An *in vitro* antimicrobial and safety study of *Lactobacillus reuteri* DPC16 for validation of probiotic concept

A thesis presented in partial fulfilment of the requirements for the degree of Master of Technology in Biotechnology at Massey University, Auckland, New Zealand.

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(2008)

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Abstract

Based on previous studies of the novel *Lactobacillus reuteri* DPC16 strain, an *in vitro* investigation on the supernatant antimicrobial activity and the culture safety against normal gastrointestinal microflora and gastric mucus was done in this thesis.

DPC16 cell-free supernatants (fresh and freeze-dried, designated as MRSc and FZMRSc) from anaerobic incubations in pre-reduced MRS broth, have shown significant inhibitory effects against selected pathogens, including *Salmonella* Typhimurium, *E. coli* O157:H7, *Staphylococcus aureus*, and *Listeria monocytogenes*. These effects were mainly due to the acid production during incubation as evidenced by the negation of such activity from their pH-neutral counterparts, and this acidic effect was shown to reduce the pathogen growth rate and decrease the total number of pathogen cells.

By incubation of concentrated (11 g/L) resting cells in glycerol-supplemented MRS broth, another DPC16 cell-free supernatant (designated as MRSg) has shown very strong antimicrobial effect against all target pathogens. As indicated by a kinetic profile, this activity developed in a sigmoidal fashion as incubation proceeded, reaching to maximum activity after 6-8h and maintained at the same level thereafter. Further study has shown that the antimicrobial activity of this supernatant was pH-independent, effective across a pH range of 4.6 to 6.5, and acted on both Gram-negative and Gram-positive pathogens. Using the minimum effective dose, a time course investigation has provided evidence that this supernatant affected the growth of the target pathogens by elongating the lag phase and lowering the total cell number at the end of the incubation. Lastly, it was found that the strong antimicrobial effect of MRSg was bactericidal at high concentrations and bacteriostatic at low concentrations. However, it also found that the viability of DPC16 cells also decreased as incubation prolonged, which suggests that this glycerol-derived supernatant had a lethal effect to its own cells. Nevertheless, this lethal effect was exerted to a much lesser extent compared with that to the pathogens.

The last DPC16 cell-free supernatant was designated as SGF, which was produced from secondary fermentation of the same resting cells in glycerol-water. SGF did not show a

significant antimicrobial activity, which suggests that this specific strain is not capable of utilising glycerol in the absence of fermentable carbohydrates.

The antimicrobial activity found in MRSg matched with previously published characteristics of reuterin, which is a unique antimicrobial substance synthesised by *L. reuteri* when incubated with glycerol. Therefore, a study on the production kinetics of reuterin by DPC16 was carried out. Supernatants of both MRSg and SGF were studied. Results showed that glycerol utilisation occurred in both MRSg and SGF, whereas the bioconversion of glycerol into reuterin was different. In MRSg, glycerol was constantly utilised by DPC16 resting cells, and by the end of an 18h incubation 85.8 mM of glycerol was utilised, where 72.8% was transformed into reuterin. The formation of reuterin initiated with an inclining production and reached the maximum rate of 10.9 mM/h after 6h of incubation, with the total production of 64 mM of reuterin at the end of the 18h incubation. This reuterin production in MRSg followed a similar pattern to that of its antimicrobial activity, which suggests a certain correlation between reuterin formation and the increase of antimicrobial activity in MRSg. Therefore, the major antimicrobial component in MRSg that accounted for its potent antimicrobial activity was very much likely to be reuterin. In SGF, however, detectable reuterin was negligible even though some glycerol may have been absorbed into the highly concentrated DPC16 resting cells. This has responded to the antimicrobial activity assay in that due to the lack of essential carbohydrate nutrient for normal cell metabolism, there was no glycerol utilisation and hence no reuterin synthesis.

Having studied the antimicrobial activity of *L. reuteri* DPC16 and the production of antimicrobial-competent reuterin, two safety issues (the impact on growth on other normal commensal probiotics and mucin degradation activity) of this strain were assessed to further evaluate its probiotic potential. By using similar *in vitro* assays as in the antimicrobial test, the same set of DPC16 supernatants have demonstrated no adverse effect on the growth of either *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, or *Bifidobacterium lactis* DR10. No stimulatory effect was found either. By incorporating purified porcine gastric mucin into classic mucin-degradation assays in both liquid and agar media, DPC16 has not exhibited the same mucinolytic activity as that of the faecal flora cultures. Thus, it can be concluded that *L. reuteri* DPC16 is as safe to the host as normal commensal microflora.

Acknowledgement

First of all, I wish to take this opportunity to express my most sincere gratitude to all my supervisors who have helped and supported me with the completion of this thesis. Without their advice and encouragement, this thesis would not have been possible.

In particular, I would like to thank my chief supervisor, Dr. Abdul Molan, for his professional guidance and continuous encouragement throughout the course of this thesis.

Equally, I am sincerely grateful to my co-supervisor, Professor Ian Maddox, for his long-term supervision, concerns and encouragements since 2005.

I will also thank Dr. Quan Shu, my third supervisor, for his leadership and for offering me this opportunity to study and research.

I wish to express my sincere gratitude to Bioactive Research New Zealand Ltd., who not only funded this research, but also provided all the stock bacteria strains and extensive laboratory supports.

I will also acknowledge my gratitude to Crop and Food Research at Mt. Albert Research Centre, Auckland, for their generous and kind support with lab facility. Specifically, I would thank Graham Fletcher, Graeme Summers, Douglas Rosendale, Muftikhar Ahmed, and the former laboratory manager Mr. Joseph Yoseph. Without their helps and kind supports, this thesis would not have been completed to the current extent. In addition, I would acknowledge my special thanks to Douglas Rosendale for not only advising me on my experiments but also proofreading the thesis.

I am also grateful to the colleagues and research fellows working at Bioactive Research, including the technical and production manager, Robert Peng, the PhD candidate Sabrina Tian and Sean Chen. The discussion forum we established has equipped me with knowledge and ideas in scientific discovery.

Finally, I would like to thank my family, my wife Sunny R. Han, my son David Bian, and my parents in China. Their endless love, support and encouragement were the fuel driving me towards the accomplishment of this research.

Abbreviations

3-HPA	3-hydroxypropionaldehyde
BbL	*B. lactis* DR10 culture
BHI	Brain heart infusion broth
EMP	Embden-Meyerhof pathway
FF	Faecal flora culture
FHEF	Facultative hetero-fermentation
FZMRSc	Aqueous solution of freeze-dried MRSc
GI	Gastrointestinal
HFF	Heat-killed faecal flora culture
LAB	Lactic acid bacteria
LR	*L. reuteri* DPC16 culture in pre-reduced MRS
LRg	*L. reuteri DPC16* glycerol fermentation
MAP	Modified atmosphere packaging
MIC	Minimum inhibition concentration
MRSc	DPC16 supernatant from incubation in MRS
MRSg	DPC16 supernatant derived from glycerol-MRS incubation
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced form of NAD+
OD	Optical density
OHEF	Obligate hetero-fermentation
OHOF	Obligate homo-fermentation
PBS	Phosphate buffered saline
PKP	Phosphoketolase pathway
PGM	Porcine gastric mucin
RPM	Revolution per minute
SCFA	Short chain fatty acids
SEM	Standard error of the mean
SGF	Sole glycerol ferment (DPC16 supernatant from glycerol-water fermentation)

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Chapter 1

General Introduction

Modern consumers are increasingly interested in their personal health, and this elevates their selection of the foods they eat beyond taste and attractiveness to a new level of being healthy and safe (Puupponen-Pimia *et al*., 2002). There is accumulating scientific evidence to support the concept that the maintenance of healthy gut microflora can provide protection against gastrointestinal disorder (Vanderhoof and Young, 1998; Salminen *et al*., 1998 b; Heselmans *et al*., 2005; Niv *et al.*, 2005). Probiotics with perceived health benefits have long been used in the food industry and now have gained greater interest in their potential to confer enhanced health and well-being for humans. The global market for probiotics products has entered an exponential phase of growth (BCC Research, 2005; Amagaze, 2008; Saxelin, 2008). Probiotic consumption in dairy products and the awareness of their health importance have a long history in New Zealand. The probiotic research focused on developing novel health-enhancing strains for New Zealand dairy industry has now stepped into a blooming era (Crittenden *et al*., 2005).

Lactobacillus reuteri DPC16 is a New Zealand patented probiotic strain (Shu and Liu, 2008), and has been intensively studied for function and production by Bioactive Research New Zealand Limited (BRNZ). Preliminary research has shown health-enhancing effects including anti-infection and immuno-modulation (Liu *et al*., 2004 a, b, c). Microencapsulation of live bacteria cells was also investigated, and it was found that microencapsulated DPC16 maintained good viability when incorporated into food ingredients (such as colostrum, whey protein, skim milk, propolis, seafood and plant extracts), hence process optimization using microencapsulation to extend shelf-life for products supplemented with this culture has been investigated (Joshi, 2005; Chang, 2006; Yin, 2007; Chen, 2007). Additionally, an industrial application has recently been examined, which involves incorporating DPC16 culture into modified atmosphere packaging (MAP) to control seafood pathogens and to extend shelf-life of seafood products (Lu, 2007).

However, to validate a probiotic concept, more characterisation of this novel strain remains to be done. For example, to evaluate the probiotic performance, a complete

antimicrobial assessment of this strain is essential. A previous study has shown that *L. reuteri* DPC16 has an antimicrobial activity against common food borne pathogens when co-cultured in modified MRS broth, and subsequent spent culture analysis revealed that such antimicrobial activity was non-proteinaceous, active at a wide range of pH values, insensitive to hydrogen peroxidase, and heat labile (Lu, 2007). These have all suggested the involvement of reuterin, a unique potent antimicrobial substance produced by *L. reuteri* species. However, in-depth investigations on DPC16 antimicrobial activity, such as establishing minimum inhibitory concentrations (MICs) and the way in which the reuterin potentially affects the growth kinetics of these pathogens have not been done. In addition, specific reuterin production kinetics of this strain during utilisation of glycerol is still unknown. Lastly, but most importantly, there has been no attempt made to assess the safety of this novel strain in human consumption in terms of its effect on the growth of other normal gut microflora and its potential to compromise the barrier function of the gastrointestinal (GI) epithelial mucus. According to the International Safety Guidelines (Mattila-Sandholm, 1999a; Mattila- Sandholm *et al*., 1999b), these safety assessments are critical for validation towards probiotic concept.

This research was built on previous findings on *Lactobacillus reuteri* and conducted as a continuing exploration focusing on fulfilling the above gaps. The main objective of this study was to provide evidence as partial contribution to the validation of this novel strain as a probiotic. The scope of this research covered the following:

- Antimicrobial activity of extracellular metabolites presented in cell-free supernatant against selected pathogens and other members of normal intestinal microflora.
- *In vitro* reuterin productivity and its relationship with corresponding antimicrobial activity.
- Safety as assessed by mucin-degradation against porcine gastric mucus, *in vitro*.

The literature relating to general probiotics concepts, the *L. reuteri* species and its antimicrobial function and safety assessments are reviewed in Chapter 2. Background of the interested *L. reuteri* DPC16 strain was also introduced. Based on the literature, hypotheses were proposed and a series of experiments was designed and conducted to test these hypotheses and to fulfil ultimate objectives of this thesis.

Chapter 2

Literature Review

2.1 Probiotics

Probiotics is a term derived from the Greek, meaning “for life”. Nobel laureate Elie Metchnikoff formulated the probiotic concept approximately 100 years ago. He proposed that consumption of certain ‘lactic bacilli’ would be beneficial to humans by maximizing health-promoting activities of the gastrointestinal microbiota and minimizing their potentially harmful effects (Metchnikoff, 1907; Casas and Dobrogosz, 2000). From then on, probiotics have been scientifically studied and its definition has evolved and emerged from the gradual progress of understanding. Lilley and Stillwell (1965) describe probiotics as substances secreted by one microorganism to stimulate the growth of another, as opposite to an antibiotic. Parker (1974) defined probiotics as organisms and substances which contribute to intestinal microbial balance, and he was the first to include microorganisms into the definition which is in the sense that it is used today. Later, Fuller (1989) recognized probiotics as “a live microbial feed supplement which beneficially affects the host (humans or animals) by improving its intestinal microbial balance”. Furthermore, the probiotics concept was broadened, as “a viable mono- or mixed- culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora” (Havenaar and Huis in’t Veld, 1992). The definition of probiotics continues to be improved, and was recently made official by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) (Araya *et al.*, 2001, 2002) as “live microorganisms which when administrated in adequate amounts confer a health benefit on the host”. Scientific findings are continuing, and the new challenge on the probiotics definition is that the beneficial effects do not have to come from viable probiotic cells; non-viable probiotics can also have health effects such as epithelial competitive exclusion, immune modulation and carcinogen binding in the host (Ouwehand and Salminen, 1998; Salminen *et al.*, 1999). Nowadays, world-wide interest in probiotics is increasing, and with continuous accumulation of knowledge the probiotics concept will surely enter a new era.

2.1.1 Common physiology of probiotics

Probiotic microorganisms are commonly from the bacterial genera of *Lactobacillus* and *Bifidobacterium*, which are also characterised as lactic acid bacteria (LAB). LAB are Gram-positive, non-sporulating, non-respiring, usually non-motile and catalase-negative bacteria (Wikipedia.org, 2008). LAB are able to adapt to grow under microaerophilic to strictly anaerobic conditions. However, they are nutritionally fastidious (heterotrophic), requiring carbohydrates, amino acids, nucleic acid derivatives and vitamins to maintain a sufficient viability (Aguirre and Collins, 1993; Klein *et al.*, 1998; Konings, 2002). LAB with probiotic activity are generally from GI microflora (Naidu *et al.*, 1999), which are acidoduric or acidophilic and share the common physiological characteristics of surviving low pH and/or bile in the GI tract (Fuller, 1989).

An essential feature of LAB is to produce lactic acid as the main end-product (>50% of sugar carbon) of metabolism, during which ATP is generated for subsequent biosynthetic purpose (Axelsson, 1998). Typical LAB metabolism, in brief, occurs *via* an anaerobic or facultatively anaerobic fermentation process, where available carbohydrate is metabolised. At the end of glycolysis, the internally-generated pyruvate acts as a hydrogen acceptor, instead of oxygen, to be directly reduced by a by-product NADH to produce lactic acid and NAD+. The resulting co-enzyme NAD+ is then recycled back to glycolysis (Elliott and Elliott, 2005).

However, variations in LAB metabolism exist due to strain-specific enzymes responsible for various metabolic pathways, and such metabolic flexibility has provided LAB with greater adaptation to their environment (Zaunmuller *et al.*, 2006). Practically, LAB are grouped into obligate homo-, facultative hetero-, or obligate hetero-fermentative organisms according to the metabolic pathway they use to ferment carbohydrate and to the end-products synthesised (Casas and Dobrogosz, 2000; Annuk *et al.*, 2003). The obligate homofermentative (OHOF) group (*e.g. L. acidophilus, L. delbrueckii, L. helveticus, L. salivarius*) use the Embden-Meyerhof pathway (EMP) and produce lactic acid exclusively as the end-product. The obligate hetero-fermentative (OHEF) group (*e.g. L. brevis, L. buchneri, L. fermentum, L. reuteri*) only metabolise through the phosphoketolase pathway, and produce significant amounts of other products such as ethanol, acetate and CO_2 in addition to lactic acid (Arskold *et al.*, 2008). The facultative hetero-fermentative (FHEF) group (*e.g., L. paracasei, L.*

curvatus, L. plantarum, L. lactis, and L. rhamnosus) can use either the Embden-Meyerhof pathway or the phosphoketolase pathway (Kleerebezem *et al.*, 2003). Additionally, various growth conditions (media composition, pH, temperature, and presence of oxygen) may as well significantly change the end-products by altering pyruvate metabolism and external electron acceptors (Axelsson, 1998; Annuk *et al*., 2003). For example, LAB are able to adapt to utilise a variety of other substances as ultimate electron acceptors (*e.g.* carbon dioxide, nitrite, sulphate, glycerol, and organic compounds) and of producing a spectrum of metabolic end-products.

