Cell immobilisation of *Lactobacillus acidophilus*, a probiotic,for potential therapeutic usage.

Submitted by

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in fulfillment of the requirements for a Masters Degree

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September 1997

DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science by Research is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work

Signed Rachel Hewitt
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Date September 1997

ACKNOWLEDGEMENTS

I wish to thank my supervisors Dr. Louise Kearney and Phil Nicholl for thorough and consistent supervision. I would also like to express my sincere thanks to Dr. Marlene Proctor for the opportunity to carry out my MSc. studies at the Dublin Institute of Technology.

I would also like to extend my thanks to all the staff and postgraduate students who made the laboratory such a friendly place to work, and to my friends and flatmates including Phil, Bronagh, Bridget, Joanne, Laura, Donna and Helen for their friendship and encouragement.

Finally, but most of all, I would like to thank my parents and family for their continuous support in everything I do.

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Abbreviations

A	Absorbance
A_w	water availability
$CaCl_2$	calcium chloride
c.f.u.	colony forming units
DNA	deoxyribonucleic acid
E.C.	enzyme collection
HCl	hydrogen chloride
in vitro	(literally in glass), in an artifical environment, outside a living organism.
MRS	de Man Rogosa Sharpe
NaCl	sodium chloride
NaOH	sodium hydroxide
pH	$-\log (H^+)$
RNA	ribonucleic acid
S.D.	standard deviation

CHAPTER ONE :

General Introduction

1.1 : GENERAL INTRODUCTION

1.1.1 : Lactic Acid Bacteria

The lactic acid bacteria comprise a group of metabolically related micro-organisms belonging to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* type N. Their name originated from their ability to ferment milk by production of lactic acid. In 1919 Orla Jensen clearly defined characteristics of this group. He described the lactic acid bacteria as a group of non-motile, sporeless, Gram-positive cocci and rods, whose main fermentation product was lactic acid. Some species of this group are associated with having 'probiotic', or health-promoting properties.

1.1.2 : *Lactobacillus acidophilus*

L.acidophilus is a member of the lactic acid bacteria group, which has been frequently associated with having probiotic properties. The term acidophilus is derived from two Greek words acidum and philus which is literally translated as 'acid loving'. As the term suggests this organism is an acid-loving bacterium. Its acid tolerance varies from 0.3% to 1.9% titratable acidity, with optimum pH 5.5 to 6.0; growth generally occurs at 5.0 or less and is often reduced at neutrality. It is a homofermentative lactobacillus which produces mainly D / L lactic acid. In appearance it is a gram positive rod with rounded ends, generally 0.6 - 0.9 X 1.5 - 6 μm, occurring singly, in pairs or as short chains. It is non-flagellated, non-motile, non-spore-forming and is intolerant to salt (Kandler and Weiss, 1992).

L.acidophilus is widely distributed in nature. They are isolated from the intestinal tract of humans and animals, human mouth (Duncan and Edberg, 1995; Mc Cartney et al., 1996)

and vagina (Kandler and Weiss, 1992). They are also isolated from milk, dairy products, other fermented foods / beverages and usually in very small numbers on plant material (Kandler and Weiss, 1992).

1.1.3 : Therapeutic aspects

The beneficial effects of *L.acidophilus* was first introduced by Ilya Metchnikoff (1908), a Russian bacteriologist who shared the Nobel prize in 1908. In his studies, he concluded that putrefaction in the large intestine produces degenerative substances in a process he named 'auto-intoxicaton'. This process he claimed was the main cause of old age, senility and natural death.

In his book, 'The prolongation of Life', he suggested elimination of putrefactive bacteria by ingesting large amounts of Bulgarian sour milk which contained the bacterium he isolated and named *Bacillus bulgaricus*, later identified as *L.acidophilus*. Metchnikoff postulated his theory on the basis that after ingestion these lactic acid producing bacteria will colonise the gut and suppress toxin formation by inhibiting the putrefactive bacteria with an undesirable acid environment. This would ultimately lead to longevity of the host. Metchnikoffs theory about prolongation of life was backed by his statistical argument that Bulgarian people of his time who consumed large amounts of sour (fermented) milk lived longer with an average age of 87 years and with 4 out of 1000 living past the century limit. However, he was unable to prove the implantation of the bacteria in the intestinal tract. It was not until 1920 that Rettger and Chaplin succeeded in proving that some strains of lactic acid bacteria had this implantation capacity.

Since Metchnikoffs era numerous therapeutic properties of *L.acidophilus* have been discovered among these include:

1. **Inhibition of pathogens:** This is accomplished through their production of a range of inhibitory substances including lactic / acetic acid production aswell as production of specific microbial inhibitors such as Nisin, Diplococcin, Lactobrevin, Bulgarican, Lactolin and Acidolin. These inhibitors can be antagonistic to a wide range of bacteria including the putrefactive type such as *Salmonella, Staphylococcus, Escherichia, Listeria and Pseudomonas* (Lindgren and Dobrogosza, 1990, Salji, 1992). Lactic acid bacteria may also prevent the establishment of pathogens by colonising the gut and thereby inhibiting the attachment of harmful bacteria.

2. **Anticarcinogenic action:** Some probiotic bacteria can inhibit formation of carcinogens by degrading nitrosamines and thus reduce their carcinogenic effect. These microorganisms can also be antagonistic to certain types of tumour cells by either inhibiting the formation of carcinogens, reducing carcinogen-promoting enzymes or indirectly by stimulating the immune system (Salji, 1992, 1994; Mital and Garg, 1995).

3. **Anticholesterolaemic effect:** Studies on both humans and animals have shown that serum cholesterol could be reduced by *L.acidophilus*. This maybe due to co-precipitation of cholesterol and bile salts or deconjugation of bile salts which lowers cholesterol levels by decreasing the digestibility of lipids and by increasing bile salt

elimination in the faeces (Salji, 1992; Noh and Gilliland, 1993; Buck and Gilliland, 1994)

4. **Stimulation of the immune system:** Lactic acid bacteria are known to enhance macrophage formation with an increase in the production of supressor cells and gamma interferon. (Lee and Salminen, 1995; Mital and Garg, 1995)

5. **Lactose utilisation:** A large proportion of the world's population is unable to utilise lactose, due to a deficiency in the enzyme lactase, also known as β-galactosidase. This common illness is associated with nausea, abdominal pain, cramps or diarrhoea after consumption of milk. Lactose intolerance could be reduced by consuming products containing lactic acid bacteria such as *L.acidophilus* as they produce the enzyme β galactosidase. Some lactic acid bacteria die after ingestion, and shed their lactase enzyme into the host gut while others colonise the gut and actively participate in further breakdown of lactose in the intestinal tract of the host (Salji, 1994).

Other health attributes of a general nature have also been reported. These include improved digestibility due to partial breakdown of proteins, fats and carbohydrates and enhancement of growth through improved bio-degradability of nutrients (Salji, 1994) and the synthesis of essential vitamins such as β-complex.

1.1.4 : Commercial Probiotic Products

Almost a hundred years have passed since the introduction of the theories on the prolongation of life by the modulation of the intestinal ecosystem. However, only recently has the scientific basis of probiotic studies been firmly established and sound clinical studies of strains like *L.acidophilus* been published giving documented examples of their ability to maintain and promote the health of the host.

With this knowledge in mind numerous probiotic products have been developed to contain high levels of lactic acid bacteria such as *L.acidophilus* cells for their therapeutic attributes. Examples include fermented foods such as Acidophilus milk, Acidophilus buttermilk, Acidophilus yeast milk, Acidophilus yoghurt, Acidophilus-bifidis yoghurt, Biogarde, Bioghurt cultura, Sweet acidophilus and Yakult (Salji, 1992, 1994). In addition concentrated preparations of probiotic lactic acid bacteria are available in tablet and capsule form.

The main disadvantage of using cultured food products over tablets / capsules is that they need to satisfy the sensory tastes of a large number of consumers. Some of these food products might have a limited regional appeal because their flavour is more of an acquired taste. Acidophilus milk, for example, although popular in eastern Europe, has a limited appeal in the western world because of its sour milk taste.

Furthermore, production of a high number of viable cells in the food products and the maintenance of this number up to consumption time are both difficult tasks to accomplish. This is due to the complex requirements of *L.acidophilus* for growth,

propagation and survival (for example the nutritional needs of *L.acidophilus* include acetate, nicotinic acid, riboflavin and calcium pantothenate). It is therefore inevitable that the viability of the organism will suffer and a decline in number will follow after about a week of storage (Salji, 1994). However, the lactic acid bacteria capsules / tablets contain concentrated numbers of freeze-dried lactic acid bacteria cells with a shelf life of over a year.

The strains of lactic acid bacteria used in these 'Acidophilus' products include *L.acidophilus*, *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Enterococcus faecium*, *Bifidobacterium bifidium* and *Streptococcus thermophilus*.

1.1.5 : Requirements for probiotic lactic acid bacteria

In order for probiotic lactic acid bacteria to be used effectively as a dietary adjunct it must colonise the gastro-intestinal tract. To reach the intestine the organism must survive its journey through the hostile environment of the stomach (Hood and Zottola, 1988). The innermost layer of the stomach contains gastric pits and glands which secrete gastric juice (Scott, 1988). Between the stomach and intestine approximately 2 litres of gastric acid and bile salts aswell as numerous enzymes, sodium, potassium and magnesium salts are secreted per day (Watson, 1987). Gastric acid is the main antibacterial factor. In studies performed by Smith (1965), large numbers of organisms were found in the anterior compartment, or body of the stomach in which pH was sufficiently high to permit multiplication, and much lower levels in the prosterior compartment in which the pH was sufficiently low to be bactericidal. The numbers increased from duodenum to ileum and the highest numbers were found in the large intestine.

Other agents such as bile salts can also be detrimental to the bacteria. At present it is not known what degree of bile resistance is needed; however studies by Gilliland et al. (1984) have shown that a strain of *L.acidophilus* possessing a high level of bile resistance produced higher numbers of lactobacilli in the intestinal tract than did a strain having lower bile resistance.

Another important factor to be considered is their antimicrobial activity against pathogens. Within the genus lactobacillus, *L.acidophilus* has been especially known to display antagonistic activity against certain pathogens (Kanatani et al., 1995).

In addition, the probiotic cells are required to play a role in re-establishing a healthy microflora in the gut after antibiotic treatment. Therefore the administered probiotic preparations should preferably exhibit high levels of antibiotic resistance thus allowing them to survive and carry out their therapeutic properties.

The strains should be safe for human use. The safety of lactic acid bacteria has been reviewed by Gasser (1994) and was concluded that the frequency and occurrence of lactic acid bacteria as opportunists is extremely rare. They have a 'generally regarded as safe' status and their use in foods has a long history.

1.1.6 : Preservation of *L.acidophilus*

Lactic acid bacteria requires some preservation in order to retain their viability during storage. A number of different preservation treatments have been reported and among them include direct transfers on culture media (agar slants), storage of cultures under oil,

suspension in distilled water, preservation by freezing, dehydration and freeze-drying (Heckly, 1978). However, the latter process is the most extensively used method for preserving bacterial cultures.

Castro et al. (1997) defined freeze-drying as a process in which a solvent (usually water) is removed from a frozen preparation by sublimation. One of the advantages of using freeze-drying over other preservation treatments is that it is a low temperature process during which the chemical alteration of the product is minimised (Rovero et al. 1991). However, despite its widespread use this method of preservation exposes micro-organisms to the stresses of both freezing and drying. The removal of bound water has been found to destabilise large macromolecular complexes such as membranes, nucleic acids and subunit enzymes (Mackey, 1984; Lievense and van't Riet, 1994). Additional losses in the viability of freeze-dried cultures during storage has been attributed to amino-carbonyl reactions, oxidation or free radical reactions (Mackey, 1984).

Following the freeze-drying treatment many cells also may become sublethally injured. Such injury maybe either metabolic, arising from damage to fundamental components that are related to their metabolic activity, or structural, resulting from damage to the permeability barriers which renders cells susceptible to many selective agents such as bile salts, NaCl and hydroxyl ions (Brennan et al., 1986). Furthermore, once administered the freeze-dried probiotic products are exposed to suboptimal conditions such as unfavourable acidic and alkaline conditions of the gastric and intestinal juice in addition to bile salt concentrations. This type of environment is not conducive to the repair of cellular structural damage (Ray et al., 1971), such as that to the cell wall and membrane.

As a result of this a portion of the inoculum maybe inactivated, thus leading to reduced therapeutic benefits. Brennan et al. (1986) correlated *L.acidophilus* sensitivity to NaCl with membrane damage, and to bile salts with cell wall damage. In commercial products freeze-drying is often used as a convenient technique and has been practised commercially for more than three decades.

One of the major advantages of freeze-drying over other preservation techniques is that cells can be kept stable over long periods of time without the need for special storage conditions.

A large range of substances have been shown to prevent loss of viability during the freeze-drying process. Positive influences on survival have been reported for sugars, polyalcohols, carboxylic acid, glycerol, milk and skim milk, culture medium, polymers (polyethylene glycol, dextran), proteins, amino acids and salts (Souzu, 1992).

De Valdez et al. (1985) tried a variety of additives on freeze-drying lactic acid bacteria. Each additive was better than water alone. They suggested that the protective effect was based on water retention by the additive.

The functionality of the cryoprotectant is based on the high number of hydroxyl groups that they contain, which alters the surface properties of the materials they mix with (Casas et al., 1990). Morichi (1970) concluded that effective cryoprotectants should have three or more hydrogen bonding and ionising groups. In addition these compounds

should have a high attraction for bacterial cells so that they may stabilise the conformation of cellular constituents in the place of water.

Loss of cell viability is also common during storage. However under favourable conditions, the percentage of organisms that die during freeze-drying is usually greater than the percentage that fail subsequent storage (Bozoglu et al., 1987). The inactivation of dried cells during storage is attributed to amino-carbonyl reactions and oxidation or free radical reactions.

The rehydration conditions of freeze-dried cultures is also an important factor in determining viability. Research by Leach and Scott (1959) and de Valdez et al. (1985) has shown that high mortality rates can result from rehydration of bacterial cultures under suboptimal conditions. Thus control over the rehydration environment is essential to ensure adequate survival of preserved cultures on regeneration.

For a dried probiotic product to be effective as a dietary adjunct, it should contain a large number of viable cells. However, limited studies have shown that *L.acidophilus* cells are quite sensitive to freeze-drying. Brennan et al. (1986) found that cells surviving freeze-drying became sensitive to bile salts and lysozyme probably from damage to the cell wall. They also became sensitive to NaCl and permeable to orthonitrophenol and ß-galactosidase resulting from cell membrane damage. A surface protein of 46-kilodalton molecular weight, that is bound to the cell wall by hydrogen bonding, was also lost from dried cells. The damaged components of the cells may result in increased sensitivity to

selective agents and result in alter metabolic activity and consequently reduced effectiveness of the dried product.

A possible means of reducing viability loss would be to immobilise the cells in a polymer gel prior to freeze-drying. The beads may afford additional protection during the freeze-drying process and provide a means of controlling the environment to which the cells are exposed during their rehydration.

1.1.8 : Immobilisation

Karel et al. (1990) defined cell immobilisation as the physical confinement of intact cells to a defined region with the preservation of their biochemical activity. A large number of immobilisation methods have been published. These techniques maybe classified into 6 different classes: covalent coupling, adsorption, affinity, confinement in liquid-liquid emulsion, capture behind semipermeable membranes and entrapment (Mattiasson, 1983; Groboillet et al., 1994). The latter method of directly entrapping cells into a 3-D gel lattice is by far the most frequently used method. The cells are free within their compartments and the pores in the material allow substrate and product to diffuse to and from the cells (Mattiasson, 1983).

A large number of polymer matrices have been employed for entrapment: collagen, gelatin, agar, alginate, carrageenan, cellulose triacetate, polyacrylamide, epoxy resin, photo-cross linkable resin, polyester, polystyrene and polyurethane. Of these matrices, polyacrylamide, alginate gel, carrageenan and photo-cross linkable resin have been used most extensively.

Alginate is produced by brown algae, principally *Macrocystis pyrifera*, but also by *Lamaria digitata*, *L.hyperborea* and *Eklonia cava*. Extracellular alginate is also produced by certain bacteria such as *Azobacter vinelandii* and several pseudomonads (Fett et al., 1986, 1995).

Chemically the alginates consist of linear polymers of 1,4- linked beta-D-mannuronic acid and 1,4- linked alpha-L- guluronic acid. There are three types of polymer groupings: blocks of mannuronic and blocks of guluronic acid and lastly blocks of alternating mannuronic and guluronic residues (Haug, 1967).

Alginate gels are formed by the bonding of dimetal or trimetal ions with polyguluronic portions of the strands which results in a cross-linking network in a process known as ionic gellation. The choice of counterion is dictated by its efficiency in forming a gel and its compatiability with the microbial cells. A number of ions have previously been used such as Sr^{2+}, Ba^{2+} , Al^{3+} and Fe^{3+} ; however Ca^{2+} ions are considered to be the most popular choice (cited by McLoughlin, 1994).

The best gel formers are those with high guluronic acid content because the guluronic acid units bind Ca^{2+} much more strongly than the mannuronic acid units (Martinsen et al., 1987). To entrap the cells within the alginate gel first the cells and the sodium alginate solution are mixed and then this alginate-cell suspension is added dropwise into the calcium chloride solution. The alginate bead formation is initiated instantaneously at the bead surface. Continued diffusion of counterions towards the centre of the beads leads to a gel formation in successively deeper layers (Mc Loughlin, 1994).

After gellation, encapsulated cells can be utilised, or placed in a nutrient solution to encourage additional cell growth inside the gel matrix prior to use. Alternatively beads can be dried after either process and stored until use.

Another feature of utilising alginate immobilisation techniques is that the process can be modified to incorporate various adjuncts such as cryoprotectants and pH modifiers thereby enhancing cell recovery under suboptimal conditions and maintaining a pH level more favourable to cells.

As mentioned previously, preservation of cells by freeze-drying exposes them to stresses which could lead to viability loss and consequently ineffectiveness of the dietary adjunct. However, by immobilising the bacteria in the polymer prior to freeze-drying, the bead may afford addition protection during the freeze-drying process and provide a means of controlling the environment to which the cells are exposed during their rehydration. Furthermore materials lost through cellular damage can be used by neighbouring cells thus reducing loss of valuable resources from the microbial environment (Mc Loughlin,1994).

The objectives of this research were to:

1. Examine the lactic acid bacteria capsules/tablets present on the market, and assess their ability to survive the hostile gastro-intestinal conditions of the stomach and intestine.

2. Immobilise cells from these commercial products in calcium alginate beads under a range of conditions, with incorporated cryoprotectants with the aim of reducing viability loss during exposure to such hostile conditions and thus enhance their potential therapeutic impact.

3. Study the antimicrobial activity of commercial free cell and the immobilised preparations against selective pathogens.

4. Determine antibiotic sensitivity of the commercial free cell and immobilised preparations.

CHAPTER TWO :

Commercial Lactic Acid Bacteria Products

2.1 : INTRODUCTION

Numerous probiotic products have been formulated to contain high levels of lactic acid bacteria for therapeutic use. Among these include capsules and tablets which contain concentrated preparations of lactic acid bacteria in freeze-dried form. *L.acidophilus* is the most widely used lactic acid bacterium found in these preparations and can be found on their own or in conjunction with other lactic acid bacteria in capsules / tablets. Oral administration of 1 X 10^6 to 1 X 10^9 cells per day over a period of several days is necessary in order to obtain beneficial effects (Gilliland, 1989; Sellars, 1991; Salminen et al., 1993; Lee and Salminen, 1995).

For probiotic lactic acid bacteria to be beneficial to the host, the bacterium supplied through dietary adjuncts must be in a viable condition. Many reports have suggested that the ineffectiveness of some commercial products could be a result of low levels of viable cells (Brennan et al., 1986; Ashton, 1996). Although freeze-drying is used as a means of preservation of bacterial cells, freeze-drying and subsequent storage and rehydration are known to be lethal to a large fraction of the population (Bozoglu et al., 1987; Souzu, 1992; Lievense et al., 1994). However, viability alone will not ensure successful establishment or activity of lactobacillus in the gastro-intestinal tract. The physiological state of the cells is an equally important factor that ultimately may affect survival of the bacteria in the gastro-intestinal environment (Duncan and Edberg, 1995).

To reach the intestine, lactic acid bacteria must survive the journey through the hostile environment of the stomach. The innermost layer of the stomach, the mucosa, contains gastric pits and glands which secrete gastric juice. The gastric juice is made up of water

(97-99%), hydrochloric acid (0.2-0.5%), enzymes (pepsinogen, rennin, gastric lipase), inorganic salts and mucus (Watson, 1987). It has been shown that the destruction of micro-organisms in gastric juice is pH dependent (Giannella et al., 1972; Conway et al, 1987). There is a high variability in the pH of the stomach contents, depending on whether or not a person has consumed food. Watson (1987) suggested that the organisms are likely to be exposed to pH ranging from 1 to 4.

Many studies have reported the bactericidal effect of gastric pH on the survival of lactic acid bacteria (Hood and Zottola, 1988; Shah and Jelen, 1990; Gupta et al., 1996). These reports show how pH Level and the choice of bacterial strain determines cellular survival.

Factors other than gastric pH can also be responsible for the ineffectiveness of dietary additives. For instance, the alkaline environment of the intestine (pH 7-9) may also be responsible for loss of cell viability. Kandler and Weiss (1986) reported reduced growth of *L.acidophilus* in pH 7.0 and higher.

In addition, the liver secretes 500-1000ml of bile daily into the hepatic ducts (Watson, 1987). Therefore the organism supplied through the dietary adjunct must be bile tolerant if it is to survive and grow in the intestinal tract. Many reports have found that the bile tolerance of *L.acidophilus* varied with the strain used (Overdahl and Zottola, 1991; Noh and Gilliland, 1993; Walker and Gilliland, 1993; Buck and Gilliland, 1994; Gupta et al., 1996), while Gilliland et al. (1984) have shown that a strain of *L.acidophilus* possessing

a high level of bile resistance produced higher numbers of lactobacilli in the gastro-intestinal tract than did a strain having lower bile resistance.

Bile tolerance is considered to be an important characteristic of *L.acidophilus* that enables it to survive, grow and exert its beneficial properties such as its anticholesterolemic effect and ß-galactosidase activity. Gilliland and Speck (1977) suggested that cholesterol assimilation may be enhanced by deconjugation of bile acids such as taurocholic or glycocholic acid by *L.acidophilus*. This organism produces a conjugated bile salt hydrolase, (E.C. 3.5.1.24), which liberates the glycine and / or taurine moiety from the steroid core (De Smet et al., 1995). This deconjugation of bile salts allows them to be more readily excreted and to compensate for this, new bile salts are synthesised from cholesterol, thus reducing cholesterol levels (Chikai et al.,1987). Gilliland et al. (1988) reported that *L.acidophilus* could assimilate pleuropneumonia-like organism (PPLO) serum as a source of cholesterol when grown anaerobically in the presence of bile. They also found that the amount of cholesterol assimilated increased with concentration of bile.

The ß-galactosidase activity of *L.acidophilus*, which improves lactose utilisation by lactose maldigestors has also been found to be enhanced in the presence of bile *in vitro*. Studies by Gilliland and Kim (1984) found that 0.5% and 1.0% bile salt increased lactase activity by approximately 3 fold. Studies by Noh and Gilliland (1993) found no relationship between bile tolerance and ß-galactosidase activity. In fact, in their study they found that one of the the least tolerant strains, *L.acidophilus* 4356, exhibited the highest ß-galactosidase activity in the presence of bile. They suggested that increased enzyme activity in the presence of bile was associated with increased cellular permeability

of the bacterial cell caused by bile which would allow lactose molecules to permeate more freely through the cell wall and be hydrolysed.

In this chapter :

- a study of the commercial probiotic products was carried out,

- the effects of simulated gastro-intestinal juice on the viability of commercial *L.acidophilus* products was assessed,

- their tolerances to a range of bile salt concentrations were investigated,

- finally levels of cell wall and membrane damage were examined.

2.2 : METHODS AND MATERIALS

2.2.1 : Examination of the viability of lactic acid bacteria in commercial probiotic products:

Commercial capsules and tablets (Product A, B, C, D, E and F) containing freeze-dried lactic acid bacteria were purchased from retail outlets and were stored at 4°C until required for use. A study was carried out on the number of lactic acid bacteria cells present in the six different commercial products. One capsule / tablet was added to a Seward Medical 4" X 6" stomacher bag which contained 9ml of sterile ringers solution, pH 6.8, (Lab M, Bury, UK). The samples were stomached using a Lab-Blender Model 80 stomacher and standard plate counts were carried out on MRS agar (Lab M). Plates were incubated at 37°C for 48 hours prior to cell enumeration. The trials were carried out in duplicate.

2.2.2 : Effect of simulated gastric juice on the viability of *L.acidophilus* in commercial products:

The survival of microorganisms from 2 of the products (Product A and B) which contained exclusively *L.acidophilus* were studied in simulated gastric juice. Simulated gastric juice at pH 1.5 and 2.5 was prepared by the dropwise addition of 1M HCl into ringers solution, pH 6.8. The simulated gastric juice was sterilised by autoclaving at 121°C for 15 minutes at 1.5kgf / cm^2 pressure using a Tomy SS-325 autoclave. One tablet / capsule from Product A and B was suspended in 9ml of simulated gastric juice at pH 1.5 and 2.5 and then incubated at 37°C for 0, 30, 60 and 90 minutes. At each time interval the supernatant was decanted and cells were resuspended in 9ml of sterile ringers

solution, (the cells released from the product into the supernatant was less than 99.97%). The samples were stomached for 5 minutes and standard plate counts were carried out on MRS agar. Plates were then incubated at 37°C for 48 hours. Duplicate trials were carried out.

2.2.3 : Effect of simulated intestinal juice on *L.acidophilus* in commercial products:

Simulated intestinal intestinal juice at pH 7.4, was prepared by the dropwise addition of 1M NaOH into a ringers solution and was then sterilised. One tablet / capsule of Product A and B was suspended in 9ml of simulated intestinal juice and incubated at 37°C for 0, 30, 60 and 90 minutes. At each time interval the supernatant was removed and cells were resuspended in 9ml of ringers solution. The sample was stomached for 5 minutes and standard plate counts were carried out on MRS agar. Plates were incubated at 37°C for 48 hours. The experiment was carried out in duplicate.

2.2.4 : Bile tolerance of *L.acidophilus* in commercial products:

The procedure of Noh and Gilliland (1993) was used. Cells from Commercial Products A and B were compared for their ability to grow in the presence of bile by inoculation of a capsule / tablet into 50ml of sterile MRS broth containing 0, 0.1, 0.2, 0.3, 0.4 and 0.5% bile salts (Oxoid) at 37°C. The cells were released from the tablets / capsules by stomaching and the absorbance measurements at A_{590nm} were monitored using a WPA Colorimeter Model C075. Measurements continued at 30 minute intervals until the absorbance reading increased by 0.3 units. Trials were carried out in triplicate.

2.2.5 : Bile Tolerance of *L.acidophilus* cells after incubation in simulated gastric juice:

Cells from Product A and B were incubated in simulated gastric juice at pH 2.5 for 90 minutes. The exposed cells were then examined for their bile tolerance using the method of Noh and Gilliland (1993), as detailed above. Trials were carried out in triplicate.

2.2.6 : Cell wall and membrane damage of *L.acidophilus* from commercial products:

To evaluate levels of cellular damage of *L.acidophilus* from Commercial Product A and B the method of Brennan et al. (1986) was used. One tablet / capsule was incubated for 30 minutes at 25°C in ringers solution containing either 8% NaCl (Oxoid, Hampshire, UK), to assess levels of cell membrane damage, or 0.1% bile salts (Oxoid), to assess the degree of cell wall damage. The cell suspension was then stomached for 5 minutes and standard plate counts were carried out on MRS agar. The plates were then incubated at 37°C for 48 hours. Relative sensitivity to the selective agent was determined from differences in colony forming units with and without the selective agent, (substituting with ringers solution as control). The experiment was carried out in duplicate.

2.3 : RESULTS

2.3.1 : Examination of the viability of lactic acid bacteria in probiotic commercial products

Table 2.1 lists commercially available lactic acid bacteria capsules and tablets studied in this project. Most of the products studied claimed to contain microbial levels ranging from 1.0 X 10^8 cells to greater than 2.0 X 10^9. When viable counts were carried out on the different products, the cell numbers ranged from 1.7 X 10^8 to 8.4 X 10^9 cells per capsule/tablet. Two of the products, (Products C and D), were found to contain microbial levels below that claimed by the manufacturer: they contained less than 34.5% and 8.5% of the claimed microbial level respectively.

More detailed studies were performed on Products A and B as representatives of products that contained only *L.acidophilus* cells.

Table 2.1: Characteristics of representative commercial products.

Product Code	A	B	C	D	E	F
Microorganisms	*L.acidophilus*	*L.acidophilus*	*L.acidophilus*	*L.acidophilus*	*L.acidophilus*	*L.acidophilus*
				L.bifidus	*L.bifidus*	*L.bifidus*
				L.rhamnosus	*L.rhamnosus*	*L.rhamnosus*
				S.faecium		*S.faecium*
Dosage form	tablet	capsule	capsule	capsule	capsule	capsule
Cell no. at encapsulation	not listed	> 1.00×10^8	> 2.00×10^9	> 2.00×10^9	> 2.00×10^9	> 2.00×10^9
CFU / capsule at time of purchase	4×10^8	8.50×10^8	6.90×10^8	1.70×10^8	8.40×10^9	3.03×10^9
Recommended daily dosage	1 to 3	2 to 4	1 to 4	1 to 4	1 to 4	2 to 4
Content	30	60	60	60	60	60

2.3.2 : Effect of simulated gastric juice on the viability of *L.acidophilus* from commercial products

A comparison of the survival of cells from Product A and B at different pH levels, pH 1.5 and pH 2.5 as shown in Figs 2.1 and 2.2, illustrates that pH is a key factor influencing survival of bacteria in gastric juice, with greatest cell reduction occurring at the lower pH value. When cells from Product A were inoculated in simulated gastric juice at pH 1.5 and 2.5, (Fig 2.1), there were 3.5 and 2.2 log cycle reductions in viability after 90 minutes respectively. Results showed that 88.1% and 53.6% of the total cell reduction occurred during the first 30 minutes exposure to simulated gastric juice at pH 1.5 and 2.5 respectively and the reduction gradually levelled off thereafter. Product B exhibited a 4.7 and 3.4 log cycle reduction in simulated gastric juice at pH 1.5 and 2.5 respectively (Fig 2.2). As observed with Product A, greatest cell reduction occurred during the first 30 minutes. The results show that 60.7 and 60.3% of the total cell reduction occurred at pH 1.5 and 2.5 respectively during this period.

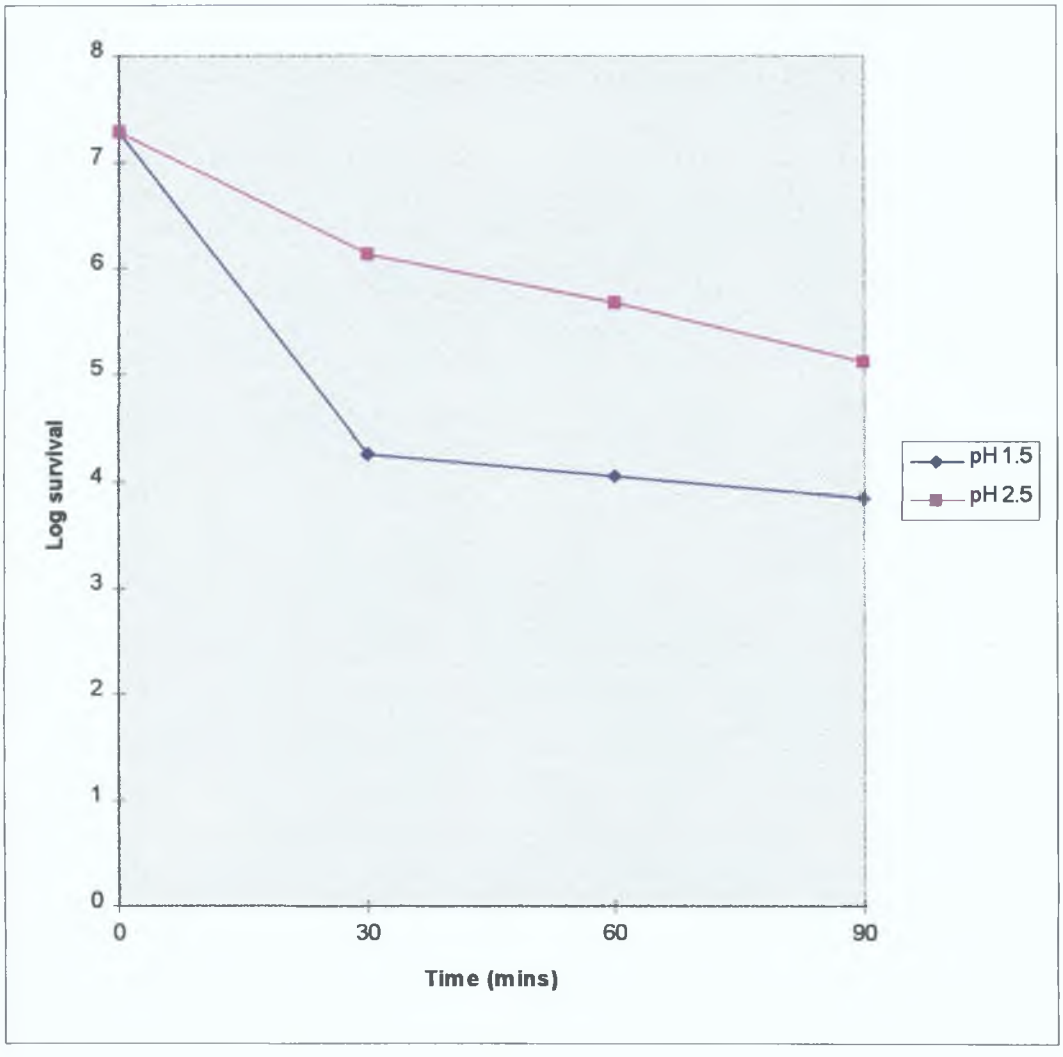


Fig. 2.1: Effect of simulated gastric juice, pH 1.5 and 2.5, on the viability of *L.acidophilus* cells from Product A.

