FACTORS AFFECTING VIABILITY OF YOGHURT AND PROBIOTIC BACTERIA IN COMMERCIAL STARTER CULTURES

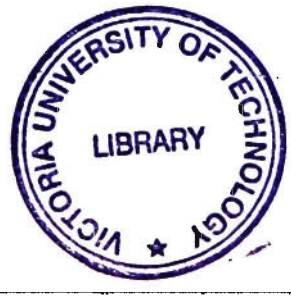
A thesis submitted for the degree of Doctor of Philosophy

By

Rajiv I. Dave
M. Sc. (Dairying)

1998

Centre for Bioprocessing and Food Technology
School of Life Sciences and Technology
Victoria University of Technology
Werribee Campus, Victoria
Australia



DEDICATED

TO

MY PARENTS

ABSTRACT

The main objective of this study was to identify the factors affecting the viability of yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) and probiotic bacteria (*Lactobacillus acidophilus* and bifidobacteria) in commercial starter cultures. To achieve the objectives of the study, it was essential to standardise the protocols for selective/differential enumeration of yoghurt and probiotic bacteria in yoghurt containing all the four organisms. Fifteen media were evaluated in order to determine their suitability for selective enumeration of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus*, and bifidobacteria. ST (*S. thermophilus*) agar was found to be suitable for selective enumeration of *S. thermophilus* when the plates were incubated aerobically at 37°C for 24 h only. The MRS (deMan Rogosa Sharpe) agar at pH 5.2 or RCA (reinforced clostridial agar) at pH 5.3 could be used for the differential enumeration of *L. delbrueckii* ssp. *bulgaricus* when the incubation was carried out at 45°C for 72 h. For selective enumeration of *L. acidophilus*, MRS-salicin agar or MRS-sorbitol agar could be used, whereas MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride and paromomycin sulfate) agar was suitable for the selective enumeration of bifidobacteria.

Viability of yoghurt and probiotic bacteria assessed in yoghurt made from four commercial starter cultures (C1-C4) was dependent on the species and strain of associative yoghurt organisms. The viability of *L. acidophilus* was adversely affected, whereas bifidobacteria exhibited better stability in yoghurt prepared from cultures that contained *L. delbrueckii* ssp. *bulgaricus*. The storage temperature of yoghurt had effect on the viability of bifidobacteria, but not on that of *L. acidophilus*. In one of the commercial cultures (C3), 3 log cycles inhibition of bifidobacteria was observed from initial counts of $>10^6$ cfu.mL^{-1}. The effect of size of inoculum on viability of yoghurt and probiotic bacteria was also studied. Yoghurts made with lower rates of inoculum showed increased rate of post-acidification. *L. acidophilus* maintained their viability to a recommended level of 10^6 cfu.g^{-1} for only up to 20-25 days storage at 4°C; after which, a sharp decline in their counts was observed. In three of four starter cultures, counts of

bifidobacteria were satisfactory, but declined dramatically to ~3 log cycles during manufacture of yoghurt with C3 starter culture at all inoculum levels.

Viability of yoghurt and probiotic bacteria was also assessed in yoghurt supplemented with four levels each of ascorbic acid (0, 50, 150 and 250 $mg.L^{-1}$) and cysteine (0, 50, 250 and 500 $mg.L^{-1}$). During storage of yoghurt, patterns of increase in oxygen content and redox potential were identical for all the four starter cultures at various levels of ascorbic acid, but different at various levels of cysteine. The oxygen content and redox potential gradually increased during storage in plastic cups and remained lower with higher levels of ascorbic acid or cysteine. The viable counts of *S. thermophilus* were lower, whereas those of *L. delbrueckii* ssp. *bulgaricus* were higher in yoghurt with increasing concentration of ascorbic acid or cysteine. The counts of *L. acidophilus* during storage decreased less rapidly with increasing concentration of acsorbic acid or cysteine for all the starter cultures. The counts of bifidobacteria remained nearly unchanged at all levels of ascorbic acid, but considerably improved with cysteine when compared with control yoghurt.

Effects of cysteine, whey powder (WP), whey protein concentrate (WPC), acid casein hydrolysates (ACH) and tryptone on viability of *S. thermophilus*, *L. acidophilus* and bifidobacteria and on textural properties of yoghurt made with C3 starter culture were also studied. The incubation time to reach pH 4.5 was considerably affected and was dependent on the added ingredients. Also, the drop in pH or increase in acidity and redox potential were dependent on the added ingredients. Addition of cysteine, WPC, ACH, and tryptone improved the viability of bifidobacteria to a variable extent, whereas WP failed to improve their viability. The morphology of *S. thermophilus* as shown by electron microscopy was affected by cysteine at 500 $mg.L^{-1}$ level, possibly as a result of reduced redox potential. SDS-PAGE and amino acid analyses suggested that nitrogen source in the form of peptides and amino acids correlated with improved viability of bifidobacteria in yoghurt made with C3 starter culture which showed a dramatic decline in the counts of bifidobacteria during yoghurt manufacture. The added ingredient had significant ($P <$

0.001) effects on the viscosity and firmness of yoghurt and the protein network of yoghurt was also altered due to added ingredients.

Antagonism between yoghurt bacteria and probiotic bacteria was also studied. Seven of 8 strains of *L. acidophilus* produced antimicrobial substances that were active against *L. delbrueckii* ssp. *bulgaricus*. Inhibition of 5 strains of *S. thermophilus* and 2 strains of bifidobacteria was found to be due to organic acids and not due to bacteriocin like inhibitory substance (BLIS). Fifteen of 19 strains of *L. acidophilus* additionally studied produced heat stable BLIS which were proteinaceous in nature. Four strains of *L. acidophilus* were selected for further studies. The bacteriocin produced by *L. acidophilus* (strains LA-1, BDLA-1, 2409, MOLA-2) inhibited *L. delbrueckii* ssp. *bulgaricus*, *L. casei*, *L. helveticus* and *L. jugurti*, but not the other lactic acid bacteria and several spoilage and pathogenic organisms studied. The BLIS showed activity over a wide range of temperature and pH. The production of BLIS increased on addition of -glycerophosphate and was highest at pH 5.5-6.0. The molecular weight of the bacteriocin produced by *L. acidophilus* (strains LA-1, BDLA-1 and LA-2409) was 50,000 daltons. The BLIS produced by *L. acidophilus* (MOLA-2) did not give a single band on silver staining of gel and 10 kDa permeate also showed considerable activity. The fractionation with ammonium sulfate was successful in purifying the BLIS produced by *L. acidophilus* (strains LA-1, BDLA-1 and LA-2409) and resulted in a single band on silver staining of SDS-PAGE gel. Some characteristics of BLIS produced by *L. fermentum* (5174) and *L. plantarum* (2903) were also studied. The BLIS produced by both of these organisms were proteinaceous in nature, heat stable and were active over a wide range of pH. The ammonium sulfate fractionation was successful in purifying the BLIS produced by *L. fermentum* (5174), but not for *L. plantarum* (2903). The antimicrobial substance produced in milk by *L. helveticus* (2700) inhibited several strains of *L. acidophilus* and was not an acid, hydrogen peroxide, bacteriophage, protein or lipid.

Dr. Nagendra P. Shah
(M. Sc., Ph. D.)
Senior Lecturer
School of Life sciences and Technology
Victoria University of Technology
Werribee Campus, Victoria
Australia

CERTIFICATE

This is to certify that the thesis entitled "FACTORS AFFECTING VIABILITY OF YOGHURT AND PROBIOTIC BACTERIA IN COMMERCIAL STARTER CULTURES" submitted by Rajiv Dave in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Food Science at the Victoria University of Technology is a record of bonafide research work carried out by him under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Werribee, Australia

Date:

(Dr. N.P. Shah)

Thesis Supervisor

ACKNOWLEDGEMENT

It is a great privilege and with an immense pleasure I express my profound gratitude to my supervisor Dr. Nagendra Shah, Senior Lecturer, School of Life Sciences and Technology, Victoria University of Technology, Werribee Campus, Werribee for his inspiring and noble guidance, constructive criticism and valuable suggestions throughout the course of study and in preparation of manuscripts.

I express my gratitude to Prof. Margaret Britz, Prof. Stirk Kyle, Mr. Nikola Popovich and Mr. Scott Currie for their support in procuring chemicals and allowing me to use the instruments and other facilities required for the successful operation of my research work. I am thankful to Australian Research Council for providing an Australian Postgraduate Research Award (Industry) for this study and I also appreciate the contribution offered by Chr. Hansen Pty. Ltd., Australia as a co-sponsor of the above scholarship. My special thanks are due to Mr. Roy Con Foo, Director, Marketing and Sales, Chr. Hansen's Pty. Ltd., Australia for active involvement and for his personal contribution in some of the technical aspects which were valuable in the course of planning of my research.

I express my sincere thanks and appreciation to my friends Dr. Warnakulasuriya Lankaputhra, Mrs. Margaret Webster and Ms. Sudarat Boonchan for their help in some aspects of my research. I am highly indebted and I appreciate the friendship of Harshul Vora and his family, Ramakanth Ravula and his family, and of Sandra McKechnie, Aslam Khan and Hisham Al-Mitubsi.

I would like to recall the love and affection of my parents, brother, in-laws and other family members; without their blessing and inspiration, this study would not have been a reality. Last, but not the least, I can not forget the reconnaissance love of my beloved wife "Trupti" and son "Kunal" who supported me throughout my research project and tolerated my long hours of working at times when they needed me the most.

Werribee, Australia

Date: RAJIV DAVE

List of Publications

REFEREED RESEARCH PAPERS

(1) Dave, R.I. and Shah, N.P. 1996. Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus,* and bifidobacteria. *Journal of Dairy Science*, **9**:1376-80.

(2) Dave, R.I. and Shah, N.P. 1997. Viability of yoghurt bacteria and probiotic bacteria in yoghurts made from commercial starter cultures. *International Dairy Journal*, 7:31-41.

(3) Dave, R.I. and Shah, N.P. 1997. Effect of level of starter culture on viability of yoghurt and probiotic bacteria. *Food Australia*, **49**:164-68.

(4) Dave, R.I. and Shah, N.P. 1997. Effectiveness of ascorbic acid as oxygen scavenger in improving the viability of probiotic bacteria in yoghurts made with commercial starter cultures. *International Dairy Journal,* 7:435-43.

(5) Dave, R.I. and Shah, N.P. 1997. Effect of cysteine on viability of yoghurt and probiotic bacteria in yoghurts made with commercial starter cultures. *International Dairy Journal*, 7:537-45.

(6) Dave, R.I. and Shah, N.P. 1997. Characteristics of bacteriocin produced by *L. acidophilus* (LA-1). *International Dairy Journal,* 7: 707-15.

(7) Dave, R.I. and Shah, N.P. 1998. The influence of ingredient supplementation on textural characteristics of yogurt. *Australian Journal of Dairy Technology,* **53**: 180-84.

(8) Dave, R.I. and Shah, N.P. 1998. Ingredient supplementation effects on viability of probiotic bacteria in yogurt. *Journal of Dairy Science,* **81**:2804-2816.

(9) Dave, R.I. and Shah, N.P. 1998. A novel antimicrobial substance produced by *L. helveticus* (2700). *Australian Journal of Dairy Technology,* (In press).

(10) Dave, R.I. and Shah, N.P. 1998. Characteristics of bacteriocin like inhibitory substances produced by *Lactobacillus acidophilus* (BDLA-1, 2409, MOLA-2), *Lactobacillus fermentum* (5174) and *Lactobacillus plantarum* (2903). *International Dairy Journal*, (Under Review)

CONFERENCE PRESENTATIONS

(1) Dave, R.I., Britz, M.L. and Shah, N.P. 1995. Evaluation of media for selective enumeration of yoghurt cultures, *L. acidophilus* and bifidobacteria. A paper presented at the Australian Society for Microbiology Conference held in Canberra, ACT, Australia, Workshop W 13, September 24-29, 1995.

(2) Dave, R.I. and Shah, N.P. 1996. Assessment of viability of yoghurt and probiotic cultures in yoghurts made from commercial starter cultures. A poster presented at the 29th Annual convention of Australian Institute of Food Science and Technology, Gold Coast, QLD, Australia, May 5-8, 1996.

(3) Shah, N.P. and Dave, R.I. 1996. Selective enumeration of yoghurt bacteria and probiotic bacteria. A poster presented at the 29th Annual convention of Australian Institute of Food Science and Technology, Gold Coast, QLD, Australia, May 5-8, 1996.

(4) Dave, R.I. and Shah, N.P. 1997. Some characteristics of bacteriocin produced by *L. acidophilus* isolated from a commercial probiotic culture. A paper presented in 30th Annual convention of Australian Institute of Food Science and Technology, Perth, WA, Australia, May 5-8, 1997.

(5) Dave, R.I. and Shah, N.P. 1997. Effect of cysteine, acid casein hydrolysate, tryptone, whey powder and whey protein concentrate on viability of yoghurt and probiotic bacteria and on textural characteristics of yogurt. A poster presented at the 92nd Annual Meetings of the American Dairy Science Association, Guelph, Canada, June 22-25, 1997.

(6) Dave, R.I. and Shah, N.P. (1998). A novel antimicrobial substance produced by *Lactobacillus helveticus* (2700). A poster presented at the Nutrition Week-1998 organised by International Dairy Federation, Wellington, NewZealand, March 9-11, 1998.

(7) Dave, R.I. and Shah, N.P. (1998). Characteristics of bacteriocins by *Lactobacillus acidophilus* strains. A poster presented at the International Dairy Congress, September 22-24, Aarhus, Denmark.

Table of Contents

Chapter.....Page

1.0 INTRODUCTION.....1

2.0 LITERATURE REVIEW.....4

2.1 **Yoghurt and Fermented milks**.....4

2.1.1 Types of yoghurt.....5

2.1.2 Starter cultures used for yoghurt and fermented milks.....7

2.2 **Probiotics**.....7

2.2.1 Definition.....7

2.2.2 Probiotics and prebiotics.....8

2.2.3 Historical background and growth characteristics of *L. acidophilus* and bifidobacteria.....10

2.2.4 Genus *Lactobacillus*.....12

2.2.4.1 Proteolytic activity of lactobacilli.....15

2.2.5 Genus *Bifidobacterium*.....15

2.2.5.1 Growth requirements of bifidobacteria.....18

2.2.5.1.1 Nutrition.....18

2.2.5.1.2 Oxygen issue.....19

2.2.5.1.3 Growth in milk.....20

2.2.5.1.4 Enhancing growth and acid production.....21

2.2.6 Selection criteria for lactobacilli and bifidobacteria for their use as dietary adjuncts.....23

2.3 **Therapeutic properties of probiotic organisms**.....24

2.4 **An overview on acidophilus and bifidobacterial products available world-wide**.....27

2.4.1 Market of acidophilus and bifidobacterial products.....28

2.4.2 Regulatory aspects of yoghurt.....28

2.5 **Viability of *L. acidophilus* and bifidobacteria in fermented products**.....30

2.5.1 Viable counts as affected by enumeration methods of yoghurt bacteria and probiotic bacteria.....30

2.5.2 Viability as affected by physico-chemical and processing parameters....34
2.5.3 Viability of yoghurt and probiotic bacteria as affected by antagonism between various groups of organisms....35
2.5.3.1 Organic acids and low pH....36
2.5.3.2 Hydrogen peroxide....37
2.5.3.3 Diacetyl....38
2.5.3.4 O-R potential....38
2.5.3.5 Competitive antagonism....39
2.5.3.6 Deconjugation of bile....39
2.5.3.7 Antibiotic-like substances....39
2.5.3.7.1 The antibacterial action of *L. acidophilus*....42
2.5.3.7.2 The antibacterial action of bifidobacteria....45
2.5.3.8 The composite effect....46
3.0 MATERIALS AND METHODS....47
3.1 **Starter cultures**....47
3.2 **Microorganisms and their maintenance**....47
3.2.1 Bacterial strains and commercial products used for antagonism studies....49
3.2.2 Isolation and identification of bacterial cultures....50
3.2.3 Maintenance of bacterial cultures....50
.3.3 **Media preparation**....50
3.3.1 Peptone and water diluent....50
3.3.2 Streptococcus thermophilus agar....51
3.3.3 MRS agar, pH modified MRS agar (pH 5.2 and 5.8), MRS-bile agar, MRS-oxgall agar and MRS-NaCl agar....51
3.3.4 MRS-maltose agar, MRS-salicin agar and MRS-sorbitol agar....51
3.3.5 MRS-NNLP agar....51
3.3.6 RCA agar (pH 6.8 and 5.3)....52
3.3.7 Rogosa acetate agar....52
3.3.8 Cellobiose esculin agar....52
3.3.9 Standardising protocols for the enumeration of yoghurt

and probiotic bacteria.....52
3.4 **Yoghurt preparation**.....53
3.4.1 Manufacture of yoghurt at the rate of starter culture recommended by the manufacturer.....53
3.4.2 Manufacture of yoghurt at various rates of inoculum of starter cultures.....53
3.4.3 Yoghurt fortified with ascorbic acid.....54
3.4.4 Yoghurt fortified with cysteine.....54
3.4.5 Yoghurt made with various ingredients.....55
3.5 **Sample preparation**.....57
3.5.1 Sample preparation for biochemical analyses.....57
3.5.2 Sample preparation for texture analyses.....57
3.6 **Time interval specification**.....57
3.7 **Analyses**.....58
3.7.1 Chemical analyses.....58
3.7.1.1 pH and titratable acidity of yoghurt.....58
3.7.1.2 Concentration of hydrogen peroxide in yoghurt.....58
3.7.1.3 Determination of organic acids by HPLC.....59
3.7.1.4 Oxygen content and redox potential measurements.....59
3.7.1.5 Determination of protein and total solids content.....59
3.7.1.6 Determination of ascorbic acid.....60
3.7.2 Microbiological analyses.....61
3.8 **Polysaccharide production by *S. thermophilus***.....61
3.9 **Electron microscopic examination of *S. thermophilus***.....62
3.10 **Texture analyses**.....63
3.11 **SDS-PAGE of casein-free fraction collected from various yoghurts**.....64
3.12 **Amino acid analysis**.....65
3.13 **Detection and assay of inhibitory activity**.....65
3.14 **pH adjustment**.....68
3.15 **Reaction with various enzymes**.....68
3.16 **Thermal resistance of bacteriocin like inhibitory substance (BLIS)**.....68

3.17 **Activity of BLIS over a range of pH**.....68
3.18 **Storage stability of BLIS**.....69
3.19 **Various sugar based MRS broth**.....69
3.20 **Effects of pH and supplementation of glucose and triple strength MRS broth on production and stability of BLIS**.....70
3.21 **Determination of viable counts, cell density and glucose concentration**.....70
3.22 **Estimation of molecular weight of BLIS**.....71
3.23 **Purification of BLIS using ammonium sulfate precipitation and SDS-PAGE**.....71
4.0 ENUMERATION OF YOGHURT AND PROBIOTIC BACTERIA.....73
4.1 **Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and bifidobacteria**.....73
4.1.1 Introduction.....73
4.1.2 Materials and methods.....74
4.1.3 Results and discussion.....74
4.1.4 Conclusion.....81
5.0 VIABILITY OF YOGHURT AND PROBIOTIC BACTERIA.....87
5.1 **Viability of yoghurt bacteria and probiotic bacteria in yoghurts made from commercial starter cultures**.....87
5.1.1 Introduction.....87
5.1.2 Materials and methods.....89
5.1.3 Results and discussion.....89
5.1.4 Conclusions.....98
5.2 **Assessment of viability of yoghurt and probiotic bacteria in yoghurts made with various sizes of inoculum of commercial starter cultures**.....114
5.2.1 Introduction.....114
5.2.2 Materials and methods.....115
5.2.3 Results and discussion.....116
5.2.4 Conclusions.....118

6.0 INGREDIENT SUPPLEMENTATION AFFECT THE VIABILITY OF YOGHURT AND PROBIOTIC BACTERIA AND THE TEXTURAL PROPERTIES OF YOGHURT.....125

6.1 **Effectiveness of ascorbic acid as an oxygen scavenger in improving viability of probiotic bacteria in yoghurts made with commercial starter cultures**.....125

6.1.1 Introduction.....125

6.1.2 Materials and methods.....126

6.1.3 Results and discussion.....126

6.1.4 Conclusions.....131

6.2 **Effect of cysteine on viability of yoghurt and probiotic bacteria in yoghurts made with commercial starter cultures**.....141

6.2.1 Introduction.....141

6.2.2 Materials and methods.....142

6.2.3 Results and discussion.....143

6.2.4 Conclusions.....148

6.3 **Effect of cysteine, whey powder, whey protein concentrates, acid casein hydrolysate and tyrptone on viability of yoghurt and probiotic bacteria**....156

6.3.1 Introduction.....156

6.3.2 Materials and methods.....158

6.3.3 Results and discussion.....159

6.3.4 Conclusions.....168

6.4 **Effect of cysteine, whey powder, whey protein concentrates, acid casein hydrolysate and tryptone on textural characteristics of yoghurt**.....184

6.4.1 Introduction.....184

6.4.2 Materials and methods.....186

6.4.3 Results and discussion.....187

6.4.4 Conclusions.....190

7.0 ANTIMICROBIAL SUBSTANCES PRODUCED BY YOGHURT AND PROBIOTIC BACTERIA.....195

7.1 **Antagonism between yoghurt bacteria and probiotic bacteria isolated from commercial starter cultures, commercial yoghurts and a probiotic capsules** 195
7.1.1 Introduction....195
7.1.2 Materials and methods....196
7.1.3 Results and discussion....197
7.1.4 Conclusions....200
7.2 **Some characteristics of bacteriocin produced by *Lactobacillus acidophilus* isolated from a commercial probiotic culture**....205
7.2.1 Introduction....205
7.2.2 Materials and methods....206
7.2.3 Results and discussion....208
7.2.4 Conclusions....215
7.3 **Antimicrobial substance produced by *Lactobacillus helveticus* (2700)**....237
7.3.1 Introduction....237
7.3.2 Materials and methods....238
7.3.3 Results and discussion....239
7.3.4 Conclusions....241
7.4 **Some characteristics of bacteriocin like inhibitory substance produced by *Lactobacillus acidophilus* (BDLA-1, 2409 and MOLA-2), *Lactobacillus fermentum* (5174) and *Lactobacillus plantarum* (2903)**....248
7.4.1 Introduction....248
7.4.2 Materials and methods....248
7.4.3 Results and discussion....250
7.4.4 Conclusions....254
8.0 SUMMARY OF RESULTS 265
9.0 FUTURE RESEARCH DIRECTION....272
10.0 REFERENCES....274

LIST OF TABLES

Serial No.	Table No.	Title	Page No.
1	2.1	Yoghurt and yoghurt-like products as known in various countries of the world.....	5
2	2.2	Fermented milks containing dietary *Lactobacilli*.....	9
3	2.3	Species of genus *Lactobacillus*.....	14
4	2.4	Species of genus bifidobacterium.....	16
5	2.5	Therapeutic properties of acidophilus and bifidus milk products.....	26
6	2.6	Bacteriocins of *Lactobacilli*.....	40
7	2.7	Bacteriocins of *Lactococci*, *Leuconostocs* and *Pediococci*	41
8	3.1	Sources of various lactic acid bacteria.....	48
9	3.2	Sources of various spoilage and pathogenic bacterial strains.....	49
10	4.1	Viable counts of *Streptococcus thermophilus* strains on different bacteriological agars	82
11	4.2	Viable counts of *Lactobacillus delbrueckii* ssp. *bulgaricus* strains on different bacteriological agars.....	83
12	4.3	Viable counts of *Lactobacillus acidophilus* strains on different bacteriological agars.....	84
13	4.4	Viable counts of various bifidobacteria on different bacteriological agars.....	85
14	4.5	Recovery of yoghurt and probiotic organisms in commercial products.....	86
15	5.1.1	Changes in the viable counts of *S. thermophilus* and *L. delbrueckii* ssp. *Bulgaricus* during manufacture and storage of yoghurt.....	100
16	5.1.2	Changes in the viable counts of *L. acidophilus* during manufacture and storage of yoghurt.....	101
17	5.1.3	Changes in the viable counts of bifidobacteria (as enumerated on MRS-NNLP agar) during manufacture and storage of yoghurt.....	102
18	5.1.4	Changes in the viable counts of bifidobacteria (as obtained by subtraction method) during manufacture and storage of yoghurt.....	103
19	5.1.5	Changes in counts of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in yoghurts stored in plastic cups at 4 and 10°C and in glass bottles at 4°C.....	104
20	5.1.6	Changes in counts of *L. acidophilus* in yoghurts stored in plastic cups at 4 and 10°C and in glass bottles at 4°C	105
21	5.1.7	Changes in counts of bifidobacteria (as enumerated on MRS-NNLP) in yoghurts stored in plastic cups at 4 and 10°C and in glass bottles at 4°C.....	106

22	5.1.8	Changes in counts of bifidobacteria (as obtained by subtraction method) in yoghurts stored in plastic cups at 4 and 10°C and in glass bottles at 4°C.....	107
23	5.2.1	Changes in the pH during manufacture and storage of yoghurt prepared with various levels of inoculum.....	119
24	5.2.2	Changes in the titratable acidity (% lactic acid) during manufacture and storage of yoghurt prepared with various levels of inoculum.....	120
25	5.2.3	Changes in the viable counts of *S. thermophilus* during manufacture and storage of yoghurt prepared with various levels of inoculum.....	121
26	5.2.5	Changes in the viable counts of *L. delbrueckii* ssp. *bulgaricus* during manufacture and storage of yoghurt prepared with various levels of inoculum.....	122
27	5.2.5	Changes in the viable counts of *L. acidophilus* during manufacture and storage of yoghurt prepared with various levels of inoculum.....	123
28	5.2.6	Changes in the viable counts of bifidobacteria during manufacture and storage of yoghurt prepared with various levels of inoculum.....	124
29	6.1.1	Changes in viable counts of *S. thermophilus* during manufacture and storage of yoghurt prepared with various levels of ascorbic acid.....	133
30	6.1.2	Changes in viable counts of *L. delbrueckii* ssp. *bulgaricus* during manufacture and storage of yoghurt prepared with various levels of ascorbic acid.....	134
31	6.1.3	Changes in viable counts of *L. acidophilus* during manufacture and storage of yoghurt prepared with various levels of ascorbic acid.....	135
32	6.1.4	Changes in viable counts of bifidobacteria during manufacture and storage of yoghurt prepared with various levels of ascorbic acid.....	136
33	6.2.1	Changes in the counts of *S. thermophilus* during manufacture and storage of yoghurt prepared with various levels of cysteine.....	149
34	6.2.2	Changes in the counts of *L. delbrueckii* ssp. *bulgaricus* during manufacture and storage of yoghurt prepared with various levels of cysteine.....	150
35	6.2.3	Changes in the counts of *L. acidophilus* during manufacture and storage of yoghurt prepared with various levels of cysteine.....	151
36	6.2.4	Changes in the counts of bifidobacteria during manufacture and storage of yoghurt prepared with various levels of cysteine.....	152
37	6.3.1	Protein and total solids contents of yoghurt supplemented with various ingredients.....	169

38	6.3.2	Changes in counts of *S. thermophilus* during manufacture of yoghurt supplemented with various ingredients.....	170
39	6.3.3	Changes in counts of *L. acidophilus* during manufacture of yoghurt supplemented with various ingredients.....	171
40	6.3.4	Changes in counts of bifidobacteria during manufacture of yoghurt supplemented with various ingredients.....	172
41	6.3.5	Changes in counts of *S. thermophilus, L. acidophilus* and bifidobacteria during storage of yoghurt supplemented with various ingredients.....	173
42	6.3.6	Molecular weight and relative area of various bands of casein free fraction of yoghurt supplemented with various ingredients after SDS-PAGE analysis.....	174
43	6.3.7	Protein content and amino acid profile of whey protein concentrates, acid casein hydrolysate and tryptone.....	175
44	6.4.1	Protein and total solids contents of yoghurt supplemented with various ingredients.....	191
45	7.1.1	Isolates of *S. thermophilus* (ST), *L. delbrueckii* ssp. *bulgaricus* (LB), *L. acidophilus* (LA) and bifidobacteria (BB) isolated from commercial starter cultures, yoghurts and a probiotic capsule.....	201
46	7.1.2	Inhibition of *S. thermophilus* (ST), *L. delbrueckii* ssp. *bulgaricus* (LB), and bifidobacteria (BB) by *L. acidophilus* (LA) isolates.....	202
47	7.2.1	Inhibitory spectrum of bacteriocin like inhibitory substance produced by *L. acidophilus* (LA-1).....	217
48	7.2.2	Sensitivity of bacteriocin like inhibitory substance produced by *L. acidophilus* (LA-1) to various enzymes...	218
49	7.2.3	Heat resistance of bacteriocin like inhibitory substance produced by *L. acidophilus*.....	219
50	7.2.4	Activity of bacteriocin like inhibitory substance (BLIS) produced by *L. acidophilus* (LA-1) over a range of pH....	220
51	7.2.5	Storage stability of bacteriocin like inhibitory substance (BLIS) produced by *L. acidophilus* (LA-1) in cell free MRS broth at various temperatures.....	221
52	7.2.6	Screening of various strains of *L. acidophilus* for the production of bacteriocin like inhibitory substance.....	222
53	7.3.1	Screening of various lactic acid bacteria during their growth in MRS broth for the production of antimicrobial substances.....	243
54	7.3.2	Inhibitory spectrum of antimicrobial substances produced by *L. helveticus* (2700) in milk.....	244
55	7.3.3	Sensitivity of antimicrobial substance produced by *L. helveticus* (2700) against various enzymes.....	245
56	7.3.4	Heat resistance of antimicrobial substance produced by *L. helveticus* (2700) in MRS broth and in milk.....	246
57	7.3.5	Activity of antibacterial substance produced by *L. helveticus* (2700) over a range of pH.....	247

58	7.4.1	Inhibitory spectrum of bacteriocin like inhibitory substances produced by *L. acidophilus* (BDLA-1, 2409, MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903)	255
59	7.4.2	Sensitivity of bacteriocin like inhibitory substances produced by *L. acidophilus* (BDLA-1, 2409, MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903) to various enzymes.....	256
60	7.4.3	Heat resistance of bacteriocin like inhibitory substances produced by *L. acidophilus* (BDLA-1, 2409, MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903).....	257
61	7.4.4	Activity of bacteriocin like inhibitory substances produced by *L. acidophilus* (BDLA-1, 2409, MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903) over a range of pH.....	258
62	7.4.5	Inhibitory activity of retentates and permeates after concentration of cell free MRS broth of *L. acidophilus* (BDLA-1, 2409, MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903).....	259

LIST OF FIGURES

Serial No.	Fig. No.	Title	Page No.
1	3.1	Flow diagram for the preparation of yoghurt	56
2	5.1.1	Changes in the titratable acidity and pH during manufacture and storage of yoghurt.....	108
3	5.1.2	Changes in oxygen (ppm) and hydrogen peroxide (μ $g.mL^{-1}$) contents during manufacture and storage of yoghurt.....	109
4	5.1.3	Changes in lactic acid and acetic acid contents during manufacture and storage of yoghurt.....	110
5	5.1.4	Changes in titratable acidity and pH in yoghurt stored in plastic cups (4 and 10°C) and glass bottles (4°C) made from C1 and C3 cultures.....	111
6	5.1.5	Changes in dissolved oxygen content (ppm) in yoghurt stored in plastic cups (4 and 10°C) and glass bottles (4° C) made from C1 and C3 cultures.....	112
7	5.1.6	Changes in hydrogen peroxide ($\mu g.mL^{-1}$) in yoghurt stored in plastic cups (4 and 10°C) and glass bottles (4° C) made from C1 and C3 cultures.....	113
8	6.1.1	Changes in TA and pH in yoghurt supplemented with various levels of ascorbic acid.....	137
9	6.1.2	Changes in the oxygen content (ppm) in yoghurt supplemented with various levels of ascorbic acid.....	138
10	6.1.3	Changes in the redox potential (mV) in yoghurt supplemented with various levels of ascorbic acid.....	139
11	6.1.4	Changes in the ascorbic acid content of yoghurt during manufacture and storage of yoghurt.....	140
12	6.2.1	Changes in pH during storage of yoghurts made using four different starter cultures with various levels of cysteine.....	153
13	6.2.2	Changes in titratable acidity during storage of yoghurts made using four different starter cultures with various levels of cysteine.....	154
14	6.2.3	Changes in redox potential during storage of yoghurts made using four different starter cultures with various levels of cysteine.....	155
15	6.3.1	Changes in pH during 24 h fermentation of yoghurt mix with C3 starter culture.....	176
16	6.3.2	Changes in redox potential (mV) during 24 h fermentation of yoghurt mix with C3 starter culture.....	177
17	6.3.3	Changes in pH and titratable acidity (% lactic acid) during refrigerated storage of yoghurt supplemented with various ingredients.....	178

18	6.3.4	Changes in redox potential (mV) of yoghurt during refrigerated storage.....	179
19	6.3.5	Polysaccharide production by *S. thermophilus* as evident by Maneval's staining.....	180
20	6.3.6	Electron micrographs of *S. thermophilus* cells in yoghurt made with various ingredients.....	181
21	6.3.7	Silver stain of SDS-PAGE of casein free fraction collected from various yoghurts.....	182
22	6.3.8	Analyses of various bands of silver stain of SDS-PAGE of casein free fraction collected from various yoghurts...	183
23	6.4.1	Firmness of yoghurt measured as penetration depth (mm) affected by supplementation with various ingredients.....	192
24	6.4.2	Changes in viscosity (Cps) during refrigerated storage (4° C) supplemented with various ingredients.....	193
25	6.4.3	Microscopic texture analysis of yoghurts supplemented with various ingredients.....	194
26	7.1.1	Zones of inhibition produced by *L. acidophilus* against *L. delbrueckii* ssp. *bulgaricus* using modified spot on lawn technique.....	203
27	7.1.2	Zones of inhibition produced by *L. acidophilus* against *L. delbrueckii* ssp. *bulgaricus* using agar well assay technique.....	204
28	7.2.1	Changes in pH during growth of *L. acidophilus* (LA-1) in various sugar based MRS broth.....	223
29	7.2.2	Zone of inhibition during growth of *L. acidophilus* (LA-1) culture in various sugar based MRS broth.....	224
30	7.2.3	Bacteriocinogenic activity of *L. acidophilus* (LA-1) as affected by its growth in milk, MRS broth and MRS broth supplemented with ß-glycero-phosphate.....	225
31	7.2.4	Changes in pH during growth of *L. acidophilus* (LA-1) in milk, MRS broth and MRS broth supplemented with ß-glycerophosphate.....	226
32	7.2.5	Viable counts of *L. acidophilus* (LA-1) in milk, MRS broth and MRS broth supplemented with ß-glycerophosphate.....	227
33	7.2.6	Changes in the cell density of *L. acidophilus* (LA-1) in MRS broth and MRS broth supplemented with ß-glycerophosphate.....	228
34	7.2.7	Utilisation of glucose by *L. acidophilus* (LA-1) in MRS broth and MRS broth supplemented with ß-glycerophosphate.....	229
35	7.2.8	Activity of bacteriocin like inhibitory substance produced by *L. acidophilus* (LA-1) in MRS broth in fermentors at various pH and after addition of glucose and triple strength MRS broth.....	230
36	7.2.9	Changes in pH during growth of *L. acidophilus* (LA-1) in MRS broth in fermentors at various pH and after addition	

		of glucose and triple strength MRS broth.....	231
37	7.2.10	Growth of *L. acidophilus* (LA-1) in MRS broth in fermentors at various pH and after addition of glucose and triple strength MRS broth.....	232
38	7.2.11	Changes in cell density of *L. acidophilus* (LA-1) in MRS broth in fermentors at various pH and after addition of glucose and triple strength MRS broth.....	233
39	7.2.12	Utilisation of glucose by *L. acidophilus* (LA-1) in MRS broth in fermentors at various pH and after addition of glucose and triple strength MRS broth.....	234
40	7.2.13	Zones of inhibition obtained by *L. acidophilus* (LA-1) against *L. delbrueckii* ssp. *bulgaricus* (2519).....	235
41	7.2.14	Silver stain of various fractions separated on SDS-PAGE during purification of BLIS.....	236
42	7.4.1	Bacteriocinogenic activity of *L. acidophilus* (LA-1, BDLA-1, 2409 and MOLA-1) as affected by their growth in MRS broth (A), MRS broth supplemented with β-glycerophosphate and MRS broth adjusted to pH 5.0 (C)..	260
43	7.4.2	Bacteriocinogenic activity of *L. fermentum* (5174) and *L. plantarum* (2903) as affected by their growth in MRS broth (A), MRS broth supplemented with β-glycerophosphate and MRS broth adjusted to pH 5.0 (C)..	261
44	7.4.3	Changes in cell density and pH during growth of *L. acidophilus* (LA-1, BDLA-1, 2409 and MOLA-1) as affected by their growth in MRS broth (A), MRS broth supplemented with β-glycerophosphate and MRS broth adjusted to pH 5.0 (C).....	262
45	7.4.4	Changes in cell density and pH during growth of *L. fermentum* (5174) and *L. plantarum* (2903) as affected by their growth in MRS broth (A), MRS broth supplemented with β-glycerophosphate and MRS broth adjusted to pH 5.0 (C).....	263
46	7.4.5	Silver stain of fractions of ammonium sulfate precipitates of *L. acidophilus* (LA-1, BDLA-1, 2409 and MOLA-1), *L. fermentum* (5174) and *L. plantarum* (2903) separated on SDS-PAGE.....	264

LIST OF ABBREVIATIONS

AFISC	Australian Food Industry Science centre
BGP	Disodium β-glycerophosphate
BLIS	Bacteriocin like inhibitory substance
BSA	Bovine Serum Albumin
CSCC	CSIRO culture collection
CSIRO	Commonwealth Scientific and Industrial Research Organisation
F6PPK	Fructose 6 phosphate phosphoketolase
MRS	deMan, Rogosa and Sharpe
NFDM	Non fat dried milk
NGYC	Non fat dry milk supplemented with glucose (2% w/v), yeast extract (1% w/v) and L-cysteine hydrochloride (0.05% w/v)
NNLP	Neomycin sulfate, nalidixic acid, lithium chloride and paromomycin sulphate
RCA	Reinforced clostridial agar
SDS-PAGE	Sodium dodecyl sulfate poly acrylamide gel electrophoresis
ST agar	*Streptococcus thermophilus* agar
TA	Titratable acidity
TS	Total solids
UNSW	University of New South Wales

1.0 INTRODUCTION

Metchnikoff (1908) in his fascinating treatise "The Prolongation of Life" suggested that the longevity of Bulgarians was in part due to ingesting large quantities of fermented milks containing lactobacilli. This observation has led to burgeoning activity on the elucidation of the role of lactic acid cultures and cultured milk products in alleviation of human and animal disorders. Recent advances in our knowledge of biosynthetic activities of lactic acid bacteria and their antagonistic action against pathogenic, toxigenic and putrefactive organisms have added a new dimension to the importance of fermented milks in human nutrition and health. The lactic acid bacteria, especially *Lactobacillus acidophilus* and bifidobacteria which normally inhabit the human intestine could be used as dietary adjuncts. The benefits offered by these organisms include improvement of intestinal disorders and lactose tolerance, antagonism against various pathogenic and spoilage organisms, altered protein and vitamin contents of milk which are beneficial with respect to their digestibility, lowering of serum cholesterol levels and anti-carcinogenic activities. A report also claims correlation of anti-hypersensitivity with consumption of fermented milks (Yamamoto *et al.*, 1994). A few studies have reported an inverse relationship between the consumption of fermented milks and the risk of breast cancer (Le *et al.*, 1986; Van't Veer *et al.*, 1989).

It has been suggested that a minimum of 10^5 to 10^6 cfu.g^{-1} viable cells of probiotic bacteria (*L. acidophilus* and bifidobacteria) should be present in a product to provide therapeutic benefits (Robinson, 1987). However, market surveys have revealed that there is a need to develop starter cultures of *L. acidophilus* and bifidobacteria which would survive under the present manufacturing and distribution practices and would offer nutritional and therapeutic benefits (Anon, 1992; Iwana *et al.*, 1993; Shah *et al.*, 1995). There is also a need to develop media to obtain counts of *L. acidophilus* and bifidobacteria in the presence of yoghurt bacteria with maximum recovery and to modify certain aspects of processing in order to improve viability and survival of these cultures in fermented milk.

The information available in the literature regarding methods for enumeration of yoghurt and probiotic bacteria is not complete and not applicable to selectively enumerate these two groups of bacteria when the fermented milk product contains all four organisms. Similarly, studies conducted for examining the viability in the past did not examine and identified all the factors (such as organic acids, pH, oxygen content, redox potential, hydrogen peroxide, nutritional factors and antagonism between yoghurt bacteria and probiotic bacteria) together. Also, no information is available on antagonism between yoghurt bacteria and probiotic bacteria. The information pertaining to effect of nutritional factors of various ingredients on the viability of youghurt and probiotic bacteria and on textural characteristics of the fermented milk product is in scant in the literature.

This study was aimed at achieving two major objectives. This included examining all the factors together that affect the viability of yoghurt bacteria and probiotic bacteria in the commercial starter cultures. This was designed to help industry partner to adopt holistic and scientific approach for improving viability and survival of *L. acidophilus* and bifidobacteria in yoghurt-like products made from commercial starter cultures manufactured by them. The second major objective was to examine antagonism between yoghurt bacteria and probiotic bacteria for which the information is not available in the literature; followed by isolation, purification and characterisation of substance/s responsible for such antagonistic effect/s between yoghurt bacteria and probiotic bacteria. The specific objectives of this project were to:

(1) assess several bacteriological media and develop methods for enumeration of *L. acidophilus,* bifidobacteria and yoghurt cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) in order to optimise selective recovery of the four groups of organisms in a product,

(2) study the viability and survival of *L. acidophilus* and bifidobacteria in a yoghurt-like (as per the legal definition, a product can not be called yoghurt in North America and some other countries, if organisms other than *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are added in the fermented milk product)

product prepared from commercial starter cultures produced by Chr. Hansen's Laboratory, the industrial partner for this project,

(3) study the effects of rate of starter additions, use of oxygen scavenging and oxygen reducing systems on viability of *L. acidophilus* and bifidobactria during manufacture and refrigerated storage of yoghurt-like fermented milk product,

(4) study the antagonism between yoghurt bacteria and probiotic bacteria and isolate, purify and characterise antibacterial substance/s produced by selected strains of lactobacilli.

Chapters 1 and 2 of this thesis contain the review of literature, Chapter 3 contains materials and methods and Chapter 4 deals with the enumeration of bacteria. Chapter 5 deals with the viability of yoghurt and probiotic bacteria in yoghurts made with commercial starter cultures and the results of effect of various ingredients on viability of yoghurt and probiotic bacteria are summarised in Chapter 6. Chapter 7 deals with the results of antimicrobial substances produced by yoghurt and probiotic bacteria, whereas Chapter 8 gives summary of results. The future research directions and list of references are given in Chapter 9 and 10, respectively.

2.0 LITERATURE REVIEW

At the beginning of this century, Nobel laureate Elie Metchnikoff at the Pasteur Institute linked health and longevity to the ingestion of bacteria present in foods such as yoghurt, kefir and sour milk. He hypothesised that these microflora control the infection caused by enteric pathogens and regulate the natural chronic toxaemia which plays a major role in ageing and mortality (Bibel, 1988). This observation and a large number of publications on lactic acid bacteria provided boosts to the manufacture and consumption of fermented products especially yoghurt type products (IDF:179, 1984).

2.1 Yoghurt and Fermented milks

Fermentation was the first technique employed by humans for food preservation from the time immemorial. It has played many important roles in human nutrition. There are various methods of carrying out the fermentation process as practised in various parts of the world and these give rise to a range of fermented milk products including yoghurt. The fermented milk products vary considerably in composition, flavour and texture, depending on the nature of fermenting organisms, the type of milk and the manufacturing process used (Rasic and Kurmann, 1978; Tamime and Deeth, 1980; Tamime and Robinson, 1985).

The word 'yoghurt' was derived from the Turkish word 'Jugurt' and Table 2.1 shows the various names by which the product is known in different countries. Some controversy still exists regarding the exact definition of yoghurt in terms of its chemical composition and types of starter organisms used. However, the most widely agreed definition of yoghurt is "the product resulting from milk by fermentation with a mixed starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*". In some countries including Australia, other organisms such as *Lactobacillus helveticus* and *Lactobacillus jugurti* are also added in yoghurt by some yoghurt manufacturers.

Table 2.1 Yoghurt and yoghurt-like products as known in various countries of the world

Traditional name	Country	Traditional name	Country
Jugurt/Eyran	Turkey	Tiaourti	Greece
Busa	Turkestan	Cieddu	Italy
Kissel Mleka	Balkans	Mezzoradu	Sicily
Urgotnic	Balkan Mountains	Gioddu	Sardinia
Leben/Leban	Lebanon and some Arab countries	Piimä/Filmjölk/Fill-bunke/Filbunk/Surmelk/ Taettem-jolk/Tettemelk	Scandinavia
Zabady	Egypt and Sudan	Tarho	Hungary
Mast/Dough	Iran and Afghanistan	Villi	Finland
Roba	Iraq	Skyr	Iceland
Dahi/Dadhi/ Dahee	India, Bangladesh, Nepal	Yoghurt/Yogurt/ Yaourt	Rest of the world
Mazun/Matzoo	Armenia	Yourt/Yaourti/ Yahourth/	('Y' is replaced by 'J' in some
Katyk	Transcaucasia	Yogur/Yaghourt	instances)

(Source: Tamime and Deeth, 1980).

2.1.1 Types of yoghurt

The manufacture of the many types of yoghurt differs according to their chemical composition, method of production, flavour and the nature of post-incubation processing. The legal standards for the chemical composition of yoghurt in various countries are based on three possible types of yoghurt classified according to the fat content (full, medium or low) of the product (FAO/WHO, 1973). Such compositional

standards are designed to facilitate standardisation of product and to protect the consumers.

In general, there are two main types of yoghurt, set and stirred, which are based on the method of production and on the physical structure of the coagulum. Set yoghurt is the product formed when fermentation/coagulation of milk is carried out in a retail container, and the yoghurt produced is in a continuous semi-solid mass. In contrast, stirred yoghurt results when the coagulum is produced from milk and the gel structure is broken before cooling and packaging. Fluid yoghurt can be considered as stirred yoghurt of low viscosity. Flavouring of yoghurt is another method often used to differentiate the various types of yoghurts. Flavoured yoghurts are basically divided into three categories. Plain or natural yoghurt is the traditional product which has a typical sharp 'nutty' flavour. Fruit yoghurts are made by addition of fruits, usually in the form of fruit preserves, puree or jam. Flavoured yoghurts are prepared by adding sugar or other sweetening agents, synthetic flavourings and colourings to plain or natural yoghurt.

The post-incubation processing of yoghurt may lead to many types of yoghurt such as pasteurised/UHT yoghurt, concentrated yoghurt, frozen yoghurt and dried yoghurt. Pasteurised/UHT yoghurt is heat treated after incubation which leads to destruction of yoghurt starter bacteria and reduction in the levels of volatile compounds which are associated with the flavour of yoghurt. Frozen yoghurt which can be either soft or hard, is a product that resembles like an ice-cream; however, its chemical composition and manufacturing details up to freezing stage is similar to yoghurt. Dried yoghurt can be produced by sun-drying, spray-drying or freeze-drying of yoghurt. The drying process transforms the junket into powder and also causes loss of some flavour compounds and the destruction of starter culture. Another type of yoghurt which may find favour among diet conscious consumers is low-calorie yoghurt in which viscosity is improved by addition of stabilisers and thickening agents such as carrageenan, gelatin etc.

2.1.2 Starter cultures used for yoghurt and fermented milks

There are several types of fermented milk products and each product is made with different lactic starter bacteria. The names of various products, the starter culture used for their manufacture and the name of the country in which they are popularly used are summarised in Table 2.2.

2.2 Probiotics

2.2.1 Definition

The term probiotic originated from two Greek words meaning 'for life' and 'against life'. According to Parker (1974) probiotics are 'organisms and substances produced by these organisms which contribute to intestinal microbial balance'. Fuller (1989) defined probiotics as a 'live microbial food supplement which beneficially affects the host by improving their intestinal microbial balance'. Lyons (1988) suggested that a successful probiotic must meet certain criteria which include the following:

(i) must be capable of reaching and colonising the intestinal tract,
(ii) must be acid resistant and rapid acid producers,
(iii) should be present in sufficient numbers to be effective,
(iv) must be quickly activated and must have a high specific growth rate and antimicrobial activity,
(v) the bacterial preparation should be stable throughout the storage period, and
(vi) must provide nutrient source which selectively promotes growth of beneficial intestinal microflora.

The selection of probiotic strains for production of fermented milks and other preparations is also based on production of acid, aroma, intensity of proteolysis, formation of L (+) or D (-) lactic acid and resistance against antagonistic substances, chemicals.

2.2.2 Probiotics and prebiotics

In recent days, 'functional foods' are increasing their market share and probiotics or prebiotics or a combination of both of these (synbiotics) are expected to increase in the 21st century. As described earlier, 'probiotic' is a mono or mixed culture of live microorganisms which when applied to man or animal beneficially affects the host by improving the properties of the indigenous microflora (Havenaar and Huis in't Veld, 1992). 'Prebiotic' is a non-digestible food that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995). 'Synbiotic' is a combination of the effects of probiotics and prebiotics to produce health-enhancing functional food ingredients (Gibson and Roberfroid, 1995). The authors state that these compounds must:

(i) be neither hydrolysed nor absorbed in the small intestine,
(ii) be a selective substrate for one or a limited number of beneficial bacteria commensal to the colon,
(iii) be able to promote a healthier gut flora, and
(iv) as a consequence induce luminal or systemic effects that are beneficial to the host.

Clinical data regarding probiotic bacteria are well reviewed (Yuan and Salminen, 1995) together with requirements for the selection of suitable probiotic cultures and ways of ensuring that the strains remain viable and effective during the shelf-life of the product. The same authors highlighted state of the art-successful strains (*L. acidophilus* LC1, *L. acidophilus* NFCO 1748, *Lactobacillus* GG, *L. casei*-Shirota, *L. gasserri*, *Bifidobacterium bifidum*, *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*) that are gaining popularity throughout the world.

Some oligosaccharides, due to their chemical structure, are resistant to digestive enzymes and therefore pass into the large intestine where they become available for

Table 2.2 Fermented milks containing dietary lactic acid bacteria

Name	Culture(s) used	Country in which the product is popular
A-38	Cultured butter milk + acidophilus milk (9:1)	Denmark
Acidophilus milk	*L. acidophilus*	Europe, North America
Acidophillin	*L. acidophilus*, *L. lactis* *Kefir grains*	Russia
Acidophilus blend	*Sour milk (90) + acidophilus milk (10)*	Czechoslovakia
Acidophilus paste	*L. acidophilus*	Russia, Japan
Acidophilus yeast milk	*L. acidophilus* and sugar fermenting yeast	Russia
ACO-yoghurt	*S. thermophilus*, *L. bulgaricus* and *L. acidophilus*	Switzerland
Bioghurt	*S. thermophilus*, *L. acidophilus*	Germany
Biogarde	*S. thermophilus, L. acidophilus* and *Bif. Bifidum*	Germany
Biolact	*L. acidophilus*	USSR
Biokys	*Bif. bifidum, Pediococcus acidilactici*, *L. acidophilus*	Czechoslovakia
Bulgarian butter milk	*L. bulgaricus*	Europe, Balkan
Cultura-AB	*L. acidophilus, Bif. Bifidum*	Denmark
Dahi	*L. lactis* and its sub sp., *S. thermophilus, L. bulgaricus*	India, Pakistan, Bangladesh
Kefir	*L. casei, L. lactis, L. acidophilus, L. bulgaricus, Kluy. fragilis, Candida kefir (Kefir* grains*)*	Middle East, North Africa, Russia
Kumiss	*L. lactis, L. bulgaricus, Candida kefir or Kluy. Lactis*	Russia, Europe, Middle East, East Asia
Pastolakt	*L. acidophilus*	Russia
Tvorog stolichnaya	*L. acidophilus*	Russia
LC1	*L. acidophilus* 1 of Nestle Ltd.	Europe and Asia-pacific
Yakult	*L. casei* (Shirota*)* and *L. acidophilus*	Japan, South East Asia
Yoghurt	*S. thermophilus* and *L. bulgaricus*	Worldwide

(Source: IDF, 1988; Mital and Garg, 1992; Patidar and Prajapati, 1997; Richardson, 1996; Yuan and Salminen, 1995)

fermentation by the saccharolytic bacteria. Compounds which are either partially degraded or not degraded by the host and are preferentially utilised by bifidobacteria as carbon and energy sources are defined as "bifidogenic factors". These substances could be used as prebiotics and could also be called 'colonic' foods, which enter the large intestine (colon) and serve as substrates for endogenous colonic bacteria, thereby indirectly providing the host with energy, metabolic substrates and essential nutrients. Some of the bifidogenic factors that are of commercial significance include fructo-oligosaccharides, lactose derivatives such as lactulose, lactitol, galacto-oligosaccharides, isomalto-oligosaccharides, xylo-oligosaccharides, gluco-oligosaccharides and soybean oligosaccharides (O'Sullivan, 1996). Resistant starch and non-starch polysaccharides are classified as colonic foods but not as prebiotics because they are not metabolised by certain beneficial bacteria (O'Sullivan, 1996).

2.2.3 Historical background and growth characteristics of L. acidophilus and bifidobacteria

In the beginning of this century, the term 'lactic acid bacteria' (LAB) was used synonymously with 'milk souring organisms. In most LAB, lactic acid is a major metabolic end product produced as a result of carbohydrate metabolism. Some members of LAB group produced acetic and propionic acids in addition to lactic acid (Bisset and Anderson, 1974; Thomas *et al.*, 1980; Kandler, 1983; Kandler and Weiss, 1986). An important progress in classification of these bacteria was made when similar characteristics between milk souring bacteria and other lactic acid producing bacteria in various habitats were recognised (Henneberg, 1904; Lohnis, 1907). Orla-Jensen (1919) classified LAB based on the characteristics such as growth temperature, cell morphology, sugar fermentation patterns and the form of lactic acid (D, L or DL) produced. LAB can be homofermentative or heterofermntative based on their patterns of carbohydrate fermentation. The group of LAB that produces lactic acid as a major end product is referred to as 'homofermentative' and that which produces CO_2, ethanol and acetic acid in addition to lactic acid is referred to as 'heterofermentative'.

Tissier (1899) at the Pasteur Institute in Paris discovered a bacterium from the stools of infants that had an unusual Y-shaped morphology. This was the first recorded observation of bifidobacteria, though the information on group or genus to which the bacteria would belong to was not known at that time by the researchers. Further, a similar bacterium was isolated from faecal matter and the organism was reported to be belonging to a member of genus *Lactobacillus* (Moro, 1900). Originally, bifidobacteria were included in genus *Lactobacillus* and the organism was referred to as *Lactobacillus bifidus* (Breed *et al.*, 1957; Denhert, 1957). De Vries and Stouthamer (1967) 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. This formed a basis for bifidobacteria to be reclassified and to be excluded from the genus *Lactobacillus* (De Vries and Stouthamer, 1967).

Although the classification of LAB into various genera was mainly based on the characteristics such as morphology and mode of sugar fermentation as used by Orla-Jensen (1919), additional characteristics such as fatty acid composition, motility, G + C content, DNA homology, hybridisation frequency etc. are used as the basis for most recent classification of microorganisms. Recent taxonomic revisions suggest that LAB could be comprised of genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus,* and *Vagococcus* (Moss, 1981; Collins *et al.*, 1987; Stackerbrandt and Teuber, 1988; Collins *et al.*, 1989, 1990).

The measurement of true phylogenic relationships using rRNA sequencing has been recommended 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 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 their DNA. Other LAB contain < 50 mol percent G + C in their DNA. The major difference in *L. acidophilus* and *Bifidobacterium* spp. is that *L. acidophilus* is rod shaped, whereas bifidobacteria show variable morphology characterised by branching and pleomorphism. Both of the organisms are Gram positive; however, *L. acidophilus* is microaerophilic and bifidobacteria prefer anaerobic conditions for their growth (Sharpe, 1979; Yang and Woese, 1989).

2.2.4 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, 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 they 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, nitrate 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, whereas 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 2.3) have been divided into 3 fundamental groups A, B, C and could be briefly described as below:

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

(ii) 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 phosphogluconate pathway are repressed.

(iii) 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 comes 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 DAN-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 < 25% DNA-DNA homology, whereas strains within each subgroup shared a similarity of 75-100%. Recent reports on the systematic studies of *L. acidophilus* employing electrophoresis of soluble proteins or lactate dehydrogenase and DNA-DNA re-association indicated that *L. acidophilus* strains include six genomic species. 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 2.3 Species of genus Lactobacillus

1	*L. 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. gasseri*	35	*L. brevis*
8	*L. helveticus*	36	*L. buchnerii*
9	*L. genseni*	37	*L. collinoides*
10	*L. johnsonii*	38	*L. fermentum*
11	*L. kefiranofaciens*	39	*L. fructivorans*
12	*L. aviarius*	40	*L. hilgardii*
13	*L. fraciminis*	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. reuteri*
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. sanfrancisco*
23	*L. coryneformis*	51	*L. confusus*
24	*L. curvatus*	52	*L. fructosus*
25	*L. graminis*	53	*L. halotolerans*
26	*L. homohiochii*	54	*L. viridescens*
27	*L. intestinalis*	55	*L. kandlery*
28	*L. murinus*	56	*L. minor*

(Source: Wood and Holzapfel, 1995).

These new techniques allow clear differentiation between six subgroups of *L. acidophilus* strains: *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.

2.2.4.1 Proteolytic activity of lactobacilli

When lactobacilli are added to milk, they primarily ferment lactose to lactic acid. These bacteria must possess very limited diversity in order to produce fermented milk products with acceptable organoleptic characteristics and increased digestibility i. e. L (+) or D (-) lactic acid. The growth of these bacteria depends on adequate supplies of suitable sources of nitrogen and carbon. If the starter organisms possess a lactose hydrolysing enzyme, then the carbon source is not a limiting factor. However, this is not so with respect to the nitrogen source because free amino acids and peptides are present only to a limited degree in milk (Thomas and Mills, 1981). So the lactic acid bacteria used must possess an efficient proteolytic system which enables them to grow to high cell densities and must have an ability to ferment lactose rapidly to lactic acid.

Law and Kolstad (1983) reported that LAB are weakly proteolytic compared with other groups of bacteria such as *Bacillus, Proteus, Pseudomonas* and *Coliforms*. Thomas (1985) observed that the proteolytic activity of lactic acid bacteria was dependent on species and strains. Amongst lactobacilli, the highest proteolytic activity was observed in *L. helveticus*, *L. bulgaricus, L. acidophilus* followed by *L. casei*. Miller and Kandler (1964) found that milk fermented with five different strains of *L. acidophilus* was characterised by a high content of free amino acids similar to that of milk cultured with *L. delbrueckii* ssp. *bulgaricus* although with a lower serine and threonine content. Sands and Hankin (1976) reported that mutants of *L. acidophilus* and *L. delbrueckii* ssp. *bulgaricus* produced large quantities of lysine. High proteolytic activity of selected *L. acidophilus* strains is reported by Singh and Sharma (1983) and Koroleva *et al.* (1983).

2.2.5 Genus Bifidobacterium

Since bifidobacteria were first discovered in the beginning of the century, a number of important discoveries were made after the advent of chemotaxonomy during 1960s. Sebald *et al.* (1965) showed that the percentage G + C content in the DNA of bifidobacteria

differed from that of *Lactobacillus, Corynebacterium* and *Propionibacterium.* In 1974, Bergey's Manual of Determinative Bacteriology (8th edn.) recognised *Bifidobacterium* as genus in its own right consisting of 11 species (Buchnan and Gibbons, 1974). Scardovi (1986) included 24 species in *Bifidobacterium* genus. 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 natural cavities and surfaces of humans. According to the latest collection of species in the genus *Bifidobacterium*, additional five species have been described (Table 2.4) making a total number of 29 species (Biavati and Mattarelli, 1991; Biavati *et al.*, 1991).

Table 2.4 Species of genus Bifidobacterium

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

(Source: 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 pleomorphism depends mainly on culture medium and 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-acetyl glucosamine and amino

acids produce 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 depends 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 different types of response 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 fructose 6 phosphate phospho ketolase (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. Such strains always require a strict anaerobic condition and low redox potential for growth and fermentation.

Optimum growth temperature of the species of human origin is around 37 $\pm$ 1°C and those of animal origin is around 42 $\pm$ 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 occurs 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 in less than 3 h (Lankaputhra and Shah, 1995; Lankaputhra *et al.*, 1996b).

Most species of bifidobacteria of human origin produce vitamins such as thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), cyanocobalbumin (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.

Relative insensitivity 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. Knowledge of resistance to antibiotics is also important due to their applicability as agents in selective media for various species of bifidobacteria. Although sufficient data 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 against these antibiotics varies from 10-500 $\mu g.mL^{-1}$ (LaVergene *et al.*, 1959; Miller and Finegold, 1967). On the contrary, bifidobacteria are strongly inhibited by ampicillin, bacitracin, chloramphenicol, clindamycin, erythromycin, lincomycin, nitrofurantoin, oleandomycin, penicillin G and vancomycin (Scardovi, 1986).

2.2.5.1 Growth requirements of bifidobacteria

2.2.5.1.1 Nutrition

After early observations that the growth of bifidobacteria is stimulated by human milk has been the object of numerous nutritional studies designed to elucidate the properties of bifidus factor/s present in human milk or to find a substitute for it (Scardovi, 1986). Some reports have appeared regarding the growth of bifidobacteria in fully synthetic media, although several of these were published before the multiplicity of bifidobacterial species was recognised. The essential factor in human milk which was lacking in cow's milk (bifidus factor) was subsequently identified as N-acetyl-D-glucosamine containing saccharides (Glick *et al.*, 1960; O'Brien *et al.*, 1960). Bifidus factor of human milk casein was further characterised by a number of workers (Bezkorovainy *et al.*, 1979; Poch and Bezkorovainy, 1988).

Bovine milk casein bifidus factor was isolated by Kehagias *et al.* (1977), Ziajka and Zbikowski (1982), Zbikowski and Ziajka (1986b), Poch and Bezkorovainy (1988) and Pahwa *et al.* (1989) and the bifidus factor was reported to stimulate the growth of *Bifidobacterium* spp. A medium prepared from faeces homogenate of breast fed infants showed bifidus stimulating activity (Yoshioka *et al.*, 1968a, b). Lactulose (4-O-β-D-galactopyranosyl-D-fructose) also has growth promoting effect on bifidobacteria (Park *et al.*, 1988; Mizota *et al.*, 1987). Various oligosaccharides that stimulated the growth of *Bifidobacterium* spp. have been prepared by the action of enzymes and yeasts on lactose containing medium, maize cob, cotton husk, malt cake and hardwood extract by Zilliken *et al.* (1954), Suwa *et al.* (1988), Matsumoto *et al.* (1987), Najivi *et al.* (1987), Dombou *et al.* (1988), Kan and Kobayashi (1988) and Kobayashi *et al.* (1988).

2.2.5.1.2 Oxygen issue

Though, bifidobacteria are anaerobic microorganisms (Scardovi, 1986), investigators ask the question as to whether they are strictly anaerobic as certain aerobic strains have been reported. In studying branched and unbranched strains of bifidobacteria, Norris *et al.* (1950) noted differences in their requirement for atmospheric CO_2 and sensitivity to atmospheric O_2. The differences in sensitivity to oxygen were found for different species of bifidobacteria by DeVries and Stouthamer (1969). Interests in producing aerobic strains of *Bifidobacterium* spp. by improving oxygen resistance have been considerably increasing in recent years. Mutai *et al.* (1978, 1980b) actively propagated a new strain of *B. bifidum* YIT 4002 under aerobic conditions in pure milk medium without adding any additional growth promoting substance or reducing agents. A drink composed of rice, lactose, cow milk, sugar and water and cultured with *Bifidobacterium adolescentis* was patented by Mutai *et al.* (1980a), who observed that this strain did not require any anaerobic condition for the growth. Misra (1988) reported that *B. bifidum* did not require any anaerobic condition for its growth in liquid medium; however, degassing the medium by heating for 15 min was observed to slightly increase their growth in liquid medium. Cheng and Sandine (1989) found satisfactory growth of a variety of *Bifidobacterium* spp. without the use of anaerobic

condition in whey based medium containing L-cysteine (0.05%) and yeast extract (0.3%). Brown and Twonsley (1970) also reported that, after original faecal isolation, no special anaerobic conditions were required for the growth of *L. bifidus*.

2.2.5.1.3 Growth in milk

Feeding of bifidobacteria is considered to be beneficial for human beings of all age groups. The most suitable medium that can be used for administration of this organism to humans is the milk (Collins and Hall, 1984). Unfortunately, milk is considered to be less than optimum medium for the growth of bifidobacteria (Kisza and Ziajka, 1973; Gilliland, 1989; Cheng and Nagasawa, 1984). Therefore, considerable studies have been carried out to improve the growth of bifidobacteria in milk.

The fermentation properties of *L. bifidus* in cow's milk was first studied by Brown and Twonsley (1970). They reported that these organisms could be cultured readily and milk coagulated in less than 24 h, but a great deal of variability in growth, consistency, vigour and acid production was observed among the various strains of bifidobacteria. Bifidobacteria were cultured in cow's milk, cow's milk with 20 per cent pepsin digested milk and cow's milk with 2 per cent lactulose syrup (Ziajka *et al.*, 1974). They reported maximum growth and acid production by most strains in pepsin supplemented milk. Kisza and Ziajka (1973) also found growth promoting effects in cow's milk on addition of pepsin or lactulose syrup. Kisza *et al.* (1974) reported increases in cell biomass of bifidobacteria in whey, whey digested with pepsin, whey plus whey proteins digested with pepsin, whey proteins digested with pepsin and skim milk digested with pepsin.

Growth of *B. bifidum* was studied in sterilised skim milk added with lysozyme (100 $mg.L^{-1}$) and incubated for 2 h followed by heating at 80°C for 15 min to inactivate lysozyme. They reported that modified milk stimulated the growth of *B. bifidum*, but direct addition of lysozyme to milk had no stimulatory effect. A starter culture composed of four bifidobacterial strains had a satisfactory milk acidification capacity as reported by Kosikowski (1978). The milk was coagulated within 20 h of incubation and had acidity of

0.77 to 0.9 per cent. In 48 h old cultured milk the number of viable cells varied from 0.6 x 10^5 to 1.5 x 10^9. High acid producing strains of bifidobacteria were isolated from infant faeces by Semenikhina and Sundukova (1982). The bifidobacterial isolates were capable of forming curd in less than 24 h. They also reported that introducing 5% of yeast extract, 0.5-1.0% of corn extract and 5% of milk hydrolysed with pancreatin into sterile milk increased the number of bifidobacteria to 10^9 to 10^{10} cells.mL^{-1} within 16 h incubation at 37°C. Kosikowski (1982) suggested the use of sterile milk supplemented with 0.5% Bacto-liver, 0.05% $MgSO_4$ and 0.001% cysteine for growth of bifidobacteria in milk. Marshall *et al.* (1982) fortified milk with whey protein and threonine to provide the bifidobacteria with nutritious medium of reduced redox potential. Anand *et al.* (1985) and Misra (1988) reported good growth of *B. bifidum* in sterile skim milk supplemented with 1% dextrose and 0.1% yeast extract, respectively. Goh *et al.* (1986) observed that acid production was increased by increasing the inoculum to 5% and by addition of 0.05% cysteine and/or 0.2% yeast extract or 0.2% tryptone.

2.2.5.1.4 Enhancing growth and acid production

The slow growth of bifidobacteria in milk may be improved by using selected fast acid producing strains and/or by the addition of growth promoting substances e.g. yeast extract, pepsin digested milk, maize extract etc. in the milk (Rasic, 1983). Several investigators and manufacturers utilised various ingredients to enhance growth and the acid producing capability of bifidobacterial species. Yakult Co. Ltd. and Snowbrand Milk Product Co., Japan introduced acidophilus drinks in 1970 with an activating material such as yeast extract, pancreatin or glutathione and ascorbic acid.

Samezina *et al.* (1970) reported enhanced acid production in whole milk or modified dried milk on addition of peptides and amino acids obtained from culture free filtrates of *L. casei*. These peptides were also found to be stimulating to the growth of bifidobacteria (Cheng and Nagasawa, 1984). Kosikowski (1982) observed that metabolic products of unwanted organisms (*Pseudomonas* spp., *Micrococcus* spp., *E. coli* etc.) in milk stimulated the growth of bifidobacteria and also enhanced acid production. The rate of growth and

acid development by bifidobacteria have been found to be stimulated by the addition of maize extract and a solution of mineral salts (Semenikhina and Sundukova, 1980; Semenikhina *et al.*, 1985b), fish meal hydrolysate (Yuguchi, 1981), 0.2% MRS-broth and 0.2% ascorbic acid (Collins and Hall, 1984), vitamins, dextrin and maltose (Semenikhina *et al.*, 1985a) in milk medium.

Inclusion of carrot juice in the diet has been found to cure for babies suffering from maldigestion and diarrhoea (Tamura, 1983). Ohta (1959) and Kuromiyo (1960) found that carrot extract increased the number of bifidobacteria in the intestine of babies. Yoshioka *et al.* (1968c) found that some strains of bifidobacteria required carrot extract suggesting that it contains unknown bifidus factors. Some strains of *B. bifidum* were reported to have no growth in semi-synthetic medium unless carrot extract was added to it (Tamura, 1983). The stimulatory effect of carrot juice on the acid development and growth of *B. bifidum* in cow's milk was investigated by Zibkowski and Ziajka (1986a). They reported the highest increase in acidity in milk added with 5% carrot juice. In a similar study, Misra (1988) concluded that addition of 5% carrot juice was optimum for the growth of bifidobacteria in fermented milk.

A β-D-galactosidase preparation from *Saccharomyces fragilis* possessing hydrolytic as well as transglycolytic activity was tested as growth factor for bifidobacteria in sterile skim milk by Khamagaeva *et al.* (1983) and Khamagaeva and Kulikova (1984). They reported increased lactic acid production by bifidobacteria with β-D-galactosidase at 0.1-0.2% concentration. A stimulatory effect of addition of β-D-galactosidase on growth and acid production in milk inoculated with *B. adolescentis* was studied by Khattab *et al.* (1986). In their study, 200 mg enzyme per litre was added at the same time of inoculation or 0.5, 1 and 2 h before inoculation at 37°C and all the treatments stimulated growth and acid production.

2.2.6 *Selection criteria for lactobacilli and bifidobacteria for their use as dietary adjuncts*

Lactobacilli and bifidobacteria establish an intimate relationship with humans from the time of birth and throughout life. The digestive tract is colonised soon after birth by a variety of micro-organisms. *Lactobacilli* commonly encountered in the intestinal tract include *L. acidophilus, B. bifidum, L. leichmanii, L. plantarum, L. casei* and *L. fermentum* (Moore and Holdman, 1970). Of these the most often mentioned as beneficial for dietary adjuncts is *L. acidophilus*, *L. casei* and *Bifidobacterium* spp. (Gilliland, 1978; Mitsuoka, 1984).

Lactobacilli and bifidobacteria are the autochthonous microflora of human alimentary tract (Savage, 1977; Patel *et al.*, 1991). Gilliland (1978), Kim (1988) and Kurmann (1988) proposed the following characteristics of candidate micro-organisms for their use as dietary adjuncts. The organism should:

(i) be normal inhabitants of the intestinal tract;
(ii) survive the upper digestive tract and maintain stable population;
(iii) produce beneficial effects, and
(iv) maintain viability and activity in the carrier food before consumption.

Manufacture of fermented milk with intestinal lactic acid bacteria is complicated compared to standard products (Tamime and Robinson, 1988; Fonden, 1989). As regards to safety, Fonden (1989) reported that selected species should not cause any infection and should not contribute to the development of dental caries.

Kurmann (1988) proposed that the strain selected should adhere to the small intestinal cells. In the large intestine, the adherence seems to be of minor importance (Freter, 1983). Giurguis and Hickey (1987) reported that selected strains should not be antagonistic to the normal flora of gastro-intestinal tract, but it should be antagonistic towards harmful micro-organisms. Hood and Zottola (1989) suggested that tolerance to low pH should be one of

the selection criteria for lactobacilli to be used as dietary adjuncts. Bile resistance is also one of the important factors enabling lactobacilli and bifidobacteria to survive and grow in the intestinal tract. At present, it is not known as to what level of bile resistance is needed (Gilliland, 1989). However, Gilliland *et al.* (1984) has shown that a strain of *L. acidophilus* possessing high level of bile resistance produced higher numbers of lactobacilli in the intestinal tract than did a strain having lower bile resistance. *L. acidophilus* strains have been reported to resist presence of 2 and 4 percent bile salts (Wheater, 1955; Rogosa and Sharpe, 1959; Muelhens, 1967).

Phenol and phenolic compounds formed in the intestinal tract are chemical agents that can influence the survival of *L. acidophilus* and bifidobacteria in the intestine. Since phenol is the product of a putrefactive process in the large intestine, the resistance to this or other similar compounds such as indole, skatol, etc. may be used as an indication of the intestinal surviving ability of bacterial cultures. Rasic and Kurmann (1978) reported that *L. acidophilus* strains were able to grow in the presence of a maximum of up to 0.5% phenol. Resistance to even 0.6% phenol in milk by a strain of *L. acidophilus* NK-10 is reported by Koroleva *et al.* (1983).

From technological point of view, the strain selected should be able to grow in milk either alone or as a part of the mixed starter. Further, the strain/s should be able to hydrolyse lactose to mainly L (+) lactic acid. During fermentation, the pH should drop to 4.7 or below in less than 20 h, with at most 10 per cent inoculum. To limit the final acidity of the product, pH during storage should not decrease to a value lower than 4.0. The strains should not release bitter peptides and the product should have more than 100 million viable cells per gram of product (Kurmann, 1988).

2.3 Therapeutic properties of probiotic organisms

Most of the work regarding nutrition therapy (or beneficial host-bacterium relationships) has been with lactobacilli or yoghurt cultures, usually *Lactobacillus acidophilus,*

Lactobacillus delbrueckii ssp. *bulgaricus* and *Streptococcus thermophilus* till 1990. Results were variable and this fuelled debates over the years. The ability to survive passage through the stomach and extent of intestinal colonisation and resistance to bile salts are factors that attenuated the perceived value of lactobacilli supplemented and lactic culture products. Another group of lactose utilising bacteria of similar performance is bifidobacteria. These were labelled as a difficult group of organism for research initially because of a perceived need for growth factors and the requirements for anaerobic conditions for their growth. In recent years, because of easy availability of anaerobic jars, more and more scientific workers are concentrating on this group of bacteria. Last decade saw a boom on research on these probiotic cultures. A large number of publications are seen on the use of autochthonous bacteria (*Lactobacillus acidophilus* and bifidobacteria) as dietary adjuncts. Following early observations of Metchnikoff (1908), further impetus to his studies was provided and strengthened by observations of Tomic-Karovic and Fanjek (1963) and Mutai *et al.* (1971). There is sufficient experimental evidence to support that oral administration of lactobacilli (preferably *L. acidophilus*) and bifidobacteria is able to restore the normal balance of microbial population in the intestine (Kopeloff, 1926; Faber and Sutton, 1930; Sanborn, 1931; Haenel, 1970; Sandine *et al.*, 1972; Poupard *et al.*, 1973; Vanovsky, 1975; Gilliland and Speck, 1977b; Rowland and Grasso, 1975; Ferreira and De, 1979; Yoshioka *et al.*, 1983; Hori, 1983; Garvie *et al.*, 1984; Gilliland, 1987; Hotta *et al.*, 1987).

The reason for the concentration of research on probiotics is the health benefits derived by consuming foods containing these bacteria. In addition to their established role in gastro-intestinal therapy, *L. acidophilus* and bifidobacteria are also claimed to offer several other nutritional and therapeutic benefits, which are beyond the scope of this review. Several authors have reviewed and reported the therapeutic roles and importance of these bacteria in detail (Metchnikoff, 1908; Le *et al.*, 1986; Van't Veer *et al.*, 1989; Modler *et al.*, 1990; Hughes and Hoover, 1991; Kanbe, 1992; Mital and Garg, 1992; Nakazawa and Hosono, 1992; Yamamoto *et al.*, 1994). Several symposia and discussions have been held and

published literature is available in plenty. As claimed and reviewed by several workers, these benefits are summarised in Table 2.5.

Table 2.5. Therapeutic properties of acidophilus and bifidus milk products

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

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

2.4 An overview on acidophilus and bifidobacterial products available world-wide

Several products of dairy origin employing these autochthonous bacteria are in the market in various countries around the world. These include sweet acidophilus milk in the USA, cultured acidophilus milks in the Russia, Kazakstan, Uzbekstan, Ukraine, Armenia, Georgia, Tadzhikistan, Yugoslavia, Bosnia, Albania, Czech Republic and Slovakia, Biolact in the Russia, Kazakstan, Uzbekstan, Ukraine, Armenia, Georgia and Tadzhikistan, Aco-yoghurt in Switzerland, dietary yoghurt in the UK, Bio-garde and Bioghurt in Germany, A-38 and Cultura in Denmark, Acidophilin and Acidophilus yeast milks in the Russia, Kazakstan, Uzbekstan, Ukraine, Armenia, Georgia and Tadzhikistan, Biokys and a blend of sour milk (90):acidophilus milk (10) in Czech Republic and Slovakia, some concentrated products like Acidophilus paste, Tvorog, Stolichnaya and Pastolact in the Russia, Kazakstan, Uzbekstan, Ukraine, Armenia, Georgia and Tadzhikistan (Mital and Garg, 1992). Other products are Bifidus milk, Bifighurt, Milmil, Progurt, Nu-trish, Biolact, cultured cream, whey drinks etc. as reviewed by Patel *et al.* (1991). In their article, they have also compiled baby food preparations which include Lantana, Bifiline, Femilact, infant powder, dried concentrates, Bifidus formula and Bifidus powder. Probiotic capsules are also recommended by physicians after heavy antibiotic dosages. Thus, products containing live lactobacilli and bifidobacteria are becoming more and more popular, thus increasing their market shares in the dairy section in supermarkets of most Asia Pacific, European and American countries. Moreover, efforts have been made to expand product application e.g. including the probiotic organisms in meat products (Dabin, 1992). Some workers have prepared cookies and confectionaries with these probiotic bacteria (Wakiguchi, 1984; Patel *et al.*, 1991). Recently, successful incorporation of bifidobacteria has been made in catfish fillet to increase their shelf life during refrigerated storage (Kim *et al.*, 1995).

2.4.1 Market of acidophilus and bifidobacterial products

More than 70 bifidus and acidophilus-containing products are in the market worldwide, mainly of dairy origin (Kim, 1988; Modler *et al.*, 1990). Several preparations employing *L. acidophilus* are well established in the market. In Japan, bifidobacteria containing products are very popular and many fermented milk recipes have been reformulated. These products account for more than one-third of the total yoghurt sales. In France, products containing bifidobacteria and *L. acidophilus* have increased by > 300% to capture 4% of total fresh milk sales. Presently, 11% of all yoghurt sold in France contain added bifidobacteria. In Germany, one of the first bifidus products to be marketed, known as Biogarde, sold more than 400 million units in 1976 and the product is well established in the market. It is produced by 45 dairy companies in Germany and the surrounding countries. In Denmark, a product called Cultura was promoted as a completely safe and easily digestible food which contains "good bacteria". The product is suggested as a daily supplement to the diet to "keep the stomach in shape". Bifidus products are also produced in Canada, Italy, Poland, UK, Czech Republic, Slovakia and Brazil. In USA and Australia, bifidus products have been introduced in the last four to five years, however, products containing *L. acidophilus* are well established (Hughes and Hoover, 1991).

2.4.2 Regulatory aspects of yoghurt

In many parts of the world, dairy manufacturers incorporate probiotic bacteria such as *L. acidophilus* and bifidobacteria in yoghurt. Yoghurt containing these two probiotic bacteria is referred to as 'AB' yoghurt. Recently, the trend is to incorporate *L. casei* in addition to *L. acidophilus* and bifidobacteria and such products are known as 'ABC' yoghurt. Traditionally, yoghurt is manufactured using *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* as starter cultures. These yoghurt organisms have been claimed to offer some health benefits; however, they are not natural inhabitants of intestine and can not survive under acidic conditions and bile concentrations usually encountered in the gastrointestinal tract. Therefore, for yoghurt to be considered as a probiotic product, *L. acidophilus* and

bifidobacteria are incorporated as dietary adjuncts. Fermented milk with only *L. acidophilus* and bifidobacteria could be manufactured; however, the longer incubation period and product quality are the two main factors that are sacrificed when fermenting milk with only 'AB' bacteria. Thus, the normal practice is to make product with both yoghurt and probiotic bacteria.

In order to obtain the desired therapeutic effects, the yoghurt and probiotic bacteria must be available in sufficient numbers. Researchers have suggested that these organisms should be present in a food to a minimum levels of 10^5-10^6 cfu.g^{-1} (Robinson, 1987; Kurmann and Rasic, 1991) or daily intake should be about 10^8 cfu.g^{-1} (Kurmann and Rasic, 1991). Such high numbers might have been suggested to compensate for the possible reduction in the numbers of the probiotic organisms during passage through the stomach and the intestine.

According to the Australian Food Standards Code (Standard H8), yoghurt must have a pH $\leq$ 4.5 and must be prepared with *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* or other suitable lactic acid bacteria. However, Australian Food Standards Code does not specify any requirements regarding the numbers of yoghurt or probiotic bacteria in the fermented products. However, in other countries standards have been developed regarding the requirement of the numbers of the probiotic bacteria in fermented products. In Japan, a standard has been developed by the Fermented Milks and Lactic Acid Bacteria Beverages Association that requires a minimum of 10^7 viable bifidobacteria cells per millilitre to be present in fresh dairy products (IDF, 1995). Also, the IDF (International Dairy Federation) recommends 10^7 cfu/mL up to the end of shelflife for yoghurt and other probiotic bacteria in fermented dairy products (IDF, 1995).

2.5 Viability of *L. acidophilus* and bifidobacteria in fermented products

2.5.1 *Viable counts as affected by enumeration methods of yoghurt bacteria and probiotic bacteria*

The need to monitor survival of yoghurt bacteria and probiotic bacteria in yoghurt has often been neglected. As a result, a number of products reach the market with few viable bacteria ranging from several hundreds to a few per gram of product (Anon, 1992; Hull and Roberts, 1984; Iwana *et al.*, 1993; Shah *et al.*, 1995). An important parameter in monitoring viable organisms during assessment of product quality is the ability of methods to count yoghurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) and probiotic bacteria (*L. acidophilus* and bifidobacteria) differentially. 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 false estimation of viability of the strains of interest.

Several media have been developed for differential enumeration of yoghurt culture organisms, including lactic acid bacteria agar (Davis *et al.*, 1971), Lee's agar (Lee *et al.*, 1974) and reinforced clostridial agar (RCA) at pH 5.5 (Johns *et al.*, 1978). Jordono *et al.* (1992) examined different M17 media for the recovery of *S. thermophilus*. Hamann and Marth (1984) evaluated four differential and two general purpose media for enumerating yoghurt culture organisms. Rybka and Kailasapathy (1996) developed a method which makes use of a new type of Reinforced Clostridial Prussian Blue agar (RCPB, pH 5), that was found to be selective for *L. delbrueckii* ssp. *bulgaricus* and *Bifidobacterium* spp.

Similarly, several media have been suggested for the enumeration of *L. acidophilus*, including bile medium (Collins, 1978), Rogosa agar, Oxygen-reduction membrane fraction (Burford, 1989), deMan Rogossa Sharpe (MRS) medium containing maltose, salicin, raffinose or melibiose in place of dextrose (Hull and Roberts, 1984), cellobiose esculin agar (Hunger, 1986) and an agar medium based on X-Glu (Kneifel and Pacher, 1993). Calicchia *et al.* (1993) developed a method for selective enumeration of *B. bifidum*,

Enterococcus faecium, and streptomycin-resistant *L. acidophilus* from a mixed probiotic product. Modified Brigg's agar with 1200 μg/mL streptomycin sulfate was used by them in a double layer diffusion technique to selectively enumerate a streptomycin-resistant strain of *L. acidophilus*. A modified LBS (*Lactobacillus* selective) agar was suggested by Nighswonger *et al.* (1996) for the enumeration of *L. acidophilus* and *L. casei*. Badran and Reichart (1993) compared 3 nutrient media (tryptone glucose yeast extract (TGE) agar, Elliker medium and modified Elliker medium) for optimising the recovery of *L. acidophilus*. It was observed that TGE media with the addition of 1% skim milk gave the highest cell numbers and addition of tomato juice to the milk media exerted a stimulatory effect on bacterial growth. IDF (1995) reviewed various culture media for detection and enumeration of *L. acidophilus* on its own or in combination with other lactic acid bacteria in the light of current interest in health benefits of *L. acidophilus* ingestion and development of dairy products containing this organism.