2.1.2 Applications of probiotics

Acid-producing probiotics have been extensively used and play an important role in various fermented foods and beverages for thousands of years, as early as 6,000 B.C (Ouwehand and Vesterlund, 2004). During the last twenty years, with accumulated scientific evidence of health-enhancing effects to human, probiotics have been increasingly incorporated into functional foods and health supplements (Fuller and Perdigon, 2003). In addition, modern processing technologies such as freeze-drying, particle immobilization and microencapsulation have provided assurance for consumers that probiotics will retain viability and functionalities not only during manufacturing but also during consumption in the GI tract (Fuller, 1997; Saarela *et al.*, 2000; Chang, 2006; Yin, 2007). Probiotics are now available to consumers in forms of milk-based dairy products (*e.g.* yoghurts and cheese), tablets and freeze-dried powders. It is noteworthy that, with continuous progress in understanding probiotics, the focus of nutrition attributes has moved from homofermentative species (*e.g. L. acidophilus*) to heterofermentative species (*e.g. L. casei, L. plantarum, L. lactis, L. rhamnosus and Bifidobacterium species*), especially the obligate heterofermentative species such as *L. reuteri* which are major components of intestinal microflora and of more prominent efficacy (Speck *et al.*, 1993; Casas and Dobrogosz, 2000; Annuk *et al*., 2003).

2.2 Function of probiotics

2.2.1 Overview of probiotic functions

Probiotics are defined as possessing valuable factors contributing to the health and well-being of hosts, including humans. It is thought that consumption of a sufficient amount [more than 10^9 CFU (colony forming units) per day of probiotics will be sufficient to balance the human GI micro-ecosystem by replacing harmful pathogens and reinforcing the natural defence mechanisms (Ouwehand *et al.*, 2002). The reported health benefits of probiotics include:

- Physiological effects, including improvement of intestinal motility (Gibson *et al.*, 2003), alleviating colon disorder symptoms (Marteau *et al.*, 1990; Motta *et al.*, 1991; Mattila-Sandholm *et al.*, 1999 b; Marteau *et al.*, 2002; Saxelin *et al.*, 2005), and reduction of serum cholesterol and lipids (Gilliland and Walker, 1990; Naidu *et al.*, 1999);

- Antimicrobial effects such as competing for nutrients and adhesion sites with pathogens, lowering intestinal pH, producing short chain fatty acids (SCFA), and synthesizing extracellular bacteriocins (Gill, 2003);

- Immuno-modulatory effects of positively stimulating both humoral and cell-mediated immunity responses. Proven effects include enhancing production and circulation of serum antibodies, augmenting secretion of cytokines, improving effectiveness of ingested vaccines and restoring immune function in immuno-compromised hosts (Perdigon and Alvares, 1992; Gill, 1998, 2003; Gill *et al.*, 2000; Maassen *et al.*, 2000; Ezendam and van Loveren, 2006).

This review focuses on the antimicrobial effects of LAB cultures and their metabolites.

2.2.2 Antimicrobial activities

The antimicrobial activity of gut lactobacilli targets pathogens of both Gram-negative and Gram–positive type; and such function has been found to be very variable and strain-specific (Jacobsen *et al*., 1999).

So far, reported antimicrobial mechanisms involve both the whole bacterial cell and the cellular metabolites. A physiological mechanism considers the whole bacterial cell in competing for available binding sites and nutrients with pathogens. LAB cells commonly express cell surface hydrophobicity and specific surface proteins to facilitate coaggregation with either cells of the same strain or related species and with the host epithelia (Wadstrom *et al.*, 1987; Strus *et al.*, 2001). These adhesive features provide steric hindrance against other microorganisms, help to compete for available nutrients, and allow extracellular LAB metabolites to have access to the host epithelial surface (Ouwehand and Salminen, 1998; Tannock, 1999). Hence, the host epithelial surface is protected with a mucosal-associated shield from a variety of harmful invasions. Additionally, some studies also reported that some *Lactobacillus* species exert *in vitro* antimicrobial activity by coaggregating with pathogens, and creating a large contact area between bacterial membranes, hence resulting in increased impact of LAB antimicrobial metabolites to the coaggregated pathogens (Reid *et al*., 1988; Drago *et al.*, 1997).

The metabolites of LAB have shown a wide inhibitory spectrum against both Gram-positive and Gram-negative bacteria. Considering the large number of different groups of chemical compounds present in LAB metabolites, it is most likely that their antimicrobial activity is not attributable to one specific mechanism but to synergistic effects targeting various components of the cell. Possible composition of LAB metabolites include SCFA (*e.g.* lactate and acetate) which lower the local pH; strain-specific bacteriocins; hydrophobic organic compounds that allow partition through the phospholipid membrane; and strong oxidative agents such as hydrogen peroxide (Ouwehand and Vesterlund, 2004). Nevertheless, the general antimicrobial effect of LAB is due to the acid production, which reduces intestinal local pH and in turn limits the growth of pathogens. For example, with a dissociation constant (pK value) of 3.86 for lactic acid and 4.75 for acetic acid, these acids are partially in their undissociated form at lower pH values. These undissociated organic acids are lipophilic and are able to penetrate the bacterial cell membrane, and at higher intracellular pH values they dissociate to produce hydrogen ions that interfere with essential bacterial metabolic functions (Ingram *et al.*, 1956; Baird-Parker, 1980; Booth, 1985). Reports have shown that LAB-produced organic acids can work in combination to display a strong inhibitory activity against many food-borne pathogens, such as *Salmonella* Typhimurium, *E. coli, Bacillus cereus, Clostridium botulinum, Clostridium perfingens,*

Listeria monocytogenes, and *Staphylococcus aureus* (Goepfert and Hicks, 1969; Adams and Hall, 1988; Wong and Chen, 1988; Kao and Frazier, 1996; Alakomi *et al*., 2000; De Keersmaecker *et al.*, 2006).

Another important factor contributing to the overall inhibition efficacy is LAB bacteriocin, a strain-specific heterogeneous substance to inhibit competition for the same niche in the micro-ecological environment (Bennison, 2005). Bacteriocins are low-molecular weight, cationic, and amphiphilic molecules released extracellularly by predominately Gram-positive bacteria (Riley and Wertz, 2002; Ouwehand and Vesterlund, 2004). A widely accepted hypothesis for bacteriocin activity is that it firstly adsorbs to specific or non-specific receptors on the target cell surface, and then alters the membrane permeability, thus disturbing membrane transport, and finally inhibiting energy production (Jung, 1991; Nissen-Meyer *et al.*, 1992). Bacteriocins are different from antibiotics in several ways: bacteriocins are ribosomally synthesised peptides, their modes of action are mainly membrane-active, and they have a narrow killing spectrum by influencing only closely-related species (Abee *et al.*, 1995). Nowadays, many bacteriocins have been discovered, and they vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties. Nisin is the best-known bacteriocin produced by *Lactobacillus lactis*, and has been approved for use in various industries throughout the world (Hurst, 1981). Other bacteriocins produced by LAB include lactacin from *L. acidophilus*, pediocin from *Pediococcus acidilactici*, and enterocin from *Enterococcus faecium*. However, fewer bacteriocins are produced by *Bifidobacterium* than by *Lactobacillus* species (De Vuyst and Vandamme, 1994). Even though bacteriocin has been widely used in food preservation and medical applications, they are still limited by their narrow spectrum of inhibition, and extrinsic parameters such as pH tolerance and thermostability.

Furthermore, another group of smaller molecules has been reported to be associated with LAB antimicrobial activity. These are low molecular weight substances sharing the common characteristics of tolerance of low pH value, thermostability and exerting activity across a broad spectrum. These include the strong oxidative agent hydrogen peroxide generated through NADH oxidase and superoxide dismutase (Batdorj *et al*., 2007); carbon dioxide from heterofermentation to fortify the anaerobic environment; and diacetyl (if citrate is fermented) which interferes with the Gram-negative bacterial utilisation of arginine (Ouwehand and Vesterlund, 2004). Amongst this group, a

newly-found substance with wide antimicrobial spectrum has caught particular attention and attracted intensive studies over the last decade. Reuterin, produced by GI-autochthonous *L. reuteri* through dissimilating glycerol, has been reported extensively as a potent antimicrobial agent against both Gram-negative and Gram-positive bacteria, and lower eukaryotic organisms including yeast, fungi and protozoa (Talarico *et al.*, 1988; Axelsson *et al.*, 1989; Chung *et al.*, 1989; Glass *et al.*, 2004). Later in this chapter, a detailed review of reuterin will be covered in aspects of production, mode of action, and safety.

Therefore, the antimicrobial activity of probiotics relies on multiple factors, and these factors may work synergistically to contribute to the overall efficacy. However, it is preferred that probiotics exert antimicrobial activity against only pathogens. As it is defined, probiotics should confer benefit to the host; and thus they should not introduce any adverse effects.

2.3 Safety of Probiotics

2.3.1 Clinical infections

Given the long history of human consumption, LAB are generally regarded as safe (GRAS). Even though this longstanding observation remains as a sound safety record, occasional reports on certain strains of LAB in association with human clinical infections have been published (Bayer *et al.*, 1978; Aguirre and Collins, 1993). The first case of lactobacilli-associated endocarditis was reported by Marschall (1938). And with the rapid development of medical science and technology during the last decade (1992-2001), 16 cases of lactobacilli endocarditis have been reported, and a few other clinical infections such as liver abscess, bacteraemia, neonatal sepsis, meningitis, pneumonia and urinary tract infection have also been documented (Bayer *et al*, 1978; Mackay *et al.*, 1999; Rautio *et al.*, 1999; Land *et al.*, 2005). Most of the *Lactobacillus* strains isolated from clinical cases belong to the species of *L. rhamnosus, L. casei, L. rhamnosus,* and *L. acidophilus* (Abgrall *et al.*, 1997) However, all reported infections were extremely rare instances, and the responsible strains are all frequent inhabitants of human indigenous intestinal flora. And also, these cases were found among immune-compromised populations with underlying disease or predisposing conditions. To date, there has been no report of clinical LAB infection in healthy people (Gasser, 1994; Donohue, 2004).

Nevertheless, with the increasing range of novel strains being introduced to food and medical applications, the safety status of the new probiotics cannot be assumed (Donohue, 2004). Hence, a comprehensive assessment is essential to evaluate novel probiotics for safety status; also, a monitoring system is necessary to track any side effects that may arise.

2.3.2 Safety Guidelines for probiotics

In 1996, the European Union initiated a program of "Demonstration of Nutritional Functionality of Probiotic Foods" (Project PROBDEMO, Fair CT-96-1028), which provides guidelines and scientific verification for claims of probiotics and associated products (Mattila-Sandholm, 1999; Mattila-Sandholm *et al.*, 1999). This program, together with the guidelines by FAO/WHO (Araya *et al.*, 2001, 2002) and other published documents (Lee and Salminen, 1995; Donohue and Salminen, 1996; Salminen *et al.*, 1996, 1998 a, 1998b, 1999; Adams, 1999; Saarela *et al.*, 2000）has established a list of safety criteria for probiotics.

Firstly, the candidate strain must have clear identity and be distinguished from other strains by well-established analytical techniques (*e.g.* 16S ribosomal RNA identification) (Salminen *et al.*, 1999). Secondly, the probiotics must be compatible with the host intestinal environment, *i.e.* surviving, colonizing and being genetically stable through the host GI tract (Saarela *et al.*, 2000). Thirdly, the probiotic efficacy on other members of the intestinal flora and interaction with the host must be determined. For instance, the potential probiotics must be non-pathogenic and non-mutagenic, must not deconjugate bile salt, must not possess enzymatic properties of detrimental degradation of epithelial mucus in the GI tract, and most of all are not associated with colon diseases (Marteau *et al.*, 1995; Saarela *et al.*, 2000; Zhou *et al*, 2001; Fernandez *et al.*, 2005). Lastly, the probiotic strain must not carry horizontally transmissible antibiotic resistance genes (Salminen *et al.*, 1998a; Saarela *et al.*, 2000). Many lactobacilli naturally display a wide range of antibiotic resistance, but in most cases, such resistance is not of the transmissible type (Charteris *et al*, 1998). Above all, the probiotic strains are preferably from healthy individual origin of similar species, in other words, probiotics for human use are preferably isolated from the GI tract of healthy human (Saarela *et al.*, 2000).

2.4 *Lactobacillus reuteri*

2.4.1 Historical background

Lactobacillus reuteri was recorded in scientific classifications of lactic acid bacteria as early as the beginning of the 20th century (Orla-Jensen, 1919), though at that time it was undistinguished from *L. fermentum*. In the 1960s, a German microbiologist Gerhard Reuter isolated *L. reuteri* from human faecal and intestinal samples, and subsequently separated it from *L. fermentum* and re-classified it as *L. fermentum* biotype II (Reuter, 1965). Kandler *et al.* (1980) eventually identified *L. reuteri* as a distinct species based on phenotypical and genetic characteristics, and proposed it being a new species of heterofermentative lactobacilli. Later, modern technologies have further confirmed the identity and clearly separated the two species. Since 1980, *L. reuteri* has been classified as a distinct species in the *Lactobacillus* genus (Kandler *et al.*, 1980).

2.4.2 Morphology

Lactobacillus reuteri strains are Gram-positive, lactic acid-producing bacteria, their cells are slightly irregular, bent rods with rounded ends, generally 0.7-1.0 x 2.0-3.0 μm in size (Kandler and Weiss, 1986), and occurring singly, in pairs and in small clusters.

2.4.3 Ecology

Lactobacillus reuteri is considered as one of the few true autochthonous lactobacilli presented regularly in host large intestine (Reuter, 2001). It has been isolated directly from the GI tract or faeces of humans, monkeys, chicken, turkeys, doves, pigs, lambs, cattle, dogs, and rodents (Mitsuoka, 1992a; Casas and Dobrogosz, 2000), and has been found to be a major component of *Lactobacillus* species found in all hosts. In addition, it is the only *Lactobacillus* species which is believed to have established a symbiotic relationship with all hosts (Casas and Dobrogosz, 2000). Therefore, all species of *L. reuteri* isolated from various hosts have exhibited probiotic efficacy to hosts, hence convincingly supporting the probiotic concept.

In animals and humans, experiments have shown that *L. reuteri* is transmitted from mothers to newborn animals or infants during birth and the nursing process *via* mainly the mammary duct (Casas and Dobrogosz, 2000). Also, orally administrated *L. reuteri*

has been shown to survive gastric acid and bile salts through the stomach and upper intestine, bind to the gut mucus and epithelial cells, and colonise the host intestine (Reuter, 1965; Casas and Dobrogosz, 2000). However, *L. reuteri* strains isolated from different hosts exhibit host-specific colonization characteristics, some of which are not crossable (Molin *et al.*, 1992; Casas and Dobrogosz, 1997).

2.4.4 Biochemistry

Lactobacillus reuteri belongs to the obligate heterofermentative group of lactobacilli, and uses the phosphoketolase-based metabolic pathway to utilise available carbohydrates (Axelsson, 1998; Casas and Dobrogosz, 2000). It can ferment glucose alone and produce lactate, ethanol and CO_2 as end-products; but an essential characteristic of *L. reuteri* is its ability to utilise glycerol (Talarico *et al.*, 1988; El-Ziney *et al.*, 1998; Luthi-Peng *et al.*, 2002 b) (Figure 2.1). When glycerol is added as a substrate, the end-products change to more acetate/less ethanol, and the NADH formed during glycolysis is reoxidized by glycerol rather than in the ethanol pathway (Talarico *et al.*, 1990). In the process involving glycerol, *L. reuteri* takes the phosphoketolase pathway (PKP) to produce lactate; in parallel with lactate formation, glycerol successfully competes with acetylphosphate as a preferred terminal hydrogen acceptor to recycle NAD+, and this results in the release of the highly energetic acetylphosphate which is subsequently channelled into the acetate kinase reaction for more ATP production. As a consequence, this glycerol metabolism allows a higher ATP generation and greater acetate production, and ultimately a higher cell growth rate and more biomass are achieved (Talarico *et al.*, 1988, 1990). Hence, it is more favourable for *L. reuteri* to utilise glycerol at the later stages of glycolysis. It should be noticed that glycerol utilisation is only initiated by completion of the first few steps of breaking down fermentable carbohydrates (*e.g.* glucose). Talarico *et al.* (1990) postulated that *L. reuteri* cannot grow on glycerol alone, so this favourable process can occur only in the presence of fermentable carbohydrates.