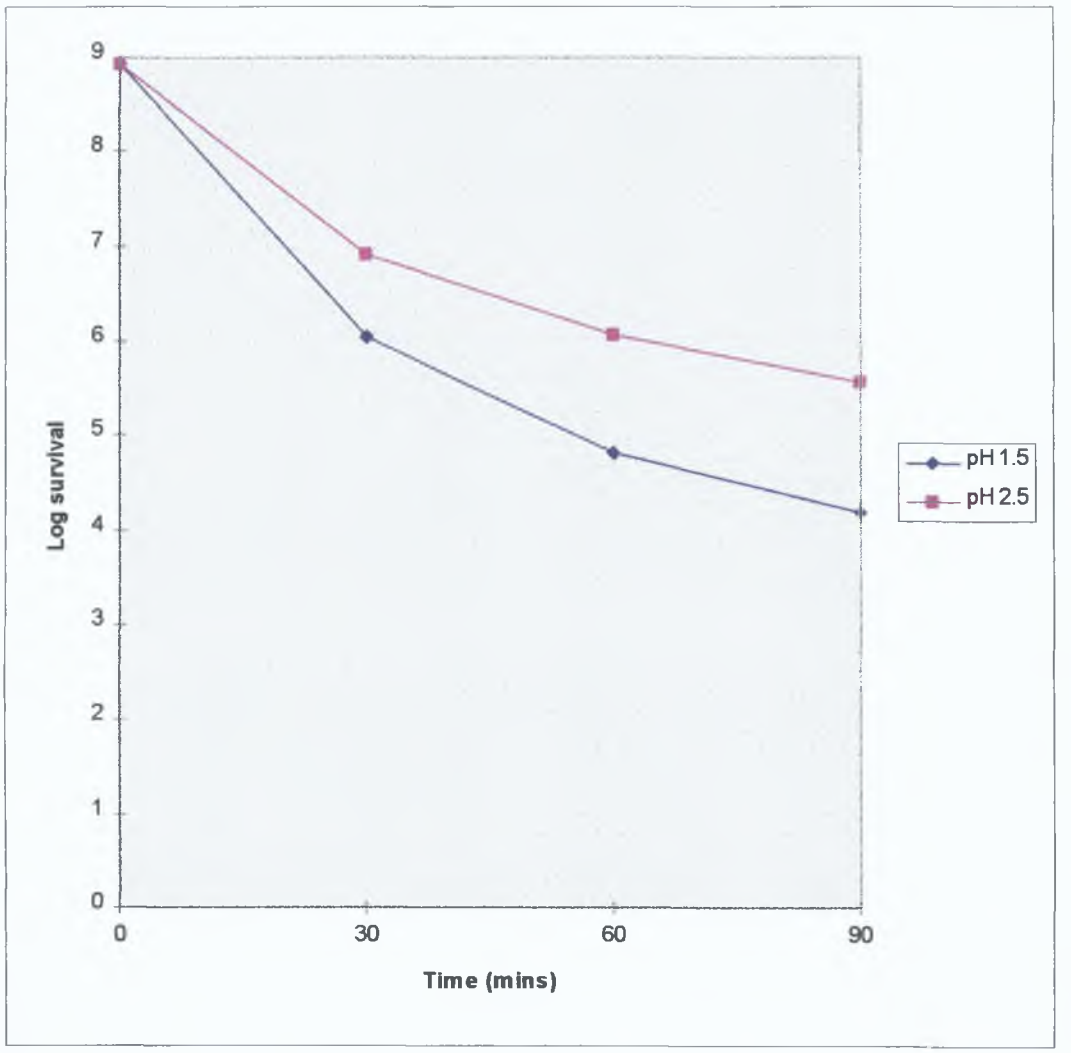


Fig 2.2: Effect of simulated gastric juice, pH 1.5 and 2.5, on the viability of *L.acidophilus* from Product B.

2.3.3 : Effect of simulated intestinal juice on the viability of *L.acidophilus* from commercial products

The effect of simulated intestinal juice on commercial Products A and B is shown in Figs 2.3 and 2.4. When cells from Product A were inoculated in simulated intestinal juice at pH 7.4 for 120 minutes they exhibited a 1.5 log reduction of which 67.8% of the total cell reduction occurred during the first 30 minutes (Fig 2.3). When cells from Product B were exposed to similar simulated intestinal conditions they exhibited a 2.3 log cycle reduction after 120 minutes (Fig 2.4). As observed with Product A, most of the cell reduction occurred during the first 30 minutes. The experiment showed that 91.8% of the total cell reduction occurred during this period.

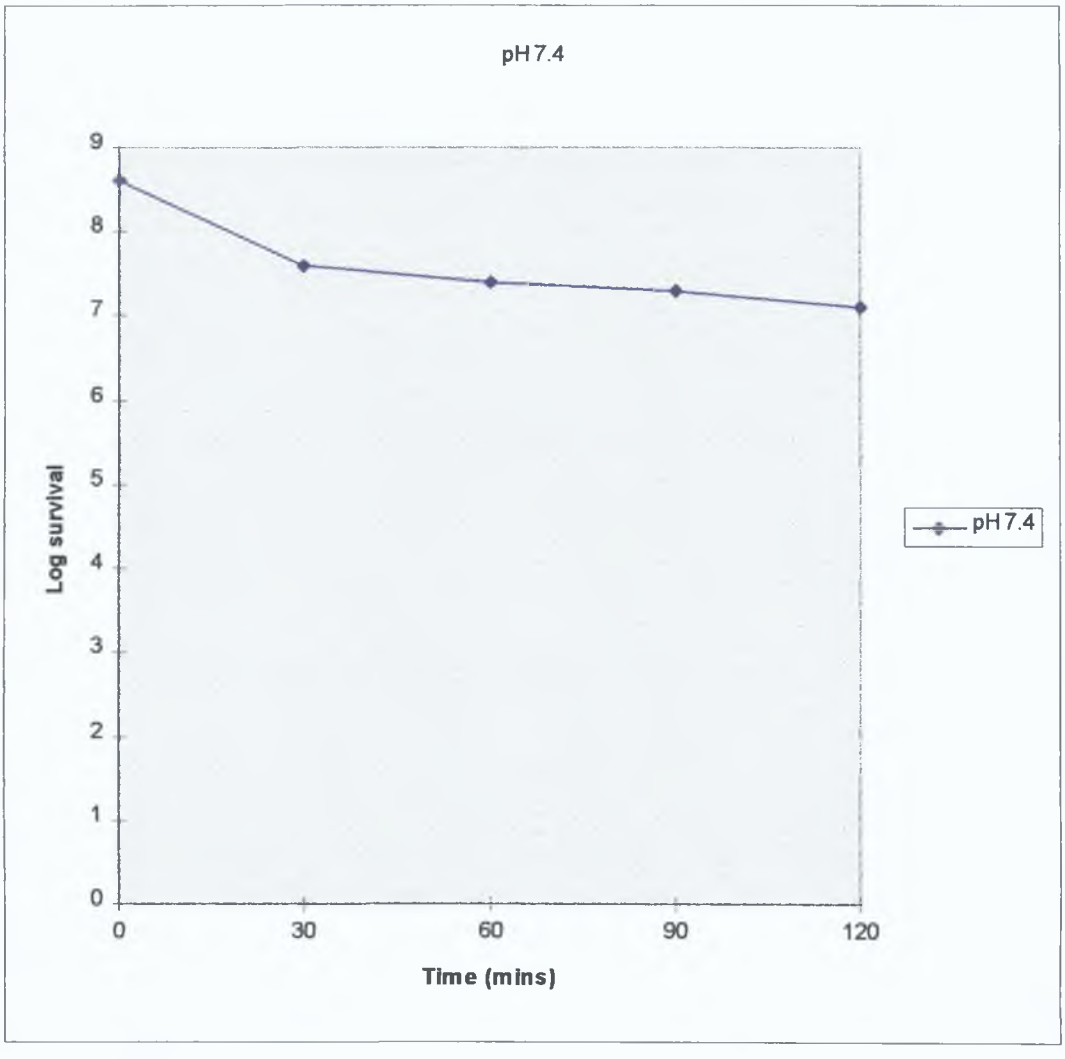


Fig 2.3: Effect of simulated intestinal juice, pH 7.4, on the viability of *L.acidophilus* from Product A.

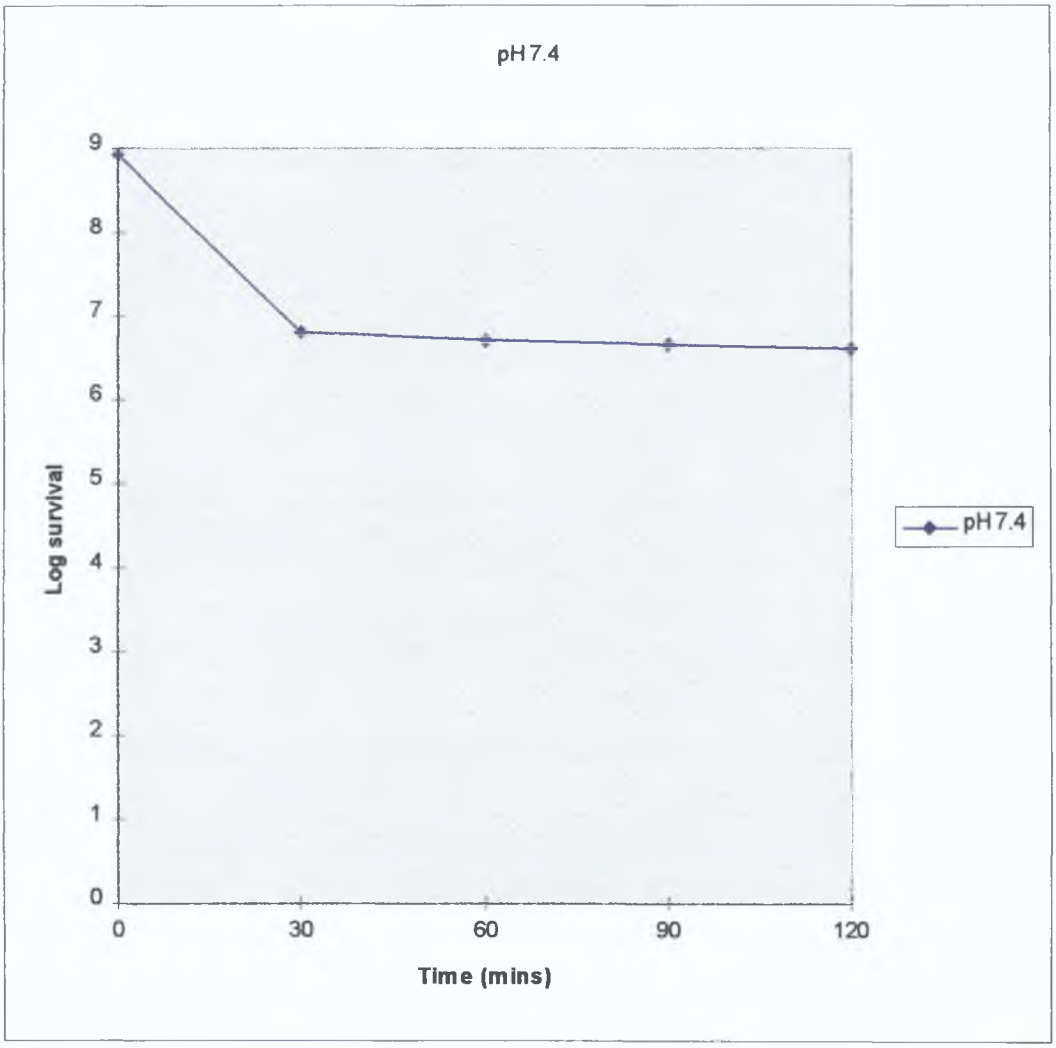
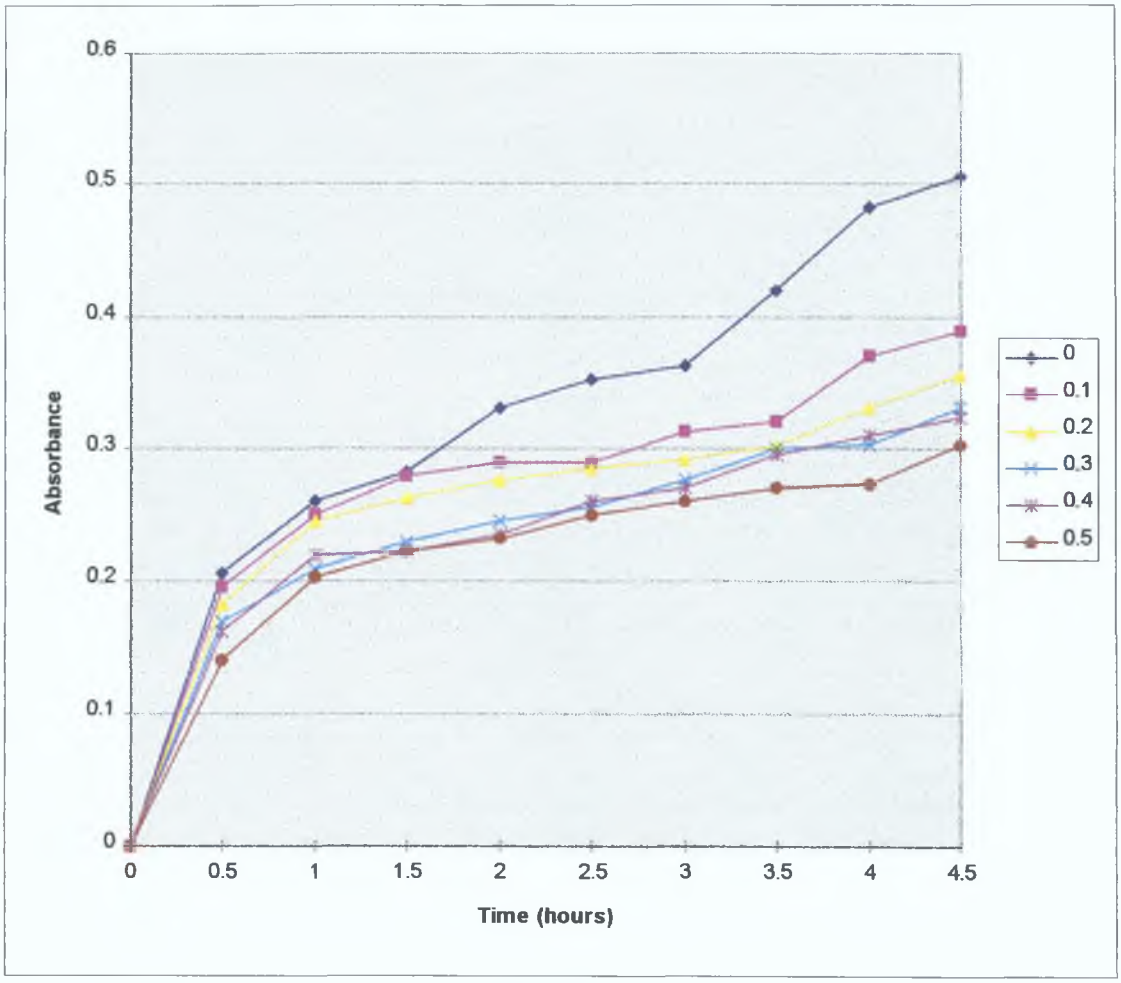


Fig 2.4: Effect of simulated intestinal juice, pH 7.4, on the viability of *L.acidophilus* from Product B.

2.3.4 : Bile tolerance of *L.acidophilus* in commercial products:

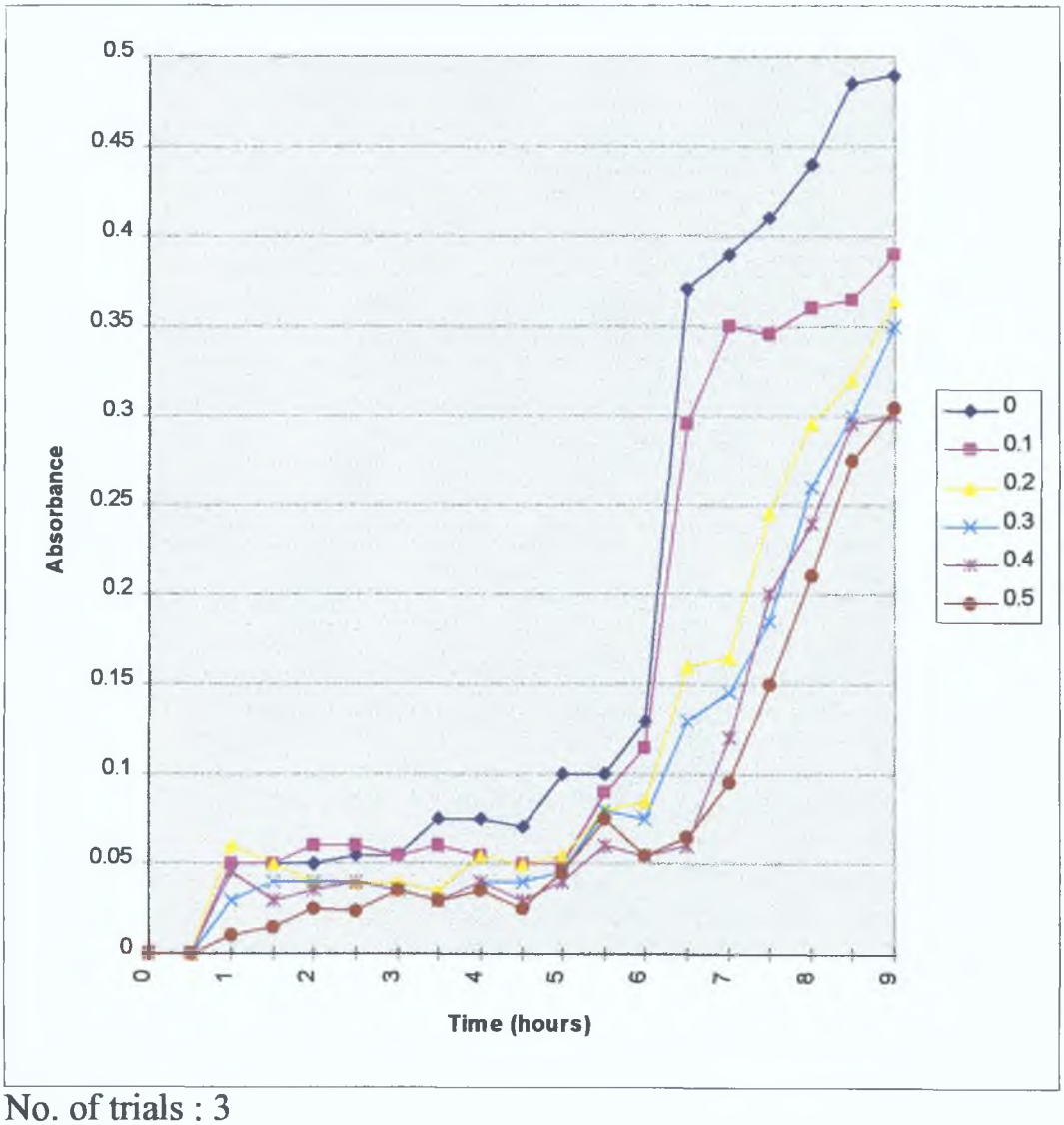
Fig 2.5 shows the absorbance readings of *L.acidophilus* from Product A obtained from 3 separate trials during growth in MRS broth containing a range of bile salt concentrations (0.1 to 0.5%). Cells from Product A reached the 0.3 absorbance level after 1.75 hours incubation in bile-free broth. The remaining broths which incorporated a range of bile salt concentrations (0.1, 0.2, 0.3, 0.4 and 0.5%) did exhibit significant ($P < 0.05$) variation in growth time from the control culture (MRS broth). Furthermore the incubation time required to reach the 0.3 absorbance level increased with increased bile salt concentration. The time required to reach the 0.3 absorbance level increased between 14.28% and 157.14% in comparison with control.

Fig 2.6 shows the absorbance readings of cells from Product B during growth in MRS broth containing a range of bile salt concentrations (0 to 0.5%). Cells from Product B reached the 0.3 absorbance level after 6.4 hours incubation in bile-free broth. However cells which were incubated in the broth containing bile salts (0.1, 0.2, 0.3, 0.4 and 0.5%) exhibited significant ($P < 0.05$) growth variation compared with their bile-free counterpart. The time required to reach the 0.3 absorbance level increased by 1.6, 30.8, 32.8, 32.8 and 40.6% in MRS broth containing 0.1, 0.2, 0.3, 0.4 and 0.5% bile salt respectively.



no. of trials = 3
S.D = 0.03

Fig 2.5 : Absorbance readings at 590nm of cells from Product A during growth in MRS broth containing a range of bile salt concentrations.



No. of trials : 3
S.D. : 0.03

Fig 2.6: Absorbance readings at 590nm of cells from Product B during growth in MRS broth containing a range of bile salt concentrations.

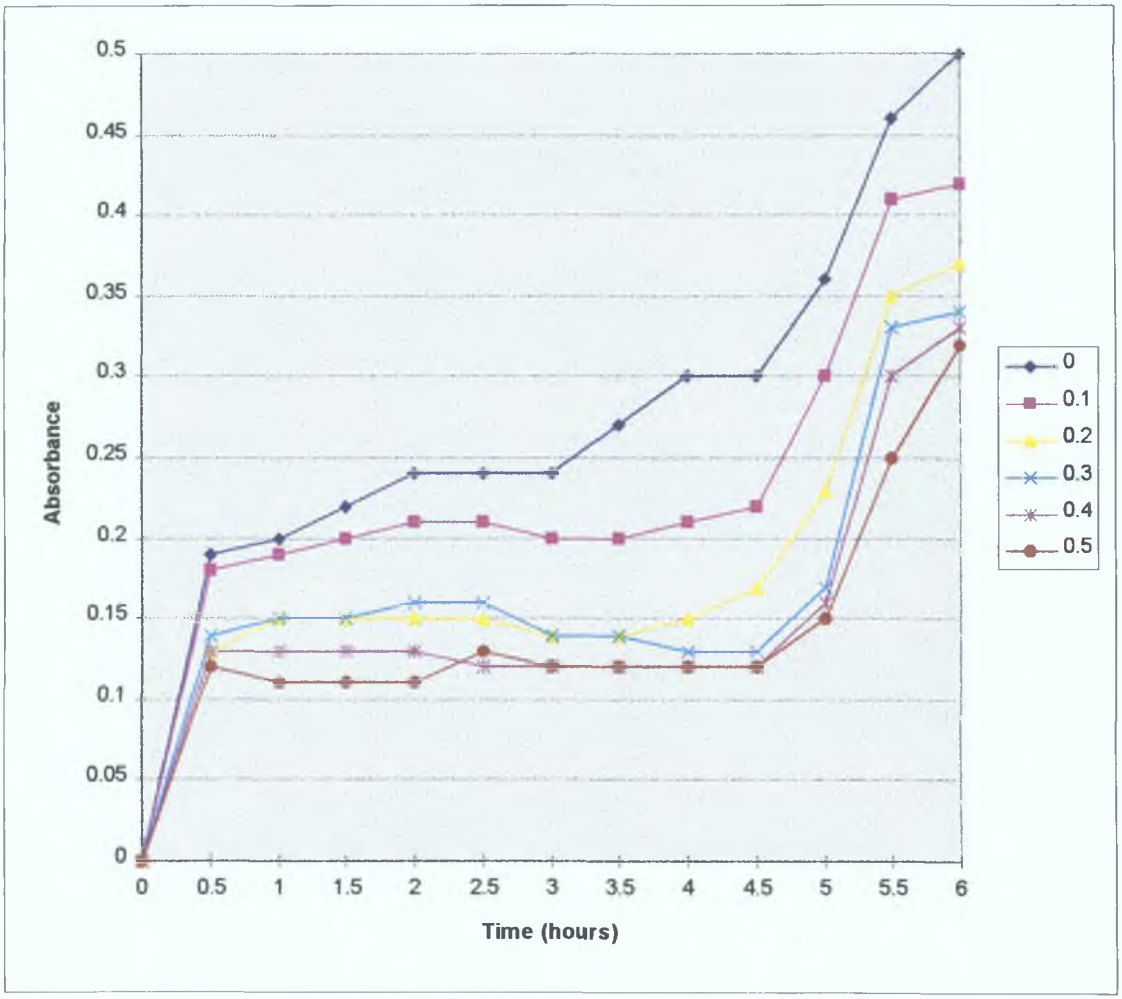
2.3.5 : Bile Tolerance of *L.acidophilus* cells after incubation in simulated gastric juice

Cells from Product A which had been exposed to simulated gastric juice did exhibit significant ($P < 0.05$) growth variation when incubated in broth containing bile salt concentrations compared with their bile-free counterpart. After 90 minutes exposure to simulated gastric juice, cells from Product A required increased incubation times in both bile-free MRS broth and in MRS broth incorporating bile salts in order to reach the 0.3 absorbance level compared with their unexposed counterparts (Fig 2.7). The time required to reach the 0.3 absorbance level increased between 25-44.2% in bile salt incorporated broth compared with bile free broth.

Incubation of Product A and B cells in simulated gastric juice did affect their growth in MRS broth and bile salt incorporated broth (Figs 2.7 and 2.8). The time required to reach the 0.3 absorbance level increased by 128, 81.8, 61.5, 51.4, 46.6 and 28.2% in MRS broth containing 0, 0.1, 0.2, 0.3, 0.4 and 0.5% respectively after exposure to simulated gastric juice compared with unexposed cells.

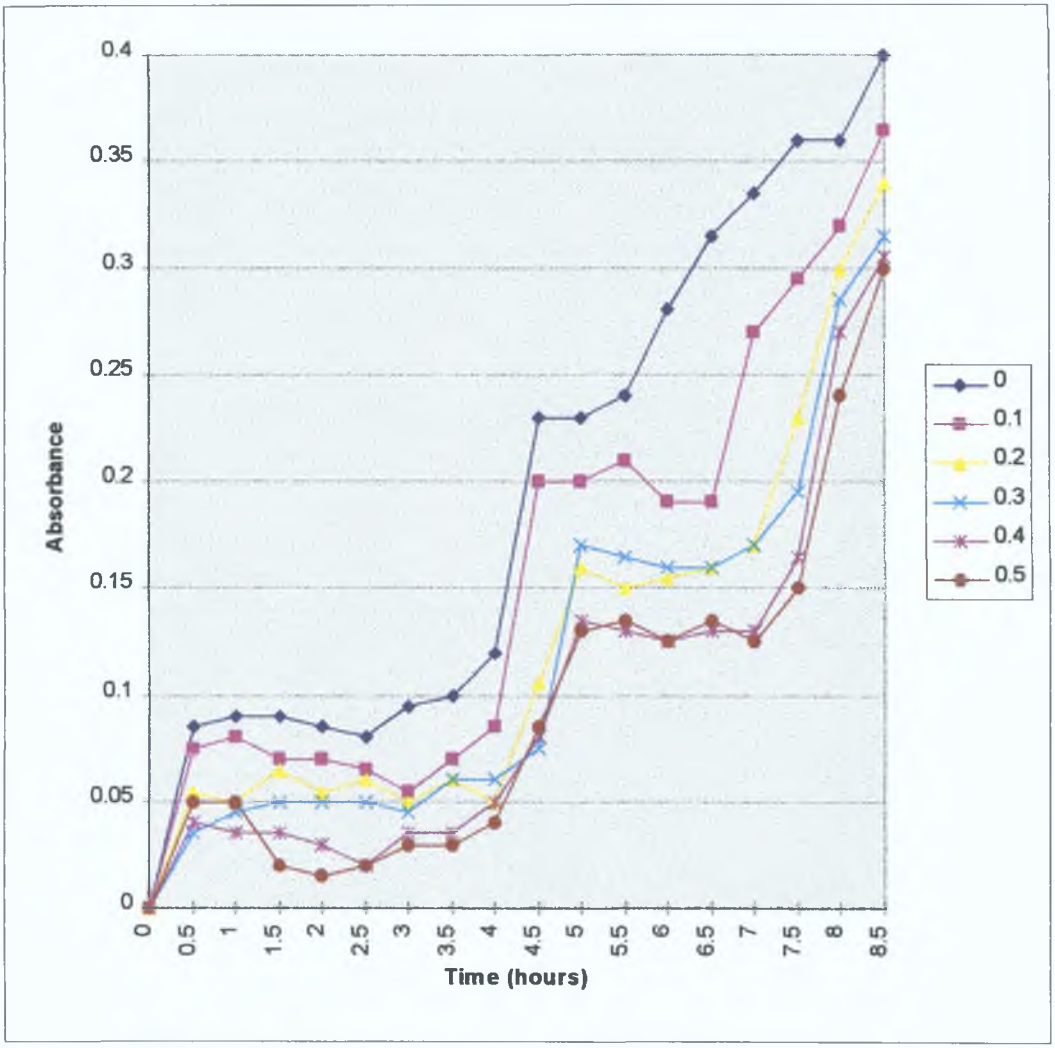
Fig 2.8 shows that when cells from Product B were exposed to similar gastric conditions, they exhibited significant ($P < 0.05$) growth variations in broth containing bile salts (0.1, 0.2, 0.3, 0.4 and 0.5%) compared with their bile free counterpart. The time required to reach the 0.3 absorbance level increased between 20-36% in MRS broth incorporating a range of bile salt concentrations compared with bile-free broth

Product B exhibited similar growth rate in bile salt incorporated broth after prior exposure to simulated gastric juice (Fig 2.8) compared with their unexposed counterparts (Fig 2.6). The time required to reach the 0.3 absorbance level decreased by 2.3, 3.0 and 5.5% in MRS broth containing 0.2, 0.3 and 0.5% bile salt, but increased by 15.4% in 0.1% bile salt and remained the same in 0.4% bile salt incorporated broth.



No. of trials: 3
S.D.: 0.01

Fig 2.7: Absorbance readings at 590nm of cells from Product A during growth in MRS broth containing a range of bile salt concentrations after incubation in simulated gastric juice.



No. of trials:3
S.D.: 0.01

Fig 2.8: Absorbance readings at 590nm of cells from Product B during growth in MRS broth containing a range of bile salt concentrations after incubation in simulated gastric juice.

2.3.6 : Cell wall and membrane damage of *L.acidophilus* cells in commercial products

The relative sensitivity of *L.acidophilus* from Products A and B to 0.1% bile salts and 8% NaCl was determined from the loss of viable cells after 30 minutes exposure to the selective agents are shown in Table 2.2. Cells from Product B exhibited greater sensitivity to 0.1% bile salts which indicates they probably had higher levels of cell wall damage. They also were more sensitive to 8% NaCl which is indicative of higher levels of cell membrane damage. Cells from Product A exhibited greater resistance to the selective agents which indicates lower levels of cellular damage.

Table 2.2: Percentage survival of *L.acidophilus* from Commercial Products A and B after exposure to selective agents.

Commercial Product	0.1% Bile salt	8% NaCl
Product A	33%	58%
Product B	6%	15%

2.4 : DISCUSSION

Of the vast array of commercial probiotic lactic acid bacteria capsules/tablets available on the market, our random selection showed a predominance of *L.acidophilus* in pure culture, (Product A, B and C), or in combination with *L.bifidus*, *L.rhamnosus* and *Streptococcus faecium*, (Product D, E, and F). Oral administration of approximately 1.0 X 10^6 to 1.0 X 10^9 cells per day over a period of several days has been advocated to overcome problems such as abusive food habits and antibiotic treatment (Salji, 1992, 1994; Lee and Salminen, 1995). The products studied claimed to contain microbial levels between 1.0 X 10^8 to in excess of 2.0 X 10^9 cells per capsule/tablet. When viable counts were carried out at the time of purchase cell counts in capsules and tablets ranged from 1.7 X 10^8 to 8.4 X 10^9 cells. The results from this study substantiated the manufacturers claims in all the products except Product C and D. These were the only products found to contain microbial levels lower than that claimed by the manufacturer.

