Microencapsulation and supply of *Bifidobacterium lactis* DSM 10140 in fermented traditional African beverages

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I declare that the content of this thesis, except where otherwise stated, represents my own work. It is submitted for the Magister Technologiae: Food Technology to the Cape Technikon. It has not been submitted for any diploma, degree or examination to any other tertiary institution. Most of the work was carried out in the Department of Food Technology (Microbiology), Cape Technikon.

The opinions and conclusions drawn are my own and not necessarily that of the Cape Technikon.

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Shaun Kokott Date

Dedicated to Beverley, Allan, Darron and Kyle

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CONGRESS PRESENTATIONS / POSTERS

1. **The supply of viable *Bifidobacterium* as a probiotic in foods for human consumption.**
   Kokott SA, Brown LD, Taylor D, and J Grove.

   Presented at:

   - Cape Biotechnology Initiative Inaugural Conference, Kirstenbosch, Cape Town.
     November 2001

2. **Strain selection and delivery of the probiotic organism, *Bifidobacterium*, in traditional fermented beverages for the South African market.**
   Kokott SA, Brown LD, Uzzanu A, Abratt VA, Trinidade M, and SA Reid.

   Presented at:

   - 17th SAAFoST International Congress and Exhibition, CSIR Conference Centre, Pretoria.
     01-04/09/2003

3. **Use of traditional African fermented beverages as delivery vehicles for microencapsulated *Bifidobacterium lactis*.**
   Kokott SA, Abratt VA, Slatter P, and LD McMaster.

   Presented at:

   - Society for Applied Microbiology Summer Conference, Cork, Ireland.
     12-15/07/2004

ABSTRACT

Probiotic foods are intended to supply selected viable microorganisms, for example *Lactobacillus acidophilus* and *Bifidobacterium*, to consumers. These organisms, when consumed at the daily intake of 10^8, provide benefits beyond basic nutrition. Probiotic (AB) foods generally include fermented dairy products such as yoghurts and cheeses, targeted at the upmarket consumer. However, due to technical problems associated with the foods and the organism, viable *Bifidobacterium* rarely occur in AB foods.

The principle aims of this study were to develop a suitable delivery system for *Bifidobacterium* to the consumer, and to supply these living organisms in the affordable traditional fermented African beverages, amasi and mahewu. This would provide the benefits of probiotics to the rural African consumer, where malnutrition and gastrointestinal diseases occur. The organism selected for this study was *Bifidobacterium lactis* DSM 10140, commonly associated with AB starter cultures for yoghurts. The delivery system selected was microencapsulation of *B. lactis* using a mixture of the *generally recognised as safe* (GRAS) edible gums, gellan and xanthan. Supply vehicles for the microcapsules to the consumer were amasi and mahewu.

Prior to microencapsulation, rheological studies were undertaken to determine whether the gellan-xanthan gum mix would provide a suitable support matrix for microencapsulated *B. lactis*. This was done using a Paar Physica MCR 300 rotational rheometer with a cone plate 50-2 measuring system. Results indicated that the hydrated gellan-xanthan gum mix behaved as a non-Newtonian material, and the flow curve fitted well to the Herschel-Bulkley model. This demonstrated that the gel was a relatively viscous material with solid properties. The average yield stress of the gel was 1.515 Pa, indicating that the gel was stable, and at lower stresses would behave as a solid. The gel mix would be disrupted by shear stresses associated with mastication and peristalsis. The minimum viscosity of the gel was constant at temperatures

between 46°C – 61°C. It was concluded from these data that the gel was suitable for microencapsulation and that microcapsules should only be included in soft foods, which do not require chewing. Temperatures associated with microencapsulation, at minimum gel viscosities, were not lethal to *B. lactis*.

Bifidobacterium lactis cells were incubated under anaerobic conditions (4% H_2, 10% CO_2, and 86% N_2) at 37°C overnight in 250 ml Tryptone-Yeast-Glucose (TYG) broth, and grown to an OD_{600} 0.9 - 1.1. Cells were harvested and washed for microencapsulation using centrifugation.

Microencapsulation of the organism was done using a mono-axial extrusion technique together with a superposed airflow, by manually extruding the aqueous gum / cell mix through a 27.5 G bevelled needle, fitted on to a 10 ml syringe. The resultant microdroplets were hardened by free fall into 0.1 M $CaCl_2$ solution. Microcapsules were separated from the $CaCl_2$ solution by filtration through Whatman No.1 filter paper. All procedures were carried out in a laminar flow hood. Results indicated that the method of microencapsulation used in this study was successful. Using a concentrated inoculum of *B. lactis*, high numbers (log_{10} 11-12 $cfu.g^{-1}$) of bacteria were incorporated into the microcapsules. Therefore the daily intake would be provided by 0.1 g microcapsules.

The diameter and size distribution of microcapsules were determined by laser diffractometry. This showed a maximum microcapsule diameter of 2.22 mm with 50% (w/v) of the microcapsules having a diameter of < 0.637 mm. Although this represents a considerable size variation, this would not adversely affect mouthfeel of the beverages, as only 0.1 g microcapsules would be required to obtain at least 10^8 *B. lactis* in any volume of amasi or mahewu.

To enumerate immobilised viable *B. lactis*, two techniques were compared. These involved the use of either a pestle and mortar, or high power ultrasound (HPUS) (20 kHz, 750 W). Results showed that HPUS was superior to the pestle and mortar technique. A short exposure (15 s) to HPUS disrupted the matrix releasing all entrapped cfus, whereas when using the pestle and mortar

technique, cells remained partially entrapped in the gel. Therefore the pestle and mortar technique yielded lower cfu values than expected.

The survival of microencapsulated *B. lactis*, in 1 M sodium phosphate buffer, was studied as a possible means of supply of microcapsules to industry for incorporation into foods. Microcapsules were stored in the buffer for 21 days at either 4°C or 22°C. Results showed that cell viability was not significantly reduced ($p>0.05$) at either temperature after 21 days. Hence this form of storage could be used to deliver viable immobilised *B. lactis* to the food industry.

In order to assess the survival of immobilised *B. lactis* in the GIT, the microcapsules were incubated at 37°C over a period of 240 min in simulated gastric juice (SGJ) (pH 1.5). Viable counts were performed by sampling at regular intervals. A similar study was done in simulated bile and pancreatic juices (BPJ) (pH 6.5). In SGJ, it was demonstrated that there was a significant reduction (3 log cycles) ($p<0.05$) of free cells after 240 min. However, this trend was not noted for microencapsulated *B. lactis*. Therefore, the gellan-xanthan gel matrix protected *B. lactis* from the lethal effect of SGJ. In BPJ, no significant difference ($p>0.05$) was noted for surviving fractions of both immobilised and free *B. lactis*.

Commercial pasteurised amasi (pH 4.4) and mahewu (pH 3.5) were selected as the supply vehicles for the microencapsulated *B. lactis*. Known numbers of viable microencapsulated and free *B. lactis* cells were added to both beverages. For most samples, incubation was at either 4°C or 22°C for 21 days in the presence of atmospheric oxygen. In addition, free cells were incubated anaerobically at 22°C. As oxygen is limiting in the microcapsules, these were not incubated under anaerobic conditions. The survival / shelf-life studies of commercial amasi indicated no significant difference ($p>0.05$) in survival rate between immobilised and free *B. lactis* cells. The reduction noted for viable counts of immobilised or free *B. lactis* cells was approximately 1.5 log cycles. Even so, after 21 days viable immobilised *B. lactis* (10^{10} 0.1 g^{-1} microcapsules) remained in excess of the daily intake 10^8, whereas in the free *B. lactis* cells,

the viable count declined to 10^6 ml^{-1}. Statistical analyses showed that temperature or oxygen presence had little effect on the survival of both immobilised or free *B. lactis* cells ($p>0.05$). In mahewu, decline in viability of cells was observed for most samples. However microencapsulation enhanced cell survival at both 4°C and 22°C when compared to free cells. The decrease in viable *B. lactis* free cells occurred more rapidly (3 log cycles) in mahewu, than in amasi, at both 4°C and 22°C. Throughout the shelf-life studies it was apparent that viable *B. lactis* cell numbers did not increase. This was advantageous as metabolites associated with *B. lactis* growth would have adversely altered the taste of both amasi and mahewu.

Sensory evaluation of the traditional fermented African beverages, enriched with either viable immobilised or free *B. lactis*, was done in order to determine consumer response to the product. An analytically trained 12-member taste panel analysed the beverages for colour, texture, and taste. The triangle taste test procedure was used. No differences were detected with regard to texture, and colour of the fermented beverages containing immobilised *B. lactis*. However, in the fermented beverages containing free cells, a change in viscosity was noted. There was a significant difference ($p<0.05$) recorded in flavour for both amasi and mahewu containing free *B. lactis* cells. In the two fermented beverages enriched with immobilised cells, significant ($p<0.05$) flavour differences were detected in mahewu. However, this was not observed in the amasi samples containing immobilised *B. lactis*. Therefore, in order to retain the sensory properties of amasi, *B. lactis* should be supplied in microcapsules. In mahewu, although flavour differences noted were not unpleasant to the panellists, results from this study indicate that the use of commercial flavoured mahewu should be considered as a supply vehicle for microencapsulated *B. lactis*.

Overall, this study demonstrated that immobilisation of *B. lactis* in gellan-xanthan gum is possible. Microcapsules produced contained high numbers of viable *B. lactis*, and were suitable for incorporation into soft foods. The gel matrix significantly protected viable cells from harsh conditions associated with SGJ. Although the surviving fraction of immobilised cells,

when compared to free cells, was not improved in amasi samples, it is recommended that for technological reasons associated with production of amasi, microencapsulation should be used. In mahewu, microencapsulation enhanced *B. lactis* survival at both 4°C and 22°C. Therefore immobilisation of *B. lactis* in mahewu is necessary in order to maintain the daily intake. Immobilised *B. lactis* should be incorporated into both beverages after fermentation, and pasteurisation.

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GLOSSARY OF ABBREVIATIONS

%	percentage
α	alpha
β	beta
κ	kappa
τ_Y	yield stress
τ_0	shear stress
(IFN)-γ	γ-interferon
µl	microlitre
µm	micrometer
(TGF)-β	transforming growth factor
κ_y	fluid consistency index
AAD	antibiotic-associated disease
AB	*Lactobacillus acidophilus*; *Bifidobacterium*
ADI	acceptable daily intake
B. lactis.ml^{-1}	*Bifidobacterium lactis* per millilitre
BHI	brain-heart infusion
BPJ	bile and pancreatic juice
$CaCl_2$	calcium chloride
CAP	cellulose acetate phthalate
cfu	colony forming unit
CO_2	carbon dioxide
CoA	coenzyme A
cysteine HCl	L-cysteine hydrochloride monohydrate
Da	Dalton
day^{-1}	per day
E	x 10
F6PPK	fructose-6-phosphate phosphoketolase
FDA	United States Food and Drug Administration
FOS	fructooligosaccharides
G	gauge

g	gram
g^{-1}	per gram
xg	gravitational force
GALT	gut-associated lymphoid tissue
GC	guanine plus cytosine
GIT	gastrointestinal tract
GRAS	generally recognised as safe
h	hour
H_2	hydrogen
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HMG	hydroxy-methylglutaryl
IgA	immunoglobulin A
IgE	immunoglobulin E
IL	interleukin
IQ	2-amino-3-methylimidazo[4,5-f]quinoline
K_2HPO_4	di-potassium hydrogen orthophosphate
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen orthophosphate
kHz	kilohertz
kJ	kilojoules
kL	kilolitres
L	litre
LAB	lactic acid bacteria
LDL	low-density lipoprotein
m	metre
M	molar
MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
$MgCl_2.6H_2O$	magnesium chloride
min	minute
min^{-1}	per minute
$ml.min^{-1}$	millilitre per minute
$ml.s^{-1}$	millilitre per second
ml	millilitre

mm	millimetre
mM	millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
MRS	de Man, Rogosa and Sharpe
n	flow behaviour index
N_2	nitrogen
NaCl	sodium chloride
$NaHPO_4$	sodium hydrogen phosphate
Na_2HPO_4	di-sodium hydrogen phosphate
NaH_2PO_4	sodium di-hydrogen phosphate
NH_4Cl	ammonium chloride
°C	degrees Celsius
OD_{600}	optical density at 600 nanometres
ORF	open reading frame
pa	per annum
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PTFE	polytetrafluoroethylene
rpm	revolutions per minute
s	second
s^{-1}	per second
SA	South Africa
SGJ	simulated gastric juice
sp.	species
ssp.	sub-species
Th1	type 1 helper lymphocyte
Th2	type 2 helper lymphocyte
Th3	type 3 helper lymphocyte
Trp-P-2	3-amino-1-methyl-5H-pyrido[2,3-b]indole
TYG	tryptone yeast glucose
USA	United States of America
v/v	volume per volume
w/v	weight per volume

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CHAPTER 1

INTRODUCTION AND AIMS

The consumption of foodstuffs containing probiotic organisms is becoming increasingly popular. Probiotic microorganisms, generally bacteria, are classified as microorganisms which, when consumed in certain numbers, exert health benefits beyond nutrition (Lactic Acid Bacteria Industrial Platform / LABIP, cited by Guarner and Schaafsma 1998). An organism that is gaining credibility as a probiotic is the obligately anaerobic bacterium, *Bifidobacterium*. Health benefits include improvement in lactose digestion (Tianan *et al.* 1996), better gut transit time for foodstuffs (Grimaud *et al.* 1994), reduction in the risk of cancer development (Orrhage *et al.* 1994; Grill *et al.* 1995), positive immunomodulating effects (Schiffrin *et al.* 1995), contribution to nutrition (Roberfroid *et al.* 1995), and a reduction in diarrhoea (Cremonini *et al.* 2002; Heyman and Menard 2002). *Bifidobacterium* is a normal commensal in both the infant and adult gastrointestinal tract (GIT) (stomach, large intestine, small intestine, and colon) constituting 95% and 25% of the normal gut microflora respectively (Yildirim and Johnson 1998).

The significance and development of probiotic foods, and their use as alternatives to antibiotics, is predicted to increase in the future (Doyle 2003; Kroger 2003). At present, *Bifidobacterium* is only available in South African foods as freeze-dried cells in AB yoghurts. In many of these bio-yoghurts the colony forming unit (cfu) value of *Bifidobacterium* is below the daily intake of 10^8, or absent (Lourens-Hattingh and Viljoen 2002). Similar trends have recently been reported in Europe (Temmerman *et al.* 2003). The use of spray-drying and freeze-drying of bifidobacteria for incorporation into foods for human consumption has shown that survival is dependant on both *Bifidobacterium* sp. and the carrier medium used for microencapsulation (Lian *et al.* 2002). Improvements in these technologies have been reported (Siuta-Cruce and Goulet 2001). However, bacteria supplied as either spray- or freeze-dried cells require time to rehydrate and reconstitute in the human host (Klaver *et al.* 1993; Lian *et al.* 2002).

Many bacteria such as *Bifidobacterium* also undergo injury during both of these processes, and as a result require time for repair in a suitable recovery medium (Ray 2001). The human GIT is not an ideal environment for resuscitation of these injured bacteria, hence it is likely that *Bifidobacterium*, present as spray- or freeze-dried cells in probiotic foods, do not respond optimally under conditions associated with the GIT.

An alternate means of delivering viable bifidobacteria directly to the GIT is through the use of microcapsules. Unlike the spray- and freeze-drying processes where the dormant microorganism is delivered directly into the environment, the capsule matrix surrounding the bacteria protects the living organisms from the external milieu, including both the food, and the gastric juices present in the stomach. Once in the colon, it is hypothesised that capsules break down to release the living organisms over a period of time (Sun and Griffiths 2000; Krasaekoopt *et al.* 2003). Microencapsulation of probiotic bifidobacteria is reported in some cases to improve survival of the organism in various foods (Krasaekoopt *et al.* 2003).

In South Africa (SA), AB yoghurts are expensive, and are not consumed by the rural population where malnutrition and gastrointestinal diseases are common. It would, therefore, be advantageous to produce affordable beverages, enriched with living immobilised bifidobacteria, and thereby expose the SA rural population to the benefits of probiotic foods. Two traditional African foods considered suitable for this type of supplementation were amasi, a soured milk product, and mahewu, a fermented, non-alcoholic maize beverage. Commercial amasi is made using *Lactococcus lactis* ssp. *lactis*, and *L. lactis* ssp. *cremoris* (Beukes *et al.* 2001). Mahewu is produced industrially, using *Lactobacillus bulgaricus* var *delbrueckii* or *Lactobacillus brevis*. At times, various fruit flavourings are added after the fermentation (Schweigart and Follingham 1963; Edwards 2003). After fermentation the starter cultures in these beverages are inactivated by pasteurisation prior to distribution and consumption. This would eliminate any potential antagonism by viable starter cultures to *Bifidobacterium*.

The annual consumption of these beverages by all age groups of black consumers is considerable; 104 000 kL amasi in 2003, and for mahewu, 77 000 kL consumed in 2001 by black miners alone (Edwards 2003; van Geems *et al.* 2003). Demand for these beverages often exceeds supply; hence commercial amasi and mahewu represent ideal vehicles for the delivery of viable probiotics.

The aims of this study were, therefore, to: -

1. Undertake rheological studies on a combination of edible gums, gellan and xanthan to:
   a. Describe the flow behaviour of the gum, by means of viscosity and yield stress, described by the Herschel-Bulkley model ($\tau_0 = \tau_Y - \kappa_y^{-n}$).
   b. Investigate the effect of temperature on the viscosity of the gum.
   c. Compare gel viscosity obtained from the temperature sweep to viscosity of time sweep.
2. Microencapsulate viable *Bifidobacterium lactis* DSM 10140 using a mixture of gellan and xanthan gum, for delivery of the microorganisms to the consumer.
3. Estimate the size range of the microcapsules important for influencing flow characteristics, and texture / mouthfeel of the beverages enriched with immobilised *B. lactis*, as well as viability of *B. lactis*.
4. Develop an enumeration technique to determine the number of viable bacteria in the microcapsules.
5. Study the survival of microencapsulated *B. lactis* in sodium phosphate buffer as a possible means of supply of microcapsules to industry, for incorporation into foods.
6. Investigate the survival of microencapsulated viable *B. lactis*, as opposed to free *B. lactis* cells, *in vitro* in both simulated gastric, and bile and pancreatic juices.
7. Compare the survival of microencapsulated viable *B. lactis*, with free *B. lactis* cells, in the traditional fermented African beverages, amasi and mahewu, as possible supply vehicles of microcapsules to rural and urban populations in SA.

8. Undertake a sensory evaluation of the traditional fermented African beverages, enriched with either microencapsulated *B. lactis* or free *B. lactis* cells, to gauge consumer response to these beverages.

CHAPTER 2

LITERATURE REVIEW

2.1 Microencapsulation:

Microencapsulation is a process by which individual units of solid, liquid, or gas are incoherently enclosed in a shell of inert polymeric material, which are known as microcapsules with diameters ranging from several nanometres to several millimetres (Franjione and Vasishtha 2001; Ukumori 2001).

Two important features of microcapsules are that microcapsules preserve / protect the core materials from contamination, impairment or alteration (such that they cannot react with other materials), and that core materials can be released, usually either by breakage of the shell material under pressure or heat, or by slow diffusion of the core materials through the shell wall (Ukumori 2001).

In the food industry, microencapsulation has been used to provide flavour, odour and colour of foods (Ukumori 2001). In addition, Rao *et al.* (1989) studied the protection of viable probiotic bacteria by microencapsulation in hydrocolloid microspheres. The aims were to improve both viability of the bacteria in food products for domestic consumption and subsequent survival of the bacteria in the intestinal tract after ingestion. Immobilisation of bacterial cells has been used in dairy fermentations to protect the bacteria from the lactic acid present in the fermented product (Champagne *et al.* 1992a; Champagne *et al.* 1992b; Champagne *et al.* 1993; Champagne *et al.* 1994). The addition of encapsulated probiotic microorganisms has also been used for continuous inoculation of milk for yoghurt manufacture (Prevost and Divies 1988) and for the fermentation of whey (Audet *et al.* 1989).

The advantages of microencapsulation of bacterial cells include: cell protection from bacteriophages (Steenson *et al.* 1987), increased cell survival during freezing and freeze drying (Kearney *et al.* 1990; Sheu and Marshall 1993; Kim and Yoon 1995; Sung 1997), and improved stability during storage (Kim *et al.* 1988; Reuter 1990; Kebary *et al.* 1998).

Microcapsules can be divided into three groups: solid spheres, coated spheres, and hollow spheres, and are produced using a variety of microencapsulation techniques (Huebner and Buccholz 1999). In the study under review, uncoated spheres were produced, consisting of viable bacteria immobilised in edible gums.

2.1.1 Techniques of microcapsule production:

There are a variety of techniques, many of which are based on a simple dropping technique. These techniques utilise a viscous liquid together with the material to be encapsulated, with a low flow velocity under pressure, breaking into individual drops when ejected from a needle. As the velocity of the viscous liquid is increased, so droplet formation increases until the maximum velocity is reached and the liquid begins to form a continuous jet (Huebner and Buccholz 1999). The method used in this study was simple dropping with a superposed air jet.

Factors influencing drop size include the force of gravity removing the drop from the needle tip, the interfacial tension of the resisting product and tip perimeter, and the viscosity and speed of flow of the liquid. Upon formation of the droplets, the droplets fall freely into a calcium chloride solution, forming solid spherical microcapsules (Refer Figure 1).

Superposing an air jet with the drop forming process significantly reduces the droplet size (Huebner and Buccholz 1999). This method is commonly used to synthesise microcapsules using hydrocolloids as the matrix (King 1995). A sterile airstream is produced such that it is

concentric to the droplet-forming needle. The exocentric air sleeve positioning combined with a bevelled droplet-forming needle is crucial for the production of very small droplets (Refer Figure 1). The distal end of the needle may be bevelled at an angle of 15° – 45° that faces the central axis of the sleeve for the airstream. This is known as the air knife technique. Small droplets are produced and have been used to successfully microencapsulate viable probiotic bacteria for incorporation into probiotic foods (Huebner and Buccholz 1999).

Aqueous solutions of edible gum powders are produced using heat to dissolve the gum whilst simultaneously producing sterile moulten gum. The moulten gum, together with bacteria of interest, is placed into a hypodermic syringe fitted with a bevelled needle. By extruding the gum into a calcium chloride solution, microcapsules are generated with a diameter of 2 mm - 3 mm (Park and Chang 2000; Krasaekoopt *et al.* 2003). This method is simple, low cost, and is associated with gentle formulation conditions that ensure high retention of cell viability (Krasaekoopt *et al.* 2003).

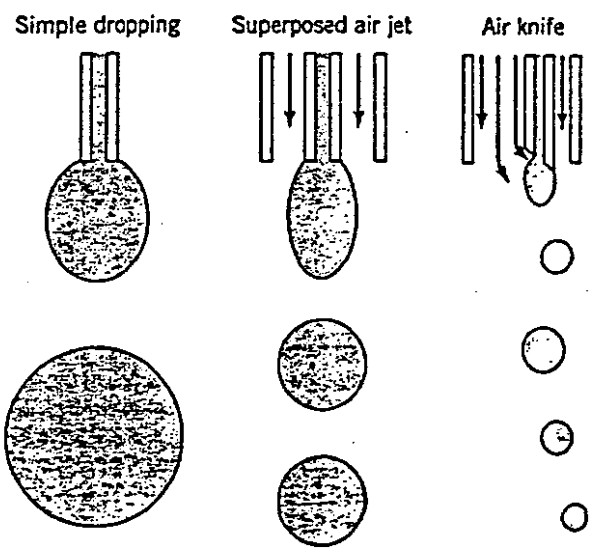

Figure 1. Schematic capsule preparation methods by mono-axial extrusion technologies based on simple dropping. (Adapted from Huebner and Buccholz 1999)

The standardisation of microencapsulation techniques used for probiotics to provide uniform microcapsules is difficult. Hence other techniques have been used and are summarised in Table 1. Authors report varying success.

Table 1. *Microencapsulation techniques used for probiotics*

Technique	**Reference**
Coacervation / Emulsion / Interfacial polymerization	Lacroix *et al.* 1990; Audet *et* al. 1992; Huebner and Buccholz 1999; Park and Chang 2000; Krasaekoopt *et al.* 2003
Pregel dissolving two-step method	Park and Chang 2000
Liquid droplet forming one-step method	Park and Chang 2000
Electrostatic extrusion	Huebner and Buccholz 1999
Spinning disc	Huebner and Buccholz 1999; Franjione and Vasishtha 2001; Heinzen 2002
Rotating cylinder	Schlameus 1995
Dipping jet	Huebner and Buccholz 1999
Vibrating jet breakage	Huebner and Buccholz 1999
Mechanical cutting	Huebner and Buccholz 1999; Schwinger *et al.* 2002
Spray-drying	Jackson and Lee 1991; Mauriello *et al.* 1999; Franjione and Vasishtha 2001; Lian *et al.* 2002; Favaro-Trindade and Grosso 2002
Spray-freezing into liquid (SFL)	Rogers *et al.* 2002

2.1.2 The coating / support material of microcapsules:

There are many substances available to produce the shell of a microcapsule. The supporting material of a microcapsule, often a hydrocolloid, can be made flexible, brittle, soft, hard, or solid by altering the thickness of the shell material chosen, or selecting a blending additive. The variety of coating materials, mainly natural macromolecules, used to produce microcapsules is shown in Table 2. These materials are used either as pure or mixed preparations. Dyes and pigments may be mixed into the shell materials to colour them (Ukumori 2001).

Table 2. Natural GRAS macromolecules used as coating (supporting) materials to produce microcapsules

Specific types of coating materials (hydrocolloids)	**References**
Carrageenan (kappa, ioda, lambda)	Audet *et al.* 1988; Thomas 1992; Adhikari *et al.* 2003; Krasaekoopt *et al.* 2003
Chitosan	Krasaekoopt *et al.* 2003
Gelatine	Mark *et al.* 1987; Poppe 1992; King 1995; Krasaekoopt *et al.* 2003
Alginate	Eikmeier and Rehm 1987; Onsoyen 1992; Sheu and Marshall 1993; Ellenton 1998; Park and Chang 2000; Ravula and Shah 2000; Schwinger *et al.* 2002; Truelstrup Hansen *et al.* 2002; Krasaekoopt *et al.* 2003
Gellan	Norton and Lacroix 1990; Sanderson 1990; Sun and Griffiths 2000
Xanthan	Sun and Griffiths 2000
Lipids	Ukumori 2001
Inorganic materials	Ukumori 2001

Coating materials, other than those of natural origin may also be used to encapsulate core materials (Refer Table 3).

Table 3. Alternate coating (supporting) materials used to produce microcapsules (Adapted from Ukumori 2001)

Class of coating material	**Specific types of coating materials**
Semi-synthetic macromolecules	Carboxymethylcellulose, ethylcellulose, methylcellulose, nitro-cellulose, acetylcellulose, cellulose acetate-phthalate, or cellulose acetate-butylate-phthalate
Synthetic macromolecules	Polyvinyl alcohol, nylon, polyurethane, polyester, epoxy, and melaninformain

In order to supply microencapsulated viable probiotics in foods for human consumption, the support substances must be *generally recognised as safe* (GRAS). In addition, these should not alter the texture or flavour of the food. Examples of suitable GRAS hydrocolloids are gums, of which many are available (Refer Table 2). In this study, two gums, gellan and xanthan, were utilised.

2.1.2.1 Gellan gum:

This name is given to the extracellular GRAS polysaccharide produced by the bacterium *Pseudomonas elodea.* The polysaccharide is produced in either a substituted or an unsubstituted manner (Refer Figures 1 and 2), and forms a gel at 0.75% w/v. The substituted form produces a soft, elastic gel, while the unsubstituted form produces a hard, brittle gel. Due to these properties, gellan has many uses in the food industry (Gibson 1992).

The chemical composition of this gum is a linear, anionic heteropolysaccharide with a molecular weight of approximately 0.5×10^6 Daltons (Da). It is composed of tetrasaccharide repeat units

(Refer Figure 2) consisting of 1,3-β-D-glucose, 1,4-β-D-glucuronic acid, 1,4-β-D-glucose and 1,4-α-L-rhamnose.

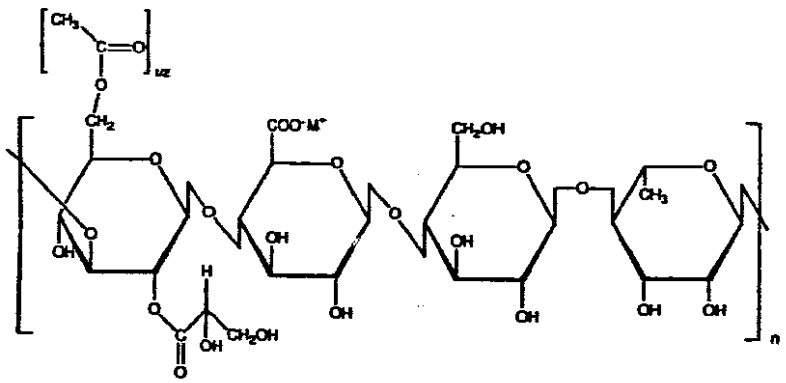

Figure 2. Substituted tetrasaccharide repeating unit of gellan gum (soft gel form).
(Adapted from Gibson 1992)

The polymer secreted by the microorganism contains approximately 1.5 acyl substituents per tetrasaccharide repeating unit, which have been identified as an L-glyceric ester on C-2 of the 3-linked D-glucose and an acetic ester on C-6 of the same glucose residue (Gibson 1992). The presence of these substituents impedes chain association and accounts for the change in gel texture brought about by de-esterification.

The de-esterified product is a polymer with a well-defined, unsubstituted, tetrasaccharide repeating unit (Refer Figure 3). In the solid state the molecule forms a parallel, half-staggered intertwined double helix in which each polysaccharide chain is a left-handed, threefold helix (Gibson 1992).

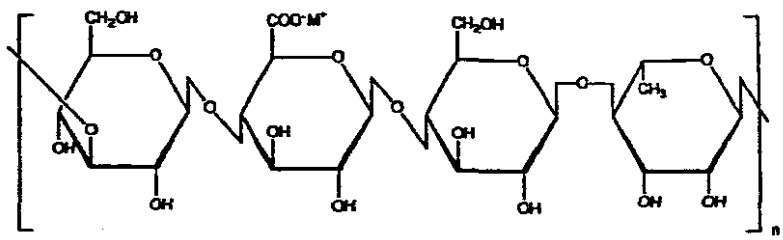

Figure 3. Unsubstituted tetrasaccharide repeating unit of gellan gum (hard / brittle gel form).
(Adapted from Gibson 1992)

2.1.2.2 Xanthan gum:

This GRAS gum was produced in the early 1960's in the United States of America (USA). It was isolated by the United States Food and Drug Administration (FDA) and labelled B-1459 (xanthan gum). It is produced by the fermentation of *Xanthomonas campestris* NRRL B-1459 (Urlacher and Dalbe 1992).

Xanthan gum has many desirable properties for the food industry, which include the production from, and subsequent availability of external factors, and consistent quality of the texturising agent.

Xanthan gum was approved for use in the USA in 1969 and in Europe in 1974; it is registered in the European list of permitted thickening and gelling agents for foods under the E-number E415 with a non-specified acceptable daily intake (ADI).

The primary structure of xanthan is a backbone comprised of 1,4-linked β-D-glucose (similar to cellulose) with side chains containing two

mannose and one glucuronic acid (Refer Figure 4). These side chains constitute about 60% of the molecule giving xanthan gum many of its unique properties. Urlacher and Dalbe (1992) showed that half of the terminal mannose units also carry a pyruvic acid residue.

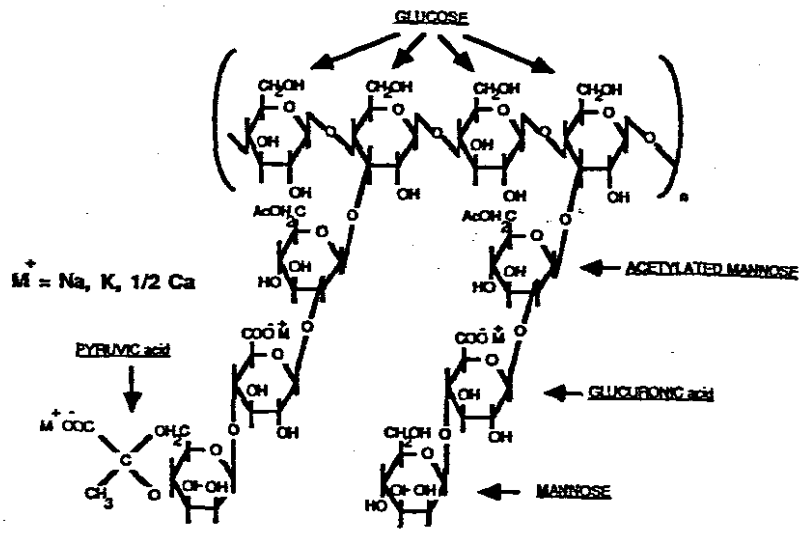

Figure 4. Primary structure of xanthan gum.
(*Adapted from Urlacher and Dalbe 1992*)

Xanthan gum has a molecular weight of 2.5 x 10^6 Da with a low polydispersity. Due to the side chains, the polymer hydrates easily, even in cold water.

2.1.3 Combinations of generally recognised as safe (GRAS) hydrocolloids:

Various studies on the microencapsulation of viable probiotics have used combinations of GRAS gums to create specialised hydrocolloid matrices in order to strengthen the outer wall of the microcapsule for incorporation into foods.

Using emulsion / coacervation microencapsulation techniques, chitosan and gelatine were used in combination to form microcapsules (Shu and Zhu 2001). Coacervation, however, may prove to be unsuitable for food

product development because the residual oil, emulsifier and surfactant in the encapsulated material are detrimental to texture and organoleptic characteristics. In addition, these substances can be toxic to live bacterial cells, and may interact with sensitive food components (Godward 2000).

Sheu and Marshall (1993) used a combination of 2% calcium alginate and 2% Hi-maize resistant starch. However, alginate is not acid resistant, and it has been reported that these spheres have a tendency to shrink, and exhibit a notable decrease in mechanical strength during lactic acid fermentation (Eikmeier and Rehm 1987; Roy *et al.* 1987; Ellenton 1998), and in simulated gastric acid suspension (Sun and Griffiths 2000). Acid resistance of microcapsules is important to protect the encapsulated material from the external environment in both foods (for example, lactic acid present in yoghurt), and the GIT (e.g. gastric acid present in the stomach). Therefore, although alginate is economically favourable to use, this hydrocolloid is no longer a popular choice for microencapsulation of probiotics.

Spheres synthesised from κ-carrageenan-locust bean gum gels (Krasaekoopt *et al.* 2003) have been used as they exhibit acid tolerance. However, the formation of κ-carrageenan-locust bean gum spheres requires the use of potassium ions for gelation, which can damage the cells of *Bifidobacterium* (Paquin *et al.* 1990). In addition, it is not recommended to use potassium ions in large amounts in the human diet, as these ions maintain the electrolyte equilibrium of the body fluid (Sun and Griffiths 2000).

Other GRAS hydrocolloids that have been used in combination include: gelatine and gellan gum (King 1995). Although both these gums carry nett negative charges and repel one another, when the pH is adjusted below the isoelectric point of gelatine, the nett charge on gelatine becomes positive, causing an interaction with the negatively charged gellan gum to form stable microcapsules (King 1995).

Due to the low entrapment stability of certain gels, such as alginate, in the presence of chelating agents, such as phosphate, lactate, and citrate, some combinations of GRAS gum capsules use a second / third hydrocolloid / polymer to form an additional membrane around the existing microcapsule membrane. The chelating agents share an affinity for calcium and destabilise the first microcapsule gel layer (Krasaekoopt *et al.* 2003). Thus *in vivo*, it is hypothesised that bacterial cell release occurs from the microcapsules (Krasaekoopt *et al.* 2003). Coated microcapsules, however, can prevent premature bacterial cell release, and are reported to show increased mechanical and chemical stability (Krasaekoopt *et al.* 2003).

The use of a combination of two gums such as gellan and xanthan, used in this study, presents an attractive option for microencapsulation of live probiotics. These two gums are acid and bile resistant, protecting the bacterial cells from acid and bile injury in the GIT (Sun and Griffiths 2000). The gum mix is solidified by calcium ions (Ca^{2+}); thus calcium in fermented milk products could assist in maintaining stability of the microcapsules in these products (Adhikari *et al.* 2003). Gellan and xanthan can be formulated so that there is timed released breakdown in the colon, thereby releasing the probiotic bacteria (Sun and Griffiths 2000).

2.1.4 Release of core materials from microcapsules:

Various methods have been used to release probiotic bacterial cells from microcapsules. Factors controlling the means by which these core materials are released from microcapsules are the (Ukumori 2001):

- ratio of the core materials to the shell materials
- components and properties of the shell materials
- size of the microcapsules
- suspending medium

In microencapsulated probiotics, using edible gums as the support material, it has been shown that the matrix of the microcapsule is weakened by acetic, lactic, and hydrochloric (HCl) acids (Sun and Griffiths 2000; Adhikari *et al.* 2003; Krasaekoopt *et al.* 2003). Regarding *in situ* release of probiotics in the human colon little is known. However, Sun and Griffiths (2000) speculate that once the microencapsulated bifidobacteria leave the human stomach, the low pH around the microcapsules will be neutralised by the intestinal juice in the human intestine. In the intestine the microcapsules might be softened at the higher pH, and broken by peristaltic movement of the small intestine, causing bifidobacteria to be released from the microcapsules.

2.2 Microencapsulation of probiotic bacteria (including *Bifidobacterium* sp.):

The ability of candidate probiotic microorganisms to survive and multiply in the host GIT influences their probiotic potential. These bacteria should be metabolically stable, survive passage through the upper digestive tract in large numbers, and once in the colon of the host, exert a beneficial effect (Krasaekoopt *et al.* 2003). However, Lankaputhra and Shah (1995) claim that survival of *Lactobacillus acidophilus* and *Bifidobacterium* sp., as free cells, is low in the presence of gastric acid and bile salts.

Many studies have shown a low viability of free probiotic cells in yoghurt and other fermented milk products (Krasaekoopt *et al.* 2003; Sun and Griffiths 2000). Ravula and Shah (1998) also reported that probiotic bacteria, at 10^{10} cells ml^{-1}, do not survive in frozen fermented dairy desserts. In that study, it was reported that the bacterial count decreased by 5-6 log cycles within 8-12 weeks of storage at –18°C. Similar studies concurred (Krasaekoopt *et al.* 2003).

In the food and pharmaceutical industries, microencapsulation of probiotic cultures is a current practice for extending their viability. Several techniques are used, including spray drying, freeze drying, and fluidised bed drying, for encapsulating the bacterial cultures and converting them into a concentrated powdered form. Bacteria encapsulated by these latter techniques are released into the product, and are not protected from the either the product environment or subsequent passage through the stomach / intestinal tract.

The two most common methods of hydrocolloid encapsulation of viable probiotic bacteria are extrusion (droplet method) and emulsion / coacervation (two-phase method). Both of these techniques can increase the survival rate of probiotic bacteria in foods by 80% - 95% (Sun and Griffiths 2000; Krasaekoopt *et al.* 2003). For the purpose of this study however, only the extrusion technique is reviewed, as this technique has advantages over the emulsion / coacervation method (Refer 2.1.3).

The extrusion method produces entrapped, rather than encapsulated core material. However, encapsulation can be achieved through co-extrusion methods or by dropping the solid gel spheres into a bath of coating material, which reacts on the surface of the spheres (Krasaekoopt *et al.* 2003). This method can prove to be difficult for large-scale production due to the slow formation of the spheres (Huebner and Buccholz 1999).

2.2.1 Advantages of microencapsulated probiotics:

Microencapsulation of probiotics is advantageous to the food industry. Firstly, it protects and enhances survival of bacteria in foods. Microencapsulation allows entrapped probiotic microorganisms to be incorporated into dairy products such as yoghurt, cheese and frozen milk products. Sheu and Marshall (1993) reported that around 40% more lactobacilli survived in frozen ice cream when entrapped in calcium alginate spheres, than did free cells. Encapsulation also protected the bacteria in batch-frozen and continuously frozen ice-milk mixes (Krasaekoopt *et al.* 2003). The encapsulation of bifidobacteria was shown by Kebary *et al.* (1998) to significantly improve survival, compared to free cells, throughout storage from 43% - 44% to 50% - 60% in frozen dairy products.

Secondly, Krasaekoopt *et al.* (2003) showed that microencapsulated *B. pseudolongum* improved survival in a simulated gastric environment when compared to free viable microorganisms. These results were reported by Truelstrup Hansen *et al.* (2002), and Sun and Griffiths (2000).

