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An immobilised cell system for the delivery of functional *Lactobacillus reuteri* DPC16 cells to their target site in a simulated gastrointestinal tract

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2012

An immobilised cell system for the delivery of functional *Lactobacillus reuteri* DPC16 cells to their target site in a simulated gastrointestinal tract

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List of publications

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3. The effect of cell immobilisation on the antibacterial activity of Lactobacillus reuteri DPC16 cells during passage through a simulated gastrointestinal tract system (submitted to *World Journal of Microbiology and Biotechnology* on 18th April, 2012).

Abstract

The objective of this study was to design and produce calcium alginate beads that can deliver immobilised cells of *Lactobacillus reuteri* DPC16 to a target site of the colon in the gastrointestinal (GI) tract, without any diminution of their important physiological characteristics. Several factors that might affect the effectiveness of calcium alginate beads for the cell delivery were investigated, using an *in vitro* GI tract model to simulate the conditions within the tract. Firstly, by varying the concentration of alginate at a constant concentration of $CaCl_2$, and combining the system with gelatin, chitosan or skim milk, the survival of immobilised DPC16 cells in simulated gastric fluid (SGF) was observed. Secondly, the physical stability of calcium alginate beads containing skim milk was observed during sequential incubation in the GI fluids using optimal concentrations of alginate. Finally, the survival of DPC16 cells immobilised within alginate beads containing skim milk was monitored when the beads were incubated for different times during sequential exposure to the simulated fluids. The results demonstrated that non-encapsulated DPC16 cells were sensitive to an acidic environment, and no viable cells were detected after 90 min exposure in SGF (pH 1.2). After appropriate experimentation, an alginate concentration of 3% (w/v) was deemed to be the optimum value and was used in subsequent investigations. When skim milk (8% (w/v)) was added to the alginate solution, the cell survival in SGF was improved markedly. The optimal concentration of calcium chloride was 0.3 M, based on the beads maintaining their integrity in SGF and simulated intestinal fluid (SIF) while disintegrating in simulated colonic fluid (SCF) to release viable cells. Hence, the beads made from 3% alginate, 8% skim milk and 0.3 M $CaCl_2$ proved to be an effective delivery and release system for DPC16 cells.

L. reuteri DPC16 has strong antimicrobial activities against pathogens, due mainly to its ability to produce reuterin. Hence this and other functional properties of the bacterial cells were studied before and after passage through the GI tract. The cells that were recovered after release from the alginate beads in the SCF showed no diminution in functional properties, including their growth kinetics, ability to adhere to epithelial cells and ability to inhibit the adhesion of *E. coli* to epithelial cells. However, the bacteriostatic and bactericidal properties of the recovered cells against some pathogens

were significantly greater ($P<0.05$) than those of the original cells. Production of reuterin by the recovered cells was significantly greater ($P<0.05$) than that of the original cells when cultured in MRS medium in the absence of its metabolic precursor, glycerol. The results demonstrate significant ($P<0.05$) consequences for the application of the encapsulation technique to protect and/or enhance the functional properties of the probiotic cells.

Subsequently, an investigation was carried out to find the reason for the antimicrobial activity enhancement. By recovering cells from different stages of the immobilisation and delivery process and examining them for their antimicrobial properties, it was found that it was the immobilisation process *per se,* rather than passage through the simulated gastrointestinal fluids, that caused the enhancement of antimicrobial activity, and that this was related to increased activity of the enzyme (diol dehydratase) that is responsible for reuterin production from glycerol.

Finally, it was demonstrated that freeze-drying of the alginate beads was not an appropriate storage technique as it resulted in a significant ($P<0.05$) diminution of the antimicrobial activities.

Based on these findings it is confirmed that the alginate-skim milk-$CaCl_2$ immobilisation system is an effective and efficient method, not only for protecting the viability of DPC16 cells, but also for maintaining the physiological characteristics.

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Abbreviations

3-HPA	3- hydroxypropionaldehyde
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
BHI	Brian heart infusion broth
cFDA	carboxy fluorescein di-acetate
c.f.u.	Colony forming units
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediamine tetra- acetic acid
FAO	Food and Agriculture Organisation of the United Nations
g	gram
GI tract	Gastrointestinal tract
h	hour
Hb	haemoglobin
IDF	International Dairy Federation
LAB	Lactic acid bacteria
MEM	Modified Eagle's medium
min	minute
MRS	De Man, Rogosa, Sharpe
MRSg	MRS broth supplemented with 250 mM glycerol
OD	Optical density
P	Provability value
PBS	Phosphate buffered saline
PI	propidium iodide
rpm	Revolutions per minute
SCFAs	Short chain fatty acids
SCF	Simulated colonic fluid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second

SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethy) amiomethane
UV	Ultra violet light
WHO	The World Health Organisation of the United Nations

Chapter 1 General Introduction

Probiotics, from the Greek "for life", is a term used in the administration of living bacteria to treat or prevent a clinical disorder (Bian *et al.*, 2011). The earliest knowledge on probiotics dates back to thousands of years ago. Nowadays, probiotic bacteria are involved in many aspects of human health, where they have been shown to provide some of the foundations of good health. Because of the important role of probiotic lactic acid bacteria in the dairy industry, this has long been the focus in New Zealand (Crittenden *et al.*, 2005). Although the majority of the early research on probiotic bacteria focused on the stability of foods such as cheese and yogurt, there is now increasing interest in personal health, and there is increased attention on the role of probiotics in animal and human nutrition and the replacement of antibiotics.

There are two types of challenges in the administration of probiotic bacteria. First, there is the chemical barrier, such as the extreme pH, bile stress and digestive enzymes in the gastrointestinal (GI) tract that may destroy the bacteria (Picot & Lacroix, 2003; Begley *et al.*, 2006). Secondly, there is the physical barrier that obstructs the orally-delivered live probiotic cells during transit to the distal part of the GI tract (Prakash & Malgorzata Urbanska, 2008). Therefore, an effective delivery system is required to cope with these challenges.

Currently, alginate beads represent a major entrapment system for bacterial cells. Their preparation does not require low pH, high temperature, high ionic strength or osmotic pressure changes, and so alginate is the most widely used encapsulation technique for lactic acid bacteria. This technique will be investigated in this thesis for the delivery of probiotic bacteria to the target site in the GI tract.

Lactobacillus reuteri DPC16 is a New Zealand-patented probiotic strain (Shu & Liu, 2008). It was isolated by Bioactive Research Limited (BRNZ), and was grouped into *Lactobacillus reuteri* by DNA sequence analysis (Lu, 2007). Some physiological and functional investigations, such as its safety assessment (Bian, 2008), growth in various stressful environments (Joshi, 2005), antimicrobial activity against food-borne pathogens (Bian *et al.*, 2011; Lu, 2007), and adhesion to mucosal cell membranes (Tian,

2010), have confirmed that DPC16 is a promising probiotic bacterium. Further, the viability of *L. reuteri* DPC16 in some microencapsulation systems has also been tested. When incorporated into food products such as skim milk, colostrum, propolis, seafood and plant extracts, the microencapsulated DPC16 maintained high viability during storage (Chang, 2006; Yin, 2006). Recently, an application that incorporates strain DPC16 into modified atmosphere packaging was investigated for the control of seafood pathogens and to prolong the shelf- life of seafood products (Lu, 2007).

In recent decades, the colon has been deemed to be a suitable site to release bioactive substances. There are several advantages of the colon: firstly, it is a site where poorly absorbed molecules may have an improved bioavailability; secondly, the environment at this site is recognied to be less hostile than those in the stomach and small intestine. Additionally, the longer transport time enhances the absorption of bioactive substances (Chourasia & Jain, 2003). In a recent study (Yin, 2006), the small intestine was chosen as the release site of encapsulated DPC16 cells, but the system proved to be ineffective. Hence, in the present project, the colon was chosen as the target release site for the encapsulated bacterial cells. A number of characteristics are considered important for probiotic activity, such as adhesion to intestinal and colonic surfaces, reduction of the colonization of pathogenic bacteria, and the production of antimicrobial substances. Preliminary experimental results (Tian, 2011) have shown that *L. reuteri* can adhere to human HT-29 and Caco-2 cells, but it is still uncertain whether DPC16 cells can replace pathogens at pre-occupied sites or compete with pathogens for these sites. Hence, further work on adhesion is reported in this thesis.

The success of any delivery system for probiotics depends on the ability to deliver viable cells to the target site (the colon) in sufficient numbers to exert their effects. Additionally, there should be no diminution in functional properties during the entire encapsulation and delivery procedure. So far, most of the literature has focused on the maintenance of viability of probiotic cells during passage through simulated gastrointestinal fluids, and few reports are available on the retention of other physiological properties. Hence, in this thesis, the latter will be investigated. Therefore the two main aims of this thesis are:

1. To develop an effective system for delivery of *L. reuteri* DPC16 cells to the colon, the site of release;

2. To recover *L. reuteri* DPC 16 cells from the target site (the colon) in a simulated GI tract, and compare their functional properties with those of the original cells.

Chapter 2 Literature review

2.1 Probiotics

2.1.1 Definition

Several definitions of probiotics have been suggested over the years (Lee & Salminen, 2009). However, the current accepted definition states that "probiotics are defined as beneficial live microorganisms that confer health promotion and disease prevention by improving the intestinal microbial balance of humans with a concentration above 10^7 c.f.u./ml at the action cite" (Salminen *et al*., 1996; Ouwehand & Salminen, 1998; Agostoni *et al*., 2004; Reid *et al*., 2008; Lee & Salminen, 2009). Modern probiotic bacterial strains are predominantly from the genera *Bifidobacterium* and *Lactobacillus*, which are important intestinal microflora contributing to the healthy human gastrointestinal tract (Reuter, 2001; Bezirtzoglou & Stavropoulou, 2011). There are many probiotic food products on the market nowadays. In the clinical medical field, probiotics have been reported to treat, or have the potential to treat, diseases such as diarrhoea, gastroenteritis, irritable bowel syndrome, inflammatory bowel disease (Crohn's disease and ulcerative colitis), cancer, inadequate lactose digestion, infant allergies and hyperlipidemia (Caramia, 2008; Kim *et al*., 2008; Shida & Nanno, 2008; Vanderhoof, 2008; Avadhani & Miley, 2011; Borowicki *et al*., 2011). It is believed that with more accumulation of knowledge on probiotics, the utilisation and application of these microorganisms will be expanded to a wider area.

2.1.2 Microorganisms used as probiotics

In general, most of the probiotic microorganisms are lactobacilli (De Peter *et al*., 2007; Matsumoto *et al*., 2010) or Bifidobacteria (Xiao *et al*., 2007; Wada *et al*., 2010; Matsuda *et al*., 2011). However, a bacterium cannot be called a probiotic unless it has been proven to be viable at the time of usage in sufficient quantity to exert a health benefit (FAO/WHO Working Group, 2002; Sahin *et al*., 2007). Similarly, simple intake of viable bacteria will not guarantee good health. A number of different studies have been carried out to determine the stability, characteristics and physiological influence of probiotics. Currently some *in vitro* examinations (Dash, 2009; Lee & Salminen, 2009) used for the study of probiotic strains are:

- Resistance to gastric acidity;
- Resistance to bile acids;
- Adhesion to human epithelial cells;
- Stability during passage to intestinal sites and during storage at 4°C or 25°C;
- Inhibitory effects on potentially pathogenic bacteria;
- Minimum effect on the normal microflora in the small intestine;
- Ability to reduce pathogen adhesion to the mucosal surface; and
- Bile salt hydrolase activity.

Lactic acid bacteria (LAB) are the most common type of microorganisms used as probiotics in the food industry (dairy, meat, and some plant fermentations). *Lactobacillus* is one of the important Gram-positive facultative anaerobic or microaerophilic genera included in the LAB group (Jafarei & Ebrahimi, 2011). It is commonly found in the GI tract of humans and animals and is considered to be among the most dominant organisms colonizing the small intestine (Marco *et al*., 2007). Some metabolic compounds of LAB, such as carboxylic acids, fatty acids and hydrogen peroxide, have antimicrobial effects (McDonell & Russell, 1999).

Currently, considerable scientific and commercial interest is focused on *Lactobacillus* owing to a myriad of claims regarding health-promoting effects on the GI tract (Young, 1998; Naidu *et al*., 1999; Horvath 2011; Yan *et al*., 2011; Whary *et al*., 2011). Furthermore, it can also induce a specific local and systemic immune response against selected pathogens, e.g. *Escherichia coli* O157:H7 (LeBlanc *et al*., 2004), *Streptococcus pneumoniae* (Vintini & Medina, 2011). Other characteristics of *Lactobacillus* include gastric acid and bile salts tolerance and adhesive ability to the GI tract epithelium cells.

One species of *Lactobacillus*, *L. reuteri,* has been studied extensively (Madhwani & McBain, 2011; Lu *et al*., 2011; Smith *et al*., 2011). It is one of the most ubiquitous members of the naturally-occurring gut bacteria, but it is not found in every individual. *L. reuteri* has antimicrobial activity against pathogens such as *Salmonella typhimurium* (Klose *et al*., 2011; Sriburi *et al*., 2001) and *E. coli* (Edens *et al*., 1997; Carey *et al*., 2011).

L. reuteri DPC16 was isolated by scientists of Bioactive Research New Zealand

(BRNZ) and patented in 2008 (Lu, 2007). The antimicrobial product from *L. reuteri* DPC16 culture supernatants has been reported to inhibit the growth of common pathogens (Bian *et al*., 2011), and *L. reuteri* DPC16 cells have been shown to adhere to human epithelial cells (Tian, 2011). However, DPC16 cells are sensitive to acid conditions, and after only a short exposure, no viable cells can be detected (Yin, 2006). The stress response of DPC16 cells to heat and salts was determined by Joshi (2005). All this evidence indicates that *L. reuteri* DPC16 is a promising probiotic.

Some other bacterial strains, such as *Bifidobacterium infantis* 35624 (McKernan *et al*., 2010), *Bifidobacterium lactis* HN019 (DR10) (Zhou & Gill, 2005; Arunachalam *et al*., 2000), *E. coli Nissle* 1917 (Adam *et al*., 2010; Angulo *et al*., 2011), *Lactobacillus acidophilus* NCFM (Sanders & Klaenhammer, 2001), *Lactobacillus casei* DN114-001 (*Lactobacillus casei* Immunitas(s)/Defensis) (Blankier *et al*., 2011; Gourbeyre *et al*., 2011) and *Lactobacillus johnsonii* La1 (NCC533) (Fukushima *et al*., 2007; Pantoflickova *et al*., 2003) have been shown to be probiotics because of their health beneficial effects. In addition, the combination of certain strains has also been demonstrated to confer health improvement activities (Hol *et al*., 2008; Martinez *et al*., 2009; Hummelen *et al*., 2010; Rerksuppaphol & Rerksuppaphol, 2010).

2.1.3 Mode of action of *L. reuteri*

Reuterin is an antimicrobial substance that has been proven to contribute to probiotic health benefits (Lu, 2007; Bian *et al*., 2011). Under anaerobic conditions, *L. reuteri* has the ability to convert glycerol to 3-hydroxypropionaldehyde (3-HPA) (Figure 2.1), which is also known as reuterin (Doleyres *et al*., 2005). Two isofunctional enzymes, glycerol dehydratase and diol dehydratase, catalyse the conversion of glycerol to 3-HPA as well as the conversions of 1,2-propanediol to propionaldehyde and ethanediol to acetaldehyde. Abeles (1961) isolated the enzyme from *Aerobacter aerogenes*, and found that vitamin B_{12} was required in this reaction. Jones (2009) reported that the biofilms formed by *L. reuteri* showed immunomodulatory activities and pathogen inhibitory effects due to the production of reuterin. Spinler (2008) studied the antimicrobial activity of four human-derived *L. reuteri* isolates and found that reuterin derived from these four strains could inhibit the growth of a range of pathogens, such as enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, *Salmonella enterica*, *Shigella sonnei* and *Vibrio cholera*.

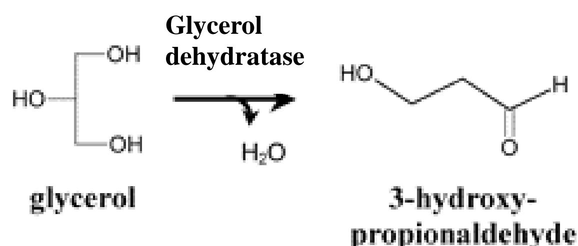


Figure 2.1 General bacterial conversion of glycerol to 3-HPA (Raynaud *et al*., 2003).

A number of mechanisms by which reuterin exerts its antibacterial effects have been postulated. One mechanism is that reuterin might inhibit the activity of bacterial ribonucleotide reductase (Cleusix *et al*., 2007), thus inhibiting the conversion of ribonucleotides to deoxyribonucleotides. Another explanation is that the aldehyde group of reuterin reacts with thiol groups and primary amines resulting in the inactivation of proteins (Schaefer *et al*., 2010).

Short-chain fatty acids (SCFAs), primarily lactic, acetic, propionic, butyric, and valeric acids, are fermentation products of *L. reuteri* (Jacobs *et al*., 2009) which have inhibitory effects on pathogens (Walker *et al*., 2005; Koning *et al*., 2008). Further, the inhibition activity increases as the medium pH decreases (Miller *et al*., 1977). Fayol-Messadoudi (2005) has confirmed that some probiotic *Lactobacillus* strains can kill *Salmonella enterica* Serovar Typhimurium by producing metabolites such as lactic and acetic acids. SCFAs are also reported to increase mucin production in the intestine, and to bind to receptors on immune cells, thus preventing pathogen adhesion (Saulnier *et al*., 2009).

Additionally, bacteriocins are heat-stable proteins, produced by some lactobacilli, that show a broad-spectrum inhibitory effect (Hardy, 1985). Many bacteriocins are active against food-borne pathogens, especially *Listeria monocytogenes* (Ogunbanwo *et al*., 2003; Xie *et al*., 2011) and *Staphylococcus aureus* (Castro *et al*., 2011). Bacteriocins

produced by lactic acid bacteria are divided into four types: type one is lanthibiotics, which are small peptides containing modified amino acids; type two is bacteriocins, which are small hydrophobic peptides containing no unusual amino acids; type three is bacteriocins that are large molecular heat-labile proteins; type four is bacteriocins that are composed of protein and other chemical components (Kabuki *et al*., 1997). Reutericin 6 is a bacteriocin produced by *L. reuteri* LA6. It induces potassium ion efflux from bacterial cells and liposomes (Kawai *et al*., 2004), and thus exerts an antimicrobial activity on pathogens.

There have been many investigations in terms of using live probiotic bacterial cells for therapeutic purposes. Table 2.1 lists some probiotic bacteria and relevant potential therapies.

Table 2.1 Potential therapies using probiotic bacteria (Prakash & Jones, 2005)

Probiotic bacteria	Action site	Disease	Mechanism	Reference
L. rhamnosus, *L. casei,* *L. reuteri,* *LGG B lactis,* *B. bifidum*	Gastrointe-stinal tract	Diarrhoea	Establishing immunity against rotavirus infections.	Caramia, 2008; Vanderhoof, 2008; Avadhani & Miley, 2011; Wen *et al*., 2011
L. lactis, *L GG*		Inflammatory bowel disease	Stimulating IgA immune response, and then promoting the gut immunological barrier; Provide an adjunct nutritional therapy.	Benjamin *et al*., 2011; Mileti *et al*., 2009
L. acidophilus *L. johnsonii La1* *B. plantarii*		Ulceration	Down-regulation of *H pylori* infection by inhibition of intestinal cell adhesion and invasion.	Medeiros *et al*., 2011; Sgouras *et al*., 2005
		Steatorrhea of lipids (malab-sorption of lipids)	Bacteria express lipolytic activity with substantial enzyme stability in human gastric juice results in the increased absorption of lipids in the small intestine.	Prakash & Jones, 2005;
Lactobacillus, *B. breve,* *B. longum*	Cancer	Colorectal cancer	Mechanisms may include: Enhancing the host's immune response; Binding and degrading potential carcinogens; Alterations in the intestinal microflora incriminated in producing Recognised carcinogens (eg, bile acid-degrading bacteria); Producing anticarcinogenic or antimutagenic compounds in the colon; Alteration of the metabolic activities of the resident microflora; Alteration of physicochemical conditions; and Effects on general physiology.	Juillerat-Jeanneret, 2011; Gueimonde *et al*., 2007
L. acidophilus, *L. casei,* *L. GG* *B. lactis* *B. bifidum*	Immune system	Enhanced immunity	By one mechanism, innate immunity is enhanced by stimulating the activity of NK cells. While antigen-feeding alone was shown to prime for an immune response, cofeeding on antigen and probiotic bacteria suppressed both antibody and cellular immune responses and may have the potential to attenuate autoimmune diseases (eg, encephalomyelitis) by jointly dosing with myelin basic protein and probiotic bacteria.	Ouwehand, 2007; Martins *et al*., 2009; Blankier *et al*., 2011; Gourbeyre *et al*., 2011 (Continued on next page)

Table 2.1 Potential therapies using probiotic bacteria (Prakash & Jones, 2005)

Probiotic bacteria	Action site	Disease	Mechanism	Reference
L. acidophilus, L. bulgaricus, L. fermentum L. reuteri	Blood	Lower cholesterol	Bacteria may bind or incorporate cholesterol directly into the cell membrane. Bile salt hydrolase (BSH) enzyme deconjugates intraluminal bile acids making them less likely to be reabsorbed into the enterohepatic circulation (ECH), causing *de novo* synthesis of bile acids from blood serum cholesterol.	Salarmoini & Fooladi, 2011; Pan *et al*., 2011; Guo *et al*., 2011
L. acidophilus, Lactic acid bacteria	Kidney	Chronic kidney failure	Small bowel bacterial overgrowth is well known to occur in end-stage kidney failure and is responsible for producing uremic toxins and contributing to decreased nutritional well-being. Certain bacteria are shown to reduce blood levels of uremic toxins produced in the intestine as bacterial putrefactive metabolites, especially that of indican, dimethylamine, and nitrosodimethylamine (a carcinogen) by inhibiting bacterial production by means of correcting the intestinal microflora.	Prakash & Jones, 2005; Brassart, 2003
L. acidophilus, L. plantarum, *L. brevis, S. thermophilus, B. infantis, O. faecalis*		Kidney stones	Urinary excretion of oxalate, a major risk factor for renal stone formation and growth in patients with idiopathic calcium-oxalate urolithiasis, can be greatly reduced with treatment using a high concentration of freeze-dried lactic acid bacteria. Oxalate-degrading enzymes produced by these microorganisms or by *Oxalobacter*-type bacteria break down the unwanted oxalate and can be used to prevent the subsequent evolution of kidney stones.	Campieri *et al*., 2001; Guo *et al*., 2010

2.1.4 Survival of probiotics in acid and bile stress

To confer the health promoting function, probiotics have to survive passage through the GI tract (Wall *et al*., 2007). As they enter the stomach, the environment suddenly changes and the probiotics are exposed to several stresses, including digestive enzymes and acidic pH. Some probiotics have been reported to have the ability to survive the harsh environment (Lee *et al*., 2004; Muthukumarasamy *et al*., 2006). Changes in gene expression and phenotype are often observed during acidic exposure. For Gram-positive bacteria, some strategies that have been reported to be involved include (Wall *et al*., 2007):

- Removal of protons from the cytoplasm;
- Production of general stress proteins and chaperones to repair and stabilise protein and DNA;
- Changes to metabolism and cell envelope composition; and
- Alkalization of the external environment *via* some signaling pathway.

After passage through the stomach, the surviving probiotic cells have to endure stress from bile acids. The chemical nature of bile acids indicates that they are able to solubilise and damage cell membranes. On exposure to bile acids, Gram-positive bacteria have been reported to alter their cellular envelope (Whitehead *et al*., 2008). The presence of bile salt hydrolase (BSH), especially in Gram-negative bacteria, can protect bacterial cells from the damage caused by bile salts (Wang *et al*., 2011). According to the microarray analysis of *L. plantarum* and *L. acidophilus* after exposure to bile stress, changes were identified to have occurred in the cell envelope gene (Bron *et al*., 2006; Pfeiler *et al*., 2007). *L. reuteri* ATCC 55730 can survive and continue growing in 0.05-0.1% bile, and viable cells could be observed at even 5% bile. Based on the gene expression assay, a multidrug resistance transporter (lr1584) is required for this ability to grow in the presence of bile, which indicates that removal of bile or toxic metabolites from the cytoplasm is required for growth (Whitehead *et al*., 2008).

2.1.5 Colonization/Adhesion inside the human body

Adhesion or colonization to the intestinal mucosa is regarded as important for probiotic microorganisms to modulate the immune system, exclude pathogens, and prolong the transit time of food (Drisko *et al*., 2003). The exact mechanism of colonization/

adhesion inside the human body, particularly in the GI tract, is still unclear.

Extracellular polysaccharides have been shown to be important substances for adhesion of *L. reuteri* (Ruzicka *et al*., 2011). The S-layer protein of some lactobacilli strains, including *L. reuteri* (Miyoshi *et al*., 2006), *L. acidophilus* (Khaleghi *et al*., 2010), *L. plantarum* (Ramiah *et al*., 2008), and *L. helveticus* (Beganovic *et al*., 2011), is also a necessary component that mediates the adhesion of probiotics to host epithelial cells (Zhang *et al*., 2010).

The most commonly used *in vitro* adhesion models employ tissue culture cells and the intestinal mucus model (Ouwehand *et al*., 2001). These models provide a rapid screening technique for recognition of probiotic strains.

2.1.6 Commercial Probiotic Products

Probiotics are presently available in commercial food products. Dairy and other fermented foods are the most common commercial sources of probiotics. In food products, the most frequently used probiotic organisms are *Lactobacillus*, *Bifidobacterium*, or *Streptococcus thermophilus*. Some commercial probiotic products are listed in Table 2.2.

Table 2.2 Some commercial probiotic products (Fasoli *et al*., 2003; Coeuret *et al*., 2004; California Dairy Research Foundation and Dairy & Food Culture Technology, 2007)

Products	Brand	Probiotics present in the product as shown on food label
Yoghurts	ABC (Sitia YOMO, Milano, Italy)	*L. acidophilus; Bifidobacterium; L. casei*
	Activia (Danone, Milano, Italy)	*B. bifidus*
	Biospega (Spega, Vicenza, Italy)	*L. acidophilus; Bifidobacterium*
	Kyr (Gigio, Reggio Emilia, Italy)	*L. acidophilus; Bifidobacterium* spp.
	Teddy (Fattoria Scaldasole®, Latina, Italy)	*Bifidobacterium*
Lyophilised products	Bifilact (Fidia Nutraceutical S.p.A., Padova, Italy)	*L. delbrueckii* subsp. *lactis*; *L. delbr.* subsp. *bulgaricus*; *L. acidophilus; S. thermophilus; B. bifidum; L. sporogenes; S. cerevidiae*
	Floraviva (Tredi farmaccutici S.r.l., Vcrona, Italy)	*Life yeast; L. bulgaricus; L. lactis; L. acidophilus; S. thermophilus; B. bifidum*
	Infloran (Istituto farmacologico BERNA, Svizzera)	*L. acidophilus; B. bifidum*
	Neolactoflorene (Newpharma S.r.l., Milano, Italy)	*L. acidophilus; B. bifidum; S. thermophilus; L. bulgaricus; L. sporogenes*
Yakult	Yakult (Tokyo, Japan)	*L. casei* Shirota *B. breve* strain Yakult
BioGaia Probiotic chewable tablets or drops	Biogaia (Stockholm, Sweden)	*L. reuteri* ATCC 55730

L. reuteri DPC16 has also been combined with natural food ingredients from New Zealand cattle, sheep, deer, seafood, colostrum and whey powder to serve as health beneficial products (Joshi, 2005).

2.2 The Gastrointestinal (GI) tract

The gastrointestinal tract starts from the mouth and proceeds to the oesophagus, stomach, duodenum, small intestine, large intestine (colon), rectum and finally ends with the anus (Figure 2.2). The major functions of the GI tract are digestion and excretion.

Oral colon-specific delivery microorganism is a novel approach that has attracted

considerable attention (Teoh *et al*., 2011; Yan *et al*., 2011). Oral bacterial cell therapy is based on oral delivery of live microorganisms which are immobilised in food grade polymeric materials to protect the cells from the adverse environment of the stomach and upper small intestine. Because of the potential health benefits of probiotics, there has been an increasing interest in delivery of probiotic bacterial cells using colon-targeted delivery systems (Prakash & Malgorzata Urbanska, 2008).

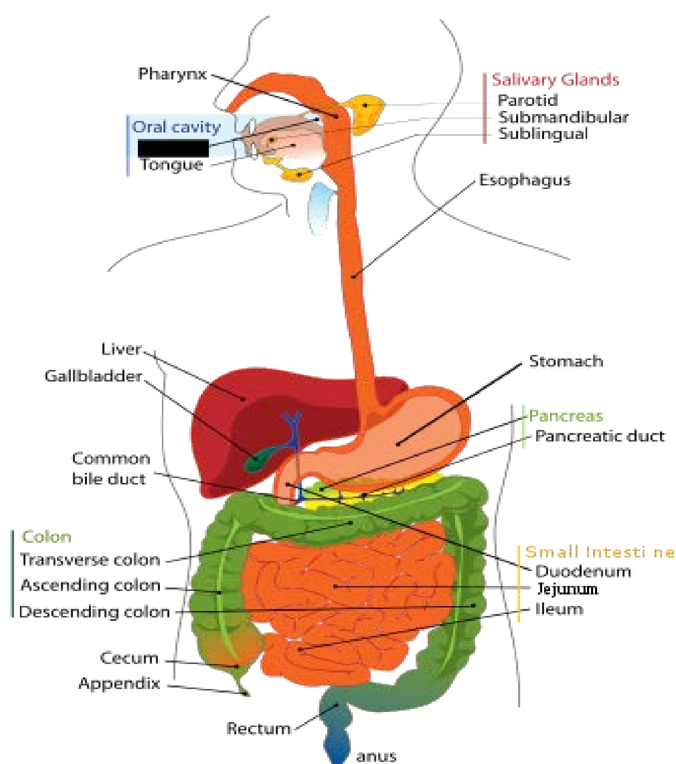


Figure 2.2 GI tract (Phloe, 2009).

2.2.1 Gastrointestinal transit time

Normally, all food should leave the stomach within 2 to 4 h (Jayaram *et al*., 1997; Ronland, 2011). Usually, glucose stays for 1 h, protein 2-3 h, and fat 5-6 h. The physical size of any solid material and the state of the stomach (empty or full) also affect the retention time of food. For small pellets or liquids, the retention time is quite short. In contrast, it takes a longer time for large pellets to go through the stomach

(McConnell *et al*., 2008a). The speed of gastric emptying determines the passage time of the probiotic (Fukui *et al*., 2000), but this varies, depending on individual circumstances (age, gender, health, dietary pattern, etc.). To enhance the efficiency of oral colon-specific delivery, the shorter the time the bacterial cells are exposed to the low pH environment in the stomach, the better the chance of viable cells passing to the next stage (Martoni *et al*., 2007).

Compared with the stomach, the transit time through the small intestine is much more regular. It is not affected by the physical state or type of food and the average transit time is 3-4 h (Davis *et al*., 1986).

Compared with the transit time through the small intestine, the time passing through the large intestine varies considerably. Liquid and small particles take a longer time than do large tablets (Hardy *et al*., 1985), which generally take 20-35 h for transit through the colon in an adult. Such a long transit time makes the colon an ideal release site for probiotic bacteria to exhibit their health- beneficial effects on the host (Kushwaha *et al*., 2010; Gangurde *et al*., 2011). However, the colon transit time may be affected by the local physiological conditions and the host's individual condition.

2.2.2 pH variation along the GI tract

The variation of pH along the GI tract directly determines the release behaviour of oral colon-specific delivery systems (Yang *et al*., 2002). The pH of an empty and a full stomach is pH 1.2-2 and pH 2-6, respectively, which will affect the efficiency of any pH-sensitive delivery system. The pH along the GI tract does not continuously increase: from the jejunum through the middle of the small intestine to the ileum, the pH increases from pH 6.6 to 7.5, then decreases slightly to pH 6.4 on reaching the right colon. It stabilises between pH 6.6 and 7.0 in the middle colon and in the left colon (McConnell *et al*., 2008a). Because there is only a small difference between the ileum and the colon, any pH-dependent delivery system may release the active component in the ileum rather than in the colon (Evans *et al*., 1988; McConnell *et al*., 2008b).

2.2.3 Intestinal flora and bile salts

The bacterial inhabitants of the human GI tract constitute a large and complex ecological system, including aerobic and anaerobic microorganisms. They are mainly *Bifidobacterium*, *Eubacterium, Peptostreptococcus, Propionibacterium, E. coli* and

lactic acid bacteria (Lau *et al*., 2004). Although under normal circumstances, the entire intestinal microflora system stays in balance both in composition and quantity, when the colon is in a pathological state, the balance might be significantly disturbed (Gorbach, 2000). Subsequently, the unbalanced condition may affect the efficiency of an oral colon-specific delivery system (Kumar *et al*., 2011).

Generally, the concentration of bile salts in bile is 0.8%, and varies between 0.2% and 2.0% in the small intestine (Whitehead *et al*., 2008). The maintenance of sufficient cell viability in the presence of bile salts is deemed to be a criterion for the selection of potential probiotics (Kailasapathy & Chin, 2000; Kosin & Rakshit, 2006). Different probiotic strains show different resistance to normal concentrations of bile salts, and it is suggested that the survival rate of probiotic strains should be between 20% and 40%. In contrast, many microorganisms have a survival rate of less than 0.1% (Bezkorovainy, 2001).

2.2.4 Colonic environment

Two characteristics of the colon, i.e. the small surface area compared to the small intestine and the lack of endogenous digestive enzymes, make it a sub-optimal site for probiotic bacteria displaying their health beneficial effects. However, the colon is the residence of many microorganisms, and the long transit time may create an ideal environment for release of probiotic cells.

2.2.5 Digestive enzymes in the GI tract

Pepsin is an enzyme that is released by the cells in the stomach and degrades food proteins into peptides. According to the United States Pharmacopeia (1990) and the International Pharmacopeia (Food and Agriculture Organisation of the United Nations, 2001), pepsin is an integral part of gastric fluid, and thus must be incorporated when designing a simulated gastric fluid. The enzymatic activity of pepsin functions best in acidic environments, particularly those with a pH of 1.2-2.

Pancreatin is a mixture of several digestive enzymes (for example, amylase, lipase and protease) produced by the exocrine cells of the pancreas. When simulating the intestinal environment, pancreatin is a necessary component. Although the pH of the small intestine (pH 7.0-8.5) is favourable towards bacterial survival, the presence of pancreatin and bile salts may have adverse effects on the probiotic cells.

Human feces contain bacterial enzymes that can degrade water-soluble A, B, and H antigens (Hoskins, 1969), which may thus affect the survival of probiotic bacterial cells in the colon. Conversely, probiotic bacterial cells may affect the activity of fecal enzymes (Goldin *et al*., 1980; Haberer *et al*., 2003).

2.2.6 Simulated GI tract

The difficulties involved in studying bacterial adhesion *in vivo* have led to the development of *in vitro* model systems for the preliminary selection of potentially probiotic strains (Saremdamerdji *et al*., 1995; Vesterlund *et al*., 2005).

Recently, various *in vitro* GI tract models have been established for probiotic research (Jacobsen *et al*., 1999; Liao *et al*., 2009). An ideal model should take into consideration the above-described physical parameters, such as the transit time, pH variation and intestinal flora (De Jong *et al*., 2007).

The most conventional GI tract model involves incubation of bacterial cells in an acidic solution, (pH 1-3); followed by incubation in a bile salts solution at pH 7 (Jacobsen *et al*., 1999). Mainville (2005) designed a dynamic human upper GI tract model which consisted of two reactors: the simulated stomach conditions (acid environment) and the simulated duodenum conditions (the presence of bile), to screen potential probiotic strains.

The simulation of the entire human GI tract may use a system that involves human fecal material (Molly *et al*., 1993). Such a model has been used to investigate interactions of probiotics with human intestinal microflora (Molly *et al*., 1993), and the effects of probiotics on the system (Gmeiner *et al*., 2000).

In addition, the release and absorption characteristics of a colon-specific delivery system may be affected by the disease status of the large intestine. In general, the absorption of many active compounds is subject to intestinal diseases such as inflammatory bowel disease (Linskens *et al*., 2001), Crohn's disease (Akerlund *et al*., 1994), constipation (McConnell *et al*., 2008a) diarrhea (Banwell *et al*., 1971) and gastroenteritis. Hence, if necessary, the disease status should also be considered when the antimicrobial activity of probiotics is evaluated.

2.3 Microencapsulation and immobilisation

2.3.1 Introduction

Microencapsulation is a technology by which liquid droplets, solid particles or gas compounds are immobilised into protective beads. In the context of the present work, the immobilisation material should be a food grade agent (Mortazavian *et al*., 2007; Rossier-Miranda, 2010) and the concept is that the probiotic bacterial cells can be immobilised by microencapsulation and thus protected from the harsh conditions of low pH, bile salts and digestive enzymes that exist in the GI tract, thus allowing safe delivery of the cells to the colon, where they will be released (Gilliland & Walker, 1990). Generally, the preparation of a microencapsulated product involves three sequential steps: first, identification of the goal of microencapsulation, including any subsequent release of the immobilised material; second, selection of the immobilisation materials that provide the desired protection and release characteristics; finally, selection of a method of preparation, e.g. whether to use the extrusion or emulsion method, to prepare the beads. The selection of a biomaterial is a critical step because both the size and shape of the formed beads depend on the materials and methods used to prepare them (Gharsallaoui *et al*., 2007).

Depending on the physicochemical properties of the bead core, the wall composition, and the microencapsulation technique, different types of beads can be obtained: (i) a simple single cell bead, in which the core material surrounds the cell with a single layer of uniform thickness (Figure 2.3 i); (ii) a particle of an irregular shape due to several cells being immobilised in a continuous matrix (Figure 2.3 ii); and (iii) a bead with several distinct layers within the same capsule, surrounded by a wall (Figure 2.3 iii).

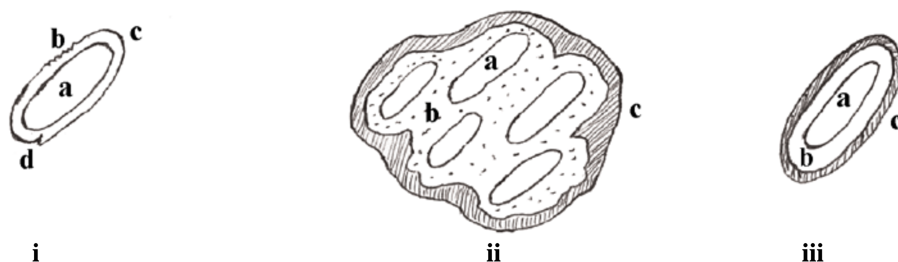


Figure 2.3 Structure of micro beads (Mortazavian *et al*., 2007). i, Single cell bead: Bacterial cell (a), uneven surface (b), even surface (c), crackled surface (d); ii, Multicell bead: Bacterial cell (a), interstitial liquid (b), capsule (c); iii, Coated bead: Bacterial cell (a), capsule (b), coat/shell (c).

Figure 2.4 shows five major methods of encapsulation/immobilisation (Yi *et al*., 2006). Of these, only entrapment and microencapsulation are relevant to the present project because of the need to protect the bacterial cells from the external environment. At present, probiotic bacterial cell entrapment in polymeric gels appears to be the most applicable immobilisation method as it allows the use of food-grade immobilisation materials and gentle techniques of immobilisation.

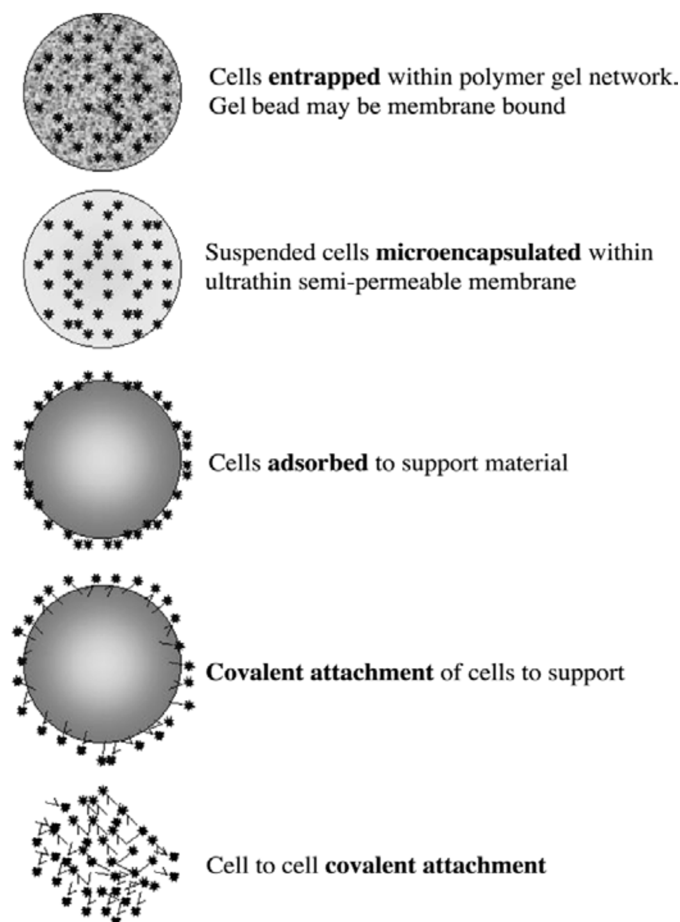


Figure 2.4 Cell immobilisation methods (Yi *et al*., 2006).

2.3.2 Commonly used immobilisation materials

Chemicals that can form a barrier to protect the viability of bacterial cells inside microbeads and block the adverse effects of the GI tract are called encapsulation wall or coating materials. Alginate (Li *et al*., 2011a; Sohail *et al*., 2011), chitosan (Ma *et al*., 2008; Chavarri *et al*., 2010), hydroxypropyl methylcellulose, gelatin (Annan *et al*., 2008), Eudragit S (Abdelkader *et al*., 2008) and starch are the most commonly used materials. The criteria for selection of an encapsulation material for probiotic cells are shown in Figure 2.5. Basically, an ideal material must be easily to form gel matrix (Desai & Park, 2005). The safety issue is also needed to be taken into consideration (Kim *et al*., 2000). Further, because of the poor survival of some probiotics in the harsh

environment along the GI tract, an immobilisation material also should have the ability to protect probiotic cells (Anal *et al.*, 2003).

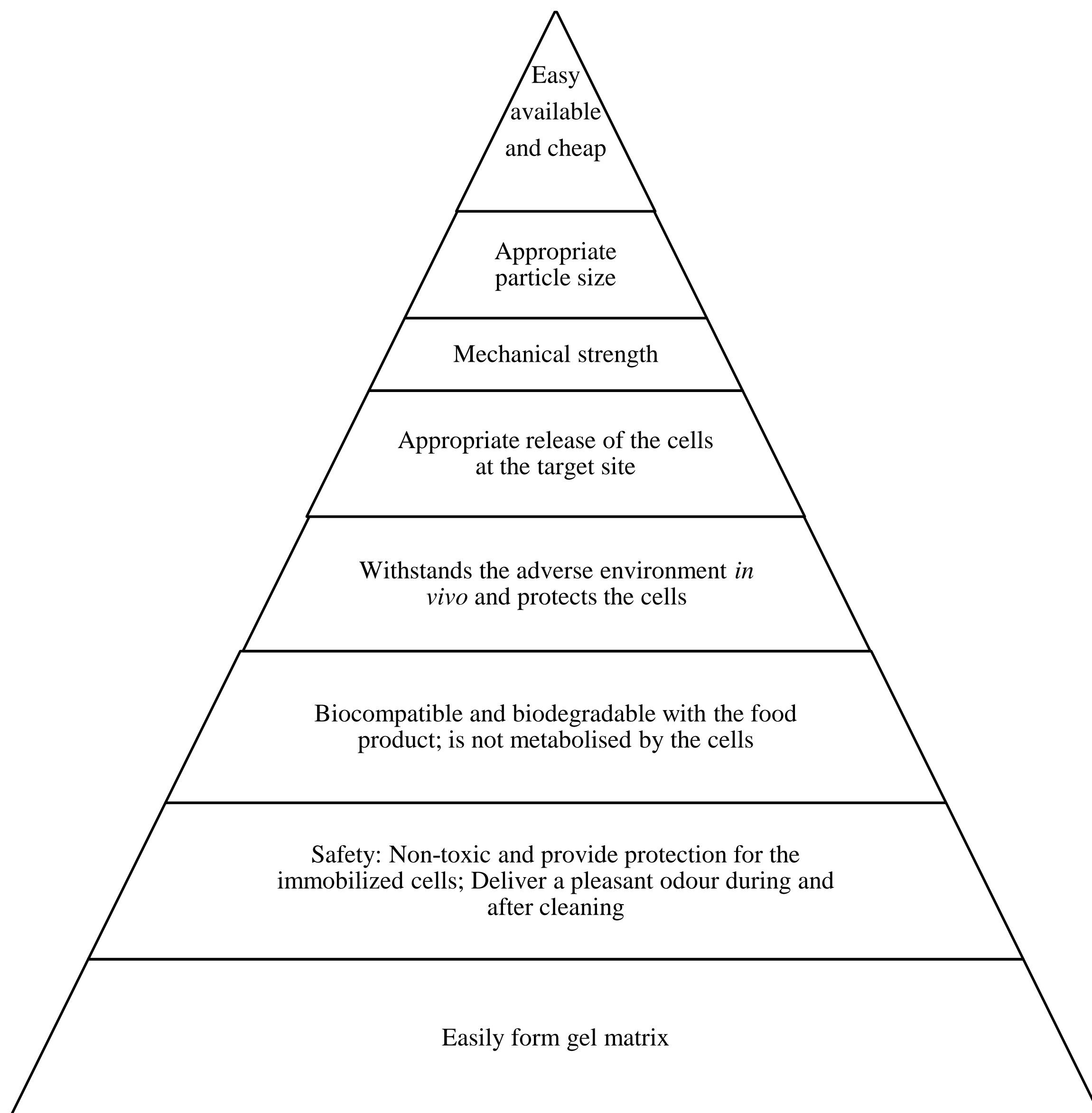


Figure 2.5 The criteria for the choice of coating material for probiotic cells (Brazel, 1999; Kim *et al*., 2000; Anal *et al.*, 2003; Desai & Park, 2005).

2.3.2.1 Carbohydrates

2.3.2.1.1 Alginate

Alginate is a viscous gum which has abundant distribution in the cell walls of brown algae. It is a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, covalently linked in different sequences or blocks (Haug *et al.*, 1967) (Figure 2.6).

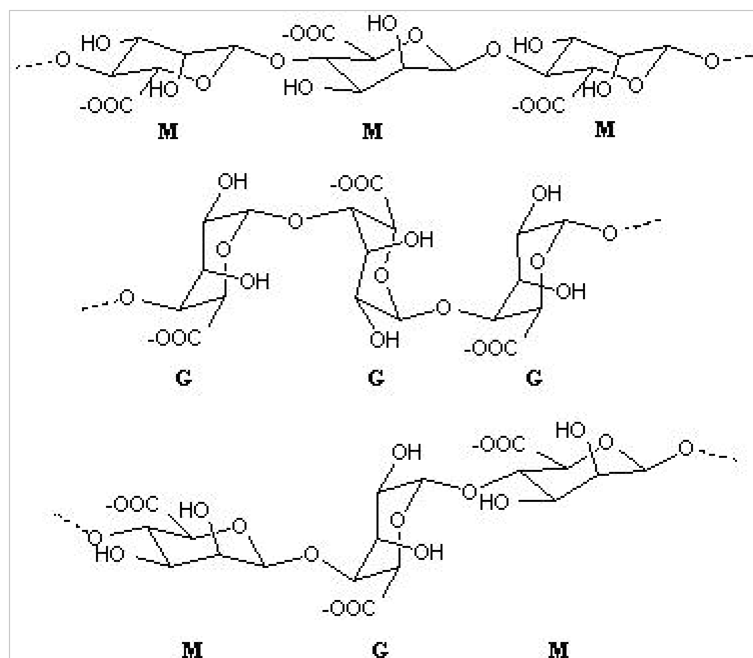


Figure 2.6 The structure of the chemical units of alginate (M = mannuronic acid and G = guluronic acid) (Haug *et al.*, 1967).

The single calcium alginate polymer is the most common type of material in cell immobilisation. Cross-linking of alginate polymer occurs so that when sodium alginate is extruded into a solution containing calcium ions, the calcium ions replace the sodium ions in the polymer. Sodium citrate and EDTA are usually used when required, to

dissolve the cross-linking between alginate and Ca^{2+}, and the same effect can be achieved using phosphate.

Alginate beads have some advantages (Klien *et al.*, 1983; Tanaka *et al.*, 1984; Martinsen *et al.*, 1989; Prevost & Divies, 1992; Dimantov *et al.*, 2003; Chandramouli *et al.*, 2004; Gouin, 2004) as they:

- easily form gel matrices around bacterial cells;
- are safe and biocompatible;
- are easily prepared; and
- dissolve in the intestine and release the cells.

However, it has been reported that alginate beads undergo shrinkage and suffer decreased mechanical strength in acidic conditions (Audet *et al.*, 1988), which might weaken the protective role of the beads. Also, as alginate gel is formed by cross-linking with calcium ions, its integrity will deteriorate if monovalent ions or chelating agents such as phosphate or citrate are present in the surrounding medium (Birnbaum *et al.*, 1981; Cekic *et al.*, 2007; Mortazavian *et al.*, 2007).

2.3.2.1.2 Chitosan

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (Figure 2.7).

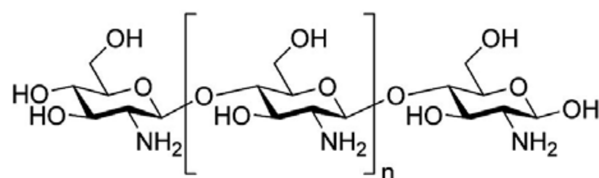


Figure 2.7 Chemical formula of chitosan (Pandya, 2008).

Chitosan is positively charged and soluble in acidic to neutral solution with a charge density dependent on pH and the degree of acetylation. Because chitosan is bioadhesive

and readily binds to negatively charged surfaces such as intestinal mucosal membranes, it is an ideal coating material for probiotics. Additionally, other properties, such as its biocompatibility, low toxicity and biodegradability (Ichikawa *et al*., 2005; Prabaharan, 2008), and the fact that it can be metabolised by some human enzymes, have made chitosan the subject of numerous investigations (Berger *et al*., 2004; Sarhan *et al*., 2010). Beads composed of layers of alginate- chitosan- alginate (ACA) can remain intact in simulated gastrointestinal fluid, while maintaining the viability of immobilised bacterial cells (Anal *et al*., 2003; Lin *et al*., 2008). The ideal ACA particle is shown in Figure 2.8 (Zhu *et al*., 2009).

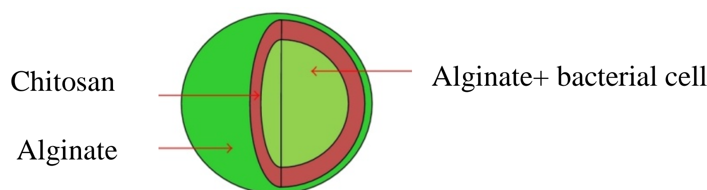


Figure 2.8 Structure of ACA beads (A = alginate and C = chitosan).

Because chitosan has low solubility in intestinal media, it can protect probiotic bacteria in transit to the colon. Calinescu & Mateescu (2008) have shown that chitosan is suitable for the colon delivery of a probiotic strain of *Lactobacillus rhamnosus*.

Unfortunately, chitosan rapidly absorbs water and swells in aqueous environments, leading to fast release of cells. To overcome these problems, a number of chemically modified chitosan derivatives have been synthesised and tested (Prabaharan, 2008).

2.3.2.1.3 Starch

Starch is another widely used coating material for immobilised cells, although a disadvantage is the propensity of amylose to swell in aqueous conditions. A combination of amylose and ethylcellulose has been shown to prevent gastric and small intestinal release while allowing release in the colon in an *in vitro* model (McConnell *et al*., 2007; Tuleu *et al*., 2002) and *in vivo* (Basit *et al*., 2004).

“Resistant” starch could fulfill the criteria for colonic delivery, not only because it

resists digestion in the small intestine, but because it can be digested by enzymes produced by colonic microorganisms (Basit, 2005).

2.3.2.2 Proteins

The most widely used proteins in microencapsulation or immobilisation are gelatin and whey protein.

Gelatin is extracted from the collagen inside animal skin and bones, and is a positively charged polymer. Because of it melts when heated and solidifies when cooled, it is widely used as a gelling agent in food, pharmaceutical, and cosmetic preparations (Carr *et al*., 1996; Karim & Bhat, 2008). At acidic pH values, gelatin is positively charged, and hence can form a complex matrix with negatively charged polysaccharides such as alginate. However, gelatin is readily hydrolysed by intestinal proteolytic enzymes.

By loading probiotic bacteria into beads based on a mixture of alginate and gelatin, probiotic bacteria have been reported to be continuously released during passage through a simulated gastrointestinal tract (Li *et al*., 2008). Annan (2008) immobilised the probiotic *Bifidobacterium adolescentis* 15703T in alginate- gelatine beads with the objective of enhancing the survival during exposure to the adverse conditions of the gastrointestinal tract. Results showed that the technique could effectively protect the cells in the simulated environment.

Because gelatin is pepsin-sensitive it may lose its integrity in only a short time when exposed to the simulated gastric fluid (Cannan & Muntwyler, 1930). For this reason, the protective effect of gelatin microencapsulation might be adversely affected.

Whey protein is a mixture of globular proteins isolated from whey, which is the liquid phase remaining after precipitation of a major milk protein fraction (caseins) in milk at pH 4.6 (Visser *et al*., 1991). The effects of whey protein on human health are being investigated in several areas to reduce disease risk, or as a supplementary treatment (Oben *et al*., 2008).

Lambert (2008) used whey protein combined with gum Arabic to form beads for delivery of a probiotic strain to the proximal region of the small intestine. Results demonstrated that the encapsulated cells survived gastric conditions as well as a pancreatin challenge. Whey protein immobilisation has been reported to protect freeze-

dried *Lactobacillus rhamnosus* R011 cells against subsequent acidic and alkaline pH conditions as well as heating and freezing of food products (Reid *et al*., 2007). The protective role of whey protein on the viability of other probiotic bacterial strains such as *Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus*, and *Bifidobacterium animalis* during storage has also been reported (Akalin *et al*., 2007).

2.3.2.3 Other food grade polymers

2.3.2.3.1 Hydroxypropyl methylcellulose (HPMC)

Hydroxypropyl methylcelluloseis a semisynthetic, inert, viscoelastic polymer used as an ophthalmic lubricant, as well as an excipient and controlled-delivery component in oral delivery systems. It can be found in a variety of commercial products (De Silva & Olver, 2005; Li *et al*., 2010).

HPMC can be directly added to food as an effective emulsification agent, adhesive and thickening agent. A combination of HPMC with phytowax as a matrix component has been reported as an immobilisation material (Sahoo *et al*., 2008).

2.3.2.3.2 Eudragit S

Enteric coating can be used to protect food from release in the stomach, and release it when exposed to the alkaline condition of the small intestine. The most commonly used polymer is the polymethacrylic acid / methyl methacrylate ester co-polymer (Eudragit S) which is designed to dissolve above pH 7. Therefore, intestinal pH variation can act as a physiological trigger mechanism for targeted release in the lower small intestine (Tyagi *et al*., 1998; Alhnan & Basit, 2011). For many pH-dependent release mechanisms, Eudragit S appears to be an ideal coating material for immobilised cells within beads (McConnell *et al*., 2008a).

2.3.2.3.3 Skim milk

Skim milk is commonly used as a protective agent during freeze-drying of bacterial cells (Khoramnia *et al*., 2011; Najafi *et al*., 2011; Zheng *et al*., 2011).

When skim milk is used in bacterial cell immobilisation, its presence can enhance the strength of alginate due to the contribution of calcium ions (Ross *et al*., 2008).

Also, because skim milk contains lactose, it can provide nutrition for the growth of the

cells. Ross (2008) used non-fat milk as an immobilisation material for probiotic cells and demonstrated that a bacterial cell suspension in a 1:1 (v/v) mixture of 20% non-fat milk and 1.8% sodium alginate was the optimal concentration for reaction with 0.1 M calcium chloride to protect cells in a simulated gastrointestinal fluid followed by their subsequent release.

2.3.2.4 Summary of the calcium alginate based immobilisation system

Calcium alginate immobilisation is the most commonly used technique in probiotic delivery and new techniques based on the gel are constantly emerging. Formation of a shell around the alginate bead has been verified to considerably improve the efficacy of the basic system. Table 2.3 lists some of the alginate-based systems.

A combination of alginate with starch has been shown to be effective in protecting cells of lactic acid bacteria in a stressful environment (Jankowski *et al*., 1997; Sultana *et al*., 2000; Sun & Griffiths, 2000; Krasaekoopt *et al*., 2003). It also has been reported that by coating a semi-permeable chitosan layer around the alginate beads, the structure became denser and more stable, and cell survival in an adverse environment was improved (Smidsrod & Skjakbraek, 1990; Krasaekoopt *et al*., 2003; Zhou *et al*., 2010). Low-molecular-weight chitosan is often chosen as a supplementary material with alginate-Ca^{2+} gel because of its rapid diffusion into the alginate matrix. The density of the resulting bead was high and it had great strength, which provided effective protection to immobilised cells (Chandramouli *et al*., 2004). Coatings of polyethylenamine and glutaraldehyde (as other types of polycationic polymers) on the alginate capsules have also been reported (Shen *et al*., 2009).

Table 2.3 Some examples of coatings of calcium alginate-based beads

Material	Reference
Alginate, $CaCl_2$, and gelatin	Annan *et al*., 2008
Alginate, $CaCl_2$, and chitosan	Li *et al*., 2008; Ma *et al*., 2008; Wang *et al*., 2001; Pandey & Khuller, 2004
Alginate, $CaCl_2$, and Phosphorylated chitin	Jayakumar *et al*., 2009
Alginate, $CaCl_2$, and PolyelectrolytePLL	Machluf *et al*., 2003
Alginate, $CaCl_2$, and Eudragit RL	Lamberti & Sefton, 1983

2.3.3 Microencapsulation/Immobilisation techniques

The technology of probiotic encapsulation or immobilisation normally includes two stages: the encapsulation stage and the drying stage. Extrusion and emulsion techniques are two basic methods for encapsulation or immobilisation of probiotic bacterial cells. Freeze drying, spray drying (Goderska & Czarnecki, 2008) and fluidised bed drying are three commonly used drying techniques for encapsulating or immobilising probiotics.

2.3.3.1 Extrusion method

Extrusion is a technology that applies pressure to a mass until it flows through an orifice under controlled conditions, thus forming a uniform product. Figure 2.9 is a sketch of the extrusion method. By mixing the immobilisation material and the probiotic bacteria, followed by passing the mixture through a particular "tunnel", a compound drop composed of a droplet of core fluid encased by a layer of shell fluid forms. The shell (and possibly the core) is then hardened in a hardening bath by appropriate means, e.g., by chemical cross-linking in the case of polymers (Kailasapathy 2002; Cellesi & Tirelli, 2005; Mealman *et al*., 2011), cooling in the case of fats or waxes, or solvent evaporation (Ahrne *et al*., 2008).

There are several factors in the extrusion system that determine the size of the beads produced, including the physical characteristics of the extruded fluids (densities, viscosities, and interfacial tensions), the processing conditions (flow rates and temperatures) and the diameters of the orifices.

Alginate based xanthan gum microencapsulation by the extrusion technique has been reported to effectively increase the survival of *Lactobacillus acidophilus* LA14 and *Bifidobacterium lactis* BI-07 in acid conditions (Albertini *et al*., 2010). Teoh (2011) introduced a double-coating encapsulation system with chitosan-coated alginate-starch, using the extrusion technique, to protect the probiotic strains *Lactobacillus acidophilus* LA-5 and *Bifidobacterium pseudocatenulatum* G4 from heat treatment and high salt stress. Experimental results showed that the extrusion method was efficient in maintaining the viability of encapsulated probiotic strains.

Recently, a new technique using centrifugally induced artificial gravity conditions to modify the extrusion method was reported (Haeberle *et al*., 2008). Compared with existing methods, this novel method offers pulse-free and reproducible droplet

generation. One advantage of the centrifugal technology is that it can encapsulate highly viscous liquids within a small diameter.

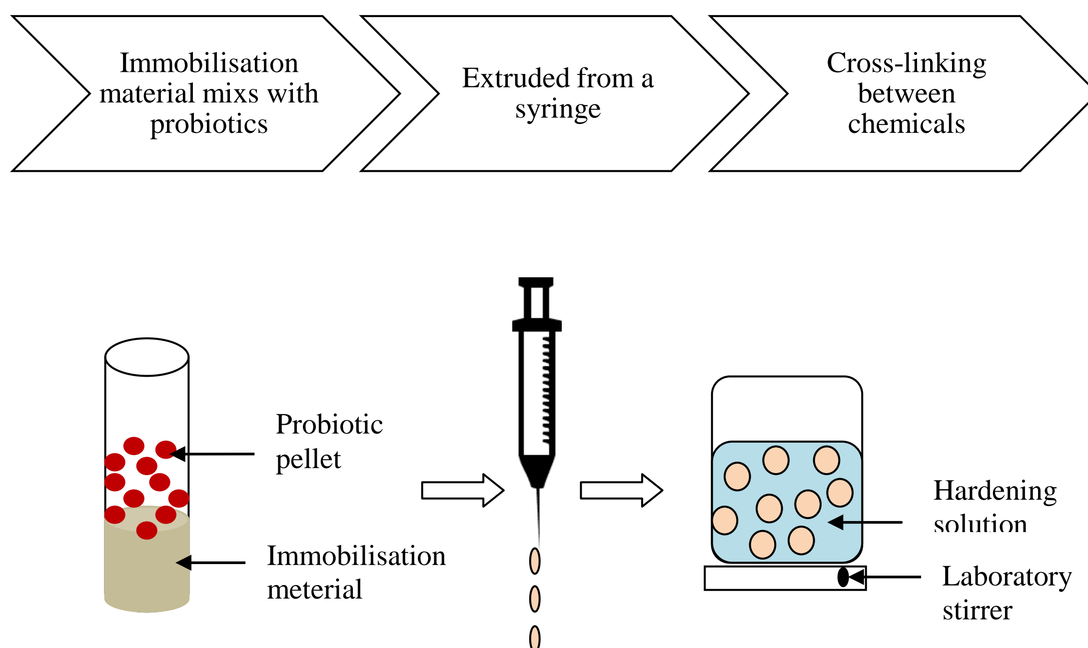


Figure 2.9 Sketch of an extrusion method (Kontturi *et al.*, 2011)**.**

2.3.3.2 Emulsion method

Emulsion or internal gelation is an alternative method to extrusion. Usually, in this technique, a small volume of cells within a microsphere polymer is added to a large volume of vegetable oil (Groboillot, *et al*., 1993). Figure 2.10 shows a preparation method of single water-in-oil (w/o) emulsions. In this case, the active ingredient, such as probiotic cells, is added to an aqueous biopolymer solution and emulsified in a hydrophobic phase such as vegetable oil to form a w/o emulsion. The biopolymer in the dispersed emulsion droplets is then cross-linked using various methods (chemical, thermal, and enzymatic), depending on the type of biopolymer employed. Following gelation, the formed beads are washed with fresh water to remove oil to accomplish encapsulation (Kailasapathy, 2002). The emulsion technique is easily scaled up and the

diameter of the produced beads can be considerably smaller than that produced by extrusion. However, this method is costly because of the use of the vegetable oil (Krasaekoopt *et al*., 2003).

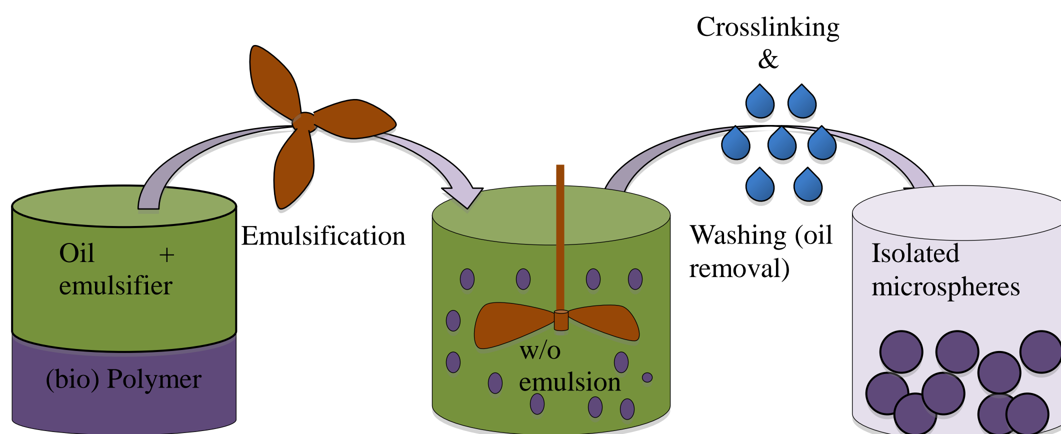


Figure 2.10 Processing scheme of single emulsion technique for encapsulation (Gate2tech, 2008).

Because the emulsion technique is easily performed, it is widely used in probiotics-related experiments. For example, it has been reported that *Lactobacillus acidophilus*, a probiotic that has poor acid resistance, entrapped in micro beads by an emulsion method can have a significantly improvement ($P<0.05$) in the survival rate in simulated gastric juice and its resistance to bile (Kailasapathy, 2002; Shima *et al*., 2009). Crittenden (2006) investigated the viability of encapsulated *Bifidobacterium infantis* using an emulsion method in an *in vitro* simulated gastrointestinal tract. The experimental result showed that the survival rate of encapsulated cells was significant higher ($P<0.05$) than that of the non-encapsulated bacterial cells. It was also reported that emulsion solvent removal method allows for the optimised release of the encapsulated material (Obeidat, 2009).

2.3.3.3 Comparison

The terms immobilisation and encapsulation are used interchangeably in most literature. Encapsulation is the process of forming a continuous coating layer around an internal

matrix of beads containing a core material, while immobilisation refers to the trapping of core material within or throughout a matrix.

In general, extrusion is a simple and low cost method. By using gentle operations, damage to the encapsulated cells is minimised, i.e. it can maintain a relatively high number of viable cells. Moreover, biocompatibility and flexibility are other advantageous characteristics of this method (Klein *et al*., 1983; Martinsen *et al*., 1989; Tanaka *et al*., 1984). However, its biggest disadvantage is the difficulty of its application to large scale production due to its relatively slow formation of microbeads. In comparison, the emulsion method can be easily scaled up and the size of beads produced is relatively small. However, this method requires more cost for performance than does the extrusion method (Krasaekoopt *et al*., 2003). Further, it is difficult to collect the resulting beads; and the involvement of the washing step to remove oil, made emulsion technique a complex procedure.

2.3.4 Drying stage

2.3.4.1 Freeze-drying

Freeze-drying is adopted to preserve material or to make material easier to transport following a dehydration process. The freeze-drying cycle consists of several steps which are demonstrated in Figure 2.11. Briefly, a freezing stage first converts most of the water contained in the product into ice which is then removed by direct sublimation (primary drying stage). Next, any unfrozen water is removed by desorption in the secondary drying stage. The water removed from the product is converted into ice in the condenser.

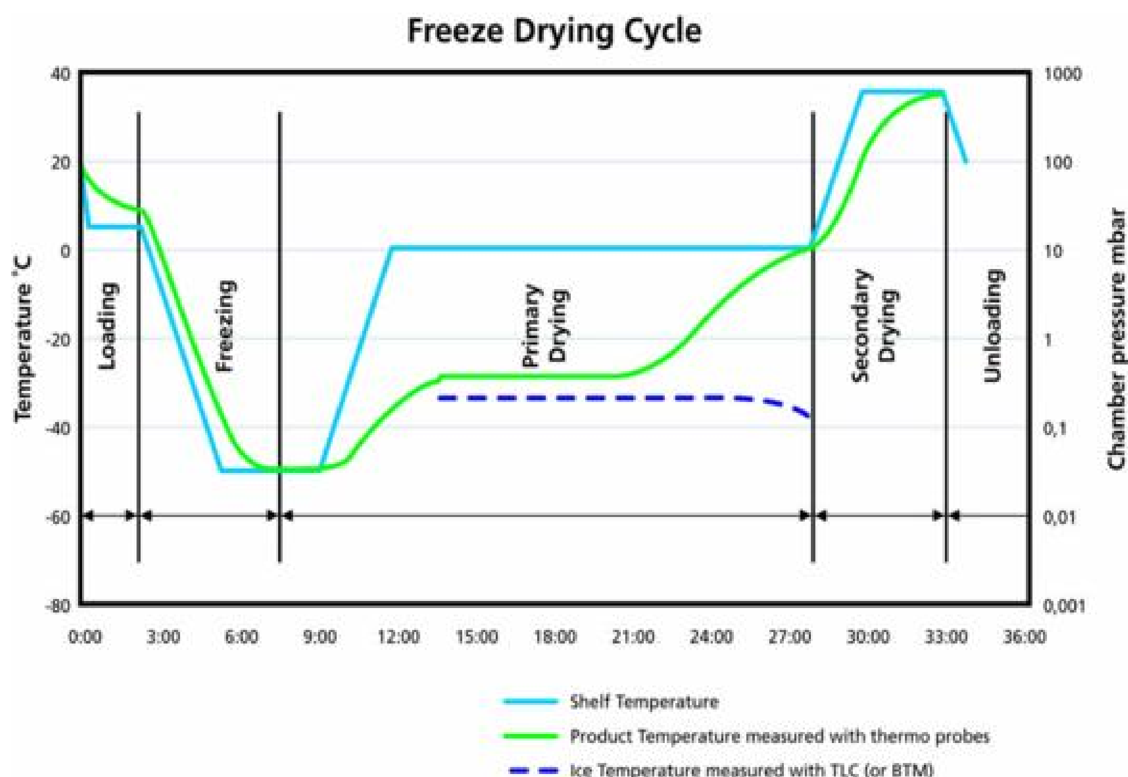


Figure 2.11 Freeze-drying cycle (GEA Pharma systems, 2009).

Freeze-drying is widely utilised in biotechnology and in the food industry (De Beer *et al.*, 2009; Siaterlis *et al.*, 2009)**,** and is widely used to preserve bacterial cultures. The performance of freeze-drying on immobilised and free *L. acidophilus* cells was studied by Tsen (2007). Results revealed that freeze-drying was an appropriate method for developing a dried product containing immobilised *L. acidophilus.*

However, freeze-drying has its drawbacks. Firstly, it is a relatively expensive process as the equipment used is several times as expensive as the equipment used in other drying processes. Secondly, freeze-drying also needs a long process time. Thirdly, the low operating temperature (between -50°C and -80°C) of the process may lead to viability loss of the cells. According to the results of Bolla (2011), freeze-drying was not suitable for several probiotic strains, including *Lactobacillus plantarum*, and *Lactococcus lactis*, for long term storage.