In order to determine the effect of acidic pH of the stomach on the survival of *L.acidophilus* cells from Product A and B, an *in-vitro* system was used. Results from this study found that the survival of *L.acidophilus* was affected by the pH of the simulated gastric juice with greater reduction in cell viability occurring at the lower pH level (pH 1.5). Product A and B exhibited a 3.5 and 4.7 log cycle reduction respectively after 90 minutes exposure to simulated gastric juice at pH 1.5 of which 61-88% of the total cell reduction occurred during the first 30 minutes. However when Product A and B were exposed to simulated gastric juice at pH 2.5 they exhibited a lower log cycle reduction. Product A and B showed a 2.2 and 3.4 log cycle reduction respectively after 90 minutes

under such conditions of which 54-60% of the total cell reduction occurred during the initial 30 minutes.

Numerous studies have also examined the effect of acidic pH of simulated gastric juice on the survival of a wide variety of strains of *L.acidophilus* and have reported an increasingly bacteriostatic and bactericidal effect at lower pH levels. Gupta et al. (1996) reported that the growth of 7 strains of *L.acidophilus* in lactic broth was pH dependent. All strains grew in lactic broth at pH 5, only 2 strains (*L.acidophilus* 1899 and 301) exhibited growth in lactic broth at pH 3 and 4 whilst no growth was recorded in lactic broth adjusted to pH 2. Hood and Zottola (1988) reported on the bacteriostatic and bactericidal properties of simulated gastric juice. They reported that *L.acidophilus* strain BG2FO4 rapidly lost viability after 45 minutes in MRS broth adjusted to pH 2, while at pH 3 and 4 they survived throughout the 2 hour study. Studies by Shah and Jelen (1990) reported a 5.03 and 6.41 reduction of *L.acidophilus* cells after 60 minutes and 2 hours incubation in pH 1.5 and a 2.70 and 5.09 log reduction after 60 minutes and 2 hours incubation in pH 2.5. Conway et al. (1987) carried out similar tests using Phosphate Buffered Saline (P.B.S.) adjusted to pH 1 and 3. They found that *L.acidophilus* from 2 human isolates, *L.acidophilus* ADH and N_2, exhibited a > 6.3 and > 6 log cycle reduction respectively in P.B.S. at pH 1, and by 2.6 and 1.7 log cycles in P.B.S. at pH 3 after 90 minutes.

Viability loss was also found when *L.acidophilus* cells from Product A and B were exposed to simulated intestinal juice at pH 7.4 for 120 minutes, however the bactericidal effect of simulated intestinal juice was less pronounced than previous simulated gastric

conditions. Product A and B exhibited a 1.5 and 2.3 log cycle reduction after exposure to such simulated intestinal conditions for 120 minutes. Most of the cell reduction was found to occur in the first 30 minutes. Kandler and Weiss (1992) also reported reduced growth of *L.acidophilus* at pH 7.0.

To survive and grow in the intestinal tract, microorganisms should also be bile resistant (Mital and Garg, 1995). Results from this study showed that the growth of cells from Product A was significantly reduced ($P < 0.05$) in MRS broth incorporating 0.2% bile salts or higher, while cells from Product B exhibited significantly ($P < 0.05$) lower growth rates in the presence of all the bile salt concentrations examined (0.1% to 0.5% bile salt) compared with the control. The additional time required for the absorbance to increase to the 0.3 absorbance level in the bile salt incorporated broth ranged from 0 to 3.0 hours for Product A and between 0.10 and 2.60 hours for Product B.

Similar studies were carried out by Noh and Gilliland (1993) using 5 *L.acidophilus* isolates of human origin (strains 107, 223, NCFM, 606 and 4356). They reported that 2 of the 5 strains tested (223 and 4356) did also exhibit significantly ($P < 0.05$) lower growth rates in the presence of 0.3% bile salt than in its absence. However, the remaining 3 strains (107, NCFM and 4356) did not exhibit any significant growth differences in the presence of bile.

Other studies by Buck and Gilliland (1994) found that the time required for the absorbance to increase by 0.3 units ranged from between 2 to 2.8 hours between different *L.acidophilus* strains from one human volunteer, and 2 to 7 hours with isolates

from 9 volunteers when they were grown in MRS broth containing 0.3% bile salt. Gilliland et al. (1985) carried out similar tests on pig isolates and they reported that the time required for *L.acidophilus* to increase absorbance under similar conditions ranged from 0 to > 2.55 hours. Many other studies have also reported that the bile tolerance of *L.acidophilus* varies among strains (Overdahl and Zottola, 1991; Walker and Gilliland, 1993; de Smet et al., 1995)

Incubation of cells from Product A and B in simulated gastric juice for 90 minutes prior to bile tolerance testing lead in some cases to increased incubation time for cells to reach the 0.3 absorbance level in MRS broth incorporating various bile salt concentrations. This maybe a result of lower levels of viability after exposure to simulated gastric conditions.

The bactericidal effect of the gastro-intestinal conditions of *L.acidophilus* is further intensified by sublethal injury incurred during the freeze-drying process. The developed sensitivity of dried cells to bile salts and NaCl has been related to damage of the cell wall and membrane components (Brennan et al., 1986; Castro et al., 1997). Therefore the degree of injury caused to the cell wall and membrane was evaluated by their resistance to bile salt and sodium chloride solutions. The commercial products exhibited variable resistance to the selective agents indicating varying degrees of cell damage. The percentage survival of cells from Product A and B after prior exposure to 0.1% bile salt and 8% NaCl for 30 minutes ranged from 6 - 33 % and from 15 - 58 % respectively. Cells from Product A showed higher levels of cell survival in 0.1% bile salt and 8% NaCl thus indicating lower levels of cell wall and membrane damage. Similar studies were

carried out by Brennan et al. (1986) using an *L.acidophilus* W strain isolated from acidophilus milk. In their studies they found that 6.5% and 39% of these freeze-dried cells survived for 30 minutes exposure to 0.1% bile salt and 8% NaCl respectively.

Other studies by Castro et al. (1997) examined the recovery of *L.bulgaricus* in MRS agar and MRS agar incorporating 6.25μgml^{-1} of NaCl following freeze-drying. They concluded that cells dried in glycerol and trehalose did not show any sensitivity towards the salt after the drying stage, while cells dried in maltodextrin and water did exhibit an increased sensitivity to NaCl post-stress. Therefore a possible explanation for the different levels of of cell wall and membrane damaged observed with *L.acidophilus* from Product A and B maybe that the cells were freeze-dried in different suspending medium which would offer different degrees of protection during the freeze-drying process. Or possibly they were different strains with different innate sensitivities.

Results from this study showed a relationship between pH sensitivity and levels of cellular damage. Cells from Product B which had higher levels of cellular damage exhibited greater sensitivity to simulated gastric juice than did Product A which had lower levels of cellular damage. Lievense et al. (1994) also found that freeze-dried cells which exhibited higher levels of cellular damage become more sensitive to HCl as hydrogen and hydroxyl ions are more freely available to permeate through the cell wall and membrane and cause death.

In order for lactic acid bacteria products to be beneficial to the host they must remain viable throughout its journey through the hostile gastro-intestinal tract. However, results

of this study showed reduced viability after exposure to simulated gastric and intestinal juice aswell as significant reduction in growth rate after exposure to various bile salt concentrations. Furthermore, it was found that different products exhibited variations in their resistance to gastro-intestinal conditions aswell as levels of cell wall and membrane damage.

CHAPTER THREE :

Immobilised Lactic Acid Bacteria

3.1 : INTRODUCTION

A possible means of solving the problem of viability loss during freeze-drying and rehydration, especially under the hostile conditions of the gastro-intestinal tract, could be to lyophilise cells after they had been immobilised by entrapment in polymers incorporating modifiers. Subsequent rehydration would then occur in the controlled microenvironment of the bead which would be more conducive to survival (Kearney et al., 1990).

The level of protection afforded through the immobilisation process is further influenced by the protectant incorporated into the bead formulation. Studies by Kearney et al. (1990) have shown that process factors such as incorporation of cryoprotectants (glycerol and adonitol), can have significant effects on the stability of the gel formed. Furthermore they found that the microenvironment created by immobilised polymers containing these protectants with skim milk increased the survival rate of immobilised *Lactobacillus plantarum* during rehydration in optimal and suboptimal acidic conditions (pH ranged from pH 3.0 to 7.0).

Other amendments, such as skim milk powder and tricalcium phosphate may also be incorporated into the bead structure as an additional protective / nutrient source. Addition of skim milk to bead formulations provides complex carbon and nitrogen sources which aids the recovery of cells after stresses such as prolonged storage aswell as enhancing cell activity and growth (Fages, 1990; Cassidy et al., 1996). Fages (1990) compared the effect of a number adjuncts (skim milk, glucose, sucrose and glycerol) on the survival of *Azospirillium lipoferum* in alginate beads and found that skim milk was

the best adjunct in terms of bacterial numbers recovered. This protectant allowed the recovery of at least twice the number of bacteria compared with other adjuncts tested. Axwell and Guzman (1987) observed increased persistence of bacteria with skim milk ammended and alginate-encapsulated bacteria after they were dried. Furthermore, Bashan (1986) suggested that incorporation of skim milk in the bead formulation could provide additional protection by regulating the internal pH of the bead.

PH modifiers such as tricalcium phosphate may also be incorporated into the bead structure thus maintaining the pH at a level more favourable to cell survival. Wang and Hettwer (1982) reported that incorporation of tricalcium phosphate into k-carrageenan beads can maintain pH at a favourable level to yeast cells during ethanol production and consequently sustain cell viability.

The objectives of this chapter were to:

- examine the effect of simulated gastric juice on the viability of *L.acidophilus* cells freeze-dried in Ca-alginate beads,

- optimise the immobilisation process through the addition of cryoprotectants / adjuncts and post entrapment incubation in order to enhance viability of *L.acidophilus* in simulated gastric juice,

- studies on the optimal bead composition to determine the effect of simulated intestinal juice and assessment of their bile tolerance,

- finally an evaluation of levels of cell wall and membrane damage on *L.acidophilus* cells from the optimal bead.

3.2 : MATERIALS AND METHODS

3.2.1 : Organism used: The bacterium used was *Lactobacillus acidophilus*, which was obtained from Commercial Products A and B. The products were stored at 4°C until required for use.

3.2.2 : Immobilisation Procedure

L.acidophilus from Commercial Product A and B were immobilised in calcium alginate beads under varying conditions (summarised in Table 3.1). The immobilisation procedure used was based on that of Kearney et al. (1990).

Table 3.1: Adjuncts incorporated into the different Bead Types and their subsequent incubation in broth.

Bead Type	Adjuncts	Incubation
1	5% skim milk	None
	0.5M glycerol	
2	5% skim milk	18 hours in Nutrient broth
	0.5M glycerol	
3	5% skim milk	18 hours in MRS broth
	0.5 M glycerol	
4	4% tricalcium phosphate	18 hours in MRS broth
	5% skim milk	
	0.5M glycerol	
5	4% tricalcium phosphate	18 hours in MRS broth
	10% skim milk	
	1M glycerol	

Type 1 A beads: Cells isolated from Commercial Products A and B were grown in 100ml of MRS broth at 37°C for 18 hours and harvested by centrifugation for 20 minutes at 1000g/5°C in a Sigma 2K15 centrifuge. Cells from 50ml of MRS broth were resuspended in 50ml of cryoprotective agent formulated to have a final concentration of 5% skim milk (Lab M) and 0.5 M glycerol (Fisons), and mixed for 30 minutes with 50ml of sodium alginate (Fisons) at a final concentration of 2%. The mixture was added dropwise with aid of a sterile 10ml syringe fitted with a pipette tip into a gently stirred sterilised 0.1M $CaCl_2$ solution (Fisons) incorporating 0.5M glycerol at room temperature. The resulting alginate beads (mean diameter 2mm) entrapped *L.acidophilus* cells. The beads remained in curing solution for 2 hours and were rinsed thoroughly using sterile deionised water. The freeze-drying ampoules were one-third filled with the beads.

Type 2 A beads: Type 1 A beads were incubated in 100ml of Nutrient broth (Lab M) for 18 hours at 37°C. The broth was decanted and the beads were washed thoroughly using sterile deionised water before dispensing into ampoules.

Type 3 A beads: Type 1 A beads were incubated in 100ml of MRS broth for 18 hours at 37°C. The broth was decanted and the beads were rinsed thoroughly using sterile deionised water and then transferred to ampoules.

Type 4 A beads: Beads were prepared as above for Type 3 A but were formulated to contain in addition 4% tricalcium phosphate (Bronagh Hall, U.C.D., personal communication), (BDH chemicals, Poole, UK). The beads were transferred to 100ml of MRS broth for 18 hours at 37°C. The MRS broth was decanted and beads were rinsed with sterile deionised water and placed in freeze-drying ampoules.

Type 5 A beads: Beads were prepared which incorporated a final concentration of 10% skim milk, 1M glycerol and 4% tricalcium phosphate. The beads were rinsed in sterile deionised water after curing and transferred into 100ml of MRS broth and incubated for 18 hours at 37°C. The MRS broth was decanted and the beads were rinsed thoroughly in sterile deionised water before dispensing into freeze-drying ampoules.

3.2.3 : Freeze-drying Procedure: Beads containing *L.acidophilus* cells were freeze-dried using a Christ Alpha 1-4 Braun freeze-drier. The sterile glass ampoules containing the immobilised beads were frozen to -26°C/1000mbar and dried to 70°C/0.02mbar pressure. The ampoules were sealed and stored in a refrigerator at 4°C until required for use.

3.2.4 : The effect of immobilisation conditions on the survival of L.acidophilus from Product A in simulated gastric juice: The immobilization procedure was optimized by comparing the survival rates of *L.acidophilus* from the 5 different bead types in simulated gastric juice at pH 1.5 and 2.5. The simulated gastric juice was prepared as outlined in Chapter 1. In each case 10 beads were added to 9ml of simulated gastric juice at pH 1.5 and 2.5, and incubated at 37°C for 0, 30, 60 and 90 minutes. The supernatant was decanted and the beads were resuspended in 9ml of sterile ringers solution, (cell leakage into the supernatant was minimal with greater than 99.9% of the cells remaining in the bead structure). The beads were stomached for 5 minutes and standard plate counts were carried out on MRS agar. Plates were incubated at 37°C for 48 hours. The experiment was carried out in duplicate.

From the results of this section the optimal bead was determined, Bead Type 5A, and further indepth studies were carried out on cell exposure to gastro-intestinal factors that the ingested cells be would likely exposed to before final implantation in the intestinal tract. Furthermore cells from a second product, Product B, were immobilised using the immobilisation procedure used to prepare Beads Type 5A. These beads (denoted Beads Type 5B) were then subjected to similar gastro-intestinal conditions and their survival was recorded.

3.2.5 : Effect of simulated gastric juice on *L.acidophilus* from Product B immobilised under Type 5 bead conditions (Type 5B beads) : Ten beads from Beads Type 5B were added to 9ml of simulated gastric juice at pH 1.5 and 2.5 and incubated at 37°C for 0, 30, 60 and 90 minutes. The supernatant was decanted and the beads were resuspended in 9ml of sterile ringers solution. The beads were stomached for 5 minutes and standard plate counts were carried out on MRS agar. The plates were incubated at 37°C for 48 hours prior to cell enumeration. The experiment was carried out in duplicate.

3.2.6 : Effect of simulated intestinal juice on *L.acidophilus* in freeze-dried Beads Type 5A and 5B: Simulated intestinal juice at pH 7.4 was prepared as described in Chapter 1. In each case 10 beads were added to 9ml of simulated gastric juice at pH 7.4 and incubated at 37°C for 0, 30, 60 and 90 minutes. The supernatant was decanted and the beads were resuspended in 9ml of sterile ringers solution. The beads were stomached and standard plate counts were carried out on MRS agar. The plates were then incubated at 37°C for 48 hours. Duplicate trials were carried out.

3.2.7 : Comparison of *L.acidophilus* cells from Beads Type 5A and 5B for bile tolerance: The procedure of Noh and Gilliland (1993) was used. Cells from immobilised beads 5A and 5B were compared for their ability to grow in the presence of bile by inoculation of ten beads from Beads Type 5A and 5B into 50 ml of MRS broth containing 0, 0.1, 0.2, 0.3, 0.4, and 0.5% bile salts and incubated at 37°C. The cells were released from the tablets / capsules by stomaching and growth was monitored by measuring the A_{590nm} every 30 minutes until the absorbance readings increased by 0.3 units. The experiment was carried out in triplicate.

3.2.8 : Bile tolerance of *L.acidophilus* cells from Beads Type 5A and 5B after incubation in simulated gastric juice: Ten type 5A and 5B beads were incubated in simulated gastric juice at pH 2.5 for 90 minutes. The exposed cells were then examined for their bile tolerance using the previous method of Noh and Gilliland (1993) as detailed above.The experiment was carried out in triplicate.

3.2.9 : Cell Wall and Membrane Damage of *L.acidophilus* cells in Beads Type 5Aand 5B: To evaluate levels of cellular damage in *L.acidophilus* cells from Beads Type 5A and 5B, the method of Brennan et al. (1986) was used. In each case ten beads of Beads Type 5A and 5B were incubated for 30 minutes at 25°C in ringers solution containing either 8% NaCl, to assess levels of cell membrane damage, or 0.1% bile salts, to assess levels of cell membrane damage. The cell suspension was stomached for 5 minutes and standard plate counts were carried out on MRS agar and plates were incubated at 37°C for 24 hours. Relative sensitivities to the selective agents was determined from the differences in CFU's with and without the selective agents.Duplicate trials were carried out.

3.3 : RESULTS

3.3.1 : Optimisation of the immobilisation process to enhance survival of *L.acidophilus* from Product A under simulated gastric conditions:

The immobilisation process was optimised so as to reduce viability loss during exposure to simulated gastric juice at pH 1.5 and 2.5.

A comparison of the survival of cells from all the different bead types at different pH levels (Fig 3.1, 3.2, 3.3, 3.4 and 3.5) illustrates that a key factor influencing survival of *L.acidophilus* in simulated gastric juice is the pH and type of bead formulation used. When Type 1A beads were exposed for 90 minutes to simulated gastric juice at pH 1.5 (Fig 3.1), they exhibited a greater than 5.5 log reduction. The greatest decrease in cell number occurred during the first 30 minutes where 65% of the total cell reduction occurred. Cell reduction was less pronounced in simulated gastric juice at pH 2.5 (Fig 3.1) where there was a 3.2 log cycle reduction after 90 minutes, 44.5% of which occurred during the first 30 minutes.

Cells in Beads Type 2A which had been incubated in Nutrient broth at 37°C for 18 hours before freeze-drying, exhibited lower survival rates than those of Bead Type 1A. Results show a > 6.8 and 5.8 log reduction in the viability of *L.acidophilus* after 90 minutes in simulated gastric juice at pH 1.5 and 2.5 respectively (Fig 3.2). Most of the cell reduction was observed during the first 30 minutes of which 56.3% and 68.6% of the total cell reduction occurred in simulated gastric juice at pH 1.5 and 2.5 respectively.

Cells in Bead Type 3A (Fig 3.3) which had been incubated in MRS broth at 37°C for 18 hours prior to freeze-drying, exhibited survival rates surpassing those of Beads Type 1A and 2A. When inoculated in simulated gastric juice at pH 1.5 and 2.5 for 90 minutes, cell viability decreased by 4.6 log cycles and 2.1 log cycles respectively of which 83.7% and 92.7% of the total cell reduction occurred during the first 30 minutes.

Beads Type 4A (Fig 3.4), which incorporated a final concentration of 4% tricalcium phosphate in the bead formulation showed enhanced survival rates in simulated gastric juice compared to the bead types previously examined (Bead Type 1A, 2A and 3A). They exhibited a 3.4 and 2.0 log cycle reduction after 90 minutes in simulated gastric juice at pH 1.5 and 2.5 respectively. The greatest reduction in cell number occurred during the first 30 minutes exposure to simulated gastric juice at pH 1.5 and 2.5, where 88.3 and 71.4% of the total cell reduction occurred respectively.

However, Beads Type 5A (Fig 3.5) exhibited survival rates surpassing those of all the bead types previously examined. They exhibited a 3.3 and a 0.5 log cycle reduction after 90 minutes incubation in simulated gastric juice at pH 1.5 and 2.5 respectively. As observed with the previous bead types, greatest cell reduction occurred during the first 30 minute interval, where 80.2 and 66.7% of the total cell reduction occurred in simulated gastric juice at pH 1.5 and 2.5 respectively. This bead formulation was subsequently used in further tests.

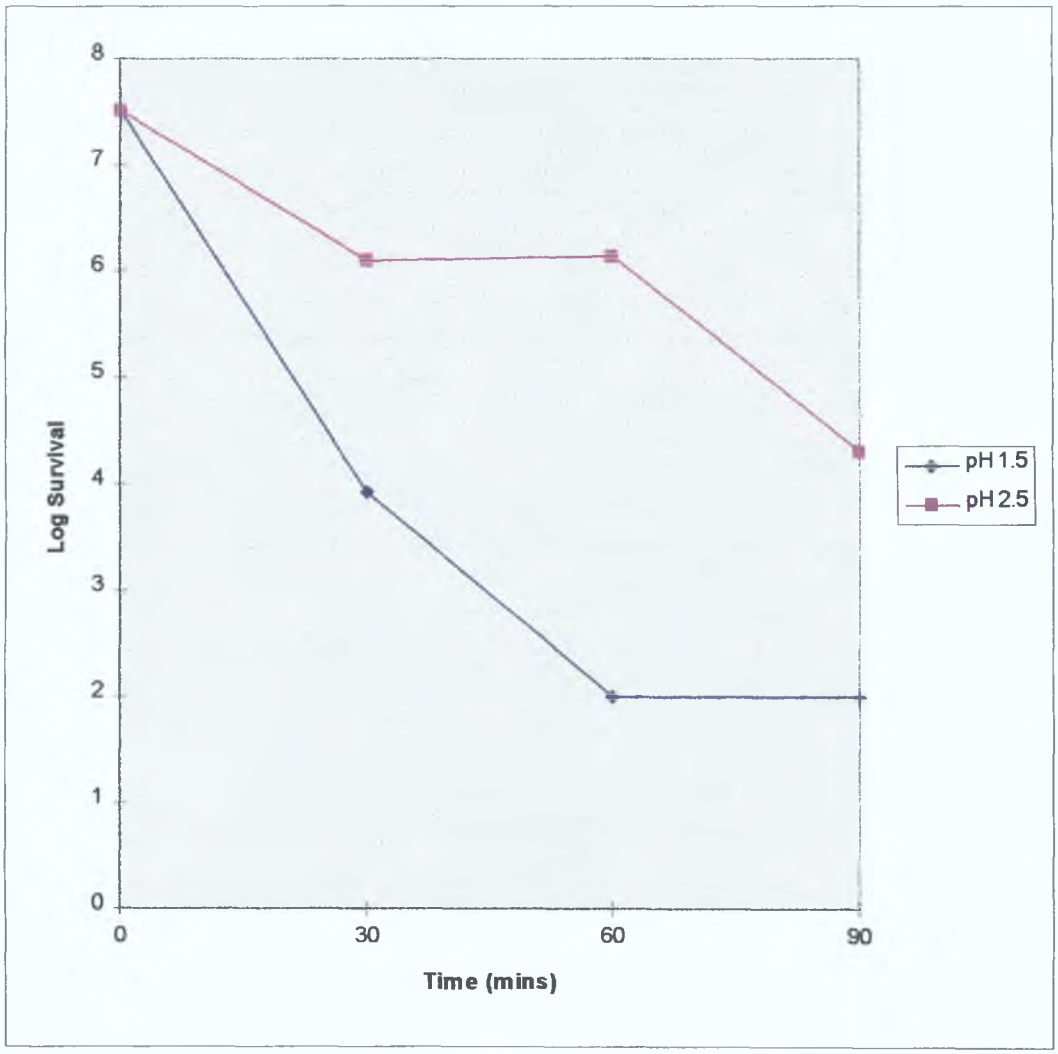


Fig 3.1: Effect of simulated gastric juice, pH 1.5 and 2.5, on the viability of *L.acidophilus* from Bead Type 1A.

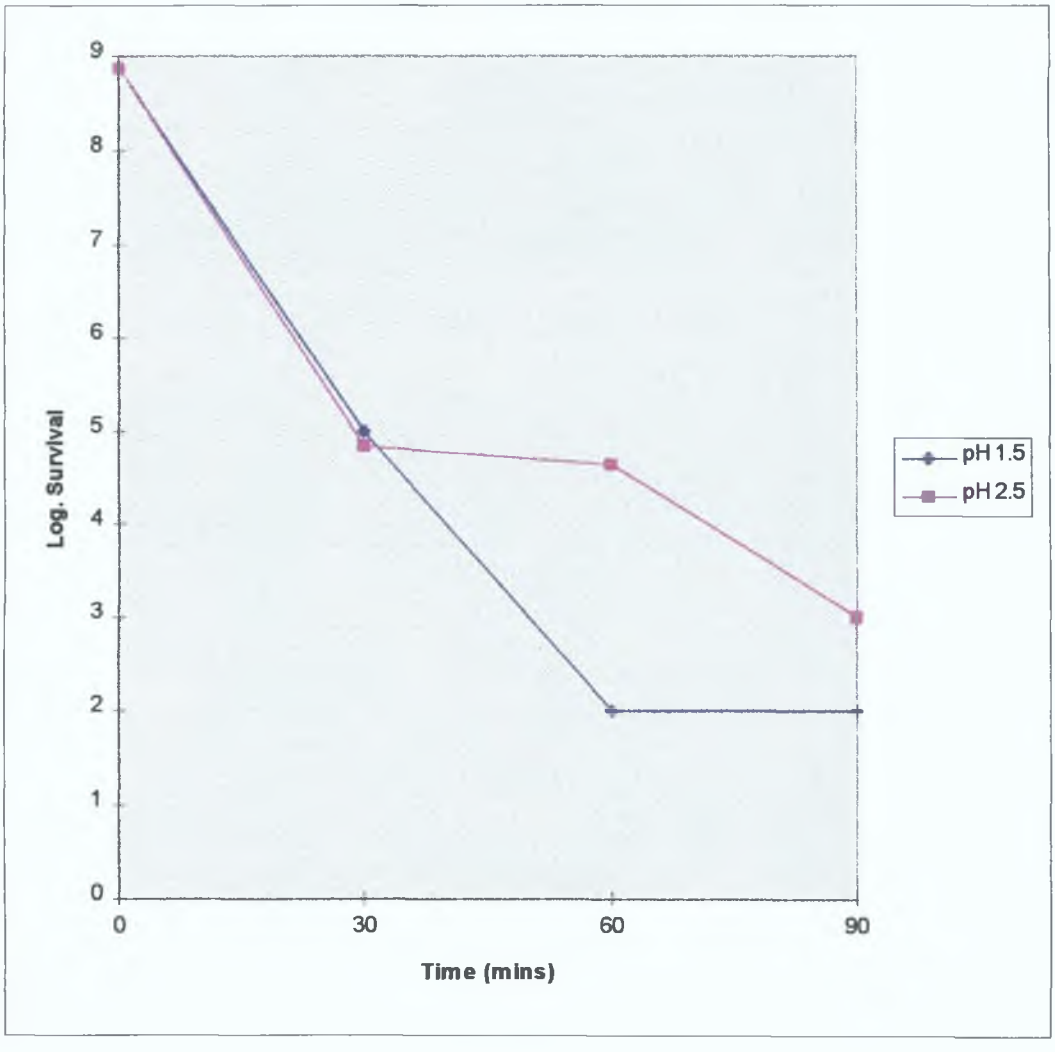


Fig 3.2: Effect of simulated gastric juice, pH 1.5 and 2.5, on the viability of *L.acidophilus* from Bead Type 2A.

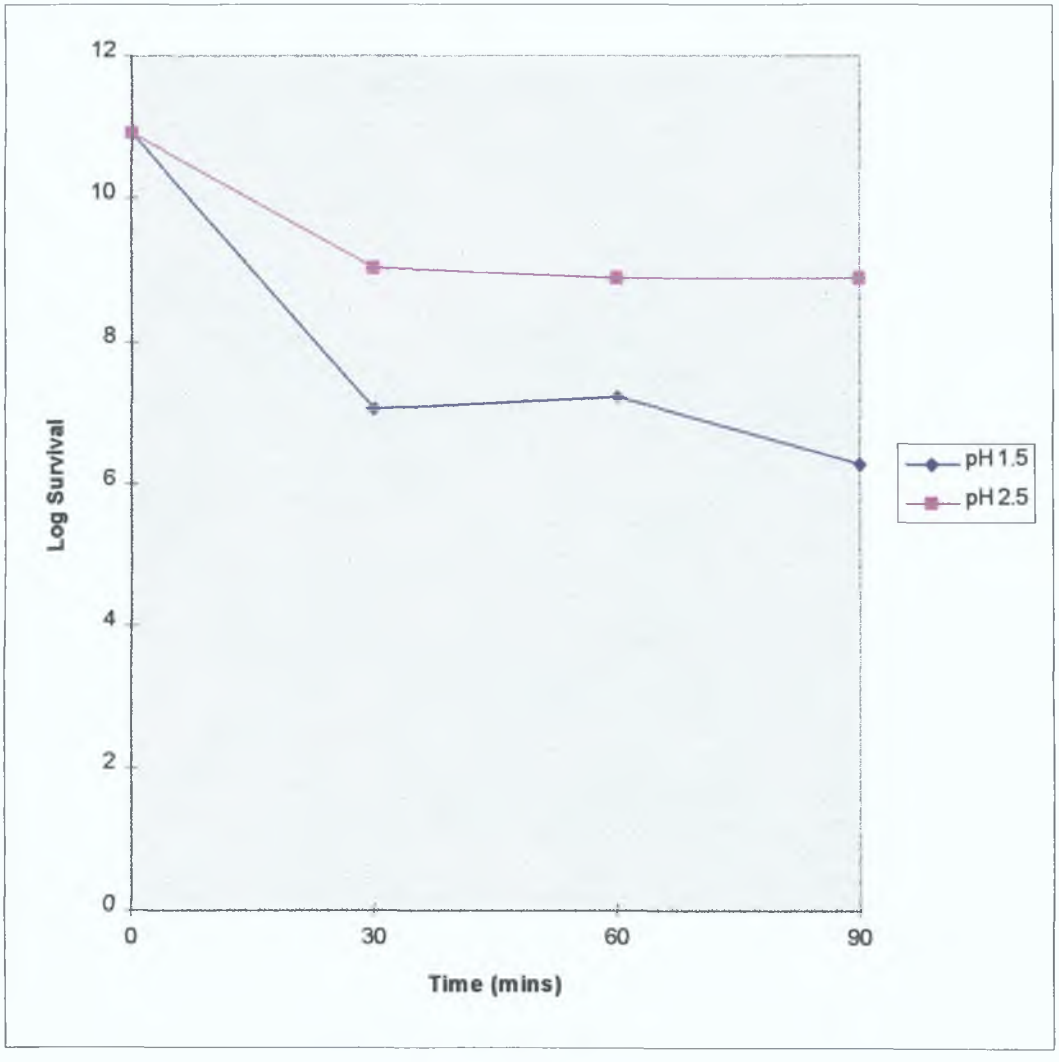


Fig 3.3: Effect of simulated gastric juice, pH 1.5 and 2.5, on the viability of *L.acidophilus* from Bead Type 3A.

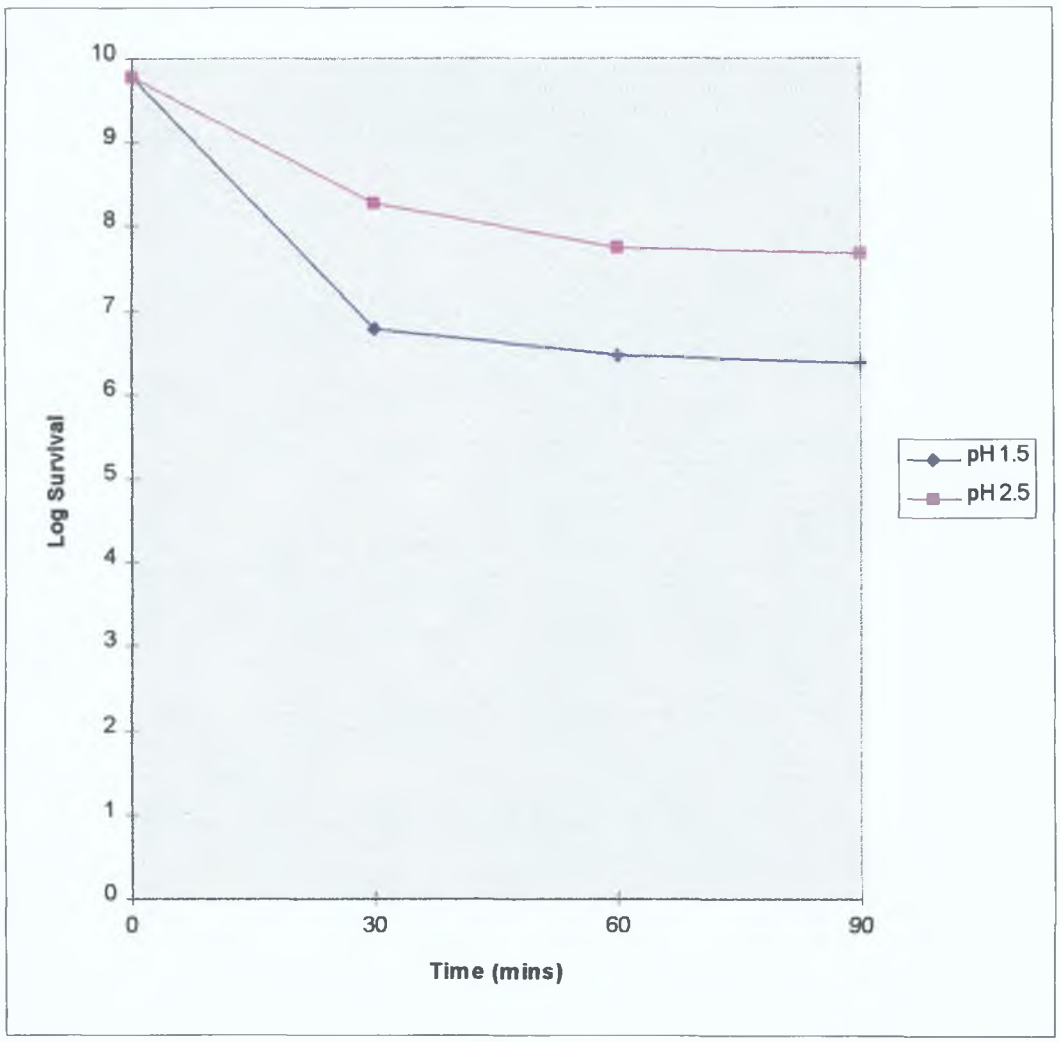


Fig 3.4: Effect of simulated gastric juice, pH 1.5 and 2.5, on the viability of *L.acidophilus* from Bead Type 4A.