Thirdly, microencapsulation of probiotics has technological advantages for the food industry. Krasaekoopt *et al.* (2003) found that the continuous manufacture of yoghurt with encapsulated microorganisms (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) was more complex than the traditional method, but advantages were noted. For example, a product with constant characteristics was obtained (detail not given) because the residence time, acidity and continuous inoculation of milk with a stable bacilli / cocci ratio was controlled at a desired pH. Encapsulated probiotic bacteria have been shown to reduce the incubation time for fresh fermented cheese production by 50% (Krasaekoopt *et al.* 2003), and cream fermentation by 60%, respectively (Krasaekoopt *et al.* 2003).

2.3 Probiotics including *Bifidobacterium* sp.:

The word 'probiotic' meaning 'for life' is derived from the Greek language, and was first used by Lilly and Stillwell (1965) to describe the growth-stimulating effect that one microorganism has on another.

Further changes in the use of this term introduced the concept of a live microbial feed supplement that improved the intestinal microbial balance of the host (Fuller 1989). The word 'probiotic' refers to the "mono- or mixed cultures of live beneficial microorganisms (usually bifidobacteria, lactobacilli and streptococci) which, when consumed, beneficially affect the host by improving the properties of the indigenous microflora" (Holtzapfel *et. al.* 1998). More recently, probiotics have been defined as, "oral probiotics are living microorganisms, which, upon ingestion in certain numbers, exert health benefits, to the human host, beyond inherent basic nutrition" (Lactic Acid Bacteria Industrial Platform / LABIP cited by Guarner and Schaafsma 1998).

Live probiotics are considered to be beneficial to the human host when consumed at a daily intake of $10^8 - 10^9$ living bacteria (Kurmann and Rasic 1991; Hull *et al.* 1992; Krasaekoopt *et al.* 2003).

2.3.1 Selecting an organism as a probiotic:

After ingestion of food, many bacterial species are killed by the acidic conditions in the stomach, as well as by bile juice released from the common bile duct into the duodenum. On reaching the colon, few ingested bacteria remain alive, where indigenous microflora predominate and displace any allochthonous bacterial species immediately (Gilliland 1989). Surviving allochthonous bacteria in the GIT are thought to be predominantly probiotic bacteria that aid digestion of ingested foods in the human body. This is done by enzyme production (such as β-galactosidase, bile hydrolase, protease, and lipase), and prevention of

adherence of pathogenic bacteria to the GIT. Preventing adherence by pathogens could be achieved either directly through a barrier effect, or indirectly, through the stimulation of mucin production. A requirement of probiotic bacteria is an ability to multiply on the surface of the gastrointestinal tract (Gilliland 1989).

For a bacterium to be considered as a viable candidate for use as a probiotic microorganism, it should be screened in accordance with recommended safety, functional and technological aspects (Saarela *et al.* 2000).

2.3.1.1 Safety aspects:

Safety aspects desirable in a probiotic organism should include the following (Saarela *et al.* 2000):

1. strains for human use must be of human origin;
2. isolation must be from a healthy human GIT;
3. non-pathogenic and non-toxic to humans;
4. no association with diseases such as infective endocarditis or other GIT-disorders;
5. no deconjugation of bile salts (bile salt deconjugation or dehydroxylation has been postulated to be potentially *detrimental in the small bowel); and*
6. no transmissible antibiotic resistance genes must be associated with the candidate probiotic

2.3.1.2 Functional requirements:

Functional requirements of probiotics include the following (Saarela *et al.* 2000):

1. adherence to epithelial surfaces and persistence in the human GIT;
2. immunostimulation;
3. antagonistic activity to pathogens, for example *Helicobacter pylori*, *Salmonella* sp., *Listeria monocytogenes* and *Clostridium difficile*;
4. antimutagenic and anticarcinogenic properties;
5. tolerance to acidity associated with human gastric juice (in the stomach); and
6. bile tolerance (an important property for survival in the duodenum and jejunum)

2.3.1.3 Technological aspects:

Technological aspects of a probiotic include (Saarela *et al.* 2000):

1. stability in the finished product particularly during storage;
2. viability during food processing;
3. retention of metabolic activity;
4. good sensory / organoleptic properties;
5. oxygen tolerance in the final product;
6. phage resistance; and
7. maintenance of viability in the appropriate food delivery system

In addition, in South Africa, foodstuffs containing these viable organisms should adhere to the requirements proposed for probiotics (recently changed from a daily intake of 10^6 to 10^8; Vanwyk, J. pers. comm. 2004) as required in the draft Labelling and Advertising regulation (1055; August 2002) (*Foodstuffs, Cosmetics and Disinfectants Act, No. 54*; 1972) (Refer Appendix A).

2.3.2 Potential and established health benefits: possible therapeutic applications of probiotic bacteria:

Probiotic bacteria (*Lactobacillus* and *Bifidobacterium*) are claimed to exert therapeutic effects in humans. Many claimed human health benefits have been derived from studies using rats. As the digestive system of rats is different from that of humans, many of these claims are spurious. Hence these studies are not considered in this review, and only those involving both humans and probiotics, with an emphasis on *Bifidobacterium*, will be reviewed. These include: lactose intolerance, strengthening of specific and non-specific immune responses, anticarcinogenic properties, hypercholesterolemic effect, treatment of diarrhoea, indigenous microflora and pathogens, and food allergy.

2.3.3 Bifidobacteria:

2.3.3.1 The organism:

Henry Tissier of the Pasteur Institute, Paris, France, working on infants with and without diarrhoea, isolated and discovered Bifidobacteria in 1899-1900. He named his isolates as *Bacillus bifidus communis* or *Bacillus bifidus*. These pleomorphic Gram-positive rods were found by Tissier to be predominant in the intestinal lumen of breast-fed infants. Later these organisms were reclassified as *Bifidobacterium*.

The genus name is derived from the observation that these rods are pleomorphic, often existing in a Y-shaped or 'bifid' form (Arunachalam 1999), although V- and X-shapes are common (Ballongue 1998).

They have a characteristic morphology, physiology, biochemical characteristics, cell-wall constituents, and DNA-base composition where the GC (guanine plus cytosine) content is 55-67 M%.

i. Morphology:

The bacteria of the genus *Bifidobacterium* are non-spore-forming and non-motile rods. While these rods are usually concave with their ends swollen to form dumbell-like structures, it is not uncommon to find rounded coccal shapes as well as long or short bacilli of different widths (Rasic and Kurmann 1983; Scardovi 1986; Ballongue 1998).

Gram-positive staining shows the frequently irregular distribution of chromatin in these bacteria, which often collects in the dumbell-like structures (Ballongue 1998). The irregular distribution of chromatin, however, does not reflect degeneration, as these bacterial forms can generate the initial forms once again (Ballongue 1998).

It is thought that the medium in which the organism is cultured is responsible for the pleomorphic shapes noted in *Bifidobacterium*. The following growth medium constituents influence morphology: N-acetylglucosamine, which is involved in cell wall peptidoglycan synthesis (Ballongue 1998), concentration / presence of certain amino acids (alanine, aspartic acid, glutamic acid, and serine) (Ballongue 1998), and calcium ions (Ca^{2+}) (Ballongue 1998).

ii. Physiology:

a. Oxygen requirements:

Bifidobacteria are anaerobic microorganisms, and will only grow in the absence of oxygen (Rasic and Kurmann 1983; Scardovi 1986; Ballongue 1998; Arunachalam 1999). However, oxygen tolerance has been reported for certain species, and this property is influenced by culture medium (Ballongue 1998).

b. Optimum temperature and pH:

The optimum temperature for the development of *Bifidobacteria* in humans is between 36°C and 38°C, while in other animals it is slightly higher at between 41°C and 43°C. At times optimum temperature may reach 46.5°C (Ballongue 1998). Growth does not occur below 20°C. Above 60°C, *B. bifidum* can be inactivated (Rasic and Kurmann 1983).

The optimum pH for growth is between pH 6.5 and 7.0. It has been reported that no growth occurs below pH 5.0 or above pH 8.0 (Scardovi 1986).

iii. Biochemical characteristics:

Major products of glucose fermentation of the genus *Bifidobacterium,* are acetate and L(+)-lactate. These products are produced in the molar ratio of 3:2 by free cells. In *Bifidobacterium* immobilised in microcapsules, where substrates for acetate and lactate are absent, neither acid is produced (Rasic and Kurmann 1983; Scardovi 1986).

The splitting of pyruvate usually produces additional acetic and formic acids, while fermentation with rhamnose, sorbose, adonitol, dulcitol, erythritol and glycerol as sole C-sources, does not produce any organic acids (i.e. the carbohydrate source influences the end-products) (Rasic and Kurmann 1983).

In *Bifidobacterium lactis*, gas (CO_2) is not produced from glucose, while catalase and nitrate reduction tests are negative. However, when the bacteria are grown in the presence of lysed red blood cells, a positive catalase reaction is noted for the organism. *Bifidobacterium* is unable to form indole and does not liquefy gelatine. Fructose-6-phosphate phosphoketolase (F6PPK) is present in cellular

extracts prepared from *Bifidobacterium* and is unique to this genus (Rasic and Kurmann 1983; Scardovi 1986; Ballongue 1998). This enzyme (F6PPK) is responsible for the production of acetic and lactic acid by *Bifidobacterium*. The presence of F6PPK has been used to separate *Bifidobacterium* from other morphologically similar bacteria, e.g. *Lactobacillus*, *Arthrobacter*, *Propionibacterium*, *Corynebacteruim*, and Actinomycetaceae.

Besides the production of the L(+)-lactic acid and acetic acid (2:3) mixture, functional probiotic characteristics of bifidobacteria are enhanced by the additional production of extracellular vitamins (B_1, B_6, B_9 and B_{12}), and amino acids (alanine, valine, aspartic acid, and threonine), which are all completely metabolised by humans (Rasic 1983; Ballongue 1998).

In 2002, Schell *et al.*, determined the 2.26 Mb genome sequence of an infant-derived strain of *Bifidobacterium longum*, and identified 1 730 possible coding sequences organised in the 60%-GC circular chromosome. Bioinformatic analysis showed that several physiological traits could partially explain the successful adaptation of *Bifidobacterium* to the colon.

There are a large number of open reading frames (ORFs) that appear to be specialised for the production of proteins that catabolise a variety of oligosaccharides, e.g. the degradation of plant polymers, such as fructooligosaccharides (FOS), or host-derived glycoproteins and glycoconjugates (Schell *et al.* 2002). This ability to scavenge from a large variety of nutrients could contribute to the competitiveness and persistence of bifidobacteria in the colon. Complete pathways for the synthesis of all amino acids, nucleotides, and some key vitamins were identified; however, routes for Asp and Cys were atypical. More significantly, genome analysis provided insight into the reciprocal interactions of bifidobacteria with their hosts. Candidate polypeptides were identified that showed

homology to most of the major proteins that are needed for production of glycoprotein-binding fimbriae. These glycoprotein-binding fimbriae structures could possibly be important for adhesion and persistence of bifidobacteria in the GIT (Schell *et al.* 2002). Genus coding for eukaryotic-type serine protein inhibitor (serpin) have also been identified, which are thought to be involved in the immunomodulatory activity of bifidobacteria (Schell *et al.* 2002).

2.3.3.2 Resistance of *Bifidobacterium* to antibiotics:

Antibiotics are commonly used to treat bacterial infections. Whilst being responsible for curing illness, there are negative aspects of antibiotic therapy. For example, they exert a major impact on the autochthonous GIT microflora. Besides an intestinal upset being an acceptable side effect of the treatment of a microbial infection, there are other undesirable side effects as a result of antibiotic therapy. These include antibiotic–associated disease (AAD), which may be life-threatening (Refer Table 4).

Table 4. Examples of antibiotic-associated disease (AAD)
(Adapted from Hill 1998)

Year	Antibiotic	Disease
1960	Neomycin	Staphylococcal enteroeditis Neomycin-induced diarrhoea
1980	Clindamycin	Pseudomembranous colitis
1914-18	Penicillin from mouldy bread	Trench mouth (*candida* infection)
Current	Broad spectrum in AIDS patients	Candidiasis (thrush)

With the ever-increasing resistance of pathogens to antibiotics, alternatives to antibiotics are being proposed. For example, it has been suggested that rather than killing the natural microflora of the GIT with antibiotics, colonisation of the GIT surface by autochthonous species is restored. It is possible that daily consumption of viable probiotics could achieve this. Resistance of *Bifidobacterium* to antibiotics is of importance with regard to the possibility of maintaining viable bifidobacteria in the digestive tract during antibiotic treatment.

Antibiotics can also be incorporated as selective agents in culture media for the isolation and identification of bifidobacteria from complex microflora obtained from medical / dietary samples / specimens (Ballongue 1998). Most bifidobacteria are resistant to a number of antibiotics such as, gentamicin, kanamycin, metronidazole, neomycin, polymyxin B, streptomycin as well as nalidixic acid. The sensitivity of the species however, varies from 10 µg - 500 µg or more antibiotic ml^{-1} depending on the antibiotic used, and *Bifidobacterium* sp. present (Ballongue 1998).

Most of the species are inhibited by penicillin, ampicillin, bacitracin, chloramphenicol, clindamycin, erythromycin, lincomycin, nitrofurantoin, oleandomycin, penicillin G, and vancomycin (Scardovi 1986). Many of these antibiotics are regularly used to control disease in humans; but while inactivating the undesirable pathogens, they simultaneously kill *Bifidobacterium* in the GIT. Restoration of a desirable microflora is therefore important after antibiotic therapy. It is arguable that in a world where antibiotic administration is common, with the associated destruction of the autochthonous GIT microflora that the natural balance of the microflora of the GIT has been permanently destroyed. The use of oral probiotic therapy in the GIT could enhance the recovery of desirable microflora after antibiotic treatment or invasion of a pathogen.

2.3.4 *Bifidobacterium* as a probiotic in humans:

2.3.4.1 Safety properties:

Bifidobacteria are normal inhabitants of the human intestine, but are also found in the human mouth and vagina, and in the alimentary tract of various animals, honeybees and birds (Rasic and Kurmann 1983).

They are the predominant autochthonous organisms in the large intestine of breast–fed infants accounting for about 99% of the faecal microflora (10^9 - 10^{11} colony forming units (cfu) g^{-1} faeces), whereas enterobacteria, enterococci and lactobacilli comprise about 1% (range 1% - 15%) of the flora, and *Bacteroides*, clostridia and other micro-organisms are absent or insignificant (Kurmann and Rasic 1991).

The faecal microflora of formula-fed infants resembles that of adolescents and adults. Bifidobacteria are approximately equal in numbers to, or may be outnumbered by *Bacteroides* and other genera, and most authors agree that apart from bifidobacteria, the number of microorganisms is higher in the stools of formula-fed infants than in breast-fed infants (Kurmann and Rasic 1991). To date, no industrial dairy milk formula has made it possible to maintain the same microflora equilibrium, including bifidobacteria, as that found in breast-fed infants (Ballongue 1998).

Table 5 shows general distribution of autochthonous *Bifidobacterium* species in the colon throughout the human life span. This distribution of the *Bifidobacterium* species will vary according to the gastric juice content of individuals (Ballongue 1998). It is noteworthy that *B. lactis*, a heteronym for *B. animalis*, used in this study, and widely used as a probiotic, is not present in Table 5. The latter two bacteria are not human isolates (Cai *et al.* 2000).

Table 5. Distribution of *Bifidobacterium* species in the human colon (Adapted from Ballongue 1998)

Population	Predominating Species	Minor Species
Breast-fed infants	*B. longum*	
	B. infantis	
	B. breve	
Bottle-fed infants	*B. adolescentis*	*B. bifidum* biovar b
Children	*B. infantis*	
	B. breve	
	B. bifidum biovar b	
	B. longum	
Adults	*B. adolescentis* biovars a and b	*B. bifidum* biovar a
	B. longum	
Old aged	*B. adolescentis* biovar b	
	B. longum	

Bifidobacteria are also present in the small intestine, but in lower numbers than occur in the large intestine (Hoover 1993). Ishibashi and Shimamura (1993) proposed a hypothetical scheme with respect to the relationship between intestinal bacteria (including *Bifidobacterium*) and human health. This relationship is complex and not yet fully understood. It is known that the composition of the microflora of the human GIT differs amongst individuals.

Of the 33 species included in the bifidobacteria genus, 12 of these species have been isolated from the GIT of humans and / or as human clinical isolates (Roy 2001). None is pathogenic, therefore these microorganisms are not affiliated with any GIT related diseases. Bifidobacteria tolerate low pH values, bile acid, and pancreatic juice.

However, gastric juice can be lethal to unencapsulated free bifidobacteria cells (Sun and Griffiths 2000; Truelstrup Hansen *et al.* 2002; Adhikari *et al.* 2003).

In addition, bifidobacteria adhere to intestinal epithelium, and increase the immune response (Refer 2.3.4.2.ii). To date, *Bifidobacterium* have not been associated with undesirable clinical side effects. In addition, bifidobacteria do not carry transmissible antibiotic resistant genes. Hence, bifidobacteria meet the safety requirements of a successful probiotic (Refer 2.3.1.1).

2.3.4.2 Claimed functional properties:

i. Adhesion of *Bifidobacterium* to the intestinal epithelium:

The maintenance of the correct balance between autochthonous bacteria in the GIT is important for optimal health. The ability of bifidobacteria to colonise the intestinal tract depends on the specific bacterium-to-host affinity (Mitsuoka 1996). In order for probiotic therapy to be effective in the long term, administered *Bifidobacterium* cells should integrate into the autochthonous population in the GIT.

It has been shown that bifidobacteria adhere to Caco-2 and HT-29-MTX human epithelial cell lines *in vitro*, thereby creating ecological niches within which the growth of bacteria is maintained. Regardless of changes in habitat, these niches enable the bacteria to produce a genuine probiotic effect (Ballongue 1998). It is postulated that the bacterial biofilm (mucous), attached to the epithelial walls, enhances the *in situ* production of various bacterial metabolites, which have a beneficial effect on the host (O'Sullivan *et al.* 1992; Ishibashi and Shimamura 1993; Saarela *et al.* 2000). Pathogenic bacteria that attach to epithelial cells have been shown to be displaced by bifidobacteria (Refer 2.3.4.2.iv).

It has been demonstrated that adherence of bifidobacteria to intestinal mucous isolated from subjects is influenced by the age group tested. The weakest attachment occurred in mucous isolated from the elderly (Fujiwara *et al.* 1997; Saarela *et al.* 2000). This phenomenon could explain the variable numbers of *Bifidobacterium* recorded from subjects in these different age groups.

ii. Stimulation of specific and non-specific immune responses:

The epithelial lining of the human GIT is a vast area for the absorption of molecules, and acts as a barrier to many foreign antigens that may pass through the gut. The exclusion of these antigens is arbitrated by the gut immune system, known as the gut-associated lymphoid tissue (GALT). The stimulation of the immune system by mucosa-associated probiotic bacteria suggests that the capacity of these organisms to stimulate a cytokine response by local mononuclear cells or lymphocytes depends, in part, on the ability of the probiotic organisms to cross over the gut epithelium before interacting with the local immune system cells. This probiotic stimulation of the immune system has been shown to prevent *Escherichia coli* 0157:H7 attachment to human GIT cells (Heyman and Menard 2002).

A number of *in vivo* and *in vitro* studies have investigated the interaction between lactic acid bacteria (LAB) and immunocompetence (O'Sullivan *et al.* 1992; Kailasapathy and Chin 2000). It was shown that by increasing the specific and non-specific immune responses of the host, LAB protected the host against infection by enteric pathogens, and tumour development (O'Sullivan *et al.* 1992; Kailasapathy and Chin 2000). It has also been documented that non-specific immune phagocyte activity of circulating blood granulocytes increases in the blood of human

volunteers after consumption of *Bifidobacterium* (Schiffrin *et al.* 1995; Tannock 1998; Kailasapathy and Chin 2000; Roberfroid 2000).

It is known that phagocytic activity is involved with natural immunity and that phagocytes are implicated in antibody immune responses acting as antigen-presenting cells (Roberfroid 2000). This latter effect might explain the stimulation of the specific intestinal surface immunoglobulin A (IgA) antibody response in the presence of *Bifidobacterium* (Kailasapathy and Chin 2000).

While probiotic bacteria stimulate the immune system by activating both macrophages and lymphocytes (O'Sullivan *et al.* 1992), they also enhance IgA levels (O'Sullivan *et al.* 1992), and stimulate (IFN)-γ (γ-interferon) production (O'Sullivan *et al.* 1992). In addition to the latter, bifidobacteria are also involved in the degradation and replacement of intestinal mucins (Ballongue 1998). Mucin (mucopolysaccharide) is the chief ingredient in mucus secreting glands, and is the lubricant that protects the internal human body surface from friction / erosion.

Recently, Kailasapathy and Chin (2000) demonstrated that probiotic bacteria, including *Bifidobacterium* sp., were confirmed to influence intestinal mucosal, and systemic immune responses against dietary antigens (Refer 2.3.3.1.iii).

iii. Metabolic effects on the host:

a. Suppression of lactose intolerance:

Lactose maldigestion / intolerance is due to unusually low activity of lactase in the human gut, thus causing various degrees of abdominal discomfort (Kailasapathy and Chin 2000). This intolerance to lactose is noted when a reduction in lactase activity

occurs during intestinal disorders such as gastroenteritis (O'Sullivan *et al.* 1992; Roberfroid 2000), and is common in Oriental and African populations (Hughes and Hoover 1991).

When lactose-intolerant individuals consume unfermented dairy products, they suffer from flatulence, abdominal pain and diarrhoea as a result of the action of the colonic bacteria on undigested lactose (O'Sullivan *et al.* 1992). Hence, such individuals are unable to consume milk and related dairy products. As a result, the daily requirement of calcium may not be consumed.

Experimental evidence suggests, however, that lactose-intolerant people are able to digest fermented milks and dairy products such as yoghurt. Heyman and Menard (2002) showed independently, in lactase-deficient subjects, that lactose was better absorbed from yoghurt than from milk. Two reasons were suggested for this. Firstly, by reducing lactose content by splitting of lactose into extracellular glucose and galactose by the starter culture bacteria in the yoghurt prior to ingestion (O'Sullivan *et al.* 1992). Secondly, it was suggested that intraluminal digestion of lactose by extracellular bacterial lactase (β-galactosidase) occurs. This enzyme is secreted by live probiotic *Lactobacillus acidophilus* associated with yoghurt production, during the ingestion of yoghurt (Heyman and Menard 2002).

Bifidobacteria, like *L. acidophilus*, could have an *in situ* effect on lactose digestion because they too produce a relatively high level of extracellular β-galactosidase, which autodigests lactose and improves tolerance to lactose (Kailasapathy and Chin 2000; Roberfroid 2000). Bifidobacteria, unlike the traditional yoghurt starter cultures *Lactobacillus bulgaricus* (now re-classified as *L. delbrueckii* ssp. *bulgaricus*) and *Streptococcus thermophilus* (now re-classified as *S. salivarius* ssp. *thermophilus*), are resistant to bile salts (Jay 1996; Ballongue 1998), and as a result

bifidobacteria could have an *in situ* effect on the metabolism of lactose.

Heyman and Menard (2002), in their review on therapeutic effects of probiotic organisms, stated that lactose content in AB yoghurt is 30% lower than in milk. This is due to β-galactosidase activity in the AB yoghurt, which decreased *in vivo* by 80% at pH 5.0 in the duodenum. The remaining 20% was functional in the terminal ileum, suggesting that the enzyme was relatively persistent along the digestive tract.

b. Food allergy:

Allergic reactions in some dairy milk-fed-infants can be triggered by casein. Kailasapathy and Chin (2000) reported that lactobacilli hydrolyse the complex casein protein forming peptides and amino acids, and hence decrease the proliferation of mitogen-induced human lymphocytes. Other studies indicate that probiotics may help to relieve intestinal inflammation and hypersensitivity reactions in infants with food allergies (Kailasapathy and Chin 2000).

In humans, food allergy can be due to the deregulation of the autoimmune lymphocyte (Th1 / Th2) balance in response to exogenous antigens. Heyman and Menard (2002) suggest that a modern style of life could be the basis of an increasing development of allergic diseases. In developed countries the prolonged use of both vaccination and antibiotics could result in the immune system not being challenged as frequently as it was before the advent of the use of these prophylactic applications. Exposure to infectious diseases generally leads to the stimulation of type 1 helper (Th1) lymphocytes, and to the release of cytokines such as (IFN)-γ (γ-interferon). T lymphocytes are classified as Th1, Th2 or Th3

types according to their profile of cytokine secretion (Heyman and Menard 2002).

Type 1 (Th1) lymphocytes produce (IFN)-γ, interleukin (IL)-2 and tumour necrosis factor (TNF)-β that activate macrophages and are responsible for cell-mediated immunity.

Type 2 (Th2) lymphocytes produce IL-4, IL-5, IL-10 and IL-13 that are responsible for antibody production, mainly immunoglobulin E (IgE), and eosinophil activation. Th2 lymphocytes predominate in response to nematode infection and in allergic disorders.

Type 3 (Th3) lymphocytes are regulatory cells mainly secreting suppressive cytokines such as transforming growth factor (TGF)-β (Heyman and Menard 2002).

It is possible that a Th1 response can prevent the development of allergic diseases, since Th1 and Th2 responses are considered mutually inhibitory. It has been suggested that in the absence of the complete immune system stimulation by infectious agents triggering Th1 cell response, the main defence mechanism for parasitic infestation, i.e. Th2-type cytokine secretion and IgE antibodies, are present, but these may be redirected against environmental substances such as food or respiratory antigens (Heyman and Menard 2002).

As the administration of AB cultures has demonstrated a reduced lactose-allergic response to dairy products, it has been reported from various studies that probiotics could represent a novel approach in the control of food allergy (Kailasapathy and Chin 2000; Heyman and Menard 2002).

c. Reduction in serum cholesterol levels:

Results from several clinical studies indicate a positive correlation between elevated serum cholesterol levels and increased incidence of coronary heart disease (O'Sullivan *et al.* 1992).

Several carefully controlled studies show a relationship between the presence of lactic microflora and a reduction in plasma cholesterol (Klaver *et al.* 1993). The administration, to hypercholesterolemic human subjects, of fermented milks containing *Bifidobacterium* at a concentration of 10^9 bacteria g^{-1} resulted in a reduction in the total cholesterol from 3 to 1.5 g ml^{-1} (Ballongue 1998). It is known that *Bifidobacterium* produces hydroxy-methylglutaryl (HMG)-CoA reductase, which is involved in the synthesis / degradation of cholesterol (Ballongue 1998). Ballongue (1998), therefore, indicated that a reduction in serum levels of cholesterol could occur in humans by the regular consumption of fermented probiotic bifidus foods.

However, results obtained from other studies on the influence of milk and other dairy products, inoculated with probiotic cultures, on blood cholesterol levels are contradictory (O'Sullivan *et al.* 1992). Various studies conducted between 1970 and 1990 consistently reported 5% - 17% reduction in human serum cholesterol concentrations after 2 - 4 weeks of daily consumption of fermented milk products containing live probiotics. These results have since been contradicted (Roberfroid 2000).

O'Sullivan *et al.* (1992) reported that many earlier studies used too few subjects, therefore statistical analyses were not done. Furthermore, it was never established whether the subjects had elevated or normal serum cholesterol levels prior to commencement of these studies. More recently Roberfroid (2000) also discredited these earlier studies stating that the major limitations were the

excessive volumes (0.5 – 8.4 L) of yoghurt consumed daily, with failure to assess or control the background diet and exercise patterns of the test subjects. Groups were not randomised, and there were no run-in periods during which time the subjects adapted to the test diet. Finally, there were neither multiple baseline measurements, nor changes in control groups. Results from these studies that claimed reduction in cholesterol levels in human subjects are therefore inaccurate.

It has been suggested, that any hypocholesterolemic effect caused by fermented milk products containing live probiotics may be due to the presence of organic acids such as uric, orotic and hydroxymethylglutaric acids, formed as a result of lactic fermentation. These acids inhibit cholesterol synthesis (O'Sullivan *et al.* 1992). Ballongue (1998) showed that metabolites produced from orotic acid during fermentation by fermented products could be responsible for a reduction in serum cholesterol levels. Ballongue (1998) also demonstrated that both orotic and hydroxymethylglutaric acids reduce serum cholesterol.

Probiotic bacteria may therefore potentially reduce the risk of heart disease by lowering blood serum cholesterol levels, increasing the resistance of low-density lipoprotein (LDL) cholesterol to oxidation, and reducing blood pressure (Tannock 1998).

Further research on the reduction of serum cholesterol is however required, to establish whether there is a definite link between the consumption of probiotic bacteria and reduced serum cholesterol levels.

iv. Antimicrobial activity:

It has been demonstrated in many studies that probiotic bacteria such as bifidobacteria have antimicrobial properties. Bifidobacteria reduce levels of pathogenic bacteria such as *Salmonella typhimurium*, *Clostridium difficile*, *Campylobacter jejuni*, *Escherichia coli* and *Shigella* sp. in the GIT (O'Sullivan *et al.* 1992).

Two hypotheses have been put forward to explain this effect. Firstly, prevention of pathogen colonization in the intestine by competing for nutrients and for adhesion sites on epithelial surfaces (Rasic 1983; O'Sullivan *et al.* 1992; Ishibashi and Shimamura 1993). When present in numbers above that of the daily intake of 10^8 g^{-1}, probiotic bacteria attach to enterocytes and prevent binding of pathogens by a process of competitive exclusion (O'Sullivan *et al.* 1992).

Secondly, the production of lactic and acetic acids during carbohydrate fermentation by bifidobacteria inhibits pathogens (Ballongue 1998). Within the intestinal lumen of humans, probiotic bacteria (including *Bifidobacterium*) inhibit the growth of enteric pathogens through the production of lactic and acetic acids (O'Sullivan *et al.* 1992). Acetic acid exerts a greater antagonistic effect against bacteria, than does lactic acid, and is produced in greater quantities by *Bifidobacterium* than by heterolactic acid bacteria (Rasic 1983). The acidic conditions induced by probiotics stimulate the peristaltic movements of the intestine, which facilitate the elimination of any pathogens present (O'Sullivan *et al.* 1992; Ishibashi and Shimamura 1993; Ballongue 1998).

In addition, probiotic bacteria (including *Bifidobacterium*) produce antimicrobial peptides, known as bacteriocins, in the human intestinal lumen, to inhibit the growth of enteric pathogens (O'Sullivan *et al.* 1992). Ballongue (1998), and Yildirim and Johnson (1998) isolated bacteriocins produced by bifidobacteria. Typically these

antimicrobial substances are peptides, heat-stable, and active at pH values between 2 and 10.

v. Anticarcinogenic properties of bifidobacteria metabolites:

Bifidobacteria can combat tumour action by the direct suppression of the procarcinogens (Ballongue 1998), activation of the immune system of the host, production of IgA (Ballongue 1998), and reduction of the intestinal pH (Ballongue 1998).

Additionally, genetically-modified, non-pathogenic bacteria have arisen as potential antitumour agents, either to provide direct tumouricidal effects, or to deliver tumouricidal molecules. Bermudes *et al.* (2002), using attenuated *Salmonella*, *Clostridium* and *Bifidobacterium* species, reported that these bacteria are capable of multiplying selectively, *in vivo* in tumours, thereby inhibiting cancer cell growth. This suggests a possible novel approach to cancer treatment (i.e. *Bifidobacterium* delivery to tumours).

There have also been reports that probiotic bacteria have the ability to inactivate dietary and intestinally generated mutagenic compounds such as azo-dyes (O'Sullivan *et al.* 1992) and nitrosamines (Rasic and Kurmann 1983). *In vitro* in the duodenum, bifidobacteria decrease the quantity of faecal microbial enzymes such as β-glucuronidase, β-glucosidase, nitroreductase and urease. The latter are possibly involved in the metabolic inactivation of miscellaneous mutagens and carcinogens (Roberfroid 2000).

Some probiotic bacteria (including *Bifidobacterium*) produce butyric acid, which can reduce the rate of apoptosis in enterocytes, and act as an anticarcinogen by neutralising the activity of mutagens. These mutagens include 4-nitroquinoline-N'-oxide, 2-nitrofluorene and benzopyrene (Kailasapathy and Chin 2000). Orrhage *et al.*

(1994) reported on the binding capacity of probiotic bacterial strains for mutagenic heterocyclic amines formed during the cooking of protein-rich food, i.e. 3-amino-1-methyl-5H-pyrido[2,3-b]indole (Trp-P-2), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). The binding capacity appeared to be a physical phenomenon, mostly due to a cation-exchange mechanism, and it has been suggested that cell wall peptidoglycans and polysaccharides are the two most important elements responsible for binding (Rafter and Glinghammar 1998). These results indicated that mutagens in cooked food could be bound to autochthonous bacteria of the normal intestinal microflora *in vitro*, thereby reducing the carcinogenic effect.

It has also been claimed that the administration of viable bifidobacteria alone or with a fermentable carbohydrate (defined as a prebiotic) can decrease the development of colonic aberrant crypts, i.e. very early preneoplastic lesions, and tumours *in vivo* (Rafter and Glinghammar 1998; Bermudes *et al.* 2002). However, several studies failed to demonstrate cancer prevention when bifidobacteria were administered to human volunteers (Rafter and Glinghammar 1998).

The precise mechanisms (if any) by which probiotic bacteria may inhibit human cancer are presently unknown.

vi. <u>Prevention and treatment of diarrhoea</u>:

It has been demonstrated that enteric infections in children could be prevented or minimised when the intestinal tract contains high numbers of administered bifidobacteria (Bullen and Willis 1971). In Japan, successful treatment of diarrhoeal diseases in children was accomplished by feeding them dairy products containing

bifidobacteria (Tojo *et al.* 1987). In addition, the feeding of viable bifidobacteria together with antibiotic therapy has been used to correct abnormal intestinal conditions, such as intractable diarrhoea (Hotta *et al.* 1987). Recent studies confirm this observation of probiotic supplementation with antibiotic treatment (Cremonini *et al.* 2002; Heyman and Menard 2002). Antibiotics used to cure many bacterial infections can alter the intestinal microflora composition and disrupt the equilibrium of the bowel ecosystem resulting in diarrhoea. It has been shown that the ingestion of probiotics (including *Bifidobacterium*) may minimise the effects of antibiotic-associated microbial alterations in the GIT (Cremonini *et al.* 2002; Heyman and Menard 2002) (Refer 2.3.3.2).

Oral probiotic therapy, including *Bifidobacterium*, in children has often been used for and described as beneficial in the treatment of acute viral diarrhoea (Heyman and Menard 2002). This is marked in children suffering from rotavirus diarrhoea. For rotavirus diarrhoea, different groups of investigators working under varying conditions have consistently reported that consuming probiotic-fermented dairy products shortened the diarrhoea episodes, or reduced the risk of diarrhoea (Kailasapathy and Chin 2000).

In children suffering with persistent diarrhoea the beneficial effect of feeding AB yoghurt to reduce the episode was noted when compared to subjects administered with milk alone (Heyman and Menard 2002). A controlled randomised single blind clinical trial revealed that treating children with chronic diarrhoea with either lactulose, reputedly a prebiotic for *Bifidobacterium*, or a probiotic preparation promoted complete remission of this intestinal disorder (Heyman and Menard 2002).

vii. Additional therapeutic benefits:

Additional claimed therapeutic applications of bifidobacteria in humans include:

1. tolerance to acidity affiliated with the gastric juice in the human stomach (Truelstrup Hansen *et al.* 2002)
2. tolerance to bile in the human duodenum and jejunum (Fooks and Gibson 2002)
3. detoxification of ammonia in the colon, thereby reducing liver stress (Arunachalam 1999)
4. maintaining a normal healthy bowel, especially in the elderly and infants, where bifidobacteria exert antifungal effects by the production of caprylic acid which inhibits *Candida albicans* (Passwater 2001)
5. increasing the synthesis of 'B-complex' vitamins (Arunachalam 1999)
6. prevention of urogenital infection (O'Sullivan *et al.* 1992)
7. alleviation of constipation (O'Sullivan *et al.* 1992), and
8. prevention of osteoporosis by the absorption of calcium (O'Sullivan *et al.* 1992; Hoover 1993)

Bifidobacterium therefore satisfies the therapeutic requirements for a probiotic organism.

2.3.4.3 Technical properties:

There are significant technical problems associated with production and supply of bifidus foods. These include a short shelf-life, e.g. refrigerated products such as yoghurt, cheese and ice cream, intolerance of the organism to oxygen during growth, and maintenance of *Bifidobacterium* at a daily intake of 10^8 (Saarela *et al.* 2000). In yoghurt, maintenance of the daily intake levels is not possible for *Bifidobacterium*, as the organism is rapidly outgrown by *L. acidophilus* and *S. thermophilus* (Klaver *et al.* 1993). In addition, cow's milk does

not support growth of *Bifidobacterium* (Klaver *et al.* 1993), and the presence of atmospheric oxygen also inhibits growth of the bacterium (Refer 2.3.3.1.ii.a). Hence many AB or ABT probiotic foods contain only *Lactobacillus* sp. and *Streptococcus* sp. (AT), and no viable bifidobacteria (B), despite claims to the contrary on food labels (Lourens-Hattingh and Viljoen 2002; Temmerman *et al.* 2003). The dairy industry needs to note that growth of *Bifidobacterium* in fermented dairy products is undesirable, as acetic acid production by this organism would result in a vinegar-flavoured product.

To obtain the therapeutic benefits of bifidobacteria it is critical that adequate numbers of living cells remain viable in fermented dairy products. The viability is influenced by the degree of acidification, bacterial strains used, fermentation conditions, storage temperature, preservation methods, and sensitivity to low pH (Shah 1997). The type and concentration of organic acid present in fermented foods also influences the *Bifidobacterium* viability (Shah 1997).

In general, the food industry aims to provide bifidobacteria at 10^6 cfu.g^{-1} of foodstuff at the time of consumption (Roy 2001; Krasaekoopt *et al.* 2003). This value appears to have been adopted to provide bacterial concentrations that are technologically attainable and cost-effective rather than to have a specific health benefit in humans (Roy 2001). Draft legislation is currently being prepared to control the probiotic food industry in South Africa (SA) (Refer Appendix A), and states that the daily intake for probiotic bacteria be 10^8 cfu in probiotic foods. This level has also been suggested elsewhere, to be desirable (Adhikari *et al.* 2003; Krasaekoopt *et al.* 2003).

In order, therefore, to enhance viability of bifidobacteria to attain the satisfactory daily intake levels without growth of the organism in dairy products, microencapsulation is the most suitable delivery system for viable *Bifidobacterium* sp. This is due to protection of the living organisms in edible hydrocolloid coatings. The organism is cultured in

synthetic media, immobilised in microcapsules, and delivered directly into foods for consumption by the consumer (Krasaekoopt *et al.* 2003) (Refer 2.1).

However, there are technical problems associated with microencapsulation. Reports on coacervation and mono-axial extrusion techniques refer mainly to small volume production of microcapsules (Huebner and Buccholz 1999; Park and Chang 2000; Sun and Griffiths 2000; Krasaekoopt *et al.* 2003). In addition, the size of microcapsules has been reported to be irregular, and in some cases microcapsules have altered the texture of the final product (Adhikari *et al.* 2003) (Refer 4.10). Although information regarding microcapsule size is limited (Adhikari *et al.* 2003), the size of microcapsules (Refer 4.4) is important when considering suitable food supply vehicles (Refer 2.3.1.3). Flavour problems (bitterness) have also been noted (Truelstrup Hansen *et al.* 2002) (Refer 4.10). Nevertheless at present, microencapsulation of *Bifidobacterium* is the most favourable means of ensuring delivery of viable *Bifidobacterium* in foods.

2.4 Traditional African beverages:

Microencapsulation of bifidobacteria is relatively recent and production methods are often patented, resulting in published data not always stating the possible variety of suitable foods. None has considered traditional fermented South African foods and beverages as potential supply vehicles for the organism.

2.4.1 Mahewu:

This non-alcoholic sour beverage, popular among the urban and rural black population of South Africa, has been brewed for centuries. The drink is available as either traditional brew, or as generic / commercial mahewu that is produced industrially (Schweigart and Follingham 1963; Edwards 2003). Traditionally, ground maize meal, together with water,

is boiled to produce a thin white gruel. The gruel is cooled to 25°C, and a small amount of wheat or corn flour is added. This is then left to ferment. A mixture of lactic acid bacteria dominates the fermentation, particularly *Lactobacillus plantarum*. *Corynebacterium* has been isolated from traditional mahewu, and is thought to hydrolyse the flour. *Saccharomyces* and *Candida* have been identified, and contribute to flavour (Caplice and Fitzgerald 1999).

