210 are not suitable for inclusion as dietary adjuncts.

All strains of *L. acidophilus* showed further reduction in viable counts at lower pH levels during storage in the absence or presence of H_2O_2. However, as shown in bifidobacterial strains, no synergistic effect was shown against *L. acidophilus*. *L. acidophilus* 2400, 2401, 2404, 2405 and 2409 showed slight improvement in viability in the presence of H_2O_2. This may be due to the ability of H_2O_2 to increase O_2 concentration in the medium by reducing to H_2O and O_2 in the presence of catalase. Generally, bifidobacteria are anaerobic and the presence of O_2 is inhibitory to these bacteria. However, as *L. acidophilus* is microaerophillic and may prefer slightly oxygenated condition.

8.6. Viability of selected strains of probiotic bacteria in yogurt prepared with commercial yogurt bacteria

Selected strains of *L. acidophilus* 2409, *B. longum* 1941, *B. pseudolongum* 20099 and *B. infantis* 1912 showed better survival in yogurt as compared to the commercial strains. Among commercial strains, all *L. acidophilus* strains showed better survival than bifidobacteria. Reduction in the viable counts of *L. acidophilus* strain in the presence of *B. longum* from supplier 1 suggested the possibility of antagonism or incompatibility between these two organisms. Organoleptic score remained 8 $\pm$ 1 out of 10 for all products suggesting that the selected strains of probiotic bacteria also could be successfully used to substitute commercial cultures of probiotic bacteria.

8.7. Presence of α-galactisidase, β-galactosidase and P-β-galactosidase in yogurt and probiotic bacteria

P-β-gal was available in small quantities in most strains of *L. acidophilus* and bifidobacteria. *L. acidophilus* 2409 and 2415, *B. infantis* 1912 and *B. longum* 20097 showed the highest activities (0.077, 0.062, 0.194 and 0.110, respectively) of this enzyme. However, yogurt bacterial strains, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* did not possess P-β-gal. This suggests that these two types of bacteria do not rely on phosphorylation. All strains of *L. acidophilus* except strain 2401 had higher activity of β-gal as compared with the levels of P-β-gal in *L. acidophilus*. *L. acidophilus* 2409 and 2415 showed the highest activities (1.027 and 0.590, respectively). All bifidobacterial strains except, *B. infantis* 1912 and *B. thermophilum* 20210 showed high β-gal activity (1.870-2.869). α-gal was present in all *L. acidophilus* strains studied; *L. acidophilus* 2409 exhibited the highest activity. All strains of bifidobacteria except *B. infantis 1912* showed high levels of activity (1.245 - 2.785). However, *B. infantis* 1912 which showed low activity of α-gal and β-gal showed high activity for P-β-gal.

β-gal and α-gal enzymes were not detected in *L. acidophilus* strains except in *L. acidophilus* 2409. Among the bifidobacterial strains, *B. breve* and *B. longum* showed β-gal and α-gal activities. Strains of bifidobacteria showed various levels of enzyme activity, while *B. longum* 1941 showed highest activity. Yogurt bacteria (*L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*) also showed β-gal activity. Yogurt bacterial strains did not show the presence of α-gal.

8.8. Probiotic yogurt fermentation with ruptured yogurt bacteria

Viable counts of yogurt bacteria were 2 log cycles lower and of probiotic bacteria 1-2 log cycles higher in yogurt made with ruptured yogurt bacteria cells and whole cells of probiotic bacteria. Higher counts of probiotic bacteria are possibly due to higher activity of β-gal released as a result of cell rupture of yogurt bacteria and/or lower level of hydrogen peroxide produced during fermentation. In general, the counts of probiotic bacteria decreased during storage but was better in yogurt prepared using ruptured cells of yogurt

bacteria and whole cells of probiotic bacteria and the level of probiotic bacteria remained above 10^6 cfu per gram. Among the bifidobacteria used in this study, the viability of *B. longum* 1941 and *B. pseudolongum* was the highest and that of *B. bifidum* the lowest. However, *B. pseudolongum* is of animal origin and thus may not provide any health benefits to humans. Production of hydrogen peroxide was higher in yogurt made with whole cells as compared with that made with ruptured cells. Although the production of acetaldehyde was slow with ruptured cells of yogurt bacteria, final amount of acetaldehyde produced was similar in yogurts made with either ruptured or whole cells of yogurt bacteria and whole cells of probiotic bacteria. Higher level of lactose was hydrolysed in yogurt made using ruptured yogurt bacteria as compared with those made using whole yogurt bacteria. During the period of fermentation, glucose was utilised rapidly by the bacteria whereas most of galactose was unused.

8.9. Two stage fermentation of probiotic yogurt

Counts of probiotic bacteria were about 5 times higher in yogurt made using two step fermentation process as compared with those made using single step fermentation process. In general, the counts of probiotic bacteria reduced in all the products during storage, however, yogurt made using two step process showed higher counts than that made using single step process. Neutralisation of the mix before fermentation also increased the initial and final counts of the two probiotic bacteria by about 4-6 times. All the products showed similar levels of acetaldehyde.