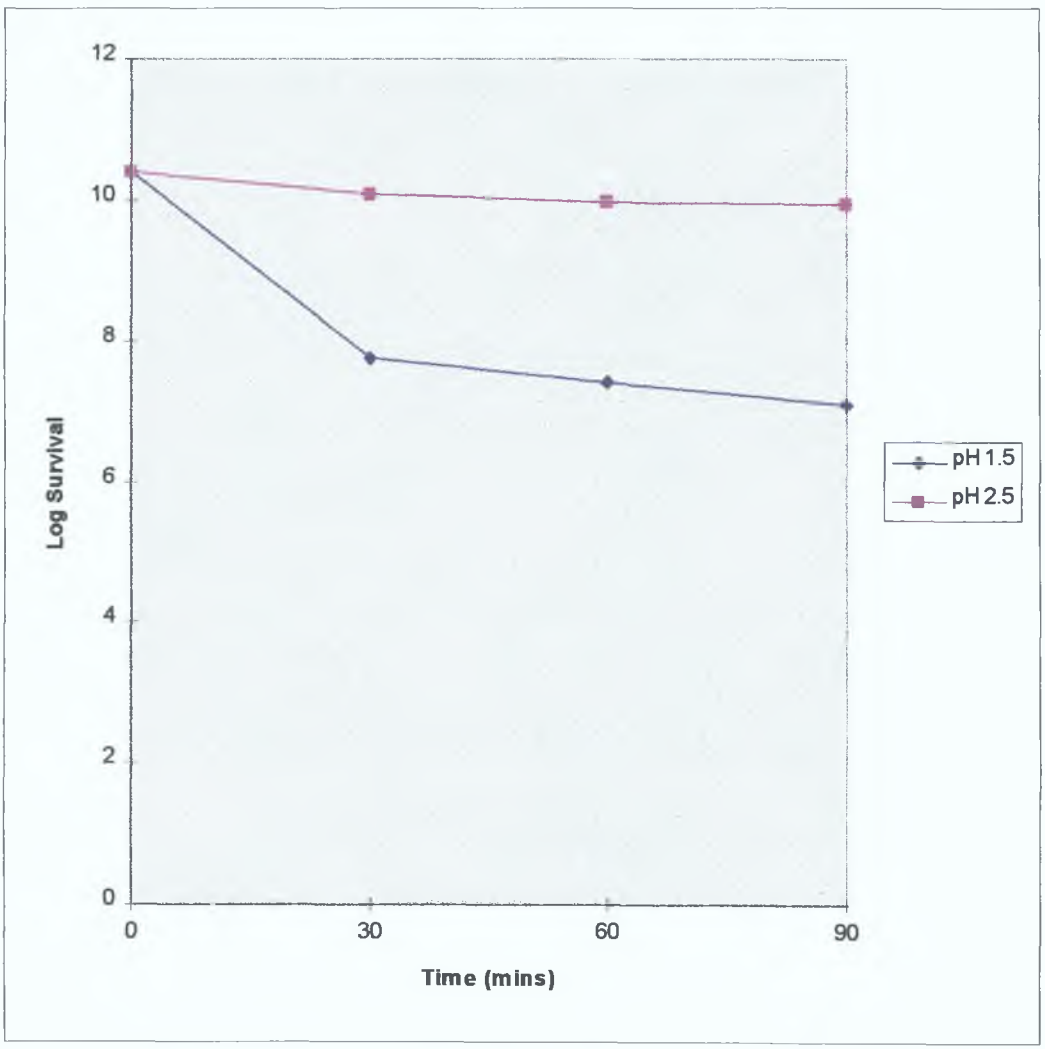


Fig 3.5: Effect of simulated gastric juice, pH 1.5 and 2.5, on the viability of *L.acidophilus* from Beads Type 5A.

3.3.2 : The effect of simulated gastric conditions on the survival of *L.acidophilus* in Beads Type 5B:

Cells from a second product (Product B) were immobilised using the immobilisation formula that was used to prepare Type 5A beads and designated Type 5B beads. When these cells were exposed to simulated gastric juice at pH 1.5 and 2.5 they exhibited a 3.4 and 2.1 log reduction respectively of which 97.1 and 82.7% of the total cell reduction occurred during the first 30 minutes (Fig 3.6).

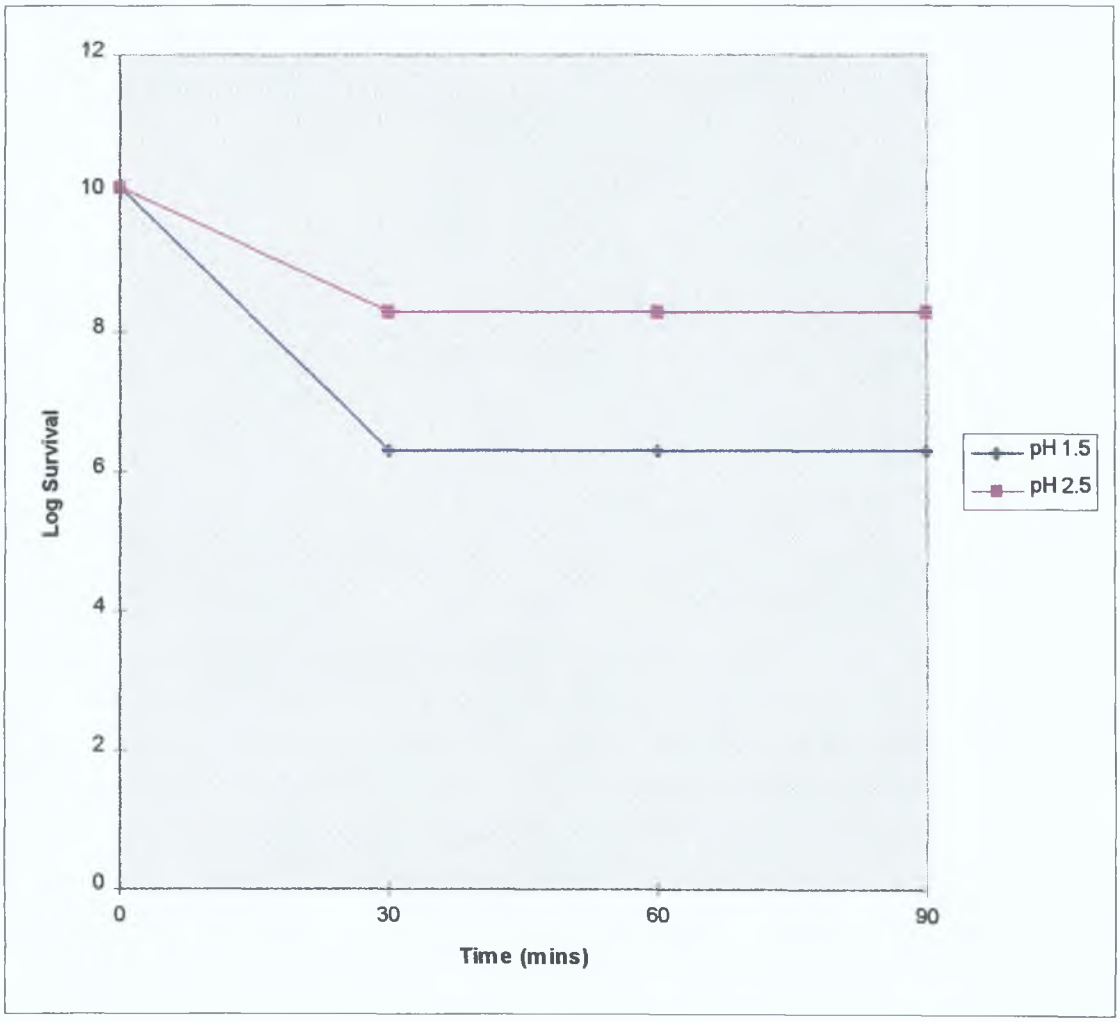


Fig 3.6: Effect of simulated gastric juice, pH 1.5 and 2.5, on the viability of *L.acidophilus*.

3.3.3 : Effect of simulated intestinal juice on the viability of *L.acidophilus* from Beads Type 5A and 5B:

The effect of simulated intestinal juice at pH 7.4 on immobilised Beads Type 5A and 5B is shown in Fig 3.7 and 3.8. A decrease in cell viability was noted for both bead types after 120 minutes incubation in simulated intestinal juice at pH 7.4. There was a 1.4 and 1.1 log cycle reduction in Bead Type 5A and 5B respectively after 120 minutes incubation in simulated intestinal juice respectively. Most of the reduction in cell viability occurred during the first 30 minutes. Results show that 47.9% and 91.9% of the total cell reduction occurred in Beads Type 5A and 5B respectively during this time.

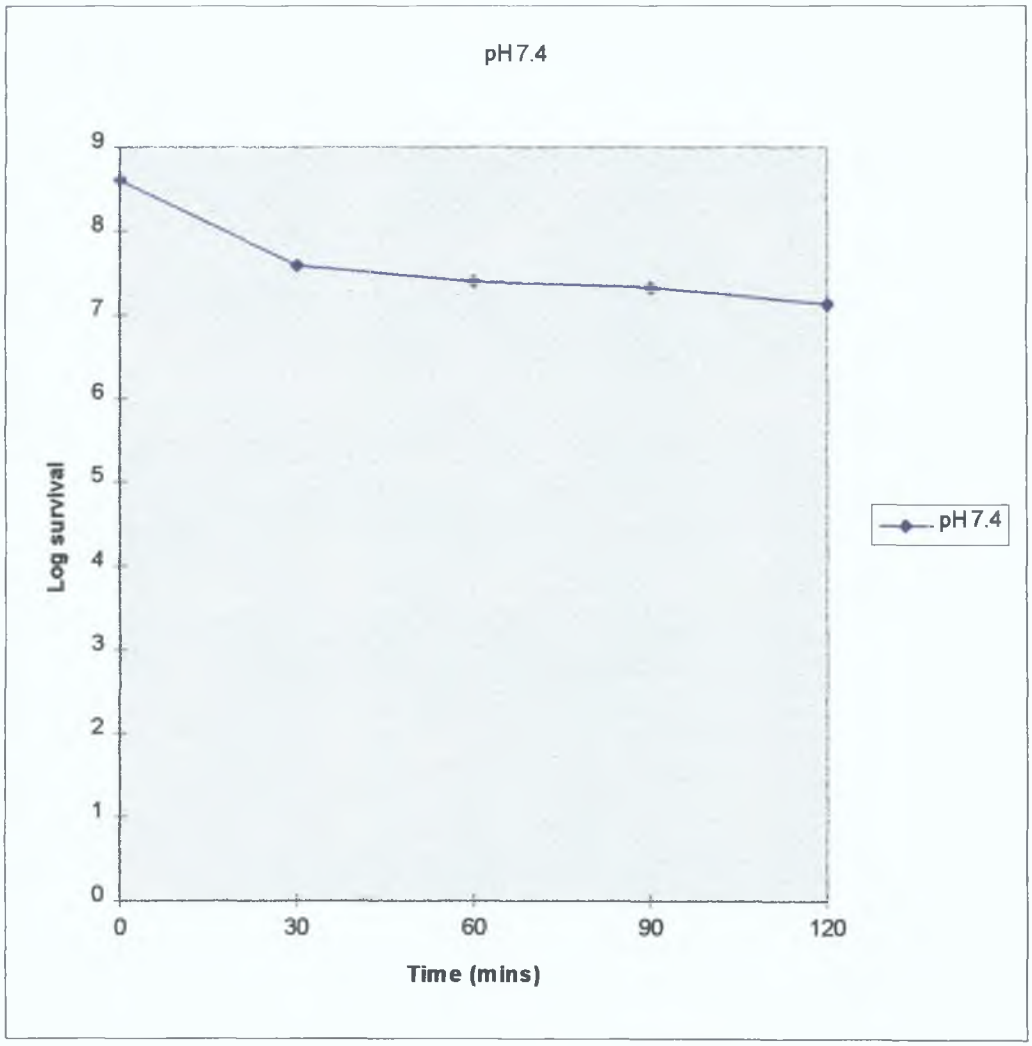


Fig 3.7: Effect of simulated intestinal juice, pH 7.4, on the viability of *L.acidophilus* from Beads Type 5A.

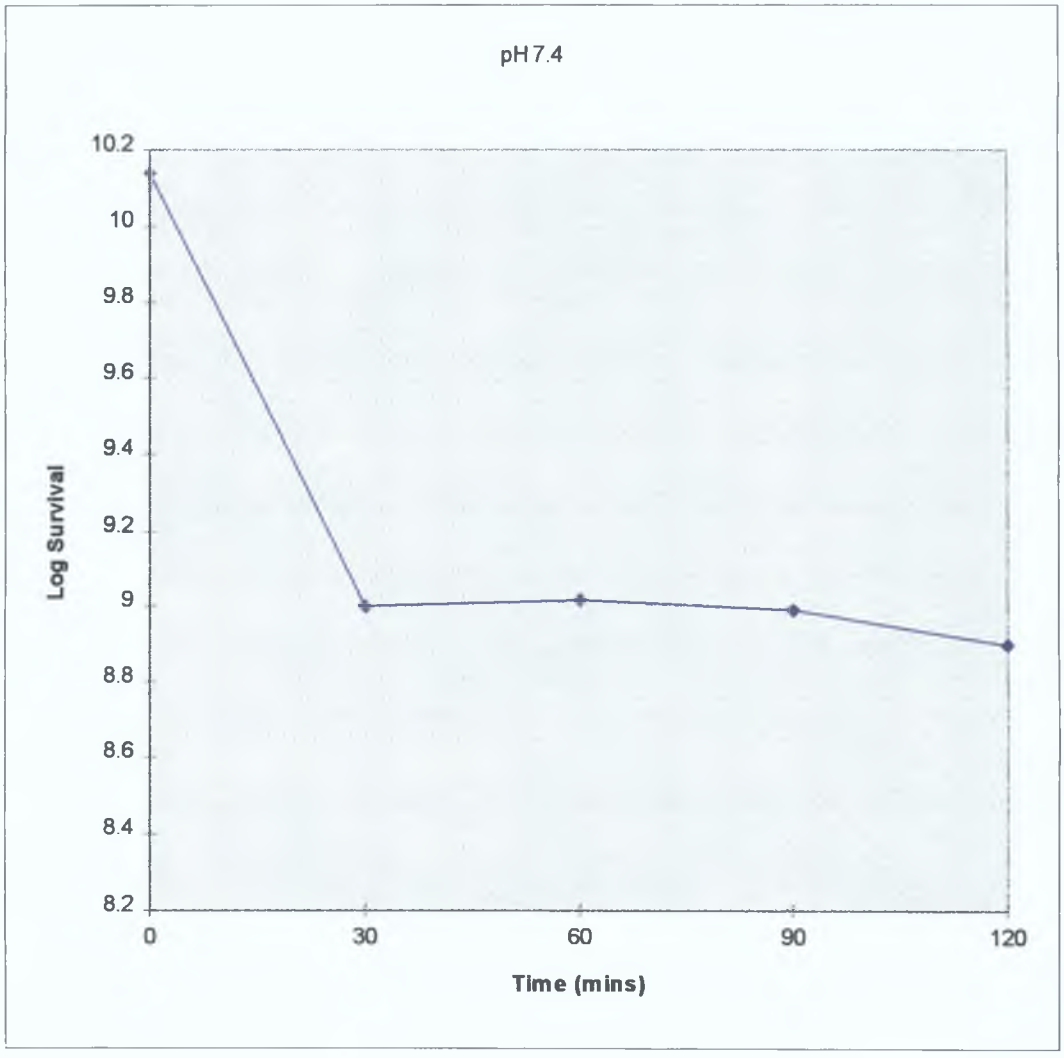
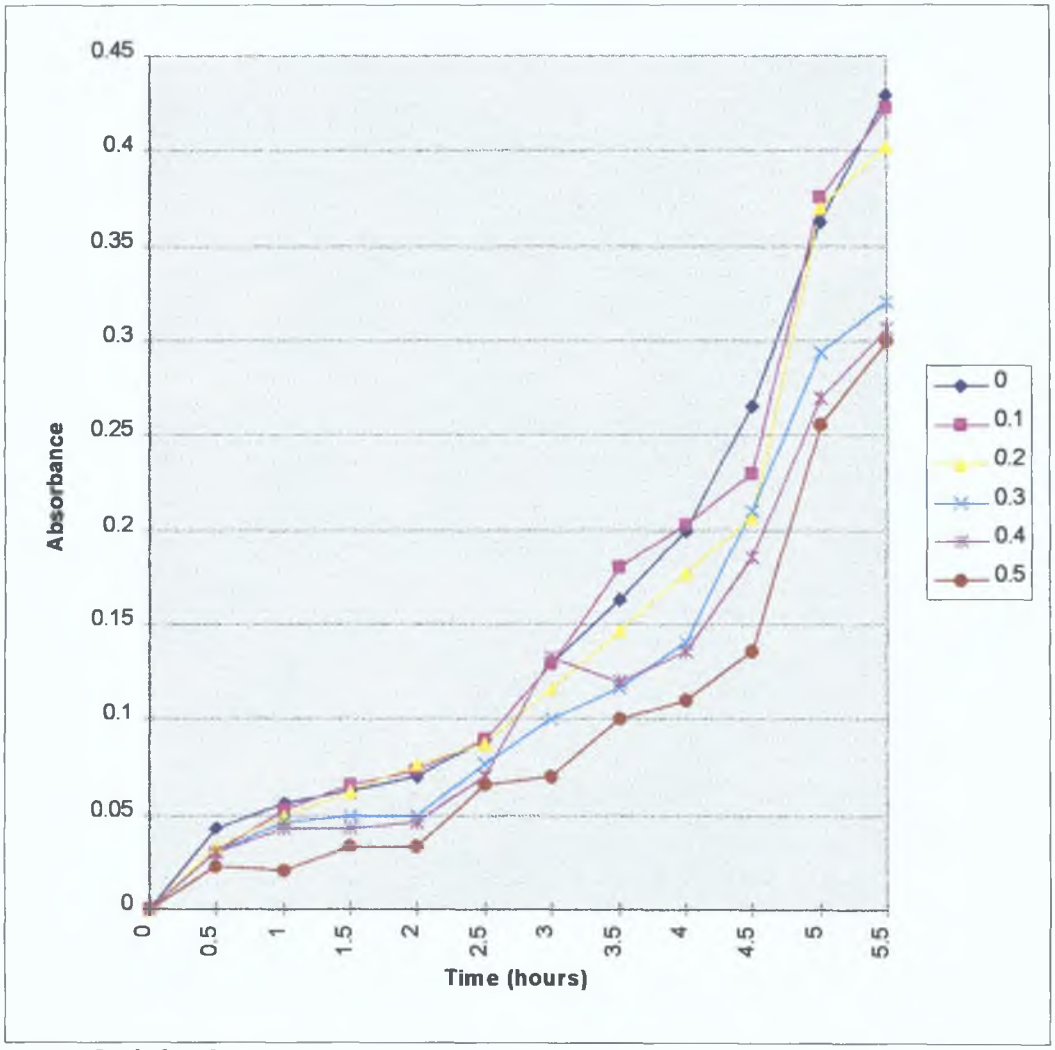


Fig 3.8: Effect of simulated intestinal juice, pH 7.4, on the viability of *L.acidophilus* from Beads Type 5B.

3.3.4 : Bile Tolerance of *L.acidophilus* cells in Beads Type 5A and 5B:

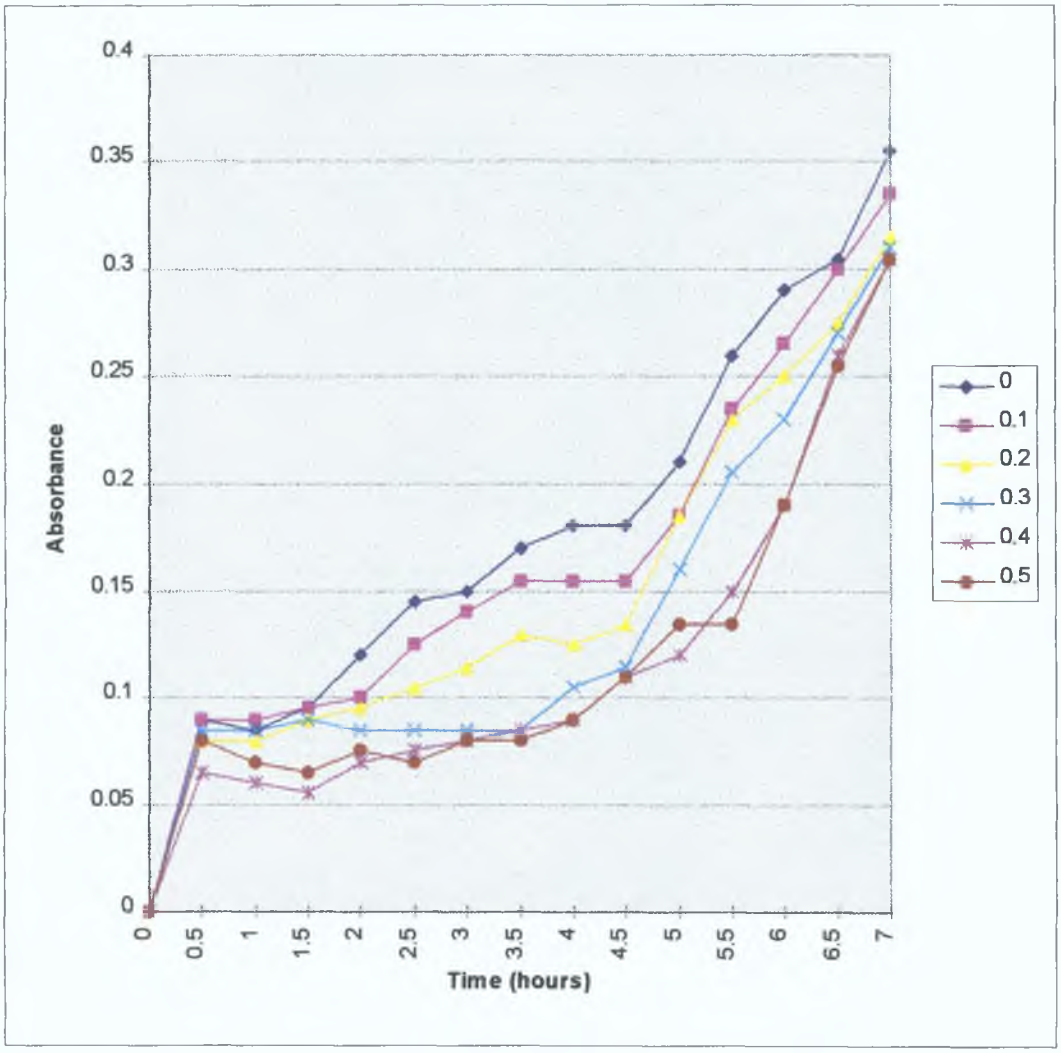
Cells from Beads Type 5A reached the 0.3 absorbance level after 4.75 hours incubation in MRS broth (Fig 3.9). Cells which had been grown in MRS broth containing 0.1 and 0.2% bile salts exhibited no significant ($P > 0.05$) variation in growth time compared with the control (bile-free broth). However, as the bile salt concentration further increased from 0.3% to 0.5% cells did exhibit significant ($P < 0.05$) growth variation with respect to the control. The time required to reach the 0.3 absorbance level increased by 10.5, 15.8 and 15% in MRS broth containing 0.3, 0.4 and 0.5% bile salt respectively compared with the control.

Cells from Beads Type 5B reached the 0.3 absorbance level after 7 hours incubation in MRS broth (Fig 3.10). Cells which had been grown in MRS broth incorporated 0.1% bile salt exhibited no significant ($P > 0.05$) variation in the growth rate. However, the remaining broths which incorporated higher concentrations of bile salts (0.2, 0.3, 0.4 and 0.5%) did exhibit significant ($P < 0.05$) variations in growth rate compared with the control. The cells incubated in these bile salt concentrations reached the 0.3 absorbance level by 7.5 hours. Therefore the time required to reach the 0.3 absorbance level increased by up to 7.1% at higher bile salt concentrations (0.2, 0.3, 0.3, 0.4 and 0.5%).



No. of trials: 3
S.D.: 0.05

Fig 3.9: Absorbance readings at 590nm of cells from Beads Type 5A during growth in MRS broth containing a range of bile salt concentrations.



No. of trials : 3
S.D. : 0.02

Fig 3.10: Absorbance readings at 590nm of cells from Beads Type 5B during growth in MRS broth containing a range of bile salt concentrations after incubation in simulated gastric juice .

3.3.5 : Bile Tolerance of *L.acidophilus* in Beads Type 5A and 5B after incubation in simulated gastric juice:

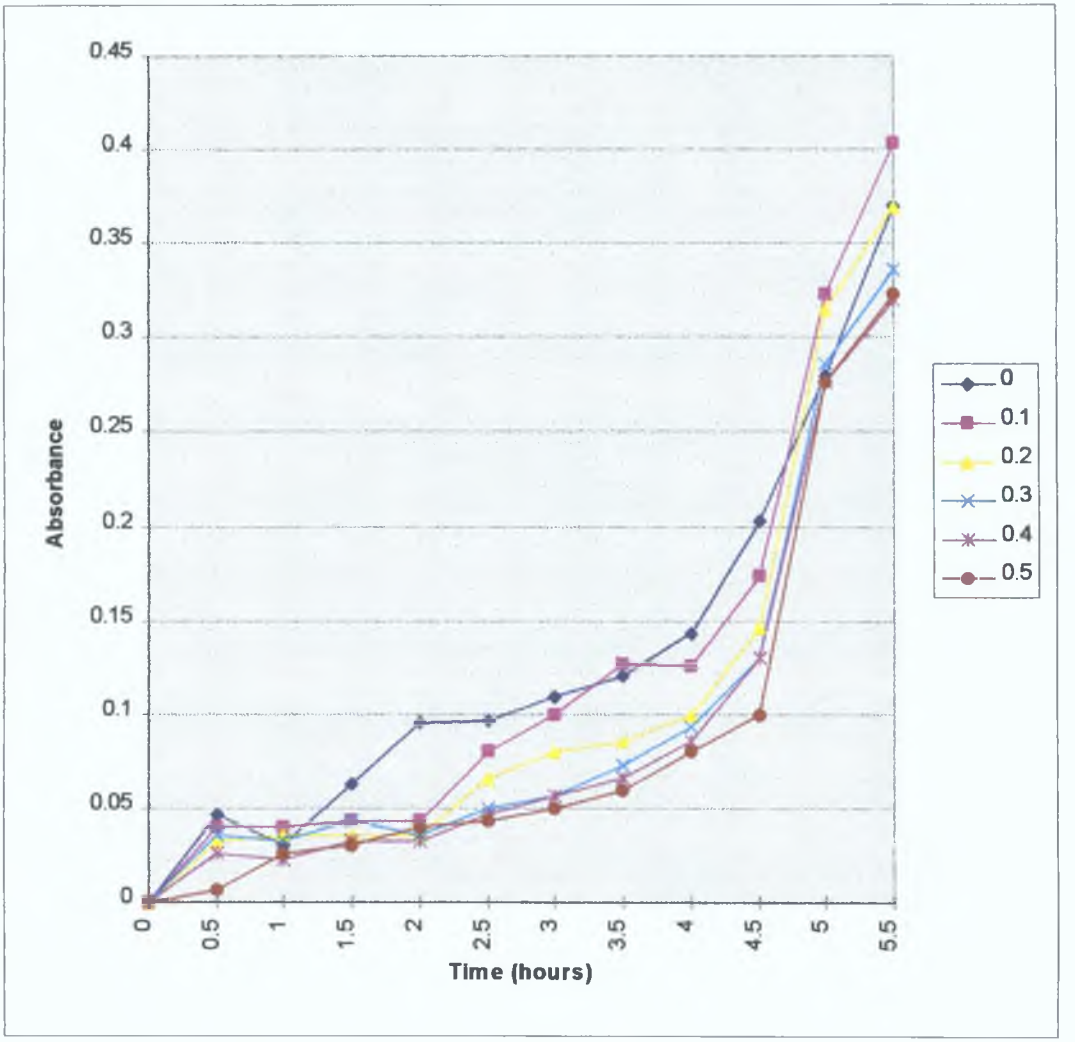
Incubation of Beads Type 5A in simulated gastric juice had minimal effect on their cell resistance to a range of bile salt concentrations (Fig 3.11). Cells which had been grown in MRS broth containing bile salt concentrations (0.1 to 0.5%) exhibited no significant ($P > 0.05$) growth variations compared with the control broth.

After 90 minutes exposure to simulated gastric juice at pH 2.5, cells from Beads Type 5A required increased incubation times in MRS broth in order to reach the 0.3 absorbance level (Fig 3.11)with respect to the unexposed cells (Fig 3.9). Results show that the incubation time of the exposed cells was increased by 7.89% in MRS broth compared with unexposed cells. However, the incubation time for cells to reach the same absorbance level grown in MRS broth containing bile salts was dependent on the concentration of bile salt incorporated into the broth. At lower bile salt concentrations the time required to reach the 0.3 absorbance level after exposure to simulated gastric juice was increased by 3.89% and 2.6% in 0.1 and 0.2% bile salt incorporated broth compared with unexposed cells, they exhibited equal incubation times in broth containing 0.3% bile salt. Whilst the incubation time was reduced by 4.5% in MRS broth incorporating 0.4% and 0.5% bile salts.

Incubation of Beads Type 5B in simulated gastric juice had also minimal effect on their resistance to a range of bile salt concentrations (Fig 3.12). Cells from Beads Type 5B which had been grown in MRS broth incorporating 0.1% bile salt exhibited no significant

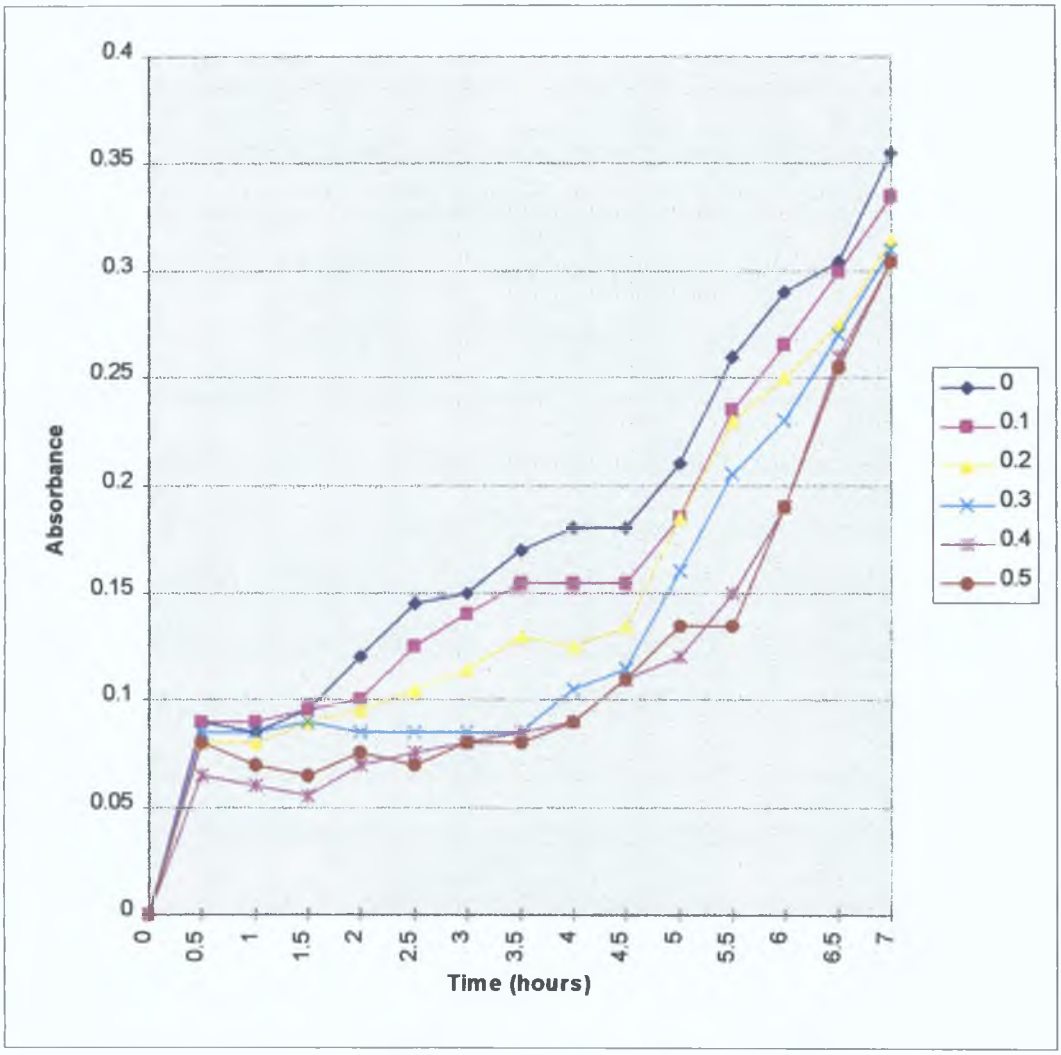
($P > 0.05$) variation in growth rate with respect to MRS broth while the remaining broths which contained higher levels of bile salts (0.2-0.5%) did exhibit significant ($P < 0.05$) variations in growth rate compared with the control broth (Fig 3.12).