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, 1994), X-gal-based medium (Chevalier *et al.*, 1991) and dicloxacillin-based medium (Sozzi *et al.*, 1990). Capon and Kiss (1991) and Beernens (1991) suggested use of propionic acid as selective agent for the enumeration of bifidobacteria. Similarly, Lapierre *et al.* (1992) used lithium chloride-sodium propionate in agar medium for the enumeration of bifidobacteria in fermented dairy products that contained *S. thermophilus* and lactobacilli. Modified VF-Bouillon agar with 0.5 mg/mL

lithium chloride, 20 μg/mL SDS, 5 mg/mL sodium propionate and 10 μg/mL neomycin sulfate with a triple layer diffusion technique were used by Calicchia *et al.* (1993) to enumerate *B. bifidum* selectively from probiotic products. Hae *et al.* (1993) suggested a method for detecting bifidobacteria by using alpha-galactosidase activity of bifidobacteria on the substrate 5-bromo-4-chloro-3-indolyl-alpha-galactoside (X-alpha-gal). Arroyo *et al.* (1994) evaluated brain heart infusion agar, modified columbia agar, RCA, modified MRS agar and modified bile agar for the enumeration of *B. adolescentis, B. infantis* and *B. longum* from pure cultures. A roll-tube repair-detection procedure was developed to enumerate injured and non-injured cells of *Bifidobacterium* spp. from water and frozen food samples (Arany *et al.*, 1995). Cho *et al.* (1995) found minimum inhibitory concentration of several antibiotics for various bifidobacteria and lactic acid bacteria in order to establish the optimum conditions for selective enumeration of bifidobacteria. Lim *et al.* (1995) successfully enumerated bifidobacteria in fermented products containing both lactobacilli and streptococci using blood glucose liver agar incorporated with oxgall (0.2 mg/mL) and gentamicin (30 μg/mL). An assay procedure for commercial probiotic cultures was developed by Mccann *et al.* (1996). They analysed commercially available probiotic cultures for viability using a re-suspension medium consisting of KH_2PO_4, Na_2HPO_4, cysteine, Tween-80, agar and anti-foam agent together with modifications of multiple-layer diffusion techniques. Kaneko and Kurihara (1997) developed a method using digoxigenin-labelled deoxyribonucleic acid probes for the enumeration of bifidobacteria in fecal samples. They concluded that the digoxigenin-labeling method and colony hybridisation assay fulfilled the pre-requisites for practical enumeration of bifidobacterial counts in the fecal samples.

Onggo and Fleet (1993) evaluated Tryptose Proteose Peptone Yeast Extract (TPPY) agar and Reinforced clostridial Prussian Blue agar (RCPB) agar for their efficacies in the isolation, enumeration and differentiation of species of the yoghurt and probiotic bacteria. Lankaputhra *et al.* (1996) examined various media to selectively enumerate *L. acidophilus* and Bifidobacterium spp.; however, with different strains of these organisms, none of the medium was satisfactory for the enumeration purposes when all four (*S. thermophilus, L.*

delbrueckii ssp. *bulgaricus*, *L. acidophilus* and bifidobacteria) organisms are present in the fermented dairy porducts. Micanel *et al.* (1997) used M17 agar for streptococci, MRS pH 5.3 agar for *L. delbrueckii* ssp. *bulgaricus*, MRS-0.2% ox-bile for *L. acidophilus*, and Bifidus-blood agar for the enumeration of bifidobacteria present in the fermented dairy products sold in the Australian markets. Information on the differential plating methods and nucleic acid technique available for the detection, enumeration and identification of probiotic bacteria in mixed population was reviewed by Charteris *et al.* (1997). Recently, Shah (1997) reviewed isolation and enumeration methods for bifidobacteria in fermented milk products and illustrated that further research is needed to develop a medium to isolate whole spectrum of species within the genus *Bifidobacterium* from products containing yoghurt bacteria, *L. acidophilus* and bifidobacteria.

It is evident that a great number of media has been suggested for the enumeration of yoghurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) and probiotic bacteria (*L. acidophilus* and bifidobacteria). However, these media may not be suitable for selective enumeration purposes when all four groups of organisms are present together in a product. Also, differences exist among the strains of the same species for tolerance to low pH, bile salts, NaCl and also in sugar fermentation characteristics (Kim, 1988). There is a growing concern that some media which contain antibiotics or bile may also restrict growth of some strains of *L. acidophilus* and bifidobacteria and that counts obtained are not necessarily representative of viable cells present in a product. Thus, there is a chance of over or under estimation of a particular group of organism in a product that contains several groups of organisms. Therefore, it is necessary to have a broader knowledge of applicability of various media and selective ingredients for the selective enumeration of yoghurt and probiotic bacteria in a product. Most researchers agree that all selective media employing antibiotics or other inhibitory substances should be validated before using them for any investigation. Pure cultures of each group of organisms should be plated individually and in a group in the selective and control (without inhibitory substances) media and the recovery of each group of organism should be compared (Dave and Shah, 1996).

2.5.2 *Viability as affected by physico-chemical and processing parameters*

As described earlier, the therapeutic benefits could only be obtained if the probiotic bacteria are present at a minimum level of 10^5 per g of product so as to ingest 10^7 to 10^8 organisms per day (Robinson, 1987) and the strain used is capable of offering these benefits. Therefore, efforts in the direction of selecting the right type of strain and improvement in viability are of commercial significance. Beneficial and proven strains could be obtained by a reputed starter culture supplier; however, the viability of organisms during manufacture and storage is the sole responsibility of the manufacturers. Several reports have shown that the viability of these organisms is often low in yoghurt (Hull *et al.*, 1984; Schioppa *et al.*, 1981; Gilliland and Speck, 1977a). Several brands of commercial yoghurts were analysed in Australia (Anon, 1992; Shah *et al.*, 1995) and in Europe (Iwana *et al.*, 1993) for the presence of *L. acidophilus* and bifidobacteria. Most of the products contained very low numbers of these organisms, especially bifidobacteria. *L. acidophilus* and bifidobacteria are affected by several inhibitory substances during production and cold storage. During production of yoghurt, yoghurt bacteria and probiotic bacteria produce organic acids and the pH is lowered to 4.5 or lower due to legal requirements and in order to produce good quality yoghurt. The amount of lactic acid could vary at the same pH in yoghurt due to the buffering effects of ingredients added to yoghurt mixes. Also, depending on the extent of growth of bifidobacteria, concentrations of acetic acid (which is more toxic compared to lactic acid) would vary in the product. By incorporation of the right types of organisms at required levels and certain process modification could help improve the viability of probiotic bacteria during manufacture and storage.

Several factors have been claimed to affect the viability of yoghurt and probiotic cultures in fermented milk products. The viability of probiotic bacteria in yoghurt depends on: the strains used, interaction between species present, culture conditions, chemical composition of the fermentation medium (carbohydrate source), hydrogen peroxide content due to bacterial metabolism, final titratable acidity, concentrations of lactic and acetic acids, pH, milk solids content, availability of nutrients, growth promoters and inhibitors,

concentration of sugars (osmotic pressure), dissolved oxygen (especially for *Bifidobacterium* spp.), level of inoculation, incubation temperature, fermentation time and storage temperature (Hamann and Marth, 1984; Costello, 1993; Bertoni *et al.*, 1994; Young and Nelson, 1978; Kneifel *et al.*, 1993). Several other researchers have reported the effects of some of these factors (Gilliland and Speck, 1977a; Hull *et al.*, 1984; Martin and Chou, 1992; Klaver *et al.*, 1993; Medina and Jordono, 1994; Patidar *et al.*, 1994; Prajapati and Dave, 1994; Samona and Robinson, 1994; Rybka and Kailasapathy, 1995). However, effects of all the parameters on the viability of probiotic bacteria have not been studied simultaneously. The factors affecting the viability of probiotic bacteria have been reviewed by Kailasapathy and Rybka (1997).

2.5.3 Viability of yoghurt and probiotic bacteria as affected by antagonism between various groups of organisms

Several reports have indicated that antibacterial substances produced by microorganisms could be utilised for food processing to inhibit the growth of pathogenic and spoilage type organisms. Considerable work on antimicrobial activity of *L. acidophilus* has been carried out (Varadaraj *et al.*, 1993; Morris, 1991; Prasad and Ghodeker, 1991; Sorbino *et al.*, 1991; Romero *et al.*, 1990; Khedkar *et al.*, 1989; Reddy *et al.*, 1983; Spillman *et al.*, 1978) and the chemical nature and structure of antibacterial substances produced by *L. acidophilus* have been studied by some workers (Brink *et al.*, 1994; Juven *et al.*, 1992; Toba *et al.*, 1991a; Lin *et al.*, 1986; Mehta *et al.*, 1984; Barefoot and Klaenhammer, 1984; Pulsani *et al.*, 1979; Hosono *et al.*, 1977; Mikolajcik and Hadman, 1975; Hadman and Mikolajcik, 1974; Vakil and Shahani, 1965; Vincent *et al.*, 1959). Few reports are available on anti-microbial activity of bifidobacteria (Ibrahim and Bezkorovainy, 1993; Vijayendra and Gupta, 1992; Misra and Kuila, 1991; Modler *et al.*, 1990; Mantere-Alhonen *et al.*, 1989; Anand *et al.*, 1985) and study on the chemical nature and structure of antibacterial substances produced by bifidobacteria is still in their infancy stage. The recent trend is to limit or eliminate the use of chemical preservatives by replacing them with biopreservatives which are believed to be less harmful than chemical preservatives

(Hammes and Tichaczek, 1994; Wang, 1993; Branby-Smith, 1992; Eckner, 1992; Medina *et al.*, 1992; Bednarski, 1991; Daeschel, 1989; Klaenhammer, 1988). These studies could also pave the way for future production and application of antibacterial substances on a commercial scale to assist in food preservation.

The precise mechanism by which LAB cause inhibition of microorganisms seem to be rather complex and has not been fully understood. The inhibitory activity of lactic acid bacteria can be attributed to the creation of hostile environment for food-borne pathogens and spoilage organisms in foods. Several mechanisms proposed for such effects include production of lactic and other organic acids, hydrogen peroxide, diacetyl, competition and nutrient depletion, altered O-R potential, bacteriocins production, antimicrobial substances production, bile deconjugation and enhanced immunocompetence. However, it is generally believed that the inhibitory activity is not a function of any one compound, but it is a composite effect of several factors.

2.5.3.1 Organic acids and low pH

While growing in milk, LAB mainly produce lactic acid and some other organic acids such as formic, acetic, propionic, etc, which help in lowering the pH and creating unfavourable environment for other micro-organisms.

Rubin *et al.* (1982) demonstrated that bacteriostatic effect of lactic acid at the intracellular levels was due to lactate anions rather than hydrogen ions. The presence of lactic acid in fermented milk foods has been shown to be inhibitory towards *Staphylococcus* (Attaie *et al.*, 1987) and bacteriocidal to *Campylobacter* (Cuk *et al.*, 1987) and Salmonella (Rubin *et al.*, 1982). These observations were based on the addition of lactic acid to milk base. In yoghurt, the decrease in *Yersinia* count was due to low pH (Ahmed *et al.*, 1986). In milk base acidified with HCl (pH <4.0), no effect on viability of *Campylobacter* was observed in 30 min However, when milk was acidified with lactic acid or propionic acid to the same pH level, it became bacteriocidal in 30 min (Cuk *et al.*, 1987). Propionic acid produced by

dairy propionibacteria demonstrates broader inhibition than lactic and acetic acids and inhibited moulds and Gram negative bacteria, but it was ineffective against Gram positive species (Barefoot and Nettles, 1993). Organic acids are generally active at low pH and the lowered pH by lactic acid increases the toxicity of acetic acid even at low concentration.

2.5.3.2 Hydrogen peroxide

It is now known that hydrogen peroxide accumulates in the cultures of lactococci, lactobacilli, leuconostocs and pediococci. Anders *et al.* (1970) observed that lactococci produced sufficient hydrogen peroxide to be auto-inhibitory. The lactobacilli have the ability to generate hydrogen peroxide during growth by several different mechanisms which are reviewed by Daeschel (1989). Accumulation of hydrogen peroxide in growth media can occur because lactobacilli do not possess the catalase enzyme (Kandler and Weiss, 1986). Dahiya and Speck (1968) identified hydrogen peroxide as inhibitor of *S. aureus* in lactobacilli. Similarly, inhibition of *Pseudomonas* spp. due to production of hydrogen peroxide by lactobacilli was reported by Price and Lee (1970). Inhibition of *S. aureus*, *E. coli*, *Sal. typhimurium* and *Cl. perfringens* was observed to be due to hydrogen peroxide produced by *L. acidophilus* (Gilliland and Speck, 1977b). *L. delbrueckii* ssp. *bulgaricus* also produced sufficient hydrogen peroxide to show inhibition of psychrotrophs (Abdel-Bar and Harris, 1984). Dairy cultures have been manipulated by protoplast fusion or mutation to over-produce hydrogen peroxide (Waxman *et al.*, 1989). However, the concentration of hydrogen peroxide produced by starters may not be sufficient to directly affect the cells in the products. Hydrogen peroxide can react with other components to form inhibitory substances. In raw milk, hydrogen peroxide generated by lactic acid bacteria can react with endogenous thiocyanate which is catalysed by lactoperoxidase to form intermediary oxidation products inhibitory to the micro-organisms. The antimicrobial effect may be greater in the intestinal tract than in the food system.

2.5.3.3 Diacetyl

Diacetyl (2,3-butanedione) is a metabolic end product of LAB that is synthesised from the intermediary metabolite, pyruvate and is primarily known as flavour compound in fermented dairy products. Certain species from genera of LAB have the ability to synthesise diacetyl. However, its inhibitory activity has also been known since early times. Jay (1982) documented the inhibitory effects of diacetyl on 4 yeasts, 10 lactic acid bacteria, 12 Gram-positive species and 14 Gram-negative cultures. The concentration required for such inhibition ranged from 100-400 $\mu g.mL^{-1}$, while lactic starters produced only 10-80 $\mu g.mL^{-1}$ of diacetyl. Although diacetyl is generally recognised as safe (GRAS), its utility as a food preservative is limited because of the relatively large amounts needed to provide preservation. The intense aroma of diacetyl would preclude its use in many foods. Jay (1982) suggested that diacetyl could be used as an antimicrobial dip for utensils and work surfaces because of its high volatility. Thus, diacetyl may be a minor contributor to the broad spectrum antagonism.

2.5.3.4 O-R potential

During fermentation, LAB produce numerous by-products which lower the O-R potential (Eh). Positive Eh favours aerobes, while negative Eh favours anaerobes. In most fermented foods, the Eh remains lower, which helps control the aerobic spoilage organisms (Fernandes and Shahani, 1989). The redox potential at very low level provides anaerobic environment and would benefit them, however, the permeation of oxygen through container would not keep the redox negative in such containers for longer time. The lowering of redox potential was also dependent on the strain of lactic acid bacteria and had variable effects on the redox potential in mixed cultures (Klaver *et al.*, 1993).

2.5.3.5 Competitive antagonism

Competitive antagonism is a general term which is the ability of certain micro-organisms to outgrow others in any particular environment. This can occur as a result of nutrient utilisation, antagonistic end products or selective colonisation. It is now well known that certain lactobacilli colonise surfaces of intestinal tract and displace other microbes (Dave and Prajapati, 1994).

2.5.3.6 Deconjugation of bile

Deconjugated bile acids are more inhibitory to pathogens than the conjugated acids. *Lactobacilli* can deconjugate bile salts in the intestine and hence may suppress the food-borne pathogens (Dave and Prajapati, 1994).

2.5.3.7 Antibiotic-like substances

It is known that LAB produce certain proteinaceous substances, which act like antibiotics, for the inhibition of food-borne pathogens and spoilage organisms. Among the antibiotic like substances, nisin is well characterised. Such substances can be better described as bacteriocins. Several review articles have appeared on various aspects of bacteriocins (Tagg *et al.*, 1976; Klaenhammer, 1988; Daeschel, 1989; Eckner, 1992; Kim, 1993; Hammes and Tichaczek, 1994).

Information on various bacteriocins from several genera of LAB as extracted from different research and review articles is presented in Tables 2.6 and 2.7. Among the bacteriocins, nisin is the most extensively characterised bacteriocin and it is widely used as a food preservative in some countries.

Table 2.6 Bacteriocins of lactobacilli

Bacteriocin	Producer	Inhibitory spectrum
Acidolin	*L. acidophilus*	Broad spectrum against spore formers, enteric pathogens
Acidophilin	*L. acidophilus*	Lactic acid bacteria, spore formers, *Salmonella* spp., *E. coli, S. aureus, Pseudomonas*
Fermenticin	*L. fermenti*	Lactobacilli
Bacteriocin	*L. fermenti*	*L. fermenti*
Plantracin B	*L. plantarum*	*L. plantarum, Pediococcus damnosus, Leuc. mesenteroides*
Brevicin	*L. brevis*	Pediococci, lactobacilli, leuconostocs, *N. carolina*
Caseicin 80	*L. casei*	*L. casei*
Curvacin A	*L. curvatus* LTH 1174	Closely related lactobacilli, *List. monocytogenes, Ent. faecalis*
Gassericin A	*L. gasseri*	*L. acidophilus, L. delbrueckii, L. helveticus, L. casei, L. brevis*
Plantaricin A	*L. plantarum*	*L. plantarum, Lactobacillus spp., Leuconostoc spp., Pediococcus spp., L. lactis, E. faecalis*
Plantaricin B	*L. plantarum*	*L. plantarum, Leuc. Mesenteroides, Pediococcus damnosus*
Bacteriocin	*L. plantarum*	Leuconostoc, *Lactobacillus, Pediococcus, Lactococcus, Streptococcus*
Plantaricin	*L. plantarum*	*L. sake*
Lactocin 27	*L. helveticus*	*L. acidophilus, L. helveticus*
Helveticin J	*L. helveticus*	*L. bulgaricus, L. lactis, L. helveticus*
Lactacin F	*L. acidophilus*	Lacobacilli, *E. faecalis*
Lactacin B	*L. acidophilus*	Lactobacilli
Lactocidin	*L. acidophilus*	Broad antibiotic spectrum, Gram + ve and Gram -ve bacteria
Protein	*L. acidophilus*	*B. subtilis, S. aureus, E. coli, Salmonella spp.*
Reuterin	*L. reuteri*	Salmonella, shigella, clostridia, staphylococci, listeria, candida, trypanosoma
Sakacin A	*L. sake*	*Car. piscicola, Enterococcus spp., L. sake, L. curvatus, Leuc. Paramesenteroides, List. monocytogenes, Aeromonas hydrophila, Staph. aureus*
Sakacin P	*L. sake* LTH 673	Closely related lactobacilli, *List. monocytogenes, Ent. faecalis*

(Sources: Tichaczek *et al.*, 1992; Klaenhammer, 1993; Hoover and Steenson, 1993)

Table 2.7 Bacteriocins of lactococci, leuconostocs and pediococci

Bacteriocin	Producer	Inhibitory spectrum
Carnocin	*Leuc. Carnosum*	lactobacilli, pediococci, enterococci, leuconostocs, carnobacteria, listeria
Diplococcin	*L. lactis* ssp. *cremoris*	*L. lactis* ssp. *lactis, L. lactis* ssp. *cremoris*
Jenseniin G	*Prop. jensenii* P 126	*L. delbrueckii* ssp. *bulgaricus* NCDO 1489
Lactostrepcins	*L. lactis* ssp. *cremoris* and *L. lactis* ssp. *diacetylactis*	Lactococci, *Group A, C* and *G s*treptococci, *B. cereus, L. helveticus, Leuc. citrovorum, Lb. paracitrovorum*
Lactococcin I	*L. lactis* ssp. *cremoris*	Lactococci, clostridia
Lacticin 481	*L. lactis* ssp. *lactis*	Lactococci, lactobacilli, leuconostocs, *Cl. perfringens*
Lacticin 3147	*L. lactis* ssp. *lactis*	Lactococci, lactobacilli, enterococci, bacilli, leuconostocs, pediococci, clostridia, staphylococci and streptococci
Lactococcin	*L. lactis* ssp. *lactis*	Lactococci, *S. aureus, B. cereus, Sal. typhi*
Leucocin S	*Leuconostoc paramesenteroides*	*L. sake, List. monocytogenes, S. aureus, A. hydrophilla, Yersinia enterocolotica*
Mesenterocin	*Leuc. Mesenteroides*	*List. monocytogenes, Brevibacterium linens, E. faecalis, Pediococcus pantosaceus*
Mesenterocin 52	*Leuc. Mesenteroides* ssp. *mesenteroides FR 52*	Other leuconostocs, Enterococcus, listeria
Nisin	*L. lactis*	Lactococci, bacilli, clostridia, micrococci, *S. aureus*
Pediocin ACH	*Pediococcus acidilactici*	Lactobacilli, *S. aureus*, leuconostocs, *Ps. putida, List. monocytogenes, Cl. perfringens*
Pediocin A	*Pediococcus pentosaceus*	Pediococci, lactobacilli, *S. aureus, Cl. botulinum, Cl. perfringens*
Propincin PLC-1	*Prop. thoenii*	*L. delbrueckii* ATCC 4797

(Sources: Hoover and Steenson, 1993; Mathieu *et al.*, 1993; Hasing and Glatz, 1996; Ryan *et al.*, 1996)

Nisin causes cellular death by affecting cytoplasmic membrane and proton motive force (Hurst, 1981). It is primarily inhibitory to Gram-positive bacteria, but when used in conjugation with EDTA, it effectively inhibits Gram-negative bacteria. Nisin is useful for

control of *Clostridial* spores in cheeses. As nisin is heat stable, it can be added before processing to heat processed foods or canned foods. Since it is a polypeptide, any residues remaining in foods are digested. Further, it acts as adjunct to heat processing and supplementary heat inactivation can be achieved even at a comparatively low temperature. All these properties have made nisin a very useful food preservative. *L. acidophilus* has attracted special attention in the last few years because of its abilities to produce a variety of bacteriocins and to control pathogens in the intestinal tract of the host.

2.5.3.7.1 The antibacterial action of *lactobacilli and related lactic acid bacteria*

L. acidophilus produces organic acids, hydrogen peroxide and antibiotics to suppress the multiplication of pathogenic or putrefying bacteria, and it shows stronger antibacterial properties against Gram-positive bacteria such as *Staphylococcus aureus* and *Clostridium perfringens* than against Gram-negative bacteria such as *Salmonella typhimurium* and *Escherichia coli* (Gilliland and Speck, 1977b). It has also been claimed that the antibacterial action of *L. acidophilus* is particularly strong in inhibiting pathogenic or potentially pathogenic bacteria (Dubois *et al.*, 1982).

The antibiotics or antimicrobial substances produced by *L. acidophilus* have shown the following characteristics:

Vincent *et al.* (1959) isolated and purified lactocidin by chromatography on a silicic acid column from an active fraction in the acid soluble fraction of cultures of *L. acidophilus* on calf liver agar medium. It was a non-volatile, non-dialysable substance soluble in ether and it had a broad antibiotic spectrum against Gram- negative and Gram-positive bacteria. A low molecular-weight peptide was extracted in methanol and acetone from acidophilus milk by Vakil and Shahani (1965) and it was named as acidophilin. The component was found to be heat stable under acidic conditions and it exerted antibacterial action against pathogenic bacteria *in vitro.* Acidolin (Hadman and Mikolajcik, 1974) was isolated and purified on Sephadex G25 from methanol extracts of acidophilus milk fermented with *L. acidophilus* 2181. It had an absorption peak at 255 nm and a molecular weight of 200 Da.

It was extremely heat stable and its solutions was strongly acidic. It had a broad antibacterial spectrum, particularly against spore-forming bacteria. Mikolajcik and Hadman (1975) found inhibitory substance of *L. acidophilus* to be dialysable and to be of low molecular weight. This substance was responsible for antibacterial activity *in vitro* against various spoilage and pathogenic type organisms. Hosono *et al.* (1977) isolated a peptide of a molecular weight of 3,500 Da from bacterial cell extracts of *L. acidophilus* ATCC 3205 that showed antibacterial activity against *E. coli* over a wide range of pH.

Lactosin B was isolated and purified from the active fraction of filtrates from cultures of *L. acidophilus* N2 by ion-exchange chromatography, ultrafiltration and gel filtration (Barefoot and Klaenhammer, 1984). It showed peak absorption at 211 nm and it had a molecular weight of 6,000-6,500 Da. The substance was claimed to have a peptide structure. Its antibacterial activity was found to be restricted to *Lactobacilli.* Mehta *et al.* (1984) purified ammonium sulphate precipitates of filtrates from cultures of *L. acidophilus* strains AC1 and AR1 on Sephadex G100 and obtained protein type antibacterial substances of molecular weight 5,400 and 5,200 Da. These showed antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Salmonella* spp. The former substance lost its activity when heated at 50°C for 20 min, but the latter substance was stable when heated for 80 min at 50°C under acidic conditions. Both substances showed an optimum pH of 4.0-5.5 and both were inactivated by treatment with trypsin. Lin *et al.* (1986) studied antibacterial activity of 91 lactic acid bacteria and they partially purified the extracts on Sephadex G25. They found the extracts resistant against treatment with pepsin; however, when it was dialysed to remove compounds of molecular weight less than 1,000 Da, all the antibacterial activity was lost. Inhibition of six strains each of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus* was found by an antibacterial substance produced by *L. acidophilus* LAPT 1060 isolated from infant faeces (Toba *et al.* 1991a). The agent was sensitive to proteolytic enzymes and heat (60°C for 10 min) and was designated as 'acidophilucin A'.

Juven *et al.* (1991) examined antagonistic effects of lactobacilli and pediococci to control intestinal colonization by human enteropathogens in live poultry. Juven *et al.* (1992) found inactivation of antibacterial substance of *L. acidophilus* LA-147 with heat (100°C for 15 min), protease and α-chymotrypsin treatments. Stoffels *et al.* (1992) reported a method of isolation and properties of bacteriocin-producing *Carnobacterium piscicola* that was isolated from fish. Gupta *et al.* (1993) studied anti-listerial activity of various lactic acid bacteria isolated from buffalo milk. Brink *et al.* (1994) screened 1,000 *Lactobacillus* strains for antibacterial activity using a target panel of spoilage and pathogenic organisms. Only eight isolates were able to produce sufficient antibacterial substances of which two isolates were studied in detail. A novel bacteriocin viz. acidocin B was isolated and characterised from *Lactobacillus acidophilus* M46. Ruiz-Barba *et al.* (1994) suggested use of bacteriocin producer *L. plantarum* LPC 010, as a starter culture in spanish-style green olive fermentations. Allison *et al.* (1995) reported utilization of the leucocin A export system in *Leuconostoc gelidum* for the production of a lactobacillus bacteriocin. The Leucocin A, a plasmid-encoded bacteriocin is reported to inhibit *E. faecalis*, *List. monocytogenes*, and some other lactic acid bacteria. In a study by Thompson *et al.* (1996), a proteinaceous antimicrobial substance that resembled like a bacteriocin was identified in culture supernatant fluids of *Lactobacillus helveticus* (strain CNR 2450).

Anti-microbial activity of bacteriocin-producing cultures in meat products were studied by Hugas *et al.* (1996). Pipek and Stankova (1996) studied the effect of bacteriocins on the lactobacilli growth in vacuum packed meat products and shown the potential application of pediocin in such products. Monfort et al. (1996) made comparison of different bacteriocin-producing lactobacilli on listeria growth in sausages and concluded that there was some inhibition of listeria due to the addition of some of the bacteriocin producing lactobacilli. However, it was mentioned that strains should be selected to be optimal for the process used. Growth and survival of *Listeria monocytogenes* in vacuum-packaged ground beef inoculated with *Lactobacillus alimentarius* Flora Carn L-2 was studied by Juven *et al.* (1998). It was observed that the anti-listerial effect observed in the laboratory media and minced beef was attributed to lactic acid produced by the growing cultures.

Isolation and partial amino acid sequence of bacteriocins produced by *L. acidophilus* (JCM 1021, JCM 1023, JCM 1028, JCM 1229, and JCM 5342) has been recently reported (Tahara and Kanatani, 1997). These bacteriocins were active against closely related lactobacilli and 2 components in were found to be responsible for inhibition of bacteria in bacteriocins produced by *L. acidophilus* JCM 1023 and JCM 1028. Jenseniin G, a heat stable bacteriocin produced by *Prop. jensenii* (strain ATCC 4872) has been reported to inhibit yoghurt starters in broth and milk culture systems (Weinbrenner *et al.*, 1997). *L. lactis* (strain LAB 3113), isolated from tradionally fermented kimchi was found to produce a bacteriocin that was active against *L. delbrueckii* ssp. *lactis*, *L. johnsonii*, *L. gasseri* and *L. curvatus*. The bacteriocin was named lactocin K3113 (Jong-Yeun, 1997).

2.5.3.7.2 The antibacterial action of bifidobacteria

(a) Anand *et al.* (1985) purified a bacteriocin named Bifidin from *Bifidobacterium bifidum* 1452. This substance was found to be heat stable at 100°C for 30 min and gave a positive reaction with ninhydrin. Its main components were phenylalanine and glutamic acid. It showed antibacterial activity against *Micrococcus flavus* and *Staphylococcus aureus* and the compound was active at pH 4.8-5.5.

(b) Mantere-Alhonen *et al.* (1989) isolated fractions of different lactobacilli and bifidobacterial cultures which were responsible for antibacterial activity by electrophoresis. Results indicated that more antibacterial activity was present in bifidobacteria compared to other strains tested.

(c) Modler *et al.* (1990), Misra and Kuila (1991) and Vijayendra and Gupta (1992) in their technical articles have illustrated antibacterial activity associated with bifidobacteria.

(d) Ibrahim and Bezkorovainy (1993) found that no antibacterial substance other than lactic and acetic acids was produced by bifidobacteria.

Shahani *et al.* (1976) found that various cultural conditions affect the production of bacteriocins. They studied the effect of the type of medium, temperature and periods of incubation on the production of antibacterial activity by *L. acidophilus* and *L. delbrueckii* ssp. *bulgaricus* cultures. All the factors significantly affected the antibacterial activity of these cultures. Therefore, it is important to study some of these parameters and to optimise the conditions for the production of bacteriocin by selected strains of bifidobacteria.

2.5.3.8 The composite effect

Even though several mechanisms have been elucidated for antagonism, the net effect in terms of antagonism between LAB and pathogens and spoilage groups or within LAB groups is the result of more than one mechanism operative against the sensitive organisms. The antimicrobial effects of LAB are indicated without assigning any special mechanism in several reports (IDF, 1991).

3.0 MATERIALS AND METHODS

3.1 Starter cultures

Four commercial starter cultures (ABY-1, ABY-2, ABT-1 and ABT-4, which will be referred to as C1, C2, C3 and C4) were used in this study. Cultures C1 and C2 contained *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lacobacillus acidophilus* and bifidobacteria as constitutive microflora, whereas cultures C3 and C4 contained *S. thermophilus*, *L. acidophilus* and bifidobacteria only. In all the commercial culture combinations studied, the strains of probiotic organisms (*L. acidophilus* and bifidobacteria) were kept the same, whereas strains of yoghurt bacteria varied. Culture C3 contained a strain of *S. thermophilus* that produces polysaccharides during fermentation. The organisms were characterised and their identity verified according to the biochemical tests specified for each organism in the Bergey's Manual of Systematic Bacteriology. The starter cultures were in freeze dried DVS (Direct Vat System) form and were obtained from Chr. Hansen Pty. Ltd., Bayswater, Australia. After procurement, the starter cultures were stored at -18°C in absence of atmospheric air.

3.2 Microorganisms and their maintenance

Various strains of lactic acid bacteria (LAB) and spoilage and pathogenic organisms used in this study and their sources are given in Tables 3.1 and 3.2. The organisms were activated by three successive transfers in suitable media. All LAB were stored as liquid stock cultures in reconstituted skim milk (11% nonfat dry milk) supplemented with glucose (1% w/v) and yeast extract (0.3% w/v). All spoilage and pathogenic organisms were stored in filter sterilised glycerol-nutrient broth. The stock cultures were stored at -18°C. After 20 transfers of the organisms, a fresh transfer of cultures was taken from the original frozen stock culture to avoid loss of plasmid associated properties.

Table 3.1. Sources of various lactic acid bacterial strains

Organism	Strain/s	Total No.	Source
Lactobacillus acidophilus	2400, 2401, 2404, 2405, 2406 2409, 2410, 2411, 2412, 2413, 2414, 2415, 2420, 2422	14	CSIRO[1]
	BDLA-1, MJLA-1, MOLA2, OLA-2, PALA-1	5	Mauri[2]
	LA-1	1	CH.H[3]
Lactobacillus delbrueckii ssp. *bulgaricus*	2501, 2505, 2515, 2517, 2519	5	CSIRO
	LB-3, LB-4	2	CH.H
Lactobacillus helveticus	2700	1	CSIRO
Lactobacillus jugurti	2819	1	CSIRO
Lactobacillus casei	2603, 2604	2	CSIRO
Lactobacillus casei ssp. *rhamnosus*	2606	1	CSIRO
Lactobacillus fermentum	5174	1	CSIRO
Lactobacillus plantarum	2903	1	CSIRO
Lactobacillus spp.	Y (Shirota strain)	1	Yakult[4]
Lactobacillus sake	LS	1	VUT[5]
Leuconostoc mesenteroides ssp. *cremoris*	4200	1	CSIRO
Leuconostoc oenos	-	1	CH.H
Streptococcus thermophilus	ST-1, ST-2, ST-3, ST-4	4	CH.H
	2000, 2002, 2008, 2010, 2013, 2014	6	CSIRO
Bifidobacterium spp.	BB-1	1	CH.H
Bifidobacterium bifidum	1900, 1901	2	CSIRO
Bifidobacterium infantis	1912	1	CSIRO
Bifidobacterium adolescentis	1920	1	CSIRO
Bifidobacterium longum	1941, 20097	1	CSIRO
Bifidobacterium pseudolongum	20099	1	CSIRO
Bifidobacterium thermophilum	20210	1	CSIRO
Pediococcus cerevisiae	2305	1	CSIRO

[1]Commonwealth Scientific and Industrial Research Organisation, Division of Food Science and Technology, Highett, Australia.
[2]Mauri Laboratories (Now Gist-brocades Australia Pty. Ltd.), Moorebank, Australia.
[3]Isolated from AB starter cultures manufactured by Chr. Hansen Pty. Ltd., Horshlom, Denmark.
[4]Isolated from 'Yakult' milk drink.
[5]Victoria University of Technology's CBFT culture collection, Werribee, Australia.

Table 3.2. Sources of various spoilage and pathogenic bacterial strains

Organism	Strain/s	Total No.	Source
Escherichia coli	VUN0100	2	VUT[1]
	Ec 1		AFISC[2]
Salmonella typhi	Sal 1	1	AFISC
Salmonella typhimurium	VUN0045	1	VUT
Enterobacter aerogenes	VUN0025	1	VUT
Aeromonas hydrophila	Ah 1	1	AFISC
Candida albicans	Ca 1	1	AFISC
Staphylococcus aureus	VUP0040	1	VUT
Micrococcus luteus	VUP0023	1	VUT
Vibrio parahaemolyticus	WP1 VUN 0300	1	VUT
Enterococcus faecalis	VUP0019	1	VUT
Pseudomonas fluorescens	VUN 0031	1	VUT
Listeria monocytogenes	Lm 1	1	AFISC
Listeria innocua	Li 1	1	AFISC
Helicobacter pylori	Hp 1, 2, 3, 4	4	UNSW[3]
Bacillus cereus	VUP0001	1	VUT
Bacillus stearothermophilus	VUP0007	1	VUT
Clostriduim perfringens	VUP 0060	1	VUT
Clostridium sporogenes	VUP 0061	1	VUT

[1]Victoria University of Technology's CBFT culture collection, Werribee, Australia.
[2]Australian Food Industry Science Centre, Werribee, Australia.
[3]School of Microbiology and Immunology, The University of New South Wales, Sydney, Australia.

3.2.1 Bacterial strains and commercial products used for antagonism studies

S. thermophilus, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and bifidobacteria were isolated from 4 freeze-dried starter cultures (C1, C2, C3 and C4), 3 commercial yoghurts namely Bulla (Regal Cream Products Pty. Ltd., North Melbourne, Australia), Eve Balance (Dairy Vale Foods Ltd., Cla. Gardens, Australia) and Yoplus (Yoplait Australia, Cheltenham, Australia) and 1 probiotic capsule (Blackmores Ltd., Balgowlah, Australia) purchased from local supermarkets. All the products were claimed to contain viable *L. acidophilus* and bifidobacteria in addition to yoghurt culture/s (*L. delbrueckii* ssp. *bulgaricus* and/or *S. thermophilus*) with the exception of the capsule which did not contain the latter. All the products were procured and stored at refrigerated temperatures (4°C) till the completion of the study.

3.2.2 Isolation and identification of bacterial cultures

Appropriate dilutions of samples were prepared in sterile 0.1% peptone and water diluent and pour plated for enumeration. *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and bifidobacteria were isolated and enumerated using *Streptococcus thermophilus* (ST) agar, deMann Rogosa Sharpe (MRS) agar (pH 5.2), MRS-salicin agar and MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride and paromomycin sulfate) agar, respectively (Dave and Shah, 1996). Selected colonies were streak-plated twice on appropriate media for purification and identification of isolated colonies was carried out by use of morphological, cultural and biochemical characteristics (Hardie,1986; Kandler and Weiss, 1986; Scardovi, 1986).

3.2.3 Maintenance of bacterial cultures

Isolated and characterised bacterial cultures were maintained at -20°C in 11% nonfat dry milk supplemented with 1% glucose and 0.3% yeast extract (NGY). L-cysteine hydrochloride (0.05%) was additionally incorporated (NGYC) for bifidobacteria. From these frozen stock cultures, working cultures were made. Sterile 100 mL batches of 11% NGY (NGYC for bifidobacteria) were inoculated with 1% of each culture and incubated at 37°C for 18 h. All the cultures were activated by subculture into fresh NGY or NGYC on a weekly basis for a maximum of twenty subcultures before a new working culture was made from frozen stock cultures. This was done to minimise loss of any plasmid associated inhibitory acitivities.

3.3 Media preparation

3.3.1 Peptone and water diluent

Peptone and water diluent (0.15%) was prepared by dissolving 1.5 g of peptone water medium (Oxoid, West Heidelberg, Australia) in 1000 mL of distilled water, adjusting the pH to 7.0 $\pm$ 0.2, followed by autoclaving 9-mL aliquots at 121°C for 15 min.

3.3.2 Streptococcus thermophilus agar

The ingredients of *Streptococcus thermophilus* (ST) agar (10.0 g of tryptone; 10.0 g of sucrose; 5.0 g of yeast extract and 2.0 g of K_2HPO_4) were dissolved in 1000 mL of distilled water, the pH was adjusted to 6.8 ± 0.1, and 6 mL of 0.5% bromocresol purple was added to the medium. Agar powder was added at the rate of 1.2% to the medium, which was sterilized at 121°C for 15 min.

3.3.3 MRS agar, pH-Modified MRS agar (pH 5.2 and 5.8), MRS-bile agar, MRS-oxgall agar and MRS-NaCl agar

Dehydrated MRS broth (Oxoid, Australia) was prepared as per the instructions of the manufacturer. To obtain pH-modified MRS agars, 1.0 **M** HCl was used to adjust the pH of the medium to 5.2 and 5.8. For the preparation of MRS-bile agar, 2.0 g of bile salts (Amyl Media, Dandenong, Australia) per L (0.2% final concentration) of MRS broth were dissolved; for MRS-oxgall agar, 10.0 g of oxgall powder (Oxoid, Australia) per L (1.0% final concentration) of MRS broth was dissolved. Twenty gram of NaCl per L (2.0% final concentration) of MRS broth was dissolved to obtain MRS-NaCl agar. After the broth was prepared, agar powder was added at the rate of 1.2%, and the media were autoclaved at 121°C for 15 min.

3.3.4 MRS-maltose agar, MRS-salicin Agar and MRS-sorbitol agar

Dehydrated MRS-maltose agar was obtained from Amyl media (Australia), and the medium was prepared as per the instructions of the manufacturer. To prepare MRS-salicin and MRS-sorbitol agars, MRS basal medium without dextrose was prepared, and 10 mL of 10% membrane-filtered solutions of salicin and D-sorbitol were added per 90 mL of basal medium (final concentration, 1.0%) just before pouring the agar medium.

3.3.5 MRS-NNLP agar

The MRS-**NNLP** (all chemicals from Sigma, Castle Hill, Australia) agar was prepared according to the method described by Laroia and Martin (1991). The basal medium

was MRS agar. Filter-sterilized NNLP was added to the autoclaved MRS base just before pouring. Filter-sterilized L-cysteine·HCl (0.05% final concentration) was also added at the same time to lower the oxidation reduction potential of the medium and to enhance the growth of anaerobic bifidobacteria.

3.3.6 RCA agar (pH 6.8 and 5.3)

Dehydrated RCA agar (Oxoid, Australia) was prepared as per the instructions of the manufacturer, and the pH of the medium was adjusted with 1.0 **M** HCl to 6.8 and 5.3. The medium was brought to boiling to dissolve all the ingredients properly and then autoclaved at 121°C for 15 min.

3.3.7 Rogosa acetate agar

Rogosa acetate agar (Oxoid, Australia) was prepared as per the instructions of the manufacturer. All contents were properly dissolved in distilled water, and the medium was brought to boiling after the pH of the medium was adjusted to 5.4 with glacial acetic acid. The medium was then filled in sterile bottles and stored at 7°C or less until used.

3.3.8 Cellobiose esculin agar

This medium was prepared according to the formula given by Hunger (1986). For enumeration purpose, spread plate technique was used, and the plates were incubated anaerobically using anaerobic jars.

3.3.9 Standardising protocols for the enumeration of yoghurt and probiotic bacteria

The activated cultures obtained after three successive transfers were used for the purpose of enumeration. One gram of each culture was 10-fold serially diluted (10^3 to 10^7) in 0.15% sterile peptone water. Enumeration was carried out using the pour plate technique, except when cellobiose esculin agar was used. For this agar, the spread-plate technique was used. Duplicate plates were incubated anaerobically (except for ST agar

plates) at 37°C for 72 h using in Oxoid jars and anaerobic kit (Oxoid, Australia). The ST agar plates were incubated aerobically (except for bifidobacteria plates) at 37°C for 24 h. Plates containing 25 to 250 colonies were enumerated and recorded as log of colony-forming units per gram of culture.

3.4 Yoghurt preparation

3.4.1 Manufacture of yoghurt at the rate of starter culture recommended by the manufacturer

The set yoghurts were manufactured using the four commercial cultures as outlined in Figure 3.1. The incubation conditions and the rate of addition of starter cultures into the milk were as per the recommendations of the manufacturer. For C1 and C4 cultures, the rate was 2 g per 10 L of milk and was 1 g per 10 L for C2 and C3 cultures. The incubation temperatures during yoghurt manufacture were 43°C for C1, 40°C for C4 and 37°C for C2 and C3 starter cultures. Viability of yoghurt bacteria and probiotic bacteria was assessed in plastic cups at 4°C during manufacture and during storage in yoghurt made from all the four commercial cultures (C1, C2, C3 and C4). The viability of yoghurt bacteria and probiotic bacteria was also assessed in yoghurts made from two cultures (C1 and C3) during manufacture and storage in plastic cups at 4°C and 10°C and in screw capped glass bottles at 4°C.

3.4.2 Manufacture of yoghurt at various rates of inoculum of starter culture

Two percent nonfat dry milk was added to homogenised and pasteurised full cream milk and the mix was heated at 85°C for 30 min and cooled to 40-43°C. The starter cultures in the freeze dried concentrated Direct to Vat Set (DVS) form were added at the level of 0.5, 1.0, 1.5 or 2.0 g/10 L in separate containers to study the effect of level of starter cultures and the inoculated mix was distributed into 100 mL cups. Incubation was carried out at the temperature recommended by the starter culture supplier as prescribed earlier (section 3.4.1) and the fermentation was terminated at pH 4.5. Yoghurt samples were stored at 4°C till the completion of the study (Fig. 3.1).

3.4.3 Yoghurt fortified with added ascorbic acid

Fifteen litres of homogenised and pasteurised milk was tempered to 45°C, fortified with 2% (w/v) skim milk powder, the mixture heated to 85°C for 30 min, and cooled to 40-43°C at which temperature the starter cultures were added. The inoculated yoghurt mix was divided into four lots. As recommended by the starter culture supplier, the rates of starter culture addition were 2 g for C1 and C4 and 1 g for C2 and C3 cultures per 10 L of the yoghurt mix. Ascorbic acid (Sigma, St. Louis, USA) was added at the rate of 0, 50, 150 or 250 $mg.kg^{-1}$ yoghurt mix and the mix filled into plastic cups. The cups were incubated at the temperatures recommended by the starter culture supplier and the fermentation was terminated at pH 4.5. The incubation temperatures during yoghurt manufacture were 43°C for C1, 40°C for C4 and 37°C for C2 and C3 starter cultures. After fermentation, yoghurt was transferred into a cold room at 4°C and stored at this temperature till the completion of the study.

3.4.4 Yoghurt fortified with cysteine

Homogenised and pasteurised milk (15 L) was tempered to 45°C, fortified with 2% (w/v) nonfat dry milk, heated to 85°C for 30 min, and cooled to 40-43°C. The starter cultures were added at 40-43°C and the mix was divided into 4 lots. As recommended

by the starter culture supplier, the rates of starter culture addition were 0.2 $g.L^{-1}$ for C1 and C4 starter cultures and 0.1 $g.L^{-1}$ for C2 and C3 starter cultures. L-cysteine·HCl (5%) (Sigma, St. Louis, USA) was added to the four lots of inoculated mix of each starter culture to achieve a final concentration of 0, 50, 250 or 500 $mg.L^{-1}$ and the contents distributed in plastic cups. Incubation was carried out at temperatures recommended by the starter culture supplier (43°C for C1, 40°C for C4 and 37°C for C2 and C3) and the fermentation was terminated at pH 4.5. The time taken to reach pH 4.5 was recorded for each sample in order to study the effect of added cysteine. After fermentation, yoghurts were transferred to a cold room at 4°C and were kept at this temperature for 35 days.

3.4.5 Yoghurt made with various ingredients

Homogenised and pasteurised full cream milk was tempered to 45°C, and fortified with 2% (w/v) skim milk powder (SMP), whey powder (WP) or whey protein concentrate (WPC 312 and WPC 392, which will be referred as WPC 1 or WPC 2) (New Zealand Dairy Board, Melbourne, Australia). The mix was heated to 85°C for 30 min, cooled to 40-43°C and the starter culture (0.1 g. L^{-1} of yogurt mix) was added. The mix supplemented with SMP was divided into 8 lots and L-cysteine·HCl (5.0% w/v) (Sigma, St. Louis, USA) was added to 4 lots to achieve a final concentration of 0, 50, 250 or 500 mg.L^{-1} of cysteine. A 5.0% solution of each of acid casein hydrolysate (ACH) (Sigma, USA) and tryptone (Oxoid, USA) was added to the remaining four lots of inoculated mix to achieve a final concentration of 250 or 500 mg per litre each of ACH and tryptone and the contents distributed in 100 mL and 500 mL plastic cups.

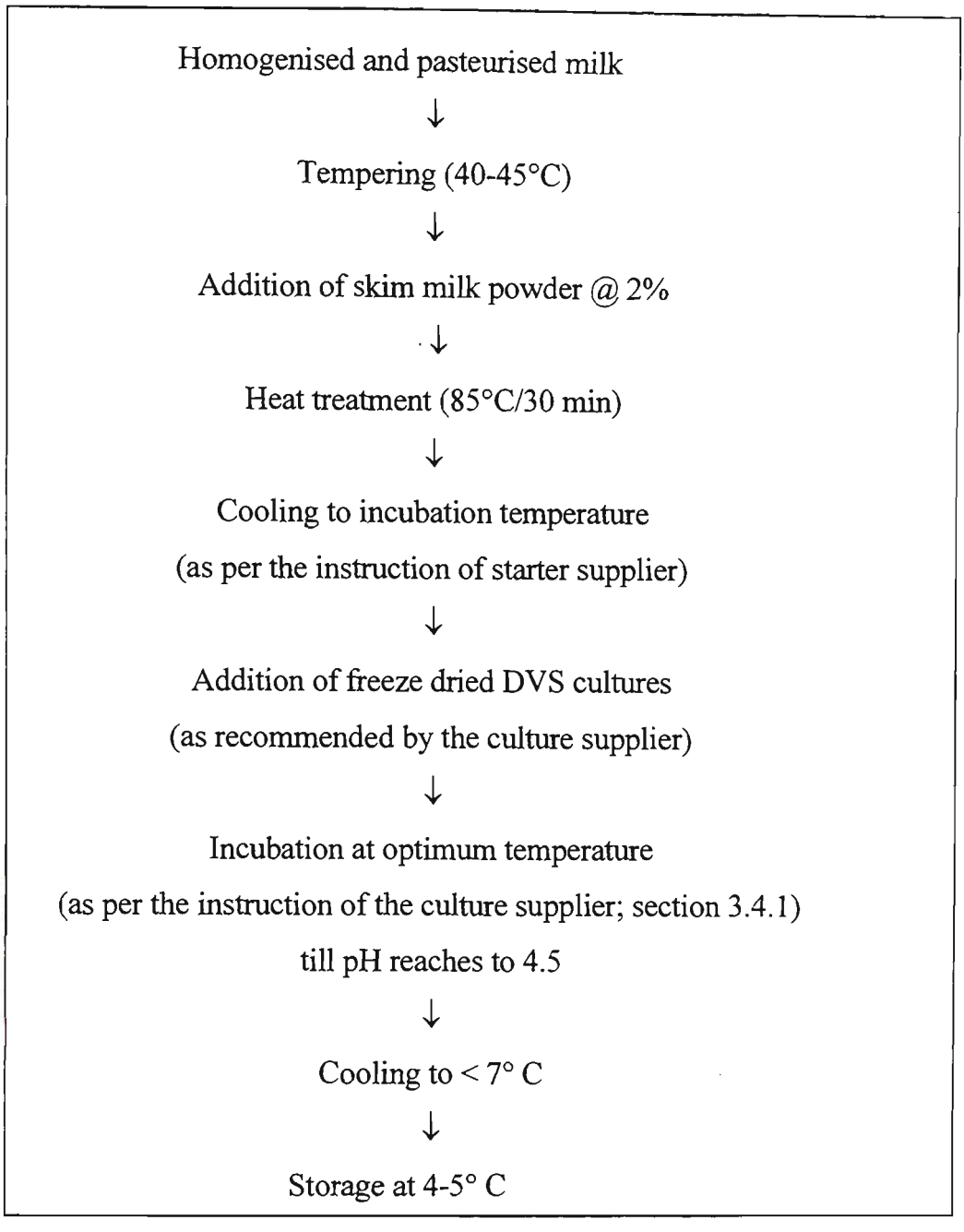


Fig 3.1 Flow diagram for the preparation of yoghurt

The incubation was carried out at 37°C and fermentation was terminated at pH 4.5. The time taken to reach a pH of 4.5 was recorded for each sample in order to study the effect of added ingredients. Samples were also kept in the incubator for 24 h to study the changes in pH, redox potential and viable counts of starter bacteria occurring during incubation. After the fermentation, yoghurts were stored in a cold room at 4°C.

3.5 Sample preparation

3.5.1 Sample preparation for biochemical analyses

For measuring the dissolved oxygen content, five cups were used from each batch of yoghurt and the dissolved oxygen concentration was estimated at 4-6°C while the yoghurt was in set form. The observations were taken aseptically in a laminar air flow unit and the tip of oxygen electrode was thoroughly washed with cold and hot water after each determination. For redox potential analyses, samples from undisturbed cups were filled to the neck of a glass bottle to minimise variations due to exposure to oxygen in the air during the measurements. After the oxygen and/or redox potential analyses, all the yoghurt samples were emptied aseptically into 500 mL sterile glass beakers and mixed homogeneously to minimise the container variations. After proper mixing under aseptic conditions, a sample was aseptically taken for microbiological analyses. Titratable acidity (TA), pH and concentrations of hydrogen peroxide and ascorbic acid were also determined using the mixed sample.

3.5.2 Sample preparation for texture analyses

Two 500 mL yogurt cups were taken at 0 day; 1 cup was used for measuring firmness with a penetrometer and microscopic examination of texture. The content from the other cup was uniformaly mixed, a sample taken aseptically for microbiological analysis and pH, TA, redox potential were measured. The remaining content was emptied into a 500 mL sterile glass beaker, mixed homogeneously and the viscosity was measured at 5°C.

3.6 Time interval specifications

The "0 h" time represents the observations taken immediately after the addition of starter culture and other ingredients such as ascorbic acid, cysteine, ACH or tryptone or the yoghurt mixes that were supplemented with various ingredients such as WP and WPC. For the measurements of hydrogen peroxide and oxygen contents, period "AF" represents the observations taken immediately after completion of the yoghurt

fermentation (i.e. at pH 4.5 ± 0.1), just before transferring the samples to cold store. The "0 d" (0 day) period represents analyses carried out after overnight cold storage of yoghurt samples, and periods 5-35 d represents analyses of yoghurt samples after 5, 10, 15, 20, 25, 30 and 35 days of storage, respectively.

3.7 Analyses

3.7.1 Chemical analyses

3.7.1.1 pH and titratable acidity of yoghurt

The pH values of the yoghurt and milk samples were measured at 17-20°C using an Orion 410A pH meter (Orion, Boston, USA) after calibrating with fresh pH 4.0 and 7.0 standard buffers. The titratable acidity was determined after mixing a yoghurt sample with 10 mL of hot distilled water and titrating with 0.1**N** NaOH using 0.5% phenolphthalein indicator to the end point of faint pink colour that persisted for at least 30 seconds.

3.7.1.2 Concentration of hydrogen peroxide in yoghurt

Concentration of hydrogen peroxide was obtained according to the method of Gilliland (1968) using horseradish peroxidase and O-dianisidine. The method is claimed to detect <1 μg of hydrogen peroxide per millilitre of milk. Ten gram of yoghurt was mixed with 0.1 **M** acetate buffer. The yoghurt and buffer mixture was diluted to 20 mL with distilled water and filtered through a Whatman No. 42 filter paper. Five millilitre of the filtrate, 1 mL of distilled water and 0.1 mL of 1% O-dionisidine in methanol were mixed into a 10 mL test tube (control sample). To another tube 5 mL of the filtrate, 1 mL of peroxidase solution (10 $\mu g.mL^{-1}$) and 0.1 mL O-dionisidine were added to measure the concentration of hydrogen peroxide (test sample). Both, the control and the test samples were incubated at 25°C in a water bath for 10 min followed by addition of 0.2 mL of 4 **N** HCl in control and test samples. After 5 min, optical density was measured using a UV-Vis spectrophotometer at 400 nm. Average of 3 readings were compared against a

standard curve prepared each time with known concentration of pure hydrogen peroxide.

3.7.1.3 Determination of lactic acid and acetic acid by HPLC

The quantification of lactic and acetic acids was carried out by High Performance Liquid Chromatography (HPLC, Varian Australia Pty. Ltd., Mulgrave, Australia) according to the method described by Garcia and McGregor (1994). For extraction of acids, 4.0 g yoghurt sample was diluted to 25 mL with 0.1**N** H_2SO_4. The suspension was mixed using a vortex for 1 min followed by centrifugation at 10,000 rpm for 5 min (Beckman J2-HS, Beckman Instruments, Fullerton, USA). The supernatant obtained after centrifugation was filtered through 0.4 μm filter papers and 1 mL of clear filtrate was pipetted into HPLC vials.

The levels of organic acids were measured using an Aminex HPX-87 H ion exclusion column and detection was carried out at 210 nm and the sample injection rate was 10 μL with a run time of 30 min. The mobile phase was 0.0075 **N** H_2SO_4 with a flow rate of 0.7 $mL.min^{-1}$. The standard solutions of organic acids were prepared in water using pure acids. The retention time for lactic acid was 9.9 to 10.1 min and for acetic acid 11.9-12.1 min. The standard curve regression coefficents were 0.98698 and 0.99300 for lactic acid and acetic acid, respectively.

3.7.1.4 Oxygen content and redox potential measurements

The dissolved oxygen in parts per million (ppm) was measured using LC82 Oxygen meter (TPS Pty. Ltd., Brisbane, Australia), after calibrating each time before use. The redox potential was measured with a platinum electrode (Model P 14805-SC-DPAS-K8S/325, Ingold (now Mettler Toledo), Urdorf, Switzerland) connected to a pH meter (Model H 18418, Hanna Instruments, Padova, Italy).

3.7.1.5 Determination of protein and total solids content

Protein content of heat treated yoghurt mixes was analysed by the Kjeldahl method

using Kjeltec system and 1002 distillation unit (Tecator ab, Hoganas, Sweden). A known quantity of yoghurt mix (~ 2.0 g) was taken in digestion tubes and two catalyst tablets were added in each sample. In these tubes, sulphuric acid (12.5 mL) was carefully added using a dispenser and the content was mixed by swirling the tube with the hand and the tubes were placed in the preheated digester, preset at 420°C with maximum air flow through the exhaust for 3-5 min. The digestion was continued until all samples were digested as indicated by the clear solution and lack of black residue in the tubes. The digestion time for most samples ranged between 45-55 min. The tubes containing digested samples were removed from the digester block along with the exhaust caps/manifold and were placed in the cooling stand. After cooling, 50 mL of nitrogen free tap water was added for dilution. The distillation was carried out and the distillates were collected into a receiver flask containing 25 mL boric acid solution. After complete distillation, the content was titrated against 0.05 **M** H_2SO_4 until the solution in the receiver flask turn to neutral grey.

The total solid content was analysed by standard gravimetric method in which the weight difference of samples before and after drying at 100°C for 2-3 h was calculated and per cent total solids were obtained.

3.7.1.6 Determination of ascorbic acid

The concentration of ascorbic acid was determined as per the method recommended by Association of Official Analytical Chemists (AOAC, 1990). The ascorbic acid was extracted by mixing equal proportion (~ 50 g) of sample and the precipitant (metaphosphoric acid-glacial acetic acid-EDTA and water mixture) solution followed by filtration through a Whatman No. 541 filter paper to obtain the clear filtrate which was designated as assay solution. Two 25 mL aliquots of assay solutions of each sample were taken into 250 mL Erlenmeyers flasks and titrated against sodium salt of 2,6, dichlorophenol-indophenol (Sigma, St. Louis, USA). Similarly, for titration of blanks, 25 mL mixture of precipitant solution and water (equal proportion) were used and titration was carried out as described earlier.

3.7.2 Microbiological analyses

One gram yoghurt sample was diluted with 9 mL of 0.15% peptone water (Oxoid, W. Heidelberg, Australia) and mixed uniformly with a vortex mixer. Subsequent serial dilutions were prepared and viable numbers enumerated using the pour plate technique. The counts of *S. thermophilus* were enumerated on ST agar by incubating the plates aerobically at 37°C for 24 ± 3 h (Dave and Shah, 1996). MRS agar (Oxoid, Australia) adjusted to pH 5.2 and anaerobic incubation at 43°C for 72 h were used for the differential enumeration of *L. delbrueckii* ssp. *bulgaricus*. MRS-salicin and MRS-sorbitol agars were used for the selective enumeration of *L. acidophilus* (Dave and Shah, 1996; Lankaputhra and Shah, 1996). Total probiotic organisms were enumerated on MRS-maltose agar, originally developed by Hull and Roberts (1984) for the differential enumeration of *L. acidophilus* from the yoghurt bacteria; however, since bifidobacteria also grow on this medium (Dave and Shah, 1996; Lankaputhra *et al.*, 1996a), a total count of *L. acidophilus* and bifidobacteria could be enumerated. The viable numbers of bifidobacteria were enumerated on MRS-NNLP agar (Laroia and Martin, 1991a) and also by subtracting the counts of *L. acidophilus* (enumerated on MRS-salicin and MRS-sorbitol agars) from the total population of probiotic organisms enumerated on MRS-maltose agar (Dave and Shah, 1996). The latter method of counting was employed to countercheck the efficacy of MRS-NNLP agar in recovering bifidobacteria in yoghurts during manufacture and storage.

3.8 Polysaccharide production by *S. thermophilus*

Maneval's capsule staining technique was used to check the polysaccharide production by *S. thermophilus*. For staining, peptone water and yoghurt were mixed in equal proportion and the filtered through a Whatman 42 filter paper to obtain the filtrate. The organisms were collected from the filtrate by centrifuging at 5000 rpm for 5 min (Model CS-15R, Beckman Instruments, Fullerton, USA). A thin blood film like smear was prepared using 1% aqueous Congo Red as the emulsifying fluid. After drying, the smear was stained with Maneval's stain (30 mL 5%, phenol, 10 mL 20% acetic acid, 4 mL 30% ferric chloride, 2 mL 1% acid fuchsin and 84 mL distilled water) (Reade, 1987) for 1 min, rinsed with water and blot dried.

Microscopic examination was carried out under Olumpus BX 50 microscope (Olumpus Optical Co. Ltd., Tokyo, Japan) using the phase contrast.

3.9 Electron microscopic examination of *S. thermophilus*

Electron microscopy of the bacteria in yoghurt made with various levels of cysteine was performed. For preparation of samples for electron microscopy, yoghurt was mixed with equal volumes of peptone water (0.15%) added with 0.9% saline and the slurry passed through a Whatman No. 42 filter paper to remove curd particles. The filtrate obtained was cooled to 4°C and centrifuged at 14,000 rpm for 2 min in Eppendorf tubes using an ultraspin centrifuge (Model Microspin 24, Sorvall® Instruments, Melbourne, Australia. The cells collected as pellets were fixed in 2.5% gluteraldehyde in 0.1**M** sodium cacodylate buffer (pH 7.4) for 2 h at room temperature (~ 20°C). After washing four times in sodium cacodylate buffer, samples were post-fixed with osmium tetroxide in the same buffer for 2 h at room temperature using a vertical rotator. The samples were then washed in distilled water twice and dehydrated in a series of graded acetone solutions (30, 50, 70, 95 and 100%) and embedded in Araldite-Epon resin (Probing & Structure, Melbourne, Australia). The blocks were polymerised at 60°C for 48 h.

Semi-thin (1μ) and ultra-thin (~80 nm) sections showing gold and silver interference colours were cut using a ultra-microtome (Model OmU2, Reichter Microtome, Vienna, Austria). Semi-thin sections were mounted on glass slides and stained with a solution of 1% methylene blue and 1% sodium tetra-borate. Ultra-thin sections were collected on acetone cleaned 200 mesh uncoated copper grids. These sections were stained with a 5% aqueous solution of uranyl acetate for 10 min and Reynold's lead citrate for 10 min. Sections so obtained were examined with a transmission electron microscope (Model 300, Philips, Einghoven, The Netherland) at 60 kV and 55,000 times magnification.

3.10 Texture analyses

The firmness of set yogurt at '0 d'was measured by a cone penetrometer (Stanhope-seta Ltd., Surray, England). The first and second angles of cone were 30° and 90°, respectively, and the total weight of the cone and its holder was 149.63 g. The penetration depth was measured in mm for 5 seconds of cone penetration. All analyses in duplicate were done at 5°C. Richter and Hartmann (1977) evaluated sour cream and yoghurt using a universal penetrometer fitted with a cone shapped penetrating device and observed that penetrometer was able to detect weak body in both products as determined by judges' evaluation.

The apparent viscosity of yogurt samples at 0, 10, 20 and 30 d was measured with a Brookfield viscometer (Model DV-II, Brookfield Engineering Laboratory, Stoughton, USA) using a Helipath D spindle at 1.5 rpm after calibrating with silicon oil (viscosity of 12,400 cps at 25°C). Yoghurt samples were maintained at 5°C. Yoghurt, being a non Newtonian fluid, gave no stable reading of viscosity, therefore, the data presented are the averages of highest and lowest viscosity readings (centipoise) obtained for each sample. The average range of fluctuations in viscosity readings was about 5-10%. For viscosity measurements, a homogenously mixed yoghurt sample was filled in a 500 mL beaker and spindle was allowed to travel in the product up and down for 5-6 times leaving ~1.5 centimetre distance from top and bottom end of beaker. The shear rate (per cent torque) was ~ 60-70% during viscosity analyses and was never below 50 per cent.

The microscopic texture analysis at '0 d' was carried out by pressing a set form of curd between a glass slide and a cover slip followed by immediate observation under an Olympus BX 50 compound light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) at 100 magnification. The pictures were taken using a camera (Model OM-4 Ti, Olympus Optical Co. Ltd., Tokyo, Japan) attached to a compound light microscope.

3.11 SDS-PAGE of whey fractions collected from various yoghurts

SDS-PAGE analysis was carried out on a 15% separating gel containing acrylamide and bisacrylamide (Schägger and Von Jagow, 1987). Standards in the range of 14,400-97,400 daltons (Bio-Rad Laboratories, Hercules, USA) were used for calibration. Yogurt samples were mixed with an equal volume of phosphate buffer and the content was filtered through an ordinary filter paper. The filtered whey fraction was refiltered through 0.45μm membranes. The filtrate so obtained were mixed with Laemmli buffer (Laemmli, 1970) containing SDS (sodium dodecyl sulfate) and the content was heated in a boiling water bath for at least 2 min. Samples were loaded in the wells of SDS-gels and electrophoresis was carried out at 30 mA for ~ 2.5-3.0 h till the bromophenol blue dye reached the bottom of the gel. The gels were stained with Coomassie blue and also silver stained. For Coomassie blue staining, the gels were fixed in fixing solution (4 parts ethanol, 1 part acetic acid and 5 parts distilled water) for 30 min followed by 30-40 min staining in staining solution (1.5 g Coomassie bule R-250, 105 mL ethanol, 30 mL acetic acid and the volume made to 300 mL with distilled water). After staining period is completed, the gels were placed in a de-staining solution (70 mL ethanol, 20 mL acetic acid and the volume made to 200 mL with distilled water) till the protein bands were clearly visible and the excess of background dye was washed off. The gels were then transferred to preserving solution (50 mL glycerol, 175 mL ethanol, 50 mL acetic acid and the volume made to 500 mL with distilled water) for 30 min and then left in distilled water. For silver staining, after the gels were fixed in fixing solution for 30 min, they were transferred into the incubation solution (75 mL ethanol, 17.0 g Na-acetate, 1.3 mL glutaraldehyde, 0.50 g sodium thio-sulphate and the volume made to 250 mL with distilled water) for 30-60 min. The gels were then washed thoroughly three to four times for 45 min in distilled water. After washing, the gels were transferred into silver solution (0.25 g silver nitrate, 50 μL formaldehyde and volume made to 250 mL with distilled water) for 40 min. After silver reaction, the gels were transferred into developing solution (6.25 g sodium carbonate, 25 μL formaldehyde and the volume made to 250 mL with distilled water) for about 15 min till the protein bands became intensively dark. Once the bands were fully developed, the stop

reaction was performed by placing the gels in stop solution (3.65 g EDTA Na_2 x $2H_2O$ in 250 mL distilled water) for 10-15 min and then into preserving solution. The relative quantification of the various bands was carried out using enhanced laser densitometer (Model Utlroscan XL, Amrad Pharmacia Biotech Ltd., Boronia, Australia).

3.12 Amino acid analysis

A complete amino acid profile of two WPC samples, ACH and tryptone was obtained. Quantification of various amino acids required three separate steps. Majority of amino acids were estimated via hydrolysis with 6**N** HCl follwed by separation on cation-exchange column in sodium form (Part No. 80002, Waters, Milford, USA) and post-column ninhydrin detection (Rayner, 1985). The sulfur containing amino acids (methionine, cysteine and cystine) are partially or completely destroyed by acid hydrolysis. Hence, these amino acids were oxidised with performic acid to form stable products prior to hydrolysis with 6**N** HCl and analysed as per the method described by Manson *et al.* (1980). Tryptophan is also destroyed by acid hydrolysis, therefore, a basic hydrolysis with barium hydroxide was performed followed by acidification and quantification with a High Performance Liquid Chromatography (HPLC) using a C18 column and UV detector (Piombi and Lozano, 1980; Nielsen and Hurrell, 1985; Delhaye and Landry, 1986). The gradient mobile phases were used for HPLC analyses. For the normal hydrolysis run, the gradient mobile phase was obtained with buffer A (0.2 **M** Na, pH 3.1) and buffer B (1.2 **M** Na, pH 6.4). For detection of cysteine and methionine, buffer A (0.2 **M** Na, pH 3.6) and buffer B (1.2 **M** Na, pH 6.4) were used. For detection of tryptophan, the mobile phase was 0.07 **M** acetate buffer and methanol (80:20) as suggested by Delhaye and Landry (1986).

3.13 Detection and assay of inhibitory activity

Several studies reported that the production of inhibitory substances was higher on solid media or inhibitory substance was only produced on solid media (Kelstrup and Gibbons, 1969; Jetten *et al.*, 1972; Mayr-Harting *et al.*, 1972; Hamada and Ooshima,

1975). Therefore, initial screening was based on measuring inhibition using the modified spot on lawn technique. Twenty five millilitre of 1.0% agar was poured into sterile petri plates, wells cut in the solidified agar using a 7.0 mm sterile metal borer and the bottom of wells was sealed with 0.9% agar. Fifty microlitre of active culture of producer organism was filled into the wells. The plates were left at room temperature for 2 h to allow migration and settling of the test cultures and incubated for 3 h at 37°C. After the initial growth, the remaining depth of well was sealed with 1% agar. Finally, the spotted plates were overlaid with ~10 mL of 0.9% agar seeded with 1% indicator organism containing approximately 1-7 x 10^7 mL^{-1} of target organism (LB-2519). The plates were left to solidify the agar and incubated for 24-48 h at 37°C. After incubation, the plates were examined for zones of inhibition around the wells.

Growth and inhibition studies of *S. thermophilus* was performed on ST agar (Dave and Shah, 1996) devoid of bromocresol purple and on MRS agar for other group of organisms. The nature of the inhibitory substance produced by the organisms in the initial screening was then determined in liquid media by the well-diffusion technique (Tagg *et al.*, 1976). Agar (0.9%) held at 45°C was inoculated with 1% of an active culture of the indicator organism. About 25 mL of the seeded agar was poured onto a sterile Petri dish and allowed to solidify. Wells were cut in the solidified agar as before. Cell-free supernatant was collected from the active broth of producer organisms by centrifuging (4500 rpm, 12 min, 4°C) (Beckman CS-15R, Beckman Instruments, Fullerton, USA) and filter sterilised by passing the supernatant through 0.45 μm Acrodisc (Gelman Sciences, Ann Arbor, USA) membranes. The supernatant was divided into three portions in sterile Eppendorf tubes: (i) untreated, (ii) neutralised to pH 6.0 using 2 **M** NaOH, and (iii) neutralised (pH 6.0) and treated with catalase (final concentration 0.05-0.1 μg/mL) (Sigma, USA) and incubated for 2 h in a water bath at 37°C. Wells were filled with 200 μL of the three test samples. The agar plates were left for 2 h at room temperature for diffusion of the test material into the agar. The plates were incubated as before and zones of inhibition measured.

In the second stage, cell-free supernatant of putative producer organisms that tested positive after the above treatments was again neutralised (pH 6.0) with 2 **M** NaOH

and treated with catalase as described before and tested for sensitivity to proteolytic enzymes, chymotrypsin and papain (final concentrations 1.0 μg. mL^{-1}) (Sigma, USA). Liquid broth without any growth of organism was used as control. The control and enzyme containing samples were incubated for 2 h at 37°C in a water bath to allow for a reaction and assayed for inhibitory zones as described before. Absence of inhibitory zones after treatment with enzymes indicated that the inhibitory substance had an active protein moiety and could be considered as a bacteriocin-like inhibitory substance (BLIS). The experiments were carried out in triplicate.

The nature of the inhibitory substance produced by organisms in the initial screening was then determined in liquid media by the well-diffusion technique (Tagg *et al.*, 1976). Suitable agar (0.9%) medium held at 45°C was inoculated with 1% of active culture of the target organism. About 25 mL of the seeded agar was poured into a sterile petri dish and wells were cut in the solidified agar as before. Cell-free supernatant was collected from the active broth of producer organisms by centrifuging (4500 rpm, 12 min, 4°C) and filter sterilising the supernatant using 0.45 μm Acrodisc (Gelman Sciences, Ann Arbor, MI, USA) membranes. The supernatant was divided into three portions in sterile Eppendorf tubes: (A) untreated, (B) neutralised to pH 6.0 with 5 **M** NaOH, and (C) neutralised and treated with catalase (final concentration 0.05-0.1 μg mL^{-1}) (Sigma, USA) and incubated for 2 h in a water bath at 37°C. Wells were filled with 200 μL of the above test samples and the agar plates were left for 2 h at room temperature for diffusion of the test material into the agar. The plates were incubated anaerobically (for bifidobacteria and *Clostridium* spp.) or aerobically (for other organisms) at 37°C for 24 h and zones of inhibition were measured.

To study the antimicrobial activity of *L. helveticus* 2700 in milk, the organism was grown in sterile 11% RSM for 14-16 h at 37°C and the fermented milk was centrifuged (4500 rpm for 15 min) in order to obtain cell free filtrate in the form of clear serum. The antimicrobial activity of the filtrate was measured using the agar well assay technique against various target organisms. Further characteristics of the antimicrobial substance produced by *L. helveticus* (2700) were studied in the filtrate.

3.14 pH Adjustment

The pH of the cell free extract was adjusted with 5 **N** lactic acid or 5 **N** sodium hydroxide using an Orion 410 A pH meter (Orion, Boston, USA).

3.15 Reaction with various enzymes

Various enzymes (catalase, trypsin, α and β chymotrypsin, papain, ficin, proteinase k, crude protease, α and β amylase, lipase, phospholipase A2 and D) (all from Sigma, USA) were dissolved in water and filter sterilised stock solutions were prepared. The solutions were stored for a maximum period of 6 months in a -18°C freezer until used. Neutralised cell free extract (900 µL) was reacted with 100 µL of stock solution of various enzymes in Eppendorf tubes for 2 h at 37°C to study the sensitivity of inhibitory substance against various enzymes.

3.16 Thermal resistance of BLIS

Cell free extract of overnight (~16-18 h) grown culture of lactobacilli was obtained by centrifuging the cold culture broth (4-5°C) in Eppendorf tubes and filter sterilised using 0.45 µm Acrodisc membranes. One millilitre aliquots of cell free extract were subjected to various heat treatments in a water bath. The temperature treatment of 121°C for 15 min was by autoclaving the filter sterilised broth in McCartney glass bottles.

The sensitivity of the inhibitory substance produced in milk by *L. helveticus* (2700) against various enzymes and the thermal resistance of the anti-microbial substance were studied as described earlier (Dave and Shah, 1997c).

3.17 Activity of BLIS over a range of pH

Cell free filtrate of overnight (~16-18 h) grown culture of lactobacilli was adjusted to various pH (2.0 to 10.0) with 5 **N** lactic acid or 5 **N** NaOH and the content was filter sterilised. The cell free filtrate at various pH was tested for bacteriocinogenic activity

using agar well assay and *L. delbrueckii* ssp. *bulgaricus* (2519) as target organism.

For measuring the activity of antimicrobial substance in milk filtrate over a range of pH, it was adjusted to various pH levels (2.0 to 10.0) by 5 **N** lactic acid or NaOH, filter sterilised and each filtrate tested for antimicrobial activity using the agar well assay and *L. delbrueckii* ssp. *bulgaricus* 2519 and *L. acidophilus* MOLA-2 as target organisms.

3.18 Storage stability of BLIS

The cell free filtrate obtained after centrigugation and filter sterilisation was adjusted to pH 6.0 and 1 mL aliquots in Eppendorf tubes were stored at 37°C, 4°C and -18°C in an incubator (37°C), a cold room (4°C) and a freezer (-18°C), respectively. Samples were taken out in duplicates at 1, 2, 4, 6, 8, 10 and 15 d from the incubator; at 1, 2, 3 ,4, 8 and 12 weeks from the cold room; and at 2, 4, 8, 12, 18, 24 and 36 weeks from the freezer, respectively. Antimicrobial activity was determined by measuring the zones of inhibitions as described earlier using *L. delbrueckii* ssp. *bulgaricus* (2519) as target organism.

3.19 Various sugar based MRS Broth

The production of inhibitory substance in various sugar (glucose, fructose, mannose, lactose, sucrose, maltose, raffinose, cellobiose and salicin) based MRS broth was monitored at 8, 16 and 24 h growth of *L. acidophilus* (LA-1). Sugar solutions (10% w/v) were prepared and filter sterilised. MRS broth devoid of glucose was prepared by dissolving all required ingredients of MRS broth (except glucose) in 800 mL distilled water, pH adjusted to 6.2 $\pm$ 0.2 and autoclaved. Twenty milliliter of each sugar solution was added to the basal medium. MRS broth containing various sugar was inoculated with 0.5 % (v/v) cells of *L. acidophilus* (LA-1) prepared by centrifuging and washing with phosphate buffer to avoid transfer of any glucose.

3.20 Effects of pH and supplementation of glucose and triple strength MRS broth on production and stability of BLIS

The effects of pH and supplementation of glucose and nutrients contained in MRS broth on the production and stability of bacteriocin were studied using Biostat®B fermentors (B. Braun, Germany). MRS broth (1.5 L) in the fermentors was inoculated with 30 mL of an active culture of *L. acidophilus* (LA-1). The fermentors were set to work at pH of 5.0, 5.5 and 6.0, the speed of the stirrer was set at 50 rpm and the temperature at 37°C. Glucose solution (50% w/v) and filter sterilised tripple strength MRS broth were added to the fermentors after 8 and 16 h of growth of LA-1 culture. Production and stability of BLIS during batch process in MRS, MRS plus ß-glycerophosphate and in milk was studied by inoculating 1 L of each medium with 2% LA-1 culture in Schott bottles.

3.21 Determination of viable counts, cell density and glucose concentration

Viable counts were monitored over a period of 48 h using the pour plate technique by serially diluting 1 mL aliquots with 9 mL peptone (0.15%) and water diluent. The viable counts of *L. acidophilus* (LA-1) were enumerated on MRS agar by incubating plates anaerobically at 37°C for 72 h. The growth (by cell density measurements) was measured at 600 nm using an Ultraspec uv/visible spectrophotometer (Pharmacia Biotech, London, England).

Glucose concentration in broth was measured colorimetrically at 505 nm using a Trinder glucose measurement kit (Sigma, St. Louis, USA). The Glucose (Trinder) reagent was prepared according to the instructions of manufacturer. Three millilitre of Glucose (Trinder) reagent was taken in a series of tubes and warmed to assay temperature. At timed intervals, 10 µL deionized water, Standard (300 mg/dL), controls and samples (cell free filtrate of MRS broth) were added to appropriately labelled test tubes and mixed by gentle inversion. The tubes were incubated for exactly 18 min at an ambient temperature (~25°C). Absorbance was recorded at 505 nm for blank, standard and samples and glucose was calculated using the formula given by the manufacturer.

3.22 Estimation of molecular weight of BLIS

The neutralised cell free extract containing active bacteriocin molecules was concentrated using ultrafiltration (UF) units with membranes of molecular weight cut off (MWCO) 5, 10, 20, 30 or 50 kDa. The concentration ratio for 5, 10 and 20 kDa membranes was 6, whereas 8-10 for 30 and 50 kDa membranes. Sartorius (Sartorius AG, Germany) Ultrasart Cell 50 unit was used for 5, 20 and 30 kDa membranes, whereas an Amicon (Amicon, Inc, Beverly, MA, USA) stirred cell unit was used for 10 and 50 kDa membranes.

For measuring the molecular weight of an anti-micribial substance produced in milk by *L. helveticus* 2700, the neutralised filtrate obtained from fermented milk containing active antimicrobial substance was passed through UF membranes of 5, 10, 30 or 50 kDa (MWCO. The concentration ratio was ~6. A Sartorius (Sartorius AG, Germany) Ultrasart Cell 50 unit was used for 5 kDa MWCO membranes, whereas an Amicon (Amicon Inc., Beverly, USA) stirred cell unit was used for the rest of the membranes.

3.23 Purification of BLIS using ammonium sulfate precipitation and SDS-PAGE

The BLIS and other proteins concentrated in the retentate of 50 kDa membrane were fractionated with ammonium sulphate at various saturation levels at ~4°C. After the mixture was stirred for at least 2 h at 4°C, the protein precipitate was centrifuged (4800 rpm, 30 min, 4°C) and the resulting pellet and pellicle were dissolved in 10-20 mL of citrate buffer (0.1 **M**, pH 6.0). The resulting suspensions and the liquid supernatant containing ammonium sulfate were dialysed using a dialysis tubing against citrate buffer (0.001 **M**, pH 6.0) for 24 h at 4°C. At each stage of purification, the resulting suspension was assayed for inhibitory activity. Protein concentration after each purification step was determined by the method of Lowry *et al.* (1951). The protein content of each fraction was estimated in order to load the right amount of protein on SDS-PAGE gel. For estimating the protein content, 0.5 mL of solution A (0.1 mL of 5% $CuSO_4$, 0.9 mL of 1% sodium-potassium tartarate and 10 mL of 10% Na_2CO_3 in 0.5 **M** NaOH) was added to 0.5 mL sample that contained 0 to 100

μg of protein. After 10 min incubation at 37°C, 1.5 mL of solution B (1 mL Folin-Ciocalteu's reagent plus 10 mL double glass distilled water) was added and the solutions were mixed immediately by vortex. Absorbance at 680 nm was recorded after incubation of the content at 52°C for 20 min. Standards containing 0 to 100 μg of protein were prepared from 0.1% bovine serum albumin (Sigma, St. Louis, USA) solution. Reagents A and B were prepared immediately before use.

SDS-PAGE analysis was carried out on a 12% separating gel containing acrylamide and bisacrylamide as described earlier (section 3.11) and stained with Coomassie brilliant blue R-250 (Sigma, USA) or silver stained to confirm the purity of BLIS.

4.0 ENUMERATION OF YOGHURT AND PROBIOTIC BACTERIA

4.1 Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus* and bifidobacteria

4.1.1 Introduction

Lactobacillus acidophilus and bifidobacteria grow slowly in milk during product manufacture. Therefore, the usual production practice is to incorporate yoghurt cultures (i.e., *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) along with probiotic cultures. It seems reasonable to assume that the beneficial effects of probiotic bacteria can be expected only when viable cells are ingested. An important parameter in monitoring viable organisms in assessing product quality is the ability to count *L. acidophilus* and bifidobacteria differentially.

Several media have been developed for differential enumeration of yoghurt and probiotic bacteria as described in Chapter 2 (section 2.5.1). However, these media may not be suitable for selective enumeration of *L. acidophilus* and *Bifidobacterium* spp. in the presence of yoghurt culture organisms (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*). Further, differences exist among the strains of the same species with respect to tolerance of low pH, bile salts, NaCl, and sugar fermentation characteristics (Kim, 1988). There is growing concern that some media that contain bile or antibiotics might also restrict the growth of *L. acidophilus* or bifidobacteria and that counts obtained are not representative of the actual number of viable cells that are present in the product. This study examined a range of media that could possibly be used in selective enumeration of yoghurt organisms and *L. acidophilus* and bifidobacteria.

A version of this chapter has been published. Dave, R. I. and Shah, N. P. (1996). *J. Dairy Sci.*, 79: 1529-1536.

4.1.2 Materials and methods

4.1.2.1 Cultures

The maintenance and source of pure cultures of 6 strains of *S. thermophilus*, 5 strains each of *L. delbrueckii* ssp. *bulgaricus* and *L. acidophilus*, 2 strains each of *Bifidobacterium bifidum* and *Bifidobacterium longum*, and 1 strain each of *Bifidobacterium adolescentis*, *Bifidobacterium pseudolongum*, *Bifidobacterium breve* and *Bifidobacterium infantis* is described in section 3.2.

4.1.2.2 Media preparation and enumeration of bacteria

The composition and details of media used in this study are described in section 3.3 and the enumeration of bacteria was carried out as described in section 3.3.9.

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

4.1.3 Results and discussion

Viable counts of *S. thermophilus* in various bacteriological media are presented in Table 4.1. As shown, ST agar gave higher recovery of *S. thermophilus* than did MRS agar. Lower recovery of *S. thermophilus* in MRS medium could possibly be due to the presence of sodium acetate in this medium. *S. thermophilus* (strain 2013) did not grow on MRS medium adjusted to pH 5.8 or pH of 5.2. This could possibly be due to increased toxicity of sodium acetate and pH for this strain that may not have permitted the growth of the organism on the MRS agar media adjusted to pH of 5.8 and 5.2. The cultures of *S. thermophilus* formed well-developed, yellow colonies on ST agar plates within 24 h of incubation; *L. delbrueckii* ssp. *bulgaricus,* either did not grow (strains 2515 and 2519) or formed tiny, white, cottony colonies (strains 2501, 2505, and 2517) (Table 4.2), that could

easily be distinguished from *S. thermophilus*. Shankar and Davies (1977) reported that the growth of *L. delbrueckii* ssp. *bulgaricus* was suppressed on pour plates, when the pH of the M17 medium was adjusted to 6.8. The pH of the ST agar is 6.8, which might play a crucial role and might have suppressed the growth of lactobacilli when the plates were incubated for 24 h. Cultures of *L. acidophilus* failed to grow on ST agar plates within 24 h of incubation under aerobic conditions (Table 4.3); however, this organism formed yellow colonies after 72 h of incubation. Bifidobacteria, which are anaerobic, did not grow on ST agar when incubated aerobically. Thus, ST agar could be used for the enumeration of *S. thermophilus* from a product containing all the 4 organisms, provided that the plates were incubated aerobically at 37°C for 24 h. Recovery of *S. thermophilus* in MRS agar at pH 5.8 was 30 to 90% less than that on ST agar or RCA agar (pH 6.8) (Table 4.1). *Lactobacillus delbrueckii* ssp. *bulgaricus, L. acidophilus,* and bifidobacteria grew on RCA agar (pH 6.8) (Tables 4.2 to 4.4). Thus, this medium could not be used for the differential enumeration of *S. thermophilus*. Growth of *S. thermophilus* on other bacteriological media did not occur (Table 4.1).

Table 4.2 shows the viable counts of *L. delbrueckii* ssp. *bulgaricus* on various bacteriological media. Recovery of *L. delbrueckii* ssp. *bulgaricus* was highest on RCA agar (pH 6.8), followed by recovery on MRS agar. The growth of *L. delbrueckii* ssp. *bulgaricus* was partially inhibited when the pH of the MRS agar was lowered to 5.8, and the growth was further inhibited at pH 5.2. A similar pattern was seen with RCA medium. Growth of 1 strain of *L. delbrueckii* ssp. *bulgaricus* (strain 2515) did not occur on RCA agar adjusted to pH 5.3. These results differed from those observed by Johns *et al.* (1978), who found complete recovery of *L. delbrueckii* ssp. *bulgaricus*. *Lactobacillus delbrueckii* ssp. *bulgaricus* did not grow on MRS-bile, MRS-oxgall, MRS-NaCl, MRS-maltose, MRS-salicin, MRS-sorbitol, MRS-NNLP, or Rogosa acetate agars. At pH 5.3, RCA agar allowed the growth of 2 strains of *L. acidophilus* (strains 2400 and 2415) (Table 4.3) and 4 strains of bifidobacteria (Table 4.4); therefore, this medium may not be suitable for differential enumeration purposes.