In the industrial process, sugar, and at times, various fruit flavourings are added after the fermentation. *Lactobacillus delbrueckii* ssp. *bulgaricus* is commonly used as the starter culture. After fermentation, the mahewu is pasteurised and packed. The beverage has a shelf life of 21 days when held at 8°C - 10°C. The nutritional constituents of 1 L generic mahewu are approximately 78.4 g carbohydrates, 5.2 g proteins, 4.1 g fat, with an energy value of 1 495 kJ. Mahewu is particularly favoured by the miners in South Africa, and in 1999, 77 000 kL were consumed by this working group alone. Consumption of mahewu is currently being promoted at schools and hospitals in South Africa (Edwards 2003).

2.4.2 Amasi:

This beverage is a soured milk product that is popular amongst the Zulu, Xhosa and Sotho people in South Africa. Amasi, or 'amazi', is produced either traditionally or commercially. In the rural areas, amasi is made using cows' milk, which is fermented in calabashes. Fermentation can occur rapidly, and a soft curd is formed. Lactic acid bacteria, including *Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Enterococcus* and *Streptococcus*, dominate the fermentation, and can reach numbers of 10^8 cfu.ml^{-1} (Beukes *et al.* 2001). Commercially, amasi is produced using *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Leuconostoc mesenteroides* ssp. *cremoris*. The latter two genera are not present in traditional amasi (Beukes *et al.* 2001). After

fermentation, commercial amasi is pasteurised, packaged and distributed for sale. The shelf-life is 21 days. In 1995, 60 000 kL of amasi was sold. This quantity was insufficient to supply the needs of the consumer, indicating the popularity of the beverage (Beukes *et al.* 2001). By 2002, the annual consumption of amasi had risen to 104 000 kL (van Geems *et al.* 2003).

Hence, the consumption of amasi and mahewu represents an ideal means of supplying the probiotic *Bifidobacterium* to a significant number of South African consumers, most of whom do not have access to the health benefits provided by ingestion of these microorganisms.

CHAPTER 3
MATERIALS AND METHODS

3.1 Isolation of *Bifidobacterium lactis* DSM 10140:

Bifidobacterium lactis DSM 10140 utilised in this study was obtained as a gift from the Department of Molecular and Cellular Biology, University of Cape Town. The organism was isolated from a yoghurt starter culture, using colony hybridisation, as described by Trinidade *et al.* (2003).

3.2 Culture and maintenance of *B. lactis*:

Bifidobacterium lactis was cultured using anaerobic conditions in Hungate tubes at 37°C in a Bactron 1.5 anaerobic chamber (Shel Lab) (Refer Figure 5). The environment consisted of hydrogen (4%), carbon dioxide (10%) and nitrogen (86%). The selective medium used to culture *Bifidobacterium* was Tryptone yeast glucose (TYG) which contained the following, in grams per litre: tryptone (Biolab), 10; yeast extract (Difco), 5; glucose (Saarchem), 5; Tween 80 (Saarchem), 1; NaCl (Saarchem), 4.5; KCl (Saarchem), 0.25; $MgCl_2.6H_2O$ (Saarchem), 0.15; KH_2PO_4 (Saarchem), 0.4; K_2HPO_4 (Saarchem), 0.2; NH_4Cl (Saarchem), 0.4; cysteine HCl (Merck), 0.5. Bacteriological agar (Biolab) (1.5% w/v) was added to solidify the media (Trinidade 2003). The final pH of TYG was 6.8. For long term storage, *B. lactis* was stored in a –80°C freezer (Snijders Scientific) as 1 ml aliquots brain-heart infusion (BHI) broth, containing 2% glycerol.

Figure 5. Bactron 1.5 anaerobic chamber (Shel Lab).

3.3 Rheology of gellan-xanthan gum:

Based on work done elsewhere, gellan and xanthan gums were selected as the microencapsulation medium, and used as a mixture of 0.75% gellan (w/v) and 1% xanthan (w/v) (Sun and Griffiths 2000). Immediately prior to the rheological studies, the gums were prepared and re-hydrated as described below (Refer 3.4).

A Paar Physica MCR 300 rotational rheometer with cone-plate 50-2 measuring system was used for the gellan-xanthan gum mix to determine a flow curve in controlled shear rate mode, at shear rates of 0.1-100 s^{-1}. In addition, two other tests were run, namely a temperature sweep in rotation at a constant shear rate of 5 s^{-1} between 30°C - 60°C, and a time

dependency test, in rotational mode at a constant shear rate of 5 s^{-1}, at constant temperatures of 30°C, 40°C and 45°C.

3.4 Microencapsulation of *B. lactis*:

A variation of the method proposed by Sun and Griffiths (2000) was used. Gellan gum (0.15 g) (Sigma) and xanthan gum (0.2 g) (Sigma) were added to distilled water (20 ml). The solution was stirred using a magnetic stirrer (Ikamag) and simultaneously heated at 80°C for 10 min. The gel mix was then autoclaved (Huxley HL 340 speedy autoclave) at 121°C for 15 min. *Bifidobacterium lactis* was grown in the Bactron 1.5 anaerobic chamber at 37°C overnight in 250 ml TYG broth. To determine the growth stage of the cells after 16 h, the OD_{600} of the culture was measured using a spectrophotometer (Gene Quant *pro*). The final OD_{600} varied between 0.9 and 1.1.

Cells were harvested from 45 ml TYG broth using centrifugation (Eppendorf 5804 R) at 6000 g for 10 min at 20°C. The pellet of cells was washed three times in 45 ml sterile distilled water using the centrifuge as described above. Finally the pellet was resuspended in sterile distilled water to give a final volume of 2.5 ml. One ml of this was added to the sterile gellan-xanthan gum at 55°C. Microcapsules were generated using a superposed airflow mono-axial extrusion technique (Hueber and Buccholz 1999; Park and Chang 2000; Krasaekoopt *et al.* 2003), by manually extruding the gum / cell mix through a 27.5 gauge bevelled needle (Teruma) fitted on to a sterile 10 ml glass syringe (Glass Van) (Refer Figure 6). Gel and sterile airflow rates were 10 ml min^{-1} and 200 ml s^{-1} respectively. Microdroplets that formed were hardened into spheres by free fall into a sterile 0.1 M $CaCl_2$ solution (400 ml) (Refer Figure 7).

After one hour in the solution, the microcapsules were separated from the solution by aseptic filtration through sterile Whatman No. 1 filter paper. The remaining free cells were removed by washing the microcapsules with

sterile 0.1 M $CaCl_2$. To minimise contamination, procedures were carried out in a laminar flow hood.

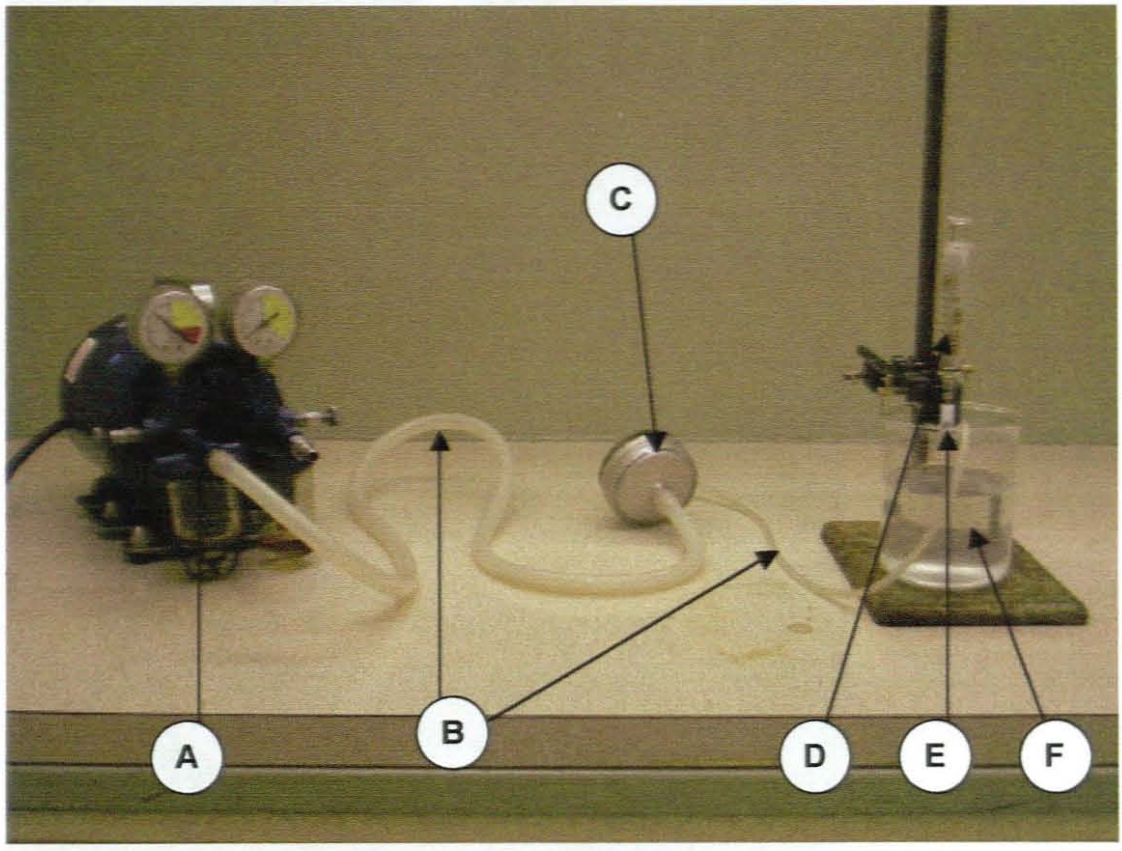

Figure 6. Equipment used to produce microcapsules, on a laminar flow bench.

A = leaf-veined air pump (General and Electric)

B = PTFE tubing

C = air filter (0.22 μm Millipore polycarbonate filter)

D = 10 ml glass syringe (Glass Van) and 27.5 gauge bevelled needle (Teruma)

E = air inlet / needle housing

F = 400 ml $CaCl_2$ solution

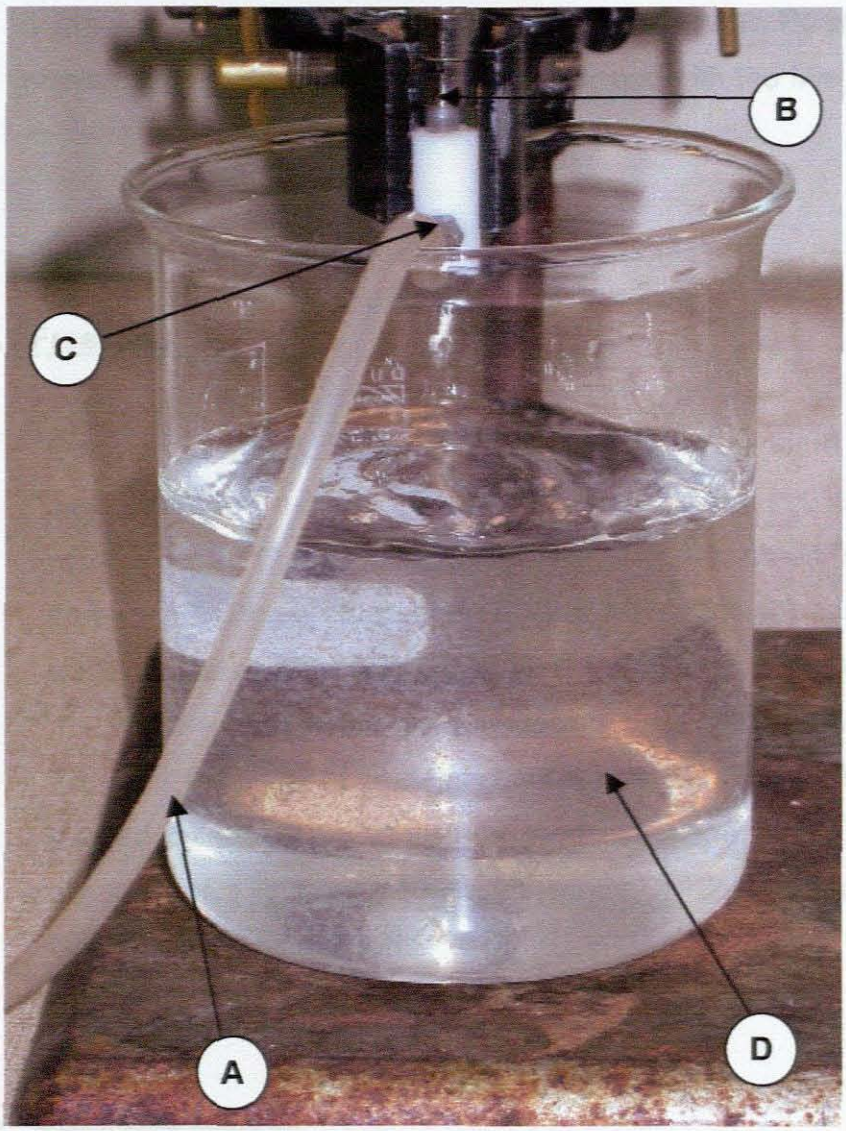

Figure 7. Formation of microcapsules in a 0.1 M $CaCl_2$ solution.

A = PTFE tubing used to supply air

B = 10 ml glass syringe (Glass Van) and 27.5 gauge bevelled needle (Teruma)

C = air inlet attached to needle housing

D = 400 ml $CaCl_2$ solution containing microcapsules

3.5 Estimation of microcapsule size:

The diameter and size distribution of 0.2 g microcapsules containing *B. lactis* were estimated by laser diffractometry using a Malvern Mastersizer S, version 2.19, calibrated to record particle diameters

5 μm – 2.5 mm. The analysis model used was Polydisperse, and the presentation – 30HD – "standard wet" for use with the Mie theory.

3.6 Enumeration of *B. lactis*:

3.6.1 Free cells:

Counting of free *B. lactis* cells was done using standard serial dilution and plate count techniques. Cells (1 ml) were diluted in 2% buffered peptone water (Biolab) (9 ml). Representative 0.1 ml volumes from dilutions were spread in triplicate onto either TYG or MRS (Biolab) agar plates. These were incubated anaerobically at 37°C for 48 h, and colonies were counted.

To check for purity of *B. lactis* cultures, samples were taken from colonies, stained by the Gram technique, and examined under the oil immersion objective of a light microscope (Olympus CHK2-F-GS) for the presence of Gram-positive bifidus rods.

3.6.2 Immobilised cells:

A comparison of two techniques was used to determine viable numbers of immobilised *B. lactis*.

3.6.2.1 Pestle and mortar:

The use of a pestle and mortar was proposed by Sun and Griffiths (2000) for the release of *B. lactis* from microcapsules. A mass of 0.2 g microcapsules, prepared as described (Refer 3.4), was softened in 10 ml 1 M sodium phosphate buffer (NaH_2PO_4 42.3 $g.l^{-1}$, Na_2HPO_4 57.7 $g.l^{-1}$) (pH 7.0) for 10 min, then ground using a sterile pestle and mortar (Pyrex). *Bifidobacterium lactis* cells (1 ml) released from the

beads were suspended in 9 ml 2% peptone water (Biolab) and standard serial dilution plate counts were done as described above (Refer 3.6.1).

3.6.2.2 High power ultrasound (HPUS) (20 kHz):

To estimate *B. lactis* numbers in the microcapsules, 0.1 g - 0.2 g capsules, from the batch prepared above (Refer 3.6.2.1), were weighed into 10 ml 1 M $NaHPO_4$ buffer. This suspension was subjected to sonication using a Vibracell Ultrasonic Processor VCX 750 (20 kHz) (Sonics and Materials). The instrument was fitted with a standard horn with a tip diameter of 13 mm (Vibracell, Sonics and Materials) (Refer Figure 8).

Microcapsules suspended in the buffer (pH 7.0) were subjected to a sound wave amplitude of 105 μm at 750 W for 15 s. All samples were treated in sterile 20 ml glass vials (Polytop) immersed in an ice water bath (Refer Figure 9).

After completion of the treatment, to estimate the number of released *B. lactis* cells, standard serial dilution plate counts were done as described above (Refer 3.6.1).

To ascertain whether the ultrasound treatment was lethal to *B. lactis*, duplicate preparations of 0.1 ml of the organism were added to 9.9 ml volumes of 1 M $NaHPO_4$. One of the pair underwent sonication as described, and the remaining sample was used as an untreated control. *Bifidobacterium lactis* cells used were from those prepared as described for microencapsulation (Refer 3.4). Viable cell numbers, before and after sonication, were estimated using standard serial dilution and plate counts.

Counts of *B. lactis* cfu.g^{-1} from both methods were compared, after which the more suitable procedure was selected and used throughout this study.

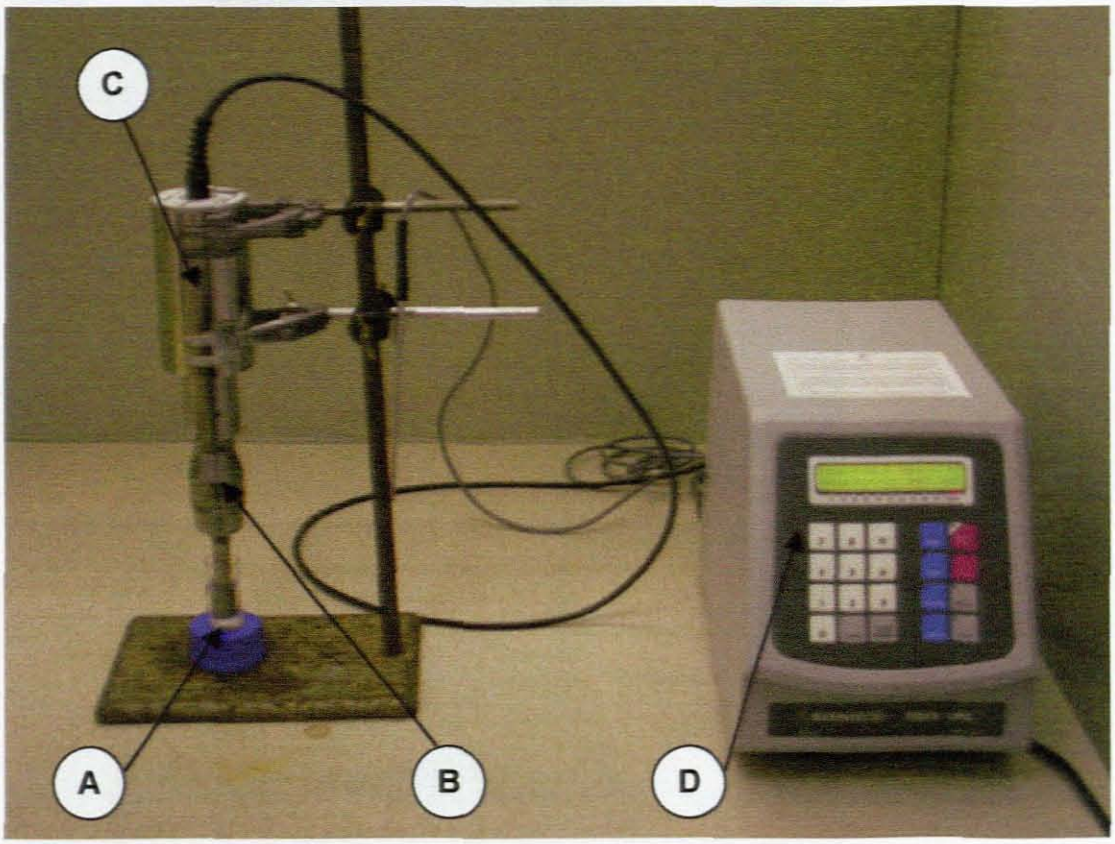

Figure 8. HPUS equipment (20 kHz, 750 W) used to enumerate *B. lactis* from gellan-xanthan microcapsules, on a laminar flow bench.

A = Polytop vial containing 10 ml buffer and microcapsules

B = 13 mm diameter standard horn (Vibracell, Sonics and Materials)

C = transducer (piezoelectric) (Vibracell, Sonics and Materials)

D = power source (Vibracell, Sonics and Materials)

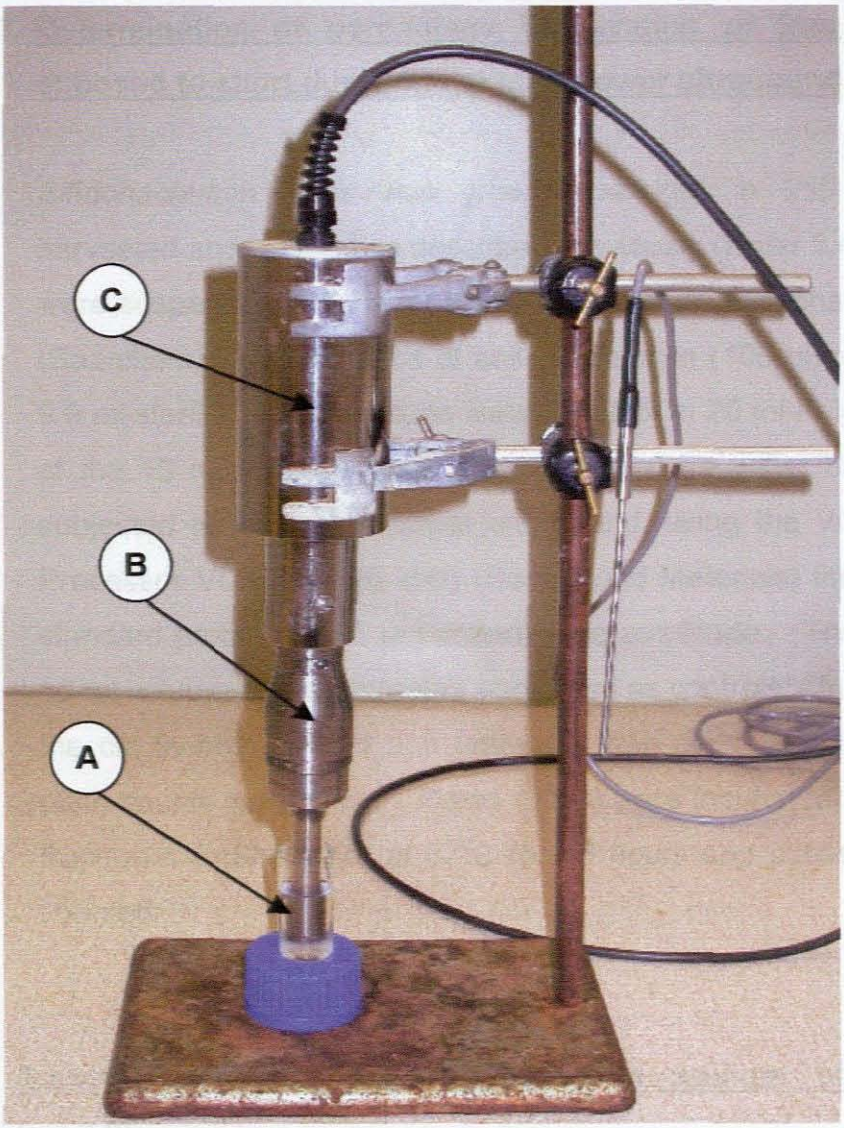

Figure 9. The standard 13 mm diameter horn (Vibracell, Sonics and Materials), attached to the transducer, immersed in 10 ml sodium phosphate buffer, on a laminar flow bench.

A = Polytop vial containing 10 ml buffer and microcapsules

B = 13 mm diameter standard horn (Vibracell, Sonics and Materials)

C = transducer (piezoelectric) (Vibracell, Sonics and Materials)

3.6.3 Determination of percentage inactivation of free *B. lactis* cells exposed to short durations of high power ultrasound:

Bifidobacterium lactis was grown overnight in 250 ml TYG broth, harvested and washed as described previously (Refer 3.4). The free cells were suspended in 4 ml sterile 1 M sodium phosphate buffer (pH 7.0) (Saarchem). Six volumes of cell suspension (100 µl) were added to six 9.9 ml sterile 2% peptone water (Biolab) in 20 ml Polytop vials. Three of the 10 ml volumes were placed into an ice-water bath, and all were subjected to 15 s high power ultrasound, using the Vibracell Ultrasonic Processor VCX 750 (20 kHz) (Sonics and Materials) fitted with a 13 mm standard probe, at 105 µm sound wave amplitude. The remaining three 10 ml volumes were untreated and acted as controls. In order to estimate the cfu in both treated and untreated free cells, standard serial dilution plate counts were done. Plates (TYG) were incubated anaerobically in a Bactron 1.5 (Shel Lab) at 37°C for 48 hours and subsequently cfu were counted.

3.7 Survival of immobilised *B. lactis* in sodium phosphate buffer (pH 7.0):

One litre sodium phosphate buffer (1 M) (Saarchem) was prepared. A known mass (0.2 g) of encapsulated *B. lactis* was added to 10 ml sterile buffer. The suspension was stored at either 4°C or 22°C for 21 days. Viable numbers of *B. lactis* were released from the microcapsules using the Vibracell Ultrasonic Processor VCX 750 (Sonics and Materials), and were subsequently determined using the serial plate dilution method (Refer 3.6.2.2). The log surviving fraction was calculated as Nt / No, where No and Nt are the average number (N) of cfu.ml^{-1} (free cells) or g^{-1} (microcapsules) at the start of the experiment (o) and at time (t) respectively.

3.8 Comparative survival of *B. lactis* in simulated gastric juices (pH 1.5):

Sterile simulated gastric juice (SGJ) (100 ml) consisting of 0.08 M HCl + 0.2% (w/v) NaCl (Saarchem) was prepared (Sun and Griffiths 2000). Nine ml of the gastric solution was placed into each of eight separate 100 ml conical flasks.

Bifidobacterium lactis cell preparation (250 ml) was grown overnight, harvested, and washed as described previously (Refer 3.4). The pellet of cells was finally suspended in a volume of 2.5 ml sterile distilled water.

For free cells, 100 µl of washed cell concentrate was added to each of four conical flasks containing 9 ml SGJ, and mixed. The four flasks were placed into an orbital shaker chamber (Psycrotherm, New Brunswick Scientific Co.) at 37°C. At start-up, and thereafter at 60, 120, and 240 min, 1 ml samples of the SGJ and bacteria were removed and serially diluted for viable counts as described elsewhere (Refer 3.6.1).

Microcapsules were prepared using 1 ml of the remaining washed cell suspension in a 20 ml gellan-xanthan mix. A measured weight of microcapsules (0.2 g) was added to each of four flasks containing 9 ml SGJ, and mixed. The four flasks were also placed into the orbital shaker chamber at 37°C.

At appropriate times, described above (Refer 3.6.2.2), immobilised bacterial cells were released using the Vibracell VCX 750 (Sonics and Materials). At start-up, and thereafter at 60, 120, and 240 min, 1 ml samples of the SGJ containing free bacteria were removed and treated as described above (Refer 3.6.1). Counts were expressed as surviving fraction (Refer 3.7).

The pH of the SGJ was measured for the duration of the experiment, using a pH meter (Hanna HI 8314 membrane pH meter).

3.9 Comparative survival of *B. lactis* in simulated bile and pancreatic juices (pH 6.5):

Pancreatin (0.5 g) (Oxoid) was dissolved in 50 ml sterile distilled water and heated to 80°C using a magnetic stirrer / hot plate (Ikamag). Bile and pancreatic juice (BPJ), consisting of 0.75 g of bile salts (Oxoid) and 25 ml (0.25 g) of pancreatin (Oxoid), was made up to 100 ml using sterile distilled water. Nine ml of the BPJ was placed into each of eight separate 100 ml conical flasks.

Bifidobacterium lactis was grown and harvested as described (Refer 3.4). The pellet of cells was suspended in a final volume of 2.5 ml sterile distilled water.

For the free cells, 100 µl washed cells were added to each of four conical flasks containing 9 ml BPJ and mixed. The four flasks were placed into an orbital shaker chamber (Psycrotherm, New Brunswick Scientific Co.) at 37°C. At start-up, and thereafter at 60, 120, and 240 min, 1 ml samples of the BPJ and bacteria were removed and serially diluted for viable counts as described elsewhere (Refer 3.6.1).

Microcapsules were prepared using 1 ml of the washed cells in a 20 ml gellan-xanthan mix. A measured weight of microcapsules (0.2 g) was added to each of four flasks containing 9 ml BPJ, and mixed. The four flasks were also placed into the orbital shaker chamber at 37°C.

At appropriate times, described above (Refer 3.6.2.2), immobilised bacterial cells were released using the Vibracell Ultrasonic Processor VCX 750 (Sonics and Materials). At start-up, and thereafter at 60, 120, and 240 min, 1 ml samples of the BPJ and bacteria were removed and treated as described above (Refer 3.6.1). The surviving fraction was calculated as described (Refer 3.7).

The pH of the BPJ was measured for the duration of the study, using a pH meter (Hanna HI 8314 membrane pH meter).

3.10 Comparative shelf life studies of *B. lactis* in fermented African beverages:

Bifidobacterium lactis was grown overnight under anaerobic conditions at 37°C in 250 ml TYG broth as described previously (Refer 3.4). For free cells, 75 µl of the culture concentrate were added in the presence of oxygen to 20 ml aliquots of either pasteurised commercial generic amasi or mahewu. Samples were stored in sterile McCartney bottles under aerobic and anaerobic conditions at ambient temperature (22°C), and under aerobic conditions at 4°C. Serial plate dilutions were done to establish levels of *B. lactis* ml^{-1} at start-up, and thereafter at 7, 14, and 21 days.

To check for contamination of samples, after counting colonies on TYG plates, random colonies were selected, and stained by the Gram technique. These stained cells were examined by light microscopy (Olympus CHK2-F-GS) for gram-positive bifidus rods.

The pH of amasi and mahewu was measured both at start-up, and at the termination of the shelf life studies, using a pH meter (Hanna HI 8314 membrane pH meter).

From the same 250 ml culture, microcapsules containing *B. lactis* were prepared as described (Refer 3.4) and 0.2 g microcapsules were added aerobically to 10 ml aliquots of either amasi or mahewu in sterile McCartney bottles. Storage and incubation conditions were identical to those described for free *B. lactis* cells, except that no microcapsules were incubated anaerobically. This was done as oxygen would be limiting within the capsules.

Viable numbers of *B. lactis* were released from the microcapsules using the Vibracell Ultrasonic Processor VCX 750 (Sonics and Materials), and were subsequently determined using the serial plate dilution method (Refer 3.6.2.2). To decrease viscosity of these samples, 10 ml sterile 1 M sodium phosphate buffer was added to both the amasi and mahewu samples immediately prior to sonication. When microcapsules were suspended in a 1:1 buffer:beverage (amasi or mahewu) mix, the sound wave amplitude was increased to 117 μm for 20 s, due to the viscosity of the beverages. After sonication, viable *B. lactis* cfu were determined using routine serial dilution plate counts. Triplicate spread plates (TYG) were prepared from relevant dilutions. Plates (TYG) were incubated anaerobically in a Bactron 1.5 (Shel Lab) at 37°C for 48 hours and subsequently all visible cfu were counted (Refer 3.6.3). The surviving fraction of cells was calculated as described (Refer 3.7).

3.11 Preparation of amasi and mahewu samples for consumption:

Three 1 L each of commercial pasteurised amasi and mahewu were used. Amasi and mahewu were aseptically dispensed into sterile 1 L Schott bottles. *Bifidobacterium lactis* was grown overnight and harvested as described (Refer 3.4).

To prevent possible adverse flavour developments, all the cells were washed by resuspending cells in 45 ml sterile distilled water and centrifuging at 6000 xg (Eppendorf 5804 R), as described (Refer 3.4). This was repeated three times. Cell pellets were finally suspended in two separate 3 ml volumes of sterile water and pooled to a final volume of 6 ml.

Two 1.5 ml volumes of clean cells were added to both 1 L volumes of amasi and mahewu, and mixed well.

Microcapsules were prepared using 3 ml of the washed cells in a 40 ml gellan-xanthan gum mix as described above. A measured weight of microcapsules (0.2 g) was added to 500 ml of either amasi or mahewu

One litre of amasi and mahewu was left sterile, and used as untreated controls for the taste trials. All samples were stored in the presence of oxygen, at 4°C.

Three samples, held for varying storage times (1, 6 or 14 days), of both beverages were prepared for sensory testing. Viable numbers of cells were determined at the start of each experiment, and at each of the sensory sampling times. Samples were then evaluated for sensory attributes by a trained analytical taste panel. The pH of the beverages was measured at the beginning and the termination of the trials.

3.12 Constitution of a 12-member analytical taste panel:

Twenty-eight Food and Consumer Science students were trained for selection as analytical taste panellists using an in-house testing schedule. They were tested on their capability of recognizing the four taste sensations namely, sweet, sour, salt and bitter. In addition to this, the panellists were tested for their ability to describe the odour sensations of 20 different odourants. Of the 28 panellists that were tested, 24 scored the required 60% in the taste sensation and odourant tests.

Panellists were then trained for descriptive analysis of a selection of 6 different food products. For the triangle tests, three randomly numbered samples were simultaneously presented to the panellists. Two samples were identical and one was different. Each panellist had to indicate which sample was different.

The requirement for the choice of the analytical panellists was that each panellist had to score above 60% in the set of 24 triangle tests in an in-house test in the Department of Food and Consumer Sciences: Food and Nutrition.

Twelve of the panellists were selected for sensory evaluation of the mahewu and amasi samples. The panel was made up as follows: seven white panellists, four black panellists and one coloured panellist. The sensory evaluation sessions were carried out under controlled conditions in tasting booths with fluorescent lighting.

The evaluation form used for the sensory evaluation sessions was made up of two sections; Section A comprised of a triangle test with three random numbered samples. Two samples in each test were identical and one was different. The two identical samples in each test were comprised of either 1 L amasi or 1 L mahewu, which had been stored for 0, 7 or 14 days. The third sample consisted of either 1 L amasi or 1 L mahewu enriched with either free cells or microcapsules, and stored for 0, 7 or 14 days (Refer Tables 21 and 22). Panellists were asked to identify the sample that was different, thereby indicating if the added free cells or microcapsules had changed the flavour of the product. In Section B of the evaluation form, panellists were asked to indicate the intensity of the colour of each of the three samples. Panellists were also required to perform a descriptive analysis of the mouth feel for each of the three samples. Panellists therefore evaluated the products not only according to the flavour, but also for colour and mouthfeel.

3.13 Statistical analyses:

Repeated Measures ANOVAs (RMA) were performed on all data obtained as outlined by Dunn and Clark (1987), using STATISTICA™. Data obtained were analysed at 5% level of significance.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of *Bifidobacterium lactis* DSM 10140:

Bifidobacterium lactis DSM 10140 was isolated from an AB yoghurt starter culture (Trinidade *et al.* 2003) and used for all subsequent studies. This bacterium was chosen as the probiotic organism for encapsulation, as it is commonly found in yoghurt starter cultures, and has characteristics that are important in selecting a probiotic for commercial use. These characteristics include fermentation of a wide variety of carbohydrates, oxygen tolerance, and survival at low pH values (Truelstrup Hansen *et al.* 2002; Trinidade 2003).

Miele *et al.* (1997) isolated *B. lactis* from fermented milk. However, after DNA hybridisation studies, Cai *et al.* (2000) stated that *B. lactis* is a later heterotypic synonym of *Bifidobacterium animalis*, initially isolated from sewage.

4.2 Rheology of gellan-xanthan gum:

Gellan (0.75% w/v) and xanthan (1% w/v) were selected as suitable immobilising materials as a combination of these gums has been reported to have better technical properties for microencapsulation than do alginate, κ-carrageenan and locust bean gums (Sun and Griffiths 2000). Although alginate is frequently used to microencapsulate probiotics, it has undesirable attributes, such as susceptibility to degradation by acids (Sun and Griffiths 2000; Truelstrup Hansen *et al.* 2002; Krasaekoopt *et al.* 2003). Hence alginate microcapsules are unlikely to remain intact in the hydrochloric acid of the stomach and reach the colon intact (Refer 2.1.3).

Rheological studies used in this study, to determine the shear stress data of the gellan-xanthan gum, indicated that the gel behaved as a non-Newtonian material, and the flow curve fitted well to the Herschel-Bulkley model $\tau_0 = \tau_Y - \kappa_y^{-n}$ (Refer Figure 10). The Herschel-Bulkley model is a model used to describe the flow behaviour of a yield pseudoplastic material.

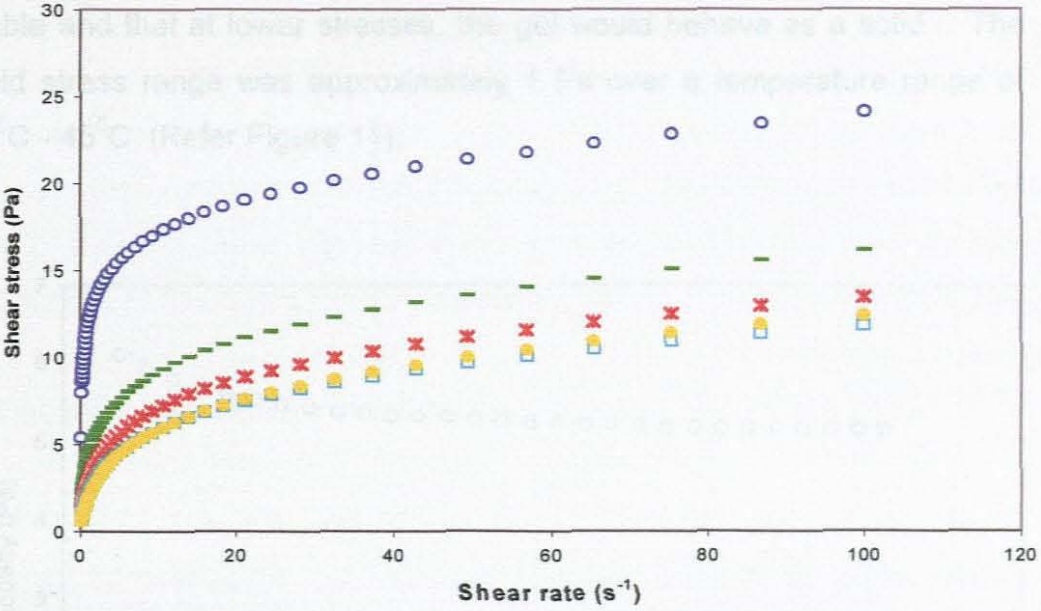

Figure 10. Flow curves of the gum mix at different temperatures, showing non-Newtonian shear thinning behaviour. Gum at 30°C (○); 35°C (-); 40°C (*); 45°C (□); 50°C (●) (Refer Tables 1a – 1g; Appendix B).

For microencapsulation, Newtonian liquids such as water or honey are unsuitable as encapsulating materials, as liquid droplets, not solid capsules, are formed during the mono-axial extrusion method. In order for successful encapsulation of microorganisms to occur, the selected material should exhibit non-Newtonian properties, i.e. be a relatively viscous material with solid properties. Hence the gum mix of gellan and

xanthan was suitable for microencapsulation, and was used for the duration of this study.

The apparent yield stress associated with the gellan-xanthan mix indicated that the gums would only flow or deform plastically when external forces are greater than the internal structural forces inherent in the gum. The average yield stress of the gum mix was 1.515 Pa, a value similar to that of raw meat batter (Steffe 1996). This value indicates that the gum mix is stable and that at lower stresses, the gel would behave as a solid. The yield stress range was approximately 1 Pa over a temperature range of 30^0C - 45^0C (Refer Figure 11).

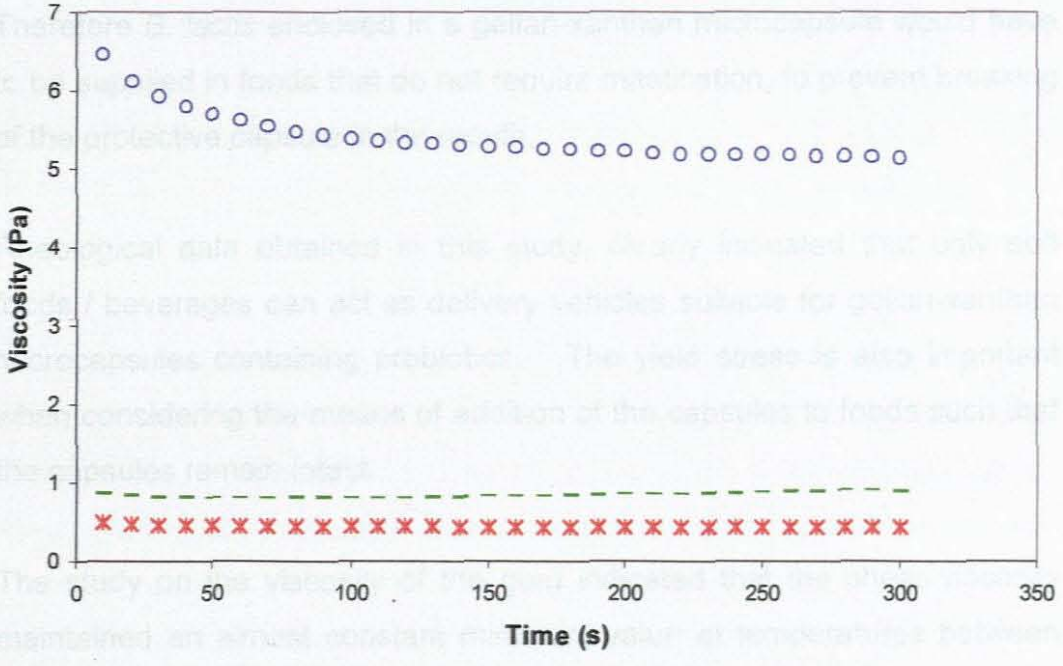

Figure 11. Time sweep of shear viscosity at 5 s^{-1} at different temperatures for the gum. Temperature range: 30^0C (○); 40^0C (-); 45^0C (*) (Refer Tables 1c-f, 1i; Appendix B).