At the end of this featured glycerol metabolism, a series of end-products, including SCFA and other organic compounds, were produced. Among these, 3-HPA (3-hydroxypropionaldehyde) has drawn the most attention, and has been found to be a potent antimicrobial substance. 3-HPA is also termed reuterin (Talarico *et al.*, 1988).

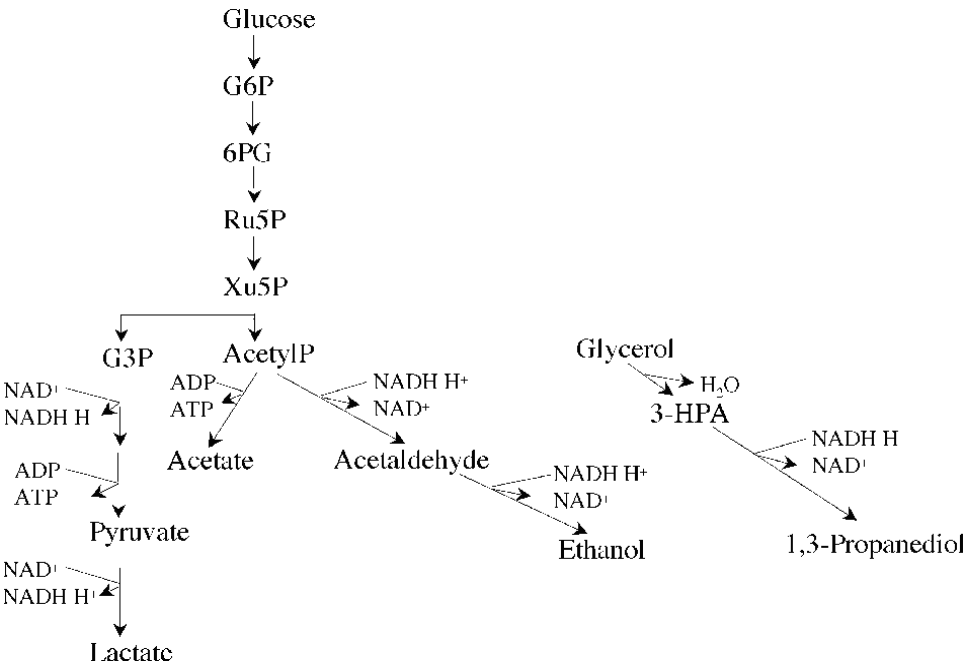

Figure 2.1 Heterofermentation of glucose by *Lactobacillus reuteri* in phosphoketolase pathway, with alternative end-products of lactate, ethanol and 3-HPA derived 1, 3-propanediol. Abbreviations: G6P, glucose 6-phosphate; 6PG, 6-phosphogluconic acid; Ru5P, ribulose 5-phosphate; Xu5P, xylulose 5-phosphate; G3P, glyceraldehyde 3-phosphate; AcetylP, acetyl phosphate; 3-HPA, 3-hydroxypropionaldehyde. Figure adapted from Talarico *et al.* (1990).

Reuterin is excreted as an intermediate during glycerol dissimilation, although some are further reduced to 1, 3-propanediol (Figure 2.2). According to Talarico *et al.* (1990), the production of reuterin involves a two-step enzymatic reaction, where glycerol is firstly dehydrated by a cobalamin (Vitamin b12)-dependent glycerol dehydratase to produce reuterin; concomitantly NADH/H+ produced from glycolysis is oxidized to NAD+. Secondly, a partial amount of reuterin is further reduced by a NAD+-dependent oxidoreductase to 1,3-propanediol, leaving excess reuterin accumulating in solution. Both of the enzymes responsible for glycerol metabolism have been isolated, purified and characterised. It has been found that glycerol dehydratase from *L. reuteri* is strictly specific for glycerol, and consists of four subunits with an overall molecular weight of 160 kDa; while the *L. reuteri* oxidoreductase is similar to those of other bacteria undertaking the same pathway in terms of structure and enzyme activity, and comprises four identical subunits (4.2 kDa each) with overall molecular weight of 180 kDa (Talarico *et al.*, 1990).

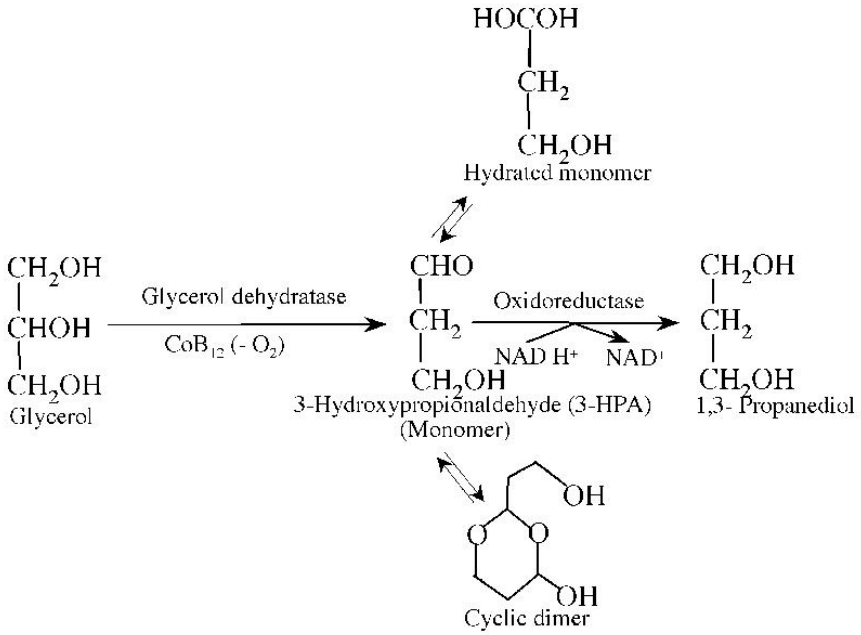

Figure 2.2 ***Lactobacillus reuteri*** **production of 3-HPA during glycerol fermentation. Three forms of 3-HPA co-exist in equimolar concentration in the aqueous solution forming the reuterin system, and with NAD-dependent oxidoreductase, 3-HPA monomers are further reduced to 1, 3-propanediol. Figure adapted from Casas and Dobrogosz (2000).**

Lactobacillus reuteri is not the only species that is known to produce 3-HPA. Other bacteria including Gram-positive *Bacillus amaracrylus* (Voisenet, 1914, 1918), *Clostridium perfringens* (Humphreys, 1924), and *Clostridium butyricum* (Malaouia and Marczak, 2000); and Gram-negative *Enterobacter agglomerans* (Barbirato *et al.*, 1996), *Klebsiella pneumoniae* (Slininger *et al.*, 1983) and *Citrobacter freundii* (Mickelson and Werkman, 1940; Barbirato *et al.*, 1996) have also been reported to utilise glycerol *via* the same pathway and produce intermediate 3-HPA (Sobolov and Smiley, 1960; Vollenweider and Lacroix, 2004; Martin *et al.*, 2005). However, these other bacteria can only produce 3-HPA as a transient metabolite, which is immediately further reduced; whereas *L. reuteri* has shown the unique capability to retain more than the required 3-HPA (Talarico *et al.*, 1988, 1990). The exact mechanism of this accumulation is still not clear, but it may be due to a strain-specific enhancement in expression of certain gene(s) encoding for responsible enzymes.

Therefore, *L. reuteri* is unique in its exclusively accumulating reuterin (3-HPA). This featured capability out-competes with other producing strains, and provides *L. reuteri* with a potent antimicrobial activity.

2.4.5 Antimicrobial activity of *Lactobacillus reuteri*

Accumulated evidence has revealed probiotic efficacy of this universally indigenous species amongst various hosts, especially its strong antimicrobial activity. It has been found that *L. reuteri* cells can strongly adhere to host GI epithelia *via* specific binding mechanisms and specialised surface proteins (Wadstrom *et al.*, 1987; Roos *et al.*, 1999; Roos and Johnsson, 2002). This strong adhesion of *L. reuteri* has assured the exclusive competition capability against other microorganisms competing for the same niche, as well as a direct impact of secreted metabolites to the host enterocytes and the gut-associated immunocytes, hence positive modulation of the host's mucosal defences can be achieved. This review focuses on the antimicrobial function of some of the *L. reuteri* metabolites.

As introduced previously, *L. reuteri* produces lactate, acetate and perhaps a variety of other SCFA along the heterofermentative metabolism pathway. Although it is certain that the acids partially contribute to the overall antimicrobial activity, this part of the review puts emphasis on those *reuteri*-specific substances and associated activities which are reported as unique to this species.

2.4.5.1 Reuterin

Reuterin is a pH neutral, water soluble, low molecular weight substance, which is non-bacteriocin, and resistant to nuclease, protease and lipolytic enzymes. It is active at a wide range of pH value, and capable of inhibiting growth of a wide spectrum of microorganisms, but it is also labile to heat (100 degrees C. for 10 minutes) (Talarico *et al.*, 1988; Axelsson *et al*., 1989; Dobrogosz and Lindgren, 1995; El-Ziney *et al.*, 1999). Reuterin was the first low molecular weight antimicrobial substance from *Lactobacillus* species ever to be chemically identified (Talarico and Dobrogosz, 1989), and has been intensively studied and relatively well understood (Vollenweider *et al.*, 2003). Reuterin has been shown to exist in solution as an equilibrium mixture of three chemical compounds derived from glycerol dissimilation, namely the 3-HPA (3-hydroxy-propionaldehyde) system, containing monomeric, hydrated monomeric and cyclic dimeric forms of 3-HPA (Talarico and Dobrogosz, 1989). Talarico and Dobrogosz (1989) were the first to chemically characterise reuterin. They reported on the basis of mass spectrometry that reuterin has a molecular weight of 148, which is double that of the

3-HPA monomer (MW=74), and this indicated that reuterin may predominantly exist in the dimeric form of 3-HPA. However, their NMR studies revealed that reuterin exists predominantly as a 3-carbon compound (either hydrate monomer or dehydrate monomer of 3-HPA) and to a lesser extent as a dimeric form. This conflict was recently re-investigated by Vollenweider *et al.* (2003), who analysed the composition of the HPA system using ESI-MS and ^{13}C NMR, and reported that distribution of the three compounds forming the HPA system was concentration-dependent in aqueous solution. At high concentrations, the HPA system predominantly comprises HPA cyclic dimer; decreasing the HPA concentration increases the mole-fraction of HPA hydrate and decreases the mole fraction of cyclic dimer until reaching equilibrium at 1.2 M HPA. The monomeric form of HPA was never detected to exist in significant amounts and always co-existed with the HPA hydrate. Hence, the three forms of 3-HPA co-exist in equilibrium in aqueous solution, and it is hard to isolate a single form and claim a certain activity for it. It is better to recognise reuterin as an equilibrium mixture of 3-HPA system, and presume that the three molecules work together to contribute to the perceived activity.

The unique and most attractive feature of reuterin is its strong antimicrobial activity. It has been found that concentrations of reuterin in the range of 15-30 μg/mL effectively inhibit growth of Gram-negative and Gram-positive bacteria, yeasts, fungi, and protozoa; while much higher concentrations are required to kill lactic acid bacteria, including *L. reuteri* itself (Axelsson *et al.*, 1989; Chung *et al.*, 1989; Casas and Dobrogosz, 2000). Studies on reuterin's mode of action have postulated that the hemiacetal structure may give rise to a reaction between the aldehyde group and the terminal hydroxyl group to produce a molecule bearing close resemblance to a pentose such as D-ribose. Hence, re-structured reuterin can be a D-ribose analogue able to compete with ribonucleotides for their ribose-recognition site(s) on ribonucleotide reductase, thus interfering with DNA synthesis by inhibiting the conversion of ribonucleotides to deoxyribonucleotides (Dobrogosz and Lindgren, 1995; Lindren and Dobrogosz, 1998). Alternatively, another proposal suggests that the hydroxyl and aldehyde group might react with sulfhydryl enzymes (*e.g.* thioredoxin and ribonucleotide reductase) and alter the enzyme functionality (Talarico and Dobrogosz, 1989).

However, the exact range of thermostability of reuterin is still not clear. Early studies have reported that reuterin in culture solution rapidly undergoes transformation to

acrolein during thermal dehydration (60°C) (Pressman and Lucas, 1942; Sobolov and Smiley, 1960). Talarico and Dobrogosz (1989) also reported reuterin was heat-sensitive at the high temperature (280°C) present in a gas chromatography injector. Dobrogosz and Lindgren (1995) claimed, in their patent, that reuterin was heat-labile at 100 °C. Recently, Lu (2007) found that the antimicrobial activity of a reuterin-containing supernatant from *L. reuteri* DPC16 culture was heat-labile (80°C). A heat-unstable reuterin could be a hindrance to some applications in the food and packaging industry.

2.4.5.2 Reutericin

Some strains of *L. reuteri* are also known to produce reutericin 6, which is a cyclic class II bacteriocin that mainly targets Gram-positive bacteria and closely related *Lactobacillus* species (Kabuki *et al.*, 1997). Reutericin 6 was first reported by Toba *et al.* (1991) as produced by a *L. reuteri* strain LA6, isolated from human infant faeces. Purified reutericin 6 was reported to be hydrophobic and have a molecular weight of 5.6 kDa (Kawai *et al.*, 2001). The antimicrobial activity of Reutericin 6 has been tested against commercial strains including *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and subsp. *lactis*, and found to be bacteriolytic. Similar to other bacteriocins, the antimicrobial mechanism of this extracellular protein is believed to be the formation of pores in the cell membrane of the target bacteria, causing membrane depolarization and efflux of small cellular components resulting in cell death (Kabuki *et al.*, 1997; Kawai *et al.*, 2001; Kawai *et al.*, 2004). However, Reutericin 6 was also reported to be sensitive to proteolytic enzymes and low pH (pH 2-3) (Toba *et al.*, 1991; Kawai *et al.*, 2004). It is nearly two decades since the discovery of reutericin, but there are still doubts and conflicts about this bacteriocin, and some published findings have failed to be reproduced. Furthermore, the heat-sensitivity of reutericin has not been reported.

2.4.5.3 Reutericyclin

Another low molecular weight compound isolated from *L. reuteri* species (strain LTH2584) is reutericyclin. It is a naturally occurring, amphiphilic, tetramic acid, and its optimal formation was observed between pH 4 and 5 (Holtzel *et al.*, 2000). It has been reported to have bactericidal and bacteriostatic activity against many gram-positive species, but does not affect Gram-negative bacteria (Ganzle *et al.*, 2000). The mode of action of reutericyclin is to act as a proton ionophore, translocating protons across the cell membrane and dissipating the transmembrane pH potential (Ganzle and Vogel,

2003). However, reutericyclin has not been identified in reuterin-producing *L. reuteri* strains (Ganzle, 2004).

Thus, it can be considered that the overall antimicrobial efficacy of *L. reuteri* results from contributions of all the above factors. Amongst these factors, reuterin has drawn the most attention and has been believed to have the most potential for industrial applications.

2.4.6 Other functions of *Lactobacillus reuteri*

In addition to its outstanding antimicrobial activity, *L. reuteri* is also believed to have physiological influence in host GI environment. As previously introduced (section 2.4.4), *L. reuteri* metabolises through the phosphoketolase pathway under the anaerobic conditions of the host GI tract. It is able to utilise a variety of substances as alternative electron acceptors in which may include potentially toxic compounds; and hence *L. reuteri* may also help with the intestinal detoxification and modulating the intestinal environment to be favourable for the host (Arskold *et al*., 2008). Furthermore, *L. reuteri* has also been used as an effective therapeutic agent to moderate acute diarrhoea among children and to significantly reduce the duration of watery diarrhoea among children suffering from rotavirus or bacterial diarrhoea (Shornikova *et al.*, 1997 a, 1997b; Weizman *et al*., 2005). It may, also, reduce *Helicobacter pylori* infections (Mukai *et al.*, 2002). An animal trail has shown that *L. reuteri* (strain ATCC 55730) has a positive influence on the development of ileal tissue, thereby enhancing absorption of nutrients (Casas *et al.*, 1998). Lastly, a strain of *L. reuteri* (ATCC 55730) has been shown to positively modulate immune responses in human GI tract (Valeur *et al.*, 2004).