It was also found that MRS agar at pH 5.2 or RCA agar at 5.3 could be used for differential enumeration of *L. delbrueckii* ssp. *bulgaricus* when the plates were incubated anaerobically at 45°C for 72 h. Some of the bifidobacteria also grew on these media; however, the colonies formed by *L. delbrueckii* ssp. *bulgaricus* could easily be differentiated from those of bifidobacteria. The recovery of *L. delbrueckii* ssp. *bulgaricus* was one to two log cycles lower than that obtained on MRS agar at 37°C.

Lactobacillus delbrueckii ssp. *bulgaricus* either did not grow (strain 2517) or formed tiny white cottony colonies (strains 2505, 2515, and 2519), except for strain 2501 on cellobiose esculin agar. The results were comparable with those reported by Hunger (1986). Cellobiose esculin agar supported the growth of *L. acidophilus* (except for strain 2415), and thus, this medium may not be suitable for the selective enumeration of *L. delbrueckii* ssp. *bulgaricus*.

A subtraction method (subtracting counts of *S. thermophilus* enumerated on ST agar and *L. acidophilus* and bifidobacteria enumerated on MRS-maltose agar from the total counts of all the four groups of organisms enumerated on MRS agar) could be used; however, the number of organisms present in a product should be in equal proportion. If the organisms are not in equal proportion, the subtraction method may not be suitable, as the plates containing 25 to 250 colonies are considered ideal for the enumeration purposes. Onggo and Fleet (1993) observed no growth of *S. thermophilus* in MRS agar. Thus, if a strain of *S. thermophilus* used in a product did not form colonies on MRS agar plates, as was observed by Onggo and Fleet (1993); counts of *L. delbrueckii* ssp. *bulgaricus* could be obtained by subtracting counts of *L. acidophilus* and bifidobacteria enumerated on MRS-maltose agar from the total counts obtained using MRS agar.

Viable counts of *L. acidophilus* enumerated on various bacteriological media are shown in Table 4.3. *Lactobacillus acidophilus* culture did not grow on ST agar (incubated at 37°C for 24 h) or on MRS-NNLP agar. Of the several media evaluated, MRS, MRS-maltose, MRS-salicin and MRS-sorbitol agars gave almost the same recovery for all the *L.*

acidophilus cultures. The growth of this organism was slightly inhibited on MRS agar at pH 5.8, and the growth on this agar was further inhibited at pH 5.2. The recovery of *L. acidophilus* was one to two log cycles lower in MRS medium containing bile or oxgall. The MRS-NaCl agar showed almost 40 to 60% inhibition.

Growth of bifidobacteria was supported by MRS-maltose agar (Table 4.4); aerobic incubation to suppress the growth of this organism resulted in 40 to 70% less recovery of some of the *L. acidophilus* cultures. Thus, this medium might not be suitable for the differential enumeration of *L. acidophilus*. However, MRS-maltose agar could be used to estimate total counts of *L. acidophilus* and bifidobacteria because *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* (Tables 4.1 and 4.2) did not grow on this medium.

The MRS-salicin and MRS-sorbitol agars supported the growth of *L. acidophilus* (Table 4.3), but bifidobacteria either did not grow or grew poorly (Table 4.4) on these complex media based on sugar. The colonies formed by bifidobacteria that grew on MRS-salicin and MRS-sorbitol agar plates were easily distinguishable from those of *L. acidophilus*. Thus, MRS-salicin agar or MRS-sorbitol agar could be used for differential enumeration of *L. acidophilus*, because *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* did not grow on these media (Tables 4.1 and 4.2). These results were similar to the findings of Hull and Roberts (1984), who observed almost the same recovery of *L. acidophilus* when dextrose in MRS agar was replaced with maltose and salicin. However, salicin was uneconomical for the routine testing, and hence the use of sorbitol to enumerate *L. acidophilus* selectively was investigated in this study. Use of MRS-sorbitol medium for differential enumeration of *L. acidophilus* has not been reported earlier.

The MRS-NNLP agar did not support the growth of *L. acidophilus* (Table 4.3). The recovery of *L. acidophilus* on RCA agar at pH 6.8 was one or two log cycles lower than that obtained on MRS agar. Three of the *L. acidophilus* cultures (2401, 2404 and 2405) were completely inhibited on RCA agar at pH 5.3. The recovery of the other 2 *L. acidophilus* cultures (2400 and 2415) was adversely affected on this medium. Similarly, 2

strains of *L. acidophilus* (2401 and 2404) did not grow on Rogosa acetate agar, and the recovery of the other three strains was adversely affected on this medium.

Cellobiose esculin agar suppressed the growth of S. *thermophilus* (Table 4.1) and *L. delbrueckii* ssp. *bulgaricus* (Table 4.2). All of the strains of *L. acidophilus* (except strain 2415) (Table 4.3) formed colonies, but bifidobacteria (except for *B. pseudolongum* 20099) formed tiny colonies (Table 4.4), which could be easily differentiated. Thus, cellobiose esculin agar could be used for differential enumeration of *L. acidophilus* (except strain 2515) from a product containing all 4 organisms, provided *B. pseudolongum* (strain 20099) was not incorporated in the product (Table 4.4). However, the recovery of *L. acidophilus* on cellobiose esculin agar was less than that on MRS, MRS-maltose, MRS-salicin or MRS-sorbitol agars.

Viable counts of bifidobacteria on different bacteriological media are presented in Table 4.4. As shown, ST agar supported the growth of *B. longum* (20097) and *B. pseudolongum* (20099) when grown anaerobically, but the other strains formed tiny colonies or failed to grow. The MRS agar provided the best recovery of bifidobacteria, but the other three groups of organisms grew as well on this medium (Tables 4.1 to 4.3). At pH 5.8, MRS agar did not show inhibition of bifidobacteria cultures. However, recovery of bifidobacteria was adversely affected on MRS agar at pH 5.2. Recovery of bifidobacteria on MRS medium containing bile, oxgall, or NaCl varied, and the counts remained less than those obtained on MRS agar. *Lactobacillus acidophilus* also grew on MRS-bile, MRS-oxgall, and MRS-NaCl agars, and these media therefore may not be suitable for the selective enumeration purposes. The MRS-maltose agar gave almost the same recovery of bifidobacteria as that obtained on MRS agar, but bifidobacteria either did not grow or formed pinpoint colonies on MRS-salicin agar plates (except for *B. pseudolongum* 20099, which formed normal colonies) and on MRS-sorbitol agar. Several media containing one or more antimicrobial substances have been suggested for the enumeration of bifidobacteria. The MRS-NNLP agar is widely used for the recovery and enumeration of bifidobacteria by researchers and quality control laboratories. This medium was originally

developed by Teraguchi *et al.* (1978). The recovery of *B. adolescentis* (1920) and *B. pseudolongum* (20099) strains in this medium was poor (Table 4.4). Four types of antibiotics are used as selective agents in MRS-NNLP medium, and one or more of these might have affected the growth of *B. adolescentis* and *B. pseudolongum*. Therefore, the counts obtained on MRS-NNLP agar may not be representative of the viable cells that are present in the product and suggests a need to countercheck the efficacy of this medium with pure cultures before adopting the medium for enumeration purposes.

To obtain a true picture of bifidobacterial strains tested in this study, subtraction of *L. acidophilus* count obtained using MRS-salicin agar or MRS-sorbitol agar from a total count of *L. acidophilus* and bifidobacteria obtained from MRS-maltose agar could be carried out, because other strains or species not tested might use salicin and sorbitol. Therefore, counterchecking of pure strains of bifidobacteria in these media before adopting them for the enumeration purposes is recommended in the presence of yoghurt cultures and *L. acidophilus*. Similarly, enumeration of bifidobacteria using MRS-NNLP agar should be carried out simultaneously, because a difference of more than one to two log cycles in the counts of bifidobacteria in a product containing *L. acidophilus* do not give a correct picture of bifidobacteria by the subtraction method.

Recovery of all the bifidobacteria obtained on RCA agar was almost identical to that on MRS agar at pH 6.8, but the recovery was slightly lower on RCA agar at pH 5.3. Bifidobacteria formed well-developed colonies on RCA agar plates at 5.3 (except for *B. bifidum* 1900 and 1901). Recovery of bifidobacteria on Rogosa acetate agar was two to three log cycles lower. *Lactobacillus acidophilus* counts on this medium were also lower (Table 4.3), which could have been due to the presence of high concentration of sodium acetate, low pH of the medium, or use of glacial acetic acid to adjust the pH.

Bifidobacteria formed tiny colonies on cellobiose esculin agar (except for *B. adolescentis* 1920, *B. longum* 1941, and *B. pseudolongum* 20099). Hunger (1986) also observed poor growth of bifidobacteria on cellobiose esculin agar. Conversely, in our study, 3 of 6 strains

of the bifidobacteria exhibited good growth. *Lactobacillus acidophilus* strain 2415 failed to show typical colony development. Therefore, cellobiose esculin agar might not be an ideal medium for the enumeration purposes.

Commercial products. Because the evaluation of media for the selective enumeration of yogurt cultures, *L. acidophilus*, and bifidobacteria was carried out using pure cultures, it was desirable to enumerate these organisms selectively from products containing mixture of these organisms. In a separate experiment, 6 samples were analysed to countercheck the efficacy of different bacteriological media in recovering the claimed organisms selectively. The samples that were analysed included: probiotic capsules (Blackmores Ltd., Balgowlah, Australia) claimed to contain *L. acidophilus* and bifidobacteria, a commercial yoghurt sample (Yo-plait, Australia), yoghurt prepared in our laboratory using a commercial starter culture supplied by Chr. Hansen (Bayswater, Australia) containing all the 4 groups of organisms, and 3 frozen Bulla brand Fruit'N Yoghurt sticks (Regal Cream Products Pty. Ltd., North Melbourne, Australia) claimed to contain AB bacteria. The results are presented in Table 4.5 as the viable counts per capsule (for Blackmores' capsules) and per gram for the rest of the samples. Enumeration of *S. thermophilus* was carried out on ST and M17 (Oxoid, Australia) agars (aerobic incubation at 37°C for 24 h), *L. delbrueckii* ssp. *bulgaricus* on MRS (pH 5.2) agar (anaerobic incubation at 45°C for 72 h), *L. acidophilus* on MRS-salicin agar, MRS-sorbitol agar, cellobiose esculin agar and MRS-maltose agar (aerobic incubation at 37°C for 72 h) and that of bifidobacteria on MRS-NNLP agar and by subtraction method (i.e., subtracting counts of *L. acidophilus* obtained on MRS-salicin or MRS-sorbitol agar from the total counts of *L. acidophilus* and bifidobacteria obtained on MRS-maltose agar). As shown in Table 4.5, ST agar was selective in enumerating *S. thermophilus;* and the counts obtained on ST agar were higher or similar to that of M17 agar. ST agar was economical over M17 media, and the presence of sucrose in ST agar could make ST agar more practical for the recovery of *S. thermophilus* in the presence of other organisms. MRS agar (pH 5.2) was suitable for counting *L. delbrueckii* ssp. *bulgaricus* differentially. MRS-salicin agar, and MRS-sorbitol agar were selective in enumerating *L. acidophilus*. Cellobiose esculin agar and MRS-maltose agar (using aerobic

incubation) also enumerated *L. acidophilus*, selectively; however counts were 40-70% less than that obtained on MRS-salicin agar or MRS-sorbitol agar. MRS-NNLP agar was selective for bifidobacteria; however, counts of bifidobacteria obtained by subtraction method were higher than those enumerated using MRS-NNLP agar.

Further, we found growth of *Bifidobacterium breve* (1930) and *Bifidobacterium infantis* (1912) on MRS-sorbitol and MRS-salicin agars (data not included). The colonies formed by these two organisms were non-differentiable from those of *L. acidophilus*. Therefore, in products containing salicin and sorbitol positive bifidobacteria, aerobic incubation of MRS-maltose agar would be useful in selectively enumerating *L. acidophilus* in the presence of other groups of organisms.

4.1.4 Conclusions

Fifteen media were evaluated to determine their suitability for selective enumeration of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and bifidobacteria. *Streptococcus thermophilus* (ST) agar was found to be suitable for selective enumeration of *S. thermophilus* under aerobic incubation at 37°C for 24 h. The MRS agar at pH 5.2 or reinforced clostridial agar at pH 5.3 could be used for the selective enumeration of *L. delbrueckii* ssp. *bulgaricus* when the incubation is carried out at 45°C for ≥ 72 h. The recovery of *L. acidophilus* and bifidobacteria on MRS-maltose agar was similar; MRS-maltose agar could be used to enumerate total counts of probiotic bacteria. For selective enumeration of *L. acidophilus,* MRS-salicin agar or MRS-sorbitol agar could be used. MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride and paromomycin sulfate) agar was suitable; however, determining counts of bifidobacteria by differential counts between *L. acidophilus* enumerated on MRS-salicin or MRS-sorbitol agar and the total counts of *L. acidophilus* and bifidobacteria obtained from MRS-maltose agar resulted in higher recovery of some strains of bifidobacteria. Other media that were evaluated in this study were not suitable for the selective enumeration.

Table 4.1. Viable counts of *Streptococcus thermophilus* strains on different bacteriological agars[1]

Strain	ST[2]	MRS	MRS (5.8 pH)	RCA (6.8 pH)
2000	53×10^7	19×10^7	85×10^{4a}	12×10^7
2002	18×10^7	16×10^7	13×10^6	15×10^7
2008	98×10^7	91×10^7	20×10^6	85×10^7
2010	120×10^7	126×10^7	11×10^6	86×10^7
2013	71×10^7	49×10^7	$<10^3$	90×10^6
2014	61×10^7	44×10^7	10×10^6	14×10^7

[1]All strains were less than 10^3 cfu/g for the following media: MRS (5.2 pH), MRS-Bile, MRS-Oxgall, MRS-NaCl, MRS-Maltose, MRS-Salicin, MRS-Sorbitol, MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride and paromomycin sulfate), RCA (reinforced clostridial agar) (5.3 pH), Rogosa acetate and cellobiose esculin.

[2]*Streptococcus thermophilus* agar;

[a]tiny colonies.

Table 4.2. Viable counts of *Lactobacillus delbrueckii* ssp. *bulgaricus* strains on different bacteriological agars[1]

Strain	ST[2]	MRS	MRS (5.8 pH)	MRS (5.2 pH)	RCA[3] (6.8 pH)	RCA (5.3 pH)	Cellobiose esculin
2501	43×10^{7a}	40×10^{7}	29×10^{7}	22×10^{6}	46×10^{7}	40×10^{6}	15×10^{6b}
2505	12×10^{8a}	12×10^{8}	14×10^{7}	10×10^{7}	190×10^{7}	15×10^{7}	104×10^{7b}
2515	$<10^{3}$	17×10^{6}	25×10^{5}	11×10^{5}	19×10^{6}	10×10^{3a}	65×10^{5a}
2517	44×10^{7a}	52×10^{7}	44×10^{6}	20×10^{6}	54×10^{7}	57×10^{6}	$<10^{3}$
2519	$<10^{3}$	16×10^{7}	12×10^{7}	76×10^{6}	16×10^{7}	17×10^{6}	88×10^{6a}

[1]All strains were less than 10^{3} cfu/g for the following media: MRS-Bile, MRS-Oxgall, MRS-NaCl, MRS-Maltose, MRS-Salicin, MRS-Sorbitol, MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate) and Rogosa acetate.

[2]ST=*Streptococcus thermophilus* agar; and

[3]RCA=reinforced clostridial agar.

[a]tiny white cottony colonies.

[b]no typical reaction of cottony colonies.

Table 4.3. Viable counts of *Lactobacillus acidophilus* strains on different bacteriological agars[1]

Strain	MRS	MRS (5.8 pH)	MRS (5.2 pH)	MRS-Bile	MRS-Oxgall	MRS-NaCl	MRS-Maltose	MRS-Salicin	MRS-Sorbitol	RCA[2] (6.8 pH)	RCA (5.3 pH)	Rogosa acetate	Cellobiose esculin
2400	44×10^7	43×10^7	40×10^7	27×10^6	24×10^6	30×10^7	43×10^7	46×10^7	45×10^7	39×10^7	35×10^6	24×10^5	38×10^7
2401	63×10^6	79×10^5	49×10^5	56×10^5	28×10^5	30×10^6	62×10^6	72×10^6	67×10^6	41×10^5	$<10^3$	$<10^3$	59×10^6
2404	32×10^6	29×10^6	28×10^5	80×10^4	16×10^5	17×10^6	33×10^6	37×10^6	37×10^6	27×10^5	$<10^3$	$<10^3$	27×10^6
2405	34×10^7	31×10^7	16×10^6	33×10^6	53×10^6	32×10^6	34×10^7	42×10^7	41×10^7	84×10^5	$<10^3$	10×10^5	35×10^7
2415	50×10^7	44×10^7	15×10^5	10×10^6	12×10^6	41×10^7	45×10^7	52×10^7	53×10^7	44×10^7	39×10^6	30×10^7	11×10^7[a]

[1]All strains were less than 10^3 cfu/g for MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride, and paromomycin sulfate) and ST (*Streptococcus thermophilus* agar); the latter showed no growth of *L. acidophilus* within 24 h of aerobic incubation at 37°C.

[2]RCA=reinforced clostridial agar.

[a]no typical reaction, i.e. dark centered 1.0-1.5 mm colonies of *L. acidophilus* with greenish/brown zone.

Table 4.4. Viable counts of various bifidobacteria on different bacteriological agars

Strain	ST[1]	MRS	MRS (5.8 pH)	MRS (5.2 pH)	MRS-Bile	MRS-Oxgall	MRS-NaCl	MRS-Maltose	MRS-Salicin	MRS-Sorbitol	MRS-NNLP[2]	RCA[3] (6.8 pH)	RCA (5.3 pH)	Rogosa acetate	Cello esc
1900	23×10^{8a}	28×10^{8}	28×10^{8}	28×10^{7}	20×10^{8}	18×10^{8}	28×10^{8}	27×10^{8}	11×10^{8a}	14×10^{6a}	27×10^{8}	23×10^{8}	22×10^{8a}	20×10^{5a}	17 x
1901	17×10^{7a}	19×10^{8}	19×10^{8}	12×10^{7}	40×10^{6}	55×10^{6}	18×10^{7}	18×10^{8}	15×10^{7a}	13×10^{7a}	18×10^{8}	15×10^{7}	11×10^{8a}	16×10^{5}	30 x
1920	$<10^{3}$	14×10^{7}	14×10^{7}	16×10^{5}	14×10^{6}	19×10^{6}	13×10^{6}	14×10^{7}	14×10^{5aa}	$<10^{3}$	13×10^{6}	18×10^{6}	16×10^{7}	11×10^{4}	12 x
1941	$<10^{3}$	34×10^{7}	39×10^{7}	65×10^{6}	70×10^{6}	78×10^{6}	35×10^{7}	37×10^{7}	25×10^{6aa}	$<10^{3}$	31×10^{7}	34×10^{7}	32×10^{7}	13×10^{5}	28 x
20097	121×10^{7}	17×10^{8}	16×10^{7}	13×10^{6}	32×10^{7}	19×10^{6}	13×10^{7}	16×10^{8}	$<10^{3}$	$<10^{3}$	16×10^{7}	18×10^{8}	17×10^{8}	11×10^{5}	61 x
20099	121×10^{7}	79×10^{8}	69×10^{8}	69×10^{6}	70×10^{7}	79×10^{8}	79×10^{8}	13×10^{8}	26×10^{7}	$<10^{3}$	79×10^{7}	78×10^{8}	65×10^{8}	11×10^{6}	48 x

ST=*Streptococcus thermophilus* agar.

[2]NNLP=nalidixic acid, neomycin sulpfate, lithium chloride, and paromomycin sulfate; and

[3]RCA=reinforced clostridial agar.

[a]tiny colonies.

[aa]pin point colonies.

[b]no typical reaction of poor growth of bifidobacteria on cellobiose esculin agar.

Table 4.5. Viable counts of yoghurt and probiotic bacteria in commercial products

Organism	Agar	Samples[1]					
		BDC	Yop	ChH	BCS	BS	BB
Streptococcus thermophilus	ST	$< 10^2$	19×10^8	7×10^8	75×10^6	79×10^7	18×10^7
	M17	$< 10^2$	17×10^8	45×10^7	54×10^6	59×10^7	16×10^7
Lactobacillus delbrueckii ssp. *bulgaricus*	MRS (pH 5.2)	$< 10^3$	$< 10^3$	15×10^6	13×10^6	15×10^6	28×10^6
Lactobacillus acidophilus	MRS-salicin	25×10^7	30×10^6	18×10^6	52×10^{3a}	89×10^{3a}	16×10^{4a}
	MRS-sorbitol	24×10^7	21×10^6	16×10^6	49×10^{3a}	69×10^{3a}	14×10^{4a}
	Cellobiose esculin	27×10^6	68×10^5	56×10^5	49×10^{2a}	34×10^{2a}	19×10^{3a}
	MRS-maltose (aerobic incubation)	91×10^6	12×10^6	82×10^5	28×10^{3a}	42×10^{3a}	68×10^{3a}
Bifidobacteria	MRS-NNLP	79×10^6	17×10^5	16×10^6	1×10^2	3×10^2	1×10^2
	Subtraction method	15×10^7	76×10^5	39×10^6	$< 10^4$	$< 10^4$	$< 10^4$

[1]BDC=Blackmores' capsule; Yop=commercial Yo-plait yoghurt sample; ChH=Yoghurt from Chr. Hansen's culture; BCS=Bulla's chocolate coated strawberry stick; BS=Bulla's strawberry stick; and BB=Bulla's banana stick.

[a]=two types of colonies; however, colonies of *L. acidophilus* were easily distinguishable.

5.0 VIABILITY OF YOGHURT AND PROBIOTIC BACTERIA

5.1 Viabilty of yoghurt bacteria and probiotic bacteria in yoghurts made from commercial starter cultures

5.1.1 Introduction

Several health benefits have been claimed to be associated with the consumption of fermented milk products (Le *et al.*, 1986; Van't Veer *et al.*, 1989; Modler *et al.*, 1990; Hughes and Hoover, 1991; Kanbe, 1992; Mital and Garg, 1992; Nakazawa and Hosono, 1992; Yamamoto *et al.*, 1994). Although yoghurt microflora (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) have been found to be beneficial for human health and nutrition (Deeth and Tamime, 1981; IDF, 1984); recent emphasis has been on development of fermented milk products with added *Lactobacillus acidophilus* and bifidobacteria (known as probiotic or AB-culture). This is due to the ability of these cultures to tolerate acid and bile (Gilliland, 1978; Kanbe, 1992; Lankaputhra and Shah, 1994); which enable them to implant in the intestinal tract. The benefits derived by the consumption of AB products are well documented and have been reviewed by several workers (Kurmann and Rasic, 1991; Misra and Kuila, 1991; Mital and Garg, 1992; Nakazawa and Hosono, 1992; Ishibashi and Shimamura, 1993). Because of the claims made in favour of probiotic bacteria, various fermented milk products have been formulated and it is estimated that more than 90 products with added AB cultures are available worldwide (Modler *et al.*, 1990; Patel *et al.*, 1991; Mital and Garg, 1992).

In order to produce the therapeutic benefits, suggested minimum level for probiotic bacteria in yoghurt is 10^5 to 10^6 viable cells per mL or g of product (Robinson, 1987; Kurmann and Rasic, 1991).

A version of section 5.1 has been published. Dave, R. I. and Shah, N. P. (1997). *Inter. Dairy J.*, 7: 31-41.

Despite the importance of viability of these beneficial micro-organisms, surveys have shown poor viability of probiotics in the market preparations (Schioppa *et al.*, 1981; Hull *et al.*, 1984; Anon, 1992; Iwana *et al.*, 1993; Shah *et al.*, 1995). Efforts to increase their viability in various products have drawn attention of the researchers in recent years.

Several factors have been claimed to affect the viability of probiotic cultures in fermented milk products. Acidity, pH and hydrogen peroxide have been identified to affect the viability during manufacture and storage of yoghurt (Lankaputhra and Shah, 1994; Lankaputhra and Shah, 1995; Lankaputhra *et al.*, 1996b). Other factors, such as temperature of storage, oxygen content, concentrations of lactic and acetic acids etc., have been presumed to affect the viability of probiotic organisms in yoghurt. Several workers have reported the effects of some of these factors (Gilliland and Speck, 1977a, b; Hull *et al.*, 1984; Martin and Chou, 1992; Klaver *et al.*, 1993; Kneifel *et al.*, 1993; Medina and Jordono, 1994; Patidar *et al.*, 1994; Prajapati and Dave, 1994; Samona and Robinson, 1994; Rybka and Kailasapathy, 1995). However, effects of all the parameters on viability of probiotic bacteria have not been studied simultaneously.

In this study, the viability of yoghurt bacteria and probiotic bacteria in yoghurts made from 4 commercial starter cultures used to manufacture AB yoghurts was assessed. Changes in titratable acidity, pH, dissolved oxygen content, hydrogen peroxide content, concentration of lactic and acetic acids and the viable counts of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and bifidobacteria were monitored during manufacture of yoghurt and during a storage period of 35 days at 4°C. The effects of altered storage temperature (10°C) and of oxygen permeation during storage on the viability of yoghurt bacteria and probiotic bacteria were also studied.

5.1.2 Materials and methods

5.1.2.1 Starter cultures and yoghurt preparation

Four starter cultures described in section 3.1 were used in this study and yoghurt was prepared as described in section 3.4.1. Samples were prepared as described in section 3.5.1.

5.1.2.2 Analyses and time interval specifications

The yoghurt samples were analysed for pH and titratable acidity (section 3.7.1.1), hydrogen peroxide (section 3.7.1.2), quantification of organic acids (section 3.7.1.3), dissolved oxygen content (section 3.7.1.4), protein and total solids contents (section 3.7.1.5). The microbiological analyses of yoghurt samples at various time intervals (section 3.6) was carried out as described in section 3.7.2.

The results of the assessment of the viability of yoghurt bacteria and probiotic bacteria are the averages of three replications, while the effects of storage temperature (4 and 10°C) and dissolved oxygen content are averages of two replications.

5.1.3 Results and Discussion

5.1.3.1 Composition

Protein content of heat treated yoghurt mix ranged from 3.55-3.65, whereas the total solid contents were in the range of 15.99-16.24%. Thus, the compositional differences were minimal in the various experimental replications and the differences in observations should not be due to the compositional factors.

5.3.1.2 Assessment of yoghurt during manufacture and storage at 4°C

Titratable acidity and pH. The changes in titratable acidity (TA) and pH in yoghurt are illustrated in Fig. 5.1.1. The initial TA of milk (0.14%) increased to 0.76-0.77% in yoghurts made from cultures C1, C2 and C4, and to 0.68% for culture C3 at 0 d. After 5 d at 4°C the TA increased to 0.82-0.84% for yoghurts made with cultures C1, C2 and C4, and to 0.70% for yoghurt made with culture C3. This increase might be attributed to the residual fermentation changes. The increase in TA was minimal for culture C1, C2 and C4, (0.03%) for these cultures as compared with culture C3 (0.07%). However, the final TA remained lower in the yoghurt made from C3 culture. The overall increase in TA after 5 d storage at 4°C was similar to that of storage for up to 35 d for all the commercial starter cultures studied.

The initial pH of milk (6.55-6.62 at 0 h) decreased to 4.33-4.41 for cultures C1, C2 and C4, and to 4.64 for C3 at 0 d. The decrease in pH of 0.17 units from the set pH of 4.5 was obviously due to continued fermentation during overnight cooling till the temperature of the product reached 4°C. After the initial drop, a gradual decrease in the pH was observed throughout the storage period of 35 d. The trend was identical for all the starter cultures, similar to that observed for TA (Fig. 5.1.1). After 35 d storage, the pH dropped to 4.16 for culture C2 and C4, 4.22 for culture C1 and to 4.40 for C3. The drop in the pH was almost the same for all the commercial starter cultures. As there was no major difference in pH values or relative drop in pH values for C1, C2 and C4 cultures at 4°C for up to 35 d, changes in microbial ecology might not be attributed to the changes in TA or pH.

Changes in oxygen content. The initial oxygen content in heat treated milk (6.50 to 6.73 ppm) dropped to 1.20 to 1.80 ppm after fermentation (AF) in yoghurts made from 4 cultures as a result of the fermentation process (Fig. 5.1.2), where several metabolic pathways require dissolved oxygen, thereby lowering the oxidation-reduction (O-R) potential. Subsequently, the oxygen content increased to 5.8 to 5.9 ppm at 0 d period,

which could be due to its permeation through the container. Further, the oxygen content stabilised at 8.13 to 8.36 ppm after 5 d period and remained ≤ 9.1 ppm for all the samples. No major differences were found amongst these cultures in terms of their behaviour to consume or absorb the oxygen. Therefore, it was presumed that the relative difference in viable counts may not be attributed to the dissolved oxygen. The dissolved oxygen content may be playing a role in affecting the viability of probiotic organisms as postulated by Ishibashi and Shimamura (1993), but equally in all the cultures. Any difference in decay of counts of probiotic organisms may not be only due to rise in oxygen. To confirm the role of oxygen, a separate study was undertaken in which yoghurt was fermented and stored in glass bottles, in place of plastic cup, as discussed below.

Changes in hydrogen peroxide content. The shift in hydrogen peroxide content in yoghurts is shown in Fig. 5.1.2. On average C1 produced the highest (16.80 $\mu g.mL^{-1}$) and C4 produced the lowest concentration (3.78 $\mu g.mL^{-1}$) of hydrogen peroxide, observed immediately after the production of yoghurt. At 0 d, the hydrogen peroxide concentration was 10.26, 8.80, 2.66 and 2.50 $\mu g.mL^{-1}$ for cultures C1, C2, C3 and C4, respectively. The higher concentration of hydrogen peroxide for C1 and C2 might be due to the presence of *L. delbrueckii* ssp. *bulgaricus*, which has been reported to produce hydrogen peroxide in yoghurt (Gilliland and Speck, 1977a). The decrease in concentration of hydrogen peroxide at 0 and 5 d could be due to its decomposition in milk affected by the organic components of milk including milk fat, thus lowering its recovery (Gilliland, 1968). After 5 d storage period, there was not much difference in hydrogen peroxide concentration for all the commercial cultures tested in this study (Fig. 5.1.2). This confirmed that no production of hydrogen peroxide occurred during storage at 4°C. The concentration of hydrogen peroxide produced by cultures C1 and C2 might have caused partial injury to the active cells of *L. acidophilus*, as *L. acidophilus* showed maximum decay in C1 culture followed by C2 culture (Table 5.1.2).

Changes in lactic and acetic acid contents. The changes as determined by HPLC are shown in Fig. 5.1.3. The lactic acid content of milk at 0 h was 0.05 $mg.g^{-1}$ which increased to 3.8, 3.7, 2.8 and 3.9 $mg.g^{-1}$ at 0 d for cultures C1, C2, C3 and C4, respectively. A noticeable increase was observed for all the cultures during first 5 d storage at refrigerated temperature, followed by gradual increase throughout the storage period. A maximum increase was observed with C3 culture during storage, however, the final concentration remained lower for this culture. After 35 d storage, the concentration of lactic acid in the product was 5.5, 5.4, 4.9 and 5.8 $mg.g^{-1}$ for C1, C2, C3 and C4 cultures, respectively. The observations showed similar pattern of increase in line with TA (Fig. 5.1.1).

The acetic acid concentration at 0 h was 5 $\mu g.g^{-1}$ of milk, increasing to 120, 86, 16 and 90 $\mu g.g^{-1}$ of yoghurt at 0 d for C1, C2, C3 and C4, respectively. Further slight increases were observed throughout the storage periods but the concentration varied considerably (190, 120, 31 and 140 $\mu g.g^{-1}$ of yoghurt for C1, C2, C3 and C4, respectively) after 35 d storage. Bifidobacteria have been reported to produce 3:1 moles of acetic:lactic acids during fermentation and these observations coincide with the changes in bifidobacterial population (Table 5.1.3) for all these cultures. The maximum increase in numbers of bifidobacteria was observed with C1 culture corresponding to maximum acetic acid concentration with this culture. Further, C3 culture showed inhibition of bifidobacteria during yoghurt making and there was only slight increase in acetic acid concentration in yoghurt made with C3 culture (Fig. 5.1.3).

Changes in the counts of yoghurt bacteria. The changes in the viable counts of *S. thermophilus* are presented in Table 5.1.1. As shown, the initial counts of *S. thermophilus* were 6 to 18 x 10^6 at 0 h increasing to 5.3-6.7 x 10^8 for cultures C1, C2 and C3 and to 23 x 10^8 for C4 at 0 d. This difference could possibly be due to the strain variation and the differences in incubation time to reach the pH of 4.5 by these cultures. For C3 and C4 cultures, major fermentation effect was that of *S. thermophilus*, whereas, fermentation in C1 and C2 cultures was carried out by *S. thermophilus* and *L.*

delbrueckii ssp. *bulgaricus*. After 0 d storage period, the counts of *S. thermophilus* increased by 15-20% in all the samples, which could be due to residual activity during this period. This finding coincides with the rise in TA and drop in pH at 5 d period (Fig. 5.1.1). After 5 d period, the counts increased slightly for C3, which is in agreement with the rise in TA and drop in pH for this culture, as is evident from Fig. 5.1.1. For other storage periods, the *S. thermophilus* counts showed a decline of 30-70%, which indicated better stability of this organism under the conditions employed in this study.

The changes in counts of *L. delbrueckii* ssp. *bulgaricus* during manufacture and storage of yoghurt are presented in Table 5.1.1. The counts of *L. delbrueckii* ssp. *bulgaricus* increased in both cultures by 12 to 22 fold during fermentation from the initial counts of 12-30 x 10^6 at 0 h after which there was a decrease of almost 50% after 5 d period, a further steady decrease up to 10 d followed by a sharp decline during the remaining storage period. Lower counts of *L. delbrueckii* ssp. *bulgaricus* have been claimed to be advantageous for the viability of probiotic organisms, otherwise *L. delbrueckii* ssp. *bulgaricus* could drop the pH during storage, as observed in market yoghurts, which has an adverse effect on the viability of probiotic organisms (Holcomb *et al.*, 1991; Laroia and Martin, 1991b; Shah *et al.*, 1995). Kim *et al.* (1993) also observed a marked decrease in the population of *L. delbrueckii* ssp. *bulgaricus* as compared to that of *S. thermophilus.*

Changes in the counts of probiotic bacteria. The changes in the counts of *L. acidophilus* in yoghurts during manufacture and storage are presented in Table 5.1.2. At 0 h period, the counts of *L. acidophilus* were similar; however, at 0 d, the counts were the highest for culture C4 and remained well within the recommended limit of one million cells per gram of yoghurt throughout the storage period. Conversely, in the products prepared from C1 and C2, the counts declined rapidly (by almost 3 log cycles) and the recommended level of 1 million cells was maintained only for 20 to 25 days.

At 0 h period, counts of bifidobacteria were 205 x 10^5, 82 x 10^5, 206 x 10^5 and 213 x 10^5 for cultures C1, C2, C3 and C4, respectively. The counts of bifidobacteria were $< 10^5$ for culture C3 for periods 0-35 d. For C3 culture, the results showed better viability of *L. acidophilus* and the viable cells remained slightly less than 1 million after 35 d storage. Sharma and Singh (1982) observed higher concentration of free amino acids, when both standard yoghurt organisms were used for making yoghurt while incorporating *L. acidophilus*. Probiotic organisms have weak proteolytic activity and require free amino acids for better multiplication. The pH and TA for C1 and C2 were almost the same as in C4 throughout the storage periods (Fig. 5.1.1). The dissolved oxygen content showed similar pattern in all the products. The possible factors that might have affected the viability of *L. acidophilus* could be either antagonism by yoghurt organisms or the higher concentration of hydrogen peroxide produced by C1 and C2 cultures (Fig. 5.1.2), causing partial injury to the cells of *L. acidophilus*. The partially injured cells might have shown faster decay in the viable counts of *L. acidophilus* in yoghurts made from C1 and C2 cultures. In general, the cells of *L. acidophilus* showed faster decay after 15 d for C1 and C2 cultures, which contained *L. delbrueckii* ssp. *bulgaricus* after 20-25 days for C3 and C4 cultures which did not contain *L. delbrueckii* ssp. *bulgaricus*. Rybka and Kailasapathy (1995) also observed less viability of *L. acidophilus* in yoghurts with *L. delbrueckii* ssp. *bulgaricus*. Similarly, Zibkowski (1981) reported better protein efficiency ratio and marked survival of *L. acidophilus* and bifidobacteria in yoghurt fermented with *L. acidophilus, S. thermophilus* and bifidobacteria only as compared to the one which was fermented with all the four types of organisms. Thus, associative yoghurt organism/s and their strains seemed to play a crucial role and may have affected the viability of *L. acidophilus* in yoghurt.

Changes in the viable counts of bifidobacteria enumerated on MRS-NNLP agar are in Table 5.1.3, and those obtained by subtraction method in Table 5.1.4. A 30-70% lower bifidobacterial counts were obtained on MRS-NNLP medium as compared with subtraction method. As shown in the tables, bifidobacteria showed 15 to 18 fold increase in the numbers for C1 and 5 to 7 fold increase for C2. The extent of increase was only

1.5 fold for C4 cultures, while C3 culture showed a decrease of 3 log cycles. The percentage decay was similar for C1, C2 and C4 cultures, which indicated that in contrast to *L. acidophilus,* bifidobacteria were not affected by hydrogen peroxide during storage. However, associative yoghurt organism/s and their strains affected the multiplication of bifidobacteria during manufacture of yoghurt. In general, for yoghurts made from C1, C2 and C4, the viable numbers were well above the recommended limit of one million.g^{-1} throughout the storage periods. This could be due to the type of bifidobacterial strain used in these cultures as observed in recent studies (Lankaputhra *et al.*, 1996b), in which, 3 bifidobacterial strains out of 9 exhibited tolerance to low pH and hydrogen peroxide concentration of 100 μg mL^{-1}. Further, Martin and Chou (1992) observed that viability of *Bifidobacterium* spp. was species and strain dependent and the viability greatly varied amongst them. The counts of bifidobacteria for C3 culture were more than one million g^{-1} at the time of addition of cultures; however, it showed 3 log cycles inhibition within 5.5 h of incubation. This could be due to lack of proteolysis by *S. thermophilus* incorporated in C3 culture and/or antagonistic effect of this *S. thermophilus* against bifidobacteria. Greater multiplication of bifidobacteria in C1 and C2 cultures could be due to the presence of *L. delbrueckii* ssp. *bulgaricus* in these cultures, which has been known for its symbiosis and proteolytic nature (Shankar and Davies, 1976). The free amino acids produced by this organism in yoghurt could be used by other organisms and would have promoted the growth of probiotic bacteria (Dutta *et al.*, 1973, Singh *et al.*, 1980). Most of the bifidobacteria have been found to be weakly proteolytic and free amino acids are essential for most of the bifidobacteria (Klaver *et al.*, 1993). Therefore, it is expected that the presence of both of these yoghurt organisms might be beneficial for multiplication of bifidobacteria during manufacture of yoghurt.

5.1.3.3 Effect of storage temperature and oxygen content on the viable counts of yoghurt and probiotic organisms in yoghurt

The one variable of concern in the first part of the study (section 5.1.3.2) was oxygen content, which increased in the plastic cups during storage. Further, the factor responsible for inhibition of bifidobacteria was unidentified in case of yoghurt made

from C3 culture. Under the commercial practices, the temperature of 3-4°C may not represent the actual temperature of storage of yoghurt in the supermarkets; on occasions the temperature may rise to 10°C (personal communication with industry partner). Therefore, in the second part of the study, yoghurt was prepared in plastic cups and in screw capped glass bottles to limit the oxygen permeation. The yoghurts prepared in plastic cups were stored at 4°C and 10°C to check the effect of temperatures of storage and the yoghurts prepared in glass bottles were stored at 4°C to compare the effects of dissolved oxygen content on TA, pH, hydrogen peroxide and viable counts of yoghurt bacteria and probiotic bacteria in yoghurts made from C1 and C3 cultures.

The changes in TA, pH, oxygen and hydrogen peroxide contents in yoghurt stored in plastic cups at 4 and 10°C and in glass bottles stored at 4°C are illustrated in Figs. 5.1.4, 5.1.5 and 5.1.6. The increase in TA and drop in pH were higher for the samples stored at 10°C, indicating continued residual fermentation at this temperature. However, the drop in pH was not high enough to lower the pH of yoghurt to <4.0, which has been reported to be more detrimental for the survival of probiotic organisms (Shah and Jelen, 1990; Laroia and Martin, 1991b; Shah *et al.*, 1995; Lankaputhra *et al.*, 1996b). For all the products, the hydrogen peroxide production was not found to be dependent on either temperature or oxygen content, but was dependent on the associative yoghurt organisms as found in the first part of this study (Figs. 5.1.2 and 5.1.6). The oxygen content was always lower throughout the storage for samples stored in glass bottles. The difference in the oxygen content was higher up to 10 d, and narrowed after 20 d storage at 4°C. Thus, any variations in microbial counts were thought to be due to the changes in oxygen content, because other factors such as TA, pH, hydrogen peroxide remained almost the same in products stored in plastic cups and in glass bottles at 4°C.

Changes in the counts of yoghurt bacteria. As shown in Table 5.1.5, *S. thermophilus* multiplied better in plastic cups than in glass bottles during manufacture of yoghurt, as observed at 0 d. The survival was almost 50% higher in plastic cups than in glass bottles, which could be due to less amount of oxygen permeation in glass bottles. The decay was

also faster at 10°C, which could be due to higher acidity and low pH (Fig. 5.1.4) at this temperature. On an average, the survival rate of *S. thermophilus* was better compared to *L. delbrueckii* ssp. *bulgaricus* and that of probiotic organisms, especially of *L. acidophilus*. These observations are in line with those observed by Kim *et al.* (1993) and Medina and Jordono (1994).

Changes in the counts of probiotic bacteria. The viable counts of *L. acidophilus* were found to be affected by the oxygen content to a greater extent than that of temperature of storage (Table 5.1.6). It is evident from the Table that the increase in numbers and survival of *L. acidophilus* during storage were directly affected by the dissolved oxygen content. For the samples stored in glass bottles, the counts remained 0.5 x 10^6 g^{-1} (for C1 culture) after 35 d storage against only 1 x 10^3 g^{-1} for the product stored in plastic cups. For C3 culture, the survival of *L. acidophilus* was 3.5 x 10^6 g^{-1} in glass bottles compared to 0.1 x 10^6 g^{-1} in plastic cups after 35 d of storage. The storage temperature of 10°C did not have any adverse effect on the survival rate of *L. acidophilus* in plastic cups for both the cultures.

Tables 5.1.7 and 5.1.8 represent shifts in population of bifidobacteria during manufacture and storage of yoghurts in plastic cups at 4 and 10°C and in glass bottles at 4°C. As shown, bifidobacteria preferred environment with less amount of dissolved oxygen content and multiplied better in glass bottles than in plastic cups. At 0 d, despite having less acidity (i.e. higher pH) for the yoghurt manufactured in glass bottles, the bifidobacterial population was 1.6 fold (in C1 samples) higher in products prepared in glass bottles than in plastic cups. Although the pH, TA and hydrogen peroxide content were similar (for C1) in products stored in glass bottles and plastic cups at 4°C, the survival rate was 30-70% higher in products fermented and stored in glass bottles compared to that in plastic cups. The difference in survival rate was greater in the initial periods (20 d), in accordance with the difference observed in the oxygen content. This confirmed the findings of Klaver *et al.* (1993), who observed better viability of bifidobacteria in de-aerated milk. The temperature of storage adversely affected the

viability of bifidobacteria (Tables 5.1.7 and 5.1.8), which was not observed with *L. acidophilus* (Table 5.1.6). The difference was again 30-70% in survival of bifidobacteria at 4 and 10°C. The possible reason could be slightly lower pH and higher acidity (Fig. 5.1.4) in yoghurts stored at 10°C. Lankaputhra *et al.* (1996b) reported that any drop in pH below 4.3, greatly affected the viability of bifidobacteria. Similarly, Sakai *et al.* (1987) concluded that the most important factor in bifidobacterial mortality was the low pH of the yoghurt, with storage temperature having a secondary effect. *L. acidophilus* has been reported to be more acid tolerant compared to bifidobacteria (Holcomb *et al.*, 1991; Hull *et al.*, 1984; Kim, 1988; Klaver and Kingma, 1992; Laroia and Martin, 1991b; Martin, 1994); in conceit the bifidobacterial population might have dropped to a greater extent than that of *L. acidophilus* at 10°C.

5.1.4 Conclusions

Viability of yoghurt and probiotic bacteria was assessed during manufacture and 35 d storage in yoghurt made from four commercial starter cultures. The titratable acidity, pH and dissolved oxygen content showed similar patterns of increase or decrease during manufacture and storage of yoghurt, whereas, concentration of hydrogen peroxide increased in the product prepared with cultures that contained *L. delbrueckii* ssp. *bulgaricus*. The increase in numbers of probiotic organisms during manufacture and the viability of these organism during storage were dependent on the species and strain of associative yoghurt organisms. The viability of *L. acidophilus* was adversely affected by the presence of *L. delbrueckii* ssp. *bulgaricus*, whereas bifidobacteria exhibited better stability in yoghurt prepared from cultures that contained *L. delbrueckii* ssp. *bulgaricus*. The viability of both probiotic organisms improved when the dissolved oxygen concentration was low in the product except for C3 starter culture for which a dramatic decline in the counts of bifidobacteria was observed. The storage temperature of yoghurt affected the viability of bifidobacteria, but not *L. acidophilus*. The variations in titratable acidity, pH, dissolved oxygen and hydrogen peroxide were almost identical at storage

temperatures 4 and 10°C. In starter culture C3, 3 log cycles inhibition of bifidobacteria was observed from initial counts of $> 10^6$ mL^{-1}.

Table 5.1.1. Changes in the viable counts of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* during manufacture and storage of yoghurt

Period[1]	C1	C2	C3	C4
Viable counts of *S. thermophilus*				
0 h	18×10^6	6×10^6	9×10^6	18×10^6
0 d	600×10^6	530×10^6	670×10^6	2300×10^6
5 d	750×10^6	680×10^6	690×10^6	2670×10^6
10 d	660×10^6	630×10^6	730×10^6	2530×10^6
15 d	640×10^6	580×10^6	810×10^6	2180×10^6
20 d	560×10^6	530×10^6	790×10^6	1760×10^6
25 d	420×10^6	430×10^6	650×10^6	1530×10^6
30 d	280×10^6	360×10^6	590×10^6	1130×10^6
35 d	220×10^6	320×10^6	690×10^6	960×10^6
Viable counts of *L. delbrueckii* ssp. *bulgaricus*				
0 h	30×10^6	12×10^6	--	--
0 d	391×10^6	287×10^6	--	--
5 d	201×10^6	138×10^6	--	--
10 d	114×10^6	26×10^6	--	--
15 d	13×10^6	6×10^6	--	--

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; C=Starter culture).

Viable counts of *L. delbrueckii* ssp. *bulgaricus* were $< 10^5$ after 20 d storage period and starter cultures C3 and C4 did not contain this organism.

Table 5.1.2. Changes in the viable counts of *L. acidophilus* during manufacture and storage of yoghurt

Period[1]	C1	C2	C3	C4
0 h	64×10^5	31×10^5	84×10^5	68×10^5
0 d	222×10^5	211×10^5	180×10^5	386×10^5
5 d	237×10^5	226×10^5	180×10^5	478×10^5
10 d	207×10^5	205×10^5	148×10^5	443×10^5
15 d	120×10^5	153×10^5	128×10^5	346×10^5
20 d	39×10^5	84×10^5	86×10^5	267×10^5
25 d	8.1×10^5	36×10^5	36×10^5	110×10^5
30 d	0.90×10^5	7.3×10^5	16×10^5	22×10^5
35 d	0.27×10^5	2.2×10^5	8.2×10^5	12×10^5

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; C=Starter culture).

Table 5.1.3. Changes in the viable counts of bifidobacteria (as enumerated on MRS-NNLP agar) during manufacture and storage of yoghurt

Period[1]	C1	C2	C3	C4
0 h	43 x 10^5	17 x 10^5	54 x 10^5	25 x 10^5
0 d	740 x 10^5	93 x 10^5	1330	42 x 10^5
5 d	660 x 10^5	93 x 10^5	960	42 x 10^5
10 d	570 x 10^5	85 x 10^5	1000	34 x 10^5
15 d	540 x 10^5	80 x 10^5	1260	31 x 10^5
20 d	450 x 10^5	68 x 10^5	1030	31 x 10^5
25 d	380 x 10^5	56 x 10^5	1030	30 x 10^5
30 d	330 x 10^5	52 x 10^5	900	29 x 10^5
35 d	300 x 10^5	49 x 10^5	760	27 x 10^5

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; C=Starter culture).

Table 5.1.4. Changes in the viable counts of bifidobacteria (as obtained by subtraction method) during manufacture and storage of yoghurt

Period[1]	C1	C2	C4
0 d	1760 x 10^5	215 x 10^5	346 x 10^5
5 d	1500 x 10^5	212 x 10^5	319 x 10^5
10 d	1340 x 10^5	189 x 10^5	300 x 10^5
15 d	1080 x 10^5	191 x 10^5	230 x 10^5
20 d	1020 x 10^5	162 x 10^5	249 x 10^5
25 d	860 x 10^5	150 x 10^5	209 x 10^5
30 d	780 x 10^5	145 x 10^5	186 x 10^5
35 d	720 x 10^5	118 x 10^5	167 x 10^5

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; C=Starter culture).

Table 5.1.5. Changes in counts of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in yoghurts stored in plastic cups at 4 and 10°C and in glass bottles at 4°C

Period[1]	C[2]1 (B, 4 C)[3]	C1 (C, 4 C)[4]	C1 (C,10 C)[5]	C[2]3 (B, 4 C)	C3 (C, 4 C)	C3 (C, 10 C)	C[2]1 (B, 4 C)[3]	C1 (C, 4 C)[4]	C1 (C,10 C)[5]
	----------Counts of *S. thermophilus* ----------						-Counts of *L. delbrueckii* ssp. *bulgaricus* -		
0 h	180×10^5	180×10^5	180×10^5	170×10^5	170×10^5	170×10^5	149×10^5	109×10^5	109×10^5
0 d	4000×10^5	6100×10^5	6100×10^5	6900×10^5	11000×10^5	11000×10^5	1890×10^5	3030×10^5	3030×10^5
5 d	6000×10^5	7500×10^5	5500×10^5	7800×10^5	120×10^5	7800×10^5	1900×10^5	2550×10^5	2800×10^5
10 d	5300×10^5	4200×10^5	3900×10^5	5800×10^5	12400×10^5	7300×10^5	1090×10^5	60×10^5	100×10^5
15 d	4500×10^5	4000×10^5	3000×10^5	5000×10^5	9000×10^5	7400×10^5	570×10^5	30×10^5	110×10^5
20 d	3900×10^5	3800×10^5	3200×10^5	5000×10^5	9200×10^5	7200×10^5	90×10^5	8×10^5	$< 10^5$
25 d	2800×10^5	3900×10^5	3200×10^5	4000×10^5	9000×10^5	7000×10^5	9×10^5	$< 10^5$	$< 10^5$
30 d	2100×10^5	4000×10^5	2200×10^5	3800×10^5	8300×10^5	6200×10^5	$< 10^5$	$< 10^5$	$< 10^5$
35 d	2000×10^5	3600×10^5	2000×10^5	2300×10^5	5600×10^5	5000×10^5	$< 10^5$	$< 10^5$	$< 10^5$

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively at 4°C; [2]C=Starter culture; [3-5]Observations of samples stored in glass bottles at 4°C (B, 4 C), in plastic cups at 4°C (C, 4 C) and 10°C (C,10 C).

Culture C3 did not contain *L. delbrueckii* ssp. *bulgaricus*.

Table 5.1.6. Changes in counts of *L. acidophilus* in yoghurts stored in plastic cups at 4 and 10°C and in glass bottles at 4°C

Period[1]	C[2]1 (B, 4 C)[3]	C1 (C, 4 C)[4]	C1 (C,10 C)[5]	C[2]3 (B, 4 C)	C3 (C, 4 C)	C3 (C, 10 C)
0 h	42×10^5	42×10^5	42×10^5	70×10^5	70×10^5	70×10^5
0 d	60×10^5	90×10^5	90×10^5	76×10^5	95×10^5	95×10^5
5 d	100×10^5	60×10^5	80×10^5	110×10^5	120×10^5	160×10^5
10 d	90×10^5	22×10^5	40×10^5	105×10^5	150×10^5	190×10^5
15 d	45×10^5	17×10^5	25×10^5	100×10^5	100×10^5	48×10^5
20 d	17×10^5	2.6×10^5	12×10^5	100×10^5	73×10^5	35×10^5
25 d	10×10^5	0.5×10^5	3.0×10^5	60×10^5	28×10^5	40×10^5
30 d	6×10^5	0.03×10^5	0.04×10^5	45×10^5	9.5×10^5	30×10^5
35 d	5×10^5	0.01×10^5	0.01×10^5	35×10^5	1.0×10^5	10×10^5

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively at 4°C; [2]C=Starter culture; [3-5]Observations of samples stored in glass bottles at 4°C (B, 4 C), in plastic cups at 4°C (C, 4 C) and 10°C (C,10 C)).

Table 5.1.7. Changes in counts of bifidobacteria (as enumerated on MRS-NNLP) in yoghurts stored in plastic cups at 4 and 10°C and in glass bottles at 4°C

Period[1]	C[2]1 (B, 4 C)[3]	C1 (C, 4 C)[4]	C1 (C,10 C)[5]	C[2]3 (B, 4 C)	C3 (C, 4 C)	C3 (C, 10 C)
0 h	67×10^5	67×10^5	67×10^5	94×10^5	94×10^5	94×10^5
0 d	320×10^5	200×10^5	220×10^5	5,000	28,000	28,000
5 d	460×10^5	220×10^5	225×10^5	6,000	25,000	10,000
10 d	530×10^5	190×10^5	110×10^5	2,500	11,000	3,100
15 d	450×10^5	84×10^5	98×10^5	2,800	15,000	4,000
20 d	275×10^5	95×10^5	115×10^5	1,800	12,000	2,000
25 d	208×10^5	70×10^5	60×10^5	1,200	20,000	1,000
30 d	173×10^5	66×10^5	20×10^5	800	20,900	400
35 d	88×10^5	30×10^5	5×10^5	900	22,000	400

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively at 4°C; [2]C=Starter culture; [3-5]Observations of samples stored in glass bottles at 4°C (B, 4 C), in plastic cups at 4°C (C, 4 C) and 10°C (C,10 C)).

Table 5.1.8. Changes in Counts of bifidobacteria (as obtained by subtraction method) in yoghurts stored in plastic cups at 4 and 10°C and in glass bottles at 4°C

Period[1]	C[2]1 (B, 4 C)[3]	C1 (C, 4C)[4]	C1 (C,10 C)[5]
0 h	220 x 10^5	220 x 10^5	220 x 10^5
0 d	930 x 10^5	530 x 10^5	530 x 10^5
5 d	1800 x 10^5	430 x 10^5	900 x 10^5
10 d	1960 x 10^5	320 x 10^5	850 x 10^5
15 d	1200 x 10^5	310 x 10^5	450 x 10^5
20 d	800 x 10^5	300 x 10^5	250 x 10^5
25 d	700 x 10^5	270 x 10^5	225 x 10^5
30 d	500 x 10^5	260 x 10^5	160 x 10^5
35 d	350 x 10^5	120 x 10^5	60 x 10^5

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively at 4°C; [2]C=Starter culture; [3-5]Observations of samples stored in glass bottles at 4°C (B, 4 C), in plastic cups at 4°C (C, 4 C) and 10°C (C,10 C)).

Counts of bifidobacteria at 0 h were 282 x 10^5 for C3 culture and < 10^5 for periods 0-35 d for the yoghurt stored in cups (4 and 10°C) and glass bottles (4°C).

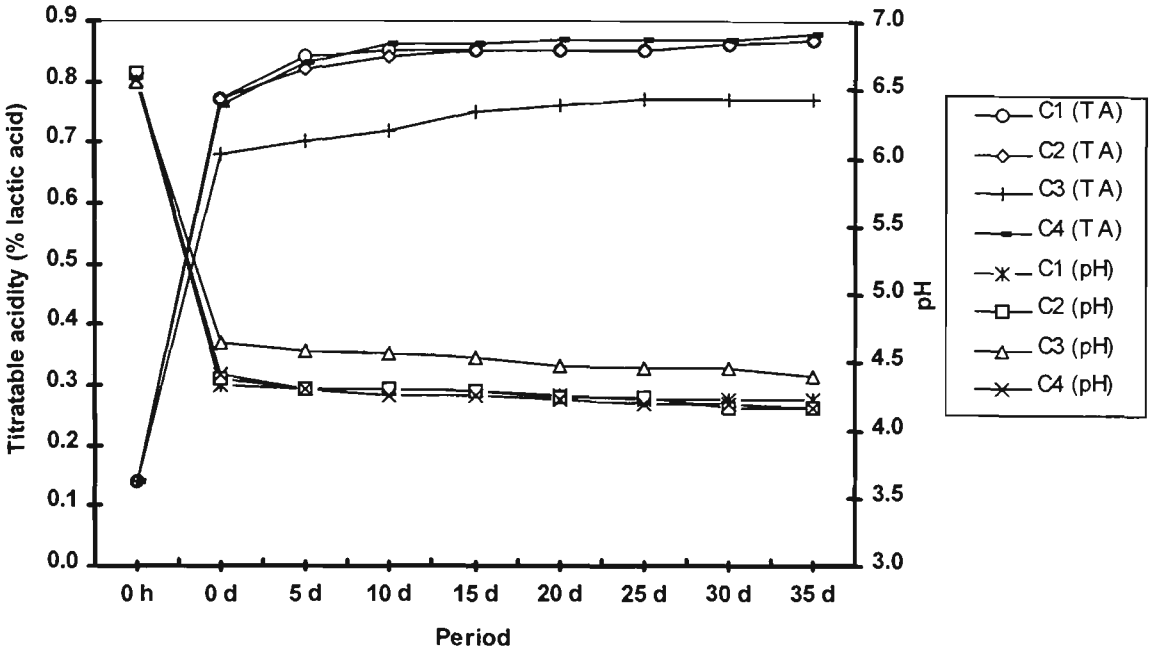


Fig.5.1.1. Changes in the titratable acidity and pH during manufacture and storage of yoghurt (C1-C4=Starter cultures 1-4; TA=Titrable acidity; 0 h, 0 d, 5 d-35=Observations taken after starter culture addition, after overnight storage and during 5 to 35 days storage of yoghurt, respectively).

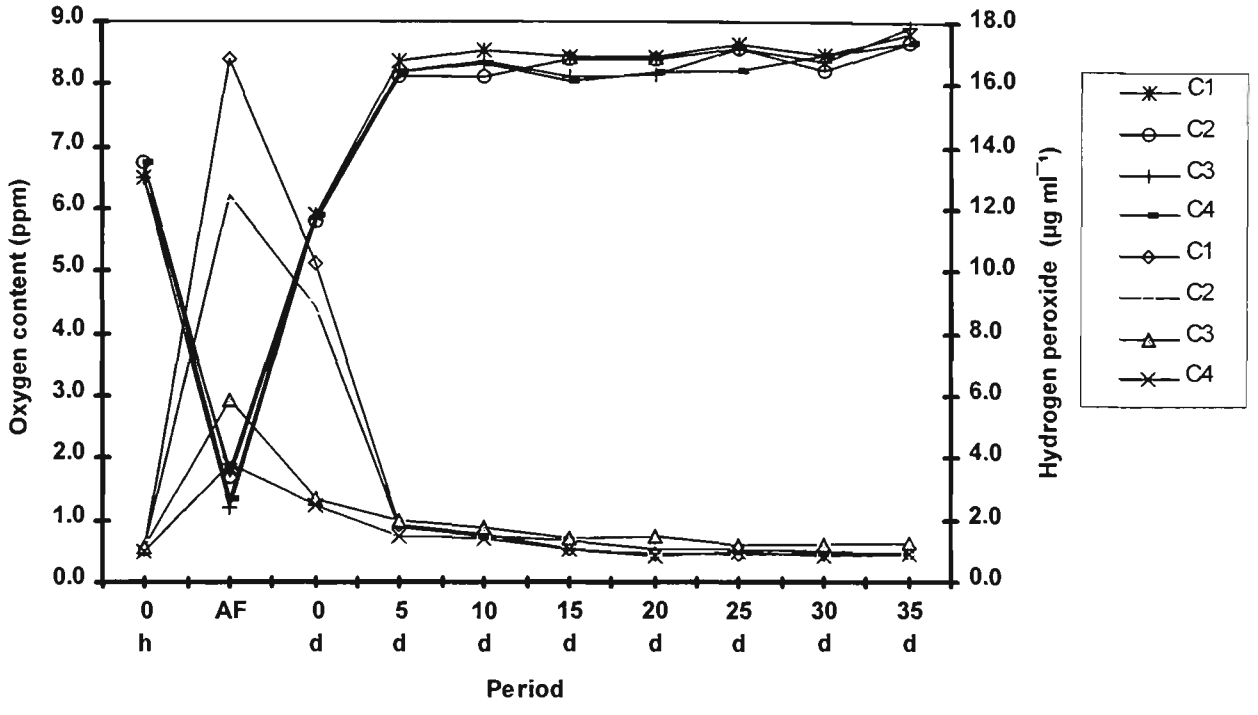


Fig. 5.1.2 Changes in oxygen (ppm) and hydrogen peroxide (µg.mL-1) contents during manufacture and storage of yoghurt (C1-C4=Starter cultures 1-4; 0 h, 0 d, 5 d-35 d=Observations taken after starter culture addition, after overnight storage and during 5 to 35 days storage of yoghurt, respectively; AF=Observations taken immediately after yoghurt making).

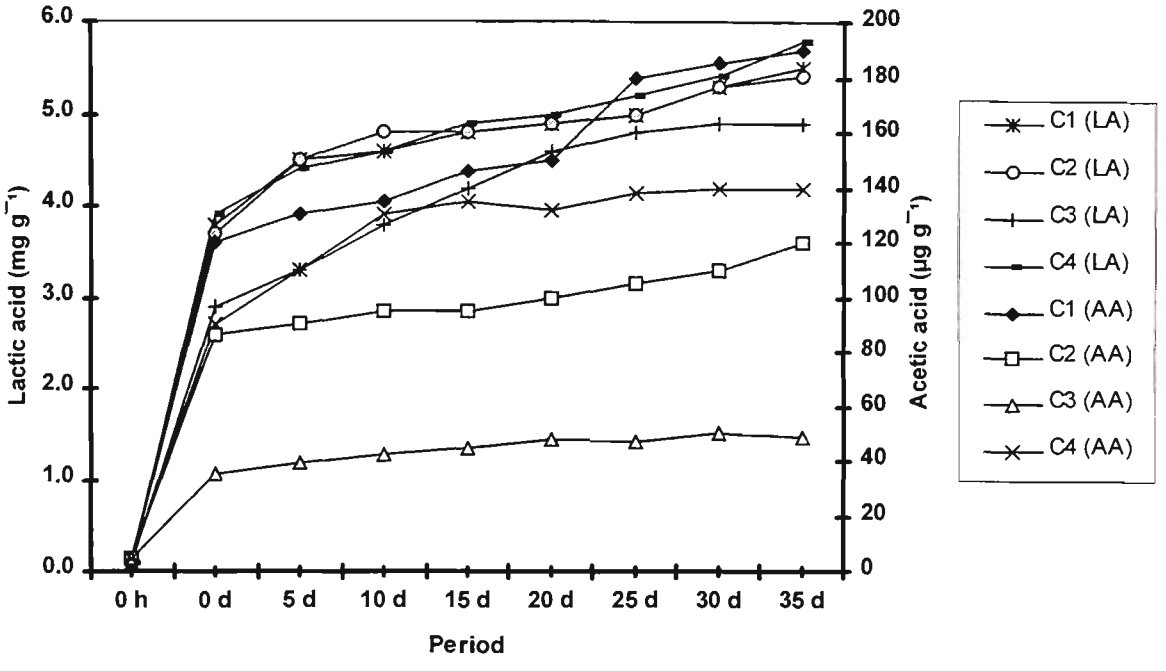


Fig. 5.1.3 Changes in lactic acid and acetic acid contents during manufacture and storage of yoghurt (C1-C4=Starter cultures 1-4; LA=Lactic acid; AA=Acetic acid; 0 h, 0 d, 5 d-35 d=Observations taken after starter culture addition, after overnight storage and during 5 to 35 days storage of yoghurt, respectively).

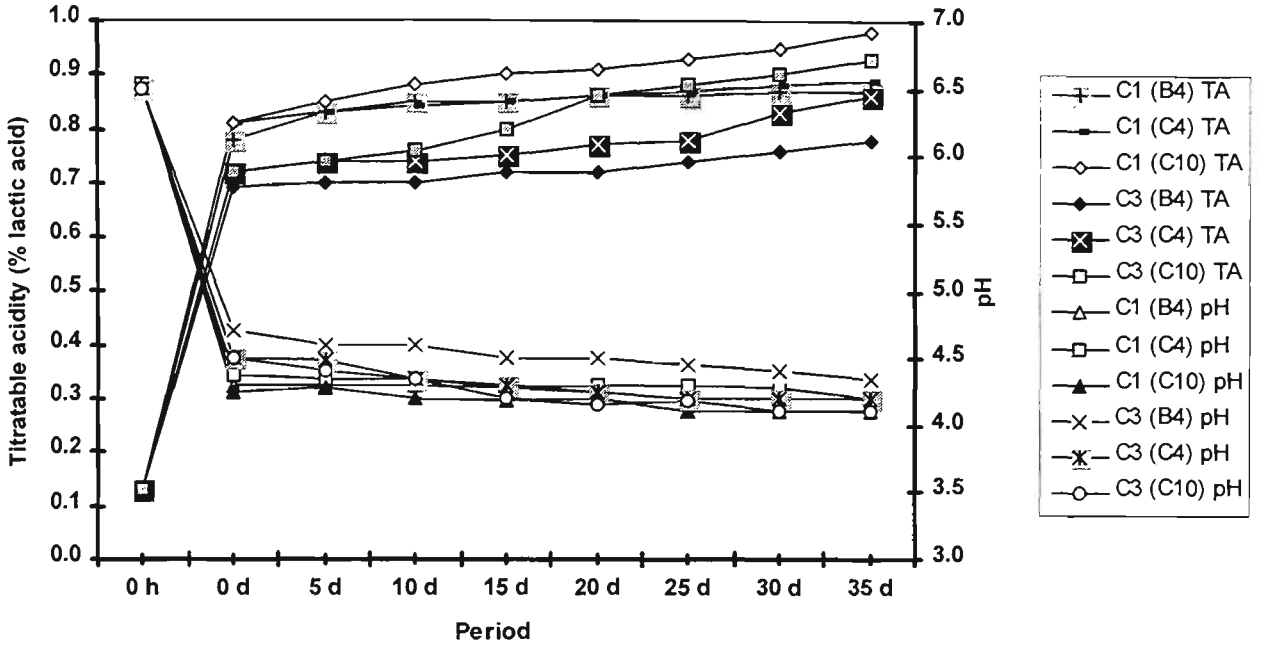


Fig. 5.1.4 Changes in titratable acidity and pH in yoghurt stored in plastic cups (4 or 10°C) or glass bottles (4°C) made from C1 or C3 cultures (C1 and C3=Starter culture 1 and 3; B4, C4=Observations taken for samples stored in glass bottles and plastic cups, respectively, at 4°C; C10= Observations taken for samples stored in plastic cups at 10°C; 0 h, 0 d, 5 d-35 d=Observations taken after starter culture addition, after overnight storage and during 5 to 35 days storage of yoghurt, respectively).

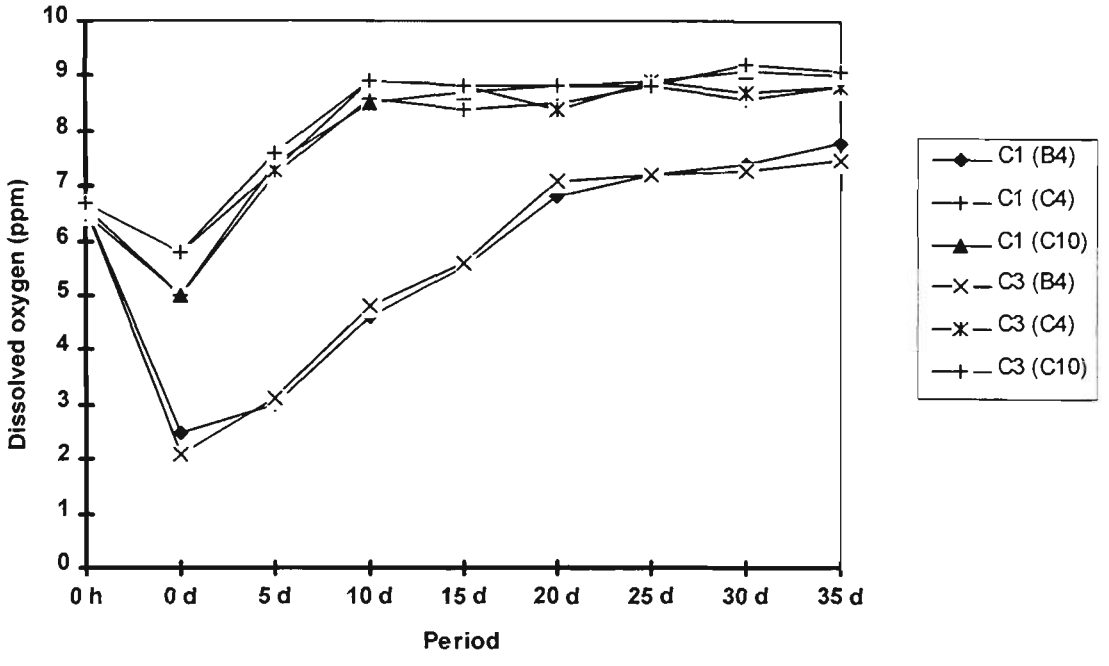


Fig. 5.1.5 Changes in dissolved oxygen content (ppm) in yoghurt stored in plastic cups (4 or 10°C) or glass bottles (4°C) made from C1 and C3 cultures (C1 and C3=Starter culture 1 and 3; B4, C4=Observations taken for samples stored in glass bottles and plastic cups, respectively, at 4°C; C10= Observations taken for samples stored in plastic cups at 10°C; 0 h, 0 d, 5 d-35 d=Observations taken after starter culture addition, after overnight storage and during 5 to 35 days storage of yoghurt, respectively).

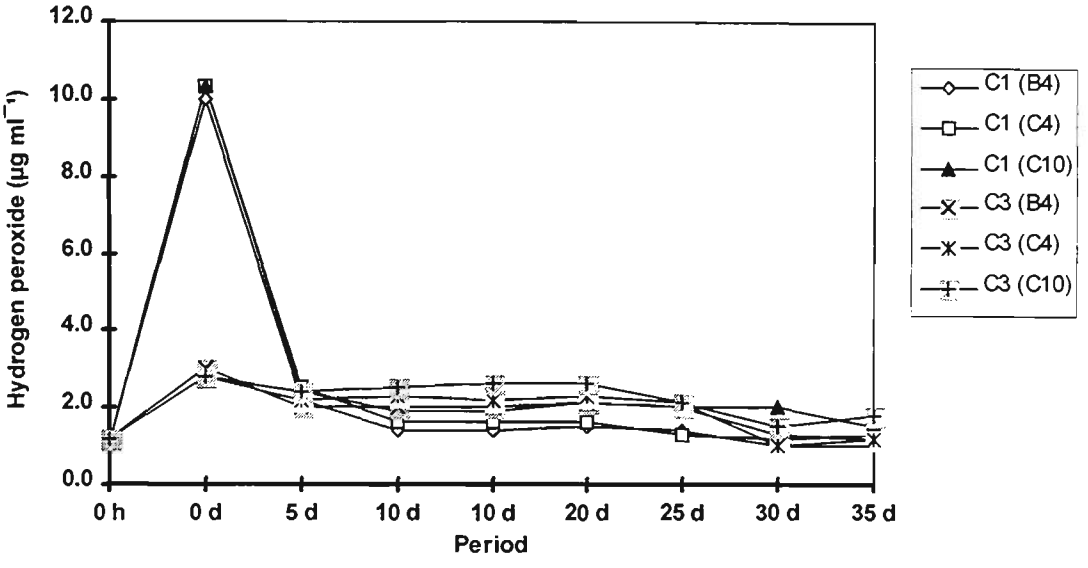


Fig. 5.1.6 Changes in hydrogen peroxide ($\mu g.mL^{-1}$) content in yoghurt stored in plastic cups (4 or 10°C) or glass bottles (4°C) made from C1 and C3 cultures (C1 and C3=Starter culture 1 and 3; B4, C4=Observations taken for samples stored in glass bottles and plastic cups, respectively, at 4°C; C10= Observations taken for samples stored in plastic cups at 10°C; 0 h, 0 d, 5 d-35 d=Observations taken after starter culture addition, after overnight storage and during 5 to 35 days storage of yoghurt, respectively).

5.2 Assessment of viability of yoghurt and probiotic bacteria in yoghurts made with various sizes of inoculum of commercial starter cultures

5.2.1 Introduction

Fermented milks have been produced in the world since time immemorial and nutritional benefits derived by consumption of these products have been well documented (Nakazawa and Hosono, 1992; Robinson, 1991). These fermented milk products have been manufactured with mesophilic (mainly *Lactococcus* spp.) and thermophilic (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) lactic bacteria. These bacteria do not survive in large numbers during passage through the digestive tract. As a result, intestinal species have been suggested to have probiotic effects. The 'probiotic effects' have been related to the maintenance of good health, stabilisation of microbial ecology in the gut, reducing risk of colon cancer, increased immune response, improvement in lactose malabsorption in lactose intolerant people and reduction in concentration of cholesterol in blood plasma (Gilliland, 1990; Hughes and Hoover, 1991; Mital and Garg, 1992; Modler, 1990; Shah and Jelen, 1990).

The Australian Food Standard Code requires the presence of viable fermenting cultures in the final product, but their populations have not been specified for yoghurt. Advisory standards for starter culture bacteria stipulates bacterial counts of $> 10^7$ cfu.g^{-1} as satisfactory level (Davis *et al.*, 1971), whereas, suggested minimum levels for probiotic bacteria in yoghurt is 10^6 cfu.g^{-1} (Kurmann and Rasic, 1991). The need to monitor survival of *L. acidophilus* and *Bifidobacterium* spp. in yoghurt has often been neglected; as a result, a number of products reaches the market with few viable bacteria (Anon, 1992; Shah *et al.*, 1995).

A version of section 5.2 has been published. Dave, R. I. and Shah, N. P. (1997) *Food Australia,* **49**: 164-168.

The yoghurt manufacturers mainly rely on starter culture suppliers for their need of starter cultures and might feel them responsible for having poor viability, especially of probiotic bacteria. On the contrary, starter culture suppliers would suspect that the manufacturers do not follow their instructions such as temperature of incubation and level of starter addition which may lead to poor viability.

In this part of study, the effect of level of starter addition on the viability of yoghurt and probiotic organisms in yoghurt made with four commercial starter cultures was monitored for 35 days of storage at 4°C. The conditions of incubation, pH and storage temperatures were strictly adhered to the recommendations of the starter culture supplier.

5.2.2 Materials and methods

5.2.2.1 Starter cultures and yoghurt preparation

Four starter cultures described in section 3.1 were used in this study and yoghurt was prepared as described in section 3.4.2. Samples were prepared as described in section 3.5.1.

5.2.2.2 Analyses and time interval specifications

The yoghurt samples were analysed for pH and titratable acidity (section 3.7.1.1). The microbiological analyses of yoghurt samples at various time intervals (section 3.6) was carried out as described in section 3.7.2.

The results presented in this study are the averages of two replicates.

5.3.3 Results and discussion

The changes in the pH values and that of titratable acidity (TA) of yoghurt during storage have been shown in Tables 5.2.1 and 5.2.2, respectively. For starter cultures C1 and C4, the trend of decrease in pH or increase in TA were identical at the recommended level of inoculation and the decrease in pH or increase in TA was higher in these cultures as compared to starter cultures C2 and C3. Starter culture C3 showed least post acidification at all the inoculum levels as compared to other starter cultures. In general, there was a gradual drop in pH of yoghurt made with all four starter cultures and the drop in pH was higher at lower rate of inoculum, especially after 20 d storage period, as compared to the recommended or higher level of inoculum.

The changes in the viable counts of *S. thermophilus* are presented in Table 5.2.3. It was observed that the organism multiplied in yoghurt many times with the lower level of inoculum than the higher level. However, the final counts of the organism remained slightly higher at the higher level of inoculation, which could possibly be due to high initial numbers. *S. thermophilus* might be biologically more active in samples prepared with lower inoculum (as the fermentation was terminated at 4.5 pH in all samples). This observation was supported by increase in their numbers for up to 10 days in the yoghurt prepared with the low level of inoculum as compared with 5 days in the yoghurt prepared with the higher rate of inoculum. This could be the reason for larger drop in pH (Table 5.2.1) or larger increase in TA (Table 5.2.2) of samples prepared with low rate of inoculum. After initial increase for up to 5-10 days, the number of these organisms decreased in all the products. However, the final numbers were well above the advisory standards of 10^7 cfu.g^{-1}. for yoghurt (Davis *et al.*, 1971). Amongst all four organisms enumerated, *S. thermophilus* was found to be the most stable and the loss of viability for this organism was least.

The viable counts of *L. delbrueckii* ssp. *bulgaricus* also increased for up to 5 d and then declined during subsequent storage (Table 5.2.4). The decay was found to be highest for

this organism and their numbers declined to < 10^5 after 15-20 d storage. Further, it was observed that the numbers of *L. delbrueckii* ssp. *bulgaricus* remained viable for longer time in yoghurt prepared with a lower level of inoculum than that prepared with a higher level (Table 5.2.4).

The counts of *L. acidophilus* declined gradually during initial period of storage of 15 d and faster decay was observed thereafter (Table 5.2.5). The viability was found to be dependent on the associative yoghurt organism. For starter cultures C3 and C4, the viability was up to a satisfactory level (10^6 cfu.g^{-1}) for 35 d in products prepared with higher rate of inoculum. For the rest of the inoculum levels for starter cultures C3 and C4 and at all inoculum levels for starter cultures C1 and C2, the viability was very poor. There was 2-3 log cycles reduction in their numbers over a period of 35 d. The pH was found to be the most crucial factor for this *L. acidophilus* culture. In a separate study, we found that if pH in yoghurt drops to < 4.4 at the time of fermentation, it would result in 3-4 log cycle decay in *L. acidophilus* numbers within 20-25 days. The associative yoghurt organisms has greater impact on the viability of *L. acidophilus*. Yoghurt prepared with starter culture C1 and C2 showed poor viability of *L. acidophilus* as compared to C3 and C4. The possible reason could be the presence of *L. delbrueckii* ssp. *bulgaricus* in the former starter cultures and this organism has been reported to produce hydrogen peroxide as observed in our earlier studies with these starter cultures (Dave and Shah, 1996b). The viability of this organism in yoghurt made with C3 and C4 starter cultures was also just near the recommended level of > 10^6 (Kurmann and Rasic, 1991) after 35 d.

Table 5.2.6 represents viable numbers of bifidobacteria in yoghurt prepared from four commercial starter cultures using various levels of inoculum. It was observed that the type of associative yoghurt organism/s had effect on initial multiplication of bifidobacteria. In yoghurt prepared with starter culture C1, the multiplication of bifidobacteria was high despite having shorter incubation time to reach a pH of 4.5, indicating better association between yoghurt and probiotic organisms. Conversely, in yoghurt prepared with starter culture C3, a dramatic decline in the population of

bifidobacteria was observed. For this starter culture, even a very high rate of inoculum (10 g per 10 L) did not help increase their numbers to a recommended level, indicating a possibility of antagonism amongst the organisms mixed in this starter culture. In all the yoghurt prepared with C1 starter culture, the number of bifidobacteria remained $\geq 10^6$ cfu.g^{-1} for up to 35 d, whereas for starter cultures C2 and C3, the counts dropped to $< 10^6$ cfu.g^{-1} in yoghurt prepared with the lower level of inoculum. For starter cultures C1 and C2, the multiplication of bifidobacteria was higher, possibly due to proteolytic activity of *L. delbrueckii* ssp. *bulgaricus* (Shankar and Davies, 1976) resulting in availability of free amino acids which have been reported to be essential growth factors for bifidobacteria (Klaver *et al.*, 1993).

5.2.4 Conclusions

The effect of size of inoculum on viability of yoghurt (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) and probiotic (*L. acidophilus* and bifidobacteria) bacteria was assesses in yoghurts made with 4 commercial starter cultures. Of the four organisms enumerated, *S. thermophilus* was found to be the most stable in yoghurt and their counts were $\geq 10^7$ cfu.g^{-1} throughout the storage for all levels of inoculum of starter cultures. *L. delbrueckii* ssp. *bulgaricus* lost their viability rapidly to $< 10^5$ cfu.g^{-1} after about 20 d storage. *L. acidophilus* maintained their viability to a recommended levels of 10^6 cfu.g^{-1} for only up to 20-25 d storage at 4°C; after which, a sharp decline in their counts was observed. In three (C1, C2 and C4) out of four starter cultures, bifidobacteria counts were satisfactory. The decay in bifidobacteria was gradual and steady during storage and the organisms exhibited good stability during storage as compared to lactobacilli. However, counts of bifidobacteria declined dramatically to about 3 log cycles during manufacture of yoghurt with the fourth starter culture (C3) and higher inoculum did not improve their numbers to a satisfactory level. The post acidification was slightly higher in yoghurt prepared with lower level of inoculum. The associative yoghurt organisms and pH of the yoghurt were found to have effect on viability of probiotic bacteria.

Table 5.2.1. Changes in the pH during manufacture and storage of yoghurt prepared with various levels of inoculum

Period[1]	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	$\mathbf{R_{2.0}}$	$R_{0.5}$	$\mathbf{R_{1.0}}$	$R_{1.5}$	$R_{2.0}$
	Starter Culture C1				**Starter Culture C2**			
0 h	6.57	6.50	6.44	**6.41**	6.55	**6.51**	6.47	6.44
0 d	4.26	4.27	4.29	**4.32**	4.29	**4.30**	4.32	4.34
5 d	4.19	4.23	4.28	**4.30**	4.26	**4.29**	4.33	4.33
10 d	4.16	4.20	4.28	**4.30**	4.26	**4.29**	4.32	4.32
15 d	4.14	4.18	4.26	**4.28**	4.24	**4.28**	4.31	4.31
20 d	4.11	4.14	4.22	**4.24**	4.17	**4.24**	4.27	4.31
25 d	4.09	4.13	4.21	**4.22**	4.14	**4.24**	4.27	4.30
30 d	4.06	4.09	4.19	**4.20**	4.11	**4.20**	4.29	4.30
35 d	4.04	4.08	4.19	**4.20**	4.09	**4.16**	4.25	4.28
	Starter Culture C3				**Starter Culture C4**			
Period	$R_{0.5}$	$\mathbf{R_{1.0}}$	$R_{1.5}$	$R_{2.0}$	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	$\mathbf{R_{2.0}}$
0 h	6.55	**6.50**	6.48	6.45	6.60	6.58	6.56	**6.56**
0 d	4.40	**4.41**	4.45	4.45	4.32	4.36	4.40	**4.41**
5 d	4.32	**4.39**	4.39	4.40	4.30	4.35	4.28	**4.34**
10 d	4.30	**4.38**	4.38	4.40	4.22	4.26	4.28	**4.27**
15 d	4.30	**4.37**	4.39	4.38	4.18	4.20	4.21	**4.24**
20 d	4.27	**4.37**	4.37	4.37	4.18	4.20	4.20	**4.24**
25 d	4.26	**4.32**	4.37	4.37	4.16	4.17	4.20	**4.20**
30 d	4.20	***4.30***	4.35	4.35	4.12	4.15	4.17	**4.19**
35 d	4.17	***4.29***	4.34	4.35	4.08	4.12	4.17	**4.19**

[1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; $R_{0.5}$, $R_{1.0}$, $R_{1.5}$ and $R_{2.0}$ =Yoghurt made with 0.5, 1.0, 1.5 and 2.0 g/10 L of yoghurt mix.

Numbers in bold represent data for the recommended rate of inoculum for the respective starter cultures.

Table 5.2.2. Changes in the titratable acidity (% lactic acid) during manufacture and storage of yoghurt prepared with various levels of inoculum

Period[1]	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	**$R_{2.0}$**	$R_{0.5}$	**$R_{1.0}$**	$R_{1.5}$	$R_{2.0}$
	Starter Culture C1				**Starter Culture C2**			
0 h	0.14	0.15	0.16	**0.16**	0.14	**0.15**	0.16	0.16
0 d	0.80	0.78	0.79	**0.78**	0.79	**0.78**	0.79	0.79
5 d	0.84	0.84	0.84	**0.83**	0.84	**0.83**	0.83	0.83
10 d	0.85	0.86	0.85	**0.85**	0.87	**0.84**	0.83	0.84
15 d	0.86	0.87	0.85	**0.86**	0.87	**0.84**	0.84	0.84
20 d	0.87	0.87	0.86	**0.86**	0.88	**0.86**	0.84	0.85
25 d	0.90	0.89	0.86	**0.87**	0.90	**0.88**	0.85	0.86
30 d	0.94	0.91	0.87	**0.87**	0.90	**0.88**	0.86	0.87
35 d	0.97	0.93	0.89	**0.88**	0.91	**0.89**	0.87	0.87
	Starter Culture C3				**Starter Culture C4**			
Period	$R_{0.5}$	**$R_{1.0}$**	$R_{1.5}$	$R_{2.0}$	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	**$R_{2.0}$**
0 h	0.14	**0.14**	0.15	0.15	0.14	0.14	0.15	**0.15**
0 d	0.76	**0.75**	0.75	0.76	0.77	0.78	0.78	**0.78**
5 d	0.77	**0.76**	0.76	0.76	0.86	0.86	0.84	**0.83**
10 d	0.78	**0.76**	0.76	0.77	0.89	0.88	0.85	**0.85**
15 d	0.80	**0.77**	0.76	0.77	0.92	0.90	0.88	**0.87**
20 d	0.80	**0.78**	0.77	0.78	0.92	0.90	0.88	**0.86**
25 d	0.83	**0.80**	0.78	0.80	0.95	0.91	0.89	**0.88**
30 d	0.86	**0.82**	0.81	0.83	0.95	0.92	0.90	**0.87**
35 d	0.89	**0.84**	0.83	0.83	0.97	0.93	0.90	**0.87**

[1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; $R_{0.5}$, $R_{1.0}$, $R_{1.5}$ and $R_{2.0}$ =Yoghurt made with 0.5, 1.0, 1.5 and 2.0 g/10 L of yoghurt mix.