The incubation time for cells from Beads Type 5B was found to be reduced in bile-free and bile incorporated broth (0.1-0.5%) compared with unexposed cells (Fig 3.10). The incubation time was reduced by between 5.02 and 10.7% in the range of bile salt concentrations examined (0.1 to 0.5%).



no. of trials: 3
S.D.: .023

Fig 3.11: Absorbance readings at 590nm of cells from Beads Type 5A during growth in MRS broth containing a range of bile salt concentrations after incubation in simulated gastric juice.



No. of trials: 3
S.D.: 0.05

Fig 3.12: Absorbance readings at 590nm from Beads Type 5B during growth in MRS broth containing a range of bile salt concentrations after incubation in simulated gastric juice.

3.3.6 : Cell wall and membrane damage of *L.acidophilus* cells from Beads Type 5A and 5B:

The relative sensitivity of *L.acidophilus* from Beads Type 5A and 5B to 0.1% bile salt and 8% NaCl was determined from the loss of viable cells after 30 minutes exposure to the selective agents as shown in Table 3.2. Cells From Beads Type 5A exhibited greater sensitivity to 0.1% bile salt and 8% NaCl compared with Beads Type 5B indicating higher levels of cell wall and membrane damage.

Table 3.2: Percentage survival of *L.acidophilus* from Beads Type 5A and 5B after exposure to selective agents.

Bead Type	0.1% Bile salt	8% NaCl
Beads Type 5 A	9%	33%
Beads Type 5 B	31%	75%

3.4 : DISCUSSION

L.acidophilus cells isolated from Commercial Products A and B were immobilised in calcium alginate and freeze-dried with the aim of enhancing cell survival under gastro-intestinal conditions. Beads Type (1A, 2A, 3A, 4A and 5A) were prepared containing different adjuncts and incubation times (Table 3.1), with the purpose of creating a bead formulation which would confer maximum protection on cell survival during incubation in simulated gastric conditions.

L.acidophilus cells from Beads Type 1A exhibited reduced survival in simulated gastric juice compared with their commercial counterparts (Chapter 2, Fig 3.1). They showed a 2.0 and 1.0 log cycle increase in cell reduction after 90 minutes incubation in simulated gastric juice at pH 1.5 and 2.5 compared with their free cell counterparts.

Beads Type 2A and 3A differed from Beads Type 1A in that they were incubated in Nutrient broth and MRS broth for 18 hours at 37°C prior to freeze-drying. The purpose of this step was to increase cell biomass within the bead matrix in order to enhance cell survival by the mutual shielding effect of the microorganisms. However, the protection afforded was dependent on the nutrient composition of the medium. Results show that incubation of the cells in Nutrient broth did not enhance cell survival while cells which had been incubated in MRS broth did improve cell survival under similar gastric conditions.

L.acidophilus cells from Beads Type 2A exhibited reduced survival under simulated gastric conditions compared with their commercial counterpart. While cells from Beads

Type 3A showed reduced survival in simulated gastric juice at pH 1.5, but exhibited similar survival after 90 minutes incubation in simulated gastric juice at pH 2.5 compared with cells from Product A.

MRS broth differs from Nutrient broth in that it contains additional ingredients such as acetate (sodium acetate, 0.5g/l), magnesium (magnesium sulphate, 0.02g/l) and manganese (manganese sulphate, 0.05g/l) which are known to be special growth factors for lactobacilli (Kandler and Weiss, 1992). Where as nutrient broth is a more general purpose medium used to cultivate micro-organisms that are not as selective in their growth requirements. The use of this broth would therefore limit the growth of fastidious organisms, like lactobacilli.

Cell growth within the bead matrix after the immobilisation procedure has been found by other researchers in some cases to enhance cell protection under suboptimal conditions. Mattiasson et al. (1982) and Keweloh et al.(1989) reported the need of cell aggregate formation to occur within the bead in order for cells to be protected and that the size of the microcolonies formed determines the extent of cell tolerance to extreme environments. Bozoglu et al (1987) also observed that the survival fraction of cells increased with increasing biomass concentration during drying. They attributed the enhanced survival to the mutual shielding effect of the microorganisms against severe conditions existing in the external environment.

L.acidophilus cells from Beads Type 4A, which incorporated 4% tricalcium phosphate into the final bead formulation, exhibited similar survival rates to their commercial counterparts under simulated gastric conditions.

Tricalcium phosphate is a white crystalline substance that reacts as follows at low pH:

$$Ca_{10}(PO_4)_6(OH)_2 \xrightarrow{H^+} 10Ca^{2+}+6H_3PO_4+2H_2O$$ (Wang and Hettwer, 1982).

While under neutral pH conditions the solubility of the crystal is low. Therefore, this substance may reduce the pH gradients within the beads when exposed to the hostile acidic conditions of the stomach.

Studies by Wang and Hettwer (1982) investigated the effect of incorporating tricalcium phosphate into k-carrageenan gels on the viability of *Saccharomyces cerevisiae* during ethanol production. They found that the cell concentration in 5% tricalcium phosphate beads was 20% higher than that in the control beads and attributed the enhanced viability to the buffering capacity of the crystals. They suggested that the tricalcium phosphate crystals would maintain the pH within the beads at a level more favourable to cells, altering the pH of the medium from between pH 2.5-3.0 to pH 4.0.

However, the optimum bead type was found to be Beads Type 5A, which incorporated higher levels of cryoprotectants (10% skim milk and 10 M glycerol), along with tricalcium phosphate. They exhibited survival rates under simulated gastric conditions surpassing those of all the beads types previously mentioned. Furthermore, a comparison of results from Chapter 2 (Fig 2.1) and Fig 3.5 show that the immobilisation procedure did confer additional protection on cells during incubation in simulated gastric juice. The

immobilisation process increased the survival of cells from Product A by 0.2 and 1.7 log cycles in simulated gastric juice at pH 1.5 and 2.5 respectively compared with free cells.

Numerous studies have also found that addition of certain agents such as skim milk and glycerol to beads resulted in enhanced cell survival during exposure to hostile conditions and preservation treatments.

For instance, the addition of skim milk to bead formulations provides complex carbon and nitrogen sources which may increase cell activity, growth and survival of cells (Bashan, 1986; Cassidy et al., 1996). Furthermore, the addition of skim milk could also enhance cell survival by regulating the internal pH of the bead (cited by McLoughlin, 1994). The addition of cryoprotectants such as glycerol has been found to provide protection during freeze-drying by reduction of the lethal effects of solute concentration, prevention of intracellular ice formation or reduction in its lethal consequences, through hydrogen bonding with water and cell structures.

Studies by Castro et al. (1997) found that the percentage survival of *L.bulgaricus* increased after freeze-drying by over 20% when cells were suspended in 11% skim milk, and by approximately 15% with 1 mol l^{-1} glycerol. While other studies by Kearney et al. (1990) reported that the incorporation of glycerol and skim milk enhanced survival of immobilised *L.plantarum* cells during freeze-drying. They found that the incorporation of 1M glycerol into 10% skim milk in calcium alginate beads enhanced their viability by greater than 44% during freeze-drying. Furthermore their results showed that

immobilising cells in calcium alginate beads incorporating 1M glycerol and 10% skim milk as cryoprotectants enhanced cell survival in a range of pH values (pH 3.0 to 7.0).

Cells from a second commercial product, Product B, were immobilised using the same procedure as used to produce Beads Type 5A. They also exhibited survival rates surpassing those of their commercial counterparts. The immobilisation process increased the survival of cells from Product B by 1.3 and 1.2 log cycles in simulated gastric juice at pH 1.5 and 2.5 respectively compared with their commercial counterparts.

Immobilised Beads Type 5A and 5B also exhibited enhanced survival rates in simulated intestinal juice, at pH 7.4 compared with free cells (Fig 2.3 and 2.4). Therefore immobilising cells from Product A and B in calcium alginate beads enhanced their survival by 0.1 and 1.2 log cycles respectively.

It maybe concluded from this section that cells from Beads Type 5A and 5B did exhibit enhanced cell survival in simulated gastric juice at pH 1.5 and 2.5 and simulated intestinal juice at pH 7.4 compared with their commercial counterparts.

To survive and grow in the intestinal tract, the probiotic cultures should also be bile resistant. Results from this chapter show that the rate of cell growth was influenced by the concentration of bile salts incorporated into the broth, with the growth being significantly ($P < 0.05$) reduced in most cases at higher bile salt concentrations. Cells from Beads Type 5A exhibited no significant ($P > 0.05$) variations in growth rate when grown in MRS broth incorporating lower concentrations of bile salts (0.1 and 0.2%) with

respect to bile-free broth. However, their cell growth was significantly reduced at higher bile salt concentrations (0.3, 0.4 and 0.5%). Cells from Beads Type 5B exhibited significant ($P < 0.05$) growth rates in the presence of 0.2, 0.3, 0.4 and 0.5% bile salts and exhibited no significant ($P > 0.05$) variation in MRS broth incorporating 0.1% bile salt compared with the control.

It may also be concluded from these results that the immobilisation process did enhance the bile tolerance of *L.acidophilus* cells. The bile tolerance of cells from immobilised Beads Type 5A and 5B and their commercial counterparts were compared by examining the added times required for the A_{590nm} to increase to the 0.3 absorbance level in MRS broth containing 0.1 to 0.5% bile salt compared with bile-free broth. The additional time required for the absorbance to reach the 0.3 level in bile salt incorporated broth ranged from 0 to 0.75 hours for Beads Type 5A compared with 0.25 to 2.75 hours for their commercial counterparts. The increased time needed for the absorbance level in bile salt (0.1 to 0.5%) incorporated broth ranged from 0 to 0.5 hours for immobilised Beads Type 5B compared with 0.1 to 2.60 hours for their commercial counterparts.

The bactericidal effect of the gastro-intestinal conditions of *L.acidophilus* is exacerbated by sublethal injury incurred during the freeze-drying process. Studies by Brennan et al. (1986) and Castro et al. (1997) correlated the developed sensitivity of dried cells to bile salt and NaCl to levels of cell wall and membrane damage. Cells from Beads Type 5B exhibited higher levels of cell survival in 0.1% bile salts and 8% NaCl, thus indicating lower levels of cell wall and membrane damage.

A comparison of results from Chapter 2 (Table 2.2) show that the immobilisation process increased the level of cell damage following freeze-drying with cells from Product A, but conferred additional protection to cells from Product B. Therefore, the enhanced survival of the immobilised beads towards the hostile conditions of the simulated gastro-intestinal conditions could not be solely attributed to reduced levels of cellular damage. Other studies by McLoughlin and Champagne (1994) reported that the percentage of cells exhibiting cell wall and membrane damage after freeze-drying was lower among immobilised *L.plantarum* cultures compared with their free cell counterparts.

In conclusion, Immobilised Cell Technology has potential in the healthcare sector in the production of probiotic preparations for therapeutic usage. Results from this chapter have shown that entrapment of *L.acidophilus* cultures in calcium alginate beads can protect them during ingestion and transport through the hostile gastro-intestinal environment. The immobilisation process enhanced their survival during exposure to the acidic and alkaline conditions of the stomach and intestine and increased their tolerance to the bactericidal concentrations of bile. The enhanced survival would consequently led to a more effective dietary adjunct.

CHAPTER FOUR :

The antimicrobial effect of Lactic Acid Bacteria on pathogens

4.1 : INTRODUCTION

Many studies have shown that lactic acid bacteria exerts antagonistic activity againsts intestinal food-borne pathogens and other related organisms (some examples are listed in Table 4.1). This antagonistic action may result not only from competition for nutrient and adhesive sites (Hood and Zottola 1989; Coconnier et al., 1992; Bernet et al., 1994) but also because of the formation of antimicrobial compounds. These compounds include organic acids such as acetic and lactic acid, hydrogen peroxide, aswell as broad range antibiotic-like compounds (Lindgren and Dobrogosza, 1990; Klaenhammer, 1993; Mital and Garg. 1995; Guillermo et al., 1996).

Organic acids such as lactic and acetic acids which are by-products of the homo and heterofermentation of hexose (Lindgren and Dobrogosza, 1990) have been found to inhibit the growth and metabolic activity of certain pathogenic micro-organisms (Mital and Garg, 1995). Their antimicrobial action appears to be the result of the ability of the lipophilic, undissociated acid molecules to penetrate the bacterial plasma membrane and interfere with essential metabolic functions such as substrate translocation and oxidative phosphorylation (Smulders et al., 1986). Their antimicrobial action is further enhanced by the low oxidation-reduction potentials that lactobacilli help to maintain in the intestine (Mital and Garg, 1995). Tramer (1966) reported that lactic acid was strongly germicidal and possibly largely responsible for the inhibition of E*scherichia coli*. Studies by Attaie (1987) found that lactic acid exhibited an inhibitory activity towards *Staphylococcus aureus*. While studies by Adam and Hall (1988) reported the growth inhibition of *E. coli* and *Salmonella enteridis* by lactic and acetic acid and their mixtures. Studies by Baird-Parker (1980) found that acetic acid is more inhibitory than lactic acid, especially against

yeasts and moulds. This maybe attributed to the extent of their dissociation since acetic acid has between two to three times more of the acid in the dissociated state at pH intervals between 4.0 and 4.6 compared to lactic acid.

Hydrogen peroxide is a primary metabolite produced by lactobacilli (Lindgren and Dobrogosza, 1990). Its bactericidal effect has been attributed to its strong oxidising action on the bacterial cell and to destruction of basic molecular structures of cell proteins (cited by Lindgren and Dobrogosza, 1990). Some of the pathogenic organisms that have been found to be inhibited by hydrogen peroxide include *S.aureus*, *Pseudomonas* species, *Clostridium perfringens, E.coli* and *S.typhimurium* (Lindgren and Dobrogosza, 1990).

The production of antagonistic substances other than metabolic by-products by lactic acid bacteria have also been reported. These include antibiotic-like substances such as bacteriocins which are directly produced as ribosomally synthesized polypeptides or precursor polypeptides (Kim, 1993; Jack et al., 1995). The bacteriocins produced by lactic acid bacteria share a common mechanism of action, which is the dissipation of the proton motive force, which increases the permeability of the cytoplasmic membrane of target cells (Jack et al., 1995, Tahara et al., 1996).

Within the genus lactobacillus, *L.acidophilus* has been especially known to display antimicrobial activity against certain organisms (Toba et al., 1991; Kanatani et al., 1995). Furthermore Barefoot and Klaenhammer (1983) found that 63% of the *L.acidophilus* strains which they examined produced bacteriocins. Examples of bacteriocins produced

by *L.acidophilus* includes Lactocin B (Barefoot and Klaenhammer, 1983, Barefoot et al., 1994), Lactacin F (Muriana and Klaenhammer, 1991), Acidophilucin A (Toba et al., 1991) and Acidocin (Tahara et al., 1992, Winkowski et al., 1993, Kanatani et al.,1995).

Bacteriocins can either have a broad or narrow spectrum of activity. Some bacteriocins-like substances produced by gram positive bacteria (especially some of those produced by lactobacilli and lactococci) do appear to have relatively narrow inhibitory spectra. For example Lactacin B produced from *L.acidophilus* N2 showed antimicrobial activity towards related lactobacilli. However, some bacteriocin and bacteriocin like compounds do possess broad spectrum activity (Lindgren and Dobrogosza, 1990). For example Mehta et al. (1984) reported that a broad spectrum antimicrobial protein (5.2 kDa) produced by *L.acidophilus* showed inhibition against *Salmonella, Shigella* and *Pseudomonas*. Other studies by Honsono et al. (1977) reported that a peptide (M_w 3500), produced by *L.acidophilus* inhibited DNA synthesis of *E.coli*. Furthermore, Lindgren and Dobrogosza (1990) and Mital and Garg (1995) reported that *L.acidophilus* produced other antibiotic-like compounds (Acidolin, Acidophilin and Lactocidin) which have a broad spectrum of activity over a range of food-borne pathogens as shown in Table 4.1.

Table 4.1: Antibiotic-like compounds produced by *L.acidophilus*.

COMPOUND	ORGANISMS INHIBITED
Lactocidin	*Escherichia coli*
	Gaffkya tetragena
	Mycobacterium phlei
	Mycobacterium smegmatis
	Proteus vulgaris
	Pseudomonas aeruginosa
	Salmonella enteridis
	Staphylococcus aureus
Acidolin	*Bacillus cereus*
	Bacillus coagulan
	Escherichia coli
	Salmonella typhimurium
	Staphylococcus aureus
Acidophilin	*Bacillus cereus*
	Bacillus subtilis
	E. coli
	Klebsiella pneumoniae
	Pseudomonas aeruginosa
	Pseudomonas fluorescens
	Salmonella typhosa
	Sarcina lutea
	Shigella dysenteriae
	Shigella paradysenteriae
	Staphylococcus aureus
	Streptococcus faecilis
	Vibrio comma

References : Mital and Garg (1995) and Lindgren and Dobrogosza (1990)

Administration of lactic acid bacteria may also stimulate the immune system and can initiate an immune response similar to that of harmful bacteria and viruses (Salji, 1994). Studies by Perdigon et al. (1986 a, b; 1987), have reported the immunoenhancing properties of some lactic acid bacteria administered orally. *L.acidophilus*, *L.casei* and *L.bulgaricus* induced the release of lysosomal enzymes from peritoneal macrophages, activated the cell population of phagocytic mononuclear systems and stimulated lymphocytes. In later studies Perdigon et al. (1990) found slightly higher levels of Immunoglobulin A to *S.typhimurium* in intestinal secretions of mice pre-treated with *L.acidophilus* compared to the control.

The objectives of this chapter were to:

- Examine the inhibitory activity of commercial lactic acid bacteria products towards *S.typhimurium* and *E.coli.*

- To assess whether the immobilisation process affected their inhibitory activity.

4.2 : MATERIALS AND METHODS

4.2.1 : Bacteria:

Commercial Products A and B, which contained *L. acidophilus* cells, were purchased from retail outlets. Their immobilised counterparts (Immobilised Beads Type 5A and 5B) were prepared using the immobilisation procedure outlined in Chapter 3. The commercial products and their immobilised counterparts were stored at 4°C until required for use.

The pathogens used were:

Salmonella typhimurium ATCC 14028 and *Escherichia coli* ATCC 35218. Cultures were maintained on nutrient agar slopes (Lab M) at 4°C until required for use.

4.2.2 : Effect of the breakdown of a tablet / capsule from commercial Products A and B on the pH of simulated gastric and intestinal juice:

Simulated gastric juice at pH 2.5 and simulated intestinal juice at pH 7.4 were prepared as described in Chapter 2. One tablet / capsule from each of the lactic acid bacteria commercial Products A and B, was added in turn to 9 ml of sterile simulated gastric juice at pH 2.5 and simulated intestinal juice at pH 7.4. The tablet / capsule was broken down by stomaching for five minutes and the resulting pH of both juices was measured using an Orion pH meter Model 410A.

4.2.3 : Effect of the breakdown of Immobilised Beads Type 5A and 5B on the pH of simulated gastro-intestinal juice:

Ten beads from Immobilised Beads Type 5A and 5B were added to 9 ml of sterile simulated gastric juice at pH 2.5 and simulated intestinal juice at pH 7.4. The beads were broken down by stomaching for five minutes and the resulting pH of the simulated gastric and intestinal juice was measured.

For subsequent experiments the pH value of the simulated gastric and intestinal juice was adjusted before inoculation with the beads by the addition of 1M NaOH and 1M HCl. This step was included so that the pH after breakdown of the immobilised beads would equal the pH value attained by that of their respective commercial product.

4.2.4 : Effect of simulated gastric and intestinal juice on the viability of *S. typhimurium*:

S. typhimurium was grown up in 50 ml of Nutrient broth at 37°C for 18 hours. A 1 ml aliquot of broth was removed and harvested by centrifugation at 1,000g / 5°C for 20 minutes. Cells from the 1 ml aliquot of broth were added to 9 ml of sterile simulated gastric and intestinal juice which had been adjusted to pH 4.6 / 4.7 and 6.4 / 6.7 by the dropwise addition of 1M NaOH and 1M HCl (this step was included due to the change in pH of simulated gastric and intestinal juice as a result of breakdown of the lactic acid bacteria tablets and capsules). Cells were incubated at 37°C in simulated gastric / intestinal juice and standard plate counts were carried out on nutrient agar at 30, 60 and 90 minutes. Plates were incubated and 37°C for 48 hours. Duplicate trials were carried

out. (Note: Nutrient Agar was used in preference to Brilliant Green agar as the colonies could be counted more clearly.)

4.2.5 : Effect of simulated gastric and intestinal juice on the viability of *E.coli*:

E.coli was propagated in 50 ml Nutrient broth at 37°C / 18 hours. A 1 ml aliquot was removed and the cells were harvested by centrifugation at 1,000g / 5°C for 20 minutes. The cells were then suspended in 9 ml of sterile simulated gastric and intestinal juice previously adjusted to pH 4.6 / 4.7 and 6.4 / 6.7, as described earlier, and incubated at 37°C. Standard plate counts were carried out on Violet Red, Bile Agar (Lab M) at 30, 60 and 90 minutes. Plates were incubated at 37°C for 48 hours.The experiment was carried out in duplicate.

4.2.6 : Effect of commercial Products A and B in simulated gastro-intestinal juice on the viability of *S.typhimurium*

S.typhimurium was grown up in 50 ml of nutrient broth at 37°C for 18 hours. A 1 ml aliquot of the broth was removed and cells were harvested by centrifugation at 1,000g / 5°C for 20 minutes. One capsule / tablet was added to 9 ml of sterile simulated gastric juice at pH 2.5, and stomached for 5 minutes. *S.typhimurium* cells from 1 ml of broth was then added to the simulated gastric and intestinal juice and incubated at 37°C for 90 minutes.1 ml aliquots were removed at 30 minute intervals and cell enumeration was carried out by standard plate count on Nutrient Agar. Plates were incubated at 37°C for 48 hours.The trial was carried out in duplicate.

4.2.7 : Effect of Commercial Product A and B in simulated gastric and intestinal juice on the viability of *E.coli*

The effect of commercial products in simulated gastric and intestinal juice on the viability of *E.coli* was examined using the procedure described above. However, for *E.coli* the standard plate counts were carried out on Violet Red Bile Agar instead of Nutrient Agar. Plates were incubated at 37°C for 48 hours. The experiment was carried out in duplicate.

4.2.8 : Effect of immobilised Beads Type 5A and 5B in simulated gastric and intestinal juice on the viability of *S.typhimurium*

S.typhimurium cells were grown up in 50 ml of nutrient broth at 37°C for 18 hours. A 1 ml aliquot containing cells was removed and cells were harvested by centrifugation for 20 minutes at 1,000g / 5°C. Ten type 5A / 5B beads were added to 9 ml of sterile simulated gastric juice at pH 2.6 and 2.7 and simulated intestinal juice at pH 7.4 and pH 7.3, and stomached for 5 minutes, (the pH of the simulated gastric and intestinal juice was adjusted to pH 2.6 and 2.7 and pH 7.3 before inoculation with Beads Type 5A and 5B so that the pH after breakdown of the beads would equal that of the commercial counterparts, to give a final pH of 4.6, 4.7 and 6.4 respectively). *S.typhimurium* cells from the 1 ml of broth was added to this solution and incubated at 37°C for 90 minutes. 1 ml aliquots were removed at 30 minute intervals and standard plate counts were carried out on Nutrient Agar. Plates were then incubated at 37°C for 48 hours.Duplicate trials were carried out.

4.2.9 : Effect of immobilised Beads Type 5A and 5B in simulated gastric and intestinal juice on the viability of *E. coli*

The effect of immobilised Beads Type 5A and 5B in simulated in gastric and intestinal juice on the viability of *E.coli* was examined by repeating the procedure detailed above. However, Violet Red Bile Agar was used instead of Nutrient Agar for enumeration purposes.Plates were incubated at 37°C for 48 hours. Th experiment was carried out in duplicate.

4.3 : RESULTS

4.3.1 : Alteration in the pH of simulated gastric and intestinal juice with the addition of commercial products:

The addition of Commercial Product A and B to simulated gastric juice altered the pH of the solution. When a tablet / capsule from Commercial Product A or B was added to simulated gastric juice at pH 2.5, the resulting pH increased to pH 4.6 and 4.7 respectively (Table 4.2). However, when Commercial Product A and B was added to simulated intestinal juice, the resulting pH of the solution decreased from pH 7.4 to 6.7 and 6.4 respectively (Table 4.3). To compensate for these variations in pH, the pH of the simulated gastric juice was adjusted prior to addition of the immobilised beads (Table 4.4 and 4.5). This step was included so that the pH of the simulated gastric juice would equal that of their commercial counterparts.

Table 4.2: Change in pH of simulated gastric juice, pH 2.5, after addition of tablet / capsule from Commercial Product A and B.

PH	Commercial Product A	Commercial Product B
Initial pH	2.5	2.5
Final pH	4.6	4.7

Table 4.3: Change in pH of simulated intestinal juice, pH 7.4, after addition of tablet / capsule from Product A and B.

PH	Commercial Product A	Commercial Product B
Initial pH	7.4	7.4
Final pH	6.7	6.4

Table 4.4: Change in pH of pH-adjusted simulated gastric juice, pH 2.6 and 2.7, after addition of Immobilised Beads Type 5A and 5B.

PH	Immobilised Bead Type 5 A	Immobilised Bead Type 5B
Initial pH	2.6	2.7
Final pH	4.6	4.7

Table 4.5: Change in pH of simulated intestinal juice, pH 7.4, after addition of Immobilised Beads Type 5A and 5B.

PH	Immobilised Bead Type 5 A	Immobilised Bead Type 5B
Initial pH	7.4	7.4
Final pH	6.7	6.5

4.3.2 : Effect of pH variations on the viability of *S.typhimurium* cells:

As a result of alterations in the pH of simulated gastric and intestinal juice with the addition of commercial products and immobilised beads, *S.typhimurium* cells were exposed to a range of pH values (pH 4.6, 4.7, 6.4, 6.7). Reduction in the cell viability of *S.typhimurium* occurred after 90 minutes exposure to each pH value (Fig 4.1). The greatest reduction in cell viability occurred at the lower pH values (pH 4.6 to 4.7). Cell numbers were reduced by 75.8% and 75% after 90 minutes exposure to pH 4.6 and 4.7 respectively. The reduction in cell viability of *S.typhimurium* cells was less pronounced at higher pH values (pH 6.4 to 6.7). Results presented in Fig 4.1 show that cell numbers were reduced by 26% and 17% after 90 minutes exposure to pH 6.4 and 6.7 respectively.

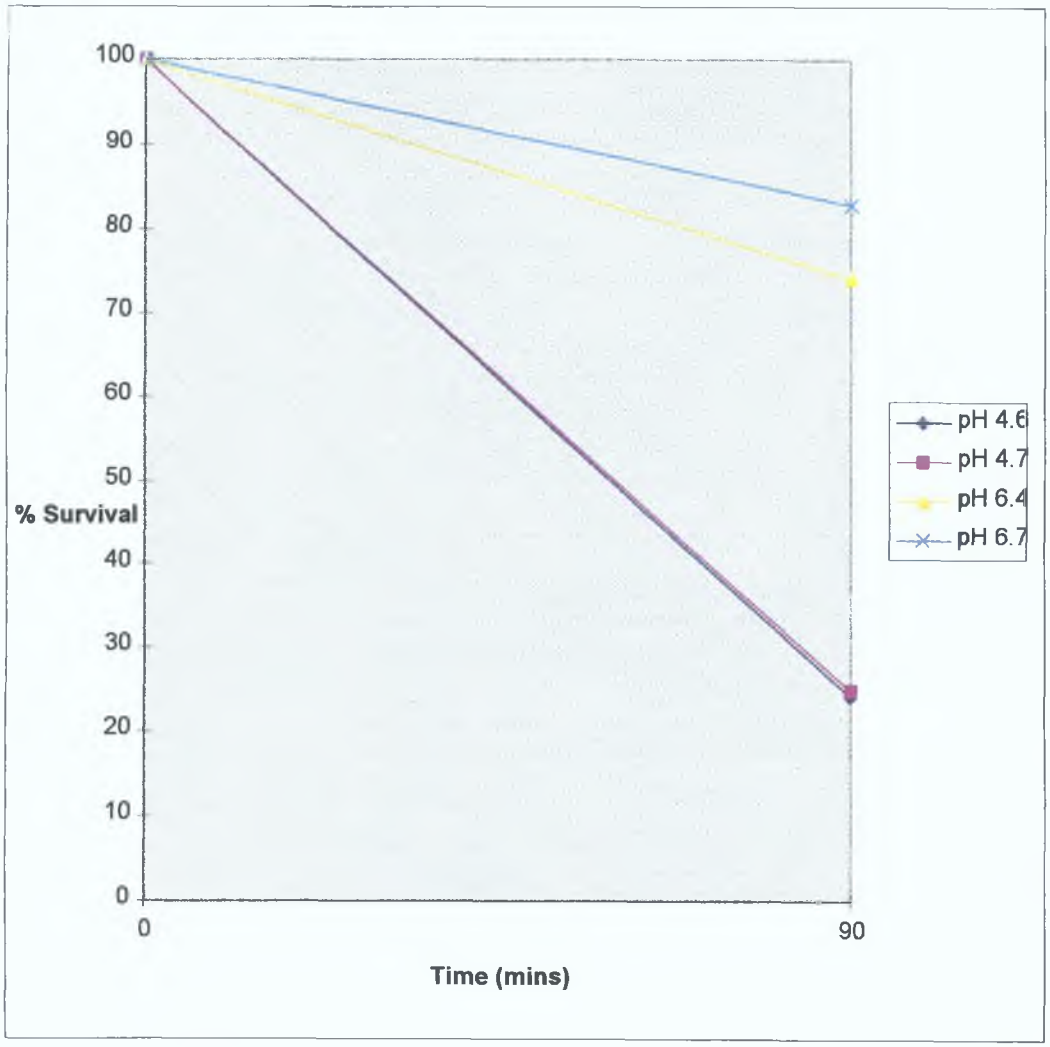


Fig 4.1: Effect of pH variations on the viability of *S.typhimurium* cells.

4.3.3 : Effect of pH variations on the viability of *E.coli* cells:

Results presented on Fig 4.2 shows that the viability of *E.coli* cells were not detrimentally affected after 90 minutes exposure to pH 4.6 and 4.7. Cell numbers actually increased by 20.4 and 24.9% after 90 minutes exposure to pH 4.6 and 4.7 respectively. The viable count increased further at higher pH levels. After 90 minutes incubation at pH 6.4 and 6.7 the cell numbers increased by 56.2% and 88.9% respectively.