In conclusion, the collected evidence suggests that *L. reuteri* can colonise the host, protect against invasive agents and toxins, secrete potent substances against harmful pathogens and positively interact with the host cells, hence offering a wide range of benefits to the host.

2.4.7 Production of reuterin

As introduced previously (section 2.4.4), reuterin production by *L. reuteri* occurs during a heterofermentative process in dissimilating glycerol involving a two-step enzymatic reaction. Talarico *et al.* (1988) introduced a two-step fermentation method using

concentrated *L. reuteri* resting cells in a condition–controlled secondary glycerol fermentation process to produce relatively pure reuterin, *in vitro*. El-Ziney *et al.* (1998) studied glucose/glycerol co-fermentation of *L. reuteri*, and reported that glucose had a repressing effect on reuterin production and only very low concentrations (glycerol at 150 mM with glucose at 60 mM or 20 mM under batch or chemostat culture condition, respectively) were favourable to reuterin production. Luthi-Peng *et al.* (2002 b) had similar findings and concluded that the end-product of glucose/glycerol co-fermentation was directed by the glucose: glycerol molar ratio, and reuterin accumulation was favoured with such molar ratio at no greater than 0.33. If excess glucose was supplied, reuterin would be further reduced to 1,3-propanediol. Hence, the impact of glucose on *L. reuteri* glycerol fermentation is significant, and glycerol as a non-fermentable carbohydrate has to be coupled with a fermentable carbohydrate source to produce reuterin.

However, due to variations among strains, some *L. reuteri* strains have been reported to produce and accumulate reuterin in glycerol-water during incubation. Luthi-Peng *et al.* (2002 a) used the two-step fermentation process introduced by Talarico *et al.* (1988) and compared reuterin production of *L. reuteri* ATCC53608 in growth medium (MRS) and non-growth medium (distilled water) both supplemented with glycerol. They reported that reuterin was produced in both media and reached a plateau after only 2h of secondary fermentation, and the reuterin production was higher when glucose was present. They also studied the effects of selected fermentation parameters (temperature, pH value, biomass, cell age and initial glycerol concentration) and determined an optimal fermentation condition for this strain to produce reuterin as incubation of 30 g/L dried *L. reuteri* resting cells in glycerol (200 mM)-water solution for 3h at 37°C. Under such conditions, they reported that 170 mM of reuterin was produced. Vollenweider *et al.* (2003) reported reuterin production from similar fermentation process by another *L. reuteri* strain SD2112, and they found that 45% of the initial glycerol concentration was converted into reuterin. The above glycerol-water fermentation to produce reuterin has provided major advantages in obtaining accumulated reuterin by simplifying the purification process and minimising contaminations from by-products. However, the ability to continue dissimilating in water seems to be highly strain-specific, and the exact mechanism by which *L. reuteri* cells reduce glycerol without the presence of glucose has never been reported.

So, *in vitro* production of relatively pure reuterin can be achieved by the application of concentrated *L. reuteri* resting cells in a condition–controlled secondary fermentation process with glycerol supplementation. But, which medium to use in the secondary fermentation remains a question, and this is highly dependent on the producing *L. reuteri* strain. Reuterin production in purer solutions of glycerol-water is preferred only if the producing strain has the required capability; otherwise reuterin is more likely to be accumulated in glucose-containing growth media with a molar concentration ratio of glucose: glycerol greater than 0.33.

The detection and quantitative determination of reuterin has been reported to use a colorimetric method introduced by Circle *et al*. (1945), which is easy to conduct and has been proved to produce accurate result (Slininger *et al.*, 1983; Talarico *et al.*, 1990; Luthi-Peng *et al.*, 2002 a, 2002 b; Vollenweider and Lacroix, 2004). This method measures a dehydration derivative of reuterin in a heated acid aqueous solution. Figure 2.4 displays the schematic pathway of glycerol reduction to reuterin, reuterin dehydration to acrolein and further reduction of reuterin to 1,3-propanediol. It shows that intermediate reuterin can establish an equilibrium with acrolein in solution under designated non-physiological conditions. During this process, acrolein is reversibly produced as a dehydration derivative in equimolar concentrations to reuterin in heated acid aqueous solution; hence, by measuring the concentration of acrolien, the quantity of reuterin can be determined.

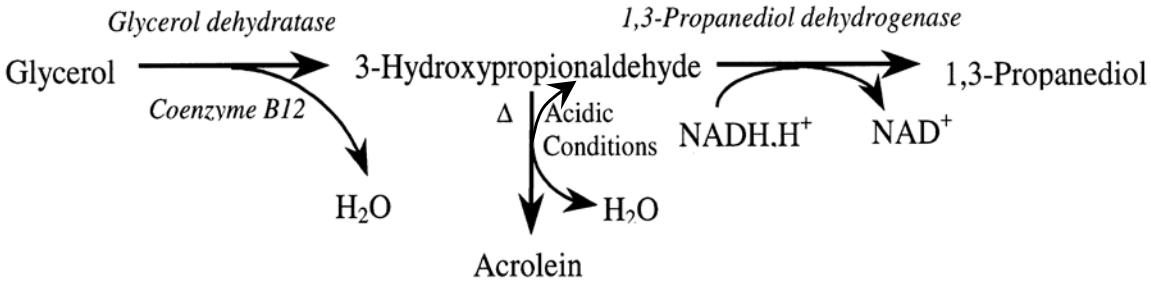

Figure 2.3 Chemical dehydration of 3-HPA (reuterin) to acrolein, followed by further oxidation to 1,3-propanediol. Adapted from Sauvageot *et al.* (2000).

The *in vivo* production of reuterin still remains to be explored. It may be produced in the more distal anaerobic regions of the gut where sufficient amounts of glycerol may become available as a product of luminal microbial fermentations, digestion of luminal

fats, sloughed mucus and exfoliated epithelial cells (Casas and Dobrogosz, 2000), and auto-diffusion of endogenous plasma glycerol. Moreover, it has been proved that reuterin production and accumulation can occur under physiological conditions of pH value and redox potential similar to those in the small and large intestine (Chung *et al.*, 1989). However, the *in vivo* measurement of reuterin production has never been reported.

2.4.8 Safety of *Lactobacillus reuteri*

According to the probiotics safety guidelines mentioned in section 2.3.2, *L. reuteri* strains should meet certain criteria to be considered as safe, such as being distinguished by genetic identification, having no history of detrimental effect on the host, being able to survive passage through the host GI tract, and not carrying horizontally transmissible antibiotic resistance genes. *Lactobacillus reuteri* belongs to the *Lactobacillus* genus, which has a long history of safe usage across various food industries. Even though opportunistic clinical infections of lactobacilli exist, there are no such reports for *L. reuteri* or the previously-classified *L. fermentum* (Wolf *et al.*, 1995). In addition, *L. reuteri* has been demonstrated, *in vitro*, to be able to resist pH 2.5 and 0.3% bile, and to adhere strongly to an intestinal epithelial model cell-line (Caco-2) indicating potential ability to colonise the host GI tract (Casa and Dobrogosz, 2000). Thirdly, a *L. reuteri* strain has been found, in a probiotics screening test, to display no adverse effect on normal GI bacterial residents (Jacobsen *et al.*, 1999). And lastly, like many other species of LAB, plasmid-associated antibiotic resistance genes have been found in some of the *L. reuteri* strains from both animal and human origin (Vescovo *et al.*, 1982; Sarra *et al.*, 1982; Tannock *et al.*, 1994; Lin *et al.*, 1996), however, there is no evidence that they are able to be transferred to other bacteria that intrinsically lack such resistance.

Results from a series of animal trials, including chicken (Dobrogosz *et al.*, 1991), turkeys (Edens *et al.*, 1997), swine (Casas *et al.*, 1998), pigs (Blanchard *et al.*, 1998) and colitis rats (Fabia *et al.*, 1993) have confirmed not only the safety of *L. reuteri* in animal feeds, but also that it possesses growth-promoting and therapeutic value to all tested animal hosts (Casas and Dobrogosz, 2000).

Speck *et al.* (1993) first postulated that *L. reuteri* is safe to be used in the food industry and can be consumed as a supplement without adverse effect. In human studies, the first

clinical safety trial of *L. reuteri* was conducted in 1995 in Finland (Casas and Dobrogosz, 2000). The strain was isolated from breast-milk of a healthy human female, and double-blind trials were conducted on infants aged from 6-36 months hospitalized with viral or bacterial infections. It was concluded that administration of *L. reuteri* was accompanied with a good GI colonization and had no adverse effect. Another trial conducted in Mexico with child subjects also confirmed that oral intake of *L. reuteri* was well tolerated and colonised in high numbers, with no significant difference amongst doses administered (Ruiz-Palacioz *et al.*, 1996). Wolf *et al.* (1995, 1998) also demonstrated that the oral administration of *L. reuteri* was safe and well tolerated in both healthy and immunodeficient adult human subjects. Recently, Connolly (2004) summarised the clinical trials ever done on *L. reuteri* strain ATCC 55730 and claimed it as a clinically proven probiotic bacterium.

The above animal studies and clinical trials indicate that *L. reuteri* is safe to use and free from side effects, even when administered in *L. reuteri* supplemented products. In addition to being safe, *L. reuteri* confers favourable physiological properties and therapeutic values for the host.

2.4.9 Applications of *Lactobacillus reuteri*

Information on *L. reuteri* as a functional food component and health supplement has been available for the two last decades. The main focus has been to take advantage of its significant efficacy in inhibiting enteropathogenic invasion, maintaining a health balance of host intestinal microflora, and reinforcing the host mucosal defence barrier and maintaining mucosal integrity.

L. reuteri was first introduced into the human functional foods market in Sweden in 1991 as a mixture of cultures (*Bifidobacterium animalis*, *L. reuteri* and *L. acidophilus*) in milk. From then on, *L. reuteri* has been incorporated into various food and drinks as a potent bioactive ingredient throughout the western world. Now, all clinically tested *L. reuteri* strains are available commercially as functional food ingredients (Casas and Dobrogosz, 2000). BioGaia, a company based on strong scientific research of *L. reuteri*, is a worldwide producer of *L. reuteri*-containing products from strains with clinically approved efficacy (Vollenweider, 2004; Wikipedia.org, 2007; BioGaia, 2008). *L. reuteri* is also reported to be a possible alternative to bio-feed in certain types of animal

farming, given its proven safe usage and significance in improving animal growth (Casas and Dobrogosz, 2000). It can be foreseen that potential replacement of antibiotics with host-specific probiotic feed in animal farming will largely reduce the threat of growing antibiotics-resistance. Recently, more attention has been put on the applications of purified reuterin. As a potent natural antimicrobial agent with low cytotoxicity, it has been used in sanitization of biological tissues (Sung *et al.*, 2002; Chen *et al.*, 2002; Liang *et al.*, 2003), and treatment of food-borne pathogens as a microbial preservative (Daeschel, 1992; El-Ziney and Debevere, 1998; El-Ziney *et al.*, 1999; Kuleasan and Cakmakci, 2002; Arques *et al.*, 2004). A very recent proposal incorporates reuterin into modified atmosphere packaging in food preservation (Lu, 2007).

2.5 *Lactobacillus reuteri* DPC16

2.5.1 Origin and identification

Lactobacillus reuteri DPC16 was originally isolated from faecal samples of a healthy Caucasian male by Bioactive Research New Zealand (BRNZ). A sugar fermentation profile established by using an API 50CH-L system (BioMerieux Inc.) was initially used to identify this strain in the *Lactobacillus* genus (Liu and Peng, 2007). In 2004, a fragment of 16S rRNA gene of this strain was sequenced (Lu, 2007), and the alignment in GenBank database revealed that this strain was affiliated to the *Lactobacillus* genus with 99.3-99.6% similarity to known species of *L. reuteri*, and at least 0.5% sequence differences in the genomic DNA from other *L. reuteri* strains. In addition, a 279 bp fragment was detected with a sequence corresponding to a gene encoding glycerol dehydratase in *L. reuteri* species. Hence, this DPC16 strain was identified as a novel *L. reuteri* strain, and registered for a patent by BRNZ in New Zealand (Shu and Liu, 2008).

2.5.2 Previous studies on *Lactobacillus reuteri* DPC16

Since its isolation, several investigations have been conducted to understand the characteristics and health-promoting properties of this new *L. reuteri* strain. And also, fermentation, storage, and a large scale production process have been investigated. Previous studies have shown that *L. reuteri* DPC16 has antimicrobial potential against

Salmonella Typhimurium infection in mice (Liu and Chen, 2004c). Preliminary tests for immunity enhancement and resistance to rotavirus infection have also been reported (Liu and Chen, 2004 a, b). Recently, as certain commercialization approaches have been attempted, DPC16 cell integrity during microencapsulation was studied to evaluate optimal processing and storage conditions for this culture (Joshi, 2005; Chang, 2006; Yin, 2007; Chen, 2007).

The most recent study (Lu, 2007) reported a strong antimicrobial activity of DPC16 against common food borne pathogens when co-cultured in glycerol-supplemented MRS broth, and subsequent analysis revealed that such antimicrobial activity was extracellular, non-proteinaceous, active at a wide range of pH values, insensitive to hydrogen peroxidase, and heat labile. By excluding the potential contributions from physiological influences, SCFA, protein-associated reutericin and reutericyclin, the remaining potent antimicrobial activity has strongly suggested the presence and involvement of reuterin. However, further investigation and characterisation of this strain remains to be done, and more importantly the exploration of this strain's safety status is still necessary.

2.6 Hypotheses

Based on the literature, several hypotheses were made towards the objectives. Firstly, this novel strain has been identified as one of the *L. reuteri* species, so it may have physiological characteristics of *L. reuteri*, including production of SCFA and reuterin. As tested in preliminary studies, this strain has inhibitory effects against selected pathogens. The first hypothesis was that this strain's antimicrobial activity was due to either the SCFA, or the production of reuterin, or both. A dose response test and subsequent time course experiment using the determined minimum dose may reveal more information on the antimicrobial activity of this strain.

Secondly, reuterin is known to be produced and accumulated in *L. reuteri* culture, and this strain has been identified to possess the gene responsible for glycerol reduction to reuterin, however, the optimal conditions for reuterin production are unknown. Thus, the secondary hypothesis was that this strain may have the ability to produce and accumulate reuterin in both growth medium (MRS) and non-growth medium (glycerol-water). By using a secondary fermentation method (Talarico *et al.*, 1988;

Luthi-Peng *et al.*, 2002 b) and a colorimetric detection technique (Circle *et al.*, 1945), the production of reuterin can be measured and the level of reuterin concentration produced in different media can be compared. Further, by determining the biomass concentration, the specific rate of reuterin production can be obtained, and by incorporating these results with the characterised antimicrobial activity, a correlation between reuterin and its antimicrobial activity can be determined.

Finally, because *L. reuteri* species is universally indigenous, it is hypothesised that this novel DPC16 strain has no adverse effects on the growth of other normal GI inhabitants. Interestingly, reuterin may have growth-stimulating effect on other probiotic bacteria, because it is structurally similar to methylglyoxal (bioactive ingredient in Manuka honey) which is proposed to stimulate growth of LAB at very low concentrations (Rosendale, 2007). Also, given that *L. reuteri* has the ability to strongly colonise host GI epithelia and there have been no safety concerns arising from either clinical trials or extensive applications, this strain of *L. reuteri* should not have any adverse effect on gastric or intestinal mucus in terms of mucinolytic activity, either.

To test the above hypotheses, a series of experiments were designed using the methods in the literature and previous studies, with some modifications to ensure compatibility with the objectives.