Numbers in bold represent data for the recommended rate of inoculum for the respective starter cultures.

Table 5.2.3. Changes in the viable counts of *S. thermophilus* during manufacture and storage of yoghurt prepared with various levels of inoculum

Period[1]	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	**$R_{2.0}$**	$R_{0.5}$	**$R_{1.0}$**	$R_{1.5}$	$R_{2.0}$
	Starter Culture C1					**Starter Culture C2**		
0 h	3.0×10^6	6.6×10^6	8.2×10^6	**12.5×10^6**	3.5×10^6	**7.2×10^6**	10.8×10^6	15.0×10^6
0 d	310×10^6	400×10^6	500×10^6	**560×10^6**	300×10^6	**510×10^6**	650×10^6	685×10^6
5 d	560×10^6	600×10^6	700×10^6	**720×10^6**	420×10^6	**620×10^6**	720×10^6	780×10^6
10 d	580×10^6	560×10^6	630×10^6	**620×10^6**	420×10^6	**590×10^6**	700×10^6	720×10^6
15 d	520×10^6	500×10^6	620×10^6	**610×10^6**	400×10^6	**570×10^6**	600×10^6	640×10^6
20 d	500×10^6	480×10^6	580×10^6	**570×10^6**	350×10^6	**550×10^6**	510×10^6	550×10^6
25 d	420×10^6	400×10^6	400×10^6	**410×10^6**	250×10^6	**440×10^6**	390×10^6	400×10^6
30 d	250×10^6	260×10^6	290×10^6	**290×10^6**	200×10^6	**320×10^6**	350×10^6	360×10^6
35 d	210×10^6	200×10^6	230×10^6	**250×10^6**	160×10^6	**290×10^6**	300×10^6	300×10^6
	Starter Culture C3					**Starter Culture C4**		
Period	$R_{0.5}$	**$R_{1.0}$**	$R_{1.5}$	$R_{2.0}$	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	**$R_{2.0}$**
0 h	4.8×10^6	**10×10^6**	16×10^6	21×10^6	5.0×10^6	9.0×10^6	12.8×10^6	**18.0×10^6**
0 d	590×10^6	**690×10^6**	800×10^6	850×10^6	1700×10^6	1900×10^6	2000×10^6	**2100×10^6**
5 d	600×10^6	**700×10^6**	860×10^6	910×10^6	2100×10^6	2200×10^6	2400×10^6	**2600×10^6**
10 d	660×10^6	**740×10^6**	840×10^6	860×10^6	2200×10^6	2300×10^6	2500×10^6	**2530×10^6**
15 d	600×10^6	**720×10^6**	790×10^6	800×10^6	2000×10^6	2000×10^6	2000×10^6	**2100×10^6**
20 d	590×10^6	**690×10^6**	760×10^6	780×10^6	1600×10^6	1700×10^6	1810×10^6	**1800×10^6**
25 d	580×10^6	**670×10^6**	700×10^6	710×10^6	1200×10^6	1380×10^6	1300×10^6	**1380×10^6**
30 d	540×10^6	**590×10^6**	680×10^6	700×10^6	980×10^6	900×10^6	900×10^6	**980×10^6**
35 d	500×10^6	**550×10^6**	640×10^6	650×10^6	830×10^6	850×10^6	880×10^6	**910×10^6**

[1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; $R_{0.5}$, $R_{1.0}$, $R_{1.5}$ and $R_{2.0}$ =Yoghurt made with 0.5, 1.0, 1.5 and 2.0 g/10 L of yoghurt mix.

Numbers in bold represent data for the recommended rate of inoculum for the respective starter cultures.

Table 5.2.4. Changes in the viable counts of *L. delbrueckii* ssp. *bulgaricus* during manufacture and storage of yoghurt prepared with various levels of inoculum

Period[1]	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	$R_{2.0}$	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	$R_{2.0}$
	Starter Culture C1				Starter Culture C2			
0 h	7.5×10^6	15.0×10^6	22.0×10^6	**31×10^6**	8.0×10^6	**15×10^6**	24×10^6	32×10^6
0 d	260×10^6	300×10^6	330×10^6	**350×10^6**	260×10^6	**300×10^6**	360×10^6	380×10^6
5 d	280×10^6	221×10^6	260×10^6	**250×10^6**	270×10^6	**150×10^6**	189×10^6	210×10^6
10 d	220×10^6	140×10^6	140×10^6	**150×10^6**	160×10^6	**24×10^6**	80×10^6	88×10^6
15 d	110×10^6	11×10^6	12×10^6	**14×10^6**	90×10^6	**11×10^6**	20×10^6	21×10^6
20 d	8.2×10^6	$< 10^5$	$< 10^5$	**$< 10^5$**	6.0×10^6	**$< 10^5$**	$< 10^5$	$< 10^5$

[1]0 h, 0 d, 5 d-20 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 20 d storage of the product, respectively, at 4°C; $R_{0.5}$, $R_{1.0}$, $R_{1.5}$ and $R_{2.0}$ =Yoghurt made with 0.5, 1.0, 1.5 and 2.0 g/10 L of yoghurt mix.

Numbers in bold represent data for the recommended rate of inoculum for the respective starter cultures.

Table 5.2.5. Changes in the viable counts of *L. acidophilus* during manufacture and storage of yoghurt prepared with various levels of inoculum

Period[1]	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	$\mathbf{R_{2.0}}$	$R_{0.5}$	$\mathbf{R_{1.0}}$	$R_{1.5}$	$R_{2.0}$
	Starter Culture C1					**Starter Culture C2**		
0 h	15×10^5	28×10^5	41×10^5	$\mathbf{58 \times 10^5}$	14×10^5	$\mathbf{30 \times 10^5}$	44×10^5	60×10^5
0 d	160×10^5	200×10^5	240×10^5	$\mathbf{260 \times 10^5}$	200×10^5	$\mathbf{242 \times 10^5}$	280×10^5	296×10^5
5 d	190×10^5	230×10^5	248×10^5	$\mathbf{280 \times 10^5}$	235×10^5	$\mathbf{260 \times 10^5}$	250×10^5	240×10^5
10 d	100×10^5	190×10^5	180×10^5	$\mathbf{200 \times 10^5}$	210×10^5	$\mathbf{235 \times 10^5}$	200×10^5	220×10^5
15 d	50×10^5	100×10^5	130×10^5	$\mathbf{140 \times 10^5}$	118×10^5	$\mathbf{165 \times 10^5}$	150×10^5	166×10^5
20 d	10×10^5	35×10^5	35×10^5	$\mathbf{40 \times 10^5}$	69×10^5	$\mathbf{88 \times 10^5}$	90×10^5	123×10^5
25 d	0.6×10^5	0.97×10^5	5×10^5	$\mathbf{7 \times 10^5}$	20×10^5	$\mathbf{39 \times 10^5}$	50×10^5	66×10^5
30 d	1×10^4	22×10^3	0.5×10^5	$\mathbf{7 \times 10^4}$	13×10^5	$\mathbf{29 \times 10^5}$	23×10^5	38×10^5
35 d	17×10^2	19×10^2	12×10^3	$\mathbf{21 \times 10^3}$	27×10^3	$\mathbf{1.8 \times 10^5}$	6×10^5	10×10^5
	Starter Culture C3					**Starter Culture C4**		
Period	$R_{0.5}$	$\mathbf{R_{1.0}}$	$R_{1.5}$	$R_{2.0}$	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	$\mathbf{R_{2.0}}$
0 h	36×10^5	$\mathbf{72 \times 10^5}$	106×10^5	138×10^5	21×10^5	40×10^5	63×10^5	$\mathbf{86 \times 10^5}$
0 d	177×10^5	$\mathbf{185 \times 10^5}$	226×10^5	298×10^5	260×10^5	290×10^5	320×10^5	$\mathbf{350 \times 10^5}$
5 d	238×10^5	$\mathbf{220 \times 10^5}$	250×10^5	255×10^5	300×10^5	330×10^5	400×10^5	$\mathbf{420 \times 10^5}$
10 d	160×10^5	$\mathbf{165 \times 10^5}$	188×10^5	198×10^5	267×10^5	300×10^5	360×10^5	$\mathbf{400 \times 10^5}$
15 d	99×10^5	$\mathbf{115 \times 10^5}$	155×10^5	150×10^5	190×10^5	200×10^5	300×10^5	$\mathbf{360 \times 10^5}$
20 d	62×10^5	$\mathbf{80 \times 10^5}$	90×10^5	98×10^5	100×10^5	130×10^5	200×10^5	$\mathbf{240 \times 10^5}$
25 d	18×10^5	$\mathbf{25 \times 10^5}$	48×10^5	45×10^5	39×10^5	58×10^5	120×10^5	$\mathbf{150 \times 10^5}$
30 d	4.0×10^5	$\mathbf{8.0 \times 10^5}$	16×10^5	20×10^5	10×10^5	20×10^5	30×10^5	$\mathbf{40 \times 10^5}$
35 d	1.0×10^5	$\mathbf{2.3 \times 10^5}$	6.0×10^5	12×10^5	0.96×10^5	2.0×10^5	8×10^5	$\mathbf{10 \times 10^5}$

[1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; $R_{0.5}$, $R_{1.0}$, $R_{1.5}$ and $R_{2.0}$ =Yoghurt made with 0.5, 1.0, 1.5 and 2.0 g/10 L of yoghurt mix.

Numbers in bold represent data for the recommended rate of inoculum for the respective starter cultures.

Table 5.2.6. Changes in the viable counts of bifidobacteria during manufacture and storage of yoghurt prepared with various levels of inoculum

Period[1]	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	$\mathbf{R_{2.0}}$	$R_{0.5}$	$\mathbf{R_{1.0}}$	$R_{1.5}$	$R_{2.0}$
	Starter Culture C1					**Starter Culture C2**		
0 h	10×10^5	20×10^5	30×10^5	$\mathbf{40 \times 10^5}$	11×10^5	$\mathbf{20 \times 10^5}$	31×10^5	44×10^5
0 d	200×10^5	300×10^5	380×10^5	$\mathbf{590 \times 10^5}$	64×10^5	$\mathbf{81 \times 10^5}$	120×10^5	150×10^5
5 d	187×10^5	280×10^5	366×10^5	$\mathbf{575 \times 10^5}$	70×10^5	$\mathbf{85 \times 10^5}$	101×10^5	140×10^5
10 d	169×10^5	250×10^5	332×10^5	$\mathbf{530 \times 10^5}$	64×10^5	$\mathbf{80 \times 10^5}$	100×10^5	130×10^5
15 d	118×10^5	190×10^5	250×10^5	$\mathbf{410 \times 10^5}$	50×10^5	$\mathbf{70 \times 10^5}$	90×10^5	120×10^5
20 d	99×10^5	145×10^5	200×10^5	$\mathbf{320 \times 10^5}$	30×10^5	$\mathbf{66 \times 10^5}$	70×10^5	90×10^5
25 d	67×10^5	100×10^5	160×10^5	$\mathbf{210 \times 10^5}$	20×10^5	$\mathbf{58 \times 10^5}$	65×10^5	88×10^5
30 d	47×10^5	50×10^5	98×10^5	$\mathbf{145 \times 10^5}$	10×10^5	$\mathbf{50 \times 10^5}$	60×10^5	78×10^5
35 d	12×10^5	30×10^5	87×10^5	$\mathbf{110 \times 10^5}$	3×10^5	$\mathbf{40 \times 10^5}$	55×10^5	65×10^5
	Starter Culture C3					**Starter Culture C4**		
Period	$R_{0.5}$	$\mathbf{R_{1.0}}$	$R_{1.5}$	$R_{2.0}$	$R_{0.5}$	$R_{1.0}$	$R_{1.5}$	$\mathbf{R_{2.0}}$
0 h	32×10^5	$\mathbf{69 \times 10^5}$	95×10^5	119×10^5	9.0×10^5	20×10^5	30×10^5	$\mathbf{38 \times 10^5}$
0 d	960	**1330**	6300	11400	15×10^5	58×10^5	80×10^5	$\mathbf{98 \times 10^5}$
5 d	900	**980**	5500	12100	13×10^5	50×10^5	70×10^5	$\mathbf{90 \times 10^5}$
10 d	880	**990**	4500	12200	10×10^5	40×10^5	65×10^5	$\mathbf{75 \times 10^5}$
15 d	960	**1100**	4100	10200	8×10^5	31×10^5	50×10^5	$\mathbf{60 \times 10^5}$
20 d	700	**1260**	3500	10000	4×10^5	17×10^5	34×10^5	$\mathbf{40 \times 10^5}$
25 d	600	**900**	3000	9100	1×10^5	14×10^5	24×10^5	$\mathbf{35 \times 10^5}$
30 d	800	**780**	2600	8000	0.7×10^5	11×10^5	16×10^5	$\mathbf{30 \times 10^5}$
35 d	660	**880**	2000	7200	0.22×10^5	9×10^5	10×10^5	$\mathbf{21 \times 10^5}$

[1]0 h, 0 d, 5 d-35 d = Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; $R_{0.5}$, $R_{1.0}$, $R_{1.5}$ and $R_{2.0}$ =Yoghurt made with 0.5, 1.0, 1.5 and 2.0 g/10 L of yoghurt mix.

Numbers in bold represent data for the recommended rate of inoculum for the respective starter cultures.

6.0 INGREDIENT SUPPLEMENTATION AFFECT THE VIABILITY OF YOGHURT AND PROBIOTIC BACTERIA AND THE TEXTURAL PROPERTIES OF YOGHURT

6.1 Effectiveness of ascorbic acid as an oxygen scavenger in improving viability of probiotic bacteria in yoghurts made with commercial starter cultures

6.1.1 Introduction

Several factors have been claimed to affect the viability of probiotic organisms in fermented milk products and accordingly viability can be different (Gilliland and Speck, 1977a; Holcomb *et al.*, 1991; Laroia and Martin, 1991b; Lankaputhra and Shah, 1994; Medina and Jordono, 1994; Samona and Robinson, 1994; Rybka and Kailasapathy, 1995; Lankaputhra *et al.*, 1996b). The efforts in improving viability of probiotic bacteria would benefit the yoghurt manufacturers. Oxygen content and redox potential were shown to be important factors for the viability of bifidobacteria during storage of fermented milks (Brunner *et al.*, 1993a, b). Ascorbic acid (vitamin C) could act as an oxygen scavenger and it has been permitted in fruit juices and other products as a food additive. Further, milk and milk products supply only 10-15% of the daily requirements of vitamin C (Webb *et al.*, 1974; Rasic and Kurmann, 1978). As a result, fortification of yoghurt with ascorbic acid would also increase its nutritive value.

In this study, effect of ascorbic acid on improving the viability of probiotic bacteria was studied in yoghurt made with four commercial starter cultures. Changes in titratable acidity, pH, oxygen content, redox potential, hydrogen peroxide content, ascorbic acid concentration and the viable counts of *Streptococcus thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus, L. acidophilus* and bifidobacteria were monitored during manufacture and storage of yoghurt for 35 days at 4°C.

A version of section 6.1 has been published. Dave, R. I. and Shah, N. P. (1997). *Inter. Dairy J.*, 7 :435-443.

6.1.2 Materials and methods

6.1.2.1 Starter cultures and yoghurt preparation

Four starter cultures described in section 3.1 were used in this study and yoghurt was prepared as described in section 3.4.3. Samples were prepared as described in section 3.5.1.

6.1.2.2 Analyses and time interval specifications

The yoghurt samples were analysed for pH and titratable acidity (section 3.7.1.1), hydrogen peroxide (section 3.7.1.2), dissolved oxygen content and redox potential (section 3.7.1.4), protein and total solids contents (section 3.7.1.5). The microbiological analyses of yoghurt samples at various time intervals (section 3.6) was carried out as described in section 3.7.2.

All the experiments and analyses were repeated twice and the results presented are the averages of two replications.

6.1.3 Results and discussion

Composition. Protein content of the heat treated yoghurt mix ranged from 3.58-3.69, whereas total solid contents were in the range of 16.00-16.21%. Thus, compositional differences were minimal in the various experimental replications.

Changes in titratable acidity and pH. After the initial increase in TA and decrease in pH, the patterns of increase or decrease were identical at all the four levels of ascorbic acid (Fig. 6.1.1). There were slight differences in the values of increase in TA or decrease in pH with various starter cultures. Starter culture C3 showed less post acidification during storage as compared with other starter cultures. There was a gradual decrease in the pH, which could be attributed to the residual fermentation. However, in any of the yoghurts,

the pH did not drop to ≤ 4.0, known to be detrimental to the viability of probiotic bacteria (Laroia and Martin, 1991b; Shah *et al.*, 1995; Lankaputhra *et al.*, 1996b). Kneifel *et al.* (1993) observed an increase in TA of 22.3% in yoghurt and 14.9% in yoghurt related products during cold storage of products made with 44 commercial starter cultures. Kim *et al.* (1993) also observed increase in TA during cold storage of yoghurt made with commercial starter cultures that contained *L. acidophilus* and bifidobacteria. However, Ilic and Ashoor (1988) and Noh *et al.* (1994) observed no significant changes in TA, pH and viscosity of the yoghurt fortified with vitamin A or C.

Changes in the oxygen content and redox potential. The changes in oxygen content and redox potential are illustrated in Fig. 6.1.2 and Fig. 6.1.3, respectively. The patterns of increase or decrease in values of oxygen content and redox potential during manufacture and storage of yoghurt were similar for all the four starter cultures. Hence, Fig. 6.1.2 and 6.1.3 show average values of two replications for all the four starter cultures. Oxygen content of the heat treated yoghurt mix was 6.5 ppm and decreased to 5.43, 4.88 and 4.65 ppm after addition of 50, 150 and 250 $mg.kg^{-1}$ ascorbic acid at '0 h', respectively. At '0 d', the oxygen content was about 0.7-1.0 ppm less in yoghurt with added ascorbic acid, whereas the redox potential was 129.8, 84.5, 67,5 and 35.6 mV for yoghurt containing 0, 50, 150 and 250 $mg.kg^{-1}$ ascorbic acid, respectively. During storage, there was a gradual increase in the oxygen content and in the redox potential of the products. The oxygen content and redox potential were initially lower in yoghurt prepared with higher levels of ascorbic acid, but the values gradually approached to that of the control yoghurt in which no ascorbic acid was added. The redox potential after 15 d storage was 163, 155, 128 and 118 mV in yoghurts with 0, 50, 150 and 250 $mg.kg^{-1}$ ascorbic acid; the redox potential was ≥ 129.8 mV, which was observed for control sample at '0 d'. After 35 d storage, the oxygen content in control yoghurt was 8.70 ppm in contrast to 8.30 ppm for yoghurt with 50 $mg.kg^{-1}$of ascorbic acid, and 7.9 ppm for yoghurts with 150 and 250 $mg.kg^{-1}$ of ascorbic acid. The redox potential was 180, 176, 171 and 169 mV after 35 d in yoghurt containing 0, 50, 150 and 250 $mg.kg^{-1}$ added ascorbic acid. Overall, it was found that at the levels used, ascorbic acid acted as oxygen scavenger;

however, oxygen scavenging was effective for up to 10-15 d in products stored in plastic cups.

Changes in hydrogen peroxide. The hydrogen peroxide concentration was the highest in yoghurt made with starter culture C1, followed by C2, both of which contained *L. delbrueckii ssp. bulgaricus*. Only small amount of hydrogen peroxide was detected in yoghurt prepared with starter culture C3 and C4. Some of the *L. delbrueckii* ssp. *bulgaricus* strains have been reported to produce hydrogen peroxide (Gilliland and Speck, 1977a). The hydrogen peroxide concentration was about 6.50, 6.00, 2.46 and 2.50 $\mu g.g^{-1}$ in yoghurt prepared with C1, C2, C3 and C4 at 0 d; in storage the level of hydrogen peroxide ranged between 1 to 2 $\mu g.g^{-1}$ in all the products. The addition of ascorbic acid did not have any marked effect on production of hydrogen peroxide.

Changes in ascorbic acid content. The changes in retention of ascorbic acid at the 4 levels of addition are illustrated in Fig. 6.1.4. On an average, about 15-20% loss occurred during fermentation and overnight storage of yoghurt. After 5 d of storage, about 30-35% loss was observed and only 15-20% of ascorbic acid was retained at the end of 35 d storage period. The possible reason could be the oxygen permeation through the container; oxygen was reported to be detrimental for vitamin C (Webb *et al.,* 1974). Overall, 5-10% variation in percentage retention of ascorbic acid was observed with all the four levels of ascorbic acid. Initial ascorbic acid level was 10.8 and 262.5 $mg.kg^{-1}$ for yoghurt made with 0 and 250 $mg.kg^{-1}$ ascorbic acid, respectively. The final concentrations of ascorbic acid were 1.62, 10.44, 31.8 and 52.4 $mg.kg^{-1}$ for yoghurt supplemented with 0, 50, 150 and 250 $mg.kg^{-1}$ levels of ascorbic acid. Thus, concentration variations were larger, whereas percentage loss or retention was identical at all the levels of ascorbic acid studied. Further, effect of starter culture was minimal and therefore, the results presented as one figure (Fig. 6.1.4) are the averages of two replications for all the four starter cultures. Czarnocka-Roczniakowa and Wojewodzka (1969) observed loss of vitamin C during prolonged fermentation of sterile milk at 45°C and concluded that the loss was mainly due to prolonged effect of exposure to heat rather than to any effects of fermentation by added micro-organisms. Similarly, loss of

vitamin C at 4°C was observed in fortified plain raspberry flavoured low fat yoghurt with various forms of vitamin C throughout 6 weeks of storage (Ilic and Ashoor, 1988).

Changes in the viable counts of yoghurt bacteria. As shown in Table 6.1.1, differences in the viability of *S. thermophilus* were minimal during manufacture and storage of yoghurt made with various concentrations of ascorbic acid in all four starter cultures studied. The counts of *S. thermophilus* remained lower in yoghurt with increasing concentration of ascorbic acid. The percentage decrease was almost the same at all the levels of ascorbic acid and the numbers were dependent on initial counts at 0 d, which were lower in yoghurt with added ascorbic acid. Conversely, counts of *L. delbrueckii* ssp. *bulgaricus* were higher and these organisms remained viable for longer periods in yoghurt supplemented with ascorbic acid (Table 6.1.2). After 20 d, counts of *L. delbrueckii* ssp. *bulgaricus* were $< 10^5$ in yoghurt prepared without ascorbic acid as compared with the counts of $> 10^7$ in yoghurt with added ascorbic acid. After 25 d, the counts of *L. delbrueckii* ssp. *bulgaricus* were $< 10^5$ in yoghurt with all levels of ascorbic acid. Kneifel *et al.* (1993) prepared yoghurt and related products using 44 commercially available starter cultures from 8 suppliers and found that approximately 80% of yoghurt had higher counts of cocci than rods. In our study, counts of *S. thermophilus* remained well above the counts of lactobacilli throughout the storage of the product.

Changes in the counts of probiotic bacteria. Changes in the viable counts of *L. acidophilus* during manufacture and storage of yoghurt are presented in Table 6.1.3. At 0 d, counts of *L. acidophilus* were the highest in yoghurt made with C4 starter culture, followed by C2, C1 and C3. For control yoghurt, counts of *L. acidophilus* were $> 10^6$ throughout the storage for C4 starter culture, whereas the viability of this organism was less than the recommended level of 10^6 g^{-1} in yoghurt made with the rest of the three starter cultures. Thus, for C4 starter culture, viability of *L. acidophilus* was satisfactory in all the yoghurts including control throughout the storage. Addition of ascorbic acid helped to improve the viability of *L. acidophilus* to a satisfactory level in yoghurt made with C2 and C3 starter cultures and the counts remained at $> 10^6$ for up to 30 d in yoghurt supplemented with ascorbic acid for C1 starter culture. The counts dropped <

10^6 g^{-1} in control yoghurt for C1 starter culture after about 20 d storage. Thus, addition of ascorbic acid helped improve the viability of *L. acidophilus* to a greater extent in yoghurt prepared with starter cultures C1, C2 and C3 and to some extent in that prepared with C4 starter culture at 150 and 250 $mg.kg^{-1}$ levels of ascorbic acid. For all the starter cultures there was improvement in viability of *L. acidophilus* and the counts were comparable at the levels of 150 and 250 $mg.kg^{-1}$ of ascorbic acid. The decrease in the counts of *L. acidophilus* was faster after about 20-25 d storage in all the products.

Changes in the viable counts of bifidobacteria are presented in Table 6.1.4. In starter culture C3, the counts of bifidobacteria were reduced dramatically by 2-3 log cycles from their initial numbers. The addition of ascorbic acid did not improve the viability of bifidobacteria to a satisfactory level of $> 10^6$ g^{-1} of yoghurt. Reduced viability of bifidobacteria could be due to anti-microbial activity of yoghurt bacteria incorporated in C3 starter culture or due to nutritional factors (Dave and Shah, 1997b). In yoghurt made with the rest of the starter cultures, the counts remained at a satisfactory level of $>10^6$ g^{-1} throughout the storage at all the levels of ascorbic acid. The multiplication of bifidobacteria was higher in yoghurt made with C1 and C2 starter cultures as compared to C4 starter culture. Starter culture C1 and C2 contained *L. delbrueckii* ssp. *bulgaricus*. This organism has been reported to be proteolytic in nature causing liberation of free amino acids (Shankar and Davies, 1976; Dutta *et al.*, 1973; Singh *et al.*, 1980). Bifidobacteria have been reported to be weakly proteolytic and they require some growth factors (Collins and Hall, 1984; Ziajka and Zbikowski, 1986; Proulx *et al.*, 1992; Klaver *et al.*, 1993). Thus, in yoghurt made with C1 and C2 starter cultures, the availability of free amino acids due to the proteolytic activity of *L. delbrueckii* ssp. *bulgaricus* might have helped bifidobacterial growth despite shorter incubation period as compared to C3 and C4 starter cultures. Further, the addition of ascorbic acid did not improve the viability of bifidobacteria, especially in yoghurt made with C3 starter culture. The possible reason could be the type of strain of bifidobacteria used in these starter cultures. Biavati *et al.* (1992) successfully screened some strains of bifidobacteria that remained viable to a satisfactory level in yoghurt at pH 4.0 during 30 d storage at 4°C. Yoghurt bacteria also act as oxygen scavengers (Ishibashi and Shimamura, 1993).

The tolerance to acid and oxygen of the bifidobacterial strain in the starter cultures used in this study and the added oxygen scavenging effect of yoghurt cultures would have been sufficient for supporting the satisfactory viability of bifidobacteria in yoghurt made with C1, C2 and C4 starter cultures. Yoghurt bacteria finish the fermentation within 5-10 h, by which time most of the dissolved oxygen in milk is utilised. The product is then transferred into a cold room before the log phase of bifidobacteria begins. Thus, with these commercial starter cultures, there was no added advantage of addition of ascorbic acid in improving the viability of bifidobacteria. However, with other oxygen sensitive bifidobacterial strains, it may help improve the viability as observed by Klaver *et al.* (1993) in aerated milk.

6.1.4 Conclusions

Viability of yoghurt and probiotic bacteria was assessed during manufacture and 35 d storage of yoghurt supplemented with four levels of ascorbic acid which is known for its oxygen scavenging effects. There was no marked differences in titratable acidity, pH and hydrogen peroxide content at various levels of ascorbic acid; however, the later was initially higher in products prepared with starter culture that contained *L. delbrueckii* ssp. *bulgaricus*. During storage of yoghurt, patterns of increase in oxygen content and redox potential and percentage retention of ascorbic acid were identical for all the four starter cultures, but different at various levels of ascorbic acid. The oxygen content and redox potential gradually increased during storage in plastic cups and remained lower with increasing levels of ascorbic acid. Loss of ascorbic acid occurred during manufacture and storage with an average retention of only 15-20% after about 35 d storage of yoghurt at 4°C. The viable counts of *Streptococcus thermophilus* were higher, whereas those of *L. delbrueckii* ssp. *bulgaricus* were higher in yoghurt with increasing concentration of ascorbic acid. The counts of *L. acidophilus* during storage decreased less rapidly with increasing concentration of ascorbic acid for all the 4 starter cultures, whereas the counts of bifidobacteria remained unchanged at all levels of ascorbic acid. In starter culture C3, counts of bifidobacteria still dramatically decreased by about 2-3 log cycles from the initial numbers during yoghurt manufacture. Use of ascorbic

acid as an oxygen scavenger did not improve the viability of bifidobacteria to a satisfactory level in yoghurt made with C3 starter culture.

Table 6.1.1. Changes in viable counts of *S. thermophilus* during manufacture and storage of yoghurt made with various levels of ascorbic acid

Period[1]	Control	AA_{50}	AA_{150}	AA_{250}
		Starter Culture	*C1*	
0 d	550 x 10^6	500 x 10^6	450 x 10^6	460 x 10^6
5 d	700 x 10^6	660 x 10^6	550 x 10^6	520 x 10^6
15 d	630 x 10^6	610 x 10^6	500 x 10^6	480 x 10^6
25 d	420 x 10^6	430 x 10^6	400 x 10^6	400 x 10^6
35 d	260 x 10^6	280 x 10^6	200 x 10^6	210 x 10^6
		Starter Culture	*C2*	
0 d	500 x 10^6	490 x 10^6	400 x 10^6	410 x 10^6
5 d	620 x 10^6	630 x 10^6	500 x 10^6	520 x 10^6
15 d	590 x 10^6	590 x 10^6	480 x 10^6	470 x 10^6
25 d	430 x 10^6	420 x 10^6	380 x 10^6	360 x 10^6
35 d	300 x 10^6	290 x 10^6	220 x 10^6	210 x 10^6
		Starter Culture	*C3*	
0 d	680 x 10^6	660 x 10^6	590 x 10^6	580 x 10^6
5 d	710 x 10^6	720 x 10^6	680 x 10^6	690 x 10^6
15 d	730 x 10^6	710 x 10^6	660 x 10^6	670 x 10^6
25 d	680 x 10^6	690 x 10^6	580 x 10^6	560 x 10^6
35 d	550 x 10^6	540 x 10^6	490 x 10^6	500 x 10^6
		Starter Culture	*C4*	
0 d	2200 x 10^6	2100 x 10^6	1900 x 10^6	1800 x 10^6
5 d	2750 x 10^6	2800 x 10^6	2100 x 10^6	2200 x 10^6
15 d	2200 x 10^6	2260 x 10^6	1800 x 10^6	1780 x 10^6
25 d	1410 x 10^6	1400 x 10^6	1300 x 10^6	1320 x 10^6
35 d	930 x 10^6	950 x 10^6	900 x 10^6	910 x 10^6

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; AA_{50-250}=Yoghurt added with 50, 150 and 250 mg kg^{-1} ascorbic acid, respectively).

At 0 h period, counts of *S. thermophilus* were 10 x 10^5, 6 x 10^5, 10 x 10^5 and 15 x 10^5 for cultures C1, C2, C3 and C4, respectively.

Table 6.1.2. Changes in viable counts of *L. delbrueckii* ssp. *bulgaricus* during manufacture and storage of yoghurt made with various levels of ascorbic acid

Period[1]	Control	AA_{50}	AA_{150}	AA_{250}
		Starter Culture C1		
0 d	290×10^6	310×10^6	400×10^6	390×10^6
5 d	140×10^6	155×10^6	230×10^6	240×10^6
10 d	20×10^6	22×10^6	110×10^6	120×10^6
15 d	10×10^6	12×10^6	48×10^6	49×10^6
20 d	$< 10^5$	4×10^6	10×10^6	14×10^6
		Starter Culture C2		
0 d	290×10^6	310×10^6	400×10^6	390×10^6
5 d	140×10^6	155×10^6	230×10^6	240×10^6
10 d	20×10^6	22×10^6	110×10^6	120×10^6
15 d	10×10^6	12×10^6	48×10^6	49×10^6
20 d	$< 10^5$	4×10^6	10×10^6	14×10^6

([1]0 h , 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; AA_{50-250}=Yoghurt added with 50, 150 and 250 mg kg^{-1} ascorbic acid, respectively; Counts of *L. delbrueckii* ssp. *bulgaricus* were $<10^5$ for periods 25-35 d).

At 0 h period, counts of *L. delbrueckii* ssp. *bulgaricus* were 32×10^5 and 15×10^5 for cultures C1 and C2, respectively.

Table 6.1.3. Changes in viable counts of *L. acidophilus* during manufacture and storage of yoghurt made with various levels of ascorbic acid

Period[1]	Control	AA_{50}	AA_{150}	AA_{250}
		Starter Culture C1		
0 d	222×10^5	250×10^5	280×10^5	290×10^5
5 d	237×10^5	270×10^5	320×10^5	335×10^5
15 d	120×10^5	168×10^5	260×10^5	255×10^5
25 d	8.1×10^5	28×10^5	78×10^5	85×10^5
35 d	0.27×10^5	1.2×10^5	2.4×10^5	3.6×10^5
		Starter Culture C2		
0 d	239×10^5	280×10^5	320×10^5	330×10^5
5 d	250×10^5	282×10^5	345×10^5	350×10^5
15 d	173×10^5	192×10^5	250×10^5	270×10^5
25 d	35×10^5	39×10^5	70×10^5	78×10^5
35 d	3.0×10^5	5×10^5	12×10^5	14×10^5
		Starter Culture C3		
0 d	190×10^5	200×10^5	269×10^5	289×10^5
5 d	210×10^5	230×10^5	320×10^5	310×10^5
15 d	120×10^5	125×10^5	220×10^5	214×10^5
25 d	30×10^5	35×10^5	163×10^5	178×10^5
35 d	8×10^5	14×10^5	69×10^5	67×10^5
		Starter Culture C4		
0 d	356×10^5	386×10^5	428×10^5	440×10^5
5 d	458×10^5	465×10^5	509×10^5	520×10^5
15 d	382×10^5	388×10^5	465×10^5	470×10^5
25 d	160×10^5	180×10^5	242×10^5	255×10^5
35 d	14×10^5	20×10^5	59×10^5	65×10^5

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; AA_{50-250}=Yoghurt added with 50, 150 and 250 mg kg^{-1} ascorbic acid, respectively).

At 0 h period, counts of *L. acidophilus* were 60×10^5, 29×10^5, 69×10^5 and 83×10^5 for cultures C1, C2, C3 and C4, respectively.

Table 6.1.4. Changes in viable counts of bifidobacteria during manufacture and storage of yoghurt made with various levels of ascorbic acid

Period[1]	Control	AA_{50}	AA_{150}	AA_{250}
		Starter Culture C1		
0 d	569×10^5	580×10^5	575×10^5	572×10^5
5 d	560×10^5	560×10^5	548×10^5	568×10^5
15 d	440×10^5	445×10^5	450×10^5	453×10^5
25 d	270×10^5	268×10^5	278×10^5	275×10^5
35 d	200×10^5	210×10^5	212×10^5	210×10^5
		Starter Culture C2		
0 d	81×10^5	85×10^5	88×10^5	90×10^5
5 d	85×10^5	81×10^5	80×10^5	85×10^5
15 d	70×10^5	73×10^5	74×10^5	72×10^5
25 d	58×10^5	59×10^5	63×10^5	62×10^5
35 d	40×10^5	42×10^5	45×10^5	44×10^5
		Starter Culture C3		
0 d	1390	1330	1460	1390
5 d	1000	980	1080	1200
15 d	1100	960	1180	1160
25 d	1260	900	980	1100
35 d	700	780	900	1000
		Starter Culture C4		
0 d	50×10^5	52×10^5	55×10^5	53×10^5
5 d	55×10^5	56×10^5	59×10^5	56×10^5
15 d	40×10^5	43×10^5	46×10^5	45×10^5
25 d	31×10^5	30×10^5	33×10^5	32×10^5
35 d	22×10^5	20×10^5	22×10^5	24×10^5

([1]0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; AA_{50-250}=Yoghurt added with 50, 150 and 250 mg kg^{-1} ascorbic acid, respectively).

At 0 h period, counts of bifidobacteria were 45×10^5, 20×10^5, 59×10^5 and 28×10^5 for cultures C1, C2, C3 and C4, respectively

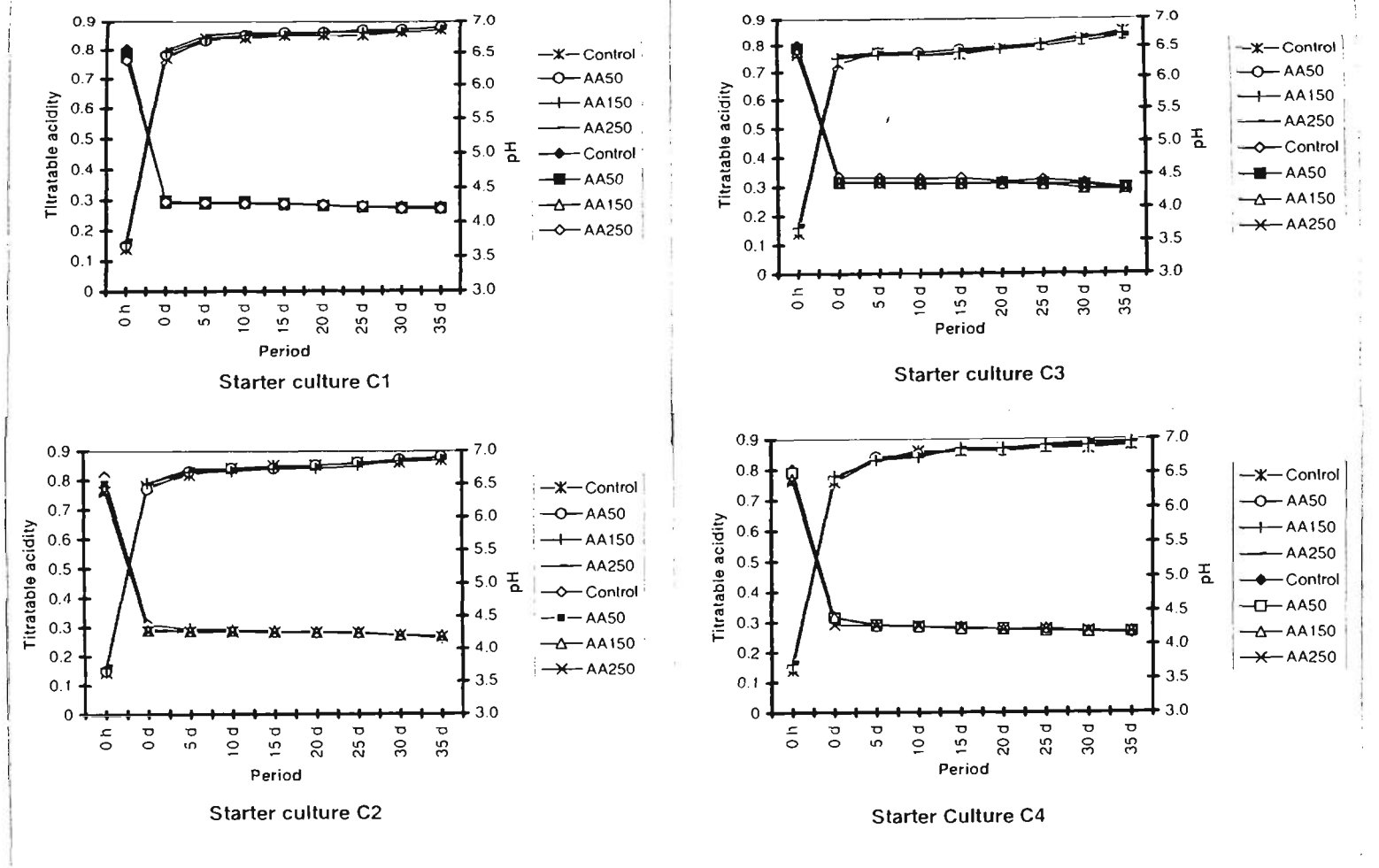


Fig. 6.1.1. Changes in titratable acidity and pH during manufacture and storage of yoghurt made with various levels of ascorbic acid (0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; AA50-250=Yoghurt added with 50, 150 and 250 mg kg^{-1} ascorbic acid, respectively).

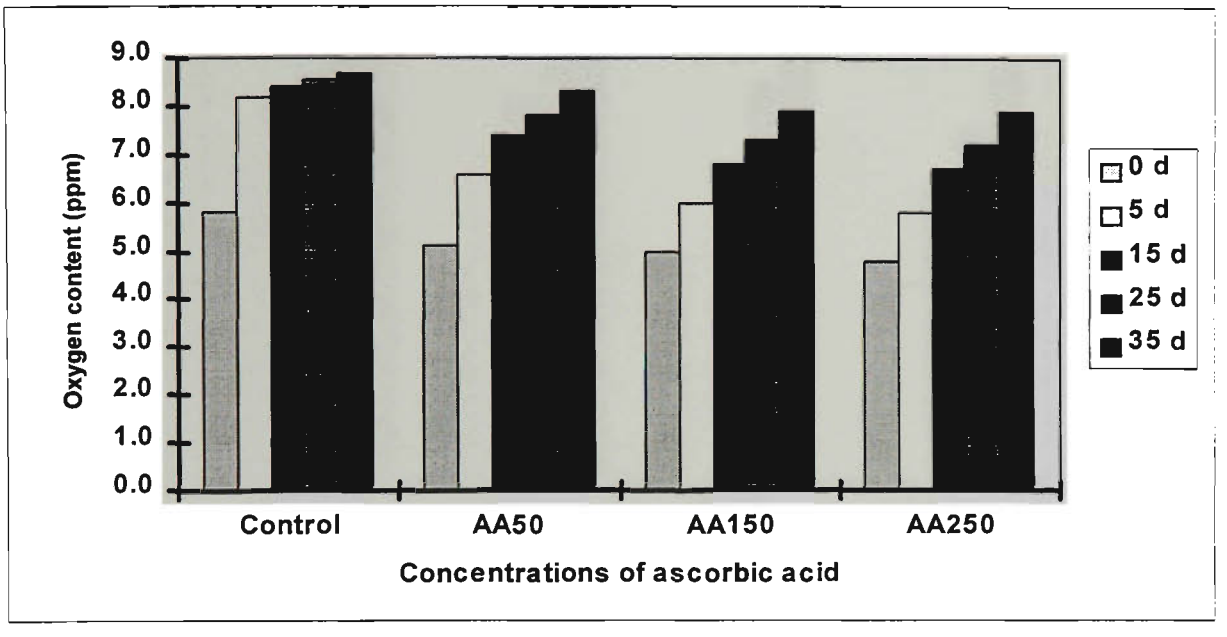


Fig. 6.1.2.Changes in oxygen content (ppm) during manufacture and storage of yoghurt made with various levels of ascorbic acid (0 d, 5 d-35 d=Observations taken after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; AA50-250=Yoghurt added with 50, 150 and 250 mg kg^{-1} ascorbic acid, respectively).

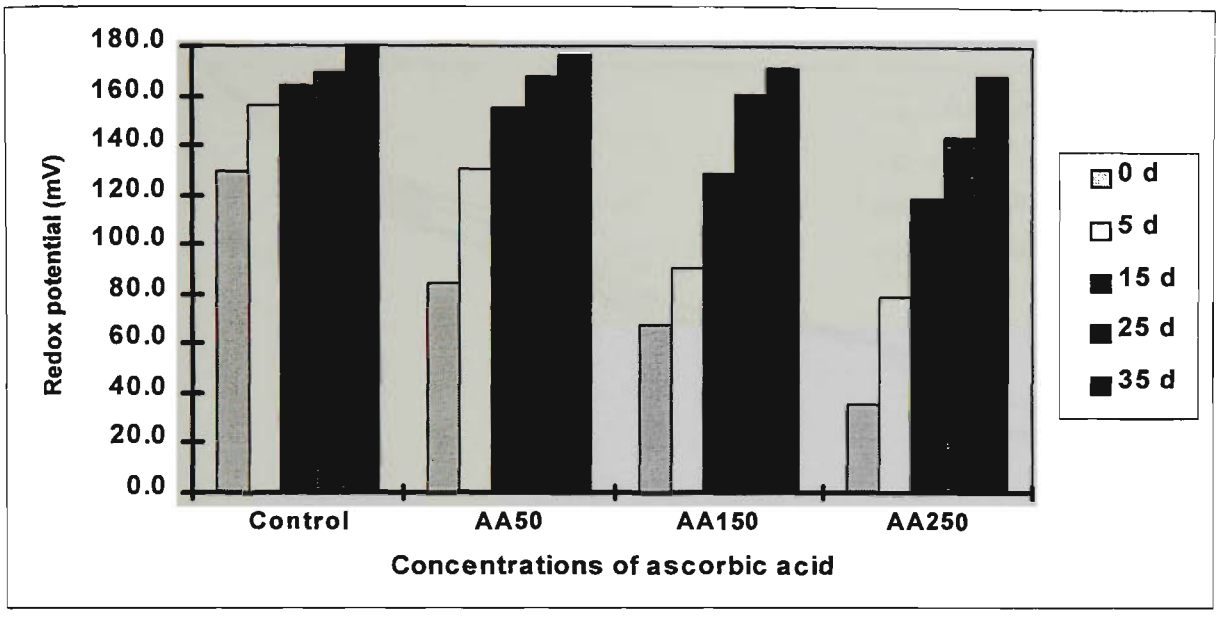


Fig. 6.1.3. Changes in redox potential (mV) during manufacture and storage of yoghurt made with various levels of ascorbic acid (0 d, 5 d-35 d=Observations taken after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; AA50-250=Yoghurt added with 50, 150 and 250 mg.kg^{-1} ascorbic acid, respectively).

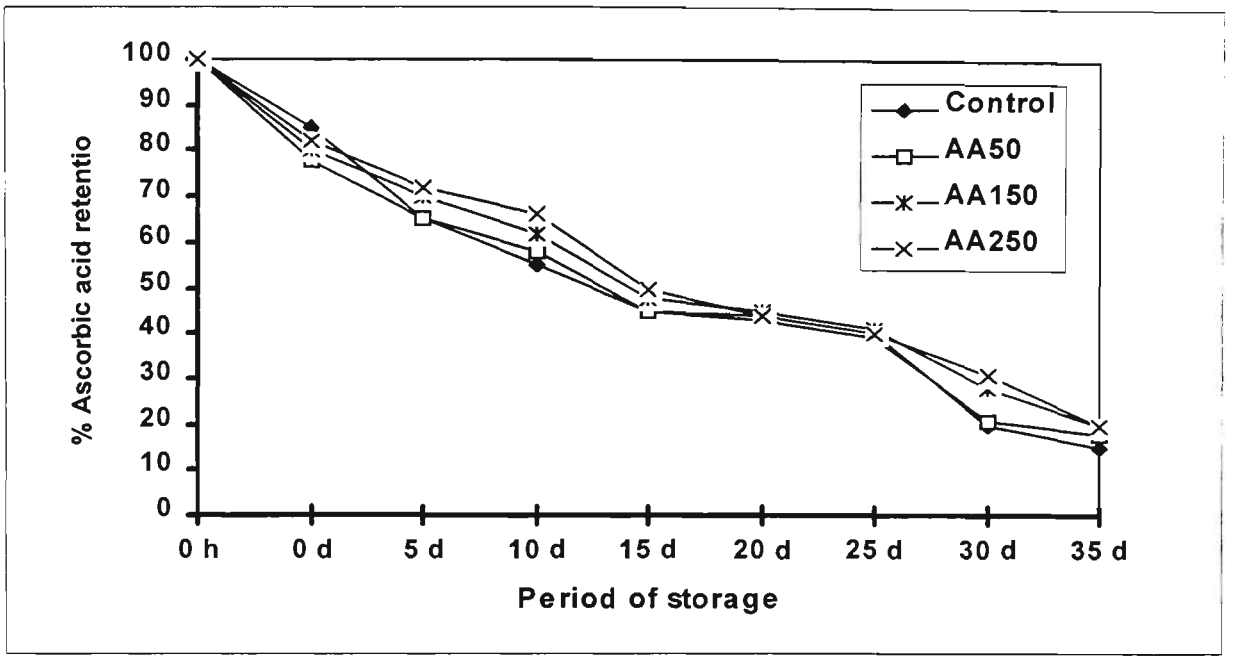


Fig. 6.1.4. Changes in ascorbic acid during manufacture and storage of yoghurt made with various levels of ascorbic acid (0 h, 0 d, 5 d-35 d=Observations taken immediately after culture addition, after overnight cooling and during 5 to 35 d storage of the product, respectively, at 4°C; AA50-250=Yoghurt added with 50, 150 and 250 $mg.kg^{-1}$ ascorbic acid, respectively).

6.2 Effect of cysteine on viability of yoghurt and probiotic bacteria in yoghurts made with commercial starter cultures

6.2.1 Introduction

Although yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) are claimed to provide certain nutritional and health benefits (Deeth and Tamime, 1981), recent interest is centred on using probiotic bacteria (*Lactobacillus acidophilus* and bifidobacteria) as dietary adjuncts because these organisms are normal inhabitants of the intestine (Hughes and Hoover, 1991; Kurmann and Rasic, 1991; Misra and Kuila, 1991; Havenaar and Huis in't Veld, 1992; Kanbe, 1992; Mital and Garg, 1992). During the past decade, probiotic products have become increasingly popular (Modler, 1990; Patel *et al.*, 1991; Mital and Garg, 1992) and consumption of such products has increased dramatically in most European, Asia Pacific and American countries. Yoghurt or yoghurt like product have been used as the most popular vehicle for incorporation of probiotic organisms. Commercially, it is not feasible to ferment milk using only probiotic organisms owing to longer fermentation time required to drop the pH of milk to 4.5 and due to objectionable taste imparted by some of the probiotic bacterial strains (Mital and Garg, 1992). Hence, yoghurt bacteria are added to reduce fermentation time and to improve taste and body and texture of a finished product. Suggested minimum numbers of probiotic bacteria at the time of consumption of a product are 10^5 to 10^6 g^{-1}(Schuler-Malyoth *et al.*, 1968; Robinson, 1987; Kurmann and Rasic, 1991).

In earlier studies, low viability of bifidobacteria in yoghurt made from one of four commercial starter cultures was reported (Dave and Shah, 1997a). It was also reported that viability of *L. acidophilus* improved in product made in glass bottles, in which

---

A version of section 6.2 has been published. Dave, R. I. and Shah, N. P. (1997). *Inter. Dairy J.*, 7: 537-545.

oxygen permeation was minimal (Dave and Shah, 1997a) and in yoghurts supplemented with vitamin C as an oxygen scavenging agent (Dave and Shah, 1997c). In these studies, a dramatic decline in the numbers of bifidobacteria in the C3 starter culture was observed and use of vitamin C or higher inoculum (Dave and Shah, 1997b) did not improve the viability of bifidobacteria to a satisfactory level.

Bifidobacteria require some growth factors for optimum growth (Klaver *et al.*, 1993; Proulx *et al.*, 1992; 1994). Media used for enumeration of bifidobacteria often incorporate L-cysteine (0.05 to 0.10%) to improve the recovery of bifidobacteria (Teraguchi *et al.*, 1978; Ervol'der *et al.*, 1980; Laroia and Martin, 1991a). Cysteine, a sulfur containing amino acid, could provide amino nitrogen as a growth factor while reducing the redox potential; both of which might favour the growth of anaerobic bifidobacterial species. Collins and Hall (1984) reported improved viability of some bifidobacterial species in 10-12% reconstituted skim milk containing 0.05% cysteine.

In this study, effect of L-cysteine on growth and viability of yoghurt and probiotic bacteria was studied in yoghurt made with four commercial starter cultures. Changes in pH, redox potential, hydrogen peroxide and viable counts of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and bifidobacteria were monitored during manufacture and storage of yoghurt for 35 days at 4°C.

6.2.2 Materials and methods

6.2.2.1 Starter cultures and yoghurt preparation

Four starter cultures described in section 3.1 were used in this study and yoghurt was prepared as described in section 3.4.4. Samples were prepared as described in section 3.5.1.

6.2.2.2 Analyses and time interval specifications

The yoghurt samples were analysed for pH and titratable acidity (section 3.7.1.1), hydrogen peroxide (section 3.7.1.2), redox potential (section 3.7.1.4), protein and total solids contents (section 3.7.1.5). The microbiological analyses of yoghurt samples at various time intervals (section 3.6) was carried out as described in section 3.7.2.

All the experiments were repeated twice and the results are averages of two replications.

6.2.3 Results and discussion

Composition. Protein content of the heat treated yoghurt mix ranged from 3.68-3.76 g 100 g^{-1} and the total solid contents were in the range of 16.20-16.35%. Thus, the compositional differences were minimal in the various experimental replications.

Changes in pH. Initial pH of milk (6.40-6.60 at 0 h) decreased to 4.32-4.50 in yoghurts containing various levels of cysteine (Fig. 6.2.1). The incubation time taken to reach pH of 4.5 varied for type of starter cultures and it was greatly affected by the concentration of cysteine. The fermentation time to reach pH 4.5 for control yoghurts was ~4.5 h, 5.5 h, 12 h and 7 h for C1, C2, C3 and C4 starter cultures, respectively. At 50 $mg.L^{-1}$ level of L-cysteine, the fermentation time decreased to ~4 h and 15 min, 5 h and 10 min, 11 h and 10 min and 6 h and 40 min for starter cultures C1, C2, C3 and C4, respectively. Thus, incubation time reduced by ~5-7% for all the starter cultures. Conversely, the incubation time increased 15-30% and 40-60% for the yoghurts supplemented with 250 and 500 $mg.L^{-1}$ of cysteine, respectively. The increase in incubation time was in the order of C3 followed by C2, C4 and C1. During cold storage, the drop in pH (Fig. 6.2.1) or an increase in titratable acidity (Fig. 6.2.2) was less during the initial period for all the 4 starter cultures at the 500 $mg.L^{-1}$ cysteine level and the extent of pH drop (Fig. 6.2.1) or increase in titratable acidity (Fig. 6.2.2) was at a faster rate after 20-25 d storage periods (Fig. 6.2.1). Overall, addition of cysteine seemed to keep the microflora

biologically more active during storage in yoghurts made with all types of starter cultures. The drop in pH remained higher in yoghurts supplemented with cysteine.

Changes in redox potential. Changes in the redox potential of yoghurt during storage at 4°C is shown in Fig. 6.2.3. In general, cysteine was found to be a powerful reducing agent. At 0 h, the redox potential of yoghurt mix dropped to about -110 to -120 mV, -180 to -190 mV and -210 to -220 mV on addition of 50, 250 and 500 $mg.L^{-1}$ of cysteine, respectively. The redox potential showed an increasing trend during cold storage of yoghurt. The type of starter culture was found to have a profound effect on the redox potential; however, the presence of cysteine had a greater effect on reducing the redox potential. At 0 d, the redox potential in control yoghurt ranged between 50 to 100 mV, whereas, for products containing 50, 250 and 500 $mg.L^{-1}$ cysteine, its level ranged between -10 to -30 mV, -25 to -80 mV, and -30 to -100 mV, respectively. The pattern of increase in redox potential during cold storage was almost identical for control yoghurts and remained positive throughout storage. Contrarily, the increases in redox potential varied for yoghurt samples incorporated with various levels of cysteine. The redox potential remained negative for up to 5 and 10 d for starter cultures C4 and C3, respectively, at 50 $mg.L^{-1}$ cysteine level, but positive in yoghurts made with the starter cultures C1 and C2. At the 250 $mg.L^{-1}$ level of cysteine, the redox potential remained negative for 5 d in yoghurts made with C1 and C4, 10 d for C2 and 25 d for C3 starter cultures. Similarly, at 500 $mg.L^{-1}$ level of cysteine, the redox potential remained negative for almost 25 d and 30 d in yoghurts made with C1 and C2, and C3 and C4 starter cultures, respectively. After 35 d storage, the redox potential was positive for all the yoghurt samples.

Changes in hydrogen peroxide concentration. Hydrogen peroxide concentration in control yoghurts at 0 d was 10.5, 8.5, 4.5 and 2.3 $\mu g.g^{-1}$ for starter culture C1, C2, C3 and C4, respectively. Addition of cysteine did not affect the hydrogen peroxide concentration. The concentration was highest in yoghurt made with starter culture C1 followed by C2, both of which contained *L. delbrueckii* ssp. *bulgaricus*. Some *L.* acidophilus are reported to produce hydrogen peroxide during fermentation of milk (Gilliland and Speck, 1977a). After 0 d, the hydrogen peroxide concentration ranged

between 1 to 2 $\mu g.g^{-1}$ in all the products throughout the storage periods and the addition of cysteine did not have any marked effect on the production or stability of hydrogen peroxide.

Changes in the counts of yoghurt bacteria. Changes in the viable counts of *S. thermophilus* during manufacture and storage of yoghurt made with 4 starter cultures and 4 levels of cysteine are presented in Table 6.2.1. The incorporation of cysteine at 250 and 500 $mg.L^{-1}$ levels made the environment unfavorable for the growth of *S. thermophilus*. At 0 d, counts of *S. thermophilus* for all the commercial starter cultures were lower in yoghurts containing 250 and 500 $mg.L^{-1}$ cysteine as compared with control yoghurt with no added cysteine. Contrarily, cysteine at 50 $mg.L^{-1}$ level was found to promote the growth of *S. thermophilus*. This observation was also supported by decrease in incubation time at 50 $mg.L^{-1}$ level and an overall increase in the incubation period to reach a pH of 4.5 at 250 and 500 $mg.L^{-1}$ cysteine levels.

During storage, the counts of *S. thermophilus* declined gradually in all yoghurts. After 35 d storage, the percentage decay in counts of *S. thermophilus* ranged between 50-70% and their numbers varied depending on starter cultures. However, the counts after 35 d remained at a suggested level of $> 10^7\ g^{-1}$ in all yoghurt samples (Davis *et al.*, 1971).

The counts of *L. delbrueckii* ssp. *bulgaricus* decreased to $<10^5\ g^{-1}$ after 25-30 d storage in yoghurts made with C1 and C2 starter cultures supplemented with various levels of cysteine (Table 6.2.2). Growth of this organism was promoted at 50 $mg.L^{-1}$ and was suppressed at 250 and 500 $mg.L^{-1}$ levels of cysteine. However, at the end of 35 d, counts of *L. delbrueckii* ssp. *bulgaricus* were higher with increasing concentration of cysteine. In general, during storage of yoghurt, the decay in counts was lower in yoghurts supplemented with cysteine. Kneifel *et al.* (1993) made yoghurt and related products using 44 commercial starter cultures and reported that approximately 80% of the products had higher counts of cocci than rods. In our study, counts of *S. thermophulus* remained well above the counts of lactobacilli throughout the storage of all yoghurts.

Changes in the counts of probiotic bacteria. Changes in the viable counts of *L. acidophilus* during manufacture and storage of yoghurt are presented in Table 6.2.3. The starter culture used had an effect on the counts of *L. acidophilus*. At 0 d, the counts of *L. acidophilus* for all the 4 commercial starter cultures were much higher in yoghurts made with 250 and 500 $mg.L^{-1}$ cysteine than those with no added cysteine or with 50 $mg.L^{-1}$of cysteine. At the 50 $mg.L^{-1}$ cysteine level, there was no marked effect on growth and multiplication of *L. acidophilus* cells which showed almost similar increase to that observed for control yoghurt as explained earlier. The incubation time to reach a pH of 4.5 was longer for yoghurts made with higher levels of cysteine (250 or 500 $mg.L^{-1}$). The yoghurt bacteria did not grow well at these cysteine levels, hence, cells of *L. acidophilus* might have multiplied several times in these yoghurts resulting in higher *L. acidophilus* counts.

In earlier studies (Dave and Shah, 1997a, b, c), the counts of *L. acidophilus* in the control yoghurts declined sharply in C1 and C2 starter cultures and their viability of > 10^5 g^{-1} could not be maintained in yoghurts made with these starter cultures. It is evident from Table 6.2.3 that the viability of *L. acidophilus* was improved during manufacture and storage upon addition of cysteine in yoghurts made from all the 4 starter cultures. Dave and Shah (1997a) reported improved viability of *L. acidophilus* in yoghurts prepared in glass bottles, where oxygen permeation was minimal. This study supported our earlier observations and confirms that redox potential plays a crucial role for the viability of *L. acidophilus*.

As shown in Table 6.2.4, the viability of bifidobacteria was adversely affected in yoghurts made with C1 and C2 starter cultures containing 250 and 500 $mg.L^{-1}$ cysteine as compared to the control yoghurts. Conversely, the viability of bifidobacteria was improved for C3 and C4 starter cultures. In earlier study (Dave and Shah, 1997a), it was reported that counts of bifidobacteria reduced dramatically in yoghurt made with C3 starter culture. Increased inoculum (Dave and Shah, 1997b) and use of ascorbic acid as an oxygen scavenging agent (Dave and Shah, 1997c) did not improve the viability of bifidobacteria in yoghurt made with this starter culture. The inhibition of this organism

was neither due to generation or presence of phage nor due to bacteriocinogenic activity. In the present investigation, viability of bifidobacteria was improved due to incorporation of cysteine in yoghurt made with C3 starter culture. A concentration of 50 $mg.L^{-1}$ cysteine seemed to be optimum for improving the viability of bifidobacteria in yoghurt made with C3 starter culture; higher levels did not further improve their viability (Table 6.2.4). For yoghurts made with C1, C2 and C4 starter cultures at 500 $mg.L^{-1}$ cysteine, the pH dropped to ~4.0, which is reported to be detrimental to the viability of probiotic bacteria (Laroia and Martin, 1991b; Shah *et al.*, 1995; Lankaputhra *et al.*, 1996b).

Bifidobacteria are reported to be weakly proteolytic thus require growth factors (Collins and Hall, 1984; Ziajka and Zbikowski, 1986; Proulx *et al.*, 1992; Klaver *et al.*, 1993). Starter cultures C1 and C2 contained *L. delbrueckii* ssp. *bulgaricus* which has been reported to be proteolytic in nature in comparison to *S. thermophilus* (Shankar and Davies, 1977; Dutta *et al.*, 1973; Singh *et al.*, 1980). Thus, in yoghurt made with C1 and C2 starter cultures, proteolytic activity of *L. delbrueckii* ssp. *bulgaricus* may have produced some essential amino acids which may likely have helped bifidobacteria multiply to some extent. The bifidobacterial strain/s used in these starter cultures seemed to tolerate oxygen for some time and hence presence of negative redox potential due to addition of cysteine did not improve their viability in yoghurts made with C1 and C2 starter cultures. Observations with C3 starter culture also indicated that 50 $mg.L^{-1}$ cysteine was sufficient for improving the viability of bifidobacteria and higher amounts did not improve counts or viability any further. This confirms that the positive role of cysteine may be due to availability of amino nitrogen from cysteine but not due to reduced redox potential. However, for oxygen sensitive bifidobacterial strains such as reported by Collins and Hall (1984) and Klaver *et al.* (1993), cysteine may also improve their viability by lowering the redox potential during manufacture and storage of products.

6.2.4 Conclusions

Viability of yoghurt bacteria and probiotic bacteria was assessed in yoghurts supplemented with 0, 50, 250 and 500 $mg.L^{-1}$ cysteine. The level of 500 $mg.L^{-1}$ cysteine was selected based on its use at this concentration in the media used for the enumeration of bifidobacteria. Cysteine is very expensive and hence, based on some initial preliminary experiments on viability and that of extent of drop in redox potential, the other two levels (50 and 250 $mg.L^{-1}$) were selected for this set of experiments. The incubation time to reach a pH of 4.5 was greatly affected by the addition of cysteine. During refrigerated storage, the relative drop in pH was higher in yoghurts containing 250 and 500 $mg.L^{-1}$ cysteine, but was similar in yoghurts prepared with 0 and 50 $mg.L^{-1}$ cysteine. The redox potential remained negative for almost 25-30 d in yoghurt supplemented with 500 $mg.L^{-1}$ cysteine, but remained positive throughout storage in yoghurt made without cysteine. The redox potential gradually increased to positive in all yoghurt samples; however, the rate of increase was affected by the level of cysteine and the type of starter culture.

Supplementation with 50 $mg.L^{-1}$ cysteine promoted the growth of yoghurt bacteria in yoghurts made with all the 4 commercial starter cultures. The growth of *S. thermophilus* was adversely affected with cysteine above 50 $mg.L^{-1}$ level. Counts of *L. acidophilus* during manufacture and storage were higher in yoghurt containing 250 $mg.L^{-1}$ cysteine, but the counts were slightly higher or lower in yoghurt containing 500 $mg.L^{-1}$ cysteine depending on the starter cultures. Addition of cysteine adversely affected the viability of bifidobacteria for C1 and C2 starter cultures that contained both yoghurt bacteria (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*), but the viability improved in yoghurt made with starter culture that contained *S. thermophilus* as yoghurt bacteria.

Table 6.2.1. Changes in the counts of *S. thermophilus* during manufacture and storage of yoghurt prepared with various levels of cysteine

Period[1]	Control	[2]Cy_{50}	Cy_{250}	Cy_{500}
		Starter Culture	*C1*	
0 d	600 x 10^6	699 x 10^6	299 x 10^6	188 x 10^6
5 d	660 x 10^6	720 x 10^6	200 x 10^6	145 x 10^6
15 d	420 x 10^6	460 x 10^6	180 x 10^6	130 x 10^6
25 d	380 x 10^6	366 x 10^6	166 x 10^6	89 x 10^6
35 d	220 x 10^6	208 x 10^6	108 x 10^6	80 x 10^6
		Starter Culture	*C2*	
0 d	580 x 10^6	880 x 10^6	172 x 10^6	107 x 10^6
5 d	660 x 10^6	890 x 10^6	199 x 10^6	150 x 10^6
15 d	410 x 10^6	580 x 10^6	175 x 10^6	90 x 10^6
25 d	400 x 10^6	530 x 10^6	171 x 10^6	79 x 10^6
35 d	250 x 10^6	270 x 10^6	69 x 10^6	66 x 10^6
		Starter Culture	*C3*	
0 d	640 x 10^6	730 x 10^6	140 x 10^6	82 x 10^6
5 d	680 x 10^6	900 x 10^6	165 x 10^6	120 x 10^6
15 d	600 x 10^6	1000 x 10^6	150 x 10^6	66 x 10^6
25 d	540 x 10^6	1070 x 10^6	140 x 10^6	48 x 10^6
35 d	490 x 10^6	1080 x 10^6	130 x 10^6	39 x 10^6
		Starter Culture	*C4*	
0 d	2510 x 10^6	2880 x 10^6	1250 x 10^6	690 x 10^6
5 d	2810 x 10^6	2910 x 10^6	1510 x 10^6	620 x 10^6
15 d	2510 x 10^6	2610 x 10^6	1200 x 10^6	600 x 10^6
25 d	1890 x 10^6	1990 x 10^6	860 x 10^6	260 x 10^6
35 d	1100 x 10^6	1300 x 10^6	490 x 10^6	40 x 10^6

[1]0 h, 0 d, 5 d-35 d=Observations taken immediately after starter culture addition, after overnight cooling and during 5 to 35 days storage of product, respectively, at 4°C; [2]Cy_{50},Cy_{250}, Cy_{500}=Yoghurts made with 50, 250 and 500 mg.L^{-1} yoghurt mix.

At 0 h period, counts of *S. thermophilus* were 145 x 10^5, 80 x 10^5, 32 x 10^5 and 65 x 10^5 for cultures C1, C2, C3 and C4, respectively.

Table 6.2.2. Changes in the counts of *L. delbrueckii* ssp. *bulgaricus* during manufacture and storage of yoghurt prepared with various levels of cysteine

Period[1]	Control	[2]Cy_{50}	Cy_{250}	Cy_{500}
		Starter Culture	*C1*	
0 d	405×10^6	450×10^6	300×10^6	270×10^6
5 d	330×10^6	390×10^6	350×10^6	300×10^6
15 d	160×10^6	210×10^6	270×10^6	200×10^6
20 d	16×10^6	90×10^6	100×10^6	160×10^6
25 d	$<10^5$	19×10^6	32×10^6	45×10^6
		Starter Culture	*C2*	
0 d	350×10^6	400×10^6	280×10^6	230×10^6
5 d	270×10^6	330×10^6	200×10^6	195×10^6
15 d	55×10^6	190×10^6	120×10^6	130×10^6
20 d	6×10^6	60×10^6	70×10^6	100×10^6
25 d	$<10^5$	6×10^6	12×10^6	45×10^6

[1]0 h, 0 d, 5 d-35 d=Observations taken immediately after starter culture addition, after overnight cooling and during 5 to 35 days storage of product, respectively, at 4°C; [2]Cy_{50},Cy_{250}, Cy_{500}=Yoghurts made with 50, 250 and 500 $mg.L^{-1}$ yoghurt mix.

At 0 h period, counts of *L. delbrueckii* ssp. *bulgaricus* were 40×10^5 and 20×10^5 for cultures C1 and C2, respectively. Starter culture C3 and C4 did not contain *L. delbrueckii* ssp. *bulgaricus* their counts were $< 10^5$ after 25 d storage.

Table 6.2.3. Changes in the counts of *L. acidophilus* during manufacture and storage of yoghurt prepared with various levels of cysteine

Period[1]	Control	$^{2}Cy_{50}$	Cy_{250}	Cy_{500}
		Starter Culture	*C1*	
0 d	150×10^5	148×10^5	290×10^5	325×10^5
5 d	158×10^5	168×10^5	280×10^5	310×10^5
15 d	90×10^5	110×10^5	180×10^5	200×10^5
25 d	35×10^5	25×10^5	50×10^5	62×10^5
35 d	0.35×10^5	0.86×10^5	14×10^5	5.1×10^5
		Starter Culture	*C2*	
0 d	550×10^5	590×10^5	990×10^5	780×10^5
5 d	540×10^5	500×10^5	850×10^5	800×10^5
15 d	278×10^5	265×10^5	346×10^5	450×10^5
25 d	32×10^5	42×10^5	104×10^5	60×10^5
35 d	0.84×10^5	0.10×10^5	15×10^5	3×10^5
		Starter Culture	*C3*	
0 d	450×10^5	389×10^5	2150×10^5	1650×10^5
5 d	400×10^5	410×10^5	2000×10^5	1800×10^5
15 d	189×10^5	177×10^5	800×10^5	820×10^5
25 d	48×10^5	75×10^5	548×10^5	489×10^5
35 d	5.1×10^5	13×10^5	59×10^5	70×10^5
		Starter Culture	*C4*	
0 d	650×10^5	650×10^5	990×10^5	1240×10^5
5 d	540×10^5	740×10^5	1200×10^5	1300×10^5
15 d	400×10^5	500×10^5	690×10^5	860×10^5
25 d	95×10^5	159×10^5	290×10^5	800×10^5
35 d	11×10^5	61×10^5	150×10^5	550×10^5

[1]0 h, 0 d, 5 d-35 d=Observations taken immediately after starter culture addition, after overnight cooling and during 5 to 35 days storage of product, respectively, at 4°C; $^{2}Cy_{50}$,Cy_{250}, Cy_{500}=Yoghurts made with 50, 250 and 500 mg.L^{-1} yoghurt mix.

At 0 h period, counts of *L. acidophilus* were 80×10^5, 45×10^5, 76×10^5 and 75×10^5 for cultures C1, C2, C3 and C4, respectively.

Table 6.2.4. Changes in the counts of bifidobacteria during manufacture and storage of yoghurt prepared with various levels of cysteine

Period[1]	Control	[2]Cy_{50}	Cy_{250}	Cy_{500}
		Starter Culture	*C1*	
0 d	850×10^5	920×10^5	389×10^5	132×10^5
5 d	726×10^5	770×10^5	298×10^5	120×10^5
15 d	505×10^5	560×10^5	250×10^5	96×10^5
25 d	210×10^5	278×10^5	188×10^5	82×10^5
35 d	107×10^5	105×10^5	95×10^5	80×10^5
		Starter Culture	*C2*	
0 d	680×10^5	520×10^5	277×10^5	335×10^5
5 d	560×10^5	500×10^5	280×10^5	300×10^5
15 d	371×10^5	450×10^5	164×10^5	222×10^5
25 d	343×10^5	299×10^5	149×10^5	139×10^5
35 d	270×10^5	223×10^5	51×10^5	60×10^5
		Starter Culture	*C3*	
0 d	700	4×10^5	2.5×10^5	4.0×10^5
5 d	800	3.9×10^5	2.6×10^5	3.0×10^5
15 d	450	2.5×10^5	2.2×10^5	2.5×10^5
25 d	300	0.9×10^5	1.25×10^5	1.8×10^5
35 d	200	0.58×10^5	1.09×10^5	1.50×10^5
		Starter Culture	*C4*	
0 d	60×10^5	102×10^5	331×10^5	217×10^5
5 d	40×10^5	90×10^5	300×10^5	200×10^5
15 d	20×10^5	50×10^5	256×10^5	168×10^5
25 d	17×10^5	45×10^5	180×10^5	158×10^5
35 d	17×10^5	35×10^5	90×10^5	95×10^5

[1]0 h, 0 d, 5 d-35 d=Observations taken immediately after starter culture addition, after overnight cooling and during 5 to 35 days storage of product, respectively, at 4°C; [2]Cy_{50},Cy_{250}, Cy_{500}=Yoghurts made with 50, 250 and 500 $mg.L^{-1}$ yoghurt mix.

At 0 h period, counts of bifidobacteria were 90×10^5, 55×10^5, 60×10^5 and 50×10^5 for cultures C1, C2, C3 and C4, respectively.

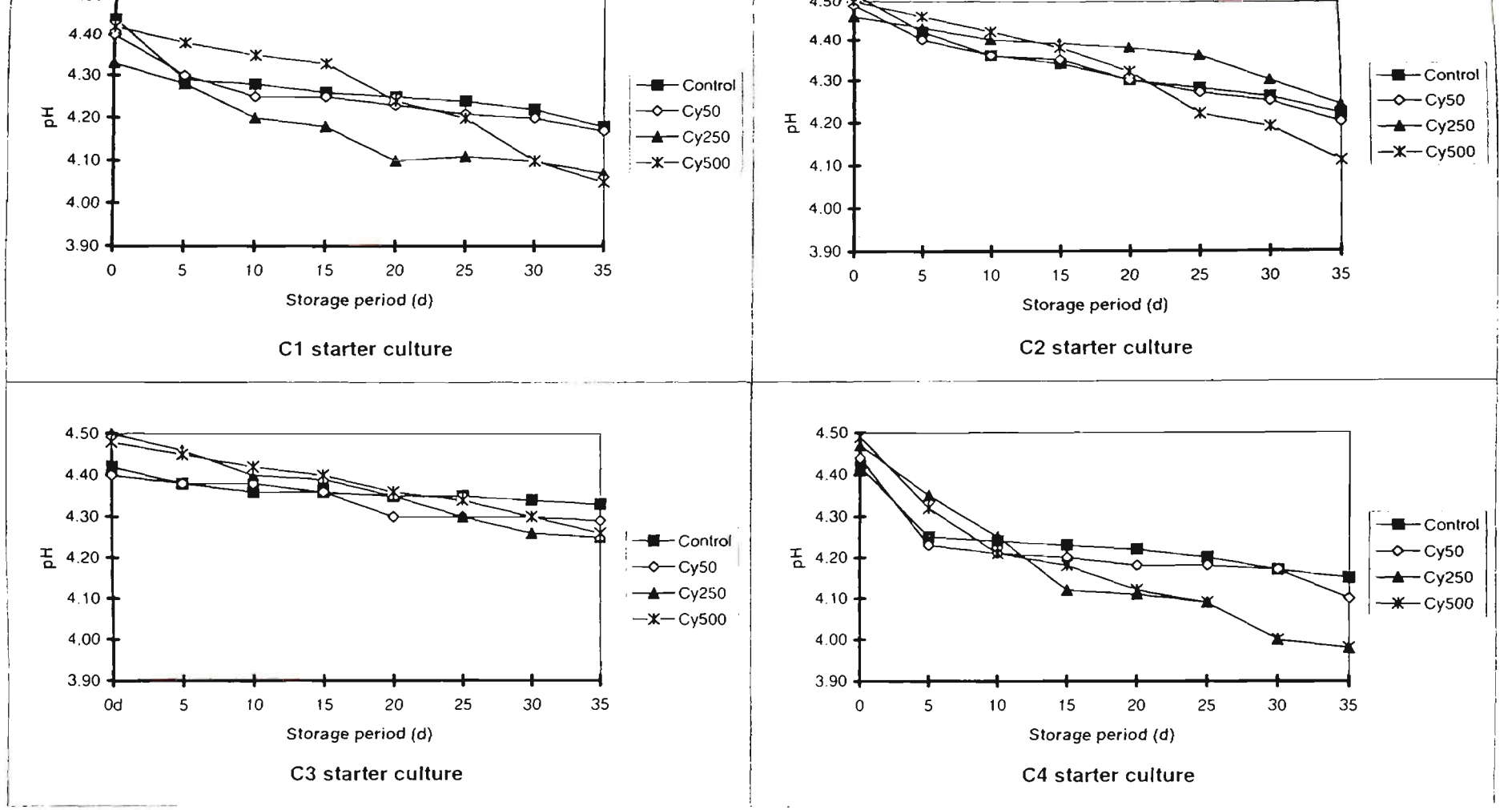


Fig. 6.2.1. Changes in pH during storage of yoghurts made using four different starter cultures with various levels of cysteine (Cy50, Cy250 and Cy500=Yoghurts made with 50, 250 and 500 mg.L^{-1} cysteine).

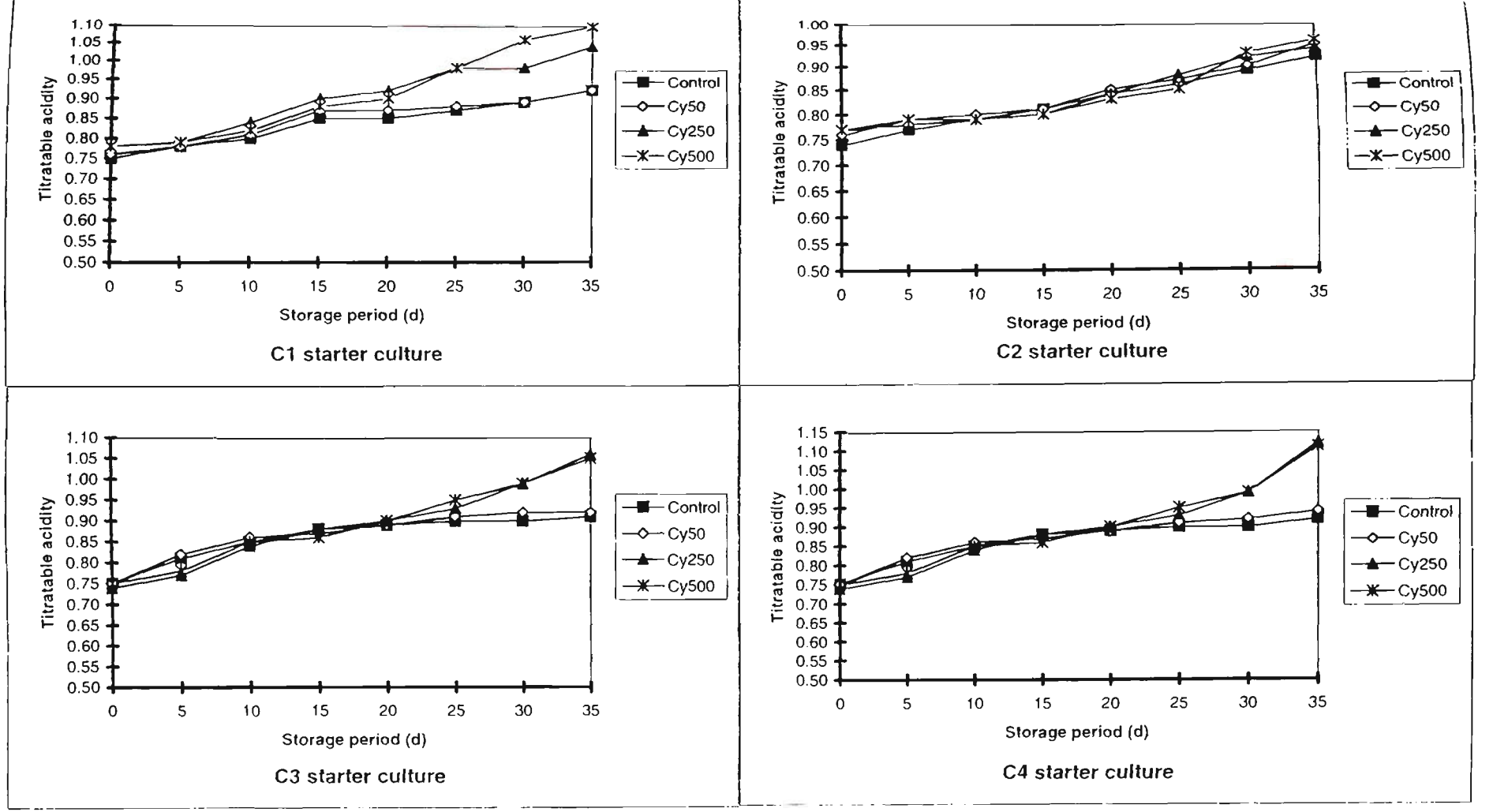


Fig. 6.2.2. Changes in titratable acidity during storage of yoghurts made using four different starter cultures with various levels of cysteine (Cy50, Cy250 and Cy500= Yoghurts made with 50, 250 and 500 mg.L^{-1} cysteine).

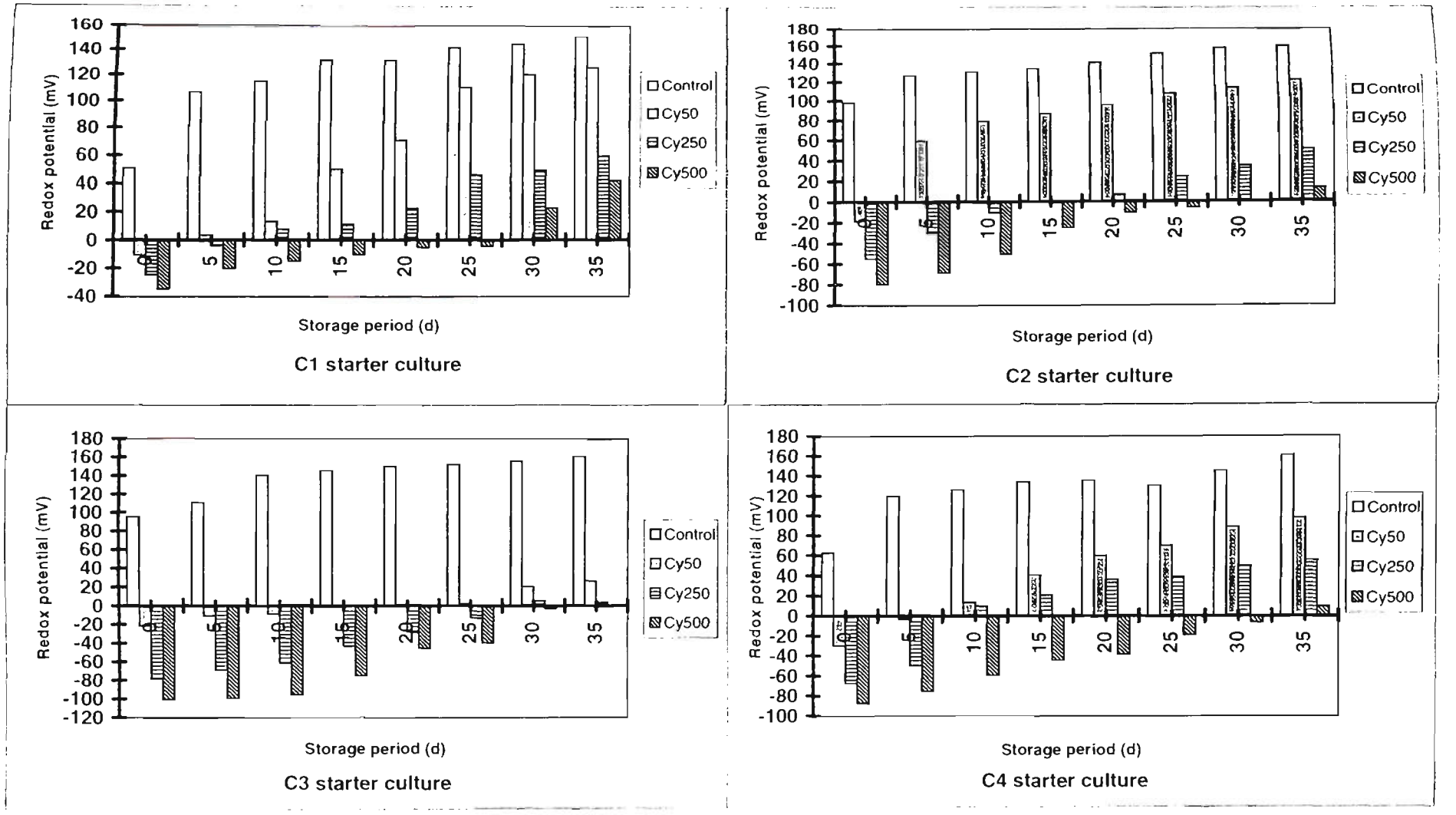


Fig. 6.2.3. Changes in redox potential during storage of yoghurts made using four different starter cultures with various levels of cysteine (Cy50, Cy250 and Cy500 = Yoghurts made with 50, 250 and 500 mg.L^{-1} cysteine).