Any material selected for microencapsulation should be able to traverse the upper gastrointestinal tract (GIT) intact to protect the bacteria, but on arrival in the colon, the yield stress of the gum should be such that the immobilised bacteria are released into the human host. The immobilising material should not have a high yield stress, such as does steel, as probiotics encapsulated in such a material would not be released. The value obtained for the gellan-xanthan gum mix indicated that with GIT movements (peristalsis), it is likely that release of the bacteria would occur in the colon.

The average yield stress of the gum indicated that gellan-xanthan would behave as a solid under low mechanical stress conditions, but would easily be broken by shear stresses such as those associated with chewing. Therefore *B. lactis* enclosed in a gellan-xanthan microcapsule would have to be supplied in foods that do not require mastication, to prevent breaking of the protective capsule in the mouth.

Rheological data obtained in this study, clearly indicated that only soft foods / beverages can act as delivery vehicles suitable for gellan-xanthan microcapsules containing probiotics. The yield stress is also important when considering the means of addition of the capsules to foods such that the capsules remain intact.

The study on the viscosity of the gum indicated that the shear viscosity maintained an almost constant minimum value at temperatures between 46^0C - 61^0C (Refer Figure 12). Hence encapsulation using gellan-xanthan should be performed between these temperatures. For the industrial production of microencapsulation it is of importance that the shear viscosity is constant over 15^0C, as opposed to, for example 1^0C - 3^0C. The latter values would require critical temperature control to maintain constant shear viscosity prior to microencapsulation. Moreover these temperatures, between 46^0C - 61^0C, are within the range for survival of *Bifidobacterium*.

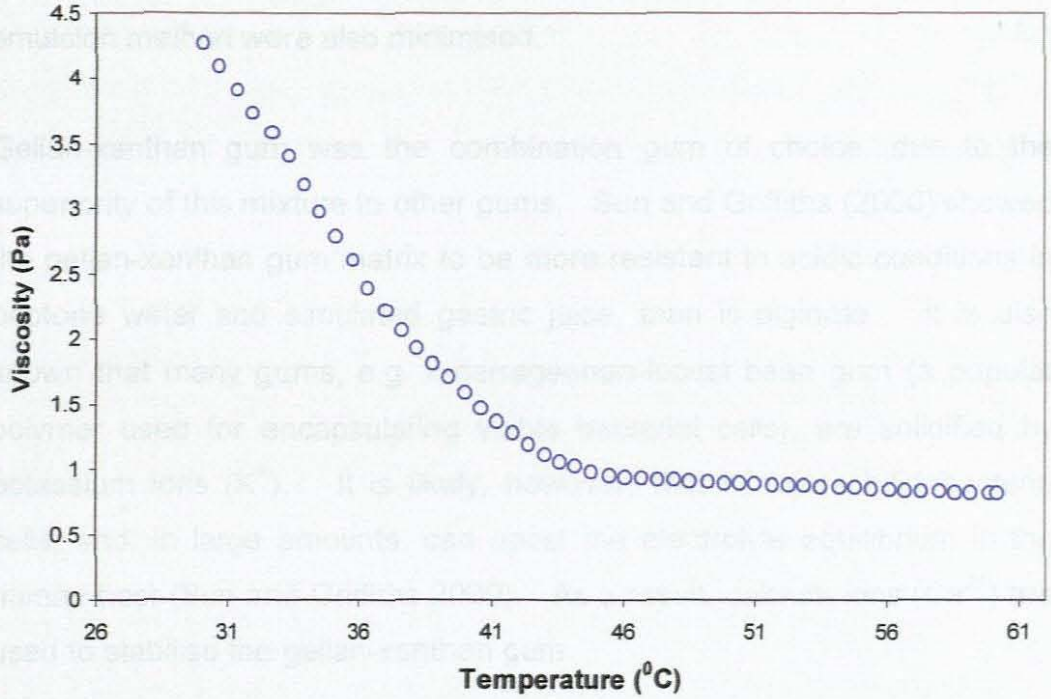

Figure 12. Shear viscosity of the aqueous gum mix at 5 s^{-1}, as a function of temperature (Refer Table 1h; Appendix B).

During microencapsulation procedures, maintenance of the constant shear viscosity obtained between 46°C - 61°C would enhance control over microcapsule size as well as permit optimization / standardization of mixing procedures necessary for even dispersal of *B. lactis* in the viscous gum held at these temperatures. These data are crucial for successful industrial production of gellan-xanthan microcapsules.

4.3 Microencapsulation of *B. lactis*:

In this study, the mono-axial extrusion technique was used together with a superposed airflow (Huebner and Buccholz 1999; Park and Chang 2000; Sun and Griffiths 2000). This rapid technique has a lower risk of microbial contamination than does the other popular microencapsulation method of

emulsion / coacervation. Potential flavour problems associated with the emulsion method were also minimised.

Gellan-xanthan gum was the combination gum of choice, due to the superiority of this mixture to other gums. Sun and Griffiths (2000) showed the gellan-xanthan gum matrix to be more resistant to acidic conditions in peptone water and simulated gastric juice, than is alginate. It is also known that many gums, e.g. κ-carrageenan-locust bean gum (a popular polymer used for encapsulating viable bacterial cells), are solidified by potassium ions (K^+). It is likely, however, that K^+ injure bifidobacteria cells, and, in large amounts, can upset the electrolyte equilibrium in the human host (Sun and Griffiths 2000). As a result, calcium ions (Ca^{2+}) are used to stabilise the gellan-xanthan gum.

Table 6. *B. lactis* cfu values recorded in microcapsules from five separate experiments (Refer Appendices I, J, L, O, and P)

Experiment number	Log_{10} cfu.g^{-1}
1	12.24 ± 0.04**
2	12.31 ± 0.07
3	11.28 ± 0.05
4	11.61 ± 0.05
5	11.37 ± 0.05

**Values represent counts, with standard deviation, from triplicate plates prepared from selected dilutions

Using methods described in this study for preparing concentrated inocula of *B. lactis* (Refer 3.4), high numbers of viable *B. lactis* were successfully microencapsulated in a gellan-xanthan gum mix such that the daily intake (10^8) is obtained in 0.1 g microcapsules (Refer Table 6; Figure 13). For the duration of the experiments, estimation of viable cfu in the

microcapsules indicated that conditions associated with the method of microencapsulation, including oxygen, were not lethal to *B. lactis* (Refer 3.4). This is in agreement with Miele *et al.* (1997), and Trinidade *et al.* (2003), who also reported a tolerance of *B. lactis* to atmospheric oxygen.

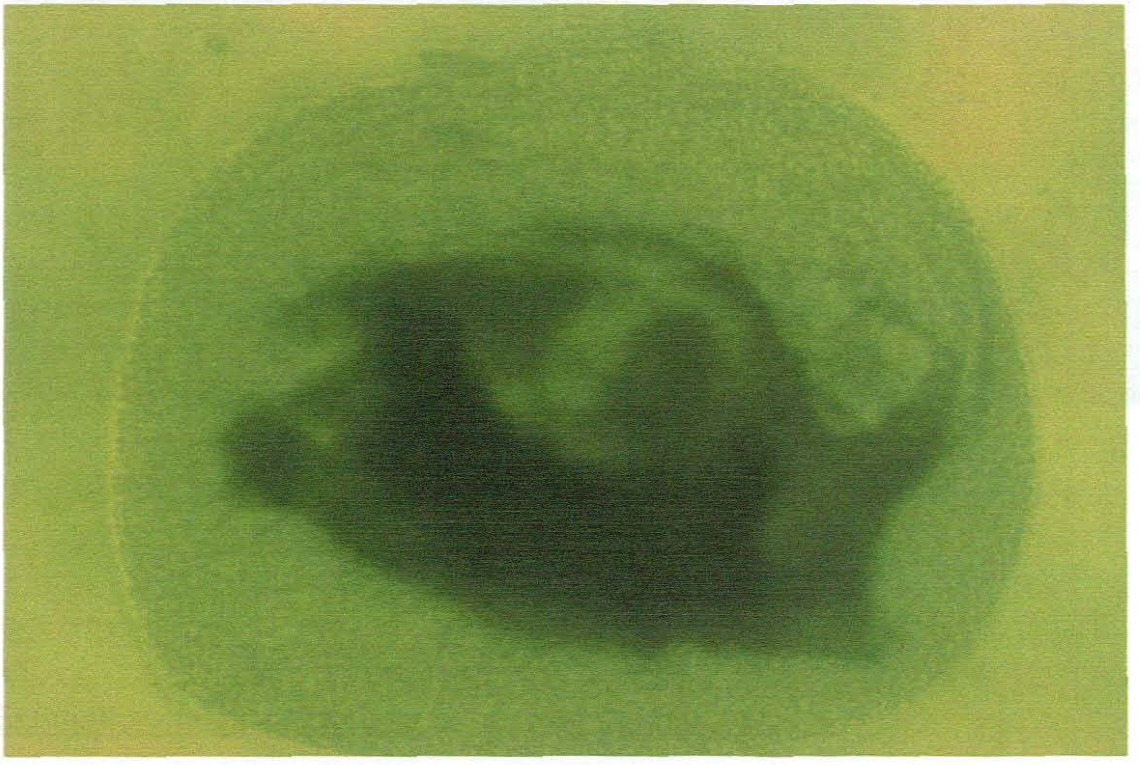

Figure 13. Phase contrast view of a single gellan-xanthan gel microcapsule, at 400x magnification, with a densely packed core of *B. lactis*.

Microscopic examination of immobilised bacteria showed oval / round microcapsules with a densely packed core of bacteria (Refer Figure 13). Gram staining of open microcapsules showed Gram-positive rods with a typical bifidus shape (Refer Figure 14).

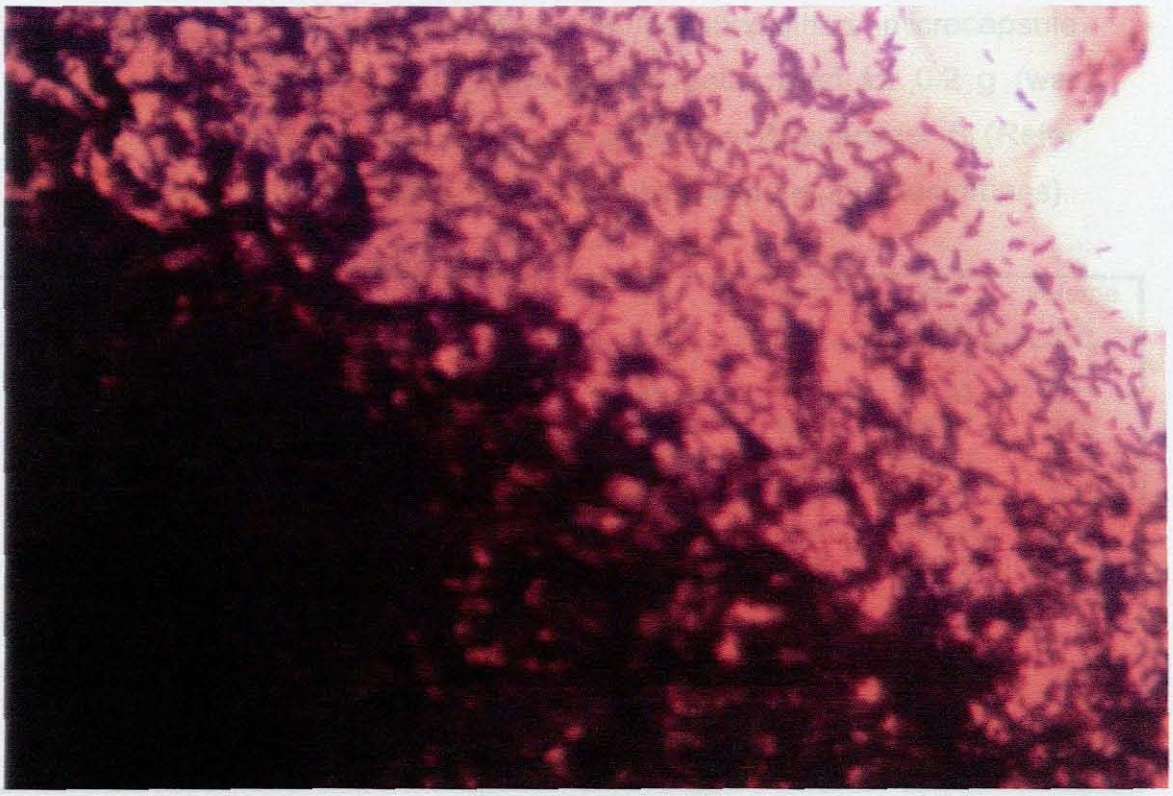

Figure 14. Gram-positive *B. lactis* trapped in the gellan-xanthan gum, viewed at 1000x magnification.

4.4 Estimation and significance of microcapsule size:

Gellan-xanthan gel was prepared as described (Refer 3.4), and a *B. lactis* cell concentrate in late exponential / early stationary phase (OD_{600} 0.9-1.1) was added immediately prior to microencapsulation. The use of a 27.5 gauge bevelled needle for mono-axial extrusion (gum flow rate of 10 $ml.min^{-1}$), and a superposed sterile airflow (airflow rate of 200 $ml.s^{-1}$), resulted in an 80% reduction in diameter, on the 3 mm diameter recorded by Sun and Griffiths (2000) who used a simple dropping technique with the same gums. Krasaekoopt *et al.* (2003) obtained microcapsules with a diameter range of 2-3 mm using the simple dropping technique, while Huebner and Buccholz (1999) recorded diameters of 25-300 µm for gum microcapsules produced by the superposed air method.

Table 7. A representative analysis of gellan:xanthan microcapsule diameter and size distribution as determined for 0.2 g (wet weight) microcapsules using a Malvern Mastersizer S (Refer Appendix C for further size analyses, and graphic illustrations).

Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%
4.19		22.49		120.67		647.41	
4.88	0.00	26.20	0.05	140.58	1.42	754.23	8.37
5.69	0.00	30.53	0.08	163.77	1.71	878.67	8.81
6.63	0.00	35.56	0.12	190.80	2.06	1023.66	8.38
7.72	0.00	41.43	0.18	222.28	2.49	1192.56	7.47
9.00	0.00	48.27	0.25	258.95	3.04	1389.33	6.17
10.48	0.00	56.23	0.35	301.68	3.71	1618.57	4.70
12.21	0.01	65.51	0.47	351.46	4.52	1885.64	3.22
14.22	0.01	76.32	0.62	409.45	5.42	2196.77	1.75
16.57	0.01	88.91	0.78	477.01	6.33	2559.23	0.27
19.31	0.02	103.58	0.97	555.71	7.16	2981.51	0.00
22.49	0.03	120.67	1.18	647.41	7.85	3473.45	0.00

D (v, 0.1) 10% of the microcapsules < 186.51 µm in diameter

D (v, 0.5) 50% of the microcapsules < 637.00 µm in diameter

D (v, 0.9) 90% of the microcapsules < 1387.15 µm in diameter

Laser diffractometry using a Malvern particle size analyser on random samples (0.2g) of the gellan-xanthan microcapsules in this study, showed a maximum size of 2.22 mm, with 50% of the capsules having a diameter < 0.637 mm. Microcapsules with diameters of less than 0.02 mm were also recorded.

Diameter of microcapsules can influence the survival of bifidobacteria. Working with nine *Bifidobacterium* species, including *B. lactis*, encapsulated in calcium alginate microcapsules with an average diameter of 100 µm, Truelstrup Hansen *et al.* (2002), showed a significant decline in survival of encapsulated cells in simulated gastric juice, pH 1-6, in all species over 120 min. However, when microcapsule diameter was > 100 µm, *B. lactis* showed an improved survival rate.

In the same study, the survival times of various immobilised bifidobacteria in milk improved over 12-15 days, compared to free cells, and capsule size did not influence these results (Truelstrup Hansen *et al.* 2002). The issue of optimum microcapsule size is complex and dependent on the encapsulation material used, the species of *Bifidobacterium* to be encapsulated, technology used to produce the microcapsule, and the environment external to the capsule.

Light microscopic investigation of the microcapsules produced in this study indicated that the capsules were generally oval / round in shape, and were densely packed with *B. lactis* cells (Refer Figure 13). The shape of the microcapsules, however, varied and not all were rounded. Irregularity of microcapsule shape is often recorded for probiotics immobilised in edible gums, and is related to the type of process utilised for production. The variation in microcapsule shape is important as it influences bacterial viability in, as well as the flow properties of the microcapsules. Determination of optimum microcapsule shape and size for microencapsulation of probiotics for use in the food industry is complex, and requires further study.

4.5 Enumeration of *B. lactis*:

Accurate enumeration of viable immobilised probiotics is difficult. Truelstrup Hansen *et al.* (2002) reported that passive solubilisation of microcapsules in 0.05 M phosphate buffer with sodium chloride or physiological saline with or without 0.05 M calcium or 0.02 M citric acid was time-consuming and unsatisfactory for enumeration of immobilised cells. Accurate and rapid enumeration was achieved with physical disruption of the capsules using a Polytron homogeniser.

For enumeration of *B. lactis* in this study (Refer 3.6.2.1; 3.6.2.2) two physical procedures were compared. These were use of a pestle and mortar as described by Sun and Griffiths (2000), and a novel method using

high power ultrasound (HPUS) (20kHz). As ultrasound can exert a lethal effect, the viability of free cells, either treated with HPUS or untreated, was compared. Results demonstrated that the ultrasound protocol used (Refer 3.6.3) was not lethal to free *B. lactis* cells (Refer Tables 10 and 11).

Using the technique proposed by Sun and Griffiths (2000), 0.2 g microcapsules were soaked in a 1 M sodium phosphate buffer solution, pH 7.0, for 10 min. Thereafter, a sterile pestle and mortar were used to release the bacterial cells from the microcapsules by grinding.

Table 8. *B. lactis* cfu recorded from microcapsules, released from the matrix by a pestle and mortar (Refer Appendix D).

Experiment number	Log_{10} cfu.g^{-1}
1	10.77 ± 0.12**
2	10.81 ± 0.24
3	10.68 ± 0.10

**Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

Table 9. *B. lactis* cfu recorded from microcapsules, released from the matrix by HPUS using the Vibracell Ultrasonic Processor VCX 750 (Refer Appendix D).

Experiment number	Log_{10} cfu.g^{-1}
1	12.01 ± 0.05**
2	11.91 ± 0.09
3	11.90 ± 0.03

**Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

Table 10. *B. lactis* cfu recorded from untreated 1 ml free cell concentrate (Refer Appendix E).

Experiment number	Log_{10} cfu.ml^{-1} concentrate
1	12.54 ± 0.14
2	11.61 ± 0.11

**Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

Table 11. *B. lactis* cfu recorded from 1 ml free cell concentrate, treated with HPUS using the Vibracell Ultrasonic Processor VCX 750 (Refer Appendix E).

Experiment number	Log_{10} cfu.ml^{-1} concentrate
1	12.07 ± 0.06**
2	11.85 ± 0.06

**Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

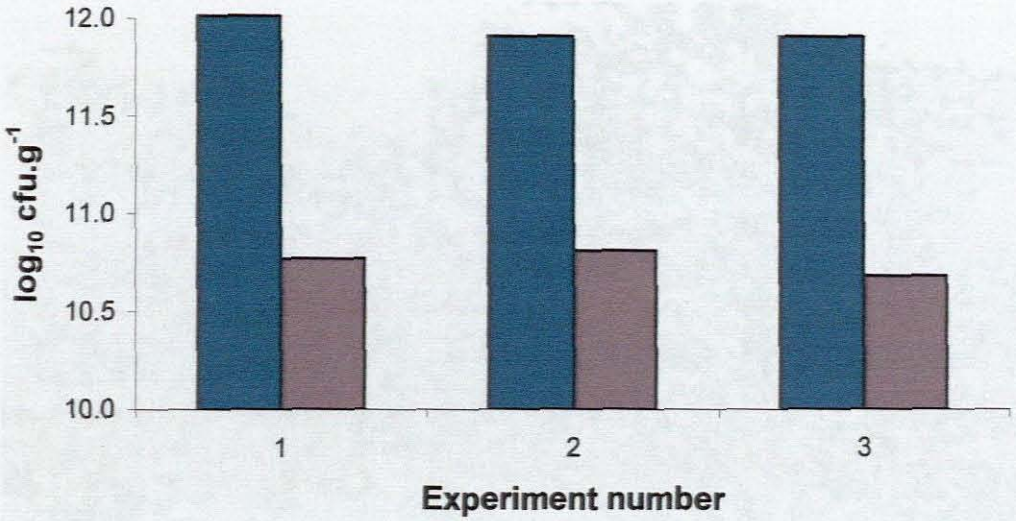

Figure 15. Comparison of enumeration techniques used to extract immobilised *B. lactis* in 1M sodium phosphate buffer (pH 7.0) at 22^0C. Ultrasound, ■, and pestle and mortar, ■ (Refer Tables 8 and 9; Appendix C).

Results indicated that when using the pestle and mortar, the gel matrix never entirely disintegrated and many bacterial cells remained trapped (Refer Figure 16). The cluster of cells observed in Figure 16 would grow only as a single colony, and not as numerous colonies had the cells been free of the matrix (Refer Figure 17). In the three experiments reported above, an average of $\log_{10}10$ cfu.g^{-1} *B. lactis* cells was released using the pestle and mortar technique (Refer Table 8; Figure 15).

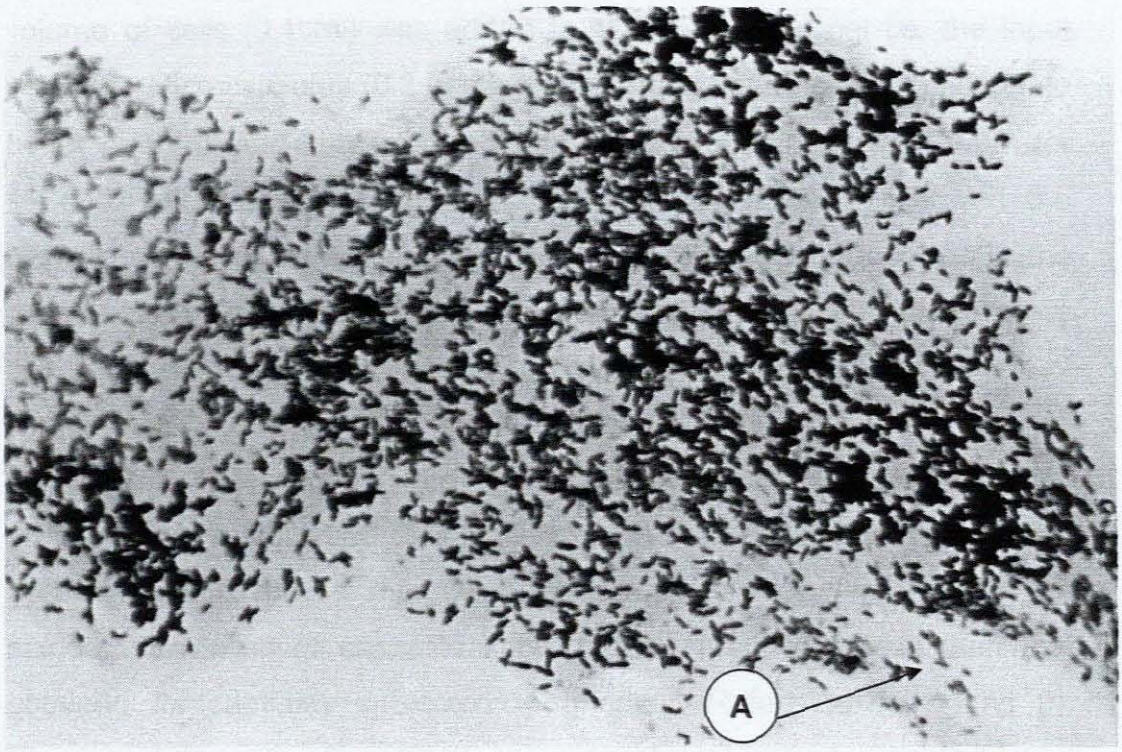

Figure 16. *B. lactis* cells trapped within the gellan-xanthan gel matrix (A), viewed at 500x magnification, after treatment with a pestle and mortar.

Having used the pestle and mortar, Sun and Griffiths (2000) reported that 1 ml of microcapsules, containing a known number of viable cells, was consistently lower than expected. In that study, equal numbers of both immobilised and free *B. lactis* cells underwent identical pestle and mortar treatment. The average cfu for the immobilised cells was $\log_{10}$ 9 cfu.ml^{-1}, while the average cfu for free cells was $\log_{10}$ 9.15 cfu.ml^{-1}. Hence, a decline in cell number was noted for the immobilised bacterial cells, possibly due to entrapment of the cells within the gel microcapsule matrix, as was observed in this study (Refer Figure 16).

The use of the Vibracell Ultrasonic Processor VCX 750 to release and detach viable bacterial cells from the encapsulating gel matrix was successful. At startup, the number of *B. lactis* in the washed cell

concentrate averaged $\log_{10}$ 11.7 cfu 0.1 ml^{-1} (Refer Table 10). This volume of cells (0.1 ml) was added to 1 g of moulten gel i.e. the input volume. Microcapsules (0.2 g) were suspended in 1 M sodium phosphate buffer (pH 7.0) and subjected to a sound wave amplitude of 105 μm for 15 s. Microscopic examination after ultrasound clearly showed the bifidobacteria free of disintegrated gel matrix (Refer Figure 17). In the three experiments reported above, an average of $\log_{10}$ 11.9 $cfu.g^{-1}$ were obtained after using HPUS (Refer Table 9). Therefore, recovery levels of immobilised *B. lactis* after sonication were similar to the input cell numbers. The recovery rate of 103% could be explained by uneven distribution of immobilised cells in the matrix. The cell recovery rate using HPUS was more reliable than when a pestle and mortar were used i.e. the standard deviation of capsules subjected to HPUS was less than that obtained for capsules subjected to grinding (Refer Tables 8 and 9). Staining of the cells after HPUS, using the Gram method, indicated intact Gram-positive bacteria with the typical pleomorphic bifidus morphology.

Hence, in this study, counts confirmed that cells were not fully released from the gum matrix, where the pestle and mortar was used. Values obtained by the latter method were consistently lower ($\log_{10}$ 10 $cfu.g^{-1}$) than those obtained after HPUS treatment ($\log_{10}$ 11.9 $cfu.g^{-1}$) from the same batch of microcapsules. In addition HPUS is a rapid process and reduces contamination risk, unlike the lengthy pestle and mortar procedure. It was therefore decided to use the HPUS method for quantitative assessment of immobilised *B. lactis*, for the duration of this study.

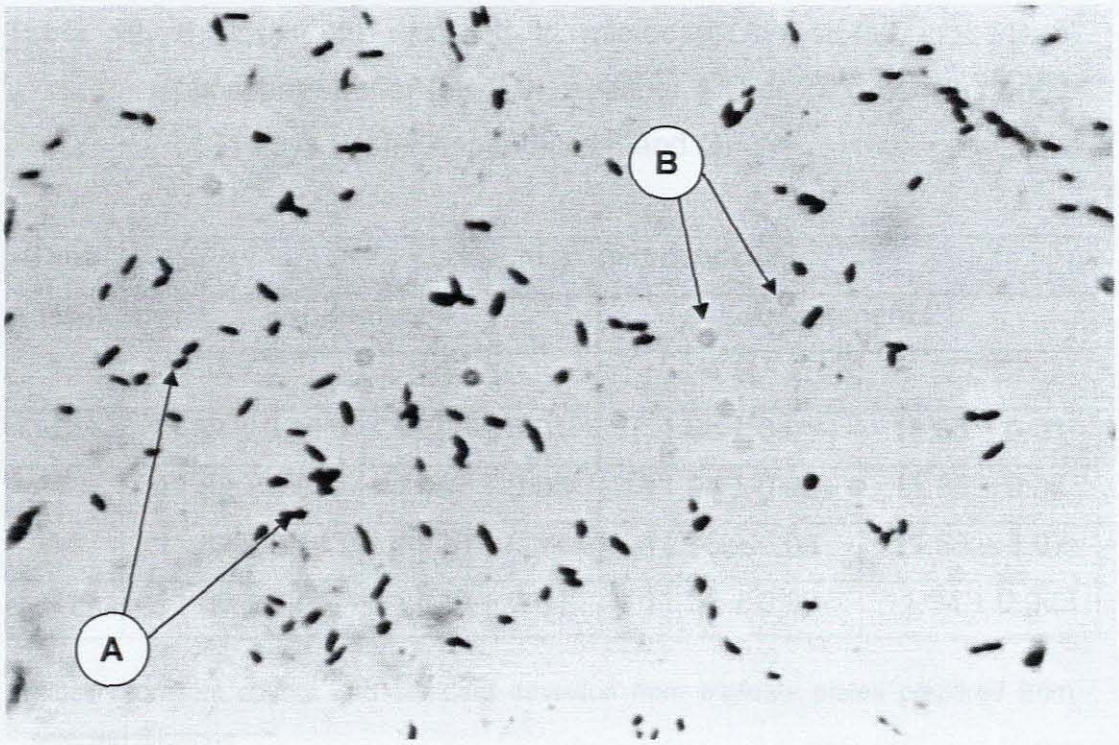

Figure 17. *B. lactis* cells (Gram-positive) released from the gellan-xanthan gel matrix, viewed at 500x magnification, after treatment with HPUS using the Vibracell Ultrasonic Processor VCX 750.

A = *B. lactis* cells

B = microcapsule gel fragments

4.6 Survival of immobilised *B. lactis* in 1 M sodium phosphate buffer (pH 7):

The viability of encapsulated *B. lactis* in buffer was examined as a means of supplying industry with microcapsules for addition to foods. Therefore, *B. lactis* was immobilised, and the capsules were stored in 1 M sodium phosphate buffer for 21 days at either 4^0C or 22^0C in the presence of atmospheric oxygen. Viable counts were done at start-up, 7, 14 and 21 days (Refer 3.7; Table 12; Figures 18 and 19).

Table 12. *B. lactis* cfu recorded in microcapsules stored in sodium phosphate buffer (pH 7.0), at either 4^0C or 22^0C over a period of 21 days (Refer Appendices F and G)

Time (days)	Log_{10} cfu.g^{-1} microcapsules			
	Experiment 1		Experiment 2	
	4°C	22°C	4°C	22°C
0	11.83 ± 0.01*	11.83 ± 0.01	11.66 ± 0.01	11.66 ± 0.01
7	11.83 ± 0.07	11.67 ± 0.05	11.94 ± 0.03	11.35 ± 0.04
14	11.82 ± 0.14	11.38 ± 0.14	11.85 ± 0.06	11.55 ± 0.07
21	11.60 ± 0.05	11.29 ± 0.01	11.53 ± 0.02	11.34 ± 0.003

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

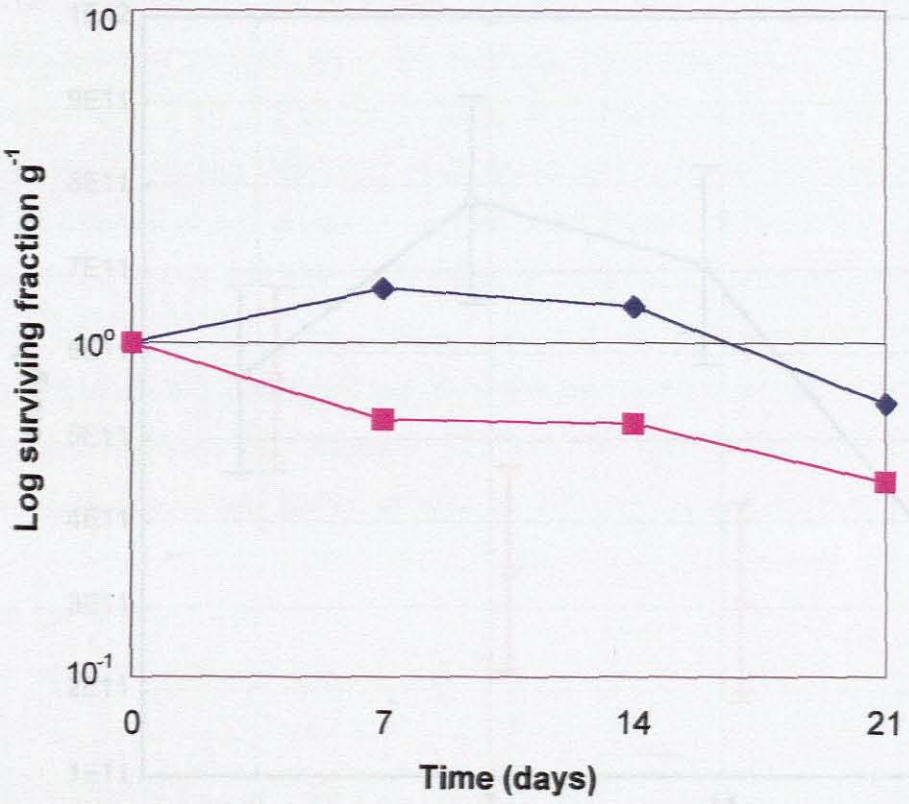

Figure 18. Log surviving fraction of immobilised *B. lactis* cells stored aerobically in sodium phosphate buffer (pH 7.0) at 4^0C, ♦, or 22^0C, ■, over 21 days. Each point on the graph represents values taken from six plate counts, at the selected dilution, from two experiments (Refer Table 12; Appendix H).

Average standard deviation $\log_{10}$ cfu ≤ 0.15.

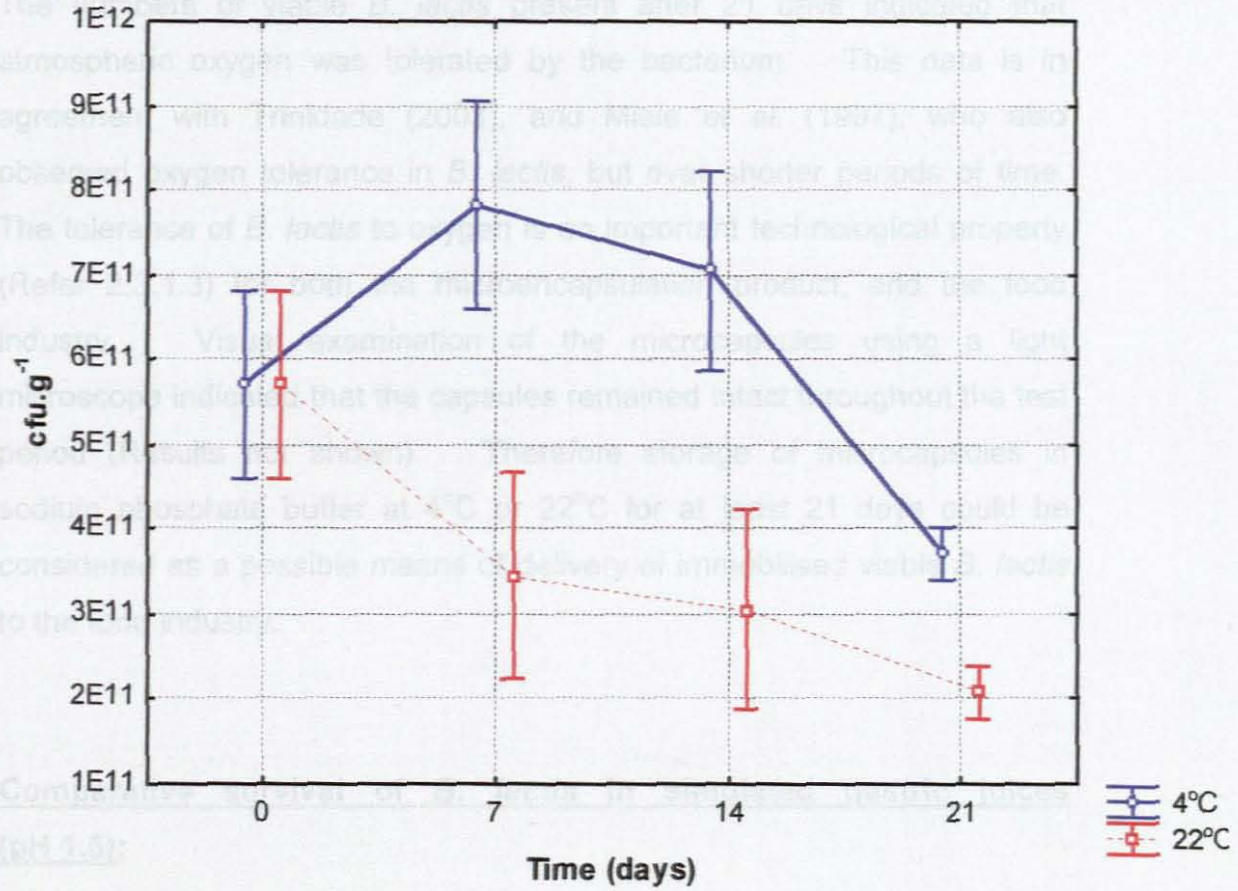

Figure 19. The survival of *B. lactis** immobilised in gellan-xanthan gum mix, stored aerobically in sodium phosphate buffer (pH 7.0) at either 4°C or 22°C over 21 days (Vertical bars denote 0.95 confidence intervals) (Refer Table 12; Appendices F and G).

*Counts expressed as $cfu.g^{-1}$ microcapsules

(1E11 = 1×10^{11}; E = x 10)

The decline in viability of immobilised *B. lactis* cells stored over the 21-day test period, in 1 M sodium phosphate buffer solution in aerobic conditions at both 4°C and 22°C was not significant ($p>0.05$). Survival at 4°C was slightly improved over storage at 22°C (Refer Figures 18 and 19). The bacterial cell number remained above $\log_{10}$ 11 $cfu.g^{-1}$ throughout the test period (Refer Table 12).

The numbers of viable *B. lactis* present after 21 days indicated that atmospheric oxygen was tolerated by the bacterium. This data is in agreement with Trinidade (2003), and Miele *et al.* (1997), who also observed oxygen tolerance in *B. lactis*, but over shorter periods of time. The tolerance of *B. lactis* to oxygen is an important technological property (Refer 2.3.1.3) for both the microencapsulation product, and the food industry. Visual examination of the microcapsules using a light microscope indicated that the capsules remained intact throughout the test period (Results not shown). Therefore storage of microcapsules in sodium phosphate buffer at 4°C or 22°C for at least 21 days could be considered as a possible means of delivery of immobilised viable *B. lactis* to the food industry.

4.7 Comparative survival of *B. lactis* in simulated gastric juices (pH 1.5):

To protect bifidobacteria from the acidic conditions in foods and the human stomach, the microcapsules must be acid-resistant. In this study, the viability of both *B. lactis* immobilised in gellan-xanthan microcapsules, and as free cells, was evaluated in simulated gastric juice (SGJ) over a period of 240 min at 37°C. Viable counts were done at start-up, 60, 120 and 240 min (Refer 3.8; Tables 13 and 14; Figures 20, 21a and 21b).