This thesis serves as a partial contribution towards the validation of the probiotic status claimed for this novel strain. Outcomes are expected to contribute as partial fulfilment of the characteristic profile of this strain with regards to health benefits and safety for human consumption and technological feasibility for commercialization.

Chapter 3

Antimicrobial activity of *Lactobacillus reuteri* DPC16 cell-free supernatants against selected food borne pathogens

3.1 Introduction

With the increasing global concerns about public health and the abuse of antibiotics, there is a growing demand for the use of naturally occurring antimicrobial agents to treat pathogenic infections where antibiotic resistance has become a serious issue. Probiotics, commonly represented by LAB, are known to inhibit enteric pathogens resulting in maintenance of a healthy microbial balance in the host GI tract, and also are generally regarded as safe (GRAS) due to their long history of safe use. Antimicrobial activities of LAB are largely due to their production of organic acids (*e.g.* lactate, acetate, citrate and butyrate), ethanol, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde and bacteriocins (Ouwehand and Salminen, 1998). These antimicrobial compounds can exert specific activities against both commensal organisms and pathogens. For example, the LAB-produced proteinaceous bacteriocins have specific inhibitory activity against closely-related probiotic species to prevent competition in the same niche (De Vuyst and Vandamme, 1994; Abee *et al*., 1995; Bennison, 2005). In contrast, pathogens are more sensitive to the organic acids produced during LAB fermentation of utilizable carbohydrates (Alakomi *et al.*, 2000; De Keersmaecker *et al.*, 2006). Additionally, some LAB strains have more strain-specific antimicrobial activity against certain or a specific group of pathogens, while some other possess the capability to produce special substances against a broad range of pathogens.

Lactobacillus reuteri is known for its potent antimicrobial activity against a wide spectrum of microorganisms including both Gram-negative and Gram-positive bacteria, yeasts, fungi, and protozoa (Axelsson *et al*., 1989). Besides the general acidic effect from SCFA, a series of other specific substances have also been reported to contribute to the overall antimicrobial activity of this species. These include the proteinaceous reutericin and reutericyclin, which are *L. reuteri*-specific bacteriocins; as well as reuterin, a hydroxyl-propanal produced from glycerol in culture media. Amongst these antimicrobial factors, reuterin has been of much interest and numerous studies have been done to investigate this potent antimicrobial compound.

Preliminary study (Lu, 2007) on this particular strain has shown that DPC16 can exert a strong antimicrobial activity against selected Gram-positive and Gram-negative pathogens when co-cultured in glycerol containing MRS broth; and this activity, from DPC16 secreted metabolites, was non-proteinaceous, active at a wide range of pH values, insensitive to hydrogen peroxidase, and heat labile. These data have provided evidence for the presence of reuterin in DPC16 culture. However, further characterisation of this DPC16 antimicrobial activity was left unfinished, and the capability of DPC16 to exhibit similar antimicrobial activity when it was cultured in glycerol-water was not demonstrated.

The antimicrobial investigation in this study was built on the previous findings, and focused on profiling the antimicrobial effects of DPC16 cell-free supernatants from a variety of media, especially ones from glycerol-supplemented media. Makras *et al.* (2006) introduced a kinetic method to study the antimicrobial activity of six lactobacilli against pathogenic *Salmonella* Typhimurium in batch fermentation under controlled conditions. In their study, the inhibitory activity of a cell-free culture supernatant of each probiotic strain was studied in a kinetic analysis. By studying the production kinetics of antibacterial activity and controlling the pH value during the antimicrobial assay, they were able to distinguish between the acid effect and effects attributed to other low-molecular-mass inhibitory metabolites produced. Additionally, by monitoring several kinetic parameters such as glucose utilisation, lactic acid production and specific growth rate of each culture, the antimicrobial activity of each *Lactobacillus* strain was comprehensively characterised.

In addition to the conventional antimicrobial assay, a similar kinetic approach based on the above method was carried out to understand the development of antimicrobial activity of DPC16 cell-free supernatants from incubations involving glycerol.

3.2 Materials and Methods

3.2.1 Growth of pathogen cultures

The pathogenic *Escherichia coli* O157:H7 strain 2988 (*E. coli*), *Salmonella enterica* serovar Typhimurium ATCC1772 (*S.* Typhimurium), *Listeria monocytogenes* Scott-A ATCC49594 (*L. monocytogenes*) and *Staphylococcus aureus* ATCC2592 (*S. aureus*) were kindly provided by BRNZ. These strains were stored as 30% glycerol stocks at -80°C. For activation, 1% (v/v) of each thawed pathogen stock was inoculated aseptically into 10 mL BHI broth (BBL, USA) and incubated at 37°C for overnight (A detailed composition of BHI broth is shown in Appendix 1). Subsequently, the activated pathogens were sub-cultured in BHI broth for another 18h and the subcultures were used as master inocula for subsequent assays (these master inocula were always freshly made from stock prior to assay).

Growth of pathogens followed the method described by Lu (2007), with enumeration using a drop-plate technique introduced by Herigstad *et al.* (2001). Briefly, 1% (v/v) of each pathogen master inoculum was inoculated in BHI, in triplicate, and incubated aerobically with shaking (250 RPM; orbital OS10 control, YellowLine; USA) at 37°C for 18h. After incubation, each pathogen culture was decimally diluted with 0.1% (w/v) sterile peptone water (trypticase peptone; BBL, USA) up to 10^7-fold, and peptone water alone was used as control. Dilutions of 10^5-, 10^6-, and 10^7-fold were used to count viable cells of each pathogen. Aliquots of each dilution of every pathogen (10 µL) were dropped, in triplicate, onto a BHI agar plate [1.5% (w/v)]. Peptone water alone was used as a control on the same BHI agar plate. All inoculated plates were allowed to dry in a laminar airflow cabinet before incubation at 37°C for 24h. Colonies were counted on a digital colony counter (SC6, Stuart Scientific; UK), and viable cells were recorded as the mean of triplicates for each countable dilution. Based on this enumeration, a culture dilution containing approximately 10^4 CFU/mL was prepared for each pathogen, as a subculture inoculum capable of producing a full growth curve (with a defined length of lag phase) during incubation.

3.2.2 Preparation of DPC16 cell-free supernatants

Lactobacillus reuteri DPC16 was provided by BRNZ in 30% glycerol stock stored at -80°C. Firstly, the thawed stock culture was activated by propagating [1% (v/v)] in 10 mL MRS broth (Difco, Michigan, USA) at 37°C overnight (A detailed composition of MRS broth is shown in Appendix 1). The activated stock culture was then aseptically streaked onto MRS agar [1.5% (w/v) agar], and incubated for 24h at 37°C. This subculture was then stored at 4°C as a DPC16 master plate until use, and this seeded storage was kept for a maximum period of one month.

The cultivation method of this strain used in previous study (Lu, 2007) was followed in the present work, with some modifications. To grow the culture, a single colony with typical morphology from the DPC16 master plate was isolated and inoculated into a pre-reduced [containing 0.05% (w/v) *L*-cysteine and keeping standing for overnight] MRS broth, and incubated anaerobically* (Gaspak system, BBL; USA) at 37°C for 20h (unless otherwise stated, this method was standardized for preparation of all probiotics cultures throughout this thesis). After incubation, the DPC16 culture was centrifuged (refrigerated centrifuge 5810R, Eppendorf, Germany) at 4,000 x *g* for 10 min at 4°C. The supernatant was filter-sterilised through a 0.2 µm syringe-end filter system (Minisart, Sartorius; Germany) to remove any remaining cells, and this cell-free supernatant was designated as MRSc (derived from pre-reduced MRS broth) and was stored at 4°C until the time of use (maximum storage one month). Additionally, part of MRSc was subjected to freeze-drying (FreeZone Plus Cascade Console Systems, Labconco; USA) and the freeze-dried MRSc was aseptically re-dissolved to the previous volume in distilled water, designated as FZMRSc. FZMRSc was subsequently filter-sterilized and stored at 4°C until use (maximum storage one month).

The cell pellet was used for secondary fermentations to prepare supernatants with glycerol supplementation. The procedure was based on the methods described by Luthi-Peng *et al.* (2002 b) in preparation of supernatant from glycerol-MRS fermentation, and Talarico *et al.* (1988) in preparation of supernatant from glycerol-water fermentation. Briefly, the cell pellet was washed twice with 1 x

*The anaerobic condition applied throughout this thesis is provided by Gaspak® Gas generating reagents contained in a BBL® sachet, which consists of inorganic carbonate, activated carbon, ascorbic acid. These reagents could produce a gas mixture with 15% or more CO_2 in humidified atmosphere. The anaerobic environment is indicated by an indicator strip coated with methylene blue solution.

Phosphate Buffer Saline solution (PBS, pH 7.2; Difco, USA) to ensure removal of metabolites, and then starved in 0.9% saline solution at 37°C for 30 min. Then, the washed and starved resting cells were harvested and subsequently freeze-dried. In the freeze-drying process, the cells were frozen at -80°C for 48h, and then lyophilized under pressure of 0.33 mbar on a bulk tray freeze-drier (FreeZone Plus Cascade Console Systems, Labconco; USA) for another 48h. The freeze-dried cells were then re-suspended in either pre-reduced MRS or distilled water to half of the original volume from which they were harvested (concentrated at twice their original concentration). The pre-reduced MRS was supplemented with 360 mM of sterile glycerol, where the glycerol to glucose molar concentration ratio was greater than 3:1 (MRS broth contains 110 mM of glucose). The distilled water was supplemented with 250 mM glycerol. The resting cells suspensions were incubated anaerobically at 37°C for up to 20h in glycerol-MRS, and up to 8h in glycerol-water. At set time intervals, samples of each suspension were harvested, cooled immediately on ice and centrifuged (4,000 x *g* for 10 min at 4°C). The sampled supernatants were collected, filter-sterilized and stored immediately at 4°C until subsequent antimicrobial assays. The supernatant series obtained from secondary fermentation of glycerol-MRS was designated as MRSg, while the supernatant series derived from glycerol-water was designated as SGF (sole glycerol ferment). These supernatants were kept for a maximum of one month. Table 3.1 summaries the supernatants prepared as above.

Table 3.1 Summary of DPC16 cell-free supernatants derived from various cultural media

Cell-free supernatants (CFS)	Source
MRSc	MRS pre-reduced with 0.05% *L*-cysteine
FZMRSc	Water (aqueous solution of freeze-dried MRSc)
MRSg	Glycerol-MRS
SGF	Glycerol-water

Finally, the pH values of the above supernatants were determined. MRSc and FZMRSc were measured using a pH meter (Orion, Thermal Scientific; USA) at room temperature. For MRSg and SGF, which were smaller volumes, pH values were measured using a portable pH meter (pH pen KS732, Shindengen; Japan). Where noted, the pH adjustments of the DPC16 supernatants were carried out by neutralising them to pH 6.5 using 0.1 M sodium hydroxide at room temperature prior to testing the antimicrobial activity

3.2.3 Primary antimicrobial assay of DPC16 supernatants

Cell-free supernatants (with and without pH adjustment) of MRSc, FZMRSc, MRSg, and SGF were primarily tested for any antimicrobial activity against selected food-borne pathogens. Briefly, an aliquot of each supernatant (20 µL) was added, in triplicate, into 180 µL of BHI broth containing 20 µL of each pathogen (approximately 10^4 CFU/mL), in 96-well microtitre plates. Negative controls contained pathogen cultures only, whereas positive controls replaced supernatant with commercial antibiotics (100 IU penicillin and 10 µg/mL streptomycin, Sigma; USA). To avoid contamination, each microtitre plate was inoculated with only one strain of pathogen. The inoculated microtitre plates were incubated aerobically at 37°C with shaking (250 RPM) for 18h. The antimicrobial activity of each supernatant was quantitatively determined by turbidity development (OD_{620}) of pathogens measured on a microtitre plate reader (Multiskan, Thermo Scientific; Finland), in comparison with untreated controls. Unless otherwise stated, this method and experimental conditions were standardised for all antimicrobial assays throughout this thesis.

3.2.4 Dose response test

Based on results from the primary antimicrobial test, the supernatants with significant antimicrobial activities were selected, binary-diluted, and subsequently tested for dose responses against respective pathogens. In brief, the binary dilution of each selected supernatant was prepared up to 512-fold in 96 well microtitre plates. Then, each dilution (20 µL) from every selected supernatant was added, in triplicate, into BHI broth (180 µL) containing 10^4 CFU/mL of each target pathogen (20 µL). Controls without supernatants were included. All microtitre plates were incubated and the OD values were measured as described above. The lowest effective dose of each selected supernatant, which still significantly inhibited the growth of target pathogens, was hence determined, and this dose was used in a following time course experiment to further study its antimicrobial activity.

3.2.5 A time course investigating the antimicrobial activity of DPC16 supernatants

A time course was designed to test pathogen growth in the presence of the above DPC16 supernatants with known minimum effective doses. In this time course, the OD value of each treated pathogen was serially measured at 3h intervals during a 21h incubation at 37°C under aerobic conditions. Untreated pathogens were used as a control.

3.2.6 An antimicrobial efficiency test using MRSg

DPC16 cell-free supernatant, MRSg (undiluted and pH-unadjusted), was further studied by a viable cell count assay to investigate its antimicrobial efficiency. By using the standardised antimicrobial assay (section 3.2.3), an undiluted 18h culture (stationary phase) of each pathogen was treated with an equal volume of undiluted and non-pH-adjusted MRSg (harvested at designated time points of secondary fermentation) in BHI broth, in triplicate, at 37°C. At predetermined time intervals, aliquots were removed, serially diluted, and plated (drop-plating) on BHI agar. Each original 18h pathogen culture was enumerated before treatment, and the number of viable cells was compared with those in MRSg-treated cultures. A survival rate of each treated pathogen was calculated and presented in a graph.

3.2.7 Viability of DPC16 resting cells in glycerol-fermenting cultures

The DPC16 resting cells from fresh incubated culture, freeze-drying preparation, and the kinetic production of MRSg and SGF were tested for viability. As mentioned in Section 3.2.2, resting cell suspensions were sampled at various time points during the glycerol fermentation in both glycerol-MRS and glycerol-water, where supernatants of MRSg and SGF were obtained. From the same suspensions, the cell pellets were harvested, washed and resuspended in PBS, and then diluted and enumerated by drop-plate, in quadruplicate, on pre-reduced MRS agar plates. Freshly harvested and washed DPC16 resting cells from the 20h culture without glycerol, and those were processed with starvation and freeze-drying were also subjected to the above procedure. Hence, the viability of DPC16 resting cell from pre-production, preparation process and post-production were compared.

3.2.8 Statistical analysis

Statistical analysis for determination of significant differences in the measured properties between groups was accomplished using a paired-sample T-test performed with 95% confidence intervals. The statistical analysis was done using a statistical program (SPSS, v.15, SPSS Inc.; Chicago, Illinois, USA). All data were presented as a mean value of replicates (generally n=3, but occasionally n>3) with the standard error of the mean (SEM) indicated. This statistical analysis method was used throughout the thesis.

3.3 Results

3.3.1 The pH values of DPC16 cell-free supernatants

The pH values of MRSc and the aqueous solution of freeze-dried MRSc (FZMRSc) were measured as 4.4 and 4.2, respectively. The pH values of MRSg and SGF measured during their production from glycerol in secondary fermentation are displayed in Figure 3.1, and show that production of MRSg was accompanied by a continuous decline of pH until 16h of incubation, after which the pH value stabilised at pH 4.6. The pH value of SGF decreased then slightly rose during incubation, finally reaching a value of 5.2. However, it was found that SGF had very low buffering strength, so the pH adjustment of this particular supernatant was not carried out and the antimicrobial activity of SGF was tested without pH adjustment. Hence, all DPC16 supernatants obtained were acidic, where MRSc and FZMRSc produced in primary fermentation had slightly lower pH values than MRSg or SGF from secondary fermentation with glycerol. At the end of the incubation, MRSg had a lower pH value than SGF and took longer to reach the final value.