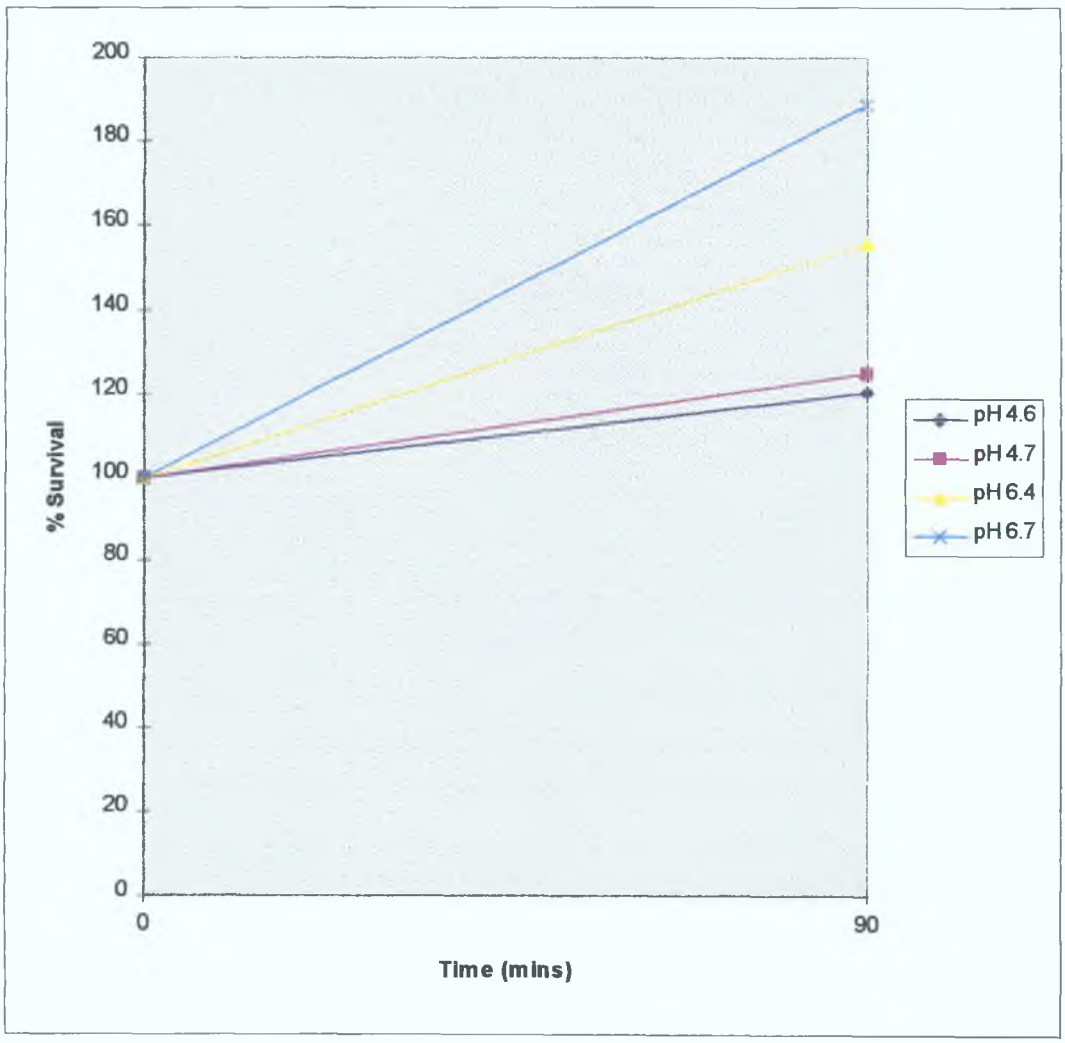


Fig 4.2: Effect of pH variations on the viability of *E.coli*.

4.3.4 : Effect of Commercial Product A and their immobilised counterparts on *S.typhimurium* under simulated gastric and intestinal conditions:

Reduction in cell numbers was observed when *S.typhimurium* cells were exposed to *L.acidophilus* from Commercial Product A and their immobilised counterpart under simulated gastric and intestinal conditions (Fig 4.3 and 4.4). However, a greater reduction in cell viability occurred when *S.typhimurium* was exposed to *L.acidophilus* cells under simulated gastric conditions at pH 2.5 (Fig 4.3), than under simulated intestinal conditions at pH 7.4 (Fig 4.4).

The effect of *L.acidophilus* cells from Product A in simulated gastric juice on the viability of *S.typhimurium* is shown in Fig 4.3. Results show that there was a 99.8% reduction in cell viability after 90 minutes of which 95.67% of the total cell reduction occurred in the first 30 minutes. When *S.typhimurium* cells were exposed to *L.acidophilus* cells from Beads Type 5A under similar simulated gastric conditions a similar death trend was observed (Fig 4.3). There was a 95.95% reduction in viability of *S.typhimurium* cells after 90 minutes, of which 97.96% of the total cell reduction occurred during the initial 30 minute period.

The effect of *L.acidophilus* from Product A in simulated intestinal juice on the viability of *S.typhimurium* is shown in Fig 4.4. Results show that there was a 33.6% reduction in the viability of *S.typhimurium* cells after 90 minutes exposure to Product A in simulated intestinal juice. The reduction in cell number decreased gradually during this time, 34.2% of the total cell death was observed in the first 30 minutes, 38.7% occurred during the

30-60 minute interval while the remaining 27.1% of the total cell death occurred in the final 30 minutes. When *S.typhimurium* cells were exposed to Bead Type 5A under similar simulated intestinal conditions, they exhibited a 12.7% reduction after 90 minutes (Fig 4.4). Cell death was more pronounced initially. Results showed that 75% of the total cell death occurred within the first 30 minutes and gradually levelled off thereafter.

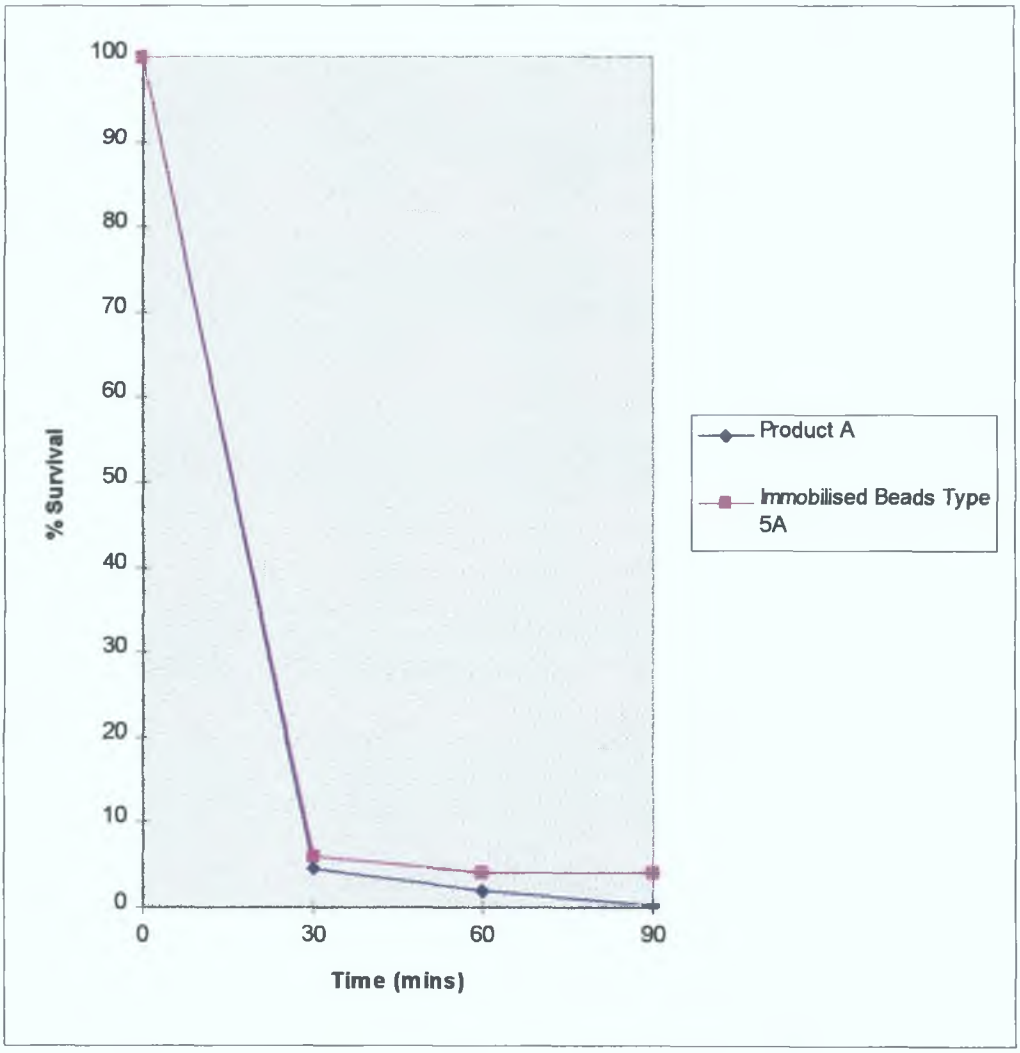


Fig 4.3 Effect of Product A and their immobilised counterparts in simulated gastric juice, pH 4.6, on *S.typhimurium*.

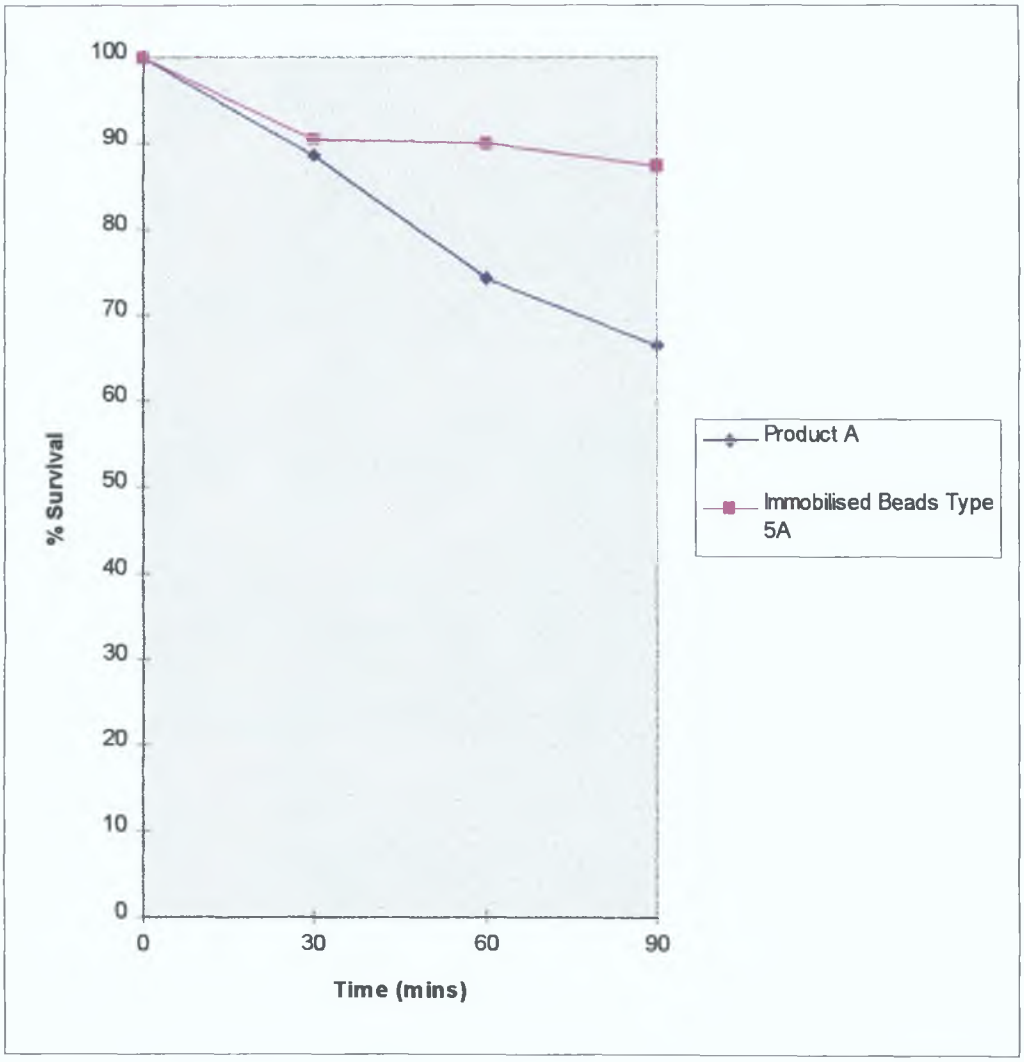


Fig 4.4: Effect of Product A and their immobilised counterparts in simulated intestinal juice, pH 6.7, on *S.typhimurium*.

4.3.5 : Effect of Commercial Product B and its immobilised counterpart on the viability of *S.typhimurium* under simulated gastro-intestinal conditions:

When *S.typhimurium* was exposed to *L.acidophilus* cells from Commercial Product B under simulated gastric conditions, they exhibited a 90.03% reduction in viability after 90 minutes (Fig 4.5). As observed previously using Commercial Product A and their immobilised counterparts, the greatest reduction in cell number was observed during the first 30 minutes where 85.5% of the total cell reduction occurred. When *S.typhimurium* cells were exposed to immobilised Beads Type 5B under similar simulated gastric conditions they showed a 90.79% reduction in viability (Fig 3.5). They also showed greatest reduction in cell viability during the first 30 minutes with 72.91% of the total cell reduction occurring during this period.

The effect of Product B cells in simulated intestinal juice on *S.typhimurium* cells is shown in Fig 4.6. Results show that there was a 55.05% reduction in cell viability after 90 minutes exposure of which 55% of the total reduction occurred during the first 30 minute interval. When *S.typhimurium* cells were exposed to *L.acidophilus* cells from immobilised Beads Type 5B under similar intestinal conditions for 90 minutes, a 59% reduction in cell viability was observed (Fig 4.6). Results show that 64.4% of the total cell reduction occurred during the first 30 minutes.

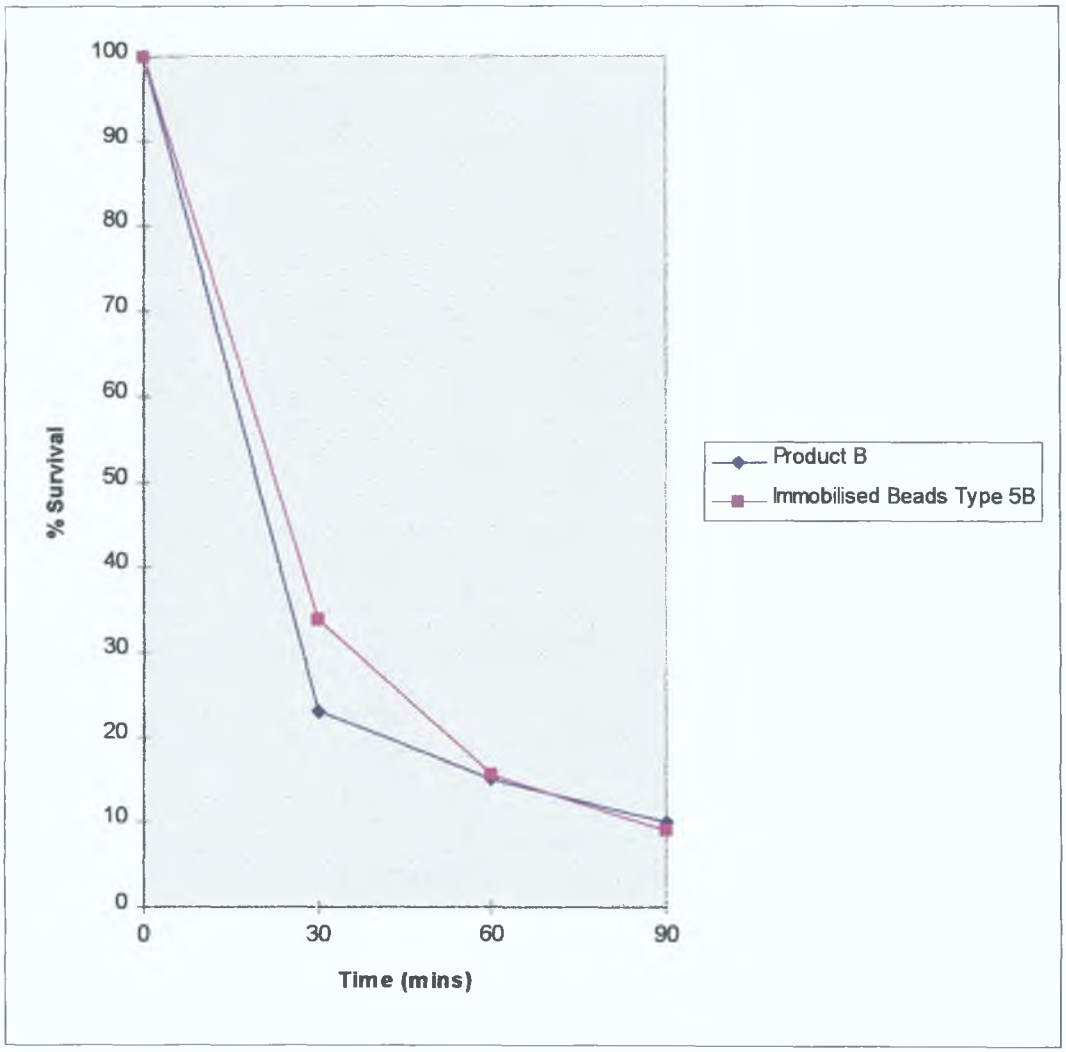


Fig 4.5: Effect of Product B and their immobilised counterparts in simulated gastric juice, pH 4.7, on *S.typhimurium*.

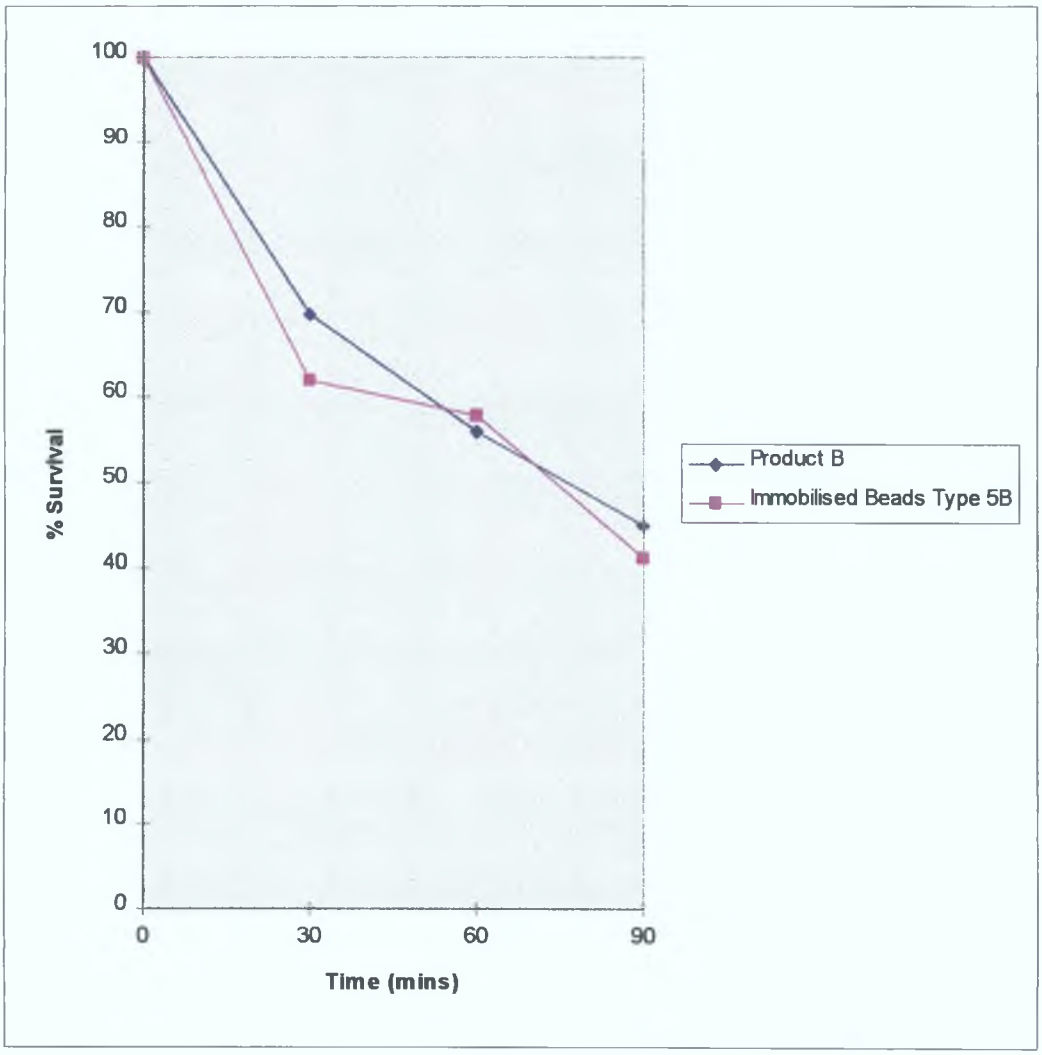


Fig 4.6: Effect of Product B and their immobilised counterpart in simulated intestinal juice, pH 6.4, on *S.typhimurium*.

4.3.6 : Effect of the Commercial Product A and their immobilised counterparts on the viability of *E.coli* under simulated gastro-intestinal conditions:

A reduction in cell viability was also observed when *E.coli* cells were exposed to Commercial Product A and Bead Type 5A under simulated gastric and intestinal conditions (Fig 4.7 and 4.8). Results show that greater cell death occurred when *E.coli* cells were exposed to Product A and Bead Type 5A under simulated gastric conditions at pH 2.5, than under simulated intestinal conditions at pH 7.4.

The effect of Product A in simulated gastric juice on the viability of *E.coli* is shown in Fig 4.7. Results show that there was an 8% decrease in cell viability after 90 minutes exposure to Product A in simulated gastric juice. The reduction in cell number decreased gradually during this time, 25% of the total cell reduction occurred during the first 30 minutes, a further 45.37% reduction was observed during the 30-60 minute interval, while the remaining 29.63% occurred during the final 30 minutes.

E.coli cells exhibited a greater reduction in cell viability when they were exposed to Bead Type 5A for 90 minutes under similar simulated gastric conditions. Results presented in Fig 4.7 show that they exhibited a 77.2% reduction in cell viability after 90 minutes exposure to such conditions. Most of the viability loss occurred during the first 30 minutes. During this period there was a 69.3% reduction in cell viability which represented 89.8% of the total cell reduction.

The effect of Product A on *E.coli* under simulated intestinal conditions is shown in Fig 4.8. After 90 minutes exposure cell viability increased by 38.5%. However, when *E.coli* cells were exposed to Bead Type 5A under such conditions they exhibited an overall decrease in cell number over the 90 minutes. Results presented in Fig 4.8 show that there was a 42% reduction in viability of *E.coli* cells during this time. There was an initial 4% increase in cell viability during the first 30 minutes, however reduction in cell number occurred thereafter.

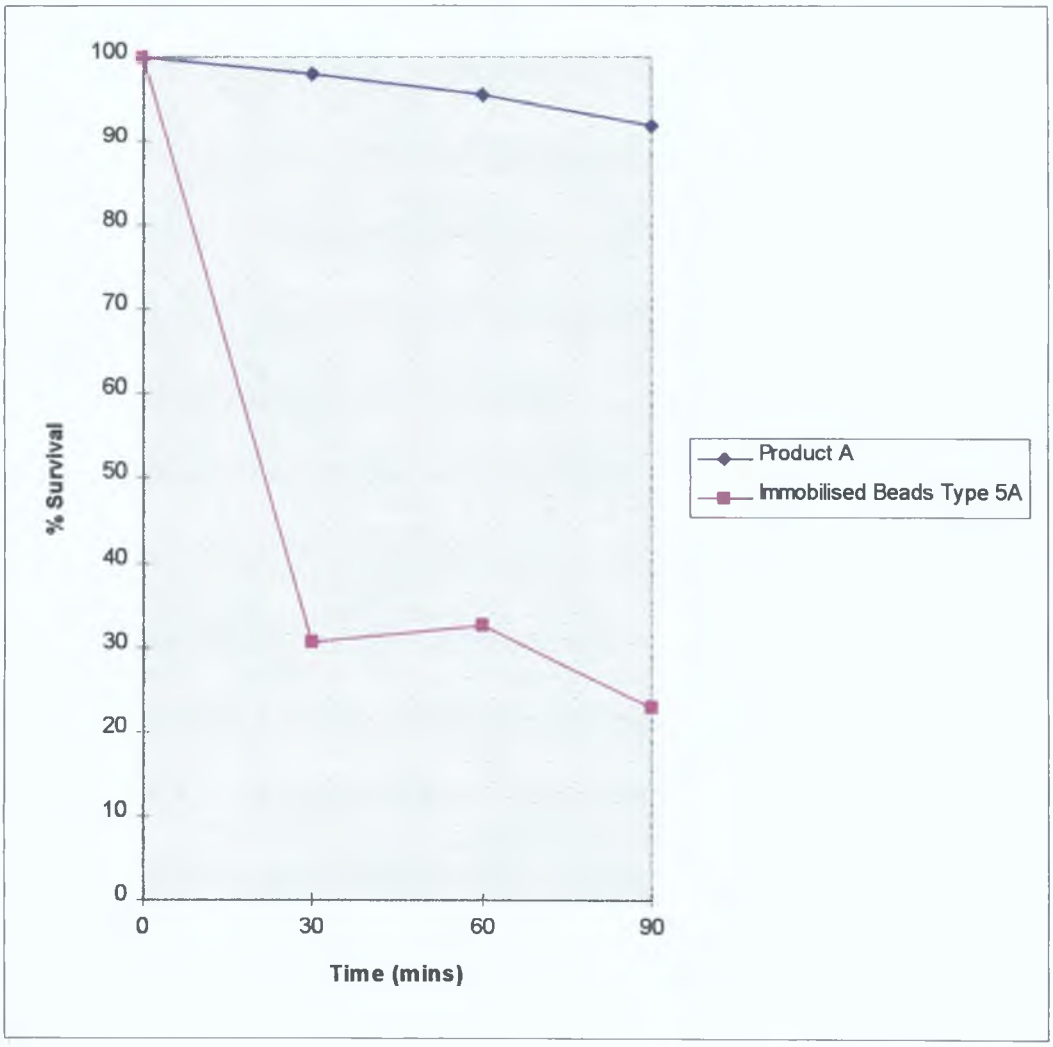


Fig 4.7: Effect of Product A and their immobilised counterparts in simulated gastric juice on *E.coli*.

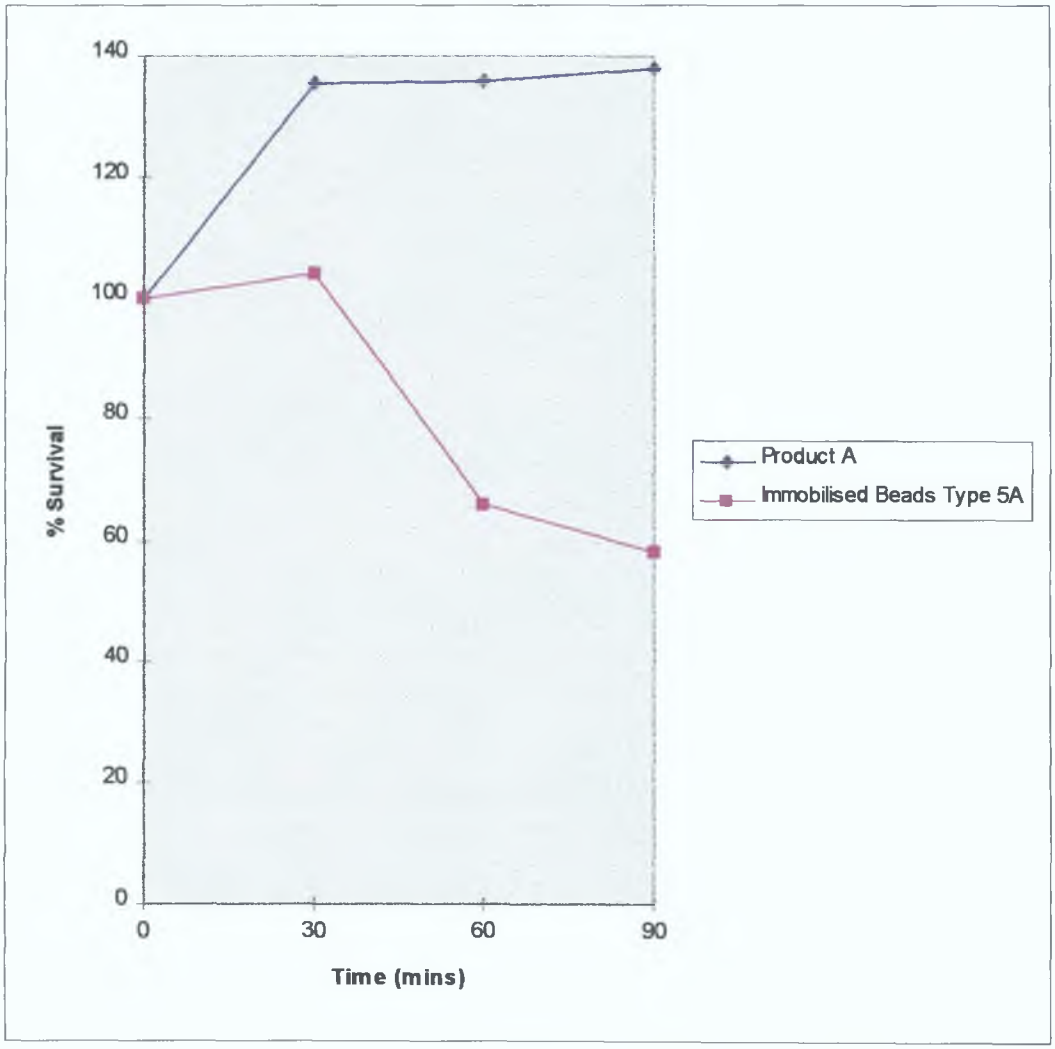


Fig 4.8: Effect of Product A and their immobilised counterpart in simulated intestinal juice,pH 6.7, on *E.coli.*

4.3.7 : Effect of Commercial Product B and its immobilised counterparts on the viability of *E.coli* under simulated gastric and intestinal conditions:

The effect of Product B on the viability of *E.coli* under simulated gastric conditions is shown in Fig 4.9. There was a 90.7% reduction in the viability of *E.coli* cells after 90 minutes exposure to such conditions. The greatest reduction in cell viability occurred during the first 30 minutes, during which 94.7% of the total cell reduction occurred. *E.coli* exhibited a greater reduction in cell viability when they were exposed for 90 minutes to Bead Type 5B under similar conditions (Fig 4.9). They exhibited a 93.7% reduction in cell number after 90 minutes exposure to Bead Type 5A in simulated gastric juice. Most of the reduction in cell number was observed during the initial 30 minutes, where 89% of the total cell reduction occurred.

The effect of Product B on the viability of *E.coli* under simulated intestinal conditions at pH 7.4 is shown in Fig 4.10. Results show that there was a 19% reduction in cell viability after 90 minutes exposure to such conditions. No reduction in cell number occurred during the first 30 minutes, 74.2% of the total cell reduction occurred during the 30-60 minute interval while the remaining 25.8% occurred in the last 30 minutes.

When *E.coli* cells were exposed for 90 minutes to Beads Type 5B under similar simulated intestinal conditions a 31% decrease in viability was observed (Fig 4.10). The greatest reduction in cell number occurred during the first 30 minutes with 83.8% of the total cell reduction occurring during this period.