Table 13. *B. lactis* cfu recorded in microcapsules in SGJ (pH 1.5), at 37°C over a period of 240 min (Refer Appendices I and J)

Time (min)	Log_{10} cfu.g^{-1} microcapsules	
	Experiment 1	Experiment 2
0	12.24 ± 0.03*	12.37 ± 0.05
60	11.41 ± 0.07	12.36 ± 0.23
120	11.15 ± 0.06	12.14 ± 0.18
240	10.21 ± 0.05	11.67 ± 0.04

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

Table 14. The numbers of *B. lactis* cfu recorded for free cells, in SGJ (pH 1.5), at 37°C over a period of 240 min (Refer Appendices I and J)

Time (min)	Log_{10} cfu.ml^{-1} free cells	
	Experiment 1	Experiment 2
0	9.36 ± 0.22*	9.58 ± 0.06
60	8.26 ± 0.11	8.98 ± 0.02
120	8.04 ± 0.04	8.26 ± 0.06
240	6.72 ± 0.03	6.43 ± 0.17

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

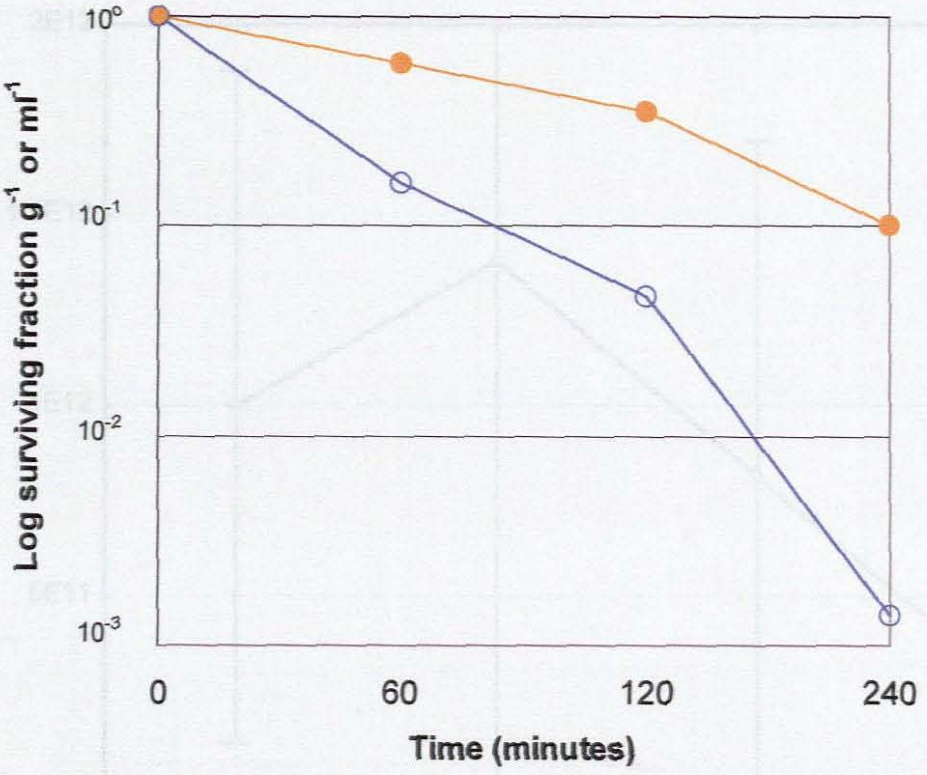

Figure 20. Log surviving fraction of immobilised *B. lactis* cells, ●, and free cells, ○, immersed in simulated gastric juice (pH 1.5) at 37°C over 240 min. Each point on the graph represents values taken from six plate counts, at the selected dilution, from two experiments (Refer Tables 13 and 14; Appendix K). Average standard deviation $\log_{10}$ cfu ≤ 0.18.

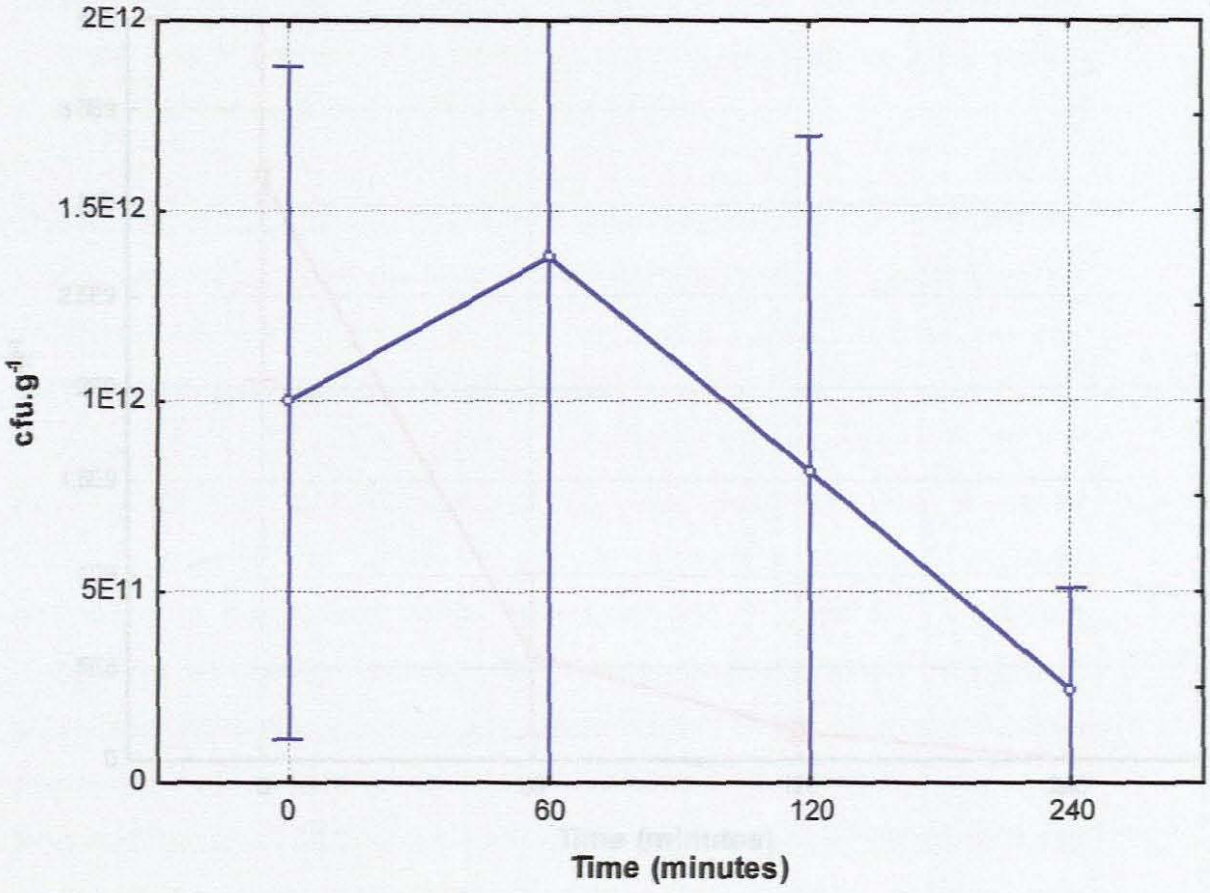

Figure 21a. The survival of *B. lactis* microcapsules[m], immersed in simulated gastric juice (pH 1.5) at 37°C over 240 min (Vertical bars denote 0.95 confidence intervals) (Refer Table 13; Appendices I and J).

[m]Counts expressed as cfu.g^{-1} microcapsules

(5 E 11 = 5 x 10^{11}; E = x 10)

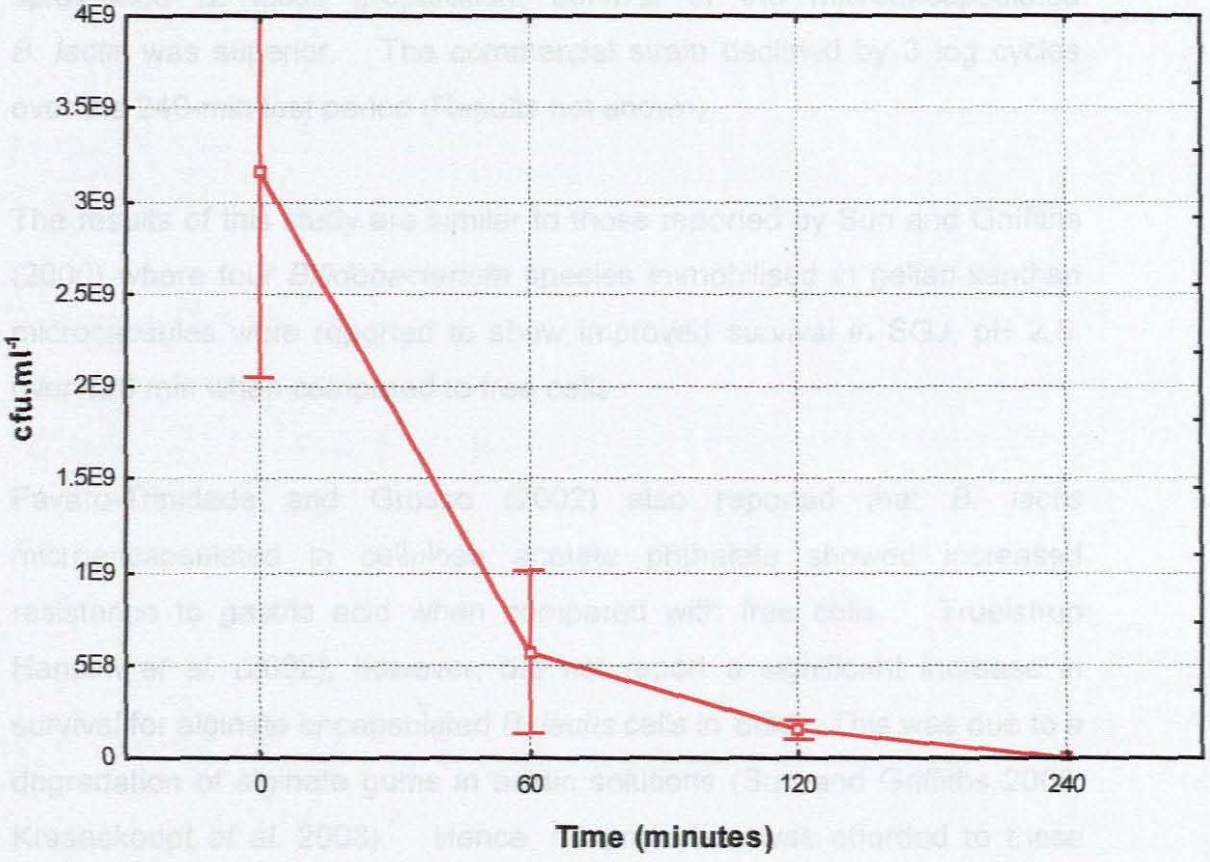

Figure 21b. The survival of *B. lactis* free cells[f], immersed in simulated gastric juice (pH 1.5) at 37°C over 240 min (Vertical bars denote 0.95 confidence intervals) (Refer Table 14; Appendices I and J).

[f]Counts expressed as cfu.ml^{-1} free cells
(5 E 8 = 5 x 10^8; E = x 10)

Survival of immobilised *B. lactis* was shown to be significantly improved ($p<0.05$) over that of free cells in SGJ (0.08M HCl + 0.2% w/v NaCl) (pH 1.5) (Refer Tables 13 and 14; Figures 20, 21a and 21b). After 240 min in SGJ, viable immobilised cfu decreased by 1 log cycle, whereas free cells declined significantly by 3 log cycles ($p<0.05$) (Refer Figures 20, 21a and 21b).

In addition, when *B. lactis*, used in this study, was immobilised in gellan-xanthan gum, and compared with a commercially available

spray-dried *B. lactis* preparation, survival of the microencapsulated *B. lactis* was superior. The commercial strain declined by 3 log cycles over the 240-min test period (Results not shown).

The results of this study are similar to those reported by Sun and Griffiths (2000) where four *Bifidobacterium* species immobilised in gellan-xanthan microcapsules were reported to show improved survival in SGJ, pH 2.5, over 120 min when compared to free cells.

Favaro-Trinidade and Grosso (2002) also reported that *B. lactis* microencapsulated in cellulose acetate phthalate showed increased resistance to gastric acid when compared with free cells. Truelstrup Hansen *et al.* (2002), however, did not report a significant increase in survival for alginate encapsulated *B. lactis* cells in SGJ. This was due to a degradation of alginate gums in acidic solutions (Sun and Griffiths 2000; Krasaekoopt *et al.* 2003). Hence, no protection was afforded to these bifidobacteria cells, and alginate should be avoided as the sole immobilising gum for probiotics.

In this study, simulated gastric conditions showed that the microencapsulation of *B. lactis* cells in gellan-xanthan gum significantly enhances survival (Refer Tables 13 and 14; Figures 20, 21a and 21b). Therefore, it is likely that *in situ*, gellan-xanthan microcapsules protect *B. lactis* from the adverse conditions associated with gastric juices.

4.8 Comparative survival of *B. lactis* in simulated bile and pancreatic juices (pH 6.5):

The viability of both *B. lactis* immobilised in gellan-xanthan microcapsules, or as free cells, was evaluated in simulated bile and pancreatic juice (BPJ) over a period of 240 min at 37^0C. Viable counts were done at start-up, 60, 120 and 240 min (Refer 3.9; Tables 15 and 16; Figures 22, 23a and 23b).

Table 15. *B. lactis* cfu recorded in microcapsules in BPJ (pH 6.5), at 37°C over a period of 240 min (Refer Appendices L and M)

Time (min)	Log_{10} cfu.g^{-1} microcapsules	
	Experiment 1	Experiment 2
0	12.31 ± 0.07*	12.38 ± 0.09
60	12.45 ± 0.10	12.39 ± 0.03
120	12.02 ± 0.04	12.49 ± 0.12
240	11.93 ± 0.04	12.70 ± 0.04

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

Table 16. The numbers of *B. lactis* cfu recorded for free cells, in BPJ (pH 6.5), at 37°C over a period of 240 min (Refer Appendices L and M)

Time (min)	Log_{10} cfu.ml^{-1} free cells	
	Experiment 1	Experiment 2
0	9.80 ± 0.14*	9.87 ± 0.03
60	9.23 ± 0.07	9.69 ± 0.05
120	9.02 ± 0.20	9.51 ± 0.09
240	8.95 ± 0.08	9.48 ± 0.01

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

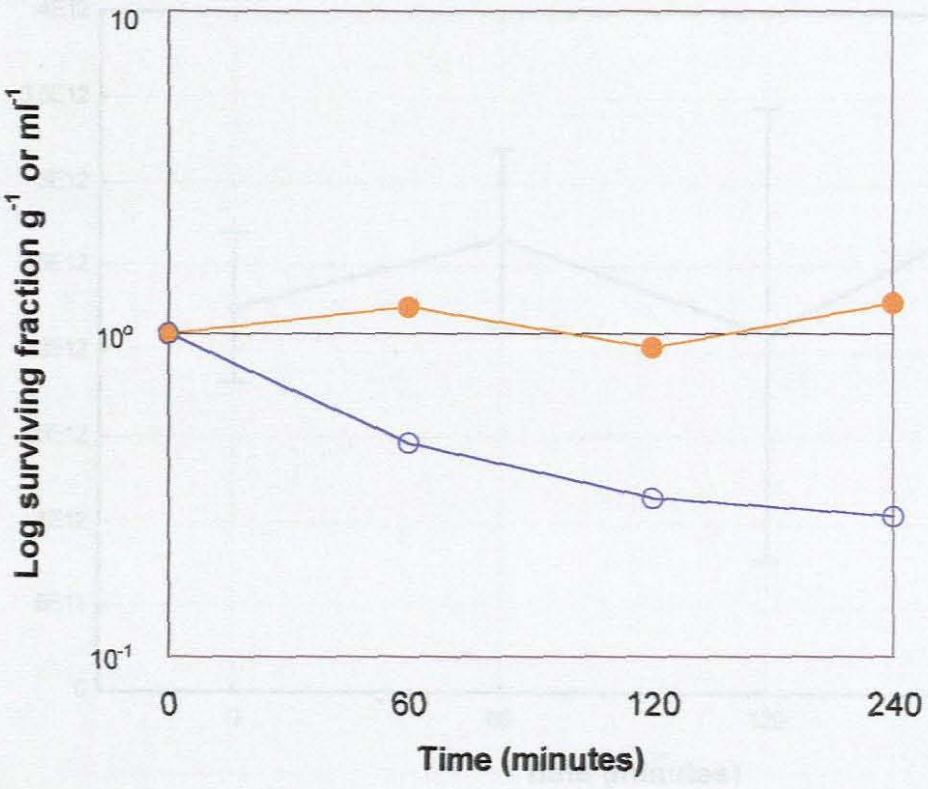

Figure 22. Log surviving fraction of immobilised *B. lactis* cells, ●, and free cells, ○, immersed in simulated bile and pancreatic solution (pH 6.5) at 37°C over 240 min. Each point on the graph represents values taken from six plate counts, at the selected dilution, from two experiments (Refer Tables 15 and 16; Appendix N).

Average standard deviation $\log_{10}$ cfu ≤ 0.15.

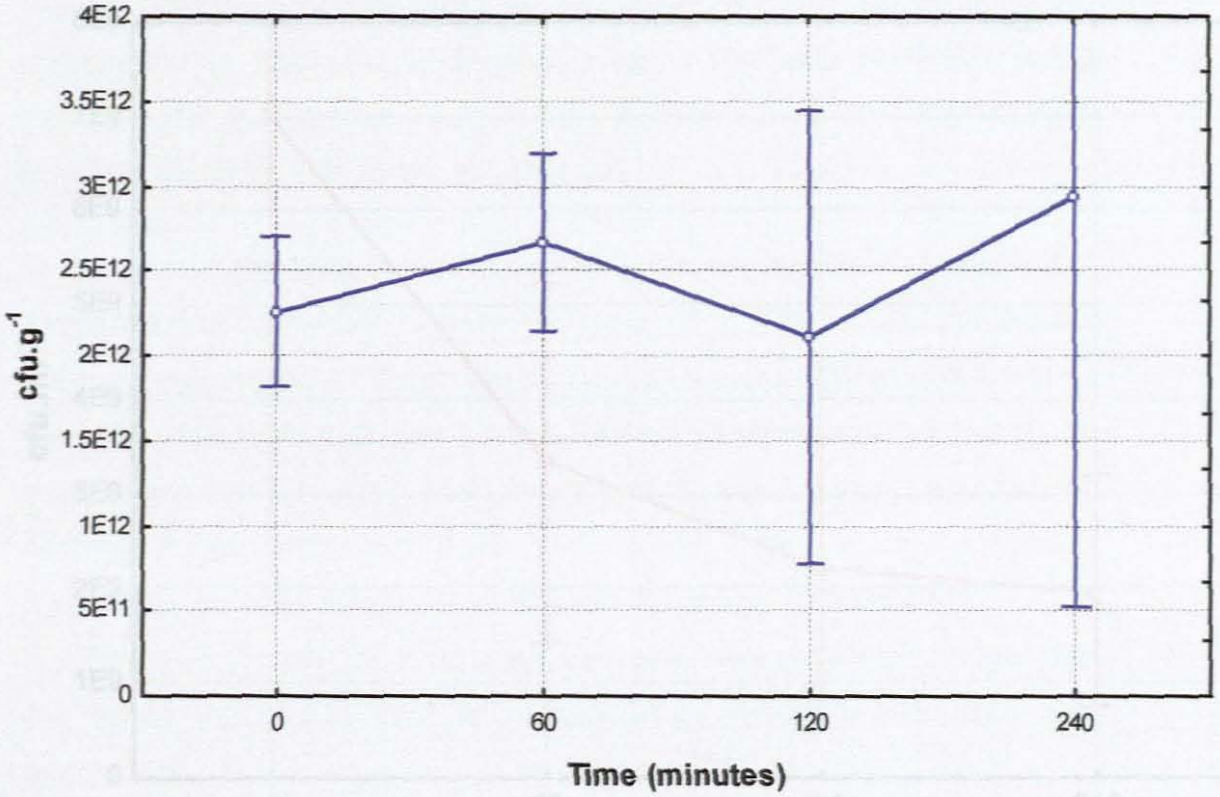

Figure 23a. The survival of *B. lactis* microcapsules[m], immersed in simulated bile and pancreatic solution (pH 6.5) at 37°C over 240 min (Vertical bars denote 0.95 confidence intervals) (Refer Table 15; Appendices L and M).

[m]Counts expressed as cfu.g^{-1} microcapsules

(1 E 12 = 1 x 10^{12}; E = x 10)

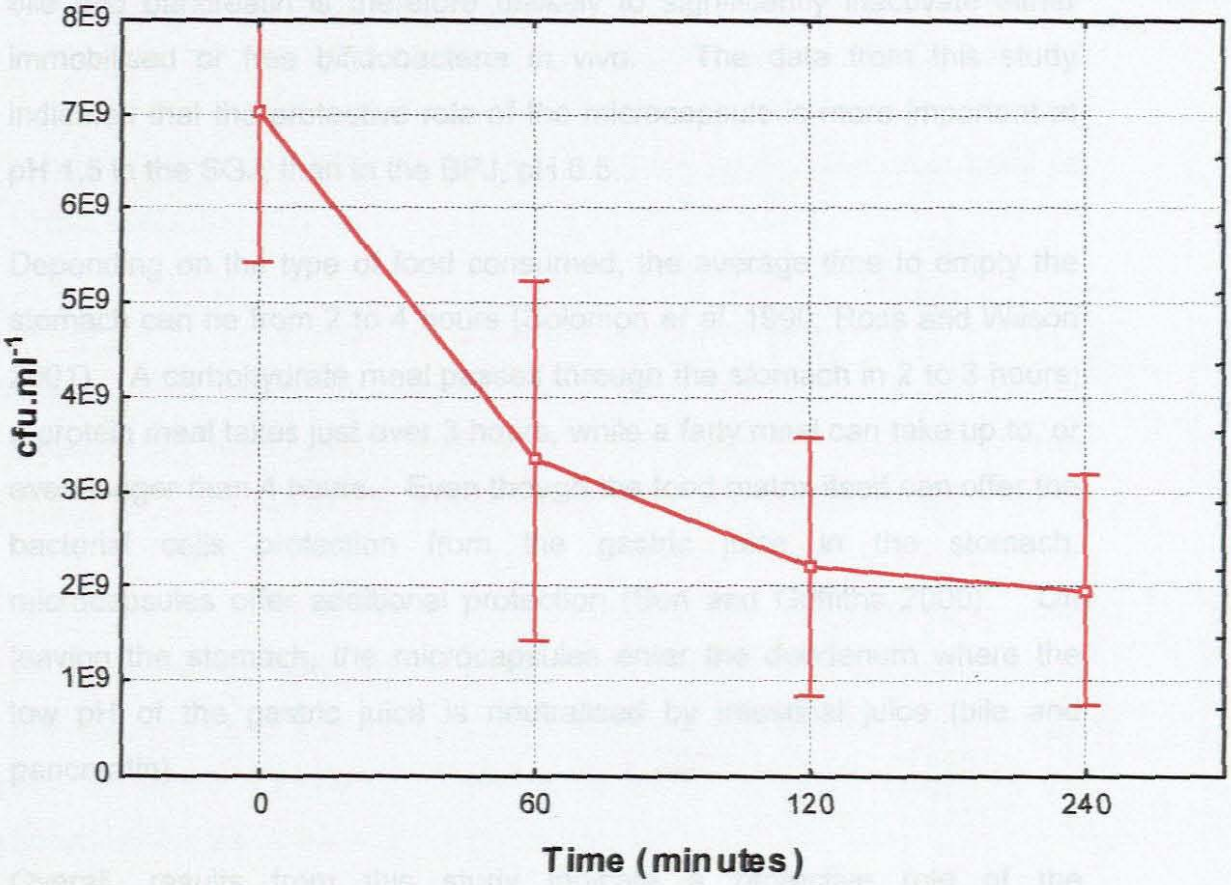

Figure 23b. The survival of *B. lactis* free cells[f], immersed in simulated bile and pancreatic solution (pH 6.5) at 37°C over 240 min (Vertical bars denote 0.95 confidence intervals) (Refer Table 16; Appendices L and M).

[f]Counts expressed as cfu.ml^{-1} free cells
(1 E 9 = 1 x 10^9; E = x 10)

Once having traversed and survived the acidic gastric conditions in the stomach, the probiotic organism encounters the BPJ. In simulated BPJ, pH 6.5, microencapsulated cells showed slightly improved survival rates over those obtained for free cells, over a time period of 4 h (Refer Tables 15 and 16; Figures 22, 23a and 23b).

Favaro-Trinidade and Grosso (2002) reported a similar resistance of both the microencapsulated and free *B. lactis* cells to bile. The presence of

bile and pancreatin is therefore unlikely to significantly inactivate either immobilised or free bifidobacteria *in vivo*. The data from this study indicated that the protective role of the microcapsule is more important at pH 1.5 in the SGJ, than in the BPJ, pH 6.5.

Depending on the type of food consumed, the average time to empty the stomach can be from 2 to 4 hours (Solomon *et al.* 1990; Ross and Wilson 2001). A carbohydrate meal passes through the stomach in 2 to 3 hours; a protein meal takes just over 3 hours, while a fatty meal can take up to, or even longer than 4 hours. Even though the food matrix itself can offer the bacterial cells protection from the gastric juice in the stomach, microcapsules offer additional protection (Sun and Griffiths 2000). On leaving the stomach, the microcapsules enter the duodenum where the low pH of the gastric juice is neutralised by intestinal juice (bile and pancreatin).

Overall, results from this study indicate a protective role of the microcapsules, permitting delivery of viable organisms to the colon. It is likely that in the colon, shear stress of gellan-xanthan gum calculated for this study is such that peristaltic movement of the colon could break the capsules open (Refer 4.2), thereby releasing viable *B. lactis*. It was also apparent from this study that the commercial freeze / spray-dried *B. lactis* tested was not adequately protected against SGJ. Hence, for both survival and delivery of viable *B. lactis* to the human colon, microencapsulation in gellan-xanthan gum is recommended.

4.9 Comparative shelf life studies of *B. lactis* in fermented African beverages:

In this study, shelf-life studies of free and immobilised *B. lactis* added to both pasteurised commercial amasi and mahewu, over a 21-day period, were carried out (Refer 3.10). A total of two independent experiments was run (Refer Tables 17 – 20; Appendices O and P). The microcapsules

and free cells were added after the fermentation and pasteurisation of the fermented commercial beverages. Regular testing throughout the experimental period showed there to be no contamination of the microcapsules, or of the beverages enriched with either free cells or microcapsules.

Within the microcapsules added to the beverages, immobilised *B. lactis* cell numbers varied between log_{10} 9-12 cfu.g^{-1} microcapsule, and when added as free cells, the cells concentration varied between log_{10} 8-9 cfu.ml^{-1} beverage (Refer Tables 17 - 20). *Bifidobacterium lactis* used was harvested from the stationary phase of growth as described (Refer 3.4). Variables included temperature of the carrier medium during storage, and the presence of atmospheric oxygen. Also important were the differing intrinsic properties of the beverages (Refer 2.4.1 and 2.4.2). The pH of both amasi and mahewu remained constant throughout the shelf-life studies, at 4.4 and 3.2 respectively.

Table 17. *B. lactis* cfu recorded in microcapsules stored in amasi (pH 4.4), in the presence of atmospheric oxygen, at either 4°C or 22°C over a period of 21 days (Refer Appendices O and P)

Time (days)	Log_{10} cfu.g^{-1} microcapsules			
	Experiment 1		Experiment 2	
	4°C	22°C	4°C	22°C
0	12.33 ± 0.10*	12.33 ± 0.10	12.97 ± 0.09	12.97 ± 0.09
7	12.34 ± 0.07	12.15 ± 0.06	12.18 ± 0.06	12.41 ± 0.04
14	11.54 ± 0.04	11.44 ± 0.09	11.94 ± 0.01	12.03 ± 0.10
21	11.24 ± 0.01	11.15 ± 0.08	11.61 ± 0.07	11.48 ± 0.04

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

Table 18a. The numbers of *B. lactis* cfu recorded for free cells stored in amasi (pH 4.4), in the presence or absence of atmospheric oxygen, at either 4°C or 22°C over a period of 21 days (Refer Appendix O)

Time (days)	Log_{10} cfu.ml^{-1} free cells		
	Experiment 1		
	4°C	22°C	
	+ O_2	+ O_2	- O_2
0	7.31 ± 0.04*	7.20 ± 0.08	7.70 ± 0.14
7	7.19 ± 0.05	7.16 ± 0.07	7.04 ± 0.07
14	7.05 ± 0.05	7.05 ± 0.08	6.95 ± 0.08
21	6.94 ± 0.06	6.88 ± 0.05	6.90 ± 0.01

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

Table 18b. The numbers of *B. lactis* cfu recorded for free cells stored in amasi (pH 4.4), in the presence or absence of atmospheric oxygen, at either 4°C or 22°C over a period of 21 days (Refer Appendix P)

Time (days)	Log_{10} cfu.ml^{-1} free cells		
	Experiment 2		
	4°C	22°C	
	+ O_2	+ O_2	- O_2
0	9.39 ± 0.06*	8.68 ± 0.61	8.73 ± 0.34
7	8.87 ± 0.04	8.40 ± 0.28	8.19 ± 0.10
14	8.22 ± 0.13	8.10 ± 0.60	7.46 ± 0.04
21	7.87 ± 0.13	5.99 ± 0.13	5.89 ± 0.07

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

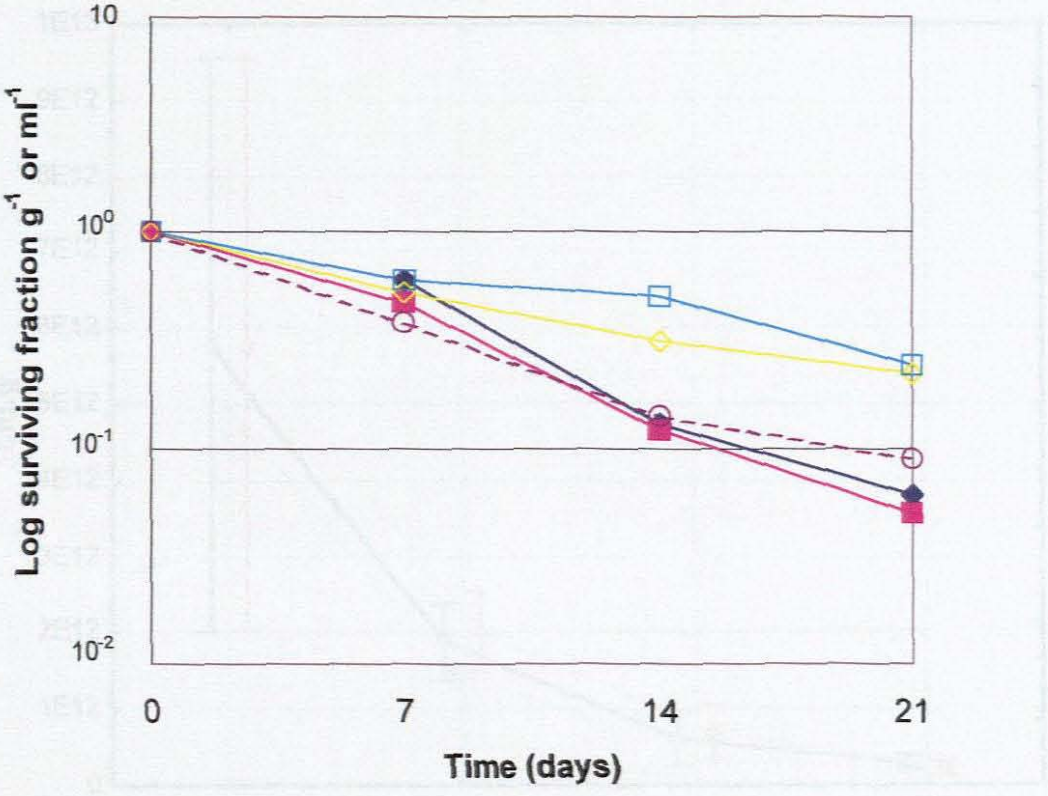

Figure 24. Log surviving fraction of immobilised *B. lactis* cells stored aerobically at 4^0C, ♦, and 22^0C, ■, and free *B. lactis* cells stored aerobically at 4^0C, ◊, and 22^0C, □, or anaerobically at 22^0C,--○--, in amasi (pH 4.4) over 21 days. Each point on the graph represents values taken from six plate counts, at the selected dilution, from two experiments (Refer Tables 17, 18a and 18b; Appendix Q). Average standard deviation $\log_{10}$ cfu ≤ 0.6.

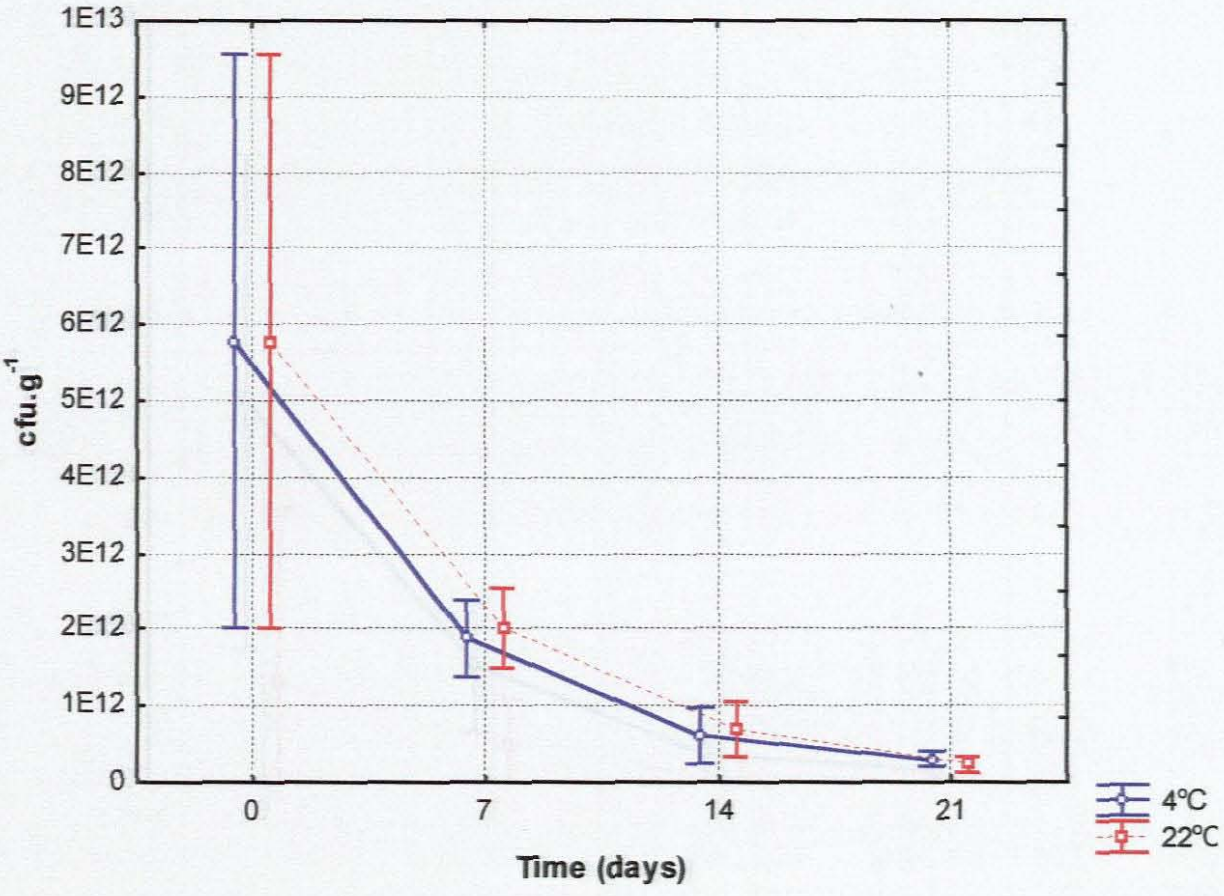

Figure 25. Comparison of *B. lactis** cfu immobilised in gellan-xanthan gum, stored aerobically in amasi (pH 4.4) at either 4°C or 22°C over 21 days (Vertical bars denote 0.95 confidence intervals) (Refer Table 17; Appendices O and P).

*Counts expressed as $cfu.g^{-1}$ microcapsules

(1 E 12 = 1 x 10^{12}; E = x 10)

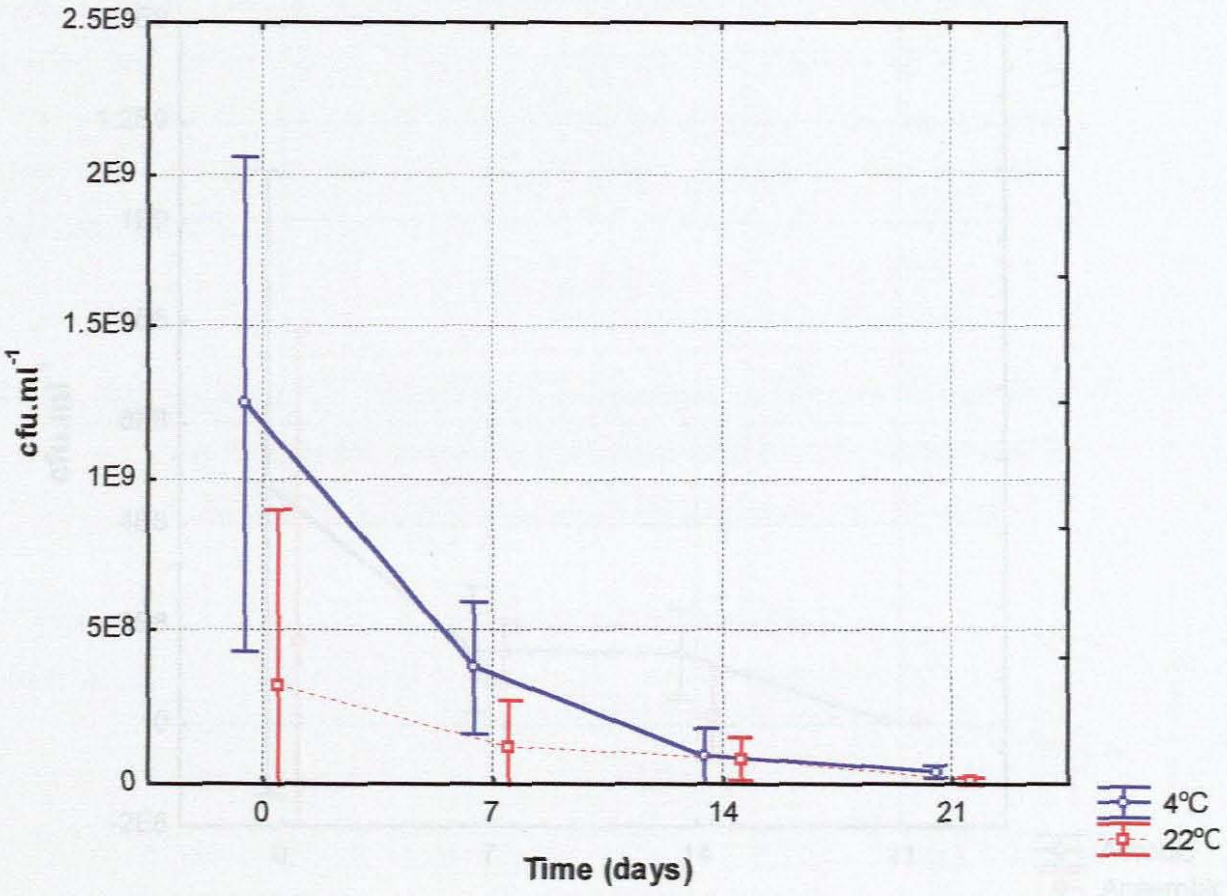

Figure 26. Comparison of *B. lactis** free cells, stored aerobically in amasi (pH 4.4) at either 4°C or 22°C over 21 days (Vertical bars denote 0.95 confidence intervals) (Refer Tables 18a and 18b; Appendices O and P).

