Cell immobilization techniques for the preservation of probiotics

BY

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Declaration

I declare that the thesis entitled "CELL IMMOBILIZATION TECHNIQUES FOR THE PRESERVATION OF PROBIOTICS", which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution

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LIST OF ABBREVIATIONS

AAD: Antibiotic Associated Diarrhoea WHO: World Health Organization

cfu: Colony forming units

CLSM: Confocal Laser Scanning Microscopy

DNA: Deoxyribonucleic acid

DVS: Direct vat set

FDA: Food and Drug Administration

FOS: Fructooligosaccharides

GI: Gastrointestinal tract

GMS: Glyceryl monostearate

GOS: Galactooligosaccharides

GRAS: Generally Regarded as Safe

HDL: High Density lipoprotein

IgA: Immunoglobulin A

IgE: Immunoglobulin E

LDL: Low Density lipoprotein

MRS: de Man Rogosa Sharpe

NDO: Non- digestible oligosaccharides

PEO-PPO-PEO: Ethylene oxide-propylene oxide triblock copolymer

PCL: Polycaprolactone

PGSS: Particles from Gas Saturated Solution

PI: Propidium iodide

PVP: Poly (vinyl pyrrolidone)

sASC: Specific Antibody Secreting Cells

$scCO_2$: Supercritical carbon dioxide

SCF: Supercritical Fluids

SEM: Scanning Electron Microscopy

SGF: Simulated Gastric Fluid

SIF: Simulated Intestinal Fluids

VA-CA: Poly (vinyl acetate-*co*-crotonic acid)

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SUMMARY

Incorporation of probiotic cultures in products in order to replenish or supplement the normal gastrointestinal microflora is a well known and accepted practice. However survival of these cultures is a problem due to a number of reasons including effects of storage conditions. Various researchers from different countries around the world have reported probiotic product instability. Microencapsulation has been used in an attempt to solve this problem. However, most methods involve the use of organic solvents which is not ideal because their toxicity may cause destruction of the microbial cells. A novel encapsulation method for probiotics, which excludes the use of organic solvents, was developed by the Council for Scientific and Industrial Research (CSIR) (US Patent Application no. 20050112205). This thesis investigated the efficiency/potential of this new method for increasing stability of sensitive probiotic cultures, specifically bifidobacteria.

Early studies using both culture dependent and culture independent techniques showed reduced numbers of viable cultures in probiotic products, mainly yoghurts, from all around the world. These results were confirmed in this study for similar products sold in South Africa. Most of the product labels did not specify viable numbers of probiotics nor the identity (genus and species names) of the microorganisms incorporated.

Successful encapsulation of bifidobacteria was achieved using the CSIR patented method. Complete encapsulation was indicated by absence of cells on surfaces of the encapsulated particles and production of a product with an acceptable particle size distribution was obtained. It was also demonstrated that the encapsulation process produced no visible morphological changes to the bacterial cells nor did it have a negative effect on cell viability over time. The potential of interpolymer complex formation in $scCO_2$ for the encapsulation of sensitive probiotic cultures was demonstrated for the first time.

Once ingested, probiotic cultures are exposed to unfavourable acidic conditions in the upper gastrointestinal tract. It is desired that these cultures be protected from this in order to increase the viability of the probiotics for efficient colonization. Interpolymer complex encapsulated *B. longum* Bb-46 cells were therefore exposed to simulated gastric fluid (SGF) and subsequently to simulated intestinal fluid (SIF).

It was found that the interpolymer complex protected bifidobacteria from gastric acidity, displaying pH-responsive release properties, with little to no release in SGF and substantial release in SIF. Thus the interpolymer complex demonstrated desirable characteristics retaining the encapsulated bacteria inside when conditions were unfavourable and only releasing them under favourable conditions. Survival was improved by the incorporation of glyceryl monostearate (GMS) in the matrix and by use of gelatine capsules. Protection efficiency of the interpolymer matrix was better when higher loading of GMS was used. Use of polycaprolactone (PCL) as an alternative to poly (vinylpyrrolidone) (PVP) and incorporation of ethylene oxide-propylene oxide triblock copolymer (PEO-PPO-PEO) affected the interpolymer complex negatively, rendering it swellable in the low pH environment exposing the bifidobacteria to gastric acidity. The use of beeswax seemed to have a more protective effect though results were inconclusive.