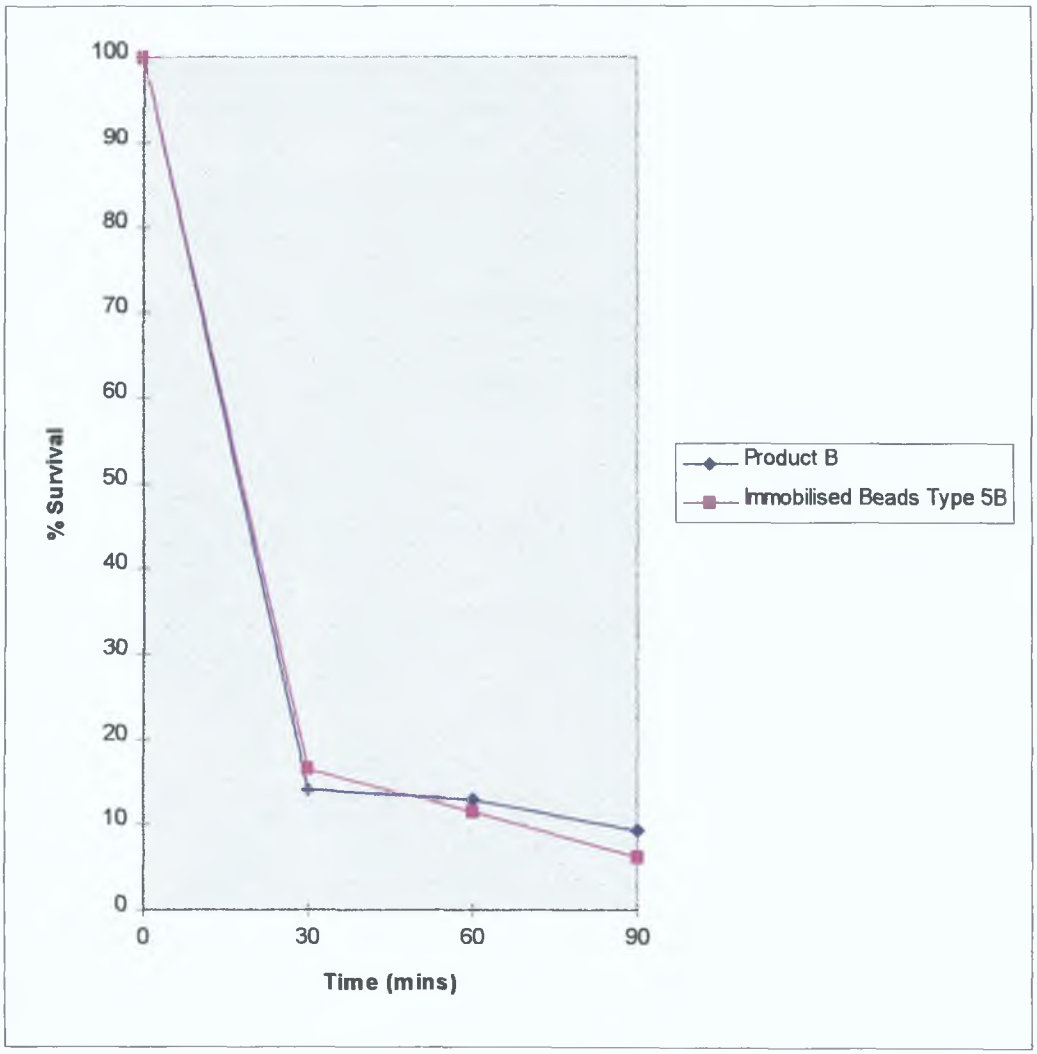


Fig 4.9: Effect of Product B and its immobilised counterparts and simulated gastric juice, pH 4.7, on *E.coli.*

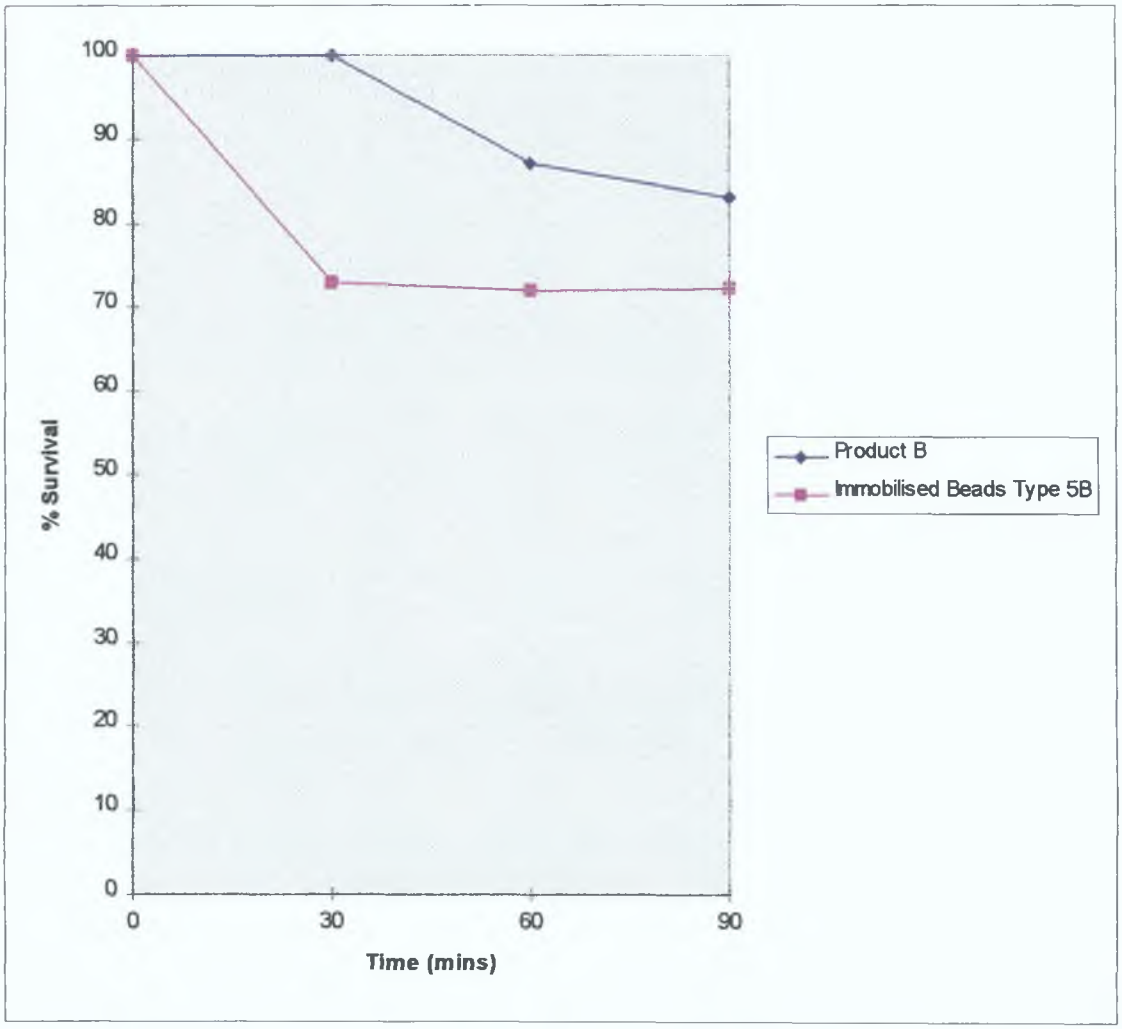


Fig 4.10: Effect of Product B and its immobilised counterpart on simulated intestinal juice, pH 6.4, on *E.coli*.

4.4 : DISCUSSION

Any successful pathogen must overcome many levels of mammalian host defences in order to be detrimental to the host. Upon entering the host, pathogens like *S.typhimurium* and *E.coli* must first survive the extreme acidity of the stomach which limits the number of viable microbes that can gain access to the small bowel (Duncan and Edberg, 1995). Furthermore, once in the intestine the organisms also faces other suboptimal conditions such as low oxygen levels, intestinal juice, bile salts, immunoglobulins, enzymes, increased osmolarity (Foster and Speck, 1995) and competition with resident microorganisms such as lactic acid bacteria for nutrient and adhesion sites (Cocconier et al., 1992; Bernet et al., 1994).

Results from this Chapter show that lactic acid bacteria products, (Products A and B), containing *L.acidophilus* cells did have an inhibitory effect on the two pathogens studied, (*S.tyhimurium* and *E.coli*), under simulated gastric and intestinal conditions. There was a greater reduction in cell viability of both pathogens when they were exposed to *L.acidophilus* under simulated gastro-intestinal conditions than under simular conditions which contained no *L.acidophilus*. Furthermore, immobilising *L.acidophilus* cells from Product A and B did in some occasions affect their antagonistic action towards both pathogens.

S. typhimurium cells were found to be detrimentally affected by the pH variations of the simulated gastric and intestinal juice, with greater reduction in cell viability occurring at the lower pH.

Studies by Foster (1991) also reported the bactericidal effect of low pH levels on survival of *S.typhimurium* cells. He found that they exhibited logarithmic death in minimal medium where the external pH was adjusted to below pH 4.0. While other studies by Foster and Spector (1995) reported that the minimal growth pH for *S.typhimurium* was 4.3 in minimal glucose media.

Further viability loss was observed when *S.typhimurium* was exposed to *L.acidophilus* cells under simulated gastric conditions. They exhibited over 95% reduction in cell number after 90 minutes exposure to Product A and their immobilised counterparts under simulated gastric conditions. The addition of Product A and Beads Type 5A to simulated gastric juice resulted in a 24% and 20.15% reduction in viability of *S.typhimurium* respectively above that for gastric conditions alone.

When *S.typhimurium* was exposed for 90 minutes to a second commercial *L.acidophilus* preparation, Product B, and their immobilised counterparts under similar simulated gastric conditions. They exhibited just over 90% reduction in cell number which represents a 15.03 and 15.79% reduction in cell number greater than when exposed to gastric juice alone.

The antagonistic action of *L.acidophilus* from the two commercial products (Product A and B) and their immobilised counterparts (Beads Type 5A and 5B) was also observed in some cases towards *S.typhimurium* under simulated intestinal conditions. Product A showed an enhanced decrease in the viability of the pathogen, but upon entrapment *L.acidophilus* did not exhibit any detrimental activity towards it (with results actually

indicating increased survival of the pathogen than when in simulated gastric juice alone). In comparison, experiments carried out using *L.acidophilus* from Product B resulted in an increase in antimicrobial activity against *S.typhimurium* by almost a third upon its incubation in either commercial free cell or immobilised preparations.

Many studies have also reported the antagonistic action of *L.acidophilus* towards *S.typhimurium*. Gilliland and Speck (1977) reported that *L.acidophilus* exerted inhibitory action towards intestinal and foodborne pathogens including *S.typhimurium*. Other studies by Alm (1983) found that oral administration of *L.acidophilus* reduced the carrier time in children and adults infected with Salmonella. Hamdan and Mikolajcik (1973) found that *S.typhimurium* was inhibited by Acidolin - an antibiotic produced by *L.acidophilus*. Other antibiotic like compounds have also been reported to inhibit other species of Salmonella: Lactocidin inhibits *Salmonella enteridis*; Acidophilin inhibits *Salmonella typhora*, and inhibiting proteins are active against *Salmonella paratyphi* and *Salmonella typhora* (Mital and Garg, 1995).

E.coli cells were found to be more tolerant to pH levels of simulated gastric and intestinal juice than *S.typhimurium*. Their cell numbers increased by over a fifth after 90 minutes exposure to simulated gastric juice in pH 4.6 and 4.7 respectively. There was a 56.2 and 88.9% increase in cell number after 90 minutes incubation in simulated intestinal juice at pH 6.4 and 6.7 respectively.

Many other studies have also reported the ability of *E.coli* to grow in a range of pH levels. Renburg et al. (1993) found that *E. coli* could manage to grow in minimal media

conditions with pH ranging from 5.0 to 8.5 which represents more than a 3,000 fold range in H^+ ion concentration and could survive at pH 4.0 (a 100,000 fold range) for extended periods. Other studies have found that *E.coli* has the ability to adapt to extremes of acid and alkali (Goodson and Rowbury, 1989a, b, c). Raja et al., (1991a) and Goodson and Rowbury, (1993) attributed this increased acid tolerance to a number of protein synthesis dependent and independent steps. Furthermore, classic studies by Gales and Epps (1942) reported that many enzymes of *E.coli* (amino acid deaminase and decarboxylase) were actually induced by high and low pH levels.

Despite their resistance to the pH variance of simulated gastric and intestinal juice, *E.coli* was found to be inhibited by *L.acidophilus* cells from Commercial Product A and B and their immobilised counterparts. However, the extent of their survival was dependent on the *L.acidophilus* preparation used (i.e. free or immobilised cells).

When *E.coli* cells were exposed for 90 minutes to Product A and their immobilised counterpart under simulated gastric conditions, they exhibited a 8 and 77.2% reduction in cell viability respectively. These results represent a 28.4 and 97.6% increase in cell reduction compared with similar tests carried out in simulated gastric juice which contained no *L.acidophilus* cells. Therefore, the immobilisation process increased the antimicrobial activity of Product A towards *E.coli* by 69.2% under simulated gastric conditions.

However, when *E.coli* cells were exposed for 90 minutes to a second product, Product B, and their immobilised counterparts under simulated gastric conditions, they exhibited

over 90% reduction in pathogen viability. The addition of Product B and its immobilised counterpart to simulated gastric juice led to a 115.6 and 118.6% increase in viability loss. Therefore, the immobilisation process increased the antagonistic activity of Product B towards *E.coli* by 3%.

The immobilised cultures, (Beads Type 5A and 5B), also exhibited enhanced antagonistic activity towards *E.coli* under simulated intestinal conditions compared with their commercial free cell counterparts. *E.coli* cells exhibited a 38.5% increase and 42% reduction in cell viability after 90 minutes exposure to Product A and their immobilised counterparts under simulated intestinal conditions. These results represent a 50.4 and 130.9% increase in cell reduction in the presence of Product A and immobilised Beads Type 5A respectively. Therefore, the immobilisation process led to a 80.5% increase in antimicrobial activity towards *E.coli* by *L.acidophilus.*

E.coli cells exhibited a 19 and 31% decrease in cell number after 90 minutes exposure to Product B and their immobilised counterparts under simulated intestinal conditions which represent a 75.2 and 87.2% increase in cell reduction of *E.coli* with respect to the control. Therefore entrapment of Product B cells in calcium alginate beads increased their antimicrobial activity by 12% towards *E.coli.*

In conclusion, the immobilised cells mostly retained their antagonistic action towards *S.typhimurium* and *E.coli*, and in some cases enhanced their antimicrobial activity. Therefore, in addition to increasing cell survival under suboptimal simulated gastro-

intestinal conditions, the immobilisation process can further enhance the effectiveness of lactic acid bacteria probiotic products by increasing their pathogenic activities.

CHAPTER FIVE :

Effect of antibiotics on *L.acidophilus*

5.1 : INTRODUCTION

The human body harbours a variety of microbes existing in ecologically balanced communities (Fuller, 1990; Mital and Garg, 1995). The bacteria in these areas are usually harmless to the host and some perform useful functions such as the synthesis of Vitamin K by coliform organisms and lactic acid bacteria (Pratt and Fekety, 1986; Mc Cann et al.,1996). Bacterial interactions also play an important role in maintaining a balance among the different species preventing some from outgrowing others and possibly harming the host (Fuller, 1990; O' Sullivan et al., 1992; Millier et al., 1992; Mc Groarty, 1993).

If the balance of these microbial communities is disturbed, as it is when an antibiotic acts upon useful indigenous flora, other microorganisms that are normally kept in check can multiply and possibly cause complications (Pratt and Fekety, 1986; Mims et al., 1993). This phenomenon is called suprainfection and the danger therefore of creating conditions that would lead to flora overgrowth should be an important consideration in the administration of antibiotics.

One of the most common infections resulting from antibiotic treatment is the development of the syndrome Pseudomembranous Colitis caused by the toxins of the anaerobic organism, *Clostridium difficile* and occasionally other Clostridia (Fuller,1990; Baron et al.,1994). This inflammatory disease of the large bowel seldom occurs except after antimicrobial treatment has altered the normal flora. When normal flora such as lactobacilli are reduced, *C. difficile*, is able to multiply and produce its toxins. Thus, this syndrome is also called antibiotic - associated colitis (Baron et al., 1994). Other micro-

organisms that maybe potentially harmful when released from the selective pressure of the normal flora include other *Clostridium* sp., *Salmonella* sp., *Candida* sp., *Staphylococci*, *Pseudomonas* sp. and various *Enterbacteriaceae* (Burr et al.,1982; Duncan and Edbury, 1995).

Amongst the hundreds of different species that make up the intestinal flora lactic acid bacteria including *L.acidophilus* present the most abundant species present (Savage, 1977; Mital and Garg, 1995), and research has shown their importance in maintaining balanced intestinal flora (Fernandes et al.,1987; Perdigon et al.,1990; Mital and Garg, 1995).

Antimicrobial drugs cannot distinguish between pathogenic organisms and those that are constituents of the normal beneficial flora of the host. The administration of broad spectrum antibiotics such as Tetracycline, Ampicillin and the Cephalosporins can significantly alter the natural competition within a given ecosystem by eliminating protective lactobacilli (Mardh, 1991; Sobel, 1992). Even narrow spectrum antibiotics have a profound effect on the normal flora of the mouth and gut (Greenwood, 1995).

The manner in which antibiotics exert their actions against susceptible organisms is varied as shown in Table 5.1. Antibiotics that interfere with metabolic systems found in micro-organisms and not in mammalian cells are the most sucessful anti-infective agents. For example, those antibiotics that interfere with the synthesis of bacterial cell walls have a high potential for selective toxicity (Greenwood, 1995), since practically all bacteria (with the exception of mycoplasmas) possess a cell wall, whilst mammalian cells lack this

feature. Cell wall active antibiotics act by interfering with the biosynthesis of peptidoglycan whilst other antibiotics notably the Penicillins and Cephalosporins act on the porin channels (Van Holde, 1990).

Table 5.1: Antibiotics, their sites of action and spectrum of activity.

Antibiotic	Main site of action	Spectrum of activity	Reference
Chloramphenicol	inhibits protein synthesis	Broad	Mims et al.(1993), Conte and Barriere(1987)
Erythromycin	inhibits protein synthesis	Gram positive and some viruses	Mims et al.(1993), Conte and Barriere(1987)
Fusidic acid	inhibits protein synthesis	Broad	Mims et al.(1993)
Methicillin	inhibits cell wall synthesis	Gram positive	Mims et al.(1993), Conte and Barriere(1987)
Novobiocin	inhibits nucleic acid synthesis	Gram positive, some Gram negative	Mims et al.(1993)
Penicillin G	inhibits cell wall formation	Gram positive	Mims et al.(1993), Van Holde (1990), Conte and Barriere(1987)
Streptomycin	inhibits protein synthesis	Broad	Mims et al.(1993), Conte and Barriere(1987)
Colistin sulphate	damages bacterial plasma membranes	Gram negative, except Proteus sp.	Mims et al.(1993), Conte and Barriere(1987)
Ampicillin	inhibits cell wall synthesis	Gram positive	Mims et al.(1993), Conte and Barriere(1987)
Ticarcillin	inhibits cell wall synthesis	Gram positive	Mims et al.(1993), Conte and Barriere(1987)
Gentamicin	inhibits protein synthesis	Broad particularly Gram negative	Mims et al.(1993), Conte and Barriere(1987)
Cephalexin	inhibits cell wall synthesis	Broad	Mims et al.(1993), Conte and Barriere(1987)
Trimethoprim	inhibits enzyme reactions	Gram negative	Mims et al.(1993), Conte and Barriere(1987)
Sulphamethoxazole	inhibits enzyme reactions	Gram negative	Mims et al.(1993), Conte and Barriere(1987)
Tetracycline	inhibits protein synthesis	Broad	Mims et al.(1993), Conte and Barriere(1987)

Another process interrupted by antibiotics is the mechanism of protein synthesis. The process as it occurs in bacterial cells is sufficiently different from mammalian synthesis to offer scope for the selective toxicity required of the therapeutically useful antimicrobial agents. The chief difference that is exploited involves the actual structure of the ribosomal workshop in both protein and RNA components with bacteria having 50S and 30S subunits and mammalians 60S and 40S subunit. Inhibitors of bacterial protein synthesis with sufficient selectivity to be useful in human therapy include Gentamicin, Streptomycin, Erythromycin and Fusidic acid (Cundliff, 1980; Conte and Barriere, 1987; Van Holde, 1990).

The process of nucleic acid synthesis is also affected by chemotherapeutic agents. Some, like the Sulphonamides, and Diaminopyrimidines, achieve their effect indirectly by interrupting metabolic pathways that lead to the manufacture of nucleic acid; others of which the Quinolones, Nitroimidazoles and Novobiocin are prime examples, exert a more direct action. For example, Novobiocin targets the DNA gyrase enzyme, which unwinds the supercoiled DNA helix prior to replication and transcription (Conte and Barriere, 1987; Greenwood, 1995).

Other antibiotics, such as Colistin sulphate, are believed to interfere with the integrity and function of the bacterial cell membrane by binding to the cell membrane and causing leakage of essential cytoplasmic contents (Greenwood, 1995).

To overcome the detrimental effects of antibiotics on the natural healthy gastrointestinal microflora, there are many commercial capsules and tablets containing freeze-dried lactic acid bacteria which can be administered to replenish the gut and therefore protect against infection.

The objectives of this section were to:

- Examine the effect of antibiotics on *L.acidophilus* cells present in commercial probiotic products.
- To assess whether exposure to simulated gastric and intestinal juice affects their resistance
- To determine whether immobilisation alters the resistance of *L.acidophilus* to the antibiotics.

5.2 MATERIALS AND METHODS

5.2.1 : Bacteria:

Commercial tablets / capsules containing *L.acidophilus* cells were purchased from a retail outlet and were stored at 4°C until required.

5.2.2 : Immobilisation procedure:

Cells isolated from Commercial Product A and B were immobilised in Type 5A and 5B Beads using the procedure described in Chapter 3.

5.2.3 : Antibiotics:

The antibiotics used in this study were as follows: Mastring-S M11 and M41 (Mast Diagnostics, Merseyside, UK). Type M11 discs contained the following antibiotics: Chloramphenicol, 25μg; Erythromycin, 5μg; Fusidic acid, 10μg; Methicillin, 10μg; Novobiocin, 5μg; Penicillin G, 1 unit; Streptomycin, 10μg and Tetracycline 25μg. Type M41 discs contained the following antibiotics: Colistin sulphate, 25μg; Ampicillin, 10μg; Ticarcillin, 75μg; Gentamicin, 10μg; Cephalexin, 30μg; Trimethoprim, 1.25μg; Sulphamethoxazole, 25μg and Tetracycline 10μg. The antibiotic discs were stored in a refrigerator (4°C) until required for use.

5.2.4 : Effect of antibiotics on *L.acidophilus* in commercial tablets / capsules:

One tablet / capsule containing *L.acidophilus* cells from Product A and B was added to a Seward Medical 4" x 6" stomacher bag which contained 9ml of sterile ringers solution at pH 6.8. The sample was stomached using a Lab-blender model 80 for 2 minutes and 0.1ml aliquot were removed and spread plated on MRS agar. Mastring-S Type M11 and

M41 were applied with a sterile forceps and in order to ensure complete contact of the discs to the agar surface, the discs were pressed down with slight pressure and incubated at 37°C for 48 hours. The diameter was measured to the nearest millimeter.

4.2.5 : Effect of antibiotics on *L.acidophilus* in freeze-dried beads:

Ten Type 5A / 5B beads were added to a stomacher bag which contained 9ml of sterile ringers solution. The beads were stomached for 2 minutes. A 0.1ml aliquot was removed and spread plated on MRS agar, Mastring- S M11 and M41 were aseptically transferred to separate indicator lawns and incubated at 37°C for 48 hours. The diameters of each zone of inhibition corresponding to different antibiotics was measured to the nearest millimeter.

4.2.6 : Effect of antibiotics on *L.acidophilus* in commercial tablets / capsules after incubation in simulated gastric and intestinal juice:

To determine the effect of simulated gastric and intestinal conditions on *L.acidophilus* resistance to antibiotics, capsules and tablets were exposed to sterile simulated gastric juice, pH 1.5 and 2.5, and intestinal juice, pH 7.4. Simulated gastric juice pH 1.5 and 2.5 and intestinal juice at pH 7.4 was prepared as described in Chapter 2. One tablet / capsule from Product A and B was resuspended in 9ml of simulated gastric juice pH 1.5 and 2.5 and simulated intestinal juice pH 7.4 for 90 minutes and then dissolved by stomaching. Aliquots of 0.1ml were removed and spread plated on MRS agar. Mastring-S M11 and M41 discs were aseptically transferred to each plate, incubated at 37°C / 48 hours and zones of inhibition measured in millimeters.

5.2.7 : Effect of antibiotics on *L.acidophilus* in freeze-dried beads after incubation in simulated gastric / intestinal juice:

The sensitivity of *L.acidophilus* from freeze-dried beads, (Beads Type 5A and 5B), to antibiotics was studied after incubation in simulated gastric / intestinal juice. Ten beads were suspended in 9ml of simulated gastric juice at pH 1.5 and 2.5 and simulated intestinal juice at pH 7.4 for 90 minutes and then dissolved by stomaching. Aliquots of 0.1ml were removed, spread plated on MRS agar and Mastring-S M11 and M41 were aseptically transferred to each plate. The plates were incubated at 37°C / 48 hours and zones of inhibition were measured to the nearest millimeter.

5.3 : RESULTS

5.3.1 : Effect of antibiotics on *L.acidophilus* cells present in commercial products and immobilised beads:

The relative sensitivity of *L.acidophilus* to 16 antibiotics was determined by the zone of inhibition measured in millimeters around each antibiotic disc. Table 5.2 lists the antibiotics assayed and their corresponding inhibition zones with cells from Product A and its immobilised counterpart, (Beads Type 5A). Cells from Commercial Product A exhibited total resistance to 2 of the 16 antibiotics tested, Trimethoprim and Sulphamethoxazole, whilst their immobilised counterpart showed total resistance to not only those mentioned above but also Methicillin, Penicillin G, Streptomycin, Colistin sulphate and Gentamicin.

Results presented in Fig. 5.2 also shows that the immobilised cells, (Beads Type 5A), were more resistant to 7 of the remaining 9 antibiotics, including Chloramphenicol, Erthromycin, Novobiocin, Ampicillin, Ticarcillin, Cephalexin and Tetracycline at a concentration of 25μg, exhibited greater sensitivity to Fusidic acid and similar sensitivity to Tetracycline (10μg).

Cells from the Commercial Product A and their immobilised counterparts exhibited different degrees of antibiotic resistance. The variation in antibiotic resistance between the 2 preparations was evident by the difference in the size of inhibition zones between the commercial product and its immobilised counterpart. Results from Table 5.2 show that 12.50% gave differences between 1-5mm, 18.75% exhibited variations between 6-10mm, 25% gave differences between 11-20mm, 6.25% showed differences between 21-

30mm, 25% showed variations greater than 31mm and the remaining 12.5% exhibited no variation in size of inhibition zone during exposure to a range of antibiotics.

The concentration of Tetracycline used also affected the size of the inhibition zone. As the concentration of the antibiotic increased from 10μg to 25μg, the size of the inhibition zone also increased. At 10μg Product A and immobilised Beads Type 5A exhibited very similar zones of inhbition, (only 1mm difference). However, at 25μg cells from the immobilised beads exhibited greater resistance, (7mm difference in size of the inhibition zone).

The relative sensitivity of *L.acidophilus* cells from a second Commercial Product, Product B and its immobilised counterpart, (Beads Type 5B), was also examined (Table 5.3). Cells from Product B exhibited total resistance to Trimethoprim and Sulphamethoxazole, whilst their immobilised counterparts showed complete resistance to those mentioned above aswell as Methicillin, Penicillin G, Streptomycin, Colistin sulphate and Gentamicin. Furthermore, results presented in Table 5.3 show that the immobilised cells were more resistant than their commercial counterparts to 7 of the remaining 9 antibiotics including Chloramphenicol, Erythromycin, Novobiocin, Ampicillin, Ticarcillin, Cephalexin and Tetracycline 25μg, but exhibited greater sensitivity to Fusidic acid and Tetracycline at a concentration of 10 μg.

When the variation in size of the inhibition zones between cells from Product B and their immobilised counterparts was examined results show that 6.25% gave differences between 1-5mm, 37.5% showed differences between 6-10mm, 12.5% gave differences

between 11-20mm, 6.25% gave differences between 21-30mm, 25% showed variations greater than 31mm, while the remaining 12.5% exhibited no variation in zone size.

As with Product A, the concentration of Tetracycline affected the size of the inhibition zone. The immobilised cells exhibited reduced resistance to Tetracycline at lower concentrations (10 μg), however at higher concentrations (25 μg), the immobilised cells showed greater cell resistance with respect to their commercial counterparts.

Table 5.2: Comparison of the effect of antibiotics on *L.acidophilus* cells from Product A and Bead 5A.

Antibiotic	Concentration	Product A	Bead 5A
Chloramphenicol	25 μg	36 mm	24 mm
Erythromycin	5 μg	36 mm	2 mm
Fusidic acid	10 μg	4 mm	14 mm
Methicillin	10 μg	36 mm	0 mm
Novobiocin	5 μg	36 mm	14 mm
Penicillin G	1 unit	36 mm	0 mm
Streptomycin	10 μg	3 mm	0 mm
Colistin sulphate	25 μg	13 mm	0mm
Ampicillin	10 μg	36 mm	26 mm
Ticarcillin	75 μg	36 mm	18 mm
Gentamicin	10 μg	36 mm	0 mm
Cephalexin	30 μg	18 mm	6 mm
Trimethoprim	1.25 μg	0 mm	0 mm
Sulphamethoxazole	25 μg	0 mm	0 mm
Tetracycline	10 μg	19 mm	20 mm
Tetracycline	25 μg	33 mm	26 mm

number of trials: 2
standard deviation: $\leq$ 1.4.

Table 5.3: Comparison of the effect of antibiotics on *L.acidophilus* from Product B and Bead 5B.

Antibiotic	Concentration	Product B	Bead 5B
Chloramphenicol	25 μg	36 mm	24 mm
Erythromycin	5 μg	36 mm	2 mm
Fusidic acid	10 μg	4 mm	14 mm
Methicillin	10 μg	36 mm	0 mm
Novobiocin	5 μg	36 mm	14 mm
Penicillin G	1 unit	36 mm	0 mm
Streptomycin	10 μg	3 mm	0 mm
Colistin sulphate	25 μg	12 mm	0 mm
Ampicillin	10 μg	36 mm	26 mm
Ticarcillin	75 μg	36 mm	26 mm
Gentamicin	10 μg	36 mm	0 mm
Cephalexin	30 μg	16 mm	6 mm
Trimethoprim	1.25 μg	0 mm	0 mm
Sulphamethoxazole	25 μg	0 mm	0 mm
Tetracycline	10 μg	14 mm	20 mm
Tetracycline	25 μg	36 mm	26 mm

number of trials: 2
standard deviation: ≤ 1.414.

5.3.2 : Effect of antibiotics on *L.acidophilus* cells present in commercial products and freeze-dried beads after prior incubation in simulated gastric juice:

Table 5.4 and 5.5 compares the effect of antibiotics on *L.acidophilus* cells from Product A and its immobilised counterpart after incubation in simulated gastric juice. Cells from Product A, which had been exposed to simulated gastric juice at pH 1.5, for 90 minutes, prior to antibiotic testing (Table 5.4), exhibited total resistance to 4 of the 16 antibiotics tested namely Fusidic acid, Streptomycin, Trimethoprim, and Sulphamethoxazole. However, when similar testing was carried out on their immobilised counterparts they exhibited complete resistance to 7 of the antibiotics: Methicillin, Penicillin G, Streptomycin, Colistin sulphate, Gentamicin, Trimethoprim and Sulphamethoxazole. Results presented in Table 5.4 also shows that immobilised cells were more resistant to 6 of the remaining antibiotics and only exhibited greater sensitivity to Fusidic acid and Tetracycline (10 μg and 25 μg).

Cells from the Commercial Product A and their immobilised counterpart exhibited different degrees of antibiotic resistance after they had been incubated in simulated gastric juice at pH 1.5. Results showed that 12.5% gave differences between 1-5mm, a further 12.5% showed differences in range 6-10mm, 18.75% exhibited variations between 11-20mm, 25% gave differences between 21-30mm, 12.5% showed variations greater than 31mm, whilst the remaining 18.75% exhibited no variation in size of inhibition zone.