6.3 Effects of cysteine, whey powder, whey protein concentrates, acid casein hydrolysates and tryptone on viability of yoghurt and probiotic bacteria

6.3.1 Introduction

Probiotic bacteria grow slowly in milk due to lack of proteolytic activity (Klaver *et al.*, 1993) and the usual practice is to add yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) to reduce the fermentation time. *L. delbrueckii* ssp. *bulgaricus* produces essential amino acids owing to proteolytic nature and the symbiotic relationship between *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is well established in which the former organism produces amino nitrogen for the latter organism. However, *L. delbrueckii* ssp. *bulgaricus* also produces lactic acid during refrigerated storage known as 'post-acidification', which is claimed to affect the viability of probiotic bacteria. To overcome the problem of post-acidification, the present trend is to use AB starter cultures devoid of *L. delbrueckii* ssp. *bulgaricus* such as ABT (*L. acidophilus*, bifidobacteria and *S. thermophilus*) (personal communication, Roy Con Foo, Director, Chr. Hansen Laboratory Pty. Ltd., Australia). Such starter cultures may necessitate incorporation of micronutrients (peptides and amino acids) through whey powder (WP), whey protein concentrate (WPC), acid casein hydrolysate (ACH) or tryptone for reducing the fermentation time and for improving the viability of probiotic bacteria as *S. thermophilus* is the main organism responsible for fermentation in ABT cultures. *S. thermophilus* is also less proteolytic than *L. delbrueckii* ssp. *bulgaricus*. ACH is a product prepared by acid hydrolysis of casein, whereas tryptone is a tryptic digest of casein, both of which are rich in peptides and essential amino acids required by *S. thermophilus*. WP and WPC are the by-products of the cheese industry and are used to partially replace skim milk powder in order to increase the solid contents of the yoghurt mix.

Several factors affect the viability of probiotic bacteria (Klaver *et al.*, 1990; 1993; Proulx *et al.*, 1994; Lankaputhra and Shah, 1995; Lankaputhara *et al.*, 1996b).

A version of section 6.3 has been published under the title "Ingredient supplementation effects on viability of probiotic bacteria in yogurt". Dave, R. I. and Shah, N. P. (1998). *J. Dairy Sci.*, **81**:2804-2816

Increase in acidity of the product during storage adversely affects the viability of probiotic bacteria. Hydrogen peroxide produced by some lactobacilli is known for its anti-microbial effects. Bifidobacteria are anaerobic in nature and therefore higher oxygen content may affect their growth and viability. The composition of product, presence of preservatives as a result of added fruits and nuts, availability of growth factors etc. are also reported to affect the growth and viability of yoghurt and probiotic bacteria. Antagonism among bacteria used in the starter culture due to production of anti-microbial substances such as bacteriocins may decrease the numbers of sensitive organisms that may be present in a product or starter culture. In earlier studies, low viability of bifidobacteria was reported in yoghurt made with one of the four commercial starter cultures (Dave and Shah, 1997a, b, c). In these studies, a dramatic decline in the numbers of bifidobacteria in the same starter culture was observed, and higher inoculum (Dave and Shah, 1997b) or use of ascorbic acid as an oxygen scavenger (Dave and Shah, 1997c) did not improve the viability of bifidobacteria to a satisfactory level. No antagonism between yoghurt bacteria and probiotic bacteria used in this starter culture was observed (Joseph *et al.*, 1998).

In this part of the study, the effect of L-cysteine, whey powder, whey protein concentrate, acid casein hydrolysate and tryptone on growth and viability of yoghurt and probiotic bacteria were examined in yoghurt made with C3 (actual name ABT-1) a commercial starter culture. Changes in pH, titratable acidity, redox potential and viable counts of *S. thermophilus, L. acidophilus* and bifidobacteria were monitored during 24 h incubation and during storage of yoghurts for 35 days at 4°C. Changes in cell morphology of *S. thermophilus* with an electron microscope were also observed. SDS-PAGE of filtrates of yoghurt samples and amino-acids analyses of the ingredients that improved the viability of bifidobacteria were also performed.

6.3.2 Materials and methods

6.3.2.1 Starter cultures and yoghurt preparation

Four starter cultures described in section 3.1 were used in this study and yoghurt was prepared as described in section 3.4.5. Samples were prepared as described in section 3.5.1

6.3.2.2 Analyses and time interval specifications

The yoghurt and yoghurt mix samples were analysed for pH and titratable acidity (section 3.7.1.1), hydrogen peroxide (section 3.7.1.2), redox potential (section 3.7.1.4), protein and total solids contents (section 3.7.1.5). The microbiological analyses of yoghurt samples at various time intervals (section 3.6) was carried out as described in section 3.7.2.

6.3.2.3 Microscopic examinations

Polysaccharide production was confirmed by Maneval's staining (section 3.8) and effect of added cysteine on *S. thermophilus* was studied by electron microscopic examination according to the procedure outlined in section 3.9.

6.3.2.4 SDS-PAGE and amino acid analyses

SDS-PAGE of filtrate collected from various yoghurts was performed as described in section 3.11 and the amino acid analyses of the ingredients that improved viability of bifidobacteria in C3 starter culture is described in section 3.12.

All experiments and analyses were repeated in duplicate at least twice. The results shown in this part of study are the averages of all available data. The standard deviation

of at least four observations was calculated using an Excel 5.0 Microsoft computer package and presented as plus or minus of the mean or as an error bar in the figures.

6.3.3 Results and discussion

6.3.3.1 Composition

The average protein and total solid contents of each sample are given in Table 6.3.1. The protein content was 0.7% higher in yoghurt supplemented with WPC, and 0.1% lower in yoghurt supplemented with WP. The total solids contents were in the range of 15.40-15.75% for all products.

6.3.3.2 Changes during fermentation of yogurt mix for 24 hours

Changes in pH and redox potential. Changes in pH during 24 h fermentation of yoghurt mix are presented in Fig. 6.3.1. The decrease in pH was faster in yoghurt containing WP, WPC, ACH and tryptone than that of control yoghurt. Cysteine at 50 $mg.L^{-1}$ level showed almost similar drop in pH during 24 h to that of control yoghurt; however, increasing concentration of cysteine above 50 $mg.L^{-1}$ level adversely affected the rate of acid production. Overall, the time taken to reach a pH of 4.5 was ~12 h for the control yoghurt and that supplemented with 50 $mg.L^{-1}$ cysteine. The incubation time prolonged to ~18 h and 20 h for yoghurt supplemented with 250 and 500 $mg.L^{-1}$ cysteine, respectively. Conversely, the fermentation time decreased to ~9 h for yoghurt supplemented with WP, ACH or tryptone, ~7.5 h and 8.5 h for that supplemented with WPC1 and WPC2, respectively. At 24 h of fermentation, the pH of the yoghurt supplemented with 250 and 500 $mg.L^{-1}$ cysteine was 4.16 and 4.19, respectively, whereas it dropped below 4.0 in the rest of the yoghurts. Changes in the pH of yoghurt supplemented with 50 $mg.L^{-1}$ cysteine were similar to that of control yoghurt. Also, changes in the pH of yoghurt supplemented with 250 $mg.L^{-1}$ cysteine and WPC1 were similar to that of yoghurt supplemented with 500 $mg.L^{-1}$ cysteine and WPC2, respectively. Yoghurt supplemented with 500 $mg.L^{-1}$ of ACH and tryptone gave similar

results to that supplemented with 250 $mg.L^{-1}$ ACH and tryptone, respectively. Champagne *et al.* (1996) tested suitability of WPC as growth medium for the production of starter cultures in comparison to milk and commercial media. They concluded that WPC could be successfully used to prepare starter cultures as it gave higher populations of bacteria than when milk was used as the medium. Supplementation of WPC with milk protein hydrolysate also stimulated starter growth.

Redox potential of control yoghurt mix after heat treatment was ~ -70 mV. The redox potential decreased to ~ -130 mV, -180 mV and -217 mV on addition of 50, 250 and 500 $mg.L^{-1}$ cysteine. The redox potential dropped to -121, -169, -143 mV in yoghurt supplemented with WP, WPC1 and WPC2, respectively, and to -80 mV on addition of ACH and tryptone (Fig. 6.3.2). Cysteine is a strong reducing agent and is known for lowering redox potential. Yoghurt supplemented with WP and WPC is expected to have higher whey protein content (which is rich in sulphur containing amino acids) than control yoghurt. The whey proteins are known to expose these sulphur containing amino acids during heat treatment. This could be the reason for having low redox potential in yoghurt mix containing cysteine, WP and WPC. Redox potential remained negative throughout the 24 h fermentation period upon addition of cysteine, for 21 h in control yoghurt, but only for 12 h in yoghurt supplemented with ACH or tryptone. The redox potential fluctuated in yoghurt supplemented with WP or WPC, and no uniform pattern of increase or decrease in redox potential was observed in these samples (Fig. 6.3.2). Changes in redox potential of yoghurt mix supplemented with 500 $mg.L^{-1}$ ACH or tryptone were similar to that of yoghurt supplemented with 250 $mg.L^{-1}$ ACH and tryptone, respectively. Overall, the redox potential remained negative in all the yoghurts until the pH reached 4.5. Thus, the loss of viability of bifidobacteria in yoghurt made with ABT-1 starter culture did not appear to be due to dissolved oxygen in the yoghurt mixes.

Changes in counts of S. thermophilus, L. acidophilus and bifidobacteria. Changes in pH during 24 h fermentation of yoghurt mix with C3 (ABT-1) starter culture are shown in Fig. 6.3.3. Changes in counts of *S. thermophilus* during fermentation of yoghurt mixes

are presented in Table 6.3.2. As shown in Fig. 6.3.3, time taken to reach pH of 4.5 was affected with addition of various ingredients. Increasing cysteine adversely affected the growth of *S. thermophilus* (Table 6.3.2), when the counts of this organism from the time of inoculation to that required to reach pH of 4.5 is taken in to consideration (Fig. 6.3.1), and their counts remained lower in yoghurts supplemented with 250 or 500 $mg.L^{-1}$ cysteine. Conversely, WP, WPC, ACH and tryptone supported the growth of *S. thermophilus* and multiplication of this organism was faster in yoghurts supplemented with these ingredients. This could have been the reason for shorter incubation time as described earlier to reach pH of 4.5 in these samples.

When the time to reach pH of 4.5 is taken in to consideration and compared (Fig. 6.3.1), and the counts of *L. acidophilus* (Table 6.3.3) are compared, it is evident that the *L. acidophilus* counts increased in yoghurt supplemented with 500 $mg.L^{-1}$ cysteine. Thus, cysteine at 500 $mg.L^{-1}$ level promoted the growth of *L. acidophilus*, unlike that of *S. thermophilus* (Table 6.3.2), but other levels had no effect. Overall, multiplication of *L. acidophilus* was faster in yoghurt supplemented with ACH and tryptone.

Bifidobacteria in control yoghurt increased for up to 6 h and then declined (Table 6.3.4). At 9 h period, one log cycle reduction in their numbers was observed followed by ~ 3 log reduction at 12 h period. The pH of control yoghurt also reached 4.5 at this period, hence, the counts of bifidobacteria in control yoghurt were very low at 0 d than their initial numbers at 0 h (Tables 6.3.4 and 6.3.5). A similar decreasing trend was observed at 12 h period in yoghurt supplemented with WP. On the other hand, the time taken to reach pH of 4.5 was considerably reduced in yoghurt supplemented with WPC, ACH or tryptone. Therefore, the counts of bifidobacteria declined by < 1 log cycle in samples other than control yoghurt and that supplemented with WP. Also, the counts of bifidobacteria never dropped below 10^5 $cfu.g^{-1}$ throughout the 24 h incubation in yoghurt supplemented with 500 $mg.L^{-1}$ cysteine, WPC1, ACH or tryptone. This indicated that the incubation period of > 12 h (similar to that required for control yoghurt and that supplemented with WP) could keep bifidobacteria viable, provided similar peptides and amino acids as present in WPC1 or tryptone are available in yoghurt mix during

incubation. It was also observed that bifidobacteria started to multiply to certain extent in some products (Table 6.3.4) after 15-18 h incubation. During fermentation, changes in counts of *S. thermophilus*, *L. acidophilus* and bifidobacteria in yoghurt mixes supplemented with 500 $mg.L^{-1}$ ACH or tryptone were similar to that of yoghurt supplemented with 250 $mg.L^{-1}$ ACH and tryptone, respectively. Conclusively, lower redox potential was not solely responsible for improving the viability of bifidobacteria, but the additional nitrogen source in the form of peptides or amino acids was required to keep bifidobacteria viable in the product during manufacture of yoghurt. The pH levels were kept almost identical in all samples during fermentation as low pH or higher acidity has been reported to affect the viability of probiotic bacteria (Robinson, 1987; Shah *et al.*, 1995; Kailasapathy and Supriadi, 1996). In a study by Klaver *et al.* (1990), no growth of *Bifidobacterium bifidum* occurred in the absence of other lactic acid bacteria *(L. acidophilus* or *S. thermophilus*) or at higher oxygen concentrations. Further, 15 of 17 strains of bifidobacteria did not grow in milk as their growth required peptides or amino acids derived from casein degradation (Klaver *et al.*, 1993). It was concluded that bifidobacteria lacked proteolytic activity and the organism could be grown by adding casein hydrolysates or by co-culturing with proteolytic species such as *L. acidophilus*.

6.3.3.3 Changes during refrigerated storage of yoghurts for 35 days

Changes in pH, TA and redox potential. Changes in pH and TA during refrigerated storage of yoghurts are shown in Fig. 6.3.3. Approximately 0.08 unit drop in pH and 0.07% increase in lactic acid were observed in the control yoghurt. The drop in pH or rise in TA in yoghurt supplemented with cysteine, WPC, ACH or tryptone was more than double than that observed with the control yoghurt. Yoghurt supplemented with WP showed almost similar trend of decrease in pH or increase in TA to that of control yoghurt. Overall, a maximum of ~0.3 unit drop in pH and ~0.1% increase in TA was observed during 35 d storage of yoghurt supplemented with ACH or tryptone, possibly due to availability of amino nitrogen source through the added ingredients. Thus, pH or TA in all yoghurt samples stabilised within a certain range and the drop in pH or

increase in acidity does not seem to be the factors that affected the viability of bifidobacteria during refrigerated storage of yoghurt supplemented with these ingredients (Table 6.3.5). During refrigerated storage, changes in pH an TA of yoghurt supplemented with 50 $mg.L^{-1}$ cysteine were similar to that of control yoghurt. Also, the changes in pH of yoghurt supplemented with 250 $mg.L^{-1}$ cysteine and WPC1 were similar to that of yoghurt supplemented with 500 $mg.L^{-1}$ cysteine and WPC2, respectively. Yoghurt supplemented with 500 $mg.L^{-1}$ of ACH and tryptone gave similar results to that supplemented with 250 $mg.L^{-1}$ ACH and tryptone, respectively.

Redox potential in yoghurt supplemented with 50 or 250 $mg.L^{-1}$ cysteine remained negative for up to 10 and 25 d, respectively (Fig. 6.3.4). At 500 $mg.L^{-1}$ cysteine, the redox potential remained negative throughout the storage period of 35 d. In control and other yoghurts, the redox potential ranged between +81 to +95 mV at 0 d which increased to +160 mV in control and between +114 to +128 mV in yoghurts supplemented with WP, WPC, ACH or tryptone during storage.

Changes in counts of S. thermophilus, L. acidophilus and bifidobacteria. At 0 d, counts of *S. thermophilus* were highest in yoghurt supplemented with ACH and tryptone and lowest in yoghurt supplemented with 500 $mg.L^{-1}$ cysteine (Table 6.3.5). During further storage, *S. thermophilus* counts declined slightly in control yoghurt and that supplemented with 250 or 500 $mg.L^{-1}$ cysteine and WP, but increased to some extent in yoghurt supplemented with 50 $mg.L^{-1}$ cysteine, WPC, ACH or tryptone. Lower counts of *S. thermophilus* especially in yoghurt supplemented with higher levels (250 or 500 $mg.L^{-1}$) of cysteine could be due to negative redox potential in the product as *S. thermophilus* is micro-aerophillic to aerobic.

Counts of *L. acidophilus* showed constant decline in all the products throughout storage. At 0 d, counts of *L. acidophilus* were lower in yoghurt supplemented with WP, WPC, ACH or tryptone than that observed in control yoghurt and yoghurt supplemented with 50 $mg.L^{-1}$ cysteine. Conversely, the counts of *L. acidophilus* were considerably higher in yoghurt supplemented with 250 or 500 $mg.L^{-1}$ cysteine, unlike those of *S. thermophilus*

in these products. This could be due to adverse effect of cysteine on *S. thermophilus* which prolonged the fermentation time and perhaps favoured multiplication of *L. acidophilus* in yoghurts supplemented with cysteine. Conversely, in other products, shorter fermentation time might not have allowed *L. acidophilus* to multiply to a greater extent, resulting in lower counts of *L. acidophilus* in finished products prepared with WPC, ACH or tryptone. Overall, the viability of *L. acidophilus* was better in yoghurts supplemented with cysteine; however, counts remained $>10^5$ cfu.g^{-1} in all the products throughout the refrigerated storage of 35 d. Kailasapathy and Supriadi (1996) examined the effect of WPC on survival of *L. acidophilus* and concluded that partial replacement of dried skim milk by WPC enabled sufficiently high numbers of *L. acidophilus* to remain viable during refrigerated storage of 21 days.

Viability of bifidobacteria was considerably low throughout the storage in control yoghurt and that supplemented with WP. Viability of this organism in yoghurt supplemented with cysteine, WPC, ACH or tryptone was improved by more than 3 log cycles as compared to the control yoghurt. Improved viability could be due to amino nitrogen present in WPC, ACH and tryptone. A considerable decline in the counts of bifidobacteria occured between 9 to 12 h of incubation in control yoghurt (Table 6.3.4), but fermentation was terminated before 9 h in yoghurt supplemented with WPC, ACH or tryptone as the pH reached 4.5 in these samples. Thus, reduction in total fermentation time due to addition of these ingredients and the favourable effects of micronutrients present in these ingredients might have been responsible for improved viability of bifidobacteria to $> 10^5$ cfu.g^{-1}. The highest viability of bifidobacteria was observed in yoghurt supplemented with WPC1. During refrigerated storage, the changes in counts of *S. thermophilus*, *L. acidophilus* and bifidobacteria in yoghurts supplemented with 500 mg.L^{-1} ACH or tryptone were similar to that in yoghurt supplemented with 250 mg.L^{-1} ACH and tryptone, respectively.

6.3.3.4 Microscopic examination of polysaccharide production by *S. thermophilus*

Maneval's staining showed polysaccharide production by *S. thermophilus* in this ABT-1 starter culture (Fig. 6.3.5), confirming the claims made by the starter culture supplier.

6.3.3.5 Cell morphology of *S. thermophilus* as shown by electron micrograph

The cell morphology of *S. thermophilus* was affected with increased concentration of cysteine as observed by electron microscopy (Fig. 6.3.6). At 500 $mg.L^{-1}$ cysteine level seemed to affect the cell wall and cell membrane of *S. thermophilus* cells. In yoghurt supplemented with WP or 250 $mg.L^{-1}$ cysteine, the morphology of *S. thermophilus* was similar and showed no clear cell membrane and cell wall. This could be due to adverse effect on the *S. thermophilus* cells. This confirmed the earlier observations of less drop in pH (Fig. 6.3.1), slow growth of *S. thermophilus* (Table 6.3.2) during fermentation of yoghurt mix and lower counts of *S. thermophilus* in yoghurt supplemented with cysteine. In previous studies (Dave and Shah, 1997a, c), counts of *S. thermophilus* were low in yoghurt prepared in glass bottles and that supplemented with ascorbic acid as an oxygen scavenger, possibly due to low redox potential. The results of electron micrographs in this study confirmed that very low redox potential caused damage to the cell wall and cell membrane of *S. thermophilus*. In the control yoghurt and that supplemented with WPC1, WPC2, ACH or tryptone, the cell structure was clearly defined and the cells of *S. thermophilus* appeared without any signs of cell damage.

6.3.3.6 SDS-PAGE and amino acids analyses

Growth of various starter bacteria (*S. thermophilus, L. acidophilus* and bifidobacteria) was affected due to addition of various ingredients. Also, the ingredients added were expected to have different intermediate proteins and peptides. Therefore, SDS-PAGE of filtrate after precipitation of casein in different yoghurt samples was performed and various protein bands obtained were analysed by a gel densitometer (Fig. 6.3.8). The

number of bands and their relative area were different in each product. At higher cysteine levels (250 and 500 $mg.L^{-1}$), the intermediate peptide products were higher as a total of 12 bands appeared in these yoghurts as compared to 8-9 bands in other yoghurts (Fig. 6.3.7). The molecular weight of each band as estimated by a laser densitometer and their relative area were different in the whey of yoghurts supplemented with various ingredients (Table 6.3.6). The starter culture used in this study contained three different bacteria and counts of these organisms varied in the finished products. *L. acidophilus* has been reported to be more proteolytic than *S. thermophilus* (Klaver *et al.*, 1990). The higher counts of *L. acidophilus* in yoghurts containing higher levels of cysteine correlated with the higher numbers of bands in yoghurts supplemented with cysteine. The degradation of casein by *L. acidophilus* might have liberated additional peptides during fermentation and may have resulted into greater number of bands. Yoghurt supplemented with WPC1 and 2, ACH or tryptone also showed difference in the number of peaks and their molecular weight. Yoghurt containing WPC2 showed some high molecular weight bands (Table 6.3.6). The rate of acid production (Fig. 6.3.1) was slower and the viability of bifidobacteria (Table 6.3.5) was lower in yoghurt supplemented with WPC2 as compared to WPC1. The change in intermediate peptide products in whey of various yoghurts could be due to the change in microbial ecology and altered proportion of various starter bacteria. After analysing the SDS-PAGE and viability results, it is presumed that intermediate peptides (15-30 kDa) might have affected the growth of starter bacteria in yoghurts during fermentation. Proteins and peptides also seemed to be responsible for the time taken to reach pH 4.5 and difference in viability of starter bacteria in yoghurts supplemented with various ingredients.

Since viability of bifidobacteria was improved with WPC1, WPC2, ACH and tryptone to a variable extent, it was desirable to know the protein and amino acids profiles of these ingredients (Table 6.3.7). Although, ACH and tryptone are the breakdown products of casein, the concentrations of protein and various amino acids were different. The total protein content was fairly similar for WPC1 and WPC2, but some differences in their amino acid profile was found. The viability of bifidobacteria was improved to a lesser extent in yoghurt containing ACH. The total protein content and cysteine were less in

ACH than tryptone. Cystine plus cysteine contents were considerably higher in WPC1 and WPC2 than tryptone or ACH (Table 6.3.7). Thus, it appears that protein and amino acid contents of WPC1, WPC2, ACH and tryptone might have been responsible for the differences in viability of probiotic bacteria in yoghurt supplemented with these ingredients. This showed that peptides and amino acids have played a role in improving the viability of probiotic bacteria, especially bifidobacteria which have been reported to be weakly proteolytic (Ziajka and Zibkowski, 1986; Klaver *et al.*, 1990; 1993; Proulx *et al.*, 1994). *L. delbrueckii* ssp. *bulgaricus* is considered to be more proteolytic than *S. thermophilus*. In ABT cultures, no symbiotic relationship exists, as a result, incubation period to reach pH 4.5 is longer for these cultures. ACH is a product prepared by acid hydrolysis of casein, whereas tryptone is a tryptic digest of casein, both of which are rich in peptides and essential amino acids required by *S. thermophilus*. Cysteine, WP, and WPC also serve as a source of peptides and amino acids when heat treated in yoghurt mix. Whey proteins are rich in sulphur containing amino acids which are liberated during heat treatment and lower the redox potential.

S. thermophilus prefers aerobic to microaerophilic environment, *L. acidophilus* is microaerophilic to anaerobic, while bifidobacteria are considered to be anaerobes. Addition of cysteine improved the viability of probiotic bacteria during storage. For ABT starter cultures, the acid production is mainly due to *S. thermophilus* which usually requires 11 to 12 h incubation period to reach a pH 4.5. Addition of cysteine above 50 $mg.L^{-1}$ level caused damage to the cell wall and cell membrane of the *S. thermophilus,* which increased the incubation time to 18-20 h. The results on changes in pH during fermentation (Fig. 6.3.1), viable counts (Table 6.3.2) and electron micrographs (Fig. 6.3.6) confirmed this. Thus, addition of cysteine up to 50 $mg.L^{-1}$ may be sufficient and commercially feasible for improving the viability of probiotic bacteria. Contrarily, addition of WPC1 and tryptone may be more economical than cysteine and these ingredients reduced incubation time due to available micronutrients and less drop in redox potential, both of which supported the growth of *S. thermophilus*. Also, WPC1 and tryptone were equally effective in improving the viability of probiotic bacteria,

especially that of bifidobacteria, which in previous studies showed a dramatic decline in their numbers.

6.3.4 Conclusions

All 11 batches of yoghurt made with a commercial ABT-1 starter culture showed different patterns of change in pH, titratable acidity and redox potential during manufacture and storage of yoghurt. Also, there was a notable difference in the counts of *S. thermophilus, L. acidophilus* and bifidobacteria. The time to reach a pH of 4.5 increased considerably on addition of 250 and 500 $mg.L^{-1}$ cysteine, whereas the incubation time decreased in yoghurt mixes supplemented with WPC, ACH or tryptone. The redox potential remained negative during refrigerated storage in yoghurt supplemented with 250 or 500 $mg.L^{-1}$ cysteine. Viability of *S. thermophilus* was adversely affected, whereas that of *L. acidophilus* was improved on addition of cysteine in yoghurt made from C3 starter culture. The counts of *L. acidophilus* remained > 10^5 $cfu.g^{-1}$ throughout the storage in all yoghurts. More than 3 log cycles reduction in counts of bifidobacteria was observed when the pH reached 4.5 in control yoghurt and that supplemented with WP. The viability of bifidobacteria improved to a variable extent in yoghurt supplemented with cysteine, WPC, ACH or tryptone and was highest in yoghurt supplemented with WPC1. Whey protein concentrates are well known for their buffering capacity which may be beneficial in reducing the toxic effects of organic acids to the bifidobacteria present in yoghurt made with C3 starter culture. Electron microscopy revealed that incorporation of higher levels of cysteine (500 $mg.L^{-1}$) in yoghurt mix affected the cell membrane and cell wall of *S. thermophilus* cells. SDS-PAGE and amino acid analyses confirmed that nitrogen source in the form of peptides and amino acids correlated with improved viability of bifidobacteria in yoghurt made with a commercial starter culture (C3) which showed a dramatic decline in the counts of this organism in our previous studies. It is concluded that the nitrogen source in the form of peptides or amino acids might be playing a crucial role for improving the viability of bifidobacteria in yoghurt made with C3 (ABT-1) starter culture.

Table 6.3.1. Protein and total solids contents of yoghurt supplemented with various ingredients

Composition	Control[1]	Cy^{2}_{50}	Cy_{250}	Cy_{500}	WP[3]	WPC[4]1	WPC2	ACH^{5}_{250}	Try^{6}_{250}
Protein (%)	3.85 ± 0.08	3.85 ± 0.06	3.87 ± 0.05	3.90 ± 0.09	3.75 ± 0.10	4.55 ± 0.12	4.50 ± 0.13	3.85 ± 0.06	3.87 ± 0.05
Total solids (%)	15.55 ± 0.12	15.50 ± 0.15	15.50 ± 0.18	15.45 ± 0.26	15.69 ± 0.23	15.75 ± 0.29	15.70 ± 0.35	15.49 ± 0.11	15.40 ± 0.15

[1]Yoghurt with 2% SMP; [2]Yoghurt containing 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; [3]Yoghurt containing 2% whey powder; [4]Yoghurt supplemented with 2% whey protein concentrates 1 and 2, respectively; [5]Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; [6]Yoghurt supplemented with 250 mg

Table 6.3.2. Changes in counts of *S. thermophilus* during manufacture of yoghurt supplemented with various ingredients

Incubation period (h)	*Control*[1]	*Cy*[2]*50*	*Cy250*	*Cy500*	*WP*[3]	*WPC*[4]*1*	*WPC2*	*ACH*[5]*250*	*Try*[6]*250*
				----cfu/g----					
0	2.2×10^6	2.2×10^6	2.2×10^6	2.2×10^6	1.99×10^6	2.1×10^6	2.0×10^6	2.3×10^6	2.3×10^6
3	57×10^6	32×10^6	12×10^6	8×10^6	133×10^6	700×10^6	415×10^6	148×10^6	159×10^6
6	482×10^6	65×10^6	39×10^6	38×10^6	1700×10^6	1400×10^6	1350×10^6	900×10^6	1000×10^6
9	1130×10^6	550×10^6	72×10^6	45×10^6	1900×10^6	1400×10^6	1400×10^6	1420×10^6	2000×10^6
12	780×10^6	1260×10^6	470×10^6	268×10^6	950×10^6	1140×10^6	1960×10^6	1350×10^6	1900×10^6
15	850×10^6	910×10^6	460×10^6	250×10^6	690×10^6	1040×10^6	1470×10^6	1200×10^6	1600×10^6
18	760×10^6	890×10^6	450×10^6	144×10^6	650×10^6	950×10^6	1630×10^6	1030×10^6	1410×10^6
21	600×10^6	680×10^6	360×10^6	125×10^6	680×10^6	620×10^6	1150×10^6	960×10^6	850×10^6
24	460×10^6	450×10^6	260×10^6	180×10^6	600×10^6	360×10^6	950×10^6	710×10^6	530×10^6

[1]Yoghurt with 2% SMP; [2]Yoghurt containing 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; [3]Yoghurt containing 2% whey powder; [4]Yoghurt supplemented with 2% whey protein concentrates 1 and 2, respectively; [5]Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; [6]Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix.

Table 6.3.3. Changes in counts of *L. acidophilus* during manufacture of yogurt supplemented with various ingredients

Incubation period (h)	*Control*[1]	*Cy*[2]*50*	*Cy250*	*Cy500*	*WP*[3]	*WPC*[4]*1*	*WPC2*	*ACH*[5]*250*	*Try*[6]*250*
				----cfu/g----					
0	2.6×10^6	2.8×10^6	2.8×10^6	2.8×10^6	2.5×10^6	2.5×10^6	2.5×10^6	2.5×10^6	2.5×10^6
3	3.4×10^6	4×10^6	3.7×10^6	3.8×10^6	3.8×10^6	3.4×10^6	3.6×10^6	4.0×10^6	3.6×10^6
6	12.8×10^6	12.2×10^6	14.3×10^6	18.1×10^6	8.5×10^6	19×10^6	15×10^6	19×10^6	16.9×10^6
9	22×10^6	29×10^6	31.5×10^6	37×10^6	11×10^6	46×10^6	28×10^6	96×10^6	120×10^6
12	64×10^6	60×10^6	58×10^6	76×10^6	31×10^6	89×10^6	62×10^6	140×10^6	268×10^6
15	80×10^6	76×10^6	61×10^6	96×10^6	44×10^6	113×10^6	79×10^6	200×10^6	300×10^6
18	126×10^6	89×10^6	97×10^6	101×10^6	90×10^6	118×10^6	104×10^6	280×10^6	344×10^6
21	165×10^6	99×10^6	113×10^6	210×10^6	138×10^6	153×10^6	116×10^6	260×10^6	350×10^6
24	222×10^6	142×10^6	174×10^6	387×10^6	166×10^6	186×10^6	170×10^6	270×10^6	289×10^6

[1]Yoghurt with 2% SMP; [2]Yoghurt containing 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; [3]Yoghurt containing 2% whey powder; Yoghurt supplemented with 2% whey protein concentrates 1 and 2, respectively; [5]Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; [6]Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix.

Table 6.3.4. Changes in counts of bifidobacteria during manufacture of yoghurt supplemented with various ingredients

Incubation period (h)	*Control*[1]	*Cy*[2]*50*	*Cy250*	*Cy500*	*WP*[3]	*WPC*[4]*1*	*WPC2*	*ACH*[5]*250*	*Try*[6]*250*
				----cfu/g----					
0	45×10^5	45×10^5	45×10^5	45×10^5	45×10^5	45×10^5	45×10^5	49×10^5	50×10^5
3	48×10^5	137×10^5	85×10^5	98×10^5	55×10^5	60×10^5	49×10^5	45×10^5	45×10^5
6	84×10^5	90×10^5	70×10^5	93×10^5	2×10^5	36×10^5	30×10^5	39×10^5	44×10^5
9	6×10^5	45×10^5	49×10^5	52×10^5	1.4×10^5	32×10^5	14×10^5	19×10^5	26×10^5
12	53×10^2	2×10^5	4.5×10^5	20×10^5	0.10×10^5	15×10^5	13×10^5	9×10^5	27×10^5
15	4.3×10^2	1×10^5	3.3×10^5	14×10^5	0.12×10^5	17×10^5	12×10^5	5×10^5	20×10^5
18	50×10^2	0.4×10^5	2.1×10^5	13×10^5	0.07×10^5	15×10^5	1.2×10^5	5×10^5	15×10^5
21	93×10^2	1.6×10^5	1.1×10^5	8×10^5	0.02×10^5	5.6×10^5	0.8×10^5	6×10^5	11×10^5
24	126×10^2	2.2×10^5	0.73×10^5	18×10^5	0.03×10^5	6.3×10^5	0.19×10^5	4.5×10^5	26×10^5

[1]Yoghurt with 2% SMP; [2]Yoghurt containing 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; [3]Yoghurt containing 2% whey powder; Yoghurt supplemented with 2% whey protein concentrates 1 and 2, respectively; [5]Yogurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; [6]Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix.

Table 6.3.5. Changes in counts of *S. thermophilus, L. acidophilus* and bifidobacteria during storage of yoghurt supplemented with various ingredients

Storage period (d)	*Control*[1]	*Cy*[2]*50*	*Cy250*	*Cy500*	*WP*[3]	*WPC*[4]*1*	*WPC2*	*ACH*[5]*250*	*Try*[6]*250*
	S. thermophilus				--------(cfu/g)---------				
0	640 x 10^6	730 x 10^6	140 x 10^6	82 x 10^6	790 x 10^6	830 x 10^6	820 x 10^6	1010 x 10^6	1000 x 10^6
10	690 x 10^6	990 x 10^6	105 x 10^6	70 x 10^6	680 x 10^6	890 x 10^6	960 x 10^6	1610 x 10^6	1790 x 10^6
25	540 x 10^6	1070 x 10^6	140 x 10^6	48 x 10^6	580 x 10^6	1040 x 10^6	1000 x 10^6	1490 x 10^6	1500 x 10^6
35	490 x 10^6	1080 x 10^6	130 x 10^6	39 x 10^6	520 x 10^6	1010 x 10^6	960 x 10^6	1500 x 10^6	1200 x 10^6
	L. acidophilus								
0	450 x 10^5	389 x 10^5	2150 x 10^5	1650 x 10^5	156 x 10^5	238 x 10^5	227 x 10^5	260 x 10^5	280 x 10^5
10	260 x 10^5	250 x 10^5	1270 x 10^5	990 x 10^5	120 x 10^5	157 x 10^5	123 x 10^5	135 x 10^5	129 x 10^5
25	48 x 10^5	75 x 10^5	548 x 10^5	489 x 10^5	59 x 10^5	27 x 10^5	44 x 10^5	52 x 10^5	50 x 10^5
35	5.1 x 10^5	13 x 10^5	59 x 10^5	100 x 10^5	2.8 x 10^5	3 x 10^5	2 x 10^5	6 x 10^5	8.9 x 10^5
	Bifidobacteria								
0	700	4.0 x 10^5	2.5 x 10^5	4.0 x 10^5	1500	33 x 10^5	8 x 10^5	0.85 x 10^5	9.0 x 10^5
10	500	3.8 x 10^5	2.1 x 10^5	2.9 x 10^5	1200	22 x 10^5	5.2 x 10^5	0.42 x 10^5	6.0 x 10^5
25	300	0.9 x 10^5	1.25 x 10^5	1.8 x 10^5	490	12 x 10^5	3.5 x 10^5	0.61 x 10^5	4.9 x 10^5
35	200	0.58 x 10^5	1.09 x 10^5	1.50 x 10^5	500	16 x 10^5	2.2 x 10^5	1.1 x 10^5	4.0 x 10^5

[1]Yoghurt with 2% SMP; [2]Yoghurt containing 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; [3]Yoghurt containing 2% whey powder; [4]Yoghurt supplemented with 2% whey protein concentrates 1 and 2, respectively; [5]Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; [6]Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix.

Table 6.3.6. Molecular weight and relative area of various bands of whey from yoghurt supplemented with various ingredients after SDS-PAGE analysis

Peak No.	*SM*[1]		*Control*[2]		*Cy*[3]*50*		*Cy250*		*Cy500*	
	MW[4] *(kD)*	*RA*[5]	*MW (kD)*	*RA*	*MW (kD)*	*RA*	*MW (kD)*	*RA*	*MW (kD)*	*RA*
1	97.4	13.5	35.8	2.3	37.3	3.3	37.9	12.8	40.1	7.4
2	66.2	19.7	27.7	17.1	33.2	3.5	33.8	5.4	36	3.6
3	45	7.7	21.8	2.5	28.7	13.1	30.4	8.3	32.3	7.3
4	31	9.8	18.8	22.6	22.4	5.6	28.4	7.2	30.5	5.5
5	21.5	22	15.8	11	18.9	18	24.4	3.7	27.7	2.2
6	14.4	27.4	14.4	7.1	15.8	7.3	23.1	10.3	26	7.2
7			13	3.5	14.1	4.8	20.3	15.2	24.5	8.6
8			11.4	10.4	13.1	2.1	16.2	2.1	21.8	13.2
9			10.5	23.5	10.3	42.3	14.5	4.6	17	1.2
10							12.3	7.2	14.5	10.5
11							10.5	23.4	13	8.3
12									11.1	25.2

Peak No.	*WP*[6]		*WPC*[7]*1*		*WPC2*		*ACH*[8]*250*		*TRY*[9]*250*	
	MW (kD)	*RA*	*MW (kD)*	*RA*	*MW (kD)*	*RA*	*MW (kD)*	*RA*	*MW (kD)*	*RA*
1	44.2	14.8	46	12.9	106	1.3	44.1	4	43.9	2.3
2	38.1	3.4	40.8	3.7	79.7	1.9	40.1	1.9	40.2	1.1
3	33	12.4	35.2	3.6	50.4	13.6	34.4	17.2	34.4	7.2
4	26.7	12.2	28.9	5.5	41.8	3.5	27.1	8.1	28.1	3.8
5	23.6	12.6	25.8	15.9	31.8	13.4	23.8	19.9	23	22.9
6	19.8	4.1	21.8	4.4	27.5	30	20.5	5.6	20.3	19.4
7	16.2	13.7	18	26.4	16.4	9.4	17.8	7	18.5	11.9
8	13.3	26.8	15.5	27.6	12.5	27	16.6	7.3	16.9	5
9							13.3	31	14.1	26.5

[1]Standard markers; [2]Yoghurt with 2% nonfat dry milk; [3]Yoghurt containing 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; [4]Molecular weight; [5]Relative area; [6]Yoghurt containing whey powder; [7]Yoghurt supplemented with whey protein concentrates 1 and 2, respectively; [8]Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; [9]Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix.

Table 6.3.7. Protein content and amino acid profile of whey protein concentrates, acid casein hydrolysate and tryptone

Content	WPC[1]1	WPC2	ACH[2]	Tryptone
% Protein	73.5	72.5	49.6	82.1
Aspartic acid	8.15	8.46	3.99	6.11
Threonine	4.58	6.25	2.24	3.99
Serine	4.11	4.76	2.65	5.48
Glutamic acid	13.01	13.86	13.07	19.11
Proline	4.32	5.01	6.09	9.39
Glycine	1.56	1.56	1.12	1.62
Alanine	4.19	4.21	2.44	2.74
Valine	4.56	5.09	4.03	6.24
Methionine	1.85	1.79	1.30	2.53
Isoleucine	4.30	5.27	2.94	4.89
Leucine	9.04	8.30	4.93	7.98
Tyrosine	2.83	2.70	2.14	1.73
Phenylalanine	2.92	2.68	2.30	4.15
Lysine	7.43	7.34	4.70	6.98
Histidine	1.50	1.40	1.47	2.42
Ammonia	1.07	1.29	0.33	1.52
Arginine	2.25	1.96	2.02	3.15
Cystine and Cysteine	2.16	2.01	0.16	0.27
Tryptophan	1.52	1.41	nd	0.91
Protein recovery	94.9	101.0	96.5	97.4

[1]Whey protein concentrates 1 and 2, respectively;
[2]Acid casein hydrolysate.

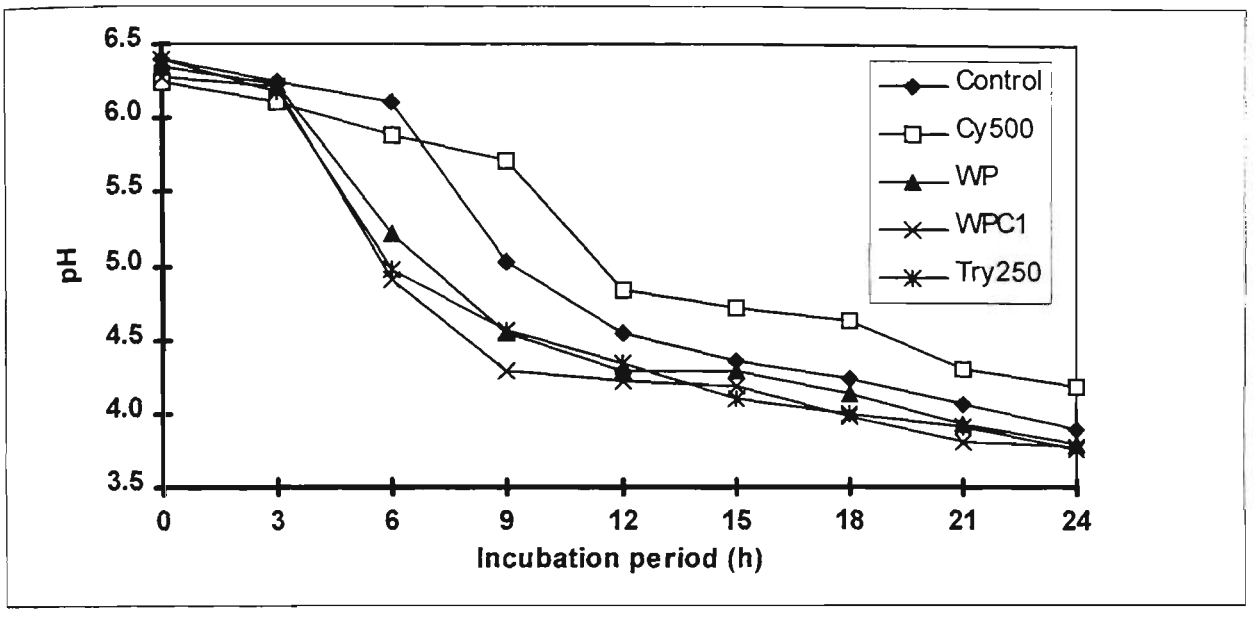


Fig. 6.3.1. Changes in pH during 24 h fermentation of yoghurt mix with C3 starter culture (Control=Yoghurt with 2% SMP; Cy_{500}=Yoghurt supplemented with 500 mg cysteine per litre of yoghurt mix; WP=Yoghurt supplemented with 2% whey powder; WPC1 =Yoghurt supplemented with 2% WPC1; Try_{250}=Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix).

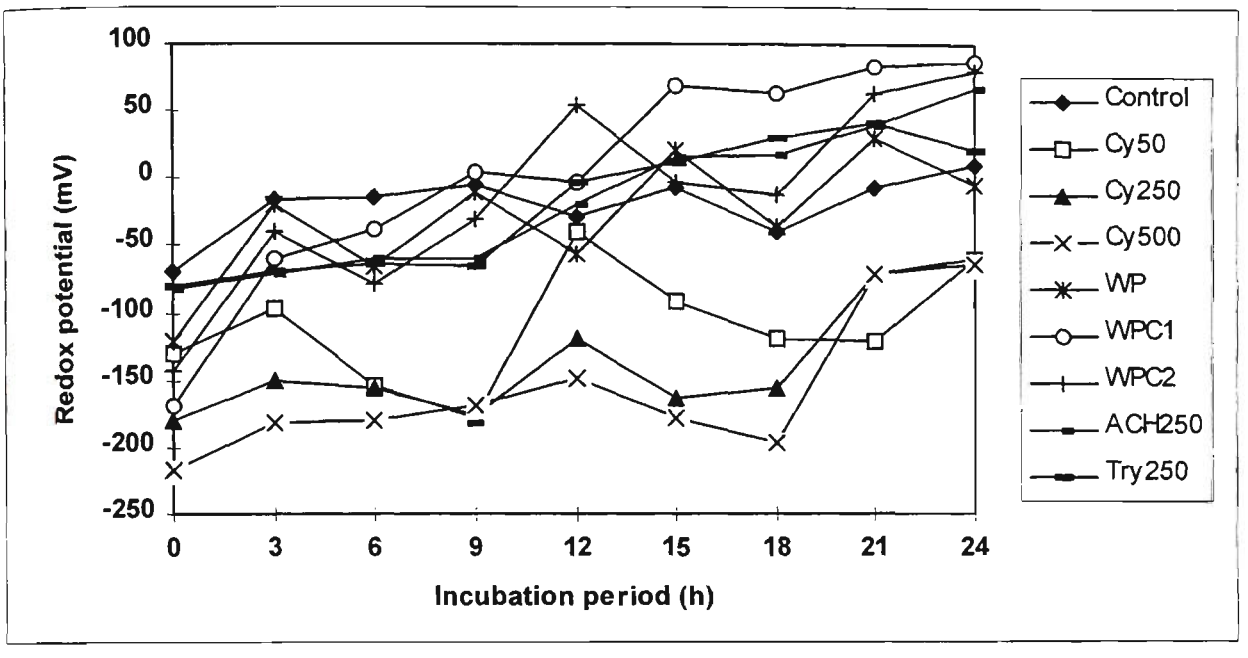


Fig. 6.3.2. Changes in redox potential (mV) during 24 h fermentation of yoghurt mix with C3 starter culture (Control=Yoghurt with 2% SMP; Cy_{500}=Yoghurt supplemented with 500 mg cysteine per litre of yoghurt mix; WP=Yoghurt supplemented with 2% whey powder; WPC1 =Yoghurt supplemented with 2% WPC1; Try_{250}=Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix).

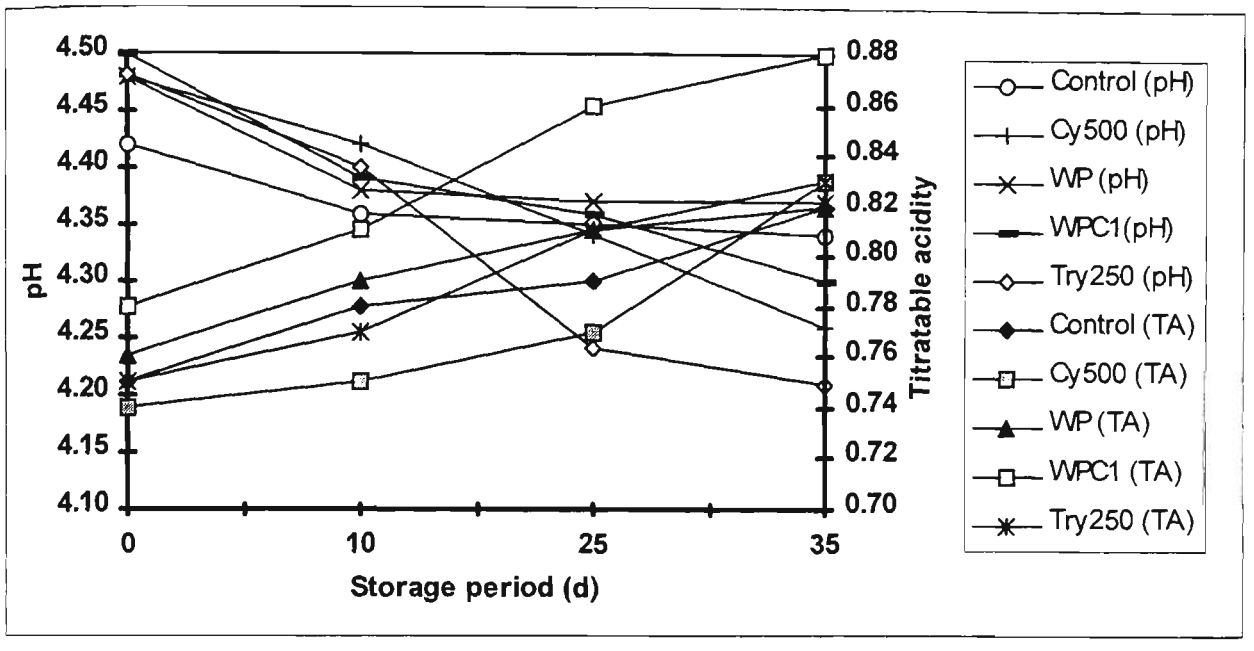


Fig. 6.3.3. Changes in pH and titratable acidity (% lactic acid) during refrigerated storage of yoghurt supplemented with various ingredients (Control=Yoghurt with 2% SMP; Cy_{500}=Yoghurt supplemented with 500 mg cysteine per litre of yoghurt mix; WP=Yoghurt supplemented with 2% whey powder; WPC1 =Yoghurt supplemented with 2% WPC1; Try_{250}=Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix).

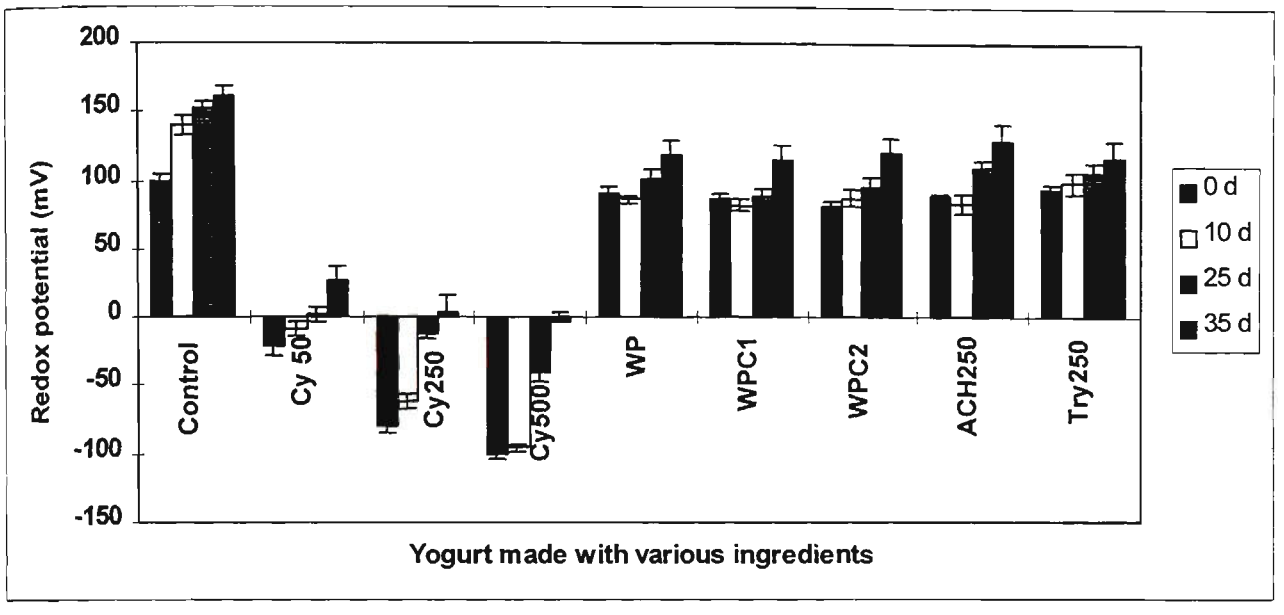


Fig. 6.3.4. Changes in redox potential (mV) of yoghurt during refrigerated storage (Control=Yoghurt with 2% SMP; Cy_{50}, Cy_{250} and Cy_{500}=Yoghurt supplemented with 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; WP=Yoghurt supplemented with 2% whey powder; WPC1 and WPC2=Yoghurt supplemented with 2% whey protein concentrates 1 and 2, respectively; ACH_{250}=Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; Try_{250}=Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix).

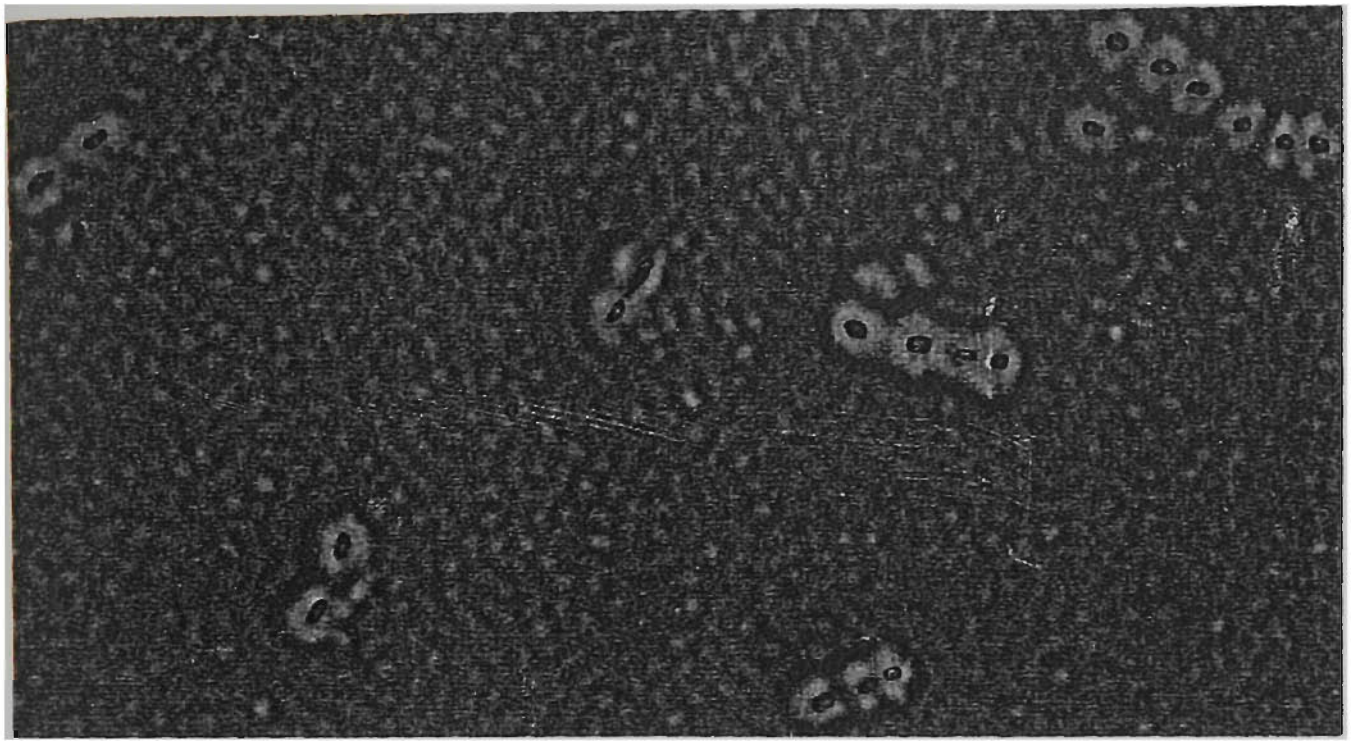


Fig. 6.3.5. Polysaccharide production by *S. thermophilus* as evident by Maneval's staining.

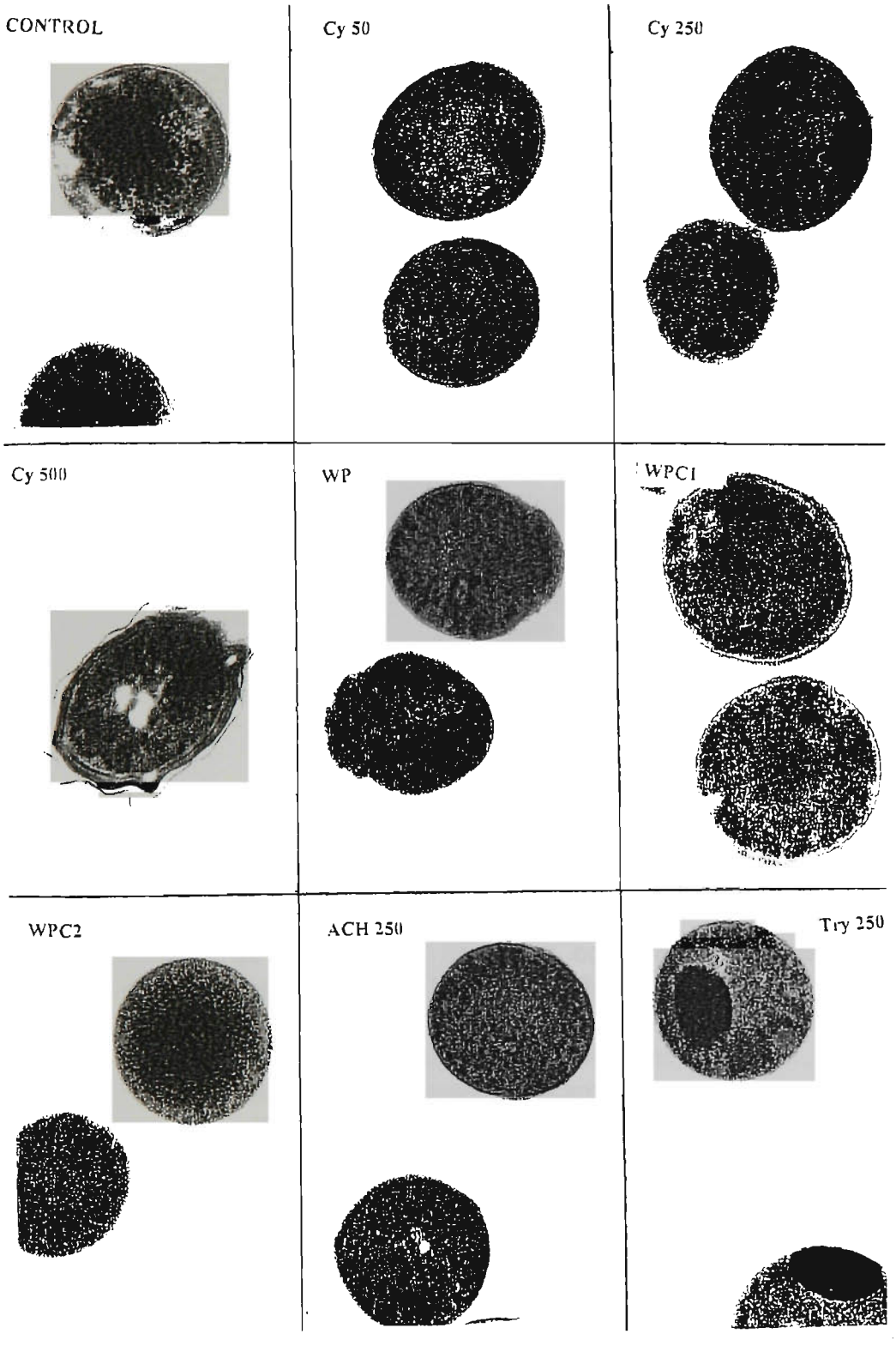


Fig. 6.3.6. Electron micrographs of *S. thermophilus* cells in yoghurt made with various ingredients (magnification=55,000 x) (Control=Yoghurt with 2% SMP; Cy_{50}, Cy_{250} and Cy_{500}=Yoghurt supplemented with 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; WP=Yoghurt supplemented with 2% whey powder; WPC1 and WPC2=Yoghurt supplemented with 2% whey protein concentrates 1 and 2, respectively; ACH_{250}=Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; Try_{250}=Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix).

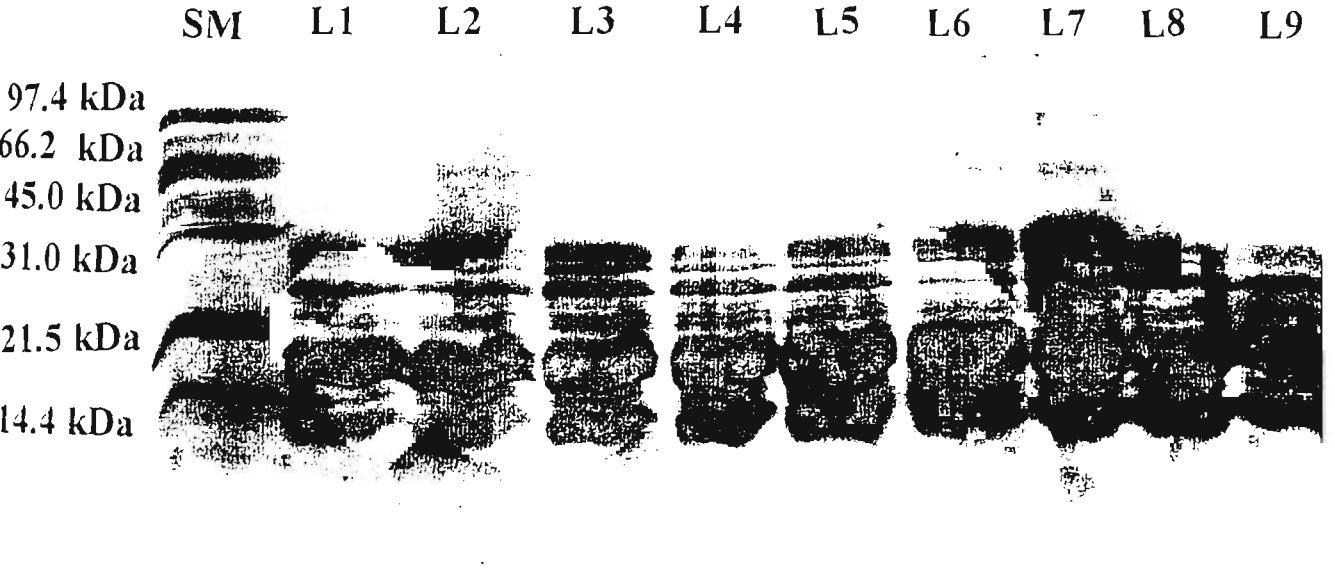


Fig. 6.3.7. Silver stain of SDS-PAGE of whey collected from various yoghurts (SM=Standard molecular weight markers; Lane 1=Yoghurt with 2% SMP; Lane 2, 3 and 4=Yogurt supplemented with 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; Lane5=Yoghurt supplemented with 2% whey powder; Lane 6 and 7=Yoghurt supplemented with 2% whey protein concentrates 1 and 2, respectively; Lane 8=Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; Lane 9=Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix).

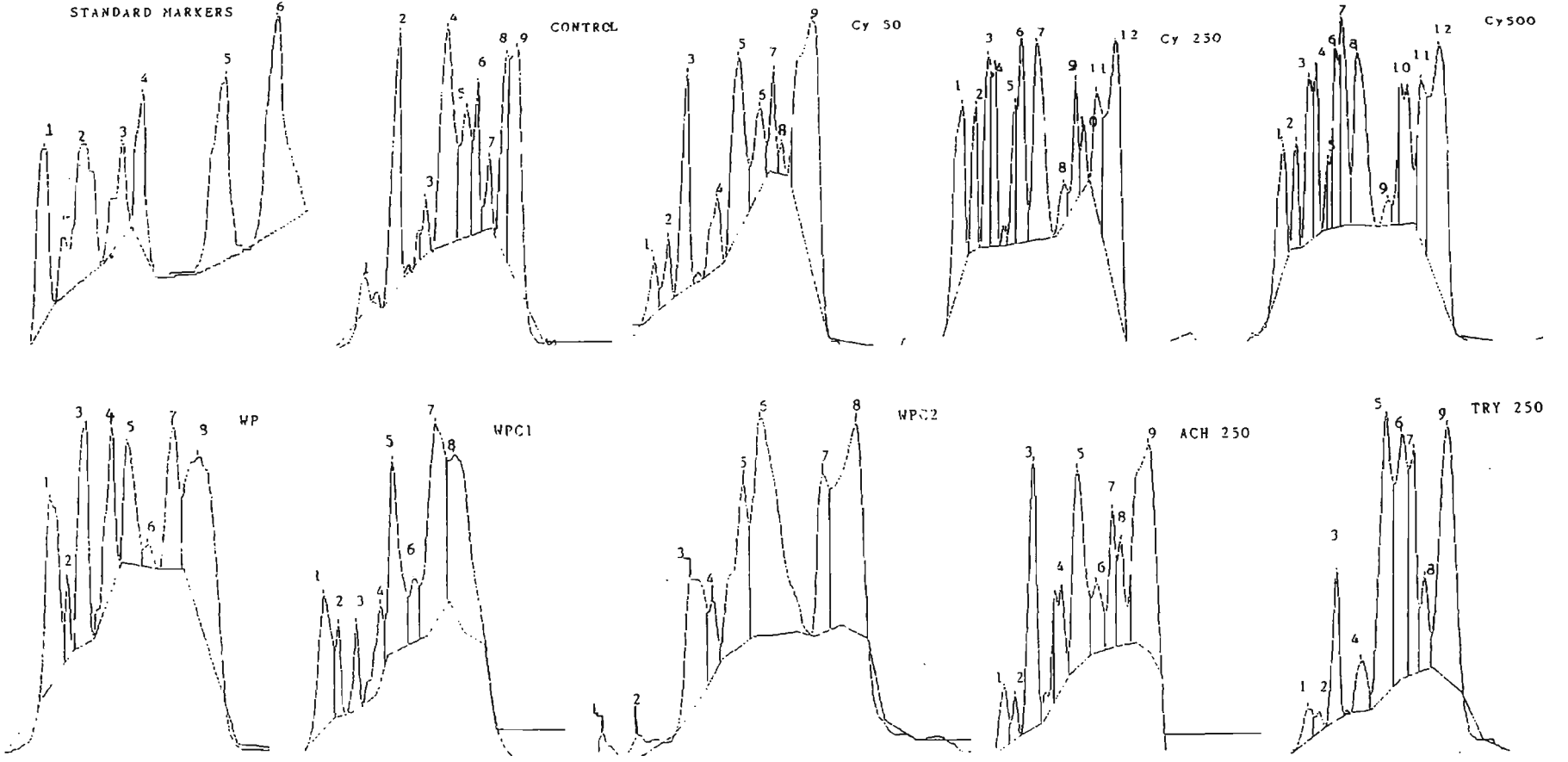


Fig. 6.3.8. Analyses of various bands of silver stain of SDS-PAGE of casein free fraction collected from various yoghurts using gel densitometer (SM=Standard molecular weight markers; Lane 1=Yoghurt with 2% SMP; Lane 2, 3 and 4=Yogurt supplemented with 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; Lane5=Yoghurt supplemented with 2% whey powder; Lane 6 and 7=Yoghurt supplemented with 2% whey protein concentrates 1 and 2, respectively; Lane 8=Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; Lane 9=Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix).

6.4 Effect of cysteine, whey powder, whey protein concentrates, acid casein hydrolysate and tryptone on textural characteristics of yoghurt

6.4.1 Introduction

Viability of AB bacteria is of paramount importance in marketing of AB products. However, in a previous study (Dave and Shah, 1997a), viability of bifidobacteria declined dramatically during manufacture of yoghurt with ABT-1 (*L. acidophilus*, bifidobacteria and *S. thermophilus*) starter culture that produces polysaccharides. Use of cysteine, whey protein concentrate (WPC) and tryptone improved the viability of the bifidobacteria in yoghurt made with the ABT-1 starter culture, possibly due to the peptides and amino acids present in these ingredients (Dave and Shah, 1998).

The type of starter cultures, incubation conditions, composition of yoghurt mix, and processing conditions influence the body and texture of yoghurt (Nielsen, 1975; Rasic and Kurmann, 1978; Chambers, 1979; Labropoulos *et al.*, 1981; Tamime and Robinson, 1985; Hassan *et al.*, 1996). Use of polysaccharide producing yoghurt starter culture is gaining popularity amongst yoghurt manufacturers (Tamime and Deeth, 1980). Gassem *et al.* (1995) and Griffin *et al.* (1996) reported that polysaccharide producing yoghurt bacteria were important determinants of yoghurt viscosity and texture. These starter cultures improve the viscosity of yoghurt leading to resistance to mechanical damage (Tamime and Deeth, 1980). The polysaccharide producing ABT yoghurt starter cultures have established their market in Australia and Europe. Hassan *et al.* (1996) studied the textural properties of yoghurt made with encapsulated ropy and non-ropy yoghurt cultures and concluded that the firmness and viscosity were dependent on the pH of yoghurt and the type of starter culture used to manufacture yoghurt. Rohm and Kovac (1994) also reported that the type of starter culture bacteria considerably affects the linear viscoelastic and physical properties of yoghurt gels.

A version of section 6.4 has been published. Dave, R. I. and Shah, N. P. (1998). *Aust. J. Dairy Technol.*, 53: 180-84..

Several studies report the potential of using whey products in fermented dairy products such as yoghurt (Tamime and Deeth, 1980). Rohm and Kneifel (1993) found that the whey seepage and gel firmness were affected in set yoghurt by the substrate used to enrich protein in yoghurt mix. Baig and Prasad (1996) reported that WPC could be used to replace dried skim milk in yoghurts as it improved the textural properties of the product without any adverse effect on the sensory properties. Marle and Zoon (1995) studied the viscosity of ropy starter cultures in stirred yoghurt. They scanned the yoghurt gels using a confocal scanning laser microscope and found that the ropy culture RR gave a regular protein network structure than a gel made by acidification with glucono-delta-lactone. Polysaccharide producing bacteria are reported to produce yoghurt gels of low fracturability and the apparent viscosity was dependent on the capsule size and bacterial counts (Kailasapathy and Supriadi, 1996). Tamime *et al.* (1995) compared the effects of whey protein-based substitute (Simplesse) and anhydrous milk fat (AMF) on microstructure, syneresis and firmness of set yoghurt and concluded that yoghurt made from Simplesse was softer than that made with AMF. No other significant difference was observed.

Cysteine, WPC, acid casein hydrolysate (ACH) and tryptone are expected to provide amino nitrogen source for *S. thermophilus* when *L. delbrueckii* ssp. *bulgaricus* is not incorporated in a mixed starter culture such as in ABT starter culture. Whey powder (WP) is the by-product of the cheese industry and is used to partially replace skim milk powder (SMP) in yoghurt. The problem of viability of bifidobacteria in the previous study (Dave and Shah, 1998) was overcome by adding cysteine, WPC, ACH or tryptone to the yogurt mix. It was observed that the incubation time was considerably affected and microbial population of yoghurt and probiotic bacteria varied due to varying action of these ingredients. These two factors have been reported to affect the texture of yoghurt (Nielsen, 1975; Rasic and Kurmann, 1978; Tamime and Deeth, 1980). The level of whey protein and casein and protein denaturation and interaction patterns will be different in yoghurt fortified with SMP to that with WP or WPC; this may have an influence on water holding capacity of yoghurt (Rasic and Kurmann, 1978; Tamime and

Deeth, 1980; Labropoulos *et al.*, 1981; Dannenberg and Kessler, 1988a, b; Mottar *et al.*, 1989). Overall, fortification of yoghurt mix with ingredients such as SMP or WPC is expected to have effects on textural properties of yoghurt, which are of commercial importance. Therefore in the present investigation, the effects of cysteine, WP, WPC, ACH and tryptone on the firmness, viscosity and protein network were studied in yoghurt made with an ABT starter culture.

6.4.2 Materials and methods

6.4.2.1 Starter cultures and yoghurt preparation

Four starter cultures described in section 3.1 were used in this study and yoghurt was prepared as described in section 3.4.5. Samples were prepared as described in section 3.5.2.

6.4.2.2 Analyses and time interval specifications

The yoghurt and yoghurt mix samples were analysed for pH (section 3.7.1.1), protein and total solids contents (section 3.7.1.5). The texture analyses of yoghurt samples at various time intervals (section 3.6) was carried out as described in section 3.10.

All the experiments and analyses presented in this part of chapter were replicated at least three times. The results shown are the averages of all data. The statistical analyses were carried out using Genstat 5 package. Significance was determined at $P < 0.001$ level.

6.4.3 Results discussion

6.4.3.1 Composition

The texture of yoghurt is reported to be dependent on the protein and solid contents of the product (Nielsen, 1975; Chambers, 1979; Tamime and Deeth, 1980; Tamime and Robinson, 1985; Rohm and Kneifel, 1993). The average protein and total solids contents of each product are given in Table 4.1. The protein content was significantly higher ($P < 0.001$) in yoghurt supplemented with WPC than that of control yoghurt containing SMP. The total solids contents were in the range of 15.50-15.75% for all the products and there was no difference in the solids content of the samples.

6.4.3.2 Texture analyses

Firmness. The firmness of yoghurt supplemented with various ingredients is shown in Fig. 6.4.1. Incorporation of WPC1 resulted in significant increase ($P < 0.001$) in the firmness of yoghurt. The depth of cone penetration was 295.5 and 304.4 mm, respectively for WPC1 and WPC2 as compared to 308.62 mm for the control yoghurt. Conversely, the firmness was significantly lower ($P < 0.001$) in yoghurt supplemented with 500 $mg.L^{-1}$ cysteine or WP.

The highest level of cysteine (500 $mg.L^{-1}$) inhibited the growth of *S. thermophilus* as reported previously (Dave and Shah, 1998) which may have produced less polysaccharides resulting in low firmness. Casein contributes to the firmness of the product and supplementation with WP decreased the ratio of casein to whey proteins which may have resulted in low firmness. The depth of cone penetration in yoghurt supplemented with ACH or tryptone was similar to that observed for control yoghurt. In general, the firmness was in the following order: WPC1 > WPC2 ≥ Cy_{50} = control = Try_{250} = ACH_{250} = Cy_{250} > WP > Cy_{500}. Increased firmness in yoghurts supplemented with WPC could be due to increase in protein content ($P < 0.001$) (Table 6.4.1) and increased water binding by denatured whey proteins in the finished product (Rasic and Kurmann, 1978; Tamime and Deeth, 1980; Labropoulos *et al.*, 1981; Dannenberg and

Kessler, 1988a, b). Labropoulos *et al.* (1981) observed that the structure or curd firmness of yoghurt were dependent on the extent of denaturation of whey proteins and reported that the yoghurt prepared with normal UHT milk gave lower firmness than the vat processed yoghurt mix due to the less denaturation of whey proteins in UHT milk.

Apparent viscosity. Changes in apparent viscosity of yoghurt samples during refrigerated storage are given in Fig. 6.4.2. As shown, significant differences ($P < 0.001$) were observed in viscosity of products supplemented with various ingredients. At 0 d, viscosity was in the following order: WPC1 > WPC2 > control = Try_{250} = ACH_{250} = Cy_{50} = Cy_{250} > Cy_{500}. At 10 d period, viscosity decreased in control yoghurt and that supplemented with WPC2 or ACH. The viscosity of control yoghurt increased ($P < 0.001$) (by ~30%) during further refrigerated storage of 10-30 d; possibly due to hydration of protein, mainly casein. With increased concentration of cysteine, the viscosity of yoghurt decreased as compared with control yoghurt. High levels of cysteine (250 and 500 $mg.L^{-1}$) affected the growth of polysaccharide producing *S. thermophilus* (Dave and Shah, 1998) which may have resulted in lower viscosity of the product. The difference in viscosity of control yoghurt and that supplemented with ACH or tryptone was not significant and followed the same pattern of increase during refrigerated storage of yoghurt. Contrarily, a significant difference ($P < 0.001$) in viscosity was observed in yoghurt supplemented with WP, WPC1 or WPC2. The viscosity was lowest in yoghurt supplemented with WP, but highest with WPC1 as compared with the control yoghurt. In general, the viscosity increased ($P < 0.001$) during 30 d refrigerated storage in all yoghurts except that supplemented with WPC2. However, the viscosity increased in yoghurt supplemented with WPC1 at 10 and 20 d period, but decreased at 30 d period and this decrease in viscosity was not significant. The differences in firmness and viscosity observed with the two types of WPC could be due to differences in their method of manufacture or due to compositional differences, especially the calcium content.

Microscopic texture analysis. Yoghurt prepared with high level of cysteine (250 or 500 $mg.L^{-1}$) and WPC had grainy texture as determined by subjective analysis of yoghurt, hence, a microscopic texture analysis was performed (Fig. 6.4.3). As shown, the control

yoghurt and that supplemented with ACH or tryptone gave a regular protein network structure with smaller flocs and pores. With increased concentration of cysteine, the flocs and pores size in yoghurt also increased, possibly due to lower production of polysaccharides by *S. thermophilus* in the finished product as discussed earlier. The texture of yoghurt supplemented with 50 $mg.L^{-1}$ cysteine and that supplemented with WP appeared similar and showed increased sizes of flocs and pores. On the other hand, incorporation of WPC1 or WPC2 resulted in very large flocs and pores. Thus, irregular protein network was observed in yoghurt supplemented with 500 $mg.L^{-1}$ cysteine, WPC1 or WPC2. Addition of WPC is expected to lower the ratio of casein to whey protein and the change in proportion of casein to whey proteins and the extent of denaturation of whey proteins have been reported to affect the texture of yoghurt (Nielsen, 1975; Chambers, 1979; Tamime and Deeth, 1980; Labropoulos *et al.*, 1981; Dannenberg and Kessler, 1988a, b; Mottar *et al.*, 1989).

Nielsen (1972) reported that heat treatment, type of stabiliser and starter culture and incubation temperature and time were the crucial factors that decided the size of the granules in yoghurt. The fermentation with ABT starter cultures is mainly due to *S. thermophilus*. Addition of high levels of cysteine inhibited the growth of polysaccharide producing *S. thermophilus* (Dave and Shah, 1998) which might have lowered polysaccharide production leading to decreased viscosity and firmness of the product. On the other hand, increased water binding by whey proteins (Rasic and Kurmann, 1978; Tamime and Deeth, 1980) might have resulted in increased viscosity of yoghurts supplemented with WPC. Whey proteins are denatured during heat treatment and β-lactoglobulin and κ-casein interact and undergo changes; this improves the body and texture of yoghurt (Rasic and Kurmann, 1978; Tamime and Robinson, 1985). Addition of WP and WPC would change the proportion of whey proteins to casein. Also, the methods of manufacture of WP and WPC will lead to differences in their composition. All these factors might have contributed to the differences in firmness, viscosity, protein network and microstructure of yoghurt supplemented with various ingredients.

Tamime *et al.* (1984) reported some differences in the microstructure of set-style yoghurt manufactured from cow's milk that was fortified by various methods to give similar protein content. Mottar *et al.* (1989) found that the proportion of β-lactoglobulin and α-lactalbumin bound to casein was dependent on the heating process and heat intensity. There was a direct relationship between the nature of casein micelle surface, as affected by adsorption of heat-denatured whey proteins, and the texture of yoghurt. Similarly, Dannenberg and Kessler (1988a, b) reported that syneresis, firmness, flow properties and gel network during fermentation of yoghurt mix was dependent on the extent of denaturation of β-lactoglobulin.

6.4.4 Conclusions

Addition of cysteine, WP, WPC, ACH and tryptone had significant effects on firmness, viscosity and microstructure of yoghurt. The firmness and viscosity of yoghurts improved on addition of WPC; however, irregular protein network resulted with WPC and high level of cysteine (500 $mg.L^{-1}$). The viscosity and firmness of yoghurt were significantly lower ($P < 0.001$) in yoghurt supplemented with cysteine and WP. The firmness, viscosity and yoghurt microstructure were similar in yoghurt supplemented with ACH and tryptone. Addition of WPC1 significantly increased ($P < 0.001$) the firmness and viscosity of yoghurt, but resulted in irregular protein network. However, this irregular network may not be that important in stirred yoghurt as the processing conditions may take care of this problem. Overall, yoghurt made with ACH or tryptone did not have any adverse effect on texture of yoghurt and also improved the viability of bifidobacteria as reported earlier.

Table 6.4.1. Protein and total solids contents of yoghurt supplemented with various ingredients

Composition	Control[1]	Cy^{2}_{50}	Cy_{250}	Cy_{500}	WP[3]	WPC[4]1	WPC2	ACH^{5}_{250}	Try[6]
Protein (%)	3.80[a]	3.82[a]	3.85[a]	3.88[a]	3.79[a]	4.50[b]	4.52[b]	3.80[a]	3.85[a]
Total solids (%)	15.50	15.50	15.45	15.40	15.66	15.70	15.75	15.40	15.5

[1]Yoghurt with 2% SMP; [2]Yoghurt containing 50, 250 and 500 mg cysteine per liter of yoghurt mix, respectively; [3]Yoghurt containing 2% whey powder; [4]Yoghurt supplemented with 2% whey protein concentrates 312 and 392, respectively; [5]Yoghurt supplemented with 250 mg acid casein hydrolysate per liter of yoghurt mix; [6]Yoghurt supplemented with 250 mg tryptone per liter of yoghurt mix.

[a,b]Means with unlike superscripts differ ($P < 0.001$).

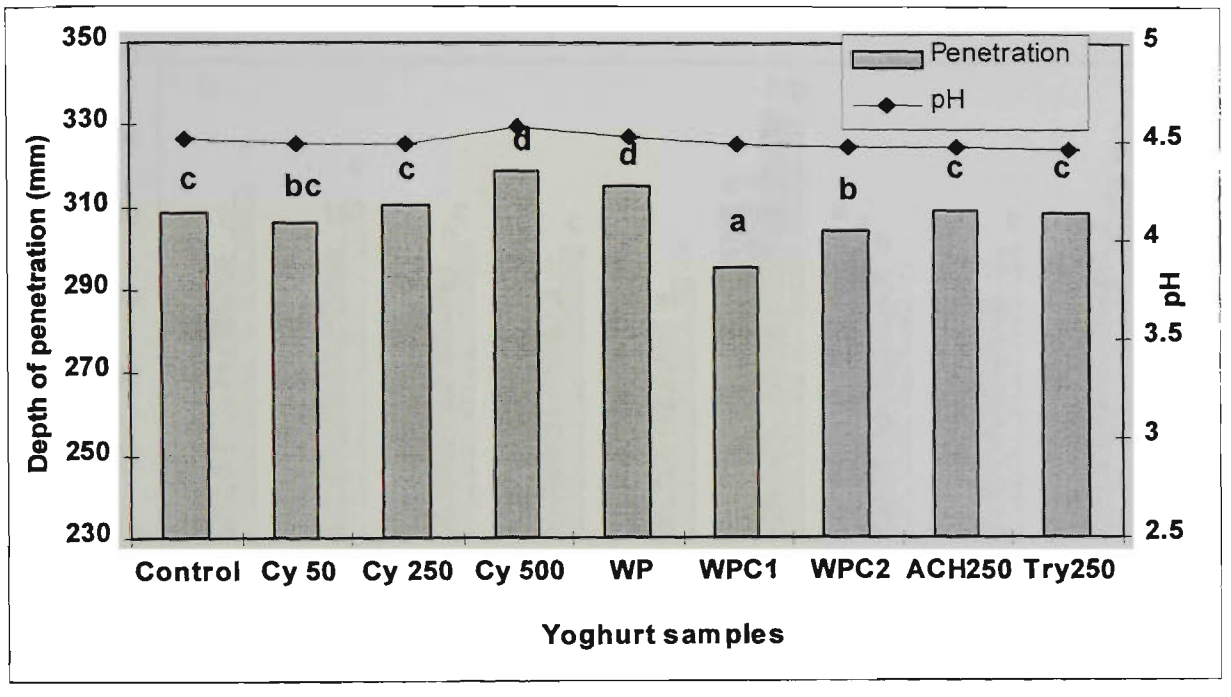


Fig. 6.4.1. Firmness of yoghurt measured as penetration depth (mm) affected by supplementation with various ingredients (Control=Yogurt with 2% SMP; Cy_{50}, Cy_{250} and Cy_{500}=Yoghurt supplemented with 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; WP=Yoghurt supplemented with 2% whey powder; WPC1 and WPC2=Yoghurt supplemented with 2% whey protein concentrates No. 312 and 392, respectively; ACH_{250}=Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; Try_{250}=Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix).

a, b, c, d = Means with unlike letters differ ($P < 0.001$).

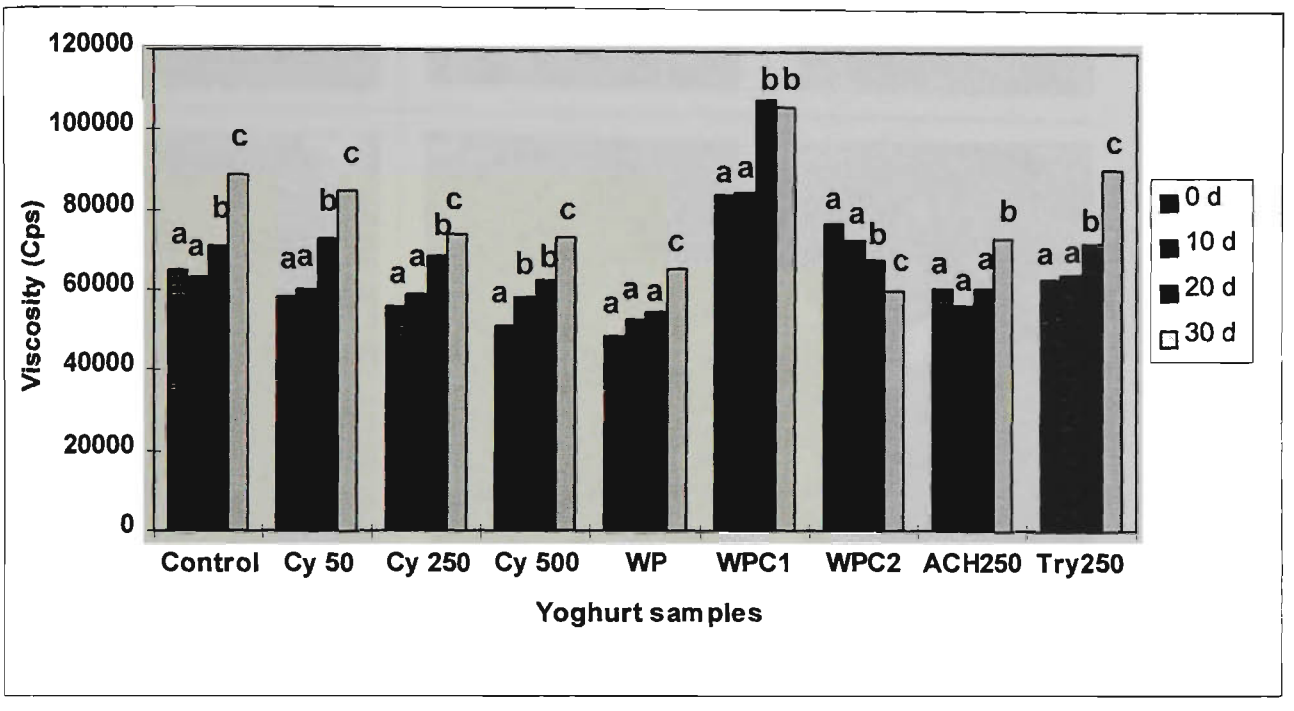


Fig. 6.4.2. Changes in viscosity (Cps) during refrigerated storage (4°C) of yoghurt supplemented with various ingredients (Control=Yoghurt with 2% SMP; Cy_{50}, Cy_{250} and Cy_{500}=Yoghurt supplemented with 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; WP=Yoghurt supplemented with 2% whey powder; WPC1 and WPC2=Yoghurt supplemented with 2% whey protein concentrates No. 312 and 392, respectively; ACH_{250}=Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; Try_{250}=Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix).

a, b, c = Means of viscosity values over 30 d storage period with unlike letters differ ($P < 0.001$).