2.3.4.2 Spray-drying

Spray-drying is another widely used drying method. It converts the feed liquid droplets, containing solids, into a powder by means of hot air (Moller & Fredsted, 2009) (Figure 2.12).

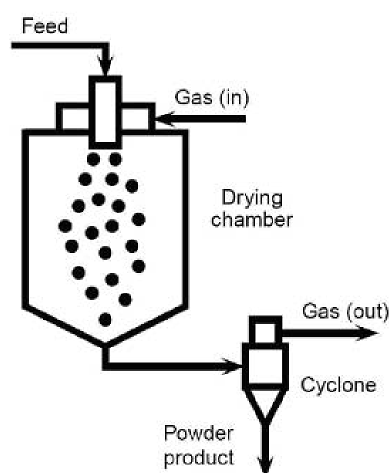


Figure 2.12 Spray drying cycle (CHEC, 2009).

To serve as a microencapsulation technique, the bacterial cells in the feed suspension become entrapped in the polymer which is also in the feed material, giving a dried particle after being dried in the drying chamber. The main advantages are the short contact time in the dryer and the economical operation.

Spray drying has been reported to protect probiotic cells from cellular injuries and long term storage damage (Ananta, 2005). While the probiotics must withstand the heat during the drying process. Goderska (2008) found that spray drying can stabilise the viability of *Lactobacillus* and *Bifidobacterium* strains during subsequent storage.

2.3.4.3 Fluidised bed drying

Generally, fluidised bed processing is involving drying, cooling, agglomeration, granulation, and coating of particulate materials. It is an ideal method both for heat sensitive and non-heat sensitive products because the temperature can be adjusted. Figure 2.13 shows a scheme of a fluidised bed drying process. In this case, the heat

pump system is served as a heat drying source, and the system was proved to be efficient for probiotic yeast (Joshi & Thorat, 2011).

Stummer (2010) compared the efficiency of drying cells by freeze-drying with drying by fluidised bed technology. The results showed that a gentle fluidised bed drying process was a suitable method for drying probiotic microorganisms.

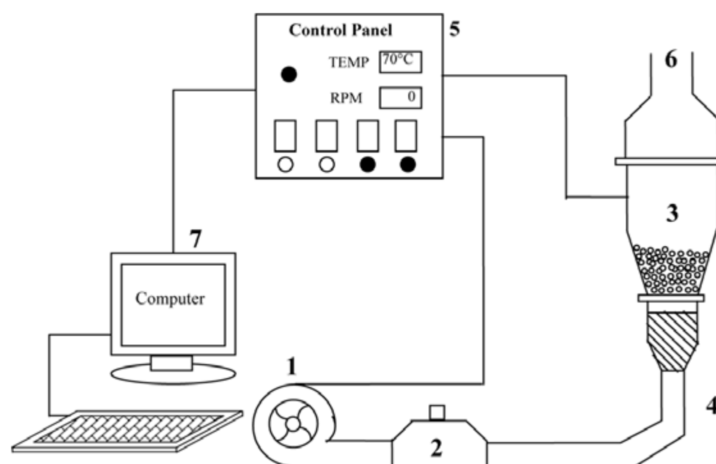


Figure 2.13 Scheme of heat pump fluidised bed drying: 1, blower; 2, heater; 3, fluidising chamber; 4, temperature sensor; 5, control panel; 6, exit air; 7, computer (Joshi & Thorat, 2011).

2.3.4.4 Comparison

Freeze-drying and spray-drying are the most commonly used industry drying techniques involved in the preservation of probiotics because of their relatively low energy consumption (Broadhead *et al*., 1994). However, the drying process can cause structural and physiological injury to the microorganisms (Prasad *et al*., 2003). The inlet air temperature of fluidised bed drying can be much lower than that for conventional spray drying, and the choice of matrix molecules in the fluidised bed drying process is broader than for traditional spray drying. But fluidised bed drying can be applied only to concentrated cultures (Champagne & Gardner, 2001).

2.3.5 Colon Release of immobilised probiotic cells

The delivery destination and release mechanism are critical issues when evaluating the

effectiveness of microencapsulation or immobilisation as a delivery technique. Thus, during the design process, it is necessary to confirm where and how delivery will take place. In addition, the mechanism of the microstructure breakdown must be taken into account. For example, using the calcium alginate gel system, by changing the concentration of the main contents the release site can be changed (Woraharn *et al.*, 2010).

Controlled release can be designed as a trigger-induced system. Several release triggers are shown in Figure 2.14. Under certain circumstances, the triggers can activate the release of the immobilised cells and allow them to reach the desired target sites.

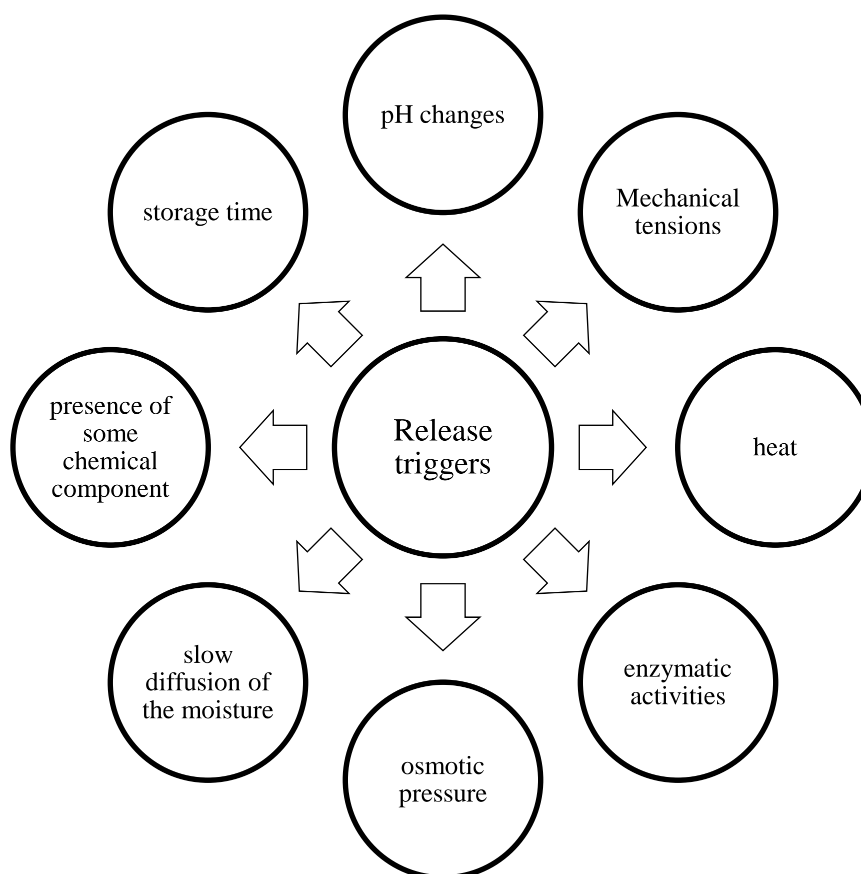


Figure 2.14 Some factors that can contribute to release of immobilised cells (Gouin, 2004; Mortazavian *et al.*, 2007).

In a healthy digestive tract, there is a shift in pH values as food goes through the system (Evans *et al.*, 1988; McConnell *et al.*, 2008a). The level of acidity and alkalinity could

act as a trigger element for pH-responsive probiotic delivery systems. Similarly, the gastrointestinal bacterial population increases along the gut, from 10^2 c.f.u./g in the stomach, to 10^4 c.f.u./g in the proximal small intestine, and to 10^7 c.f.u./g in the ileum (Bernhardt & Knoke, 1997; Mentula *et al*., 2005). There is a dramatic increase upon reaching the large intestine, to 10^{11}-10^{12} c.f.u./g (Topping & Clifton, 2001). This clear difference between the environments, e.g. pH change, can be used as a release trigger. In addition, the colonic bacteria produce a variety of enzymes which can also serve as release agents (McConnell *et al*., 2008b).

Depending on how the immobilised cells are administered, free cells may be released by diffusion, wall dissolution or by the biodegradation of the encapsulating polymer (Figure 2.15) (Moyano *et al*., 2002).

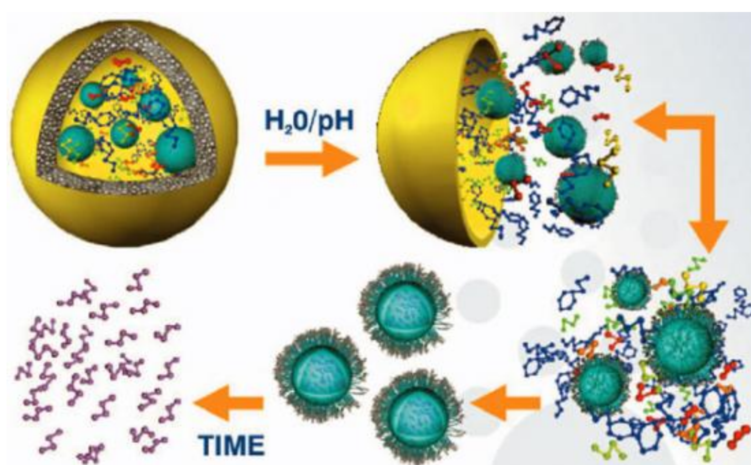


Figure 2.15 The controlled-release encapsulation system by H_2O or pH. Nanospheres (blue) containing the active ingredients (purple) are encapsulated with a microsphere (yellow). When been exposed to water or pH, the microsphere releases its contents, and over an extended period the nanosphere starts to release the active ingredients to the target site (ComputeScotland, 2010).

For alginate-$CaCl_2$ beads, two mechanisms may be involved in the release of immobilised cells: diffusion through the pore-network and/ or degradation/dissolution of the alginate (Raj & Sharma, 2003; Leonard *et al*., 2004). The pore size distribution on the surface of the beads determines the diffusion speed and therefore controls the rate of cell release (Choudhury & Kar, 2005). Finally, smaller beads release cells faster than do larger beads (Krasaekoopt *et al*., 2003).

2.3.6 Methods of studying adhesion

The adhesion of probiotic bacterial cells to the intestinal mucosal surface is a critical prerequisite for exerting beneficial effects on their hosts and is considered one of the main selection criteria for potential probiotics (Collins *et al*., 1998; Klaenhammer, 1982; Mattila-Sandholm *et al*., 1999; Wang *et al*., 2008; Dhanani *et al*., 2011). The longer that cells can adhere to the mucosal surface, the longer the time that the probiotics can exert their health-promoting effects (Mattila-Sandholm *et al*., 1999; Kailasapathy & Chin, 2000). The difficulties involved in studying bacterial adhesion *in vivo*, especially in humans, have led to the development of *in vitro* model systems for the preliminary selection of potentially adhesive strains (Saremdamerdji *et al*., 1995; Vesterlund *et al*., 2005). One of these *in vitro* models is the HT-29 cell line, which originates from human colon adenocarcinoma (Chauviere *et al*., 1992; Coconnier *et al*., 1992; Fogh *et al*., 1977; Pinto *et al*., 1983). The HT-29 cell model expresses not only its own characteristics, but also those of mature enterocytes, including polarisation, a functional brush border, and apical intestinal hydrolases (Lesuffleur *et al*., 1990). Hence, the HT-29 cell line is one of the best available models to study the adhesion of probiotic strains.

The CaCo-2 cell line is another well-characterised *in vitro* adhesion model (Chen *et al*., 2009). CaCo-2 cells express several characteristics of normal small intestinal villus cells and, additionally, can differentiate into intestinal epithelium cells. Therefore this cell line is widely used to study the mechanisms of adhesion and invasion of many pathogenic bacteria, including *Salmonella typhimurium* (Li *et al*., 2011b; Oliveria *et al*., 2011), *Listeria monocytogenes* (Lorentzen *et al*., 2011; Walecka *et al*., 2011), enteropathogenic (Liu *et al*., 2011) and enterotoxigenic *E. coli* (Laparra *et al*., 2010), and *Vibrio cholera* (Senoh *et al*., 2010). Hence, CaCo-2 cells *in vitro* provide an excellent environment for studying not only the mechanisms by which normal intestinal flora adhere to the intestine, but also how these bacteria may interact with pathogenic bacteria to compete for the same receptor (Li *et al*., 2011b).

Figures 2.16 and 2.17 show a simulated probiotic adhesion model and a competitive adhesion model for *in vitro* studies. During the assay of probiotic adhesion, the pre-stained probiotics are added onto the intestinal epithelial monolayer cells. The adhesion of the probiotic strain is observed under a functional microscope (Tian, 2011). Regarding to the inhibitory activity of a probitic strain on the adhesion of pathogens,

the probiotics compete with pathogens for the attachment sites on the monolayer model (Encarnacao, 2011).

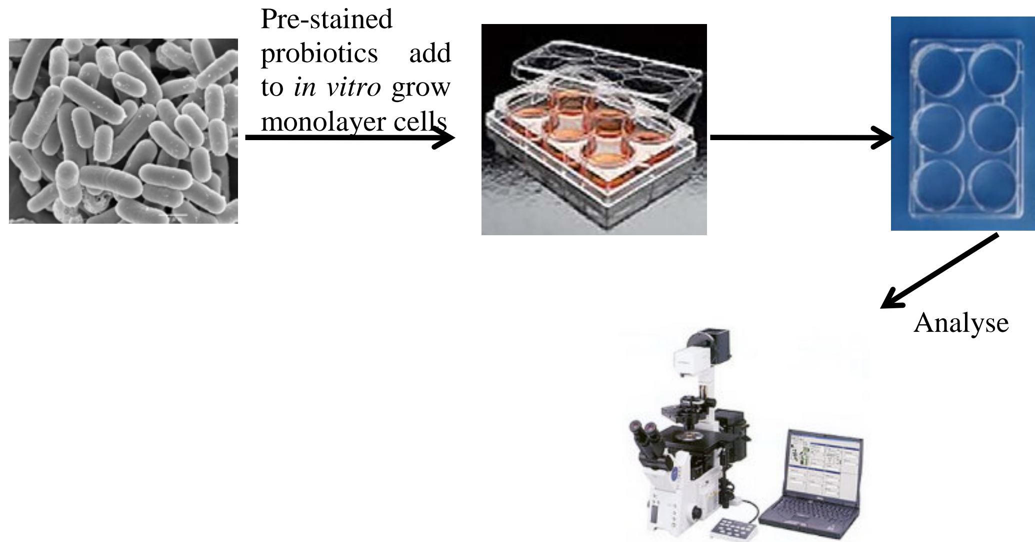


Figure 2.16 Assay of probiotic bacterial cells adhesion (the method used in the experimental part of this study).

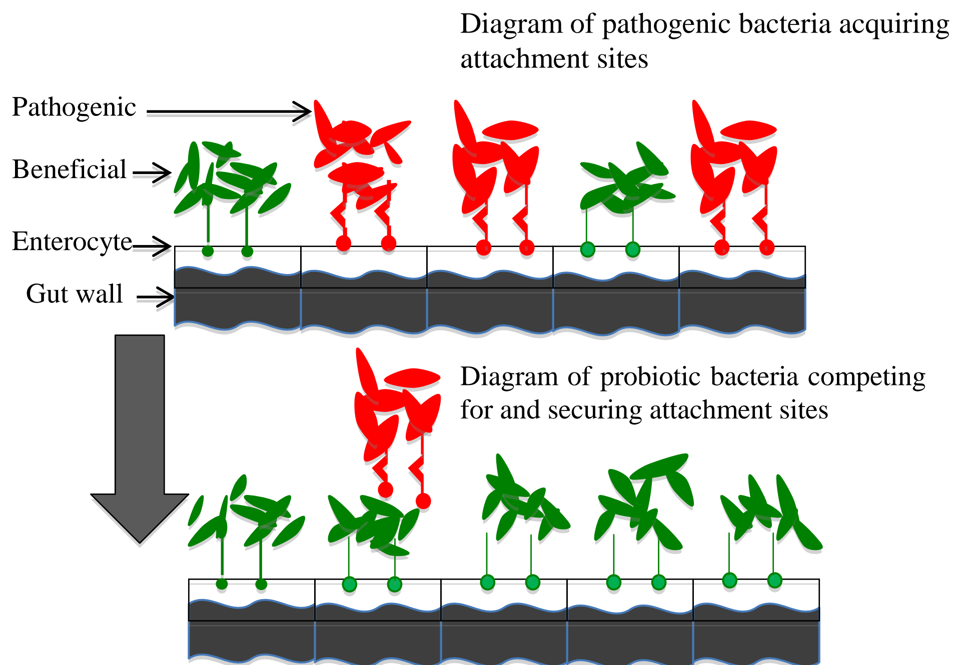


Figure 2.17 Pathogens compete with probiotics for receptor sites (Encarnacao, 2011).

2.4 Summary

Probiotics are live bacteria that, once administered, can provide a health benefit to the host (FAO/WHO working group, 2002). Because of the environmental sensitive properties of some potential probiotic strains, their utilisation in foods and other types of supplements has been limited. Generally, in a commercial probiotic food, the food itself is assumed to protect the bacterial cells from the adverse environment in the stomach (low pH) and the small intestine (presence of bile salts). However, studies on the survival of probiotic bacteria in foods such as yoghurt and other fermented milks, have shown that the food may not adequately protect the bacteria from the acidic pH in the stomach (Homayouni *et al.*, 2008; Kailasapathy, 2006). Therefore, technologies that protect the viability of probiotics during manufacture, storage, and gastrointestinal delivery are highly desired.

Up to now, numerous microencapsulation strategies have been studied for their ability to protect probiotic bacteria from environmental stresses (Kailasapathy, 2002; Krasaekoopt *et al.*, 2003), but there is still considerable work that is required for the development of a successful encapsulation system. It includes protection against the environmental stresses during formulation, drying, storage, and the critical protection against low pH values, bile acids and proteases during GI transit, followed by efficient release of the bacterial cells at the desired target site within the GI tract (Crittenden *et al.*, 2006). New coating materials and techniques are continuously emerging in microencapsulation technology, for example, coating the micro beads with a second layer or multi layers can increase microencapsulation efficacy. Delivery of viable bacterial cells to the colon and maintenance of viability and activity is still the main target.

The challenge in the present work, therefore, was to develop a technique by which a sufficient number of viable bacteria could be delivered to the large intestine, without any adverse effects on their beneficial physiological properties.

2.5 Previous studies on the physiological and functional characteristics of free and encapsulated *L. reuteri* DPC16 related to this project

- Survival of DPC16 cells in sequential simulated gastric and intestinal fluids (Yin, 2006)

Calcium alginate-encapsulated DPC16 cells were well protected during 1 h incubation in simulated gastric fluid compared with free cells, which demonstrated complete loss of viability. On exposure to a neutral pH environment, the viability of free DPC16 cells decreased only slightly, and the alginate-Ca^{2+} beads continuously released viable probiotic cells to the surrounding simulated intestinal fluid. However, Yin (2006) did not measure the survival rate of probiotic cells inside the beads.

- Viability of encapsulated DPC16 during storage (Joshi, 2005)

The shelf life of free, calcium alginate- encapsulated and freeze-dried encapsulated DPC16 cells was measured. The viability of DPC16 cells in alginate beads was higher than that of non-encapsulated cells at both room temperature and at -20°C. After storage for 120 days, the survival rate of encapsulated cells was above 60%, while the free cells were below 20%.

After 90-day-storage, the survival rate of DPC16 cells in freeze-dried alginate beads was less than 20%, which indicated a harmful effect of freeze-drying on this strain.

- Antimicrobial activity of *L. reuteri* DPC16 (Bian, 2008)

The inhibitory effect of cell-free supernatants from *L. reuteri* DPC16 cultures was examined on both Gram-positive and Gram-negative pathogens. It revealed that the pH-unadjusted (approximately pH 4.4) supernatants had significant inhibitory effects ($P<0.05$) against pathogens, whereas their pH-adjusted (pH 6.5) counterparts, while still having inhibitory effects, showed less activity. It was concluded that reuterin contributed to the activity at both pH levels, while short chain fatty acids contributed at pH 4.4 but not at pH 6.5. The Gram-positive pathogens (*L. monocytogenes* and *S. aureus*) tested were more sensitive to DPC16 supernatants than were the Gram-negative pathogens (*S. Typhimurium* and *E. coli*).

2.6 Scope of this project

Based on preliminary experiments using *L. reuteri* DPC16 cells, and the literature review, the objectives of this project were as follows:

i. Selection of an effective immobilisation technique to maintain the viability of DPC16 cells during passage through simulated gastric and intestinal fluids, followed by release in a simulated colonic fluid.

ii. Comparison of the physiological and functional characteristics of the cells recovered from the colonic fluid with the original DPC16 cells.

Chapter 3 Development of an optimal immobilisation system for *L. reuteri* DPC16 for passage through a simulated GI tract

3.1 Introduction

Health benefits associated with probiotics include stimulation of the immune system, alleviation of lactose intolerance and inflammatory bowel disease, prevention of infectious diarrhea and allergic diseases, prevention of carcinogenesis and tumor growth, and reduction of blood cholesterol level (De Roos & Katan, 2000; Desmond *et al*., 2002; Kailasapathy & Chin, 2000; Krasaekoopt *et al*., 2003; Cruttenden & Playne, 2004; Roberfroid, 2000). To achieve these beneficial health effects, the International Dairy Federation recommends the consumption of foods with at least 10^7 c.f.u./g (Ouwehand & Salminen, 1998). Moreover, probiotics must be able to survive the harsh acidic conditions during transit through the gastrointestinal (GI) tract (Gardiner *et al*., 1998; Kailasapathy & Chin, 2000; Picot & Lacroix, 2003). A significant proportion of probiotics is destroyed by low stomach pH and high concentrations of intestinal bile acids (Krasaekoopt *et al*., 2003; Sultana *et al*., 2000). Consequently, protective delivery systems need to be applied to protect probiotic bacteria from adverse conditions.

The aim of a manufacturing process for probiotics is to produce a stable product which can maintain a stable level of viable cells during storage, while the number of non-viable cells should be less than 1 $\log_{10}$ c.f.u./g after 1 year of storage (Hekmat *et al*., 2009; Karimi *et al*., 2011).

Product instability, or lack of a sufficiently long shelf life, is a common problem faced by many probiotic products. Previous research on the survival rate of freeze-dried non-encapsulated *L. reuteri* DPC16 cells showed that the number of viable cells decreased rapidly during storage at -20°C (Joshi, 2005) (Table 3.1).

Table 3.1 Survival (c.f.u./g) of non-encapsulated freeze-dried *L. reuteri* DPC16 cells during storage for 90 days at -20°C (Joshi, 2005)

Batch No.	Storage period (day)			
	0	30	60	90
1	$(1.15\pm0.09)\times10^{10}$	$(8.6\pm0.9)\times10^{9}$	$(3.2\pm0.3)\times10^{9}$	$(7.0\pm0.4)\times10^{8}$
2	$(1.28\pm0.18)\times10^{10}$	$(9.1\pm0.7)\times10^{9}$	$(4.8\pm0.8)\times10^{9}$	$(1.0\pm0.3)\times10^{8}$
3	$(1.26\pm0.09)\times10^{10}$	$(9.7\pm1.1)\times10^{9}$	$(3.9\pm0.5)\times10^{9}$	$(3.5\pm0.1)\times10^{8}$

Microencapsulation or immobilisation techniques have been widely recommended to maintain viability during processing and storage as well as to deliver the sufficient number ($\geq 10^7$c.f.u./g) of live probiotic bacteria to their sites of action (Ouwehand *et al*., 2002). These methods involve a process of enclosing or coating bacterial cells with a protective layer or entrapping them within a protective wall matrix of biopolymers which provides a barrier to stressful conditions such as oxygen, moisture, temperature, light, acids and enzymes (Kailasapathy & Sureeta, 2004; Picot & Lacroix, 2003; Krasaekoopt *et al*., 2003; McMaster & Kokott, 2005). Encapsulation or immobilisation can also be applied as a delivery system for conveying live probiotics to a targeted organ of the recipient, thus maximising the beneficial effects of probiotics (Doleyres *et al*., 2005). Among the different systems of encapsulation or immobilisation of probiotics, calcium alginate gel systems that form when sodium alginate is combined with calcium ions are the most widely utilised (Albertini *et al*., 2010; Bajaj *et al*., 2010; Ding & Shah, 2009a). Some advantages of calcium alginate gel systems as a delivery system include their physical stability in low pH solutions and their ability to swell in weakly basic solutions, thus enabling the release of bacterial cells at a target site in the intestine. The formation and physicochemical properties of calcium alginate gel beads are affected by various factors including type and concentration of alginate, amount of core materials (e.g. bacterial cells) loaded, calcium chloride concentration and hardening time in calcium chloride (Chavarri *et al*., 2010). Therefore, it is important to understand the behaviour of calcium alginate gel beads in gastrointestinal (GI) fluids and the factors that influence the properties of alginate gel systems as a delivery system. The concentrations of alginate and calcium chloride affect not only the extent of cross-linking density of the alginate gel matrix but also the release of encapsulated or immobilised cells at the target site (Nokhodchi & Tailor, 2004; El-Kamel *et al*., 2003).

Chandramouli (2004) reported that the number of surviving *Lactobacillus acidophilius* cells immobilised in sodium alginate increased with increasing alginate concentration while no difference was obtained with variable concentrations of $CaCl_2$ in simulated gastric fluid (SGF).

Compared with the transit time through the small intestine, which is relatively constant at 5-6 h, the process in the large intestine varies considerably. Liquid and small particles take a longer time to transit through the colon than large tablets do (McConnell *et al*., 2008a). Generally, it takes 20-35 h for tablets and capsules to transit through the colon in an adult. Such a long transit time benefits the delivery of substances that interact with the colon microflora. Due to the important role of the colon in the human GI tract, oral colon-specific delivery systems are becoming an efficient way for bioactive substances to exert their bioactive roles in the host, including those for probiotic bacterial cells.

L. reuteri DPC16 was isolated at Bioactive Research New Zealand (BRNZ) and patented in 2004. It is a promising potential probiotic due to its inhibitory activities against a range of pathogenic bacteria (Lu, 2007). In this investigation, the delivery of viable *L. reuteri* DPC16 cells immobilised in calcium alginate beads to a target site (the colon) in the GI tract was studied by varying the concentrations of alginate and $CaCl_2$ used in the formation of calcium alginate beads. Roy *et al*. (2009) and Li *et al*. (2010) reported that the addition of subsidiary materials inside calcium alginate gel systems improved the viability of cells. Gelatin is one of the most widely used polymers for probiotics (Annan *et al*., 2008; Brown *et al*., 1998). Gelatin can combine with alginate through electrostatic interaction, and hence can fill up the porous structure of alginate beads. However, gelatin is sensitive to degradation by pepsin during exposure to simulated gastric fluid (SGF) and the beads lose their integrity within a short period of time (Yanek & Stanley, 1997). Recently, chitosan has been used as a coating material to improve the encapsulation efficiency of alginate-Ca^{2+} beads. Further coating with chitosan can increase the stability of alginate calcium gel system through ionic cross-linking with alginate to decrease the loss of encapsulated material (Graff *et al*., 2008). Skim milk is commonly used as a protective agent for microorganisms in probiotic productions, especially during freeze-drying (Chavez & Ledeboer, 2007; Desmond *et al*., 2002). Lactose and protein present in skim milk may serve as a source of nutrients required for probiotics during storage or delivery inside the body, and the protein may

act as a pH buffer. The addition of gelatin, chitosan and skim milk to calcium alginate gels was therefore also studied in this part of work.

The residence time of probiotic cells in each organ (stomach, intestine and colon) during sequential transit through the GI tract is also a crucial factor that determines the viability and function of probiotics, as is the buffering capacity of any ingested food. The survival rate of encapsulated cells is expected to be higher with shorter exposure time in SGF. Therefore, in this work, the effect of different transit times in simulated gastric, intestinal and colonic fluids on the viability of immobilised bacterial cells during their sequential passage through the GI fluids was also studied. However, it is noted that in the *in vivo* situation, these transit times are determined entirely by the host.

Freeze-drying was selected to protect the encapsulated DPC16 cells during storage. Although microencapsulation can protect probiotic bacterial cells during transit through the GI tract, the shelf life of freeze-dried encapsulated cells is unknown. Hence, in this part of the research, the stability (viability) of freeze-dried alginate-skim milk-Ca^{2+} gel encapsulated DPC16 cells was monitored continuously for 6 months, and two fluorescent dyes were used to indicate the survival and death of DPC16 cells inside the freeze-dried beads.

3.2 Materials and methods

3.2.1 Materials

Sodium alginate and gelatin (CNC Grade) were provided by local ingredient suppliers: FMC BioPolymer (New Zealand) and GELITANZ Ltd. (New Zealand), respectively. Skim milk powder (PAMS, New Zealand), containing 35% protein, was purchased from a local supermarket in Auckland, New Zealand. Enzymes (pepsin and pancreatin) and bile salts were purchased from Sigma-Aldrich Chemical Company (St. Louis, Mo, USA). As stated by the manufacturer, the bile salts contained a mixture of cholic acid and deoxycholic acid sodium salt. Unless otherwise specified, all other chemicals were of analytical grade obtained from either Sigma Aldrich Chemical Company (St. Louis, Mo, USA) or BDH Chemicals (BHD Ltd, Poole, England). All the solutions were prepared using freshly distilled water.

3.2.2 Preparation of simulated gastrointestinal fluids

Simulated gastric fluid (SGF) was prepared by dissolving 3.2 g/l of pepsin (the method used to detect the enzymatic activity was described in Appendix I) in 0.1 M hydrochloric acid and the pH was adjusted to 1.2 (Krishnamachari *et al*., 2007). Simulated intestinal fluid (SIF), containing 10g/l of glucose (Ajax FineChem Pty Ltd., New Zealand), 5 g/l of yeast extract (Becton, Dickinson and Company, France), 10 g/l of pancreatin and 8 g/l of bile salts, was prepared in 0.1M Tris-HCl buffer at pH 7.4 (Wijnands *et al*., 2006). Simulated colonic fluid (SCF), containing 4 g/l pectin, 8 g/l glucose, 6 g/l starch, 2 g/l yeast extract, 4 g/l bile salts, and 1 g/l KH_2PO_4 was prepared in fecal water at pH 5.8 (Probert *et al*., 2004; Walker *et al*., 2005). The fecal water was prepared using fresh fecal sample from a healthy non-vegetarian, non-smoking male with no history of gastrointestinal disease who consumed a normal balanced diet. The fecal sample was homogenised in a stomacher with Tris-HCl buffer (pH 5.8) at a ratio of 1:5 (w/w) and centrifuged (Heraeus Biofuge primo R, Kendro Laboratory Products, Germany) at 3,200×g for 30 min at 4°C. After fecal particles were discarded, the supernatant was centrifuged using a Micromax microcentrifuge (Thermo Electron Corporation) at 13,000×g for 10 min to separate the fecal flora from the aqueous phase. The top layer of fecal water was sterilised by filtration through a 0.22 µm pore filter (Global Science, New Zealand) and stored at -20°C until required for use.

3.2.3 *L. reuteri* DPC16 culture

Lactobacillus reuteri DPC16 cells, obtained from Bioactive Research New Zealand Ltd. (Auckland, New Zealand), were grown in MRS broth (Difco, Michigan, USA) under anaerobic conditions using the GasPak Plus systemat 37°C for 18 h to obtain a cell density of about 10^9-10^{10} c.f.u./ml. Cells were harvested by centrifugation (Heraeus Biofuge primo R, Kendro Laboratory Products, Germany) at 3,200×g for 10 min at 4°C. The pellet of cells was washed twice by re-suspension in peptone water (0.1%) (MERCK, Germany) followed by further centrifugation before immobilisation.

3.2.4 Survival of free *L. reuteri* DPC16 cells in immobilisation-related materials and simulated gastrointestinal fluids

The survival of free bacterial cells in simulated gastric, intestinal and colonic fluids was determined by measuring the number of viable cells, using the drop plate method on

MRS agar, after incubation for the required length of time (Kramer & Gilbert, 1978).

To determine any killing effects due to immobilisation-related materials, the viability of free cells in 10% sodium citrate and 1% chitosan-0.4% acetic acid solutions was monitored (Nokhodchi & Tailor, 2004; Li *et al*., 2008; Ma *et al*., 2008). Before each incubation, DPC16 cells were washed twice with 0.1% peptone. The viability of cells was then measured using the drop plate method.

3.2.5 Immobilisation of *L. reuteri* DPC16 cells in calcium alginate gel beads

3.2.5.1 Comparison of 5 different types of beads based on an alginate-Ca^{2+} system

The extrusion technique, by using a 5 ml dropper (Global Science, New Zealand), was used during immobilisation of the cells, using the alginate-$CaCl_2$ gel as the base system. Gelatin, chitosan, gum Arabic, and skim milk were chosen as possible subsidiary materials (Hyndman *et al*., 1993; Annan, *et al*., 2008; Ross *et al*., 2008; Sarhan *et al*., 2010). All glassware and reagents used in the experiments were sterilised at 120°C for 15 min. Five types of immobilisation systems (beads) were prepared:

(i) **Alginate-$CaCl_2$ beads.** Washed *L.reuteri* DPC16 cell pellets were suspended in sterile sodium alginate solutions of different concentrations (2%, 3% and 4%, w/v) to a final concentration of 2×10^8 c.f.u./ ml. The alginate cell suspension (40 ml) was then added drop-wise, using a sterile dropper, into 150 ml of 1 M sterile $CaCl_2$ (Sigma) solution at 25°C while being stirred at 30 rpm using a magnetic stirring bar (Joshi, 2005; Yin, 2006).

(ii) **Alginate-skim milk-$CaCl_2$ beads.** Skim milk was dissolved in 3% alginate solution to a concentration of 4% or 8% (w/v). Washed DPC16 cell pellets were suspended in the prepared solutions, as described above, and then the mixture was added drop-wise into 1 M $CaCl_2$ solution (Ross *et al*., 2008).

(iii) A**lginate-gelatin-$CaCl_2$ beads.** Gelatin (1%, 2%, and 4%, w/v) -alginate based beads were prepared following essentially the same procedure described above for the alginate-skim milk-$CaCl_2$ beads, but using gelatin instead of skim milk (Annan, *et al*., 2008).

(iv) **Alginate-gelatin-gum Arabic-$CaCl_2$ beads.** Washed DPC16 cell pellets were suspended into an 18% gelatin, 18% gum Arabic and 4.8% $CaCl_2$ mixture, and then the

suspension was added drop-wise into alginate solution (1%) (Hyndman *et al*., 1993).

(v) **Alginate-$CaCl_2$-chitosan-alginate beads.** Washed DPC16 pellets were suspended in 3% alginate solution. The suspension was then extruded into 1 M $CaCl_2$ solution and stirred for 1 h. The wet beads were then transferred into a solution of 1% chitosan (Aldrich) that was dissolved in 0.4% (w/v) acetic acid (multiple-coated beads) (Ma *et al*., 2008). After 30 min of coating, the beads were separated and added into a 1% alginate solution and stirred for 30 min. Similarly, alginate-($CaCl_2$-chitosan)-alginate beads were prepared as follows: alginate-DPC16 mixture was added drop-wise into 1M $CaCl_2$ solution which contained 1% chitosan in acetic acid (Pandey & Khuller, 2004). The beads formed instantaneously and were transferred into 1% alginate solution at ambient temperature.

The beads formed using the above methods were harvested, washed twice with sterile peptone water (0.1%), and stored at 4°C until required for use (within 24 h).

The effectiveness of the above five different types of beads for the protection of viable DPC16 cells in SGF was compared.

3.2.5.2 Optimisation of the concentration of calcium chloride

The effect of calcium chloride concentration, in the absence of bacterial cells but in the presence of skim milk, on the integrity of alginate-skim milk-$CaCl_2$ beads during passage through the simulated gastrointestinal tract was determined. Skim milk powder (final concentration 8%, w/v) was dissolved in 3% sodium alginate solution. The mixture (40 ml) was added drop-wise into stirred $CaCl_2$ solutions (150 ml) at different concentrations (0.05, 0.1, 0.2, 0.3, 0.5 and 1 M). It was found that the optimum concentration of calcium chloride was 0.3 M.

3.2.5.3 Determination of the optimal immobilisation system

For the study on the viability of immobilised cells during passage through the simulated GI tract, beads containing skim milk (8%, w/v) were prepared at the concentrations of sodium alginate and calcium chloride of 3% and 0.3 M, respectively, using the method (ii) described above.

3.2.6 Visual observation of beads

De novo calcium alginate beads prepared without bacterial cells but cross-linked with the different concentrations of $CaCl_2$ (0.05-1.0 M) as described above, were analysed for changes in their physical integrity under the simulated GI conditions in order to determine an optimum concentration of $CaCl_2$ required to form calcium alginate beads that could remain physically intact during passage through the simulated stomach and small intestine, but then lose their integrity in the simulated colon. Incubation time in the simulated GI tract followed the sequence: SGF for 3 h, SIF for 6 h and SCF for 24 h. Images of beads were taken using a digital camera (Model S850, Samsung, 5× optical zoom). The method used to detect diffusion and leakage of alginate-skim milk-Ca^{2+} system was described in Appendix II.

3.2.7 Viability of immobilised *L. reuteri* DPC16 cells in simulated GI fluids

Washed calcium alginate beads containing entrapped cells were placed in SGF or other simulated GI fluids at 37°C for various time intervals. Samples (aqueous liquid for free cells and beads for immobilised cells) were withdrawn and examined for the viable cell counts. The beads were dissolved in 10% (w/v) sodium citrate solution, pH 7.2, prior to enumeration of viable cell counts.

Immobilised *L. reuteri* DPC16 cells were also measured for their viability by placing the beads sequentially in sterile SGF, SIF and SCF at 37°C for incubation times of three different patterns (SGF/SIF/SCF; 1 h/6 h/24 h, 2 h/5 h/24 h and 3 h/6 h/24 h). After incubation in each fluid, the beads were collected and transferred into the next GI fluid. A portion of the beads was collected at each step and analysed for the viability of the immobilised DPC16 cells.

The viable cell counts were enumeration by serial dilution plating on MRS agar, using the drop plate method. Three replicate samples were prepared each time.

3.2.8 Viability of freeze-dried encapsulated DPC16 cells during storage

Freshly prepared calcium alginate beads containing skim milk (8%) and DPC16 cells (Section 3.2.5.3) were collected in six sterilised plastic boxes. Protective reagent, containing 140 g/l skim milk powder, 60 g/l glucose and 10 g/l glycerol, was poured evenly into the plastic boxes to cover the beads. After covering with foil, the boxes

were frozen for 18h at -20°C, followed by storage at -80°C until required for freeze-drying. The freeze drier was a refurbished General Purpose Freeze Drier from Cuddon Ltd. (New Zealand) fitted with an Edwards High vacuum pump (Model number D2M-18). Before drying the samples, the freeze drier was cooled to -50°C and a vacuum of 80 mmHg was applied. The frozen samples were then mounted on the machine and freeze-drying was conducted at 80 mmHg for 4 days, after which the drier was stopped and the vacuum pump was degassed for approximately 30 min. The freeze-dried beads were transferred to either 4°C or room temperature for storage, and the number of viable DPC16 cells was determined at 30, 60, 90, 120, 150 and 180 days during storage.

At the end of the six-month viability study, the fluorescent stains, carboxy fluorescein di-acetate (cFDA) and propidium iodide (PI), were used to assess the viability of the DPC16 cells inside the freeze-dried beads. In particular, cFDA is utilised to assay intracellular enzyme activity. It readily diffuses across the cell membrane, while PI is excluded by viable cells with their intact membranes, and is used as a probe for cell membrane integrity (Amor *et al.*, 2002). cFDA/ PI dual staining has been successfully applied in determination of the viable and dead cells amount for *L. reuteri* DPC16 by flow cytometry (Chen *et al.*, 2012). Firstly, the freeze-dried beads were dissolved using sodium citrate, and then centrifuged (Heraeus Biofuge primo R, Kendro Laboratory Products, Germany) at 3200×g for 10 min. The bacterial pellets were washed twice with 0.1% peptone, and then mixed with carboxy fluorescein di-acetate (cFDA) and propidium iodide (PI). When viable bacterial cells are stained by cFDA, they appear green under the fluorescence microscope. Non-viable cells appear red in color, stained by PI (Ulmer *et al.*, 2000; Ananta *et al.*, 2005).

3.2.9 Statistical analysis

All experiments and sample analyses were conducted in duplicate or triplicate. The results were analysed by one-way ANOVA and Two-Samples T-test using Minitab™ 15.0 (Minitab, Pennsylvania State University, USA).

3.3 Results

3.3.1 Images of immobilised beads

Calcium alginate gel beads were prepared as described in Section 3.2.5.1. In all the formed beads, the average diameter was in the range of 2-5 mm. However, the actual

diameter varied depending on the wall materials.

The diameter of the simple freshly made calcium alginate wet bead was about 3 mm, although it shrank to approximately 2 mm after incubation in SGF for 6 h (Figure 3.1).

Shrinkage of the alginate-skim milk based beads was also observed, but was not as pronounced as that without the skim milk (Figure 3.2).

The alginate-based gelatin beads are shown in Figure 3.3. After exposure to air, the two types of beads shrank in different ways. Alginate-gelatin-gum Arabic-$CaCl_2$ beads shrank from the inside because of the low concentrations of gel-forming materials (1% alginate and 4.8% $CaCl_2$), whereas the alginate-gelatin-$CaCl_2$ beads shrank thoroughly.

The diameter of the chitosan alginate beads increased with the second coating of alginate (Figure 3.4).

Figure 3.1 Alginate-$CaCl_2$ beads before and after incubation in SGF.

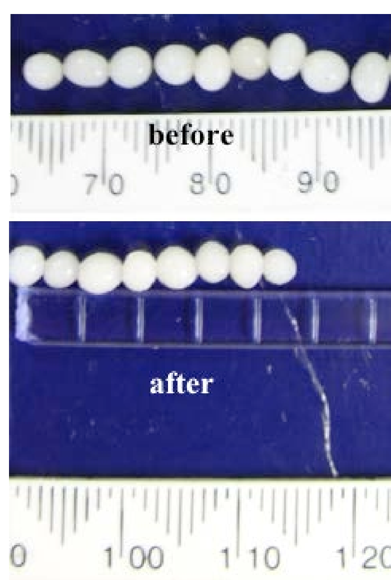


Figure 3.2 Alginate-skim milk-$CaCl_2$ beads before and after incubation in SGF.

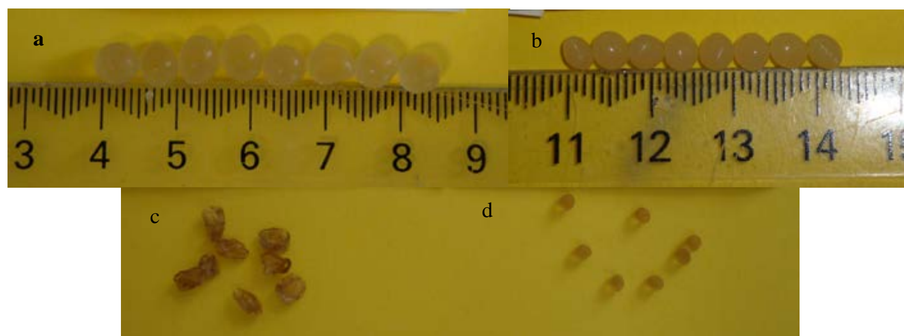


Figure 3.3 Alginate-gelatin beads. (a) beads with 18% gelatin, 18% gum Arabic, 4.8% $CaCl_2$ and 1% alginate; (b) beads with 3% alginate, 4% gelatin and 1M $CaCl_2$; (c) Air dried beads of type (a); (d) Air-dried beads of type (b).

Figure 3.4 Alginate-Ca^{2+}-chitosan (chitosan dissolved in calcium chloride first) beads before and after coating with 1% alginate. (a) alginate-($CaCl_2$-chitosan) beads; (b) (alginate-($CaCl_2$-chitosan))-alginate beads

3.3.2 Survival of free and immobilised *L. reuteri* DPC16 cells during passage through the GI tract

3.3.2.1 Viability of free DPC16 cells in immobilisation-related chemicals and simulated GI fluids

The viability of free *L. reuteri* DPC16 cells in sodium citrate and chitosan- acetic acid was determined. The results (Figure 3.5) showed that when incubated in 10% sodium

citrate solution for 90 min, the number of viable cells dropped from 2.0×10^{8} c.f.u./ml after 90 min incubation at 37°C. In contrast, during incubation in chitosan-acetic acid solution, the viability of DPC16 dropped from 3.8×10^{7} c.f.u./ml to 1.2×10^{6} c.f.u./ml after 30 h incubation at 37°C after 90 min incubation at 37°C.

The survival of DPC16 in SGF (37°C) was also determined. Although the pH of the gastric fluid *in vivo* is normally higher than 1.2, due to the buffering capacity of the food, this value was selected to represent an extreme situation. Figure 3.6 shows that when exposed to SGF of pH 1.2, the cell viability decreased rapidly from 1×10^{9} c.f.u./ml to 5×10^{3} c.f.u./ml after 60 min, and no viable cells were detected after 90 min.

When incubated in different solutions at pH 7.2, (i.e. Tris-HCl buffer, Tris-HCl buffer with 0.8% bile salts, and SIF), the number of viable DPC16 cells varied with the solution (Figure 3.7). No loss of viability occurred when DPC16 cells were incubated in Tris-HCl buffer. However, with the addition of 0.8% bile salts, the number of viable cells dropped markedly. When nutrients were added to the Tris+0.8% bile salts solution (i.e. SIF), only a slight decrease in the number of viable cells was observed after 6 h incubation at 37°C.

During incubation in SCF, the number of viable cells decreased from 4.3×10^{7} c.f.u./ml to 1.8×10^{6} c.f.u./ml after 30 h incubation at 37°C (Figure 3.8).

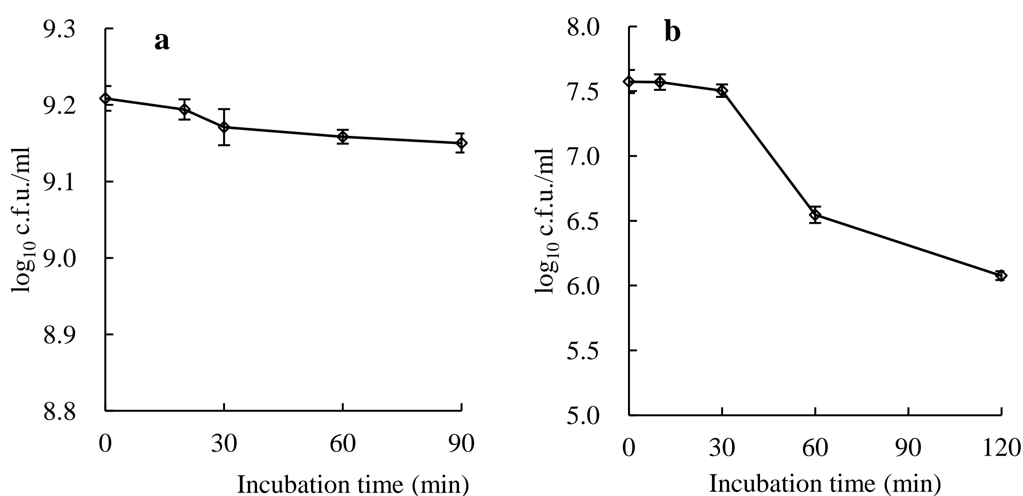


Figure 3.5 Viable free *L. reuteri* DPC16 cells in (a) 10% sodium citrate and (b) chitosan (1%) - acetic acid (0.4%) solution during incubation at 37°C for 90 and 120 min, respectively. This experiment is done triplicate.

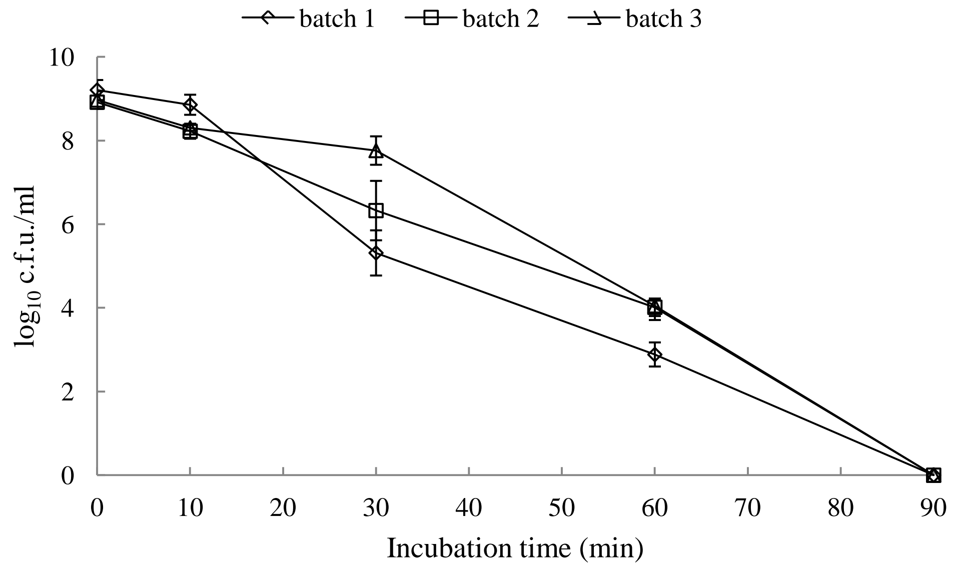


Figure 3.6 Viable free *L. reuteri* DPC16 cells in SGF (pH 1.2) during incubation at 37°C for 90 min. This experiment is done in triplicate.

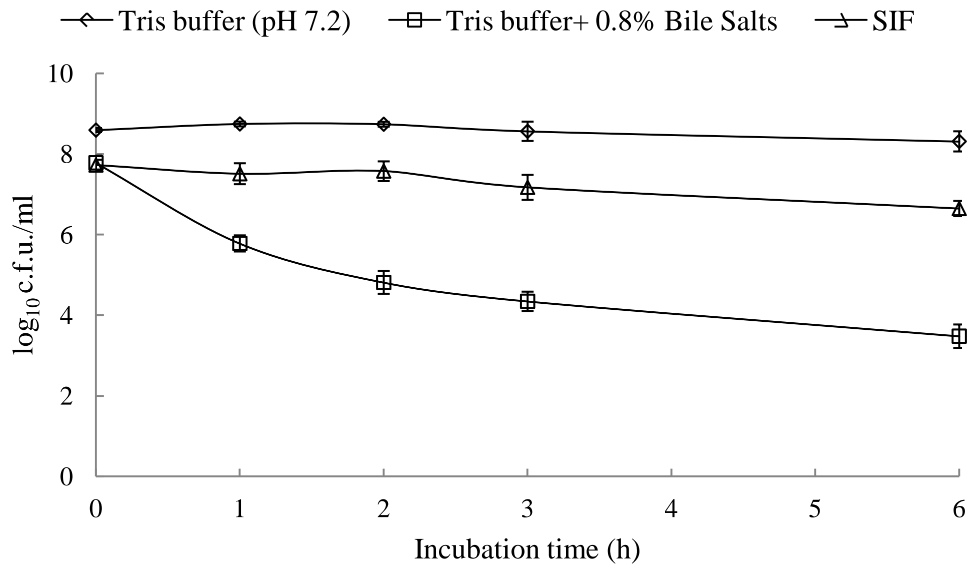


Figure 3.7 Viable free *L. reuteri* DPC16 cells in Tris-HCl, Tris-HCl + bile salts, and SIF (pH 7.2) during incubation at 37°C for 6 h. This experiment is done in duplicate.

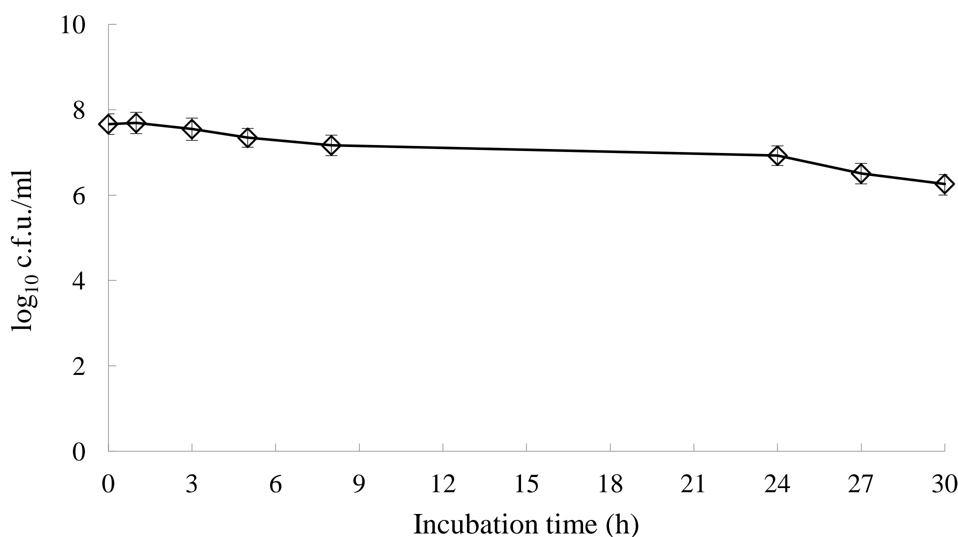


Figure 3.8 Viable free *L. reuteri* DPC16 cells in SCF at 37°C for 30 h. This experiment is done in triplicate.

3.3.2.2 Survival of *L. reuteri* DPC16 in different types of immobilisation systems during incubation in simulated gastric fluid at 37°C

The purpose of this section of the work was to evaluate the five immobilisation systems described in Section 3.2.5.1 for their effectiveness in protecting viable cells of strain DPC16 during incubation in simulated gastric fluid (SGF). One of the most efficient systems would then be selected for further study. On occasions, the SGF was prepared without pepsin so that its influence on the efficiency of wall materials could be assessed.

Firstly, the simple alginate-Ca^{2+} system was evaluated, using three different alginate concentrations (2, 3, and 4%) at 1.0 M $CaCl_2$. In addition to counting the number of viable cells, any effects on the integrity of the beads were noted. The results (Figure 3.9) showed that, in contrast to the results using free cells, the immobilised cells maintained some viability after 90 min of incubation in SGF. Fewer cells died with increasing alginate concentration. Figure 3.9 shows that the viable cell numbers in SGF after 90 min incubation decreased from 8×10^8 to 7×10^1 c.f.u./g for 2% alginate, from 7×10^8 to

3×10^2 c.f.u./g for 3% alginate, and from 5×10^8 to 1×10^3 c.f.u./g for 4% alginate. Although the 4% alginate concentration conferred the greatest protection, the irregular shape of the beads eliminated it from further investigation. Overall, the results showed that the alginate-$CaCl_2$ bead does not offer sufficient protection to the cells during incubation in SGF at 37°C.

The number of viable DPC16 cells during incubation of gelatin-alginate immobilisation systems in SGF is shown in Figure 3.10. Using immobilisation system (iii) (Section 3.2.5.1), where the concentration of alginate was constant at 3% (w/v) and the concentration of gelatin varied from 1-4% (w/v), it was clear that even when SGF does not contain pepsin, a rapid loss of viability was observed (Figure 3.10a). When pepsin was present in the SGF, there were no detectable viable cells after 3 h of incubation, even in 4% (w/v) gelatin. Using the immobilisation system (iv) (Section 3.2.5.1), there was a considerable reduction in the number of viable cells after 3 h incubation at 37°C (Figure 3.10b). Figure 3.11 shows the results when skim milk was incorporated in the alginate-$CaCl_2$ gel (immobilisation system (ii), Section 3.2.5.1). The addition of 4% skim milk was probably insufficient to protect the viability of DPC16. However, the addition of 8% skim milk significantly reduced the cell death (e.g. less than 1 log reduction in 90 min) in SGF, both with and without the presence of pepsin. After 30 and 90 min in SGF, the viable cell counts decreased from 5×10^8 c.f.u./g to 3×10^8 and 2×10^8 c.f.u./g, respectively. Thus, the presence of skim milk (8%) in alginate beads increased the survival of cells to about 50% after incubation in SGF for 90 min.

The effect of chitosan as a component of the immobilisation system was evaluated (system (v), Section 3.2.5.1). There was only a 35% decrease in the number of viable cells in (alginate-($CaCl_2$+chitosan))-alginate beads (Figure 3.12), while in the ((alginate-$CaCl_2$)+chitosan)- alginate beads coating system, the number of viable cells decreased from 5.4×10^8 to 1.7×10^7 c.f.u./g during 3 h incubation in SGF.

The gradient of the curves in Figures 3.9, 3.10, 3.11 and 3.12 were measured and recorded as an arbitrary “rate of viability decrease”. These rates are shown in Table 3.2. Based on these data, and the ease of bead preparation, skim milk proved to be an optimal subsidiary material for alginate-$CaCl_2$ beads, and the immobilisation system was therefore used in the subsequent experiments.

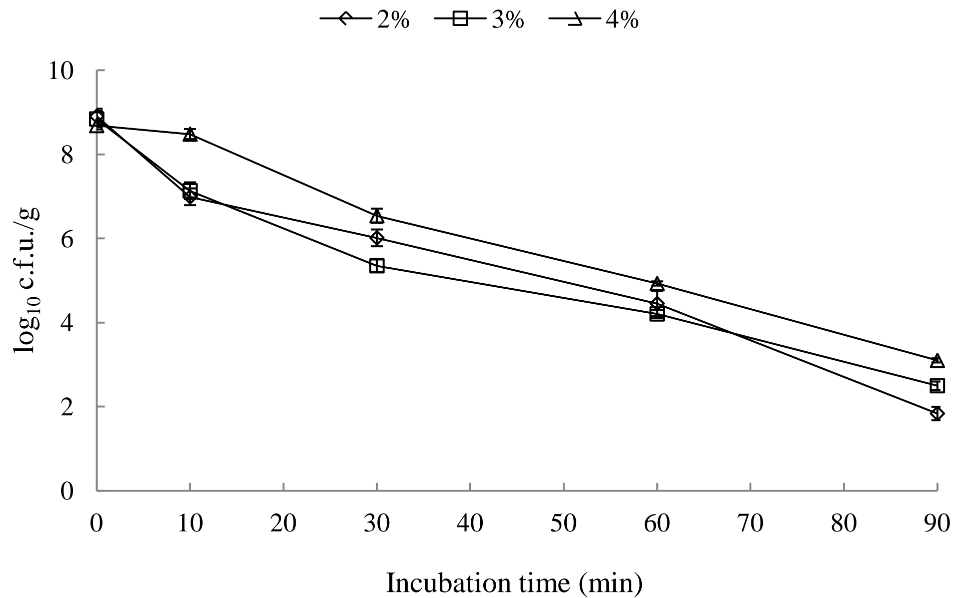


Figure 3.9 Number of viable DPC16 cells immobilised in alginate-$CaCl_2$ beads at different alginate concentrations during incubation in SGF at 37°C (the concentration of $CaCl_2$ was maintained at 1 M). This experiment is done in triplicate.

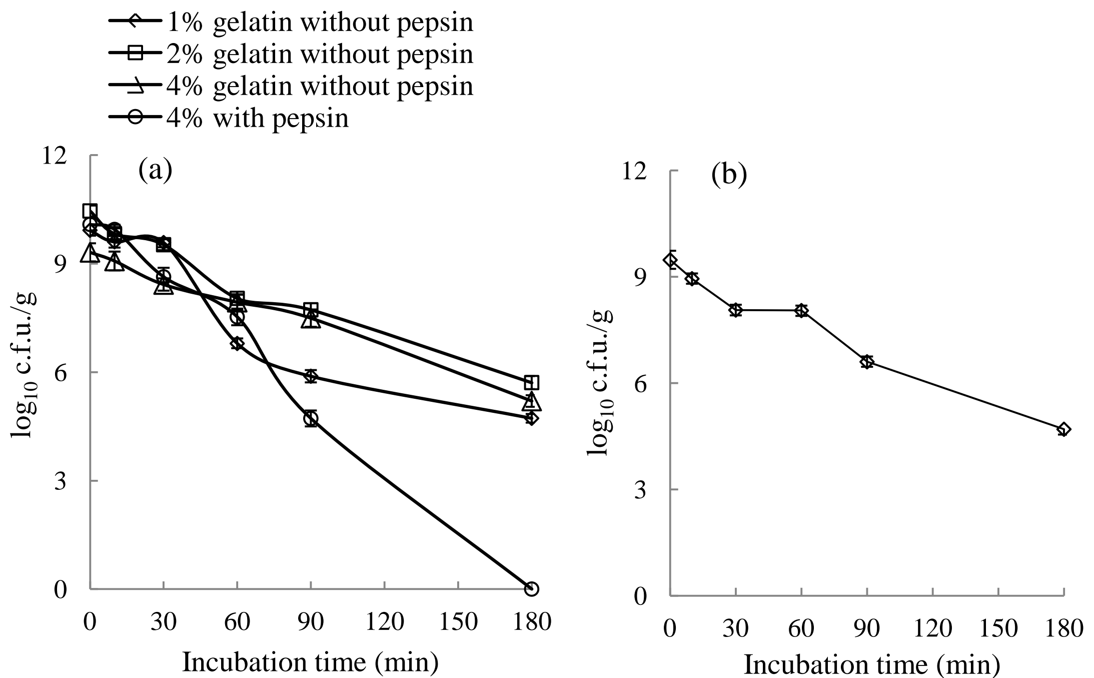


Figure 3.10 Number of viable DPC16 cells during incubation of (a) 3% alginate-$CaCl_2$ immobilisation system at different concentrations of gelatin; (b) 3% alginate-18% gelatin-18% gum Arabic-4.8% $CaCl_2$ immobilisation system, in SGF at 37°C. SGF did not contain pepsin unless specified. This experiment is done in triplicate.

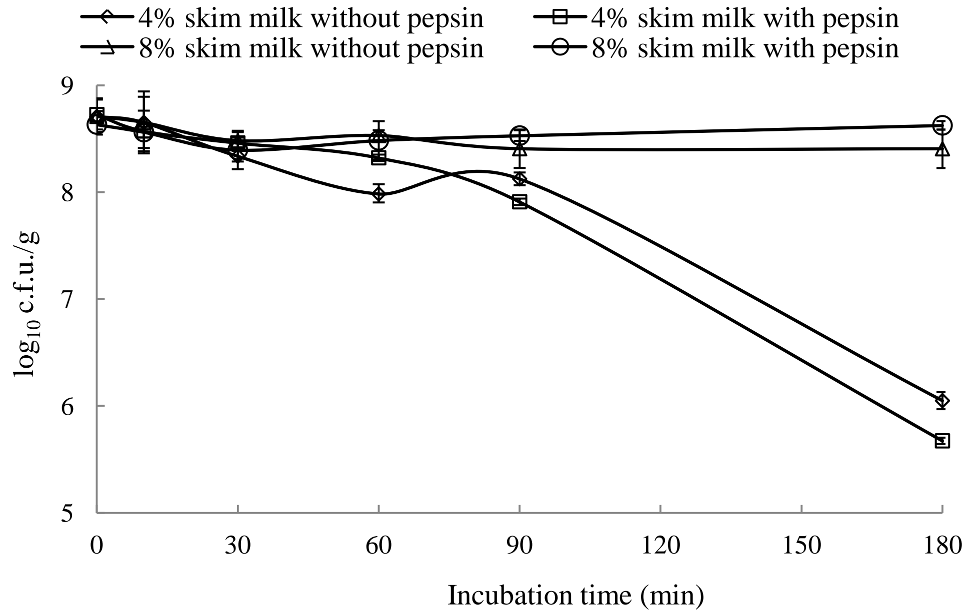


Figure 3.11 Number of viable cells immobilised in alginate-$CaCl_2$ incorporating skim milk during incubation in SGF at 37°C, in the presence and absence of pepsin. This experiment is done in triplicate.

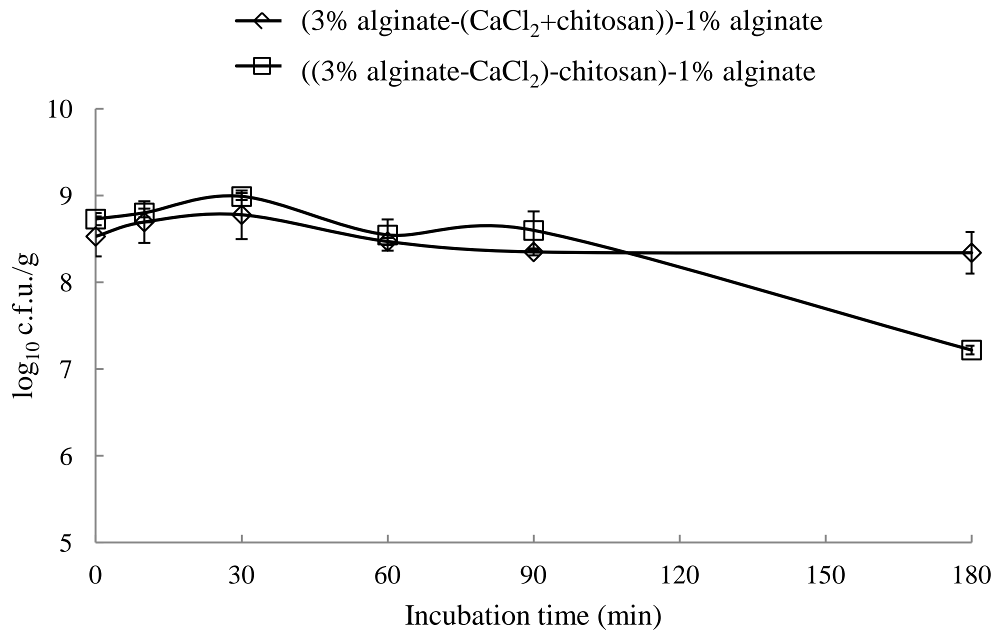


Figure 3.12 Number of viable DPC16 cells in alginate-chitosan-$CaCl_2$ beads during incubation in SGF at 37°C. This experiment is done in triplicate.

Table 3.2 A summary of the arbitrary "rates of viability decrease" during incubation in SGF of free and immobilised DPC16 cells

Types of beads (Section 3.2.5.1)		Pepsin	
		Absence	Presence
Free cells (n=9)		0.17±0.014	0.12±0.037
Alginate-$CaCl_2$ beads	2% alginate, 1 M $CaCl_2$	0.080±0.011	-
	3% alginate, 1 M $CaCl_2$	0.079±0.009	0.082±0.005
	4% alginate, 1 M $CaCl_2$	0.073±0.005	-
Alginate-gelatin-$CaCl_2$ beads	3% alginate-1% gelatin-1 M $CaCl_2$	0.031±0.005	-
	3% alginate, 2% gelatin, 1 M $CaCl_2$	0.031±0.007	-
	3% alginate, 4% gelatin, 1 M $CaCl_2$	0.022±0.008	0.058±0.011
Alginate-skim milk-$CaCl_2$ beads	3% alginate, 4% skim milk, 1 M $CaCl_2$	0.016	0.024±0.015
	3% alginate, 8% skim milk, 1 M $CaCl_2$	0.0018±0.001	0.0003±0.000
	3% alginate, 5% gelatin, 8% skim milk, 1 M $CaCl_2$	0.0088±0.009	-
Alginate-gelatin-gum Arabic-$CaCl_2$ beads	3% alginate, 18% gelatin, 18% gum Arabic, 4.8% $CaCl_2$	0.026±0.003	-
Alginate-$CaCl_2$-chitosan-alginate beads	(3% alginate-$CaCl_2$)-chitosan-1% alginate	-	0.0074±0.002
	3% alginate-($CaCl_2$-chitosan)-1% alginate	-	0.0005±0.000

(-) indicates "not determined"

3.3.3 Physical characteristics of calcium alginate beads

Possible changes in the physical properties that alginate beads would undergo during passage through SIF, SGF and SCF were investigated. The purpose of this experiment was to identify an optimum concentration of $CaCl_2$ to produce alginate beads which can maintain their physical integrity during passage from SGF through SIF to SCF and then release bacterial cells in the SCF. For this experiment, *de novo* calcium alginate beads were prepared without immobilising bacterial cells, at fixed concentrations of 3% sodium alginate and 8% skim milk but with varying concentrations of $CaCl_2$ (0.05, 0.1, 0.2, 0.3, 0.5 and 1 M).

The size of formed beads was in the range of 3-4 mm diameter (Figure 3.13). No

markedly size difference that could be attributed to $CaCl_2$ concentration was observed. When beads were placed in SGF, those made with a low concentration of $CaCl_2$ had a higher tendency to float on the surface of SGF (Figure 3.14a). Nonetheless, all beads remained intact in SGF (Figure 3.14a) irrespective of $CaCl_2$ concentration, and no discernable changes were observed. When immersed in the SIF, however, the beads made using a low concentration of $CaCl_2$ (0.05, 0.1, 0.2 and 0.3 M) started to swell and after 6 h began to lose their integrity, especially the beads prepared using 0.05-0.2 M $CaCl_2$ which were very fragile and easily broken. In contrast, the beads made using concentrations of 0.5 M and 1 M $CaCl_2$ remained stable without much swelling (Figure 3.15a). In this study, the diffusion of skim milk from the beads was not noticeable in SGF but it occurred in SIF (Figure 3.14b). There were no observed differences for the different $CaCl_2$ concentrations used.

Further experiments were performed to observe the physical changes occurring when beads had undergone sequential exposure in SGF, SIF and SCF. It was observed that only the beads made using 0.3 M $CaCl_2$ maintained their integrity in SIF but disintegrated during subsequent exposure to SCF. Since the target of the delivery system was to release viable DPC16 cells in SCF, a concentration of 0.3 M $CaCl_2$ was chosen as the optimum concentration for the preparation of calcium alginate beads (Figure 3.15b). The dark colour of the beads was probably due to the presence of faecal water in the SCF.

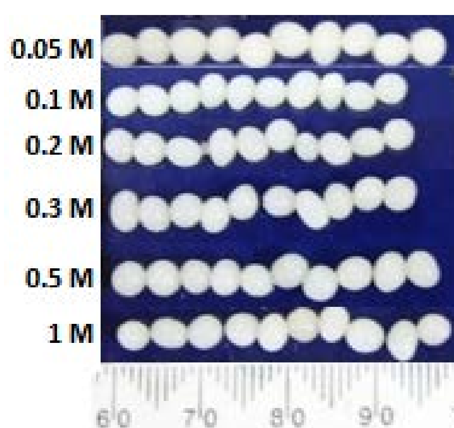


Figure 3.13 The initial sizes of alginate (3%) beads (containing skim milk at a final concentration of 8% (w/v) made at different $CaCl_2$ concentrations (0.05-1 M).