Probiotic cultures must also remain viable in products during storage. Encapsulated bacteria were either harvested from the reactor after 2 h of equilibration followed by depressurization, and then ground to a fine powder or after 2 h of equilibration the liquefied product was sprayed through a capillary tube with a heated nozzle at the end, into the product chamber. Encapsulated bacteria were stored in either sterile plastic bags or glass bottles under different conditions and then viable counts were determined over time. Survival of bacteria was generally better when the products were stored in glass bottles than in plastic bags. Bacteria encapsulated in an interpolymer complex formed between PVP and vinyl acetate-crotonic acid copolymer (VA-CA), (PVP:VA-CA) survived better than non-encapsulated bacteria under all storage conditions when the

product was recovered from the reaction chamber. When the product was recovered from the product chamber, numbers of viable non-encapsulated bacteria were higher than the encapsulated bacteria for all interpolymer complex formulations. This was probably due to some exposure to high shear during spraying into the product chamber. The interpolymer complex between PCL and VA-CA i.e. PCL:VA-CA seemed weaker than the PVP:VA-CA interpolymer complex as viable counts of bacteria released from it were lower than those from the latter complex. Addition of PEO-PPO-PEO to both the PVP:VA-CA and PCL:VA-CA complexes decreased the protection efficiency. However, results indicated that sufficient release of encapsulated bacteria from the interpolymer complexes was obtained when the encapsulated material was incubated in SIF rather than in Ringer's solution. When SIF was used for release of encapsulated bacteria, the shelf life of *B. longum* Bb-46 was doubled. Encapsulation in an interpolymer complex therefore provided protection for encapsulated cells and thus has potential for improving shelf life of probiotic cultures in products. Further studies will investigate the effects of encapsulating probiotics together with prebiotics in the interpolymer complex as well as effects of encapsulating combinations of different probiotic strains together, both on survival in simulated gastrointestinal tract and during storage.

The unique particles produced using the patented encapsulation technique increased the stability of probiotic cultures. This technique may find significant application in industries manufacturing probiotic products, especially food and pharmaceuticals, thereby improving the well being of consumers.

INTRODUCTION

There are twenty times more bacteria in the human body than cells. The large intestine alone contains about 10^{10}- 10^{11} bacteria g^{-1} of intestinal contents. This is made up of approximately 400-500 species, making the large intestine the most densely populated area in the whole body. These autochthonous bacteria have profound effects on the anatomical, physiological and immunological development of the host. Some members, good bacteria, are vital for good health while others, pathogens, are harmful and can cause infections. The good bacteria help promote digestion of food and absorption of nutrients. They also stimulate the host immune system to respond more quickly to pathogen challenge and inhibit colonization of the gastrointestinal (GI) tract by pathogens through bacterial antagonism. Pathogens produce chemicals toxic to the body and are frequently responsible for common digestive complaints such as constipation, diarrhoea and inflammation and for chronic conditions such as irritable bowel syndrome (Berg, 1996).

The GI tracts of healthy individuals maintain a balance between the good bacteria and pathogens. In these individuals intestines are colonized by favourable Gram positive microorganisms, notably lactobacilli and bifidobacteria (Fooks et al., 1999; Bin-Nun et al., 2005). When the balance between the two groups of bacteria is disturbed, the microflora population shifts towards prevalence of potentially detrimental microorganisms like *Clostridia*, sulphate reducing bacteria and *Bacteroides* (Fooks et al., 1999).

Factors contributing to imbalance include host physiology, microbial interactions (Richardson, 1996), lack of food or poor diet, travelling, antibiotics, cytostatics radiation, immune disorders, emotional stress and ageing (Havenaar and Huis in'tVeld, 1992; Richardson, 1996). Sites on the intestinal epithelium that were inhabited by beneficial microbes become empty. Occupation of these sites by potential pathogens increases the risk for outbreak of opportunistic infectious disease (Havenaar and Huis in'tVeld, 1992). Transient enteropathogens such as *Salmonella*, *Campylobacter*, *Escherichia coli* and

Listeria cause disorders such as cancer and ulcerative colitis. Susceptibility of the individual to infections is increased (Fooks et al., 1999) and other diseases e.g. liver and kidney disorders, atherosclerosis and hypertension may occur (Mitsuoka, 1996).