8.10. Antimicrobial activity of probiotic bacteria against pathogenic bacteria

L. acidophilus and bifidobacteria showed antimicrobial activity against *A. hydrophila*, *C. albicans*. *E. coli* and *S. typhimurium*. However, this antimicrobial activity was due to acidity only. The probiotic bacteria did not show bacteriocin activity against the pathogens studied. In general *L. acidophilus* produced more lactic acid and bifidobacteria produced more acetic acid. When *A. hydrophila* and *C. albicans* were grown in a co-culture, the growth of the former pathogens was inhibited in the presence of probiotic

bacteria. Therefore, the selected probiotic strains (*L. acidophilus* 2409, *B. infantis* 1912 and *B. longum* 1941) could be used as probiotic dietary adjuncts.

8.11. Antimutagenic activity of probiotic bacteria

Strains of probiotic bacteria showed different levels of antimutagenic activity and binding of mutagens. Generally, most strains of *L. acidophilus* and bifidobacteria were effective in inhibiting NF (nitrofluorene), NPD (4-nitro-O-phenylenediamine), and AFTB (aflatoxin-B). Similarly, most strains of bifidobacteria showed antimutagenic activity against AMIQ (2-amino-3-methyl-3H-imidazoquinoline).

Live probiotic bacteria exhibited higher antimutagenic activity and greater binding of mutagens as compared with killed cells of probiotic bacteria. Binding of mutagens to probiotic bacteria appeared to be permanent for live cells and temporary for killed cells. Killed cells released bound mutagens when extracted with DMSO. The results emphasised the importance of consuming live probiotic bacteria and of maintaining viability of these bacteria in the intestine so that efficient inhibition of mutagens can be achieved in order to provide benefit to consumers.

Acetic and butyric acids reduced mutagenicity of the mutagens studied. Butyric acid inhibited all mutagens, while acetic acid showed antimutagenic effect against 3 of 8 mutagens studied. Thus, it appears that organic acids, especially butyric and acetic acids produced by probiotic bacteria contributed to the antimutagenic activity

8.12. Adherence of probiotic bacteria to human colon cancer Ht-29 cells

Bifidobacteria showed better adherence to Ht-29 colonic monolayer cells as compared with *L. acidophilus*. Among bifidobacteria, *B. infantis* 1912 and *B. longum* 1941 showed highest levels of adherence. Among the strains of *L. acidophilus*, 2400 and 2415 showed better adherence. Thus, in general, bifidobacteria may be proffered as dietary adjuncts as compared with *L. acidophilus*.

Spent broth proteins of all strains of adhering bacteria involved in adherence of probiotic bacteria to Ht-29 cells. However, involvement of polysaccharides from bacteria and Ht-29 cells in adherence varied from strain to strain of probiotic bacteria. Polysaccharides from Ht-29 cell surfaces contributed to adherence more than the polysaccharides originating from the bacterial cells. Polysaccharides of both bacterial and Ht-29 origin were involved in adherence for *B. infantis* 1912 and *B. longum* 1941.

The molecular size of proteins involved in adherence varied among the strains of probiotic bacteria. The molecular size fraction of 30,000-50,0000 kD was responsible for adherence for *B. longum* 1941, and several size fractions of proteins > 30,000 kD were involved in adherence for *B. infantis* 1912 while the fraction of 30,000-50,000 kD produced highest effect. Electron micrographs exhibited that adherence was mediated by a bridging structure (possibly protein-polysaccharide) formed between bacterial and Ht-29 cells.

9.0. FUTURE RESEARCH DIRECTIONS

As more information about probiotic products and their benefits become available to public, the popularity of these products have increased in many countries. With increased demand for such products, manufacturers of probiotic products have started introducing new products. Some products are of dairy origin such as yogurt, fermented milk and other dairy products incorporated with probiotic bacteria whereas other products are non-dairy based mixed cultures in freeze dried form, as capsules or tablets. However, with the complexity of these mixed cultures, the process of enumeration becomes difficult.

Selective enumeration of probiotic bacteria in a product is one of the fundamental yet important requirement in assessing the viability of probiotic bacteria. As probiotic yogurt contains yogurt bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) as well as probiotic bacteria, it will be important to develop and validate the media for enumerating not only *L. acidophilus* or bifidobacteria but also other organisms such as *Lactobacillus gasseri*, *Enteroccoccus faecium* and *Lactobacillus caseii* selectively. Further research will be required to develop new methods to enumerate the organisms in such mixed products selectively. Use of chromogenic substrates to differentiate the colonies of different bacterial species and strains in agar plates would need more fundamental research regarding the availability of various enzymes and by-products. Resistance to antibiotics and effective concentrations of these antibiotics against probiotic bacteria could become important tools too.

In order to claim health benefits and effectiveness of these organisms *in vivo*, clinical experiments will be required with the selected strains based on their other characteristics such as acid, bile and hydrogen peroxide tolerance and anti-microbial, anti-mutagenic and anti-carcinogenic properties. The strains which have been found to adhere to Ht-29 cells could be evaluated by clinical research.

Our work has shown that anti-carcinogenic and anti-mutagenic effects of these bacteria are due to short chain fatty acids such as butyric acid. It may be useful to further study the molecular mechanisms which suppressed the growth of cancer cells and mutagenicity of various mutagens.

10.0. REFERENCES

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