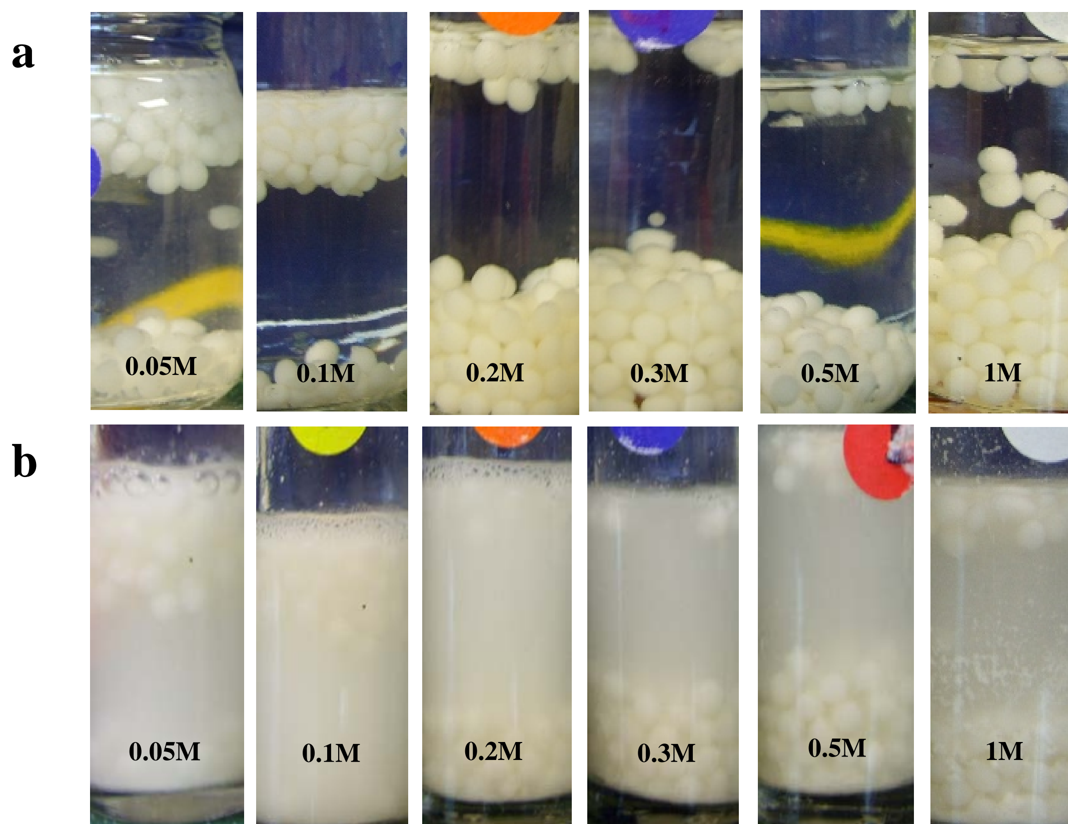


Figure 3.14 Calcium alginate beads containing skim milk prepared at different concentrations of $CaCl_2$ (0.05-1 M). (a) after 1 h incubation in SGF; (b) after1 h incubation in SGF followed by 2 h in SIF.

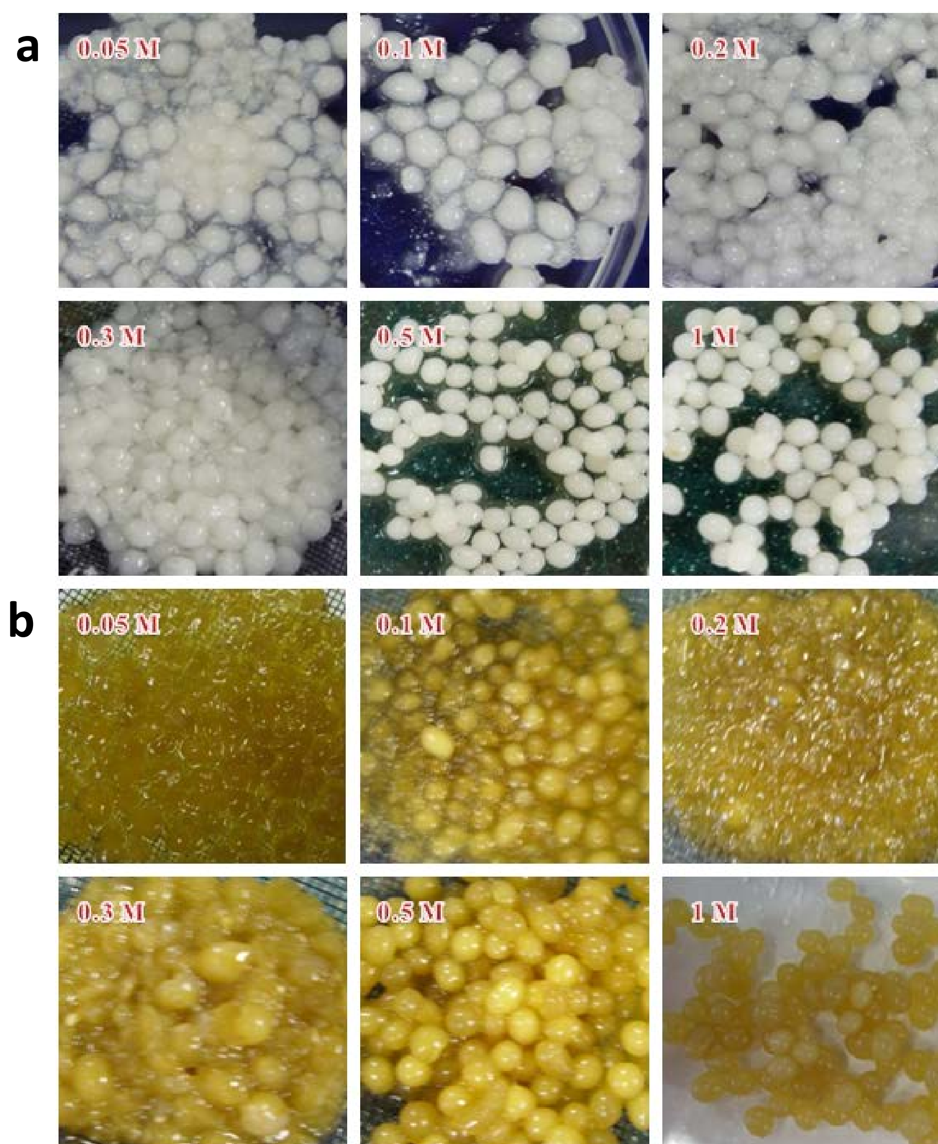


Figure 3.15 Photographs of calcium alginate beads containing skim milk (8%) prepared at different concentrations of $CaCl_2$ (0.05-1 M). (a) After 6 h incubation in SIF; (b) after 33 h of sequential incubation in the simulated GI tract (3 h in SGF, 6 h in SIF and 24 h SCF).

3.3.4 Sequential incubation of immobilised *L. reuteri* DPC16 in the simulated GI tract

Different time patterns in the GI tract were chosen to aid the identification of an effective calcium alginate/skim milk-based delivery system by comparing the survival rates of immobilised *L. reuteri* DPC16 cells in the different patterns.

The study found that the incubation time in SGF is one of the main factors that determined the effectiveness of the calcium alginate/skim milk immobilisation system (Table 3.3). The viable cell numbers decreased with increasing incubation time in SGF. This showed that when the incubation time in SGF was prolonged to 3 h, the number of viable immobilised DPC16 cells after the subsequent incubation in SIF for 6 h followed by SCF for 24 h was considerably less than when the SGF incubation time was 1 or 2 h. For beads incubated in SGF for 2 h followed by SIF for 5 h and SCF for 24 h, the survival rate of immobilised *L. reuteri* DPC16 cells was 88% after their exposure in SGF. Interestingly, when incubation in SGF was for 1 h only, there was some apparent cell growth in the SCF after incubation in the SIF. The significance of this is unclear (Table 3.3).

Regarding the targeted release of immobilised cells, the calcium alginate/skim milk system allowed the release of viable cells when exposed to the SCF. Experiments were performed using two different batches of immobilised cells. While viable cells were occasionally detected in the SGF and SIF, presumably due to leakage from the beads, these numbers were always low (Table 3.4). However, in the SCF, a constant level of viable cells was detected. The immobilised beads remained intact in SGF and swelled in SIF, and then disintegrated in SCF. The detectable viable *L. reuteri* DPC16 cell numbers in the SCF were maintained at 10^4 c.f.u./ml during the monitored incubation time (Table 3.4).

Upon plating on MRS agar, the colonial appearance of the DPC16 cells that had been released in the SCF was identical to that of the original cells (Figure 3.16). Further, no differences in microscopy appearance could be detected in Gram stains (Figure 3.17).

Table 3.3 Viable cell numbers (log_{10} c.f.u./g) of *L. reuteri* DPC16 cells in calcium alginate beads (3%) incorporated with skim milk (8%) during sequential incubation (37°C) in SGF, SIF and SCF.

Residence time in SGF/SIF/SCF	Control	SGF	Survival (%)	SIF	Survival (%)	SCF	Survival (%)
1 h/6 h/24 h	8.9 ± 0.02	8.7 ± 0.00	66.5	6.1 ± 0.03	0.16	7.3 ± 0.02	2.34
2 h/5 h/24 h	8.3 ± 0.02	8.3 ± 0.02	88.4	7.6 ± 0.01	20.0	7.1 ± 0.02	6.14
3 h/6 h/24 h	8.4 ± 0.03	5.2 ± 0.03	<0.1	3.1 ± 0.03	<0.1	2.8 ± 0.03	<0.1

Table 3.4 Viable cell counts of *L. reuteri* DPC16 cells immobilised in calcium alginate beads (3%) containing skim milk (8%) during sequential incubation in simulated GI fluids (SGF 2 h, SIF 5 h and SCF 24 h) at 37°C.

		log_{10} viable cells in the beads and simulated solutions										
		SGF (0-2 h)			SIF (2-7 h)				SCF (7-31 h)			
		0	0.5	1	2	3	4	7	8	9	26	31
Batch 1	inside	8.36±0.03	-	8.47±0.02	8.28±0.02	8.19±0.02	8.05±0.04	7.64±0.01	7.60±0.02	7.58±0.04	-	7.13+0.02
	outside	-	4.91±0.02	-	<1	1.41±0.03	1.73±0.03	1.92±0.04	4.36±0.04	4.53±0.03	-	4.23±0.04
Batch 2	inside	8.89±0.01	-	-	7.98±0.02	8.012±0.05	-	6.17±0.06	-	-	-	5.35±0.02
	outside	-	-	-	<1	<1	-	<1	<1	-	3.59±0.03	4.42±0.02

(-) indicates " not determined"

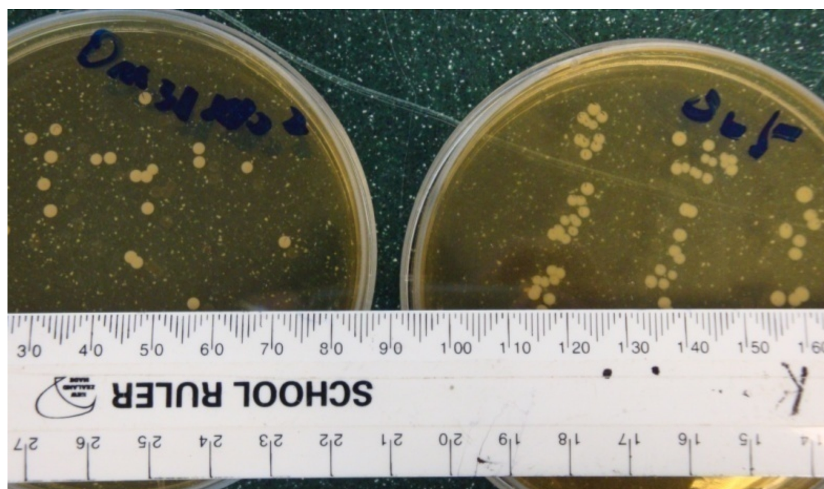


Figure 3.16 Colony appearance on MRS agar of the original and recovered DPC 16 cells after sequential incubation in simulated GI fluids.

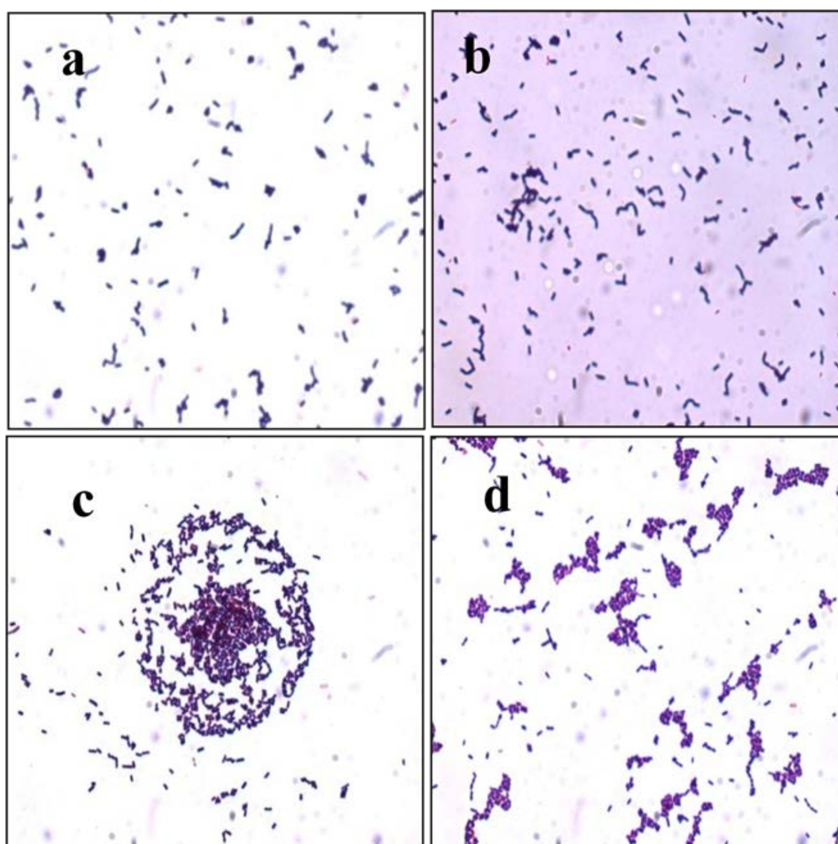


Figure 3.17 Gram stained cells from different simulated solutions. (a) original sample; (b) after 2 h in SGF; (c) after an additional 5 h in SIF; (d) after an additional 24 h in SCF. ×1000 magnification, using an Axiostar® plus (Carl Zeiss, USA).

3.3.5 Survival of DPC16 in freeze-dried beads

After freeze-drying, there was almost no change in the diameter of the alginate-skim milk-Ca^{2+} beads (Figure 3.18). This was as expected because of the high viscosity of the immobilisation materials, although drying would normally cause shrinkage of the beads. Figure 3.19 shows photomicrographs after staining with fluorescent chemicals, indicating that some cell death had occurred after 6 months storage of the freeze-dried beads.

At the end of the six-month storage period, the number of viable DPC16 cells was measured in 3 separate batches of freeze-dried beads. The results (Table 3.5) showed less than 1 log reduction in viability over the six-month period.

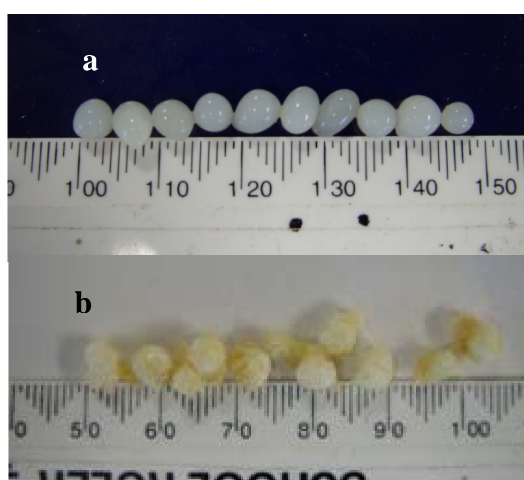


Figure 3.18 Alginate-skim milk-Ca^{2+} beads before and after freeze-drying. (a): wet alginate-skim milk-Ca^{2+} beads; (b): freeze-dried alginate-skim milk-Ca^{2+} beads.

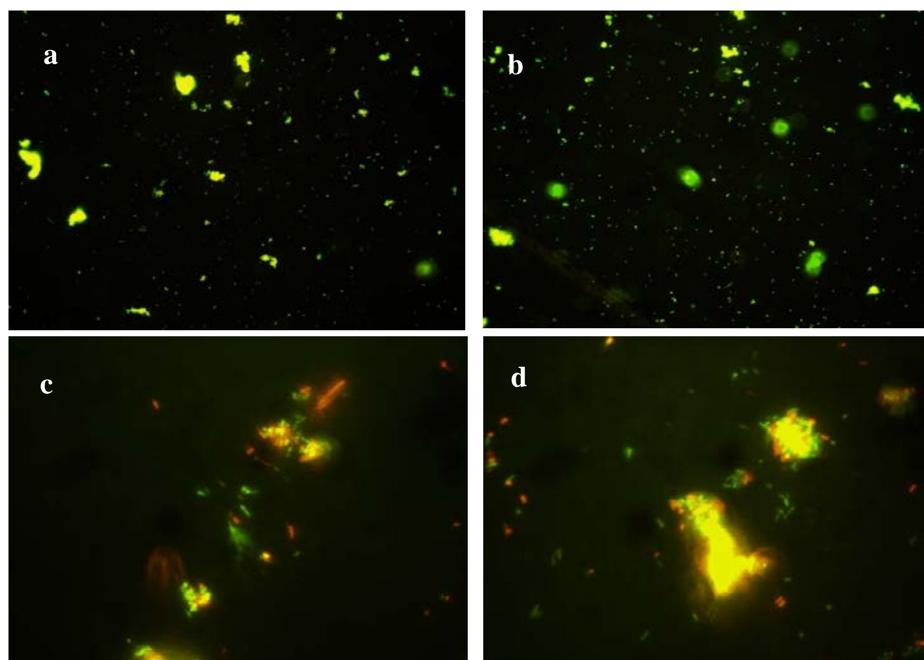


Figure 3.19 Photomicrographs of DPC16 cells in freeze-dried beads at the beginning and after six months, under 200× magnification, using a fluorescence microscope (Olympus, model CKX41, Japan). The cells were released from the beads prior to staining by immersion in sodium citrate solution (10%). The live cells were dyed green using cFDA, while the dead cells appeared red by PI. (a) and (b) were stained at zero time; (c) and (d) were stained at the end of the six months storage.

Table 3.5 Viable cells (c.f.u./bead) of freeze-dried alginate-skim milk-$CaCl_2$ immobilised DPC16 cells during storage at 4°C

	Storage time (month)	Batch No. 1	2	3
Before drying	-	$(2.11\pm0.14)\times10^7$	$(2.43\pm0.12)\times10^7$	$(2.23\pm0.25)\times10^7$
After drying	0	$(1.51\pm0.15)\times10^7$	$(1.61\pm0.09)\times10^7$	$(1.42\pm0.13)\times10^7$
	1	$(1.14\pm0.10)\times10^7$	$(1.23\pm0.12)\times10^7$	$(1.18\pm0.13)\times10^7$
	2	$(1.14\pm0.07)\times10^7$	$(1.22\pm0.05)\times10^7$	$(1.14\pm0.07)\times10^7$
	3	$(1.07\pm0.23)\times10^7$	$(1.28\pm0.07)\times10^7$	$(1.15\pm0.21)\times10^7$
	4	$(8.16\pm0.12)\times10^6$	$(9.83\pm1.00)\times10^6$	$(9.04\pm0.19)\times10^6$
	5	$(6.55\pm0.12)\times10^6$	$(8.25\pm0.14)\times10^6$	$(7.58\pm0.33)\times10^6$
	6	$(3.11\pm0.29)\times10^6$	$(5.38\pm0.57)\times10^6$	$(3.20\pm0.40)\times10^6$

3.4 Discussion

L. reuteri DPC16 is a promising probiotic bacterium because of its beneficial antimicrobial activity. However, the present results revealed that this strain is very sensitive to a low pH environment and bile salts stress, at least in the absence of nutrients, thus creating problems of delivery to the target site in the GI tract. Similar results have been observed for other probiotic bacteria, at pH values of 2.7 (Wall *et al*., 2007) and 1.8 (Woraharn *et al*., 2010) and in 3% (w/v) taurocholic acid (Ding & Shah, 2009b). *L. bulgaricus KFRI* 673 exhibited no survival after 60 min in SGF at pH 2.0 (Lee *et al*., 2004). *L. reuteri* ATCC 55730 and DSM 16666 were eliminated by SGF after 90 min from an initial cell concentration of 10^9 c.f.u./ml. In contrast, some *L. reuteri* strains, such as *L. reuteri* PTA 4965 and ATCC 53608 have been reported to survive in the gastric environment although there was a 7 $\log_{10}$ c.f.u./ml reduction after 2 h (Muthukumarasamy *et al*., 2006). In addition, it is known that *L. reuteri* DPC16 has a longer survival time at low pH values in the presence of nutrients (Tian *et al*., 2010). Therefore, an immobilisation technique was developed to protect the DPC16 cells from the unfriendly environment, using a simulated gastrointestinal tract model system.

When simulating the GI tract *in vitro*, several factors, such as pH variation, bile stress, distribution of nutrients, and digestive enzymes, should be considered. Gastric pH of an empty stomach and a full stomach is 1.2-2 and 2-6 respectively, which affect the efficiency of a pH sensitive delivery system. Moreover, pepsin is a digestive enzyme released by stomach cells. Therefore, during investigation of the effectiveness of immobilisation systems, an extreme environment of pH 1.2 (Krishnamachari *et al*., 2007), with or without the presence of pepsin, was used in the simulated gastric fluid. *In vivo*, the concentration of bile salts in bile is approximately 0.8%, and varies between 0.2% and 2.0% in the small intestine (Whitehead *et al*., 2008). The pH varies from 6.6 to 7.5 through the middle of the small intestine to the ileum. When simulating the small intestine environment *in vitro*, glucose acts as a sugar source (Vertzoni, 2010) and yeast extract acts as a nutrient for bacteria. The colon is a site for the absorption of nutrients, and hence during simulation of the colonic fluid some nutrients should be present (Probert *et al*., 2004, Walker *et al*., 2005). Faecal water was also incorporated in this simulated fluid, as it may contain toxic compounds.

Initially, a calcium alginate gel, without the addition of other materials, was tested to

determine if this could protect cells in the SGF. After incubation in the fluid, the cells were released using a citrate solution that had been shown to be non-toxic, and the number of viable cells was counted. Unfortunately, even though the concentrations of sodium alginate and calcium ions were optimised, there was little protection conferred upon the cells in the SGF. In the experiment to find the optimum alginate concentration, it was observed that as the concentration of alginate increased, the number of viable immobilised cells also increased. The reason for this can be attributed to the formation of a more compact, dense gel network of calcium alginate matrix, making it less permeable to SGF. Haeberle (2008) reported that the viscosity of alginate solution increases with increasing alginate concentration. The high viscosity might reduce the diffusion rate of SGF, which could explain the observed results. Nonetheless, the overall survival of immobilised *L. reuteri* DPC16 in SGF was still too low, implying that cell immobilisation within calcium alginate gel matrix alone is not sufficient to protect *L. reuteri* DPC16 cells from the acidic SGF. Hence, supplementary materials that could be added to the calcium alginate gel were investigated, including gelatin, chitosan and skim milk.

Incorporation of other biopolymers in calcium alginate beads has been reported, including gelatin (Annan *et al.*, 2008), whey protein (Gbassi *et al.*, 2009) and chitosan (Graff *et al.*, 2008). The mechanism and the possible effects on the encapsulated probiotic cells when they are combined with alginate include:

- Chitosan is positively charged, which allows bonding to alginate and other negatively charged materials, thus strengthening the matrix (George & Abraham, 2006). Below pH 6.5, the amino groups are positively charged and can interact with anionic groups of the microbial cell surface (Calinescu & Mateescu, 2008);
- At acidic pH, gelatin is positively charged and hence can complex with negatively charged alginate. By loading probiotic bacteria in microcapsules based on alginate and gelatin, probiotic bacteria could be continuously released from the microcapsules in the GI tract as gelatin is hydrolysed by pepsin (Li *et al.*, 2009);
- Skim milk contains lactose, which can provide a nutrient for the growth of probiotics, and protein, which, at low pH values, can interact with alginate and strengthen the matrix; the proteins within the skim milk have a pH buffering effect.

The survival of immobilised cells has been shown to be improved by incorporating

additional materials into the alginate gel matrix or by coating the surface of alginate beads with a protective layer of polymers. However, the survival of DPC16 cells in gelatin beads was still low. When 8% skim milk or 1% chitosan were added to the alginate, the number of viable cells that survived during incubation in SGF was higher than 50%. In Table 3.6, the advantages and disadvantages of each immobilisation system are listed.

Because of the ease of preparation, and its effectiveness in protecting cell viability, the system incorporating skim milk (8%) in calcium alginate gel (prepared using 3% alginate and 0.3 M $CaCl_2$) was selected as the optimal system for further research. In addition, this gel system disintegrated in the simulated colonic fluid to release free viable cells at the target site.

Table 3.6 Advantages and disadvantages of different immobilisation materials

Immobilisation systems	Advantages	Disadvantages
Alginate	The simple bead is easy to prepare; the immobilised materials are safe and edible.	The protection effect is not significant.
Alginate+ gelatin	At room temperature, gelatin solidifies, which forms a gel that fills the porous structure of the alginate cross-linking.	Gelatin is sensitive to pepsin, which results in the low survival rate of probiotic cells when pepsin is present in SGF.
Alginate+ skim milk	Skim milk is relatively cheap and efficient; it is widely used in freeze-drying process; it is effective in the protection of immobilised cells	The high viscosity of coating material can possibly block the nozzle used during extrusion.
Alginate+ chitosan	Chitosan is positively charged, it therefore readily binds to negatively charged surfaces such as mucosal membranes (Tapia *et al*., 2005); double coating can improve the efficiency of encapsulation system	Chitosan must be dissolved in acidic solution; the acidic pH can cause viability loss of bacterial cells.

All foods normally leave the stomach within 2 to 4 h. In the small intestine, it usually takes 5 to 6 h for food to be digested and absorbed. The time spent in the colon prior to defecation ranges from 12-24 h (Camilleri *et al*., 1989; Charles *et al*., 1995). Although lactobacilli normally interact with the host in the lower part of the small intestine, there is increasing interest in using the colon as the target site for probiotic bacterial cells (Prakash & Malgorzata Urbanska, 2008). One of the major reasons for this is that during the long transit time in the colon, the probiotic cells have more opportunity to inhibit the growth of intestinal pathogens and/or adhere to the epithelial cells that line the intestine (Kaur *et al*., 2010; Prakash & Malgorzata Urbanska, 2008). The aim of the present work was to achieve a stable release of bacterial cells in the colon.

The effectiveness of the calcium alginate immobilisation system was found to depend on the concentration of alginate, the presence of skim milk and the incubation times during sequential exposure to the GI fluids. Varying the concentration of Ca^{2+} ions in the alginate beads affected the integrity of beads in the simulated GI tract. Calcium alginate prepared at low calcium ion concentration (0.05M) was the weakest, whereas at highest calcium ion concentration (1M), the alginate beads were most stable. The incubation time of immobilised beads in SGF is a critical factor that determines the survival rate of immobilised *L.reuteri* DPC16 cells in the simulated GI tract. When the incubation time of immobilised beads in separate chambers followed the following time pattern: in SGF for 2 h, in SIF for 5 h, followed by SCF 24 h at 37 °C, the immobilisation system could maintain the viability of immobilised *L. reuteri* DPC16 efficiently while releasing viable cells in the SCF.

In conclusion, the optimal concentrations of alginate and calcium chloride for this particular delivery system were 3% and 0.3 M, respectively, in the presence of skim milk (8%). The latter was particularly important in maintaining cell viability. Compared with non-encapsulated *L. reuteri* DPC16 cells, the alginate-based skim milk beads significantly improved the viability of cells during transit through the simulated GI tract. However, the longer the time that beads were in the SGF, the lower the survival rate of encapsulated probiotic cells.

Additionally, the alginate-skim milk-$CaCl_2$ beads were also effective in maintaining the viability of DPC16 cells after freeze-drying and storage, hence allowing a simple

technique for the long-term storage of immobilised probiotic cells.

The purpose of this project was to find an effective method for delivering viable *L. reuteri* DPC16 to the colon and maintaining their beneficial effects after release at the target site. Hence, the next stage of the work was to investigate whether there are any physiological changes to the DPC16 cells during transit through the simulated GI tract.

Chapter 4 Some functional properties of free and immobilised *L. reuteri* DPC16 during and after passage through a simulated GI tract

4.1 Introduction

The previous chapter demonstrated that *L. reuteri* DPC16 is able to survive the passage through the simulated gastrointestinal tract by virtue of the protection conferred by the alginate-skim milk-$CaCl_2$ immobilisation system. In the present chapter, the cells that were released into the simulated colonic fluid were isolated and characterised.

The purpose of this study was to recover the released cells from the simulated colonic fluid for comparison of their functional properties with those of the original strain. The strains were examined for their growth kinetics, bactericidal and bacteriostatic activities, production of selected metabolites, survival in the presence of bile salts, adhesion to epithelial cells, and ability to inhibit adhesion of pathogens to epithelial cells. Ideally, the recovered cells should show no diminution in functional properties.

Previous research (Lu, 2007) on this probiotic strain showed that its cell-free supernatant can inhibit the growth of food-borne pathogens when glycerol was present in the culture medium. The antimicrobial activity of *L. reuteri* can be attributed to reuterin, bacteriocins and organic acids, such as lactic and acetic acids (Schaefer *et al*., 2010; Cleusix *et al*., 2007; Saulnier *et al*., 2009). Reuterin is reported to have anti-pathogen activity and inhibit a wide range of microorganisms including both Gram-positive and Gram-negative bacteria (Bian *et al*., 2011; Spinler *et al*., 2008). When grown in the presence of glycerol, *L. reuteri* can convert glycerol into reuterin (3-hydroxypropionaldehyde) using a cobalamin-dependent glycerol dehydratase. Hence, reuterin and short chain fatty acids (SCFAs) production were compared between the recovered and original DPC16 strains.

To compare the antimicrobial activities of the original and the recovered strains, the target pathogens were grown in the presence of the DPC16 cell-free supernatants and

their growth was monitored using optical density changes. To evaluate the bactericidal properties of the DPC16 supernatants against the specific pathogens, the traditional viable cell plate counting method was used after exposure of the pathogens to the supernatants.

Some recent studies have reported that an immobilisation system can alter the stress tolerance ability of immobilised probiotic bacteria (Doleyres *et al*., 2004; Demattos *et al*., 1994). To test this ability of DPC16, the survival of the recovered and the original cells were compared at different concentrations of bile salts.

To study the adhesion of the DPC16 cells to human intestinal epithelial cells, two human carcinoma cell lines, HT-29 and Caco-2 were used. These two cellular models express morphological and functional differentiation *in vitro* and show characteristics of mature enterocytes, including polarisation, a functional brush border, and apical intestinal hydrolases (Hauri *et al*., 1985; Meunier *et al*., 1995; Zwelbaum *et al*., 2011). To simulate the human colonic epithelial cells, HT-29 and Caco-2 cells form two clearly distinguishable domains as an apical membrane and a basolateral membrane separated by tight junctions. Because the bacterial cells need to bind with specific receptors of mucosal cells, Caco-2 and HT-29 cells are considered to be the best available models to study intestinal attachment of bacteria and viruses (Coconnier *et al*., 1992; Resta-Lenert *et al*., 2011). Adhesion of DPC16 to HT-29 and Caco-2 cells was examined at two pH values as adhesion can occur in both the small intestine (pH 7.2) and the colon (pH 5.8). A fluorescent probe was also used to show the cell adhesion.

Adhesion of pathogenic bacteria to mucosal surfaces is the first step of intestinal infections, but this may be inhibited by blocking the receptors. To determine the inhibitory effect of DPC16 on the adhesion of a pathogen (*E. coli* O157:H7), three different adhesion patterns (i.e. competition, displacement and exclusion) to Caco-2 cells were examined, and the inhibitory abilities of the original and recovered DPC16 cells were compared.

4.2 Methods and Materials

4.2.1 Bacterial strains and culture growth

Probiotic strains *Lactobacillus plantarum* DPC206, *Pediococcus acidilactici* DPC209, *Lactobacillus reuteri* DPC16 and *Bifidobacterium lactis* HN0196 were provided by Bioactive Research New Zealand Ltd. (Auckland, New Zealand) as a 20% (v/v) glycerol stock in MRS broth (Difco, Michigan, USA) at -80°C. The cells were activated in MRS broth at 37°C overnight, and then purified by streaking on MRS agar with anaerobic incubation (Gaspak system, BBL, USA). The streaked MRS plates were anaerobically incubated at 37°C for 48 h.

Frozen stocks of pathogens were provided by the microbiology laboratory of Massey University (Auckland, New Zealand) and grown in BHI broth (MERCK, Germany). *E. coli* O157:H7 strain 2988 and *Salmonella derby* were incubated at 37°C for 24 h; *Staphylococcus aureus* was incubated at 35°C for 24 h, and *Listeria monocytogenes* was cultured at 30°C for 24 h.

The recovered DPC16 cells were collected from the simulated colonic fluid after passage through the simulated GI tract (SGF 2 h, SIF 5 h, and SCF 24 h). SCF solution (1 ml), which contained viable DPC16 cells released from the alginate-skim milk-$CaCl_2$ immobilisation system, was centrifuged (Heraeus Biofuge primo R, Kendro Laboratory Products, Germany) at 3200×g (4°C) for 10 min and the cells were washed twice before being plated on MRS agar. After 48 h incubation, the colonies were examined and compared with the original DPC16 cells, and the cells were examined microscopically. Colonies were randomly selected to prepare the cell-free supernatants after growth in MRS medium (in the presence and absence of glycerol). Isolates were stored at 4°C and sub-cultured every month. Both first and third generation cultures were tested for antimicrobial activity to check the stability of this strain.

4.2.2 Preparation of cell-free supernatants of *L. reuteri* DPC16

L. reuteri DPC16 was inoculated into MRS broth, with or without the presence of 250 mM glycerol, and incubated at 37°C for 16 h. Cultures were harvested by centrifugation (3200×g, 10 min, at 4°C). The supernatants were collected and adjusted to pH 7.0 or 4.4 as appropriate, and then sterilised by passage through a 0.22 µm (Global Science, New Zealand) syringe-filter system.

4.2.3 Antimicrobial assay of strain DPC16 supernatants

The antimicrobial activities of the cell-free supernatants were assayed against the target pathogens using 96-well microtitre plates using the modified method of Bian (2011). Briefly, a 20 µl aliquot of each supernatant was added into 180 µl of BHI broth containing each pathogen (at a concentration of 10^4 c.f.u./ml) prepared on a 96-well plate. The control group contained 180 µl pathogen cultures and 20 µl MRS or MRSg (pH adjusted to 4.4). The inoculated plates were incubated at 37˚C, 35˚C or 30˚C for 24 h, according to the optimum growth temperature of each pathogen. The optical density was measured every 3600 sec as a cycle (30 sec additional double orbital shaking mode was set before each cycle) at 620 nm. The inhibitory effects were examined from three aspects: the length of the lag phase, the maximum specific growth rate, and the endpoint optical density reading.

The maximum specific growth rate of each pathogen response curve is given by the slope of the line when the organism grows exponentially (Figure 4.1).

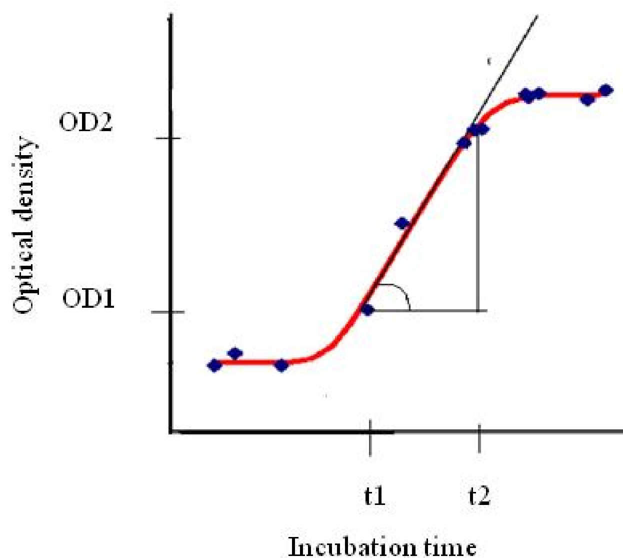


Figure 4.1 Typical growth curve (where growth rate $=\frac{OD2-OD1}{t2-t1}$).

Notes: OD1, the optical density at t1; OD2, the optical density at t2.

4.2.4 Bactericidal effects of strain DPC16 supernatants

The survival of the target pathogens was examined in cell-free supernatants from *L. reuteri* DPC16 cultures. The pathogen cells (50 µl at a concentration of approximately 10^9 c.f.u./ml) were washed twice with 0.1% peptone water, and then incubated in 1 ml of DPC16 supernatant (pH adjusted to either 7.0 or 4.4) for 4 h at the appropriate temperature. For control samples, cells were incubated in 0.1% peptone water alone. The numbers of surviving pathogen cells were enumerated on an hourly basis using plate count agar.

4.2.5 Effect of bile salts on the two DPC16 strains

The original and recovered DPC16 strains were incubated in the presence of 0%, 0.2%, 0.4%, 0.8%, 1.6%, 3.2% (w/v) bile salts (Sigma, dissolved in Tris-HCl, pH 7.2). The numbers of surviving cells were determined at 0, 1, 3 and 5 h of incubation, by plating

on MRS agar.

4.2.6 Determination of reuterin and short chain fatty acids

Reuterin was determined using a colorimetric method (Circles, 1945). Samples were diluted appropriately in 95% ethanol, and 2 ml was incubated with 0.5 ml of 0.01 M tryptophan solution (Sigma, USA) and 6.3 ml of 12 M HCl (37%, J. T. Baker Chemical Co., USA). The mixtures were held for 50 min at 40°C to allow maximum colour development, followed by reading the absorbance at 560 nm. A standard curve was prepared using acrolein (BioChemika, Fluka, Sigma, EU), diluted as required in 95% ethanol.

Lactate was measured using a YSI 2700 Select Biochemistry Analyser with a lactate membrane. Concentrations of short chain fatty acids were measured using high performance liquid chromatography (Dionex ICS 2000 Ion Chromatography System) with an AS11-HC column. The acids were eluted using a concentration gradient of KOH from 0.7 mM to 95 mM.

4.2.7 *In vitro* enterocyte cells model

The human carcinoma cell line, HT-29, and human colon adenocarcinoma, Caco-2, cells were provided by Plant & Food Research Institute (Auckland, New Zealand). HT-29 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) containing 10% (v/v) fetal bovine serum (heat inactivated at 60°C, for 45 min) (Invitrogen, USA) and 1% antibiotics (including streptomycin and penicillin) (Invitrogen, USA) at 37°C in an atmosphere of 5% CO_2 in air. As the monolayers reached 80-90% confluence, the cells were transferred into a 12-well plate. The seed concentration was 3.5×10^5 cells/ml. After seeding, the culture medium was refreshed daily until HT-29 monolayers were at 90% confluence.

Caco-2 cells were cultured in Modified Eagle Medium (MEM, Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 60°C, for 45 min) (Invitrogen, USA), 1% antibiotics (including streptomycin and penicillin) (Invitrogen, USA), 1% Non-Essential Amino Acid (Invitrogen, USA) and 1% sodium pyruvate

(Invitrogen, USA). After the monolayer was at 90% confluence, the cells were seeded in 12-well plates at 37°C in an atmosphere of 5% CO_2 in air for 15 days to allow their differentiation. During the incubation, the MEM medium was replaced every other day.

4.2.8 Adhesion of strain DPC16 cells to HT-29 and Caco-2 cells

Adhesion of *L. reuteri* DPC16 to HT-29 cells was determined as described by Chauviere (1992). DPC16 cells were grown in MRS broth for 18 h, followed by centrifugation at 3200×g for 10 min. After washing with PBS, the cells were suspended in DMEM medium without added antibiotics or fetal bovine serum. The monolayers of HT-29 were washed twice with PBS (pH 7.4) before the addition of *L. reuteri* DPC16 cells and the plates were incubated at 37°C in 5% CO_2 in air for 1.5 h to allow adhesion to take place. After incubation, each well was washed three times with PBS (pH 7.4) to remove the non-adhering *L. reuteri* DPC16 cells. The monolayers were trypsinised to separate the monolayer HT-29 cells and the adhered bacteria from the well. *L. reuteri* DPC16 cells were enumerated on MRS agar, and the cell counts were compared with the HT-29 cell number to obtain the adhesion efficiency (c.f.u./cell).

The adhesion of DPC16 cells to Caco-2 cells used similar methodology. However, because it was difficult to separate the differentiated Caco-2 cells into single cells, the results were expressed as Adhesion Percentage (attached bacterial cells per total bacterial cells in the well).

A separate batch of DPC16 cells at a concentration of 1×10^9 c.f.u./ ml was prepared for a fluorescence-based cell adhesion assay using HT-29 and Caco-2 cells. Firstly, DPC16 cells were washed and re-suspended in 0.1% peptone to 1 ml. Then a fluorescent probe carboxyfluorescein diacetate (CFDA) (Sigma, USA) from stock solution was dissolved in 0.1% peptone to a concentration of 10 μM. An equal volume of CFDA solution was added to the cell suspension. The solutions were mixed gently and were incubated for 15 min at 37˚C. Dyed cell mixtures were immediately centrifuged at 3000 rpm for 5 min and re-suspended with DMEM OR MEM to 1 ml prior to performing the adhesion assay. The resulting adhesion was observed using a fluorescence microscope (Olympus,

model CKX41, Japan).

4.2.9 Inhibition of *E. coli* adhesion to Caco-2 cells by probiotic bacteria

For these experiments, in addition to strain DPC16 cells (both the original and those recovered after passage of encapsulated cells through the simulated GI tract), three other probiotic strains were tested for comparative purposes. Three different types of experiments were performed: (i) competition assay, where the probiotic bacterial cells, at a concentration of 10^8 c.f.u./ml, and *E. coli* (10^7 c.f.u./ml) were incubated simultaneously with Caco-2 monolayers; (ii) displacement assay, where *E. coli* (10^7 c.f.u./ml) was added to Caco-2 monolayers and incubated for 1 h at 37°C prior to addition of the probiotic (10^8 c.f.u./ml); (iii) exclusion assay, where probiotic cells (10^8 c.f.u./ml) were incubated with Caco-2 cells for 1h, prior to addition of *E. coli* cells (10^7 c.f.u./ml) to each well. Plates were then incubated at 37°C for 1 h before washing three times with PBS (pH 7.4) to remove the non-adhering cells. The monolayers were trypsinised to separate the monolayer Caco-2 cells and the adhered cells from the well. Samples were plated on MacConkey agar, and the viable cells of *E. coli* were compared with those obtained in the control group, in which *E. coli* had been allowed to adhere to Caco-2 monolayers in the absence of any probiotic cells.

After the displacement assay, the samples were stained using the fluorescent dye hexidium iodide (Sigma, USA) which stains Gram-positive cells in particular and confers a red or orange colour, and SYTO 13 (Sigma, USA), which stains cells green. Results were observed using a fluorescence microscope (Olympus, model CKX41, Japan).

4.2.10 Statistical analysis

To determine if there were any significant differences between the growth kinetics and antimicrobial activities of the original DPC16 cells and the recovered cells, a Two-Sample T-Test and one-way ANOVA test were used for statistical analysis with 95% confidence intervals. All the statistical analysis were done using Minitab 15.0. The data

presented in the tables are the mean values of replicates of at least 2 independent experiments.

4.3 Results

4.3.1 Growth kinetics of the original and recovered strains of DPC16

The original and recovered cultures were grown in MRS broth, at an initial concentration of 10^6 c.f.u./ml (Figure 4.2). There was virtually no lag phase in the growth curves. The cells quickly entered exponential phase, and the optical density increased from below 0.1 to above 1.2. After approximately 21 h, the optical density started to decline, indicating the cells entered the decline phase.

The experimental data showed no significant statistical differences ($P>0.05$) in growth rate or endpoint reading between the original and recovered DPC16 cells.

The experimental results in Table 4.1 show the growth rates of the cultures as they progressed from the exponential phase, through the stationary phase to the decline phase. No significant differences ($P>0.05$) were observed between the two cultures at any of the growth phases, nor in the endpoint optical density values (Table 4.2). There was also no significant difference ($P>0.05$) in the pH changes between these two strains (Table 4.3) as they progressed through the growth.

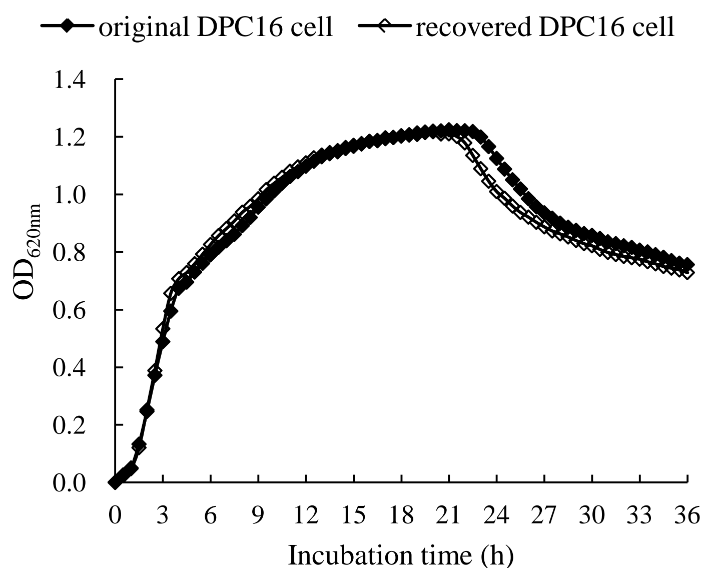


Figure 4.2 Growth curves of original and recovered *L. reuteri* DPC16 cells.

Table 4.1 Growth rates of two *L. reuteri* DPC16 cultures in different phases. The rates were calculated from triplicate OD_{620nm} readings at different time intervals. A Two-Sample T-test was performed and P-values were calculated (significant difference at $P<0.05$).

Growth rate	Exponential phase	Stationary phase	Decline phase
Original cell	0.22±0.03	0.038±0.03	-0.03±0.02
Recovered cell	0.24±0.06	0.034±0.02	-0.03±0.02
P-value	0.42	0.57	0.79

Table 4.2 Optical density reading at the endpoint (36 h) for two *L. reuteri* DPC16 cultures. A Two-Sample T-test was performed and P-values ($P=0.05$) were calculated.

Endpoint reading (OD_{620nm})	Mean
Original cell	0.76±0.04
Recovered cell	0.73±0.02
P-value	0.53

Table 4.3 pH changes during the growth of two *L. reuteri* DPC16 cultures. A Two- Sample T-test was performed and P-values ($P=0.05$) were calculated.

	Growth period (h)					
	0	1	18	24	28	30
Original cell	6.38±0.000	6.29±0.010	4.48±0.010	4.39±0.005	4.47±0.000	4.46±0.005
Recovered cell	6.40±0.005	6.29±0.005	4.46±0.005	4.39±0.005	4.43±0.000	4.43±0.005
P-value	1.00	0.73	0.41	1.00	1.00	0.052

4.3.2 Antimicrobial activities of cell-free supernatants from *L. reuteri* DPC16 cultures

The original and recovered strains of DPC16 were grown in MRS broth, in the presence and absence of glycerol, to obtain the culture supernatants. The antimicrobial activity against the pathogens was compared from three aspects: the length of lag phase, the maximum growth rate and the endpoint OD_{620nm} reading.

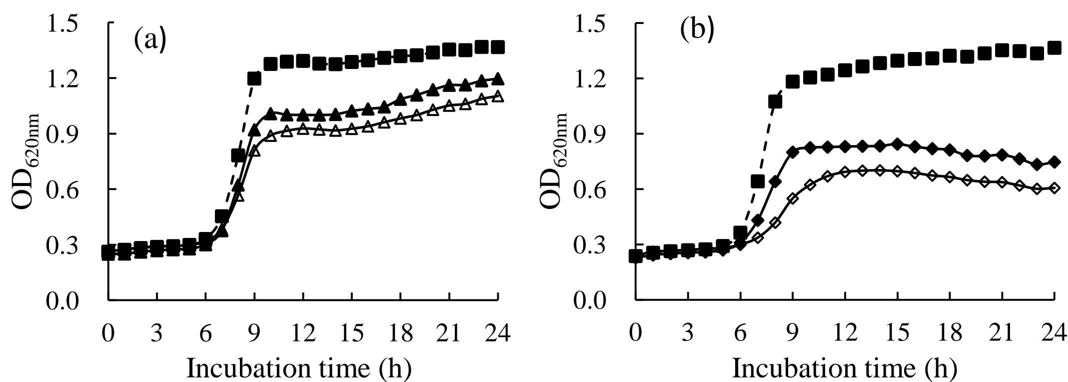
In Figures 4.3 to 4.4, the abbreviations used are:

Abbreviation	Stands for
BHI	Pathogen incubated in BHI (control)
So	Pathogen incubated in BHI with supernatant from original DPC16 culture
Sr	Pathogen incubated in BHI with supernatant from recovered DPC16 culture
So/g	Pathogen incubated in BHI with supernatant from original DPC16 culture grown in the presence of glycerol
Sr/g	Pathogen incubated in BHI with supernatant from recovered DPC16 culture grown in the presence of glycerol

4.3.2.1 The length of lag phase

Figure 4.3 shows the growth response curves of the tested pathogens grown in the presence of supernatants (pH 4.4) from the different DPC16 cultures. Regarding the effect of the supernatants on the respective lag phases of the pathogens, there was little effect when either strain of DPC16 was grown in MRS alone, but when grown in the presence of glycerol, the DPC16 supernatants caused an extension of the lag phase of all pathogens, especially those of *S. derby* and *S. aureus*.

E. coli



S. derby

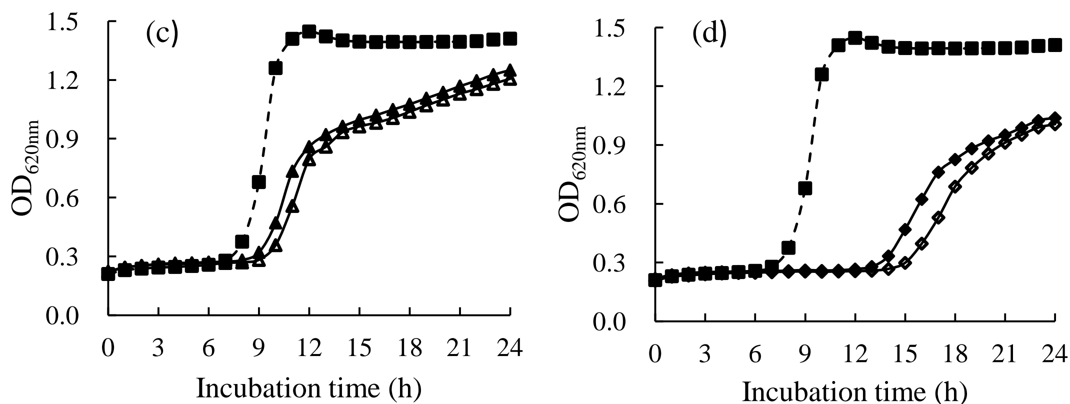
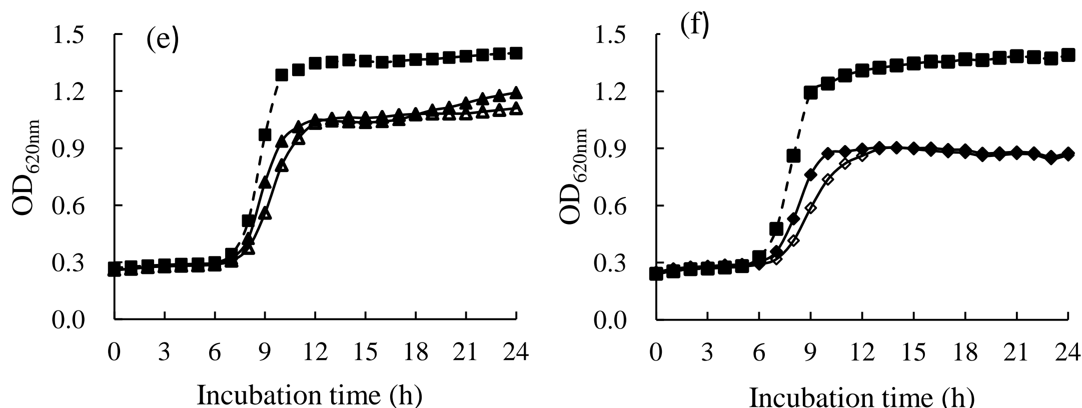


Figure 4.3 Antimicrobial activities of DPC16 supernatants (pH 4.4) against growth of selected pathogens.

Symbols: ■control; ▲original DPC16 grown in the absence of glycerol; Δ recovered DPC16 grown in the absence of glycerol; ♦ original DPC16 grown in the presence of glycerol; ◊ recovered DPC16 grown in the presence of glycerol. In experiments (a), (c), (e) and (g), the supernatants were without glycerol, and in experiments (b), (d), (f) and (h), the supernatants were with glycerol.

L. monocytogenes



S. aureus

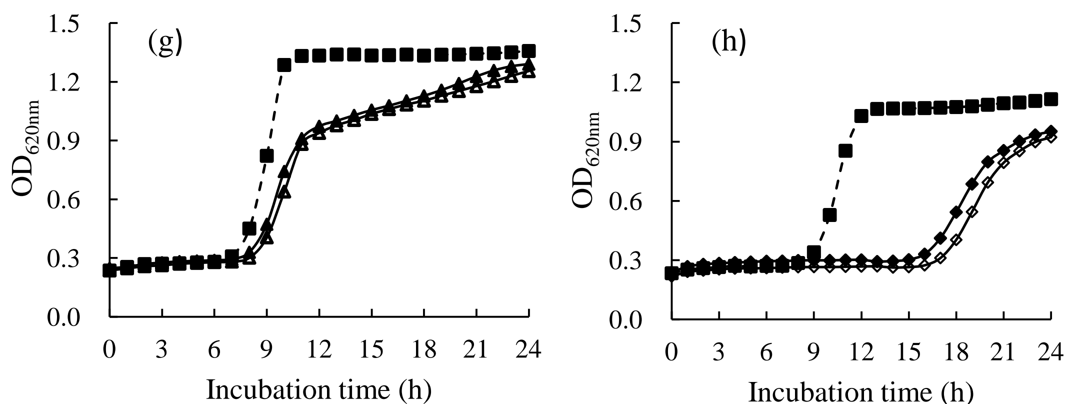


Figure 4.4 Antimicrobial activities of DPC16 supernatants (pH 4.4) against growth of selected pathogens.

Symbols: ■control; ▲original DPC16 grown in the absence of glycerol; Δ recovered DPC16 grown in the absence of glycerol; ♦ original DPC16 grown in the presence of glycerol; ◊ recovered DPC16 grown in the presence of glycerol. In experiments (a), (c), (e) and (g), the supernatants were without glycerol, and in experiments (b), (d), (f) and (h), the supernatants were with glycerol.

4.3.2.2 The maximum specific growth rate

Table 4.4 summarises the observed growth rates of selected pathogens in BHI with DPC16 supernatants. All the samples of the DPC16 supernatants had significant inhibitory effects ($P<0.05$) on the growth of the pathogens. Further, when DPC16 was grown in MRS containing glycerol, the recovered DPC16 culture had a significantly stronger ($P<0.05$) inhibitory effect on the pathogens than did the original culture, except on *L. monocytogenes*. When grown in MRS without glycerol, there was significantly greater ($P<0.05$) antimicrobial activity of the recovered culture against *E. coli* and *L. monocytogenes*.

Table 4.4 Growth rates of tested pathogens in BHI and in the additional presence of DPC16 supernatants. One-way ANOVA and Two-Sample T-test were performed, with $P<0.05$ indicating statistical significance.

(a) Growth rates of pathogens in BHI and in the presence of DPC16 supernatants grown in MRS alone

	E. coli	*S. derby*	*L. monocytogenes*	*S. aureus*
BHI	0.37±0.040	0.35±0.021	0.31±0.008	0.33±0.001
So	0.27±0.029*	0.18±0.010*	0.26±0.020*	0.15±0.020*
Sr	0.21±0.038*#	0.17±0.009*	0.19±0.022*#	0.12±0.015*

(b) Growth rate of pathogens in BHI and in the presence of DPC16 supernatants grown in MRS containing glycerol

	E. coli	*S. derby*	*L. monocytogenes*	*S. aureus*
BHI	0.36±0.033	0.35±0.021	0.29±0.019	0.23±0.013
So/g	0.18±0.021*	0.12±0.002*	0.17±0.011*	0.12±0.008*
S r/g	0.11±0.023*#	0.10±0.004*#	0.14±0.016*	0.11±0.001*#

(*) indicates significant difference ($P<0.05$) between the BHI group and the supernatants group; (#) indicates significant difference ($P<0.05$) between the original DPC16 group and the recovered DPC16 group.

4.3.2.3 Endpoint optical density reading

The endpoint optical density readings were compared between the groups (Figure 4.4)

and the experimental results are summarised in Table 4.5. The results demonstrate similar trends to those observed with the growth rate; all of the DPC16 supernatants showed significant (P<0.05) inhibitory effects, while the supernatant produced from the recovered strain had, in some cases, significantly greater (P<0.05) inhibitory effects than the supernatant produced from the original strain.

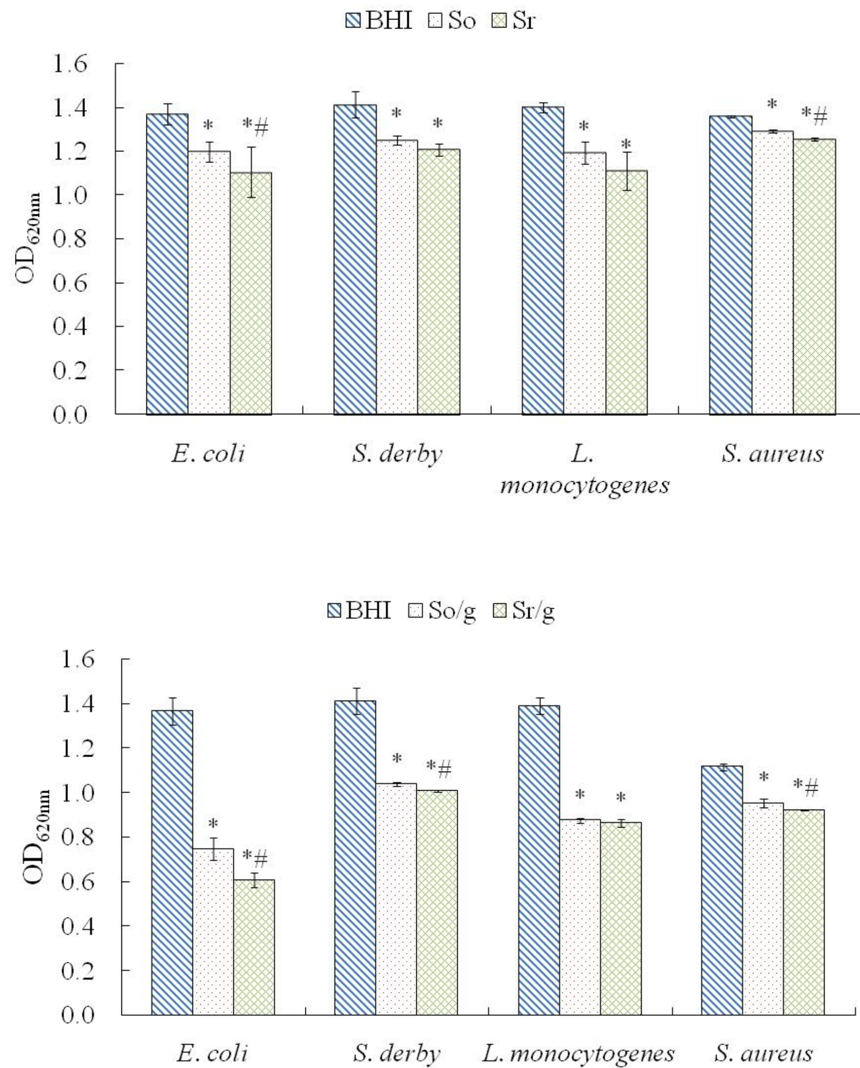


Figure 4.5 OD_{620nm} of pathogens grown in BHI and in the presence of DPC16 supernatants, at the end of incubation (24 h). Error bars are standard deviation from triplicate OD_{620nm} readings. The symbol (*) above the standard error bar indicates the supernant groups are significantly different from the BHI group (P<0.05), and (#) indicates that the recovered cultures are more inhibitory than are the original cultures (P<0.05).

Table 4.5 A summary of the inhibitory effects of the DPC16 supernatants when grown in the presence and absence of glycerol, on the growth of pathogens

Pathogen	Glycerol	Supernatant source The original culture	 The recovered culture
E. coli	+	i	*
	-	i	*
S. derby	+	i	*
	-	i	N
L. monocytogenes	+	i	N
	-	i	*
S. aureus	+	i	*
	-	i	N

(i) indicates a significant (P<0.05) inhibitory effect of the original DPC16 supernatant on the growth of pathogen.
(*) indicates a significant (P<0.05) up-regulation of the inhibitory effect by the recovered DPC16 culture supernatant.
(N) indicates no significant difference (P<0.05) between the antimicrobial activities of the recovered cells compared with those of the original cells.
(+) indicates DPC16 supernatant from the spent culture with the presence of glycerol.
(-) indicates DPC16 supernatant from the spent culture without the presence of glycerol.

4.3.3 Bactericidal effects of DPC16 supernatants on selected pathogens

Two sets of experiments were performed. In one experiment, the culture supernatants were used at their original values (pH 4.4), while in the other, the pH value was adjusted to pH 7.0. The results are shown in Figure 4.5 and Table 4.6 and summarised in Table 4.7. All the supernatants, at both pH values, had a bactericidal effect on all the pathogens used. The effect was much more pronounced at pH 4.4 than at pH 7.0. In addition, the bactericidal effect was stronger in those supernatants that had been prepared from cultures growing in the presence of glycerol compared to those grown in MRS alone. Finally, the bactericidal (killing) activity of the recovered DPC16 culture was significantly greater (P<0.05) than that of the original strain.

It was noted that, in some cases, the killing effects of DPC16 supernatants (pH 7.0) were obvious during the first 1 h after which there was no significant change (P>0.05). The reason for this is not clear, but it could be due to the degradation of the active bactericidal compound(s) or to the presence of resistant cells.

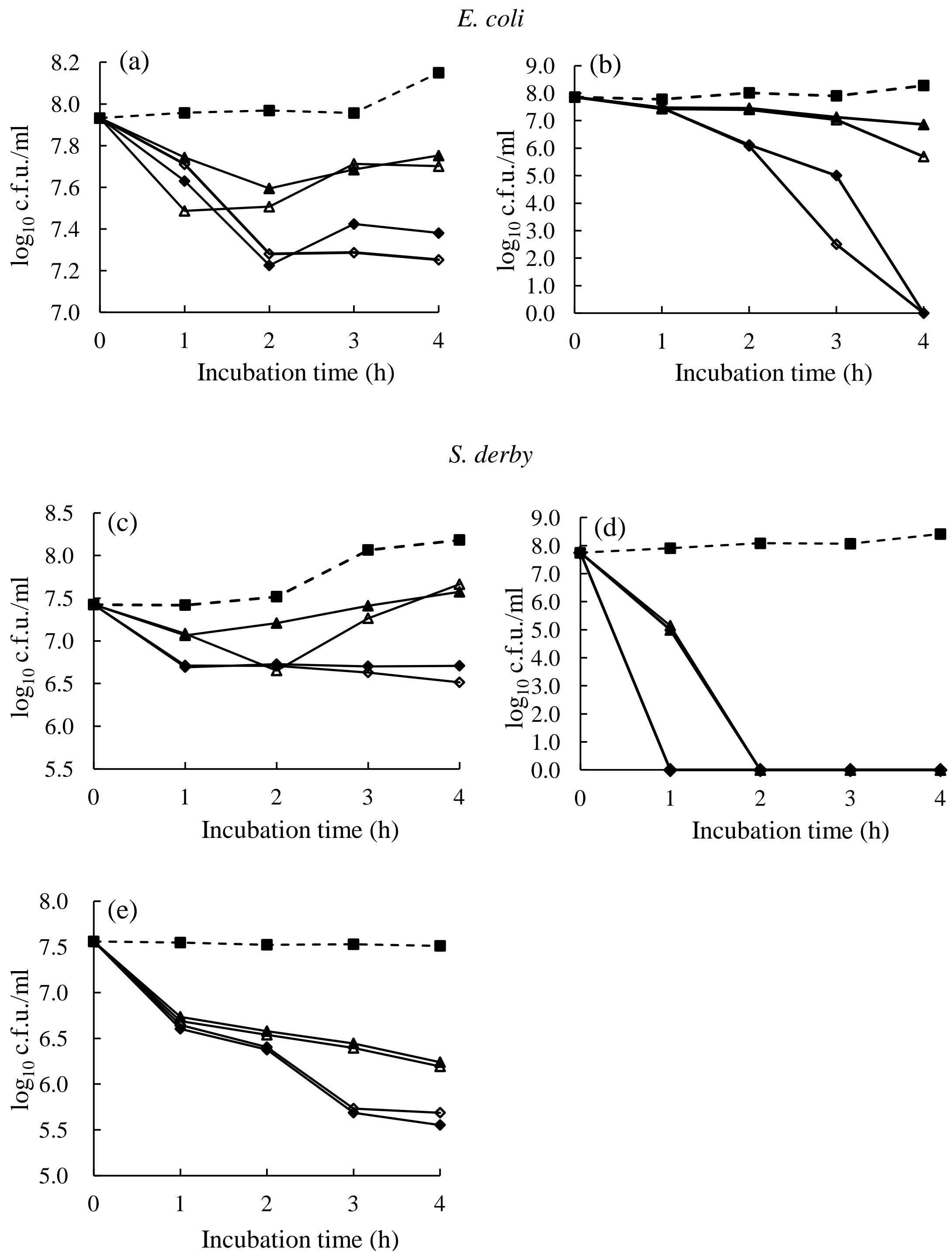


Figure 4.6 Bactericidal activity of DPC16 cell-free supernatants (pH adjusted to 7.0 and unadjusted (approximately pH 4.4)) on growth of *E. coli*, *S. derby*, *L. monocytogenes* and *S. aureus.*

Symbols: ■control; ▲original DPC16 grown in the absence of glycerol; Δ recovered DPC16 grown in the absence of glycerol; ♦ original DPC16 grown in the presence of glycerol; ◊ recovered DPC16 grown in the presence of glycerol. In experiments (a), (c), (f) and (h), the supernatants were adjusted to pH 7.0, and in experiments (b), (d), (e), (g) and (i), the pH was unadjusted (approximately pH 4.4). For *S. derby*, experiment (d) used the original concentration, but in experiment (e), the supernatants were diluted ×10.

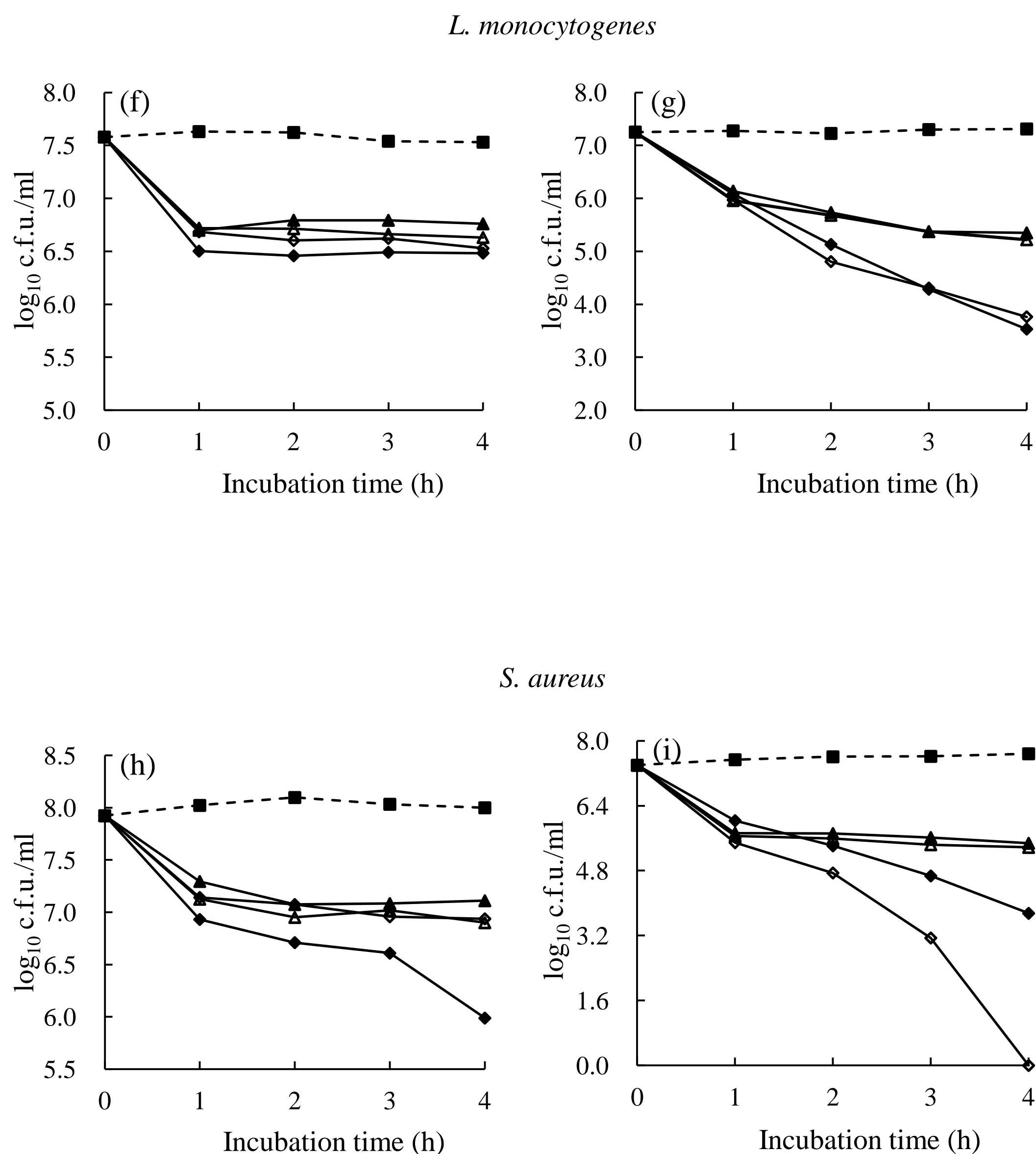


Figure 4.7 Bactericidal activity of DPC16 cell-free supernatants (pH adjusted to 7.0 and unadjusted (approximately pH 4.4)) on growth of *E. coli*, *S. derby*, *L. monocytogenes* and *S. aureus.*

Symbols: ■control; ▲original DPC16 grown in the absence of glycerol; Δ recovered DPC16 grown in the absence of glycerol; ♦ original DPC16 grown in the presence of glycerol; ◊ recovered DPC16 grown in the presence of glycerol. In experiments (a), (c), (f) and (h), the supernatants were adjusted to pH 7.0, and in experiments (b), (d), (e), (g) and (i), the pH was unadjusted (approximately pH 4.4). For *S. derby*, experiment (d) used the original concentration, but in experiment (e), the supernatants were diluted ×10.