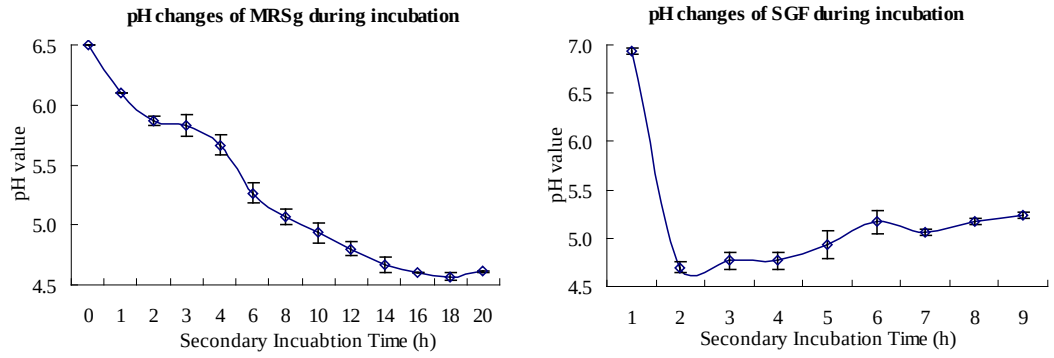

Figure 3.1 The pH values of MRSg and SGF measured during secondary fermentation with glycerol. Data points are the mean of triplicate pH values, with SEMs represented as vertical bars.

3.3.2 Primary antimicrobial assay against selected pathogens

The antimicrobial activities exhibited by different cell-free supernatants of *L. reuteri* DPC16 are shown in Figures 3.2, 3.3, and 3.4. The percentage of inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{OD\ untreated\ control - OD\ treatment}{OD\ untreated\ control} \times 100.$$

Figure 3.2 shows the inhibition of pathogens treated with MRSc and FZMRSc, with and without pH adjustment, in comparison with untreated controls. It is found that only the unadjusted supernatants of MRSc and FZMRSc had significant inhibitory effects ($P<0.05$ or $P<0.01$) against pathogens, whereas their pH-adjusted counterparts did not show any significant antimicrobial activity in comparison to the untreated controls. The Gram-negative pathogens (*S.* Typhimurium and *E. coli*) were more sensitive to MRSc or FZMRSc than the Gram-positive pathogens (*L. monocytogenes* and *S. aureus*) in terms of percentage of inhibition relative to untreated controls. Furthermore, the inhibition by FZMRSc was a little higher than that of the MRSc, but the difference did not reach a significant level. This also indicates that the freeze-drying process did not have a significant impact on the antimicrobial activity of the MRSc supernatant (statistics data are presented in Appendix 2).

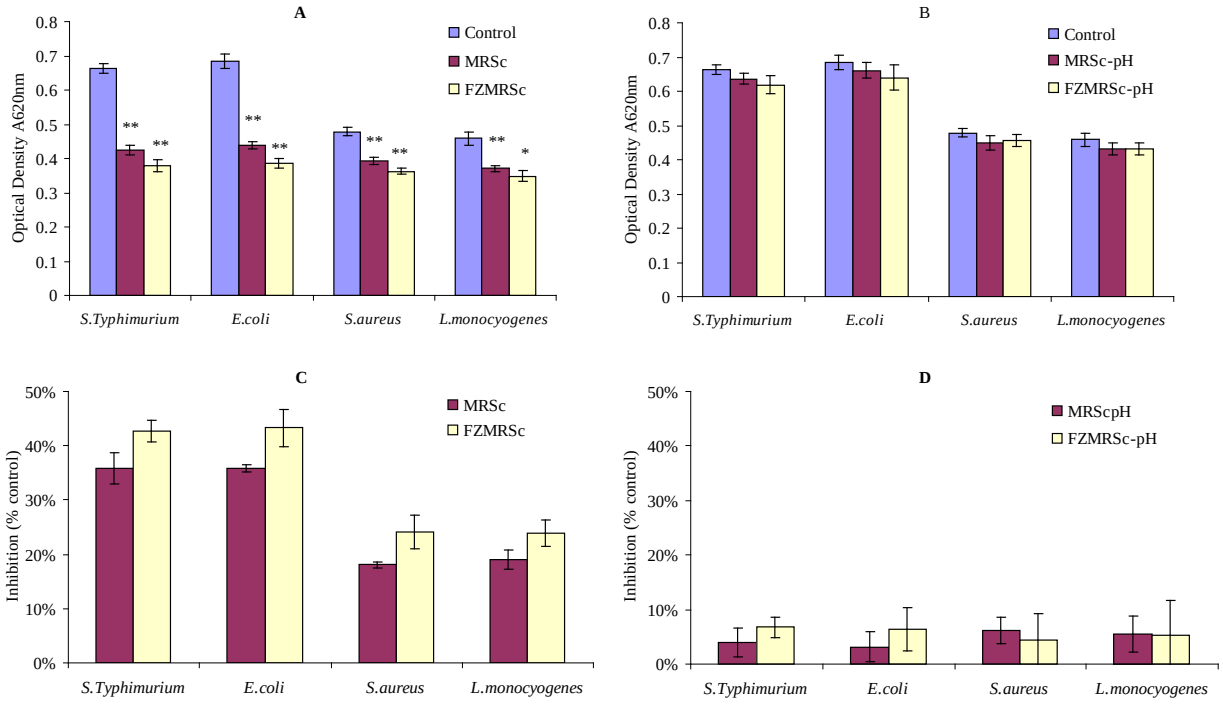

Figure 3.2 Primary antimicrobial tests of MRSc and FZMRSc, and their pH adjusted counterparts. A, growth of pathogens with or without treatment of original MRSc and FZMRSc; B, growth of pathogens with and without treatment of pH-adjusted MRSc and FZMRSc (MRSc-pH and FZMRSc-pH); C: Percentage of inhibition of each pathogen as shown in A; D: Percentage of inhibition of each pathogen as shown in B. Bar values are presented as means calculated from triplicate OD_{620} readings with SEM shown by vertical bars (n=3). Percentage of inhibition was calculated by comparing OD_{620} of treated pathogens with untreated controls. Pathogen growth is significantly inhibited when symbol * P <0.05 or ** P <0.01 is presented.

Figure 3.3 illustrates percentage inhibition of growth of each pathogen by original MRSg (non-pH-adjusted) harvested during the 20h secondary fermentation with glycerol. It is clear that antimicrobial activity of MRSg, against all selected pathogens, increased with time of incubation, and a sudden jump was observed between 6h-8h (except for *S. aureus*, where a similar increase occurred sooner during incubation), which raised the inhibition percentage to a level of 100%. Table 3.2 shows an example of statistical analysis on antimicrobial activity of MRSg (at certain points of incubation) against target pathogens, by comparing growth of treated-pathogens with untreated controls as well as commercial antibiotics. It suggests that MRSg exerted significant inhibition against all pathogens from 2h of incubation, and by 8h of incubation the antimicrobial effect of MRSg has no significant difference from the antibiotics. Hence, during the production of MRSg, 6h can be considered as a threshold in terms of its antimicrobial efficacy against target pathogens (4h in case of *S. aureus*), where before this threshold MRSg exerts its antimicrobial activity similar to MRSc or FZMRSc, and beyond this threshold nearly 100% inhibition of all tested pathogens were observed similar to antibiotic.

Table 3.2 Antimicrobial activity of the MRSg (non-pH-adjusted), from selected time points during incubation, against target pathogens. Percentage of inhibition and P values are calculated from OD_{620} of treated pathogens comparing to untreated controls (upper panel), or comparing to commercial antibiotics (lower panel). Data are means of multiple replicates (n=6), with P<0.05 indicating significance.

Antimicrobial Activity				
Growth inhibition	**1h**	**P-value**	**2h**	**P-value**
S. Typhimurium	20.0% ± 0.7%	0.0003	23.3% ± 0.9%	0.0004
E. coli	8.2% ± 1.7%	0.0878	13.7% ± 1.9%	0.0334
S. aureus	12.0% ± 0.6%	0.0006	23.1% ± 1.4%	0.0010
L. monocytogenes	0.5% ± 0.4%	0.6633	5.6% ± 0.5%	0.0070
Antibiotics Analogue				
Growth inhibition	**8h**	**P-value**	**10h**	**P-value**
S. Typhimurium	99.4% ± 0.1%	1.00	99.6% ± 0.1%	0.16
E. coli	99.2% ± 0.1%	0.18	99.0% ± 0.1%	0.26
S. aureus	98.0% ± 0.4%	0.31	99.4% ± 0.1%	0.14
L. monocytogenes	99.4% ± 0.1%	0.26	99.5% ± 0.0%	0.09

A post-treatment viability test was used to count viable pathogen cells after incubation with MRSg collected at various time points during the glycerol incubation. Figure 3.4 shows a sharp decrease in numbers of viable cells after MRSg treatments, especially for *S. aureus* where no viable cells were found after treatment with MRSg harvested after 8h of secondary fermentation. In the viable counts of 10h MRSg-treated pathogen cultures, no viable pathogen cell was detected in any cultures. These results agree well with those of the antimicrobial assay in that the antimicrobial activity of MRSg has largely increased between 6-8h of secondary fermentation and stabilised thereafter. Also, it confirms that *S. aureus* was the most susceptible to MRSg; and suggests that this potent effect of MRSg is bactericidal in nature.

The pH-adjusted MRSg-8h and its unadjusted form were also tested in an antimicrobial assay against the above pathogens. Statistical analysis showed that pH adjustment did not make any significant difference to the antimicrobial activity of MRSg-8h against any target pathogen (statistics data are shown in Appendix 2). Hence, the inhibitory effect of MRSg was pH-independent, effective across a pH range of 4.6 to 6.5, and impacted on both Gram-negative and Gram-positive pathogens.

Figure 3.5 shows % inhibition of pathogens treated with SGF (non-pH adjusted) sampled during the 8 h incubation. Unlike the pattern of MRSg, the kinetic series of SGF showed very limited antimicrobial activity, and it was only, at a transient moment, effective against *S. typhi* and *S. aureus*, where a statistic analysis showed that 2 h SGF had the highest inhibition against both ($P = 0.007$ and 0.002, respectively) comparing to the untreated controls.

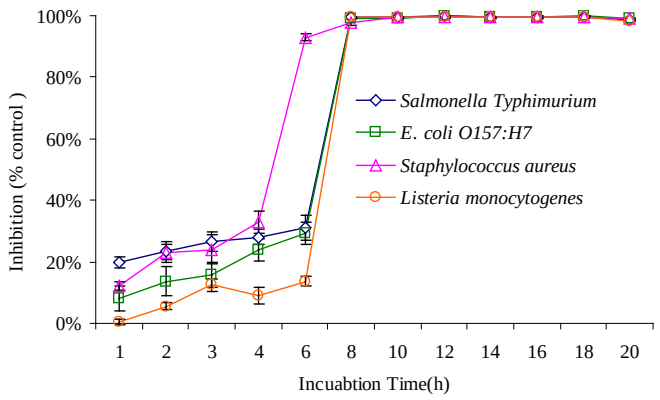

Figure 3.3 Primary antimicrobial test of MRSg harvested during secondary fermentation. Values are expressed as percentage of inhibition relative to controls, calculated from mean OD_{620} of MRSg-treated pathogens and untreated controls, with SEMs represented by vertical bars (n=3).

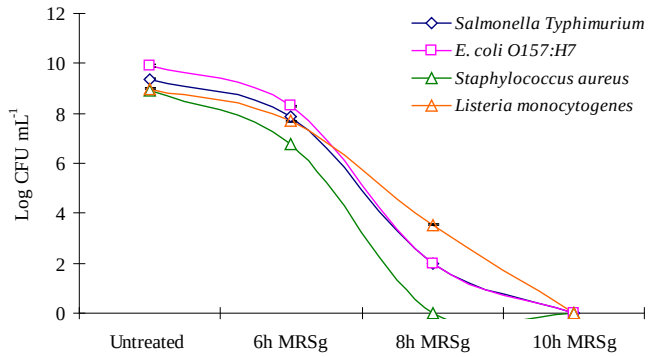

Figure 3.4 Post-treatment viability test of pathogens treated with MRSg harvested from selected time points during secondary fermentation with glycerol.

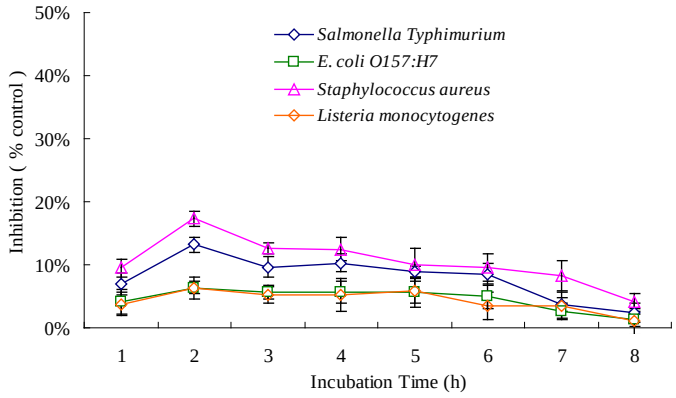

Figure 3.5 Primary antimicrobial test of SGF harvested during secondary fermentation. Values are mean percentage of inhibition relative to controls, calculated from OD_{620} of treated pathogens comparing to untreated controls, with standard errors represented by vertical bars (n=3).

3.3.3 Dose response test

From the primary antimicrobial test, DPC16 cell-free supernatants with significant inhibitory effects against pathogens were selected for a dose response assay. These included the original (pH-unadjusted) MRSc, FZMRSc and MRSg harvested at 8h of the secondary fermentation, denoted as MRSg-8h. SGF from a 2h incubated glycerol-water culture (SGF-2h) was also selected but only assayed against *S.* Typhimurium and *S. aureus* for dose response. By comparing the growth of pathogens treated by selected DPC16 supernatants with their corresponding untreated controls, a series of inhibition bar charts were developed to reveal dose responses of each selected cell-free supernatant against target pathogens.

Figure 3.6 displays the dose-response of MRSc dilutions and the decreasing antimicrobial activities against selected pathogens. It is evident that the inhibitory effects of MRSc against both the Gram-negative and the Gram-positive pathogens are dose dependent, and the antimicrobial activity declines along the binary dilution. Statistical analysis shows that significant inhibitions of MRSc against *E. coli* (P=0.01) and *S.* Typhimurium (P=0.049) were detected at dilutions as far as 80- and 160-fold, respectively. However, the minimum effective dose of MRSc against *S. aureus* (P=0.001) and *L. monocytogenes* (P=0.006) were higher at the 10-fold and 20-fold dilutions of MRSc, respectively. This suggests that Gram-negative pathogens are more susceptible to acidic MRSc than the Gram-positive pathogens.

A similar trend of dose response was observed using FZMRSc against the same pathogens (Figure 3.7). It was also noted that, at the initial dose (10-fold dilution), the FZMRSc had higher inhibitory capability against all pathogens than had MRSc, which agrees with the result of the primary tests. But, the minimum effective doses of both MRSc and FZMRSc are similar.