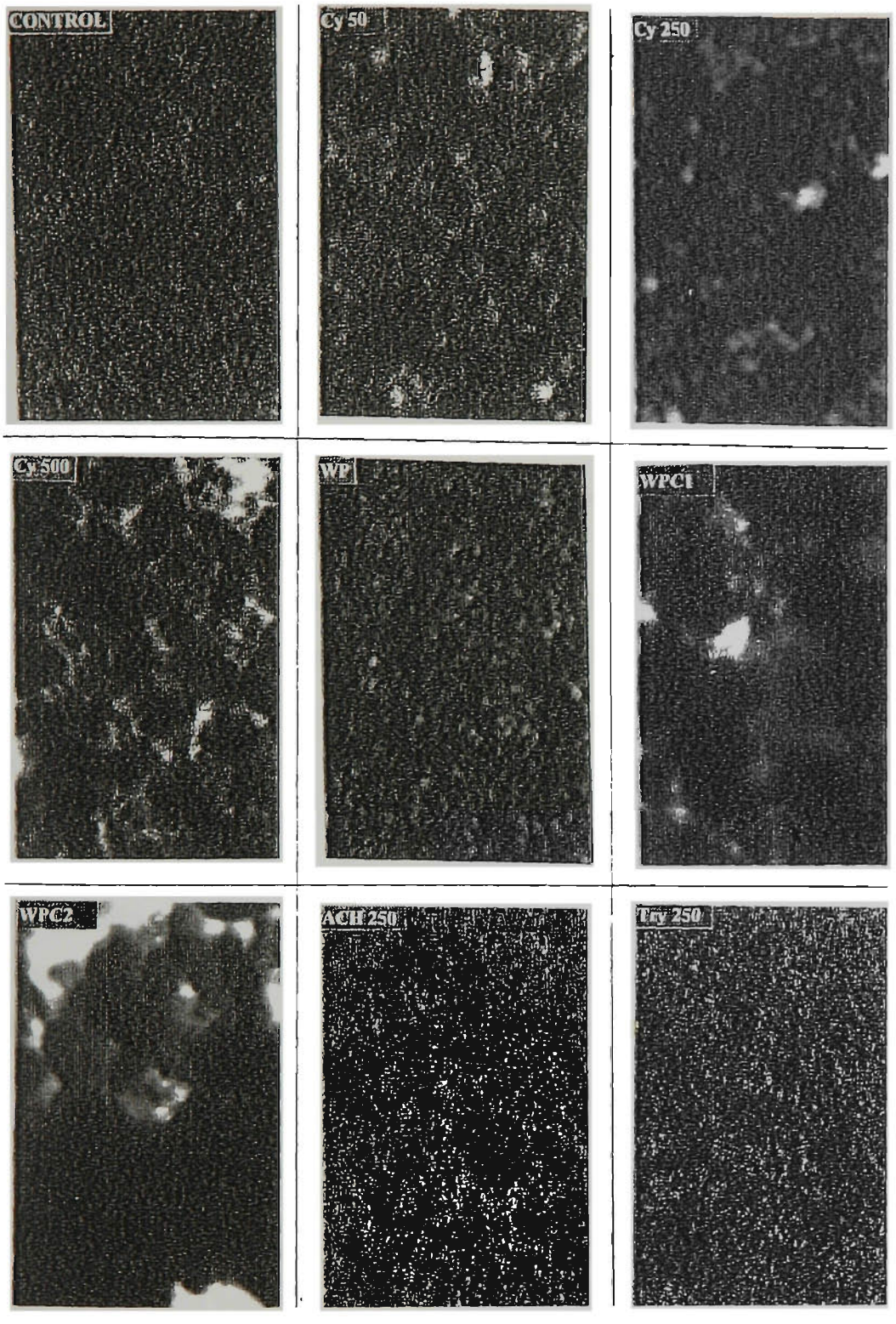


Fig. 6.4.3. Microscopic texture analysis of yoghurts supplemented with various ingredients (Control=Yoghurt with 2% SMP; Cy_{50}, Cy_{250} and Cy_{500}=Yoghurt supplemented with 50, 250 and 500 mg cysteine per litre of yoghurt mix, respectively; WP=Yoghurt supplemented with 2% whey powder; WPC1 and WPC2=Yoghurt supplemented with 2% whey protein concentrates No. 312 and 392, respectively; ACH_{250}=Yoghurt supplemented with 250 mg acid casein hydrolysate per litre of yoghurt mix; Try_{250}=Yoghurt supplemented with 250 mg tryptone per litre of yoghurt mix).

7.0 ANTIMICROBIAL SUBSTANCES PRODUCED BY YOGHURT AND PROBIOTIC BACTERIA

7.1 Antagonism between yoghurt bacteria and probiotic bacteria isolated from commercial starter cultures, commercial yoghurts and a probiotic capsule

7.1.1 Introduction

Well before the existence of bacteria was recognised, bacterial activities were used in preserving dairy foods. During growth in fermented products, dairy starters including lactobacilli, lactococci, leuconostocs, streptococci, propionibacteria and bifidobacteria are reported to produce inhibitory metabolites, including organic acids, hydrogen peroxide and bacteriocins (Barefoot and Nettles, 1993).

According to Tagg *et al.* (1976), bacteriocins are bactericidal or bacteriostatic compounds containing a biologically active protein moiety. Lactic acid bacteria (LAB) have been studied extensively for bacteriocinogenic activity. Over the past few years, much attention has been given to biochemical and genetic characterisation of bacteriocins produced by LAB (Barefoot and Nettles, 1993). Many strains of LAB associated with dairy, meat, and vegetable fermentations have been isolated and their bacteriocins characterised.

In previous studies (Dave and Shah, 1997a, b; Shah *et al.*, 1995), it was reported that viability of probiotic bacteria was dependent on associative yoghurt organisms. In these studies the counts of bifidobacteria were dramatically reduced in one of four commercial starter cultures during yoghurt manufacture and increased inoculum did not improve their viability (Dave and Shah, 1997a, b). The inhibition was not due to pH or hydrogen peroxide. The viability of the same strain of bifidobacteria was satisfactory in three other commercial starter cultures, in which the yoghurt bacterial strains differed.

A version of section 7.1 has been published. Joseph, P., Dave, R. I. and Shah, N. P. (1998). *Food Australia*, **50**: 20-23.

Information pertaining to bacteriocins produced by organisms in probiotic products available on Australian markets is limited. The aims of the present study were to isolate yoghurt bacteria and probiotic bacteria from some probiotic starter cultures and probiotic products sold in Australia, and to screen these organisms for inhibitory activity against each other.

7.1.2 Materials and methods

7.1.2.1 Bacterial strains and commercial products

The details of bacterial strains and commercial strains used are given in section 3.2.1.

7.1.2.2 Isolation, identification and maintenance of bacterial cultures

Appropriate dilutions of samples were prepared in sterile 0.1% peptone and water diluent and pour plated for enumeration. *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and bifidobacteria were isolated and enumerated using *Streptococcus thermophilus* (ST) agar, deMann Rogosa Sharpe (MRS) agar (pH 5.2), MRS-salicin agar and MRS-NNLP (nalidixic acid, neomycin sulfate, lithium chloride and paromomycin sulfate) agar, respectively (Dave and Shah, 1996). Selected colonies were streak-plated twice on appropriate media for purification and isolated colonies were identified by use of morphological, cultural and bio-chemical characteristics (Hardie, 1986; Kandler and Weiss, 1986; Scardovi, 1986). The maintenance of pure cultures was carried out as described in section 3.2.

7.1.2.3 Detection and assay of inhibitory activity

Preliminary screening of inhibitory substance/s was carried on solid media using a modification of the spot on lawn technique and in liquid media by the well-diffusion technique (section 3.13). The cell-free supernatant of putative producer organisms that

tested positive after the above treatments was again neutralised (pH 6.0) with 2 M NaOH, treated with catalase and tested for sensitivity to proteolytic enzymes such as chymotrypsin and papain (final concentrations 1.0 $mg.mL^{-1}$). Liquid broth without any growth of organism was used as control. The control and enzyme containing samples were incubated for 2 h at 37C in a water bath to allow for a reaction and assayed for inhibitory zones.

The experiments were carried out in triplicates and the results reported in this part of study were based on all available data of three replications.

7.1.3 Results and discussion

From the 4 freeze-dried commercial yoghurt starter cultures (C1, C2, C3 and C4), 3 commercial yoghurts and 1 probiotic capsule, 7 isolates of *S. thermophilus* (ST 1-7), 5 isolates of *L. delbrueckii* ssp. *bulgaricus* (LB 1-2, 5-7), 8 isolates of *L. acidophilus* (LA 1-8) and 8 strains of bifidobacteria (BB 1-8) were isolated (Table 7.1.1). The protocols given by Dave and Shah (1996) successfully isolated the four groups of organisms selectively. All the isolates agreed with the published results of Gram stain, catalase reaction, cell morphology and fermentation of esculin, fructose, galactose, glucose, lactose, maltose, salicin and sucrose (Hardie,1986; Kandler and Weiss, 1986; Scardovi, 1986).

All the 28 isolates were screened against the same 28-isolate panel as indicator organisms. Inhibition of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* or bifidobacterial strains was not observed by any of the *S. thermophilus* strains. Inhibition of one strain of *S. thermophilus* (ST-6) was observed by 2 strains of *L. delbrueckii* ssp. *bulgaricus* (LB-5 and LB-7). Amongst *L. delbrueckii* ssp. *bulgaricus*, LB-5 strain was inhibited by LB-7 and two of the bifidobacteria (BB-7 and 8). These inhibitions were observed during initial screening with modified spot on lawn technique, but not observed with agar well assay technique.

Inhibition of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and bifidobacteria by *L. acidophilus* strains is given in Table 7.1.2. These are results of inhibition zones that were well-defined during the initial screening of cultures using modified spot on lawn technique. The inhibition caused by the producer organisms could have been due to hydrogen peroxide, organic acids, or due to a BLIS.

Inhibition of 4 strains of *S. thermophilus* (ST-1, ST-2, ST-3 and ST-7) was observed by all the 8 isolates of *L. acidophilus*. Inhibition of 2 *S. thermophilus* isolates (ST-5 and ST-6) with any isolates of *L. acidophilus* was not observed. Inhibition of *S. thermophilus* (ST-4) by 2 isolates of *L. acidophilus* (LA-6 and LA-7) was also not observed and the zones of inhibition with the other *L. acidophilus* were smaller and unclear for 3 *S. thermophilus* isolates (ST-1, ST-2 and ST-7). The inhibitory zones for *S. thermophilus* by *L. acidophilus* were not as clear as observed for *L. delbrueckii* ssp. *bulgaricus*. The diameter of zones was different with various *L. acidophilus* strains for all target *S. thermophilus* isolates. *S. thermophilus* isolates ST-5 and ST-6 did not show inhibition by any of the *L. acidophilus*.

Inhibition of all 5 isolates of *L. delbrueckii* ssp. *bulgaricus* was observed with 7 out of 8 isolates of *L. acidophilus*. The zones of inhibition were clear and distinct for *L. delbrueckii* ssp. *bulgaricus* isolates as shown in Figures 7.1.1 and 7.1.2. Inhibition of *L. delbrueckii* ssp. *bulgaricus* isolates was strong as compared to *S. thermophilus* or bifidobacterial strains, based on the size of zone of inhibition. Amongst *L. delbrueckii* ssp. *bulgaricus*, the sensitivity was found to be different and also varied with various *L. acidophilus* strains. All the isolates of *L. acidophilus* were resistant and did not show inhibition by any of the *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus, L. acidophilus* or bifidobacterial isolates. Bifidobacterial strains BB-5 and BB-7 were inhibited by 7 out of 8 strains of *L. acidophilus*. *L. acidophilus* (strain LA-7) did not show inhibition of 1 *S. thermophilus* (ST-4) isolate or any of the *L. delbrueckii* ssp. *bulgaricus* or bifidobacterial strains. Inhibition zones for 3 *S. thermophilus* isolates (ST-1, ST-3 and ST-7) were

unclear. Zones of inhibition for 2 bifidobacterial isolates (BB-5 and BB-7) were < 9.0 mm with all *L. acidophilus* strains.

It was observed that all inhibitory activities (except for *L. delbrueckii* ssp. *bulgaricus* by *L. acidophilus* strains) was due to organic acids, but not due to a bacteriocin. Lowering pH of MRS broth to 4.0 with lactic acid resulted in similar inhibition of 5 *S. thermophilus* and 2 bifidobacterial isolates. Contrarily, *L. delbrueckii* ssp. *bulgaricus* were not inhibited by MRS broth at pH 4.0.

Inhibitory activity of *L. acidophilus* was lost when the MRS broth was treated with proteolytic enzymes, chymotrypsin and papain. This confirmed that an active protein moiety was involved in the inhibition of all 5 *L. delbrueckii* ssp. *bulgaricus* isolates by 7 isolates of *L. acidophilus.* Thus, *L. acidophilus* strains isolated from commercial starter cultures, commercial yoghurts and a probiotic capsule produced a bacteriocin like inhibitory substance (BLIS) which was active against *L. delbrueckii* ssp. *bulgaricus* strains incorporated into the products. Zones of inhibition were larger with modified spot on lawn technique as compared with agar well assay technique (Figures 7.1.1 and 7.1.2). Maintaining the pH of the culture medium at 5.5-6.0 resulted in increased production of the inhibitory substance (BLIS) by *L. acidophilus*. A possible explanation for this difference could be the effect of pH for growth of organism or due to production of BLIS. Organisms in the early stationary phase (18 h cultures) when transferred into a fresh medium containing optimum nutrients and favourable pH condition could have enhanced the production of the BLIS on the solid agar medium. Eckner (1992) reported that antimicrobial substances sometimes are produced on solid media only.

Dave and Shah (1997a, b) reported that a strain of bifidobacteria lost its viability in yoghurts made with commercial starter cultures that contained yoghurt bacteria and probiotic bacteria. A possible reason for such inhibition could not be due to the production of antimicrobial substances in yoghurt by associative organisms; because this was not observed in this study. *S. thermophilus* and some bifidobacteria have been

reported to be sensitive to low pH (Davis *et al.*, 1971; Martin and Chou, 1992; Lankaputhra *et al.*, 1996b). The inhibition observed for some of the *S. thermophilus* and bifidobacterial isolates could be due to sensitivity of these strains to acid. Other strains might be resistant to low pH (4.0) of broth or agar during assay using well assay technique or modified spot on lawn technique. Inhibition of bifidobacteria during yoghurt manufacture with one of the starter culture (Dave and Shah, 1997a, b) was not due to the production of antimicrobial substance by *S. thermophilus*.

7.1.4 Conclusions

Antagonism between yoghurt bacteria and probiotic bacteria isolated from 4 commercial starter cultures, 3 commercial yoghurts and a probiotic capsule was studied. *S. thermophilus* (7 strains), *L. delbrueckii* ssp. *bulgaricus* (5 strains), *L. acidophilus* (8 strains) and bifidobacteria (8 strains) were isolated and the isolates were used as both producer and indicator organisms for inhibitory activity. Seven of 8 strains of *L. acidophilus* produced antimicrobial substances that were active against all 5 strains of *L. delbrueckii* ssp. *bulgaricus*. The inhibitory substances produced by *L. acidophilus* were sensitive to proteolytic enzymes such as chymotrypsin and papain, and were active at neutral pH. When the pH of the culture broth was maintained between 5.5 and 6.0, there was increased production of a bacteriocin like inhibitory substance (BLIS) indicating that the pH of the culture broth had an effect on the production of BLIS. Inhibition of 5 strains of *S. thermophilus* and 2 strains of bifidobacteria was found to be due to organic acids (mainly lactic acid produced by these lactic acid bacteria) and not due to BLIS.

Table 7.1.1. Isolates of yoghurt and probiotic bacteria isolated from commercial starter cultures, yoghurts and a probiotic capsule

	Isolates			
Product	**ST[1]**	**LB[2]**	**LA[3]**	**BB[4]**
C1	+	+	+	+
C2	+	+	+	+
C3	+	-	+	+
C4	+	-	+	+
Bulla	+	+	+	+
Yo-plait	+	+	+	+
Eve Balance	+	+	+	+
Blackmore capsule	-	-	+	+

[1]ST= *S. thermophilus*; [2]LB= *L. delbrueckii* ssp. *bulgaricus*; [3]LA= *L. acidophilus*; [4]BB= bifidobacteria
+= isolated
= no isolate

Table. 7.1.2. Inhibition of *S. thermophilus, L. delbrueckii* ssp. *bulgaricus* and bifidobacteria by *L. acidophilus* isolates.

	Target Organism											
Producer Organism	*ST[1]-1*	*ST-2*	*ST-3*	*ST-4*	*ST-7*	*LB[2]-1*	*LB-2*	*LB-5*	*LB-6*	*LB-7*	*BB[4]-5*	*BB-7*
LA[3]-1	++	++	+(uc)	+	+(uc)	++++	++++	+++	+++	++++	+	+
LA-2	++	++	+(uc)	+	+(uc)	++++	++++	+++	+++	++++	+	+
LA-3	++	++	+(uc)	+	+(uc)	++++	++++	+++	+++	++++	+	+
LA-4	++	++	+(uc)	+	+(uc)	++++	++++	+++	+++	++++	+	+
LA-5	++	++	++	+	++	++	++	++	++	+++	+	+
LA-6	++	++	+(uc)	-	+(uc)	+++	+++	+++	+++	+++	+	+
LA-7	+(uc)	++	+(uc)	-	+(uc)	-	-	-	-	-	-	-
LA-8	++	+	++	+	++	++++	++++	+++	+++	++++	+	+

[1]ST= *S. thermophilus*; [2]LB= *L. delbrueckii* ssp. *bulgaricus*; [3]LA= *L. acidophilus*; [4]BB= bifidobacteria.

1 to 8 represents strains isolated from commercial starter cultures C1, C2, C3 and C4, and products of Bulla, Yo-plait, Eve Balance and Blackmore digestive capsules respectively.

Zone of inhibitions: +=up to 9.0 mm; ++=> 9.0 to 11.0 mm; +++=>11.0 to 13.0 mm; ++++=>13.0mm; - =no inhibition and uc= unclear inhibition.

Inhibition of ST-1, ST-2, all 8 strains of LA and BB-2, BB-4,5,6,7 was not observed by any of the LA isolates.

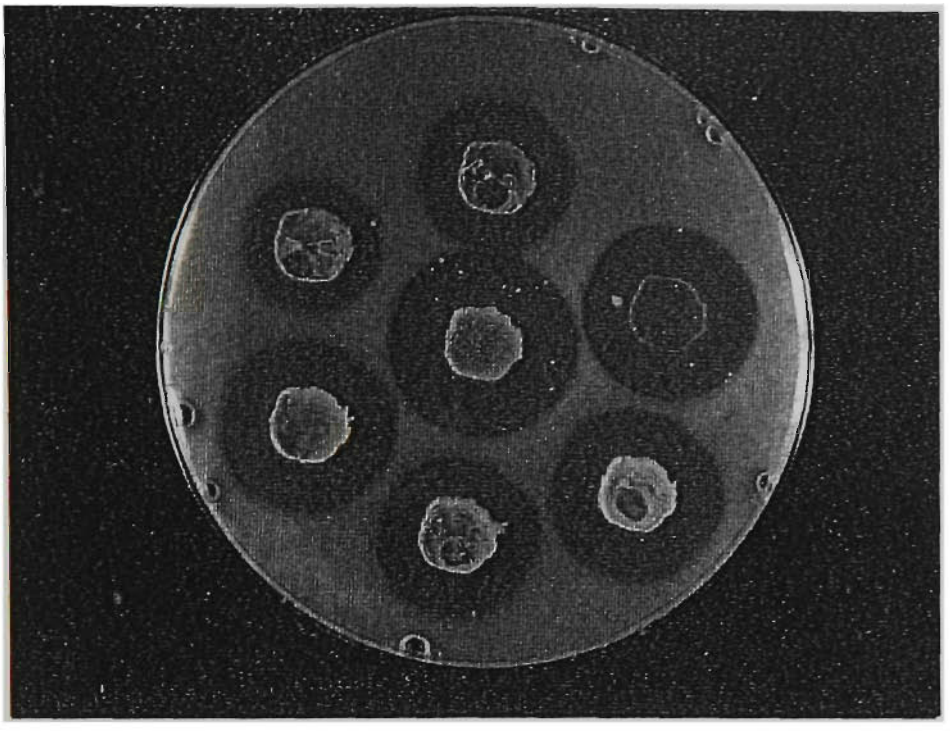


Figure 7.1.1. Zones of inhibition produced by various *L. acidophilus* strains against *L. delbrueckii* ssp. *bulgaricus* (LB- 1) using modified spot on lawn technique.

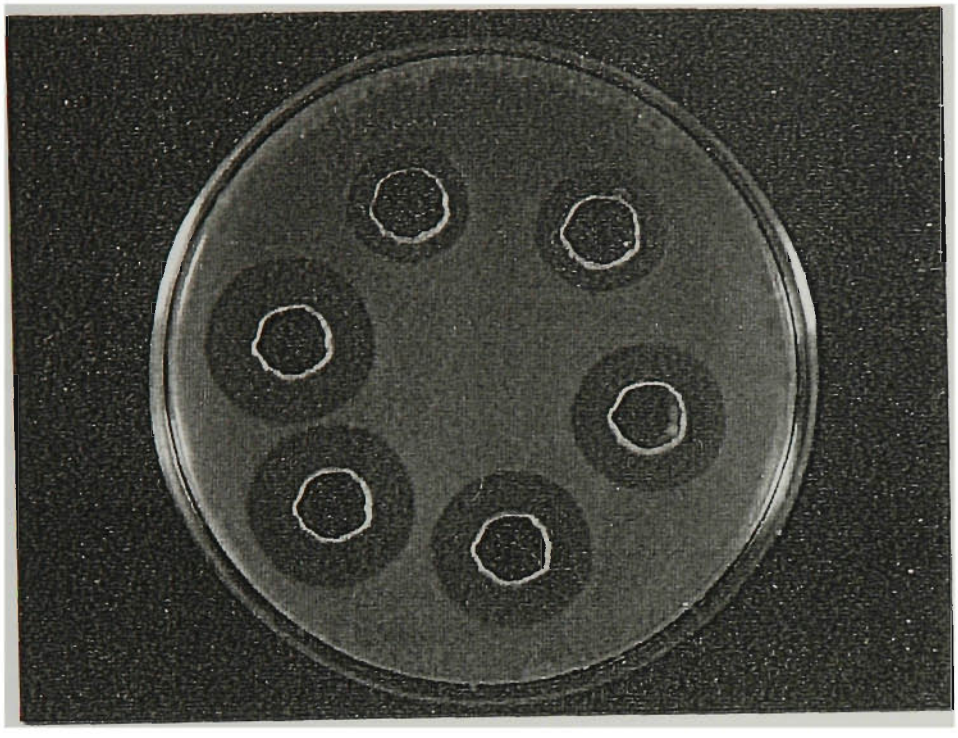


Figure 7.1.2. Zones of inhibition produced by various *L. acidophilus* strains against *L. delbrueckii* ssp. *bulgaricus* (LB-1)using agar well assay technique.

7.2 Some characteristics of bacteriocin produced by *Lactobacillus acidophilus* isolated from a commercial probiotic culture

7.2.1 Introduction

Lactic acid bacteria (LAB) have been used to produce safe and wholesome foods since time immemorial (Hammes and Tichaczek, 1994). The contribution of LAB to human health and nutrition is well documented (Robinson, 1991; Nakazawa and Hosono, 1992). The role of LAB in the inhibition of several spoilage and pathogenic bacteria has attracted considerable attention (Kanbe, 1992; Hoover and Steenson, 1993). A number of substances such as diacetyl (Daeschel, 1989), ammonia (Rogul and Carr, 1972), fatty acids (Walstad *et al.*, 1974), organic acids including lactic acid (Tramer, 1966), hydrogen peroxide (Su, 1948; Wheater *et al.*, 1952; Holmberg and Hollander, 1973; Malke *et al.*, 1974) and bacteriocins (Hoover and Steenson, 1993) produced by various micro-organisms are found to be inhibitory to other microbes.

The potential for use of bacteriocins in the food industry has spurred research in this area (Kim, 1993). Bacteriocins are proteinaceous compounds that kill or inhibit closely related bacteria (Jacob *et al.*, 1953; Tagg *et al.*, 1976). However, research has shown that bacteriocins may exhibit bactericidal activity beyond species that are closely related. Various organisms belonging to species such as *Lactococcus, Pediococcus, Lactobacillus, Leuconostoc, Carnobacterium, Propionibacterium, Enterococcus, Bacillus* and *Escherichia* have been reported to produce bacteriocins or bacteriocin like inhibitory substances (BLIS) (Hoover and Steenson, 1991; Branby-Smith, 1992; Klaenhammer, 1988; 1993).

A version of section 7.2 has been published under the title "Characteristics of bacteriocin produced by *L. acidophilus* (LA-1)". Dave, R. I. and Shah, N. P. (1997). *Inter. Dairy J.*, 7: 707-15.

Presently, several bacteriocins have been characterised, of which nisin is best studied with regard to its application in the food industry (Eckner, 1992). Microgard and Reuterin are gaining most attention for their usage in cottage cheese and the former has received the FDA approval (Daeschel, 1989). Products that have been targeted for application of bacteriocins include meat, fish, dairy, cereals, fruits and vegetables and beverages (Eckner, 1992).

In our earlier study, we evaluated several yoghurt and probiotic bacterial strains isolated from probiotic products sold in Australia for antagonism (Joseph *et al.*, 1998). We reported that BLIS was produced only by *L. acidophilus* strains and was active against *L. delbrueckii* ssp. *bulgaricus.*

In the present investigation, some characteristics of a bacteriocin produced by one strain of *L. acidophilus* (LA-1) that has been used in commercial probiotic starter cultures (C1, C2, C3 and C4) were studied in detail. In addition, 20 *L. acidophilus* strains were tested for their inhibitory activities against the same target organisms which were inhibited by LA-1.

7.2.2 Materials and methods

7.2.2.1 Microorganisms and their maintenance

The range of microorganisms including lactic acid bacteria, spoilage and pathogenic type of microorganisms were obtained from various sources (Table 3.1 and 3.2) and were maintained as described in section 3.2.

7.2.2.1 Determination of antimicrobial activity

The detection and assay of inhibitory activity was carried out according to the procedure outlined in section 3.13.

7.2.2.1 Reaction with various enzymes

The cell free supernatant was reacted with various enzymes and the nature of antimicrobial substance was detected as described in section 3.15

7.2.2.1 Thermal resistance, stability and activity of BLIS over a range of pH

The thermal resistance (section 3.16), its stability at various temperatures (section 3.18) and activity over a range of pH (section 3.17) was also studied.

7.2.2.1 Effect of various sugar based media

The production of BLIS in various sugar based media was studied as described in section 3.19.

7.2.2.1 Effects of pH and supplementation of glucose and triple strength MRS broth on production and stability of BLIS

The effects of pH and supplementation of glucose and nutrients contained in MRS broth on the production and stability of BLIS was studied in fermentors and is described in section 3.20.

7.2.2.1 Determination of viable counts, cell density and glucose concentration

The changes in viable counts, cell density and glucose concentration were monitored over a period of 48-72 h; the details of which are described in section 3.21.

7.2.2.1 Estimation of molecular weight

The molecular weight of neutralised cell free extract was estimated using ultrafiltration (UF) techniques as described in section 3.22.

7.2.2.1 Purification of BLIS using ammonium sulfate precipitation and SDS-PAGE

The purification of BLIS by ammonium sulfate precipitation and SDS-PAGE of various fractions are described in section 3.23.

For these set of experiments, unless otherwise indicated, all experiments and analyses were repeated at least twicw. The results shown are the averages + standard deviation (Excel 5.0 Microsoft package) of all available data.

7.3.1 Results and discussion

In the earlier study (Joseph *et al.*, 1998), it was reported that several strains of *L. acidophilus* produced BLIS against *L. delbrueckii* ssp. *bulgaricus*. In the present investigation, one strain of *L. acidophilus* (LA-1) was selected to systematically study the characteristics of the BLIS produced by this bacterium.

Inhibitory spectrum of BLIS. The inhibitory activity of BLIS produced by LA-1 against various LAB and spoilage and pathogenic groups of organisms is presented in Table 7.2.1. The organisms that were inhibited included *L. delbrueckii* ssp. *bulgaricus* (2501, 2505, 2515, 2517, 2519, LB-3 and LB-4), *L. helveticus* (2700), *L. jugurti* (2819) and *L.*

casei (2603). Inhibition of other strains of *L. casei* (2604, 2606 and Shirota strain) was not observed, indicating a possibility of strain specificity of the BLIS. Other LAB and the spoilage and pathogenic groups of organisms were not affected by the BLIS. Some spoilage and pathogenic groups of organisms were inhibited with un-neutralised MRS broth; however, when the pH was adjusted to >5.5, no effect was observed. This indicated that pH might have been responsible for the inhibition of these groups of organisms.

Vincent *et al.* (1959) first described a bacteriocin like inhibitor 'lactocidin' produced by *L. acidophilus* in veal liver agar. This compound was non-volatile, insensitive to catalase, and was active at neutral pH. Both Gram positive and Gram negative genera were inhibited by the lactocidin. However, no information is available on the purification of this compound. 'Acidophilin', a low molecular weight peptide was extracted from acidophilus milk using methanol and acetone (Vakil and Shahani, 1965; Shahani *et al.*, 1976; 1977). This substance was heat stable under acidic conditions and showed antibacterial activity against pathogenic bacteria *in vitro.* Hadman and Mikolajcik (1974) isolated and purified 'Acidolin' on Sephadex G25 from methanol extracts of acidophilus milk fermented with *L. acidophilus* 2181; the substance showed a broad spectrum antibacterial activity particularly against spore forming bacteria. Hosono *et al.* (1977) isolated a peptide from bacterial cell extracts of *L. acidophilus* ATCC 3205 of molecular weight of 3500 Da, which showed antibacterial activity against *Escherichia coli* over a wide range of pH. Mehta *et al.* (1984) purified ammonium sulfate precipitates of filtrates from *L. acidophilus* AR1 on Sephadex G100 and obtained protein type antibacterial substance with molecular weight of ~5200 Da. The substance showed antibacterial activity against *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Salmonella spp.* These substances were inactivated by trypsin treatment. 'Lactacin B' inhibited only 4 species of Lactobacilli (Barefoot and Klaenhammer, 1983) and 'Lactacin F' inhibited *L. fermentum* and *Streptococcus faecalis* in addition to the lactacin B target organisms (Muriana and Klaenhammer, 1987; 1991). BLIS produced by other lactobacilli has been reported to have broader inhibitory

spectrum. Brevicin 37 (Rammelsberg and Radler, 1990) was active against *Pediococcus damnosus, Lactobacillus brevis* and *Leuconostoc oenos.* Carnocin U149 (Stoffels *et al.*, 1992) was active against *Carnobacterium, Pediococcus, Enterococcus* and *Listeria.* Sakacin A produced by *Lactobacillus sake* (Schillinger and Luke, 1989) and bacteriocin produced by *Lactobacillus* strains 75 and 592 (Lewus *et al.*, 1991; Okereke and Montville, 1991; Schillinger *et al.*, 1991) were found to be active against *Carnobacterium piscicola, Enterococcus spp., L. sake, L. curvatus, Leuconostoc paramesenteroides, Listeria monocytogenes, Aeromonas hydrophila* and *Staphylococcus aureus.*

Sensitivity of BLIS to various enzymes. The inhibitory activity of BLIS produced by LA-1 was lost after reaction with proteolytic enzymes such as trypsin, α or β-chymotrypsin, papain, ficin, Proteinase K and crude protease. However, the activity was not affected when reacted with enzymes such as α or β-amylase, lipase and phospholipase A2 and D (Table 7.2.2). This confirmed that active protein moiety of the BLIS was responsible for the inhibition of the target organisms.

Stability of BLIS. The BLIS produced by LA-1 retained full activity after various heat treatments including autoclaving (Table 7.2.3) and was active over a wide range of pH (Table 7.2.4). The BLIS reported in our study was also stable for > 15 d at 37°C, > 3 months at 4°C and > 8 months at -18°C (Table 7.2.5).'Lactacin B', a bacteriocin produced by *L. acidophilus* N2 was sensitive to protease, but was heat stable during heating for 1 h at 100°C (Barefoot and Klaenhammer, 1983; 1984). 'Lactacin F' also retained full activity after heat treatment of 121°C for 15 min, but was sensitive to ficin, proteinase k, trypsin and subtilicin (Muriana and Klaenhammer, 1987; 1991). Conversely, 'Acidophilucin A', a bacteriocin produced by *L. acidophilus* LAPT 1060 and active against *L. delbrueckii ssp. bulgaricus* and *L. helveticus,* was sensitive to heat (60°C for 10 min), unlike lactacin B and F (Toba *et al.*, 1991).

Production of BLIS by L. acidophilus (LA-1) in various sugar based MRS broth. Changes in pH and production of BLIS by LA-1 in various sugar based MRS broth are illustrated in Figures 7.2.1 and 7.2.2, respectively. As shown, LA-1 produced BLIS in all fermentable sugar based MRS broth. Acid production by LA-1 was slow in mannose and raffinose (Fig. 7.2.1) and the production of BLIS was therefore slow in each of these two sugar based media (Fig. 7.2.2). The propagation of LA-1 before inoculation to various sugar based media was in MRS broth that contained glucose. This might have delayed initiation of fermentation of complex sugars in the media. *L. acidophilus* (LA-1) grew at a slower rate in these media (Fig. 7.1.1) which might have also delayed production of BLIS. In the basal medium, which did not contain any sugar, no growth of LA-1 occurred and hence no production of BLIS was observed.

Production of BLIS as affected by growth of organism in various media. The initial screening for production of bacteriocin was carried out on MRS agar. The organism also produced BLIS in MRS broth (Fig. 7.2.3). The production of BLIS was higher in MRS broth supplemented with 1.9 % sodium β-glycerophosphate (BGP). Conversely, inhibitory effect was not produced until 22 h in milk (Fig. 7.2.3) and the zone of inhibition produced in milk was unclear. Similar observations were found during growth of LA-1 in whey. The activity of BLIS increased during intial growth of LA-1 for 12-14 h and remained similar for almost 20-22 h, after which the activity declined in MRS broth and MRS broth supplemented with BGP (Fig. 7.2.3). The pH of MRS broth increased from 6.3 to ~7.1 due to addition of BGP (Fig. 7.2.4); higher pH could be responsible for the increased production of BLIS in MRS broth with BGP. The drop in pH was faster in MRS broth during 48 h growth of LA-1 than in milk or MRS broth supplemented with BGP (Fig. 7.2.4), however, viable counts of LA-1 remained almost comparable in these media (Fig. 7.2.5). On an average, the viable counts of LA-1 remained higher throughout 48 h incubation in MRS broth as compared to milk and MRS broth supplemented with BGP. The changes in optical density (Fig. 7.2.6) and depletion of glucose (Fig. 7.2.7) also confirmed that multiplication of LA-1 was faster in MRS broth as compared to MRS broth supplemented with BGP. The possible reason

could be the buffering effect of BGP that altered the pH which might not be optimum for the growth of LA-1 (Fig. 7.2.4). The glucose in MRS broth or MRS broth supplemented with BGP was completely depleted after 20-22 h (Fig. 7.2.7).

This set of experiments indicated that pH and glucose content might be important for the production of BLIS. Further, the cells of LA-1 started to settle at the bottom of the Schott bottles after 14-16 h of growth in the broth medium. Hence, subsequent studies were undertaken in fermentors to confirm the role of pH and nutrients on production of BLIS by LA-1 using *L. delbrueckii* ssp. *bulgaricus* (LB-2519) as target organism.

Effect of pH and supplementation of glucose and triple strength MRS broth on production of BLIS in fermentors. The growth of LA-1 was considerably faster in fermentors compared to batch fermentation in bottles. Changes in the inhibitory activity of BLIS against LB-2519 during growth of LA-1 over a period of 48 h are presented in Fig. 7.2.8. The production of BLIS was greatly affected by pH, being maximum at pH 5.5 and 6.0, whereas, its production was considerably lower at pH 5.0. Conversely, the stability of BLIS after its production, measured as zone of inhibition at every 2 h intervals for up to 48 h, varied with pH. The stability was highest at pH 5.0 followed by 5.5 and 6.0 (Fig. 7.2.8). The pH of broth increased by 0.15 to 0.29 units after 24 h of growth of LA-1 in fermentors set at various pH. This may be due to death of LA-1 cells and liberation of cell bound proteases and peptidases (Fig. 7.2.9). On an average, the growth of LA-1 was faster at pH 5.0, however, the final cell population of the organism was almost similar at pH 5.0, 5.5 and 6.0 (Fig. 7.2.10 and 7.2.11). This confirmed that pH was most crucial for the production of BLIS and the pH range of 5.5-6.0 was optimum for its production as compared to pH 5.0.

The glucose was completely utilised within 12-14 h in fermentors set at various pH (Fig. 7.2.12). The activity of BLIS declined after stationary growth phase of LA-1 and its production seemed to occur during log phase and early stationary phase. Once the organism entered into death phase, the activity of BLIS also started to declined. This is

supported by an increase in pH units of 0.15-0.29 after 24 h in fermentors set at various pH (Fig. 7.2.9) and stability of BLIS in cell free filtrate at 37°C as described earlier (Table 7.2.5). The log phase of the organism was between 10-12 h and the maximum activity of BLIS was also found during this period. Therefore, addition of glucose or triple strength MRS broth was made in fermentors set at pH 6.0 after 8 and 16 h so as to continue fermentation process and to favour the growth of LA-1 as the growth may have been affected due to un-availability of glucose as a source of energy. Supplementation of glucose did not increase the cell numbers of LA-1 to a considerable extent (Fig. 7.2.10 and 7.2.11). However, the pH of fermentor did not increase throughout the 48 h incubation as observed earlier in MRS broth in fermentors set at various pH without supplementation of extra glucose (Fig. 7.2.9).

The rate of glucose utilisation declined to a considerable extent after 20 h and about 688 $mg.dL^{-1}$ glucose was still available after 48 h of fermentation. The stability of BLIS was improved with additional glucose; however, no increase in zone of inhibition was observed. Thus, production of BLIS did not increase by additional glucose (Fig. 7.2.8). The possible reason could be depletion of amino acids and other growth factors in MRS broth. To confirm this, production of BLIS with supplemented glucose after 8 and 16 h of incubation was compared against addition of triple strength MRS broth. It is evident from Fig. 7.2.8 that the stability of BLIS was considerably improved due to addition of all nutrients of MRS broth than that of glucose only. The production of BLIS at pH 6.0 was maximum after about 12 h and remained stable for almost 24 h due to additional MRS broth as compared to 16 h due to addition of glucose. The viable counts (Fig. 7.2.10) and optical density (Fig. 7.2.11) also showed that counts were highest with additional MRS broth after 8 and 16 h. In fermentor added with triple strength MRS broth, the glucose was completely utilised in ~30 h, whereas a considerable amount of glucose (1000 mg dL^{-1}) was left unutilised at this period in fermentor added with glucose only (Fig. 7.2.12).

Molecular weight of BLIS. The zones of inhibition of retentate and permeate fractions after concentration using various membranes are illustrated in Fig. 7.2.13. The activity of BLIS was not found in the permeate of 5, 10, 20 or 30 kDa membranes. Permeates of 50 kDa membranes showed some inhibitory activity; however, major activity was found in the retentate (Fig. 7.2.13). Thus, molecular weight of BLIS appeared to be ~ 50,000 daltons. It is known that MWCO is not as accurate as specified by the manufacturers and 50 kDa membranes may be sieving molecules in the range of 50 $\pm$ 5 kDa and some BLIS molecules might have passed through the membranes.

A crude lactacin B produced by *L. acidophilus* N2 and existed as large aggregate was estimated to be of >100 kDa; however, purified lactacin B resolved as a protein of 6.0 to 6.5 kDa (Barefoot and Klaenhammer, 1984). Lactacin F was reported to have a molecular weight of 6.3 kDa (Muriana and Klaenhammer, 1991). BLIS produced by various *L. acidophilus* strains as reported by several workers (Vincent *et al.,* 1959; Vakil and Shahani, 1965; Hadman and Mikolajcik, 1974; Hosono *et al.,* 1977; Mehta *et al.* 1984) had a molecular weight of < 10.0 kDa. Conversely, Caseicin 80 produced by *L. casei* had a molecular weight of 40 kDa (Rammelsberg and Ralder, 1990).

Purification of BLIS. A judicious application of ammonium sulphate precipitation has been the most viable purification process for small quantity of proteins. After preliminary concentration of the BLIS by ultrafiltration, various saturation levels of ammonium sulphate were tried to obtain purest form of BLIS. The purity of the BLIS was checked by SDS-PAGE and standard molecular weight markers were also applied to the gel to estimate the molecular weight of the BLIS. The SDS-PAGE results (Fig. 7.2.14) showed that a two step ammonium sulphate precipitation gave most pure form of BLIS. Precipitation of 50 kDa retentate with single stage 40 or 70% ammonium sulphate saturation level showed 2 or more bands on silver staining of SDS-PAGE gels. However, two stage fractionation (first stage, 0-40%; second stage, 40-70%) resulted in a single band (Lane 9, Fig. 7.2.14) on silver staining of gel. The BLIS activity was found to be lost to a considerable extent after each step of purification. The single band obtained was

~54 kDa when compared with the standard molecular weight markers. Thus, it was concluded that the BLIS produced by *L. acidophilus* LA-1 had a molecular weight of ~54 kDa.

Screening of various L. acidophilus strains. The BLIS produced by LA-1 showed narrow spectrum of inhibition against only 4 species of lactobacilli (Table 7.2.6). Therefore, screening of further 19 strains of *L. acidophilus* was carried out to assess the bacteriocinogenic activity using *L. delbrueckii* ssp. *bulgaricus* (2519), *L. helveticus* (2700), *L. casei* (2603) and *L. jugurti* (2819) as target organisms. Four strains of *L. acidophilus* (2401, 2420, MJLA-1 and PALA-1) did not produce any inhibitory substance against 4 target organisms tested. *L. acidophilus* strains OLA-2 and 2400 showed little inhibition and the zones were not as large as produced by LA-1 or other *L. acidophilus* (Table 7.2.6). Further, inhibitory substance produced by all these *L. acidophilus* strains showed activity even after autoclaving the neutralised and catalase treated broth and the activity was lost after reacting with β-chymotrypsin. This confirmed that 15 strains of *L. acidophilus* also produced heat resistant BLIS as an active protein moiety was involved in the inhibition of targeted organisms. Barefoot and Klaenhammer (1983) tested 52 strains of *L. acidophilus* for the production of bacteriocin. A majority (63%) of *L. acidophilus* cultures produced bacteriocin like compound that inhibited closely related lactobacilli; no broad spectrum bacteriocin was found.

7.2.4 Conclusions

Bacteriocin like inhibitory substance (BLIS) produced by *L. acidophilus* (LA-1) was active against seven strains of *L. delbrueckii* ssp. *bulgaricus* (2501, 2505, 2515, 2517, 2519, LB-3 and LB-4), one strian each of *L. casei* (2603), *L. helveticus* (2700) and *L. jugurti* (2819), but not against *L. casei* ssp. *rhamnosus* (2606), *L. casei* (2604 and Shirota strain), *L. plantarum* (2903), *Leuconostoc mesenteroides* ssp. *cremoris* (4200), *Pediococcus cerevisiae* (2305), *L. sake* and several spoilage and pathogenic organisms tested. The

production of BLIS was found to occur in MRS broth and MRS broth containing various sugars in place of glucose. In milk or whey based medium, the zone of inhibition was unclear. The BLIS showed activity over a wide range of temperature and pH. The production of BLIS increased on addition of β-glycerophosphate and the production was highest at pH 5.5-6.0. The BLIS activity declined when the organism entered into death phase; however, its stability was better in fermentors upon supplementation with glucose or tripple strength MRS broth after 8 and 16 h growth. The molecular weight of BLIS produced by *L. acidophilus* (LA-1) was ~50,000 daltons. Two stage fractionation with ammonium sulfate was successful in purifying the BLIS and resulted in a single band on silver staining of SDS-PAGE gel. Further, 19 *L. acidophilus* strains were tested for BLIS activity against *L. delbrueckii* ssp. *bulgaricus*, *L. helveticus*, *L. jugurti* and *L. casei* using the spot plate method and agar well assay technique. Fifteen of 19 strans of *L. acidophilus* produced heat stable BLIS. The BLIS reported in this study was different from those reported earlier.

Table 7.2.1. Inhibitory spectrum[1] of bacteriocin like inhibitory substance produced by *L. acidophilus* (LA-1)

Target organism	Strain	Agar/Medium	Zone of inhibition[2] (mm)
Lactic acid bacteria			
Lactobacillus delbrueckii ssp. *bulgaricus*	2501, 2505, 2515, 1517, 2519, LB-3 and LB-4	MRS	14-17.0
Lactobacillus helveticus	2700	MRS	16.0
Lactobacillus jugurti	2819	MRS	14.0
Lactobacillus casei	2603	MRS	12.0
Lactobacillus casei	2604	MRS	nil
Lactobacillus casei ssp. *rhamnosus*	2606	MRS	nil
Pediococcus cerevisiae	2305	MRS plus 2% glucose	nil
Leuconostoc mesenteroids ssp. *cremoris*	4200	MRS	nil
Leuconostoc oenos			
Lactobacillus fermentum	5174	MRS	nil
Lactobacillus spp.	Y	MRS	nil
Lactobacillus sake	LS	MRS	nil
Lactobacillus plantarum	2903	MRS	nil
Streptococcus thermophilus	St-1,2,3,4	M17	nil
Lactobacillus acidophilus	2400, 2404, 2405,2409, 2410, 2415	MRS	nil
Bifidobacteria	BB1, 1901, 1902, 1912, 1920 1941, 20099, 20210	MRS plus cysteine	nil
Spoilage and pathogenic organisms			
Escherichia coli	Ec 1, VUN 0100	NA & BHI	nil**
Candida albicans	Ca 1	NA & BHI	nil
Aeromonas hydrophila	Ah 1	NA & BHI	nil**
Salmonella typhi	Sal 1	NA & BHI	nil**
Staphylococcus aureus	VUP 0040	NA & BHI	nil
Pseudomonas fluorescens	VUN 0031	NA & BHI	nil**
Salmonella typhimurium	VUN 0045	NA & BHI	nil**
Enterobacter aerogenes	VUN 0025	NA & BHI	nil**
Bacillus stearothermophilus	VUP 0007	NA & BHI	nil**
Bacillus cereus	VUP 0001	NA & BHI	nil**
Enterococcus faecalis	VUP 0019	NA & BHI	nil
Micrococcus luteus	VUP0023	NA & BHI	nil
Helicobacter pylori	Hp 1,2,3,4	Helicobacter agar[3]	nil**
Listeria innocua	Li 1	BHI	nil**
Listeria monocytogenes	Lm 1	BHI	nil**
Clostridum perfringens	VUP 0060	RCA	nil**
Clostridium sporogenes	VUP 0061	RCA	nil**
Vibrio parahaemolyticus	WP1 VUN 0300	BHI plus horse blood	nil**

MRS=deMan Rogossa Sharpe's agar; NA=Nutrient agar; BHI=Brain Heart Infusion agar; RCA=Reinforced clostridial agar.

[1]Inhibitory spectrum was measured after neutralising and treating MRS broth with catalase.

[2]Zone of inhibition includes 7.0 mm bore diameter.

[3]The Helicobacter agar was not added with antibiotic inhibitor solution. **indicates inhibiton of organisms with unneutralised MRS broth (pH <4.0).

Table 7.2.2. Sensitivity of bacteriocin like inhibitory substance produced by *L.acidophilus* (LA-1) to various enzymes

Enzyme	Concentration[1]	Zone of inhibition[2] (mm)			
		Target organism			
		LB-2519	**LH-2700**	**LC-2603**	**LJ-2819**
Control	No enzyme	22.0 ± 0.71	24.0 ± 0.71	18.0 ± 1.4	21.0 ± 1.1
Trypsin	1.0 mg	nil	nil	nil	nil
α-chymotrypsin	0.1 mg	nil	nil	nil	nil
β-chymotrypsin	1.0 mg	nil	nil	nil	nil
Papain	0.5 mg	nil	nil	nil	nil
Ficin	100 U	nil	nil	nil	nil
Proteinase k	0.2 mg	nil	nil	nil	nil
Protease (crude)	1.0 mg	nil	nil	nil	nil
α-amylase	1000 U	20.5 ± 0.71	22.5 ± 0.71	16.0 ± 0.71	20.0 ± 1.4
β-amylase	100 U	20.5 ± 0.71	22.5 ± 0.35	15.0 ± 0.0	20.0 ± 0.71
Lipase	250 U	19.5 ± 0.71	22.5 ± 0.71	15.0 ± 0.57	19.0 ± 0.0
Phospholipase A2	0.1 mg	19.0 ± 0.25	21.5 ± 0.71	16.0 ± 0.71	18.5 ± 0.7
Phospholipase D	500 U	20.5 ± 0.35	23.5 ± 0.71	18.0 ± 0.71	20.0 ± 1.4

LB=*L. delbrueckii* ssp. *bulgaricus*; LH=*L. helveticus*; LC=*L. casei* and LJ=*L. jugurti*

[1]Final concentration of enzyme per millilitre of neutralised and catalase treated MRS broth.

[2]Zone of inhibition includes 7.0 mm bore diameter.

Table 7.2.3. Heat resistance of bacteriocin like inhibitory substance produced by *L. acidophilus* (LA-1)[1]

Treatment	Zone of inhibition[2] (mm) against various *L. delbrueckii* ssp. *bulgaricus* strains						
	2501	*2505*	*2515*	*2517*	*2519*	*LB-3*	*LB-4*
MRS broth (pH 4.22)	13.0 ± 0.35	13.2 ± 0.35	12.9 ± 0.35	13.5 ± 0.45	15.0 ± 0.35	14.3 ± 0.21	14.6 ± 0.35
MRS broth (neutralised to pH 6.2)	12.9 ± 0.35	13.0 ± 0.25	12.8 ± 0.35	13.4 ± 0.45	14.5 ± 0.45	14.0 ± 0.45	14.0 ± 0.45
MRS broth (50°C for 10 and 30 mins)	12.6 ± 0.21	13.0 ± 0.21	12.7 ± 0.50	12.9 ± 0.35	14.5 ± 0.35	14.1 ± 0.30	14.2 ± 0.35
MRS broth (60°C for 10 and 30 mins)	12.6 ± 0.35	13.1 ± 0.21	12.7 ± 0.60	13.0 ± 0.35	14.6 ± 0.35	14.1 ± 0.45	14.1 ± 0.25
MRS broth (70°C for 10 and 30 mins)	12.7 ± 0.21	13.2 ± 0.35	12.8 ± 0.35	13.0 ± 0.25	14.8 ± 0.25	14.2 ± 0.35	14.3 ± 0.45
MRS broth (80°C for 10 and 30 mins)	12.8 ± 0.35	11.2 ± 0.50	12.8 ± 0.35	13.0 ± 0.35	14.8 ± 0.25	14.2 ± 0.35	14.3 ± 0.35
MRS broth (90°C for 10 and 30 mins)	12.8 ± 0.30	13.2 ± 0.35	12.8 ± 0.35	13.3 ± 0.25	14.9 ± 0.30	14.2 ± 0.35	14.4 ± 0.35
MRS broth (100°C for 10 and 30 mins)	12.8 ± 0.25	13.4 ± 0.35	12.9 ± 0.21	13.5 ± 0.45	14.8 ± 0.35	14.3 ± 0.25	14.4 ± 0.25
MRS broth (121°C for 15 mins)	12.8 ± 0.35	13.2 ± 0.25	12.7 ± 0.31	13.3 ± 0.45	14.5 ± 0.45	14.1 ± 0.45	14.1 ± 0.45

[1]The results are averages of 5 replicates.

[2]Zone of inhibition includes 7.0 mm bore diameter.

Table 7.2.4. Activity of bacteriocin like inhibitory substance (BLIS) produced by *L. acidophilus* (LA-1) over a range of pH

	-------pH of the medium--------									
	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
BLIS activity[1]	24.0**	22.0**	20.0	17.0	17.0	16.0	16.0	16.0	15.5	15.5
Control activity[2]	19.0**	13.0**	9.0**	nil	nil	nil	nil	nil	nil	nil

[1]presented as zone of inhibition (mm) using 2519 as target organism and zone includes 7.0 mm bore diameter.

[2]activity of uninoculated MRS broth adjusted to various pH and zone includes 7.0 mm bore diameter.

** zone of inhibition was not very clear as observed with BLIS at other pH.

Table 7.2.5. Storage stability of bacteriocin like inhibitory substance (BLIS) produced by *L. acidophilus* (LA-1) in cell free MRS broth at various temperatures

At 37°C	Zone of Inhibition[1]	At 4°C	Zone of Inhibition	At -18°C	Zone of Inhibition
(Days)	*(mm)*	*(Weeks)*	*(mm)*	*(Weeks)*	*(mm)*
0	17.0 ± 0.85	0	17.0 ± 0.87	0	17.0 ± 0.75
1	17.0 ± 0.75	1	17.0 ± 0.95	2	17.0 ± 1.10
2	17.0 ± 0.78	2	17.0 ± 0.85	4	17.0 ± 1.45
4	16.5 ± 0.90	3	17.0 ± 0.95	8	17.0 ± 1.10
6	17.0 ± 1.00	4	17.0 ± 1.10	12	17.0 ± 0.55
8	16.5 ± 0.85	8	16.5 ± 0.77	18	17.0 ± 0.45
10	16.5 ± 0.65	10	16.5 ± 0.94	24	16.5 ± 0.65
15	16.0 ± 0.75	12	16.0 ± 0.85	36	16.5 ± 0.75

[1]Zone of inhibition includes 7.0 mm bore diameter.

Table 7.2.6. Screening of various strains of *L. acidophilus* for the production of bacteriocin like inhibitory substance

Producer organism	Target organisms				
L. acidophilus	LB-2519	LH-2700	LC-2603	LJ-2819	LB-2519[1]
		Zone of inhibition[2] (mm)			
2404	16.5 ± 0.65	19.0 ± 1.1	14.0 ± 1.2	17.0 ± 1.2	15.5 ± 1.4
2405	20.5 ± 0.65	23.5 ± 0.85	18.5 ± 1.1	16.5 ± 1.4	18.5 ± 1.7
2406	15.0 ± 0.65	16.0 ± 0.45	8.5 ± 0.35	9.5 ± 0.85	15.0 ± 1.2
2409	20.0 ± 1.1	22.0 ± 0.85	17.0 ± 0.85	18.5 ± 1.2	20.0 ± 1.5
2410	20.0 ± 0.65	22.0 ± 0.35	19.0 ± 0.65	18.0 ± 0.65	20.0 ± 1.7
2411	20.0 ± 0.45	22.5 ± 0.46	18.0 ± 0.65	19.0 ± 0.85	20.0 ± 1.7
2412	16.5 ± 0.65	20.0 ± 0.35	12.0 ± 0.35	20.0 ± 1.2	17.0 ± 1.6
2413	14.5 ± 1.1	16.0 ± 0.45	10.5 ± 0.25	12.5 ± 1.0	14.5 ± 0.65
2414	17.5 ± 0.35	19.5 ± 0.25	15.5 ± 0.65	16.5 ± 0.35	16.5 ± 0.45
2415	19.5 ± 0.25	23.0 ± 0.25	17.5 ± 0.45	18.0 ± 0.45	19.5 ± 0.45
2422	16.0 ± 0.25	18.0 ± 0.25	13.0 ± 0.25	13.5 ± 0.25	16.0 ± 0.35
BDLA-1	16.0 ± 0.45	20.0 ± 0.45	14.5 ± 0.25	17.5 ± 0.25	16.0 ± 0.45
OLA-2	8.0 ± 0.85	8.5 ± 0.85	nil	nil	nil
MOLA-2	22.0 ± 1.1	26.5 ± 1.2	20.0 ± 1.1	20.0 ± 1.6	21.5 ± 1.9

[1]Inhibition of 2519 was measured after autoclaving the neutralised and catalase treated MRS broth.

[2]Zone of inhibition includes 7.0 mm bore diameter.

LB=*L. delbrueckii* ssp. *bulgaricus*; LH=*L. helveticus*; LC=*L. casei* and LJ=*L. jugurti* *(L. acidophilus* (strains 2401, 2420, MJLA-1 and PALA-1) did not show inhibition of any of the four target organism; MRS broth in which various *L. acidophilus* strains were grown; Inhibition of 2519 was not observed after treating neutralised cell free MRS broth with β-chymotrypsin (1.0 $mg.mL^{-1}$) for all of the *L. acidophilus).*

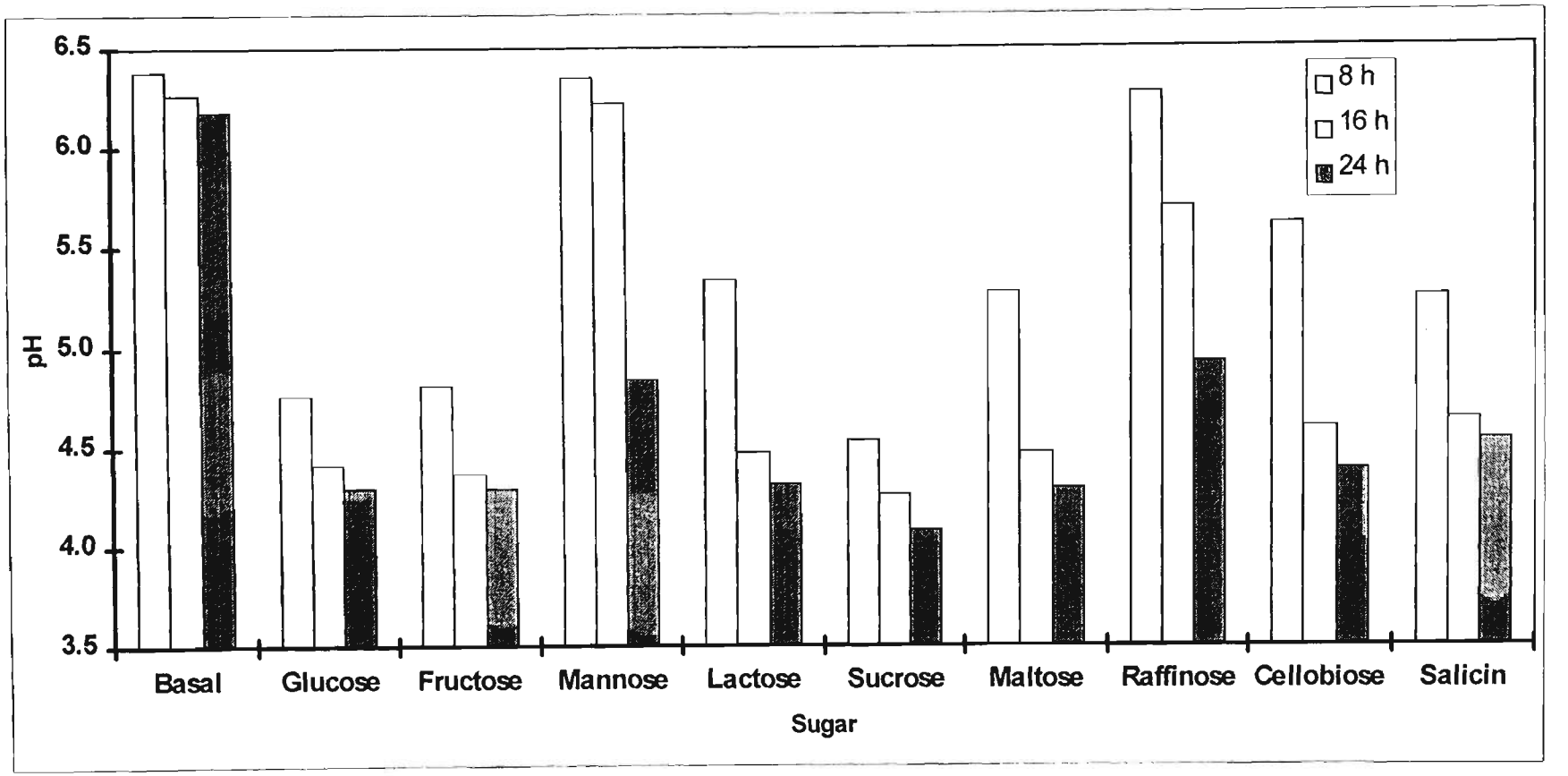


Fig. 7.2.1. Changes in pH during growth of *L. acidophilus* (LA-1) in various sugar based MRS broth.

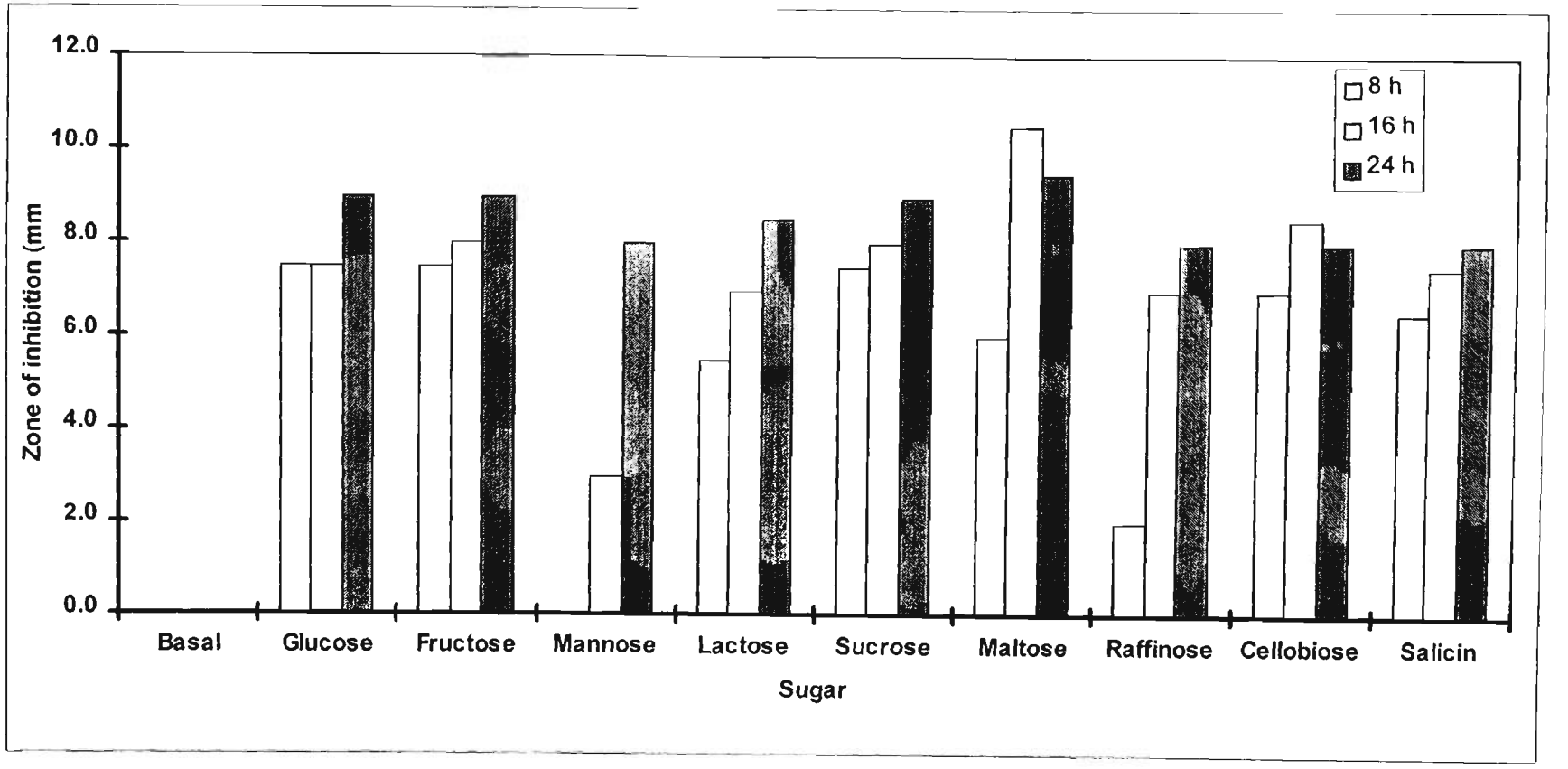


Fig. 7.2.2. Zone of inhibition during growth of *L. acidophilus* (LA-1) culture in various sugar based MRS broth (Zone of inhibition does not include 7.0 mm bore diameter).

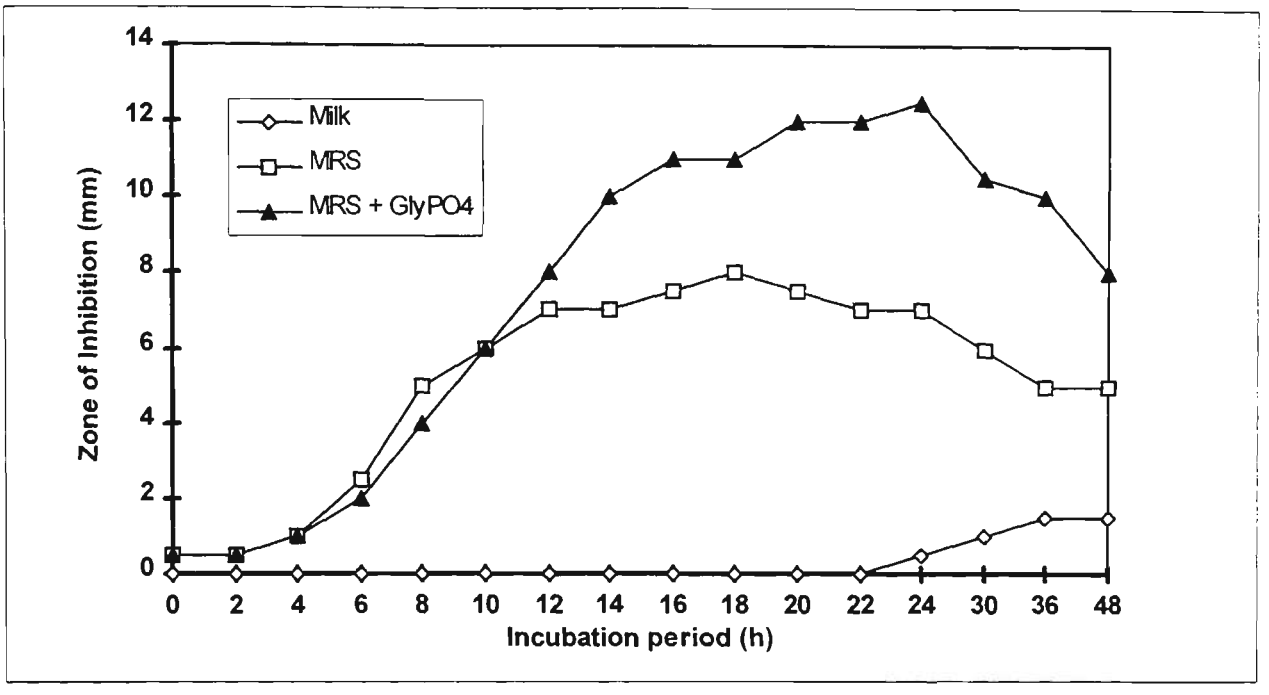


Fig. 7.2.3. Bacteriocinogenic activity of *L. acidophilus* (LA-1) as affected by its growth in milk, MRS broth and MRS broth supplemented with ß-glycerophosphate (MRS + $GlyPO_4$=MRS (deMan Rogossa Sharpe's) broth supplemented with β-glycero-phosphate).

Zone of inhibition does not include 7.0 mm bore diameter.

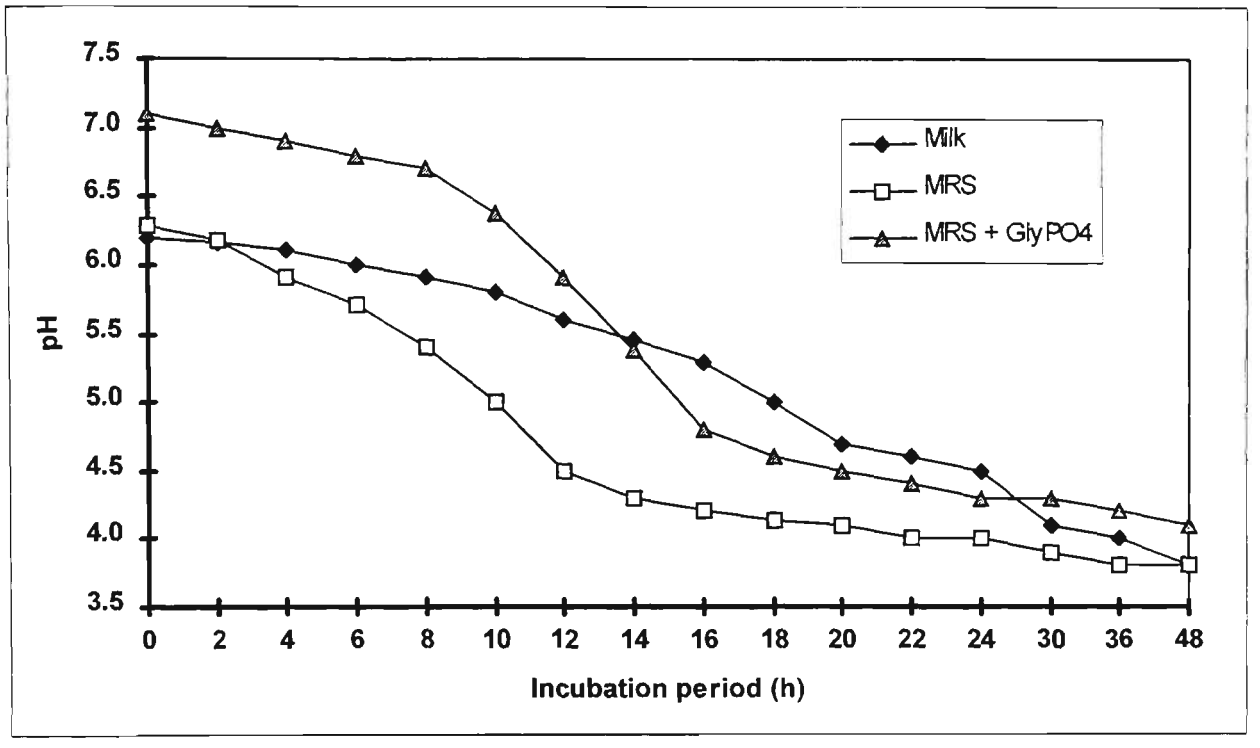


Fig. 7.2.4. Changes in pH during growth of *L. acidophilus* (LA-1) in milk, MRS broth and MRS broth supplemented with ß-glycerophosphate (MRS + $GlyPO_4$=MRS broth supplemented with β-glycerophosphate).

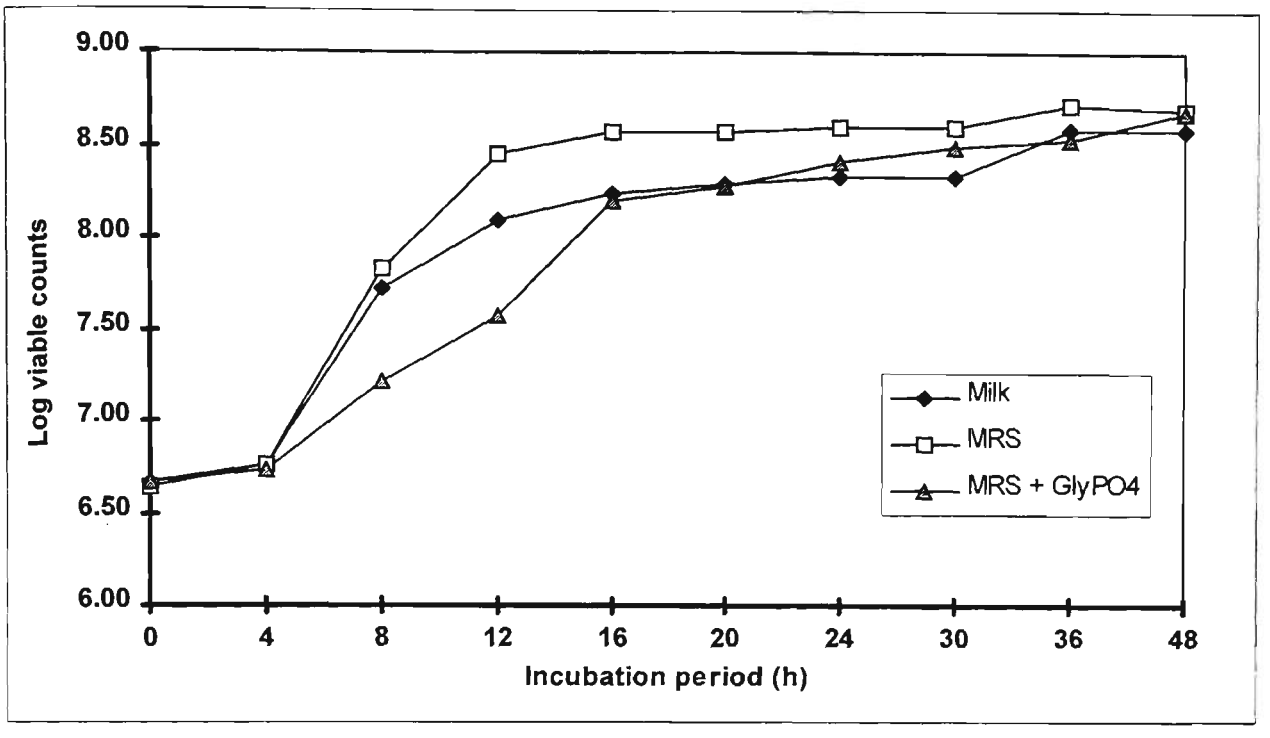


Fig. 7.2.5. Viable counts of *L. acidophilus* (LA-1) in milk, MRS broth and MRS broth supplemented with ß-glycerophosphate (MRS + $GlyPO_4$=MRS broth supplemented with β-glycerophosphate).

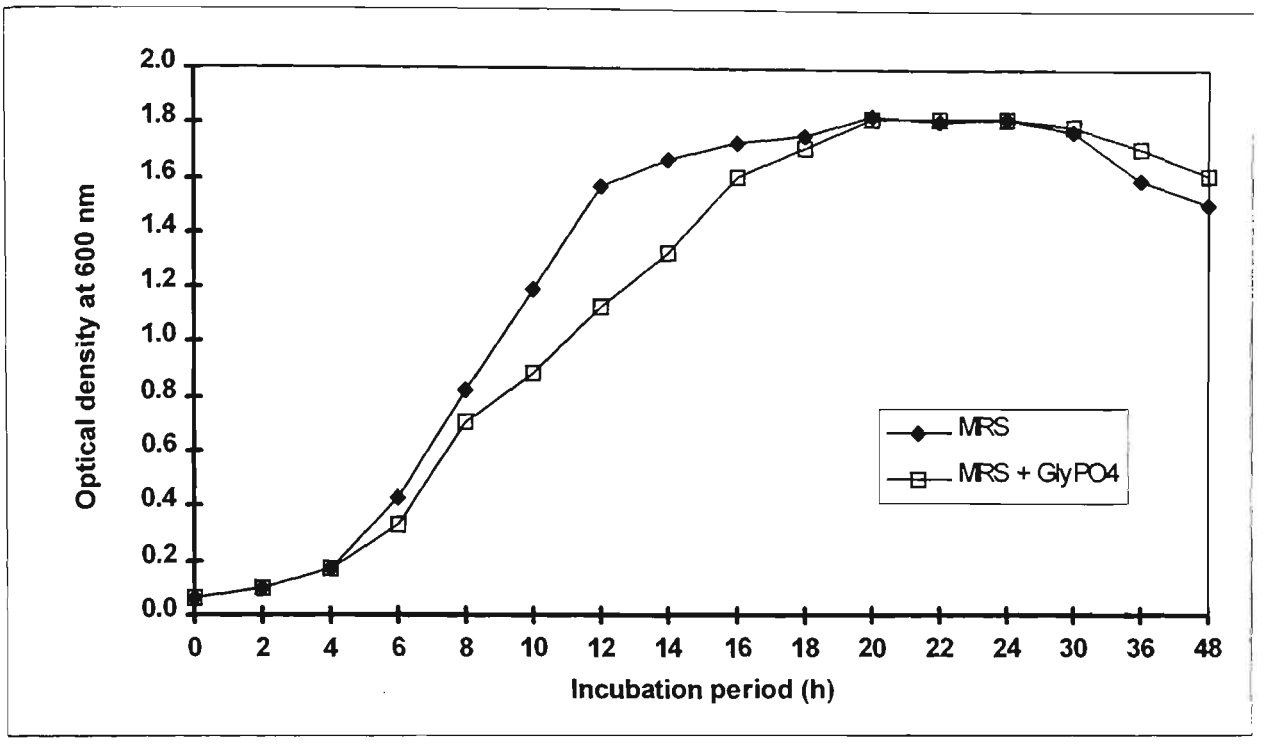


Fig. 7.2.6. Changes in the cell density of *L. acidophilus* (LA-1) in MRS broth and MRS broth supplemented with ß-glycerophosphate (MRS + $GlyPO_4$=MRS broth supplemented with β-glycerophosphate).

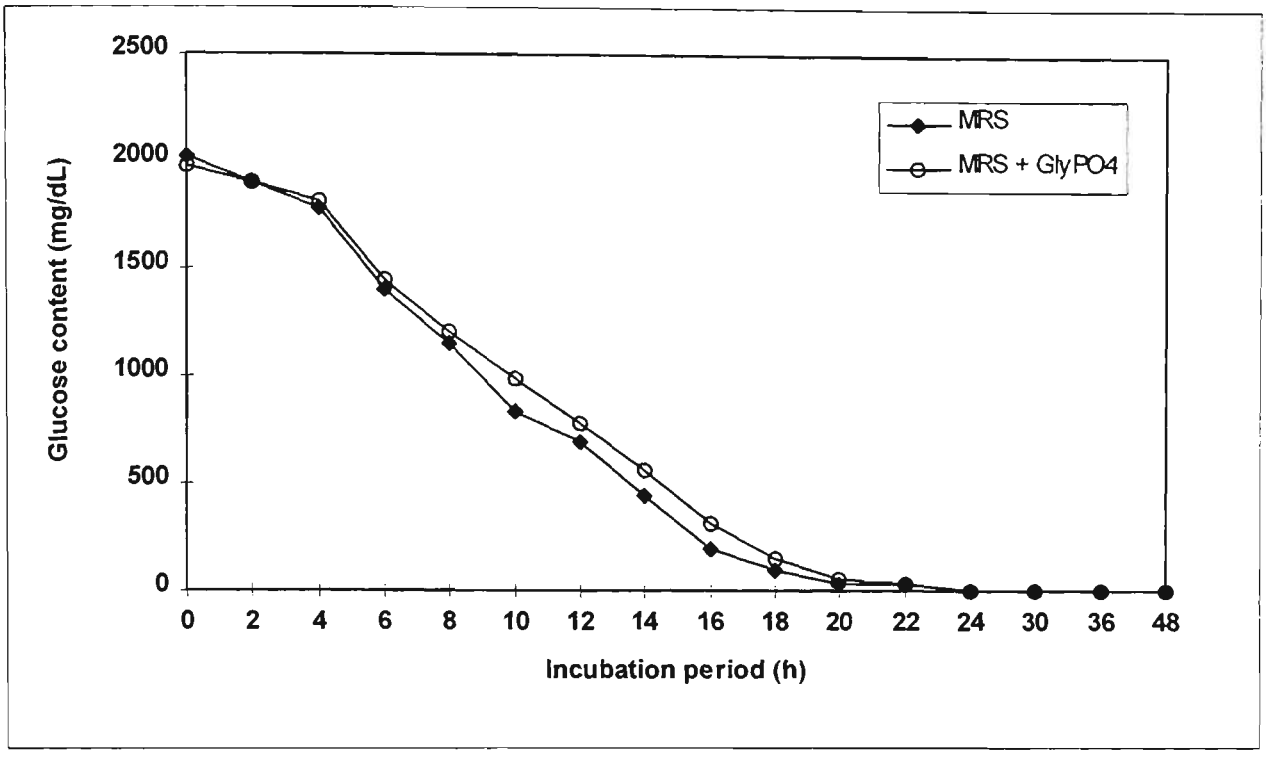


Fig. 7.2.7. Utilisation of glucose by *L. acidophilus* (LA-1) in MRS broth and MRS broth supplemented with ß-glycerophosphate (MRS + $GlyPO_4$=MRS broth supplemented with β-glycerophosphate).

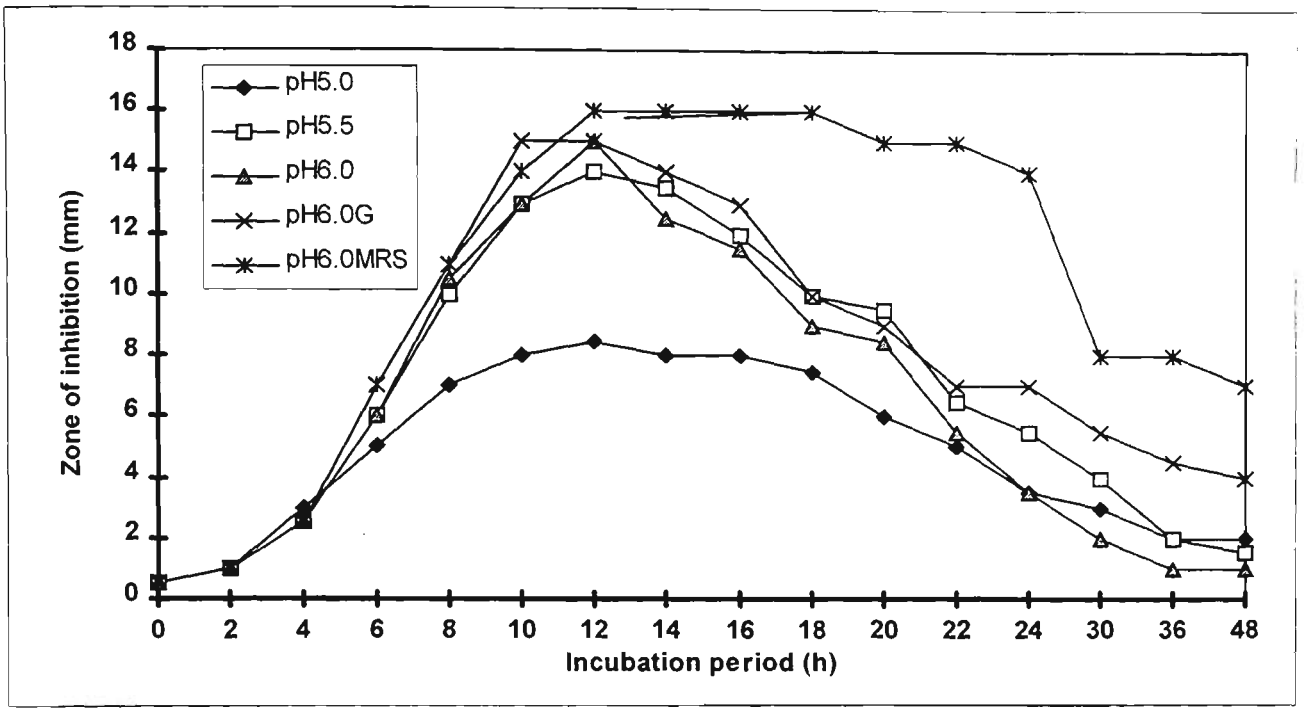


Fig. 7.2.8. Activity of bacteriocin like inhibitory substance produced by *L. acidophilus* (LA-1) in MRS broth in fermentors at various pH and after addition of glucose and triple strength MRS broth (pH6.0G and pH6.0MRS=Fermentors set at pH 6.0 supplemented with glucose and triple strength MRS broth after 8 and 16 h of growth of LA-1, respectively).

Zone of inhibition does not include 7.0 mm bore diameter.