Table 4.6 Bactericidal effects of *L. reuteri* DPC16 supernatants on selected pathogens. The viable cell counts (c.f.u./ml) were the average of triplicate counts of each sample at different time intervals. One-way ANOVA and Two-Sample T-test were performed, and P-values were calculated, with P <0.05 indicating significance.

Incubation time (h)	Group	Pathogens (c.f.u./ml)							
		E. coli	*S. derby*	*S. aureus*	*L. monocytogenes*	*E. coli*	*S. derby*	*S. aureus*	*L. monocytogenes*
		pH 7				pH 4.4			
	peptone	9.07×10^{7}	2.63×10^{7}	1.05×10^{8}	4.27×10^{7}	6.57×10^{7}	3.52×10^{7}	3.43×10^{7}	1.88×10^{7}
	supernatant from the original cell	5.53×10^{7}*$^{\Delta}$	1.16×10^{7}*	1.97×10^{7}*$^{\Delta}$	4.99×10^{6}*	2.95×10^{7}*	5.44×10^{6}*$^{\Delta}$	5.31×10^{5}*	1.37×10^{6}*
	supernatant from the recovered cell	3.07×10^{7}*	1.21×10^{7} *	1.33×10^{7}*	5.23×10^{6} *	2.71×10^{7}*	4.88×10^{6}*	4.55×10^{5}*	9.07×10^{5} *
1	supernatant from the original cell with the presence of glycerol	5.13×10^{7}*	5.15×10^{6}*	1.39×10^{7}*#	4.83×10^{6} *#	2.95×10^{7}*	4.43×10^{6}*	1.08×10^{6}*	9.33×10^{5}*#
	supernatant from the recovered cell with the presence of glycerol	4.27×10^{7}*	4.92×10^{6}*	8.55×10^{6}*	3.17×10^{6}*	2.91×10^{7}*	4.01×10^{6}*	3.09×10^{5} *	1.21×10^{6}*
	peptone	9.31×10^{7}	3.29×10^{7}	1.25×10^{8}	4.19×10^{7}	5.94×10^{7}	3.33×10^{7}	4.04×10^{7}	1.68×10^{7}
	supernatant from the original cell	3.93×10^{7}*$^{\Delta}$	1.61×10^{7}*$^{\Delta}$	1.19×10^{7}*$^{\Delta}$	6.21×10^{6}*$^{\Delta}$	2.83×10^{7}*	3.79×10^{6}*	5.20×10^{5}*	5.40×10^{5}*
	supernatant from the recovered cell	3.21×10^{7}*	7.20×10^{6}*	8.93×10^{6}*	5.16×10^{6}*	2.55×10^{7}*	3.45×10^{6}*	3.89×10^{5}*	4.77×10^{5}*
2	supernatant from the original cell with the presence of glycerol	1.91×10^{7}*	5.11×10^{6}*#	1.19×10^{7}*#	4.00×10^{6}*	1.20×10^{6}*	2.53×10^{6}*	2.59×10^{5}*	6.40×10^{4}*
	supernatant from the recovered cell with the presence of glycerol	1.68×10^{7} *	5.33×10^{6}*	5.13×10^{6}*	2.87×10^{6}*	1.31×10^{6}*	2.37×10^{6}*	5.55×10^{4}*	1.35×10^{5}*
	peptone	9.05×10^{7}	1.16×10^{8}	1.08×10^{8}	3.46×10^{7}	6.08×10^{7}	3.37×10^{7}	4.15×10^{7}	1.99×10^{7}
	supernatant from the original cell	4.84×10^{7}*	2.57×10^{7}*$^{\Delta}$	1.21×10^{7}*$^{\Delta}$	6.20×10^{6}*$^{\Delta}$	1.33×10^{7}*	2.79×10^{6}*	4.15×10^{5}*	2.35×10^{5}*
3	supernatant from the recovered cell	5.16×10^{7}*	1.84×10^{7}*	1.04×10^{7}*	4.59×10^{6}*	1.08×10^{7}*	2.48×10^{6}*	2.75×10^{5}*	2.35×10^{5}*
	supernatant from the original cell with the presence of glycerol	1.93×10^{7}*#	4.27×10^{6}*#	9.07×10^{6}*#	4.19×10^{6}*#	3.20×10^{2}*#	5.40×10^{5}*#	4.69×10^{4}*#	2.03×10^{4}*
	supernatant from the recovered cell with the presence of glycerol	2.65×10^{7}*	5.04×10^{6}*	4.07×10^{6}*	3.09×10^{6}*	1.01×10^{5}*	4.87×10^{6}*	1.39×10^{3}*	1.92×10^{4}*
	peptone	1.41×10^{8}	1.52×10^{8}	1.00×10^{8}	3.39×10^{7}	6.02×10^{7}	3.24×10^{7}	4.80×10^{7}	2.04×10^{7}
	supernatant from the original cell	5.65×10^{7}*	4.60×10^{7}*$^{\Delta}$	1.29×10^{7}*$^{\Delta}$	5.75×10^{6}*$^{\Delta}$	7.20×10^{6}*$^{\Delta}$	1.73×10^{6}*	2.99×10^{5}*	2.23×10^{5}*
	supernatant from the recovered cell	5.03×10^{7}*	3.75×10^{7}*	7.95×10^{6}*	4.27×10^{6}*	4.93×10^{5}*	1.56×10^{6}*	2.36×10^{5}*	1.65×10^{5}*
4	supernatant from the original cell with the presence of glycerol	1.79×10^{7}*#	3.27×10^{6}*#	8.67×10^{6}*#	3.37×10^{6}*#	0*	4.87×10^{5}*#	5.61×10^{3}*#	5.80×10^{3}*#
	supernatant from the recovered cell with the presence of glycerol	2.40×10^{7}	5.11×10^{6}*	9.73×10^{5}*	3.04×10^{6}*	0*	3.57×10^{5}*	0*	3.40×10^{3}*

(*) indicates significant differences (P<0.05) between the supernatant groups and the peptone group;

(Δ) indicates significant difference (P<0.05) between the supernatant from the original *L. reuteri* DPC16 cells and the supernatant from the recovered *L. reuteri* DPC16 cells in inhibiting pathogens when *L. reuteri* DPC16 was grown in MRS without glycerol;

(#) indicates significant difference (P<0.05) between the supernatant from the original *L. reuteri* DPC16 cells and the supernatant from the recovered *L. reuteri* DPC16 cells when they were grown in MRS containing glycerol.

Table 4.7 A summary of the bactericidal effects of the original DPC16 culture and the recovered DPC16 culture

	Growth in MRS				Growth in MRS + glycerol			
Time (h)	*E. coli*	*S. derby*	*S. aureus*	*L. monocytogenes*	*E. coli*	*S. derby*	*S. aureus*	*L. monocytogenes*
				pH 7.0				
1	*	N	*	N	N	N	*	*
2	*	*	*	*	N	*	*	N
3	N	*	*	*	*	*	*	*
4	N	*	*	*	*	*	*	*
				pH 4.4				
1	N	*	N	N	N	N	N	*
2	N	N	N	N	N	N	N	N
3	N	N	N	N	*	*	*	N
4	*	N	N	N	N	N	*	*

The viable cells were counted at different time intervals and compared. In comparison with the control, all the supernatants demonstrated significant (P<0.05) bactericidal effects on the pathogens.
(*) indicates that the bactericidal effect of the recovered DPC16 cells was significantly greater (P<0.05) than that of the original cells.
(N) indicates that there was no significant difference (P>0.05) between the bactericidal effects of the two DPC16 cultures.

4.3.4 Viability of DPC16 cells in different concentrations of bile salts

Both the original and the recovered strains were sensitive to bile salts under the experimental conditions used, even at a concentration as low as 0.2% (w/v) (Figure 4.6 and Table 4.8). However, the recovered DPC16 culture displayed higher tolerance to bile salts than did the original strain. Interestingly, the recovered strain showed less viability when incubated alone in Tris buffer.

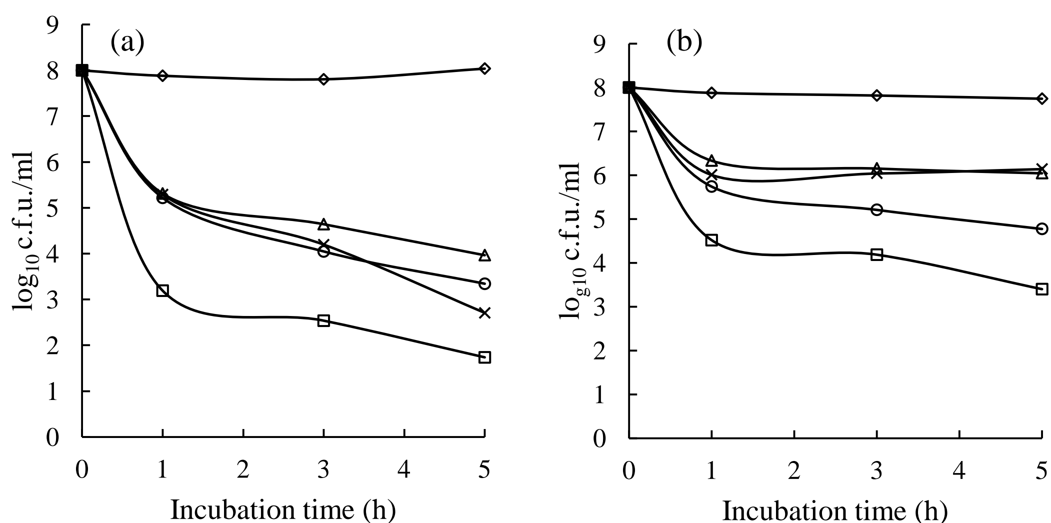


Figure 4.8 Viable cell counts of the two DPC16 strains in different concentrations of bile salts. (a) original DPC 16 culture; (b) recovered DPC 16 culture. Symbols: ◊ Tris-HCl buffer; □ 1.6% bile salts; Δ 0.8% bile salts; ○ 0.4% bile salts; × 0.2% bile salts.

Table 4.8 Viable cell counts (log_{10} c.f.u./ml) of the original and recovered DPC16 cultures in different concentrations of bile salts. A Two-Sample T-test was performed to compare the viabilities of the two strains after 5 h incubation, and P-values were calculated, with P <0.05 indicating significance.

	Time (h)	Tris-HCl bufer	Bile salt (%, w/v)			
			1.6	0.8	0.4	0.2
Initial counts	0	7.99±0.040	7.99±0.040	7.99±0.040	7.99±0.040	7.99±0.040
Original cell	5	8.04±0.027	1.73±0.102	3.97±0.0589	3.34±0.064	2.71±0.063
Recovered cell	5	7.74±0.069*	3.40±0.005*	6.05±0.091*	4.77±0.013*	6.14±0.018*
P-value		0.004	0.000	0.013	0.000	0.001

(*) indicates significant differences (P<0.05) between the original and the recovered culture.

4.3.5 Reuterin and short chain fatty acid production by the original and recovered cells of *L. reuteri* DPC16

The data shown in Table 4.9 indicate that when grown in the absence of glycerol, the recovered DPC16 cells produced significantly more (P<0.05) reuterin than did the original cells. However, there was no significant difference (P>0.05) in the concentration of reuterin when the cultures were grown in the presence of glycerol, although the amounts produced were markedly higher. For the short chain fatty acids and lactate, no significant difference (P>0.05) was found.

Table 4.9 Production of reuterin and short chain fatty acids by the original and recovered cells of *L. reuteri* DPC16

	Original *L. reuteri* DPC16	Recovered *L. reuteri* DPC16	P - value
Reuterin concentration (µg/ml) in the supernatant from cell growth in MRS alone	13.01 ± 0.22	16.66 ± 1.17*	0.008
Reuterin concentration (µg/ml) in the supernatant from cell growth in MRSg	26.70 ± 2.39	29.54 ± 2.07	0.22
Lactate (g/L)	4.94±0.58	5.66±0.29	0.06
Acetate (g/L)	5.37±0.39	5.66±0.16	0.07
Propionate (mg/L)	46.07±1.49	46.72±2.62	0.65
Iso-butyrate (mg/L)	152.7±20.7	147.9±32.6	0.79
Butyrate (mg/L)	20.50±6.50	26.40±1.35	0.12
Iso-valerate (mg/L)	13.52±5.48	18.40±3.08	0.13
Valerate (mg/L)	17.07±9.85	21.00±3.21	0.54

(*) indicates significant difference (P<0.05) in the concentration of reuterin and short-chain fatty acids produced by the recovered cells compared to the original cells.

4.3.6 Adhesion of DPC16 cells to HT-29 and Caco-2 cells

Figures 4.7 and 4.9 show the images of HT-29 and Caco-2 cells, respectively, prior to the adhesion assay. The data on the ability of *L. reuteri* DPC16 to adhere to HT-29 and Caco-2 epithelial cells are shown in Table 4.10. Two different batches of recovered cells were assessed. No significant difference (P>0.05) was observed between the

original and recovered strains when the assay was performed at either pH 5.8 or pH 7.2.

Accoring to Walter *et al*. (1996), the monolayers formed by Caco-2 are morphologically polar and develop brush borders at the apical suface. HT-29 cells are goblet cells, which produces mucin molecules by forming a human mucus layer. In contrast with Caco-2 cells, HT-29 only develops sparse microvilli on the apical side (Karjalainen *et al*., 1994; Gopal *et al*., 2001; Pontier *et al*., 2001). The fluorescent stain results showed that due to the different features of HT-29 and Caco-2 cells when grown *in vitro*, *L. reuteri* DPC16 bacterial cells showed different distributions: unevenly distributed on HT-29 (Figure 4.8), while evenly attached to Caco-2 cells (Figure 4.10).

Table 4.10 Adhesion of *L. reuteri* DPC16 to HT-29 cells (c.f.u./cell) and Caco-2 cells. Adhesion percentage = (attached bacterial cells per well/total bacterial cells per well) ×100%.

Strain	HT-29 Adhesion efficiency (c.f.u./cell)		Caco-2 Adhesion percentage (%)	
	pH 5.8	pH 7.2	pH 5.8	pH 7.2
Original *L. reuteri* DPC16	14.18 ± 0.36	12.14 ± 0.41	2.55 ± 0.047	2.47 ± 0.042
Batch 1 Recovered *L. reuteri* DPC16	14.31 ± 1.96	12.69 ± 0.54	2.56 ± 0.089	2.54 ± 0.032
Batch 2 Recovered *L. reuteri* DPC16	15.77±3.90	9.23±2.59	2.60±0.040	2.50±0.018

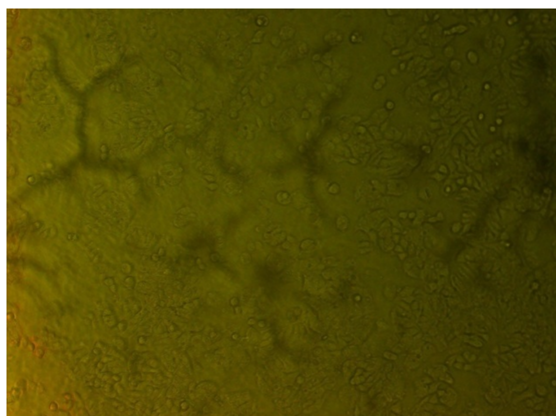


Figure 4.9 HT-29 cells at 90% confluence before the adhesion experiment, under 400× magnification, using a fluorescence microscope (Olympus, model CKX41, Japan).

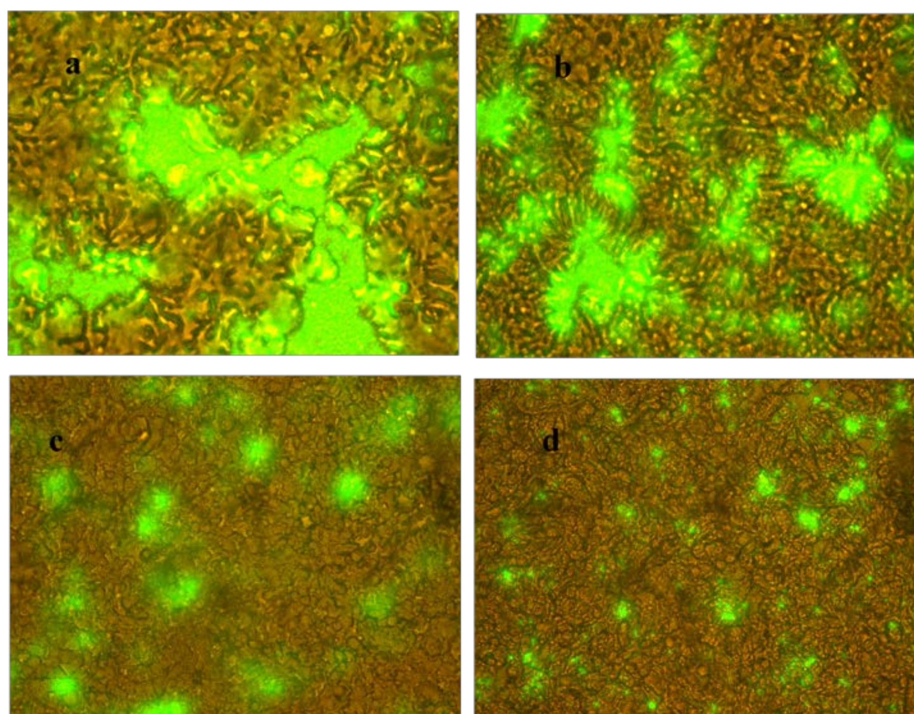


Figure 4.10 Fluorescent stains of *L. reuteri* DPC16 cells adhering to HT-29 cells, under 200× magnification, using a fluorescence microscope (Olympus, model CKX41, Japan). DPC16 cells appear a green colour because of the fluorescent stain. (a): the original DPC16 strain adhering to HT-29 cells at pH 5.8; (b): the original DPC16 strain adhering to HT-29 cells at pH 7.2; (c): the recovered DPC16 strain adhering to HT-29 cells at pH 5.8; (d): the recovered DPC16 strain adhering to HT-29 cells at pH 7.2.

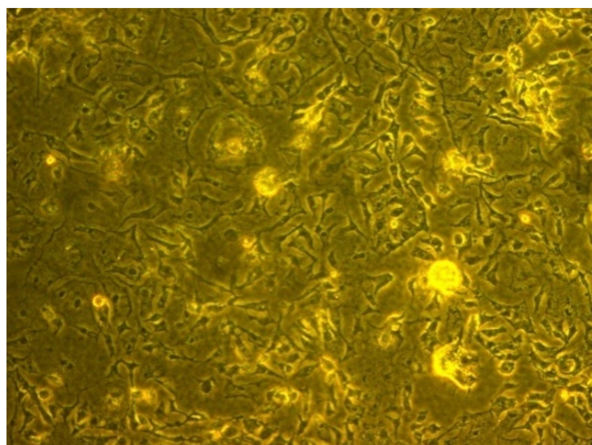


Figure 4.11 Caco-2 cells at >90% confluence before the adhesion experiment, under 200× magnification, using a fluorescence microscope (Olympus, model CKX41, Japan).

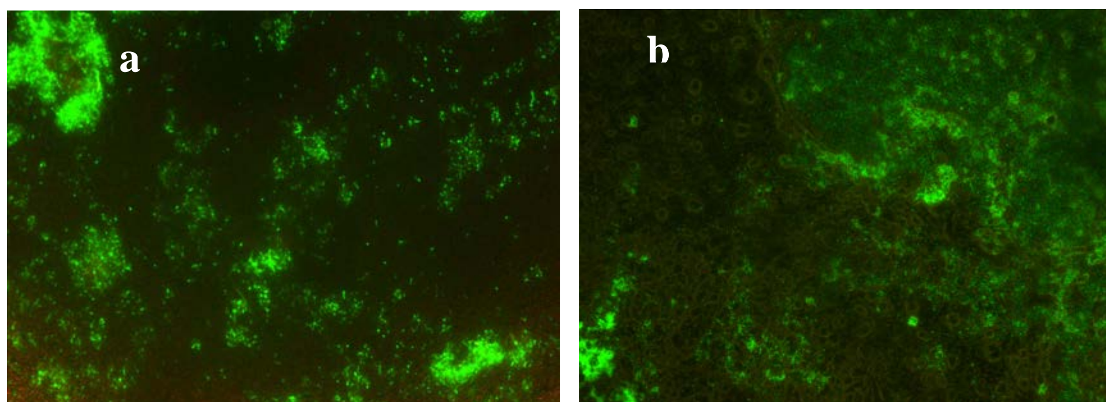


Figure 4.12 Fluorescent stains of *L. reuteri* DPC16 adhering to Caco-2 cells, under 200× magnification, using a fluorescence microscope (Olympus, model CKX41, Japan). DPC16 cells appear green.
(a): original *L. reuteri* DPC16 cell adhering to Caco-2 cells at pH 5.8; (b): recovered *L. reuteri* DPC16 cells adhering to Caco-2 cells at pH 5.8.

4.3.7 Inhibition of adhesion of *E. coli* to Caco-2 cells

The data for the three types of assays are shown in Table 4.11. The probiotic strains significantly inhibited ($P<0.05$) the adhesion of *E. coli* in all the assays. In the displacement assay, *L. reuteri* DPC16 performed the best, while in the exclusion assay, *L. plantarum* DPC206 was the most effective with a highly significant reduction ($P<0.05$) in the numbers of *E. coli* (3.8 $\log_{10}$ c.f.u./ml) adhering to the Caco-2 cells. In the competition assay, the results of different probiotic strains were similar.

In the displacement and exclusion assays the recovered DPC16 strain was significantly more ($P<0.05$) effective than the original strain in preventing adhesion of *E. coli*.

During the displacement assay, the fluorescent stain was used to compare the adhesion difference before and after adding DPC16 cells to *E. coli* pre-occupied Caco-2 cell monolayers (Figure 4.11). The result clearly showed that DPC16 cells could replace *E. coli* at their receptor sites on Caco-2 cells.

Table 4.11 Effect of probiotic bacterial cells on the adhesion of *E. coli* to Caco-2 cells

	$\log_{10}$ c.f.u./ml of *E. coli*		
Probiotic strain	Competition assay	Displacement assay	Exclusion assay
Control	6.14±0.009	6.14±0.011	6.17±0.058
Bifidobacterium lactis HN0196	4.95±0.017*	6.03±0.011*	4.91±0.049*
Pediococcus acidilactici DPC209	4.89±0.056*	5.29±0.079*	4.07±0.011*
Lactobacillus plantarum DPC206	5.04±0.101*	5.73±0.052*	3.83±0.026*
L.reuteri DPC16 original cell	4.97±0.040*	5.11±0.032*Δ	4.73±0.024*Δ
L.reuteri DPC16 recovered cell	4.90±0.020*	5.01±0.017*	4.43±0.014*

(*) indicates significant difference ($P<0.05$) comparing the probiotics treatment groups with control group;
(Δ) indicates significant difference ($P<0.05$) between the original *L. reuteri* DPC16 cell and the recovered *L. reuteri* DPC16 cell in inhibiting the adhesion of *E. coli*.

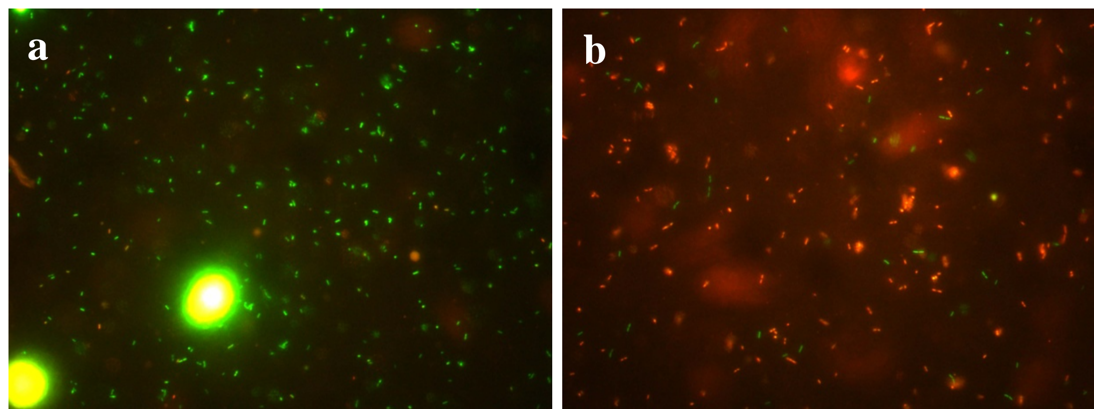


Figure 4.13 Fluorescence microscopy of the displacement assay of DPC16 cells with *E. coli*, under 400× magnification, using a fluorescence microscope (Olympus, model CKX41, Japan). The green stained bacterial cells are *E. coli*, and the red stained ones are *L. reuteri* DPC16 cells: (a) the control, without added probiotic cells, showing *E. coli* adhering to Caco-2 cells; (b) the test group with *L. reuteri* DPC16.

4.4 Discussion

The success of any delivery system for probiotics depends on the ability of the system to deliver viable cells to the colon in sufficient numbers to exert their effects. Further, the functional properties of the bacteria should not be compromised during transit in the GI tract. In the previous chapter, it was shown that *L. reuteri* DPC16 cells could be delivered through the simulated gastrointestinal tract to release viable cells in the colon, by virtue of the protection conferred by the alginate-skim milk-$CaCl_2$ gel system. The purpose of this chapter was to isolate the DPC16 cells that had been released in the colonic fluid and to determine if any of the important functional properties had been compromised in any way. Two separate batches of recovered cells were isolated and tested, and the results were virtually identical.

Firstly, the growth kinetics of these two strains were compared. No significant differences ($P>0.05$) were observed in the growth rates, endpoint OD readings or pH profiles. In the case of bile stress tolerance, the recovered strain was more resistant than the original strain to all the bile salt concentrations.

The production of selected metabolites, and antimicrobial and bactericidal activity against several pathogens were then compared between these two strains. In no case was there any diminution of any of these properties, confirming that the encapsulation

delivery system had no adverse effects on strain DPC16. In fact, one of the major functional properties, antimicrobial activity, was significantly and reproducibly enhanced. Previous research has shown that *L. reuteri* DPC16 produces the antimicrobial compound, reuterin, and that production is enhanced when the cells are grown in the presence of glycerol, its metabolic precursor (Bian *et al*., 2011). It has also been shown that short chain fatty acids are produced and that these contribute to antimicrobial activity at low pH values (Arques *et al*., 2008; Bian *et al*., 2011; Cleusix *et al*., 2007; Jones & Versalovic, 2009). In the current work, the increased antimicrobial activity of the recovered cells appears to be due to increased production of reuterin, as its concentration in the cell-free supernatant, when grown in MRS alone, was significantly higher ($P<0.05$) than in the original cells. This observation has been made twice on two different batches of cells recovered after passage through the simulated GI tract, showing reproducibility. The reason for this increased production is unclear, but it is likely to be in response to a stress factor during encapsulation or passage through the tract. There is some evidence in the literature to suggest the former (Doleyres *et al*., 2004). At the pH 7.0, it is unlikely that the fatty acid salts contributed to the antimicrobial activity.

Adhesion to the intestinal mucosal surface is a prerequisite for probiotic bacterial cells exerting beneficial effects on their hosts (Kailasapathy & Chin 2000; Wang *et al*., 2008). In the current work, cells of *L. reuteri* DPC16 were shown to adhere to both HT-29 and Caco-2 cells and no significant difference ($P>0.05$) was observed in their adhesion ability after passage through the simulated GI tract. Previous work done in this laboratory has shown that the ability of strain DPC16 cells to adhere to Caco-2 cells is similar to that of other probiotic bacteria (Tian, 2011). Some published data for adhesion of other probiotic strains to Caco-2 cells are shown in Table 4.12. The adhesion of strain DPC 16 is a little less than for the other strains, but is of a similar order of magnitude. Further, the ability to inhibit the adhesion of *E. coli*, a crucial property of any probiotic bacteria, was unimpaired. Three *in vitro* models were used to examine this inhibitory effect. The displacement assay simulates the host which suffers from intestinal disease caused by *E. coli*; the exclusion assay simulates pretreatment with probiotics to prevent disease; while the competition assay simulates the host who may be attacked by *E. coli* O157:H7 and dosed with probiotic cells at the same time. The results showed that all the tested probiotics provided a barrier which could reduce

the number of *E. coli* attached to the simulated intestinal epithelium. These results support those obtained with other strains of probiotics (Yu *et al*., 2011). The explanation for this phenomenon may be the steric hindrance of the probiotic cell that prevents the adhesion of the pathogen (Bernet *et al*., 1994). Among the tested probiotic strains, *L. reuteri* DPC16 was not the most effective in inhibiting *E. coli* attachment, but its effect was still significant ($P<0.05$), particularly in the displacement assay. Interestingly, the recovered DPC16 cells were significantly more ($P<0.05$) effective than the original cells in the exclusion and displacement assays, but not in the competition assay. The reason for this is unclear.

Table 4.12 Published data for adhesion of some probiotic bacteria to Caco-2 cells

Bacterial strain	Adhesion percentage (%)	References
Lactobacillus plantarum 9	7.4	Kaushik *et al*. (2009)
Lactobacillus plantarum ATCC 8014	6.7	Kaushik *et al*. (2009)
Lactobacillus plantarum 122E	8.5	Kaushik *et al*. (2009)
Bifidobacterium lactis Bb12	6.9	Guglielmetti *et al*. (2008)
Bifidobacterium lactis NCC189	4.1	Preising *et al*. (2010)
Bifidobacterium lactis S16	1.5	Preising *et al*. (2010)
Pediococcus acidilactici LMG P-21927	0.9	Speelmans *et al*. (2006)
Lactobacillus reuteri DPC16	2.5	Zhao *et al*. (2011)

In conclusion, an alginate-skim milk encapsulation system has been demonstrated to be an efficient delivery system for *L. reuteri* DPC16. Viable cells were released in the simulated colonic fluid, and there was no apparent loss of the functional properties tested. Indeed, the recovered DPC16 cells demonstrated a stronger antimicrobial activity than did the original cells and this was probably due to the higher concentration of reuterin that was produced.

The next step was to determine the cause(s) of the enhancement of the antimicrobial properties. During the immobilisation process and subsequent passage through the GI tract, several factors, including the immobilisation process itself (Doleyres *et al*., 2004; Muthukumarasamy & Holley, 2007), the acidic stress in SGF (Wall *et al*., 2007), and

bile stress in the SIF and SCF (Whitehead *et al*., 2008) may affect the functional characteristics of *L. reuteri* DPC16. Hence, in the following Chapter, an investigation was made into the possible reasons for the phenomenon.

Chapter 5 Investigation into the factors that cause the changes to the bacteriostatic and bactericidal activities of DPC16 cells during passage of immobilised cells through simulated GI tract

5.1 Introduction

In the previous chapter, it was demonstrated that the DPC16 cells that were recovered from the SCF after immobilisation and passage through the simulated GI tract displayed enhanced bacteriostatic and bactericidal activities.

To determine the factor(s) that are responsible for this enhancement, it was now proposed to recover cells from different stages of the process and examine them for their antimicrobial properties. In addition, the properties of freeze-dried immobilised cells would be studied. In this way, each "strain" of recovered cells would be influenced by a minimum number of factors during immobilisation, freeze-drying and passage through the GI tract. The process and the corresponding isolated "strains" are outlined in Figure 5.1 and described in Section 5.2.1.

Reuterin has been shown to play a major role in mediating the antimicrobial activity of *Lactobacillus reuteri* DPC16 cells. The results in chapter indicated that the increased production of reuterin was the cause of the enhanced antimicrobial activity. Therefore, reuterin production was compared among the different isolated DPC16 "strains", and then compared with their corresponding antimicrobial activities. During the production of reuterin from glycerol by *L. reuteri*, it has been suggested that there are two different isofunctional cobalamin-dependent dehydratases, glycerol dehydratase and diol dehydratase, that participate during the reaction (Figure 5.2). Diol dehydratase is a coenzyme B_{12} or Coα-[α-(5,6-dimethylbenzimidazolyl)]-Coβ-adenosylcobamide)-requiring enzyme which catalyses the conversion of 1,2-propanediol, 1,2-ethanediol, and glycerol to propionaldehyde, acetaldehyde, and 3-hydroxypropionaldehyde, respectively.

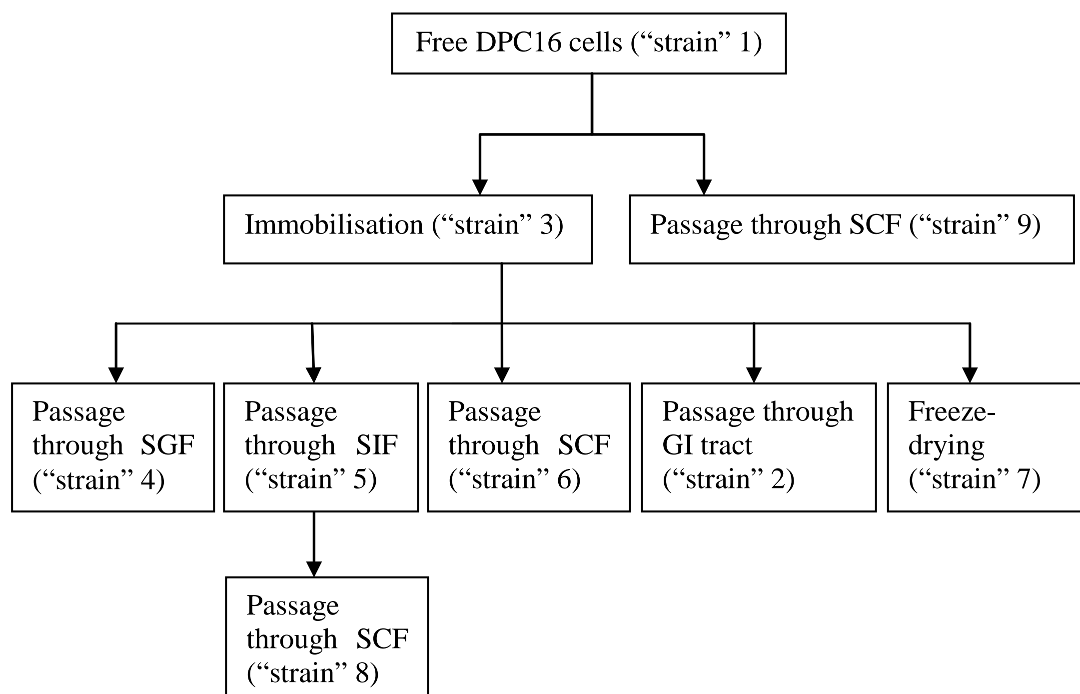


Figure 5.1 Overview of approach to “strain” selection for identification of factors that contribute to enhanced antimicrobial activity of cells recovered from simulated colonic fluid after passage of immobilised cells through a simulated gastrointestinal tract.

To confirm that the enhancement of antimicrobial activity is related to increased reuterin production, the diol dehydratase expression was also compared among the different "strains" of DPC16 cells.

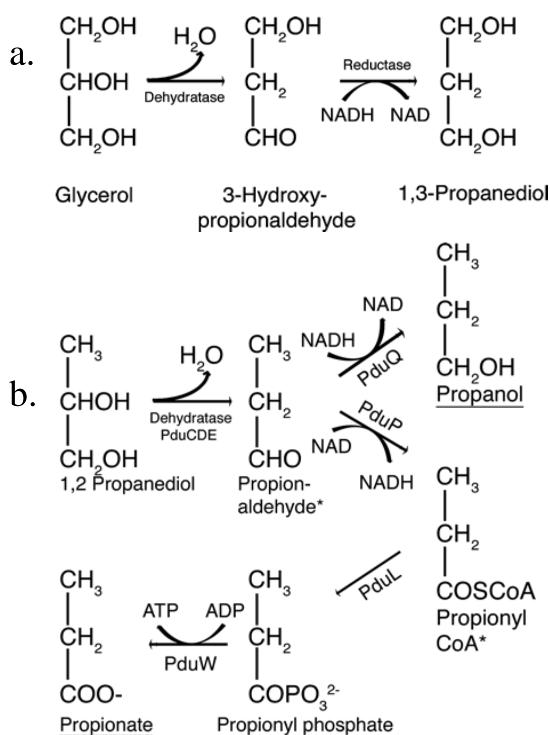


Figure 5.2 Reuterin production in *L. reuteri*. (a) 3-HPA production and metabolism in *L. reuteri*; (b) Proposed pathway of cobalamin-dependent 1,2-propanediol metabolism in *L. reuteri*. Metabolic endpoints are underlined; (*) indicate metabolic intermediates retained within the metabolosome (Sriramulu *et al*., 2008).

5.2 Materials and Methods

5.2.1 Isolation of different DPC16 "strains"

Different "strains" of DPC16 cells were isolated for further study. The following "strains" are referred to in Figure 5.1:

(1) the original DPC16 "strain" which had not been treated by immobilisation, freeze-drying or passage through the GI tract;

(2) the DPC16 cells which had been immobilised in Ca alginate-skim milk and then passed through the entire simulated GI tract before recovery of cells from the simulated colonic fluid;
(3) the DPC16 cells that had been immobilised in Ca alginate-skim milk, and then released (using Na citrate) and recovered immediately;
(4) the DPC16 cells that had been immobilised in Ca alginate-skim milk followed by incubation in SGF for 2 h followed by release (using Na citrate) and recovery of cells;
(5) the DPC16 cells that had been immobilised in Ca alginate-skim milk followed by incubation in SIF for 5 h followed by release (using Na citrate) and recovery of cells;
(6) the DPC16 cells that had been immobilised in Ca alginate-skim milk followed by incubation in SCF for 24 h followed by recovery of the cells that had been released naturally into the SCF;
(7) the DPC16 cells that had been immobilised in Ca alginate–skim milk, freeze-dried, and then stored for 3 months at 4°C, followed by release (using Na citrate) and recovery of cells;
(8) the DPC16 cells that had been immobilised in Ca alginate-skim milk followed by sequential incubation in SIF for 5 h and SCF for 24 h, followed by recovery of free cells from the SCF;
(9) free DPC16 cells that had been incubated in SCF for 24 h.

This classification is summarised in Table 5.1, and the “strain” numbers are also used in the following sections.

During the isolation and testing of the different “strains”, the alginate beads were dissolved using sodium citrate and the viable cells were inoculated on MRS agar. A single colony showing typical morphology was isolated, inoculated into MRS broth, and incubated anaerobically for 18 h in both the presence and absence of glycerol.

Table 5.1 A summary of the isolated DPC16 "strains"

Strain	Type of DPC 16 cell
1	Original
2	Recovered
3	Immobilised
4	SGF
5	SIF
6	SCF
7	Freeze-dried combined with long term storage
8	SIF + SCF
9	Free SCF

5.2.2 Growth curve, reuterin production and SCFAs production of the different DPC16 "strains"

The methods used in this section were similar to those described in Chapter 4.

Reuterin and SCFAs production were measured during growth in MRS broth, in the presence and absence of glycerol.

Reuterin production during a secondary fermentation was also determined. Cells from an 18 h culture in MRS broth were harvested, washed, and then suspended in a glycerol solution (250 mM) at the original cell concentration. The reuterin concentration was determined after incubation for 2 h at 37°C (Talarico *et al*., 1988).

5.2.3 Antimicrobial assay of strain DPC16 supernatants

The DPC16 cell cultures were harvested by centrifugation at 3,200×*g* for 10 min at 4°C. The supernatants were filter-sterilised through a 0.22 µm syringe-filter to remove the remaining cells, and these cell-free supernatants were stored at -20°C until required for use.

The details of the assays have been described in Chapter 4.

5.2.4 Diol dehydratase assay

Each *L. reuteri* "strain" was grown anaerobically at 37°C in 1 L MRS broth containing 7.6 g 1,2-propanediol (Sigma-Aldrich Chemical Co., St. Louis, Mo, USA). The pH of

the medium was adjusted to 7.1 using 1 M KOH. Cells were harvested in the late exponential phase, resuspended in 0.05 M potassium phosphate buffer (pH 8.0) and disrupted by sonication at 20 kHz for 5 min using an ultrasonic disintegrator (Sonicator^{TM} Heat System-ultrosonics, Inc. Nicholas Watson Victor Ltd.). The homogenate was centrifuged (Sorvall RC 6+ centrifuge, USA) at 20,000×g for 30 min, and the supernatant solution was used as cell free extract. Protein concentration was determined by the Coomassie blue protein assay using bovine serum albumin (BSA) (Sigma-Aldrich Chemical Co., St. Louis, Mo, USA), at concentrations 0, 2, 4, 6, 8, 10 μg/ml, as a standard. The Coomassie R-250 (BDH, New Zealand) was allowed to be warmed to room temperature before the assay. A series of diluted BSA standard solutions were then prepared. The mixture, consisting of 0.1 ml of each standard or unknown sample (i.e. cell free extract) and 5.0 ml of Coomassie Reagent, was incubated at room temperature for 10 min before measuring the optical density at 595 nm, using an UV spectrophotometer (UV mini 1240, Japan). A standard curve of the absorbance values measured at 595 nm against the concentration in μg/ml was created and used to calculate the protein concentration of each cell-free extract.

The diol dehydratase activities were assayed using the 3-methyl-2-benzothiazolinone hydrazone (MBTH) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) method (Toraya *et al*., 1977). The assay mixture contained an appropriate amount of diol dehydratase preparation, 0.2 M 1,2-propanediol, 0.05 M KCl, 0.035 M potassium phosphate buffer (pH 8.0), and 15 μM cyanocobalamin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), in a total volume of 1.0 ml. After incubation at 37°C for 2, 4, 5 and 10 min, the enzyme reaction was terminated by adding 1 ml of 0.1 M potassium citrate buffer (pH 3.6) and 0.5 ml of 0.1% MBTH hydrochloride. After 15 min at 37°C, 1 ml of water was added and the amount of propionaldehyde was determined spectrophotometrically by measuring absorbance at 305 nm. The apparent molar extinction coefficient at 305 nm (UV mini 1240, Japan) for the coloured product formed from propionaldehyde by the MBTH method is 13.3×10^3 M^{-1} cm^{-1}. One unit is defined as the amount of enzyme activity catalysing the formation of 1 μmol of propionaldehyde per min under the standard assay conditions. For routine assays, the amount of enzyme to be assayed is between 0.003 and 0.03 units. In experiments in which more than 0.03 units of enzyme were used, the absorbance was measured after dilution. The amount of propionaldehyde produced under these conditions varies

linearly with at least 0.001 to 0.3 unit of enzyme (Toraya *et al*., 1977).

5.2.5 Protein analysis of cell-free extract (from Section 5.2.4) using SDS-PAGE

The DPC16 cell-free extracts were also analysed by SDS-PAGE to determine the presence of diol hydratase. In this study, SDS-gels consisting of 5% stacking gel and 12% resolving gel were prepared according to the methods described by Schagger (2006). The resolving gel contained 1.6 ml H_2O, 2.0 ml 30% acrylamide mix (Fluka, USA), 1.3 ml 1.5 M Tris buffer (pH 8.8), 0.05 ml 10% SDS (Fluka, USA), 0.05 ml 10% ammonium persulfate (BDH, New Zealand) and 0.002 ml TEMED (J.T.Baker, USA) per 5 ml, paired with 5% stacking gel, which consisted of 0.68 ml H_2O, 0.17 ml 30% acrylamide mix, 0.13 ml 1.0 M Tris buffer (pH 6.8), 0.01 ml 10% SDS, 0.01 ml 10% ammonium persulfate and 0.001 ml TEMED, in a total volume of 1 ml. After the preparation of SDS-gels (i.e. polymerisation), the gels were put in the electrophoresis apparatus (Bio-Rad, New Zealand) and Tris-glycine electrophoresis buffer, containing 25 mM Tris, 250 mM glycine (electrophoresis grade, pH 8.3), and 0.1% SDS, was added to the top of the reservoirs. For the SDS-PAGE sample preparation, the cell-free extracts were mixed with 1× SDS gel-loading buffer (Bio-Rad, New Zealand) at 10:1 to denature the proteins (Schagger, 2006).

The boiled sample (15 µl) and molecular weight markers were loaded onto each well in the SDS-gels. The electrophoresis apparatus was attached to an electric power supply to start protein separation, and continued until the bromophenol bands had reached the bottom of the gels. The gel was finally stained with Coomassie Brilliant Blue G250.

5.2.6 Statistical analysis

All experiments and sample analyses were conducted in duplicate or triplicate. The results were analysed by one-way ANOVA and Two-Samples T-test using Minitab^{TM} 15.0 (Minitab, Pennsylvania State University, USA), with $P<0.05$ indicating the significance of differences.

5.3 Results

5.3.1 Comparison of colony morphology and growth curves of different DPC16 "strains"

The colony morphology of all DPC16 "strains" was observed on MRS agar plates after

incubation at 37°C for 48 h. The yellow colonies had uniform shape and size, with smooth and opaque surfaces, and no obvious differences observed among them (Figure 5.3). All “strains” showed flocculation and sedimentation when cultured in MRS broth, with no membrane or bubbles on the liquid surface. The Gram stains showed that the “strains” were Gram-positive rods occurring as single or double cells, and no differences were observed among them.

The growth curve of each *L. reuteri* DPC16 “strain” was determined in MRS broth in the absence (Figure 5.4a) and in the presence of glycerol (MRSg) (Figure 5.4b). Table 5.2 shows that there was no significant difference ($P>0.05$) in the growth rate among the different DPC16 “strains” except for the freeze-dried/ long term storage “strain” (“strain” 7) which displayed a significantly lower value ($P<0.05$). The growth rate of some “strains” grown in the presence of glycerol was lower than the corresponding rate in its absence, indicating that glycerol, or a metabolite of glycerol, is inhibitory to the growth of *L. reuteri* DPC16.

Comparing the growth curves in the presence of glycerol tothose in its absence, the former entered the decline phase after a short stationary phase, while the latter remained in the stationary phase at the end of the incubation period. In terms of the final OD_{620nm} value at 36 h (Table 5.3), the presence of glycerol resulted in a significantly lower value ($P<0.05$) than in its absence, indicating again that glycerol, or a metabolite thereof, is inhibitory to the cells.

From these results, it can be concluded that the process of immobilisation and passage through the simulated GI tract did not change the growth kinetics or morphology of DPC16 cells. However, the process of immobilisation and freeze drying combined with long term storage highly weakened the growth of this probiotic bacterial cell. Additionally, results showed that glycerol, or one of its metabolites, is inhibitory to strain DPC16. Given that glycerol itself is not inhibitory (Tian, 2011), it may be concluded that reuterin is slightly inhibitory to the producing organism.

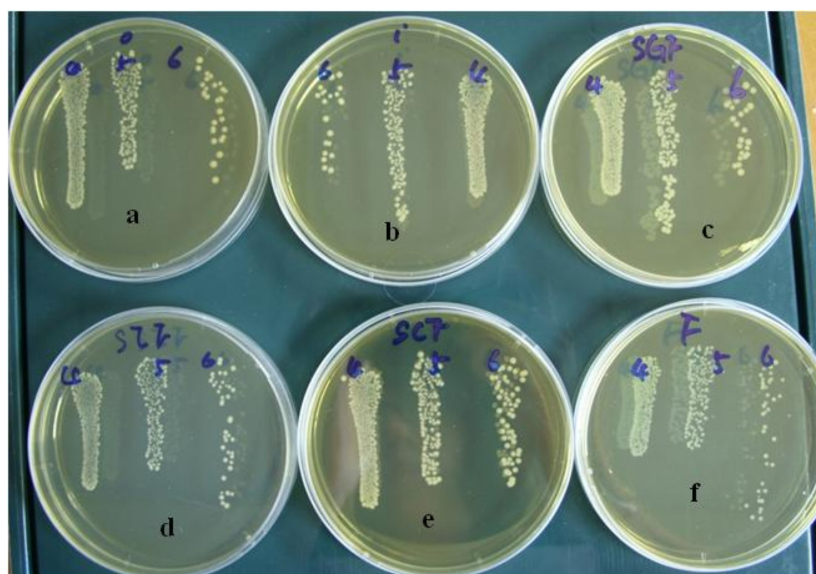


Figure 5.3 Different DPC16 “strains” on MRS agar. (a) original (“strain” 1), (b) immobilised (“strain” 2), (c) SGF (“strain” 4), (d) SIF (“strain” 5), (e) SCF (“strain” 6), (f) Freeze-dried with long term storage (“strain” 7). “Strain” numbers are described in Table 5.1.

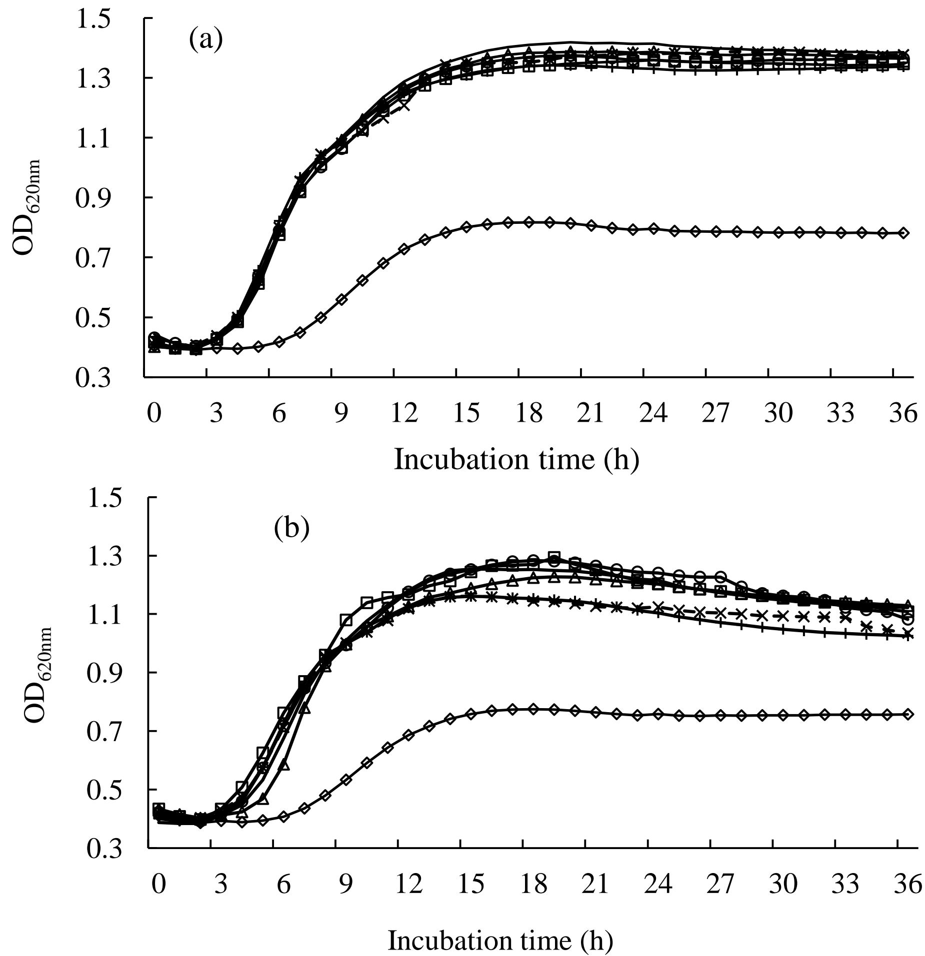


Figure 5.4 Growth curves of *L.reuteri* DPC16 “strains”. (a) in the absence of glycerol in MRS (MRS); (b) in the presence of glycerol in MRS (MRSg).The initial concentration of all the bacteria was 2×10^6 c.f.u./ml. Legend: × original “strain” (“strain” 1); □ recovered “strain” (“strain” 2); Δ immobilised “strain” (“strain” 3); + SGF “strain” (“strain” 4); ○ SIF “strain” (“strain” 5); - SCF “strain” (“strain” 6); ◊ freeze-dried combined with long term storage “strain” (“strain” 7). “Strain” numbers are described in Table 5.1.

Table 5.2 Growth rate of *L. reuteri* DPC16 cells grown in MRS with and without the presence of glycerol

”Strain” (Table 5.1)	Growth rate	
	Glycerol absent	Glycerol present
Original (1)	0.088±0.003	0.075±0.005#
Recovered (2)	0.090±0.007	0.077±0.005#
Immobilised (3)	0.086±0.013	0.082±0.004
SGF (4)	0.086±0.003	0.075±0.002#
SIF (5)	0.081±0.004	0.074±0.006
SCF (6)	0.094±0.006	0.086±0.007
Freeze-dried combined with long term storage (7)	0.040±0.002*	0.036±0.001*#

(*) indicates that there is significant difference ($P<0.05$) between the treated DPC16 “strains” and the original “strain”.
(#) indicates that there is significant difference ($P<0.05$) between the glycerol-absent group and the glycerol-present group.

Table 5.3 Endpoint OD_{620nm} readingS of *L. reuteri* DPC16 cells grown in MRS with and without glycerol at 36 h

”Strain” (Table 5.1)	OD620nm at 36 h	
	Glycerol absent	Glycerol present
Original (1)	1.28±0.05	1.10±0.01#
Recovered (2)	1.27±0.03	1.11±0.05#
Immobilised (3)	1.23±0.07	1.13±0.04
SGF (4)	1.24±0.02	1.09±0.02#
SIF (5)	1.26±0.03	1.08±0.06#
SCF (6)	1.29±0.04	1.05±0.06#
Freeze-dried combined with long term storage (7)	0.78±0.04*	0.76±0.02*

(*) indicates that there is significant difference ($P<0.05$) between the treated DPC16 “strains” and the original “strain”.
(#) indicates that there is significant difference ($P<0.05$) between the glycerol-absent group and the glycerol-present group.

5.3.2 Reuterin and short chain fatty acids production by different DPC16 “strains”

Reuterin production by the different DPC16 “strains” is shown in Table 5.4. Notably, the production of the metabolite after the secondary fermentation was much higher than that observed during the primary fermentation. Also, when glycerol was present in MRS during the primary fermentation, the production was often (but not always) higher than when glycerol was absent.

In all three tested fermentation conditions, the immobilised "strain" ("strain" 3, Table 5.1) was the most effective in producing reuterin. After passage through SGF, the immobilised "strain" ("strain" 4, Table 5.1) still produced more reuterin than did the original "strain" ("strain" 1, Table 5.1), despite exposure to the low pH value.

However, passage of the immobilised cells through SIF alone ("strain" 5, Table 5.1) resulted in a marked reduction in the ability to produce reuterin. This ability appeared to be restored on subsequent passage through the simulated colonic fluid ("strain" 8, Table 5.1), and the colonic fluid itself ("strain" 6, Table 5.1) had no adverse effect.

The free cells that had been incubated in SCF alone ("strain" 9, Table 5.1), produced the same amount of reuterin as did the original "strain", indicating that SCF *per se*, had no significant ($P>0.05$) effect on reuterin production.

The immobilised cells that had been freeze-dried and stored for 3 months ("strain" 7, Table 5.1) produced markedly less reuterin than any other "strain", perhaps reflecting the poorer growth of this strain.

In terms of the SCFAs production (Table 5.5), when grown in the absence of glycerol, there was no significant difference ($P>0.05$) among the "strains", except for the freeze-dried/long term storage "strain" ("strain" 7, Table 5.1), where the production of lactate was markedly lower than by the original. Also, neither valerate nor 2-methyl propionate was detected in "Strain" 7. When grown in the presence of glycerol, lactate production from the immobilised, SGF, and SIF "strains" ("strains" 3, 4 and 5, Table 5.1) was significantly higher ($P<0.05$) than that of the original "strain". However, the SCF and recovered "strain" ("strains" 6 and 2) showed no difference from the original, indicating, perhaps, that any differences that may be due to the immobilisation process or passage through the SGF and SIF are nullified by passage through the SCF. Interestingly, the immobilised/freeze-dried/long term storage strain ("strain" 7) showed no difference from the original. However, it is noticeable that lactate production by the original cells when grown in the presence of glycerol was markedly lower than when the cells were grown in its absence, but there was also less growth in the former (Table 5.3).

Regarding the production of acetate, there were no differences observed among the "strains". The only other differences observed among the "strains" were in the

production of propionate and valerate, but these metabolites were produced in relatively low concentrations.

Table 5.6 summarises the reuterin and SCFAs production by the various "strains" grown in MRS in both the presence and absence of glycerol, and also the data from the secondary fermentation. The most significant result appears to be the up-regulation of reuterin production upon immobilisation in alginate-skim milk, and the down-regulation during passage of the immobilised cells through the simulated intestinal fluid. In addition, long term storage of freeze dried immobilised cells down-regulates reuterin production, but this may be a consequence of the lesser growth in the conditions used.

Table 5.4 Reuterin production (μg/ml) by different DPC16 "strains"

"Strain" (Table 5.1)	Cell growth in MRS alone	Cell growth in MRS+ glycerol	Secondary fermentation
Original (1)	16.94±0.22	26.33±3.14#	70.81±5.14#
Recovered (2)	17.62±0.34*	27.41±1.55#	76.04±4.54#
Immobilised (3)	18.47±0.75*	34.17±1.45*	85.42±6.29*
SGF (4)	18.04±0.71*	32.26±2.19*	80.65±7.86*
SIF (5)	16.63±0.87#	3.22±0.27*#	5.23±0.06*#
SCF (6)	17.45±2.32	29.47±2.69#	73.67±3.14#
Freeze-dried combined with long term storage (7)	13.69±0.46*#	0.88±0.05*#	2.88±0.35*#
Free SCF (9)	16.32±1.35#	28.20±3.47#	65.12±7.64#

(*) indicates that there is significant difference (P<0.05) in reuterin production between the treated DPC16 "strain" and the original "strain".
(#) indicates that there is significant difference (P<0.05) between the immobilised cell "strain" and the other "strains"

Table 5.5 Short chain fatty acids production by different DPC16 "strains"

"Strain" (Table 5.1)	Lactate (g/l)	Acetate (g/l)	Propionate (mg/l)	2-methyl propionate (mg/l)	Valerate (mg/l)
SCFAs production in the absence of glycerol					
Original (1)	5.65±0.37	8.50±0.08	231.00±32.53	36.70±0.57	38.13±3.60
Recovered (2)	5.41±0.18	8.28±0.20	226.00±26.87	37.20±3.68	41.70±13.44
Immobilised (3)	5.42±0.21	8.68±0.59	220.00±12.73	38.25±5.02	51.80±0.57
SGF (4)	4.85±0.89	8.38±0.002	231.00±31.11	35.85±0.79	51.20±13.15
SIF (5)	3.37±0.98	8.16±0.002	201.00±18.38	33.50±1.56	47.03±3.31
SCF (6)	5.033±0.01	8.95±0.81	233.00±29.70	37.15±1.77	50.10±11.88
Freeze-dried combined with long term storage (7)	1.65±0.70*	8.37±0.28	268.50±21.92	0*	0*
SCFAs production in the presence of glycerol					
Original (1)	1.50±0.18	6.39±1.62	251.50±28.99	28.30±2.83	38.45±3.89
Recovered (2)	1.66±0.16	7.40±1.92	210.00±26.87	38.10±2.12	49.80±8.34
Immobilised (3)	3.69±0.02*	11.80±0.26	240.00±31.11	37.95±4.03	52.70±7.92*
SGF (4)	4.19±0.29*	11.22±2.69	238.50±12.02	29.60±0.28	60.10±7.92
SIF (5)	3.18±0.50*	6.03±1.95	155.00±38.18*	29.85±2.48	33.30±8.77
SCF (6)	2.67±0.86	10.45±0.11	189.00±45.25	29.75±3.18	57.20±6.08
Freeze-dried combined with long term storage (7)	1.33±0.06	7.21±0.02	216.50±7.78	25.10±4.81	26.50±1.98

(*) indicates that there is significant difference (P<0.05) between the treated "strain" and the original "strain".

Table 5.6 A summary of reuterin and SCFAs production by the various "strains" when grown in the presence/absence of glycerol and during secondary fermentation

"Strain" (Table 5.1)	Reuterin production			$SCFA_S$ production	
	Glycerol present	Glycerol absent	Second fermentation	Glycerol present	Glycerol absent
Original (1)	Origin	Origin	Origin	Origin	Origin
Immobilised (3)	+	+	+	+	N
SGF (4)	+	N	+	+	N
SIF (5)	-	N	-	+	N
SCF (6)	N	N	N	N	N
Freeze-dried combined with long storage (7)	-	-	-	N	-
Recovered (2)	N	+	N	N	N

The symbols show the up-regulation (+) or down-regulation (-) of reuterin and SCFAs production of DPC16 "strains" compared with the original DPC16 "strain" (P<0.05). (N) means there is no difference between the treatment DPC16 "strain" and the original "strain".

5.3.3 The antimicrobial effects of the different DPC16 "strain" culture supernatants on the growth of selected pathogens

In this part of experiment, the various "strains" were examined for their antimicrobial effects against selected pathogens.

In the following Figures, the abbreviations used are:

Abbreviation	Description
Control	Pathogen incubated in BHI +MRS or MRSg
S1	Pathogen incubated in BHI plus supernatant from original DPC16 "strain" ("strain" 1)
S1/g	Pathogen incubated in BHI plus supernatant from original DPC16 "strain" ("strain" 1) grown in the presence of glycerol
S2	Pathogen incubated in BHI plus supernatant from recovered DPC16 "strain" ("strain" 2)
S2/g	Pathogen incubated in BHI plus supernatant from recovered DPC16 "strain" ("strain" 2) grown in the presence of glycerol
S3	Pathogen grown in BHI plus supernatant from immobilised DPC16 "strain" ("strain" 3)
S3/g	Pathogen grown in BHI plus supernatant from immobilised DPC16 "strain" ("strain" 3) grown in the presence of glycerol
S4	Pathogen grown in BHI plus supernatant from SGF DPC16 "strain" ("strain" 4)
S4/g	Pathogen grown in BHI plus supernatant from SGF DPC16 "strain" ("strain" 4) grown in the presence of glycerol
S5	Pathogen grown in BHI plus supernatant from SIF DPC16 "strain" ("strain" 5)
S5/g	Pathogen grown in BHI plus supernatant from SIF DPC16 "strain" ("strain" 5) grown in the presence of glycerol
S6	Pathogen grown in BHI plus supernatant from SCF DPC16 "strain" ("strain" 6)
S6/g	Pathogen grown in BHI plus supernatant from SCF DPC16 "strain" ("strain" 6) grown in the presence of glycerol
S7	Pathogen grown in BHI plus supernatant from freeze-dried/long term storage DPC16 "strain" ("strain" 7)
S7/g	Pathogens grow in BHI plus supernatant from freeze dried/long term storage DPC16 "strain" ("strain" 7) in presence of glycerol
S9	Pathogen grown in BHI plus supernatant from free cells SCF DPC16 "strain" ("strain" 9)
S9/g	Pathogen grown in BHI plus supernatant from free cells SCF DPC16 "strain" ("strain" 9) grown in the presence of glycerol

5.3.3.1 Immobilisation effect (comparison of the antimicrobial activities of "strains" 1, 2 and 3).

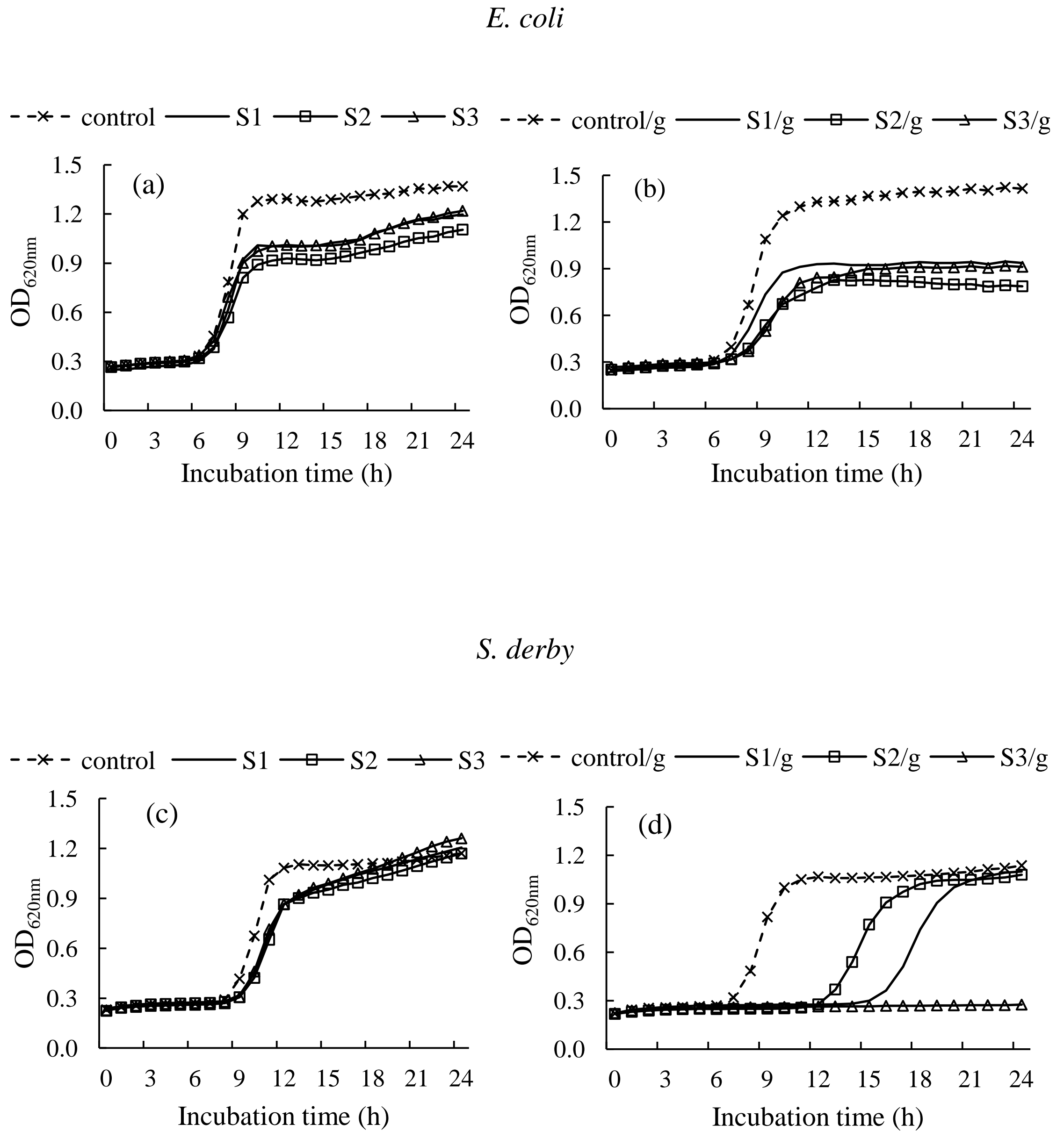
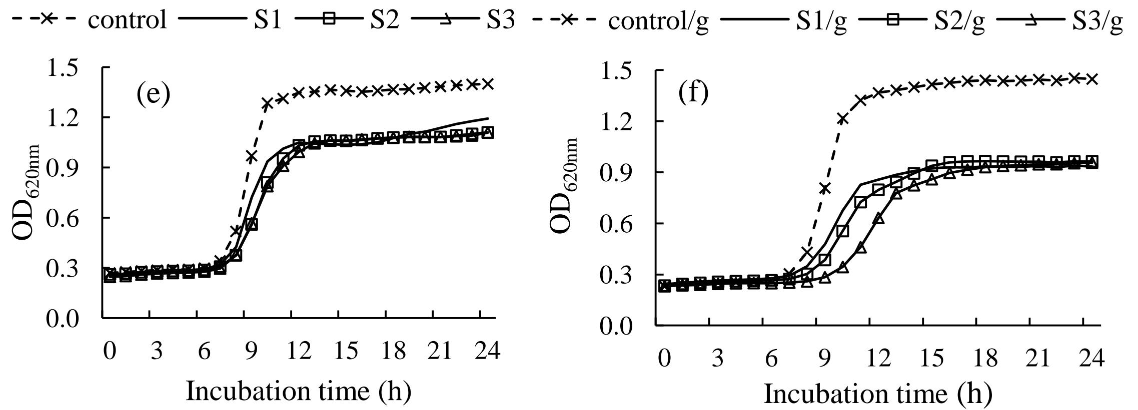


Figure 5.5 Growth curves of pathogens in the presence of cell-free culture supernatants from "strains" 1, 2 and 3. Data points are means (n=12) of OD_{620nm} readings. In experiments (a), (c), (e), (g), the DPC16 supernatants were from the spent culture without the presence of glycerol, and (b), (d), (f), (h), the DPC16 supernatants were from the spent culture with the presence of glycerol.There was significant inhibition ($P<0.05$) by the DPC16 supernatants against the growth of pathogens. For *S. derby* and *S. aureus*, the lag phase was prolonged by the DPC16 supernatants with the presence of glycerol.