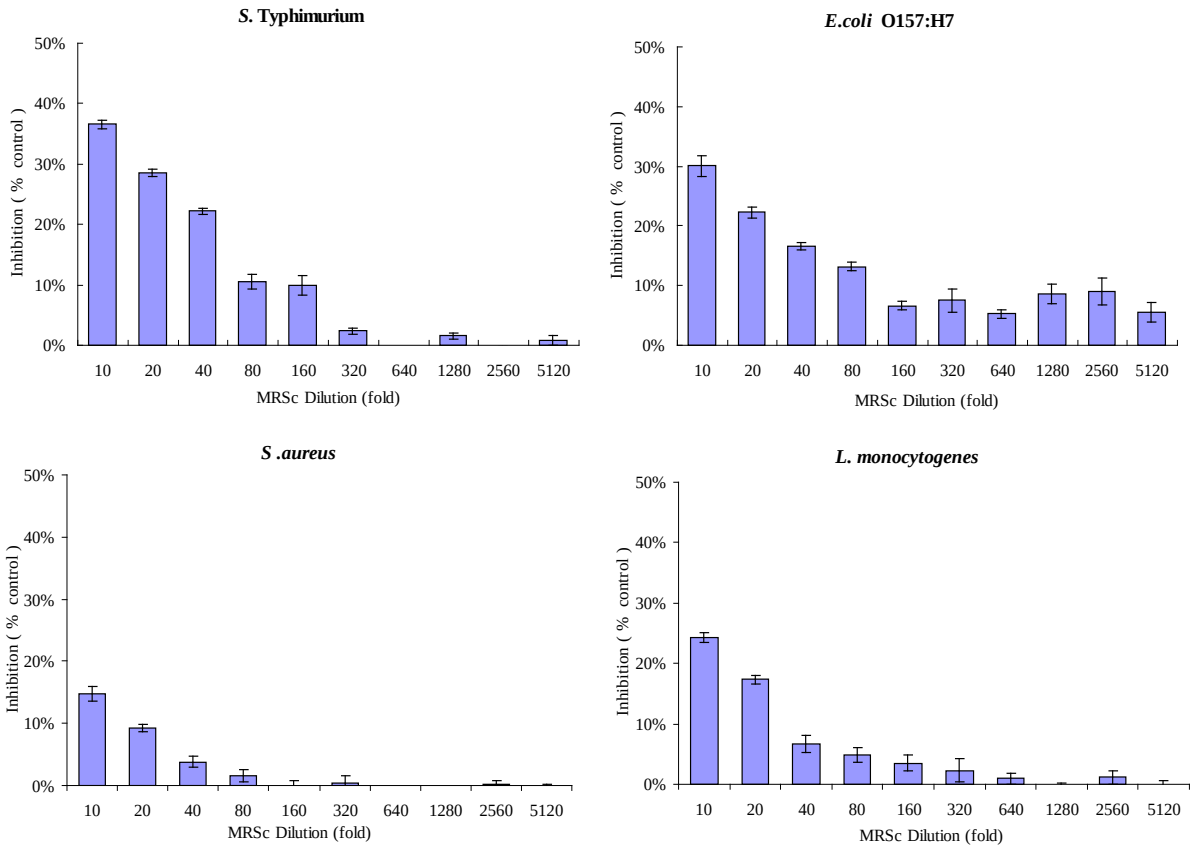

Figure 3.6 Dose response of original MRSc against pathogens. Values are expressed as percentage of inhibition relative to controls, calculated from mean OD_{620} of MRSc-treated pathogens and untreated controls, with SEMs represented by vertical bars (n=3).

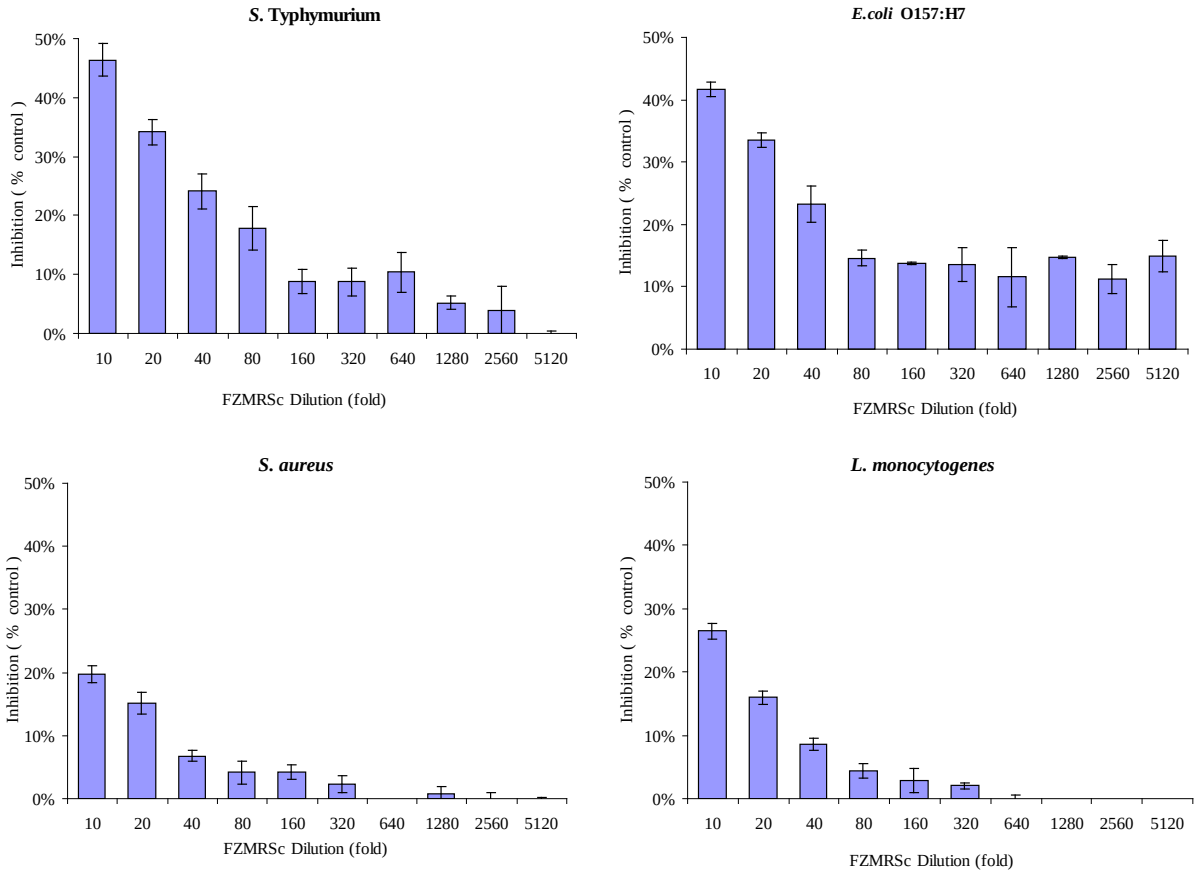

Figure 3.7 Dose response of original FZMRSc against pathogens. Values are expressed as percentage of inhibition relative to controls, calculated from mean OD_{620} of FZMRSc-treated pathogens and untreated controls, with SEMs represented by vertical bars (n=3).

Figure 3.8 demonstrates the dose response for the antimicrobial activity of the original MRSg-8h. It is observed that the antimicrobial activity of MRSg is dose dependent against all assayed pathogens regardless of whether Gram-negative or Gram-positive, and more importantly the dose response patterns were almost identical across pathogens (except for a little difference with *L. monocytogenes* at lower doses). The results show that the first few dilutions retained the strong inhibitory effect against every pathogen. The minimum effective dose of MRSg-8h were at 320-fold dilution for both *S.* Typhimurium and *E. coli* (p= 0.01 and 0.039, respectively). MRSg treatment continued to completely suppress the growth of *S. aureus* until it was diluted up to 160-fold, and the minimum effective dose was determined as the 640-fold dilution (P=0.028). For *L. monocytogenes*, significant inhibition was continued until the minimum effective dose of the 640-fold dilution (P=0.0153). SGF-2h, however, did not show any significant dose response in its antimicrobial activity against *S.* Typhimurium or *S. aureus* (Figure 3.9).

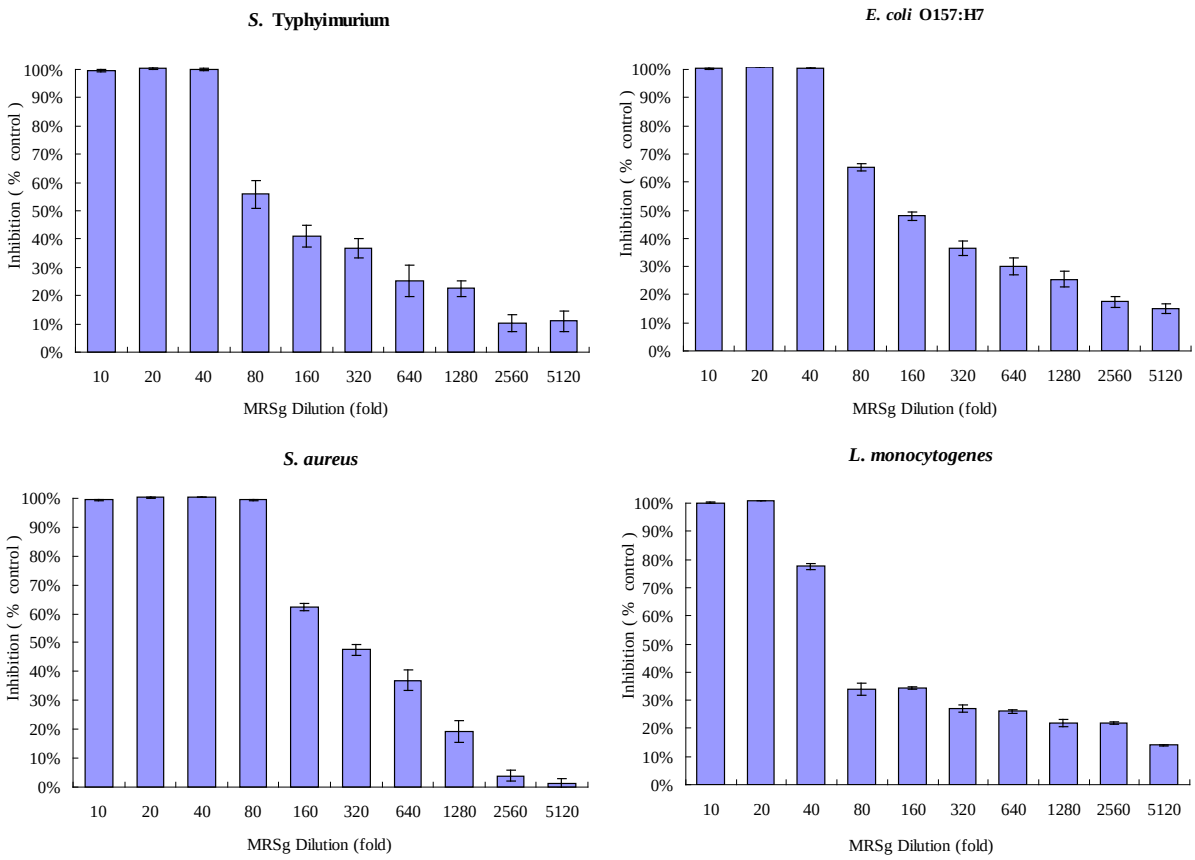

Figure 3.8 Dose response of original MRSg-8h against pathogens. Values are expressed as percentage of inhibition relative to controls, calculated from mean OD_{620} of MRSg-8h-treated pathogens and untreated controls, with SEMs represented by vertical bars (n=3).

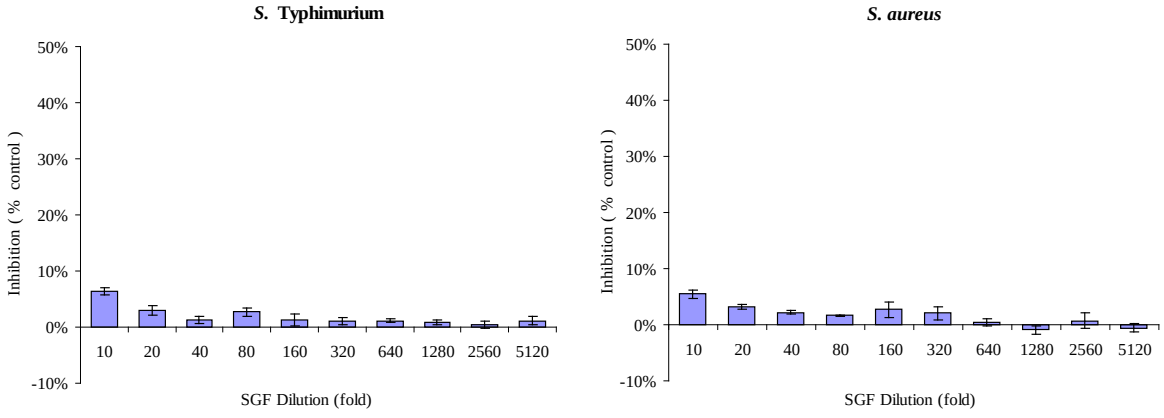

Figure 3.9 Dose response of SGF-2h against pathogens. Values are expressed as percentage of inhibition relative to controls, calculated from mean OD_{620} of SGF-2h-treated pathogens and untreated controls, with SEMs represented by vertical bars (n=3).

From the dose response assays, it is possible to determine the lowest effective dose of each selected cell-free supernatant, which still significantly inhibits the growth of target pathogens (Table 3.3).

Table 3.3 The lowest effective dose of each selected original supernatant (non-pH adjusted) against target pathogens.

Dose (fold)	*S.* Typhimurium	*E. coli*	*S. aureus*	*L. monocytogenes*
MRSc	160	80	10	10
FZMRSc	80	160	20	20
MRSg (8h)	320	320	640	640
SGF (2h)	1	-	1	-

3.3.4 A time course investigation of DPC16 antimicrobial activity on pathogen growth

The purpose of this time course experiment was to investigate the inhibitory effect of DPC16 cell-free supernatants on pathogen growth from the following three aspects: the length of the lag phase, the growth rate during exponential phase, and the total number of pathogen cells at the endpoint of incubation.

Figures 3.10 to 3.13 show the growth curves of the selected pathogens with and without treatments of selected DPC16 supernatant at their minimum effective dose as determined previously. Firstly, pathogens treated with the original (pH unadjusted) MRSc (Figure 3.10) and FZMRSc (Figure 3.11) had similar patterns of growth with no effect on the length of the lag phase. SGF treatment (Figure 3.13) had no significant effect on the growth of *S.* Typhimurium or *S. aureus*, at all. However, the MRSg-8h (Figure 3.12) apparently extended the lag phase of all pathogens from 3h to 6h. Since pathogen growth continued, the MRSg, at the dilution used, did not exhibit bactericidal activity, but rather might have introduced impaired division or suppression to growth.

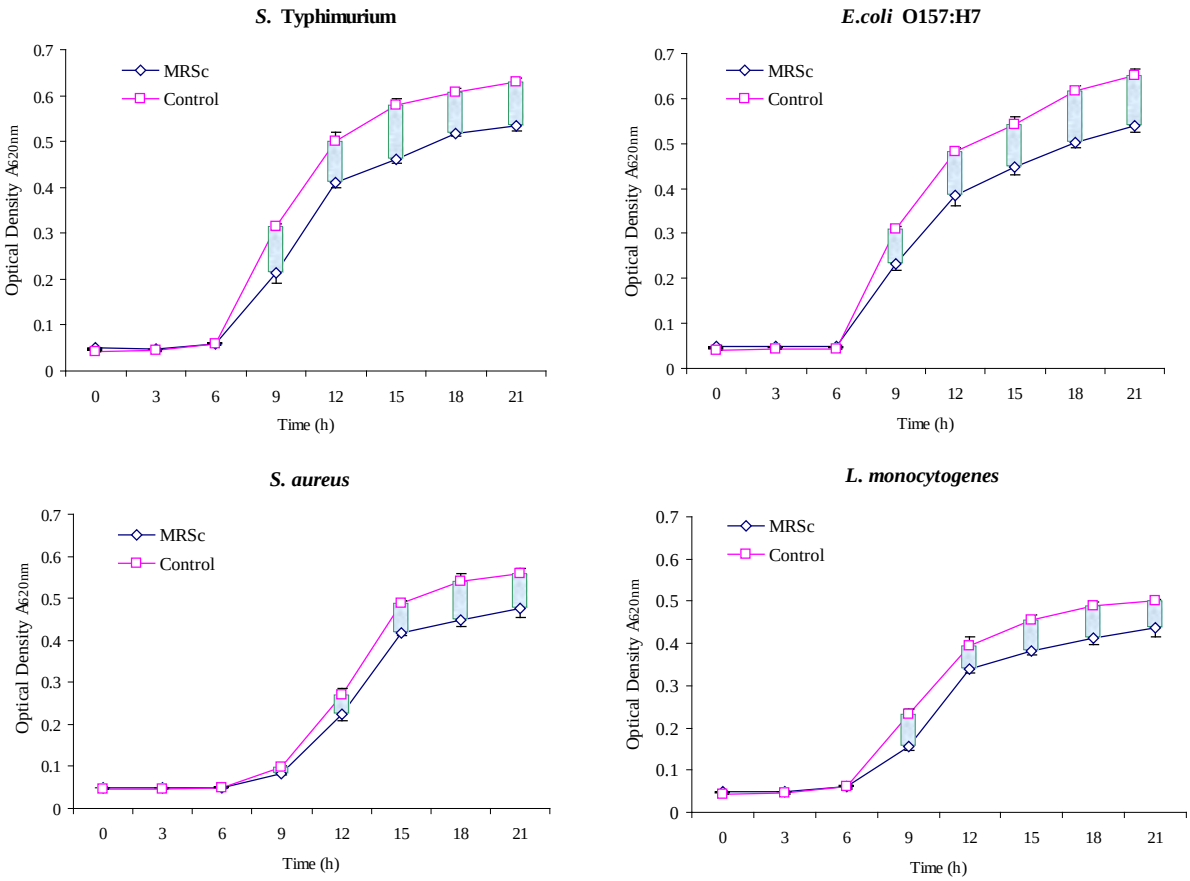

Figure 3.10 A time course of original MRSc treatment, in minimum effective dose, against selected pathogens. Data points are means of triplicate OD_{620} readings measured along a series of time intervals during incubation, with SEMs represented as vertical bars. Drop-down bars between growth curves indicated growth differences of MRSc-treated pathogen compared with untreated controls.