*Counts expressed as cfu.ml^{-1} free cells

(5 E 8 = 5 x 10^8; E = x 10)

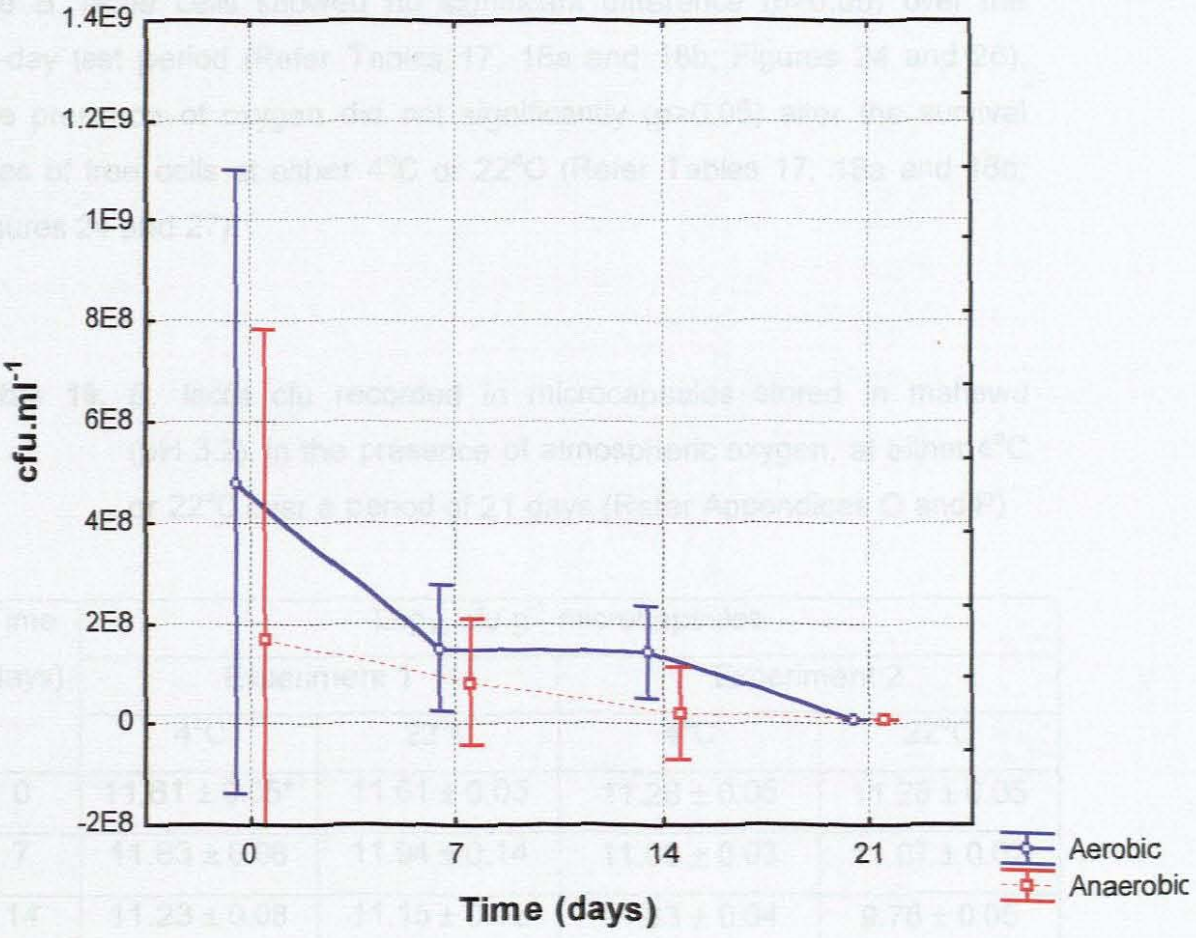

Figure 27. Comparison of *B. lactis** free cells, stored aerobically or anaerobically in amasi (pH 4.4) at 22°C over 21 days (Vertical bars denote 0.95 confidence intervals) (Refer Tables 18a and 18b; Appendices O and P).

*Counts expressed as cfu.ml^{-1} free cells

(2 E 8 = 2 x 10^8; E = x 10)

A gradual decline in all cell populations not exceeding two log cycles, was noted in amasi, irrespective of treatment, over the 21-day test period (Refer Tables 17, 18a and 18b; Figures 24 - 27). Survival of microencapsulated *B. lactis* cells, in aerobic conditions, was similar at both 4°C and 22°C. The surviving fraction of these bacterial cells showed a gradual significant decline (1.2 log cycles) over the test period ($p<0.05$) (Refer Tables 17, 18a and 18b; Figures 24 and 25). Comparison of temperature influence, at both 4°C and 22°C, on microencapsulated and

free *B. lactis* cells showed no significant difference ($p>0.05$) over the 21-day test period (Refer Tables 17, 18a and 18b; Figures 24 and 26). The presence of oxygen did not significantly ($p>0.05$) alter the survival rates of free cells at either 4°C or 22°C (Refer Tables 17, 18a and 18b; Figures 24 and 27).

Table 19. *B. lactis* cfu recorded in microcapsules stored in mahewu (pH 3.2), in the presence of atmospheric oxygen, at either 4°C or 22°C over a period of 21 days (Refer Appendices O and P)

Time (days)	Log_{10} cfu.g^{-1} microcapsules			
	Experiment 1		Experiment 2	
	4°C	22°C	4°C	22°C
0	11.61 ± 0.05*	11.61 ± 0.05	11.28 ± 0.05	11.28 ± 0.05
7	11.83 ± 0.06	11.94 ± 0.14	11.09 ± 0.03	11.07 ± 0.02
14	11.23 ± 0.08	11.15 ± 0.02	11.63 ± 0.04	9.76 ± 0.05
21	10.97 ± 0.02	10.73 ± 0.04	11.55 ± 0.26	9.29 ± 0.08

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

Table 20a. The numbers of *B. lactis* cfu recorded for free cells stored in mahewu (pH 3.2), in the presence or absence of atmospheric oxygen, at either 4°C or 22°C over a period of 21 days (Refer Appendix O)

Time (days)	Log_{10} cfu.ml^{-1} free cells		
	Experiment 1		
	4°C	22°C	
	+ O_2	+ O_2	- O_2
0	7.63 ± 0.03*	7.27 ± 0.04	7.19 ± 0.07
7	7.18 ± 0.12	6.89 ± 0.07	7.12 ± 0.15
14	6.26 ± 0.05	5.73 ± 0.04	6.80 ± 0.08
21	5.24 ± 0.01	3.64 ± 0.13	6.31 ± 0.24

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

Table 20b. The numbers of *B. lactis* cfu recorded for free cells stored in mahewu (pH 3.2), in the presence or absence of atmospheric oxygen, at either 4°C or 22°C over a period of 21 days (Refer Appendix P)

Time (days)	Log_{10} cfu.ml^{-1} free cells		
	Experiment 2		
	4°C	22°C	
	+ O_2	+ O_2	- O_2
0	10.35 ± 0.05*	10.36 ± 0.08	9.72 ± 0.03
7	9.39 ± 0.08	9.18 ± 0.05	9.23 ± 0.03
14	7.35 ± 0.09	6.51 ± 0.04	7.77 ± 0.04
21	6.07 ± 0.13	5.83 ± 0.13	5.87 ± 0.05

*Values represent counts with standard deviation from triplicate plates prepared from selected dilutions

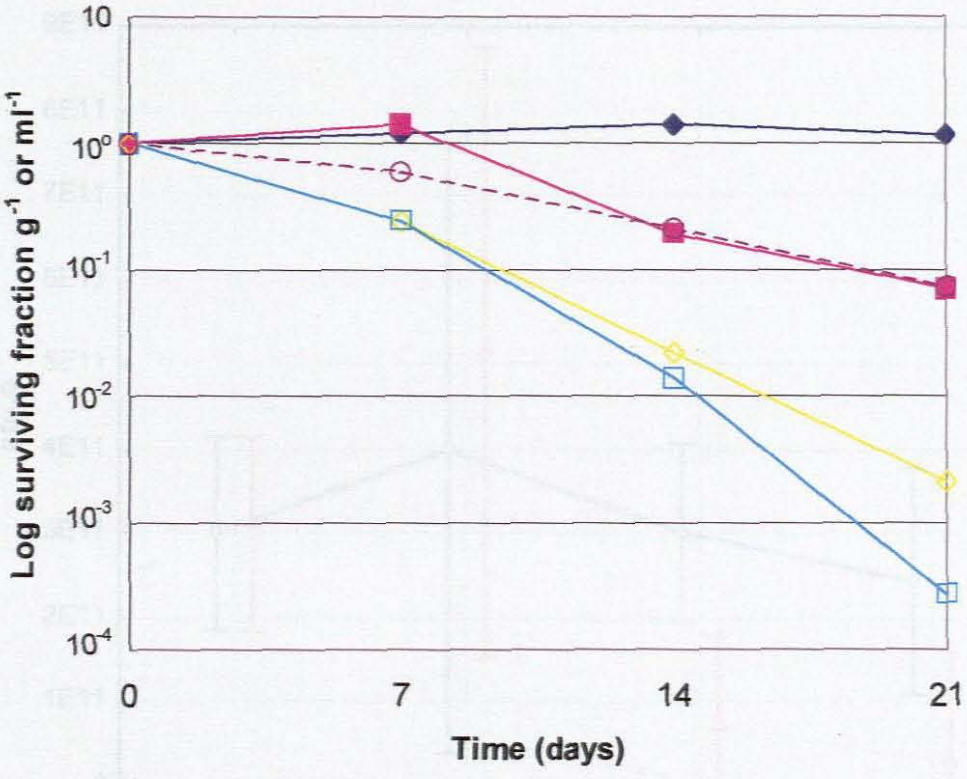

Figure 28. Log surviving fraction of immobilised *B. lactis* cells stored aerobically at 4^0C, ♦, and 22^0C, ■, and free *B. lactis* cells stored aerobically at 4^0C, ◊, and 22^0C, □, or anaerobically at 22^0C,--○--, in mahewu (pH 3.2) over 21 days. Each point on the graph represents values taken from six plate counts, at the selected dilution, from two experiments (Refer Tables 19, 20a and 20b; Appendix R). Average standard deviation $\log_{10}$ cfu ≤ 0.26.

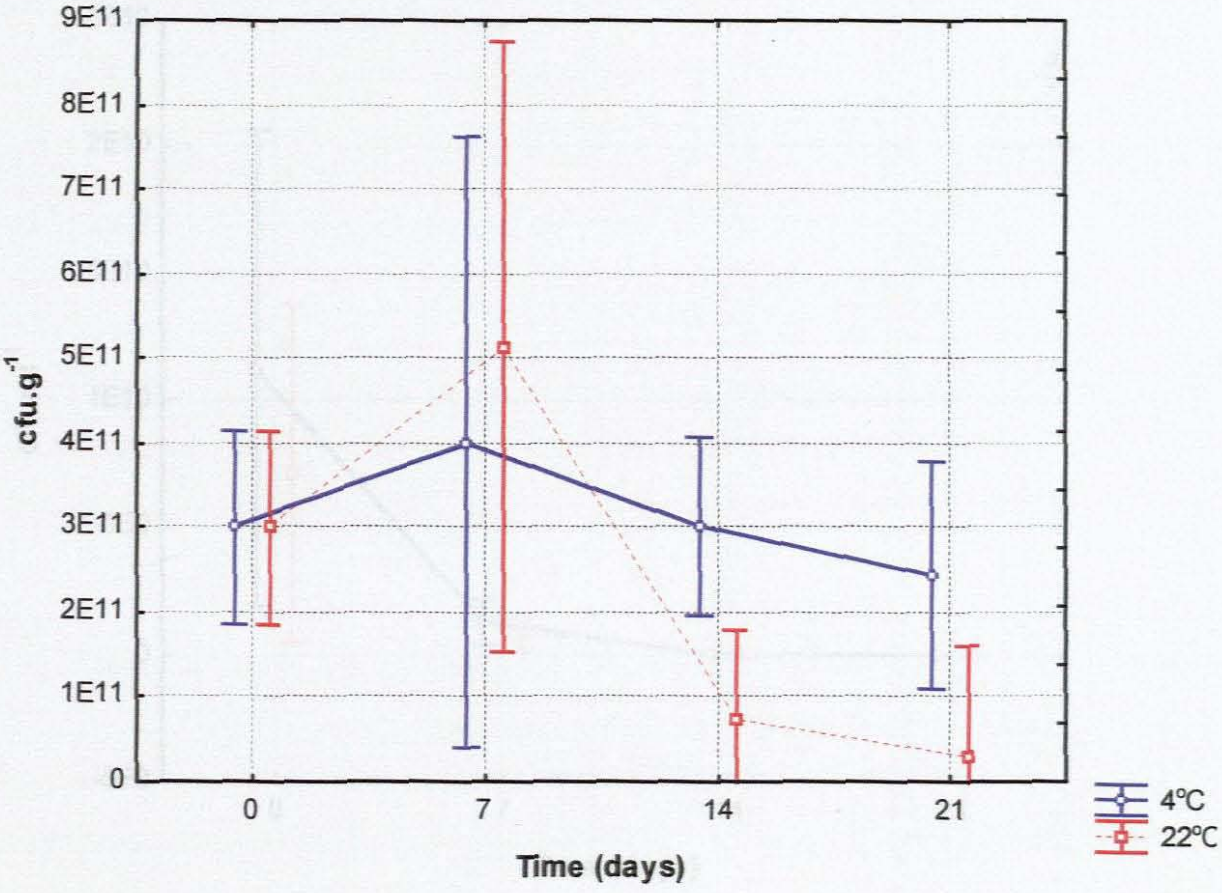

Figure 29. Comparison of *B. lactis** cfu immobilised in gellan-xanthan gum, stored aerobically in mahewu (pH 3.2) at either 4°C or 22°C over 21 days (Vertical bars denote 0.95 confidence intervals) (Refer Table 19; Appendices O and P).

*Counts expressed as $cfu.g^{-1}$ microcapsules

(1 E 11 = 1 x 10^{11}; E = x 10)

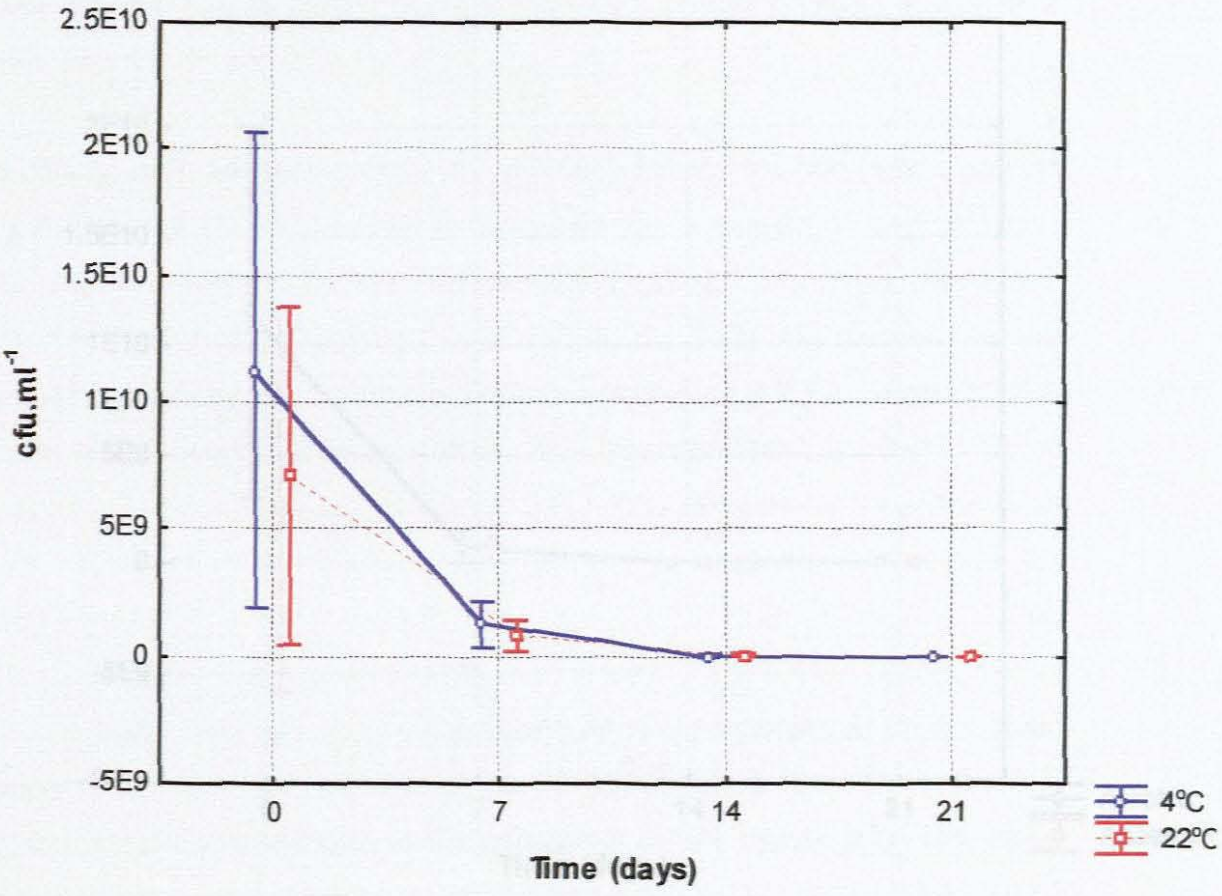

Figure 30. Comparison of *B. lactis** free cells, stored aerobically in mahewu (pH 3.2) at either 4°C or 22°C over 21 days (Vertical bars denote 0.95 confidence intervals) (Refer Tables 20a and 20b; Appendices O and P).

*Counts expressed as cfu.ml^{-1} free cells

(5 E 9 = 5 x 10^9; E = x 10)

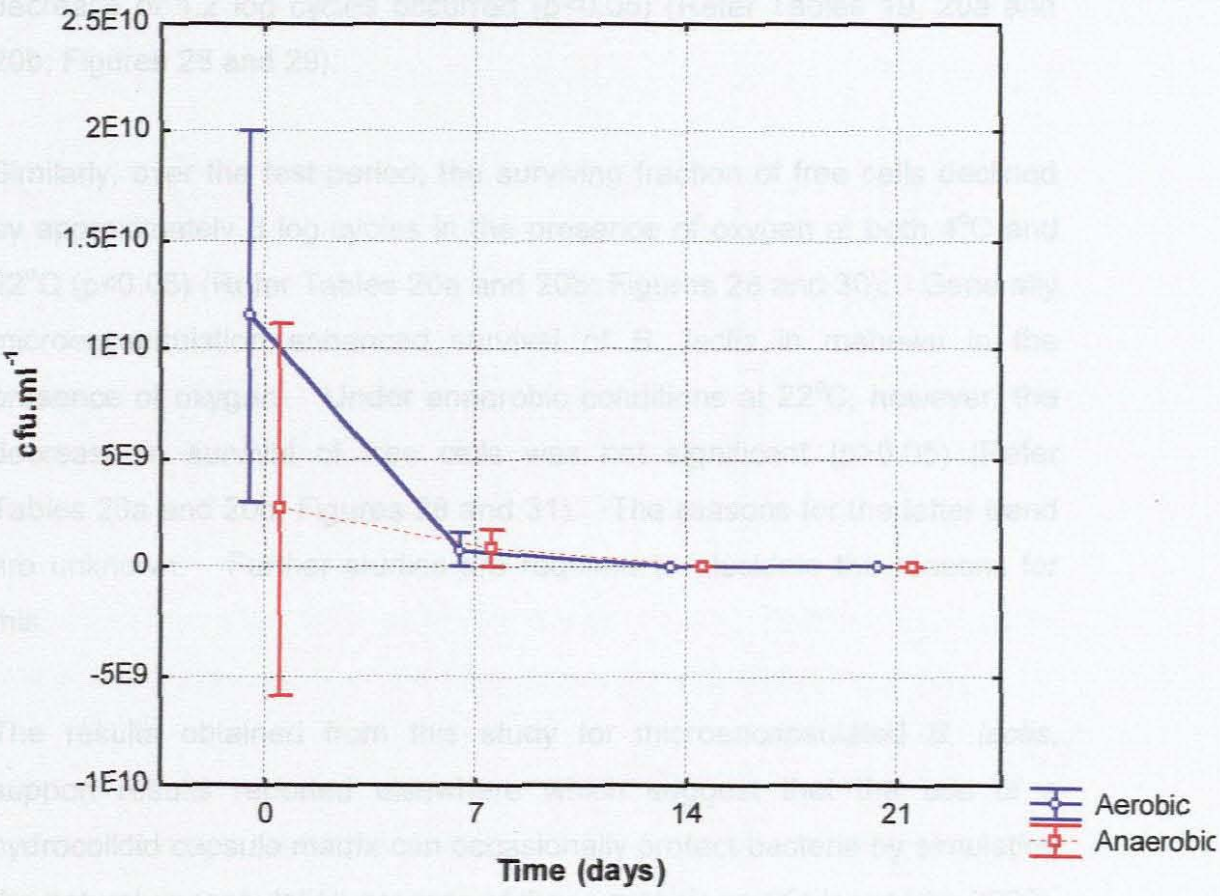

Figure 31. Comparison of *B. lactis** free cells, stored aerobically or anaerobically in mahewu (pH 3.2) at 22°C over 21 days (Vertical bars denote 0.95 confidence intervals) (Refer Tables 20a and 20b; Appendices O and P).

*Counts expressed as $cfu.ml^{-1}$ free cells
($5\ E\ 9 = 5 \times 10^9$; E = x 10)

In mahewu, the surviving fraction of *B. lactis* showed that both microencapsulation of the cells, and refrigeration at 4°C, were important for optimal survival (Refer Tables 19, 20a and 20b; Figures 28 - 30). No significant decrease in surviving fraction, and associated cfu of immobilised *B. lactis* cells at 4°C occurred over the 21-day test period ($p>0.05$) (Refer Tables 19, 20a and 20b; Figures 28 and 29). At 22°C after the first seven days storage, there was no significant decrease in the surviving fraction of immobilised cells. Thereafter, however, a rapid

decrease of 1.2 log cycles occurred ($p<0.05$) (Refer Tables 19, 20a and 20b; Figures 28 and 29).

Similarly, over the test period, the surviving fraction of free cells declined by approximately 3 log cycles in the presence of oxygen at both 4°C and 22°C ($p<0.05$) (Refer Tables 20a and 20b; Figures 28 and 30). Generally microencapsulation enhanced survival of *B. lactis* in mahewu in the presence of oxygen. Under anaerobic conditions at 22°C, however, the decrease in survival of free cells was not significant ($p>0.05$) (Refer Tables 20a and 20b; Figures 28 and 31). The reasons for the latter trend are unknown. Further studies are required to elucidate the reasons for this.

The results obtained from this study for microencapsulated *B. lactis*, support results reported elsewhere which suggest that the use of a hydrocolloid capsule matrix can occasionally protect bacteria by simulating the natural encapsulation process of these organisms (Kailasapathy 2002). Foods in which survival of probiotics, including bifidobacteria, have been improved due to microencapsulation, include dairy products such as yoghurts, ice cream and cheese (Sun and Griffiths 2000; Kailasapathy 2002; Adhikari *et al.* 2003; Krasaekoopt *et al.* 2003).

Adhikari *et al.* (2003) showed that immobilised *B. longum*, where κ-carrageenan was used as an encapsulation material, improved the survival rates in stirred unpasteurised yoghurt at 4°C over 30 days. It was proposed that the shell of the microcapsule formed a protective barrier against the acidity (0.10% - 0.14% lactic acid) of the yoghurt.

Generally, results obtained in this study, support the view that microencapsulation of viable bifidobacteria is possible. High numbers of cells were immobilised. Results also support the view that microencapsulation is a complex process influenced by the bifidobacterial species, gel matrix, temperature during storage, presence of oxygen and

external food matrix (Sun and Griffiths 2000; Truelstrup Hansen *et al.* 2002; Adhikari *et al.* 2003).

In this study, microcapsules were stored in pasteurised fermented African beverages, amasi and mahewu, where any potential antagonistic effects of living AB cultures were absent. The viability of *B. lactis* in microcapsules was not tested anaerobically, as oxygen would naturally be limiting within the microcapsule. However, it was evident from the results obtained that oxygen presence did not significantly influence survival of free cells in amasi. In mahewu, however, a significant difference was noted. Although anaerobic conditions are important for cell growth, they are not essential for the maintenance of cell viability. *Bifidobacterium lactis* grows anaerobically, but survives in the presence of atmospheric oxygen.

The pH of amasi and mahewu, 4.4 and 3.2 respectively, remained constant throughout the shelf-life studies. Results obtained from this study suggest that these external environmental pH values could adversely affect survival of both immobilised and free cells. In amasi, pH 4.4, at 4°C and 22°C, both the immobilised and free cells decreased in viability over the 21-day test period. Free cells had improved survival values over immobilised cells (Refer Figures 24 - 27). In a study lasting eight weeks, similar trends for microencapsulated bifidobacteria in fermented pasteurised dairy products were noted by Sun and Griffiths (2000) when the protective function of the microcapsules only became apparent after 35 days of storage.

In mahewu, pH 3.2, microencapsulated *B. lactis* stored at 4°C and 22°C, showed improved survival rate over that of the free cells at the same temperature over 21 days. The decline in free cells could be due to the presence of organic acids, and / or the lack of buffering proteins in mahewu. In amasi, milk proteins could act as a buffer, ensuring a longer shelf life for both microcapsules and free cells. In mahewu, there are few proteins and the major carbon source is starch (carbohydrate); thus the buffering capacity of this beverage is poor. Hence, the decrease in

viability noted in mahewu for free cells at 4°C and 22°C, and immobilised cells at 22°C, could be attributed to the lengthy time for which the organism was exposed to pH 3.2 in the absence of an appropriate buffering system. The lack of buffering proteins and the presence of inorganic acid could, therefore, explain the decline recorded for immobilised and free *B. lactis* in SGJ (pH 1.5) over 240 min. Truelstrup Hansen *et al.* (2002) suggested that calcium ions in fermented dairy products could stabilise a gum matrix of calcium alginate (Ca-alginate), thereby enhancing the integrity and therefore protection by the microcapsule matrix. Results obtained from this study regarding viability of immobilised *B. lactis* in amasi over 21 days, do not support those findings, as both immobilised and free *B. lactis* cells showed a decline in survival rate. Further studies are required to determine the reason/s for this.

In both amasi and mahewu, over the 21-day test period, high levels of viable cfu were maintained in excess of the daily intake of 10^8. Using the mono-axial technique with superposed airflow (air knife technique), for microencapsulation, it is possible to deliver viable *B. lactis* in excess of the daily intake, in 0.1g microcapsules. After pasteurisation the capsules may be added to any volume of amasi or mahewu. In mahewu, however, optimum conditions for delivery of viable cells appears to require refridgeration at 4°C.

Bacterial numbers showed that *B. lactis* cells did not increase in number in either amasi or mahewu during the 21-day period, even under anaerobic conditions (Refer Appendices O and P). Adhikari *et al.* (2003) noted the same incidence in unpasteurised stirred yoghurt when *B. longum* was added to the food after fermentation.

The difficulty of culturing *Bifidobacterium* in various dairy products has been reported by many (Klaver *et al.* 1993; Truelstrup Hansen *et al.* 2002; Adhikari *et al.* 2003). In amasi, an increase in *B. lactis* viable cell number was therefore not expected during storage under the conditions used in this study. Mahewu lacks any sustainable fermentable carbohydrates for

B. lactis, as this bacterium cannot ferment starch (Trinidade *et al.* 2003). It is also likely that oxygen presence in either amasi or mahewu would inhibit the growth of *B. lactis*. These results are advantageous, as growth of *B. lactis* could alter the sensory properties of these African beverages. *Bifidobacterium* produces acetic and lactic acids at a ratio of 2:1 when fermenting carbohydrates (Ray 2001). The presence of acetic acid in either amasi or mahewu is undesirable, as it would negatively affect the sensory properties.

4.10 Consumption of amasi and mahewu samples enriched with *B. lactis*:

Twelve panellists were selected and trained for analytical sensory evaluation of the mahewu and amasi samples. The panel was made up as follows: seven white panellists, four black panellists and one coloured panellist (Refer 3.12). Panellists were asked to evaluate the products according to flavour, texture and colour.

Table 21. Viable *B. lactis* cfu present g^{-1} microcapsules, added to amasi or mahewu, prepared for sensory analysis after 1 or 6 or 14 days storage at 4^0C, prior to tasting (Refer Appendix S)

Storage time (days)	1	6	14
Amasi $\log_{10}$ cfu.g^{-1}	11.76 ± 0.07*	11.36 ± 0.02	12.38 ± 0.07
Mahewu $\log_{10}$ cfu.g^{-1}	11.82 ± 0.06	11.11 ± 0.04	12.15 ± 0.06

*Standard deviation $\log_{10}$ cfu

Table 22. Viable *B. lactis* free cells present in 1 ml^{-1} amasi or mahewu, prepared for sensory analysis after 1 or 6 or 14 days storage at 4^0C, prior to tasting (Refer Appendix S)

Storage time (days)	1	6	14
Log_{10} cfu.ml^{-1} amasi	7.80 ± 0.09*	7.00 ± 0.04	7.77 ± 0.06
Log_{10} cfu.ml^{-1} mahewu	7.63 ± 0.09	7.05 ± 0.02	7.77 ± 0.06

*Standard deviation log_{10} cfu

Table 23. Flavour differences detected in amasi and mahewu stored at 4^0C containing *B. lactis*, by 12 taste panellists for samples prepared 1 or 6 or 14 days prior to tasting

Storage time (days)	Amasi		Mahewu	
	Free cells	Microcapsules	Free cells	Microcapsules
1	11*	5	8	10*
6	10*	1	6	7
14	11*	7	12*	11*

*Significance $p \leq 0.05$

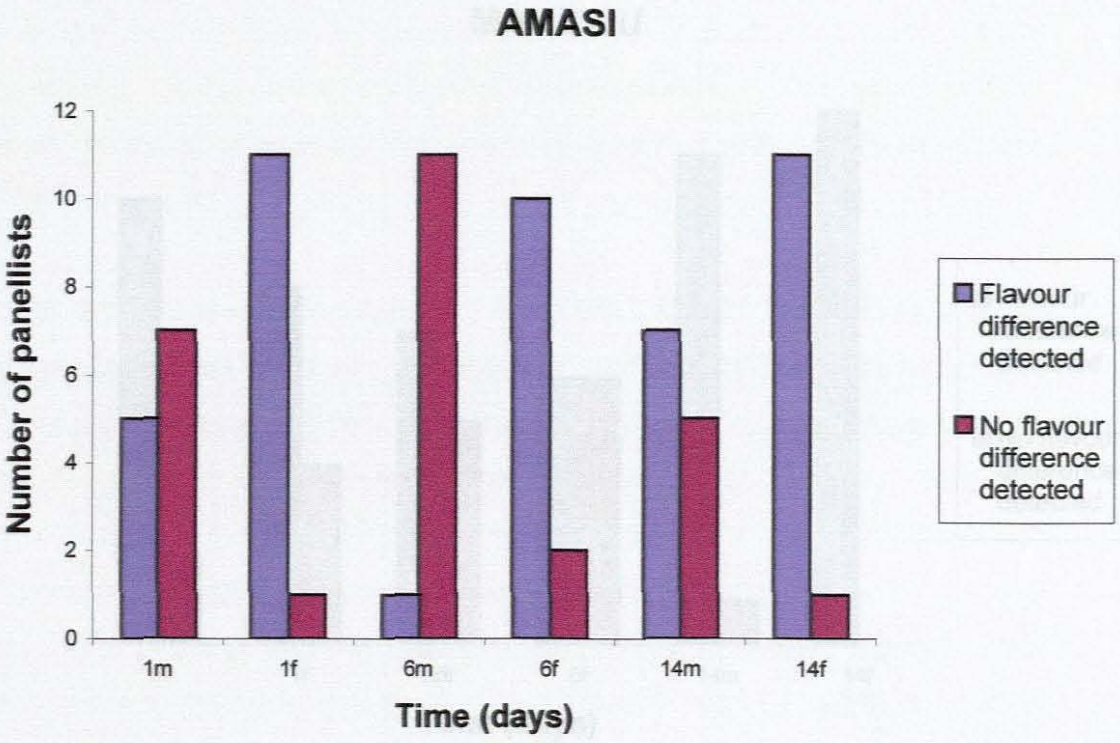

Figure 32. Flavour differences detected by a 12-member taste panel in amasi supplemented with *B. lactis* microcapsules[m] and free cells[f] for samples prepared 1 or 6 or 14 days prior to tasting.

[m] *B. lactis* microcapsules

[f] *B. lactis* free cells

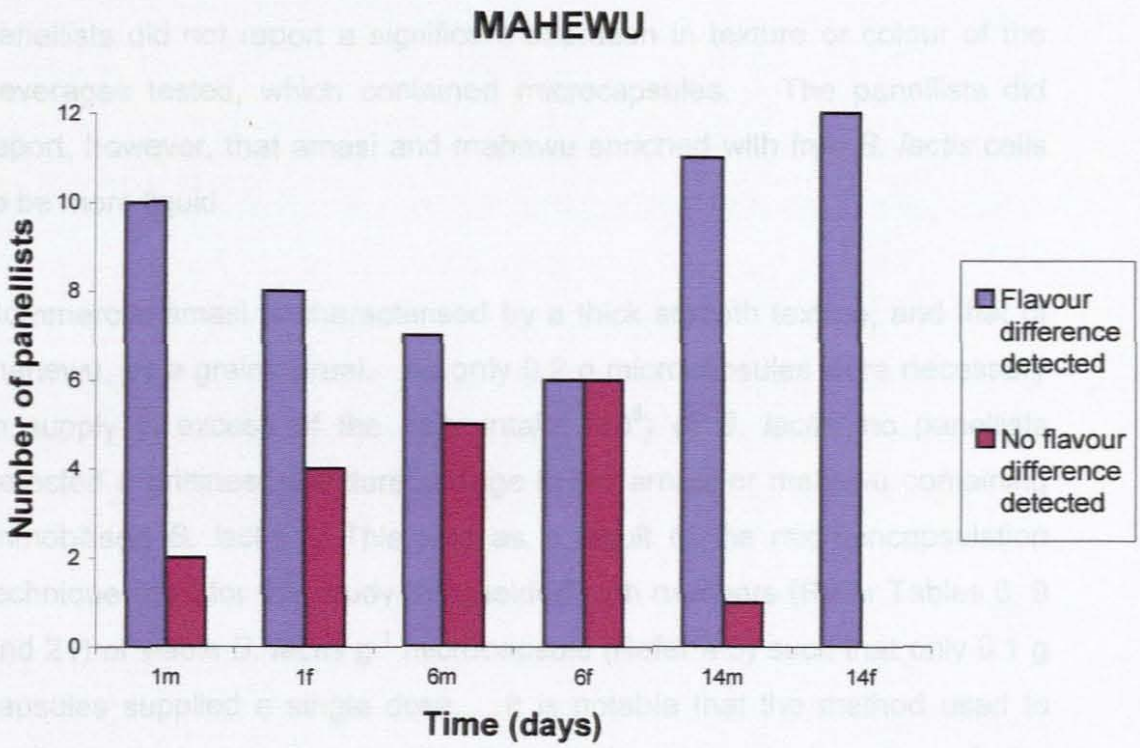

Figure 33. Flavour differences detected by a 12-member taste panel in mahewu supplemented with *B. lactis* microcapsules[m] and free cells[f] for samples prepared 1 or 6 or 14 days prior to tasting.

[m] *B. lactis* microcapsules

[f] *B. lactis* free cells

No differences in the colour of the beverages were detected by the panellists (Results not shown).

With regard to texture, Singer and Dunn (1990) reported that particles with a diameter below 3 μm are undetected by the human tongue. Therefore above this value, microcapsules can impart a gritty texture to foods normally not associated with this sensation. Others reported that alginate microcapsules were added to yoghurt at 10% (w/v), which resulted in an unfavourable consumer response due to the grainy texture (Adhikari *et al.* 2003). The concentration of 10% w/v microencapsulated *B. longum* was

required to provide the daily intake (10^8) of the organism. In this study, panellists did not report a significant alteration in texture or colour of the beverages tested, which contained microcapsules. The panellists did report, however, that amasi and mahewu enriched with free *B. lactis* cells to be more liquid.

Commercial amasi is characterised by a thick smooth texture, and that of mahewu, as a grainy gruel. As only 0.2 g microcapsules were necessary to supply in excess of the daily intake (10^8) of *B. lactis*, no panellists detected a grittiness / texture change in the amasi or mahewu containing immobilised *B. lactis*. This was as a result of the microencapsulation technique used for this study that yielded high numbers (Refer Tables 6, 9 and 21) of viable *B. lactis* g^{-1} microcapsule (Refer 4.5) such that only 0.1 g capsules supplied a single dose. It is notable that the method used to produce the microcapsules in this study reduced the size of the microcapsules by 80% when compared to Sun and Griffiths (2000) who reported an average capsule size of 2 mm for *B. lactis* immobilised in gellan-xanthan gum.

Microcapsules produced by coacervation / emulsion methods (Krasaekoopt *et al.* 2003) are smaller than those produced by the simple dropping technique, as used in this study. Adhikari *et al.* (2003) produced microcapsules using the coacervation method, with 91% of the microcapsules in the size range of 22 µm - 350 µm diameter. This size range is far smaller than the range reported in this study. However, coacervation is known to trap residual oil within the capsules during phase separation. As a result, flavour problems occur (Godward 2000). In addition, the presence of residual oil, emulsifier and surfactant in the microcapsules, proved to be toxic to the probiotic bacteria (Godward 2000).

The flavour responses of the 12-member analytically trained taste panel partaking in the triangle taste test (Refer 3.12) are recorded in Table 23. A significant difference ($p<0.05$) was detected by panellists in all amasi

samples with free cells. However, no significant difference in flavour was perceived in amasi containing microcapsules (Refer Figure 32). Microencapsulation of *B. lactis* in amasi is therefore recommended to retain the traditional flavour of amasi, even though survival of the bacterium is not enhanced when compared to free cells (Refer Figure 24).

In mahewu, taste difference was significant ($p<0.05$) for both microencapsulated and free cells in the 14-day sample, and a significant taste difference was even detected by panellists in the 1-day old sample containing microcapsules (Refer Figure 33). Although all the panellists did not object to the altered taste of the mahewu, the use of a fruit-flavoured mahewu for the delivery of microencapsulated *B. lactis* should be assessed as a preferred supply vehicle. Truelstrup Hansen *et al.* (2002) reported changes in flavour (bitterness) after microencapsulated *B. lactis* was stored for 12 days in AB yoghurt.

CHAPTER 5

CONCLUSION

Rheological studies, used to determine the shear stress data of the gellan-xanthan gum in this study, indicated that the gel behaved as a non-Newtonian material, and the flow curve fitted well to the Herschel-Bulkley model ($\tau_0 = \tau_Y - \kappa_y^{-n}$). The rheological studies indicated that gellan-xanthan is suitable for microencapsulation of *Bifidobacterium*. It also provided useful data for application to large-scale industrial production of the microcapsules, as well as for the type of foods suitable as delivery vehicles.

Bifidobacterium lactis DSM 10140 was successfully isolated from a freeze-dried AB culture, and microencapsulated in gellan-xanthan gum (GRAS) using the mono-axial extrusion method with superposed airflow (air knife technique). Microscopic examination of immobilised bacteria showed oval / round microcapsules with a densely packed core of bacteria.

The size of the microcapsules was determined using laser diffractometry, which resulted in an 80% reduction in diameter over the 3 mm diameter recorded by Sun and Griffiths (2000) who used a gellan-xanthan gum mix together with a simple dropping technique. Random samples (0.2 g) of the gellan-xanthan microcapsules in this study, showed a maximum size of 2.22 mm, with 50% of the capsules having a diameter ≤ 0.650 mm. Microcapsules with diameters of less than 0.02 mm were also recorded.

Enumeration of viable *B. lactis* from the microcapsules using HPUS (20kHz) provided a more accurate viable count for immobilised cells than did a pestle and mortar technique. Tests conducted in this study, indicated that the sonication applied was not lethal to *B. lactis*. At a level of 0.1 g encapsulated *B. lactis*, a single dose of living cells consisting of $\log_{10}$ 10-12 cfu.g^{-1}, provides an excess of the daily intake probiotic of 10^8, and did not alter the texture of the beverages.

Hence, 0.1 g microcapsules can be added to any volume of amasi or mahewu, after fermentation and pasteurisation.

In vitro studies, simulating gastric juices in the human stomach, and bile and pancreatic juices in the human colon, were carried out on both microencapsulated and free *B. lactis* cells over a period of 4 h. Survival of microencapsulated *B. lactis* in SGJ at 37°C, proved superior to that of free cells. Therefore, the gellan-xanthan matrix protected *B. lactis* against the acidity of the simulated gastric juices.

Although the organism requires an anaerobic environment for growth, it survives in the presence of oxygen. In this study it was demonstrated that *B. lactis* survived in atmospheric oxygen without nutrient supplementation, for at least 21 days in a sodium phosphate buffer or amasi or mahewu. Viable *B. lactis*, once encapsulated, could be supplied to industry in a 1 M sodium phosphate buffer (pH 7.0).

For use as a probiotic in amasi or mahewu, *B. lactis* should be added in the form of microcapsules. Although survival rate of immobilised *B. lactis* was not significantly improved in amasi, microencapsulation is still recommended as the flavour difference in the beverage was not as noticeable for capsules as for free cells. In addition, it is technically easier to add 0.1 g microcapsules to thickened pasteurised amasi, than free cells, to reach a concentration of 10^6 ml^{-1}. Refrigeration (4°C) of microcapsules in mahewu is recommended for maintaining probiotic viability. For amasi, 4°C did not improve survival of immobilised *B. lactis*. Therefore this enriched beverage could be stored at 22°C. This would be important for rural African homes where refridgeration is lacking. Both amasi and mahewu, enriched with immobilised *B. lactis*, can be used as supply vehicles of the probiotic to urban and rural populations in South Africa.

Although *B. lactis* ferments a wide variety of carbohydrates, the organism was unable to grow in amasi (lactose) or mahewu (starch) under anaerobic conditions. Growth of *Bifidobacterium* in these fermented beverages, under conditions of oxygen limitation is undesirable, due to formation of unpleasant

flavours and odours, e.g. acetic and butyric acids, not normally found in either amasi or mahewu. Although in some cases there was a significant difference noted in taste, panellists in an open unstructured interview stated that this difference was not unpleasant. Delivery of *B. lactis* in microcapsules is thus recommended, to rural South Africans, particularly in amasi.