L. monocytogenes



S. aureus

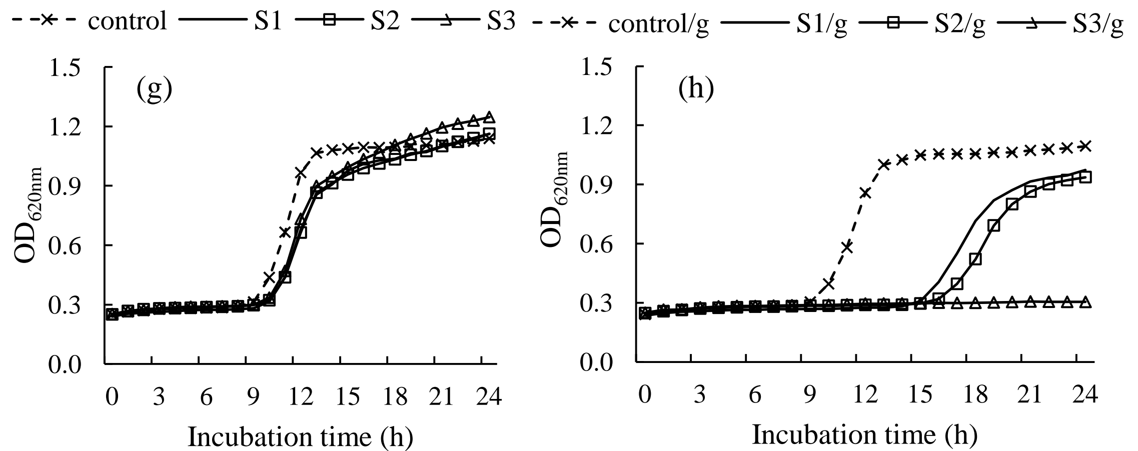
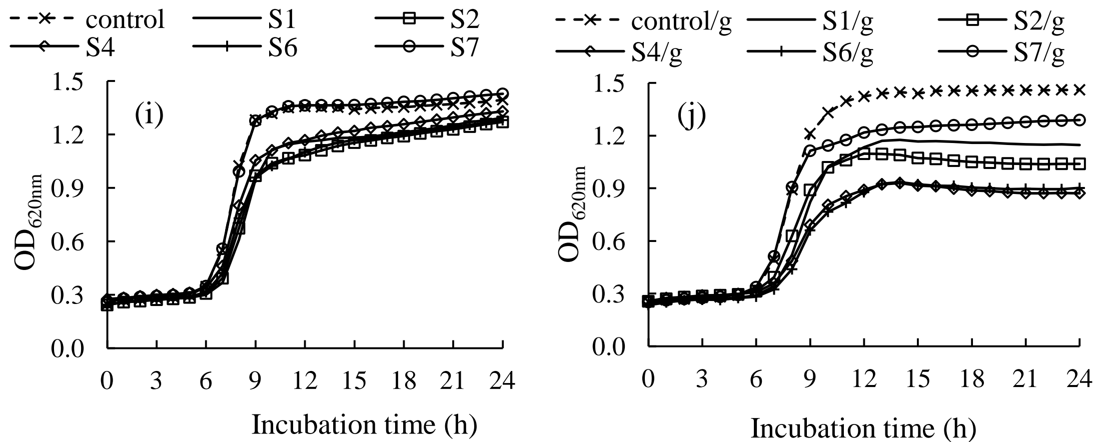


Figure 5.6 Growth curves of pathogens in the presence of cell-free culture supernatants from "strains" 1, 2 and 3. Data points are means (n=12) of OD_{620nm} readings. In experiments (a), (c), (e), (g), the DPC16 supernatants were from the spent culture without the presence of glycerol, and (b), (d), (f), (h), the DPC16 supernatants were from the spent culture with the presence of glycerol.There was significant inhibition ($P<0.05$) by the DPC16 supernatants against the growth of pathogens. For *S. derby* and *S. aureus*, the lag phase was prolonged by the DPC16 supernatants with the presence of glycerol.

5.3.3.2 Effect of incubation of immobilised cells in SGF and SCF, and effect of freeze-drying/storage of immobilised cells (comparison of "strains" 1, 2, 4, 6 and 7)

E. coli



S. derby

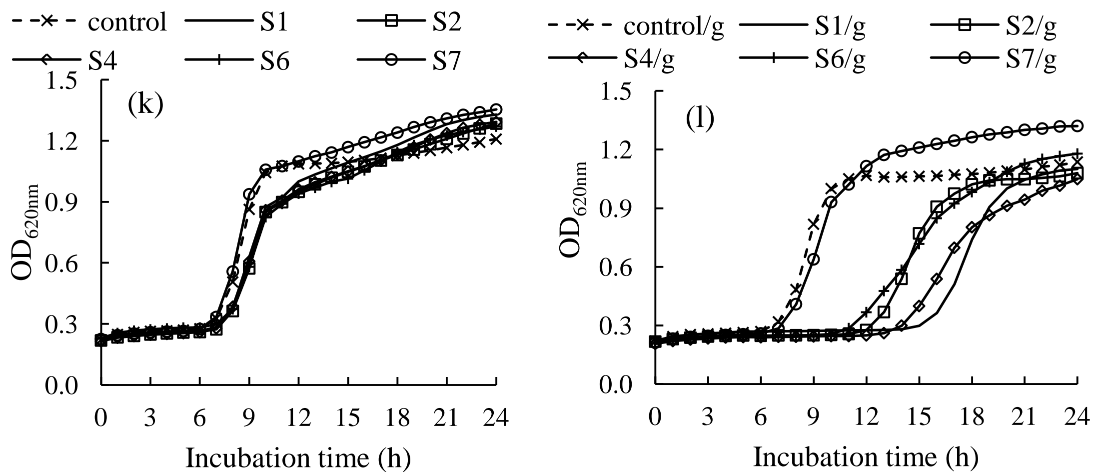
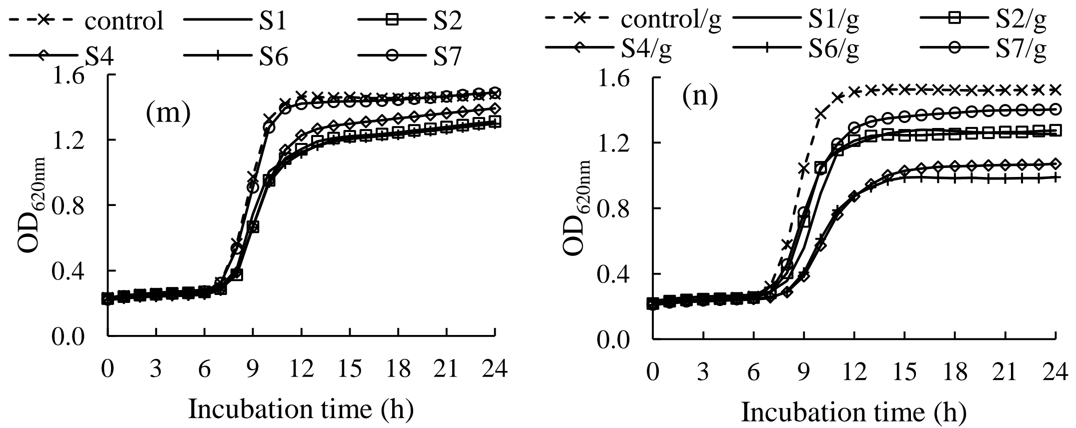


Figure 5.7 Growth curves of pathogens in the presence of cell-free culture supernatants from "strains" 1, 2, 4, 6 and 7. Data points are means (n=12) of OD_{620nm} readings. In experiments (i), (k), (m), (o), the DPC16 supernatants were from the spent culture without the presence of glycerol, and (j), (l), (n), (p), the DPC16 supernatants were from the spent culture with the presence of glycerol. There was significant inhibition ($P<0.05$) by the DPC16 supernatants, except the supernatant from "strain" 7, against the growth of pathogens. For *S. derby* and *S. aureus*, the lag phase was prolonged by the DPC16 supernatants with the presence of glycerol.

L. monocytogenes



S. aureus

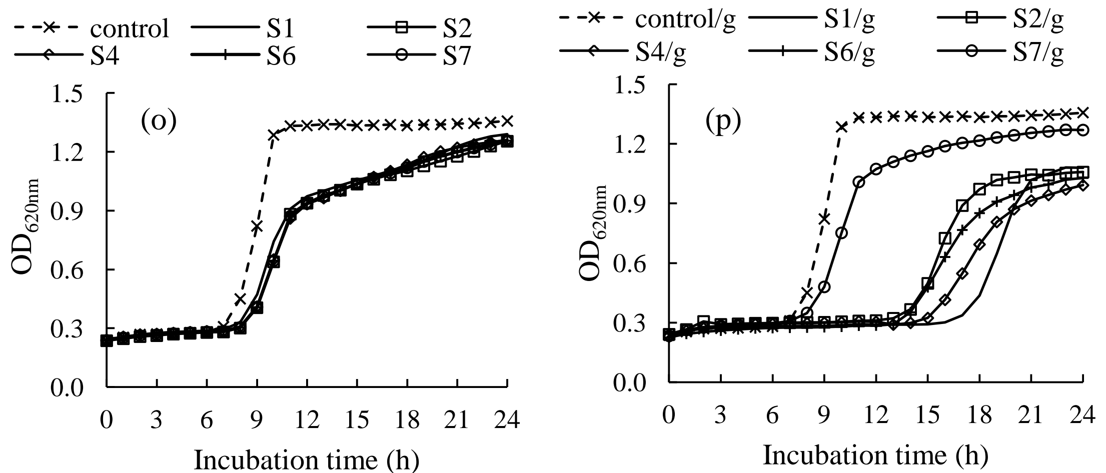


Figure 5.8 Growth curves of pathogens in the presence of cell-free culture supernatants from "strains" 1, 2, 4, 6 and 7. Data points are means (n=12) of OD_{620nm} readings. In experiments (i), (k), (m), (o), the DPC16 supernatants were from the spent culture without the presence of glycerol, and (j), (l), (n), (p), the DPC16 supernatants were from the spent culture with the presence of glycerol. There was significant inhibition ($P<0.05$) by the DPC16 supernatants, except the supernatant from "strain" 7, against the growth of pathogens. For *S. derby* and *S. aureus*, the lag phase was prolonged by the DPC16 supernatants with the presence of glycerol.

5.3.3.3 Effect of incubation of immobilised cells in SIF (comparison of “strains” 1, 2 and 5)

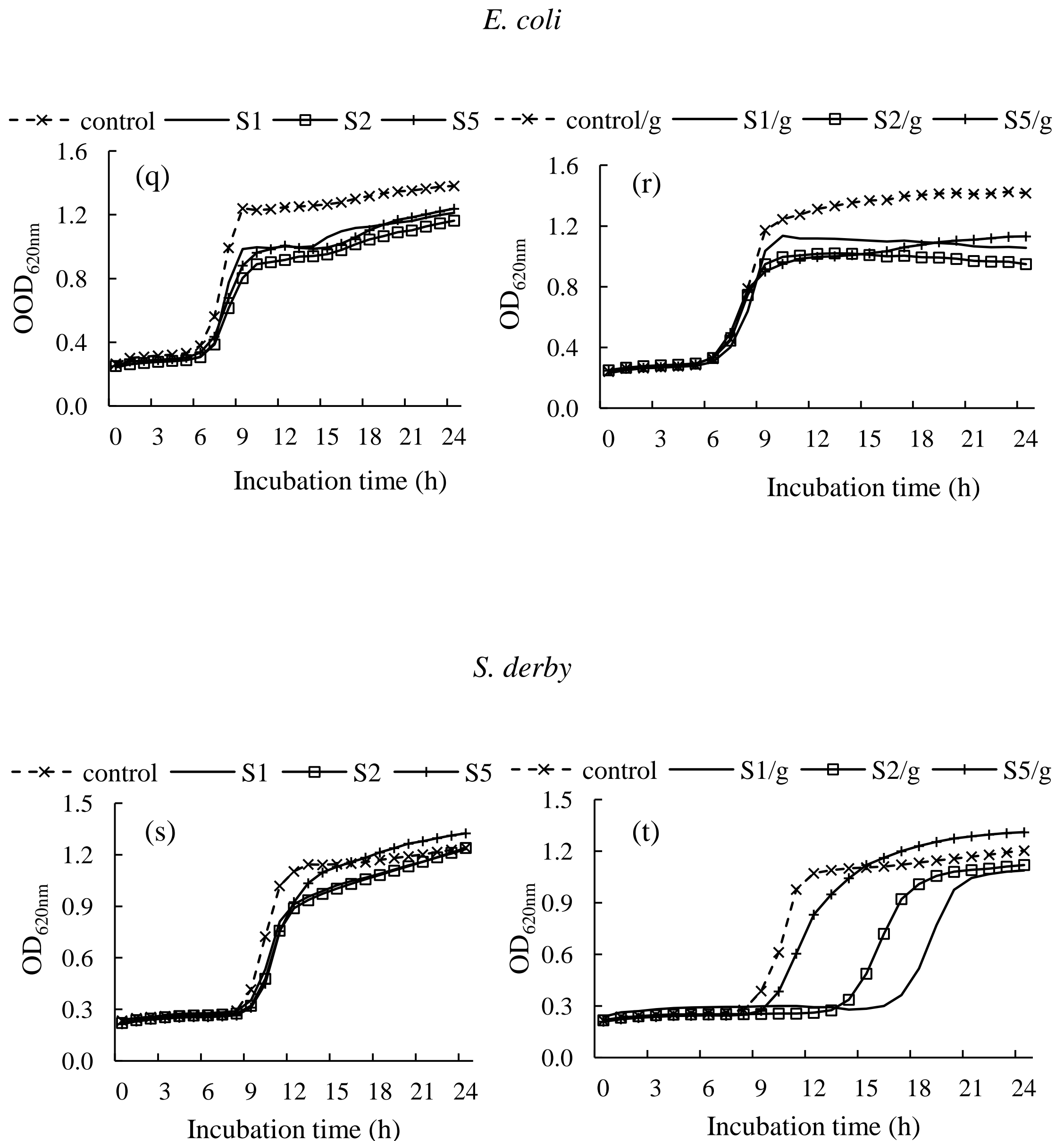
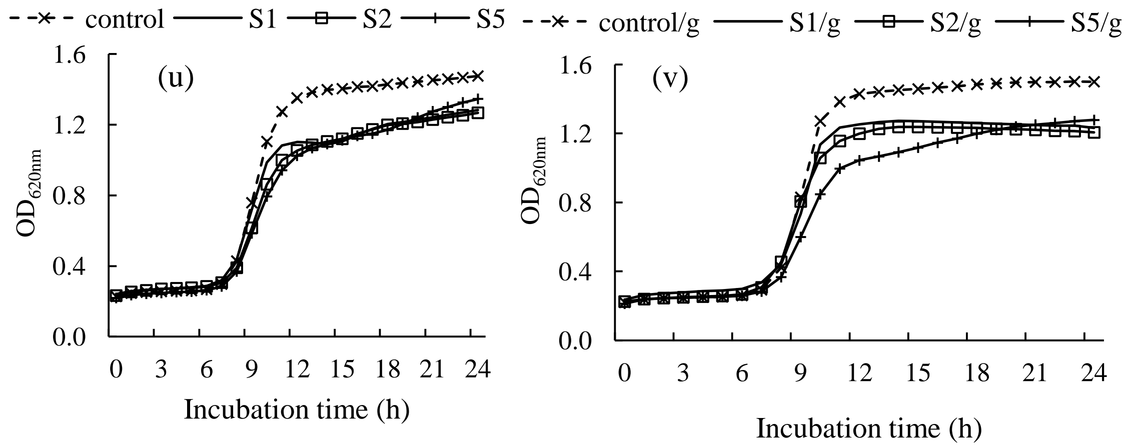


Figure 5.9 Growth curves of pathogens in the presence of cell-free culture supernatants from “strains” 1, 2 and 5. Data points are means (n=12) of OD_{620nm} readings. In experiments (q), (s), (u), (w), the DPC16 supernatants were from the spent culture without the presence of glycerol, and (r), (t), (v), (x), the DPC16 supernatants were from the spent culture with the presence of glycerol. There was significant inhibition ($P<0.05$) by the DPC16 supernatants against the growth of pathogens. For *S. derby* and *S. aureus*, the lag phase was prolonged by the DPC16 supernatants with the presence of glycerol.

L. monocytogenes



S. aureus

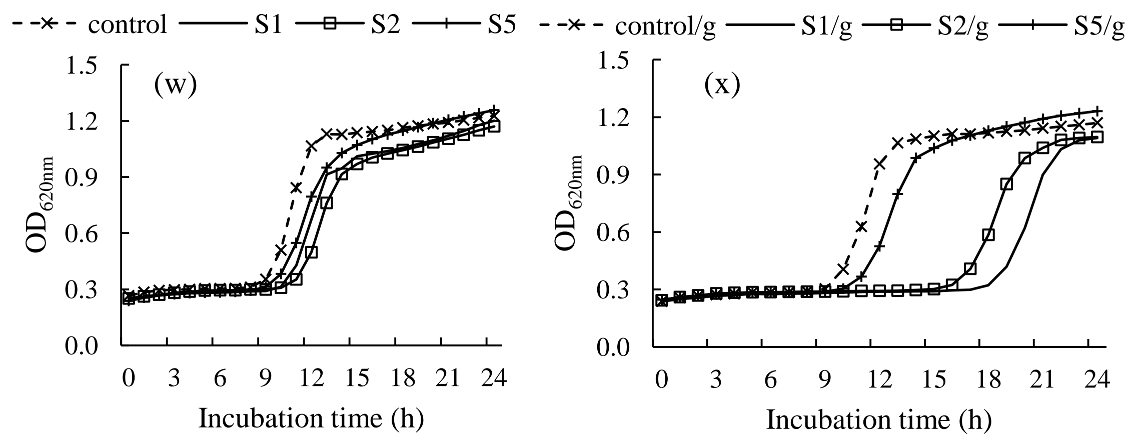


Figure 5.10 Growth curves of pathogens in the presence of cell-free culture supernatants from "strains" 1, 2 and 5. Data points are means (n=12) of OD_{620nm} readings. In experiments (q), (s), (u), (w), the DPC16 supernatants were from the spent culture without the presence of glycerol, and (r), (t), (v), (x), the DPC16 supernatants were from the spent culture with the presence of glycerol. There was significant inhibition ($P<0.05$) by the DPC16 supernatants against the growth of pathogens. For *S. derby* and *S. aureus*, the lag phase was prolonged by the DPC16 supernatants with the presence of glycerol.

5.3.3.4 Effect of incubation of free cells in SCF (comparison of "strains" 1 and 9)

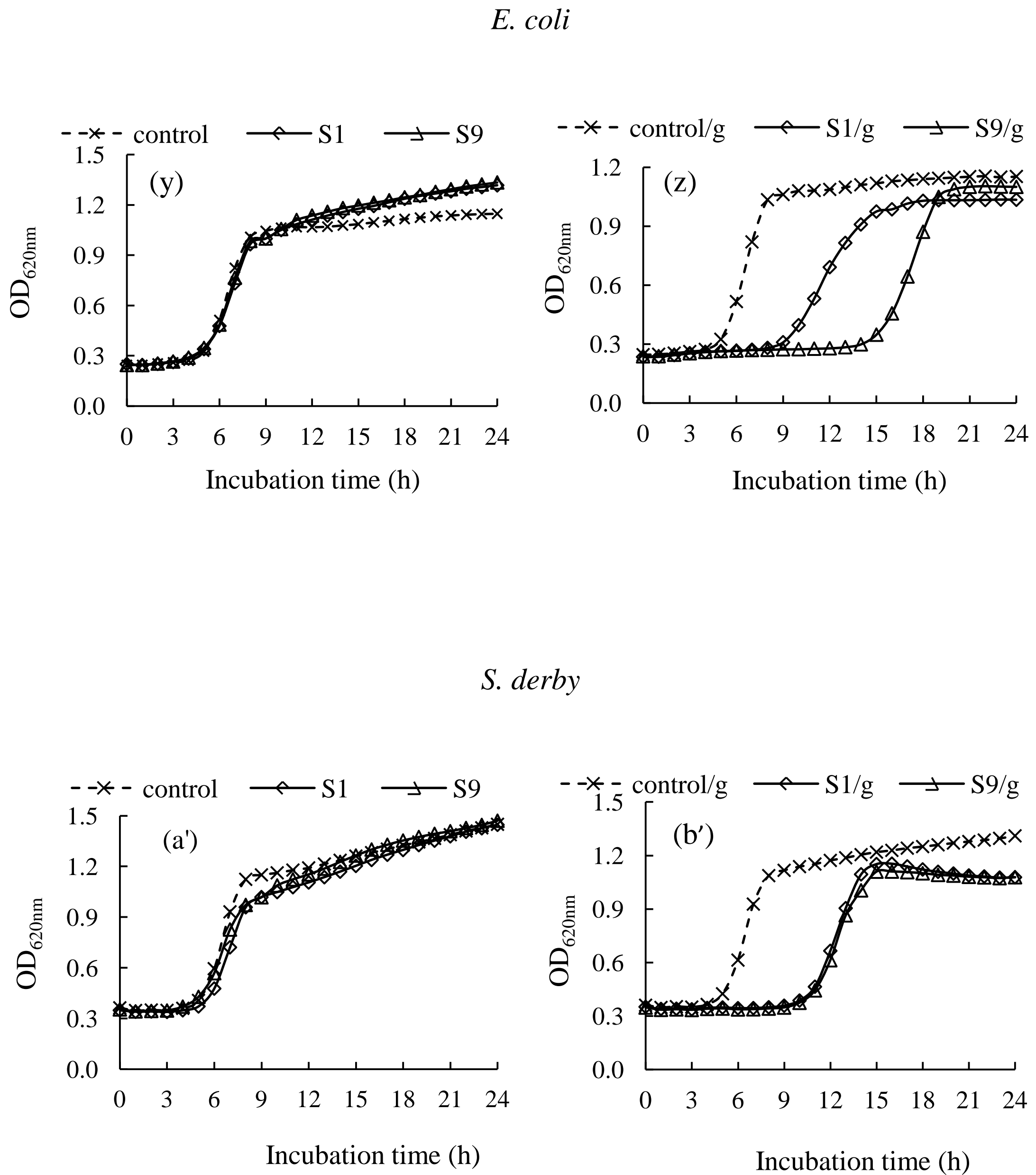
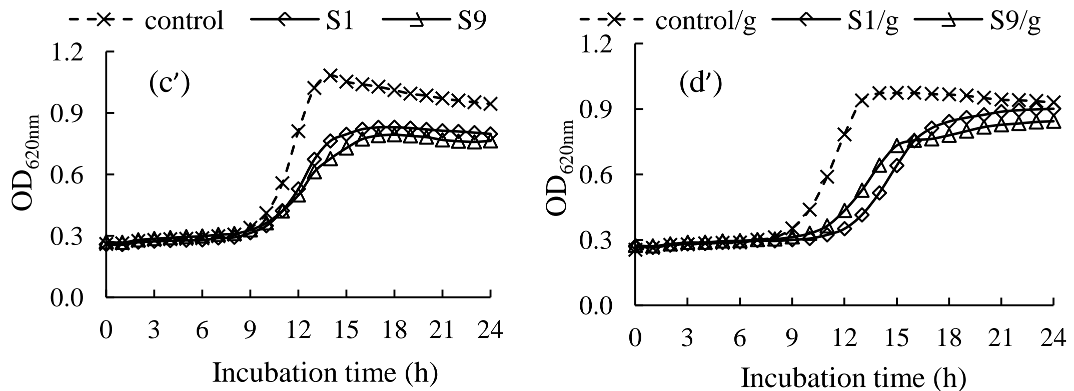


Figure 5.11 Growth curves of pathogens in the presence of cell-free culture supernatants of "strains" 1 and 9. Data points are means (n=12) of OD_{620nm} readings. In experiments (y), (a'), (c'), (e'), the DPC16 supernatants were from the spent culture without the presence of glycerol, and (z), (b'), (d'), (f'), the DPC16 supernatants were from the spent culture with the presence of glycerol. There was significant inhibition ($P<0.05$) by the DPC16 supernatants against the growth of pathogens. For *S. derby* and *S. aureus*, the lag phase was prolonged by the DPC16 supernatants with the presence of glycerol.

L. monocytogenes



S. aureus

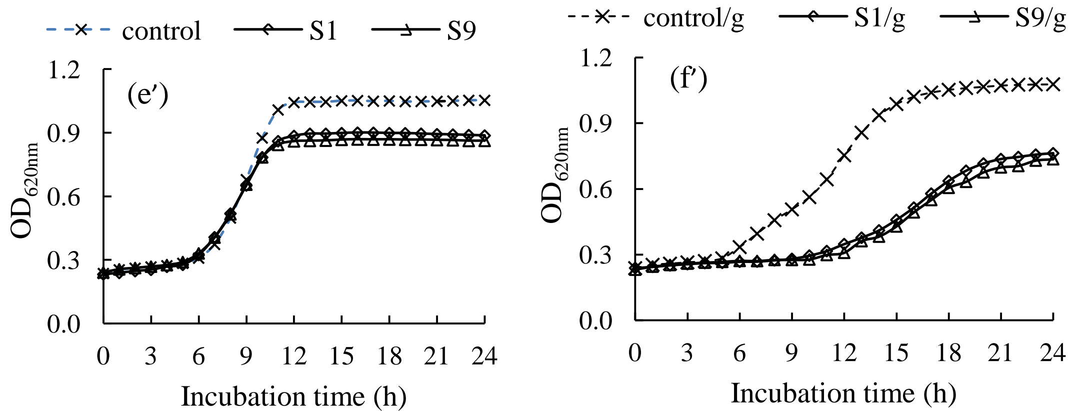


Figure 5.12 Growth curves of pathogens in the presence of cell-free culture supernatants of "strains" 1 and 9. Data points are means (n=12) of OD_{620nm} readings. In experiments (y), (a'), (c'), (e'), the DPC16 supernatants were from the spent culture without the presence of glycerol, and (z), (b'), (d'), (f'), the DPC16 supernatants were from the spent culture with the presence of glycerol. There was significant inhibition ($P<0.05$) by the DPC16 supernatants against the growth of pathogens. For *S. derby* and *S. aureus*, the lag phase was prolonged by the DPC16 supernatants with the presence of glycerol.

5.3.3.5 The length of lag phase

Figures 5.5, 5.6, 5.7, and 5.8 show the growth curves of selected pathogens grown in the presence of supernatants from the different DPC16 cultures. Table 5.7 summarises the results.

Regarding the effect of the supernatants on the lag phase of the pathogens, there was no significant ($P>0.05$) effect when DPC16 "strains" were grown in MRS alone, but when grown in the presence of glycerol, there was an extension of the lag phase of selected pathogens, especially *S. derby* and *S. aureus*. Upon immobilisation ("strain" 3), the inhibitory effect was strengthened. However, passage through the simulated intestinal fluids, and long-term storage of immobilised cells appeared to negate this inhibition.

5.3.3.6 The specific growth rate

The summary in Table 5.7 indicates that, as expected, the supernatants from the original "strain" ("strain" 1) inhibited the growth rate of selected pathogens. Upon immobilisation ("strain" 3), there was, in some cases, an up-regulation of this inhibition. Table 5.8 compares the inhibition caused by all other immobilised "strains" with that caused by "strain" 3, i.e. after passage through one or more simulated intestinal fluids, and clearly indicates that there is either no change or a down-regulation in these circumstances. Hence, this is strong evidence that it is the immobilisation process itself, rather than incubation of the immobilised cells in the simulated fluids, that is responsible for the enhanced inhibitory activity compared to the original "strain". Freeze drying followed by long term storage of immobilised cells ("strain" 7) resulted in a clear down-regulation of the inhibitory activity.

Incubation of free cells in SCF ("strain" 9) had no effect on the magnitude of the inhibitory activity.

5.3.3.7 Endpoint optical density reading

The endpoint optical density readings were compared among the different DPC16 "strains". As expected, the supernatants had an inhibitory effect, and in some cases this was enhanced by the immobilisation process alone ("strain" 3). Incubation of the immobilised cells in the simulated gastric fluid led, in some cases, to a reversion of the increased inhibitory effect, e.g. "strain" 4 against *S. aureus*. However, incubation of immobilised cells in SIF often led to a down-regulation of the inhibitory activity

("strain" 5). Incubation of free cells in SCF ("strain" 9) had no effect on the inhibitory activity. Table 5.8 compares the inhibition caused by all other immobilised "strains" with that caused by "strain" 3. The results reveal that there is either no change or a down-regulation when immobilised cells are incubated in the simulated fluids. Hence, as with the inhibitory effect on the growth rate, it appears to be the immobilisation process itself that enhances this effect rather than incubation in the simulated fluids. Freeze-drying and long term storage of the immobilised cells is clearly detrimental to the inhibitory activity.

Table 5.7 A summary of the antimicrobial activities of the different "strains" of DPC16 against pathogens

			"Strain" (Table 5.1)							
			Original (1)	Immobilised (3)	SGF (4)	SIF (5)	SCF (6)	Freeze-dried combined with long term storage (7)	Free SCF (9)	Recovered (2)
Growth rate	*E. coli*	Glycerol present	i	+	+	+	+	+	N	+
		Glycerol absent	i	N	N	N	+	N	N	+
	S. derby	Glycerol present	i	++	N	N	N	-	N	+
		Glycerol absent	i	N	N	N	N	-	N	N
	L. monocytogenes	Glycerol present	i	N	N	N	N	-	N	N
		Glycerol absent	i	+	N	N	N	-	N	+
	S. aureus	Glycerol present	i	++	+	N	+	-	N	+
		Glycerol absent	i	N	N	N	N	-	N	N
Endpoint OD reading	*E. coli*	Glycerol present	i	+	+	-	+	+	N	+
		Glycerol absent	i	N	N	N	N	HC	N	N
	S. derby	Glycerol present	i	+	N	-	N	HC	N	+
		Glycerol absent	HC	HC	HC	HC	HC	HC	HC	HC
	L. monocytogenes	Glycerol present	i	N	+	-	+	-	N	N
		Glycerol absent	i	N	N	-	+	-	N	N
	S. aureus	Glycerol present	i	+	N	N	N	HC	N	+
		Glycerol absent	i	+	N	-	N	N	N	+
Length of lag phase	*E. coli*	Glycerol present	EC	EC	EC	EC	EC	EC	EC	EC
		Glycerol absent	EC	EC	EC	EC	EC	EC	EC	EC
	S. derby	Glycerol present	i	++	-	-	-	EC	i	-
		Glycerol absent	EC	EC	EC	EC	EC	EC	EC	EC
	L. monocytogenes	Glycerol present	EC	EC	EC	EC	EC	EC	EC	EC
		Glycerol absent	EC	EC	EC	EC	EC	EC	EC	EC
	S. aureus	Glycerol present	i	++	-	-	-	EC	i	-
		Glycerol absent	EC	EC	EC	EC	EC	EC	EC	EC

Notes: The symbols show the up-regulation (+) or down-regulation (-) of the antimicrobial activities of DPC16 supernatants compared with the original DPC16 group ($P<0.05$); (i) inhibition of the growth of pathogens by the orginal supernatant;. (++) more than 10-fold up-regulation of antimicrobial activity; (HC) the endpoint OD value wais higher than that of the control group; (N) no effect on the antimicrobial activity compared with the original group; (EC) equal to control.

Table 5.8 A summary of the immobilisation effect compared with the effect of incubating the immobilised "strain" in different simulated gastrointestinal fluids, and freeze-drying storage of the immobilised cells

			Groups					
			Immobilised (3)	Recovered (2)	SGF (4)	SIF (5)	SCF (6)	Freeze-dried combined with long term storage (7)
Growth rate	*E. coli*	Glycerol present	i	N	+	-	+	-
		Glycerol absent	i	N	N	N	N	-
	S. derby	Glycerol present	i	-	-	-	-	-
		Glycerol absent	i	N	N	N	N	-
	L. monocytogenes	Glycerol present	i	N	N	-	N	-
		Glycerol absent	i	N	N	N	N	-
	S. aureus	Glycerol present	i	-	-	-	-	-
		Glycerol absent	i	N	N	N	N	-
Endpoint Reading	*E. coli*	Glycerol present	i	N	+	-	+	-
		Glycerol absent	i	N	N	N	N	-
	S. derby	Glycerol present	i	-	-	-	-	-
		Glycerol absent	i	N	N	N	N	-
	L. monocytogenes	Glycerol present	i	N	N	-	N	-
		Glycerol absent	i	N	N	N	N	-
	S. aureus	Glycerol present	i	-	-	-	-	-
		Glycerol absent	i	N	N	-	N	-

Notes: The symbol (-) down-regulation of the inhibitory activities of DPC16 supernatants compared with the immobilised "strain" 3 ($P<0.05$); (+) up-regulation of the inhibitory activities of DPC16 supernatants compared with the immobilised "strain" 3 ($P<0.05$); (i) inhibited the growth of pathogens by the "strain" 3 supernatant; (N) no change of the inhibitory activity compared with the immobilised "strain" 3.

5.3.4 Diol dehydratase activity assay

The results of SDS-PAGE showed a single band around 200 kDa to 210 kDa in each sample. According to Sauvageot (2002), the molecular weight of diol dehydratase is 207 kDa.

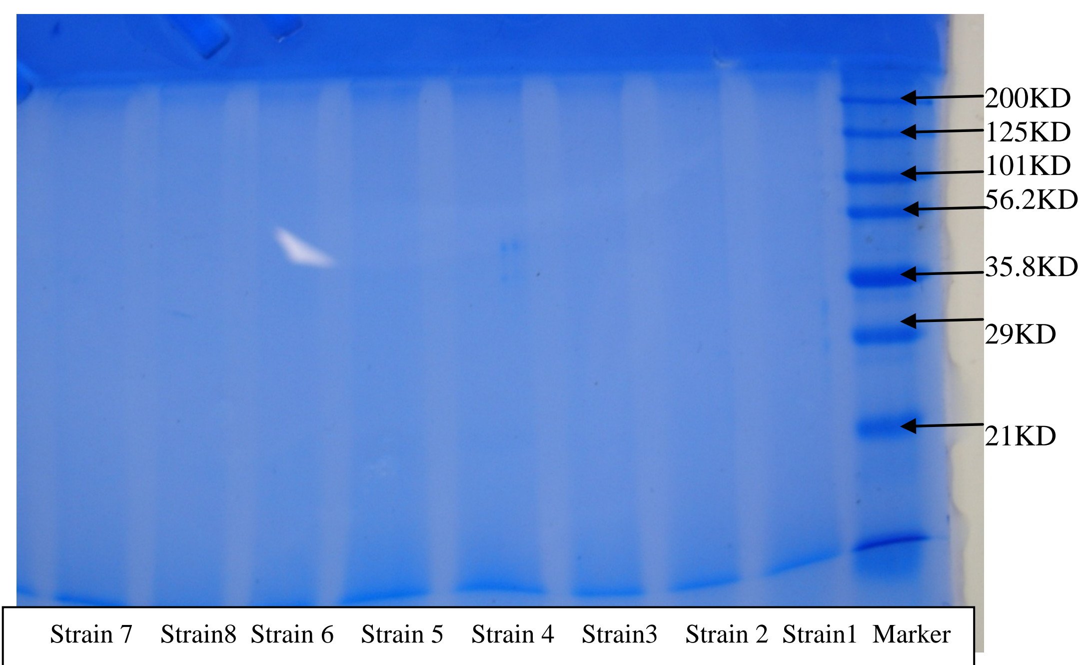


Figure 5.13 SDS-PAGE of the cell-free extract from different *L. reuteri* DPC16 "strains" (strain numbers were listed in Table 5.1).

Table 5.9 shows the activity of diol dehydratase in the different DPC16 "strains". The highest activity was detected in the immobilised "strain" ("strain" 3). All the different "strains" showed significantly different ($P<0.05$) enzyme activities compared with the original "strain" ("strain" 1). However, in the case of "strains" 5 and 7, the results were significantly lower ($P<0.05$), indicating, again, that both freeze drying/long term storage and incubation in SIF had an adverse effect on reuterin production and, hence, the inhibitory activity. "Strain" 8, in which the immobilised cells had been incubated in SIF followed by SCF had an increased activity compared to "strain" 5, indicating that the conditions in SCF allowed a recovery of this enzyme activity. Table 5.10 compares the individual enzyme activities with that of "strain" 3, showing that after passage

through one or more simulated intestinal fluids, or freeze-drying, the increased enzyme expression resulting from immobilisation was significantly ($P<0.05$) reduced.

Figure 5.10 shows the relationship between diol dehydratase activity and reuterin production (during the secondary fermentation). The R^2 value was 0.72, indicating a strong correlation between these two indices. However, it should be noted that these experimental values were obtained after cell culture under different conditions.

Table 5.9 Diol dehydratase activities in cell-free extracts of different *L. reuteri* DPC16 “strains”. Values are initial rates, taken after 2 min incubation

"Strain"(Table 5.1)	Diol dehydratase (unit/mg protein)
Original (1)	18.86±0.24
Recovered (2)	31.10±0.21*
Immobilised (3)	40.21±0.22*
SGF (4)	25.05±0.41*
SIF (5)	13.33±0.17*
SCF (6)	31.63±0.03*
Freeze dried combined with long term storage (7)	8.74±0.47*
SIF+SCF (8)	39.23±0.14*

(*) indicates that there is a significant difference ($P<0.05$) in diol dehydratase activity between the “strain” and the original strain.

Table 5.10 A summary of the immobilisation effect compared with the effect of incubating the immobilised “strain” in different simulated gastrointestinal fluids

“Strain” (Table 5.1)	Diol dehydratase activity
Immobilisation (3)	control
Recovered (2)	-
SGF (4)	-
SIF (5)	-
SCF (6)	-
Freeze dried combined with long term storage (7)	-
SIF+SCF (8)	-

(-) indicates significant ($P<0.05$) down-regulation of activity compared to the control.

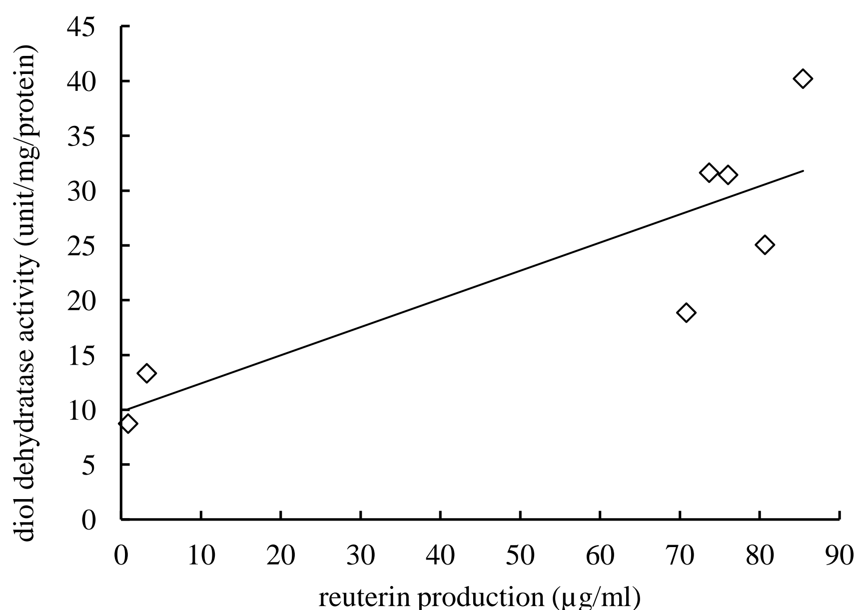


Figure 5.14 A plot of diol dehydratase activity against reuterin production.

5.4 Discussion

According to the experimental results of Chapter 4, the DPC16 "strain" that was recovered from the SCF after immobilisation and passage through the simulated GI tract displayed enhanced bacteriostatic and bactericidal activities. To identify the factor(s) that caused this functional change, several different DPC16 "strains" were isolated after exposure to different technological and environmental stresses. The rationale was as follows:

- A "strain" was isolated from wet alginate beads ("strain" 3) immediately after immobilisation. During and after immobilisation, the cells are maintained at a high cell density, and the close contact between the cells may increase the expression of certain genes (Lacroix & Yildirim, 2007);

- Cells were isolated after the alginate beads had been incubated separately in the simulated gastric fluid ("strain" 4), the simulated intestinal fluid ("strain" 5) and the simulated colonic fluid ("strain" 6). Different challenge factors were present during incubation in the different solutions. The presence of pepsin and acids in gastric juice is reported to be detrimental to some microorganisms (Holzapfel *et al.*, 1998); bile and pancreatic juices are present in the small intestine, although

the environment is not as extreme as that in the stomach. Cell growth within gel beads is limited by diffusion limitations of nutrients as well as by the presence of potentially toxic substances such as the acidic fluid and the bile salts. Further, acidic shock (Moslehi- Jenabian *et al*., 2009; Walter *et al*., 2008) and bile stress (Bron *et al*., 2004; Whitehead *et al*., 2008) may induce gene mutation, and provide the possibility to change the functional characteristics of probiotic cells;

- The freeze-dried/ long term storage “strain” (“strain” 7) was isolated because the removal of water from the bacteria during the freeze-drying process may result in irreversible changes to the structural and functional integrity of bacterial membranes and proteins (Ananta *et al*., 2005). Although the results reported in Chapter 3 showed that a less than 1 $\log_{10}$ c.f.u./bead viability drop occurred after freeze-drying and storage, it was considered important to examine whether freeze-drying is an appropriate way to preserve and store the immobilised probiotic bacteria.

After isolation of the various strains, their growth kinetics were compared. No significant differences ($P>0.05$) were observed either in the growth rates or in the endpoint OD readings except in those of the freeze-dried/long term storage “strain” (“strain” 7), where the values were considerably lower.

When glycerol was present in the DPC16 cultures, the growth rates and optical density values at 36 h observation were often lower than those observed in the absence of glycerol. To confirm this result, the plate count method was used to measure the final numbers of viable cells of several selected DPC16 “strains” and the results (data not shown) confirmed the observation. Glycerol has been reported to inhibit the proliferation of some human cell lines (Wiebe & Dinsdale, 1991), but as it has been previously shown to be non-toxic to *L. reuteri* DPC 16 (Tian 2011), it is more likely that it is a metabolite of glycerol (e.g. reuterin) that is slightly toxic to the producing organism. Tian (2011) has demonstrated that culture supernatants of strain DPC16 are slightly inhibitory to other probiotic bacteria, although to a much lesser extent than to pathogens.

Reuterin has been identified as a product of glycerol fermentation by *L. reuteri* under anaerobic conditions (Talarico *et al*., 1988). Therefore, when glycerol was present in MRS during the primary fermentation, reuterin production was higher than when

glycerol was absent. This has been confirmed in the present results. Further, Doleyers (2005) have reported that a two-step process whereby *L. reuteri* cells are first propagated in optimal conditions for cell growth followed by incubation in a pure glycerol solution, results in a higher reuterin concentration. Again, this has been confirmed in the present work. It has been reported that reuterin at a certain threshold concentration is toxic to the producing strain itself (Vollenweider & Lacroxic, 2004), and this is the most probable reason for the current observations. Reuterin, at a concentration of 15 to 30 µg/ml, inhibits growth of Gram-negative and most Gram-positive bacteria, yeast, fungi, and protozoa (Edens *et al*., 1997). From the experimental results of this chapter and the previous one, *L. reuteri* DPC16 demonstrated both bacteriostatic and bactericidal effects on selected food-borne pathogens. But whether the mechanism of action of reuterin is bacteriostatic or bactericidal, or both, is still not clear. The definition of a bactericidal effect is considered as a 99.9% reduction of viable cells within a 24 h period of exposure, while a bacteriostatic agent inhibits growth and reproduction of bacteria without killing them (Peters *et al*., 1992). However, there is not an accurate distinction between them, as a high concentration of some bacteriostatic agents may also be bactericidal, while a low concentration of some bacteriocidal agents may be bacteriostatic. According to the report of Arques (2008), reuterin at a concentration of 8 AU/ml, exhibited bacteriostatic activity against *Listeria monocytogenes*, while its activity was bactericidal against *Staphylococcus aureus*, *E. coli* O157: H7, and *Salmonella choleraesuis* subsp. *choleraesuis*. A number of mechanisms by which reuterin exerts its inhibitory effects has been postulated. One of the explanations is that reuterin may inhibit the activity of bacterial ribonucleotide reductase (Cleusix *et al*., 2007); another is that after being treated with reuterin, cells undergo oxidative stress (Schaefer *et al*., 2010).

Short-chain fatty acids are widely used as feed additives for the control of pathogens in animals (Van Immerseel *et al*., 2004). It has been suggested that whether or not SCFAs are bacteriostatic or bactericidal *in vitro* also depends on if there is a sufficiently long contact time with the bacteria (Thompson & Hinton, 1997). It is recognised that the SCFAs diffuse into bacterial cells in the undissociated form. Inside the bacterial cell, the acid dissociates, resulting in reduction of the intracellular pH and accumulation of the anion (Russell & DiezGonzalez, 1998; Van Der Wielen *et al*., 2000; Van Immerseel *et al*., 2004). In different pH environments, short-chain fatty acids have different effects

on bacteria. For example, at pH 6.0, 12.5 mM butyrate is bactericidal for *Campylobacter jejuni*, while 50 mM propionate and acetate have a bacteriostatic effect on this organism. However, none of these short-chain fatty acids at a concentration of 50 mM had a bactericidal effect at pH 7.5 (Van Deun *et al*., 2008).

According to the experimental results, the immobilised "strain" ("strain" 3) demonstrated the highest reuterin production, the strongest antimicrobial activity and the highest diol dehydratase activity. After passage through the simulated fluids, especially the simulated intestinal fluid, the enhanced abilities were weakened. Therefore, the evidence strongly suggests that it was the process of cell immobilisation, rather than passage through the simulated gastrointestinal fluids, that caused the enhancement of the inhibitory effect of the recovered DPC16 "strain" ("strain" 2) compared to the original. However, freeze-drying and long term storage of the immobilised cells ("strain" 7) resulted in a loss of antimicrobial activity and a very low reuterin production, which was probably due to the low diol dehydratase expression.

Zamudio-Jaramillo (2009) proposed a hypothesis that immobilisation could stimulate cell to cell interactions and could generate a microenvironment in alginate immobilised beads, and these conditions were responsible for higher rates of reuterin production compared with free *L. reuteri* cells. Doleyres (2004) reported that the tolerance of *Bifidobacterium longum* and *Lactococcus lactis* to various chemical and physicochemical stresses during continuous fermentation was increased after cell immobilisation. Barakat (2009) compared *E. coli* counts in stored cheese after using immobilised and non-immobilised lactobacilli during the cheese making process, and the results indicated that the inhibitory effects of the lactobacilli could be enhanced by the immobilisation technique.

In general, passage of the immobilised cells through the simulated gastrointestinal fluids resulted in a weakening of the antimicrobial activity of the immobilised DPC16 cells. However, passage through the SCF appeared to have a positive effect on immobilised DPC16 cells to regain their reuterin production ability. The reason for this is unclear, but it may be due to the presence of toxins in the faecal water component of the SCF. However, incubation of free cells in SCF resulted in no such change to the antimicrobial activity.

The weak antimicrobial activity of the freeze-dried immobilised cells, followed by long

term storage ("strain" 7), requires explanation. Clearly, water removal could have an adverse impact, while exposure to oxygen may cause damage to the bacterial cytoplasmic membrane (Israeli *et al*., 1974). During the freeze drying process, it is difficult to provide an anaerobic environment. From a practical viewpoint, storage of immobilised DPC16 cells by freeze-drying is not recommended if retention of antimicrobial activity is to be achieved.

During the process of producing reuterin from glycerol and other carbohydrates, two different isofunctional dehydratases, glycerol dehydratase and diol dehydratase catalyse this reaction (Talarico & Dobrogosz, 1990). In the present study, the activity of diol dehydratase was measured, and the results showed a high correlation with the result of reuterin production, i.e. the immobilised DPC16 cells ("strain" 3) showed the highest dehydratase enzyme activity and the highest reuterin production among all the tested strains, while, in contrast, the freeze- dried "strain" ("strain" 7) gave the lowest values of each. Knowledge of the mechanism by which reuterin production and diol dehydratase respond to immobilisation, acid and bile stress conditions, and freeze-drying is still missing. However, there are several reports on the effect of freeze-drying combined with long-term on enzyme stability, and whose results are similar to the present ones. Dianawati & Shah (2011) showed that the enzyme activities of β-galactosidase, β-glucosidase, lactate dehydrogenase, and ATPase from *Bifidobacterium animalis* ssp. *lactis* Bb12 were significantly ($P<0.05$) decreased after freeze drying and 10 weeks of storage.

In conclusion, the results of this Chapter have demonstrated that the immobilisation process is the key factor that induces the enhancement of antimicrobial activity of the cells that were recovered after passage through a simulated GI tract. However, it must be cautioned that laboratory conditions do not exactly reflect the real environment in nature, and the environmental conditions are also strongly dependent on the host. Thus, *in vivo* experiments are needed to confirm these i*n vitro* results in the future. Nevertheless, freeze drying is not recommended as a means of long-term storage of alginate immobilised cells.

Chapter 6 Final discussions and conclusions

This research has developed an effective immobilisation method to protect the probiotic strain *Lactobacillus reuteri* DPC16 during passage through a simulated GI tract, followed by release at the target site, i.e. the colon. The inclusion of skim milk in the calcium alginate beads proved to be a simple technique to allow protection of the cells during passage through the simulated gastric juice where the conditions can be lethal to unprotected cells. According to the experimental results, the DPC16 cells that were recovered from the target site showed no adverse physiological changes compared to the original cells. In fact, the antimicrobial activity of the recovered cells was significantly enhanced ($P<0.05$). The process of immobilisation in the Ca alginate/skim milk was confirmed to be the main step causing the enhanced inhibitory effects on pathogens.

Enhanced production of reuterin was identified as the cause of the enhanced antimicrobial effect, and this was linked to increased diol dehydratase activity, whereby reuterin is synthesised from glycerol. The exact nature, or mechanism, of the increased enzyme activity remains unclear, but is probably in response to the stressful environmental conditions imposed on the cells during immobilisation.

The effect of freeze-drying of immobilised cells of *L. reuteri* DPC16 on their viabilities and antimicrobial activity was also investigated in this research. Although the viability of freeze-dried immobilised probiotic cells did not decrease significantly during a six-month storage period, however the antimicrobial activity decreased markedly and there was an adverse effect on the growth kinetics of the cells. Also, after passage through the simulated GI tract, no viable cells could be recovered from either the colonic fluid or inside the alginate-skim milk beads, indicating that the cells had been "weakened" in some way. One conclusion to be drawn from this is that a simple viability test on freeze-dried immobilised cells does not give a true reflection of their physiological characteristics. In the current context, storage of immobilised cells by freeze-drying is not a satisfactory technique for the maintenance of important physiological characteristics.

In addition to the above, this work has provided further information on the adhesion of strain DPC16 to epithelial cells and its ability to compete with pathogens for the

receptor sites. In this case, the positive results have contributed to the potential benefits of this strain as a probiotic organism.

Finally, it must be cautioned that all the experiments in this project were carried out *in vitro*, which is less complex than the *in vivo* condition. Therefore, the results might not accurately reflect the real status inside the human body. Thus, *in vivo* investigation is recommended. In addition, the reason why the immobilisation process causes the enhancement of the antimicrobial activity of the probiotic strain still needs to be clarified. In terms of storage of the immobilised cells, freeze-drying is not an appropriate technique for *L. reuteri* DPC16, hence it is necessary to find an effective technique to store this probiotic strain.

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Appendix

Appendix I Pepsin Enzymatic activity assay

Method 1: activity assay with haemoglobin

A standard curve was prepared: a series of standard solutions containing different concentrations of tyrosine (0, 50, 100, 200, 250, 500 μg/ml) were prepared and then the optical density at 280nm was measured to create the tyrosine standard curve.

3 g/l human haemoglobin was prepared by dissolving 0.03 g haemoglobin powder (Sigma, USA) into 10 ml distilled H_2O. The solution was used as a substrate in this method and the testing conditions were kept acidic at 37 °C. Firstly, 3.2 g/l pepsin and haemoglobin solution were incubated; after 10 min, the reaction was stopped by the addition of 5 % TCA (Sigma-Aldrich, USA). The two mixtures were filtered through Whatman #45 filter paper; the resulting supernatant containing the small peptides produced by the proteolytic digestion was analysed for absorbance at 280 nm using spectrophotometer (UV mini 1240, Japan).

Method 2: gelatin liquification

In order to test the enzymatic activity of pepsin on gelatin, three different solutions, i.e. 5 ml H_2O, 5 ml 0.1 M HCl, and 5 ml SGF (containing pepsin), were mixed with 5 ml 5% (w/v) gelatin solutions seperately. The mixtures were then incubated at 37°C for 20 min, and the results were compared at room temperature.

Result

Table A.1 showed the OD_{280nm} readings of different testing groups. From the tyrosine-standard curve (Figure A.1), it could be deduced that the unit of pepsin (3.2 g/L) was 17.6 μg tyrosine/ml/ min.

Compared with the gel structure formed in water, gelatin lost the solidification characteristics in 0.1 M HCl solution with the presence of pepsin, and the mixture was liquid. In the absence of pepsin, gelatin formed a semi-solid gel in HCl solution (Figure A.2).

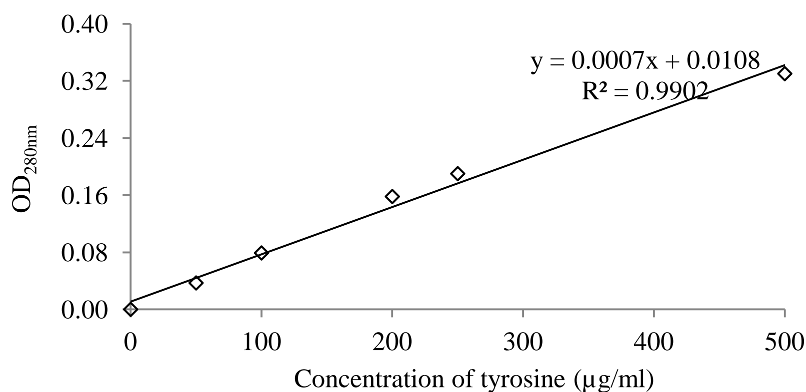


Figure A.1 Tyrosine standard curve.

Table A.1 Absorbance of different groups of pepsin in the pepsin activity assay

Group	Contents	OD_{280nm}
Blank	0.5 ml HCl (pH 1.2) + 2.5ml Hb (3.0g/L) +2.5ml TCA	0.055
Pepsin test	0.25 ml HCl (pH 1.2) + 0.25 ml SGF (with 32 g/L pepsin) + 2.5 ml Hb (3.0 g/L)+ 2.5 ml TCA	0.872
	0.25 ml HCL (pH 1.2) + 0.25 ml SGF (with 3.2 g/L pepsin) + 2.5 ml Hb (3.0 g/L)+ 2.5 ml TCA	0.189
Skim milk + pepsin	0.25 ml skim milk+0.25 ml SGF (with 3.2 g/L pepsin) +2.5 ml Hb (3.0 g/L)+ 2.5 ml TCA	0.565

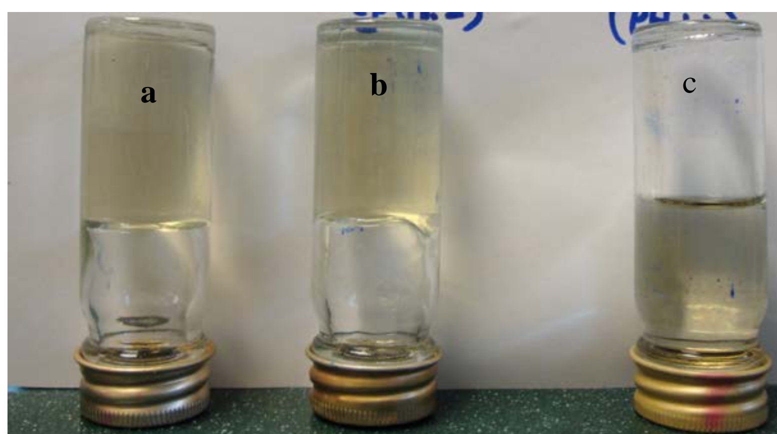


Figure A.2 Pepsin induced degradation of gelatin. (a) 5% gelatin mixed with H_2O, (b) 5% gelatin mixed with HCl, (c) 5% gelatin mixed with SGF pepsin. The mixing ratio was 1:1.

Appendix II Diffusion & leakage test of alginate-skim milk-Ca^{2+} system

Method

Methyl Red was chosen as a specific dye to indicate the diffusion and leakage that occurred from the alginate-skim milk-$CaCl_2$ immobilisation system during incubation in SGF. In acid solutions (pH<4.4), methyl red is in a red colour and appears yellow at pH values higher than 6.2. Orange is the middle colour. In this experiment, two different techniques were utilised. (a) Leakage test: 1% (w/v) methyl red was firstly mixed with 3% alginate, 8% skim milk and probiotic cells at the concentration of 0.4%, and then dropped into 0.3 M calcium chloride solution. The color changes both inside the beads and in the surrounding solution were observed. (b) Diffusion test: the SGF was first dyed with 1% methyl red. The prepared alginate-skim milk-$CaCl_2$ beads were then immerged in the SGF. At 5 min, 30 min, 120 min, several beads were harvested and cut into two parts to observe the diffusion phenomenon. Photographs were taken during the process.

Result

Before adding alginate, skim milk, DPC16 cells and methyl red mixture into calcium chloride, the solution was orange, indicating that the pH value was between 4.4 and 6.2 (see Figure A.3). The calcium chloride solution changed from colourless to yellow after the immobilisation occurred (Figure A.4), which might result from the immobilised contents coming out of the beads. When the formed beads were incubated in SGF, as showed in Figure A.5, the colour of SGF quickly changed from colourless to red (pH< 4.4).

Using the other method, the dyed SGF permeated through the freshly made beads in approximately 30 min: the color of the beads changed from colourless to red (Figure A.6).

Figure A.3 Mixture of alginate, skim milk, DPC16 cells and methyl red.

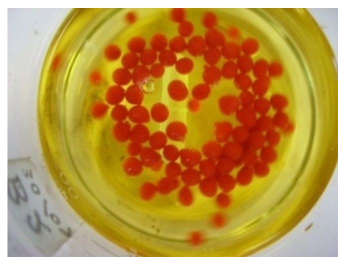


Figure A.4 Dyed Alginate-skim milk beads formed in $CaCl_2$.

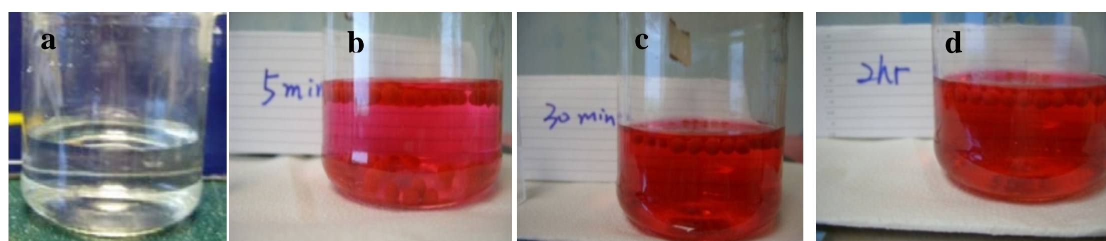


Figure A.5 Colour change of the SGF (pH 1.2) after putting alginate- skim milk- $CaCl_2$ beads in: (a) colourless SGF; (b) beads in SGF for 5 min, SGF changed colour from colourless to light red; (c) beadsin SGF for 30 min, the colour of surrounding SGF changed to dark red; (d) beads in SGF for 2 h: the colour becoming more dark.

Figure A.6 Colour changing inside the beads after being put in methyl red-stained SGF: the colour inside the beads varied from colourless to red.

Appendix III Publications

Publication 1 published by World Journal of Microbiology and Biotechnology: Functional properties of free and encapsulated *Lactobacillus reuteri* DPC16 during and after passage through a simulated gastrointestinal tract

Publication 2 published by Beneficial Microbes: Viability and delivery of immobilised *Lactobacillus reuteri* DPC16 within calcium alginate gel systems during sequential passage through simulated gastrointestinal fluids

Appendice iv Statistic analysis

Chapter 4

4.3.1 Growth kinetics of the original and recovered strains of DPC16

- Exponential phase

Two-Sample T-Test and CI: expO, expR

```
Two-sample T for expO vs expR

      N     Mean    StDev  SE Mean
expO  5  0.21774   0.0326    0.015
expR  5   0.2434   0.0568    0.025


Difference = mu (expO) - mu (expR)
Estimate for difference:  -0.02564
95% CI for difference:  (-0.0973, 0.0460)
T-Test of difference = 0 (vs not =): T-Value = -0.88  P-Value = 0.415  DF = 6
```

- Stationary phase

Two-Sample T-Test and CI: statO, statR

```
Two-sample T for statO vs statR

       N      Mean     StDev  SE Mean
statO  5    0.0379    0.0302   0.0053
statR  5    0.0340    0.0224   0.0042


Difference = mu (statO) - mu (statR)
Estimate for difference:  0.00387
95% CI for difference:  (-0.00961, 0.01734)
T-Test of difference = 0 (vs not =): T-Value = 0.57  P-Value = 0.568  DF = 61
```

- Decline phase

Two-Sample T-Test and CI: DecO, DecR

```
Two-sample T for DecO vs DecR

      N       Mean     StDev  SE Mean
DecO  5   -0.0290    0.0245   0.0043
DecR  5   -0.0306    0.0241   0.0043


Difference = mu (DecO) - mu (DecR)
Estimate for difference:  0.00159
95% CI for difference:  (-0.01054, 0.01373)
T-Test of difference = 0 (vs not =): T-Value = 0.26  P-Value = 0.794  DF = 61
```

- Optical density reading at the endpoint (36 h) for two *L. reuteri* DPC16 Cultures

Two-Sample T-Test and CI: OD620 Orig, OD620 Rev

```
Two-sample T for OD620 Orig vs OD620 Rev

            N    Mean   StDev  SE Mean
```

```
OD620 Orig  2  0.7558  0.0363    0.026
OD620 Rev   2  0.7282  0.0215    0.015


Difference = mu (OD620 Orig) - mu (OD620 Rev)
Estimate for difference:  0.0276
95% CI for difference:  (-0.3518, 0.4070)
T-Test of difference = 0 (vs not =): T-Value = 0.92  P-Value = 0.525  DF = 1
```

- pH changes during the growth phases of two *L. reuteri* DPC16 cultures

Two-Sample T-Test and CI: 18Ori, 18Rec

```
Two-sample T for 18Ori vs 18Rec

       N     Mean    StDev  SE Mean
18Ori  2   4.4800   0.0141    0.010
18Rec  2  4.46000  0.00707   0.0050


Difference = mu (18Ori) - mu (18Rec)
Estimate for difference:  0.0200
95% CI for difference:  (-0.1221, 0.1621)
T-Test of difference = 0 (vs not =): T-Value = 1.79  P-Value = 0.325  DF = 1
```

Two-Sample T-Test and CI: 24Ori, 24Rec

```
Two-sample T for 24Ori vs 24Rec

       N     Mean    StDev  SE Mean
24Ori  2  4.39000  0.00707   0.0050
24Rec  2  4.39000  0.00707   0.0050


Difference = mu (24Ori) - mu (24Rec)
Estimate for difference:  0.00000
95% CI for difference:  (-0.03042, 0.03042)
T-Test of difference = 0 (vs not =): T-Value = 0.00  P-Value = 1.000  DF = 2
```

Two-Sample T-Test and CI: 30Ori, 30Rec

```
Two-sample T for 30Ori vs 30Rec

       N     Mean    StDev  SE Mean
30Ori  2  4.46000  0.00707   0.0050
30Rec  2  4.43000  0.00707   0.0050


Difference = mu (30Ori) - mu (30Rec)
Estimate for difference:  0.03000
95% CI for difference:  (-0.00042, 0.06042)
T-Test of difference = 0 (vs not =): T-Value = 4.24  P-Value = 0.051  DF = 2
```

4.3.2 Antimicrobial activities of cell-free supernatants from *L. reuteri* DPC16 cultures

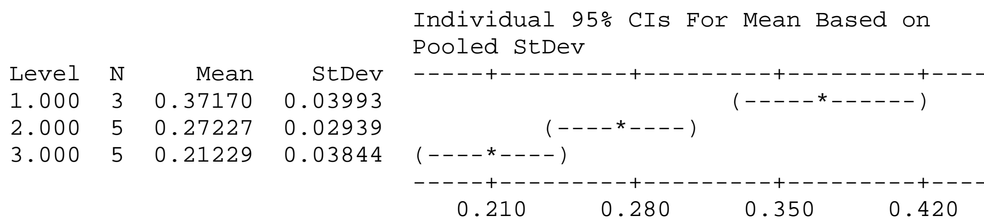
Level	Group
1	BHI
2	Original
3	Recovered

Growth rates

- *E. coli* without glycerol

One-way ANOVA: growth rate e.coli versus group e.coli

```
Source         DF       SS       MS      F      P
group e.coli    2  0.04765  0.02382  18.98  0.000
Error          10  0.01255  0.00126
Total          12  0.06020

S = 0.03543   R-Sq = 79.15%   R-Sq(adj) = 74.98%
```



```
                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N     Mean    StDev   -----+---------+---------+---------+----
1.000  3  0.37170  0.03993                         (-----*------)
2.000  5  0.27227  0.02939           (----*----)
3.000  5  0.21229  0.03844   (----*----)
                             -----+---------+---------+---------+----
                                0.210     0.280     0.350     0.420

Pooled StDev = 0.03543
```

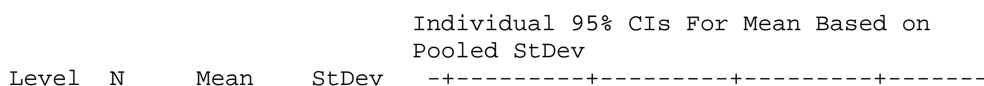
Two-Sample T-Test and CI: O growth rate e.coli, R growth rate e.coli

```
Two-sample T for O growth rate e.coli vs R growth rate e.coli

                      N    Mean   StDev  SE Mean
O growth rate e.coli  5  0.2723  0.0294    0.013
R growth rate e.coli  5  0.2123  0.0384    0.017


Difference = mu (O growth rate e.coli) - mu (R growth rate e.coli)
Estimate for difference:  0.0600
95% CI for difference:  (0.0088, 0.1111)
T-Test of difference = 0 (vs not =): T-Value = 2.77  P-Value = 0.028  DF = 7
```

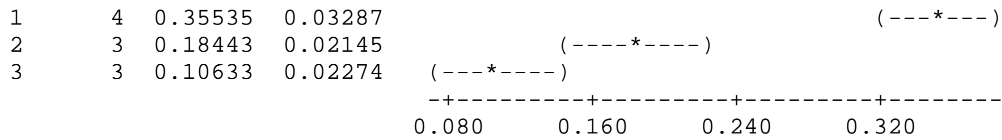
- *E. coli* with glycerol

One-way ANOVA: growth rate e.coli/g versus group e.coli/g

```
Source           DF        SS        MS      F      P
group e.coli/g    2  0.114956  0.057478  77.45  0.000
Error             7  0.005195  0.000742
Total             9  0.120151

S = 0.02724   R-Sq = 95.68%   R-Sq(adj) = 94.44%
```



```
                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N     Mean    StDev    -+---------+---------+--------+--------
```



```
1      4  0.35535  0.03287                                  (---*---)
2      3  0.18443  0.02145            (----*----)
3      3  0.10633  0.02274   (---*----)
                             -+---------+---------+---------+--------
                            0.080     0.160     0.240     0.320
```

Pooled StDev = 0.02724

Two-Sample T-Test and CI: O/g growth rate e.coli, R/g growth rate e.coli

Two-sample T for O/g growth rate e.coli vs R/g growth rate e.coli

	N	Mean	StDev	SE Mean
O/g growth rate e.coli	3	0.1844	0.0215	0.012
R/g growth rate e.coli	3	0.1063	0.0227	0.013

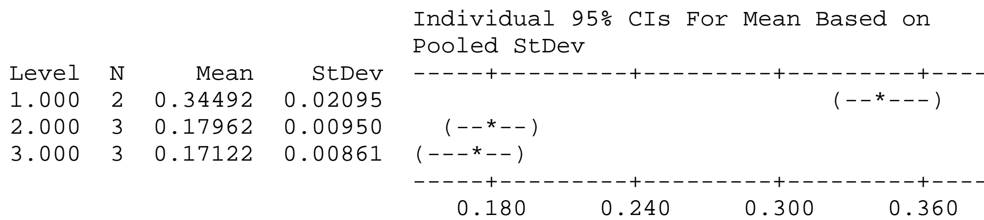
Difference = mu (O/g growth rate e.coli) - mu (R/g growth rate e.coli)
Estimate for difference: 0.0781
95% CI for difference: (0.0207, 0.1355)
T-Test of difference = 0 (vs not =): T-Value = 4.33 P-Value = 0.023 DF = 3

- *S. derby* without glycerol

One-way ANOVA: growth rate s.der versus group s.der

Source	DF	SS	MS	F	P
group s.der	2	0.043198	0.021599	140.64	0.000
Error	5	0.000768	0.000154		
Total	7	0.043966			

S = 0.01239   R-Sq = 98.25%   R-Sq(adj) = 97.55%



```
                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N     Mean    StDev    -----+---------+---------+---------+----
1.000  2  0.34492  0.02095                               (--*---)
2.000  3  0.17962  0.00950      (--*--)
3.000  3  0.17122  0.00861    (---*--)
                              -----+---------+---------+---------+----
                                 0.180     0.240     0.300     0.360
```

Pooled StDev = 0.01239

Two-Sample T-Test and CI: O growth rate s.der, R end reading s.der

Two-sample T for O growth rate s.der vs R end reading s.der

	N	Mean	StDev	SE Mean
O growth rate s.der	3	0.17962	0.00950	0.0055
R end reading s.der	3	1.2058	0.0256	0.015

Difference = mu (O growth rate s.der) - mu (R end reading s.der)
Estimate for difference: -1.0261
95% CI for difference: (-1.0939, -0.9584)
T-Test of difference = 0 (vs not =): T-Value = -65.17 P-Value = 0.000 DF = 2

- *S. derby* with glycerol

One-way ANOVA: growth rate s.der/g versus group s.der/g

```
Source         DF         SS         MS       F      P
group s.der/g   2  0.0810715  0.0405357  421.99  0.000
Error           5  0.0004803  0.0000961
Total           7  0.0815518

S = 0.009801   R-Sq = 99.41%   R-Sq(adj) = 99.18%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -------+---------+---------+---------+--
1.000  2  0.34492  0.02095                                   (-*--)
2.000  3  0.12328  0.00212     (-*-)
3.000  3  0.10331  0.00402  (-*-)
                            -------+---------+---------+---------+--
                                 0.140     0.210     0.280     0.350

Pooled StDev = 0.00980
```

Two-Sample T-Test and CI: O/g growth rate s.der, R/g growth rate s.der

```
Two-sample T for O/g growth rate s.der vs R/g growth rate s.der

                       N     Mean    StDev  SE Mean
O/g growth rate s.der  3  0.12328  0.00212   0.0012
R/g growth rate s.der  3  0.10331  0.00402   0.0023


Difference = mu (O/g growth rate s.der) - mu (R/g growth rate s.der)
Estimate for difference:  0.01998
95% CI for difference:  (0.01164, 0.02832)
T-Test of difference = 0 (vs not =): T-Value = 7.62  P-Value = 0.005  DF = 3
```

- *L. monocytogenes* without glycerol

One-way ANOVA: growth rate l.mono versus group l.mono

```
Source        DF        SS        MS      F      P
group l.mono   2  0.026048  0.013024  36.98  0.000
Error          8  0.002817  0.000352
Total         10  0.028866

S = 0.01877   R-Sq = 90.24%   R-Sq(adj) = 87.80%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  ------+---------+---------+---------+---
1.000  3  0.31411  0.00789                       (----*----)
2.000  4  0.26110  0.02023              (---*----)
3.000  4  0.19249  0.02209  (---*----)
                            ------+---------+---------+---------+---
                                0.200     0.250     0.300     0.350

Pooled StDev = 0.01877
```