The normal balance of intestinal flora may be restored from an unbalanced state by deliberate ingestion of beneficial bacteria. Intestinal strains of lactic acid bacteria or bifidobacteria are used to fulfil this purpose (Mitsuoka, 1996). These strains of bacteria used to restore the balance of indigenous microflora of the gut are called probiotics.

The importance of autochthonous bacteria in the GI tract as a resistance factor against potential pathogens was already recognised in the 19th century by Metchnikoff. Research on probiotics started in 1950, although it was overshadowed and largely ignored due to introduction of antibiotics (Havenaar and Huis in'tVeld, 1992). Research in the field re-emerged in the 1960s due to the increased interest of people in health and natural ways of promoting health. The increase in numbers of bacteria acquiring resistance to multiple drugs, especially those causing nosocomial infections, the demand of consumers for natural substitutes for drugs, and the emergence of scientific and clinical evidence proving health benefits related to consumption of probiotic strains also made a contribution (Havenaar and Huis in'tVeld, 1992; Reid et al., 2003; Leahy et al., 2005).

Probiotics have been defined differently by various researchers, with the changes in the definition based on observations made when these particular microorganisms are studied. Fuller (1989) defined probiotics as "live microbial food supplements with health benefits to the host by improving the intestinal microbiota". Probiotics were later defined as "microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well being of the host" (Salminen et al., 1999). The World Health Organization and Food and Agriculture Organization of the United Nations (FAO/WHO, 2001) agreed on the definition of probiotics as: "live microorganisms which, when administered in adequate amounts confer a health benefit on the host" (Leahy et al., 2005).

The potential benefits of probiotic foods include: (Wahlqvist, 2002)

- Prevention and treatment of diarrhoea caused by rotavirus, especially in children
- Immune system enhancement
- Reducing some allergic reactions
- Treating and preventing respiratory infections, especially in children
- Decreased faecal mutagenicity
- Decrease in the level of pathogenic bacteria
- Decreased faecal bacterial enzyme activity
- Prevention of the recurrence of superficial bladder cancer
- The restoration of the correct balance of natural microflora after stress, antibiotic treatment, alcohol use and chemotherapy.

Today consumers are very cautious of their health and they expect the food that they consume to be healthy or even able to prevent illness (Mattila-Sandholm et al., 2002). Probiotics are available as tablets or capsules, powders, liquid suspensions and sprays (Marcon, 1997, Fooks et al., 1999). Some can be obtained from pharmacies as over-the-counter products (Marcon, 1997). In some countries like Japan, probiotics are incorporated into confectionery and fruit drinks. South Africa also has probiotic products on the market, comprising of different fermented milks and lyophilised preparations in the form of tablets or capsules (Theunissen and Witthuhn, 2004). The probiotic industry in South Africa is worth approximately R45 million per annum, with over 11 million doses taken annually. This means that over 30 000 doses of probiotics are taken daily in South Africa. The market for probiotics is developing and it is estimated to be growing at a rate between 8 and 15% for dietary supplements (Health 24, 2004).

The market for probiotics offers a great potential for manufacturers and is increasing although there are complex processing challenges of formulating products incorporating probiotics. The biggest challenge associated with the use of probiotics is the retention of viability of probiotic cultures during processing and storage. Probiotic products have to be efficient and reliable, i.e. they must contain sufficient numbers of viable microorganisms up to the expiry date (Fasoli et al., 2003). Their eventual success thus

depends on their survival in the products during their storage and their resistance to acidity in the upper GI tract, leading to establishment, colonization and ultimate efficiency (Sun and Griffiths, 2000: Picot and Lacroix, 2003). These bacteria often die during food manufacturing or during passage to the intestine. Shelf life has been unpredictable for probiotics, and the industry has had difficulty backing up label claims (Fasoli et al., 2003).