5.1 Recommendations:

Further rheological studies should include tensile strength of the solidified gellan-xanthan gum, as well as microcapsules. Knowledge of changes in viscosity in the gum mix at various temperatures would assist in attaining constant viscosity and flow rate. This would also assist in obtaining microcapsules of a smaller, and more uniform shape and size. Rheology could be used to test whether storage of the microcapsules in a sodium phosphate buffer, pasteurised amasi, mahewu, SGJ or BPJ weakens the gum matrix. Additionally, it is recommended to test calcium (Ca) influence in dairy products on the shear stress of the gum mix.

A probiotic bacterium should be of human origin. As *Bifidobacterium lactis* is not a human isolate, other *Bifidobacterium* species should be isolated, and studied with regard to suitability as a probiotic. Microencapsulation techniques different from those used in this study should be considered, e.g. atomisation, which could be used to reduce the size, as well as standardise the shape, of microcapsules.

Pasteurised amasi and mahewu are ideal for the supply of immobilised *B. lactis* as no possible antagonistic effects from live starter cultures are likely. The use of unpasteurised products, such as bio-yoghurts (Truelstrup Hansen *et al.* 2002; Adhikari *et al.* 2003) that contain *L. acidophilus* and *S. thermophilus*, may not be suited as supply vehicles of immobilised *B. lactis*. The latter bacteria could be antagonistic to *Bifidobacterium* through the production of various metabolites, e.g. H_2O_2 (Ray 2001). In this study, as pasteurised beverages were used, the effect of live probiotic

cultures on *B. lactis*, immobilised in gellan-xanthan gum, was not investigated. Hence, it is recommended that, as *Bifidobacterium* is absent from many AB foods in SA (Lourens-Hattingh and Viljoen 2002), bifidobacteria immobilised in a gellan-xanthan gum matrix be added to SA bio-yoghurts, which contain viable *L. acidophilus*. Viability studies on the organism stored under these conditions should be done.

With regard to the structure of the analytical taste panel, it is recommended that members of this panel are black, i.e. are representative of the target market for amasi and mahewu.

This study suggests that acidic pH values decrease survival of *B. lactis*. Hence alternate foods with neutral pH should be considered as alternate supply sources (e.g. dairy milk).

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APPENDIX A

Probiotic and prebiotic claims

20. (a) Claims for "Probiotic" and "Prebiotic" shall not be made on the label of a foodstuff unless the claim complies with the conditions specified in paragraphs (b), (c) and (d) and (e) in Table 5.

(b) No claim for Probiotics or Prebiotics may have additional information added to it, except the information permitted in column I of Table 5 and the wording of the information may not be altered in a way which will result in a change of meaning or which will result in a change of emphasis;

(c) No claim for Probiotics or Prebiotics may attribute any degree of a disease risk reduction to specific dietary guidelines.

(d) An indication to the nearest kilojoule of the energy content (as per Annexe 2) of a serving and per 100 g or per 100 ml shall be provided on the label; and

(e) The total amount of proteins, carbohydrates, fats and dietary fibre in grams and sodium in milligrams, (as per Annex 2) present in a ready-to-eat serving or when packed, whatever is appropriate, and per 100 g/ml, and an indication of what percentage of the RDA protein represent per serving and per 100 g/ml, shall be provided on the label;

(f) Foodstuffs and nutritional supplements for which a Probiotic claim is mad shall bear on the main panel of the label the expression "KEEP REFRIGERATED" or 'KEEP FROZEN", as the case may be, in capital (upper-case) letters not less than 3 mm in height.

(d)

TABLE 5: PROBIOTIC CLAIM

I PERMISSIBLE INFORMATION TO ACCOMPANY CLAIM	II CONDITIONS	III FOODSTUFFS
	For foods for persons older than 1 year	
Probiotics such as *Bifidobacteria* And the *Lactobacilli* improve the Intestinal microbial balance, and Consequently the health and functioning of the digestive tract. They manufacture B vitamins, inhibit the growth of harmful pathogens and may, when ingested on a regular basis As part of a prudent, balanced diet, assist In improving the immune status, The digestion of lactose and may Help reduce the risk of colon cancer	The probiotic microbial count should exceed $1x10^6$ colony forming units per gram product** for foodstuffs Permitted species are: * *Lactobacillus* spp. * *Bifidobacterium* spp. * *Lactococcus* spp. * *Streptococcus salivarius* subspecies *thermophilus.* * *Lactobacillus delbrueckii* subspecies B*ebulgaricus*	Chilled foods

TABLE 5: PROBIOTIC CLAIM

I PERMISSIBLE INFORMATION TO ACCOMPANY CLAIM	II CONDITIONS	III FOODSTUFFS
	For foods, formulae for infants younger than 1 year	
Do	The probiotic bacterial Count should exceed 10^6 colony forming units per gram product** for foodstuffs Permitted organism is *Bifidobacterium infantis* only.	Infant formulae *and infant foods*

** As determined by .....method(s)

(e)

TABLE 5: PREBIOTIC CLAIM

PERMISSIBLE INFORMATION TO ACCOMPANY CLAIM	CONDITIONS	FOODSTUFF SOURCE
Prebiotics are food components that escape digestion by normal human digestive enzymes *and reach the large intestine where they* may create conditions that will promote the growth of indigenous, colonic bacteria, also referred to as Probiotics and are considered to be beneficial.	At least 3 g "prebiotic" *per daily serving.* The amount and source of "Prebiotics" such as Fructo-oligosaccharides, or *Galactosylsucrose, or* Galacto-oligosaccharides *Galacto-oligosaccharidesfrom whey and* Shall be declared onGalactosylsucrose. the label.	Fructo-oligosaccharides mainly from chicory, onion, garlic,asparagus, Jerusalem artichoke, and soya beans, Galacto-oligosaccharides from whey *and Galactosyl*-sucrose.

APPENDIX B

Rheological data on gellan-xanthan gel

Table 1a Yield stress, τ_Y (Pa) values by method of Herschel-Bulkley

	Yield stress, τ_Y (Pa)
Gum 1	1.467
Gum 2	2.029
Gum 3	1.048
Average	**1.515**

Table 1b Herschel Bulkley coefficients

	τ_Y, yield stress (Pa)	K	n
30^{0}C	8.67	4.68	0.258
35^{0}C	1.84	3.68	0.30
40^{0}C	1.12	2.79	0.33
45^{0}C	0.81	2.16	0.36
50^{0}C	0.67	2.16	0.38

Table 1c Velocity ($m.s^{-1}$) and Flow rate ($m^3.s^{-1}$) at corresponding pressure drop ($T=30^0C$)

$T=30^0C$

D 0.0004 m
L 0.013 m

τ_Y	8.672
K	4.675
n	0.258

τ_0 (Pa)	ΔP (Pa)	V ($m.s^{-1}$)	Q ($m^3.s^{-1}$)
8.672	1127.36	0	0
8.78	1141.4	6.17E-12	7.75E-19
9.02	1172.6	1.78E-09	2.24E-16
9.29	1207.7	2.80E-08	3.52E-15
9.58	1245.4	1.74E-07	2.19E-14
9.87	1283.1	6.42E-07	8.07E-14
10.2	1326	2.00E-06	2.51E-13
10.5	1365	4.58E-06	5.76E-13
10.8	1404	9.21E-06	1.16E-12
11.1	1443	1.68E-05	2.11E-12
11.4	1482	2.85E-05	3.58E-12
11.7	1521	4.56E-05	5.73E-12
12	1560	6.97E-05	8.75E-12
12.3	1599	1.02E-04	1.29E-11
12.6	1638	1.46E-04	1.83E-11
13	1690	2.23E-04	2.81E-11
13.3	1729	3.00E-04	3.77E-11
13.6	1768	3.95E-04	4.96E-11
13.9	1807	5.11E-04	6.42E-11
14.2	1846	6.51E-04	8.19E-11
14.5	1885	8.19E-04	1.03E-10
14.8	1924	1.02E-03	1.28E-10
15.1	1963	1.25E-03	1.57E-10
15.4	2002	1.52E-03	1.91E-10
15.7	2041	1.83E-03	2.30E-10
16	2080	2.19E-03	2.75E-10
16.3	2119	2.60E-03	3.27E-10
16.7	2171	3.23E-03	4.06E-10

17	2210	3.78E-03	4.74E-10
17.3	2249	4.39E-03	5.51E-10
17.6	2288	5.07E-03	6.37E-10
18	2340	6.10E-03	7.66E-10
18.3	2379	6.96E-03	8.75E-10
18.7	2431	8.26E-03	1.04E-09
19	2470	9.35E-03	1.18E-09
19.4	2522	1.10E-02	1.38E-09
19.8	2574	1.28E-02	1.61E-09
20.2	2626	1.48E-02	1.86E-09
20.5	2665	1.65E-02	2.07E-09
20.9	2717	1.89E-02	2.38E-09
21.4	2782	2.23E-02	2.81E-09
21.8	2834	2.54E-02	3.19E-09
22.3	2899	2.96E-02	3.73E-09
22.8	2964	3.44E-02	4.32E-09
23.4	3042	4.08E-02	5.13E-09
24.1	3133	4.94E-02	6.21E-09

Table 1d Velocity ($m.s^{-1}$) and Flow rate ($m^3.s^{-1}$) at corresponding pressure drop ($T=35^0C$)

$T=35^0C$

D 0.0004 m
L 0.013 m

τ_Y	1.835
K	3.675
n	0.298

τ_0 (Pa)	ΔP (Pa)	V ($m.s^{-1}$)	Q ($m^3.s^{-1}$)
1.835	238.55	0	0
1.84	239.2	2.73E-16	3.43E-23
2.16	280.8	1.61E-08	2.02E-15
2.37	308.1	1.20E-07	1.51E-14
2.37	308.1	1.20E-07	1.51E-14
2.47	321.1	2.37E-07	2.98E-14
2.57	334.1	4.21E-07	5.28E-14
2.69	349.7	7.56E-07	9.50E-14
2.82	366.6	1.30E-06	1.63E-13
2.98	387.4	2.30E-06	2.89E-13
3.16	410.8	3.99E-06	5.01E-13
3.34	434.2	6.41E-06	8.05E-13
3.53	458.9	9.93E-06	1.25E-12
3.74	486.2	1.52E-05	1.91E-12
3.96	514.8	2.27E-05	2.85E-12
4.2	546	3.34E-05	4.20E-12
4.47	581.1	4.93E-05	6.19E-12
4.7	611	6.65E-05	8.35E-12
4.93	640.9	8.75E-05	1.10E-11
5.23	679.9	1.22E-04	1.53E-11
5.48	712.4	1.56E-04	1.96E-11
5.76	748.8	2.03E-04	2.55E-11
5.99	778.7	2.48E-04	3.12E-11
6.23	809.9	3.02E-04	3.80E-11
6.5	845	3.72E-04	4.68E-11
6.8	884	4.63E-04	5.82E-11
7.09	921.7	5.65E-04	7.10E-11
7.37	958.1	6.77E-04	8.51E-11

7.69	999.7	8.23E-04	1.03E-10
8.03	1043.9	1.00E-03	1.26E-10
8.33	1082.9	1.18E-03	1.48E-10
8.64	1123.2	1.39E-03	1.74E-10
9	1170	1.66E-03	2.09E-10
9.31	1210.3	1.92E-03	2.41E-10
9.67	1257.1	2.26E-03	2.84E-10
10	1300	2.61E-03	3.28E-10
10.4	1352	3.07E-03	3.86E-10
10.7	1391	3.46E-03	4.35E-10
11.1	1443	4.03E-03	5.06E-10
11.5	1495	4.66E-03	5.86E-10
11.9	1547	5.36E-03	6.73E-10
12.3	1599	6.13E-03	7.70E-10
12.7	1651	6.97E-03	8.76E-10
13.1	1703	7.89E-03	9.91E-10
13.6	1768	9.15E-03	1.15E-09
14	1820	1.03E-02	1.29E-09
14.5	1885	1.18E-02	1.48E-09
15	1950	1.35E-02	1.69E-09
15.6	2028	1.57E-02	1.97E-09
16.1	2093	1.77E-02	2.22E-09

Table 1e Velocity ($m.s^{-1}$) and Flow rate ($m^3.s^{-1}$) at corresponding pressure drop ($T=40^0C$)

$T=40^0C$

D 0.0004 m
L 0.013 m

τ_Y	1.123
K	2.789
n	0.328

τ_0 (Pa)	ΔP (Pa)	V ($m.s^{-1}$)	Q ($m^3.s^{-1}$)
1.123	145.99	0	0
1.21	157.3	3.80E-10	4.77E-17
1.27	165.1	2.89E-09	3.63E-16
1.35	175.5	1.49E-08	1.87E-15
1.43	185.9	4.55E-08	5.71E-15
1.53	198.9	1.26E-07	1.58E-14
1.63	211.9	2.74E-07	3.45E-14
1.73	224.9	5.13E-07	6.45E-14
1.85	240.5	9.53E-07	1.20E-13
1.97	256.1	1.60E-06	2.01E-13
2.1	273	2.58E-06	3.24E-13
2.24	291.2	4.01E-06	5.04E-13
2.39	310.7	6.07E-06	7.62E-13
2.55	331.5	8.94E-06	1.12E-12
2.71	352.3	1.26E-05	1.58E-12
2.89	375.7	1.78E-05	2.24E-12
3.09	401.7	2.51E-05	3.16E-12
3.29	427.7	3.42E-05	4.30E-12
3.51	456.3	4.65E-05	5.85E-12
3.72	483.6	6.08E-05	7.64E-12
3.97	516.1	8.13E-05	1.02E-11
4.19	544.7	1.03E-04	1.29E-11
4.44	577.2	1.32E-04	1.65E-11
4.7	611	1.67E-04	2.10E-11
4.95	643.5	2.06E-04	2.59E-11
5.21	677.3	2.53E-04	3.18E-11
5.48	712.4	3.10E-04	3.89E-11
5.76	748.8	3.76E-04	4.73E-11

6.04	785.2	4.52E-04	5.68E-11
6.33	822.9	5.41E-04	6.79E-11
6.63	861.9	6.44E-04	8.09E-11
6.93	900.9	7.60E-04	9.55E-11
7.23	939.9	8.89E-04	1.12E-10
7.55	981.5	1.04E-03	1.31E-10
7.87	1023.1	1.21E-03	1.52E-10
8.19	1064.7	1.40E-03	1.76E-10
8.52	1107.6	1.61E-03	2.03E-10
8.87	1153.1	1.86E-03	2.34E-10
9.23	1199.9	2.14E-03	2.69E-10
9.59	1246.7	2.45E-03	3.08E-10
9.96	1294.8	2.80E-03	3.52E-10
10.3	1339	3.15E-03	3.96E-10
10.7	1391	3.59E-03	4.52E-10
11.2	1456	4.21E-03	5.29E-10
11.6	1508	4.75E-03	5.96E-10
12	1560	5.33E-03	6.70E-10
12.5	1625	6.12E-03	7.70E-10
12.9	1677	6.81E-03	8.56E-10
13.4	1742	7.75E-03	9.74E-10

Table 1f Velocity ($m.s^{-1}$) and Flow rate ($m^3.s^{-1}$) at corresponding pressure drop ($T=45^0C$)

$T=45^0C$

D 0.0004 m
L 0.013 m

τ_Y	0.814
K	2.164
n	0.360

τ_0 (Pa)	ΔP (Pa)	V ($m.s^{-1}$)	Q ($m^3.s^{-1}$)
0.814	105.82	0	0
0.89	115.7	1.10E-09	1.38E-16
0.933	121.29	5.41E-09	6.79E-16
0.979	127.27	1.68E-08	2.11E-15
1.03	133.9	4.18E-08	5.26E-15
1.1	143	1.06E-07	1.33E-14
1.17	152.1	2.14E-07	2.69E-14
1.24	161.2	3.78E-07	4.75E-14
1.32	171.6	6.44E-07	8.10E-14
1.41	183.3	1.06E-06	1.33E-13
1.5	195	1.62E-06	2.04E-13
1.6	208	2.43E-06	3.05E-13
1.71	222.3	3.57E-06	4.48E-13
1.83	237.9	5.14E-06	6.46E-13
1.96	254.8	7.29E-06	9.16E-13
2.11	274.3	1.04E-05	1.30E-12
2.26	293.8	1.42E-05	1.78E-12
2.43	315.9	1.95E-05	2.45E-12
2.61	339.3	2.63E-05	3.30E-12
2.81	365.3	3.55E-05	4.46E-12
3	390	4.59E-05	5.77E-12
3.21	417.3	5.95E-05	7.47E-12
3.42	444.6	7.54E-05	9.48E-12
3.64	473.2	9.48E-05	1.19E-11
3.87	503.1	1.18E-04	1.49E-11
4.1	533	1.45E-04	1.82E-11
4.34	564.2	1.77E-04	2.22E-11
4.58	595.4	2.13E-04	2.68E-11

4.83	627.9	2.56E-04	3.21E-11
5.1	663	3.07E-04	3.86E-11
5.37	698.1	3.65E-04	4.58E-11
5.64	733.2	4.29E-04	5.39E-11
5.93	770.9	5.05E-04	6.35E-11
6.22	808.6	5.90E-04	7.42E-11
6.52	847.6	6.87E-04	8.64E-11
6.83	887.9	7.98E-04	1.00E-10
7.15	929.5	9.23E-04	1.16E-10
7.48	972.4	1.06E-03	1.34E-10
7.81	1015.3	1.22E-03	1.53E-10
8.16	1060.8	1.40E-03	1.76E-10
8.51	1106.3	1.60E-03	2.00E-10
8.88	1154.4	1.82E-03	2.29E-10
9.26	1203.8	2.07E-03	2.60E-10
9.64	1253.2	2.34E-03	2.95E-10
10	1300	2.62E-03	3.30E-10
10.5	1365	3.04E-03	3.83E-10
10.9	1417	3.41E-03	4.29E-10
11.3	1469	3.80E-03	4.78E-10
11.8	1534	4.34E-03	5.45E-10

Table 1g Velocity ($m.s^{-1}$) and Flow rate ($m^3.s^{-1}$) at corresponding pressure drop ($T=50^0C$)

$T=50^0C$

D 0.0004 m
L 0.013 m

τ_Y	0.674
K	2.155
n	0.376

τ_0 (Pa)	ΔP (Pa)	V ($m.s^{-1}$)	Q ($m^3.s^{-1}$)
0.674	87.62	0	0
0.724	94.12	5.27E-10	6.62E-17
0.763	99.19	3.85E-09	4.83E-16
0.81	105.3	1.58E-08	1.99E-15
0.867	112.71	4.91E-08	6.17E-15
0.932	121.16	1.21E-07	1.53E-14
1	130	2.47E-07	3.10E-14
1.07	139.1	4.38E-07	5.51E-14
1.15	149.5	7.46E-07	9.38E-14
1.24	161.2	1.22E-06	1.53E-13
1.34	174.2	1.92E-06	2.41E-13
1.45	188.5	2.92E-06	3.67E-13
1.56	202.8	4.18E-06	5.26E-13
1.68	218.4	5.89E-06	7.41E-13
1.82	236.6	8.36E-06	1.05E-12
1.97	256.1	1.16E-05	1.46E-12
2.13	276.9	1.58E-05	1.99E-12
2.3	299	2.12E-05	2.67E-12
2.48	322.4	2.81E-05	3.53E-12
2.68	348.4	3.71E-05	4.67E-12
2.88	374.4	4.78E-05	6.01E-12
3.1	403	6.16E-05	7.74E-12
3.32	431.6	7.76E-05	9.75E-12
3.55	461.5	9.69E-05	1.22E-11
3.79	492.7	1.20E-04	1.51E-11
4.02	522.6	1.45E-04	1.82E-11
4.28	556.4	1.77E-04	2.23E-11
4.53	588.9	2.12E-04	2.67E-11

4.8	624	2.54E-04	3.19E-11
5.07	659.1	3.01E-04	3.78E-11
5.35	695.5	3.55E-04	4.46E-11
5.63	731.9	4.15E-04	5.22E-11
5.94	772.2	4.88E-04	6.14E-11
6.24	811.2	5.66E-04	7.12E-11
6.55	851.5	6.55E-04	8.23E-11
6.89	895.7	7.62E-04	9.57E-11
7.22	938.6	8.75E-04	1.10E-10
7.58	985.4	1.01E-03	1.27E-10
7.94	1032.2	1.16E-03	1.45E-10
8.32	1081.6	1.33E-03	1.67E-10
8.71	1132.3	1.52E-03	1.90E-10
9.11	1184.3	1.73E-03	2.17E-10
9.53	1238.9	1.97E-03	2.47E-10
9.96	1294.8	2.23E-03	2.80E-10
10.4	1352	2.53E-03	3.17E-10
10.9	1417	2.89E-03	3.63E-10
11.4	1482	3.28E-03	4.13E-10
11.9	1547	3.71E-03	4.66E-10
12.4	1612	4.17E-03	5.24E-10

Table 1h Apparent Shear Viscosity, η (Pa.s) at different temperatures, T (^{0}C) for Gum

Temperature (^{0}C)	Apparent Shear Viscosity
30.10	4.270
30.70	4.090
31.40	3.910
32.00	3.730
33.30	3.400
33.90	3.180
34.50	2.970
35.10	2.790
35.80	2.600
36.40	2.390
37.00	2.220
37.60	2.070
38.20	1.930
38.80	1.810
39.40	1.710
40.00	1.590
40.60	1.470
41.20	1.370
41.80	1.280
42.40	1.190
43.00	1.110
43.60	1.060
44.20	1.020
44.80	0.982
45.50	0.959
46.10	0.944
46.70	0.932

47.30	0.922
47.90	0.917
48.50	0.911
49.20	0.903
49.80	0.897
50.40	0.895
51.00	0.890
51.70	0.883
52.30	0.881
52.90	0.877
53.50	0.870
54.20	0.864
54.80	0.859
55.40	0.851
56.10	0.844
56.70	0.839
57.30	0.832
58.00	0.828
58.60	0.825
59.90	0.818

Table 1i Apparent Shear Viscosity, η (Pa.s) values

	Apparent Shear Viscosity, η (Pa.s)
Gum at 30^0C	5.152
Gum at 40^0C	0.896
Gum at 45^0C	0.426

APPENDIX C

Malvern data on microcapsule sizes

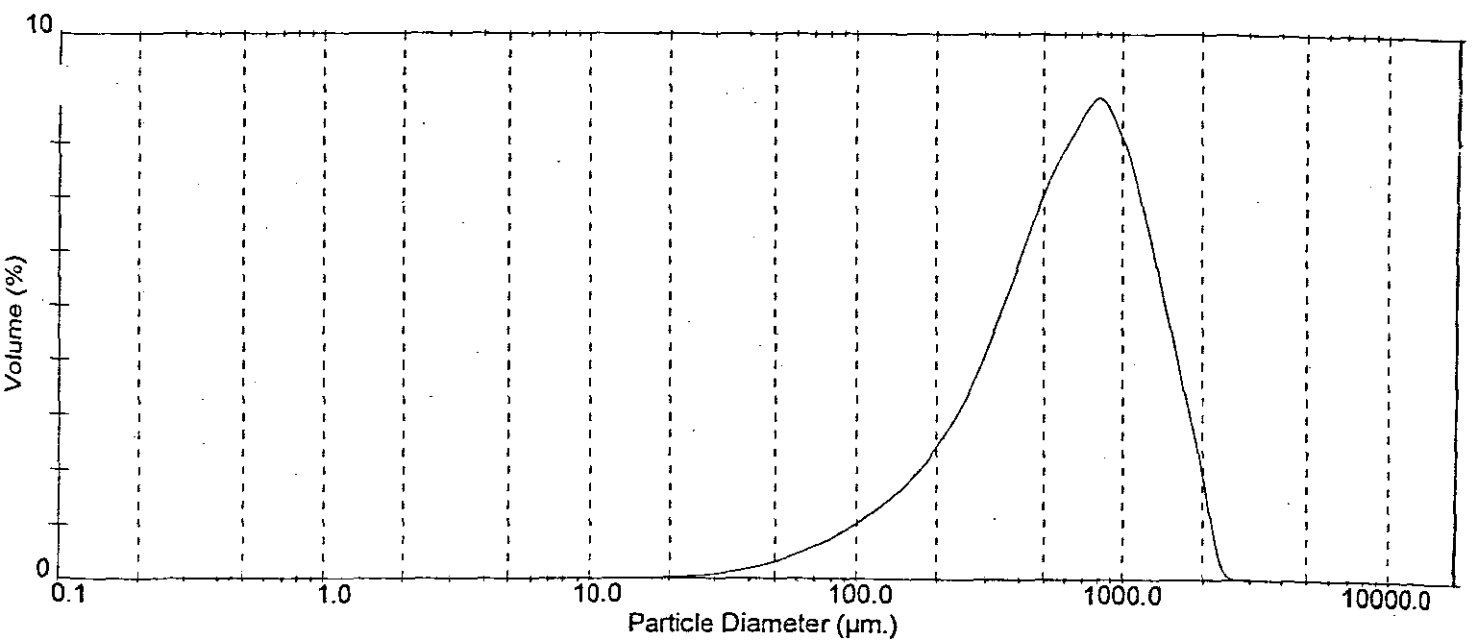
Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%
4.19		22.49		120.67		647.41	
4.88	0.00	26.20	0.05	140.58	1.42	754.23	8.37
5.69	0.00	30.53	0.08	163.77	1.71	878.67	8.81
6.63	0.00	35.56	0.12	190.80	2.06	1023.66	8.38
7.72	0.00	41.43	0.18	222.28	2.49	1192.56	7.47
9.00	0.00	48.27	0.25	258.95	3.04	1389.33	6.17
10.48	0.00	56.23	0.35	301.68	3.71	1618.57	4.70
12.21	0.01	65.51	0.47	351.46	4.52	1885.64	3.22
14.22	0.01	76.32	0.62	409.45	5.42	2196.77	1.75
16.57	0.01	88.91	0.78	477.01	6.33	2559.23	0.27
19.31	0.02	103.58	0.97	555.71	7.16	2981.51	0.00
22.49	0.03	120.67	1.18	647.41	7.85	3473.45	0.00

D (v, 0.1) 186.51; D (v, 0.5) 637.00; D (v, 0.9) 1387.15

D (v, 0.1) 10% of the microcapsules < 186.51 µm in diameter

D (v, 0.5) 50% of the microcapsules < 637.00 µm in diameter

D (v, 0.9) 90% of the microcapsules < 1387.15 µm in diameter



APPENDIX C

Malvern data on microcapsule sizes

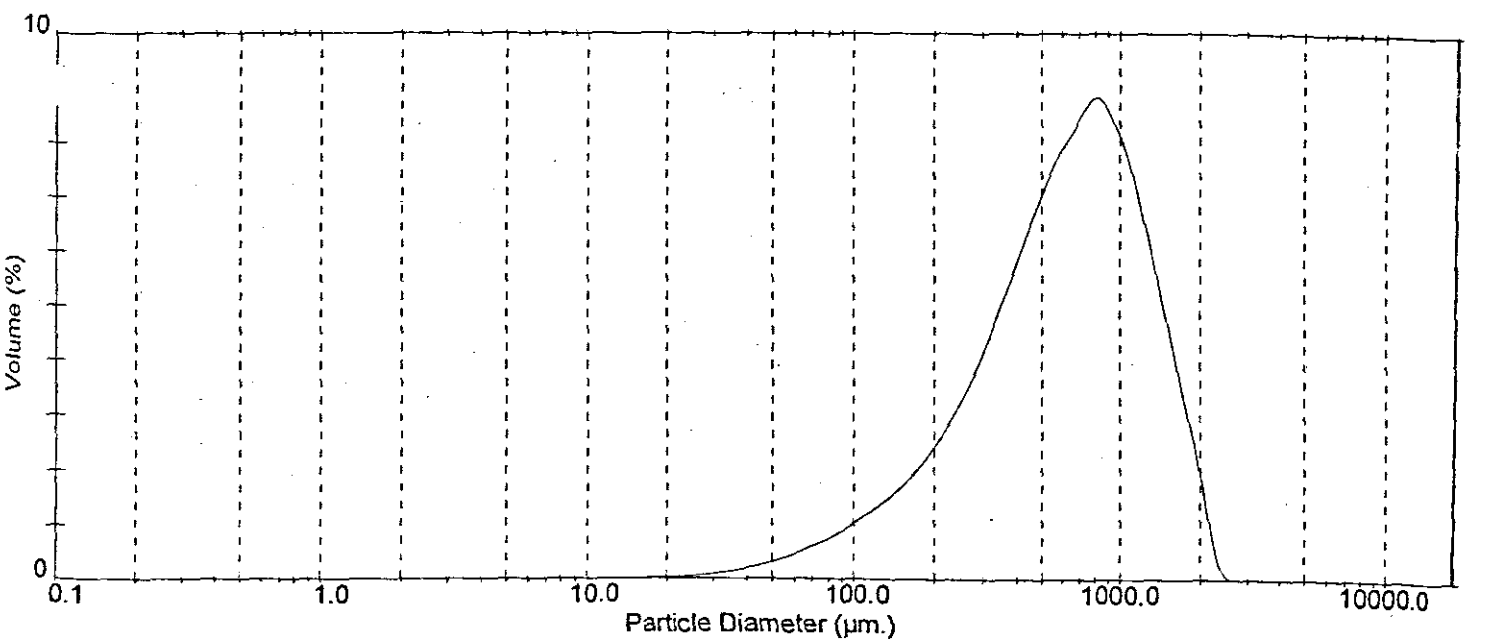
Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%
4.19		22.49		120.67		647.41	
4.88	0.00	26.20	0.05	140.58	1.42	754.23	8.37
5.69	0.00	30.53	0.08	163.77	1.71	878.67	8.81
6.63	0.00	35.56	0.12	190.80	2.06	1023.66	8.38
7.72	0.00	41.43	0.18	222.28	2.49	1192.56	7.47
9.00	0.00	48.27	0.25	258.95	3.04	1389.33	6.17
10.48	0.00	56.23	0.35	301.68	3.71	1618.57	4.70
12.21	0.01	65.51	0.47	351.46	4.52	1885.64	3.22
14.22	0.01	76.32	0.62	409.45	5.42	2196.77	1.75
16.57	0.01	88.91	0.78	477.01	6.33	2559.23	0.27
19.31	0.02	103.58	0.97	555.71	7.16	2981.51	0.00
22.49	0.03	120.67	1.18	647.41	7.85	3473.45	0.00

D (v, 0.1) 186.51; D (v, 0.5) 637.00; D (v, 0.9) 1387.15

D (v, 0.1) 10% of the microcapsules < 186.51 µm in diameter

D (v, 0.5) 50% of the microcapsules < 637.00 µm in diameter

D (v, 0.9) 90% of the microcapsules < 1387.15 µm in diameter



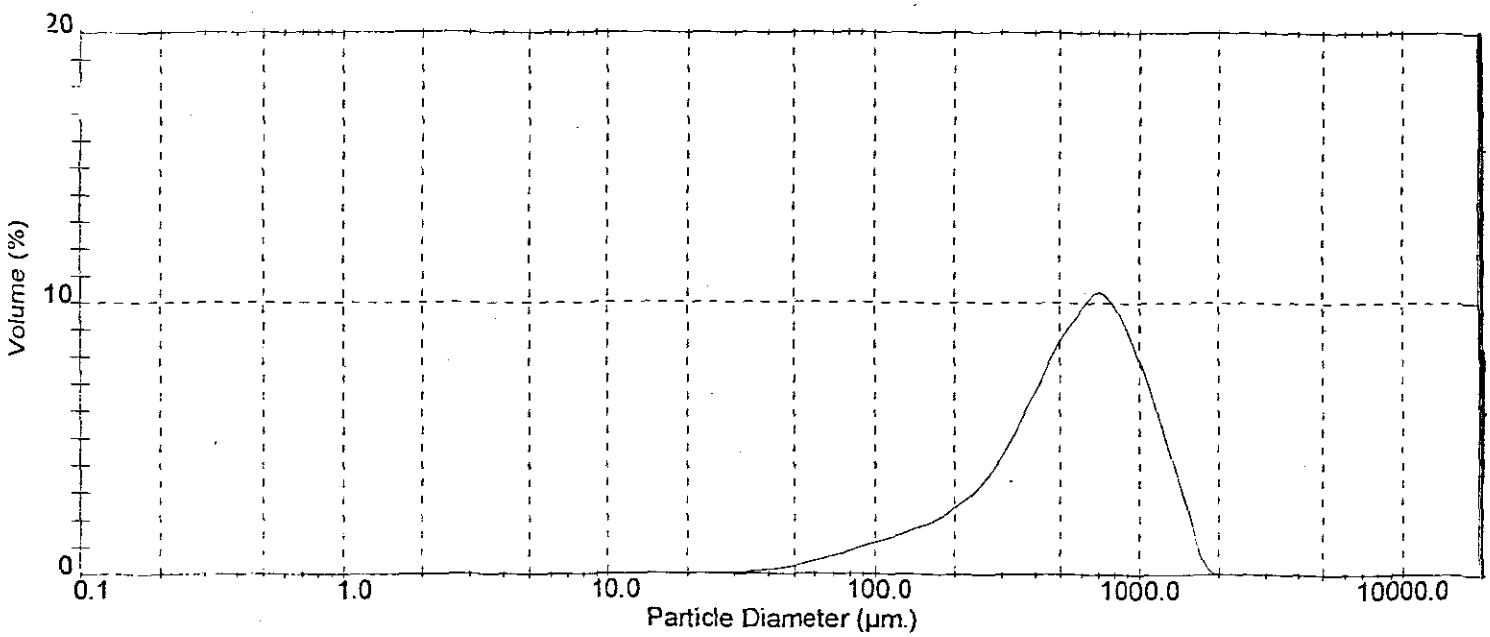
Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%
4.19		22.49		120.67		647.41	
4.88	0.00	26.20	0.03	140.58	1.56	754.23	10.36
5.69	0.00	30.53	0.05	163.77	1.81	878.67	9.76
6.63	0.01	35.56	0.08	190.80	2.11	1023.66	8.42
7.72	0.01	41.43	0.14	222.28	2.51	1192.56	6.55
9.00	0.01	48.27	0.23	258.95	3.05	1389.33	4.53
10.48	0.01	56.23	0.35	301.68	3.81	1618.57	2.51
12.21	0.01	65.51	0.5	351.46	4.87	1885.64	0.49
14.22	0.01	76.32	0.69	409.45	6.18	2196.77	0.00
16.57	0.01	88.91	0.9	477.01	7.55	2559.23	0.00
19.31	0.01	103.58	1.12	555.71	8.74	2981.51	0.00
22.49	0.02	120.67	1.33	647.41	9.69	3473.45	0.00

D (v, 0.1) 178.11; D (v, 0.5) 577.00; D (v, 0.9) 1119.33

D (v, 0.1) 10% of the microcapsules < 178.11 µm in diameter

D (v, 0.5) 50% of the microcapsules < 577.00 µm in diameter

D (v, 0.9) 90% of the microcapsules < 1119.33 µm in diameter



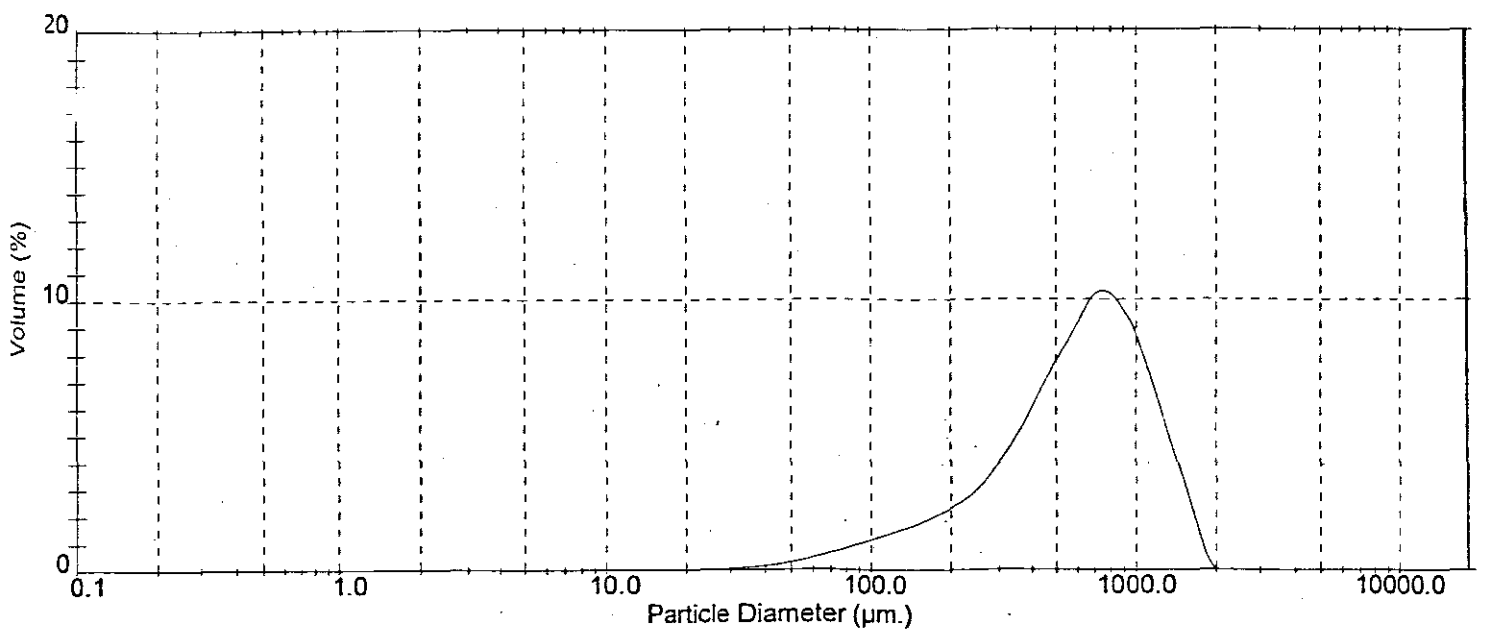
Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%
4.19		22.49		120.67		647.41	
4.88	0.00	26.20	0.03	140.58	1.5	754.23	10.14
5.69	0.00	30.53	0.04	163.77	1.73	878.67	10.11
6.63	0.00	35.56	0.08	190.80	2.01	1023.66	9.19
7.72	0.00	41.43	0.13	222.28	2.38	1192.56	7.5
9.00	0.01	48.27	0.22	258.95	2.88	1389.33	5.4
10.48	0.01	56.23	0.33	301.68	3.55	1618.57	3.31
12.21	0.01	65.51	0.48	351.46	4.45	1885.64	1.22
14.22	0.01	76.32	0.66	409.45	5.56	2196.77	0.00
16.57	0.01	88.91	0.86	477.01	6.76	2559.23	0.00
19.31	0.01	103.58	1.07	555.71	7.95	2981.51	0.00
22.49	0.02	120.67	1.29	647.41	9.08	3473.45	0.00