Two-Sample T-Test and CI: O growth rate l.mon, R growth rate l.mono

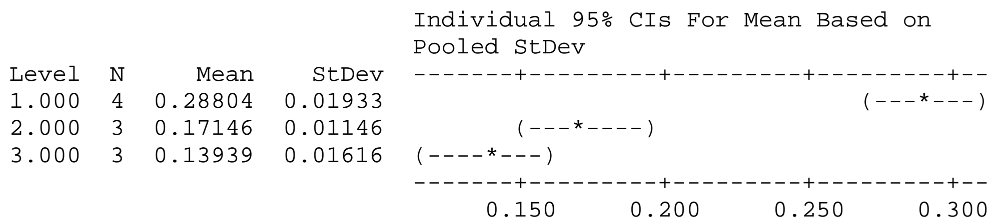
```
Two-sample T for O growth rate l.mon vs R growth rate l.mono

                      N    Mean   StDev  SE Mean
O growth rate l.mon   4  0.2611  0.0202    0.010
R growth rate l.mono  4  0.1925  0.0221    0.011
```

```
Difference = mu (O growth rate l.mon) - mu (R growth rate l.mono)
Estimate for difference:  0.0686
95% CI for difference:  (0.0301, 0.1071)
T-Test of difference = 0 (vs not =): T-Value = 4.58  P-Value = 0.006  DF = 5
```

- *L. monocytogenes* with glycerol

One-way ANOVA: growth rate l.mono/g versus L.mono group/g

```
Source          DF        SS        MS      F      P
L.mono group/g   2  0.043753  0.021877  80.34  0.000
Error            7  0.001906  0.000272
Total            9  0.045659

S = 0.01650   R-Sq = 95.83%   R-Sq(adj) = 94.63%
```



```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -------+---------+---------+---------+--
1.000  4  0.28804  0.01933                               (---*---)
2.000  3  0.17146  0.01146         (---*----)
3.000  3  0.13939  0.01616  (----*---)
                            -------+---------+---------+---------+--
                                 0.150     0.200     0.250     0.300
```

```
Pooled StDev = 0.01650
```

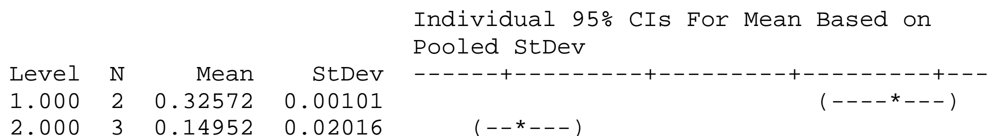
Two-Sample T-Test and CI: O/g growth rate l.mono, r/g growth rate l.mon

```
Two-sample T for O/g growth rate l.mono vs r/g growth rate l.mon

                        N    Mean   StDev  SE Mean
O/g growth rate l.mono  3  0.1715  0.0115   0.0066
r/g growth rate l.mon   3  0.1394  0.0162   0.0093


Difference = mu (O/g growth rate l.mono) - mu (r/g growth rate l.mon)
Estimate for difference:  0.0321
95% CI for difference:  (-0.0043, 0.0685)
T-Test of difference = 0 (vs not =): T-Value = 2.80  P-Value = 0.068  DF = 3
```

- *S. aureus* without glycerol

One-way ANOVA: growth rate s.au2 versus group s.au2

```
Source       DF        SS        MS       F      P
group s.au2   2  0.056288  0.028144  112.91  0.000
Error         5  0.001246  0.000249
Total         7  0.057534

S = 0.01579   R-Sq = 97.83%   R-Sq(adj) = 96.97%
```



```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  ------+---------+---------+---------+---
1.000  2  0.32572  0.00101                            (----*---)
2.000  3  0.14952  0.02016      (--*---)
```

```
3.000  3  0.11924  0.01470  (--*--)
                            ------+---------+---------+---------+---
                                0.140     0.210     0.280     0.350

Pooled StDev = 0.01579
```

Two-Sample T-Test and CI: O growth rate s.au, R growth rate s.au

```
Two-sample T for O growth rate s.au vs R growth rate s.au

                    N    Mean   StDev  SE Mean
O growth rate s.au  3  0.1495  0.0202    0.012
R growth rate s.au  3  0.1192  0.0147   0.0085


Difference = mu (O growth rate s.au) - mu (R growth rate s.au)
Estimate for difference:  0.0303
95% CI for difference:  (-0.0156, 0.0761)
T-Test of difference = 0 (vs not =): T-Value = 2.10  P-Value = 0.126  DF = 3
```

- *S. aureus* with glycerol

One-way ANOVA: growth rate s.au/g versus group s.au/g

```
Source        DF         SS         MS       F      P
group s.au/g   2  0.0276215  0.0138107  158.38  0.000
Error          6  0.0005232  0.0000872
Total          8  0.0281447

S = 0.009338   R-Sq = 98.14%   R-Sq(adj) = 97.52%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  --------+---------+---------+---------+-
1      3  0.22991  0.01294                                (--*---)
2      4  0.11682  0.00792      (--*--)
3      2  0.10478  0.00064  (---*---)
                            --------+---------+---------+---------+-
                                  0.120     0.160     0.200     0.240

Pooled StDev = 0.00934
```

Two-Sample T-Test and CI: O/g growth rate s.au, r/g growth rate s.au

```
Two-sample T for O/g growth rate s.au vs r/g growth rate s.au

                      N      Mean     StDev  SE Mean
O/g growth rate s.au  4   0.11682   0.00792   0.0040
r/g growth rate s.au  2  0.104775  0.000636  0.00045


Difference = mu (O/g growth rate s.au) - mu (r/g growth rate s.au)
Estimate for difference:  0.01204
95% CI for difference:  (-0.00064, 0.02472)
T-Test of difference = 0 (vs not =): T-Value = 3.02  P-Value = 0.057  DF = 3
```

Endpoint reading

- *E. coli* without glycerol

One-way ANOVA: end reading e.coli versus group e.coli

```
Source         DF       SS       MS     F      P
group e.coli    2  0.13074  0.06537  9.82  0.004
Error          10  0.06655  0.00665
Total          12  0.19729

S = 0.08158   R-Sq = 66.27%   R-Sq(adj) = 59.52%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -----+---------+---------+---------+----
1.000  3  1.3681  0.0473                      (---------*--------)
2.000  5  1.1973  0.0473          (------*------)
3.000  5  1.1041  0.1153  (------*------)
                          -----+---------+---------+---------+----
                             1.08      1.20      1.32      1.44

Pooled StDev = 0.0816
```

Two-Sample T-Test and CI: O end reading e.coli, R end reading e.oli

```
Two-sample T for O end reading e.coli vs R end reading e.oli

                      N    Mean   StDev  SE Mean
O end reading e.coli  5  1.1973  0.0473    0.021
R end reading e.oli   5   1.104   0.115    0.052


Difference = mu (O end reading e.coli) - mu (R end reading e.oli)
Estimate for difference:  0.0932
95% CI for difference:  (-0.0500, 0.2364)
T-Test of difference = 0 (vs not =): T-Value = 1.67  P-Value = 0.155  DF = 5
```

- *E. coli* with glycerol

One-way ANOVA: end reading e.coli/g versus group e.coli/g

```
Source           DF       SS       MS       F      P
group e.coli/g    2  1.16978  0.58489  223.25  0.000
Error             7  0.01834  0.00262
Total             9  1.18812

S = 0.05119   R-Sq = 98.46%   R-Sq(adj) = 98.02%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ---------+---------+---------+---------+
1      4  1.3652  0.0610                                (--*-)
2      3  0.7462  0.0504        (--*--)
3      3  0.6056  0.0324  (--*--)
                          ---------+---------+---------+---------+
                                0.75      1.00      1.25      1.50

Pooled StDev = 0.0512
```

Two-Sample T-Test and CI: O/g end reading e.coli, R/g end reading e.coli

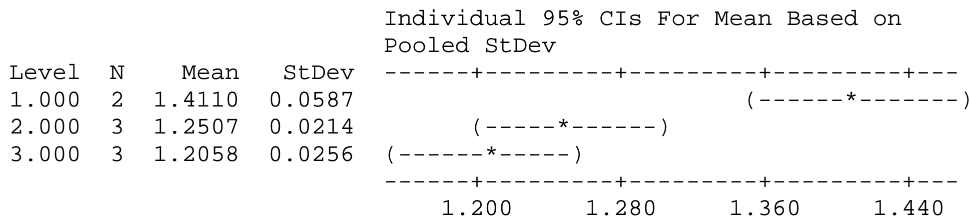
```
Two-sample T for O/g end reading e.coli vs R/g end reading e.coli

                        N    Mean   StDev  SE Mean
O/g end reading e.coli  3  0.7462  0.0504    0.029
```

```
R/g end reading e.coli  3  0.6056  0.0324    0.019


Difference = mu (O/g end reading e.coli) - mu (R/g end reading e.coli)
Estimate for difference:  0.1406
95% CI for difference:  (0.0305, 0.2507)
T-Test of difference = 0 (vs not =): T-Value = 4.07  P-Value = 0.027  DF = 3
```

- *S. derby* without glycerol

One-way ANOVA: end reading s.der versus group s.der

```
Source        DF       SS       MS      F      P
group s.der    2  0.05314  0.02657  23.43  0.003
Error          5  0.00567  0.00113
Total          7  0.05881

S = 0.03367   R-Sq = 90.36%   R-Sq(adj) = 86.50%
```



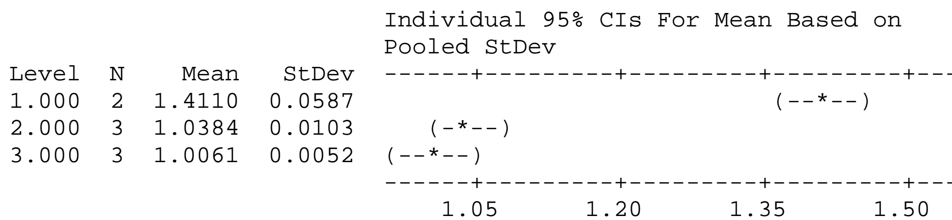
```
                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N    Mean   StDev  ------+---------+---------+---------+---
1.000  2  1.4110  0.0587                           (------*-------)
2.000  3  1.2507  0.0214        (-----*------)
3.000  3  1.2058  0.0256  (------*-----)
                          ------+---------+---------+---------+---
                              1.200     1.280     1.360     1.440
```

```
Pooled StDev = 0.0337
```

- *S.derby* with glycerol

One-way ANOVA: end reading s.der/g versus group s.der/g

```
Source          DF        SS        MS       F      P
group s.der/g    2  0.228294  0.114147  153.83  0.000
Error            5  0.003710  0.000742
Total            7  0.232004

S = 0.02724   R-Sq = 98.40%   R-Sq(adj) = 97.76%
```



```
                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N    Mean   StDev  ------+---------+---------+---------+---
1.000  2  1.4110  0.0587                             (--*--)
2.000  3  1.0384  0.0103     (-*--)
3.000  3  1.0061  0.0052  (--*--)
                          ------+---------+---------+---------+---
                              1.05      1.20      1.35      1.50
```

```
Pooled StDev = 0.0272
```

Two-Sample T-Test and CI: O/g end reading s.der, R/g end reading s.der

```
Two-sample T for O/g end reading s.der vs R/g end reading s.der

                       N     Mean    StDev  SE Mean
O/g end reading s.der  3   1.0384   0.0103   0.0059
R/g end reading s.der  3  1.00607  0.00517   0.0030
```

```
Difference = mu (O/g end reading s.der) - mu (R/g end reading s.der)
Estimate for difference:  0.03230
95% CI for difference:  (0.00367, 0.06093)
T-Test of difference = 0 (vs not =): T-Value = 4.85  P-Value = 0.040  DF = 2
```

- *L. monocytogenes* without glycerol

One-way ANOVA: end reading l.mono versus group l.mono

```
Source        DF       SS       MS      F      P
group l.mono   2  0.15354  0.07677  36.75  0.000
Error          8  0.01671  0.00209
Total         10  0.17025

S = 0.04571   R-Sq = 90.18%   R-Sq(adj) = 87.73%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --+---------+---------+---------+-------
1.000  3  1.3992  0.0210                         (----*----)
2.000  4  1.1718  0.0245       (----*---)
3.000  4  1.1096  0.0684  (---*----)
                          --+---------+---------+---------+-------
                          1.08      1.20      1.32      1.44

Pooled StDev = 0.0457
```

Two-Sample T-Test and CI: O end reading l.mono, R end reading l.mono

```
Two-sample T for O end reading l.mono vs R end reading l.mono

                      N    Mean   StDev  SE Mean
O end reading l.mono  4  1.1718  0.0245    0.012
R end reading l.mono  4  1.1096  0.0684    0.034


Difference = mu (O end reading l.mono) - mu (R end reading l.mono)
Estimate for difference:  0.0622
95% CI for difference:  (-0.0534, 0.1778)
T-Test of difference = 0 (vs not =): T-Value = 1.71  P-Value = 0.185  DF = 3
```

- *L. monocytogenes* with glycerol

One-way ANOVA: end reading L.mono/g versus L.mono group/g

```
Source          DF        SS        MS       F      P
L.mono group/g   2  0.652028  0.326014  454.73  0.000
Error            7  0.005019  0.000717
Total            9  0.657047

S = 0.02678   R-Sq = 99.24%   R-Sq(adj) = 99.02%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -----+---------+---------+---------+----
1.000  4  1.3902  0.0373                                   (-*-)
2.000  3  0.8744  0.0096   (-*--)
3.000  3  0.8636  0.0183  (--*-)
                          -----+---------+---------+---------+----
```

```
                             0.90      1.05      1.20      1.35

Pooled StDev = 0.0268
```

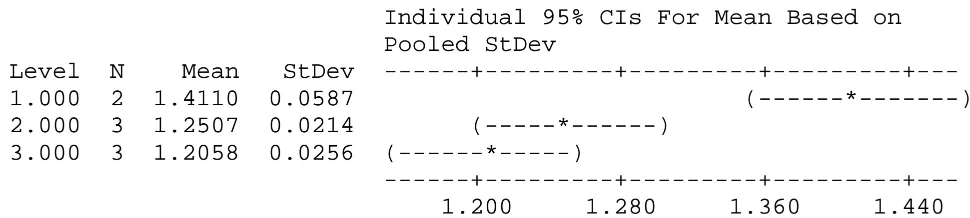
Two-Sample T-Test and CI: O/g end reading l.mono, r/g end reading l.mono

```
Two-sample T for O/g end reading l.mono vs r/g end reading l.mono

                        N     Mean    StDev  SE Mean
O/g end reading l.mono  3  0.87440  0.00956   0.0055
r/g end reading l.mono  3   0.8636   0.0183    0.011


Difference = mu (O/g end reading l.mono) - mu (r/g end reading l.mono)
Estimate for difference:  0.0108
95% CI for difference:  (-0.0272, 0.0487)
T-Test of difference = 0 (vs not =): T-Value = 0.90  P-Value = 0.433  DF = 3
```

- *S. derby* without glycerol

One-way ANOVA: end reading s.der versus group s.der

```
Source       DF       SS       MS      F      P
group s.der   2  0.05314  0.02657  23.43  0.003
Error         5  0.00567  0.00113
Total         7  0.05881

S = 0.03367   R-Sq = 90.36%   R-Sq(adj) = 86.50%
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ------+---------+---------+---------+---
1.000  2  1.4110  0.0587                           (------*-------)
2.000  3  1.2507  0.0214        (-----*------)
3.000  3  1.2058  0.0256  (------*-----)
                          ------+---------+---------+---------+---
                              1.200     1.280     1.360     1.440

Pooled StDev = 0.0337
```

Two-Sample T-Test and CI: O end reading s.der, R end reading s.der

```
Two-sample T for O end reading s.der vs R end reading s.der

                     N    Mean   StDev  SE Mean
O end reading s.der  3  1.2507  0.0214    0.012
R end reading s.der  3  1.2058  0.0256    0.015


Difference = mu (O end reading s.der) - mu (R end reading s.der)
Estimate for difference:  0.0449
95% CI for difference:  (-0.0164, 0.1062)
T-Test of difference = 0 (vs not =): T-Value = 2.33  P-Value = 0.102  DF = 3
```

- *S. derby* with glycerol

One-way ANOVA: end reading s.au/g versus group s.au/g

```
Source        DF        SS        MS       F      P
group s.au/g   2  0.061026  0.030513  116.68  0.000
Error          6  0.001569  0.000262
Total          8  0.062595

S = 0.01617   R-Sq = 97.49%   R-Sq(adj) = 96.66%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --+---------+---------+---------+-------
1      3  1.1149  0.0164                              (--*---)
2      4  0.9518  0.0185       (--*--)
3      2  0.9222  0.0018  (---*---)
                          --+---------+---------+---------+-------
                          0.910     0.980     1.050     1.120

Pooled StDev = 0.0162
```

Two-Sample T-Test and CI: O/g end reading s.der, R/g growth rate s.der

```
Two-sample T for O/g end reading s.der vs R/g growth rate s.der

                       N     Mean    StDev  SE Mean
O/g end reading s.der  3   1.0384   0.0103   0.0059
R/g growth rate s.der  3  0.10331  0.00402   0.0023


Difference = mu (O/g end reading s.der) - mu (R/g growth rate s.der)
Estimate for difference:  0.93506
95% CI for difference:  (0.90760, 0.96252)
T-Test of difference = 0 (vs not =): T-Value = 146.49  P-Value = 0.000  DF = 2
```

- *S. aureus* without glycerol

One-way ANOVA: end reading s.au2 versus group s.au2

```
Source       DF         SS         MS       F      P
group s.au2   2  0.0128185  0.0064092  103.07  0.000
Error         5  0.0003109  0.0000622
Total         7  0.0131294

S = 0.007886   R-Sq = 97.63%   R-Sq(adj) = 96.68%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -----+---------+---------+---------+----
1.000  2  1.35755  0.00488                               (---*---)
2.000  3  1.29047  0.00848            (---*--)
3.000  3  1.25440  0.00847  (--*---)
                            -----+---------+---------+---------+----
                               1.260     1.295     1.330     1.365

Pooled StDev = 0.00789
```

Two-Sample T-Test and CI: O end reading s.au, R end reading s.au

```
Two-sample T for O end reading s.au vs R end reading s.au

                    N     Mean    StDev  SE Mean
O end reading s.au  3  1.29047  0.00848   0.0049
R end reading s.au  3  1.25440  0.00847   0.0049
```

```
Difference = mu (O end reading s.au) - mu (R end reading s.au)
Estimate for difference:  0.03607
95% CI for difference:  (0.01405, 0.05808)
T-Test of difference = 0 (vs not =): T-Value = 5.21  P-Value = 0.014  DF = 3
```

- *S. aureus* with glycerol

One-way ANOVA: endreading s.au/g2 versus group s.au/g2

```
Source         DF        SS        MS      F      P
group s.au/g2   2  0.045501  0.022751  72.83  0.000
Error           5  0.001562  0.000312
Total           7  0.047063

S = 0.01767   R-Sq = 96.68%   R-Sq(adj) = 95.35%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ---+---------+---------+---------+------
1.000  2  1.1139  0.0230                             (---*----)
2.000  4  0.9518  0.0185        (--*--)
3.000  2  0.9222  0.0018  (----*---)
                          ---+---------+---------+---------+------
                           0.910     0.980     1.050     1.120

Pooled StDev = 0.0177
```

Two-Sample T-Test and CI: O/g end reading s.au, r/g rend reading s.au

```
Two-sample T for O/g end reading s.au vs r/g rend reading s.au

                       N     Mean    StDev  SE Mean
O/g end reading s.au   4   0.9518   0.0185   0.0093
r/g rend reading s.au  2  0.92220  0.00184   0.0013


Difference = mu (O/g end reading s.au) - mu (r/g rend reading s.au)
Estimate for difference:  0.02960
95% CI for difference:  (-0.00018, 0.05938)
T-Test of difference = 0 (vs not =): T-Value = 3.16  P-Value = 0.051  DF = 3
```

4.3.3 Bactericidal activity of DPC16 cell-free supernatants (pH adjusted to 7.0 and unadjusted (approximately pH 4.4)) on growth of *E. coli*, *S. derby*, *L. monocytogenes* and *S. aureus.*

Level	Group
1	peptone
2	supernatant from the original cell
3	supernatant from the recovered cell
4	supernatant from the original cell with the presence of glycerol
5	supernatant from the recovered cell with the presence of glycerol

pH value of supernatant is 7.0

- Inhibition of *E. coli*

One-way ANOVA: 1 hour versus GROUP

```
Source  DF           SS           MS      F      P
GROUP    4  6.07840E+15  1.51960E+15  96.58  0.000
Error   10  1.57333E+14  1.57333E+13
Total   14  6.23573E+15

S = 3966527   R-Sq = 97.48%   R-Sq(adj) = 96.47%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  -------+---------+---------+---------+--
1      3  90666667  2309401                                (-*--)
2      3  55333333  4163332                 (--*-)
3      3  30666667  1154701  (-*--)
4      3  51333333  4163332               (--*-)
5      3  42666667  6110101       (-*--)
                             -------+---------+---------+---------+--
                             40000000  60000000  80000000  1.00E+08

Pooled StDev = 3966527
```

One-way ANOVA: 2 hour versus GROUP

```
Source  DF           SS           MS        F      P
GROUP    4  1.15644E+16  2.89111E+15  4235.02  0.000
Error   10  6.82667E+12  6.82667E+11
Total   14  1.15713E+16

S = 826236   R-Sq = 99.94%   R-Sq(adj) = 99.92%


                             Individual 95% CIs For Mean Based on Pooled StDev
Level  N      Mean    StDev      --+---------+---------+---------+-------
1      3  93066667   832666                                          (*
2      3  39333333   611010                  (*
3      3  32133333   832666              *)
4      3  19066667  1006645       (*
5      3  16800000   800000      *)
                                 --+---------+---------+---------+-------
                             20000000  40000000  60000000  80000000

Pooled StDev = 826236
```

One-way ANOVA: 3 hour versus GROUP

```
Source  DF           SS           MS       F      P
GROUP    4  9.30662E+15  2.32666E+15  491.27  0.000
Error   10  4.73600E+13  4.73600E+12
Total   14  9.35398E+15

S = 2176235   R-Sq = 99.49%   R-Sq(adj) = 99.29%


                             Individual 95% CIs For Mean Based on Pooled StDev
Level  N      Mean    StDev      --+---------+---------+---------+-------
1      3  90533333  2722744                                        (*-)
2      3  48400000  3274141                    (*-)
3      3  51600000  1600000                     (-*)
4      3  19333333   832666      (-*)
5      3  26533333  1514376          (*-)
                                 --+---------+---------+---------+-------
```

```
                            20000000  40000000  60000000  80000000

Pooled StDev = 2176235
```

One-way ANOVA: 4 hour versus GROUP

```
Source  DF          SS          MS       F      P
GROUP    4  2.93193E+16  7.32981E+15  134.40  0.000
Error   10  5.45387E+14  5.45387E+13
Total   14  2.98646E+16

S = 7385030   R-Sq = 98.17%   R-Sq(adj) = 97.44%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N       Mean     StDev  --------+---------+---------+---------+-
1      3  141333333  16165808                                (-*--)
2      3   56533333   3028751            (-*--)
3      3   50266667    832666          (--*-)
4      3   17866667    611010  (-*--)
5      3   24000000   1058301    (-*-)
                               --------+---------+---------+---------+-
                                40000000  80000000  1.20E+08  1.60E+08

Pooled StDev = 7385030
```

Two-Sample T-Test and CI: group2,1, group3,1

```
Two-sample T for group2,1 vs group3,1

          N      Mean    StDev  SE Mean
group2,1  3  55333333  4163332  2403701
group3,1  3  30666667  1154701   666667


Difference = mu (group2,1) - mu (group3,1)
Estimate for difference:  24666667
95% CI for difference:  (13933965, 35399368)
T-Test of difference = 0 (vs not =): T-Value = 9.89  P-Value = 0.010  DF = 2
```

Two-Sample T-Test and CI: group4,1, group5,1

```
Two-sample T for group4,1 vs group5,1

          N      Mean    StDev  SE Mean
group4,1  3  51333333  4163332  2403701
group5,1  3  42666667  6110101  3527668


Difference = mu (group4,1) - mu (group5,1)
Estimate for difference:  8666667
95% CI for difference:  (-4918399, 22251733)
T-Test of difference = 0 (vs not =): T-Value = 2.03  P-Value = 0.135  DF = 3
```

Two-Sample T-Test and CI: group2,2, group3,2

```
Two-sample T for group2,2 vs group3,2

          N      Mean   StDev  SE Mean
group2,2  3  39333333  611010   352767
group3,2  3  32133333  832666   480740
```

```
Difference = mu (group2,2) - mu (group3,2)
Estimate for difference:  7200000
95% CI for difference:  (5302356, 9097644)
T-Test of difference = 0 (vs not =): T-Value = 12.07  P-Value = 0.001  DF = 3
```

Two-Sample T-Test and CI: group4,2, group5,2

```
Two-sample T for group4,2 vs group5,2

          N      Mean    StDev  SE Mean
group4,2  3  19066667  1006645   581187
group5,2  3  16800000   800000   461880


Difference = mu (group4,2) - mu (group5,2)
Estimate for difference:  2266667
95% CI for difference:  (-95881, 4629215)
T-Test of difference = 0 (vs not =): T-Value = 3.05  P-Value = 0.055  DF = 3
```

Two-Sample T-Test and CI: group2.3, group3,3

```
Two-sample T for group2.3 vs group3,3

          N      Mean    StDev  SE Mean
group2.3  3  48400000  3274141  1890326
group3,3  3  51600000  1600000   923760


Difference = mu (group2.3) - mu (group3,3)
Estimate for difference:  -3200000
95% CI for difference:  (-12252629, 5852629)
T-Test of difference = 0 (vs not =): T-Value = -1.52  P-Value = 0.268  DF = 2
```

Two-Sample T-Test and CI: group4,3, group5,3

```
Two-sample T for group4,3 vs group5,3

          N      Mean    StDev  SE Mean
group4,3  3  19333333   832666   480740
group5,3  3  26533333  1514376   874325


Difference = mu (group4,3) - mu (group5,3)
Estimate for difference:  -7200000
95% CI for difference:  (-10375366, -4024634)
T-Test of difference = 0 (vs not =): T-Value = -7.22  P-Value = 0.005  DF = 3
```

Two-Sample T-Test and CI: group2.4, group3,4

```
Two-sample T for group2.4 vs group3,4

          N      Mean    StDev  SE Mean
group2.4  3  56533333  3028751  1748650
group3,4  3  50266667   832666   480740


Difference = mu (group2.4) - mu (group3,4)
Estimate for difference:  6266667
95% CI for difference:  (-1536321, 14069654)
T-Test of difference = 0 (vs not =): T-Value = 3.46  P-Value = 0.075  DF = 2
```

Two-Sample T-Test and CI: group4,4, group5,4

```
Two-sample T for group4,4 vs group5,4

          N      Mean    StDev  SE Mean
group4,4  3  17866667   611010   352767
group5,4  3  24000000  1058301   611010


Difference = mu (group4,4) - mu (group5,4)
Estimate for difference:  -6133333
95% CI for difference:  (-8378656, -3888010)
T-Test of difference = 0 (vs not =): T-Value = -8.69  P-Value = 0.003  DF = 3
```

- Inhibition of *S. derby*

One-way ANOVA: 1 hour versus group

```
Source  DF           SS           MS      F      P
group    4  9.02428E+14  2.25607E+14  65.38  0.000
Error   10  3.45056E+13  3.45056E+12
Total   14  9.36933E+14

S = 1857568   R-Sq = 96.32%   R-Sq(adj) = 94.84%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  ------+---------+---------+---------+---
1      3  26266667  3257811                                (---*--)
2      3  11600000  2227106           (---*--)
3      3  12133333  1285820            (--*---)
4      3   5146667   122202  (--*---)
5      3   4920000   105830  (--*--)
                             ------+---------+---------+---------+---
                             7000000  14000000  21000000  28000000

Pooled StDev = 1857568
```

One-way ANOVA: 2 hour versus group

```
Source  DF           SS           MS       F      P
group    4  1.68389E+15  4.20972E+14  531.89  0.000
Error   10  7.91467E+12  7.91467E+11
Total   14  1.69180E+15

S = 889644   R-Sq = 99.53%   R-Sq(adj) = 99.35%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev   -----+---------+---------+---------+----
1      3  32933333  1890326                                     (*-)
2      3  16133333   611010                  (*-)
3      3   7200000        0     (*)
4      3   5106667    83267   (*-)
5      3   5333333    61101   (-*)
                              -----+---------+---------+---------+----
                             8000000  16000000  24000000  32000000

Pooled StDev = 889644
```

One-way ANOVA: 3 hour versus group

```
Source  DF           SS           MS        F      P
group    4  2.62752E+16  6.56879E+15  1295.22  0.000
Error   10  5.07157E+13  5.07157E+12
Total   14  2.63259E+16

S = 2252015   R-Sq = 99.81%   R-Sq(adj) = 99.73%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N       Mean    StDev  +---------+---------+---------+---------
1      3  116000000  4000000                                      (*)
2      3   25733333  1890326          (*)
3      3   18400000  2400000       (*)
4      3    4266667   140475  (*)
5      3    5040000    69282   (*)
                              +---------+---------+---------+---------
                              0  30000000  60000000  90000000

Pooled StDev = 2252015
```

One-way ANOVA: 4 hour versus group

```
Source  DF           SS           MS       F      P
group    4  4.43067E+16  1.10767E+16  122.21  0.000
Error   10  9.06400E+14  9.06400E+13
Total   14  4.52131E+16

S = 9520504   R-Sq = 98.00%   R-Sq(adj) = 97.19%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N       Mean     StDev  --+---------+---------+---------+-------
1      3  152000000  21166010                               (-*--)
2      3   37466667   1803700          (-*--)
3      3   46000000   1385641            (-*--)
4      3    3266667     83267  (--*-)
5      3    5106667    140475   (-*-)
                               --+---------+---------+---------+-------
                                 0  50000000  1.00E+08  1.50E+08

Pooled StDev = 9520504
```

Two-Sample T-Test and CI: 2,1, 3,1

```
Two-sample T for 2,1 vs 3,1

     N      Mean    StDev  SE Mean
2,1  3  11600000  2227106  1285820
3,1  3  12133333  1285820   742369


Difference = mu (2,1) - mu (3,1)
Estimate for difference:  -533333
95% CI for difference:  (-5258430, 4191763)
T-Test of difference = 0 (vs not =): T-Value = -0.36  P-Value = 0.743  DF = 3
```

Two-Sample T-Test and CI: 4,1, 5,1

```
Two-sample T for 4,1 vs 5,1

     N     Mean   StDev  SE Mean
4,1  3  5146667  122202    70553
5,1  3  4920000  105830    61101
```

```
Difference = mu (4,1) - mu (5,1)
Estimate for difference:  226667
95% CI for difference:  (-70362, 523695)
T-Test of difference = 0 (vs not =): T-Value = 2.43  P-Value = 0.093  DF = 3
```

Two-Sample T-Test and CI: 2,2, 3,2

```
Two-sample T for 2,2 vs 3,2

     N      Mean   StDev  SE Mean
2,2  3  16133333  611010   352767
3,2  3   7200000  100000    57735


Difference = mu (2,2) - mu (3,2)
Estimate for difference:  8933333
95% CI for difference:  (7395306, 10471360)
T-Test of difference = 0 (vs not =): T-Value = 24.99  P-Value = 0.002  DF = 2
```

Two-Sample T-Test and CI: 4,2, 5,2

```
Two-sample T for 4,2 vs 5,2

     N     Mean  StDev  SE Mean
4,2  3  5106667  83267    48074
5,2  3  5333333  61101    35277


Difference = mu (4,2) - mu (5,2)
Estimate for difference:  -226667
95% CI for difference:  (-416431, -36902)
T-Test of difference = 0 (vs not =): T-Value = -3.80  P-Value = 0.032  DF = 3
```

Two-Sample T-Test and CI: 2,3, 3,3

```
Two-sample T for 2,3 vs 3,3

     N      Mean    StDev  SE Mean
2,3  3  25733333  1890326  1091380
3,3  3  18400000  2400000  1385641


Difference = mu (2,3) - mu (3,3)
Estimate for difference:  7333333
95% CI for difference:  (1720026, 12946641)
T-Test of difference = 0 (vs not =): T-Value = 4.16  P-Value = 0.025  DF = 3
```

Two-Sample T-Test and CI: 4,3, 5,3

```
Two-sample T for 4,3 vs 5,3

     N     Mean   StDev  SE Mean
4,3  3  4266667  140475    81104
5,3  3  5040000   69282    40000


Difference = mu (4,3) - mu (5,3)
Estimate for difference:  -773333
95% CI for difference:  (-1162427, -384240)
T-Test of difference = 0 (vs not =): T-Value = -8.55  P-Value = 0.013  DF = 2
```

Two-Sample T-Test and CI: 2,4, 3,4

```
Two-sample T for 2,4 vs 3,4

     N      Mean    StDev  SE Mean
2,4  3  37466667  1803700  1041367
3,4  3  46000000  1385641   800000


Difference = mu (2,4) - mu (3,4)
Estimate for difference:  -8533333
95% CI for difference:  (-12712461, -4354205)
T-Test of difference = 0 (vs not =): T-Value = -6.50  P-Value = 0.007  DF = 3
```

Two-Sample T-Test and CI: 4,4, 5,4

```
Two-sample T for 4,4 vs 5,4

     N     Mean   StDev  SE Mean
4,4  3  3266667   83267    48074
5,4  3  5106667  140475    81104


Difference = mu (4,4) - mu (5,4)
Estimate for difference:  -1840000
95% CI for difference:  (-2140044, -1539956)
T-Test of difference = 0 (vs not =): T-Value = -19.52  P-Value = 0.000  DF = 3
```

- Inhibition of *S. aureus*

One-way ANOVA: 1 hour versus group

```
Source  DF           SS           MS       F      P
group    4  2.02663E+16  5.06658E+15  637.00  0.000
Error   10  7.95381E+13  7.95381E+12
Total   14  2.03459E+16

S = 2820251   R-Sq = 99.61%   R-Sq(adj) = 99.45%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N       Mean    StDev  --------+---------+---------+---------+-
1      3  105333333  6110101                                (*)
2      3   19733333   832666     (-*)
3      3   13333333   611010   (*-)
4      3   13866667  1154701   (-*)
5      3    8546667   189033  (*)
                              --------+---------+---------+---------+-
                               30000000  60000000  90000000  1.20E+08

Pooled StDev = 2820251
```

One-way ANOVA: 2 hour versus group

```
Source  DF           SS           MS        F      P
group    4  3.23212E+16  8.08030E+15  1037.18  0.000
Error   10  7.79061E+13  7.79061E+12
Total   14  3.23991E+16

S = 2791167   R-Sq = 99.76%   R-Sq(adj) = 99.66%


                              Individual 95% CIs For Mean Based on
```

```
                              Pooled StDev
Level  N       Mean    StDev  +---------+---------+---------+---------
1      3  125333333  6110101                                   (*)
2      3   11866667   461880    (*)
3      3    8933333   832666    (*)
4      3   11866667   832666    (*)
5      3    5133333   140475  (*)
                              +---------+---------+---------+---------
                              0  35000000  70000000  1.05E+08

Pooled StDev = 2791167
```

One-way ANOVA: 3 hour versus group

```
Source  DF           SS           MS        F      P
group    4  2.36703E+16  5.91757E+15  1769.44  0.000
Error   10  3.34432E+13  3.34432E+12
Total   14  2.37037E+16

S = 1828748   R-Sq = 99.86%   R-Sq(adj) = 99.80%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N       Mean    StDev  ---------+---------+---------+---------+
1      3  108000000  4000000                                  (*)
2      3   12133333   400666    (*)
3      3   10400000   692820    *)
4      3    9066667   230940   (*)
5      3    4066667   166533  *)
                              ---------+---------+---------+---------+
                                30000000  60000000  90000000  1.20E+08

Pooled StDev = 1828748
```

One-way ANOVA: 4 hour versus group

```
Source  DF           SS           MS       F      P
group    4  2.07003E+16  5.17507E+15  123.38  0.000
Error   10  4.19447E+14  4.19447E+13
Total   14  2.11197E+16

S = 6476476   R-Sq = 98.01%   R-Sq(adj) = 97.22%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N       Mean     StDev  --+---------+---------+---------+-------
1      3  100000000  14422205                                  (-*--)
2      3   12906667    567568     (-*--)
3      3    7946667   1012982    (--*-)
4      3    8666667    611010    (--*--)
5      3     973333     46188  (-*--)
                               --+---------+---------+---------+-------
                                 0  30000000  60000000  90000000

Pooled StDev = 6476476
```

Two-Sample T-Test and CI: 2.1, 3.1

```
Two-sample T for 2.1 vs 3.1

     N      Mean   StDev  SE Mean
2.1  3  19733333  832666   480740
3.1  3  13333333  611010   352767
```

```
Difference = mu (2.1) - mu (3.1)
Estimate for difference:  6400000
95% CI for difference:  (4502356, 8297644)
T-Test of difference = 0 (vs not =): T-Value = 10.73  P-Value = 0.002  DF = 3
```

Two-Sample T-Test and CI: 4.1, 5.1

```
Two-sample T for 4.1 vs 5.1

     N      Mean    StDev  SE Mean
4.1  3  13866667  1154701   666667
5.1  3   8546667   189033   109138


Difference = mu (4.1) - mu (5.1)
Estimate for difference:  5320000
95% CI for difference:  (2413382, 8226618)
T-Test of difference = 0 (vs not =): T-Value = 7.88  P-Value = 0.016  DF = 2
```

Two-Sample T-Test and CI: 2.2, 3.2

```
Two-sample T for 2.2 vs 3.2

     N      Mean   StDev  SE Mean
2.2  3  11866667  461880   266667
3.2  3   8933333  832666   480740


Difference = mu (2.2) - mu (3.2)
Estimate for difference:  2933333
95% CI for difference:  (1183792, 4682875)
T-Test of difference = 0 (vs not =): T-Value = 5.34  P-Value = 0.013  DF = 3
```

Two-Sample T-Test and CI: 4.2, 5.2

```
Two-sample T for 4.2 vs 5.2

     N      Mean   StDev  SE Mean
4.2  3  11866667  832666   480740
5.2  3   5133333  140475    81104


Difference = mu (4.2) - mu (5.2)
Estimate for difference:  6733333
95% CI for difference:  (4635646, 8831021)
T-Test of difference = 0 (vs not =): T-Value = 13.81  P-Value = 0.005  DF = 2
```

Two-Sample T-Test and CI: 2.3, 3.3

```
Two-sample T for 2.3 vs 3.3

     N      Mean   StDev  SE Mean
2.3  3  12133333  400666   231325
3.3  3  10400000  692820   400000


Difference = mu (2.3) - mu (3.3)
Estimate for difference:  1733333
95% CI for difference:  (262812, 3203855)
T-Test of difference = 0 (vs not =): T-Value = 3.75  P-Value = 0.033  DF = 3
```

Two-Sample T-Test and CI: 4.3, 5.3

```
Two-sample T for 4.3 vs 5.3

     N     Mean   StDev  SE Mean
4.3  3  9066667  230940   133333
5.3  3  4066667  166533    96148


Difference = mu (4.3) - mu (5.3)
Estimate for difference:  5000000
95% CI for difference:  (4476856, 5523144)
T-Test of difference = 0 (vs not =): T-Value = 30.42  P-Value = 0.000  DF = 3
```

Two-Sample T-Test and CI: 2.4, 3.4

```
Two-sample T for 2.4 vs 3.4

     N      Mean    StDev  SE Mean
2.4  3  12906667   567568   327685
3.4  3   7946667  1012982   584846


Difference = mu (2.4) - mu (3.4)
Estimate for difference:  4960000
95% CI for difference:  (2826521, 7093479)
T-Test of difference = 0 (vs not =): T-Value = 7.40  P-Value = 0.005  DF = 3
```

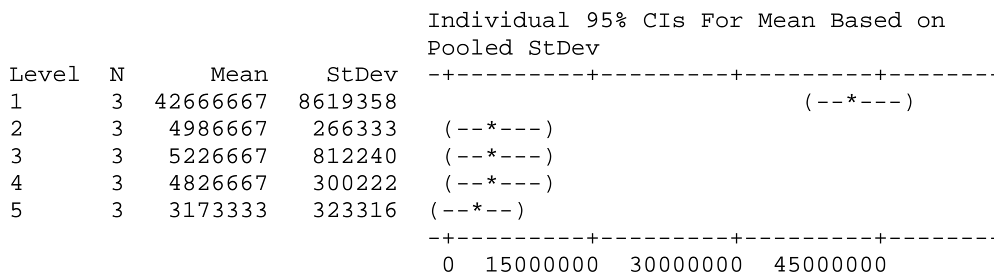
Two-Sample T-Test and CI: 4.4, 5.4

```
Two-sample T for 4.4 vs 5.4

     N     Mean   StDev  SE Mean
4.4  3  8666667  611010   352767
5.4  3   973333   46188    26667


Difference = mu (4.4) - mu (5.4)
Estimate for difference:  7693333
95% CI for difference:  (6171170, 9215497)
T-Test of difference = 0 (vs not =): T-Value = 21.75  P-Value = 0.002  DF = 2
```

- Inhibition of *L. monocytogenes*

One-way ANOVA: 1 hour versus group

```
Source  DF           SS           MS      F      P
group    4  3.49416E+15  8.73541E+14  58.07  0.000
Error   10  1.50437E+14  1.50437E+13
Total   14  3.64460E+15

S = 3878625   R-Sq = 95.87%   R-Sq(adj) = 94.22%
```



```
                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  -+---------+---------+---------+--------
1      3  42666667  8619358                           (--*---)
2      3   4986667   266333   (--*---)
3      3   5226667   812240   (--*---)
4      3   4826667   300222   (--*---)
5      3   3173333   323316  (--*--)
                             -+---------+---------+---------+--------
                              0  15000000  30000000  45000000
```

```
Pooled StDev = 3878625
```

One-way ANOVA: 2 hour versus group

```
Source  DF           SS           MS        F      P
group    4  3.35911E+15  8.39778E+14  1848.10  0.000
Error   10  4.54400E+12  4.54400E+11
Total   14  3.36366E+15

S = 674092   R-Sq = 99.86%   R-Sq(adj) = 99.81%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean   StDev   --------+---------+---------+---------+-
1      3  41866667  1222020                                 (*)
2      3   6213333   244404    (*)
3      3   5160000   454313    *)
4      3   4000000   692820   *)
5      3   2866667   180370  *)
                             --------+---------+---------+---------+-
                              12000000  24000000  36000000  48000000

Pooled StDev = 674092
```

One-way ANOVA: 3 hour versus group

```
Source  DF           SS           MS        F      P
group    4  2.18694E+15  5.46734E+14  3205.52  0.000
Error   10  1.70560E+12  1.70560E+11
Total   14  2.18864E+15

S = 412989   R-Sq = 99.92%   R-Sq(adj) = 99.89%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N      Mean  StDev   -------+---------+---------+---------+--
1      3  34600000  600000                               (*
2      3   6200000  341760     *)
3      3   4586667  227450   (*
4      3   4186667  468757   *)
5      3   3093333  323316  *)
                            -------+---------+---------+---------+--
                            10000000  20000000  30000000  40000000

Pooled StDev = 412989
```

One-way ANOVA: 4 hour versus group

```
Source  DF           SS           MS        F      P
group    4  2.13875E+15  5.34688E+14  1272.26  0.000
Error   10  4.20267E+12  4.20267E+11
Total   14  2.14295E+15

S = 648280   R-Sq = 99.80%   R-Sq(adj) = 99.73%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean   StDev   --------+---------+---------+---------+-
1      3  33866667  1404754                                (*)
2      3   5746667   323316     (*)
3      3   4266667    46188   (*)
4      3   3373333   100664   *)
5      3   3040000   105830  (*)
```

```
                             --------+---------+---------+---------+-
                              10000000  20000000  30000000  40000000

Pooled StDev = 648280
```

Two-Sample T-Test and CI: 2.1, 3.1

```
Two-sample T for 2.1 vs 3.1

     N     Mean   StDev  SE Mean
2.1  3  4986667  266333   153768
3.1  3  5226667  812240   468947


Difference = mu (2.1) - mu (3.1)
Estimate for difference:  -240000
95% CI for difference:  (-2363417, 1883417)
T-Test of difference = 0 (vs not =): T-Value = -0.49  P-Value = 0.675  DF = 2
```

Two-Sample T-Test and CI: 4.1, 5.1

```
Two-sample T for 4.1 vs 5.1

     N     Mean   StDev  SE Mean
4.1  3  4826667  300222   173333
5.1  3  3173333  323316   186667


Difference = mu (4.1) - mu (5.1)
Estimate for difference:  1653333
95% CI for difference:  (842659, 2464007)
T-Test of difference = 0 (vs not =): T-Value = 6.49  P-Value = 0.007  DF = 3
```

Two-Sample T-Test and CI: 2.2, 3.2

```
Two-sample T for 2.2 vs 3.2

     N     Mean   StDev  SE Mean
2.2  3  6213333  244404   141107
3.2  3  5160000  454313   262298


Difference = mu (2.2) - mu (3.2)
Estimate for difference:  1053333
95% CI for difference:  (105460, 2001206)
T-Test of difference = 0 (vs not =): T-Value = 3.54  P-Value = 0.038  DF = 3
```

Two-Sample T-Test and CI: 4.2, 5.2

```
Two-sample T for 4.2 vs 5.2

     N     Mean   StDev  SE Mean
4.2  3  4000000  692820   400000
5.2  3  2866667  180370   104137


Difference = mu (4.2) - mu (5.2)
Estimate for difference:  1133333
95% CI for difference:  (-645096, 2911763)
T-Test of difference = 0 (vs not =): T-Value = 2.74  P-Value = 0.111  DF = 2
```

Two-Sample T-Test and CI: 2.3, 3.3

```
Two-sample T for 2.3 vs 3.3

     N     Mean   StDev  SE Mean
2.3  3  6200000  341760   197315
3.3  3  4586667  227450   131318


Difference = mu (2.3) - mu (3.3)
Estimate for difference:  1613333
95% CI for difference:  (859035, 2367632)
T-Test of difference = 0 (vs not =): T-Value = 6.81  P-Value = 0.006  DF = 3
```

Two-Sample T-Test and CI: 4.3, 5.3

```
Two-sample T for 4.3 vs 5.3

     N     Mean   StDev  SE Mean
4.3  3  4186667  468757   270637
5.3  3  3093333  323316   186667


Difference = mu (4.3) - mu (5.3)
Estimate for difference:  1093333
95% CI for difference:  (47044, 2139622)
T-Test of difference = 0 (vs not =): T-Value = 3.33  P-Value = 0.045  DF = 3
```

Two-Sample T-Test and CI: 2.4, 3.4

```
Two-sample T for 2.4 vs 3.4

     N     Mean   StDev  SE Mean
2.4  3  5746667  323316   186667
3.4  3  4266667   46188    26667


Difference = mu (2.4) - mu (3.4)
Estimate for difference:  1480000
95% CI for difference:  (668684, 2291316)
T-Test of difference = 0 (vs not =): T-Value = 7.85  P-Value = 0.016  DF = 2
```

Two-Sample T-Test and CI: 4.4, 5.4

```
Two-sample T for 4.4 vs 5.4

     N     Mean   StDev  SE Mean
4.4  3  3373333  100664    58119
5.4  3  3040000  105830    61101


Difference = mu (4.4) - mu (5.4)
Estimate for difference:  333333
95% CI for difference:  (64966, 601701)
T-Test of difference = 0 (vs not =): T-Value = 3.95  P-Value = 0.029  DF = 3
```

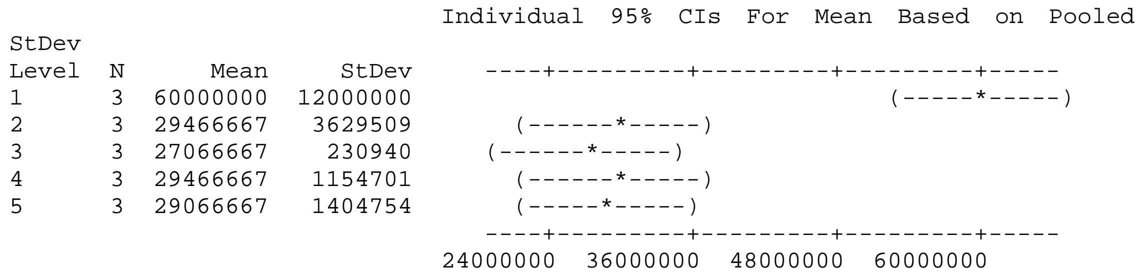
Supernatant pH = 4.4

- Killing *E. coli*

One-way ANOVA: 1 hour versus group

```
Source  DF           SS           MS      F      P
group    4  2.35313E+15  5.88283E+14  18.32  0.000
```

```
Error   10  3.21067E+14  3.21067E+13
Total   14  2.67420E+15

S = 5666274   R-Sq = 87.99%   R-Sq(adj) = 83.19%
```

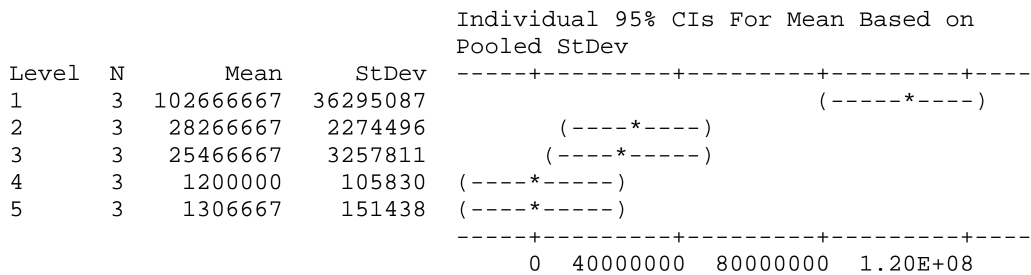


```
                              Individual 95% CIs For Mean Based on Pooled
StDev
Level  N      Mean     StDev    ----+---------+---------+---------+-----
1      3  60000000  12000000                                (-----*-----)
2      3  29466667   3629509      (------*-----)
3      3  27066667    230940    (------*-----)
4      3  29466667   1154701      (------*-----)
5      3  29066667   1404754      (-----*-----)
                                ----+---------+---------+---------+-----
                              24000000  36000000  48000000  60000000
```

```
Pooled StDev = 5666274
```

One-way ANOVA: 2 hour versus group

```
Source  DF           SS           MS      F      P
group    4  2.08226E+16  5.20566E+15  19.52  0.000
Error   10  2.66631E+15  2.66631E+14
Total   14  2.34890E+16

S = 16328834   R-Sq = 88.65%   R-Sq(adj) = 84.11%
```

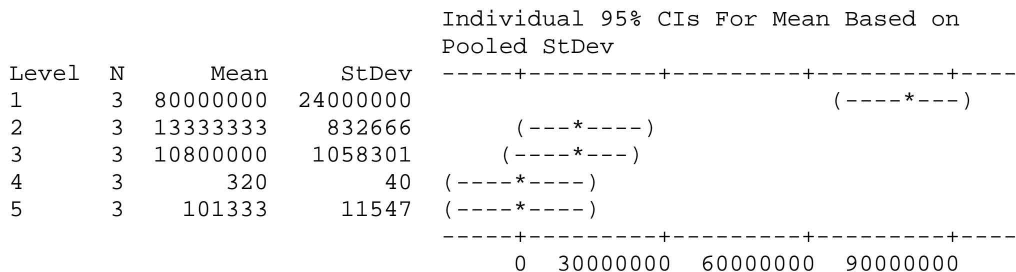


```
                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N       Mean     StDev  -----+---------+---------+---------+----
1      3  102666667  36295087                          (-----*----)
2      3   28266667   2274496        (----*----)
3      3   25466667   3257811       (----*-----)
4      3    1200000    105830  (----*-----)
5      3    1306667    151438  (----*-----)
                               -----+---------+---------+---------+----
                                    0  40000000  80000000  1.20E+08
```

```
Pooled StDev = 16328834
```

One-way ANOVA: 3 hour versus group

```
Source  DF           SS           MS      F      P
group    4  1.35643E+16  3.39108E+15  29.34  0.000
Error   10  1.15563E+15  1.15563E+14
Total   14  1.47200E+16

S = 10750009   R-Sq = 92.15%   R-Sq(adj) = 89.01%
```



```
                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N      Mean     StDev  -----+---------+---------+---------+----
1      3  80000000  24000000                           (----*---)
2      3  13333333    832666      (---*----)
3      3  10800000   1058301     (----*---)
4      3       320        40  (----*----)
5      3    101333     11547  (----*----)
                              -----+---------+---------+---------+----
                                   0  30000000  60000000  90000000
```

```
Pooled StDev = 10750009
```

One-way ANOVA: 4 hour versus group

```
Source  DF           SS           MS       F      P
group    4  8.44059E+16  2.11015E+16  100.73  0.000
Error   10  2.09483E+15  2.09483E+14
Total   14  8.65007E+16

S = 14473520   R-Sq = 97.58%   R-Sq(adj) = 96.61%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N       Mean     StDev  ---+---------+---------+---------+------
1      3  189333333  32331615                                 (---*--)
2      3    7200000   1442221   (--*--)
3      3     493333     23094  (--*--)
4      3          0         0  (--*--)
5      3          0         0  (--*--)
                               ---+---------+---------+---------+------
                                  0  60000000  1.20E+08  1.80E+08

Pooled StDev = 14473520
```

Two-Sample T-Test and CI: 2.1, 3.1

```
Two-sample T for 2.1 vs 3.1

     N      Mean    StDev  SE Mean
2.1  3  29466667  3629509  2095498
3.1  3  27066667   230940   133333


Difference = mu (2.1) - mu (3.1)
Estimate for difference:  2400000
95% CI for difference:  (-6634432, 11434432)
T-Test of difference = 0 (vs not =): T-Value = 1.14  P-Value = 0.371  DF = 2
```

Two-Sample T-Test and CI: 4.1, 5.1

```
Two-sample T for 4.1 vs 5.1

     N      Mean    StDev  SE Mean
4.1  3  29466667  1154701   666667
5.1  3  29066667  1404754   811035


Difference = mu (4.1) - mu (5.1)
Estimate for difference:  400000
95% CI for difference:  (-2941148, 3741148)
T-Test of difference = 0 (vs not =): T-Value = 0.38  P-Value = 0.729  DF = 3
```

Two-Sample T-Test and CI: 2.2, 3.2

```
Two-sample T for 2.2 vs 3.2

     N      Mean    StDev  SE Mean
2.2  3  28266667  2274496  1313181
3.2  3  25466667  3257811  1880898


Difference = mu (2.2) - mu (3.2)
Estimate for difference:  2800000
95% CI for difference:  (-4500384, 10100384)
T-Test of difference = 0 (vs not =): T-Value = 1.22  P-Value = 0.309  DF = 3
```

Two-Sample T-Test and CI: 4.2, 5.2

```
Two-sample T for 4.2 vs 5.2

     N     Mean   StDev  SE Mean
4.2  3  1200000  105830    61101
5.2  3  1306667  151438    87433


Difference = mu (4.2) - mu (5.2)
Estimate for difference:  -106667
95% CI for difference:  (-446128, 232794)
T-Test of difference = 0 (vs not =): T-Value = -1.00  P-Value = 0.391  DF = 3
```

Two-Sample T-Test and CI: 2.3, 3.3

```
Two-sample T for 2.3 vs 3.3

     N      Mean    StDev  SE Mean
2.3  3  13333333   832666   480740
3.3  3  10800000  1058301   611010


Difference = mu (2.3) - mu (3.3)
Estimate for difference:  2533333
95% CI for difference:  (59108, 5007559)
T-Test of difference = 0 (vs not =): T-Value = 3.26  P-Value = 0.047  DF = 3
```

Two-Sample T-Test and CI: 4.3, 5.3

```
Two-sample T for 4.3 vs 5.3

     N    Mean  StDev  SE Mean
4.3  3   320.0   40.0       23
5.3  3  101333  11547     6667


Difference = mu (4.3) - mu (5.3)
Estimate for difference:  -101013
95% CI for difference:  (-129698, -72329)
T-Test of difference = 0 (vs not =): T-Value = -15.15  P-Value = 0.004  DF = 2
```

Two-Sample T-Test and CI: 2.4, 3.4

```
Two-sample T for 2.4 vs 3.4

     N     Mean    StDev  SE Mean
2.4  3  7200000  1442221   832666
3.4  3   493333    23094    13333


Difference = mu (2.4) - mu (3.4)
Estimate for difference:  6706667
95% CI for difference:  (3123533, 10289800)
T-Test of difference = 0 (vs not =): T-Value = 8.05  P-Value = 0.015  DF = 2
```

- Killing *L. monocytogenes*

One-way ANOVA: 1 hour versus group

```
Source  DF           SS           MS        F      P
group    4  7.51787E+14  1.87947E+14  1137.51  0.000
Error   10  1.65227E+12  1.65227E+11
Total   14  7.53440E+14
```

```
S = 406481   R-Sq = 99.78%   R-Sq(adj) = 99.69%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N      Mean   StDev  ---------+---------+---------+---------+
1      3  18800000  692820                                    (*)
2      3   1373333  509640   (*)
3      3    906667  128582  (*)
4      3    933333  166533  (*)
5      3   1213333  205264  (*)
                            ---------+---------+---------+---------+
                               5000000  10000000  15000000  20000000

Pooled StDev = 406481
```

One-way ANOVA: 2 hour versus group

```
Source  DF           SS           MS       F      P
group    4  6.53599E+14  1.63400E+14  182.27  0.000
Error   10  8.96453E+12  8.96453E+11
Total   14  6.62564E+14

S = 946812   R-Sq = 98.65%   R-Sq(adj) = 98.11%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  --+---------+---------+---------+-------
1      3  16800000  2116601                                  (--*-)
2      3    540000    28000   (-*--)
3      3    477333     8327   (-*-)
4      3     64000    10583  (-*--)
5      3    134667    36074  (-*--)
                             --+---------+---------+---------+-------
                               0   5000000  10000000  15000000

Pooled StDev = 946812
```

One-way ANOVA: 3 hour versus group

```
Source  DF           SS           MS        F      P
group    4  9.35290E+14  2.33823E+14  1684.86  0.000
Error   10  1.38778E+12  1.38778E+11
Total   14  9.36678E+14

S = 372530   R-Sq = 99.85%   R-Sq(adj) = 99.79%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N      Mean   StDev  -+---------+---------+---------+--------
1      3  19866667  832666                                 (*)
2      3    234667   12858   *)
3      3    234667   19732   *)
4      3     20267    1665  (*)
5      3     19200    1058  (*)
                            -+---------+---------+---------+--------
                             0   6000000  12000000  18000000

Pooled StDev = 372530
```

One-way ANOVA: 4 hour versus group

```
Source  DF           SS           MS       F      P
group    4  9.89197E+14  2.47299E+14  406.46  0.000
```

```
Error   10  6.08421E+12  6.08421E+11
Total   14  9.95281E+14

S = 780014   R-Sq = 99.39%   R-Sq(adj) = 99.14%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  --+---------+---------+---------+-------
1      3  20400000  1743560                                  (-*-)
2      3    222667    40266   (*-)
3      3    165333    22030   (*-)
4      3      5800      501  (-*-)
5      3      3400      472  (-*-)
                             --+---------+---------+---------+-------
                               0   6000000  12000000  18000000

Pooled StDev = 780014
```

Two-Sample T-Test and CI: 2.1, 3.1

```
Two-sample T for 2.1 vs 3.1

     N     Mean   StDev  SE Mean
2.1  3  1373333  509640   294241
3.1  3   906667  128582    74237


Difference = mu (2.1) - mu (3.1)
Estimate for difference:  466667
95% CI for difference:  (-839023, 1772356)
T-Test of difference = 0 (vs not =): T-Value = 1.54  P-Value = 0.264  DF = 2
```

Two-Sample T-Test and CI: 4.1, 5.1

```
Two-sample T for 4.1 vs 5.1

     N     Mean   StDev  SE Mean
4.1  3   933333  166533    96148
5.1  3  1213333  205264   118509


Difference = mu (4.1) - mu (5.1)
Estimate for difference:  -280000
95% CI for difference:  (-765664, 205664)
T-Test of difference = 0 (vs not =): T-Value = -1.83  P-Value = 0.164  DF = 3
```

Two-Sample T-Test and CI: 2.2, 3.2

```
Two-sample T for 2.2 vs 3.2

     N    Mean  StDev  SE Mean
2.2  3  540000  28000    16166
3.2  3  477333   8327     4807


Difference = mu (2.2) - mu (3.2)
Estimate for difference:  62667
95% CI for difference:  (-9900, 135233)
T-Test of difference = 0 (vs not =): T-Value = 3.72  P-Value = 0.065  DF = 2
```

Two-Sample T-Test and CI: 4.2, 5.2

```
Two-sample T for 4.2 vs 5.2

     N    Mean  StDev  SE Mean
4.2  3   64000  10583     6110
5.2  3  134667  36074    20827


Difference = mu (4.2) - mu (5.2)
Estimate for difference:  -70667
95% CI for difference:  (-164056, 22723)
T-Test of difference = 0 (vs not =): T-Value = -3.26  P-Value = 0.083  DF = 2
```

Two-Sample T-Test and CI: 2.3, 3.3

```
Two-sample T for 2.3 vs 3.3

     N    Mean  StDev  SE Mean
2.3  3  234667  12858     7424
3.3  3  234667  19732    11392


Difference = mu (2.3) - mu (3.3)
Estimate for difference:  0
95% CI for difference:  (-43273, 43273)
T-Test of difference = 0 (vs not =): T-Value = 0.00  P-Value = 1.000  DF = 3
```

Two-Sample T-Test and CI: 4.3, 5.3

```
Two-sample T for 4.3 vs 5.3

     N   Mean  StDev  SE Mean
4.3  3  20267   1665      961
5.3  3  19200   1058      611


Difference = mu (4.3) - mu (5.3)
Estimate for difference:  1067
95% CI for difference:  (-2559, 4692)
T-Test of difference = 0 (vs not =): T-Value = 0.94  P-Value = 0.418  DF = 3
```

Two-Sample T-Test and CI: 2.4, 3.4

```
Two-sample T for 2.4 vs 3.4

     N    Mean  StDev  SE Mean
2.4  3  222667  40266    23247
3.4  3  165333  22030    12719


Difference = mu (2.4) - mu (3.4)
Estimate for difference:  57333
95% CI for difference:  (-27000, 141666)
T-Test of difference = 0 (vs not =): T-Value = 2.16  P-Value = 0.119  DF = 3
```

Two-Sample T-Test and CI: 4.4, 5.4

```
Two-sample T for 4.4 vs 5.4

     N  Mean  StDev  SE Mean
4.4  3  5800    501      289
5.4  3  3400    472      272
```

```
Difference = mu (4.4) - mu (5.4)
Estimate for difference:  2400
95% CI for difference:  (1136, 3664)
T-Test of difference = 0 (vs not =): T-Value = 6.04  P-Value = 0.009  DF = 3
```

- Killing of *S. derby* by diluted supernatant

One-way ANOVA: 1 hour versus group

```
Source  DF           SS           MS       F      P
group    4  2.23744E+15  5.59360E+14  607.02  0.000
Error   10  9.21493E+12  9.21493E+11
Total   14  2.24666E+15

S = 959944   R-Sq = 99.59%   R-Sq(adj) = 99.43%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  -------+---------+---------+---------+--
1      3  35200000  2116601                                (*)
2      3   5440000   138564   (*-)
3      3   4880000    40000   (*)
4      3   4426667   166533  (*-)
5      3   4013333   280951  (*)
                             -------+---------+---------+---------+--
                             10000000  20000000  30000000  40000000

Pooled StDev = 959944
```

One-way ANOVA: 2hour versus group

```
Source  DF           SS           MS       F      P
group    4  2.20722E+15  5.51805E+14  207.37  0.000
Error   10  2.66101E+13  2.66101E+12
Total   14  2.23383E+15

S = 1631261   R-Sq = 98.81%   R-Sq(adj) = 98.33%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  +---------+---------+---------+---------
1      3  33333333  3629509                                (-*-)
2      3   3786667   115470    (-*-)
3      3   3453333   230940   (-*--)
4      3   2533333   220303  (--*-)
5      3   2373333   128582  (-*-)
                             +---------+---------+---------+---------
                             0  10000000  20000000  30000000

Pooled StDev = 1631261
```

One-way ANOVA: 3 hour versus group

```
Source  DF           SS           MS       F      P
group    4  2.49587E+15  6.23966E+14  879.58  0.000
Error   10  7.09392E+12  7.09392E+11
Total   14  2.50296E+15

S = 842254   R-Sq = 99.72%   R-Sq(adj) = 99.60%


                             Individual 95% CIs For Mean Based on
```

```
                              Pooled StDev
Level  N      Mean    StDev  -+---------+---------+---------+--------
1      3  33733333  1847521                                  (*)
2      3   2786667   272274     (*)
3      3   2480000   243311    (*-)
4      3    540000    12000  (-*)
5      3    486667    12220  (*-)
                             -+---------+---------+---------+--------
                              0  10000000  20000000  30000000

Pooled StDev = 842254
```

One-way ANOVA: 4 hour versus group

```
Source  DF           SS           MS        F      P
group    4  2.36570E+15  5.91425E+14  4424.72  0.000
Error   10  1.33664E+12  1.33664E+11
Total   14  2.36704E+15

S = 365601   R-Sq = 99.94%   R-Sq(adj) = 99.92%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean   StDev  +---------+---------+---------+---------
1      3  32400000  800000                                 *)
2      3   1733333   83267   (*
3      3   1560000  144222   (*
4      3    486667   22030  *)
5      3    357333   10066  *)
                            +---------+---------+---------+---------
                            0  10000000  20000000  30000000

Pooled StDev = 365601
```

Two-Sample T-Test and CI: 2.1, 3.1

```
Two-sample T for 2.1 vs 3.1

     N     Mean   StDev  SE Mean
2.1  3  5440000  138564    80000
3.1  3  4880000   40000    23094


Difference = mu (2.1) - mu (3.1)
Estimate for difference:  560000
95% CI for difference:  (201733, 918267)
T-Test of difference = 0 (vs not =): T-Value = 6.73  P-Value = 0.021  DF = 2
```

Two-Sample T-Test and CI: 4.1, 5.1

```
Two-sample T for 4.1 vs 5.1

     N     Mean   StDev  SE Mean
4.1  3  4426667  166533    96148
5.1  3  4013333  280951   162207


Difference = mu (4.1) - mu (5.1)
Estimate for difference:  413333
95% CI for difference:  (-186754, 1013421)
T-Test of difference = 0 (vs not =): T-Value = 2.19  P-Value = 0.116  DF = 3
```

Two-Sample T-Test and CI: 2.2, 3.2

```
Two-sample T for 2.2 vs 3.2

     N     Mean   StDev  SE Mean
2.2  3  3786667  115470    66667
3.2  3  3453333  230940   133333


Difference = mu (2.2) - mu (3.2)
Estimate for difference:  333333
95% CI for difference:  (-308068, 974735)
T-Test of difference = 0 (vs not =): T-Value = 2.24  P-Value = 0.155  DF = 2
```

Two-Sample T-Test and CI: 4.2, 5.2

```
Two-sample T for 4.2 vs 5.2

     N     Mean   StDev  SE Mean
4.2  3  2533333  220303   127192
5.2  3  2373333  128582    74237


Difference = mu (4.2) - mu (5.2)
Estimate for difference:  160000
95% CI for difference:  (-308684, 628684)
T-Test of difference = 0 (vs not =): T-Value = 1.09  P-Value = 0.357  DF = 3
```

Two-Sample T-Test and CI: 2.3, 3.3

```
Two-sample T for 2.3 vs 3.3

     N     Mean   StDev  SE Mean
2.3  3  2786667  272274   157198
3.3  3  2480000  243311   140475


Difference = mu (2.3) - mu (3.3)
Estimate for difference:  306667
95% CI for difference:  (-364252, 977585)
T-Test of difference = 0 (vs not =): T-Value = 1.45  P-Value = 0.242  DF = 3
```

Two-Sample T-Test and CI: 4.3, 5.3

```
Two-sample T for 4.3 vs 5.3

     N    Mean  StDev  SE Mean
4.3  3  540000  12000     6928
5.3  3  486667  12220     7055


Difference = mu (4.3) - mu (5.3)
Estimate for difference:  53333
95% CI for difference:  (21864, 84802)
T-Test of difference = 0 (vs not =): T-Value = 5.39  P-Value = 0.012  DF = 3
```

Two-Sample T-Test and CI: 2.4, 3.4

```
Two-sample T for 2.4 vs 3.4

     N     Mean   StDev  SE Mean
2.4  3  1733333   83267    48074
3.4  3  1560000  144222    83267
```

```
Difference = mu (2.4) - mu (3.4)
Estimate for difference:  173333
95% CI for difference:  (-132653, 479319)
T-Test of difference = 0 (vs not =): T-Value = 1.80  P-Value = 0.169  DF = 3
```

Two-Sample T-Test and CI: 4.4, 5.4

```
Two-sample T for 4.4 vs 5.4

     N    Mean  StDev  SE Mean
4.4  3  486667  22030    12719
5.4  3  357333  10066     5812


Difference = mu (4.4) - mu (5.4)
Estimate for difference:  129333
95% CI for difference:  (69165, 189502)
T-Test of difference = 0 (vs not =): T-Value = 9.25  P-Value = 0.011  DF = 2
```

- Killing *S. aureus*

One-way ANOVA: 1 hour versus group

```
Source  DF           SS           MS       F      P
group    4  2.72231E+15  6.80578E+14  377.43  0.000
Error   10  1.80320E+13  1.80320E+12
Total   14  2.74034E+15

S = 1342832   R-Sq = 99.34%   R-Sq(adj) = 99.08%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  -+---------+---------+---------+--------
1      3  34266667  3002221                                 (*-)
2      3    530667    12220  (-*)
3      3    454667    26633  (*-)
4      3   1080000    40000  (-*-)
5      3    309333    14048  (*-)
                             -+---------+---------+---------+--------
                              0  10000000  20000000  30000000

Pooled StDev = 1342832
```

One-way ANOVA: 2 hour versus group

```
Source  DF           SS           MS        F      P
group    4  3.85845E+15  9.64612E+14  1586.02  0.000
Error   10  6.08198E+12  6.08198E+11
Total   14  3.86453E+15

S = 779870   R-Sq = 99.84%   R-Sq(adj) = 99.78%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  -+---------+---------+---------+--------
1      3  40400000  1743560                                 (*)
2      3    520000    26230   *)
3      3    389333     6110  (*)
4      3    258667    16166  (*)
5      3     55467     1405  (*)
                             -+---------+---------+---------+--------
                              0  12000000  24000000  36000000
```

```
Pooled StDev = 779870
```

One-way ANOVA: 3 hour versus group

```
Source  DF           SS           MS       F      P
group    4  4.09048E+15  1.02262E+15  388.09  0.000
Error   10  2.63498E+13  2.63498E+12
Total   14  4.11683E+15

S = 1623263   R-Sq = 99.36%   R-Sq(adj) = 99.10%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  --+---------+---------+---------+-------
1      3  41466667  3629509                                   (-*)
2      3    414667    20526   (*-)
3      3    274667    34020  (-*-)
4      3     46933     3107  (-*-)
5      3      1387      234  (-*-)
                             --+---------+---------+---------+-------
                               0  12000000  24000000  36000000

Pooled StDev = 1623263
```

One-way ANOVA: 4 hour versus group

```
Source  DF           SS           MS        F      P
group    4  5.49874E+15  1.37468E+15  3302.72  0.000
Error   10  4.16228E+12  4.16228E+11
Total   14  5.50290E+15

S = 645158   R-Sq = 99.92%   R-Sq(adj) = 99.89%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean    StDev  -+---------+---------+---------+--------
1      3  48000000  1442221                                (*)
2      3    298667    32083   *)
3      3    236000    10583   *)
4      3      5613      306  (*)
5      3         0        0  (*)
                             -+---------+---------+---------+--------
                              0  15000000  30000000  45000000

Pooled StDev = 645158
```

Two-Sample T-Test and CI: 2.1, 3.1

```
Two-sample T for 2.1 vs 3.1

     N    Mean  StDev  SE Mean
2.1  3  530667  12220     7055
3.1  3  454667  26633    15377


Difference = mu (2.1) - mu (3.1)
Estimate for difference:  76000
95% CI for difference:  (3207, 148793)
T-Test of difference = 0 (vs not =): T-Value = 4.49  P-Value = 0.046  DF = 2
```

Two-Sample T-Test and CI: 4.1, 5.1

```
Two-sample T for 4.1 vs 5.1
```

```
     N     Mean  StDev  SE Mean
4.1  3  1080000  40000    23094
5.1  3   309333  14048     8110


Difference = mu (4.1) - mu (5.1)
Estimate for difference:  770667
95% CI for difference:  (665352, 875982)
T-Test of difference = 0 (vs not =): T-Value = 31.49  P-Value = 0.001  DF = 2
```

Two-Sample T-Test and CI: 2.2, 3.2

```
Two-sample T for 2.2 vs 3.2

     N    Mean  StDev  SE Mean
2.2  3  520000  26230    15144
3.2  3  389333   6110     3528


Difference = mu (2.2) - mu (3.2)
Estimate for difference:  130667
95% CI for difference:  (63764, 197569)
T-Test of difference = 0 (vs not =): T-Value = 8.40  P-Value = 0.014  DF = 2
```

Two-Sample T-Test and CI: 4.2, 5.2

```
Two-sample T for 4.2 vs 5.2

     N    Mean  StDev  SE Mean
4.2  3  258667  16166     9333
5.2  3   55467   1405      811


Difference = mu (4.2) - mu (5.2)
Estimate for difference:  203200
95% CI for difference:  (162891, 243509)
T-Test of difference = 0 (vs not =): T-Value = 21.69  P-Value = 0.002  DF = 2
```

Two-Sample T-Test and CI: 2.3, 3.3

```
Two-sample T for 2.3 vs 3.3

     N    Mean  StDev  SE Mean
2.3  3  414667  20526    11851
3.3  3  274667  34020    19641


Difference = mu (2.3) - mu (3.3)
Estimate for difference:  140000
95% CI for difference:  (66996, 213004)
T-Test of difference = 0 (vs not =): T-Value = 6.10  P-Value = 0.009  DF = 3
```

Two-Sample T-Test and CI: 4.3, 5.3

```
Two-sample T for 4.3 vs 5.3

     N   Mean  StDev  SE Mean
4.3  3  46933   3107     1794
5.3  3   1387    234      135


Difference = mu (4.3) - mu (5.3)
```

```
Estimate for difference:  45547
95% CI for difference:  (37807, 53287)
T-Test of difference = 0 (vs not =): T-Value = 25.32  P-Value = 0.002  DF = 2
```

Two-Sample T-Test and CI: 2.4, 3.4

```
Two-sample T for 2.4 vs 3.4

     N    Mean  StDev  SE Mean
2.4  3  298667  32083    18523
3.4  3  236000  10583     6110


Difference = mu (2.4) - mu (3.4)
Estimate for difference:  62667
95% CI for difference:  (-21257, 146590)
T-Test of difference = 0 (vs not =): T-Value = 3.21  P-Value = 0.085  DF = 2
```

- Killing *S. derby*

One-way ANOVA: 1 hour versus group

```
Source  DF           SS           MS      F      P
group    4  1.58530E+16  3.96326E+15  58.06  0.000
Error   10  6.82668E+14  6.82668E+13
Total   14  1.65357E+16

S = 8262371   R-Sq = 95.87%   R-Sq(adj) = 94.22%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N      Mean     StDev  ----+---------+---------+---------+-----
1      3  81333333  18475209                              (--*---)
2      3     98667      6110  (---*---)
3      3    140000     22271   (--*---)
4      3         0         0  (---*---)
5      3         0         0  (---*---)
                              ----+---------+---------+---------+-----
                                  0  30000000  60000000  90000000

Pooled StDev = 8262371
```

One-way ANOVA: 2 hour versus group

```
Source  DF           SS           MS       F      P
group    4  3.53323E+16  8.83307E+15  295.75  0.000
Error   10  2.98667E+14  2.98667E+13
Total   14  3.56309E+16

S = 5465040   R-Sq = 99.16%   R-Sq(adj) = 98.83%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N       Mean     StDev  --+---------+---------+---------+-------
1      3  121333333  12220202                                    (-*-)
2      3          0         0  (-*-)
3      3          0         0  (-*-)
4      3          0         0  (-*-)
5      3          0         0  (-*-)
                               --+---------+---------+---------+-------
                                 0  35000000  70000000  1.05E+08
```

```
Pooled StDev = 5465040
```

One-way ANOVA: 3 hour versus group

```
Source  DF          SS          MS        F      P
group    4  3.30411E+16  8.26027E+15  1936.00  0.000
Error   10  4.26667E+13  4.26667E+12
Total   14  3.30837E+16

S = 2065591   R-Sq = 99.87%   R-Sq(adj) = 99.82%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N       Mean    StDev  -+---------+---------+---------+--------
1      3  117333333  4618802                                 (*
2      3          0        0  (*)
3      3          0        0  (*)
4      3          0        0  (*)
5      3          0        0  (*)
                              -+---------+---------+---------+--------
                               0  35000000  70000000  1.05E+08

Pooled StDev = 2065591
```

One-way ANOVA: 4 hour versus group

```
Source  DF          SS          MS      F      P
group    4  1.62240E+17  4.05600E+16  95.30  0.000
Error   10  4.25600E+15  4.25600E+14
Total   14  1.66496E+17

S = 20630075   R-Sq = 97.44%   R-Sq(adj) = 96.42%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N       Mean     StDev  ---+---------+---------+---------+------
1      3  260000000  46130250                                (---*--)
2      3          0         0  (--*--)
3      3          0         0  (--*--)
4      3          0         0  (--*--)
5      3          0         0  (--*--)
                               ---+---------+---------+---------+------
                                  0  80000000  1.60E+08  2.40E+08

Pooled StDev = 20630075
```

Two-Sample T-Test and CI: 2.1, 3.1

```
Two-sample T for 2.1 vs 3.1

     N    Mean  StDev  SE Mean
2.1  3   98667   6110     3528
3.1  3  140000  22271    12858


Difference = mu (2.1) - mu (3.1)
Estimate for difference:  -41333
95% CI for difference:  (-98702, 16035)
T-Test of difference = 0 (vs not =): T-Value = -3.10  P-Value = 0.090  DF = 2
```

4.3.4 Viable counts (log_{10}(c.f.u./ml)) of the original and recovered DPC16 cultures in different concentrations of bile salts.