Probiotic bacteria perform best when they find suitable environmental conditions and when they are protected against stresses (e.g extreme temperatures, high pressure, shear forces) that they may encounter during their production at the industry level or in the gastrointestinal tract (gastric acids and bile salts) (Siuta-Cruce and Goulet, 2001). Harsh environments including exposure issues related to transport logistics, extended storage and the acidic conditions in the human stomach can kill live bacteria rendering probiotic supplements worthless by the time they are consumed or reach the intestines. This has been illustrated by numerous studies showing that most commercially available probiotic products do not deliver what they promise (Micanel et al., 1997; Vinderola et al., 2000; Huff, 2004).

This problem may be alleviated by use of probiotic encapsulation technology to ensure probiotic viability (Mattila-Sandholm, 2002). Development of delivery forms such as encapsulation techniques and coatings for protection of probiotics from detrimental factors leading to death is a significant area in probiotic research (Sun and Griffiths, 2000). Encapsulation of bifidobacteria for maintenance of viability has been investigated by various researchers (Hsiao et al., 2004). The most commonly used materials for immobilization of cells are alginate beads and κ-carrageenan (Sun and Griffiths, 2000). The encapsulation methods used typically employ organic solvents which are not favourable for use in this regard as solvents are generally toxic to microbial cells (Sardessai and Bhosle, 2002; Matsumoto et al., 2004). Probiotics are microbes and are therefore sensitive to these solvents. Solvents accumulate in the cytoplasmic membrane of cells changing its structure (Fernandes et al., 2003) and stopping the cell from

performing its normal functions (Kashket, 1987; Fernandes et al., 2003), which ultimately lead to cell lysis and death (Fernandes et al., 2003).

The technologies developed to produce gel beads present serious difficulties for large-scale production such as low production capacity and large bead diameters for the droplet extrusion methods and transfer from organic solvents and large size distribution for the emulsion techniques. Moreover, addition of some of the polysaccharides used is not permitted in yoghurts or fermented milk in some countries (Picot and Lacroix, 2004). Even though encapsulation of bifidobacteria for protection of viability has been investigated by various researchers, the methods and formulations still need to be refined.

Supercritical fluids have been widely used in extraction and recovery of high value compounds. A supercritical fluid is a substance that, at temperatures and pressures greater than its critical temperature and pressure, is a gas-like, compressible fluid that takes the shape of its container and fills it (Demirbaş, 2001). Experience accumulated in recent years on the use of supercritical fluids and their processes have indicated that it is possible to explore and envision their uses beyond the common practice of extraction (Sarrade et al., 2003). Supercritical fluid technologies can also be applied in making new innovative products. One of the very promising areas of research is microencapsulation of drug molecules, used for controlled drug release in the human body (Sihvonen et al., 1999). Supercritical fluids have the potential to contribute towards elimination of solvent toxicity problems as some of them (in particular carbon dioxide) can be used as a low temperature, stable, unreactive, environmentally benign solvent in encapsulation processes.

This work was part of a project entitled: “Supercritical fluid encapsulation of sensitive actives” where the main aim was to develop an encapsulation method using supercritical fluid, for protection and preservation of sensitive substances (like probiotics) in order to improve their viability, effectiveness and shelf life. If the method could overcome the problems posed by encapsulation methods using currently known technologies, it could benefit health care in South Africa in general, and particularly rural and remote areas of

the country. It could also be used for veterinary vaccines and other products if successfully developed.

The main objective of this research was to investigate the efficiency of the novel encapsulation technique for encapsulation of probiotics. The specific objectives were to:

- Determine viability of cultures in commercial South African probiotic yoghurts and to determine whether product labels specified the probiotic cultures and their levels in colony forming units (cfu) by the end of the shelf life**.**
- Investigate the efficiency of the novel method of probiotic encapsulation in interpolymer complexes and the effect of the encapsulation process on bacterial cells
- Determine the yield of probiotics that can be obtained after encapsulation into the polymer material.
- Determine whether encapsulation provides protection for probiotics in the gastrointestinal tract.
- Evaluate the effect of the encapsulation method on the shelf life of different probiotic microorganisms.
- Compare survival rates of the same *Bifidobacterium* strain when immobilised in different polymer complex formulations.
- Test the effect of incorporation of prebiotics on the stability (shelf life) of probiotics.
- Determine the effect of combining different probiotic strains on the survival rate.

REFERENCES

Berg, R.D., 1996. The indigenous gastrointestinal microflora. Trends in Microbiology 4, 430-435.