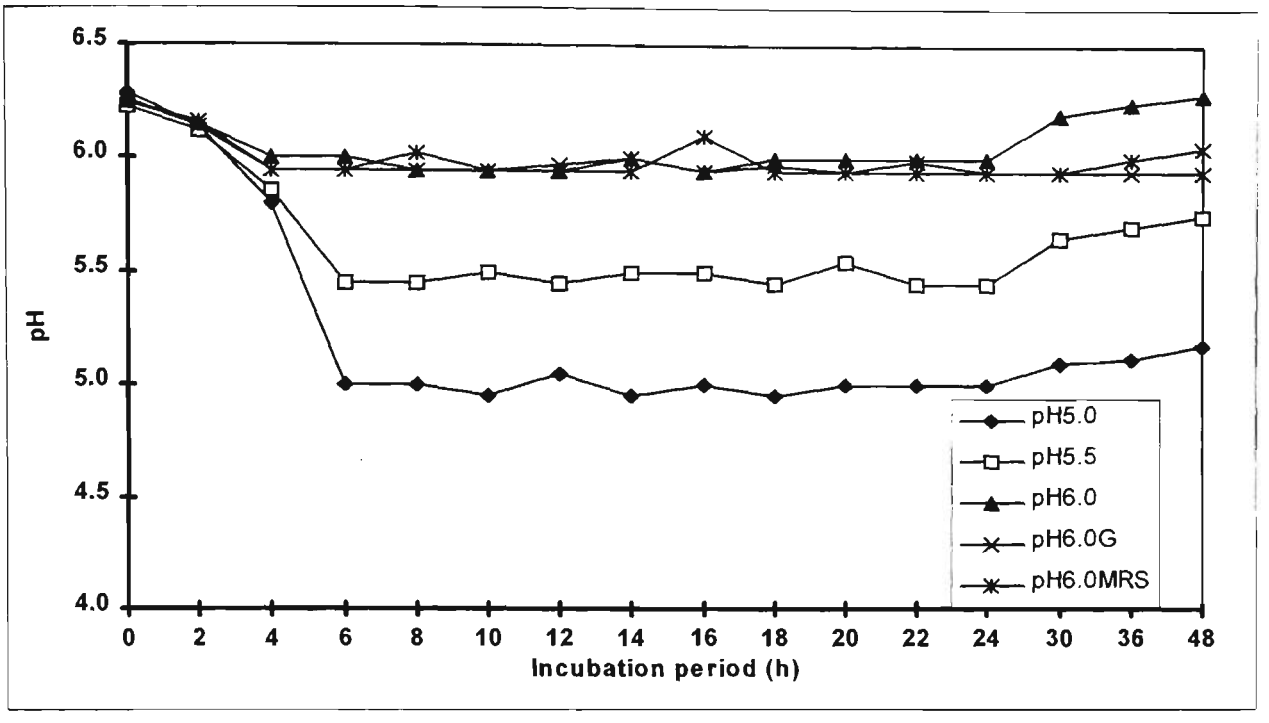


Fig. 7.2.9. Changes in pH during growth of *L. acidophilus* (LA-1) in MRS broth in fermentors at various pH and after addition of glucose and triple strength MRS broth (pH6.0G and pH6.0MRS=Fermentors set at pH 6.0 supplemented with glucose and triple strength MRS broth after 8 and 16 h of growth of LA-1, respectively).

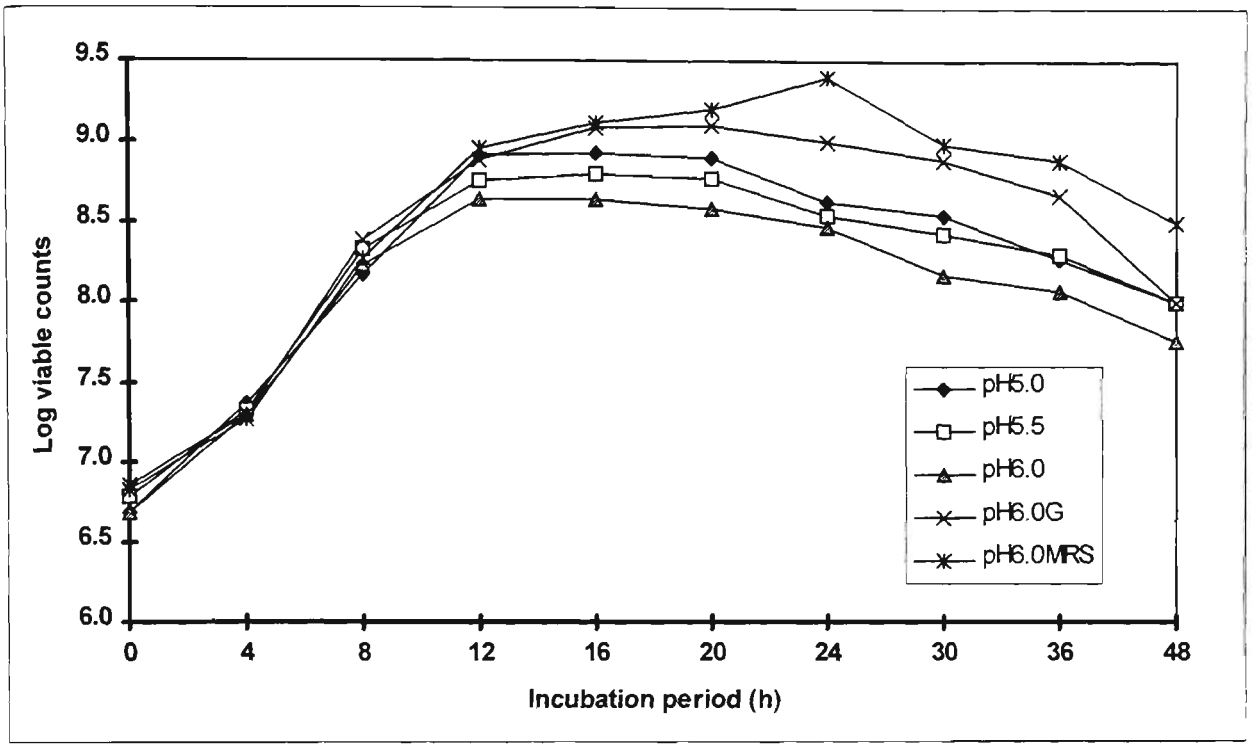


Fig. 7.2.10. Growth of *L. acidophilus* (LA-1) in MRS broth in fermentors at various pH and after addition of glucose and triple strength MRS broth (pH6.0G and pH6.0MRS=Fermentors set at pH 6.0 supplemented with glucose and triple strength MRS broth after 8 and 16 h of growth of LA-1, respectively).

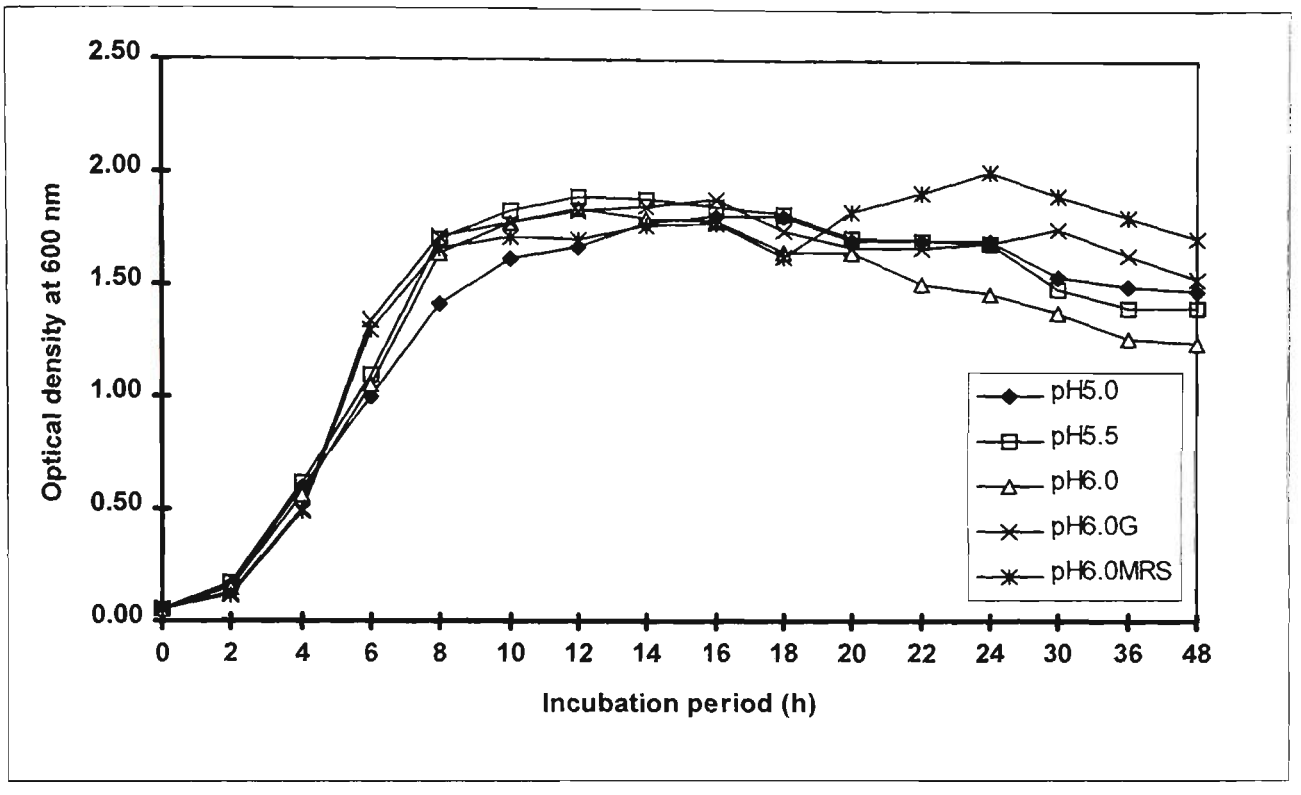


Fig. 7.2.11. Changes in cell density of *L. acidophilus* (LA-1) in MRS broth in fermentors at various pH and after addition of glucose and triple strength MRS broth (pH6.0G and pH6.0MRS=Fermentors set at pH 6.0 supplemented with glucose and triple strength MRS broth after 8 and 16 h of growth of LA-1, respectively).

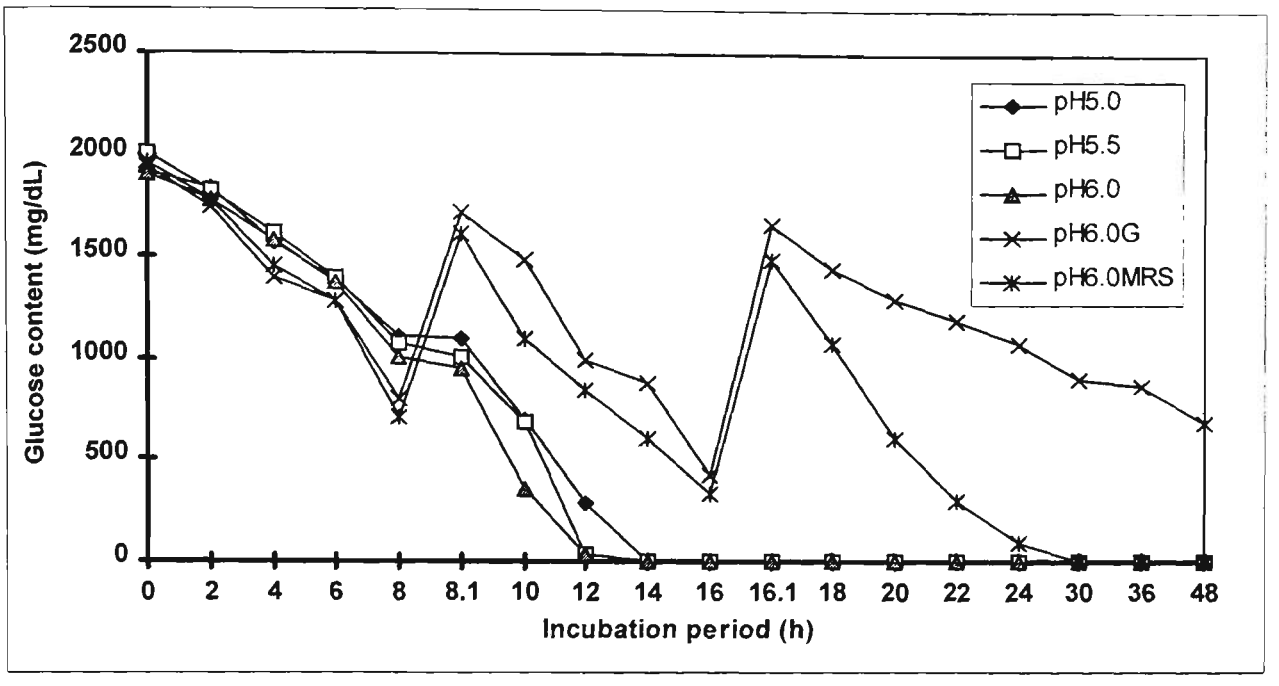


Fig. 7.2.12. Utilisation of glucose by *L. acidophilus* (LA-1) in MRS broth in fermentors at various pH and after addition of glucose and triple strength MRS broth (pH6.0G and pH6.0MRS=Fermentors set at pH 6.0 supplemented with glucose and triple strength MRS broth after 8 and 16 h of growth of LA-1, respectively).

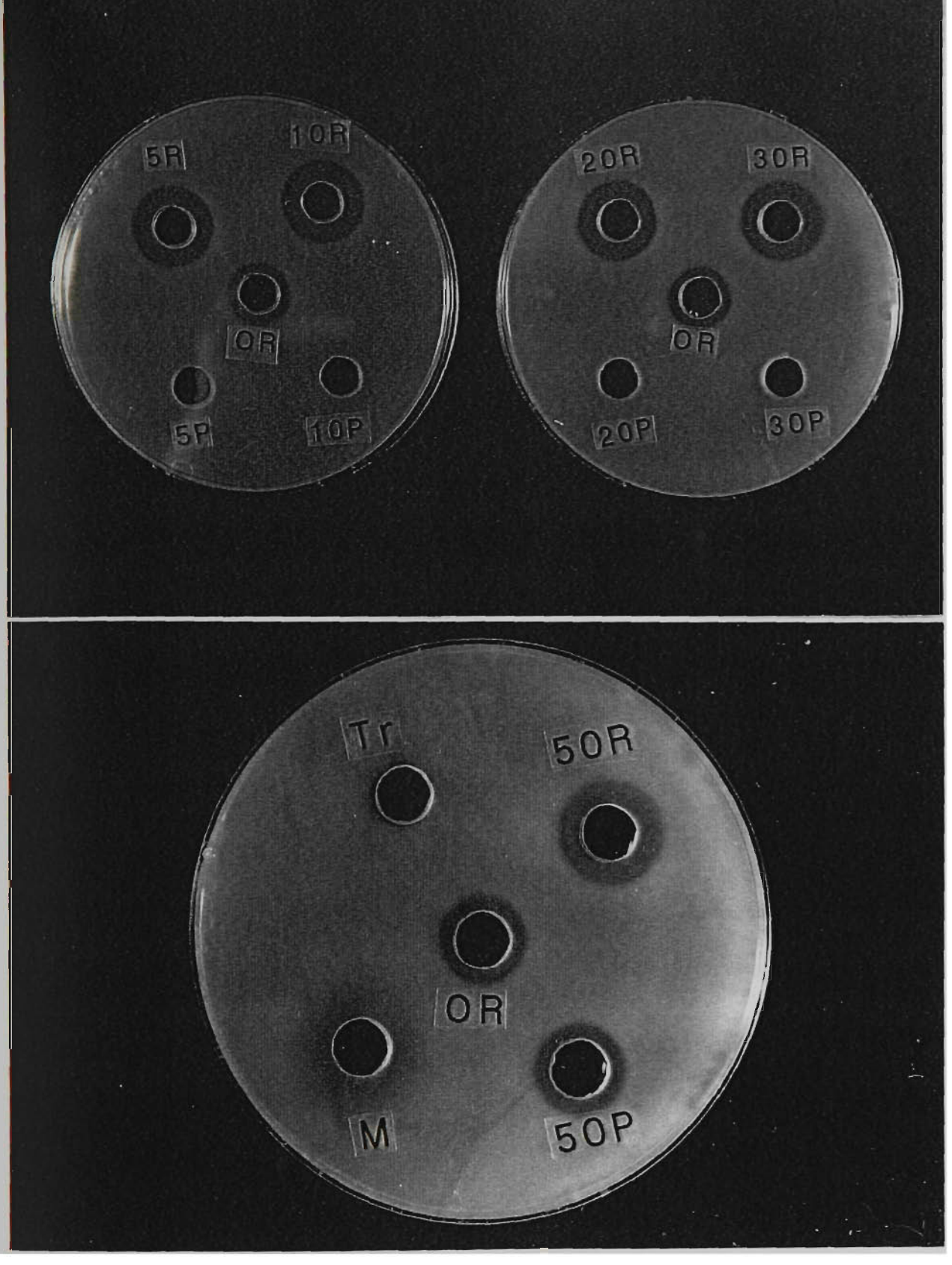


Fig. 7.2.13. Zones of inhibition obtained by *L. acidophilus* (LA-1) against *L. delbrueckii* ssp. *bulgaricus* 2519 (5, 10, 20, 30 and 50 R=Activities in retentates obtained after passing BLIS in MRS broth through 5, 10, 20, 30 and 50 kDa MWCO membranes, respectively; 5, 10, 20, 30 and 50 P=Activities in permeates obtained after passing BLIS in MRS broth through 5, 10, 20, 30 and 50 kDa MWCO membranes, respectively; OR=Original sample before ultrafiltration; Tr=Activity after reacting BLIS in MRS broth with trypsin; M=Activity observed after 96 h growth of LA-1 in milk).

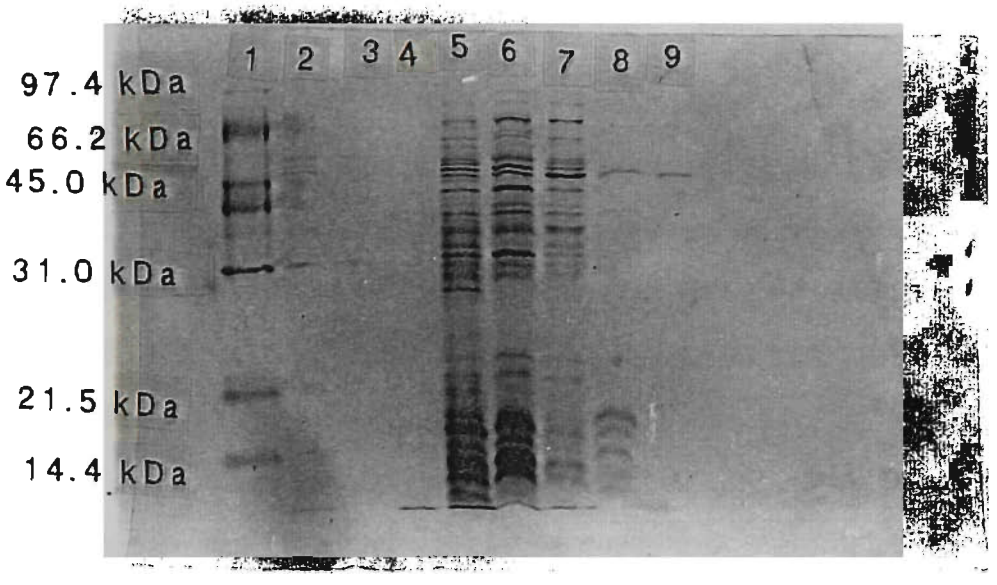


Fig. 7.2.14. Silver stain of various fractions separated on SDS-PAGE during purification of BLIS (Lane 1=Standard molecular weight markers; Lane 2=50 kDa retentate; Lane 3=Supernatant after 40% precipitation with ammonium sulfate; Lane 4=Supernatant after 70% precipitation with ammonium sulfate; Lane 5=10 kDa retentate; Lane 6=20 kDa retentate; Lane 7=30 kDa retentate; Lane 8=Precipitates obtained after 70% saturation of ammonium sulfate; Lane 9= Two stage precipitates obtained after 70 and 40% saturation of ammonium sulfate).

7.3 Antimicrobial substance produced by *Lactobacillus helveticus (2700)*

7.3.1 Introduction

A number of antimicrobial substances such as diacetyl (Daeschel, 1989), ammonia (Rogul and Carr, 1972), fatty acids (Walstad *et al.*, 1974), lactic acid (Tramer, 1966), hydrogen peroxide (Holmberg and Hollander, 1973; Malke *et al.*, 1974) and bacteriocins (Hoover and Steenson, 1993) are produced by various microorganisms. Lactic acid bacteria (LAB) have been extensively studied for their bacteriocinogenic activity. Many bacteriocins isolated from LAB associated with dairy, meat and fermented vegetables have been characterised. According to Tagg *et al.* (1976), bacteriocins are antimicrobial substances containing a biologically active protein moiety that are inhibitory to closely related species of bacteria. However, several researchers (Hoover and Steensen, 1993; Branby-Smith, 1992) have shown that bacteriocin-like inhibitory substances (BLIS) may exhibit bactericidal activity beyond species that are closely related or confined within the same ecological niche. Various microorganisms belonging to the species of *Lactococcus, Pediococcus, Lactobacillus, Leuconostoc, Carnobacterium, Propionibacterium, Enterococcus, Bacillus* and *Escherichia* are reported to produce bacteriocins or BLIS (Hoover and Steenson, 1993; Branby-Smith, 1992; Klaenhammer, 1988, 1993).

Probiotic bacteria such as *Lactobacillus acidophilus* and bifidobacteria are incorporated in fermented dairy foods for their potential therapeutic benefits. It is believed that a minimum of 10^5-10^6 cfu.g^{-1} organisms should be available at the time of consumption in order to avail therapeutic effects (Kurmann and Rasic, 1991).

In earlier studies, several strains of *L. acidophilus* produced BLIS (Dave and Shah, 1997e; Joseph *et al.*, 1998). A bacteriocin produced by *L. acidophilus* LA-1 was

---

A version of section 7.3 has been submitted for publication under the title "Antimicrobial substance produced by *Lactobacillus helveticus (2700)*. Dave, R. I. and Shah, N. P. (1998). *Aust. J. Dairy Technol.* : (In press).

active against *L. delbrueckii* ssp. *bulgaricus, L. jugurti, L. helveticus* and *L. casei* (Dave and Shah, 1997e). Some yoghurt manufacturers in Australia incorporate *L. helveticus* and *L. jugurti* in addition to the traditional yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus).* The information on antagonism between these yoghurt bacteria and probiotic bacteria is scant.

In this study, fifteen LAB were screened for their ability to produce antimicrobial substances against *L. delbrueckii* ssp. *bulgaricus* (2519), *L. helveticus* (2700), *L. jugurti* (2819) and *L. casei* (2603) using the agar well assay technique. Out of three lactobacilli exhibiting antimicrobial activity, the characteristics of the antibacterial substance produced by *L. helveticus* (2700) were studied in detail.

7.3.2 Materials and Methods

7.3.2.1 Microorganisms and their maintenance

The range of microorganisms including lactic acid bacteria, spoilage and pathogenic type of microorganisms were obtained from various sources (Table 3.1 and 3.2) and were maintained as described in section 3.2.

7.3.2.2 Determination of antimicrobial activity

The detection and assay of inhibitory activity was carried out according to the procedure outlined in section 3.13.

7.3.2.3 Reaction with various enzymes

The cell free supernatant was reacted with various enzymes and the nature of antimicrobial substance was detected as described in section 3.15

7.3.2.4 Thermal resistance and activity of the antimicrobial substance over a range of pH

The thermal resistance (section 3.16) and activity over a range of pH (section 3.17) was also studied.

7.3.2.5 Estimation of mloecular weight

The molecular weight of neutralised cell free extract was estimated using ultrafiltration (UF) techniques as described in section 3.22.

All analyses and experiments were repeated at least twice and the results are means of all available data.

7.3.3 Results and Discussion

In a previous study, several *L. acidophilus* strains showed antibacterial activity against four target organisms (*L. delbrueckii* ssp. *bulgaricus* (2519), *L. helveticus* (2700), *L. jugurti* (2819) and *L. casei* (2603)) (Dave and Shah, 1997e). In the present investigation, the initial screening of fifteen additional lactic acid bacteria was carried out using the same target organisms. Three of fifteen organisms studied *L. helveticus* (2700), *L. plantarum* (2903), *L. fermentum* (5174)) produced antimicrobial substances (Table 7.3.1). The antimicrobial substance produced by *L. helveticus* (2700) in MRS broth inhibited various strains of *L. delbrueckii* ssp. *bulgaricus* only, whereas those produced by *L. plantarum* (2903) and *L. fermentum* (5174) were active against all the four target organisms. *L. helveticus* (2704), *Pediococcus cerevisiae* (2305), *Leuconostoc mesenteroides* ssp. *cremoris* (4200), *L. jugurti* (2819), *L. helveticus* (2704), *L. casei* (2603, 2604), *L. casei* ssp. *rhamnosus* (2606) and *L. delbrueckii* ssp. *bulgaricus* (2501, 2505, 2515, 2517 and 2519) did not produce any antimicrobial substance against any of the four target organisms.

After the initial screening, the nature of the antibacterial substance produced by *L. helveticus* (2700) was studied in detail. In an earlier study, *L. acidophilus* (LA-1) showed only slight inhibition of the target organism (*L. delbrueckii* ssp. *bulgaricus*-

2519) when grown in milk (Dave and Shah, 1997e). Contrarily, in the present investigation, *L. helveticus* (2700) produced higher amount of antimicrobial substance in RSM during fermentation than MRS broth. Thus, further studies on the characteristics of the antibacterial substance produced by *L. helveticus* (2700) during fermentation were carried out in RSM. The antimicrobial substance of *L. helveticus* (2700) inhibited seven strains of *L. delbrueckii* ssp. *bulgaricus* (2501, 2505, 2515, 2517, 2519, LB-3 and LB-4), *L. plantarum* (2903), *L. jugurti* (2819), fourteen strains of *L. acidophilus* (2401, 2404, 2405, 2406, 2409, 2410, 2412, 2414, 2415, 2422, BDLA-1, LA-1, MOLA-2, MJLA-1) and the producer organism *L. helveticus* (2700), but not *L. acidophilus* (2400, 2420, OLA-2 and PALA-1), bifidobacteria (1900, 1901, 1912, 1920, 1930, 1941, 20097, 20099, 20210, BB-1), *S. thermophilus (2000, 2002, 2008, 2010, 2013, 2014)*, *L. casei* (2603, 2604, 2606), *L. helveticus* (2704) or *L. fermentum* (5174) (Table 7.3.2). Inhibition of several spoilage and pathogenic groups of organisms was observed at pH < 4.0 only (Table 7.3.2) suggesting the role of organic acids such as lactic acid in the inhibition of these groups of organisms. Spillmann *et al.* (1978) could not detect any antimicrobial activity other than that of lactic acid in yoghurt or in cultures of *L. acidophilus*, *L. casei* or *L. helveticus* against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens* and *Staphylococcus aureus*. The inhibition of 14 of 18 strains of *L. acidophilus* by the antimicrobial substance produced by *L. helveticus* (2700) may be of concern to the Australian yoghurt manufacturers as they incorporate *L. helveticus* in addition to yoghurt starter bacteria to manufacture probiotic or AB (**A**cidophilus-**B**ifidus) yoghurt. Viability of *L. acidophilus* could be adversely affected if *L. helveticus* used by the yoghurt manufacturers produces antimicrobial substance similar to that produced by *L. helveticus* (2700).

The antimicrobial activity associated with *L. helveticus* (2700) was not lost when the neutralised cell-free milk filtrate was reacted with catalase, proteolytic enzymes, lipase, phospholipases or amylases (Table 7.3.3). This indicated that the inhibitory substance was not an organic acid, hydrogen peroxide, protein, fatty acid or a starch molecule. Also, the antimicrobial activity was not due to bacteriophages or diacetyl.

The antimicrobial substance produced by *L. helveticus* (2700) lost its activity at a temperature of 70°C with 30 min holding time (Table 7.3.4). However, it is interesting to note that the antimicrobial activity against *L. delbrueckii* ssp. *bulgaricus* (2519) was not completely lost even after autoclaving. Acidophilucin A produced by *L. acidophilus* (Toba *et al.*, 1991a) and Lacticin A produced by *L. delbrueckii* (Toba *et al.*, 1991b) were reported to be heat labile, whereas Lactacin B (Barefoot and Klaenhammer, 1983), and Lactacin F (Muriana and Klaenhammer, 1987) were heat stable.

The antimicrobial substance produced by *L. helveticus* (2700) was active over a pH range of 3.0 to 10.0 against *L. delbrueckii* ssp. *bulgaricus* (2519) and *L. acidophilus* (MOLA-2) (Table 7.3.5). The maximum activity of the substance was observed at a pH < 4.0; when the pH was adjusted above 6.0, the activity was considerably reduced.

The molecular weight of the antimicrobial substance produced by *L. helveticus* (2700) appeared to be around 5000 daltons as considerable activity was found in the permeate of 5 kDa membrane. *L. helveticus* produced Lactocin 27 which existed as a protein-lipopolysaccharide complex with a molecular weight of > 200 kDa. After purification, the molecular weight was 12.4 kDa (Upreti and Hinsdill, 1975). Helveticin J that inhibited several lactobacilli also existed as a complex aggregate of > 300 kDa and was purified to 37 kDa (Joerger and Klaenhammer, 1986).

7.3.4 Conclusions

Fifteen lactic acid bacteria (LAB) were screened for their ability to produce antimicrobial substances against *L. delbrueckii* ssp. *bulgaricus* (2519), *L. helveticus* (2700), *L. casei* (2603) and *L. jugurti* (2819). Of the fifteen LAB, *L. helveticus* (2700), *L. plantarum* (2903) and *L. fermentum* (5174) exhibited antimicrobial activity. The antimicrobial substance produced in milk by *L. helveticus* (2700) inhibited seven strains of *L. delbrueckii* ssp. *bulgaricus*, the producer organism *L. helveticus* (2700), fourteen strains of *L. acidophilus* and one strain each of *L.*

plantarum (2903) and *L. jugurti* (2819), but not the other LAB, spoilage and pathogenic microorganisms tested. The antimicrobial activity of *L. helveticus* (2700) was not due to organic acids, hydrogen peroxide, diacetyl or bacteriocin like inhibitory substance. The molecular weight of the substance was around 5000 daltons. The substance was active over a pH range of 3.0-10.0, and inactivation occurred at > 70°C.

Table 7.3.1. Screening of various lactic acid bacteria during their growth in MRS broth for the production of antimicrobial substances

Organism	Target organisms			
	LB-2519	LH-2700	LJ-2819	LC-2603
	----Zone of inhibition (mm)---			
L. helveticus (2700)	13.5	nil	nil	nil
L. helveticus (2704)	nil	nil	nil	nil
L. plantarum (2903)	23.5	26.5	22.5	20.5
L. fermentum (5174)	16.5	17.0	15.5	14.5
Pediococcus cereviciae (2305)	nil	nil	nil	nil
Leuconostoc mesenteroides ssp. *cremoris* (4200)	nil	nil	nil	nil
L. jugurti (2819)	nil	nil	nil	nil
L. casei (2603)	nil	nil	nil	nil
L. case (2604)	nil	nil	nil	nil
L. casei ssp. *rhamnosus* (2606)	nil	nil	nil	nil
L. delbrueckii ssp. *bulgaricus* (2501, 2505, 2515, 2517, 2519)	nil	nil	nil	nil

LB=*L. delbrueckii* ssp. *bulgaricus*; LH=*L. helveticus*; LC=*L. casei* and LJ=*L. jugurti.*

Table 7.3.2. Inhibitory spectrum[1] of antimicrobial substance produced by *L. helveticus* (2700) in milk

Target organisms	Numbers positive/Numbers tested
Lactobacillus delbrueckii ssp. *bulgaricus* (2501, 2505, 2515, 2517, 2519, LB-3, LB-4)	7/7
Lactobacillus helveticus (2700, 2704)	1/2
Lactobacillus jugurti (2818)	1/1
Lactobacillus plantarum (2903)	1/1
Lactobacillus acidophilus (2400, 2401, 2404, 2405, 2406, 2409, 2410, 2411, 2412, 2414, 2415, 2420, 2422, LA-1, BDLA-1, MJLA-1, MOLA-2, OLA-2, PALA-1)	14/18
L. casei (2603, 2604)	0/2
L. casei ssp. *rhamnosus* (2606)	0/1
L. fermentum (5174)	0/1
S. thermophilus (2000, 2002, 2008, 2010, 2013, 2014)	0/6
Bifidobacteria (1900,1901, 1912, 1920, 1930, 1941, 20097, 20099, 20210, BB-1)	0/10
Spoilage and pathogenic organisms	
*Escherichia coli*** (Ec 1, VUN 0100)	0/2
Candida albicans (Ca 1)	0/1
*Aeromonas hydrophila*** (Ah 1)	0/1
*Salmonella typhi***(Sal 1)	0/1
Staphylococcus aureus (VUP 0040)	0/1
*Pseudomonas fluorescens*** (VUN 0031)	0/1
*Salmonella typhimurium*** (VUN 0045)	0/1
*Enterobacter aerogenes*** (VUN 0025)	0/1
*Bacillus stearothermophilus*** (VUP 0007)	0/1
*Bacillus cereus*** (VUP 0001)	0/1
Enterococcus faecalis (VUP 0019)	0/1
Micrococcus luteus (VUP0023)	0/1
*Helicobacter pylori*** (Hp 1,2,3,4)	0/4
*Listeria innocua*** (Li 1)	0/1
*Listeria monocytogenes*** (Lm 1)	0/1
*Clostridum perfringens*** (VUP 0060)	0/1
*Clostridium sporogenes*** (VUP 0061)	0/1
*Vibrio parahaemolyticus*** (WP1, VUN 0300)	0/2

[1]Inhibitory spectrum was measured using the agar well assay technique.
**inhibition of organisms was found with unneutralised (pH <4.0) filtrate and the spoilage and pathogenic microorganisms were not inhibited by the antimicrobial substance produced by *L. helveticus* (2700), but due to the acid produced by this organism.

Table7.3.3. Sensitivity of antimicrobial substance produced by *L. helveticus* (2700) against various enzymes

Treatment	Concentration	Target Organisms	
		LB-2519	*LA-2409*
		...Zone of inhibition[1] (mm)...	
Control[2]	No Enzyme	18.5	16.0
Catalase	0.1 mg/mL	18.0	15.0
Trypsin	1.0 mg/mL	18.0	15.5
α-chymotrypsin	1.0 mg/mL	18.5	15.5
β-chymotrypsin	1.0 mg/mL	18.0	15.5
Papain	0.5 mL/mL	18.0	15.0
Proteinase-K	0.2 mg/mL	18.5	15.0
Crude protease	1.0 mg/mL	18.0	15.5
Ficin	10 Units/mL	18.0	16.0
Phospholipase A2	0.1 mg/mL	18.5	14.5
Phospholipase D	50 Units/mL	17.5	14.5
α-amylase	1000 Units/mL	18.0	15.5
β-amylase	100 Units/mL	18.0	15.5
Lipase	250 Units/mL	17.5	14.5

LB=*L. delbrueckii* ssp. *bulgaricus*; LA=*L. acidophilus*.

[1]Zone of inhibition was measured using *L. delbrueckii* ssp. *bulgaricus-2519* as target organism.

[2]Filtrate without any treatment.

Table 7.3.4. Heat resistance of antimicrobial substance produced by *L. helveticus* (2700) in MRS broth and in milk

Treatment	Target organism					
	LB 2519	LA-1	BDLA-1	LA2409	MOLA2	LH-2700
	------Zone of inhibition[1] (mm)----					
MRS broth (pH 4.0)	14.5	nil	nil	nil	nil	nil
MRS broth (pH 6.0)	14.0	nil	nil	nil	nil	nil
MRS broth subjected to autoclaving	11.0	nil	nil	nil	nil	nil
Filtrate[2] at pH 3.48	23.5	16.5	16.5	17.5	17.0	13.5
Filtrate neutralised to pH 6.2	22.5	16.0	16.0	16.5	17.0	13.5
Filtrate heated to 50°C for 10 and 30 min	22.5	16.0	16.0	16.0	16.5	13.5
Filtrate heated to 60°C for 10 min	22.0	15.0	15.5	15.5	16.0	13.5
Filtrate heated to 60°C for 30 min	20.0	12.0	13.0	12.0	13.0	12.0
Filtrate heated to 70°C for 10 min	19.0	12.0	12.0	12.0	12.5	12.0
Filtrate heated to 70°C for 30 min	16.0	nil	nil	nil	nil	nil
Filtrate heated to 100°C for 10 and 30 min	13.0	nil	nil	nil	nil	nil
Filtrate subjected to autoclaving	11.5	nil	nil	nil	nil	nil

LB=*L. delbrueckii* ssp. *bulgaricus*; LA=*L. acidophilus* and LH=*L. helveticus*.

[1]Zone of inhibition includes 7.0 mm bore diameter.

[2]Casein free serum after growth of *L. helveticus* 2700 in RSM.

Table 7.3.5. Activity of antibacterial substance produced by *L. helveticus* (2700) over a range of pH

	-------pH of the medium--------							
Target organisms	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
	·····Zone of inhibition·····							
L. delbrueckii ssp. *bulgaricus* (2519)	25.0	25.0	24.5.	24.0	22.0	20.0	20.0	19.0
L. acidophilus (MOLA-2)	16.5	16.0	16.0	15.5	15.0	15.0	13.5	8.0
Control[1]	9.0**	nil	nil	nil	nil	nil	nil	nil

[1]Activity of un-inoculated sterile reconstituted skim milk adjusted to various pH and using *L. delbrueckii* ssp. *bulgaricus* as a target organism.
**Zone of inhibition was not very clear as observed at other pH.

7.4 Characteristics of bacteriocin like inhibitory substances produced by *Lactobacillus acidophilus* (BDLA-1, 2409 and MOLA-2), *Lactobacillus fermentum* (5174) and *Lactobacillus plantarum* (2903)

7.4.1 Introduction

Bacteriocins are proteinaceous compounds that kill or inhibit closely related bacteria (Tagg *et al.*, 1976). However, studies have shown that bacteriocins may exhibit bactericidal activity beyond species that are closely related. The information on antagonistic effects due to bacteriocins between yoghurt bacteria *(Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) and probiotic bacteria (*Lactobacillus acidophilus* and bifidobacteria) is scant.

In the previous studies, characteristics of acidophilicin LA-1 (Dave and Shah, 1997e) and the antimicrobial substance produced by *L. helveticus* (2700) (section 7.3) were reported. It was found that 15 of 19 strains of *L. acidophilus, L. fermentum* (5174) and *L. plantarum* (2903) also produced antimicrobial substances that were active against *L. delbrueckii* ssp. *bulgaricus* (2519) which was also inhibited by *L. acidophilus* (LA-1) and *L. helveticus* (2700). In the present investigation, some characteristics of a bacteriocin produced by three strains of *L. acidophilus* (BDLA-1, LA-2409 and MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903) were studied in detail and the results were compared with the antimicrobial substance produced by *L. helveticus* (2700) and the bacteriocin like inhibitory substance (BLIS) produced by *L. acidophilus* (LA-1) which was named as acidophilicin LA-1 (Dave and Shah, 1997e).

7.4.2 Materials and methods

7.4.2.1 Microorganisms and their maintenance

The range of microorganisms including lactic acid bacteria, spoilage and pathogenic type of microorganisms were obtained from various sources (Tables 3.1 and 3.2) and

---

A version of section 7.4 has been submitted for publication. Dave, R. I. and Shah, N. P. (1998). *Inter. Dairy J.*: (Under review).

were maintained as described in section 3.2.

7.4.2.2 Determination of antimicrobial activity

The detection and assay of inhibitory activity was carried out according to the procedure outlined in section 3.13.

7.4.2.3 Reaction with various enzymes

The cell free supernatant was reacted with various enzymes and the nature of antimicrobial substance was detected as described in section 3.15

7.4.2.4 Thermal resistance and activity of BLIS over a range of pH

The thermal resistance (section 3.16) and activity over a range of pH (section 3.17) was also studied.

7.4.2.5 Determination of cell density

The changes in viable counts were monitored over a period of 48 h; the details of which are described in section 3.21.

7.4.2.6 Estimation of mloecular weight

The molecular weight of neutralised cell free extract was estimated as described in section 3.22.

7.4.2.7 Purification of BLIS using ammonium sulfate precipitation and SDS-PAGE

The purification of BLIS by ammonium sulfate precipitation and SDS-PAGE of various fractions are described in section 3.23.

All analyses and experiments were repeated at least twice and the results are means of all available data.

7.4.3 Results and discussion

Inhibitory spectrum of BLIS produced by the lactobacilli. The inhibitory activity of BLIS produced by *L. acidophilus* (BDLA-1, 2409 and MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903) against various LAB, spoilage and pathogenic groups of organisms is presented in Table 7.4.1. The organisms that were inhibited by *L. acidophilus* (BDLA-1, 2409, MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903) included *L. delbrueckii* ssp. *bulgaricus* (2519), *L. helveticus* (2700), *L. jugurti* (2819), *L. casei* (2603) and *L. acidophilus* (MJLA-1). *L. acidophilus* (MOLA-2) also showed some inhibition of *L. acidophilus* (OLA-2); however the zone of inhibition was not very clear. Inhibition of other strains of *L. casei* (2604, 2606 and Shirota strain) or *L. acidophilus* was not observed, indicating a possibility of strain specificity of the BLIS (Table 7.4.1). Other LAB and the spoilage and pathogenic groups of organisms were not affected by the BLIS produced by any of the 5 lactobacilli tested in this study. Some spoilage and pathogenic groups of organisms were inhibited with unneutralised MRS broth; however, when the pH was adjusted to >5.5, no effect was observed (Table 7.4.1). This indicated that organic acid might have been responsible for the inhibition of these groups of organisms. Thus, inhibitory spectrum of the 5 lactobacilli studied was similar to that of acidophilicin LA-1, but not similar to that observed by the antimicrobial substance produced by *L. helveticus* (2700).

Sensitivity of BLIS to various enzymes. The inhibitory activity of BLIS produced by *L. acidophilus* (BDLA-1, 2409 and MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903) was lost after reaction with proteolytic enzymes such as trypsin, α or β-chymotrypsin, papain, ficin, proteinase K and crude protease. However, the activity was not affected when reacted with enzymes such as α or β-amylase, lipase and phospholipase A2 and D (Table 7.4.2). This confirmed that active protein moiety was responsible for the inhibition of the target organisms in the BLIS produced by *L.*

acidophilus (BDLA-1, 2409, MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903). Thus, sensitivity of BLIS produced by all 5 lactobacilli studied to various enzymes was similar to that reported for acidophilicin LA-1 but not similar to the antimicrobial substance produced by *L. helveticus* (2700).

Stability of BLIS. The BLIS produced by *L. acidophilus* (BDLA-1, 2409 and MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903) retained full activity after various heat treatments including autoclaving (Table 7.4.3) and was active over a wide range of pH; similar to that observed for acidophilicin LA-1 (Table 7.4.4).

Production of BLIS as affected by growth of organism in various media

The pH was found to be most crucial for the production of acidophilicin LA-1 (Dave and Shah, 1997e); therefore, *L. acidophilus* (LA-1, BDLA-1, 2409 and MOLA2), *L. fermentum* (5174), *L. plantarum* (2903) were grown in Schott bottles in MRS broth, MRS broth supplemented with 1.9 % sodium β-glycerophosphate (BGP) and MRS broth adjusted to pH 5.0 and the changes in pH, cell density and the zone of inhibition were monitored over a period of time at various time intervals. The results were compared with acidophilicin LA-1 which was reported to be produced by *L. acidophilus* (LA-1) (Dave and Shah, 1997e). The production of BLIS was considerably higher in MRS broth supplemented with 1.9 % sodium β-glycerophosphate (BGP) than MRS broth for cultures *L. acidophilus* (LA-1, BDLA-1, 2409) and *L. fermentum* (5174), but the production of BLIS was almost similar in these two media for cultures *L. acidophilus* (MOLA-2) and *L. plantarum* (2903). The production of BLIS was almost negligible in MRS broth adjusted to pH 5.0 for *L. acidophilus* (LA-1 and BDLA-1). For other lactobacilli, it was considerably lower in this medium (Fig. 7.4.1 and 7.4.2). The activity of BLIS increased during initial growth of LA-1 for 18-24 h and remained similar or declined to some extent during further incubation in MRS broth and that supplemented with BGP (Fig. 7.4.1 and 7.4.2). The pH of MRS broth increased from 6.3 to ~7.0 due to addition of BGP (Fig. 7.4.3 and 7.4.4); higher pH could be responsible for the increased production of BLIS in MRS broth with BGP. The drop in pH and growth of organisms was relatively faster in MRS broth than in MRS broth supplemented with BGP or that adjusted to

pH 5.0, initially for up to 16-20 h (Fig. 7.4.3 and 7.4.4). On an average, the increase in optical density was considerably lower at pH 5.0 for most of the cultures than at pH 6.2 of MRS broth (Fig. 7.4.3 and 7.4.4). This confirmed that multiplication of bacterial cells of these lactobacilli was faster in MRS broth as compared to MRS broth supplemented with BGP or that adjusted to pH 5.0. The possible reason could be the buffering effect of BGP that altered the pH to ~ 7.0 and the pH of 5.0 in MRS broth might not be optimum for the growth of these lactobacilli.

This set of experiments confirmed that the pH and the total cell population might be important for the production of BLIS. Further, the cells of lactobacilli started to settle at the bottom of the Schott bottles after 14-16 h of growth in the broth medium. Hence, great variations were observed in the amount of BLIS produced by these lactobacilli during their growth under batch conditions.

Molecular weight of BLIS. The zones of inhibition of retentate and permeate fractions after concentration using membranes of various molecular weight cut offs showed that the molecular weight of *L. acidophilus* (BDLA-1 and 2409) and *L. fermentum* (5174) was almost similar to that of *L. acidophilus* (LA-1) reported earlier. Conversely, the molecular weight of BLIS produced by *L. acidophilus* (MOLA-2) and *L. plantarum* (2903) was considerably lower than that produced by *L. acidophilus* (LA-1) (Table 7.4.5). The activity of BLIS produced by *L. acidophilus* (LA-1, BDLA-1) was not found in the permeate of 10, 20 or 30 kDa membranes. Permeates of 50 kDa membranes showed some inhibitory activity; however, major activity was found in the retentate. Further, some activity in the permeates was observed for *L. acidophilus* (2409) and *L. fermentum* (5174). Contrarily, almost equal activity was observed in retantates and permeates of 10kDa membranes during concentration of crude BLIS produced by *L. acidophilus* (MOLA-2) and *L. plantarum* (2903).

Purification of BLIS. Precipitation of 50 kDa retentate with single stage 40 or 70% ammonium sulphate saturation level showed 2 or more bands on silver staining of SDS-PAGE gels for *L. acidophilus* (LA-1). However, two stage fractionation (first

stage, 0-40%; second stage, 40-70%) resulted in a single band on silver staining of gel. Thus, the highest activity containing retantates of these lactobacilli were fractionated with two stage ammonium sulfate and each fraction was silver stained on SDS-PAGE to check the purity of protein band. SDS-PAGE results showed that a two step ammonium sulphate precipitation is useful in achieving the purest form of BLIS produced by *L. acidophilus* (LA-1, BDLA-1, 2409) and *L. fermentum* (5174). The purity was variable in each fraction and the activity was found to be lost to a considerable extent after each step of purification for these lactobacilli (Fig 7.4.5). The ammonium sulfate fractionation was not successful in purifying the BLIS produced by *L. acidophilus* (MOLA-2) and *L. plantarum* (2903) and more than one band resulted for various ammonium sulfate fractions. However, the ammonium sulfate fractions of fltered and concentrated broth of both of these organisms showed one band (~54 kDa) similar to that observed for other lactobacilli which gave single band which was also similar to that observed for acidophilicin LA-1 (Dave and Shah, 1997e). This indicated that these two organisms also produced similar protein to that produced by *L. acidophilus* (LA-1).

Lactacin B, Lactacin F and Acidophilucin A have been reported to be produced by various *L. acidophilus*. The former two were heat stable, but the later one was heat labile. Lactacin B and Lacticin F were high molecular weight complex and showed different inhibitory spectrum against a range of target organisms (Hoover and Steenson, 1993).

The bacteriocin produced by *L. fermenti* that inhibited *Lactobacillus fermenti* was a macromolecular complex that had lipocarbohydrate moiety (de Klerk and Smit, 1967). *L. plantarum* have been reported to produce Plantaricin A (Daeschel *et al.*, 1990), Plantacin B (West and Warner, 1988) and an unnamed bacteriocin (Jimenez-Diaz *et al.*, 1990). Plantaricin A has been reported to be active against *L. plantarum*, *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Lactococcus lactis* and *Enterococcus faecalis*. The substance was proteinaceous and had > 8 kDa molecular mass, stable at 100°C for 30 min and fully active over a pH range of 4.0-6.5 (Daeschel *et al.*, 1990). Plantacin B is reported to inhibit *L. plantarum*, *Leuconostoc*

mesenteroides and *Pediococcus damnosus* and an active protein moiety was responsible for their inhibition (West and Warner, 1988). An unnamed bacteriocin of *L. plantarum* was active against *Leuconosoc*, *Lactobacillus*, *Pediococcus*, *Lactococcus* and *Streptococcus* was sensitive to proteases, α-amylase and lipase, but stable at 100°C for 30 min. Thus, the BLIS produced by the 5 lactobacilli and reported in this chapter were different from those reported by other workers, but similar to acidophilicin LA-1 as reported by Dave and Shah (1997e).

7.4.4 Conclusions

Some characteristics of bacteriocin like inhibitory substance (BLIS) produced by *L. acidophilus* (BDLA-1, 2409, MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903) were studied. The BLIS produced by all of these lactobacilli showed a narrow spectrum of inhibition, similar to that observed for acidophilicin LA-1. The BLIS produced by all of these lactobacilli were sensitive to proteolytic enzymes, but not to amylases, lipase or phospholipases, confirming proteinaceous nature of the inhibitory substance/s. The crude BLIS produced by these lactobacilli were stable over a wide range of pH and temperature and remained active after autoclaving. The production of BLIS occurred in the log and early stationary phase and was greatly influenced by the pH of the growth medium. The fractionation with ammonium sulfate of retentates was successful in purifying the BLIS produced by *L. acidophilus* (BDLA-1, 2409) and *L. fermentum* (5174) and resulted in a single band on silver staining of SDS-PAGE gel. Conversely, ammonium sulfate fractionation of retentates of *L. acidophilus* (MOLA-2) and *L. plantarum* (2903) was not successful in completely purifying the BLIS produced by them and more than one band resulted with the range of ammonium sulfate fractions; however, a band of ~54 kDa was common for all 5 lactobacilli studied and acidophilicin LA-1. Thus, it is finally concluded that the production of ~54 kDa protein was one of the responsible factor in all 5 lactobacilli studied for the inhibition of target organisms and most of the characteristics were similar to that of acidophilicin, but not similar to the antimicrobial substance produced by *L. helveticus* (2700).

Table 7.4.1. Inhibitory spectrum[1] of bacteriocin like inhibitory substance produced by *L. acidophilus* (BDLA-1, LA-2409, MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903)

Target organism	Zone of inhibition[2] (mm)				
Lactic acid bacteria	***BDLA-1***	***LA-2409***	***MOLA-2***	***LF-5174***	***LP-2903***
Lactobacillus delbrueckii ssp. *bulgaricus* (2519)	21.0	20.0	24.0	19.0	25.0
Lactobacillus helveticus (2700)	24.0	20.5	27.0	20.0	26.0
Lactobacillus jugurti (2819)	20.0	18.5	23.0	19.0	25.0
Lactobacillus casei (2603)	18.0	17.0	20.0	18.0	22.0
Lactobacillus casei (2604)	nil	nil	nil	nil	nil
Lactobacillus casei ssp. *rhamnosus* (2606)	nil	nil	nil	nil	nil
Pediococcus cerevisiae (2305)	nil	nil	nil	nil	nil
Leuconostoc mesenteroides ssp. *cremoris* (4200)	nil	nil	nil	nil	nil
Lactobacillus fermentum (5174)	nil	nil	nil	nil	nil
Lactobacillus plantarum (2903)	nil	nil	nil	nil	nil
Streptococcus thermophilus (St-1,2,3,4)	nil	nil	nil	nil	nil
Lactobacillus acidophilus (MJLA-1)	20.0	17.0	24.0	17.0	24.0
Lactobacillus acidophilus (OLA-2)	nil	nil	13.0*	nil	11.0*
Lactobacillus acidophilus (2400, 2409, 2420, BDLA-1, PALA-1, MOLA-2)	nil	nil	nil	nil	nil
Bifidobacteria (BB1, 1901, 1902, 1912, 1920 1941, 20099, 20210)	nil	nil	nil	nil	nil
	nil	nil	nil	nil	nil
Spoilage and pathogenic organisms					
Escherichia coli (Ec 1, VUN 0100)**	nil	nil	nil	nil	nil
Candida albicans (Ca 1)	nil	nil	nil	nil	nil
Aeromonas hydrophila (Ah 1)**	nil	nil	nil	nil	nil
Salmonella typhi (Sal 1)**	nil	nil	nil	nil	nil
Staphylococcus aureus (VUP 0040)	nil	nil	nil	nil	nil
Pseudomonas fluorescens (VUN 0031)**	nil	nil	nil	nil	nil
Salmonella typhimurium (VUN 0045)**	nil	nil	nil	nil	nil
Enterobacter aerogenes (VUN 0025)**	nil	nil	nil	nil	nil
Bacillus stearothermophilus (VUP 0007**)	nil	nil	nil	nil	nil
Bacillus cereus (VUP 0001)**	nil	nil	nil	nil	nil
Enterococcus faecalis (VUP 0019)	nil	nil	nil	nil	nil
Micrococcus luteus (VUP0023)	nil	nil	nil	nil	nil
Helicobacter pylori (Hp 1,2,3,4)**	nil	nil	nil	nil	nil
Listeria innocua (Li 1)**	nil	nil	nil	nil	nil
Listeria monocytogenes (Lm 1)**	nil	nil	nil	nil	nil
Clostridum perfringens (VUP 0060)**	nil	nil	nil	nil	nil
Clostridium sporogenes (VUP 0061)**	nil	nil	nil	nil	nil
Vibrio parahaemolyticus (WP1, VUN 0300)**	nil	nil	nil	nil	nil

BDLA-1, LA-2409 and MOLA-2=*L. acidophilus* strains BDLA-1, 2409 and MOLA-2, respectively; LF-5174=*L. fermentum* (5174); LP-2903=*L. plantarum* (2903).

[1]Inhibitory spectrum was measured after neutralising and treating MRS broth with catalase.

[2]Zone of inhibition includes 7.0 mm bore diameter.

*indicates unclear zone of inhibition; **indicates inhibiton of organisms with unneutralised MRS broth (pH <4.0).

Table 7.4.2. Sensitivity of bacteriocin like inhibitory substance produced by *L. acidophilus* (LA-1) to various enzymes

Enzyme	Zone of inhibition (mm)				
	Producer organism				
	BDLA-1	LA-2409	MOLA-2	LF-5174	LP-2903
Control	20.5	19.0	23.5	17.5	25.5
Catalase	19.5	18.5	23.5	17.0	24.5
Trypsin	nil	nil	nil	nil	nil
α-chymotrypsin	nil	nil	nil	nil	nil
β-chymotrypsin	nil	nil	nil	nil	nil
Papain	nil	nil	nil	nil	nil
Ficin	nil	nil	nil	nil	nil
Proteinase k	nil	nil	nil	nil	nil
Protease (crude)	nil	nil	nil	nil	nil
α-amylase	20.5	19.0	23.5	17.0	24.0
β-amylase	19.5	18.5	23.0	16.5	24.5
Lipase	20.0	19.5	23.0	17.0	24.0
Phospholipase A2	19.5	19.0	23.5	16.0	23.5
Phospholipase D	19.5	19.5	22.5	16.5	24.5

BDLA-1, LA-2409 and MOLA-2=*L. acidophilus* strains BDLA-1, 2409 and MOLA-2, respectively; LF-5174=*L. fermentum* (5174); LP-2903=*L. plantarum* (2903).

Final concentration of enzyme per millilitre of neutralised and catalase treated MRS broth was same as used before (Table 7.2.2); Zone of inhibition includes 7.0 mm bore diameter and the target organism was *L. delbrueckii* ssp. *bulgaricus* (2519).

Table 7.4.3. Heat resistance of bacteriocin like inhibitory substance produced by *L. acidophilus* (BDLA-1, 2409 and MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903)

Treatment	Zone of inhibition[1] (mm) against *L. delbrueckii* ssp. *bulgaricus* (2519) by various lactobacilli				
	BDLA-1	*LA-2409*	*MOLA-2*	*LF-5174*	*LP-2903*
MRS broth (pH 4.22)	21.0	19.5	24.0	19.0	25.0
MRS broth (neutralised to pH 6.2)	20.5	19.0	23.5	18.5	24.5
MRS broth (50°C for 10 and 30 mins)	20.0	19.5	23.5	18.5	24.0
MRS broth (60°C for 10 and 30 mins)	21.0	19.5	23.5	18.0	23.5
MRS broth (70°C for 10 and 30 mins)	20.5	19.0	23.0	18.5	24.0
MRS broth (80°C for 10 and 30 mins)	21.0	20.0	24.0	19.0	24.5
MRS broth (90°C for 10 and 30 mins)	20.5	19.5	23.5	18.5	25.5
MRS broth (100°C for 10 and 30 mins)	20.0	19.5	24.0	19.0	25.0
MRS broth (121°C for 15 mins)	19.5	19.0	23.0	19.0	25.0

BDLA-1, LA-2409 and MOLA-2=*L. acidophilus* strains BDLA-1, 2409 and MOLA-2, respectively; LF-5174=*L. fermentum* (5174); LP-2903=*L. plantarum* (2903).
[1]Zone of inhibition includes 7.0 mm bore diameter.

Table 7.4.4. Activity of bacteriocin like inhibitory substance (BLIS) produced by *L. acidophilus* (BDLA-1, 2409 and MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903) over a range of pH

	-------pH of the medium--------								
	2.0	***3.0***	***4.0***	***5.0***	***6.0***	***7.0***	***8.0***	***9.0***	***10.0***
BLIS activity[1] by BDLA-1	24.5	24.0	21.5	21.5	21.0	21.0	21.0	20.0	18.5
BLIS activity by LA-2409	24.5	22.0	2.0	19.5	19.0	18.0	18.0	17.5	16.5
BLIS activity by MOLA-2	27.5	26.5	26.0	25.0	25.0	25.0	25.0	23.5	22.0
BLIS activity by LF-5174	23.5	22.0	19.5	19.0	18.5	18.5	18.0	17.5	17.0
BLIS activity by LP-2903	27.5	27.0	25.5	25.0	24.5	24.0	23.5	23.0	22.0
Control activity[2]	13.0**	9.0**	nil	nil	nil	nil	nil	nil	nil

BDLA-1, LA-2409 and MOLA-2=*L. acidophilus* strains BDLA-1, 2409 and MOLA-2, respectively; LF-5174=*L. fermentum* (5174); LP-2903=*L. plantarum* (2903).
[1]presented as zone of inhibition (mm) using 2519 as target organism and zone includes 7.0 mm bore diameter.
[2]activity of uninoculated MRS broth adjusted to various pH.
** zone of inhibition was not very clear as observed with BLIS at other pH.

Table 7.4.5 Inhibitory activity[1] of retentates and permeates after concentration of cell free MRS broth of *L. acidophilus* (BDLA-1, 2409 and MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903)

Membrane	Activity in...	Zone of inhibition (mm) for				
		BDLA-1	*LA-2409*	*MOLA-2*	*LF-5174*	*LP-2903*
10 kDa	Control	20.0	22.0	24.0	18.0	25.0
	Retentates	27.5	27.0	25.0	24.5	27.0
	Permeates	8.0	9.5	18.0	8.0	23.0
30 kDa	Control	19.5	21.5	23.0	17.0	24.0
	Retentates	27.0	27.5	24.5	23.5	26.0
	Permeates	9.0	11.5	21.0	10.0	23.0
50 kDa	Control	20.5	22.5	24.0	18.0	24.5
	Retentates	23.5	23.5	22.0	24.0	25.0
	Permeates	16.0	18.0	21.0	15.0	22.0

BDLA-1, LA-2409 and MOLA-2=*L. acidophilus* strains BDLA-1, 2409 and MOLA-2, respectively; LF-5174=*L. fermentum* (5174); LP-2903=*L. plantarum* (2903).

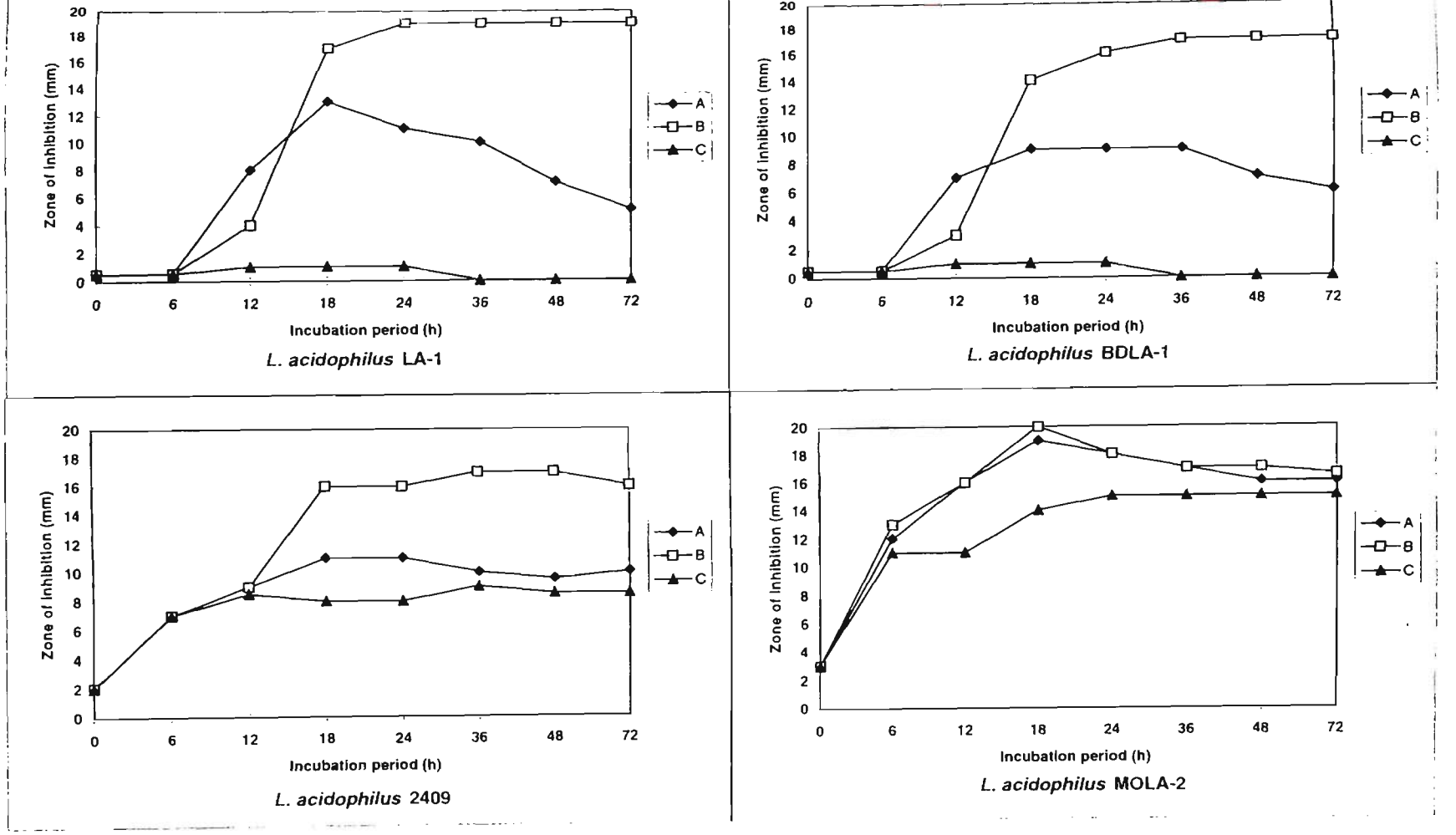


Fig. 7.4.1. Bacteriocinogenic activity of *L. acidophilus* (LA-1, BDLA-1, 2409 and MOLA-2) as affected by their growth in MRS broth (A), MRS broth supplemented with ß-glycerophosphate (B) and MRS broth adjusted to pH 5.0 (C) (Zone of inhibition does not include 7.0 mm bore diameter).

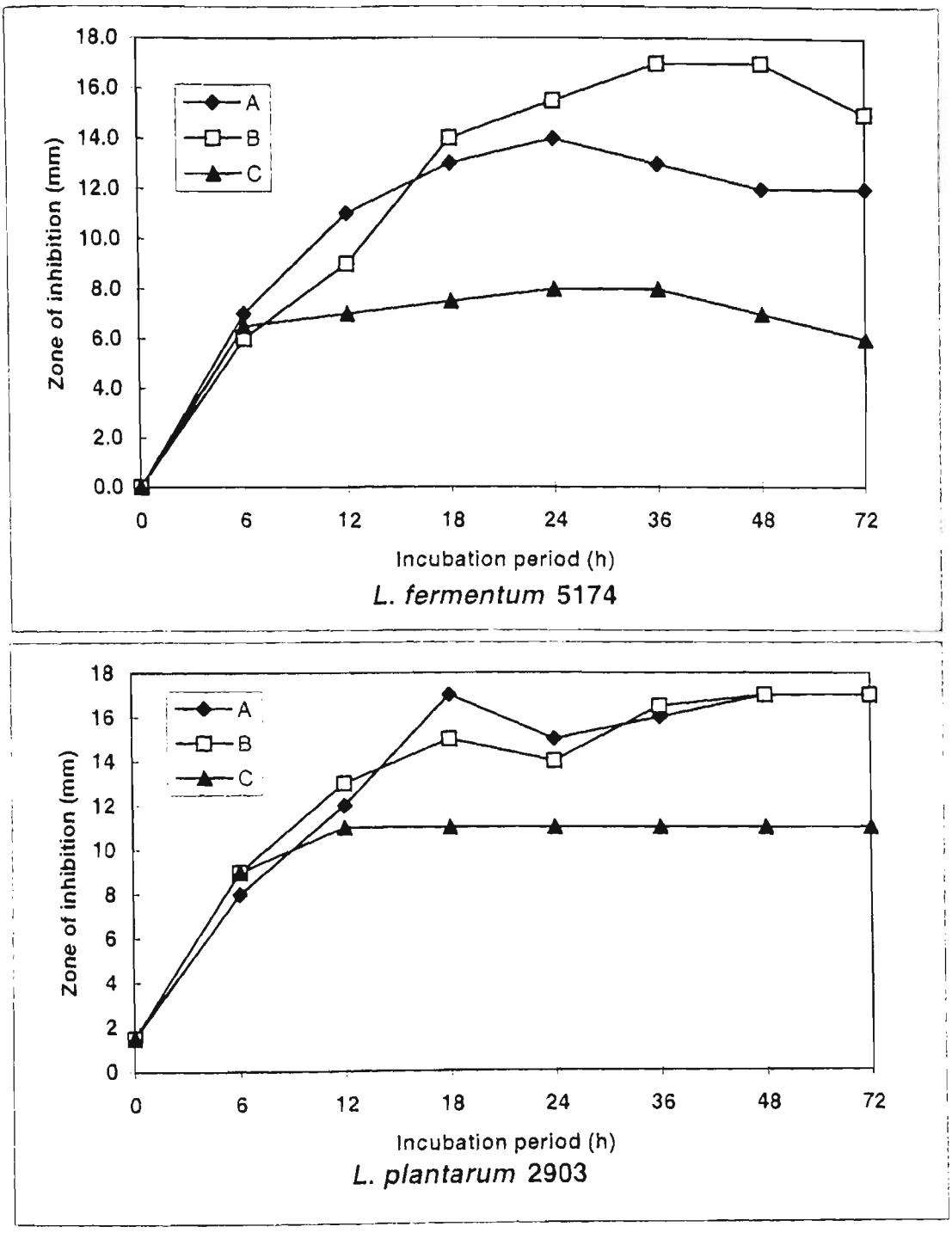


Fig. 7.4.2. Bacteriocinogenic activity of *L. fermentum* (5174) and *L. plantarum* (2903) as affected by their growth in MRS broth (A), MRS broth supplemented with β-glycerophosphate (B) and MRS broth adjusted to pH 5.0 (C) (Zone of inhibition does not include 7.0 mm bore diameter).

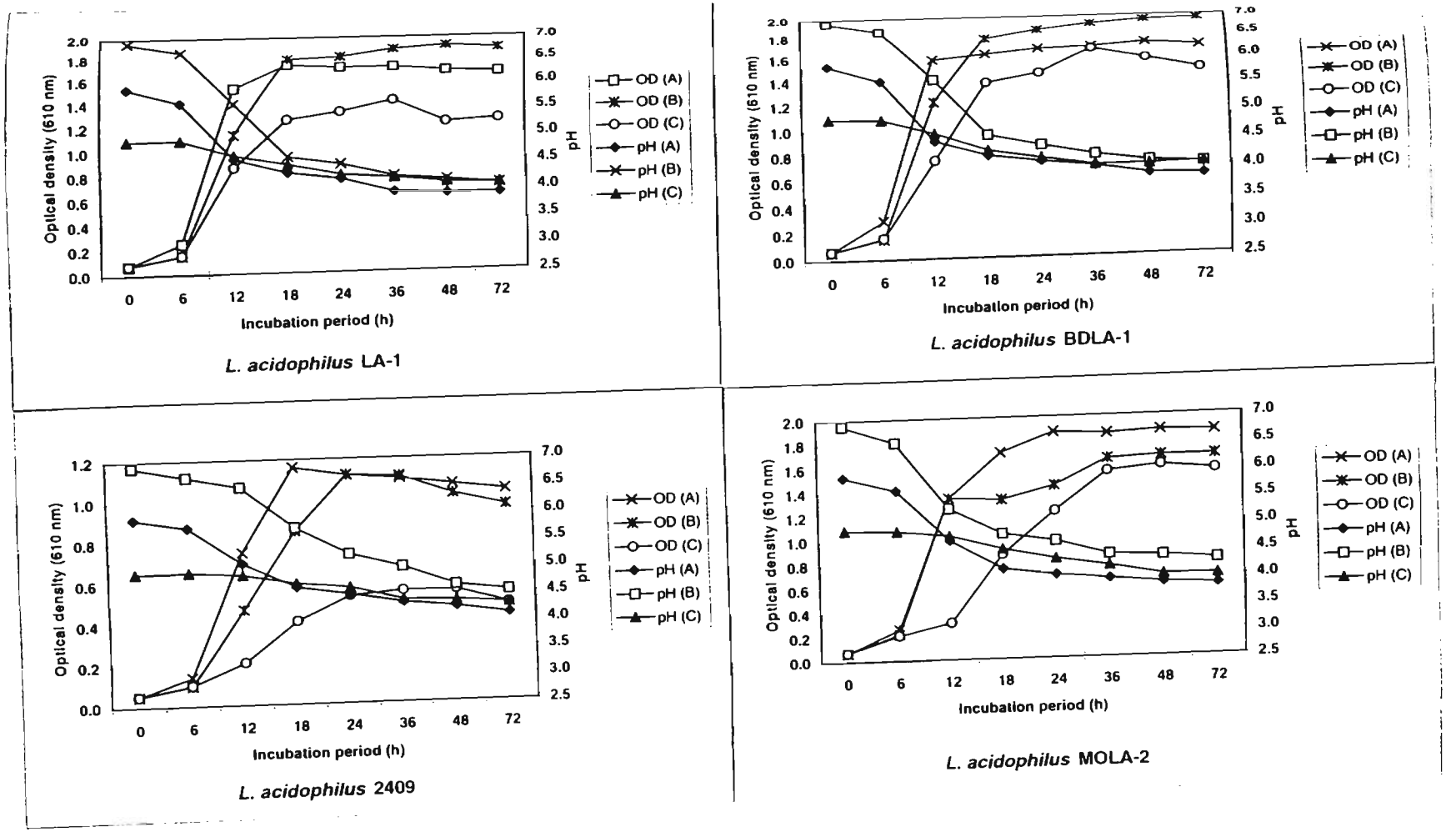


Fig. 7.4.3. Changes in cell density and pH during growth of *L. acidophilus* (LA-1, BDLA-1, 2409 and MOLA-2) in MRS broth (A), MRS broth supplemented with ß-glycerophosphate (B) and MRS broth adjusted to pH 5.0 (C).

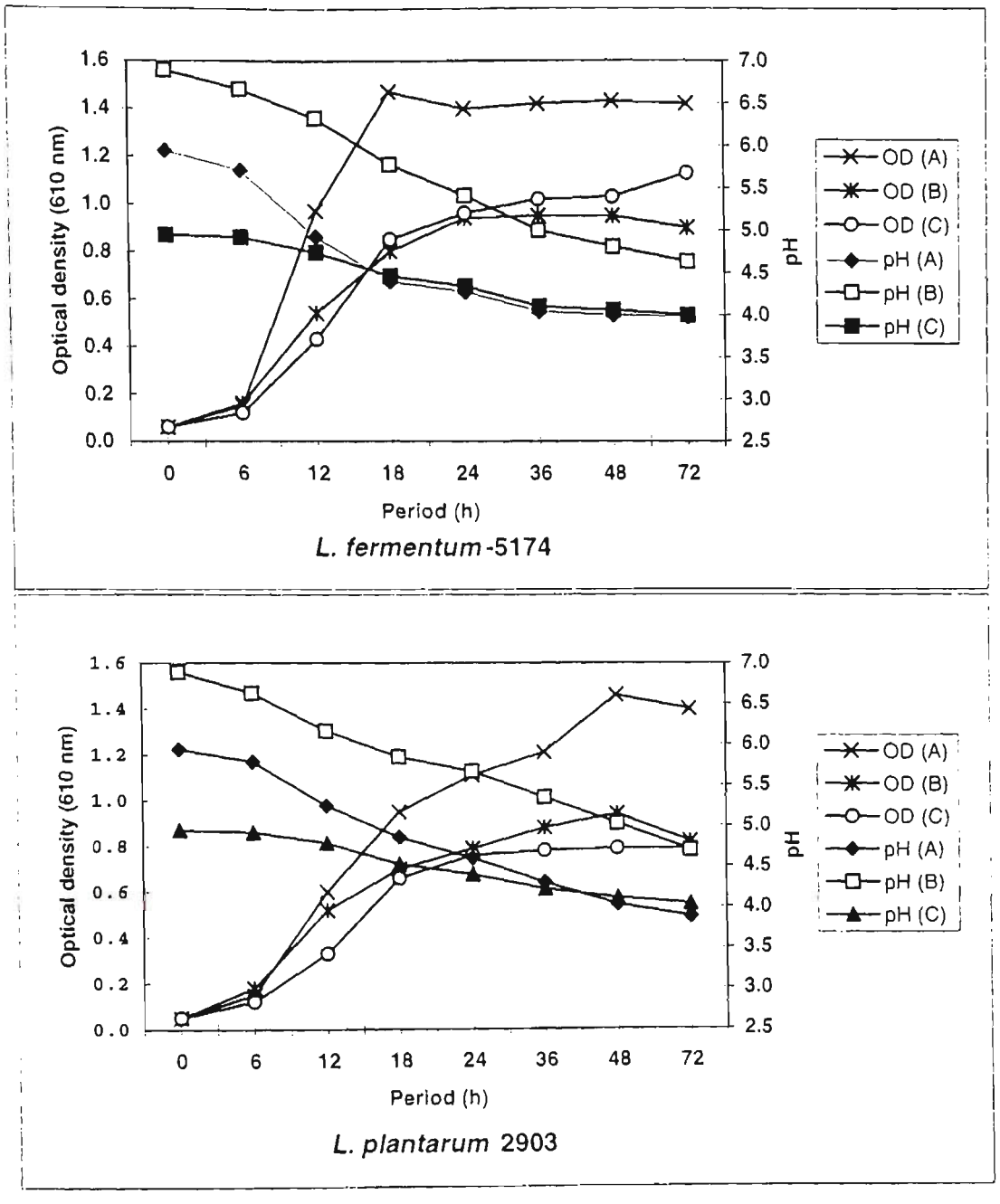


Fig. 7.4.4. Changes in cell density and pH during growth of *L. fermentum* (5174) and *L. plantarum* (2903) in MRS broth (A), MRS broth supplemented with ß-glycerophosphate (B) and MRS broth adjusted to pH 5.0 (C).

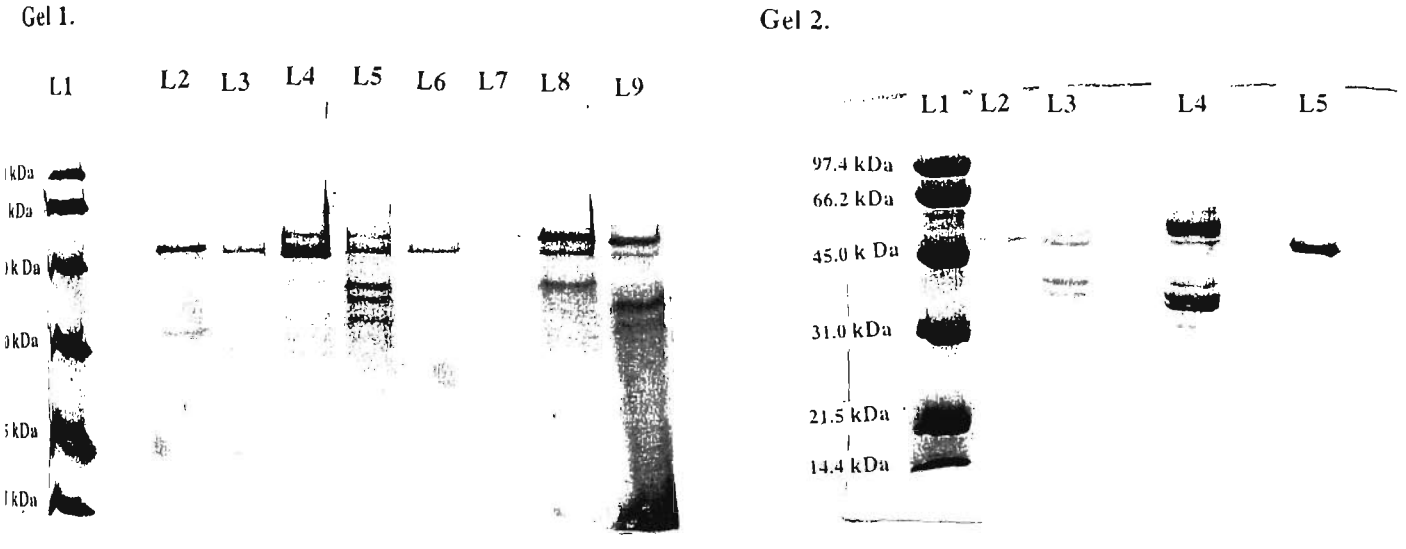


Fig. 7.4.5. Silver stain of fractions of ammonium sulfate precipitates of *L. acidophilus* (LA-1, BDLA-1, 2409 and MOLA-2), *L. fermentum* (5174) and *L. plantarum* (2903) separated on SDS-PAGE during purification of BLIS (Lane 1=Standard molecular weight markers; Lane 2=; Lane 3=; Lane 4=Supernatant after 70% precipitation with ammonium sulfate; Lane 5=10 kDa retentate; Lane 6=20 kDa retentate; Lane 7=30 kDa retentate; Lane 8=Precipitates obtained after 70% saturation of ammonium sulfate; Lane 9= Two stage precipitates obtained after 70 and 40% saturation of ammonium sulfate).

8.0 SUMMARY OF RESULTS

Fifteen media were evaluated for their suitability to selectively recover and count *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus,* and bifidobacteria. Provided the incubation was carried out aerobically at 37°C for 24 h, ST agar was suitable for the selective enumeration of *S. thermophilus* from a mixed culture containing *S. thermophilus, L. delbrueckii* ssp. *bulgaricus, L. acidophilus,* and bifidobacteria. MRS agar (pH 5.2) or RCA agar (pH 5.3) could be used for the enumeration of *L. delbrueckii* ssp. *bulgaricus* when the plates were incubated at 45°C for 72 h. These conditions did not allow complete recovery of *L. delbrueckii* ssp. *bulgaricus,* and colonies of *L. delbrueckii* ssp. *bulgaricus* needed to be differentiated from those of bifidobacteria on the basis of colony characteristics. In a product containing yoghurt organisms, *L. acidophilus* and bifidobacteria, MRS-maltose agar can be used to estimate total counts of *L. acidophilus* and bifidobacteria; MRS-salicin agar or MRS-sorbitol agar could be used to enumerate *L. acidophilus* differentially, provided the product did not contain salicin- or sorbitol-positive bifidobacteria. In such products, aerobic incubation of MRS-maltose agar plates would inhibit the anaerobic bifidobacteria and could selectively enumerate *L. acidophilus*. A 40 to 70% inhibition of *L. acidophilus* was observed under aerobic incubation. Bifidobacteria count could be obtained either by selective enumeration on MRS-NNLP agar or by subtraction of the counts of *L. acidophilus* obtained on MRS-salicin agar or MRS-sorbitol agar from the total counts obtained from MRS-maltose agar, provided the population of bifidobacteria was not less than one log cycle to that of *L. acidophilus*. The subtraction method could be useful, because some strains of bifidobacteria were inhibited (40 to 60%) on MRS-NNLP agar. The MRS medium containing bile salts, oxgall, NaCl, and Rogosa acetate agar showed gross inhibition of *L. acidophilus* and bifidobacteria and might not be suitable for the enumeration purposes. Cellobiose esculin agar gave variable results with the cultures used in this study. Moreover, use of salicin, cellobiose, and esculin in enumeration media were uneconomical for routine testing of *L. acidophilus* compared with that of D-sorbitol.

All the starter cultures (C1-C4) showed similar patterns of increase or decrease in titratable acidity, pH or dissolved oxygen during manufacture and storage of yoghurt. However, there was notable difference in the viability of probiotic organisms. The dissolved oxygen content decreased during manufacture due to bacterial metabolic activity, increased again during overnight storage of yoghurt and stabilised within 5 d storage. The hydrogen peroxide production was higher in products manufactured from C1 and C2, which contained *L. delbrueckii* ssp. *bulgaricus* along with *S. thermophilus* and probiotic bacteria. The numbers of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* multiplied considerably. *S. thermophilus* could maintain its viability to the highest level, but *L. delbrueckii* ssp. *bulgaricus* rapidly lost the viability in the product and a reduction in the viable counts of more than 4 log cycles was observed during cold storage of the product. The multiplication and viability of probiotic bacteria were also influenced by the associative strains and species of yoghurt organism/s. In C1 and C2 cultures, counts of *L. acidophilus* rapidly decreased, possibly due to the production of hydrogen peroxide by *L. delbrueckii* ssp. *bulgaricus*. Conversely, bifidobacteria were able to multiply in products prepared with these cultures (C1 and C2). In C3 culture, bifidobacteria were found to be inhibited and their counts were reduced by 3 log cycles during manufacture of yoghurt. The storage temperature of 10°C was found to affect the viability of bifidobacteria, whereas, *L. acidophilus* counts did not show much variations. There was a slight decrease in the pH and a slight increase in the titratable acidity at 10°C.

The reduced level of inoculum in commercial DVS probiotic yoghurt cultures resulted in slightly higher post-acidification, which was found to have adverse effect on viability of probiotic organisms. Any alteration in the inoculum level and incubation temperature might change this ratio of yoghurt and probiotic bacteria and might adversely affect the viability of probiotic organisms as found in this study. Further, pH was found to be a crucial factor for the viability of *L. acidophilus*. Overall, the viability of *L. acidophilus* needed to be improved in two of the starter cultures (C1 and C2) that contained *L. delbrueckii* ssp. *bulgaricus*, whereas that of bifidobacteria needed to be improved in one of the starter cultures (C3) that

contained polysaccharide producing *S. thermophilus* and did not contain *L. delbrueckii* ssp. *bulgaricus*.

The four commercial starter cultures studied showed similar patterns of increase or decrease in titratable acidity, pH, dissolved oxygen, redox potential and retention of ascorbic acid during manufacture and storage of yoghurt. The addition of ascorbic acid did not have any influence on increase in titratable acidity or drop in pH, however, oxygen content and redox potential varied in yoghurt fortified with ascorbic acid. The storage of yoghurt in plastic cups limited the role of ascorbic acid as an oxygen scavenger and an increase in concentration of oxygen content and redox potential was observed during storage. At the highest level of ascorbic acid used (250 $mg.kg^{-1}$), the purpose of oxygen scavenging and maintaining low redox potential seems to be fulfilled for the initial 15-20 d storage period after which the differences in oxygen content and redox potential narrowed down to that of control samples. Addition of ascorbic acid adversely affected the multiplication of *S. thermophilus* and favoured the viability of *L. delbrueckii* ssp. *bulgaricus*. The decrease in counts of *L. acidophilus* was faster in C1 and C2 starter cultures and fortification with ascorbic acid helped improve their viability. *L. acidophilus* counts remained $\geq 10^6$ throughout the storage in yoghurt prepared with C4 starter culture with or without ascorbic acid. During storage, the decrease in bifidobacterial count was about 60-70% in yoghurt made with C1, C2 and C4 starter cultures and addition of ascorbic acid did not have any influence on the viability of the organism. For C3 starter culture, counts of bifidobacteria were decreased dramatically by about 3 log cycles from their initial numbers and ascorbic acid did not improve their viability to a desirable level.

Yoghurts made with four commercial starter cultures with various levels of cysteine showed different patterns of decrease in pH and of viability of yoghurt and probiotic bacteria. On an average, addition of 50 $mg.L^{-1}$ cysteine reduced the fermentation time by 5-7%, whereas 250 and 500 $mg.L^{-1}$ cysteine levels increased the incubation period by ~15-30% and 40-60%, respectively. The increase in incubation time was in the order of C3, followed by C2, C4 and C1 starter culture. The drop in pH was identical for almost 20-25 d at various cysteine concentrations, after which a greater drop in

pH were observed in yoghurts containing 250 and 500 $mg.L^{-1}$ cysteine. The redox potential was greatly affected by added cysteine and remained positive throughout the storage for control yoghurt and shifted to positive in 5 d in yoghurt containing 50 $mg.L^{-1}$ cysteine. At 250 $mg.L^{-1}$ cysteine, the redox potential remained negative for up to 5 d in yoghurts made with C1 and C4 starter cultures, for up to 15 d for C2 and for 25 d for C3 starter culture. At 500 $mg.L^{-1}$ level of cysteine, the redox potential remained negative for up to 25-30 d.