The immobilised cells showed reduced resistance to Tetracycline, (10 and 25μg), with respect to their commercial counterparts.The concentration of Tetracycline affected the

size of the inhibition zones for cells from Product A However, their immobilised counterparts exhibited no variation in zone size when exposed to 10 and 25µg of Tetracycline

A comparison of results from Table 5 2 and 5 4 show that exposure of cells from Product A and their immobilised counterparts to simulated gastric juice at pH 1 5 does affect their resistance to antibiotics with respect to unexposed cells

Cells from Product A which had been incubated in simulated gastric juice at pH 1 5 exhibited smaller inhibition zones for 8 of the 16 antibiotics tested namely Fusidic acid, Methicillin, Novobiocin, Streptomycin, Colistin sulphate, Gentamicin, Cephalexin and Tetracycline at concentrations of 10 µg and 25 µg compared with unexposed cells They exhibited equal resistance to the remaining 7 antibiotics tested

Exposure of cells from Beads Type 5A to simulated gastric juice at pH 1 5 prior to antibiotic testing did also affect the relative size of the inhibition as shown in Table 5 2 and 5 4 They exhibited enhanced resistance to 3 of the antibiotics including Fusidic acid, Ticarcillin, and Cephalexin and reduced resistance to 3 including Chloramphenicol, Erythromycin and Tetracycline at concentrations of 10 and 25 µg However, their variations in zone size between exposed and unexposed cells was less pronounced than for its commercial counterpart

The pH of the simulated gastric juice was also found to be an important factor in the cells resistance to antibiotics Table 5 5 compares the effect of antibiotics on

L acidophilus cells from Product A and its immobilised counterpart after incubation in simulated gastric juice at pH 2 5 for 90 minutes The pattern of sensitivities of free versus immobilised cultures was similar to pH 1 5, but some variations in the sizes of the zones of inhibition was observed Comparison of results from Table 5 4 and 5 5 show that when cells from Product A were exposed to simulated gastric juice, at pH 2 5 instead of pH 1 5, they exhibited less sensitivity to 2 of the antibiotics, Erythromycin and Novobiocin and reduced resistance to 4 of the antibiotics, Colistin sulphate, Gentamicin, Cephalexin and Tetracycline at concentrations of 10 and 25 μg, similar resistance to Methicillin and equal resistance to the remaining antibiotics

When their immobilised counterparts, (Beads Type 5A), were subjected to similar conditions they exhibited enhanced resistance to Cephalexin, similar resistance to Chloramphenicol and reduced resistance to Ticarcillin and Tetracycline at concentrations of 10 μg and 25 μg, compared with other cells that had been previously exposed to gastric juice of lower pH However, their variations in antibiotic resistance was not as pronounced as that observed for their commercial free cell counterparts 31 25% of the immobilised cells exhibited variations in zone size between 1-5mm, while the remaining 68 75% exhibited no differences in size of the inhibition zones

Table 5 6 compares the effect of antibiotics on *L acidophilus* cells from Product B and its immobilised counterparts after incubation in simulated gastric juice at pH 1 5 Cells from Product B, which had been exposed to simulated gastric juice at pH 1 5 for 90 minutes, prior to antibiotic testing exhibited total resistance to 5 of the 16 antibiotics

tested, Fusidic acid, Streptomycin, Colistin sulphate, Trimethoprim and Sulphamethoxazole

When similar testing was carried out using their immobilised counterparts they also exhibited complete resistance to the same 5 antibiotics However results presented in Table 5 6 shows that the immobilised beads also exhibited enhanced resistance to 3 of the remaining 11 antibiotics, namely Methicillin, Cephalexin and Tetracycline at 10μg and only showed reduced resistance to Gentamicin, similar resistance to Novobiocin and equal resistance to the remaining 6

Cells from Commercial Product B and their immobilised counterparts exhibited different degrees of antibiotic resistance after incubation in simulated gastric juice at pH 1 5 Results show that 12 5% gave differences between 1 and 5mm, 12 5% exhibited variations between 6-10mm, 6 25% showed differences in range 11-20mm, while the remaining 68 75% exhibited no variation in antibiotic resistance

A comparison of results from Table 5 3 and 5 6 shows that exposure of cells from Product B and their immobilised counterparts to simulated gastric juice at pH 1 5 did affect their resistance to antibiotics with respect to unexposed cells Cells from Product B which had been incubated in simulated gastric juice at pH 1 5 exhibited enhanced resistance to 5 antibiotics including Fusidic acid, Novobiocin, Streptomycin, Colistin sulphate, Gentamicin and only increased sensitivity to Cephalexin and Tetracycline (10μg)

Exposure of the immobilised Beads Type 5B to simulated gastric juice at pH 1 5 did also affect the relative size of the inhibition zone as shown in Table 4 6 with respect to unexposed cells (Table 5 3) They exhibited enhanced resistance to 2 of the 16 antibiotics studied, Fusidic acid and Novobiocin and increased sensitivity to Chloramphenicol, Erythromycin, Methicillin, Penicillin G, Ampicillin, Ticarcillin, Gentamicin, Cephalexin and Tetracycline (25μg)

Table 5 7 compares the effect of antibiotics on *L acidophilus* cells from Product B and its immobilised counterpart after 90 minutes in simulated gastric juice at pH 2 5 A comparison of results from Table 5 6 and 5 7 shows that when cells from Product B were exposed to simulated gastric juice at pH 2 5 instead of pH 1 5 for 90 minutes prior to antibiotic testing they exhibited enhanced resistance to Tetracycline at concentrations of 10 and 25 μg and increased sensitivity to 3 antibiotics including Novobiocin, Colistin sulphate and Gentamicin

When their immobilised counterparts, (Beads Type 5B), were exposed to simulated gastric juice, pH 2 5, instead of pH 1 5 they also exhibited different levels of antibiotic resistance They exhibited enhanced resistance to 3 of the antibiotics including Erythromycin, Methicillin, Gentamicin, similar resistance to Cephalexin and equal resistance to the remaining antibiotics

Table 5 4 Comparison of the effect of antibiotics on *L acidophilus* cells from Product A and Bead 5A after incubation in simulated gastric juice, pH 1 5

Antibiotic	Concentration	Product A	Bead 5A
Chloramphenicol	25 µg	36 mm	27 mm
Erythromycin	5 µg	36 mm	4 mm
Fusidic acid	10 µg	0 mm	12 mm
Methicillin	10 µg	25 mm	0 mm
Novobiocin	5 µg	25 mm	14 mm
Penicillin G	1 unit	36 mm	0 mm
Streptomycin	10 µg	0 mm	0 mm
Colistin sulphate	25 µg	1 mm	0 mm
Ampicillin	10 µg	36 mm	26 mm
Ticarcillin	75 µg	36 mm	15 mm
Gentamicin	10 µg	22 mm	0 mm
Cephalexin	30 µg	8 mm	4 mm
Trimethoprim	1 25 µg	0 mm	0 mm
Sulphamethoxazole	25 µg	0 mm	0 mm
Tetracycline	10 µg	7 mm	28 mm
Tetracycline	25 µg	14 mm	28 mm

number of trials 2
standard deviations $\leq 0\ 707$

Table 5 5 Comparison of the effect of antibiotics on *L acidophilus* cells from Product A and Bead 5A after incubation in simulated gastric juice, pH 2 5

Antibiotic	Concentration	Product A	Bead 5A
Chloramphenicol	25 µg	36 mm	26 mm
Erythromycin	5 µg	34 mm	4 mm
Fusidic acid	10 µg	0 mm	12 mm
Methicillin	10 µg	26 mm	0 mm
Novobiocin	5 µg	22 mm	14 mm
Penicillin G	1 unit	36 mm	0 mm
Streptomycin	10 µg	0 mm	0 mm
Colistin sulphate	25 µg	7 mm	0 mm
Ampicillin	10 µg	36 mm	26 mm
Ticarcillin	75 µg	36 mm	18 mm
Gentamicin	10 µg	36 mm	0 mm
Cephalexin	30 µg	16 mm	2 mm
Trimethoprim	1 25 µg	0 mm	0 mm
Sulphamethoxazole	25 µg	0 mm	0 mm
Tetracycline	10 µg	9 mm	29 mm
Tetracycline	25 µg	23 mm	30 mm

number of trials 2
standard deviation $\leq 0\ 707$

Table 5 6 Comparison of the effect of antibiotics on *L acidophilus* cells from Product B and Bead 5B after incubation in simulated gastric juice, pH 1 5

Antibiotic	Concentration	Product B	Bead 5B
Chloramphenicol	25 µg	36 mm	36 mm
Erythromycin	5 µg	36 mm	36 mm
Fusidic acid	10 µg	0 mm	0 mm
Methicillin	10 µg	36 mm	30 mm
Novobiocin	5 µg	12 mm	11 mm
Penicillin G	1 unit	36 mm	36 mm
Streptomycin	10 µg	0 mm	0 mm
Colistin sulphate	25 µg	0 mm	0 mm
Ampicillin	10 µg	36 mm	36 mm
Ticarcillin	75 µg	36 mm	36 mm
Gentamicin	10 µg	1 mm	6 mm
Cephalexin	30 µg	36 mm	29 mm
Trimethoprim	1 25 µg	0 mm	0 mm
Sulphametoxazole	25 µg	0 mm	0 mm
Tetracycline	10 µg	36 mm	20 mm
Tetracycline	25 µg	36 mm	36 mm

number of trials 2
standard deviation $\leq 0\ 707$

Table 5 7 Comparison of the effect of antibiotics on *L acidophilus* cells from Product B and Bead 5B after incubation in simulated gastric juice, pH 2 5

Antibiotic	Concentration	Product B	Bead 5B
Chloramphenicol	25 µg	36 mm	36 mm
Erythromycin	5 µg	36 mm	30 mm
Fusidic acid	10 µg	0 mm	0 mm
Methicillin	10 µg	36 mm	27 mm
Novobiocin	5 µg	20 mm	11 mm
Penicillin G	1 unit	36 mm	36 mm
Streptomycin	10 µg	0 mm	0 mm
Colistin sulphate	25 µg	6 mm	0 mm
Ampicillin	10 µg	36 mm	36 mm
Ticarcillin	75 µg	36 mm	36 mm
Gentamicin	10 µg	4 mm	4 mm
Cephalexin	30 µg	36 mm	28 mm
Trimethoprim	1 25 µg	0 mm	0 mm
Sulphamethoxazole	25 µg	0 mm	0 mm
Tetracycline	10 µg	31 mm	20 mm
Tetracycline	25 µg	34 mm	36 mm

number of trials 2
standard deviation $\leq 0\ 707$

5.3.3 :Effect of antibiotics on *L.acidophilus* cells present in commercial products and in freeze-dried beads after prior incubation in simulated intestinal juice.

Table 5 8 compares the effect of antibiotics on *L acidophilus* cells from Commercial Product A and its immobilised counterpart (Bead Type 5A) after incubation in simulated intestinal juice at pH 7 4 Cells from Commercial Product A which had been exposed to simulated intestinal juice at pH 7 4 for 90 minutes, exhibited complete resistance to 4 of the 16 antibiotics tested, Fusidic acid, Streptomycin, Trimethoprim and Sulphamethoxazole Cells from their immobilised counterparts also exhibited total resistance to 4 of the 16 antibiotics tested namely Streptomycin, Colistin sulphate, Trimethoprim and Sulphamethoxazole However, results presented in Table 5 8 show that the immobilised cells exhibited enhanced resistance to 9 of the remaining antibiotics Chloramphenicol, Erythromycin, Methicillin, Novobiocin, Penicillin G, Ampicillin, Ticarcillin, Gentamicin and Cephalexin, and only showed greater sensitivity to Fusidic acid and Tetracycline at concentrations of 10 and 25 µg

Cells from commercial Product A and their immobilised counterpart exhibited different degrees of antibiotic resistance after they had been incubated in simulated gastric juice Results show that 25% showed differences in range 6-10mm, 25% exhibited variations between 11-20mm, 18 75% gave differences between 21-30mm, 12 5% showed variations greater than 31mm and the remaining 18 75% exhibited no variation in antibiotic resistance

A comparison of the results from Table 5 2 and 5 8 show that exposure of Product A cells and their immobilised counterparts to simulated intestinal juice for 90 minutes prior

to antibiotic testing does affect their resistance to antibiotics compared with unincubated cells When cells from Product A were incubated in simulated intestinal juice (Table 5 8), prior to antibiotic testing they exhibited increased resistance to Colistin sulphate, Chloramphenicol, Fusidic acid, Methicillin, Streptomycin, Cephalexin and Tetracycline (25 μg), similar resistance to Tetracycline 10μg and equal resistance to the remaining antibiotics compared with immobilised cells

Exposure of their immobilised counterparts to simulated intestinal juice prior to antibiotic testing did also affect the relative size of the inhibition zone compared with unincubated cells (Table 5 2 and 5 8) The exposure of cells to simulated intestinal juice resulted in increased resistance to Fusidic acid, Ampicillin, Ticarcillin and Cephalexin, similar resistance to Novobiocm and increased sensitivity to 7 of the antibiotics including Chloramphenicol, Erythromycin, Methicillin Penicillin G, Gentamicin and Tetracycline at a concentration of 10 and 25μg)

Results from Table 5 9 compares the effect of antibiotics on *L acidophilus* cells from Product B and its immobilised counterpart (Beads Type 5B) after incubation in simulated intestinal juice at pH 7 4 When cells from Commercial Product B were exposed to simulated intestinal juice at pH 7 4 for 90 minutes, they exhibited total resistance to 4 of the 16 antibiotics tested, Fusidic acid, Streptomycin, Trimethoprim and Sulphamethoxazole However, cells from their immobilised counterparts showed complete resistance to 5 of the 16 antibiotics, the 4 of those listed above as well as

Colistin sulphate In addition, the immobilised beads exhibited enhanced resistance to 4 of the antibiotics including Methicillin, Novobiocin, Cephalexin and Tetracycline (10 µg) and only reduced resistance to Chloramphenicol and equal resistance to the remaining antibiotics compared with their immobilised counterparts

Cells from Commercial Product B and their immobilised counterpart exhibited different degrees of antibiotic resistance after exposure to simulated gastric juice Results from Table 5 9 show that 12 5% exhibited variations in the 1-5mm range, 18 75% exhibited variations between 6-10mm, 6 25% showed differences between 11-20mm, while the remaining 62 5% exhibited no variation in antibiotic resistance

A comparison of results from Table 5 3 and 5 9 shows that exposure of Product B cells and their immobilised counterparts to simulated gastric juice prior to antibiotic testing does affect their resistance compared with unincubated cells When cells from Product B were incubated in simulated intestinal juice (Table 5 9), prior to antibiotic testing they exhibited greater resistance to Chloramphenicol, Fusidic acid, Novobiocin, Streptomycin, Colistin sulphate and Gentamicin and only increased susceptibility to Cephalexin and Tetracycline (10µg)

Exposure of their immobilised counterparts to simulated intestinal conditions prior to antibiotic tests did also affect the size of the inhibition zone compared with unexposed cells, (Table 5 3 and 5 9) Cells which had been incubated in simulated intestinal juice exhibited increased resistance to Fusidic acid Novobiocin and Tetracycline 10 µg and increased sensitivity to 9 antibiotics including Chloramphenicol, Erythromycin,

Methicillin, Penicillin G, Ampicillin, Ticarcillin, Gentamicin, Cephalexin and Tetracycline 25 µg

Table 5 8 Comparison of the effect of antibiotics on *L acidophilus* cells from Product A and Bead 5A after incubation in simulated intestinal juice, pH 7 4

Antibiotic	Concentration	Product A	Bead A
Chloramphenicol	25 µg	34 mm	27 mm
Erythromycin	5 µg	36 mm	4 mm
Fusidic acid	10 µg	0 mm	12 mm
Methicillin	10 µg	22 mm	2 mm
Novobiocin	5 µg	36 mm	13 mm
Penicillin G	1 unit	36 mm	2 mm
Streptomycin	10 µg	0 mm	0 mm
Colistin sulphate	25 µg	6 mm	0 mm
Ampicillin	10 µg	36 mm	22 mm
Ticarcillin	75 µg	36 mm	15 mm
Gentamicin	10 µg	36 mm	2 mm
Cephalexin	30 µg	14 mm	2 mm
Trimethoprim	1 25 µg	0 mm	0 mm
Sulphamethoxazole	25 µg	0 mm	0 mm
Tetracycline	10 µg	18 mm	28 mm
Tetracycline	25 µg	23 mm	30 mm

number of trials 2
standard deviation $\leq$ 1 414

Table 5 9 Comparison of the effect of antibiotics on *L acidophilus* from Product B and Bead 5B after incubation in simulated intestinal juice, pH 7 4

Antibiotic	Concentration	Product B	Bead 5B
Chloramphenicol	25 µg	31 mm	36 mm
Erythromycin	5 µg	36 mm	36 mm
Fusidic acid	10 µg	0 mm	0 mm
Methicillin	10 µg	36 mm	30 mm
Novobiocin	5 µg	20 mm	12 mm
Penicillin G	1 unit	36 mm	36 mm
Streptomycin	10 µg	0 mm	0 mm
Colistin sulphate	25 µg	6 mm	0 mm
Ampicillin	10 µg	36 mm	36 mm
Ticarcillin	75 µg	36 mm	36 mm
Gentamicin	10 µg	6 mm	6 mm
Cephalexin	30 µg	34 mm	29 mm
Trimethoprim	1 25 µg	0 mm	0 mm
Sulphamethoxazole	25 µg	0 mm	0 mm
Tetracycline	10 µg	30 mm	18 mm
Tetracycline	25 µg	36 mm	36 mm

number of trials 2
standard deviation $\leq$ 1 060

5.4 DISCUSSION

In recent years there has been increasing recognition of the importance of lactobacilli in its role of maintaining ecological balance within dynamic ecosystems such as the gut in the prevention of infection caused by colonisation of pathogens (McGroarty, 1993, Mital and Garg, 1995) However, antibiotic treatment has been shown to reduce the level of these beneficial micro-organisms, thus leaving the host more susceptible to gastro-intestinal infection Therefore after treatment it is important to reconstitute the gut with lactic acid bacteria preparations, preferably containing high antibiotic resistance, thus allowing them to survive and carry out their therapeutic properties

In order to determine the effect of antibiotics on *L acidophilus* cells from two representative commercial products, an in-vitro system was used Results showed the sensitivity was dependent on the antibiotic assayed and in the case of Tetracycline, the concentration used Cells from both products exhibited varying levels of sensitivity to 14 of the 16 antibiotics screened and showed complete resistance to the remaining 2 including Trimethoprim and Sulphamethoxazole, which are known to be effective against Gram negative organisms (Mims et al , 1993)

The process of immobilisation altered the cells resistance to antibiotics The immobilised cells exhibited complete to the antibiotics listed above as well as Methicilin, Penicillin G, Streptomycin, Colistin sulphate and Gentomycin

Exposure of *L acidophilus* cells to simulated gastric juice altered the cells response for bot6h free and immobilised cells from both products This indicates the importance of assessing microbial resistance following typical exposure treatments

Results by Champagne (1992) also found that immobilised cells exhibited enhanced resistance to Penicillin compared with their free cell counterparts He studied the effect of Penicillin on milk acidification by immobilised and free lactic acid bacteria and found that the entrapped cells were less affected by Penicillin producing higher levels of lactic acid than their free cell counterparts

Entrapped cells are exposed to environments of lower water availability (Rosevear, 1984) the enhanced resistance of immobilised cells to antibiotics may be related to these alterations in osmotic conditions as it is shown that the resistance of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* to Penicillin increased when aw was lowered with glycerol from 0 992 to 0 985 and from 0 998 to 0 955 respectively (Larsen and Aon, 1982)

However, other studies by Champagne (1992) found an insignificant difference in aw between immobilised and free cell systems Champagne (1992) suggested that the improved resistance of immobilised cells towards some antibiotics may result from altered growth patterns In immobilised systems, a steady state occurs whereby cell growth is limited The inhibitory effect of some antibiotics would then be reduced since the effect is maximised with growing cells However, this explanation is only applicable in the case of antibiotics that are metabolic inhibitors

In conclusion, immobilisation of *L acidophilus* in calcium-alginate beads was found to enhance the survival of cells during exposure to a number of antibiotics tested possibly as a result of factors associated with the altered cell microenvironment Therefore Immobilised Cell Technology has potential in increasing the effectiveness of lactic acid bacteria probiotic preparations by enhancing cell resistance during antibiotic treatment

CHAPTER SIX :

General Discussion

6.1 : DISCUSSION

Results from this research showed that immobilising *L acidophilus* cells in calcium-alginate beads afforded cells additional protection

1 During exposure to the hostile gastro-intestinal environment,

- the immobilised cells exhibited enhanced survival to the pH variations of the simulated gastric and intestinal juice
- furthermore, the immobilised cells also showed enhanced tolerance to a range of bile salt concentrations

2 During exposure to a number of antibiotics,

cells which had been entrapped, upon release, exhibited greater resistance to many of the antibiotics tested

The immobilised cells also generally retained their antimicrobial activity towards pathogenic micro-organisms, (*S typhimurium* and *E coli*), and in some cases the immobilisation procedure enhanced this antagonistic activity, thereby increasing their therapeutic impact

The behavioural differences between free and immobilised cells can be attributed to direct microenvironmental effects and their influence on cell metabolism.

6.1.1 : IMMOBILISED AND FREE CELL MICROENVIRONMENTS

The process of immobilisation induces many changes to the microenvironment Some of these changes include exposure to high polymer concentrations, altered water activity, oxygen tension and enhanced cell-cell communication

6.1.1.1. : High polymer concentrations:

One of the fundamental features of an immobilised cell microenvironment is that cells are exposed to high polymer concentrations Many studies have found that cell survival after freeze-drying and rehydration is influenced by the volume and composition of the suspending medium (Heckly, 1978, Mackey, 1984, Bozoglu et al , 1987, Souzu, 1992, Lievense and van't Riet, 1994) Therefore, immobilising the cells in a polymer gel with incorporated protectants may afford additional protection on cells during freeze-drying and subsequent rehydration under simulated gastro-intestinal conditions

The diffusional limitations within the calcium alginate microenvironment may also offer advantages during rehydration of the freeze-dried cultures McLoughlin (1994) reported that the diffusion of water soluble molecules is controlled by gel composition and gelation conditions, while Renneberg et al (1988) found that gels have a limited water content Studies by Leach and Scott (1959) also found that slower rates of fluid addition resulted in higher survival during rehydration, while Souzu (1992) suggested that rapid reabsorption of water molecules by freeze-dried cells could result in disruption and inaccurate rearrangement of the membrane constituents Furthermore, de Valdez et al

(1985) found that controlled rehydration conditions were important in protecting lactic acid bacteria They also found that lower quantities of water yielded higher survival rates, which they attributed to reduced osmotic shock Therefore, the rehydration properties of the calcium alginate beads could contribute to higher viability

Therefore, by having cells immobilised, would control the rate of fluid diffusion into the calcium alginate bead and the volume of fluid reabsorbed during rehydration and could contribute to less environmental shock when exposed to hostile rehydration conditions

Levels of cellular damage resulting from freeze-drying have been found to differ between free and immobilised cells Mc Loughlin and Champagne (1994) reported that the percentage of cells exhibiting cell wall and membrane damage was lower among immobilised lactic acid bacteria than their free cell counterparts Results from Chapter 2 and 3 also showed that in some cases immobilisation can reduce levels of cellular damage Structural damage results in leakage of molecular materials from cells However, the high polymer concentrations within the beads limits the diffusion rates of substrate into the macroenvironment (de Alteriis et al, 1990, Cassidy et al, 1996) Therefore, any material lost through cell breakdown or damage can be quickly utilised by neighbouring cells, thus preventing loss of valuable nutrients from the microbial environment (Mc Loughlin and Champagne, 1994)

6.1.1.2. : Reduced water activity and oxygen tension

A variety of hypothesis have been proposed to explain the altered physiological state of immobilised cells Many reports have suggested that the transfer limitations result in decreased water activity and oxygen deficiency which have been reported responsible for alterations in immobilised cell behaviour (Mattiasson, 1983, Mattassion and Hahn-Hagerdahl, 1982)

The polymer macromolecules are capable of organising water and consequently may alter the available water of immobilised cells This property of the gel might result in altered cell metabolism as water activity influences enzyme stability, activity and specificity (Hahns-Hagerdahl, 1986, 1990) While other reports by Mattiasson et al (1984) also hypothesised that microenvironmental conditions incurred during immobilisation, such as low a_w, influences their physiological status and consequently their metabolic behaviour They found that *Candida tropicalis* switched its metabolism from xylitol to ethanol production, altered ethanol production was obtained for *Saccharomyces cerevisiae* while glycerol production of the algal *Dunaliella* was enhanced under simulated immobilisation conditions

Keweloh et al (1989) also indicated that it is not the effects of the beads, but rather reduced water activity or oxygen deficiency in microcolonies that influences the dynamic and not the structural aspects of the membrane since after liberation the bacteria were as sensitive to phenol as cells grown in the free state

Other studies by Karel and Robertson (1989) examined the bacterial growth in the confines of an entrapped microenvironment and suggested that the cells exert pressure on the polymers by accumulation of intracellular solutes This would lead to an alteration in the osmotic pressure which may also be responsible for altered cell behaviour

For example, Champagne (1992) attributed the lower inhibitory effect of Penicillin on immobilised cells to the altered osmotic levels of the beads Therefore, reducing the a_w by immobilisation has the potential of enhancing their tolerance to antibiotics

Exposing cells to osmotic stresses has been found to induce the production of stress proteins (Hecker et al , 1988) Thus, immobilisation could stimulate the production of these stress proteins resulting in altered cell composition of free and immobilised cells The production of these stress proteins could consequently increase their tolerance to freeze-drying and subsequent exposure to hostile conditions

6.1.1.3. : Enhanced cell-cell communication

Reports by Wirth et al (1996) and Kaprelyants and Kells, (1996) have suggested that communication between bacterial cells through excretion of pheromones could affect their survival and metabolic behaviour For example, the A factor produced by *Streptomyces griseus* triggers the expression of Streptomycin production and Streptomycin resistance, while a RNA III activating protein secreted by *Staphylococcus aureus* regulates production of toxic exoproteins (enterotoxins B, hemolysin and toxic shock syndrome toxin 1) Furthermore, data on the regulation of bacteriocin production

in lactobacilli by pheromonal signals was described at the Beijerinck Centennial Meeting held at The Hague, Netherlands (1995) Therefore secretion of these chemical signals may enhance the survival of *L acidophilus* under simulated gastro-intestinal conditions and may also influence its therapeutic behaviour such as production of antimicrobial compounds

The immobilised microenvironment may improve the potential of these chemical signals by the nature of their close cell-cell contact, in addition to their diffusional constraints which would restrict the diffusion of the pheromones into the bulk macroenvironment

6.1.2 : MODIFICATION OF CELL COMPOSITION AND METABOLISM

The behavioural differences between free and immobilised *L acidophilus* cells can be attributed to microenvironmental influences and their effects on cellular metabolism

6.1.2.1. : Tolerance to Toxic compounds and hostile environments

Immobilisation of microbial cells has been observed to confer protection from toxic compounds and adverse environments This could consequently affect survival during the transit through the hostile gastro-intestinal conditions and enhance antibiotic resistance

Bettmann and Rehm (1984) observed that immobilisation of *Pseudomonas sp* conferred protection from phenol toxicity The authors postulated two mechanisms for their observations (a) that cell growth into microcolonies inside beads may protect the cells

or (b) the colonies around the outside edge of beads form a diffusion barrier to protect cells inside the beads Keweloh et al (1989) observed that immobilisation of *E coli* in calcium alginate beads enhanced their tolerance to bacteriostatic concentrations of phenol They attributed this enhanced tolerance to the formation of an envelope which surrounded microcolonies of entrapped cells, which was not present with free cells They suggested that this protective envelope may act by binding toxic agents or by inhibiting diffusion of toxic compounds into the cell aggregate

Subsequent studies by Keweloh et al (1990) suggested that enhanced phenol tolerance may result from alterations in the cell membrane of immobilised cells They found higher protein-lipid ratios in cell membranes of immobilised cells compared to free cells, with different protein patterns in the outer membrane

Diefenbach et al (1992) also attributed the enhanced resistance of immobilised cells to hostile agents to the altered membrane composition of the immobilised cells They suggested that palmetic and oleic fatty acid impurities in the alginate were probably incorporated in membranes of immobilised cells The incorporation of these fatty acids would have a stiffening effect on the cell membrane and consequently provide protection against bactericidal compounds/environments

Results from this study also found that the immobilisation procedure can enhance the survival of *L acidophilus* under suboptimal conditions encountered during transit through the gastro-intestinal tract This enhanced tolerance might also be attributed to altered composition of the cell

Doran and Bailey's (1986) analysis of cell composition showed that the immobilised cells contained 4 times the polysaccharide content of suspended cells The average DNA content of immobilised cells was 3 8 times that of free cells, while that of released progeny was 3 1 fold higher than that of free cells They also found that the immobilised cells contain a quarter of the amount of stable double-stranded RNA found in suspended cells However, the protein content of the free cells, the immobilised cells, and their released progeny were similar

Many other studies have found that immobilised cells exhibit morphological changes compared to their free cell counterparts For example, Doran and Bailey (1986) observed a number of changes in *Saccharomyces cerevisiae* cells attached to gelatin The main size of the immobilised cells was about two-thirds that of suspended cells Furthermore evidence from a number of studies on different immobilised cell systems indicate that the effects of immobilisation can be maintained for some time after cell release (cited by Mc Loughlin, 1994)

Barbotin et al (1990) also observed morphological changes of immobilised cells In their studies they found that the mycelial growth of *Gibberella fujikuroi* within the bead was affected by the physical barriers imposed in the alginate network structures resulting in zig-zag shaped immobilised mycelia having a rough cell wall They suggested that the changes of the cell wall appearance may support the idea of more varied protein biosynthesis in the suboptical area of the immobilised fungi

6.1.2.2. : Internal pH

Using entrapped *L acidophilus* in calcium alginate beads as a dietary adjunct would have an additional advantage of providing cells with a buffer environment Therefore, immobilisation would provide protection during exposure to the pH variations of simulated gastric and intestinal juice Studies by Loureiro-Dias and Stanos (1990) used ^{31}P NMR to show that immobilised cells exhibited a higher cytoplasmic pH during glucose fermentation compared to free cells They reported that the free cells were unable to maintain efficient pumping of protons to the exterior of cells when they were exposed to strong acidic conditions of the external medium The protective effect of immobilisation was also observed for *S cerevisiae* immobilised m alginate Buzas et al (1989) reported that the immobilised cells exhibited enhanced tolerance to external pH values (pH 2 5 - 6 5) compared with their free cell counterparts Improved tolerance to changes in the external pH was also observed for fermentation m dairy waste by *Candida pseudotropicalis* (Marwana et al , 1990) Results from Chapter 3 also showed that the immobilisation process enhanced survival of *L acidophilus* under simulated gastric and intestinal conditions

6.1.2.3. : Metabolite Production

Lactic acid bacteria produce important primary metabolites (eg lactic acid, acetic acid and H_2O_2) and secondary metabolites (bacteriocins and antibiotic-like compounds) As discussed previously, in Chapter 4, these substances have important therapeutic implications in that they prevent the establishment of food-borne pathogens in the gut

Many literature reports have shown that entrapment of cells within a gel environment can induce changes in metabolic behaviour Hilge-Rotmann and Rehm (1990) found that cells grown in the form of microcolonies in the alginate beads showed faster glucose uptake and ethanol productivity compared with free cells in suspension under identical culture conditions They observed that the immobilised cells exhibited altered enzyme activity compared with their free cell counterparts Increased specific hexokinase and phosphofrucokinase activities could be determined from these immobilised cells Other studies by Kearney et al (1990b) found that immobilisation of *Lactobacillus plantarum* within calcium alginate beads yielded cultures with increased fermentation potential Their results showed that the lactic acid fermentation time required to reach a pH value of 5 0 was reduced by approximately 30% with respect to free cell cultures Furthermore, immobilisation also appeared to protect cells from the initial shock experience on innoculation, thus resulting in decreased lag phase of both growth and acidification (McLoughlin and Champagne, 1994)

Furthermore, positive and negative effects of immobilisation on secondary metabolite production was reported by Bringi and Shuler (1990) The immobilisation process often alters production of these secondary metabolites as they are produced by different pathways that depend largely on environmental conditions Therefore, the calcium-alginate matrix may act as an elicitor resulting in enhanced metabolite production in the external medium

Studies by Pertot et al (1988) suggested that immobilisation shifts from growth towards metabolite production In their studies they found that alkalloid production was 7 times

higher for alginate encapsulated *Claviceps paspali* than their free cells Other studies by Wan et al (1995) found that immobilised L A B (*Lactococcus lactis*, *Pediococcus acidilactici* and *Lactobacillus brevis*) could produce bacteriocin concentrations at least equivalent to those obtained from free cells

Therefore, increased production of primary and secondary metabolites by entrapped cells may be a contributing factor to the enhanced antimicrobial activity of immobilised *L acidophilus* cells towards *S typhimurium* and *E coli* as observed in Chapter 4

6.1.3 : OTHER ADVANTAGES OF USING IMMOBILISED CULTURES

An additional advantage of using immobilised cultures is that immobilisation enhances plasmid stability Research by Oriel (1988) and Barbotin et al (1990) found that immobilisation of *E coli* cultures resulted in higher plasmid stability and copy numbers They also found that immobilising the cells within a gel matrix eliminated the need for selective pressure

Other studies by McLoughlin and Champagne (1994) reported that immobilisation reduces plasmid loss in lactic acid bacteria This has obvious advantages for example in bacteriocin production as genes for many of the bacteriocins of lactic acid bacteria are found on plasmids (Jack et al , 1996) For example, studies by Huang et al (1996) found

that immobilisation considerably improved the stability of a plasmid related to Pediocin 5 production in *Pediococcus acidilactici* UL5 cells

The improved plasmid stability observed with immobilised cells cannot be explained by one factor alone Kumar and Schugerl (1990) reported that the nature of the matrices, matrix concentration, inoculum size, gel bead volume, nutritional limitations, temperature, pH and oxygen concentrations have implications in the microbial stability Other studies by De Taxis du Poet et al (1986) and Oriel (1988) reported that the microenvironment may play an important role in promoting the higher level of plasmid stability following immobilisation

One explanation for the increased plasmid stability conferred through immobilisation is the possibility that immobilisation influences copy number Sayadi et al (1988) have demonstrated an increased copy number of TG201 in immobilised *E coli* compared with free cells In a similar manner Huang et al (1993) attributed the reduced pediocin production of some bacteriocin-producing isolates of free cells, compared with their immobilised counterparts, to a reduction in their plasmid copy number

Huang et al (1993) also described other hypotheses including physical separation arising from gel structure which isolated the plasmid containing and plasmid-free cells and thus eliminating competition, the close proximity of immobilised cells plus restricted freedom to move could promote transfer of plasmid DNA between populations by either conjugated or transformation

Another possible reason for the enhanced plasmid stability of immobilised cells is that the nutritional and oxygen limitations found within the gel matrix restricts growth to the outer layers of the bead The recombinant cells grow in the outer layers of the bead for approximately 10 - 16 generations, forming microcolonies, which results in destabilisation of outer layers of the polymer resultmg in loss of mechanical rigidity Cell leakage follows which makes oxygen and nutrients available for cells which reside deeper in the gel bead Thus plasmid containing cells are continuously being replenished by new ones deeper in the bead Thus there is a continuous renewal of plasmid containing cells as growth rates do not exceed a maximum of approximately 16 generations which is less than that required in free cell systems in order to regenerate plasmid free cells (Mc Loughlin, 1994)

Immobilisation could also have important implications in protection from bacteriophage infection Klaenhammer (1984) reported that bacteriophage attack of starter cultures is a major problem in the dairy industry However, studies by Steenson et al (1987) and Champagne et al (1988) found that the immobilisation process afforded cells protection from attack from lytic bacteriophage

6.2 : GENERAL CONCLUSIONS

It maybe concluded from this research work that immobilised cell technology has potential in the healthcare industry for therapeutic functions in lactic acid bacteria probiotic preparations

The results of this work showed that by manipulating the immobilisation process, cells with enhanced survival to exposure to hostile simulated gastro-intestinal conditions such as simulated gastric/intestinal juice and bile salts could be produced The cells which had been entrapped also exhibited enhanced resistance to many of the antibiotics tested compared to their commercial counterparts Finally the entrapped cells generally retained their antagonistic activity and in some cases increased antimicrobial activity towards the pathogens tested probably due to the enhanced survival level resulting in increased therapeutic impact

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