- In Tris-HCl buffer

Two-Sample T-Test and CI: O tris, R tris

```
Two-sample T for O tris vs R tris

         N       Mean    StDev  SE Mean
O tris5  3  109153713  6662280  3846469
R tris5  3   55882353  8823529  5094267


Difference = mu (O tris) - mu (R tris)
Estimate for difference:  53271360
95% CI for difference:  (32956760, 73585961)
T-Test of difference = 0 (vs not =): T-Value = 8.35  P-Value = 0.004  DF = 3
```

- In 1.6% Bile salts

Two-Sample T-Test and CI: O 1.6, R 1.6

```
Two-sample T for O 1.6  vs C 1.6

         N    Mean  StDev  SE Mean
O 1.6    3    55.3   12.0      6.9
R 1.6    3  2500.0   29.4       17


Difference = mu (O 1.6) - mu (R 1.6)
Estimate for difference:  -2444.7
95% CI for difference:  (-2523.6, -2365.9)
T-Test of difference = 0 (vs not =): T-Value = -133.36  P-Value = 0.000  DF =
2
```

- In 0.8% Bile salts

Two-Sample T-Test and CI: O 0.8, R 0.8

```
Two-sample T for O 0.8 vs C 0.8

         N     Mean   StDev  SE Mean
O 0.8    3     9326    1294      747
R 0.8    3  1127451  224636   129694


Difference = mu (O 0.8) - mu (R 0.8)
Estimate for difference:  -1118125
95% CI for difference:  (-1676161, -560088)
T-Test of difference = 0 (vs not =): T-Value = -8.62  P-Value = 0.013  DF = 2
```

- In 0.4% Bile salts

Two-Sample T-Test and CI: O 0.4, R 0.4

```
Two-sample T for O 0.4 vs C 0.4

         N   Mean  StDev  SE Mean
O 0.4    3   2211    317      183
R 0.4    3  59412   1789     1033


Difference = mu (O 0.4) - mu (R 0.4)
Estimate for difference:  -57201
95% CI for difference:  (-61714, -52688)
```

```
T-Test of difference = 0 (vs not =): T-Value = -54.53  P-Value = 0.000  DF = 2
```

- In 0.2% Bile salts

Two-Sample T-Test and CI: O 0.2, R 0.2

```
Two-sample T for O 0.2  vs R 0.2

         N     Mean  StDev  SE Mean
O 0.2.5  3    511.2   72.8       42
C 0.2.5  3  1382353  58824    33962


Difference = mu (O 0.2.5) - mu (R 0.2.5)
Estimate for difference:  -1381842
95% CI for difference:  (-1527968, -1235716)
T-Test of difference = 0 (vs not =): T-Value = -40.69  P-Value = 0.001  DF = 2
```

4.3.5 Reuterin and short chain fatty acid production by the original and recovered cells of *L. reuteri*DPC16

- **Reuterin production in MRS**

Two-Sample T-Test and CI: Orig, Rec

```
Two-sample T for Orig vs Rec

    N    Mean  StDev  SE Mean
Orig  7  13.009  0.224    0.085
Rec  4   16.66   1.17     0.58


Difference = mu (Orig) - mu (Rec)
Estimate for difference:  -3.653
95% CI for difference:  (-5.530, -1.776)
T-Test of difference = 0 (vs not =): T-Value = -6.19  P-Value = 0.008  DF = 3
```

- **Reuterin production in MRS+glycerol**

Two-Sample T-Test and CI: Go, Gr

```
Two-sample T for Go vs Gr

      N     Mean   StDev  SE Mean
Orig  7  26.7012    2.39      1.4
Rec   4  29.5356    2.07    0.034


Difference = mu (Go) - mu (Gr)
Estimate for difference:  2.53
95% CI for difference:  (-0.80, 5.85)
T-Test of difference = 0 (vs not =): T-Value = 1.86  P-Value = 0.112  DF = 6
```

- lactate production

Two-Sample T-Test and CI: Lactate g/L, group scfa

```
Two-sample T for Lactate g/L
```

```
group
scfa    N   Mean  StDev  SE Mean
1       5  4.935  0.582     0.26
2       5  5.655  0.290     0.13


Difference = mu (1) - mu (2)
Estimate for difference:  -0.720
95% CI for difference:  (-1.467, 0.027)
T-Test of difference = 0 (vs not =): T-Value = -2.48  P-Value = 0.056  DF = 5
```

- acetate production

Two-Sample T-Test and CI: Acetate g/L, group scfa

```
Two-sample T for Acetate g/L

group
scfa    N   Mean  StDev  SE Mean
1       5  5.373  0.394     0.18
2       5  5.659  0.156    0.070


Difference = mu (1) - mu (2)
Estimate for difference:  -0.286
95% CI for difference:  (-0.773, 0.202)
T-Test of difference = 0 (vs not =): T-Value = -1.51  P-Value = 0.192  DF = 5
```

- propionate production

Two-Sample T-Test and CI: Propionate mg/L, group scfa

```
Two-sample T for Propionate mg/L

group
scfa    N   Mean  StDev  SE Mean
1       5  46.07   1.49     0.67
2       5  46.72   2.62      1.2


Difference = mu (1) - mu (2)
Estimate for difference:  -0.65
95% CI for difference:  (-3.95, 2.65)
T-Test of difference = 0 (vs not =): T-Value = -0.48  P-Value = 0.646  DF = 6
```

- iso-butyrate production

Two-Sample T-Test and CI: iso-Butyrate mg/L, group scfa

```
Two-sample T for iso-Butyrate mg/L

group
scfa    N   Mean  StDev  SE Mean
1       5  152.7   20.7      9.3
2       5  147.9   32.6       15


Difference = mu (1) - mu (2)
Estimate for difference:  4.8
```

```
95% CI for difference:  (-37.5, 47.1)
T-Test of difference = 0 (vs not =): T-Value = 0.28  P-Value = 0.791  DF = 6
```

- butyrate production

Two-Sample T-Test and CI: Butyrate mg/L, group scfa

```
Two-sample T for Butyrate mg/L

group
scfa   N   Mean  StDev  SE Mean
1      5  20.50   6.50      2.9
2      5  26.40   1.35     0.60


Difference = mu (1) - mu (2)
Estimate for difference:  -5.90
95% CI for difference:  (-14.15, 2.35)
T-Test of difference = 0 (vs not =): T-Value = -1.99  P-Value = 0.118  DF = 4
```

- iso-valerate production

Two-Sample T-Test and CI: iso-Valerate mg/L, group scfa

```
Two-sample T for iso-Valerate mg/L

group
scfa   N   Mean  StDev  SE Mean
1      5  13.52   5.48      2.5
2      5  18.40   3.08      1.4


Difference = mu (1) - mu (2)
Estimate for difference:  -4.88
95% CI for difference:  (-11.76, 2.00)
T-Test of difference = 0 (vs not =): T-Value = -1.73  P-Value = 0.133  DF = 6
```

- valerate production

Two-Sample T-Test and CI: Valerate mg/L, group scfa

```
Two-sample T for Valerate mg/L

group
scfa   N   Mean  StDev  SE Mean
1      5   17.0   10.9      4.9
2      5  20.42   3.68      1.6


Difference = mu (1) - mu (2)
Estimate for difference:  -3.42
95% CI for difference:  (-17.73, 10.89)
T-Test of difference = 0 (vs not =): T-Value = -0.66  P-Value = 0.543  DF = 4
```

4.3.6 Adhesion of *L. reuteri* DPC16 to HT-29 cells (c.f.u./cell) and Caco-2 cells
Adhere to HT-29
pH 5.8

One-way ANOVA: adhesion of Ori, adheison of Rec1, adhesion of Rec2

```
Source  DF     SS    MS     F      P
Factor   2   5.28  2.64  0.32  0.739
```

```
Error     6  49.66  8.28
Total     8  54.94

S = 2.877   R-Sq = 9.61%   R-Sq(adj) = 0.00%


                                    Individual 95% CIs For Mean Based on
                                    Pooled StDev
Level              N    Mean  StDev ---+---------+---------+---------+------
adhesion of Ori    3  14.179  0.364    (----------------*---------------)
adheison of Rec1   2  14.308  1.958 (-------------------*-------------------)
adhesion of Rec2   4  15.769  3.897             (-------------*-------------)
                                    ---+---------+---------+---------+------
                                    10.0      12.5      15.0      17.5

Pooled StDev = 2.877
```

pH 7.2

One-way ANOVA: adhesion of Orig, adhesion of Rec1, adhesion of Rec2

```
Source  DF     SS     MS     F      P
Factor   2  23.33  11.66  3.90  0.073
Error    7  20.92   2.99
Total    9  44.24

S = 1.729   R-Sq = 52.72%   R-Sq(adj) = 39.22%


                                    Individual 95% CIs For Mean Based on
                                    Pooled StDev
Level              N    Mean  StDev  -+---------+---------+---------+--------
adhesion of Orig   4  12.135  0.409             (--------*-------)
adhesion of Rec1   2  12.692  0.544            (-----------*----------)
adhesion of Rec2   4   9.231  2.590  (-------*-------)
                                     -+---------+---------+---------+--------
                                    7.5      10.0      12.5      15.0

Pooled StDev = 1.729
```

Adhere to Caco-2

pH 5.8

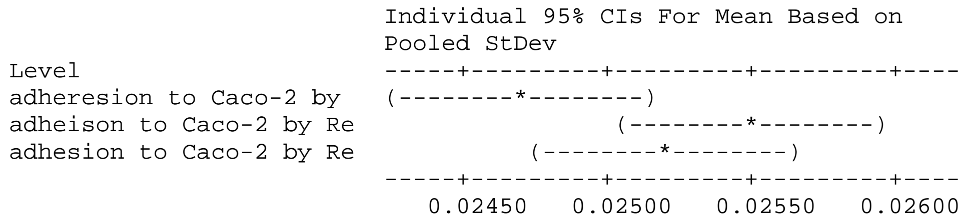
One-way ANOVA: adhesion of Orig, adhesion of Rec1, adhesion of Rec2

```
Source  DF         SS         MS     F      P
Factor   2  0.0000004  0.0000002  0.45  0.674
Error    3  0.0000012  0.0000004
Total    5  0.0000015

S = 0.0006229   R-Sq = 23.12%   R-Sq(adj) = 0.00%



Level              N      Mean     StDev
adhesion of Orig   2  0.025452  0.000470
adhesion of Rec1   2  0.025617  0.000887
adhesion of Rec2   2  0.026027  0.000396

                   Individual 95% CIs For Mean Based on Pooled StDev
Level              ---------+---------+---------+---------+
adhesion of Orig   (-------------*-------------)
adhesion of Rec1    (-------------*-------------)
adhesion of Rec2        (-------------*-------------)
                   ---------+---------+---------+---------+
                          0.0250    0.0260    0.0270    0.0280

Pooled StDev = 0.000623
```

pH 7.2

One-way ANOVA: adheresion to Caco-2 by original DPC16 cells, adheison to Caco-2 by batch 1 recovered cells, adhesion to Caco-2 by batch 2 recovered cells

```
Source  DF         SS         MS     F      P
Factor   2  0.0000009  0.0000005  4.46  0.065
Error    6  0.0000006  0.0000001
Total    8  0.0000015

S = 0.0003195   R-Sq = 59.78%   R-Sq(adj) = 46.37%



Level                          N      Mean     StDev
adheresion to Caco-2 by Orig   3  0.024717  0.000417
adheison to Caco-2 by Rec1     3  0.025485  0.000318
adhesion to Caco-2 by Rec2     3  0.025215  0.000178
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level                     -----+---------+---------+---------+----
adheresion to Caco-2 by   (--------*--------)
adheison to Caco-2 by Re                  (--------*--------)
adhesion to Caco-2 by Re            (--------*--------)
                          -----+---------+---------+---------+----
                             0.02450   0.02500   0.02550   0.02600

Pooled StDev = 0.000319
```

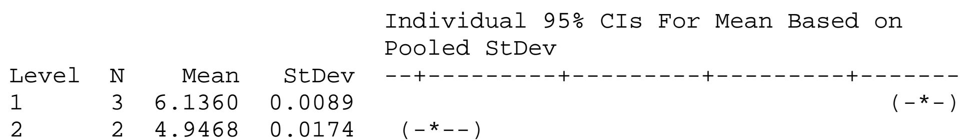
4.3.7 Effect of probiotic bacterial cells on the adhesion of *E. coli* to Caco-2 cells

Level	Group
1	Control
2	*Bifidobacterium lactis*HN0196
3	*Pediococcus acidilactici*DPC209
4	*Lactobacillus plantarum*DPC206
5	*L.reuteri DPC16* original cell
6	*L.reuteri DPC16* recovered cell

- competition assay of probiotics cells on the adhesion of *E. coli* to Caco-2 cells

One-way ANOVA: competition assay versus group

```
Source  DF       SS       MS       F      P
group    5  3.39059  0.67812  285.13  0.000
Error    8  0.01903  0.00238
Total   13  3.40962

S = 0.04877   R-Sq = 99.44%   R-Sq(adj) = 99.09%
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --+---------+---------+--------+-------
1      3  6.1360  0.0089                                  (-*-)
2      2  4.9468  0.0174   (-*--)
```

```
3      3  4.8915  0.0562  (-*-)
4      2  5.0355  0.1014      (-*-)
5      2  4.9676  0.0397    (-*-)
6      2  4.8946  0.0196  (-*-)
                          --+---------+---------+---------+-------
                          4.90      5.25      5.60      5.95

Pooled StDev = 0.0488
```

Two-Sample T-Test and CI: Co, Cr

```
Two-sample T for Co vs Cr

    N    Mean   StDev  SE Mean
Co  2  4.9676  0.0397    0.028
Cr  2  4.8946  0.0196    0.014


Difference = mu (Co) - mu (Cr)
Estimate for difference:  0.0729
95% CI for difference:  (-0.3246, 0.4704)
T-Test of difference = 0 (vs not =): T-Value = 2.33  P-Value = 0.258  DF = 1
```

- Displacement assay of probiotics cells on the adhesion of *E. coli* to Caco-2 cells

One-way ANOVA: displacement assay versus group

```
Source  DF       SS       MS       F      P
group2   5  3.61102  0.72220  494.08  0.000
Error    9  0.01316  0.00146
Total   14  3.62417

S = 0.03823   R-Sq = 99.64%   R-Sq(adj) = 99.44%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ---+---------+---------+---------+------
1      2  6.1351  0.0113                                   (*-)
2      3  6.0252  0.0108                                (-*)
3      2  5.2914  0.0862              (*-)
4      2  4.7268  0.0518  (*-)
5      3  6.1098  0.0324                                  (-*)
6      3  6.0084  0.0170                                (*)
                          ---+---------+---------+---------+------
                           4.80      5.20      5.60      6.00

Pooled StDev = 0.0382
```

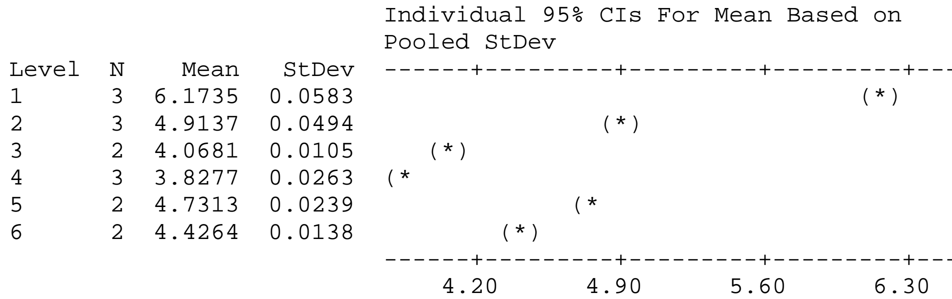
Two-Sample T-Test and CI: Do, Dr

```
Two-sample T for Do vs Dr

    N    Mean   StDev  SE Mean
Do  3  6.1098  0.0324    0.019
Dr  3  6.0084  0.0170   0.0098


Difference = mu (Do) - mu (Dr)
Estimate for difference:  0.1014
95% CI for difference:  (0.0342, 0.1686)
T-Test of difference = 0 (vs not =): T-Value = 4.80  P-Value = 0.017  DF = 3
```

- Exclusion assay of probiotics cells on the adhesion of *E. coli* to Caco-2 cells

One-way ANOVA: exclusion assay versus group

```
Source  DF       SS       MS        F      P
2group   5  9.85119  1.97024  1272.31  0.000
Error    9  0.01394  0.00155
Total   14  9.86513

S = 0.03935   R-Sq = 99.86%   R-Sq(adj) = 99.78%
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ------+---------+---------+---------+---
1      3  6.1735  0.0583                                 (*)
2      3  4.9137  0.0494                   (*)
3      2  4.0681  0.0105     (*)
4      3  3.8277  0.0263  (*
5      2  4.7313  0.0239                 (*
6      2  4.4264  0.0138          (*)
                          ------+---------+---------+---------+---
                              4.20      4.90      5.60      6.30

Pooled StDev = 0.0394
```

Two-Sample T-Test and CI: Eo, Er

```
Two-sample T for Eo vs Er

    N    Mean   StDev  SE Mean
Eo  2  4.7313  0.0239    0.017
Er  2  4.4264  0.0138   0.0098


Difference = mu (Eo) - mu (Er)
Estimate for difference:  0.3049
95% CI for difference:  (0.0566, 0.5532)
T-Test of difference = 0 (vs not =): T-Value = 15.60  P-Value = 0.041  DF = 1
```

Chapter 5

5.3.1 Comparison of colonial morphology and growth curves of different DPC16 “strains”

- Growth rate of different DPC16 "strains" without glycerol

One-way ANOVA: OD620nm versus group

```
Source  DF         SS         MS      F      P
group    6  0.0112785  0.0018798  46.65  0.000
Error   27  0.0010880  0.0000403
Total   33  0.0123665

S = 0.006348   R-Sq = 91.20%   R-Sq(adj) = 89.25%


                              Individual 95% CIs For Mean Based on
```

```
                                Pooled StDev
Level  N      Mean     StDev  --+---------+---------+---------+-------
1      5  0.087869  0.003318                        (--*--)
2      3  0.089778  0.007302                        (---*---)
3      5  0.086002  0.012978                       (--*--)
4      5  0.085620  0.002774                       (--*--)
5      5  0.081202  0.004388                     (--*--)
6      5  0.094056  0.005799                           (--*--)
7      6  0.040454  0.002063  (-*--)
                              --+---------+---------+---------+-------
                              0.040     0.060     0.080     0.100

Pooled StDev = 0.006348
```

- Growth rate of different DPC16 "strains" with glycerol

One-way ANOVA: OD620nm versus Group

```
Source  DF         SS         MS      F      P
Group2   6  0.0091008  0.0015168  68.62  0.000
Error   29  0.0006410  0.0000221
Total   35  0.0097419

S = 0.004702   R-Sq = 93.42%   R-Sq(adj) = 92.06%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N      Mean     StDev  --------+---------+---------+---------+-
1      6  0.074589  0.004499                          (--*-)
2      6  0.076607  0.005336                           (--*--)
3      4  0.081506  0.004234                              (--*---)
4      5  0.075136  0.001956                          (--*--)
5      4  0.073786  0.006230                         (--*--)
6      5  0.085969  0.007175                                 (--*--)
7      6  0.036463  0.001101  (-*--)
                              --------+---------+---------+---------+-
                                    0.045     0.060     0.075     0.090

Pooled StDev = 0.004702
```

5.3.2 Reuterin and short chain fatty acids production by different DPC16 "strains"

- Lactate production in the absence of glycerol

One-way ANOVA: Lac ori, Lac rec, Lac Immo, Lac sgf, Lac sif, Lac scf, ...

```
Source  DF      SS     MS      F      P
Factor   6  25.604  4.267  12.18  0.002
Error    7   2.453  0.350
Total   13  28.057

S = 0.5920   R-Sq = 91.26%   R-Sq(adj) = 83.76%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level       N    Mean   StDev  ------+---------+---------+---------+---
Lac ori     2  5.6450  0.3748                           (------*-----)
```

```
Lac rec      2  5.4050  0.1768                         (------*------)
Lac Immo     2  5.4200  0.2121                          (-----*------)
Lac sgf      2  4.8500  0.8910                      (-----*------)
Lac sif      2  3.3700  0.9758            (-----*------)
Lac scf      2  5.0273  0.0103                       (------*-----)
Lac freeze   2  1.6450  0.7000  (------*------)
                                ------+---------+---------+---------+---
                                    1.5       3.0       4.5       6.0

Pooled StDev = 0.5920


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 99.46%
```

- Lactate production in the presence of glycerol

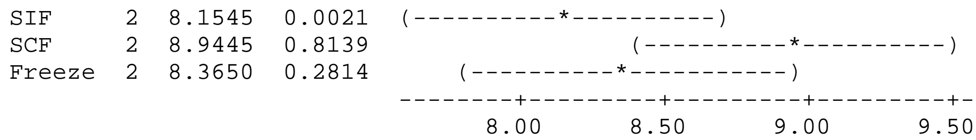
One-way ANOVA: Lac Ori/g, Lac Rec/g, Lac Immo/g, Lac sgf/g, Lac sif/g, ...

```
Source  DF      SS     MS      F      P
Factor   6  15.473  2.579  16.08  0.001
Error    7   1.123  0.160
Total   13  16.596

S = 0.4005   R-Sq = 93.23%   R-Sq(adj) = 87.44%


                                 Individual 95% CIs For Mean Based on
                                 Pooled StDev
Level         N    Mean   StDev  ----+---------+---------+---------+-----
Lac Ori/g     2  1.5000  0.1838   (-----*----)
Lac Rec/g     2  1.6600  0.1556    (-----*----)
Lac Immo/g    2  3.6850  0.0212                   (-----*----)
Lac sgf/g     2  4.1850  0.2899                       (-----*----)
Lac sif/g     2  3.1800  0.4950               (-----*----)
Lac scf/g     2  2.6650  0.8556           (----*-----)
Lac freeze/g  2  1.3300  0.0566  (----*-----)
                                 ----+---------+---------+---------+-----
                                   1.2       2.4       3.6       4.8

Pooled StDev = 0.4005
```

- Acerate production in the absence of glycerol

One-way ANOVA: Orig, Rec, Immo, SGF, SIF, SCF, Freeze

```
Source  DF     SS     MS     F      P
Factor   6  0.848  0.141  1.24  0.386
Error    7  0.795  0.114
Total   13  1.644

S = 0.3370   R-Sq = 51.62%   R-Sq(adj) = 10.16%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  --------+---------+---------+---------+-
Orig    2  8.4945  0.0813         (----------*----------)
Rec     2  8.2760  0.1980    (-----------*----------)
Immo    2  8.6760  0.0877            (-----------*----------)
SGF     2  8.3815  0.0021      (-----------*----------)
```



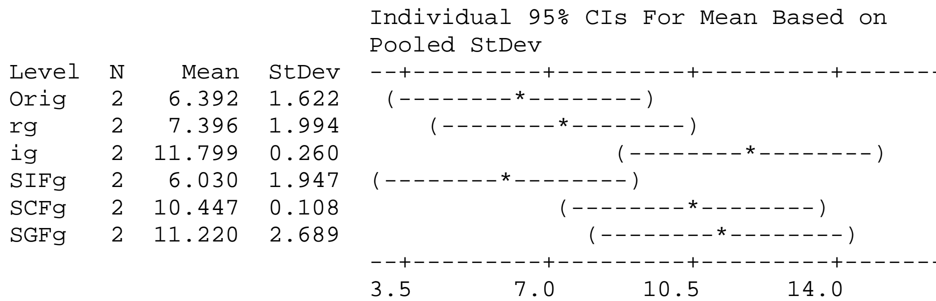
```
SIF     2  8.1545  0.0021  (----------*----------)
SCF     2  8.9445  0.8139                 (----------*----------)
Freeze  2  8.3650  0.2814      (----------*-----------)
                           --------+---------+---------+---------+-
                                 8.00      8.50      9.00      9.50
```

```
Pooled StDev = 0.3370
```

- Acerate production in the presence of glycerol

One-way ANOVA: Orig, recg, immog, SIFg, SCFg, SGFg, SGFg

```
Source  DF      SS     MS     F      P
Factor   6   75.30  12.55  3.52  0.062
Error    7   24.94   3.56
Total   13  100.25

S = 1.888   R-Sq = 75.12%   R-Sq(adj) = 53.79%
```



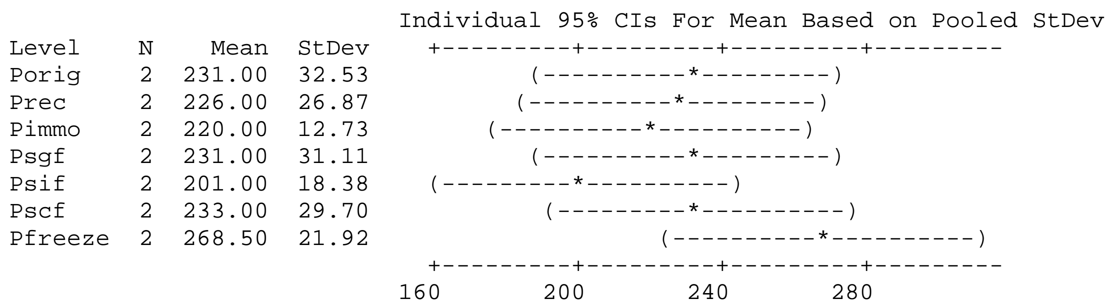
```
                         Individual 95% CIs For Mean Based on
                         Pooled StDev
Level  N    Mean  StDev  --+---------+---------+---------+-------
Orig   2   6.392  1.622   (--------*--------)
rg     2   7.396  1.994      (--------*--------)
ig     2  11.799  0.260                   (--------*--------)
SIFg   2   6.030  1.947  (--------*--------)
SCFg   2  10.447  0.108               (--------*--------)
SGFg   2  11.220  2.689                 (--------*--------)
                         --+---------+---------+---------+-------
                         3.5       7.0      10.5      14.0
```

```
Pooled StDev = 1.888
```

- Propionate production in the absence of glycerol

One-way ANOVA: Porig, Prec, Pimmo, Psgf, Psif, Pscf, Pfreeze

```
Source  DF    SS   MS     F      P
Factor   6  4900  817  1.24  0.388
Error    7  4611  659
Total   13  9511

S = 25.66   R-Sq = 51.52%   R-Sq(adj) = 9.97%
```



```
                           Individual 95% CIs For Mean Based on Pooled StDev
Level    N    Mean  StDev    +---------+---------+---------+---------
Porig    2  231.00  32.53           (----------*---------)
Prec     2  226.00  26.87          (----------*---------)
Pimmo    2  220.00  12.73        (----------*----------)
Psgf     2  231.00  31.11           (----------*---------)
Psif     2  201.00  18.38    (---------*----------)
Pscf     2  233.00  29.70            (---------*----------)
Pfreeze  2  268.50  21.92                    (----------*----------)
                             +---------+---------+---------+---------
                           160       200       240       280
```

```
Pooled StDev = 25.66
```

- Propionat production in the presence of glycerol

One-way ANOVA: Orig, recg, immog, SIFg, SCFg, SGFg, Freezeg

```
Source  DF     SS    MS     F      P
Factor   6   7114  1186  1.94  0.149
Error   13   7950   612
Total   19  15065

S = 24.73   R-Sq = 47.22%   R-Sq(adj) = 22.87%


                             Individual 95% CIs For Mean Based on Pooled StDev
Level      N    Mean  StDev   +---------+---------+---------+---------
Orig       3  241.67  29.50             (--------*--------)
recg       3  212.00  30.81     (--------*-------)
immog      3  208.00  22.65    (-------*--------)
SIFg       3  225.33  24.09         (-------*--------)
SCFg       3  205.67  15.31   (--------*--------)
SGFg       3  226.00  24.25         (--------*-------)
Freezeg    2  268.50  21.92                   (----------*----------)


Pooled StDev = 24.73
```

- 2-methylpropionate production in the absence of glycerol

One-way ANOVA: 2-orig, 2-rec, 2-Immo, 2-SGF, 2-SIF, 2-SCF, 2-Freeze

```
Source  DF      SS     MS      F      P
Factor   6  2303.4  383.9  29.69  0.000
Error    7    90.5   12.9
Total   13  2393.9

S = 3.596   R-Sq = 96.22%   R-Sq(adj) = 92.98%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level     N    Mean  StDev  ----+---------+---------+---------+-----
2-orig    2  36.700  0.806                       (---*---)
2-rec     2  37.200  5.204                        (---*---)
2-Immo    2  38.250  7.099                        (----*---)
2-SGF     2  35.850  1.117                       (---*---)
2-SIF     2  33.500  2.206                     (---*---)
2-SCF     2  37.150  2.503                        (---*---)
2-Freeze  2   0.000  0.000  (---*---)
                            ----+---------+---------+---------+-----
                                0        15        30        45

Pooled StDev = 3.596
```

- 2-methylpropionate production in the presence of glycerol

One-way ANOVA: 2-Orig/g, 2-Rec/g, 2-Immo/g, 2-SIF/g, 2-SCF/g, 2-SGF/g, ...

```
Source  DF     SS    MS     F      P
Factor   6  290.5  48.4  2.64  0.115
Error    7  128.4  18.3
Total   13  419.0

S = 4.283   R-Sq = 69.35%   R-Sq(adj) = 43.07%
```

```
                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level        N    Mean  StDev  ----+---------+---------+---------+-----
2-Orig/g     2  28.300  2.828      (---------*----------)
2-Rec/g      2  38.100  2.998                    (---------*----------)
2-Immo/g     2  37.950  5.699                    (---------*---------)
2-SIF/g      2  29.850  3.507        (----------*---------)
2-SCF/g      2  29.750  4.497        (----------*---------)
2-SGF/g      2  29.600  0.396        (---------*----------)
2-Freeze/g   2  25.100  6.802  (---------*---------)
                               ----+---------+---------+---------+-----
                                21.0      28.0      35.0      42.0

Pooled StDev = 4.283
```

- Valerate production in the absence of glycerol

One-way ANOVA: v-orig, v-rec, v-immo, v-sgf, v-sif, v-scf, v-freeze

```
Source  DF      SS     MS     F      P
Factor   6  4096.4  682.7  7.17  0.005
Error    9   857.0   95.2
Total   15  4953.4

S = 9.758   R-Sq = 82.70%   R-Sq(adj) = 71.16%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level     N    Mean   StDev  ------+---------+---------+---------+---
v-orig    3  38.133   3.595                 (----*----)
v-rec     2  41.700  13.435                 (------*-----)
v-immo    2  51.800   0.806                     (------*-----)
v-sgf     2  51.200  18.597                     (-----*------)
v-sif     3  47.033   3.308                     (----*----)
v-scf     2  50.100  16.801                     (-----*-----)
v-freeze  2   0.000   0.000  (-----*-----)
                             ------+---------+---------+---------+---
                                   0        25        50        75

Pooled StDev = 9.758
```

- Valerate production in the presence of glycerol

One-way ANOVA: v-orig/g, v-rec/g, v-immo/g, v-sif/g, v-scf/g, v-sgf/g, ...

```
Source  DF      SS     MS     F      P
Factor   6  1812.0  302.0  3.27  0.073
Error    7   645.6   92.2
Total   13  2457.6

S = 9.604   R-Sq = 73.73%   R-Sq(adj) = 51.21%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level       N    Mean   StDev  --+---------+---------+---------+-------
v-orig/g    2  38.450   5.501        (---------*---------)
v-rec/g     2  49.800  11.795               (---------*---------)
v-immo/g    2  52.700  11.201                 (---------*---------)
v-sif/g     2  33.300  12.403     (---------*---------)
v-scf/g     2  57.100   8.457                    (---------*---------)
```

```
v-sgf/g     2  60.100  11.201                      (---------*---------)
v-freeze/g  2  28.480   0.000  (---------*---------)
                               --+---------+---------+---------+-------
                                16        32        48        64

Pooled StDev = 9.604
```

- Reuterin production in MRS

One-way ANOVA: orig MRS, Rec MRS, Immo MRS, SGF MRS, SIF MRS, SCF MRS, ...

```
Source  DF     SS    MS     F      P
Factor   6  29.84  4.97  2.30  0.151
Error    7  15.16  2.17
Total   13  45.01

S = 1.472   R-Sq = 66.31%   R-Sq(adj) = 37.43%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level       N    Mean  StDev  -----+---------+---------+---------+----
orig MRS    2  16.940  0.311               (---------*---------)
Rec MRS     2  17.620  0.481                  (---------*---------)
Immo MRS    2  18.470  1.061                     (---------*---------)
SGF MRS     2  18.040  1.004                   (---------*---------)
SIF MRS     2  16.630  1.230              (---------*---------)
SCF MRS     2  17.450  3.281                 (---------*---------)
Freeze MRS  2  13.690  0.651  (---------*---------)
                              -----+---------+---------+---------+----
                                12.5      15.0      17.5      20.0

Pooled StDev = 1.472
```

- Reuterin production in MRS plus glycerol

One-way ANOVA: orig MRS/g, Rec MRS/g, Immo MRS/g, SGF MRS/g, SIF MRS/g, ...

```
Source  DF       SS      MS      F      P
Factor   7  2379.93  339.99  35.31  0.000
Error    8    77.03    9.63
Total   15  2456.96

S = 3.103   R-Sq = 96.86%   R-Sq(adj) = 94.12%


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level         N    Mean  StDev  ---+---------+---------+---------+------
orig MRS/g    2  26.330  4.441                      (---*---)
Rec MRS/g     2  27.410  2.192                       (---*---)
Immo MRS/g    2  34.170  2.051                            (---*----)
SGF MRS/g     2  32.260  3.097                           (---*---)
SIF MRS/g     2   3.220  0.382   (----*---)
SCF MRS/g     2  29.470  3.804                        (----*---)
Freeze MRS/g  2   0.880  0.071  (---*---)
free scf/g    2  28.200  4.907                       (----*---)
                                ---+---------+---------+---------+------
                                   0        12        24        36

Pooled StDev = 3.103
```

- Reuterin production by second fermentation

One-way ANOVA: sec orig, sec rec, sec Immo, sec SGF, sec SIF, sec SCF, ...

```
Source  DF       SS      MS      F      P
Factor   7  15744.3  2249.2  41.36  0.000
Error    8    435.0    54.4
Total   15  16179.3

S = 7.374   R-Sq = 97.31%   R-Sq(adj) = 94.96%


                                 Individual 95% CIs For Mean Based on
                                 Pooled StDev
Level         N    Mean   StDev  ---+---------+---------+---------+------
sec orig      2  70.810   7.269                         (---*---)
sec rec       2  76.040   6.421                          (---*---)
sec Immo      2  85.420   8.895                             (---*---)
sec SGF       2  80.650  11.116                            (---*---)
sec SIF       2   5.230   0.085   (---*---)
sec SCF       2  73.670   4.441                          (---*---)
sec Freeze    2   2.880   0.495  (---*---)
sec free scf  2  65.170  10.875                      (---*---)
                                 ---+---------+---------+---------+------
                                    0        30        60        90

Pooled StDev = 7.374
```

5.3.3.1 Immobilization effect (comparison of the antimicrobial activities of “strains” 1, 2 and 3).

Level	Group
1	BHI
2	Original ("strain" 1)
3	Recorved ("strain" 2)
4	Immobilized ("strain" 3)

Growth rate

- *E. coli* without the presence of glycerol

One-way ANOVA: growth rate e.coli versus group e.coli

```
Source         DF       SS       MS      F      P
group e.coli    3  0.05177  0.01726  15.28  0.000
Error          14  0.01581  0.00113
Total          17  0.06759

S = 0.03361   R-Sq = 76.61%   R-Sq(adj) = 71.59%


                                Individual 95% CIs For Mean Based on Pooled StDev
Level  N     Mean    StDev    +---------+---------+---------+---------
1.000  3  0.37170  0.03993                             (------*------)
2.000  5  0.27227  0.02939              (----*-----)
3.000  5  0.21229  0.03844    (----*-----)
4.000  5  0.23834  0.02853        (-----*----)
```

```
                               +---------+---------+---------+---------
                             0.180     0.240     0.300     0.360
```

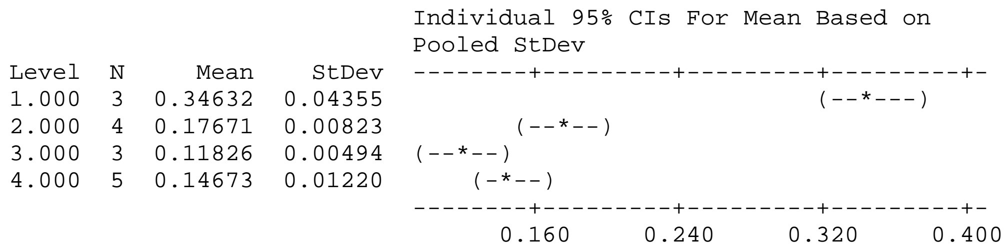
Pooled StDev = 0.03361

- *E. coli* with the presence of glycerol

One-way ANOVA: growth rate e.coli/g versus group e.coli/g

Source	DF	SS	MS	F	P
group e.coli/g	3	0.098799	0.032933	78.05	0.000
Error	11	0.004641	0.000422		
Total	14	0.103441			

S = 0.02054   R-Sq = 95.51%   R-Sq(adj) = 94.29%



```
                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level  N     Mean    StDev  --------+---------+---------+---------+-
1.000  3  0.34632  0.04355                           (--*---)
2.000  4  0.17671  0.00823         (--*--)
3.000  3  0.11826  0.00494  (--*--)
4.000  5  0.14673  0.01220      (-*--)
                            --------+---------+---------+---------+-
                                  0.160     0.240     0.320     0.400
```

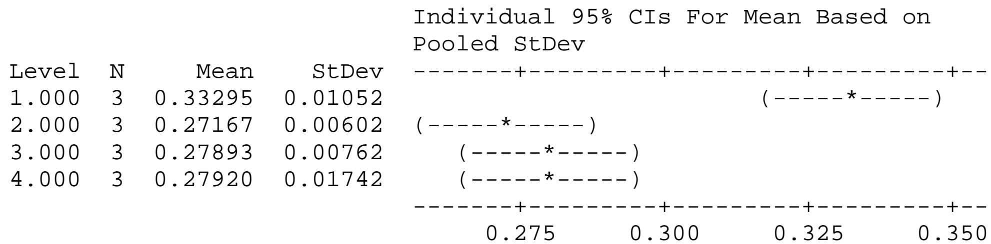
Pooled StDev = 0.02054

- *S. derby* without the presence of glycerol

One-way ANOVA: growth rate s.der versus group s.der

Source	DF	SS	MS	F	P
group s.der	3	0.007254	0.002418	19.02	0.001
Error	8	0.001017	0.000127		
Total	11	0.008271			

S = 0.01127   R-Sq = 87.70%   R-Sq(adj) = 83.09%



```
                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level  N     Mean    StDev  -------+---------+---------+---------+--
1.000  3  0.33295  0.01052                       (-----*-----)
2.000  3  0.27167  0.00602  (-----*-----)
3.000  3  0.27893  0.00762     (-----*-----)
4.000  3  0.27920  0.01742     (-----*-----)
                            -------+---------+---------+---------+--
                                 0.275     0.300     0.325     0.350
```

Pooled StDev = 0.01127

- *S. derby* with the presence of glycerol

One-way ANOVA: growth rate s.der/g versus group s.der/g

Source	DF	SS	MS	F	P
group s.der/g	3	0.219646	0.073215	605.40	0.000
Error	12	0.001451	0.000121		
Total	15	0.221098			

S = 0.01100   R-Sq = 99.34%   R-Sq(adj) = 99.18%

```
                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
1.000  8  0.27477  0.01325                                   (*)
2.000  2  0.11299  0.00233               (-*-)
3.000  2  0.09232  0.01468            (--*-)
4.000  4  0.00120  0.00067   (*-)
                             -+---------+---------+---------+--------
                             0.000     0.080     0.160     0.240

Pooled StDev = 0.01100
```

- *L. monocytogenes* without the presence of glycerol

One-way ANOVA: growth rate l.mono versus group l.mono

```
Source         DF        SS        MS      F      P
group l.mono    3  0.038038  0.012679  40.83  0.000
Error          11  0.003416  0.000311
Total          14  0.041454

S = 0.01762   R-Sq = 91.76%   R-Sq(adj) = 89.51%


                             Individual 95% CIs For Mean Based on Pooled StDev
Level  N     Mean    StDev   ---------+---------+---------+---------+
1.000  3  0.31411  0.00789                           (----*---)
2.000  5  0.25572  0.02126                 (--*---)
3.000  4  0.19249  0.02209       (--*---)
4.000  3  0.17759  0.00311   (----*---)
                             ---------+---------+---------+---------+
                                    0.200     0.250     0.300     0.350

Pooled StDev = 0.01762
```

- *L. monocytogenes* with the presence of glycerol

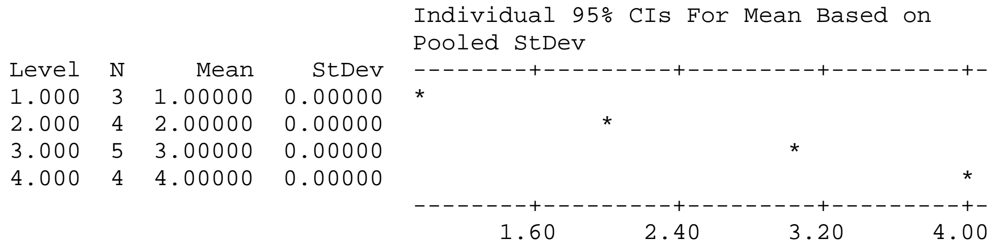
One-way ANOVA: growth rate l.mono/g versus group l.mon/g

```
Source          DF        SS        MS      F      P
group l.mon/g    3  0.146349  0.048783  69.61  0.000
Error            8  0.005607  0.000701
Total           11  0.151955

S = 0.02647   R-Sq = 96.31%   R-Sq(adj) = 94.93%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
1.000  3  0.39372  0.04575                            (--*---)
2.000  3  0.15937  0.01505       (---*--)
3.000  3  0.14058  0.02181      (--*---)
4.000  3  0.12168  0.00279    (--*---)
                             -+---------+---------+---------+--------
                             0.10      0.20      0.30      0.40

Pooled StDev = 0.02647
```

- *S. aureus* without the presence of glycerol

One-way ANOVA: group s.au versus group s.au

```
Source       DF        SS       MS  F  P
group s.au    3  17.75000  5.91667  *  *
Error        12   0.00000  0.00000
Total        15  17.75000

S = 0   R-Sq = 100.00%   R-Sq(adj) = 100.00%
```

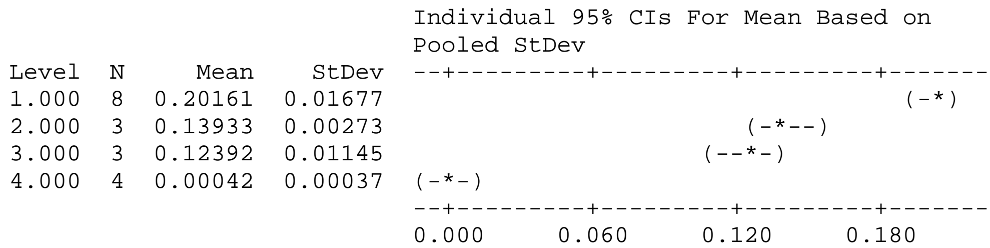


```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N    Mean    StDev   --------+---------+---------+---------+-
1.000  3  1.00000  0.00000  *
2.000  4  2.00000  0.00000              *
3.000  5  3.00000  0.00000                          *
4.000  4  4.00000  0.00000                                      *
                            --------+---------+---------+---------+-
                                 1.60      2.40      3.20      4.00

Pooled StDev = 0.00000
```

- *S. aureus* with the presence of glycerol

One-way ANOVA: growth rate s.au/g versus group s.au/g

```
Source         DF        SS        MS       F      P
group s.au/g    3  0.108336  0.036112  225.05  0.000
Error          14  0.002246  0.000160
Total          17  0.110583

S = 0.01267   R-Sq = 97.97%   R-Sq(adj) = 97.53%
```



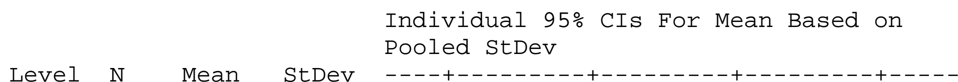
```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N    Mean    StDev   --+---------+---------+---------+-------
1.000  8  0.20161  0.01677                                  (-*)
2.000  3  0.13933  0.00273                       (-*--)
3.000  3  0.12392  0.01145                    (--*-)
4.000  4  0.00042  0.00037  (-*-)
                            --+---------+---------+---------+-------
                            0.000     0.060     0.120     0.180

Pooled StDev = 0.01267
```

End point reading

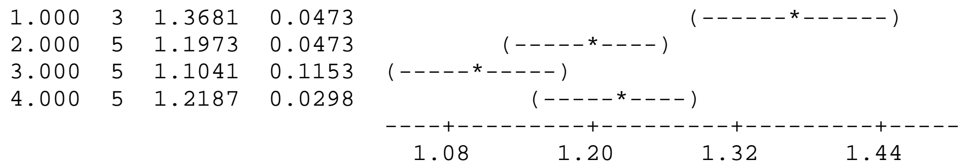
- *E. coli* without the presence of glycerol

One-way ANOVA: end reading e.coli versus group e.coli

```
Source         DF       SS       MS     F      P
group e.coli    3  0.13189  0.04396  8.78  0.002
Error          14  0.07011  0.00501
Total          17  0.20199

S = 0.07076   R-Sq = 65.29%   R-Sq(adj) = 57.86%
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N   Mean   StDev   ----+---------+---------+---------+-----
```



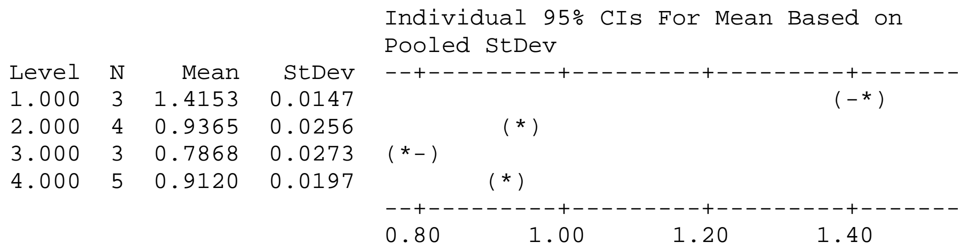
```
1.000  3  1.3681  0.0473                       (------*------)
2.000  5  1.1973  0.0473           (-----*----)
3.000  5  1.1041  0.1153  (-----*-----)
4.000  5  1.2187  0.0298             (-----*----)
                          ----+---------+---------+---------+-----
                            1.08      1.20      1.32      1.44
```

```
Pooled StDev = 0.0708
```

- *E. coli* with the presence of glycerol

One-way ANOVA: end reading e.coli/g versus group e.coli/g

```
Source           DF        SS        MS       F      P
group e.coli/g    3  0.708180  0.236060  477.08  0.000
Error            11  0.005443  0.000495
Total            14  0.713623

S = 0.02224   R-Sq = 99.24%   R-Sq(adj) = 99.03%
```

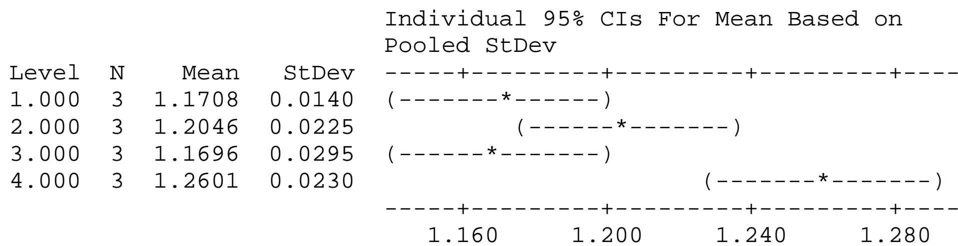


```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --+---------+---------+---------+-------
1.000  3  1.4153  0.0147                                (-*)
2.000  4  0.9365  0.0256          (*)
3.000  3  0.7868  0.0273  (*-)
4.000  5  0.9120  0.0197         (*)
                          --+---------+---------+---------+-------
                          0.80      1.00      1.20      1.40

Pooled StDev = 0.0222
```

- *S. derby* without glycerol

One-way ANOVA: end reading s.der versus group s.der

```
Source       DF        SS        MS      F      P
group s.der   3  0.016210  0.005403  10.29  0.004
Error         8  0.004200  0.000525
Total        11  0.020410

S = 0.02291   R-Sq = 79.42%   R-Sq(adj) = 71.70%
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -----+---------+---------+---------+----
1.000  3  1.1708  0.0140  (-------*------)
2.000  3  1.2046  0.0225           (------*-------)
3.000  3  1.1696  0.0295  (------*-------)
4.000  3  1.2601  0.0230                        (-------*-------)
                          -----+---------+---------+---------+----
                             1.160     1.200     1.240     1.280

Pooled StDev = 0.0229
```

- *S. derby* with glycerol

One-way ANOVA: end reading s.der/g versus group s.der/g

```
Source         DF        SS        MS        F      P
group s.der/g   3  2.090954  0.696985  1139.13  0.000
Error          12  0.007342  0.000612
Total          15  2.098296
```

```
S = 0.02474   R-Sq = 99.65%   R-Sq(adj) = 99.56%


                          Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev    +---------+---------+---------+---------
1.000  8  1.1452  0.0287                                    (*)
2.000  2  1.0293  0.0276                               (*-)
3.000  2  0.9826  0.0246                             (*-)
4.000  4  0.2747  0.0083    (*)
                            +---------+---------+---------+---------
                          0.25      0.50      0.75      1.00

Pooled StDev = 0.0247
```

- *L. monocytogenes* without the presence of glycerol

One-way ANOVA: end reading l.mono versus group l.mono

```
Source         DF       SS       MS      F      P
group l.mono    3  0.17662  0.05887  24.84  0.000
Error          11  0.02607  0.00237
Total          14  0.20268

S = 0.04868   R-Sq = 87.14%   R-Sq(adj) = 83.63%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ---+---------+---------+---------+------
1.000  3  1.3992  0.0210                        (-----*----)
2.000  5  1.1921  0.0501          (---*---)
3.000  4  1.1096  0.0684   (---*----)
4.000  3  1.1088  0.0237  (----*-----)
                          ---+---------+---------+---------+------
                           1.08      1.20      1.32      1.44

Pooled StDev = 0.0487
```

- *L. monocytogenes* with the presence of glycerol

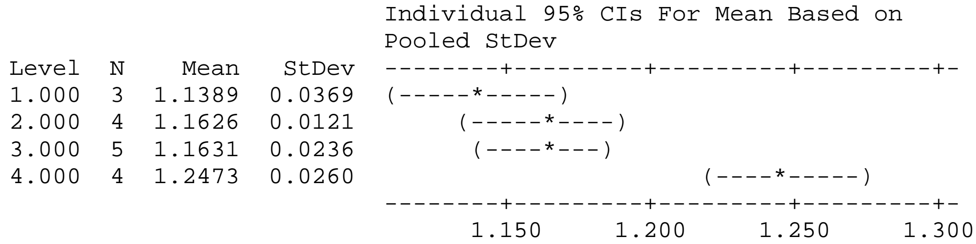
One-way ANOVA: end reading l.mon/g versus group l.mon/g

```
Source          DF         SS         MS        F      P
group l.mon/g    3  0.5531739  0.1843913  2502.85  0.000
Error            8  0.0005894  0.0000737
Total           11  0.5537633

S = 0.008583   R-Sq = 99.89%   R-Sq(adj) = 99.85%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  ---------+---------+---------+---------+
1.000  3  1.44687  0.01247                                       *)
2.000  3  0.93387  0.00327  (*)
3.000  3  0.96447  0.01126     *)
4.000  3  0.95680  0.00128    (*)
                            ---------+---------+---------+---------+
                                  1.05      1.20      1.35      1.50

Pooled StDev = 0.00858
```

- *S. aureus* without the presence of glycerol

One-way ANOVA: end reading s.au versus group s.au

```
Source      DF        SS        MS      F      P
group s.au   3  0.025834  0.008611  13.95  0.000
Error       12  0.007406  0.000617
Total       15  0.033241

S = 0.02484   R-Sq = 77.72%   R-Sq(adj) = 72.15%
```

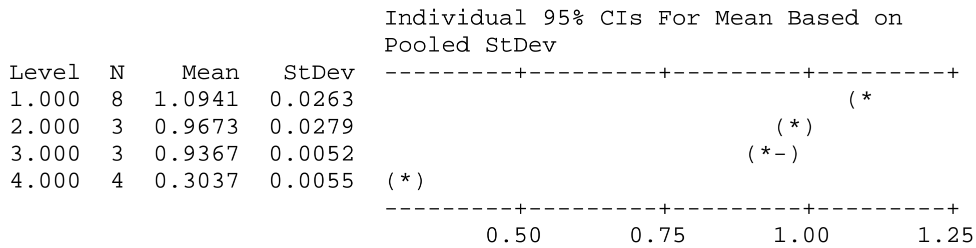


```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --------+---------+---------+---------+-
1.000  3  1.1389  0.0369  (-----*-----)
2.000  4  1.1626  0.0121       (-----*----)
3.000  5  1.1631  0.0236        (----*---)
4.000  4  1.2473  0.0260                      (----*-----)
                          --------+---------+---------+---------+-
                              1.150     1.200     1.250     1.300

Pooled StDev = 0.0248
```

- *S. aureus* with the presence of glycerol

One-way ANOVA: end reading s.au/g versus group s.au/g

```
Source        DF        SS        MS        F      P
group s.au/g   3  1.726302  0.575434  1231.15  0.000
Error         14  0.006544  0.000467
Total         17  1.732845

S = 0.02162   R-Sq = 99.62%   R-Sq(adj) = 99.54%
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ---------+---------+---------+---------+
1.000  8  1.0941  0.0263                                 (*
2.000  3  0.9673  0.0279                            (*)
3.000  3  0.9367  0.0052                          (*-)
4.000  4  0.3037  0.0055  (*)
                          ---------+---------+---------+---------+
                               0.50      0.75      1.00      1.25

Pooled StDev = 0.0216
```

5.3.3.2 Effect of incubation of immobilized cells in SGF and SCF, and effect of freeze-drying/storage of immobilized cells (comparison of "strains" 1, 2, 4, 6 and 7)

Level	Group
1	BHI
2	Original ("strain" 1)
3	Recorved ("strain" 2)
4	SGF ("strain" 4)

5	SCF ("strain" 6)
6	Freeze-dried ("strain" 7)

Growth rate

- *E. coli* without the presence of glycerol

One-way ANOVA: Growth rate E. coli versus group

```
Source  DF        SS        MS      F      P
group    5  0.049805  0.009961  64.99  0.000
Error   23  0.003525  0.000153
Total   28  0.053330

S = 0.01238   R-Sq = 93.39%   R-Sq(adj) = 91.95%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  --+---------+---------+---------+-------
1      4  0.31913  0.01907                               (--*---)
2      5  0.24191  0.01655          (--*--)
3      5  0.21439  0.00899  (--*---)
4      5  0.23953  0.00639         (--*---)
6      5  0.30954  0.00854                            (--*---)
5      5  0.21451  0.01183  (--*---)
                            --+---------+---------+---------+-------
                            0.210     0.245     0.280     0.315

Pooled StDev = 0.01238
```

- *E. coli* with the presence of glycerol

One-way ANOVA: Growth rate E. coli/g versus group/g

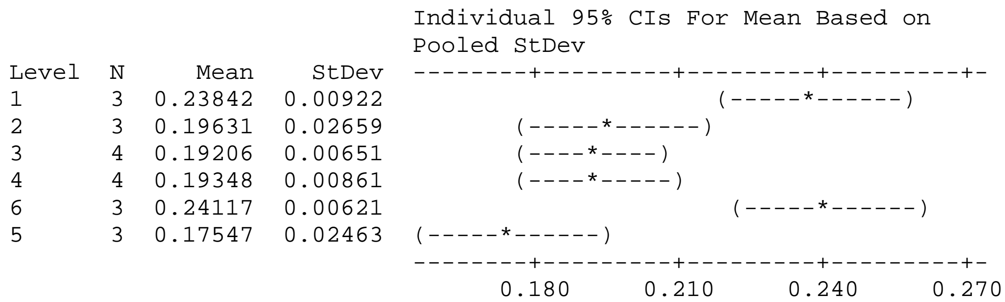
```
Source   DF        SS        MS      F      P
group/g   5  0.051248  0.010250  87.80  0.000
Error    22  0.002568  0.000117
Total    27  0.053816

S = 0.01080   R-Sq = 95.23%   R-Sq(adj) = 94.14%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -----+---------+---------+---------+----
1      4  0.27942  0.00587                                 (--*--)
2      5  0.22744  0.01231                     (--*-)
3      5  0.20857  0.00830                 (-*--)
4      5  0.14866  0.01568  (-*--)
6      5  0.21048  0.00432                 (--*-)
5      4  0.15369  0.01323   (-*--)
                            -----+---------+---------+---------+----
```

```
                              0.160     0.200     0.240     0.280

Pooled StDev = 0.01080
```

- *S. derby* without the presence of glycerol

One-way ANOVA: growth rate s.de versus group s.de

```
Source       DF        SS        MS     F      P
group s.de    5  0.011319  0.002264  9.83  0.000
Error        14  0.003224  0.000230
Total        19  0.014543

S = 0.01517   R-Sq = 77.83%   R-Sq(adj) = 69.91%
```

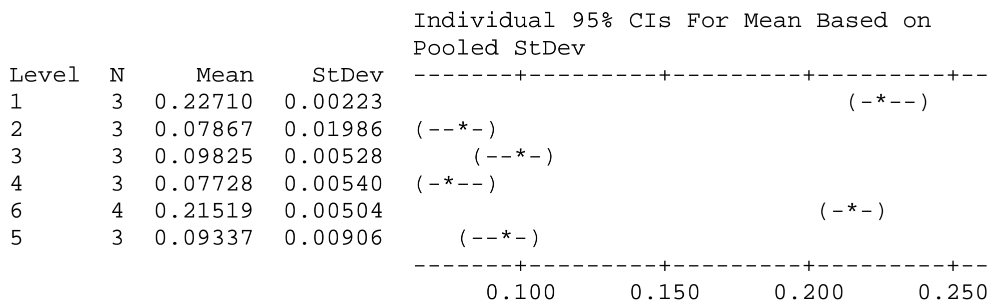


```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  --------+---------+---------+---------+-
1      3  0.23842  0.00922                      (-----*------)
2      3  0.19631  0.02659         (-----*------)
3      4  0.19206  0.00651         (----*----)
4      4  0.19348  0.00861         (----*-----)
6      3  0.24117  0.00621                       (-----*------)
5      3  0.17547  0.02463  (-----*------)
                            --------+---------+---------+---------+-
                                0.180     0.210     0.240     0.270

Pooled StDev = 0.01517
```

- *S. derby* with the presence of glycerol

One-way ANOVA: growth rate s.de/g versus group s.de/g

```
Source         DF         SS         MS       F      P
group s.de/g    5  0.0799180  0.0159836  180.22  0.000
Error          13  0.0011529  0.0000887
Total          18  0.0810709

S = 0.009417   R-Sq = 98.58%   R-Sq(adj) = 98.03%
```



```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -------+---------+---------+---------+--
1      3  0.22710  0.00223                               (-*--)
2      3  0.07867  0.01986  (--*-)
3      3  0.09825  0.00528      (--*-)
4      3  0.07728  0.00540  (-*--)
6      4  0.21519  0.00504                             (-*-)
5      3  0.09337  0.00906     (--*-)
                            -------+---------+---------+---------+--
                               0.100     0.150     0.200     0.250

Pooled StDev = 0.00942
```

- *S. aureus* without the presence of glycerol

One-way ANOVA: growth rate s.au versus group s.au

```
Source       DF        SS        MS      F      P
group s.au    5  0.077241  0.015448  34.84  0.000
Error        11  0.004877  0.000443
Total        16  0.082118
```

```
S = 0.02106   R-Sq = 94.06%   R-Sq(adj) = 91.36%


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level  N     Mean    StDev  -------+---------+---------+---------+--
1      2  0.32572  0.00101                             (----*---)
2      4  0.14399  0.01983      (---*--)
3      3  0.11924  0.01470  (---*---)
4      3  0.12151  0.02497   (--*---)
6      2  0.21417  0.01683              (----*---)
5      3  0.11529  0.02944  (--*---)
                            -------+---------+---------+---------+--
                                 0.140     0.210     0.280     0.350

Pooled StDev = 0.02106
```

- *S. aureus* with the presence of glycerol

One-way ANOVA: growth rate s.au/g versus group s.au/g

```
Source         DF        SS        MS      F      P
group s.au/g    5  0.047411  0.009482  53.68  0.000
Error          17  0.003003  0.000177
Total          22  0.050414

S = 0.01329   R-Sq = 94.04%   R-Sq(adj) = 92.29%


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level  N     Mean    StDev  -------+---------+---------+---------+--
1      4  0.21235  0.01194                           (--*---)
2      3  0.14343  0.00391           (---*---)
3      4  0.12595  0.02434       (--*---)
4      4  0.12092  0.00366      (--*---)
6      4  0.22003  0.00856                             (--*---)
5      4  0.10778  0.01301  (---*--)
                            -------+---------+---------+---------+--
                                 0.120     0.160     0.200     0.240

Pooled StDev = 0.01329
```

- *L. monocytogenes* without the presence of glycerol

One-way ANOVA: growth rate L. monocytogenes versus group l

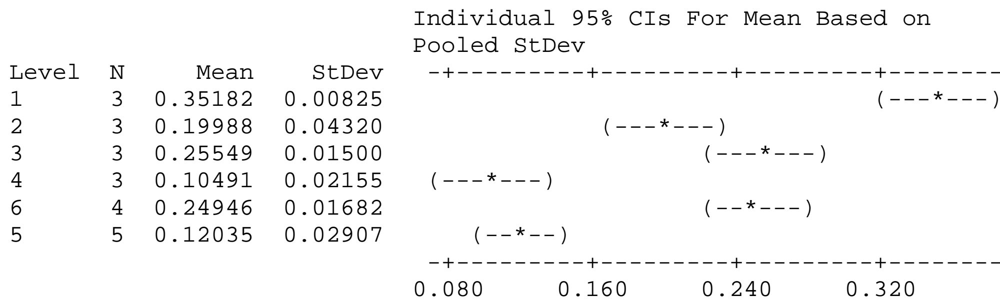
```
Source   DF        SS        MS      F      P
group l   5  0.044248  0.008850  21.19  0.000
Error    18  0.007519  0.000418
Total    23  0.051767

S = 0.02044   R-Sq = 85.48%   R-Sq(adj) = 81.44%


                            Individual 95% CIs For Mean Based on Pooled StDev
Level  N     Mean    StDev    +---------+---------+---------+---------
1      2  0.33350  0.00717                        (-----*-----)
2      4  0.24368  0.01851      (----*---)
3      4  0.22102  0.00658    (---*---)
4      5  0.22122  0.01737    (---*---)
6      4  0.31721  0.01524                      (---*----)
5      5  0.21975  0.03319    (---*---)
                              +---------+---------+---------+---------
```

```
                            0.200     0.250     0.300     0.350

Pooled StDev = 0.02044
```

- *L. monocytogenes* with the presence of glycerol

One-way ANOVA: growth rate L. monocytogenes/g versus group l/g

```
Source      DF        SS        MS      F      P
group l/g    5  0.146136  0.029227  46.26  0.000
Error       15  0.009478  0.000632
Total       20  0.155614

S = 0.02514   R-Sq = 93.91%   R-Sq(adj) = 91.88%
```