The counts of yoghurt bacteria were adversely affected by cysteine at 250 and 500 $mg.L^{-1}$ levels. The counts of *S. thermophilus* were considerably lower in these samples despite longer incubation time. During storage, the counts of *L. delbrueckii* ssp. *bulgaricus* remained higher in yoghurts incorporated with cysteine in comparison to control; however, the counts dropped to $<10^5$ g^{-1} within 20-25 d storage of yoghurt at 4°C. The viability of *L. acidophilus* was improved at higher levels of cysteine (250 or 500 $mg.L^{-1}$). The viability of bifidobacteria was adversely affected on addition of 250 or 500 $mg.L^{-1}$ cysteine in yoghurts made with C1 and C2 starter cultures, whereas the viability of this organism improved in yoghurts made with C3 and C4, in particular C3, in which the viability of bifidobacteria was a major problem.

All the 11 batches of yoghurt made with a commercial ABT starter culture showed different patterns of change in pH, titratable acidity and redox potential during manufacture and storage of yoghurt. Also, there was a notable difference in the counts of *S. thermophilus, L. acidophilus* and bifidobacteria. The time to reach a pH of 4.5 increased considerably on addition of 250 and 500 $mg.L^{-1}$ cysteine, whereas the incubation time decreased in yoghurt mixes supplemented with WPC, ACH or tryptone. The redox potential remained negative during refrigerated storage in yoghurt supplemented with 250 or 500 $mg.L^{-1}$ cysteine.

Viability of *S. thermophilus* was adversely affected, whereas that of *L. acidophilus* was improved on addition of cysteine in yoghurt. The counts of *L. acidophilus* remained $> 10^5$ $cfu.g^{-1}$ throughout the storage in all yoghurts. More than 3 log cycles

reduction in counts of bifidobacteria was observed when the pH reached 4.5 in control yoghurt and that supplemented with WP. The viability of bifidobacteria improved to a variable extent in yoghurt supplemented with cysteine, WPC, ACH or tryptone and was highest in yoghurt supplemented with WPC1.

Electron microscopy showed that incorporation of high levels of cysteine (500 $mg.L^{-1}$) in yoghurt mix affected the cell membrane and cell wall of *S. thermophilus* cells. SDS-PAGE and amino acid analyses suggested that nitrogen source in the form of peptides and amino acids correlated with improved viability of bifidobacteria in yoghurt made with a commercial starter culture which showed a dramatic decline in the counts of this organism in the previous experiments. It was concluded that the nitrogen source in the form of peptides or amino acids might be playing a crucial role for improving the viability of bifidobacteria in yoghurt made with ABT starter culture.

Addition of cysteine, whey powder, whey protein concentrate, acid casein hydrolysate and tryptone had considerable effects on firmness, viscosity and micro-structure of yoghurt. The firmness and viscosity of yoghurts improved on addition of WPC; however, irregular protein network resulted with WPC and high level of cysteine (500 $mg.L^{-1}$). The viscosity and firmness were also low in yoghurt supplemented with cysteine and WP. The firmness, viscosity and yoghurt micro-structure were similar in yoghurt supplemented with ACH and tryptone. Addition of WPC1 increased the firmness and viscosity of yoghurt, but resulted in irregular protein network.

A total of 28 strains of yoghurt bacteria and probiotic bacteria were isolated from commercial starter cultures, commercial yoghurts and a probiotic capsule. Inhibition of 5 *S. thermophilus* strains and 2 bifidobacterial strains observed was due to organic acids but not due to a bacteriocin or bacteriocin like inhibitory substance (BLIS). The other 2 strains of *S. thermophilus* and 6 bifidobacterial strains were not inhibited by organic acids/low pH. *L. delbrueckii* ssp. *bulgaricus* strains were not inhibited by most *S. thermophilus, L. delbrueckii* ssp. *bulgaricus* or bifidobacterial strains isolated

from the market preparations. However, these organisms showed strong inhibition due to BLIS produced by 7 of 8 strains of *L. acidophilus*. All *L. acidophilus* strains were resistant and did not show inhibition by any group of organism tested. The yoghurt and probiotic bacteria varied in their resistance to inhibitory substances. The production of BLIS was enhanced by neutralisation of MRS broth (pH 6.0) during growth of *L. acidophilus* cultures.

Some characteristics of BLIS produced by *L. acidophilus* (LA-1) used in the commercial probiotic culture (C1 to C4) were studied. The bacteriocin produced by LA-1 showed a narrow spectrum of inhibition and was sensitive to proteolytic enzymes, but not to amylases, lipase or phospholipases. The crude bacteriocin was stable over a wide range of pH and temperature and remained active after autoclaving. It was also stable after storage at 37°C, 4°C and -18°C. The production of BLIS occured in the log and early stationary phase and was influenced by pH, being highest in the range of pH 5.5-6.0. The activity declined considerably when the cells entered into death phase. The glucose of MRS medium in the fermentors was exhausted within 12 h at various pH. Incorporation of additional glucose and triple strength MRS broth after 8 and 16 h of growth of LA-1 in fermentors at pH 6.0 did not increase bacteriocin production; however, supplementation of glucose or triple strength MRS broth increased its stability to a considerable extent. The glucose was exhausted within 30 h in fermentor added with triple strength MRS broth, but it was available throughout 48 h in fermentor supplemented with additional glucose. Further, degradation of bacteriocin was observed due to lysis of cells of producer organism *L. acidophilus* (LA-1). The bacteriocin produced by LA-1 had a molecular weight of ~50,000 Da. Two stage fractionation with ammonium sulfate resulted in a single band on silver staining of SDS-PAGE gel. The bacteriocin produced by *L. acidophilus* (LA-1) was different from those reported earlier and was designated as acidophilicin LA-1.

Screening of 19 additional *L. acidophilus* strains and 15 other lactic acid bacteria was carried out to study their ability to produce BLIS. Out of 19 strains of *L. acidophilus*, 15 strains produced heat stable BLIS. Characteristics of BLIS produced by *L.*

acidophilus (stains BDLA-1, LA-2409 and MOLA-2) were studied and *L. acidophilus* (BDLA-1 and LA-2409) produced bacteriocins that were very similar to acidophilicin LA-1, but that produced by MOLA-2 differed, especially the molecular weight of BLIS produced was < 10 kDa. *L. plantarum* (2903) and *L. fermentum* (5174) also produced bacteriocin like inhibitory substances against the four target organisms and a single protein band was obtained in the ammonium sulfate precipitates of *L. fermentum* (5174), but not with *L. plantarum* (2903). *L. helveticus* (2700) produced a higher level of antibacterial substance in RSM during fermentation than in MRS broth and was not a protein, organic acid, hydrogen peroxide, lipid or bacteriophage. The antimicrobial substance produced by *L. helveticus* (2700) in milk inhibited seven strains of *L. delbrueckii* ssp. *bulgaricus*, the producer *L. helveticus* (2700), fourteen strains of *L. acidophilus, L. plantarum* (2903) and *L. jugurti* (2819), but not *L. fermentum* (5174), *L helveticus* (2704), *L. casei* (2603, 2604), *L. casei* ssp. *rhamnosus* (2606), five strains of *S. thermophilus*, ten strains of bifidobacteria and several spoilage and pathogenic microorganisms. The antibacterial substance was not sensitive to catalase, various proteolytic enzymes, lipase, phospholipases or amylases and was active over a pH range of 3.0 to 10.0. The substance lost its antimicrobial activity during heat treatment at 70°C for 30 min and above. *L. helveticus* 2700 produced antibacterial substance in MRS broth that inhibited *L. delbrueckii* ssp. *bulgaricus*, but not *L. acidophilus* or the producer organism *L. helveticus* 2700. The antimicrobial substance appeared to be less than 5000 Da as considerable activity was obtained in the permeate of 5000 Da membranes.

9.0 FUTURE RESEARCH DIRECTIONS

The popularity of probiotic products is rapidly increasing due to consumer awareness and encouraging evidence of health benefits of probiotic bacteria. Due to increased demand for these products, yoghurt manufacturers have started introducing more than one species of probiotic bacteria (*Lactobacillus acidophilus*, *Lactobacillus casei*, propionibacteria and bifidobacteria) in addition to yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) in fermented milk products. Due to the diversity and complexity of these mixed cultures and strain variations within the same species of bacteria, there is still a scope of work to standardise method for various groups of organisms for selective or differential enumeration of these bacteria when they are present together in a product. The products containing *L. acidophilus*, *L. casei*, bifidobacteria and yoghurt bacteria are presently available in the supermarkets. There will be a need to count the above mentioned five groups of organisms selectively with their optimum recovery.

Our study demonstrated that the added ingredients (such as cysteine, whey protein concentrates, tryptone and acid casein hydrolysates) had effect on viability of probiotic bacteria. The nutritional/growth factors in the form of amino acids and peptides are one of the crucial factors which improved the viability of bifidobaccteria in the commercial starter cultures. However, there is a need to extend this study in order to find out the exact amino acid(s) or peptide(s) required for the improved viability of bifidobacteria. In this study, it was also observed that *L. delbrueckii* ssp. *bulgaricus* provided sufficient growth factors for improving viability of bifidobacteria, possibly due to higher proteolytic activity that provided essential amino acids and peptides to bifidobacteria. Further study could be undertaken to examine the effects of selected strains of *L. delbrueckii* ssp. *bulgaricus* with highest proteolytic activity on improving the viability of probiotic bacteria.

In this study, it was observed that inhibition of pathogens was mainly due to acid produced by lactic acid bacteria but not due to bacteriocin like inhibitory substance/s. There is a need to screen more lactic acid bacteria that show inhibition of pathogenic

and spoilage groups of organisms to support the claims made in favour of probiotic bacteria in suppressing the growth of spoilage and pathogenic bacteria and restoration of normal intestinal balance of microflora. Bacteriocins produced by bifidobacteria need to be studied in more detail as only few reports are available on this interesting aspect. The information related to the antimicrobial substance produced by *L. helveticus* (2700) in milk that inhibited several *L. acidophilus* strains appears to be novel. More strains of such antimicrobial substance/s producing bacteria can be identified and the substances purified and characterised in future. This anticipated study would provide some information and would form a basis for the antagonism between yoghurt bacteria and probiotic bacteria; the information which is scant in literature. This would also benefit starter culture manufacturers for developing new range of cultures that contain yoghurt and probiotic bacteria.

10.0 REFERENCES

Abdel-Bar, N.M. and Harris, N.D. (1984). Inhibitory effect of *Lactobacillus bulgaricus* on psychrotrophic bacteria in associative cultures and in refrigerated foods. *J. Food Prot.*, **47**: 61.

Ahmed A.A.H., Moustafa, M.K. and El-Bassiony, T.A. (1986). Growth and survival of *Yersinia enterocolitica* in yogurt. *J. Food Prot.*, **49**: 983.

Allison, G.E., Ahn, C., Stiles, M.E. and Kleanhammer, T.R. (1995). Utilization of the leucocin A export system in *Leuconostoc gelidum* for production of a *Lactobacillus* bacteriocin. *FEMS-Microbiol.-Lett.*, **131**(1): 87.

Anand, S.K., Srinivasan, R.A. and Rao, L.K. (1985). Antibacterial activity associated with *Bifidobacterium bifidum* - II. *Cult. Dairy Prod. J.*, **20**(1): 21.

Anders, R.F., Hogg, D.M. and Jago, G.R. (1970). Formation of hydrogen peroxide by group N streptococci and its effect on their growth and metabolism. *Appl. Microbiol.*, **19**: 608.

Anon (1992). Yoghurt and probiotics. *Choice,* **11**: 32.

AOAC. Official Methods of Analysis (1990). 15th ed. Vitamin C (Reduced Ascorbic acid) in Ready to Feed Milk Based infant Formula, No. 985.33, pp. 1108.

Arany, C.B., Hackney, C.R., Duncan, S.E., Kator, H., Webster, J., Pierson, M., Boling, J.W. and Eigel, W.N. (1995). Improved recovery of stressed *Bifidobacterium* from water and frozen yogurt. *J. Food Prot.*, **58**(10): 1142.

Arroyo, L., Cotton L.N., and Martin, J.H. (1994). Evaluation of media for enumeration of *Bifidobacterium adolescentis, B. infantis* and *B. longum* from pure culture. *Cult. Dairy Prod. J.*, **29**(5): 20.

Attaie, R., Whalen, P.J., Shahani, K.M. and Amer, M.A. (1987). Inhibition of growth of *Staphylococcus aureus* during production of acidophilus yogurt. *J. Food Prot.*, **50**: 224.

Badran, I.I. and Reichart, O. (1993). Comparative study of some fermentation properties of *Streptococcus thermophilus* and *Lactobacillus acidophilus* in milk and modified media. I. Effect of the nutrient additives and fermentation temperature. *Acta-Alimentaria*, **22**(4): 359.

Baig, M.I. and Prasad, V. (1996). Effect of incorporation of cottage whey solids and *Bifidobacterium bifidum* in freshly made yoghurt. *J. Dairy Res.*, **63**: 467.

Barefoot, S.F. and Klaenhammer, T.R. (1983). Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.*, **45**: 1808.

Barefoot, S.F. and Klaenhammer, T.R. (1984). Purification and characterization of the *Lactobacillus acidophilus* bacteriocin lactacin B. *Antimicro. Agent. Chemothe.*, **26**(3): 328.

Barefoot, S.F. and Nettles, C.G. (1993). Antibiosis revisted: Bacteriocins produced by dairy starter cultures. *J. Dairy Sci.*, **76**: 2366.

Bednarski, W. (1991). Antimicrobial compounds synthesized by lactic acid bacteria and their utilization in food technology. *Przemysl- Spozywczy,* **45**(7): 177.

Bertoni, J., Calamary, L., Maiamti, M. G. and Azzoni, A. (1994). Factors modifying the acidification rate of milk. *Lait,* **17**: 941.

Bezkorovainy, A., Grohlich, D. and Nichols, J. H. (1979). Isolation of a glycopeptide fraction with *Lactobacillus bifidus* var. *pennsylvanicus* growth promoting activity from whole milk casein. *Amer. J. Clin. Nutr.*, (Cited from *Dairy Sci. Abstr.*, **38**: 1725).

Biaveti, B. and Mattarelli, P. (1991). *Bifidobacterium ruminatum* and *Bifidobacterium merycicum* from the rumen of cattle. *Inter. J. Systm. Bacteriol.*, **41:**163.

Biaveti, B., Sgorbati, B. and Scardovi, V. (1991). The genus *Bifidobacterium*. In The Prokarryotes, 2nd edn. (eds Balows, A.). Springer-Verlag, Berlin, Germany.

Beerens, H. (1991). Detection of bifidobacteria by using propionic acid as a selective agent. *Appl. Environ. Microbiol.*, **57**: 2418.

Biavati, B, Sozzi, T., Mattarelli, P. and Trovatelli, L. D. (1992). Survival of bifidobacteria from human habitat in acidified milk. *Microbiologica,* **15:** 197.

Bibel, D.J. (1988). Elie Metchnikoff's bacillus of long life. *ASM News,* **54**(12): 661.

Bissett, D.L. and Anderson, R.L. (1974). Lactose and D-galactose metabolism in Group N streptococci: presence of enzymes for both, the D-galactose-1- phosphate and D-tagatose-6-phosphate pathways. *J. Bacteriol.*, **117:** 318.

Branby-Smith, F.M. (1992). Bacteriocins: applications in food preservation. *Trends in Food Sci. and Technol.*, **3**(6): 133.

Breed, R.S., Murray, E.G.D. and Smith, N.R. eds. (1957). Bergey's Manual of Determinative Bacteriology, 7th ed., Williams and Wilkins, Baltimore, MD, USA.

Brink B., Minekus, M., Vossen JMBM-Van-der, Leer, R.J. and Huis-in't-Veld-J.H.J. (1994). Antimicrobial activity of lactobacilli: Preliminary characterization and optimization of production of acidocin B, a novel bacteriocin produced by *Lactobacillus acidophilus* M46. *J. Appl. Bact.*, **77**(2): 140.

British Standards (1974), BS: 4020. Method for the estimation and differentiation of nisin in processed cheese.

Brown, C.D. and Twonsley, P.M. (1970). Fermentation of milk by *Lactobacillus bifidus*. *J. Inst. Can. Technol. Aliment.*, **3**: 121.

Brunner, J.C., Spillmanns, H. and Puhan, Z. (1993a) Metabolism and survival of bifidobacteria in fermented milk during cold storage. *Milchwirtschaftliche-Forschung*, **22**: 19.

Brunner, J.C., Spillmanns, H. and Puhan, Z. (1993b). Changes in pH, free sulphydryl groups, oxygen and redox potential during fermentation of milk with *Bifidobacterium longum*. *Milchwirtschaftliche-Forschung*, **22**: 26.

Buchnan, R E. and Gibbons, N.E. (1974). Bergey's Manual of Deterinative Bacteriology, 8th ed., Williams and Wilkins, Baltimore, MD, USA.

Burford, M.Y. (1989). Enumeration of *Lactobacillus acidophilus* and *Bifidobacterium* in milk using oxygen-reducing membrane fraction. *Cult. Dairy Prod. J.*, **24**(4): 21.

Calicchia, M.L., Wang, C.I.E., Nomura, T., Yotsuzuka, F. and Osato, D.W. (1993). Selective enumeration of *Bifidobacterium bifidum*, *Enterococcus faecium* and streptomycin-resistant *Lactobacillus acidophilus* from a mixed probiotic product. *J. Food Prot.*, **56**(11): 954.

Chambers, J.V. (1979). Culture and processing techniques important to the manufacture of good yoghurt. *Cult. Dairy Prod. J.*, **14**(2): 28.

Champagne, C.P., Gelais, D.ST. and Andet, P. (1996). Starters produced on whey protein concentrates. *Milchwissenschaft*, **51**: 561.

Chapon, J.L. and Kiss, K. (1991). Enumeration of bifidobacteria in cultured milks. Proposal of a microbiological method. *Mitteilungen-aus-dem-Gebiete-der-Lebensmittelunterschung-und-Hygiene*, **82**(3): 264.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K. (1997). Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. *Inter. J. Food Microbiol.*, **35**(1): 1.

Cheng C.C. and Nagasawa, T. (1983). Associative relationships between bifidobacteria and lactobacilli. *Jpn. J. Zootech. Sci.*, **54**: 740.

Cheng C.C. and Nagasawa, T. (1984). Effect of peptides and amino acids produced by *Lactobacillus casei* in milk on the acid production of bifidobacteria. *Jpn. J. Zootech. Sci.*, **55:** 339.

Cheng, R. and Sandine, W.E. (1989). Growth characteristics of bifidobacteria species in whey base medium. J. Dairy Sci., **72** (Suppl 1): 148.

Chevalier, P., Denis, R. and Savoie, L. (1991). X-a-Gal-based medium for simultaneous enumeration of bifidobacteria in milk. *J. Microbiol. Meth.*, **13**: 75.

Cho, Y.K., Cho, K.H., Hong, S.S. and Lee, H.S. (1995). Studies on the selective screening method for bifidobacteria used in yoghurt. *Kor. J. Appl. Microbiol. Biotechnol.*, **23**(2): 214.

Collins, E.B. (1978). Enumeration of *Lactobacillus acidophilus* with the agar plate count. *J. Food Prot.*, **41**(6): 439.

Collins, E.B. and Hall, B.J. (1984). Growth of bifidobacteria in milk and preparation of *Bifidobacterium infantis* for a dietary adjunct. *J. Dairy Sci.*, **67**: 1376.

Collins, M.D., Farrow, J.A.E., Phillips, B.A., Ferusu, S. and Jones, D. (1987). Classification of *Lactobacillus divergens*, *Lactobacillus piscicola* and some catalase negative asporogenous rod shaped bacteria from poultry in a new genus, *Carnobacterium*. *Int. J. Syst. Bacteriol.*, **37**: 310.

Collins, M.D., Farrow, J.A.E., Phillips, B.A., Ferusu, S. and Jones, D. (1989). 16-S Ribosomal ribonucleic acid sequence analysis of lactococci and related taxa. Description of *Vagococcus fluvialis*. *J. Appl. Bacteriol.*, **67**: 453.

Collins, M.D., Williams, A.M. and Wallbanks, S. (1990). The phylogeny of Aerococcus and Pediococcus as determined by 16 S RNA sequence analysis: description of tetragenococcus gen. Nov. *FEMS Microbiol. Let.*, **70**: 225.

Costello, M. (1993). Probiotic Foods. In The Food Industry Conference Proceedings, Sydney Convention and Exhibition Centre. Publ. Food Pro.-93, July 12-14, 1993.

Cuk, Z., Annan-Prah, A., Janc, M. and Zajc-Salter, J. (1987). Yogurt: an unlikely source of *Campylobacter jejuni/coli*. *J. Appl. Bacteriol.*, **63**: 201.

Czarnocka-Roczniakowa, B. and Wojewodzka, M. (1969). Changes in Vitamin C content of vitaminized milk during fermentation induced by yoghurt microflora. *Zeszyty-Naukowe-Wyzszzej-Szkoly-Rdniczej-W-Olsztynie,* **25**: 455.

Dabin, E. (1992). Meat products containing bifidobacteria. *French Pat. Appl.*, No. FR 90-01128 (400 131).

Daeschel. M.A. (1989). Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technol.*, **43**(1): 164.

Daeschel, M.A., Mc Kenney, M.C. and Mc Donald, L.C. (1990). Bactericidal activity of Lactobacillus plantarum C11. *Food Microbiol.*, 7: 91.

Dahiya, R.S. and Speck, M.L. (1968). Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. *J. Dairy Sci.*, **51**: 1568.

Dannenberg, F., and Kessler, H.G. (1988a). Effect of denaturation of beta-lactoglobulin on texture properties of set-style nonfat yoghurt. I. Syneresis. *Milchwissenschaft,* **43**: 632.

Dannenberg, F., and Kessler, H.G. (1988b). Effect of denaturation of beta-lactoglobulin on texture properties of set-style nonfat yoghurt. II. Firmness and flow properties. *Milchwissenschaft,* **43**: 700.

Dave, J.M. and Prajapati, J.B. (1994). Lactic acid bacteria as antibacterials against foodborne pathogens and food spoilage organisms. A paper presented in a special session on "Microbes for better living" in MICON-94 and 35th AMI conference held at Mysore, India, Nov. 9-12, 1994.

Dave, R.I. and Shah, N.P. (1996). Evaluation of media for selective enumeration of *Streptococcus thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus acidophilus* and *Bifidobacterium* spp., *J. Dairy Sci.*, **79**: 1529.

Dave, R.I. and Shah, N.P. (1997a). Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter culture. *Int. Dairy J.*, 7: 31.

Dave, R.I. and Shah, N.P. (1997b). Effect of level of starter culture on viability of yoghurt and probiotic bacteria in yoghurts. *Food Australia*, **49**: 164.

Dave, R.I. and Shah, N.P. (1997c). Effectiveness of ascorbic acid as an oxygen scavenger in improving viability of probiotic bacteria in yoghurts made with commercial starter cultures. *Int. Dairy J.*, 7: 435.

Dave, R.I. and Shah, N.P. (1997d). Effect of cysteine on the viability of yoghurt and probiotic bacteria in yoghurts made with commercial starter cultures. *Inter. Dairy J.*, 7: 537.

Dave, R.I. and Shah, N.P. (1997e). Characteristics of bacteriocin produced by *L. acidophilus* LA-1. *Inter. Dairy J.*, 7: 707.

Dave, R.I. and Shah, N.P. (1998). Ingredients supplementation affects viability of probiotic bacteria in yogurt. J. Dairy Sci.: (In press).

Davis, J.G., Ashton, T.R. and MacCaskill, M. (1971). Enumeration and viability of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yoghurts. *Dairy Ind.*, **36**(10): 569.

de Klerk, H.C. and Smit, J.A. (1967). Properties of a Lactobacillus fermenti bacteriocin. *J. Gen. Microbiol.*, **48**: 309.

Deeth, H.C. and Tamime, A.Y. (1981). Yoghurt: Nutritive and therapeutic aspects. *J. Food Prot.*, **44:** 78.

Deguchi, Y., Morishita, T. and Mutai, M. (1985). Comparative studies on synthesis of water soluble vitamins among human species of bifidobacteria. *Agril. Biol. Chem.*, **49**: 13.

Delhaye, S. and Landry J. (1986). High Performance Liquid Chromatography and Ultraviolet Spectrophotometry for quantitation of tryptophan in barytic hydrolysates. *Anal. Biochem.*, **159**: 175.

Denhert, J. (1957). Untersuchungen uber die Gram-positive Stuhlflora des Brustmilchkinder. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infections-krankheiten und Hygiene (Abteilung Originale),* A **169**: 66.

DeVries, W. and Stouthamer, A.H. (1967). Fermentation of glucose, lactose, galactose, mannitol and xylose by bifidobacteria. *J. Bacteriol.*, **96**: 472.

DeVries, W. and Stouthamer, A.H. (1969). Factors determining the degree of anaerobiosis of *Bifidobacterium* strains. *Arch. Mikrobiol.*, **65**: 275.

Dombou, M., Tomioka, I. and Jsurutau, R. (1988). Production of growth factor for *Bifidobacterium* spp., European Patent EP 0 262 858 AZ 12PP. (Cited from *Dairy Sci. Abstr.*, **50**: 5725).

Dubois, G., Smoragiewicz, W., Chabonneau, R. and Gagnon, M. (1982). *Le Lait,* **62** (621/622): 681. (Cited by Kanbe, 1992).

Dutta, S.M., Kuila, R.K. and Rangnathan, B. (1973). Effect of different heat treatments on acid and flavour production by single strain starter cultures. *Milchwissenschaft,* **28**: 231.

Eckner, K.F. (1992). Bacteriocins and food application. *Dairy Food and Environ. Sanitation,* **12**(3): 204.

Ervol'der, T.M., Gudkov, A.V., Semenova, L.P. and Goncharova, G.I. (1980). Effect of enzymic hydrolysates on accumulation of *Bifidobacterium* biomass. *Molchnaya-Promyshlennost,* **12**: 15.

Faber, H.K. and Sutton, R.L. (1930). Brest fed and bottle fed babies during the first year; comparision in weight and morbidity. *Amer. J. Diseased Child.,* **40**: 1163.

FAO/WHO (1973). Code of principles concerning milk and milk products. CX 5/70, 16th Session, Rome, Italy.

Fernandes, C.F. and Shahani, K.M. (1989). Inhibitory effect of fermented milk cultures on the gastro-intestinal pathogens. In Fermented Milks-Current Research, John Libbey Eurotext Ltd., London, pp 105.

Ferreira, C.L. and De, L.F. (1988). *Infro. Agropec.*, **13**: 43. (Cited from *Dairy Sci. Abstr.*, **51**: 296).

Fonden, R. (1989). *Lactobacillus acidophilus*. Current Research in Fermented Milks. Proceedings of International Congress held in Paris, France. Ed. John Libbey and Company Ltd., England.

Fox, P.F., Lucey, J.A. and Cogan, T.M. (1990). Glycosis and related reactions during cheese manufacture and ripening. *Crit. Rev. Food Sci. Nutr.*, **29**: 237.

Freter, R. (1983). Mechanism that control the microflora in the large intestine. In Human intestinal microflora in health and disease. Ed. Hentges, D. J., Academic Press, New York, USA.

Fuller, R. (1989). Probiotics in man and animals: A review. *J. Appl. Bacteriol.*, **66**: 365.

Fuller, R. (1992). Probiotics-The Scientific basis. Champman and Hall, London.

Garcia, E.F.and McGregor, J.U. (1994). Determination of organic acids during the fermentation and cold storage of yoghurt. *J. Dairy Sci.*, **77**: 2934.

Garvie, E.I. (1986a). Genus *Leuconostoc*. In Bergey's Manual of Systematic Bacteriology, Vol. 2, edited by: Sneath, P.H.A., Sharpe, M.E. and Holt, J.G., Williams and Wilkins, Baltimore, MD, USA.

Garvie, E.I. (1986b). Genus *Pediococcus*. In Bergey's Manual of Systematic Bacteriology, Vol. 2 (edited by: Sneath, P. H. A., Sharpe, M. E. and Holt, J. G.), Williams and Wilkins, Baltimore, MD, USA.

Garvie, E.I., Cole, C.B., Fuller, R. and Hewitt, D. (1984). The effect of yoghurt on some component of the gut microflora and on metabolism of lactose in the rat. *J. Appl. Bact.*, **56**: 237.

Gassem, M.A., Schmidt, K.A. and Frank, J.F. (1995). Exopolysaccharide production in different media by lactic acid bacteria. *Cult. Dairy Prod. J.*, **30**(3): 18.

Gibson, G.R. and Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.*, **125**: 1401.

Gilliland, S.E. (1968). Enzymatic determination of residual hydrogen peroxide in milk. *J. Dairy Sci.*, **52**(3): 321.

Gilliland, S.E. (1978). Beneficial interrelationships between certain microorganisms and humans: Candidate microorganisms for use as dietary adjuncts. *J. Food Prot.*, **42:** 164.

Gilliland, S.E. (1987). *XXII Inter. Dairy Congr.* held in Tokyo, Japan. 623. (Cited by Patel *et al.*, 1992).

Gilliland, S.E. (1989). Acidophilus milk products: A review of potential benefits to consumers. *J. Dairy Sci.*, **72**: 2483.

Gilliland, S.E. (1990). Health and nurtritional benefits from lactic acid bacteria. *FEMS Microbiol. Rev.*, **87**: 175-88.

Gilliland, S.E. and Speck, M.L. (1977a). Instability of *L. acidophilus* in yoghurt. *J. Dairy Sci.*, **60**(8): 1394.

Gilliland, S.E. and Speck, M.L. (1977b). Antagonistic action of *L. acidophilus* towards intestinal and food borne pathogens in associative cultures. *J. Food Prot.*, **40**(12): 820.

Gilliland, S.E., Speck, M.L. and Morgan, C.G. (1975). Detection of *Lactobacillus acidophilus* in faeces of humans, pigs and chickens. *Appl. Microbiol.*, **30**: 541.

Gilliland, S.E., Staley, T.E. and Bush, L.J. (1984). Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjuncts. *J. Dairy Sci.*, **67**: 3045.

Giurguis, N and Hickey, M.W. (1987). Factors affecting the performance of thermophilic starters II. Sensitivity to the lactoperoxidase system. *Aust. J. Dairy Technol.*, **42**: 14.

Glick, M.C., Sall, T. Zilliken, F. and Mudd, S. (1960). Morphological changes of *Lactobacillus bifidus* var. *pennsylvanicus* produced by a cell wall precursor. *Biochem. Biophy. Acta.* **37**: 361.

Goh, J.S., Kwon, I.K and Kim, Y.O. (1986). Studies on the growth of *Bifidobacterium bifidum* ATCC 11603 in milk. *Korean J. Dairy Sci.*, 8: 48.

Griffin, A.M., Morris, V.J. and Gasson, M.J. (1996). The cpsABCDE genes involved in polysaccharide production in *Streptococcus salivarius* ssp. *thermophilus* strain NCBF 2393. *Gene*, 183: 23.

Gupta, R.K., Grover, S. and Batish, V.K. (1993). Anti-listerial activity of lactic acid bacteria isolated from buffalo market milk. *Cult. Dairy Prod. J.*, 28(2): 21.

Hadman, I.Y. and Mikolajcik, E.M. (1974). Acidolin: an antibiotic produced by *Lactobacillus acidophilus*. *J. Antibio.*, 27: 631.

Hae-Ki-Min, See-Kyung-Lee and Kook-Hee-Kang. (1993). Detection of bifidobacteria by alpha-galactosidase activity. *J. Kor. Agril. Chem. Soc.*, 36(3): 191.

Haenel, H. (1970). Human normal and abnormal gastrointestinal flora. *Amer. J. Clin. Nutr.*, 23: 1433.

Hamada, S. and Ooshima, T. (1975). Inhibitory spectrum of a bacteriocin like substance (mutacin) produced by some strains of *Streptococcus mutans*. *J. Dental Res.*, 54 :140.

Hamann, W.T., and Marth, E.H. (1984). Comparison of four differential and two general purpose media to enumerate *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. *Milchwissenschaft*, 39(3): 147.

Hammes, W.P. and Tichaczek, P.S. (1994). The potential of lactic acid bacteria for the production of safe and wholesome food. *Zeitschrift-Fuer-Lebensmittel-Untersuchung-und-Forschung*, 198 (3): 193.

Hammes, W.P., Weiss, N. and Holzapfel, W.H. (1991). The genera *Lactobacillus* and *Carnobacterium*. In 'The Prokaryotes'. Handbook on the biology of bacteria. Springer, New York, USA.

Hardie, J.M. (1986). Other streptococci. In *Bergey's Manual of Systemic Bacteriology*, Vol 2, P. H. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt eds. The William and Wilkins Co., Baltimore, pp. 1069-70.

Hassan, A.N., Frank, J.F., Schmidt, K.A. and Shalabi, S.I. (1996). Textural properties of yogurt made with encapsulated nonropy lactic cultures. *J. Dairy Sci.* 79: 2098.

Havenaar, R. and Huis in't Veld, J.H.J. (1992). Probiotics : a general view. The lactic acid bacteria. In The Lactic Acid Bacteria in Health and Disease, Vol. 1, edited by: Wood B. J. B., Elsevier Appl. Sci. Publ., London, pp. 151.

Henneberg, W. (1904). Zur Kenntnis der Milchsaurebakterien der Brennerei-Maische, der Milch, des Bieres, der Presshefe, der Melasse, des Sauerkohls, der Sauren Gurken und des Sauerteigs; sowie einige Bemerkungen uber die Milchsaurebakterien des mensschlishen Magens. *Zentbl. Bakt. ParasitKde Abt. II.*, **11**: 154.

Holcomb, J.E., Frank, J.F. and McGregor, J.U. (1991). Viability of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in soft serve frozen yoghurt. *Cult. Dairy Prod. J.*, 26: 4.

Holmberg, K. and Hollaner, H.O. (1973). Production of bacteriocidal concentration of hydrogen peroxide of *Streptococcus sanguis*. *Arch. Oral Biol.*, **18**: 423.

Hood, S.K. and Zottola, E.A. (1989). An electron microscopic study of adherence of *L. acidophilus* to human intestinal cells *in vitro*. *Food Microstr.*, 8: 91.

Hoover, D.G. and Steenson, L.R. (1993). Bacteriocins of Lactic Acid Bacteria. Academic Press, Inc., San Diego, California, USA.

Hori, S. (1983). Characteristics of "Mil-Mil E'- a bifidobacterium fermented milk. *New Food Ind.*, 25 (3): 25.

Hosono, A, Yastuki, K and Tokita, F. (1977). Isolation and characterization of an inhibitory substance against *E. coli* produced by *L. acidophilus*. *Milchwissenschaft*, 32 (12): 727.

Hotta, M., Sato, Y., Iwata, S., Yamashita, N., Suakawa, K, Oikawa, T., Tanaka, R., Wstanabe, K., Takyama, H., Sakurai, T., and Mutai, M. (1987). Clinical effects of *Bifidobacterium* preparations on pediatric intractable diarrhea. *Keio J. Medi.*, **12**: 298.

Hsing-Yi-Hsieh and Glatz, B.A. (1996). Long-term storage stability of the bacteriocin propioncin PLC-1 produced by *Propeonibacterium thoenii* and potential as a food preservative. *J. Food Prot.*, 59(5): 481.

Hugas, M., Neumeyer, B., Pages, F., Garriga, M. and Hammes, W.P. (1996). Antimicrobial activity of bacteriocin-producing cultures in meat products. II. Comparison of antilisterial potential of bacteriocin-producing lactobacilli in fermenting sausages. *Fleischwirtschaft*, 76(6): 649.

Hughes, D.B. and Hoover, D.G. (1991). Bifidobacteria- their potential for use in American dairy products. *Food Technol.*, April, 1991.

Hull, R.R., Roberts, A.V. and Mayes, J.J. (1984). Survival of *L. acidophilus* in yoghurt. *Aust. J. Dairy Technol.*, **39**: 164.

Hull, R.R. and Roberts, A.V. (1984). Differential enumeration of *Lactobacillus acidophilus* in yoghurt. *Aust. J. Dairy Technol.* 3: 160.

Hunger, W. 1986. Aesculin-cellobiose agar for the isolation and counting of *Lactobacillus acidophilus. Milchwissenschaft,* 41: 283.

Hurst, A. (1981). Nisin. *Adv. Appl. Microbiol.*, 27: 85.

Ibrahim, S.A. and Bezkorovainy, A. (1993). Inhibition of *E. coli* by bifidobacteria. *J. Food Prot.,* 56 (8): 713.

IDF Bulletin-179. (1984). Fermented Milks- Proceedings of an IDF seminar in Avignon (France), 14-16 May, 1984.

IDF Bulletin 227. (1988). Fermented milks-Science and Technology. Chapter 2, Section 5: Starters with selected intestinal bacteria. International Dairy Federation Bulletin No. 227. pp. 41.

IDF Bulletin No. 255. (1991). Cultured products in human nutrition. Bull. Int. Dairy Fed., No. 255.

IDF Bulletin No. 306. (1995). Fermented and non-fermented milk products. Detection and enumeration of Lactobacillus acidophilus. Culture media. Bull. Int. Dairy Fed., No. 306.

Ilic, D B. and Ashoor, S.H. (1988). Stability of vitamin A and C in fortified yoghurt. *J. Dairy Sci.,* 71: 1492.

Ishibashi, N. and Shimamura, S. (1993). Bifidobacteria: Research and development in Japan. *Food Technol.*, 47: 126.

Iwana, H, Masuda, H., Fujisawa, T., Suzuki, H. and Mitsuoka, T. (1993). Isolation of *Bifidobacterium* spp. in commercial yoghurts sold in Europe. *Bifidobacteria Microflora,* 12: 39.

Jacob, F., Lwoff, A., Siminoritch, A. and Wollman, E. (1953). Definition de quelques termes relatifs a la lysogenie. *Annual Rep. Inst. Pasteur*, Paris, 84: 22.

Jay, J.M. (1982). Antimicrobial properties of diacetyl. *Appl. Environ. Microbiol.*, 44: 525.

Jetten, A.M., Vogels, G.D. and De Windt, F. (1972). Production and purification of *Staphylococcus epidermidis* bacteriocin. *J. Bact.*, 112: 235.

Jimenez-Diaz, R., Piard, J.C., Ruiz-Barba, J.L. and Desmazeand, M.J. (1990). Isolation of a bacteriocin producing Lactobacillus plantarum from a green olive fermentation. *FEMS Microbiol. Rev.*, **87**: 91.

Joerger, M.C. and Klaenhammer, T.R. (1986). Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. *J. Bacteriol.*, **167**: 439.

Johns, F.E., Gordon, J.F. and Shapton, N. (1978). The separation from yoghurt cultures of lactobacilli and streptococci using reinforced clostridial agar at pH 5.5 and plate count agar incorporating milk. *J. Soc. Dairy Technol.*, **31**(4): 209.

Jong-Yeun-Shin and Cheol Ahn. (1997). Characterization of bacteriocin production by *Lactobacillus lactis* LAB3113 isolated from kimchi. *J. Food Sci. Nutr.*, **2**(2): 101.

Jordono, R., Serrano, C.E., Torres, M. and Salmaron, J. (1992). Comparison of three M17 media for the enumeration of *Streptococcus thermophilus* in fermented dairy products. *J. Food Prot.*, 55(12): 999.

Joseph, P., Dave, R.I. and Shah, N.P. (1998). Antagonism between yoghurt bacteria and probiotic bacteria isolated from commercial starter cultures, commercial yoghurts and a probiotic capsule. *Food Australia.*, **50**: 20.

Juven, B.J., Barefoot, S.F., Pierson, M.D., McCaskill, L.H. and Smith, B. (1998). Growth and survival of *Listeria monocytogenes* in vacuum-packaged ground beef inoculated with *Lactobacillus alimentarius* FloraCarn L-2. *J. Food Prot.*, **61**(5): 551.

Juven, B.J., Meinersmann, R.J. and Stern, N.J. (1991). Antagonistic effects of lactobacilli and pediococci to control intestinal colonization by human enteropathogens in live poultry. *J. Appl. Bacteriol.*, **70**(2): 95.

Juven, B.J., Schved, F. and Linder, P. (1992). Antagonestic compounds produced by a chicken intestinal strain of *L. acidophilus*. *J. Food Prot.*, **55**(3): 157.

Kailasapathy, K. and Rybka, S. (1997). *L. acidophilus* and *Bifidobacterium* spp. -their therapeutic potential and survival in yogurt. *Aust. J. Dairy Technol.*, **52**: 28.

Kailasapathy, K. and Supriadi, D. (1996). Effect of whey protein concentrate on the survival of *Lactobacillus acidophilus* in lactose hydrolysed yoghurt during refrigerated storage. *Milchwissenschaft*, **51**: 565.

Kanbe, M. (1992). *Functions of fermented milks: Challanges for the health sciences.* Edited by: Yugii Nakazawa and Akiyoshi Hosono; Elsevier Applied Science Publishers, 1992. pp. 289.

Kandler, O. (1983). Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwen.*, **49**: 209.

Kandler, O. and Weiss, N. (1986). Regular non-sporing Gram-positive rods. In Bergey's Manual of Systematic Bacteriology, Vol. 2, edited by: Sneath, P. H. A., Sharpe, M. E. and Holt, J. G., Williams and Wilkins, Baltimore, MD, USA.

Kaneko, T. and Kurihara, H. (1997). Digoxigenin-labeled deoxyribonucleic acid probes for the enumeration of bifidobacteria in fecal samples. *J. Dairy Sci.*, **80**: 1254.

Kan, T. and Kobayashi, Y. (1988). Methods for producing oligosaccharides. European Patent Appl. EP O 226 AZ 7 pp. (Cited from *Dairy Sci. Abstr.*, **50**: 6848).

Kehagias, C., Jao, Y.C., Mikolajcik, E.M. and Hansen, P.M.T. (1977). Growth response of *Bifidobacterium bifidum* to a hydrolytic product isolated from bovine casein. *J. Food Sci.*, **42**: 146.

Kelstrup, J. and Gibbons, R.J. (1969). Bacteriocins from human and rodent streptococci. *Arch. Oral Biol.*, **14**: 251.

Khamagaeva, I.S. and Kulikova, A.K. (1984). Volatile fatty acids in culture milk products containing hydrolysed lactose. *Izvestiya Viysshikhi Vchebnykh* Zavedenii *Pischevaya Technologia,* **4**: 113. (Cited from *Dairy Sci. Abstr.*, **47**: 2301).

Khamagaeva, I.S., Sharobaiko, V.I., Naumetova, S.S., Vestya, I.Z., Vysshikh uchebnyclh. (1983). Cultivation of bifidobacteria in milk. *Zavedenii Pishchevaya TeKhnologie.* (Cited from *Dairy Sci. Abstr.*, **46**: 2316).

Khattab, A.A., Attia, I.A., Gudd, E. (1986). Stimulation of *Lactobacillus acidophilus* and *Bifidobacteium adolescentis* in milk by β-galactosidase. *Egypt. J. Dairy Sci.*, **14**: 155.

Khedkar, C.D., Mantri, J.M. and Kulkarni, S.A. (1989). Therapeutic properties of acidophilus milk. *Indian Dairyman,* **41**(11): 562.

Kim, C.R., Hearnsberger, J.O., Vickry. A.P., White, C.H. and Marshall, D.L. (1995). Sodium acetate and bifidobacteria increase shelf life of refrigerated catfish fillets. *J. Food Sci.*, **60** (1): 25.

Kim, E.R., Lee, K.W., Park, Y.H. and Kwak, H.S. (1993). The study of lactic acid bacteria in yoghurt during delivery and storage. *Korean J. Dairy Sci.*, **14**: 260.

Kim, H.S. (1988). Characterization of lactobacilli and bifidobacteria as applied to dietary adjuncts. *Cult. Dairy Prod. J.*, **23**: 6.

Kim, W.J. (1993). Bacteriocins of lactic acid bacteria: Their potentials as food preservative. *Food Rev. Inter.*, **9**: 299.

Kisza, V.J. and Ziajka, S. (1973). Studies on *Lactobacillus bifidus* cultivation in cow's milk. *Milchwissenschaft*, **28**: 696.

Kisza, J., Ziajka, S. and Panfil, H. (1974). Intensified growth of *Bifidobacterium bifidum*. XIX Int. Dairy Congr. E. 578.

Klaenhammer, T.R. (1988). Bacteriocins of lactic acid bacteria. *Biochimie,* **70** (3): 337

Klaenhammer, T.R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.*, **12**: 39.

Klaver, F.A.M. and Kingma, F. (1992). Mild-souring yoghurt. *Voedings-middelen-technologie,* **25**: 11.

Klaver, F.A.M., Kingma, F. and Bolle, A.C. (1990). Growth relationships between bifidobacteria and lactobacilli in milk. *Voedingsmiddelentechnologie,* **23**: 13.

Klaver, F.A.M., Kingma, F. and Weerkamp, A.H. (1993). Growth and survival of bifidobacteria in milk. *Neth. Milk Dairy J.,* **47**: 151.

Kneifel, W., Jaros, D. and Erhard, F. (1993). Microflora and acidification properties of yoghurt and yoghurt related products fermented with commercially available starter cultures. *Int. J. Food Microbiol.*, **18**: 179.

Kneifel, W. and Pacher, B. (1993). An X-Glu based agar medium for the selective enumeration of *L. acidophilus* in yoghurt related milk products. *Int. Dairy J.,* **3**: 277.

Kobayashi, Y., Kan, T. and Terashima, T. (1988). Method for producing oligosaccharides. European Patent 1988. Ep. 0263 700 A2 pp. (Cited from *Dairy Sci. Abstr.,* **51**: 1230).

Kopeloff, L.M. (1926). *Lactobacillus acidophilus.* The Williams and Wilkins Co., Baltimore, USA.

Koroleva, N.S., Lagoda, I.V., Bannikova, L.A., Kosareva,. L.V., Popelova, V.V., Rakhimova, N.G., Koroleva, D.D., Gudachkova, V.M. and Shkundova, Y.V. (1983). Methods of producing acidophilus cultures. USSR Patent, S. U. 1004 474.

Kosikowski, M. (1978). Biochemical activity of *Bifidobacterium.* XX Int. Dairy. Congr. E. 549.

Kosikowski, M. (1982). Influence of technologically harmful microflora of milk in the development of bifidobacteria. XXI Inter. Dairy Congr., **1**: 324.

Kurmann, J.A. (1988). Starters with selected intestinal bacteria. Fermented milks: Science and Technology. In IDF Bulletin, No.227, Brussels.

Kurmann, J.A. and Rasic, J.L. (1991). The health potential of products containing bifidobacteria. In Therapeutic properties of Fermented milks. Robinson, R.K. (ed). Elsevier Applied Food Science Series, London, pp.117.

Kuromiyo, M. (1960). The study on the effect of cinderlike especially carrot powder in dyspeptic infants. *Ochanomizu Igaku Zasshi*, **8**: 88.

Labropoulos, A.E., Palmer, J.K. and Lopez, A. (1981). Whey protein denaturation of UHT processed milk and its effect on rheology of yoghurt. *J. Texture Studies*, **12**:365.

Laemmli, U.R. (1970). Most commonly used discontinuous buffer system for SDS electrophoresis. *Nature*, 227: 680.

Lankaputhra, W.E.V. and Shah, N.P. (1994). Investigation of factors affecting viability of *Lactobacillus acidophilus* and *Bifidobacteria* in yoghurt. 24th Inter. Dairy Congress, Melbourne, Australia, Sept. 18-22, 1994.

Lankaputhra, W.E.V. and Shah, N.P. (1995). Survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in the presence of acid and bile salts. *Cult. Dairy Prod. J.*, **30**: 2.

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.

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.

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.

Lapierre, L., Undeland, P. and Cox, L.J. (1992). Lithium chloride-sodium propionate agar for the enumeration of bifidobacteria in fermented dairy products. *J. Dairy Sci.*, **75**: 1192.

Laroia, S. and Martin, J.H. (1991a). Methods for enumerating and propagating bifidobacteria. *Cult. Dairy Prod. J.*, **26**: 32.

Laroia, S. and Martin, J.H. (1991b). Effect of pH on survival of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in frozen fermented dairy desserts. *Cult. Dairy Prod. J.*, **26**: 13.

LaVergene, E., Burdin, J.C., Schmitt, J. and Manciaux, M. (1959). Sensibilite de *B. bifidum* a onze antibiotiques. *Annales de l' Institut Pasteur*. **97**(1): 104.

Law, B.A. and Kolstad, J. (1983). Proteolytic systems in lactic acid bacteria. *Antoine Leeuwenhock*, **49**: 225.

Le, M.G., Moulton, L.H., Hill, C. and Kramar, A. (1986). Consumption of dairy produce and alcohol in a case control study of breast cancer. *J. Nat. Cancer Insti.*, 77: 633.

Lee, S.Y., Vedamuthu, E.R. and Washam, C.J. (1974). An agar medium for the differential enumeration of yoghurt starter bacteria. *J. Milk Food Technol.*, **37**(5): 272.

Lewus, C.B., Kaiser, A. and Montville, T.J. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Appl. Environ. Microbiol.*, **57**: 1683.

Lin, C.W., Shih, C.H. and Su, H.P. (1986). Studies on the natural antimicrobial agents from lactic acid bacteria I -Primary screening of lactic cultures for antimicrobial activitity. *J. Chinese Agri. Chem. Soc.*, **24** (4): 384.

Lohnis, F. (1907). Versuch einer Gruppierung der Milchsaurebakterien. *Zentbl. Bakt. ParasitKde*. Abt ll, **18**: 97.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**: 265.

Lyons, T.P. (1988). A biological approach to productivity improvement in the calf and dairy cow. *Indian Dairyman*, **40**: 115.

Malke, H.R., Starke, R.O., Jacob, H.E. and Kohler, W. (1974). Bacteriocin like activity of group A streptococci due to the production of peroxide. *J. Med. Microbiol.*, 7: 367.

Manson, V., Bech-Anderson, S. and Rudemo, M. (1980). *Z. Tierphysil, Tierernahrg u. Futtermittelkde,* **43**: 146.

Mantere-Alhonen, S., Noro, K. and Sippola, L. (1989). Antibacterial effect of bifidobacteria and lactobacilli. *Meijeritieteellinen-Aikakauskirja,* **47**(1): 19.

Marle, M. E-van, and Zoon, P. (1995). Structure of yoghurt gels. *Voedings-middelentechnologie,* **28**: 13.

Marshall, V.M., Cole, W.M. and Mabbit, L.A (1982). Fermentation of specially formulated milk with single strains of bifidobacteria. *J. Soc. Dairy Technol.*, 35: 143.

Martin, J.H. (1994). Resistance of bifidobacteria to pH changes in yoghurt during refrigerated storage. 24th International Dairy Congress, Australia, Melbourne, Sept. 18-22, 1994.

Martin, J.H. and Chou, K.M. (1992). Selection of *Bifidobacterium* spp. for use as dietary adjuncts in cultured dairy foods. I. Tolerance to pH of yoghurt. *Cult. Dairy Prod. J.*, **27**(4): 21.

Mathieu, F., Sudirman-Suwandhi, I., Rekhif, N., Milliere, J.B. and Lefebvre, G. (1993). Mesenterocin 52, a bacteriocin produced by *Leuconostoc mesenteroides* ssp. *mesenteroides* FR 52. *J. Appl. Bacteriol.*, **74**(4): 372.

Matsumoto, K., Komiyama, S., Watanabe, T., Kyroda, K. and Mutai, K. (1987). Method for bifidobacteria Japanese Patent JP 62208 293 6PP. (Cited from *Dairy Sci. Abstr.*, 50: 2637).

Mayr-Harting, A., Hedges, A.J. and Barkeley, R.C.W. (1972). Methods for studying bacteriocins. In "Methods in Microbiology" (T. Bergen and J. R. Norris, eds.), Vol. 7A, pp. 315-422, Academic Press, Inc., London.

Mccann, T., Egan, T. and Weber, G.H. (1996). Assay procedures for commercial probiotic cultures. *J. Food Prot.*, **59**(1): 41.

Medina, L.M. and Jordono, R. (1994). Survival of constitutive microflora in commercially fermented milk containing bifidobacteria during refrigerated storage. *J. Food Prot.*, **56**: 731.

Medina, M., Gaya, P. and Nunez, M. (1992). Bacteriocins produced by lactic acid bacteria. *Revista- Espanola-de-Lecheria.* No. **34**: 28.

Mehta, A.M., Patel, K.A. and Dave, P.J. (1984). Purification and some properties of an inhibitory protein isolated from *L. acidophilus AR1. Milchwissenschaft,* **39**(2): 86.

Metchnikoff, E. (1908). The prolongation of life. G.P. Putman's Sons, New York.

Micanel, N., Haynes, I.N. and Playne, M.J. (1997). Viability of probiotic cultures in commercial Australian yogurts. *Aust. J. Dairy. Technol.*, **52**(1): 24.

Mikolajcick, E.M. and Hadman, I.Y. (1975). *Lactobaacillus acidophilus* II: Antimicrobial agents. *Cult. Dairy Prod. J.*, **10**(1): 18.

Miller, I. and Kandler, O. (1964). Breakdown of protein in sour milks. Free amino acids in yoghurt, bioghurt and acidophilus milk. *Medizin Emahr*, **5**: 100.

Miller, L.G. and Finegold, S.M. (1967). Antibacterial sensitivity of *Bifidobacterium (Lactobacillus bifidus)*. *J. Bact.*, **93**: 125.

Misra, A.K. (1988). Studies on *Bifidobacterium bifidum* based fermented milk products. Ph.D thesis submitted to Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, India.

Misra, A.K. and Kuila, R.K. (1991). Bifidus milk: Potential for developing countries. *Indian Dairyman,* **43** (9): 390.

Mital, B.K. and Garg, S.K. (1992). Acidophilus milk products- Manufacture and Therapeutics. *Food Rev. Inter.*, **8** (3): 347.

Mitsuoka, T. (1984). Taxonomy and ecology of bifidobacteria. *Bifidobacteria Microflora*, **3**: 11.

Mizota, T., Tamura, Y., Tomota, M. and Okanogi, S. (1987). Lactulose as a sugar with physiological significance. IDF Bulletin, No. 212: 69.

Modler, H.W., Mckeller, R.C. and Yaguchi, M. (1990). Bifidobacteria and bifidogenic factors. *Canad. Inst. Food Sci. Technol. J.*, **23**(1): 29.

Monfort, J.M., Hugas, M., Pages, F. and Garriga, M. (1996). Comparison of different bacteriocin-producing lactobacilli on Listeria growth in sausages. Eurocrane, 6(48): 51.

Moore, W.E.C. and Holdman, I.V. (1970). Gram positive nonsporeforming anaerobic bacilli. In Manual of chemical microbiology (edited by: Blair, J. E. , Lennette, E. H. and Truant, J. P.), American Soc, Microbiol., Bethesda, MD, USA.

Moro, E. (1900). Uber die Gram farbbaren bacillen des sauglingsstuhles. Wien. Lin. Wschr., **13**: 114.

Morris, C.E. (1991). Dairy ingredients boost shelf life, replace fat. *Food Engg.*, **63**(2): 99.

Moss, C.W. (1981). Gas liquid chromatography as an analytical tool in Microbiology. *J. Chromatography*, **203**: 337

Mottar, J., Bassier, A., Joniau, M. and Baret, J. (1989). Effect of heat-induced association of whey proteins and casein micelles in yoghurt texture. *J. Dairy Sci.*, **72**: 2247.

Muelhens, K. (1967). *Medizin und. Ernahrung*, **8**: 11. (Cited from Rasic and Kurmann, 1978).

Munoa, F.J., and Pares, R. (1988). Selective medium for isolation and enumeration of *Bifidobacterium spp. Appl. Environ. Microbiol.* **54**(7): 1715.

Muriana, P.M. and Klaenhammer, T.R. (1987). Conjugal transfer of plasmid encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. *Appl. Environ. Microbiol.*, **53:** 553.

Muriana, P.M. and Klaenhammer, T.R. (1991). Purification and partial characterization of lactacin F, a bacteriocin produced by *L. acidophilus* 11088. *Appl. Environ. Microbiol.*, **57:** 114.

Mutai, M., Aso, K, and Shirota, M. (1971). International symposium on manufacture of food held at Kyoto, Japan.

Mutai, M., Mada, M., Nakajama, K., Shimada, K. and Tiqima, T. (1978). Fermented milk containing viable bifidobacteria. U. S. Patent 4087 559.

Mutai, M., Mada, M. and Shimada, K. (1980a). A food stuff from rice by the action of bifidobacteria. UK Patent Appl., 2043 10/A. (Cited from *Dairy Sci. Abstr.*, **43**: 8116).

Mutai, M., Mada, M. and Shimada, K. (1980b). Method for producing food containing bifidobacteria. U. S. Patent, 4187 321.

Najivi, K., Takasaki, K., Deie, H. and Igarashi, K. (1987). Novel oligosaccharide and method of making it. Japanese Patent. JP 62 205 793 5 PP. (Cited from Dairy Sci. Abstr., **50**: 2636).

Nakazawa, Y. and Hosono, A. (1992). Functions of Fermented Milk: Challenges for Health Sciences. Elsevier Sci. Publ., London.

Nielsen, V.H. (1972). Smooth-textured yogurts. Processing methods that remove granules with minimum damage to the firmness of the product. *Amer. Dairy Rev.*, **34**(2): 26.

Nielsen, V.H. (1975). Factors which control the body and texture of commercial yoghurt. Amer. Dairy *Rev.*, **37**(11): 36.

Nielson, H.K. and Hurrell, R.F. (1985). Tryptophan determination of food proteins by HPLC after alkaline hydrolysis. *J. Food Sci. Agric.*, **36**: 893.

Nighswonger, B.D., Brashears, M.M. and Gilliland, S.E. (1996). Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in fermented milk products during refrigerated storage. *J. Dairy Sci.*, **79**: 212.

Noh, W.S., Shin, H.S. and Lim, J.W. (1994). A study on the fortification of yoghurt with vitamin A and C. *Korean J. Dairy Sci.*, **16**: 385.

Norris, R.F., Flanders, T., Tomarelli, R.M. and Gyorgy, P. (1950). The isolation and cultivation of *Lactobacillus bifidus.* A comparison of branched and unbranched strains. *J. Bacteriol.*, **60**: 681.

O'Brien, P.J., Glick, M.C. and Zilliken, F. (1960). Acidic amino sugars from bacteria. I Incorporation of (1-14C), β-methyl-N-acetyl-D-glycosaminide into Muramic acid. *Biochim. Biophys. Acta.*, **37**: 357.

Ohta, K. (1959). Dietary treatment of dyspepsia of infants. *Shonika Shinnyo*, **22**: 854. Cited from Misra (1988).

Okereke, A. and Montville, T.J. (1991). Bacteriocin inhibition of *Clostridium botulinum* spores by lactic acid bacteria. *J. Food Prot.*, **54:** 349.

Olson, N.F. (1990). The impact of lactic acid bacteria on cheese flavour. *FEMS Microbiol.* Rev., **87**: 131.

Onggo, I., and Fleet, G.H. (1993). Media for the isolation and enumeration of lactic acid bacteria from yoghurts. *Aust. J. Dairy Technol.*, **48**: 89.

Orla-Jensen, S. (1919). The lactic acid bacteria. Host, Copenhegen, Denmark.

O'Sullivan, M.G. (1996). Metabolism of bifidogenic factors by gut flora-An overview. IDF Bulletin No. 313. 23.

Pahwa, A., Mathur, B.N. and Hansen, P.M.J. (1989). Fractionation of bifidus growth stimulating factors from casein and rennet whey. *Indian J. Dairy Sci.*, **42**: 348.

Parker, R.B. (1974). Probiotics: the other half of the antibiotic story. *Animal Nutr. Health*, **29**: 4.

Park, I.S., Lee, H.K. and Kang, K.H. (1988). A study on the effect of oligosaccharides on growth of intestinal bacteria. *Korean J. Dairy Sci.*, **10**: 159.

Patel, J.R., Dave, R.I., Sannabhadti, S.S. and Dave, J.M. (1991). Use of bifidobacteria in fermented dairy products. *Indian Dairyman,* **43**(4): 181.

Patel, J.R., Dave, J.M., Dave, R.I. and Sannabhadti, S.S. (1992). Effect of feeding milk fermented with mixed culture of lactobacilli on faecal lactobacilli and coliform counts in human test subjects. *Indian J. Dairy Sci.,* **45**(7): 379.

Patidar, S.K. and Prajapati, J.B. (1997). Methods for assessing the immunostimulating properties of dietary lactobacilli-A critical appraisal. *J. Food Sci. Technol.*, **34**: 181.

Patidar, S.K., Prajapati, J.B. and Dave, R.I. (1994). Standardization and evaluation of acidophilus lassi. 24th Inter. Dairy Congress, Melbourne, Australia, Sept. 18-22, 1994.

Piombi, G., and Lozano, Y. (1980). Automated procedure for routine analysis of tryptophan in cereal and legume food samples. *J. Agric. Food Chem.*, **28**: 489.

Pipek, P. and Stankova, L. (1996). The effect of bacteriocins on the lactobacilli growth in vacuum packed meat products. *Potravinarske-Vedy,* **14**(2): 109.

Poch, M. and Bezkorovalny, A. (1988). Growth enhancing supplements for various species of the genus *Bifidobacterium. J. Dairy Sci.*, **71**: 3214.

Pot, B., Descheemaeker, P, Roeges, N. and Kersters, K. (1994). Identification of lactic acid bacteria by sodium dodecyl sulphate polyacrylamide gel electrophoresis of whole cell proteins. In Lactic-91: Lactic Acid Bacteria, Research and Industrial applications in the agro-food industries, Symposium Abstracts, Caen, France.

Poupard, J.A., Husain, J. and Norris, R.F. (1973). Biology of the bifidobacteria. *Bact. Rev.*, **37**: 136.

Prajapati, J.B. and Dave, R.I. (1994). Associative growth of *L. acidophilus* and *S. thermophilus* in buffalo milk. 24th Inter. Dairy Congress, Melbourne, Australia, Sept. 18-22, 1994.

Prasad, M.M. and Ghodeker, D.R. (1991). Antimicrobial activity of lactobacilli isolated from fermented milk products. *Cult. Dairy Prod. J.,* **26**(2): 22.

Price, R.J. and Lee, J.S. (1970). Inhibition of Pseudomonas species by hydrogen peroxide producing lactobacilli. *J. Milk Food Technol.*, **33**: 13.

Proulx, M., Gultier, S.F. and Roy, D. (1992). Utilisation d'hydrolysats enzymatique de ca'eine pour la croissance des bifidobacteries. *Lait,* **72**: 393.

Proulx, M., Ward, P., Gauthier, S.F. and Roy. D. (1994). Comparison of bifidobacterial growth promoting activity of ultrafiltered casein hydrolysate fractions. *Lait,* **74**: 139.

Pulsani, S.R., Rao, D.R. and Sunki, G.R. (1979). Antimicrobial activity of lactic cultures: Partial purification and characterization of antimicrobial compound(s) produced by *Streptococcus thermophilus. J. Food Sci.,* **44**(2): 575.

Rammelsberg, M. and Radler, F. (1990). Antibacterial polypeptides of *Lactobacillus* species. *J. Appl. Bact.*, **69**: 177.

Rasic, J.L. (1983). The role of dairy foods containing bifido and acidophilus bacteria in nutrition and health. *North European Dairy J.*, **4**: 80.

Rasic, J.L. and Kurmann, J.A. (1978). Yoghurt: Scientific Grounds, Technology, Manufacture and Preparations. Technical Dairy Publishing House, Vanlose, Copenhagen, Denmark.

Rasic, J.L. and Kurmann, J.A. (1983). Bifidobacteria and Their Role. Birkhauser Verlag, Basel, Switzerland.

Rayner, C. (1985). Protein hydrolysis of animal feeds for amino acid content. *J. Agric. Food Chem.*, **33**: 722.

Reade, E. (1987). Staining techniques. In Microbiological techniques. Ed., Reade,E. Published by the School of Microbiology, University of Melbourne, Australia. pp. 55.

Reddy, G.V., Shahani, K.M., Friend, B.A. and Chandan, R.C. (1983). Natural antibiotic activity of *Lactobacillus acidophilus* and *bulgaricus* III Production and partial purification of bulgarican from *Lactobacillus bulgaricus*. *Cult. Dairy Prod. J.*, **18**(2): 15.

Resnick, I.G., and Levin, M.A. (1981). Quantitative procedure for enumeration of bifidobacteria. *Appl. Environ. Microbiol.*, **42**(3): 427.

Richardson, D. (1996). Probiotics and product innovation. *Nutr. Food Sci.*, **4**: 27.

Richter, R.L. and Hartmann, G.H. (1977). Penetrometer evaluation of sour cream and yogurt at 1977 Kultures and Kurds Klinic. *Cult. Dairy Prod. J.*, **12**(4): 14.

Robinson, R.K. (1987). Survival of *Lactobacillus acidophilus* in fermented products. *Suid Afrikaanse Tydskrif Vir Suiwelkunde*, **19**: 25.

Robinson, R.K. (1991). Therapeutic Properties of Fermented Milks. Elsevier Applied Food Science Series, London

Rogosa, M. and Sharpe, M.E. (1959). An approach to the classification of lactobacilli. *J. Appl. Bact.*, **22**: 329.

Rogul, M. and Carr, S.R. (1972). Viable ammonia production among smooth and rough strains of *Pseudomonas pseudomallei*: resemblence to bacteriocin production. *J. Bact.*, **112**: 372.

Rohm, H. and Kovac, A. (1994). Effects of starter cultures on linear viscoelastic and physical properties of yogurt gels. *J. Text. Studies*. **25**: 311.

Rohm, H. and Kneifel, W. (1993). Physical properties of set yoghurt enriched with different substrates. *DMZ-Lebensmittelindustrie-und-Milchwirtschaft*. **114**: 272.

Romero, N.C., Nader-de-Macias, M.E., Apella, M.C. and Oliver, G. (1990). Inhibition of *Listeria monocytogenes* by strains of lactic acid bacteria used as ferments. *Microbiologie Aliments Nutr.*, **8**(4): 335.

Rowland, I.R. and Grasso, P. (1975). Degradation of N-nitrosamines by intestinal bacteria. *Appl. Microbiol.*, **29**: 7.

Roy, D., and Ward, P. (1990). Evaluation of rapid methods for differentiation of *Bifidobacterium* species. *J. Appl. Bacteriol.*, **69**: 738.

Rubin, H.E., Nerad, T. and Vaughan, F. (1982). Lactic acid inhibition of *Salmonella typhimurium* in yogurt. *J. Dairy Sci.*, **65**: 197.

Ruiz-Barba, J.L., Cathcart, D.P., Warner, P.J. and Jimenez-Diaz-R. (1994). Use of *Lactobacillus plantarum* LPC010, a bacteriocin producer, as a starter culture in Spanish-style green olive fermentations. *Appl. Environ. Microbiol.*, **60**(6): 2059.

Ryan, M.P., Ross, R.P. and Rea, M.C. (1996). Bacteriocins. PCT-International-Patent-Application, WO 96/32482 A1.

Rybka, S. and Kailasapathy, K. (1995). The survival of culture bacteria in fresh and freeze-dried AB yoghurts. *Aust. J. Dairy Technol.*, **50**: 51.

Rybka, S. and Kailasapathy, K. (1996). Media for the enumeration of yoghurt bacteria. *Inter. Dairy J.*, **6**: 839.

Sakai, K., Mishima, C., Tachiki, T., Kumagai, H. and Tochikura, T. (1987). Mortality of bifidobacteria in boiled yoghurt. *J. Fermented Technol.*, **65**: 215.

Salminen, S. and Wright, A. (1993). Bifidobacteria and probiotic in action. In Lactic Acid Bacteria. Marcel Dekker, Inc., New York, USA.

Samezina, H., Furukawa, H. and Deguechi, T. (1970). Method for enriching dairy products. Japanese Patent, 3189/70. (Cited from *Dairy Sci. Abstr.*, **33**: 3964).

Samona, A. and Robinson, R.K. (1994). Effect of yoghurt cultures on the survival of bifidobacteria in fermented milks. *J. Soc. Dairy Technol.*, **47**: 58.

Sanborn, A.G. (1931). *J. Infect. Dis.*, **49**: 37. Cited by Patel *et al.*, 1992.

Sandine, W.E., Murlidhara, K.S., Elliker, P.R. and England, D.C. (1972). Lactic acid bacteria in food and health: A review with special reference to enteopathogenic *E. coli* as well as certain enteric diseases and their treatment with antibiotics and lactobacilli. *J. Milk Food Technol.*, **35**: 691.

Sands, D.C. and Hankin, L. (1976). Fortification of foods by fermentation with lysine excreting mutants of lactobacilli. *J. Agr. Food Chem.*, **24**: 1104.

Savage, D.C. (1977). Introduction to mechanism of association of indigenous micro-organisms. *Am. J. Clin. Nutr.*, **32**: 113.

Scardovi, V. (1986). Genus *Bifidobacterium*. Page 1418 In Bergey's Manual of Systematic Bacteriology. Vol. 2. P. H. Sheath, N. S. Mair, M. E. Sharpe, and J. G. Holt, ed. Williams and Wilkins Publ., Baltimore, MD.

Schagger, H. and Von Jagow, G. (1987). Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 200 kDa. *Anal. Biochem.*, **166**: 368.

Schillinger, U. and Luke, F.K. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl. Environ. Microbiol.*, **55:** 1901.

Schillinger, U., Kaya, M. and Luke, F.K. (1991). Behavior of *Listeria monocytogenes* in meat and its control by a bacteriocin producing strain of *Lactobacillus sake. J. Appl. Bact.*, **70:** 473.

Schioppa, F., Prete, V. and diel Montanaro, D. (1981). Addition of lactobacillus to yoghurt. *Rivista della Scienta Italiana di Scienza dell Alimentazione,* **10**: 247.

Schuler-Malyoth, R., Ruppert, A. and Muller, F. (1968). The microorganisms of the bifidus group (*Lactobacillus bifidus*). I. Historical review, nutritional, physiological and therapeutic aspects, morphology, culture procedures and taxonomy. *Milchwissenschaft,* **23:** 356.

Sebald, M., Gasser, F. and Werner, H. (1965). DNA base composition and classification: Application to group of bifidobacteria and to related genera. *Ann. Ins. Pasteur,* **109**: 251.

Semenikhina, V.F. and Sundukova, M.B. (1980). Use of bifidobacteria in the manufacture of cultured products for children. *Molochnoya Promysklennost*, **3**: 33.

Semenikhina, V.F. and Sundukova, M.B. (1982). The use of bifidobacteria in the production of cultured food products for babies. XXI Int. Dairy Congr., Vol. 1, pp. 132.

Semenikhina, V.F., Granina, V.I. and Sundukova, M.B. (1985a). The strain *Bifidobacterium adolescentis* B for preparation of starters. USSR Patent, SUI 169 989 A. (Cited from Dairy Sci. Abstr., 49: 6551).

Semenikhina, V.F., Khorkova, E.A., Ivonova, L.N. and Ladodo, K.S. (1985b). Effect of mixture component of mylyulka infants formula on biochemical activity of bifidobacteria in production of cultured formula. *Dairy Sci. Abstr.*, **47**: 6373.

Sgorbati, B., Biavati, B. and Palenzona, D. (1995). The genus *Bifidobacterium*. In The Genera of Lactic Acid Bacteria. edited by Wood, B. J. B. and Holzapfel, W. H., Blackie Academic and Professional, London, UK.

Shah, N. and Jelen, P. (1990). Survival of lactic acid bacteria and their lactases under acidic conditions. *J. Food Sci.*, **55**: 506.

Shah, N., Lankaputhra, W.E.V., Britz, M. and Kyle, W.S.A. (1995). Survival of *L. acidophilus* and *Bifidobacterium bifidum* in commercial yoghurt during refrigerated storage. *Inter. Dairy J.*, **5**: 515.

Shah, N.P. (1997). Isolation and enumeration of bifidobacteria in fermented milk products: a review. *Milchwissenschaft*, **52**(2): 72.

Shahani, K.M., Vakil, J.R. and Kilara, A. (1976). Natural antibiotic activity of *L. acidophilus* and *bulgaricus*. I. Cultural conditions for the production of antibiotics. *Cult. Dairy Prod. J.*, **11:** 14.

Shahani, K.M., Vakil, J.R. and Kilara, A. (1977). Natural antibiotic activity of *L. acidophilus* and *bulgaricus*. II. Isolation of acidophillin from *L. acidophilus*. *Cult. Dairy Prod. J.*, **12:** 8.

Shankar, P. and Davies, F. (1976). Associative bacterial growth in yoghurt starters: Initial observations on stimulatory factors. *J. Soc. Dairy Technol.*, **30**: 31.

Shankar, P.A. and Davies, F.L. (1977). A note on the suppression of *Lactobacillus bulgaricus* in media containing β-glycerophosphate and application of the media to selective isolation of *Streptococcus thermophilus* from yoghurt. *J. Soc. Dairy Technol.*, **30**: 28.

Sharma, D.K. and Singh, J. (1982). Yoghurt starters in skim milk II. Behaviour of *L. acidophilus* in yoghurt starters. *Cult. Dairy Prod. J.*, **27**(4): 10.

Sharpe, M.E. (1979). Identification of the lactic acid bacteria. In Identification Methods for Microbiologists, 2nd ed., Soc. Appl. Bacteriol., Technical Series, No. 14, edited by: Skinner, F. A. and Lovelock, D. W., Academic Press, London, UK.

Singh, J., Khanna, A. and Chandar, H. (1980). Effect of incubation temperature and heat treatments of milk from milk of cow and buffalo on acid and flavour production by *S. thermophilus* and *L. bulgaricus*. *J. Food. Prot.*, **43**: 399.

Singh, J. and Sharma, D.K. (1983). Proteolytic breakdown of casein and its fraction by lactic acid bacteria. *Milchwissenschaft*, **38**: 148.

Sorbino, O.J., Rodriguez, J.M., Moreira, W.L., Fernandez, M.F., Sanz, B. and Hernandez, P.E. (1991). Antibacterial activity of *Lactobacillus sake* isolated from dry fermented sausages. *Inter. J. Food Microbiol.*, **13**(1): 1.

Sozzi, T., Brigidi, P. Mignot, O. and Matteuzzi, D. (1990). Use of dicloxacillin for the isolation and counting of *Bifidobacterium* from dairy products. *Lait,* **70**: 357.

Spillmann, H., Puhan, Z. and Banhegy, M. (1978). Antibacterial activity of thermophillic lactobacilli. *Milchwissenschaft,* **33**(3): 148.

Stackerbrandt, E. and Teuber, M. (1988). Molecular taxonomy and phylogenetic position of lactic acid bacteria. *Biochimie,* **70**: 317.

Stoffels, G., Nes, I.F. and Guomundsdottir, A. (1992a). Isolation and properties of a bacteriocin-producing *Carnobacterium piscicola* isolated from fish. *J. Appl. Bacteriol.*, **73**(4): 309.

Stoffel, G., Nissen-Meyer, J., Gudmundsdottir, A., Sletten, K. Holo, H. and Nes, I.F. (1992b). Purification and characterization of a new bacteriocin isolated from a *Carnobacterium* spp. *Appl. Environ. Microbiol.*, **58**: 1417.

Su, T.L. (1948). Micrococcin. An antibacterial substance formed by a strain of Micrococcus. *British J. Expet. Path.*, **30**: 466.

Suwa, Y., Koga, K., Kawas, Fuji, Okazak, M, Irie, T. and Nakada, T. (1988). *Lactobacillus bifidus* proliferation promoting composition. European Patent, Ep. 0265 970 AI 20 PP (EN). (Cited from *Dairy Sci. Abstr.*, **51**: 316).

Tagg, J.R., Dajani, A.S. and Wannamaker, L.W. (1976). Bacteriocins of Gram positive bacteria. *Microbiol. Rev.*, **40:** 722.

Tahara, T. and Kanatani, K. (1997). Isolation and partial amino acid sequence of bacteriocins produced by *Lactobacillus acidophilus*. Bioscience-Biotechnology and Biochemistry, **61**(5): 884.

Tamime, A.Y. and Deeth, H.C. (1980). Yoghurt: Technology and Biochemistry. *J. Food Prot.*, **43**: 939.

Tamime, A.Y. and Robinson, R.K. (1985). Yoghurt-Science and Technology. Pergamon Press, Oxford, UK.

Tamime, A.Y. and Robinson, R.K. (1988). Technology of thermophillic starter cultures. In Fermented Milks: Science and Technology, IDF Bulletin, No. 227, Brussels.

Tamime A.Y., Kalab, M. and Davies, G. (1884). Microstructure of set-style yoghurt manufactured from cow's milk fortified by various methods. *Food Microstr.*, **3**: 83.

Tamime, A.Y., Kalab, M., Muir, D.D. and Barrantes, E. (1995). The microstructure of set-style natural yoghurt made by substituting micro-particulate whey protein for milk fat. *J. Soc. Dairy Technol.*, **48**: 107.

Tamura, Z. (1983). Nutriology of bifidobacteria. *Bifidobacteria Microflora*, **2**: 3.

Teraguchi, S., Uehara, M., Ogasa, K. and Mitsuoka, T. (1978). Enumeration of bifidobacteria in dairy products. *Jpn. J. Bacteriol.*, **33**(6): 753.

Thomas, T.D. (1985). Role of latic acid bacteria and their improvement for production of better fermented animal products. *NZ. J. Dairy Sci. Technol.*, **20**: 1.

Thomas, T.D. and Mills, O.E. (1981). Proteolytic enzymes of starter bacteria. *Neth. Milk Dairy J.*, **35**: 255.

Thomas, T.D., Turner, K.W. and Crow, V.L. (1980). Galactose fermentation by *Streptococcus lactis* and *Streptococcus cremoris*: pathways products and regulation. *J. Bacteriol.*, **144**: 672.

Thompson, J.K., Collins, M.A. and Mercer, W.D. (1996). Characterization of a proteinaceous antimicrobial produced by *Lactobacillus helveticus* CNRZ450. *J. Appl. Bacteriol.*, **80**(3): 338.

Tichaczek, P.S., Nissen-Mayer. J., Nes. I.F., Vogel, R.F. and Hammes, W.P. (1992). Characterization of the bacteriocin curvacin A from *Lactobacillus curvatus* LTH1174 and sakacin P from *L. sake* LTH673. *Syst. Appl. Microbiol.*, **15**(3): 460.

Tissier, H. (1899). Recherches sur la flore intestinale normale et pathologique du nourission. Thesis, University of Paris, Paris, France.

Toba, T., Yoshioka, E. and Itoh, T. (1991a). Acidophilucin A- a new heat labile bacteriocin produced by *Lactobacillus acidophilus* LAPT 1060. *Lett. Appl. Microbiol.*, **12**(4): 106.

Toba, T., Yoshioka, E. and Itoh, T. (1991b). Lacticin, a bacteriocin produced by *Lactobacillus delbrueckii* ssp. *lactis*. *Let. Appl. Microbiol.*, **12**: 43.

Tomic-Karovic, V.K. and Fanjek, J. (1963). Acidophilus milch in der therapie der sauglings diarrhoen verursacht durch pathogene *E. coli*. *Annal. Paediatrics,* **199**: 625.

Trammer, J. (1966). Inhibiting effect of *Lactobacillus acidophilus*. *Nature*, **211**: 204.

Upreti, G.C. and Hinsdill, R.D. (1975). Production and mode of action of lactocin 27: bacteriocin from a homofermentative *Lactobacillus. Antimicro. Agent. Chemothe.*, **7:** 139.

Vakil, J.R. and Shahani, K.M. (1965). Partial purification of antibacterial activity of *L. acidophilus. Bact. Proceed.* **9.** (Cited in *Dairy Sci. Abst.*, **27**: 2175).

Van't Veer, P., Dekker, J.M., Lemers, J.W.J., Kok, F.J., Schouten, E.G., Brants, HAM, Sturmans, F. and Hermus, R.J.J. (1989). Consumption of fermented milk products and breast cancer: a case control study in the Netherlands. *Cancer Res.*, **49**: 4020.

Vanovsky, B. (1975). Godishen Zbornik, **21**: 405. (Cited from *Dairy Sci. Abstr.*, **39**: 5909).

Varadaraj, M.C., Devi N., Keshava, N. and Manjrekara, S.P. (1993). Antimicrobial activity of neutralized extracellular culture filtrates of lactic acid bacteria isolated from a cultured Indian milk product (dahi). *Inter. J. Food Microbiol.*, **20**(4): 259.

Vijayendra, S.V.N. and Gupta, R.C. (1992). Therapeutic importance of bifidobacteria and *L. acidophilus* in fermented milks. *Indian Dairyman,* **44**(12): 595.

Vincent, J.G., Veomett, R.C. and Riley, R.F. (1959). Antibacterial activity associated with *Lactobacillus acidophilus. J. Bact.*, **78**: 477.

Wakiguchi, H. (1984). *Japanese J. Dairy Food Sci.,* **33** (6):A203. Cited by Kanbe, 1992.

Walstad, D.L., Reitz, R.C. and Sparling, P.F. (1974). Growth inhibition among strains of *Neisseria gonorrhoeae* due to production of inhibitory free fatty acids and lysophosphatidylethanolamine: absence of bacteriocins. *Infect. Immun.*, **10**: 481.

Wang J.K. (1993). Bacteriocins of lactic acid bacteria: their potentials as biopreservatives. *Food Rev. Inter.*, **9**(2): 299.

Warburg, O. and Kaplan, N.O. (1957). Methods in enzymology 3. Academic Press Inc., NY., pp. 451.

Waxman, A.M., Harmon, K.S. and Bishop, J.R. (1989). Inhibition of *Pseudomonas* by hydrogen peroxide producing lactic acid starter cultures. *J. Dairy Sci.*, **72**: 2208.

Webb, H.W., Johnson, A.H. and Alford, J.A. (1974). Fundamentals of Dairy Chemistry, 2nd ed., AVI Publishing Co., Westport, Connecticut. pp. 373-80.

Weinbrenner, D.R., Barefoot, S.F. and Grinstead, D.A. (1997). Inhibition of yogurt starter cultures by jenseniin C, a Propionibacterium bacteriocin. *J. Dairy Sci.*, **80**: 1246.

West, C. and Warner, P.J. (1988). Plantacin B, a bacteriocin produced by *Lactobacillus plantarum* NCDO 1193. *FEMS Microbiol. Lett.*, **49**: 163.

Wheater, D.M. (1955). The characteristics of *Lactobacillus acidophilus*. *J. Gen. Microbiol.*, **12**: 123.

Wheater, D.M., Hirsch, A. and Mattick, A.T.R. (1952). Possible identification of "lactobacillin" with hydrogen peroxide produced by lactobacilli. *Nature*, **170**: 623.

Wood, B.J.B. and Holzapfel, W.H. (1995). The Genera of Lactic Acid Bacteria. In The Lactic Acid Bacteria, Vol. 2, Blackie Academic and Professional, London, UK.

Yakult Co. Ltd. (1970). Acidophilus drink. Japanese Patent, JP 5026/70. (Cited from *Dairy Sci. Abstr.*, **38**: 3278).

Yamamoto, N., Akino, A. and Takano, T. (1994). Antihypersensitive effects of different kinds of fermented milk in spontaneously hypersensitive rats. *Bio-Sci. Biotechnol. Biochem.*, **58**: 776.

Yang, D. and Woese, C.R. (1989). Phylogenetic structure of the Leuconostocs: an interesting case of a rapidly evolving organism. *Syst. Appl. Microbiol.*, **12**: 145.

Yoshioka, H.Y., Iseki, K. and Fujita, K. (1983). Development and differences of intestinal flora in the neonatal period in brest fed and bottle fed infants. *Pediatrics*, **72**: 317.

Yoshioka, Y., Iwasaki, T. and Kanauchi, T. (1968a). Study on the metabolism of *Lactobacillus bifidus*. Part-I. Sugar in the infant faeces and its utilization by strain *L. bifidus*. *J. Agric. Chem. Soc. Japan*, **42**: 651.

Yoshioka, Y., Iwasaki, T. and Kanauchi, T. (1968b). Study on the metabolism of *Lactobacillus bifidus*. Part-II. Bifidus factor activity of infant faeces and utilization of faeces sugar by *L. bifidus* var. *pennslyvanicus*. *J. Agric. Chem. Soc. Japan*, **42**: 659.

Yoshioka, M., Yoshioka, S., Tamura, Z. and Ohta, K. (1968c). Growth responses of *Bifidobacterium bifidus* to co-enzymes A, its precaursors and carrot extract. *Jap. J. Microbiol.*, **12**: 395.

Young, C.K. and Nelson, F.E. (1978). Survival of *Lactobacillus acidophilus* in 'sweet acidophilus milk' during refrigerated storage. *J. Food Prot.*, **41**: 248.

Yuan-Kun-Lee and Salminen, S. (1995). The coming age of probiotics. *Trends in Food Sci. Technol.*, **6**(7): 241.

Yuguchi, H. (1981). Utilization of fish meal hydrolysate for fermented milk product. III. Comparative study of fish meal hydrolysate, Brewer yeast extract on stimulation of growth of bifidobacteria. *Jpn. J. Dairy Food Sci.*, **30**: 145.

Ziajka, S., and Zibkowski, Z.(1986). Hydrolysed casein as a source of bifidus factor. *Die Nahrung,* **30:** 415.

Zibkowski, Z. (1981). Study of use of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in yoghurt production. *Technologia Zywnosci*, **16**: 3.

Zibkowski, Z. and Ziajka, S. (1986a). Technology of obtaining humanized infant formula with enhanced bifidogenic properties by adding carrot juice. *Indian J. Dairy Sci.*, **39**: 69.

Zibkowski, Z. and Ziajka, S. (1986b). Hydrolysed casein as a source of bifidus factor. *Die Nahrung,* **30:** 415.

Ziajka, S., Kisza, J. and Roskosz, D. (1974). Growth of *Lactobacillus bifidus* cultures in cow milk. *Zeszyty Naukowe Akademii Rolniczo Technicznejw Olsztynie,* **121**: 171.

Ziajka, S. and Zibkowski, Z. (1982). Characteristics of *Bifidobacterium* factor depending on the way of its obtaining. XX Int. Dairy Congr., Vol I, pp. 138.

Zilliken, F., Smith, P.N., Rose, C.S. and Gyorgy, P. (1954). Enzymatic synthesis of a growth factor for *Lactobacillus bifidus. J. Biol. Chem.*, **208**: 299.