Bin-Nun, A., Bromiker, R., Wilschanski, M., Kaplan, M., Rudensky, B., Caplan, M., Hammerman, C., 2005. Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. The Journal of Paediatrics 147, 192-196.

Demirbaş, A., 2001. Supercritical fluid extraction and chemicals from biomass with supercritical fluids. Energy Conversion and Management 42, 279-294.

Fasoli, S., Marzotto, M., Rizzotti, L., Rossi, F., Dellaglio, F., Torriani, S., 2003. Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. International Journal of Food Microbiology 82, 59-70.

Fernandes, P., Ferreira, B.S., Cabral, J.M S., 2003. Solvent tolerance in bacteria: role of efflux pumps and cross-resistance with antibiotics. International Journal of Food Microbiology 22, 211-216.

Fooks, L.J., Fuller, R., Gibson, G.R., 1999. Prebiotics, probiotics and human gut microbiology. International Dairy Journal 9, 53-61.

Health News 24. 2004. Natural health. Labels on SA supplements lie.
Website: http://www.health24.com/news/Natural_health/1-932,26696.aspFood. Access date: 02/06/2004.

Hsiao, H.-C., Lian, W.-C., Chou, C.-C., 2004. Effect of packaging conditions and temperature on viability of microencapsulated bifidobacteria during storage. Journal of the Science of Food and Agriculture 84, 134-139.

Huff, B.A., 2004. "Probiotics" might not be what they seem. Canadian Family Physician 50, 583-587.

Kashket, E.R., 1987. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiology Letters 46, 233-244.

Leahy, S.C., Higgins, D.G., Fitzgerald, G.F., van Sinderen, D., 2005. Getting better with bifidobacteria. Journal of Applied Microbiology 98, 1303-1315.

Marcon, M.J., 1997. Probiotics in health and medicine: Moving from fashion to scientific validity. Clinical Microbiology Newsletter 19, 89-96.

Matsumoto M., Mochiduki, K., Kondo, K. 2004. Toxicity of ionic liquids and organic solvents to lactic acid producing bacteria. Journal of Bioscience and Bioengineering 98, 344-347.

Mattila-Sandholm, T., Myllärinen, P., Crittenden, R., Mogensen, G., Fondén, R., Saarela, M., 2002. Technological challenges for future probiotic foods. International Dairy Journal 12, 173-182.

Micanel, N., Haynes, I.N., Playne, M.J., 1997. Viability of probiotics cultures in commercial Australian yoghurts. Australian Journal of Dairy Technology 52, 24-26.

Mitsuoka, T., 1996. Intestinal flora and human health. Asia Pacific Journal of Clinical Nutrition 5, 2-9.

Picot, A., Lacroix, C., 2003. Effects of micronization on viability and thermotolerance of probiotic freeze-dried cultures. International Dairy Journal 13, 455-462.

Richardson, D., 1996. Probiotics and product innovation. Nutrition and Food Science 4, 27-33.

Sardessai Y., Bhosle S., 2002. Tolerance of bacteria to organic solvents. Research in Microbiology 153, 263-268.

Sarrade S., Guizard, C., Rios, G.M., 2003. New applications of supercritical fluids and supercritical processes in separation. Separation and Purification Technology 32, 57-63.

Sihvonen, M., Järvenpää E., Hietaniemi, V., Huopalahti, R., 1999. Advances in supercritical carbon dioxide technologies. Trends in Food Science and Technology 10, 217-222.

Siuta-Cruce, P., Goulet, J., 2001. Improving probiotic microorganisms in food systems. Food Technology 55, 36-42.

Sun, W., Griffiths, M.W., 2000. Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan-xanthan beads. International Journal of Food Microbiology 61, 17-25.

Theunissen, J, Witthuhn, C., 2004. Probiotic content: truth or fiction? South African Food Review 31, 15-17.

Vinderola, C.G., Bailo, N., Reinheimer, J.A, 2000. Survival of probiotic microflora in Argentinian yoghurts during refrigerated storage. Food Research International 33, 97-102.

Wahlqvist, M., 2002. Prebiotics and Probiotics.
Internet: www.healthyeatingclub.com. Access date: 5/10/2003