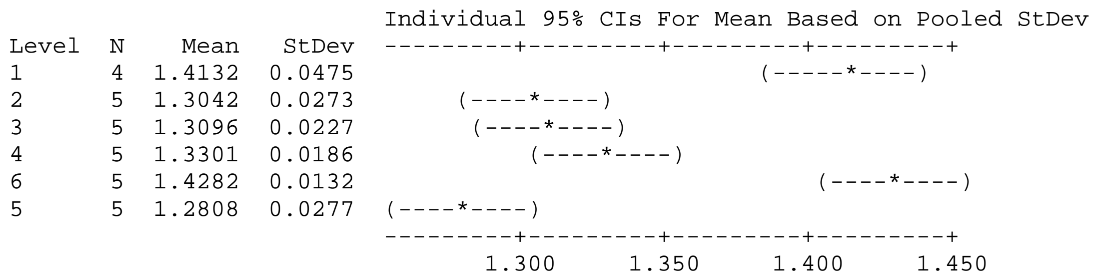
```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
1      3  0.35182  0.00825                               (---*---)
2      3  0.19988  0.04320              (---*---)
3      3  0.25549  0.01500                     (---*---)
4      3  0.10491  0.02155   (---*---)
6      4  0.24946  0.01682                     (--*---)
5      5  0.12035  0.02907      (--*--)
                             -+---------+---------+---------+--------
                            0.080     0.160     0.240     0.320

Pooled StDev = 0.02514
```

<u>End point reading</u>

- *E. coli* without glycerol

One-way ANOVA: End E. coli versus group

```
Source  DF        SS        MS      F      P
group    5  0.089283  0.017857  24.21  0.000
Error   23  0.016964  0.000738
Total   28  0.106247

S = 0.02716   R-Sq = 84.03%   R-Sq(adj) = 80.56%
```

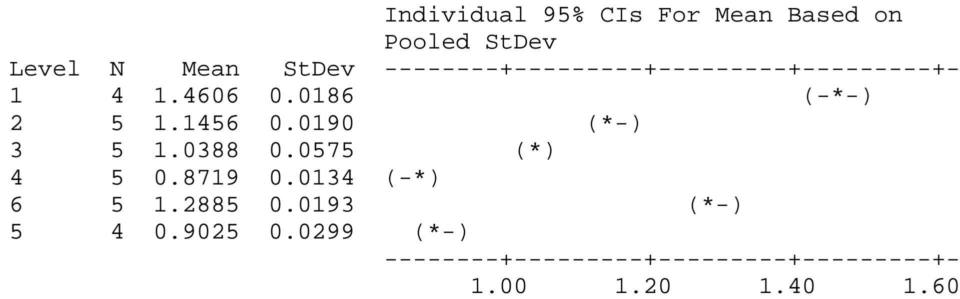


```
                          Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev  ---------+---------+---------+---------+
1      4  1.4132  0.0475                            (-----*----)
2      5  1.3042  0.0273       (----*----)
3      5  1.3096  0.0227        (----*----)
4      5  1.3301  0.0186            (----*----)
6      5  1.4282  0.0132                                (----*----)
5      5  1.2808  0.0277  (----*----)
                          ---------+---------+---------+---------+
                                 1.300     1.350     1.400     1.450

Pooled StDev = 0.0272
```

- *E. coli* with glycerol

One-way ANOVA: End E. coli/g versus group/g

```
Source    DF        SS        MS       F      P
group/g    5  1.137956  0.227591  243.30  0.000
Error     22  0.020579  0.000935
Total     27  1.158535

S = 0.03058   R-Sq = 98.22%   R-Sq(adj) = 97.82%
```

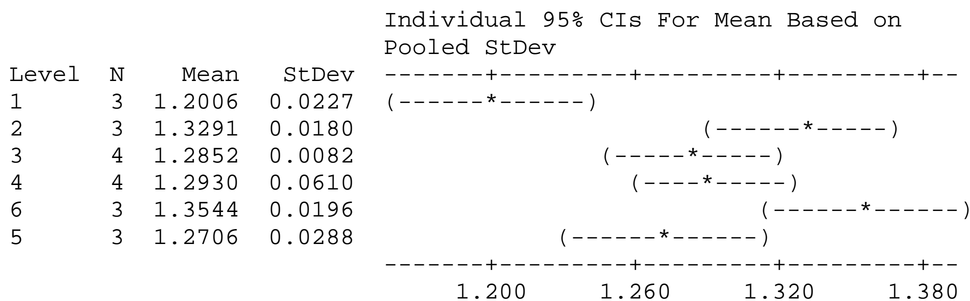


```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --------+---------+---------+---------+-
1      4  1.4606  0.0186                               (-*-)
2      5  1.1456  0.0190                 (*-)
3      5  1.0388  0.0575            (*)
4      5  0.8719  0.0134  (-*)
6      5  1.2885  0.0193                        (*-)
5      4  0.9025  0.0299    (*-)
                          --------+---------+---------+---------+-
                               1.00      1.20      1.40      1.60

Pooled StDev = 0.0306
```

- *S. derby* without glycerol

One-way ANOVA: S. derby versus group

```
Source  DF       SS       MS     F      P
group   5  0.04223  0.00845  7.64  0.001
Error   14  0.01547  0.00111
Total   19  0.05770

S = 0.03325   R-Sq = 73.18%   R-Sq(adj) = 63.60%
```

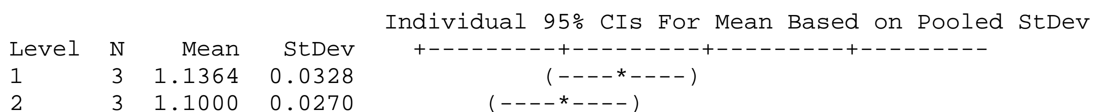


```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -------+---------+---------+---------+--
1      3  1.2006  0.0227  (------*------)
2      3  1.3291  0.0180                      (------*-----)
3      4  1.2852  0.0082               (-----*-----)
4      4  1.2930  0.0610                 (----*-----)
6      3  1.3544  0.0196                          (------*------)
5      3  1.2706  0.0288            (------*------)
                          -------+---------+---------+---------+--
                             1.200     1.260     1.320     1.380

Pooled StDev = 0.0332
```

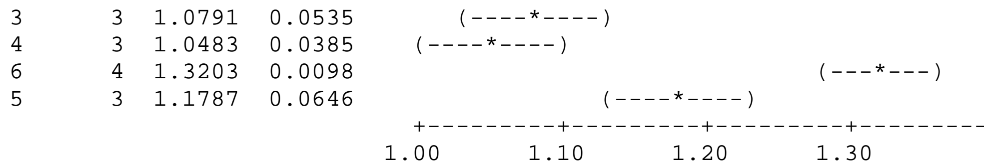
- *S. derby* with glycerol

One-way ANOVA: S. derby/g versus group3

```
Source  DF       SS       MS      F      P
group3   5  0.17254  0.03451  21.45  0.000
Error   13  0.02091  0.00161
Total   18  0.19345

S = 0.04011   R-Sq = 89.19%   R-Sq(adj) = 85.03%
```



```
                          Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev    +---------+---------+---------+---------
1      3  1.1364  0.0328             (----*----)
2      3  1.1000  0.0270         (----*----)
```

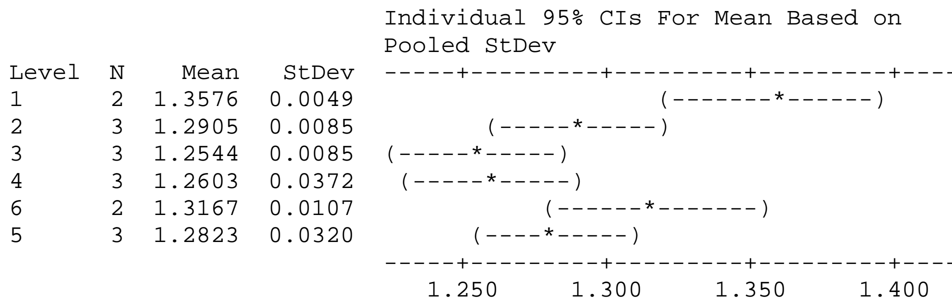


```
3      3  1.0791  0.0535      (----*----)
4      3  1.0483  0.0385   (----*----)
6      4  1.3203  0.0098                           (---*---)
5      3  1.1787  0.0646               (----*----)
                           +---------+---------+---------+---------
                          1.00      1.10      1.20      1.30

Pooled StDev = 0.0401
```

- *S. aureus* without glycerol

One-way ANOVA: S. aureus versus group

```
Source  DF        SS        MS     F      P
group    5  0.017114  0.003423  6.53  0.006
Error   10  0.005243  0.000524
Total   15  0.022358

S = 0.02290   R-Sq = 76.55%   R-Sq(adj) = 64.82%
```

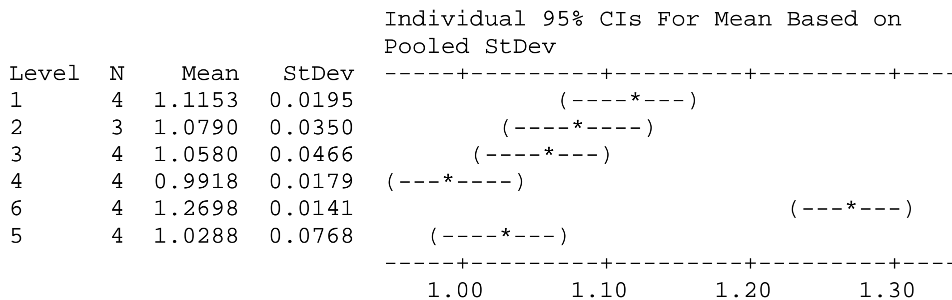


```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -----+---------+---------+---------+----
1      2  1.3576  0.0049                     (-------*------)
2      3  1.2905  0.0085         (-----*-----)
3      3  1.2544  0.0085  (-----*-----)
4      3  1.2603  0.0372   (-----*-----)
6      2  1.3167  0.0107            (------*-------)
5      3  1.2823  0.0320       (----*-----)
                          -----+---------+---------+---------+----
                             1.250     1.300     1.350     1.400

Pooled StDev = 0.0229
```

- *S. aureus* with glycerol

One-way ANOVA: S. aureus/g versus group

```
Source  DF       SS       MS      F      P
group    5  0.18987  0.03797  21.99  0.000
Error   17  0.02936  0.00173
Total   22  0.21923

S = 0.04156   R-Sq = 86.61%   R-Sq(adj) = 82.67%
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -----+---------+---------+---------+----
1      4  1.1153  0.0195            (----*---)
2      3  1.0790  0.0350        (----*----)
3      4  1.0580  0.0466      (----*---)
4      4  0.9918  0.0179  (---*----)
6      4  1.2698  0.0141                            (---*---)
5      4  1.0288  0.0768     (----*---)
                          -----+---------+---------+---------+----
                             1.00      1.10      1.20      1.30

Pooled StDev = 0.0416
```

- *L. monocytogenes* without glycerol

One-way ANOVA: End L. monocytogenes versus group I

```
Source    DF        SS        MS      F      P
group l    5  0.098661  0.019732  35.52  0.000
Error     18  0.010000  0.000556
Total     23  0.108661

S = 0.02357   R-Sq = 90.80%   R-Sq(adj) = 88.24%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -------+---------+---------+---------+--
1      2  1.4810  0.0021                             (-----*-----)
2      4  1.3714  0.0188            (----*---)
3      4  1.3757  0.0094             (---*---)
4      5  1.3917  0.0075                (---*---)
6      4  1.4881  0.0177                                (---*---)
5      5  1.2990  0.0433  (---*--)
                          -------+---------+---------+---------+--
                               1.320     1.380     1.440     1.500

Pooled StDev = 0.0236
```

- *L. monocytogenes* with glycerol

One-way ANOVA: End L .monocytogenes/g versus group l/g

```
Source      DF        SS        MS       F      P
group l/g    5  0.750573  0.150115  433.05  0.000
Error       15  0.005200  0.000347
Total       20  0.755773

S = 0.01862   R-Sq = 99.31%   R-Sq(adj) = 99.08%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -----+---------+---------+---------+----
1      3  1.5226  0.0144                                   (-*)
2      3  1.2541  0.0072                 (-*)
3      3  1.2758  0.0224                   (*-)
4      3  1.0711  0.0277     (*-)
5      4  1.4043  0.0079                             (-*)
6      5  0.9896  0.0221  (*)
                          -----+---------+---------+---------+----
                             1.05      1.20      1.35      1.50
```

5.3.3.3 Effect of incubation of immobilized cells in SIF (comparison of "strains" 1, 2 and 5)

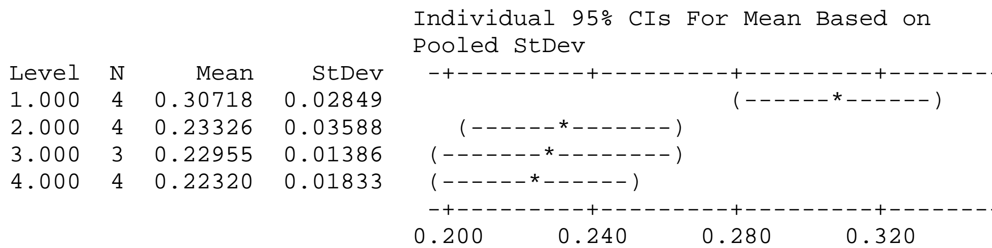
Level	Group
1	BHI
2	Original ("strains" 1)
3	Immobilized ("strains" 3)
4	SIF ("strains" 5)

Growth rate

- *E. coli* without glycerol

One-way ANOVA: growth rate e.coli versus group e.coli

```
Source         DF        SS        MS     F      P
group e.coli    3  0.018321  0.006107  8.74  0.003
Error          11  0.007688  0.000699
Total          14  0.026009

S = 0.02644   R-Sq = 70.44%   R-Sq(adj) = 62.38%
```

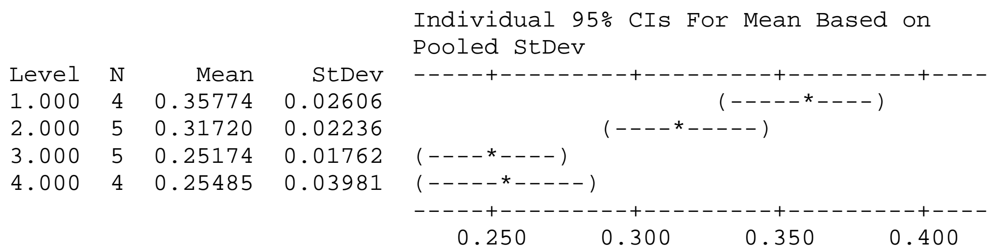


```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
1.000  4  0.30718  0.02849                       (------*------)
2.000  4  0.23326  0.03588     (------*-------)
3.000  3  0.22955  0.01386   (-------*--------)
4.000  4  0.22320  0.01833   (------*------)
                             -+---------+---------+---------+--------
                            0.200     0.240     0.280     0.320

Pooled StDev = 0.02644
```

- *E. coli* with glycerol

One-way ANOVA: growth rate e.coli/g versus group e.coli/g

```
Source           DF        SS        MS      F      P
group e.coli/g    3  0.034001  0.011334  15.81  0.000
Error            14  0.010034  0.000717
Total            17  0.044035

S = 0.02677   R-Sq = 77.21%   R-Sq(adj) = 72.33%
```

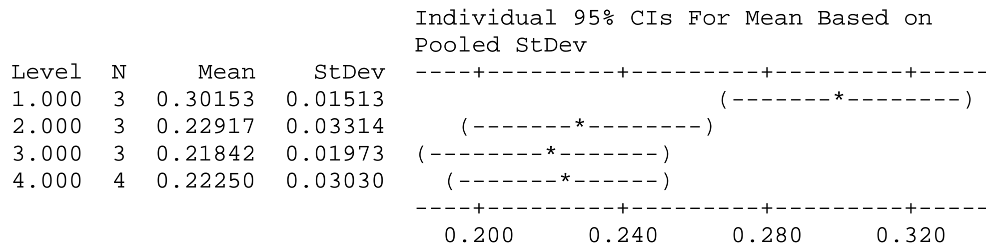


```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -----+---------+---------+---------+----
1.000  4  0.35774  0.02606                          (-----*----)
2.000  5  0.31720  0.02236                  (----*-----)
3.000  5  0.25174  0.01762  (----*----)
4.000  4  0.25485  0.03981  (-----*-----)
                            -----+---------+---------+---------+----
                               0.250     0.300     0.350     0.400

Pooled StDev = 0.02677
```

- *S. derby* without glycerol

One-way ANOVA: growth rate s.der versus group s.der

```
Source       DF        SS        MS     F      P
group s.der   3  0.014311  0.004770  6.94  0.010
Error         9  0.006187  0.000687
Total        12  0.020497

S = 0.02622   R-Sq = 69.82%   R-Sq(adj) = 59.75%
```

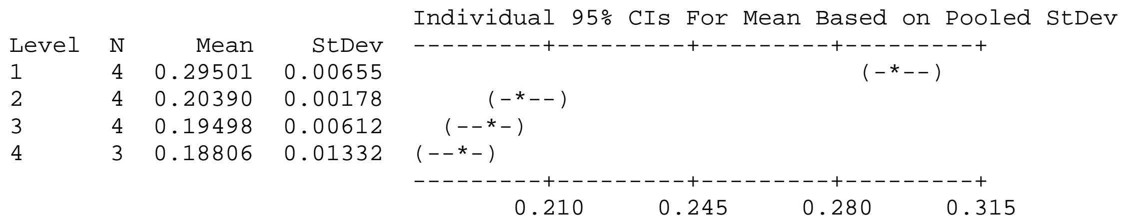


```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  ----+---------+---------+---------+-----
1.000  3  0.30153  0.01513                      (-------*--------)
2.000  3  0.22917  0.03314     (-------*--------)
3.000  3  0.21842  0.01973  (--------*-------)
4.000  4  0.22250  0.03030    (-------*------)
                            ----+---------+---------+---------+-----
                              0.200     0.240     0.280     0.320

Pooled StDev = 0.02622
```

- *S. derby* with glycerol

One-way ANOVA: growth rate s.der/g versus group s.der/g

```
Source          DF          SS          MS       F      P
group s.der/g    3   0.0290037   0.0096679  175.69  0.000
Error           11   0.0006053   0.0000550
Total           14   0.0296090

S = 0.007418   R-Sq = 97.96%   R-Sq(adj) = 97.40%
```

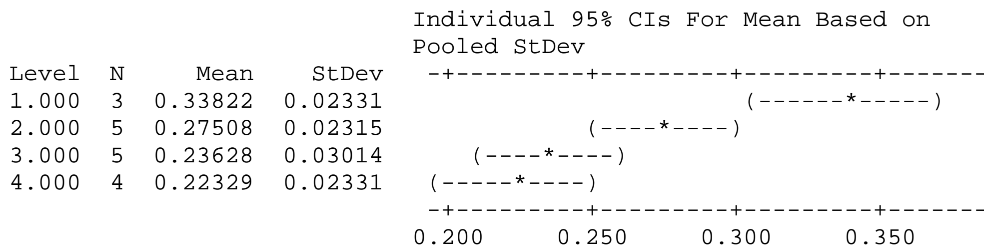


```
                            Individual 95% CIs For Mean Based on Pooled StDev
Level  N     Mean    StDev  ---------+---------+---------+---------+
1      4  0.29501  0.00655                               (-*--)
2      4  0.20390  0.00178       (-*--)
3      4  0.19498  0.00612    (--*-)
4      3  0.18806  0.01332  (--*-)
                            ---------+---------+---------+---------+
                                   0.210     0.245     0.280     0.315

Pooled StDev = 0.00742
```

- *L. monocytogenes* without glycerol

One-way ANOVA: growth rate l.mono versus group l.mono

```
Source        DF        SS        MS      F      P
group l.mono   3  0.027578  0.009193  14.07  0.000
Error         13  0.008493  0.000653
Total         16  0.036071

S = 0.02556   R-Sq = 76.45%   R-Sq(adj) = 71.02%
```

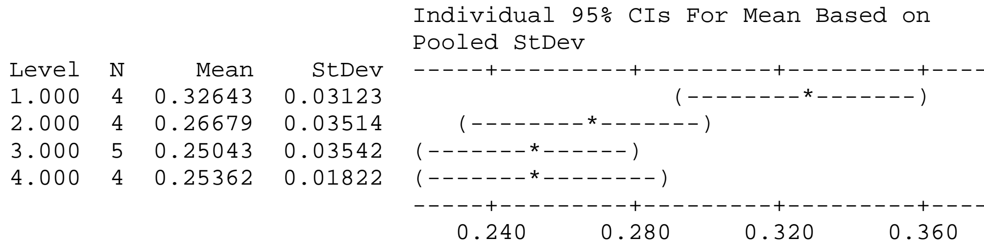


```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
1.000  3  0.33822  0.02331                        (------*-----)
2.000  5  0.27508  0.02315             (----*----)
3.000  5  0.23628  0.03014     (----*----)
4.000  4  0.22329  0.02331   (-----*----)
                             -+---------+---------+---------+--------
                            0.200     0.250     0.300     0.350

Pooled StDev = 0.02556
```

- *L. monocytogenes* with glycerol

One-way ANOVA: growth rate l.mono/g versus group l.mono/g

```
Source           DF        SS        MS     F      P
group l.mono/g    3  0.015624  0.005208  5.35  0.013
Error            13  0.012646  0.000973
Total            16  0.028270

S = 0.03119   R-Sq = 55.27%   R-Sq(adj) = 44.94%
```

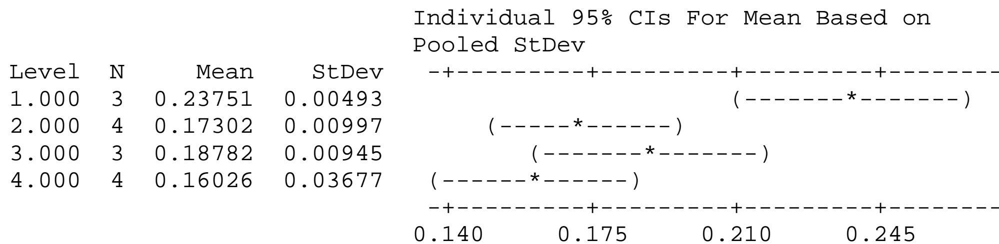


```
                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N     Mean    StDev  -----+---------+---------+---------+----
1.000  4  0.32643  0.03123                     (--------*-------)
2.000  4  0.26679  0.03514     (--------*-------)
3.000  5  0.25043  0.03542  (-------*------)
4.000  4  0.25362  0.01822  (-------*--------)
                            -----+---------+---------+---------+----
                               0.240     0.280     0.320     0.360

Pooled StDev = 0.03119
```

- *S. aureus* without glycerol

One-way ANOVA: growth rate s.au versus group s.au

```
Source     DF        SS        MS     F      P
group s.au  3  0.011293  0.003764  8.22  0.005
Error      10  0.004582  0.000458
Total      13  0.015875

S = 0.02141   R-Sq = 71.14%   R-Sq(adj) = 62.48%
```

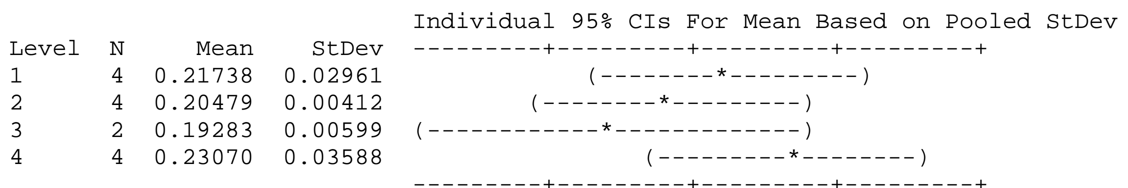


```
                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
1.000  3  0.23751  0.00493                       (-------*-------)
2.000  4  0.17302  0.00997       (-----*------)
3.000  3  0.18782  0.00945          (-------*-------)
4.000  4  0.16026  0.03677   (------*------)
                             -+---------+---------+---------+--------
                            0.140     0.175     0.210     0.245

Pooled StDev = 0.02141
```

- *S. aureus* with glycerol

One-way ANOVA: growth rate s.au/g versus group s.au/g

```
Source       DF        SS        MS     F      P
group s.au/g  3  0.002396  0.000799  1.21  0.354
Error        10  0.006579  0.000658
Total        13  0.008975

S = 0.02565   R-Sq = 26.70%   R-Sq(adj) = 4.71%
```



```
                            Individual 95% CIs For Mean Based on Pooled StDev
Level  N     Mean    StDev  ---------+---------+---------+---------+
1      4  0.21738  0.02961              (--------*---------)
2      4  0.20479  0.00412          (--------*---------)
3      2  0.19283  0.00599  (------------*-------------)
4      4  0.23070  0.03588                  (---------*--------)
                            ---------+---------+---------+---------+
```

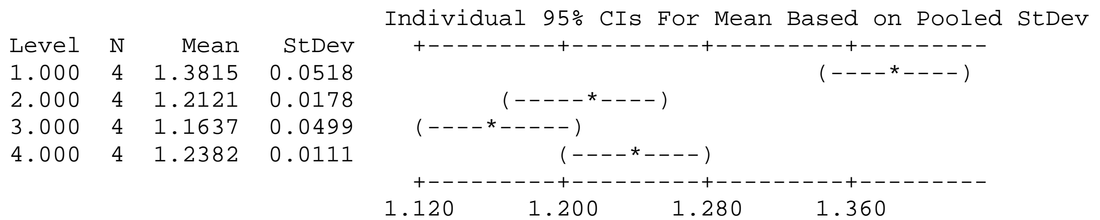
```
                                  0.180     0.210     0.240     0.270

Pooled StDev = 0.02565
```

Endpoint reading

- *E. coli* without glycerol

One-way ANOVA: end reading e.coli versus group e.coli

```
Source        DF       SS       MS      F      P
group e.coli   3  0.10530  0.03510  25.00  0.000
Error         12  0.01685  0.00140
Total         15  0.12215

S = 0.03747   R-Sq = 86.21%   R-Sq(adj) = 82.76%
```

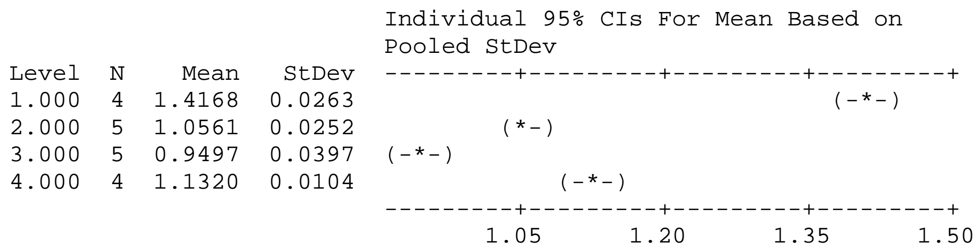


```
                          Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev    ---------+---------+---------+---------
1.000  4  1.3815  0.0518                               (----*----)
2.000  4  1.2121  0.0178          (-----*----)
3.000  4  1.1637  0.0499    (----*-----)
4.000  4  1.2382  0.0111              (----*----)
                            ---------+---------+---------+---------
                          1.120     1.200     1.280     1.360

Pooled StDev = 0.0375
```

- *E. coli* with glycerol

One-way ANOVA: end reading e.coli/g versus group e.coli/g

```
Source          DF        SS        MS       F      P
group e.coli/g   3  0.518229  0.172743  214.89  0.000
Error           14  0.011254  0.000804
Total           17  0.529483

S = 0.02835   R-Sq = 97.87%   R-Sq(adj) = 97.42%
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ---------+---------+---------+---------+
1.000  4  1.4168  0.0263                                (-*-)
2.000  5  1.0561  0.0252          (*-)
3.000  5  0.9497  0.0397  (-*-)
4.000  4  1.1320  0.0104              (-*-)
                          ---------+---------+---------+---------+
                                1.05      1.20      1.35      1.50

Pooled StDev = 0.0284
```

- *S. derby* without glycerol

One-way ANOVA: end reading s.der versus group s.der

```
Source       DF        SS        MS      F      P
group s.der   3  0.020941  0.006980  22.47  0.000
Error         9  0.002796  0.000311
Total        12  0.023736

S = 0.01762   R-Sq = 88.22%   R-Sq(adj) = 84.30%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ----+---------+---------+---------+-----
1.000  3  1.2406  0.0205    (-----*------)
2.000  3  1.2337  0.0210  (-----*------)
3.000  3  1.2400  0.0153    (-----*------)
4.000  4  1.3249  0.0142                          (-----*----)
                          ----+---------+---------+---------+-----
                            1.225     1.260     1.295     1.330

Pooled StDev = 0.0176
```

- *S. derby* without glycerol

One-way ANOVA: end reading s.der/g versus group s.der/g

```
Source         DF        SS        MS      F      P
group s.der/g   3  0.099880  0.033293  42.93  0.000
Error          11  0.008531  0.000776
Total          14  0.108411

S = 0.02785   R-Sq = 92.13%   R-Sq(adj) = 89.98%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --------+---------+---------+---------+-
1      4  1.2030  0.0367                (--*---)
2      4  1.0880  0.0330  (---*---)
3      4  1.1198  0.0055      (---*---)
4      3  1.3098  0.0236                          (----*---)
                          --------+---------+---------+---------+-
                                1.120     1.200     1.280     1.360

Pooled StDev = 0.0278
```

- *L. monocytogenes* without glycerol

One-way ANOVA: end reading l.mono versus group l.mono

```
Source        DF        SS        MS      F      P
group l.mono   3  0.095090  0.031697  48.08  0.000
Error         13  0.008571  0.000659
Total         16  0.103660

S = 0.02568   R-Sq = 91.73%   R-Sq(adj) = 89.82%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ---+---------+---------+---------+------
1.000  3  1.4749  0.0271                            (----*---)
2.000  5  1.2830  0.0255     (--*---)
3.000  5  1.2666  0.0310  (---*--)
```

```
4.000  4  1.3465  0.0147             (---*---)
                           ---+---------+---------+---------+------
                            1.260     1.330     1.400     1.470

Pooled StDev = 0.0257
```

- *L. monocytogenes* with glycerol

One-way ANOVA: end reading l.mono/g versus group l.mono/g

```
Source          DF        SS        MS       F      P
group l.mono/g   3  0.225404  0.075135  103.29  0.000
Error           13  0.009456  0.000727
Total           16  0.234860

S = 0.02697   R-Sq = 95.97%   R-Sq(adj) = 95.04%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --+---------+---------+---------+-------
1.000  4  1.5001  0.0448                               (--*--)
2.000  4  1.2328  0.0215    (--*--)
3.000  5  1.2062  0.0207  (--*-)
4.000  4  1.2715  0.0104        (--*--)
                          --+---------+---------+---------+-------
                          1.20      1.30      1.40      1.50

Pooled StDev = 0.0270
```

- *S. aureus* without glycerol

One-way ANOVA: growth rate s.au versus group s.au

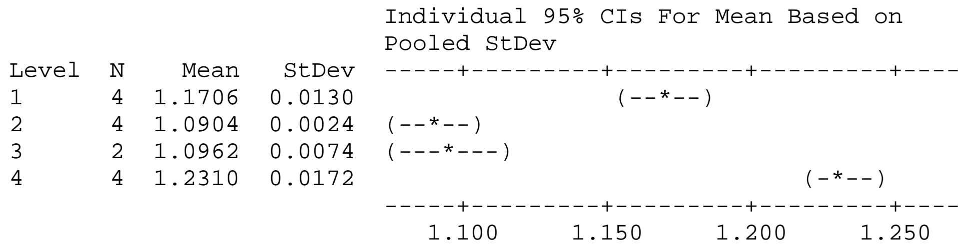
```
Source      DF        SS        MS     F      P
group s.au   3  0.011293  0.003764  8.22  0.005
Error       10  0.004582  0.000458
Total       13  0.015875

S = 0.02141   R-Sq = 71.14%   R-Sq(adj) = 62.48%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
1.000  3  0.23751  0.00493                         (-------*-------)
2.000  4  0.17302  0.00997       (-----*------)
3.000  3  0.18782  0.00945          (-------*-------)
4.000  4  0.16026  0.03677   (------*------)
                             -+---------+---------+---------+--------
                            0.140     0.175     0.210     0.245

Pooled StDev = 0.02141
```

- *S. aureus* with glycerol

One-way ANOVA: end reading s.au/g versus group s.au/g

```
Source        DF        SS        MS       F      P
group s.au/g   3  0.047663  0.015888  108.15  0.000
Error         10  0.001469  0.000147
```

```
Total         13  0.049132

S = 0.01212   R-Sq = 97.01%   R-Sq(adj) = 96.11%
```



```
                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N    Mean   StDev   -----+---------+---------+---------+----
1      4  1.1706  0.0130                   (--*--)
2      4  1.0904  0.0024   (--*--)
3      2  1.0962  0.0074   (---*---)
4      4  1.2310  0.0172                                (-*--)
                           -----+---------+---------+---------+----
                              1.100     1.150     1.200     1.250

Pooled StDev = 0.0121
```

5.3.3.4 Effect of incubation of free cells in SCF (comparison of "strains" 1 and 9)

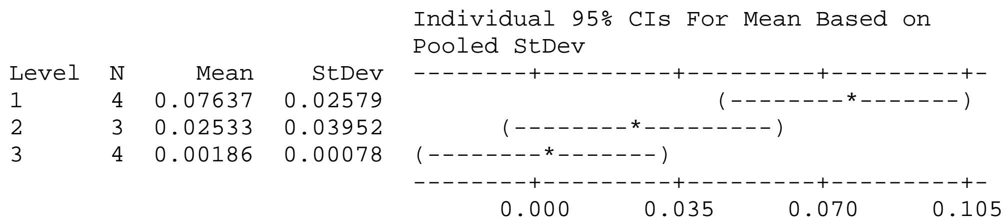
Level	Group
1	BHI
2	Original ("strain" 1)
3	Free SCF("strain" 9)

Growth rate

- *E. coli* without glycerol

One-way ANOVA: growth rate e.coli versus group e.coli

```
Source        DF        SS        MS     F      P
group e.coli   2  0.011516  0.005758  8.99  0.009
Error          8  0.005122  0.000640
Total         10  0.016638

S = 0.02530   R-Sq = 69.22%   R-Sq(adj) = 61.52%
```



```
                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N     Mean    StDev   --------+---------+---------+---------+-
1      4  0.07637  0.02579                        (--------*-------)
2      3  0.02533  0.03952         (--------*---------)
3      4  0.00186  0.00078   (--------*-------)
                             --------+---------+---------+---------+-
                                 0.000     0.035     0.070     0.105

Pooled StDev = 0.02530
```

- *E. coli* with glycerol

One-way ANOVA: growth rate e.coli/g versus group e.coli/g

```
Source          DF       SS       MS     F      P
group e.coli/g   2  0.00119  0.00059  0.51  0.618
Error            9  0.01051  0.00117
```

```
Total           11  0.01169

S = 0.03417   R-Sq = 10.14%   R-Sq(adj) = 0.00%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
1      4  0.04765  0.01982       (------------*------------)
2      4  0.03691  0.04129   (------------*------------)
3      4  0.06120  0.03747          (-----------*------------)
                             -+---------+---------+---------+--------
                            0.000     0.030     0.060     0.090

Pooled StDev = 0.03417
```

- *S. derby* without glycerol

One-way ANOVA: growth rate versus group s.derby

```
Source         DF        SS        MS      F      P
group s.derby   2  0.016981  0.008490  19.95  0.000
Error           9  0.003830  0.000426
Total          11  0.020811

S = 0.02063   R-Sq = 81.60%   R-Sq(adj) = 77.51%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  ---+---------+---------+---------+------
1      7  0.31373  0.02280                               (----*----)
2      2  0.23320  0.00453  (---------*--------)
3      3  0.24048  0.01857      (-------*------)
                            ---+---------+---------+---------+------
                             0.210     0.245     0.280     0.315

Pooled StDev = 0.02063
```

- *S. derby* with glycerol

One-way ANOVA: growth rate s.derby/g versus group s.derby/g

```
Source           DF        SS        MS      F      P
group s.derby/g   2  0.019478  0.009739  36.24  0.003
Error             4  0.001075  0.000269
Total             6  0.020553

S = 0.01639   R-Sq = 94.77%   R-Sq(adj) = 92.16%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -----+---------+---------+---------+----
1      3  0.31407  0.02053                      (----*----)
2      2  0.20498  0.01305  (-----*-----)
3      2  0.21012  0.00785   (-----*-----)
                            -----+---------+---------+---------+----
                               0.200     0.250     0.300     0.350

Pooled StDev = 0.01639
```

- *L. monocytogenes* without glycerol

One-way ANOVA: growth rate l.mono versus group l.mono

```
Source         DF        SS        MS     F      P
group l.mono    2  0.000449  0.000224  0.90  0.438
Error          10  0.002497  0.000250
Total          12  0.002946

S = 0.01580   R-Sq = 15.24%   R-Sq(adj) = 0.00%


                            Individual 95% CIs For Mean Based on Pooled StDev
Level  N     Mean    StDev    +---------+---------+---------+---------
1      6  0.12619  0.02024                 (-----------*-----------)
2      3  0.11692  0.00338    (----------------*----------------)
3      4  0.11310  0.01193    (-------------*--------------)
                              +---------+---------+---------+---------
                            0.096     0.108     0.120     0.132

Pooled StDev = 0.01580
```

- *L. monocytogenes* with glycerol

One-way ANOVA: growth rate l.mono/g versus group l.mon/g

```
Source         DF        SS        MS     F      P
group l.mon/g   2  0.000563  0.000281  0.40  0.690
Error           6  0.004273  0.000712
Total           8  0.004836

S = 0.02669   R-Sq = 11.64%   R-Sq(adj) = 0.00%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -----+---------+---------+---------+----
1      3  0.11781  0.02285         (--------------*--------------)
2      3  0.11324  0.02583       (--------------*--------------)
3      3  0.09922  0.03078  (--------------*--------------)
                            -----+---------+---------+---------+----
                               0.075     0.100     0.125     0.150

Pooled StDev = 0.02669
```

- *S.aureus* without glycerol

One-way ANOVA: growth rate s.au versus group s.au

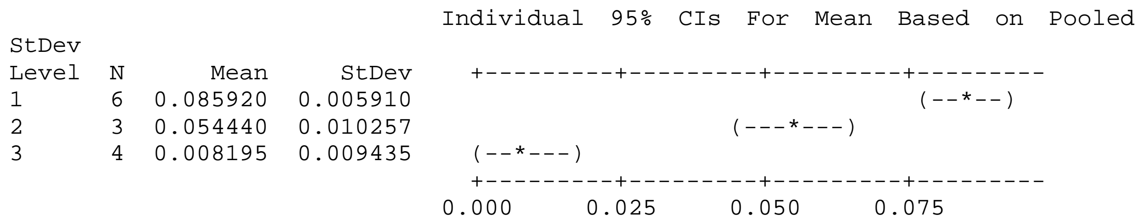
```
Source      DF         SS         MS      F      P
group s.au   2  0.0028437  0.0014219  25.82  0.001
Error        6  0.0003304  0.0000551
Total        8  0.0031741

S = 0.007420   R-Sq = 89.59%   R-Sq(adj) = 86.12%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  ---+---------+---------+---------+------
1      2  0.16742  0.01346                      (------*-----)
2      4  0.12366  0.00694  (----*---)
3      3  0.12619  0.00156   (----*----)
                            ---+---------+---------+---------+------
```

```
                            0.120     0.140     0.160     0.180

Pooled StDev = 0.00742
```

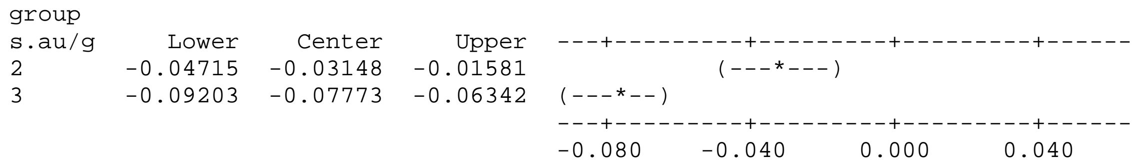
- *S.aureus* with glycerol

One-way ANOVA: growth rate s.au/g versus group s.au/g

```
Source         DF         SS         MS       F      P
group s.au/g    2  0.0144992  0.0072496  111.17  0.000
Error          10  0.0006521  0.0000652
Total          12  0.0151513

S = 0.008075   R-Sq = 95.70%   R-Sq(adj) = 94.84%
```



```
                              Individual 95% CIs For Mean Based on Pooled
StDev
Level  N      Mean     StDev    +---------+---------+---------+---------
1      6  0.085920  0.005910                                 (--*--)
2      3  0.054440  0.010257                    (---*---)
3      4  0.008195  0.009435    (--*---)
                                +---------+---------+---------+---------
                              0.000     0.025     0.050     0.075

Pooled StDev = 0.008075


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of group s.au/g

Individual confidence level = 97.93%


group s.au/g = 1 subtracted from:

group
s.au/g     Lower    Center     Upper  ---+---------+---------+---------+------
2       -0.04715  -0.03148  -0.01581             (---*---)
3       -0.09203  -0.07773  -0.06342  (---*--)
                                      ---+---------+---------+---------+------
                                      -0.080    -0.040     0.000     0.040
```



```
group s.au/g = 2 subtracted from:
```

Ending point reading

- *E. coli* without glycerol

One-way ANOVA: end reading e.coli versus group e.coli

```
Source        DF        SS        MS      F      P
group e.coli   2  0.082591  0.041296  90.74  0.000
Error          8  0.003641  0.000455
Total         10  0.086232

S = 0.02133   R-Sq = 95.78%   R-Sq(adj) = 94.72%
```

```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ---+---------+---------+---------+------
1      4  1.1471  0.0180  (---*---)
2      3  1.3169  0.0088                            (---*----)
3      4  1.3341  0.0290                               (---*---)
                          ---+---------+---------+---------+------
                           1.140     1.200     1.260     1.320

Pooled StDev = 0.0213
```

- *E. coli* with glycerol

One-way ANOVA: end reading e.coli/g versus group e.coli/g

```
Source          DF       SS       MS      F      P
group e.coli/g   2  0.02826  0.01413  11.22  0.004
Error            9  0.01134  0.00126
Total           11  0.03959

S = 0.03549   R-Sq = 71.37%   R-Sq(adj) = 65.00%


                          Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev   -+---------+---------+---------+--------
1      4  1.1526  0.0340                         (--------*-------)
2      4  1.0340  0.0459   (-------*-------)
3      4  1.0998  0.0227                (-------*-------)
                           -+---------+---------+---------+--------
                          1.000     1.050     1.100     1.150

Pooled StDev = 0.0355
```

- *S. derby* without glycerol

One-way ANOVA: end reading s.der versus group s.der

```
Source       DF       SS       MS     F      P
group s.der   2  0.00413  0.00207  0.66  0.538
Error         9  0.02799  0.00311
Total        11  0.03212

S = 0.05577   R-Sq = 12.87%   R-Sq(adj) = 0.00%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ----+---------+---------+---------+-----
1      5  1.1852  0.0157  (----------*----------)
2      3  1.2252  0.0660      (--------------*--------------)
3      4  1.2209  0.0781        (-----------*------------)
                          ----+---------+---------+---------+-----
                            1.150     1.200     1.250     1.300

Pooled StDev = 0.0558
```

- *S. derby* with glycerol

One-way ANOVA: end reading/g versus group s.der

```
Source       DF       SS       MS      F      P
group s.der   2  0.06889  0.03445  14.21  0.002
```

```
Error          9  0.02182  0.00242
Total         11  0.09071

S = 0.04924   R-Sq = 75.95%   R-Sq(adj) = 70.60%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -------+---------+---------+---------+--
1      5  1.1544  0.0079                           (------*------)
2      3  0.9949  0.0856  (--------*--------)
3      4  1.0055  0.0480     (-------*-------)
                          -------+---------+---------+---------+--
                               0.980     1.050     1.120     1.190

Pooled StDev = 0.0492
```

- *L. monocytogenes* without glycerol

One-way ANOVA: end reading l.mono versus group l.mono

```
Source         DF        SS        MS      F      P
group l.mono    2  0.090846  0.045423  61.23  0.000
Error          10  0.007418  0.000742
Total          12  0.098264

S = 0.02724   R-Sq = 92.45%   R-Sq(adj) = 90.94%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  --------+---------+---------+---------+-
1      6  0.94410  0.03323                               (---*---)
2      3  0.79753  0.01643       (-----*-----)
3      4  0.76375  0.02126  (----*----)
                            --------+---------+---------+---------+-
                                  0.780     0.840     0.900     0.960

Pooled StDev = 0.02724
```

- *L. monocytogenes* with glycerol

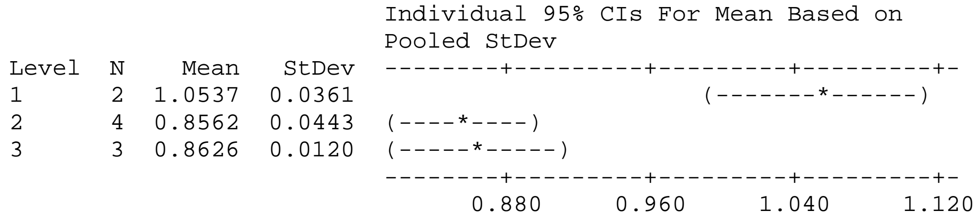
One-way ANOVA: end reading l.mon/g versus group l.mon/g

```
Source          DF        SS        MS      F      P
group l.mon/g    2  0.011614  0.005807  11.47  0.009
Error            6  0.003039  0.000506
Total            8  0.014653

S = 0.02251   R-Sq = 79.26%   R-Sq(adj) = 72.35%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -------+---------+---------+---------+--
1      3  0.93017  0.01999                        (-------*------)
2      3  0.90123  0.00659                (-------*-------)
3      3  0.84373  0.03281  (-------*-------)
                            -------+---------+---------+---------+--
                                 0.840     0.880     0.920     0.960

Pooled StDev = 0.02251
```

- *S.aureus* without glycerol

One-way ANOVA: end reading s.au versus group s.au

```
Source      DF       SS       MS      F      P
group s.au   2  0.05905  0.02953  23.68  0.001
Error        6  0.00748  0.00125
Total        8  0.06653

S = 0.03531   R-Sq = 88.75%   R-Sq(adj) = 85.01%
```

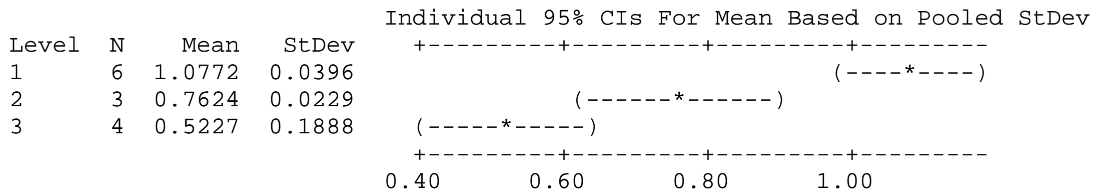


```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --------+---------+---------+---------+-
1      2  1.0537  0.0361                      (-------*------)
2      4  0.8562  0.0443  (----*----)
3      3  0.8626  0.0120  (-----*-----)
                          --------+---------+---------+---------+-
                                0.880     0.960     1.040     1.120

Pooled StDev = 0.0353
```

- *S.aureus* with glycerol

One-way ANOVA: end reading s.au/g versus group s.au/g

```
Source          DF      SS      MS      F      P
group s.au/g     2  0.7578  0.3789  32.71  0.000
Error           10  0.1159  0.0116
Total           12  0.8737

S = 0.1076   R-Sq = 86.74%   R-Sq(adj) = 84.09%
```



```
                          Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev    +---------+---------+---------+---------
1      6  1.0772  0.0396                                 (----*----)
2      3  0.7624  0.0229              (------*------)
3      4  0.5227  0.1888    (-----*-----)
                            +---------+---------+---------+---------
                          0.40      0.60      0.80      1.00

Pooled StDev = 0.1076
```

Comparison the immobilization effect with the effect of incubating the immobilized "strain" in different simulated gastrointestinal fluids, and freeze-drying storage of the immobilized cells

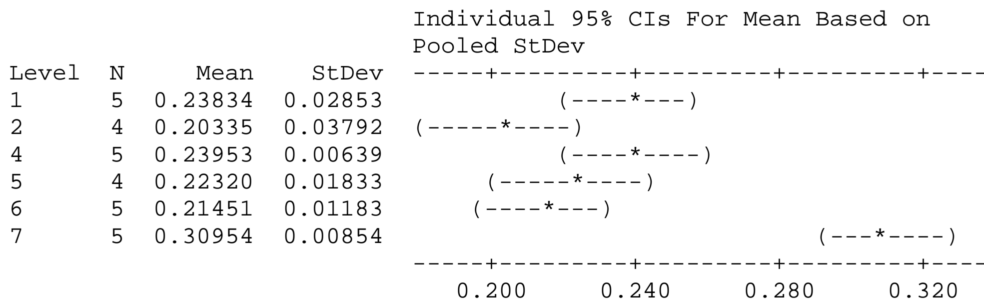
Level	Group
1	Immobilized "strain"
2	Recovered "strain"
4	SGF
5	SIF
6	SCF
7	Freeze-dried combined with long term storage

Growth rate

- *E. coli* without glycerol

One-way ANOVA: growth rate e.coli versus group e.coli

```
Source        DF        SS        MS      F      P
group e.coli   5  0.033943  0.006789  15.57  0.000
Error         22  0.009593  0.000436
Total         27  0.043536

S = 0.02088   R-Sq = 77.97%   R-Sq(adj) = 72.96%
```

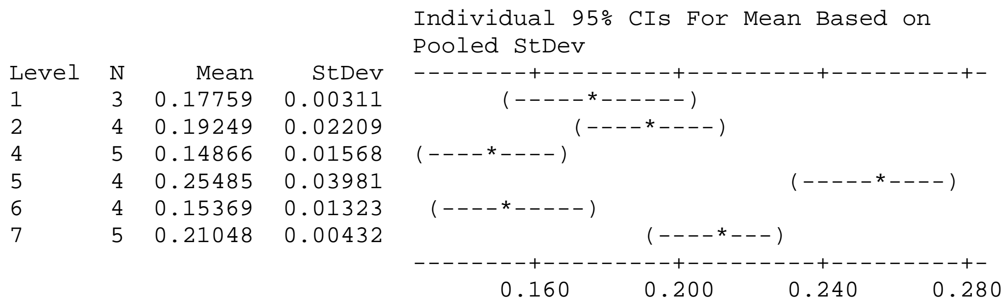


```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -----+---------+---------+---------+----
1      5  0.23834  0.02853            (----*---)
2      4  0.20335  0.03792  (-----*----)
4      5  0.23953  0.00639            (----*----)
5      4  0.22320  0.01833       (-----*----)
6      5  0.21451  0.01183      (----*---)
7      5  0.30954  0.00854                             (---*----)
                            -----+---------+---------+---------+----
                               0.200     0.240     0.280     0.320

Pooled StDev = 0.02088
```

- *E. coli* with glycerol

One-way ANOVA: growth rate e.coli/g versus group e.coli/g

```
Source          DF        SS        MS      F      P
group e.coli/g   5  0.033212  0.006642  16.14  0.000
Error           19  0.007821  0.000412
Total           24  0.041034

S = 0.02029   R-Sq = 80.94%   R-Sq(adj) = 75.92%
```



```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  --------+---------+---------+---------+-
1      3  0.17759  0.00311        (-----*------)
2      4  0.19249  0.02209             (----*----)
4      5  0.14866  0.01568  (----*----)
5      4  0.25485  0.03981                           (-----*----)
6      4  0.15369  0.01323   (----*-----)
7      5  0.21048  0.00432                 (----*---)
                            --------+---------+---------+---------+-
                                  0.160     0.200     0.240     0.280

Pooled StDev = 0.02029
```

- *S. derby* without glycerol

One-way ANOVA: growth rate s.derby versus group s.derby

```
Source         DF        SS        MS     F      P
group s.derby   5  0.009194  0.001839  5.50  0.005
Error          14  0.004678  0.000334
Total          19  0.013872

S = 0.01828   R-Sq = 66.28%   R-Sq(adj) = 54.24%


                            Individual 95% CIs For Mean Based on Pooled StDev
Level  N     Mean    StDev  ---------+---------+---------+---------+
1.000  3  0.19224  0.01199        (------*-------)
2.000  3  0.19263  0.00785        (------*-------)
4.000  4  0.19348  0.00861         (-----*------)
5.000  4  0.22250  0.03030                   (-----*------)
6.000  3  0.17547  0.02463  (------*-------)
7.000  3  0.24117  0.00621                        (------*-------)
                            ---------+---------+---------+---------+
                                   0.180     0.210     0.240     0.270

Pooled StDev = 0.01828
```

- *S. derby* with glycerol

One-way ANOVA: growth rate s.der/g versus group s.derby/g

```
Source           DF         SS         MS       F      P
group s.derby/g   5  0.1144871  0.0228974  342.16  0.000
Error            13  0.0008700  0.0000669
Total            18  0.1153570

S = 0.008180   R-Sq = 99.25%   R-Sq(adj) = 98.96%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev   -+---------+---------+---------+--------
1      4  0.00120  0.00067   (*-)
2      2  0.09232  0.01468                 (-*-)
4      3  0.07728  0.00540               (-*-)
5      3  0.18806  0.01332                               (*-)
6      3  0.09337  0.00906                  (-*)
7      4  0.21519  0.00504                                   (-*)
                             -+---------+---------+---------+--------
                            0.000     0.060     0.120     0.180

Pooled StDev = 0.00818
```

- *L. monocytogenes* with glycerol

One-way ANOVA: growth rate l.mono versus group l.mono

```
Source        DF        SS        MS      F      P
group l.mono   5  0.031920  0.006384  15.69  0.000
Error         20  0.008140  0.000407
Total         25  0.040060

S = 0.02017   R-Sq = 79.68%   R-Sq(adj) = 74.60%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev   -+---------+--------+--------+--------
1      5  0.21707  0.00424    (---*----)
```

```
2      3  0.22061  0.00800   (-----*-----)
4      5  0.22122  0.01737     (---*----)
5      4  0.22329  0.02331     (----*----)
6      5  0.21975  0.03319    (----*----)
7      4  0.31721  0.01524                            (----*-----)
                             -+---------+---------+---------+--------
                            0.200     0.240     0.280     0.320

Pooled StDev = 0.02017
```

- *L. monocytogenes* with glycerol

One-way ANOVA: growth rate l.mono/g versus group l.mono/g

```
Source           DF        SS        MS      F      P
group l.mono/g    5  0.087834  0.017567  39.47  0.000
Error            16  0.007121  0.000445
Total            21  0.094956

S = 0.02110   R-Sq = 92.50%   R-Sq(adj) = 90.16%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  ----+---------+---------+---------+-----
1      3  0.12168  0.00279     (----*-----)
2      3  0.14058  0.02181         (----*----)
4      3  0.10491  0.02155  (----*----)
5      4  0.25362  0.01822                             (----*---)
6      5  0.12035  0.02907      (---*---)
7      4  0.24946  0.01682                            (----*---)
                            ----+---------+---------+---------+-----
                              0.100     0.150     0.200     0.250

Pooled StDev = 0.02110
```

- *S. aureus* without glycerol

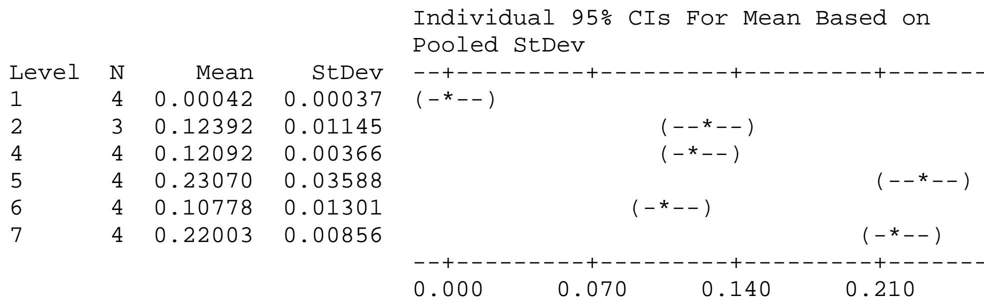
One-way ANOVA: growth rate s.au versus group s.au

```
Source      DF        SS        MS     F      P
group s.au   5  0.017466  0.003493  3.89  0.022
Error       13  0.011685  0.000899
Total       18  0.029151

S = 0.02998   R-Sq = 59.91%   R-Sq(adj) = 44.50%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -----+---------+---------+---------+----
1.000  3  0.11459  0.00846  (-------*------)
2.000  4  0.13702  0.03752        (-----*------)
4.000  3  0.12151  0.02497    (------*-------)
5.000  4  0.16026  0.03677            (-----*------)
6.000  3  0.11529  0.02944   (------*-------)
7.000  2  0.21417  0.01683                   (--------*--------)
                            -----+---------+---------+---------+----
                               0.100     0.150     0.200     0.250

Pooled StDev = 0.02998
```

- *S. aureus* with glycerol

One-way ANOVA: growth rate s.au/g versus group s.au/g

```
Source         DF        SS        MS      F      P
group s.au/g    5  0.142125  0.028425  98.79  0.000
Error          17  0.004892  0.000288
Total          22  0.147017

S = 0.01696   R-Sq = 96.67%   R-Sq(adj) = 95.69%
```



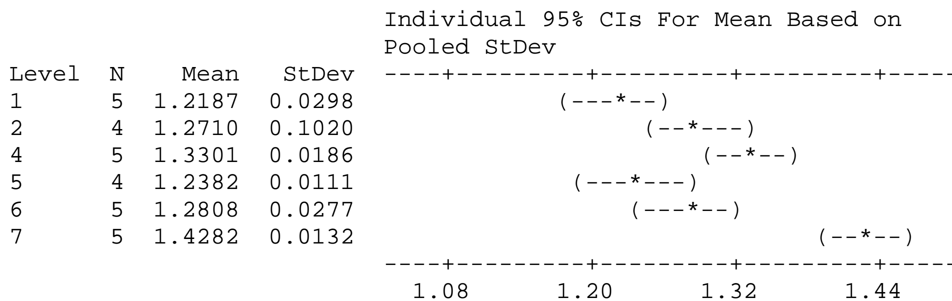
```
                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  --+---------+---------+---------+-------
1      4  0.00042  0.00037  (-*--)
2      3  0.12392  0.01145                 (--*--)
4      4  0.12092  0.00366                 (-*--)
5      4  0.23070  0.03588                                (--*--)
6      4  0.10778  0.01301               (-*--)
7      4  0.22003  0.00856                               (-*--)
                            --+---------+---------+---------+-------
                            0.000     0.070     0.140     0.210

Pooled StDev = 0.01696
```

Endpoint reading

- *E. coli* without glycerol

One-way ANOVA: end reading e.coli versus group e.coli

```
Source         DF       SS       MS      F      P
group e.coli    5  0.31933  0.06387  34.88  0.000
Error          22  0.04029  0.00183
Total          27  0.35962

S = 0.04279   R-Sq = 88.80%   R-Sq(adj) = 86.25%
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ----+---------+---------+---------+-----
1      5  1.2187  0.0298             (---*--)
2      4  1.2710  0.1020                   (--*---)
4      5  1.3301  0.0186                       (--*--)
5      4  1.2382  0.0111              (---*---)
6      5  1.2808  0.0277                  (---*--)
7      5  1.4282  0.0132                               (--*--)
                          ----+---------+---------+---------+-----
                            1.08      1.20      1.32      1.44

Pooled StDev = 0.0428
```

- *E. coli* with glycerol

One-way ANOVA: end reading e.coli/g versus group e.coli/g

```
Source           DF        SS        MS       F      P
group e.coli/g    5  0.557838  0.111568  114.93  0.000
Error            20  0.019414  0.000971
Total            25  0.577253
```

```
S = 0.03116   R-Sq = 96.64%   R-Sq(adj) = 95.80%


                          Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev    +---------+---------+---------+---------
1      3  1.0381  0.0222                 (---*--)
2      5  1.0388  0.0575                  (--*-)
4      5  0.8719  0.0134    (--*-)
5      4  1.1320  0.0104                         (-*--)
6      4  0.9025  0.0299       (-*--)
7      5  1.2885  0.0193                                      (-*--)
                            +---------+---------+---------+---------
                          0.84      0.96      1.08      1.20

Pooled StDev = 0.0312
```

- *S. derby* without glycerol

One-way ANOVA: end reading s.derby versus group s.derby

```
Source          DF       SS       MS     F      P
group s.derby    5  0.01968  0.00394  3.57  0.027
Error           14  0.01544  0.00110
Total           19  0.03512

S = 0.03320   R-Sq = 56.04%   R-Sq(adj) = 40.34%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ------+---------+---------+---------+---
1.000  3  1.2601  0.0230  (-------*-------)
2.000  3  1.2838  0.0094       (-------*-------)
4.000  4  1.2930  0.0610         (-------*------)
5.000  4  1.3249  0.0142                (------*------)
6.000  3  1.2706  0.0288    (-------*-------)
7.000  3  1.3544  0.0196                     (-------*-------)
                          ------+---------+---------+---------+---
                              1.250     1.300     1.350     1.400

Pooled StDev = 0.0332
```

- *S. derby* with glycerol

One-way ANOVA: end reading s.derby/g versus group s.derby/g

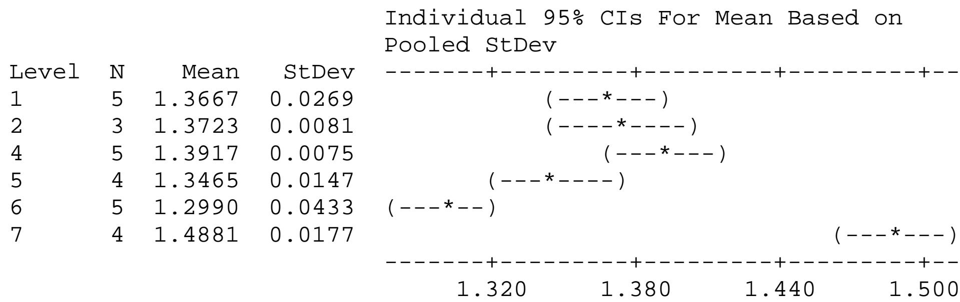
```
Source            DF       SS       MS       F      P
group s.derby/g    5  2.90575  0.58115  558.79  0.000
Error             13  0.01352  0.00104
Total             18  2.91927

S = 0.03225   R-Sq = 99.54%   R-Sq(adj) = 99.36%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --+---------+---------+---------+-------
1      4  0.2747  0.0083  (*)
2      2  0.9826  0.0246                       (-*)
4      3  1.0483  0.0385                          (*)
5      3  1.3098  0.0236                                  (-*)
6      3  1.1787  0.0646                              (*-)
7      4  1.3203  0.0098                                   (*)
                          --+---------+---------+---------+-------
                          0.30      0.60      0.90      1.20
```

```
Pooled StDev = 0.0322
```

- *L. monocytogenges* without glycerol

One-way ANOVA: end reading l.mono versus group l.mono

```
Source         DF        SS        MS      F      P
group l.mono    5  0.085034  0.017007  27.55  0.000
Error          20  0.012348  0.000617
Total          25  0.097382

S = 0.02485   R-Sq = 87.32%   R-Sq(adj) = 84.15%
```



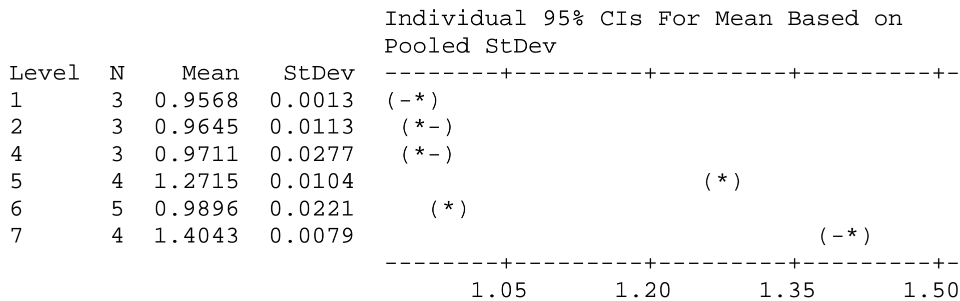
```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -------+---------+---------+---------+--
1      5  1.3667  0.0269             (---*---)
2      3  1.3723  0.0081             (----*----)
4      5  1.3917  0.0075                 (---*---)
5      4  1.3465  0.0147         (---*----)
6      5  1.2990  0.0433  (---*--)
7      4  1.4881  0.0177                               (---*---)
                          -------+---------+---------+---------+--
                               1.320     1.380     1.440     1.500
```

```
Pooled StDev = 0.0248
```

- *L. monocytogenes* with glycerol

One-way ANOVA: end reading l.mon/g versus group l.mono/g

```
Source           DF        SS        MS       F      P
group l.mono/g    5  0.659633  0.131927  495.60  0.000
Error            16  0.004259  0.000266
Total            21  0.663893

S = 0.01632   R-Sq = 99.36%   R-Sq(adj) = 99.16%
```



```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --------+---------+---------+---------+-
1      3  0.9568  0.0013  (-*)
2      3  0.9645  0.0113   (*-)
4      3  0.9711  0.0277   (*-)
5      4  1.2715  0.0104                       (*)
6      5  0.9896  0.0221     (*)
7      4  1.4043  0.0079                                (-*)
                          --------+---------+---------+---------+-
                               1.05      1.20      1.35      1.50
```

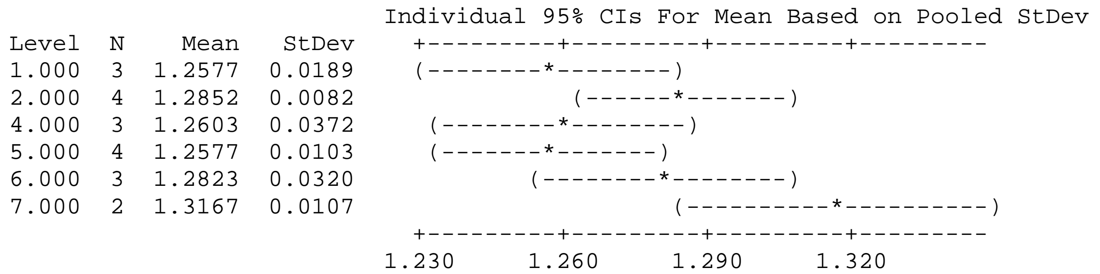
```
Pooled StDev = 0.0163
```

- *S. aureus* without glycerol

One-way ANOVA: end reading s.au versus group s.au

```
Source      DF        SS        MS     F      P
group s.au   5  0.006764  0.001353  2.85  0.059
Error       13  0.006169  0.000475
```

```
Total        18  0.012933

S = 0.02178   R-Sq = 52.30%   R-Sq(adj) = 33.96%
```

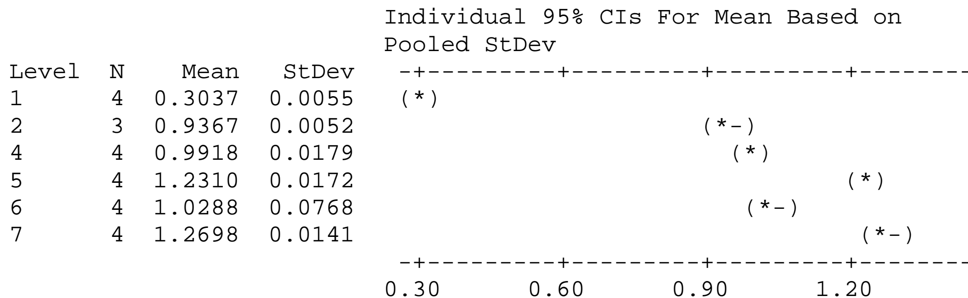


```
                          Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev    +---------+---------+---------+---------
1.000  3  1.2577  0.0189    (--------*--------)
2.000  4  1.2852  0.0082               (------*-------)
4.000  3  1.2603  0.0372     (--------*--------)
5.000  4  1.2577  0.0103     (-------*-------)
6.000  3  1.2823  0.0320            (--------*--------)
7.000  2  1.3167  0.0107                      (----------*----------)
                            +---------+---------+---------+---------
                          1.230     1.260     1.290     1.320

Pooled StDev = 0.0218
```

- *S. aureus* with glycerol

One-way ANOVA: end reading s.au/g versus group s.au/g

```
Source         DF       SS       MS       F      P
group s.au/g    5  2.42528  0.48506  406.34  0.000
Error          17  0.02029  0.00119
Total          22  2.44557

S = 0.03455   R-Sq = 99.17%   R-Sq(adj) = 98.93%
```

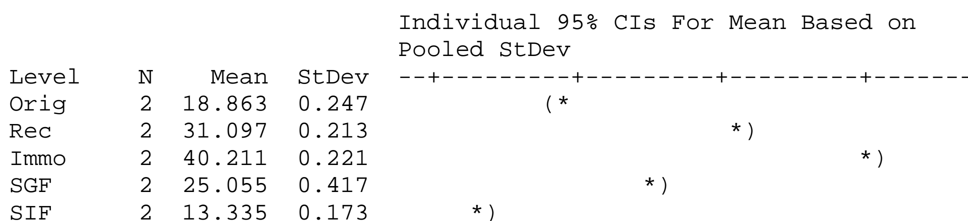


```
                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  -+---------+---------+---------+--------
1      4  0.3037  0.0055  (*)
2      3  0.9367  0.0052                     (*-)
4      4  0.9918  0.0179                       (*)
5      4  1.2310  0.0172                               (*)
6      4  1.0288  0.0768                        (*-)
7      4  1.2698  0.0141                                (*-)
                          -+---------+---------+---------+--------
                          0.30      0.60      0.90      1.20

Pooled StDev = 0.0346
```

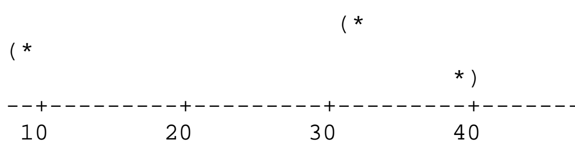
5.3.4 Diol dehydratase actibvity assay

One-way ANOVA: Orig, Rec, Immo, SGF, SIF, SCF, Freeze, SIF+SCF

```
Source  DF        SS       MS        F      P
Factor   7  1889.332  269.905  3586.49  0.000
Error    8     0.602    0.075
Total   15  1889.934

S = 0.2743   R-Sq = 99.97%   R-Sq(adj) = 99.94%
```



```
                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level    N    Mean  StDev  --+---------+---------+---------+-------
Orig     2  18.863  0.247            (*
Rec      2  31.097  0.213                        *)
Immo     2  40.211  0.221                                 *)
SGF      2  25.055  0.417                  *)
SIF      2  13.335  0.173       *)
```



```
SCF       2  31.633  0.031                        (*
Freeze    2   8.744  0.471  (*
SIF+SCF   2  39.230  0.141                                *)
                            --+---------+---------+---------+-------
                             10        20        30        40

Pooled StDev = 0.274
```