D (v, 0.1) 183.98; D (v, 0.5) 615.65; D (v, 0.9) 1190.87

D (v, 0.1) 10% of the microcapsules < 183.98 µm in diameter

D (v, 0.5) 50% of the microcapsules < 615.65 µm in diameter

D (v, 0.9) 90% of the microcapsules < 1190.87 µm in diameter



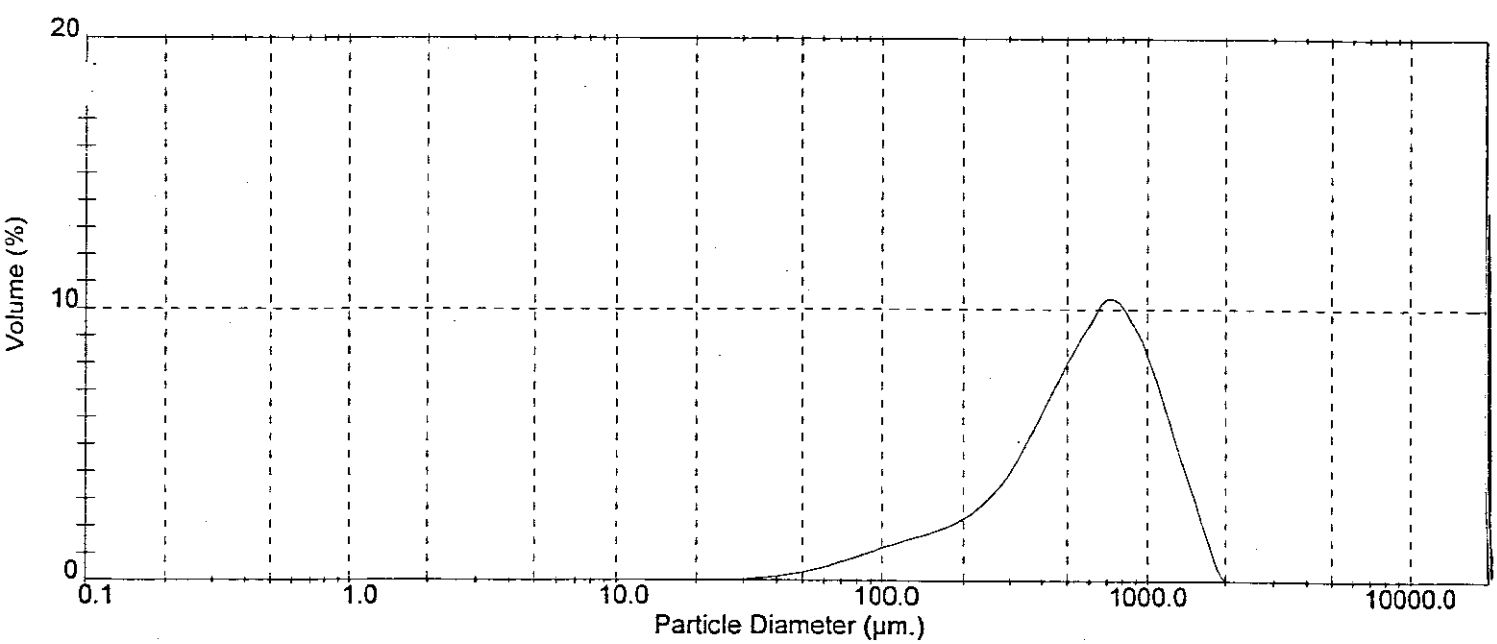
Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%	Size (µm)	Volume in%
4.19		22.49		120.67		647.41	
4.88	0.00	26.20	0.03	140.58	1.57	754.23	10.31
5.69	0.00	30.53	0.05	163.77	1.78	878.67	9.99
6.63	0.00	35.56	0.08	190.80	2.03	1023.66	8.84
7.72	0.01	41.43	0.14	222.28	2.37	1192.56	7.05
9.00	0.01	48.27	0.23	258.95	2.85	1389.33	4.99
10.48	0.01	56.23	0.35	301.68	3.55	1618.57	3.09
12.21	0.01	65.51	0.52	351.46	4.5	1885.64	1.20
14.22	0.01	76.32	0.72	409.45	5.69	2196.77	0.00
16.57	0.01	88.91	0.94	477.01	6.96	2559.23	0.00
19.31	0.02	103.58	1.16	555.71	8.22	2981.51	0.00
22.49	0.02	120.67	1.37	647.41	9.33	3473.45	0.00

D (v, 0.1) 176.60; D (v, 0.5) 602.56; D (v, 0.9) 1171.51

D (v, 0.1) 10% of the microcapsules < 176.60 µm in diameter

D (v, 0.5) 50% of the microcapsules < 602.56 µm in diameter

D (v, 0.9) 90% of the microcapsules < 1171.51 µm in diameter



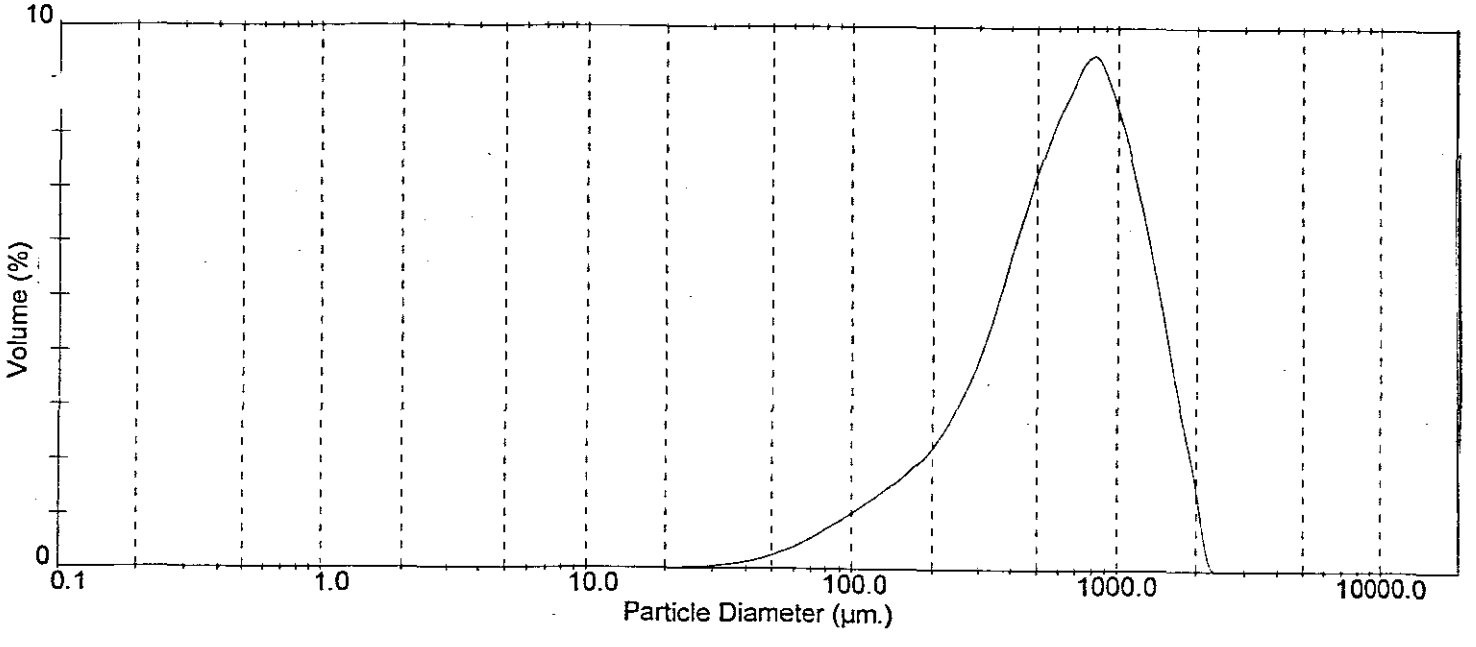
Size (μm)	Volume in%	Size (μm)	Volume in%	Size (μm)	Volume in%	Size (μm)	Volume in%
4.19		22.49		120.67		647.41	
4.88	0.00	26.20	0.03	140.58	1.42	754.23	8.95
5.69	0.00	30.53	0.04	163.77	1.66	878.67	9.46
6.63	0.00	35.56	0.07	190.80	1.94	1023.66	8.84
7.72	0.01	41.43	0.11	222.28	2.31	1192.56	7.70
9.00	0.01	48.27	0.18	258.95	2.81	1389.33	6.20
10.48	0.01	56.23	0.28	301.68	3.46	1618.57	4.56
12.21	0.01	65.51	0.42	351.46	4.30	1885.64	2.93
14.22	0.01	76.32	0.58	409.45	5.31	2196.77	1.30
16.57	0.01	88.91	0.78	477.01	6.38	2559.23	0.00
19.31	0.01	103.58	0.98	555.71	7.41	2981.51	0.00
22.49	0.02	120.67	1.2	647.41	8.29	3473.45	0.00

D (v, 0.1) 193.70; D (v, 0.5) 646.76; D (v, 0.9) 1344.33

D (v, 0.1) 10% of the microcapsules < 193.70 μm in diameter

D (v, 0.5) 50% of the microcapsules < 646.76 μm in diameter

D (v, 0.9) 90% of the microcapsules < 1344.33 μm in diameter



APPENDIX D

Pestle and mortar, and ultrasound data on encapsulated *Bifidobacterium lactis* DSM 10140

Pestle and mortar

Encapsulated cells

Experiment 1

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
7.98E+10	10.90173069	0.116048816	10.76921058	6.03E+10
4.85E+10	10.68574174			
5.25E+10	10.7201593			

Experiment 2

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.23E+11	11.08990511	0.244294738	10.80874658	7.21E+10
4.45E+10	10.64836001			
4.88E+10	10.68797462			

Experiment 3

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
3.65E+10	10.56229286	0.102041044	10.67885459	4.86E+10
5.28E+10	10.72222246			
5.65E+10	10.75204845			

Ultrasound

Encapsulated cells

Experiment 1

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.11E+12	12.04336228	0.054315409	12.01447762	1.04E+12
1.12E+12	12.04824753			
8.95E+11	11.95182304			

Experiment 2

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
7.78E+11	11.8907004	0.08763134	11.90739008	8.19E+11
1.01E+12	12.00216606			
6.75E+11	11.82930377			

Experiment 3

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
8.28E+11	11.917768	0.027534903	11.90387025	8.03E+11
8.35E+11	11.92168648			
7.45E+11	11.87215627			

APPENDIX E

Ultrasound data on free *B. lactis*

Untreated

Free cells

Experiment 1

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
3.60E+11	11.5563025	0.137628588	11.54211346	3.60E+11
2.50E+11	11.39794001			
4.70E+11	11.67209786			

Experiment 2

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
5.20E+10	10.71600334	0.105541287	10.60773777	4.13E+10
3.20E+10	10.50514998			
4.00E+10	10.60205999			

Ultrasound

Free cells

Experiment 1

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.03E+11	11.01283722	0.053875132	11.06719901	1.17E+11
1.32E+11	11.12057393			
1.17E+11	11.06818586			

Experiment 2

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
6.00E+10	10.77815125	0.063210107	10.84619124	7.07E+10
8.00E+10	10.90308999			
7.20E+10	10.8573325			

APPENDIX F

Shelf-life of encapsulated *B. lactis* cells in 1 M sodium phosphate buffer (pH 7.0) (Experiment 1)

0 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
7.08E+11	11.84972644	0.011047687	11.83168962	6.79E+11
6.83E+11	11.83410266			
6.48E+11	11.81123977			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
7.08E+11	11.84972644	0.011047687	11.83168962	6.79E+11
6.83E+11	11.83410266			
6.48E+11	11.81123977			

7 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
6.50E+11	11.81291336	0.06523876	11.83183655	6.84E+11
6.00E+11	11.77815125			
8.03E+11	11.90444504			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
5.15E+11	11.71180723	0.051244333	11.66467757	4.64E+11
4.70E+11	11.67209786			
4.08E+11	11.61012761			

14 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
5.95E+11	11.77451697	0.139931095	11.82426385	6.92E+11
5.20E+11	11.71600334			
9.60E+11	11.98227123			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
3.23E+11	11.50852972	0.141069001	11.38325196	2.50E+11
2.58E+11	11.41077723			
1.70E+11	11.23044892			

21 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
3.53E+11	11.54715912	0.049777866	11.60017581	4.00E+11
4.05E+11	11.60745502			
4.43E+11	11.64591328			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.88E+11	11.27300127	0.011761233	11.28620099	1.93E+11
1.98E+11	11.2955671			
1.95E+11	11.29003461			

APPENDIX G

Shelf-life of encapsulated *B. lactis* cells in 1 M sodium phosphate buffer (pH 7.0) (Experiment 2)

0 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
4.51E+11	11.65417654	0.009997712	11.6641734	4.62E+11
4.72E+11	11.67417197			
4.62E+11	11.66417171			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
4.51E+11	11.65417654	0.009997712	11.6641734	4.62E+11
4.72E+11	11.67417197			
4.62E+11	11.66417171			

7 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
9.03E+11	11.95544721	0.031257821	11.94497314	8.83E+11
8.13E+11	11.90982337			
9.33E+11	11.96964884			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.10E+11	11.32221929	0.044802397	11.3506132	2.25E+11
2.53E+11	11.40226138			
2.13E+11	11.32735893			

14 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
6.18E+11	11.79063696	0.061623439	11.85342424	7.18E+11
8.20E+11	11.91381385			
7.18E+11	11.85582191			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
3.60E+11	11.5563025	0.072536026	11.54927919	3.58E+11
4.15E+11	11.6180481			
2.98E+11	11.47348697			

21 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
3.28E+11	11.5152113	0.016099435	11.53234367	3.41E+11
3.53E+11	11.54715912			
3.43E+11	11.53466058			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.20E+11	11.34242268	0.002833251	11.34405846	2.21E+11
2.20E+11	11.34242268			
2.23E+11	11.34733002			

APPENDIX H

Surviving fraction of encapsulated *B. lactis* cells in 1 M sodium phosphate buffer (pH 7.0)

4°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	6.79E+11	1.00	1.00	1.00E+00
2		4.62E+11	1.00		
1	7	6.84E+11	1.01	1.46	1.46E+00
2		8.83E+11	1.91		
1	14	6.92E+11	1.02	1.29	1.29E+00
2		7.18E+11	1.55		
1	21	4.00E+11	0.59	0.66	6.60E-01
2		3.41E+11	0.74		

22°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	6.79E+11	1.00	1.00	1.00E+00
2		4.62E+11	1.00		
1	7	4.64E+11	0.68	0.59	5.90E-01
2		2.25E+11	0.49		
1	14	2.50E+11	0.37	0.57	5.70E-01
2		3.58E+11	0.77		
1	21	1.93E+11	0.28	0.38	3.80E-01
2		2.21E+11	0.48		

APPENDIX I

Shelf-life of encapsulated and free *B. lactis* cells in simulated gastric juice (SGJ) (pH 1.5) (Experiment 1)

Encapsulated cells

0 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.93E+12	12.28443073	0.034881149	12.24415345	1.76E+12
1.68E+12	12.22401481			
1.68E+12	12.22401481			

60 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.58E+11	11.41077723	0.073420313	11.41084544	2.60E+11
2.18E+11	11.33745926			
3.05E+11	11.48429984			

120 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.68E+11	11.22401481	0.063549787	11.15063384	1.43E+11
1.30E+11	11.11394335			
1.30E+11	11.11394335			

240 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.73E+10	10.2367891	0.049807211	10.211214	1.63E+10
1.43E+10	10.15381486			
1.75E+10	10.24303805			

Free cells

0 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
3.33E+09	9.522444234	0.221228461	9.358937411	2.47E+09
2.80E+09	9.447158031			
1.28E+09	9.10720997			

60 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.45E+08	8.161368002	0.111190778	8.255884203	1.84E+08
1.69E+08	8.227886705			
2.39E+08	8.378397901			

120 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.05E+08	8.021189299	0.036134822	8.037735923	1.09E+08
1.03E+08	8.012837225			
1.20E+08	8.079181246			

240 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
5.73E+06	6.758154622	0.034951079	6.71893543	5.25E+06
4.91E+06	6.691081492			
5.10E+06	6.707570176			

APPENDIX J

Shelf-life of encapsulated and free *B. lactis* cells in simulated gastric juice (SGJ) (pH 1.5) (Experiment 2)

Encapsulated cells

0 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.25E+12	12.35218252	0.051440703	12.37145775	2.36E+12
2.69E+12	12.42975228			
2.15E+12	12.33243846			

60 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
3.98E+12	12.59933713	0.225399432	12.35672337	2.49E+12
2.08E+12	12.3170181			
1.43E+12	12.15381486			

120 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.20E+12	12.34192888	0.182351343	12.14464912	1.48E+12
1.29E+12	12.10974724			
9.60E+11	11.98227123			

240 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
4.18E+11	11.62065648	0.04333971	11.67068492	4.70E+11
4.98E+11	11.69679309			
4.95E+11	11.6946052			

Free cells

0 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
3.27E+09	9.514547753	0.060890415	9.582296039	3.85E+09
4.29E+09	9.632457292			
3.98E+09	9.599883072			

60 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
8.96E+08	8.95230801	0.02108805	8.975858114	9.47E+08
9.84E+08	8.992995098			
9.60E+08	8.982271233			

120 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.56E+08	8.193124598	0.059119452	8.260625988	1.83E+08
2.01E+08	8.303196057			
1.93E+08	8.285557309			

240 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
3.93E+06	6.59439255	0.174296555	6.432403559	2.85E+06
1.77E+06	6.247973266			
2.85E+06	6.45484486			

APPENDIX K

Surviving fraction of encapsulated and free *B. lactis* cells in simulated gastric juice (SGJ) (pH 1.5)

Encapsulated cells

37°C (aerobic)

Experiment number	Time (minutes)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	1.76E+12	1.00	1.00	1.00E+00
2		2.36E+12	1.00		
1	60	2.60E+11	0.15	0.60	6.00E-01
2		2.49E+12	1.06		
1	120	1.43E+11	0.081	0.35	3.50E-01
2		1.48E+12	0.63		
1	240	1.63E+10	0.0093	0.10	1.00E-01
2		4.70E+11	0.20		

Free cells

37°C (aerobic)

Experiment number	Time (minutes)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	2.47E+09	1.00	1.00	1.00E+00
2		3.85E+09	1.00		
1	60	1.84E+08	0.074	0.16	1.60E-01
2		9.47E+08	0.25		
1	120	1.09E+08	0.044	0.046	4.60E-02
2		1.83E+08	0.048		
1	240	5.25E+06	0.0021	0.0014	1.40E-03
2		2.85E+06	0.00074		

APPENDIX L

Shelf-life of encapsulated and free *B. lactis* cells in simulated bile and pancreatic juice (BPJ) (pH 6.5) (Experiment 1)

Encapsulated cells

0 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.20E+12	12.34242268	0.065595476	12.31206439	2.07E+12
2.28E+12	12.3569814			
1.73E+12	12.2367891			

60 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.30E+12	12.36172784	0.102632687	12.44731951	2.86E+12
3.64E+12	12.56110138			
2.63E+12	12.41912931			

120 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.18E+12	12.07188201	0.043737391	12.02141406	1.05E+12
9.88E+11	11.9945371			
9.95E+11	11.99782308			

240 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
7.93E+11	11.89899927	0.037914879	11.92787491	8.49E+11
9.35E+11	11.97081161			
8.20E+11	11.91381385			

Free cells

0 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
4.58E+09	9.660865478	0.144714385	9.79852844	6.53E+09
6.10E+09	9.785329835			
8.90E+09	9.949390007			

60 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
2.05E+09	9.311753861	0.071618204	9.230655034	1.72E+09
1.50E+09	9.176091259			
1.60E+09	9.204119983			

120 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
7.10E+08	8.851258349	0.201085986	9.020926449	1.13E+09
1.75E+09	9.243038049			
9.30E+08	8.968482949			

240 minutes
37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
9.10E+08	8.959041392	0.083352981	8.950582677	9.03E+08
1.07E+09	9.029383778			
7.30E+08	8.86332286			

APPENDIX M

Shelf-life of encapsulated and free *B. lactis* cells in simulated bile and pancreatic juice (BPJ) (pH 6.5) (Experiment 2)

Encapsulated cells

0 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.90E+12	12.2787536	0.092558018	12.38131054	2.44E+12
2.55E+12	12.40654018			
2.88E+12	12.45863785			

60 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.35E+12	12.37106786	0.027060363	12.39300849	2.48E+12
2.65E+12	12.42324587			
2.43E+12	12.38471174			

120 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
3.58E+12	12.55327605	0.123437471	12.4896026	3.17E+12
3.70E+12	12.56820172			
2.23E+12	12.34733002			

240 minutes
37°C (aerobic)

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
5.38E+12	12.73037847	0.035955639	12.70015448	5.03E+12
5.13E+12	12.70969387			
4.58E+12	12.6603911			

Free cells

0 minutes

37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
7.94E+09	9.899820502	0.026209933	9.874921502	7.51E+09
7.04E+09	9.847572659			
7.54E+09	9.877371346			

60 minutes

37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
5.19E+09	9.715167358	0.049386715	9.693065504	4.95E+09
5.34E+09	9.727541257			
4.33E+09	9.636487896			

120 minutes

37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
3.50E+09	9.544068044	0.094278302	9.509750643	3.28E+09
3.82E+09	9.582063363			
2.53E+09	9.403120521			

240 minutes

37°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
3.08E+09	9.488550717	0.010101921	9.478488181	3.01E+09
2.94E+09	9.46834733			
3.01E+09	9.478566496			

APPENDIX N

Surviving fraction of encapsulated and free *B. lactis* cells in simulated bile and pancreatic juice (BPJ) (pH 6.5)

Encapsulated cells

37°C (aerobic)

Experiment number	Time (minutes)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	2.07E+12	1.00	1.00	1.00E+00
2		2.44E+12	1.00		
1	60	2.86E+12	1.38	1.20	1.20E+00
2		2.48E+12	1.02		
1	120	1.05E+12	0.51	0.90	9.00E-01
2		3.17E+12	1.30		
1	240	8.49E+11	0.41	1.24	1.24E+00
2		5.03E+12	2.06		

Free cells

37°C (aerobic)

Experiment number	Time (minutes)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	6.53E+09	1.00	1.00	1.00E+00
2		7.51E+09	1.00		
1	60	1.72E+09	0.26	0.46	4.60E-01
2		4.95E+09	0.66		
1	120	1.13E+09	0.17	0.30	3.10E-01
2		3.28E+09	0.44		
1	240	9.03E+08	0.14	0.27	2.70E-01
2		3.01E+09	0.40		

APPENDIX O

Shelf-life of encapsulated and free *B. lactis* cells in amasi (pH 4.4) and mahewu (pH 3.2) (Experiment 1)

Amasi

Encapsulated cells

0 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.19E+12	12.34044411	0.09573281	12.32887737	2.17E+12
2.62E+12	12.41830129			
1.69E+12	12.2278867			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.19E+12	12.34044411	0.09573281	12.32887737	2.17E+12
2.62E+12	12.41830129			
1.69E+12	12.2278867			

7 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.91E+12	12.28103337	0.067300803	12.34415548	2.23E+12
2.60E+12	12.41497335			
2.17E+12	12.33645973			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.41E+12	12.14921911	0.057720874	12.15277224	1.43E+12
1.63E+12	12.2121876			
1.25E+12	12.09691001			

14 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
3.20E+11	11.50514998	0.037328259	11.54300054	3.50E+11
3.80E+11	11.5797836			
3.50E+11	11.54406804			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.62E+11	11.41830129	0.094142767	11.44073494	2.80E+11
2.29E+11	11.35983548			
3.50E+11	11.54406804			

21 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.74E+11	11.24054925	0.00654231	11.24300512	1.75E+11
1.78E+11	11.25042			
1.73E+11	11.2380461			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.27E+11	11.10380372	0.084684774	11.14558229	1.42E+11
1.75E+11	11.24303805			
1.23E+11	11.08990511			

Amasi

Free cells

0 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
2.23E+07	7.348304863	0.039101893	7.314809642	2.07E+07
2.11E+07	7.324282455			
1.87E+07	7.271841607			

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.53E+07	7.184691431	0.083171003	7.19694089	1.59E+07
1.32E+07	7.120573931			
1.93E+07	7.285557309			

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
6.30E+07	7.799340549	0.139884843	7.704753535	5.23E+07
5.90E+07	7.770852012			
3.50E+07	7.544068044			

7 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.35E+07	7.130333768	0.054102645	7.187168157	1.55E+07
1.56E+07	7.193124598			
1.73E+07	7.238046103			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.21E+07	7.08278537	0.065259103	7.155162218	1.44E+07
1.62E+07	7.209515015			
1.49E+07	7.173186268			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
9.60E+06	6.982271233	0.067026686	7.035232723	1.09E+07
1.03E+07	7.012837225			
1.29E+07	7.11058971			

14 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.13E+07	7.053078443	0.045892376	7.054016387	1.14E+07
1.26E+07	7.100370545			
1.02E+07	7.008600172			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
9.30E+06	6.968482949	0.080925485	7.049344913	1.13E+07
1.12E+07	7.049218023			
1.35E+07	7.130333768			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
8.30E+06	6.919078092	0.081797326	6.952165225	9.07E+06
7.80E+06	6.892094603			
1.11E+07	7.045322979			

21 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
8.80E+06	6.944482672	0.056401723	6.935438557	8.67E+06
7.50E+06	6.875061263			
9.70E+06	6.986771734			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
8.50E+06	6.929418926	0.051918079	6.880661485	7.63E+06
6.70E+06	6.826074803			
7.70E+06	6.886490725			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
8.10E+06	6.908485019	0.005428999	6.903067366	8.00E+06
7.90E+06	6.897627091			
8.00E+06	6.903089987			

Mahewu

Encapsulated cells

0 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
4.04E+11	11.60638137	0.05402465	11.61053597	4.10E+11
3.62E+11	11.55870857			
4.64E+11	11.66651798			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
4.04E+11	11.60638137	0.05402465	11.61053597	4.10E+11
3.62E+11	11.55870857			
4.64E+11	11.66651798			

7 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
7.40E+11	11.86923172	0.062296366	11.82747969	6.77E+11
7.20E+11	11.8573325			
5.70E+11	11.75587486			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.16E+12	12.06445799	0.138978922	11.94485776	9.10E+11
6.20E+11	11.79239169			
9.50E+11	11.97772361			

14 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.77E+11	11.24797327	0.078974586	11.23418125	1.73E+11
1.41E+11	11.14921911			
2.02E+11	11.30535137			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.40E+11	11.14612804	0.020115989	11.14685006	1.40E+11
1.34E+11	11.1271048			
1.47E+11	11.16731733			

21 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
8.90E+10	10.94939001	0.019502372	10.97129512	9.37E+10
9.50E+10	10.97772361			
9.70E+10	10.98677173			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
4.80E+10	10.68124124	0.039383056	10.72582626	5.33E+10
5.50E+10	10.74036269			
5.70E+10	10.75587486			

Mahewu

Free cells

0 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
4.53E+07	7.656098202	0.028119501	7.628463624	4.26E+07
3.98E+07	7.599883072			
4.26E+07	7.629409599			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.94E+07	7.28780173	0.041463482	7.274394824	1.89E+07
1.69E+07	7.227886705			
2.03E+07	7.307496038			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.61E+07	7.206825876	0.065376577	7.194510183	1.58E+07
1.33E+07	7.123851641			
1.79E+07	7.252853031			

7 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.13E+07	7.053078443	0.114997843	7.180575951	1.55E+07
1.63E+07	7.212187604			
1.89E+07	7.276461804			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
7.30E+06	6.86332286	0.066209092	6.888653259	7.80E+06
6.90E+06	6.838849091			
9.20E+06	6.963787827			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.25E+07	7.096910013	0.144592313	7.116231397	1.36E+07
1.86E+07	7.269512944			
9.60E+06	6.982271233			

14 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.81E+06	6.257678575	0.053082264	6.255523912	1.81E+06
1.59E+06	6.201397124			
2.03E+06	6.307496038			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
4.80E+05	5.681241237	0.039383056	5.725826261	5.33E+05
5.50E+05	5.740362689			
5.70E+05	5.755874856			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
6.50E+06	6.812913357	0.075779506	6.804083497	6.43E+06
5.30E+06	6.72427587			
7.50E+06	6.875061263			

21 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.74E+05	5.240549248	0.00654231	5.243005118	1.75E+05
1.78E+05	5.250420002			
1.73E+05	5.238046103			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
3.20E+03	3.505149978	0.132876787	3.636490149	4.47E+03
5.90E+03	3.770852012			
4.30E+03	3.633468456			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.10E+06	6.041392685	0.242793053	6.314827557	2.27E+06
3.20E+06	6.505149978			
2.50E+06	6.397940009			

APPENDIX P

Shelf-life of encapsulated and free *B. lactis* cells in amasi (pH 4.4) and mahewu (pH 3.2) (Experiment 2)

Amasi

Encapsulated cells

0 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
8.99E+12	12.95375969	0.088631754	12.96641763	9.39E+12
7.67E+12	12.88479536			
1.15E+13	13.06069784			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
8.99E+12	12.95375969	0.088631754	12.96641763	9.39E+12
7.67E+12	12.88479536			
1.15E+13	13.06069784			

7 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.72E+12	12.23552845	0.057736694	12.1812077	1.53E+12
1.54E+12	12.18752072			
1.32E+12	12.12057393			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.82E+12	12.45024911	0.036956957	12.41168868	2.59E+12
2.56E+12	12.40823997			
2.38E+12	12.37657696			

14 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
8.70E+11	11.93951925	0.007676464	11.93780695	8.67E+11
8.50E+11	11.92941893			
8.80E+11	11.94448267			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.16E+12	12.06445799	0.102909774	12.02962052	1.09E+12
1.29E+12	12.11058971			
8.20E+11	11.91381385			

21 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
3.41E+11	11.53275438	0.071979069	11.61418423	4.15E+11
4.67E+11	11.66931688			
4.37E+11	11.64048144			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
3.27E+11	11.51454775	0.035253866	11.47953316	3.02E+11
2.78E+11	11.4440448			
3.02E+11	11.48000694			

Amasi

Free cells

0 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
2.50E+09	9.397940009	0.063652458	9.389621874	2.47E+09
2.10E+09	9.322219295			
2.81E+09	9.44870632			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
2.41E+09	9.382017043	0.611275195	8.677687445	9.45E+08
1.93E+08	8.285557309			
2.32E+08	8.365487985			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
4.20E+08	8.62324929	0.260347946	8.399863547	2.80E+08
1.30E+08	8.113943352			
2.90E+08	8.462397998			

7 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
7.51E+08	8.875639937	0.035575707	8.87177223	7.46E+08
6.83E+08	8.834420704			
8.04E+08	8.905256049			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
3.11E+08	8.492760389	0.282018947	8.39959835	2.84E+08
4.20E+08	8.62324929			
1.21E+08	8.08278537			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.25E+08	8.096910013	0.089852826	8.188832139	1.57E+08
1.89E+08	8.276461804			
1.56E+08	8.193124598			

14 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.32E+08	8.120573931	0.12896957	8.222302267	1.72E+08
2.33E+08	8.367355921			
1.51E+08	8.178976947			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
2.61E+08	8.416640507	0.041686385	8.432682664	2.72E+08
3.02E+08	8.480006943			
2.52E+08	8.401400541			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
2.61E+07	7.416640507	0.039828589	7.460695604	2.90E+07
3.12E+07	7.494154594			
2.96E+07	7.471291711			

21 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
5.30E+07	7.72427587	0.129506031	7.872494208	7.67E+07
9.20E+07	7.963787827			
8.50E+07	7.929418926			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
6.30E+06	6.799340549	0.128646928	6.827942281	6.93E+06
5.20E+06	6.716003344			
9.30E+06	6.968482949			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
7.60E+06	6.880813592	0.049176709	6.867403989	7.40E+06
6.50E+06	6.812913357			
8.10E+06	6.908485019			

Mahewu

Encapsulated cells

0 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.70E+11	11.23044892	0.045906445	11.27714061	1.90E+11
2.10E+11	11.32221929			
1.90E+11	11.2787536			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.70E+11	11.23044892	0.045906445	11.27714061	1.90E+11
2.10E+11	11.32221929			
1.90E+11	11.2787536			

7 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.31E+11	11.1172713	0.033092837	11.09492195	1.25E+11
1.14E+11	11.05690485			
1.29E+11	11.11058971			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.16E+12	12.06445799	0.138978922	11.94485776	9.10E+11
6.20E+11	11.79239169			
9.50E+11	11.97772361			

14 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
4.50E+11	11.65321251	0.040229582	11.63292155	4.31E+11
4.56E+11	11.65896484			
3.86E+11	11.5865873			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
6.50E+09	9.812913357	0.052731898	9.758786129	5.77E+09
5.10E+09	9.707570176			
5.70E+09	9.755874856			

21 Days

4°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
5.78E+11	11.76192784	0.258427489	11.54668244	3.92E+11
1.82E+11	11.26007139			
4.15E+11	11.6180481			

22°C

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.42E+09	9.383815366	0.079401613	9.292405882	1.98E+09
1.79E+09	9.252853031			
1.74E+09	9.240549248			

Mahewu

Free cells

0 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
2.51E+10	10.39967372	0.051504325	10.34821459	2.24E+10
2.23E+10	10.34830486			
1.98E+10	10.29666519			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
2.39E+10	10.3783979	0.08440091	10.357656	2.31E+10
1.84E+10	10.26481782			
2.69E+10	10.42975228			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
5.68E+09	9.754348336	0.032096981	9.722940095	5.29E+09
4.90E+09	9.69019608			
5.30E+09	9.72427587			

7 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
2.61E+09	9.416640507	0.077082761	9.388276178	2.47E+09
2.00E+09	9.301029996			
2.80E+09	9.447158031			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.55E+09	9.190331698	0.049774814	9.180915259	1.52E+09
1.68E+09	9.225309282			
1.34E+09	9.127104798			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.55E+09	9.190331698	0.034508332	9.229547417	1.70E+09
1.80E+09	9.255272505			
1.75E+09	9.243038049			

14 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
2.71E+07	7.432969291	0.089558199	7.350617925	2.27E+07
1.80E+07	7.255272505			
2.31E+07	7.36361198			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
3.52E+06	6.546542663	0.043665889	6.508651482	3.24E+06
2.89E+06	6.460897843			
3.30E+06	6.51851394			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
6.50E+07	7.812913357	0.044337903	7.769347079	5.90E+07
5.30E+07	7.72427587			
5.90E+07	7.770852012			

21 Days

4°C

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.23E+06	6.089905111	0.071557644	6.070488319	1.19E+06
9.80E+05	5.991226076			
1.35E+06	6.130333768			

22°C (aerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
6.30E+06	6.799340549	0.128646928	6.827942281	6.93E+06
5.20E+06	6.716003344			
9.30E+06	6.968482949			

22°C (anaerobic)

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
7.60E+06	6.880813592	0.049176709	6.867403989	7.40E+06
6.50E+06	6.812913357			
8.10E+06	6.908485019			

APPENDIX Q

Surviving fraction of encapsulated and free *B. lactis* cells in amasi (pH 4.4)

Encapsulated cells

4°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	2.17E+12	1.00	1.00	1.00E+00
2		9.39E+12	1.00		
1	7	2.23E+12	1.03	0.60	6.00E-01
2		1.53E+12	0.16		
1	14	3.50E+11	0.16	0.13	1.30E-01
2		8.67E+11	0.092		
1	21	1.75E+11	0.08	0.06	6.00E-02
2		4.15E+11	0.044		

22°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	2.17E+12	1.00	1.00	1.00E+00
2		9.39E+12	1.00		
1	7	1.43E+12	0.66	0.47	4.70E-01
2		2.59E+12	0.28		
1	14	2.80E+11	0.13	0.12	1.20E-01
2		1.09E+12	0.12		
1	21	1.42E+11	0.07	0.05	5.00E-02
2		3.02E+11	0.032		

APPENDIX Q

Surviving fraction of encapsulated and free *B. lactis* cells in amasi (pH 4.4)

Encapsulated cells

4°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	2.17E+12	1.00	1.00	1.00E+00
2		9.39E+12	1.00		
1	7	2.23E+12	1.03	0.60	6.00E-01
2		1.53E+12	0.16		
1	14	3.50E+11	0.16	0.13	1.30E-01
2		8.67E+11	0.092		
1	21	1.75E+11	0.08	0.06	6.00E-02
2		4.15E+11	0.044		

22°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	2.17E+12	1.00	1.00	1.00E+00
2		9.39E+12	1.00		
1	7	1.43E+12	0.66	0.47	4.70E-01
2		2.59E+12	0.28		
1	14	2.80E+11	0.13	0.12	1.20E-01
2		1.09E+12	0.12		
1	21	1.42E+11	0.07	0.05	5.00E-02
2		3.02E+11	0.032		

Free cells

4°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	2.07E+07	1.00	1.00	1.00E+00
2		2.47E+09	1.00		
1	7	1.55E+07	0.75	0.53	5.30E-01
2		7.46E+08	0.30		
1	14	1.14E+07	0.55	0.31	3.10E-01
2		1.72E+08	0.070		
1	21	8.67E+06	0.42	0.22	2.20E-01
2		7.67E+07	0.031		

22°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	1.59E+07	1.00	1.00	1.00E+00
2		9.45E+08	1.00		
1	7	1.44E+07	0.91	0.60	6.00E-01
2		2.84E+08	0.30		
1	14	1.13E+07	0.71	0.50	5.00E-01
2		2.72E+08	0.29		
1	21	7.63E+06	0.48	0.24	2.40E-01
2		6.93E+06	0.0073		

Free cells

22°C (anaerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	5.23E+07	1.00	1.00	1.00E+00
2		2.80E+08	1.00		
1	7	1.09E+07	0.21	0.38	3.80E-01
2		1.57E+08	0.56		
1	14	9.07E+06	0.17	0.14	1.40E-01
2		2.90E+07	0.10		
1	21	8.00E+06	0.15	0.09	9.00E-02
2		7.40E+06	0.026		

APPENDIX R

Surviving fraction of encapsulated and free *B. lactis* cells in mahewu (pH 3.2)

Encapsulated cells

4°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	4.10E+11	1.00	1.00	1.00E+00
2		1.90E+11	1.00		
1	7	6.77E+11	1.65	1.15	1.20E+00
2		1.25E+11	0.66		
1	14	1.73E+11	0.42	1.35	1.40E+00
2		4.31E+11	2.27		
1	21	9.37E+10	0.23	1.15	1.15E+00
2		3.92E+11	2.06		

22°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	4.10E+11	1.00	1.00	1.00E+00
2		1.90E+11	1.00		
1	7	9.10E+11	2.22	1.42	1.40E+00
2		1.17E+11	0.62		
1	14	1.40E+11	0.34	0.19	1.90E-01
2		5.77E+09	0.030		
1	21	5.33E+10	0.13	0.07	7.00E-02
2		1.98E+09	0.010		

Free cells

4°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	4.26E+07	1.00	1.00	1.00E+00
2		2.24E+10	1.00		
1	7	1.55E+07	0.36	0.24	2.40E-01
2		2.47E+09	0.11		
1	14	1.81E+06	0.042	0.022	2.20E-02
2		2.27E+07	0.0010		
1	21	1.75E+05	0.0041	0.0021	2.10E-03
2		1.19E+06	0.000053		

22°C (aerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	1.89E+07	1.00	1.00	1.00E+00
2		2.31E+10	1.00		
1	7	7.80E+06	0.41	0.24	2.40E-01
2		1.52E+09	0.066		
1	14	5.33E+05	0.028	0.014	1.40E-02
2		3.24E+06	0.00014		
1	21	4.47E+03	0.00024	0.00027	2.70E-04
2		6.93E+06	0.00030		

Free cells

22°C (anaerobic)

Experiment number	Time (days)	Average titre	Surviving fraction (Nt/No)	Average surviving fraction	Log surviving fraction
1	0	1.58E+07	1.00	1.00	1.00E+00
2		5.29E+09	1.00		
1	7	1.36E+07	0.86	0.59	5.90E-01
2		1.70E+09	0.32		
1	14	6.43E+06	0.41	0.21	2.10E-01
2		5.90E+07	0.011		
1	21	2.27E+06	0.14	0.073	7.30E-02
2		7.40E+06	0.0014		

APPENDIX S

Taste panel data of encapsulated and free *B. lactis* cells in amasi (pH 4.4) and mahewu (pH 3.2)

Amasi

Encapsulated cells

Day 1

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
5.40E+11	11.73239376	0.073047979	11.75673762	5.77E+11
5.00E+11	11.69897			
6.90E+11	11.83884909			

Day 6

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.27E+11	11.35602586	0.018242547	11.35513253	2.27E+11
2.17E+11	11.33645973			
2.36E+11	11.372912			

Day 14

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
2.14E+12	12.33041377	0.069629729	12.38358221	2.44E+12
2.90E+12	12.462398			
2.28E+12	12.35793485			

Amasi

Free cells

Day 1

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
6.80E+07	7.832508913	0.08517387	7.796270471	6.33E+07
5.00E+07	7.698970004			
7.20E+07	7.857332496			

Day 6

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.08E+07	7.033423755	0.039750399	6.995888755	9.93E+06
1.00E+07	7.000000000			
9.00E+06	6.954242509			

Day 14

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
5.30E+07	7.72427587	0.05685683	7.76832427	5.90E+07
6.80E+07	7.832508913			
5.60E+07	7.748188027			

Mahewu
Encapsulated cells

Day 1

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
7.00E+11	11.84509804	0.059796527	11.81687285	6.60E+11
7.20E+11	11.8573325			
5.60E+11	11.74818803			

Day 6

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.28E+11	11.10720997	0.037426009	11.10613688	1.28E+11
1.39E+11	11.1430148			
1.17E+11	11.06818586			

Day 14

cfu.g^{-1}	Log cfu.g^{-1}	Standard deviation	Average log cfu.g^{-1}	Average cfu.g^{-1}
1.23E+12	12.08990511	0.059475544	12.15468277	1.44E+12
1.47E+12	12.16731733			
1.61E+12	12.20682588			

Mahewu
Free cells

Day 1

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
3.50E+07	7.544068044	0.090165752	7.632244281	4.35E+07
4.25E+07	7.62838893			
5.30E+07	7.72427587			

Day 6

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
1.07E+07	7.029383778	0.019503723	7.047630873	1.12E+07
1.17E+07	7.068185862			
1.11E+07	7.045322979			

Day 14

cfu.ml^{-1}	Log cfu.ml^{-1}	Standard deviation	Average log cfu.ml^{-1}	Average cfu.ml^{-1}
5.30E+07	7.72427587	0.05685683	7.76832427	5.90E+07
6.80E+07	7.832508913			
5.60E+07	7.748188027			