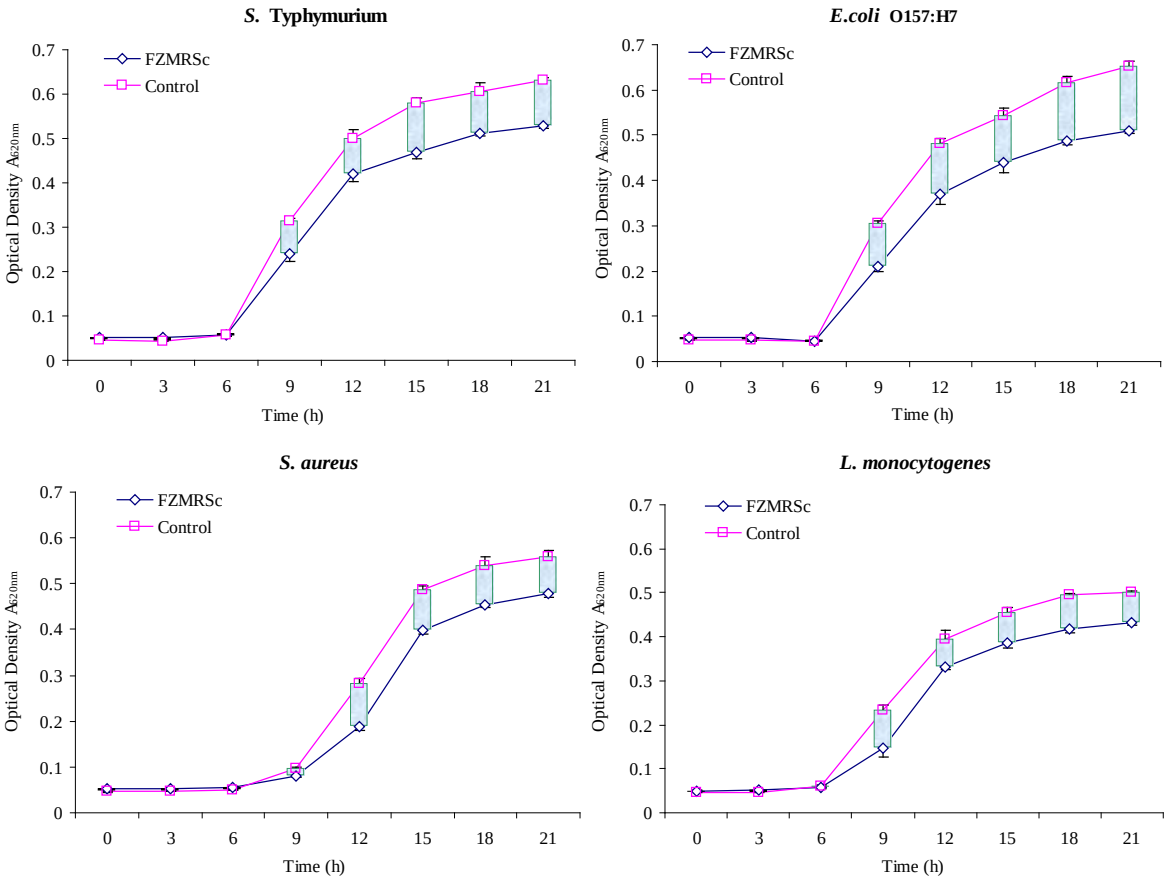

Figure 3.11 A time course of original FZMRS treatment, in minimum effective dose, against selected pathogens. Data points are means of triplicate OD_{620} readings measured along a series of time intervals during incubation, with SEMs represented as vertical bars. Drop-down bars between growth curves indicated growth differences of FZMRSc-treated pathogen compared with untreated controls.

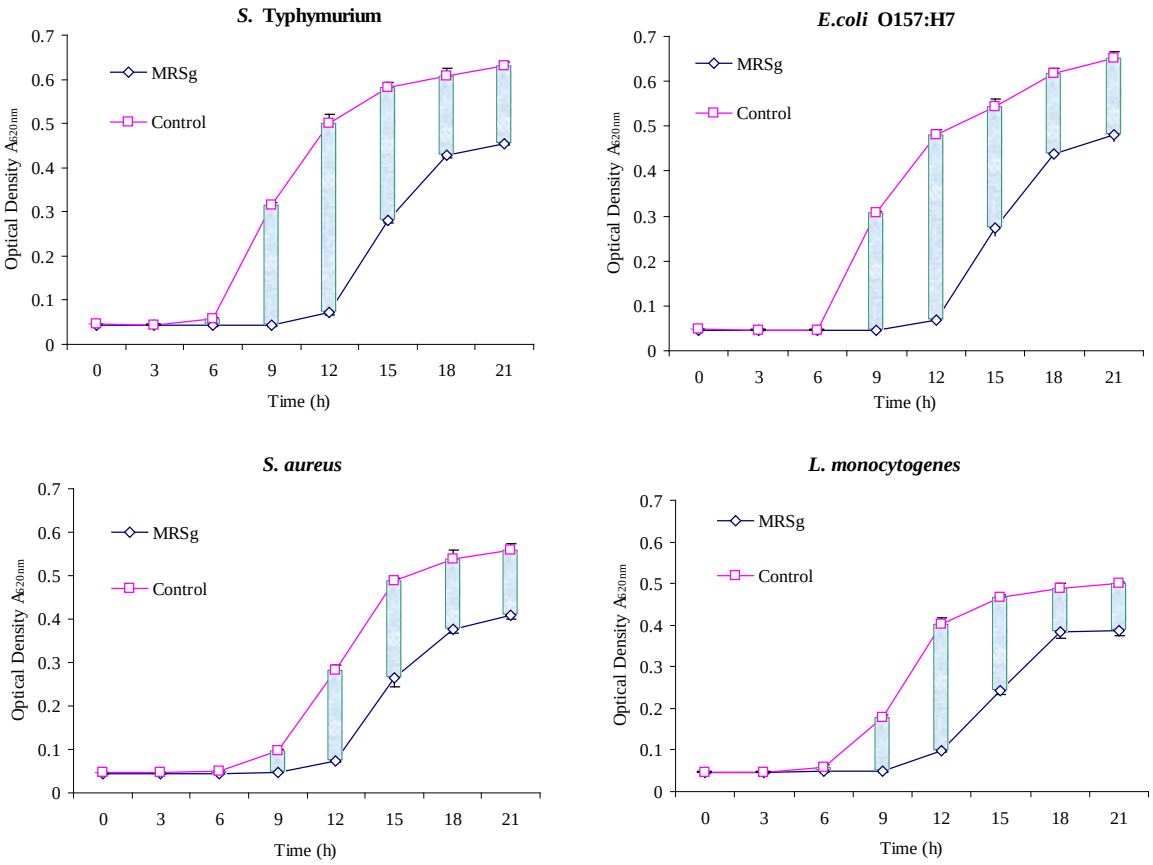

Figure 3.12 A time course of MRSg treatment, in minimum effective dose, against selected pathogens. Data points are means of triplicate OD_{620} readings measured along a series of time intervals during incubation, with SEMs represented as vertical bars. Drop-down bars between growth curves indicated growth differences of MRSg-treated pathogen compared with untreated controls.

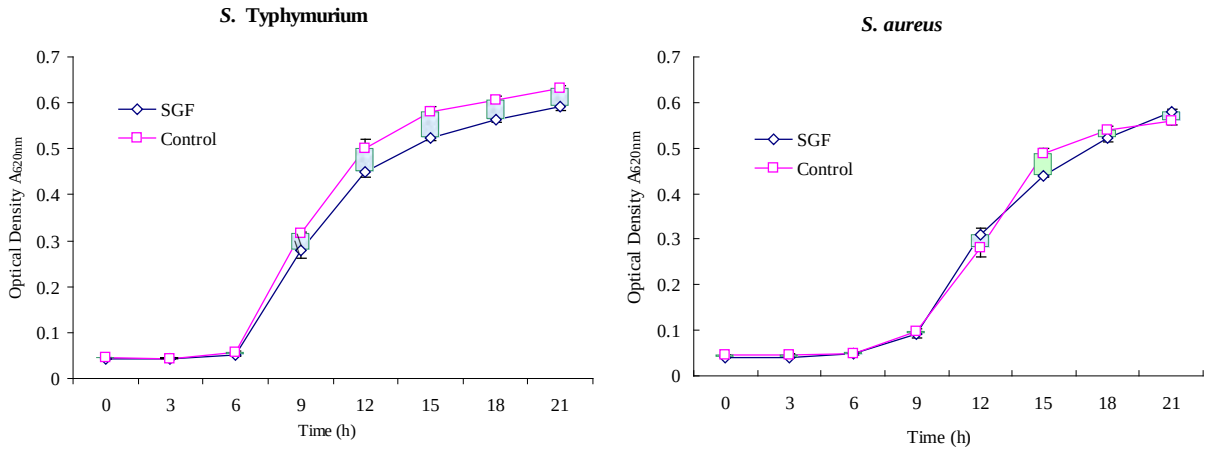

Figure 3.13 A time course of undiluted SGF treatment against *S.* Typhimurium and *S. aureus*. Data points are means of triplicate OD_{620} readings measured along a series of time intervals during incubation, with SEMs represented as vertical bars. Drop-down bars between growth curves indicated growth differences of SGF-treated pathogen compared with untreated controls.

The pathogens' growth rates were calculated, based on the development of optical density (OD_{620}) in the exponential phase (6h), as:

$$\text{Growth rate} = \frac{OD2 - OD1}{t2 - t1}.$$

The growth rate was determined and compared for each pathogen culture with and without the treatments of DPC16 supernatants. Table 3.4 summarises the pathogen growth rate with treatment of designated DPC16 supernatants; and the statistical significances of DPC16 supernatants affecting the growth rates in comparison with the untreated controls were indicated. It is clear that the MRSc and FZMRSc from non-glycerol incubation significantly lowered the pathogens' growth rate (except for *E. coli* O157:H7, this could be due to experimental error since the OD_{620} data deviated greater than the others), whereas the less acidic MRSg from glycerol incubation did not have significant effect on the growth rate of pathogens (except for *L. monocytogenes*). SGF did not affect the target pathogens' growth rate.

Table 3.4 A statistical analysis of pathogen growth rate with and without treatments from the designated DPC16 supernatants. Panel A, pathogen growth rate with MRSc and FZMRSc treatment; panel B, pathogen growth rate with MRSg and SGF treatment. The growth rates were calculated from triplicate OD_{620} readings at the beginning and end point of the exponential phase, and by comparing with those in untreated control, a paired sample T-test was performed and P values were presented, where P <0.05 indicating significance.

A.

Growth Rate (h^{-1})	control	MRSc	P value	control	FZMRSc	P value
S. Typhimurium	0.074 ± 0.002	0.059 ± 0.001	0.015	0.074 ± 0.002	0.060 ± 0.002	0.026
E. coli	0.073 ± 0.001	0.056 ± 0.003	0.074	0.073 ± 0.001	0.054 ± 0.004	0.085
S. aureus	0.065 ± 0.001	0.056 ± 0.000	0.011	0.065 ± 0.001	0.053 ± 0.001	0.002
L. monocytogenes	0.056 ± 0.001	0.047 ± 0.001	0.036	0.056 ± 0.001	0.046 ± 0.002	0.101

B.

Growth Rate (h^{-1})	control	MRSg	P value	control	SGF	P value
S. Typhimurium	0.053 ± 0.004	0.059 ± 0.001	0.446	0.074 ± 0.002	0.067 ± 0.001	0.071
E. coli	0.072 ± 0.001	0.062 ± 0.001	0.053	-	-	-
S. aureus	0.065 ± 0.001	0.050 ± 0.002	0.121	0.065 ± 0.001	0.058 ± 0.001	0.105
L. monocytogenes	0.057 ± 0.001	0.047 ± 0.000	0.002	-	-	-

Finally, the optical density at the endpoint of incubation, reflecting the total number of pathogen cells, was measured and compared with those of the untreated controls. A statistical analysis shows that, except for SGF, almost every selected DPC16 supernatant has significantly decreased the final concentration cells produced during pathogen growth (Table 3.5).

Hence, at the minimum effective dose, MRSc and FZMRSc inhibited the pathogen growth by effectively decreasing the growth rate; while MRSg, a derivative from DPC16 glycerol fermentation, exerted its strong antimicrobial activity by elongating the lag phase of pathogen growth and significantly reducing the total number of pathogen cell in stationary phase. SGF did not show any effective inhibition activity during this time course.

Table 3.5 A statistical analysis comparing the number of total pathogen cells at endpoint of the incubation, between pathogens treated with respective DPC16 supernatants and the untreated controls. Data are mean OD_{620} from triplicate readings, which reflect the cell concentration at the end of the incubation. The P values are calculated from a paired sample T-test (n=3), with P<0.05 indicating significance.

A.

OD_{620}	Control	MRSc	P-value	FZMRSc	P-value
S. Typhimurium	0.631 ± 0.007	0.533 ± 0.011	0.027	0.529 ± 0.006	0.004
E. coli	0.651 ± 0.014	0.529 ± 0.001	0.027	0.510 ± 0.007	0.008
S. aureus	0.559 ± 0.012	0.447 ± 0.001	0.027	0.480 ± 0.001	0.019
L. monocytogenes	0.501 ± 0.003	0.421 ± 0.010	0.018	0.433 ± 0.007	0.016

B.

OD_{620}	Control	MRSg	P-value	SGF	P-value
S. Typhimurium	0.631 ± 0.007	0.455 ± 0.004	0.005	0.592 ± 0.007	0.110
E. coli	0.651 ± 0.014	0.478 ± 0.013	0.021	-	-
S. aureus	0.559 ± 0.012	0.410 ± 0.001	0.011	0.596 ± 0.006	0.094
L. monocytogenes	0.501 ± 0.003	0.388 ± 0.09	0.004	-	-

3.3.5 An antimicrobial efficiency test on MRSg

The strong antimicrobial activity of DPC16 supernatant MRSg was further studied using a viable cell count assay to investigate its efficiency in killing pathogen cells. Table 3.6 shows the survival rate of pathogen cells when in contact with MRSg-8h for a certain period of time. The survival rate was calculated by comparing viable pathogen cells enumerated at each time point to that in its original 18h culture counted prior to treatment. It is clear that viability of all pathogens decreased with increasing contact time with MRSg-8h. The impact on bacterial cells was almost immediate for all pathogens. It is interesting to note that *S. aureus* was the most susceptible, and the number of viable cells hugely declined as the contact with MRSg continued, resulting in 99.9999% (a reduction of 6 log scale) of the cells killed after 180min of treatment. Another Gram-positive pathogen, *L. monocytogenes*, also largely lost its viability during

the MRSg treatment, however it had higher survival rate of 0.221% than any other tested pathogens. A steady decrease of cell viability was observed in both *S.* Typhimurium and *E. coli* O157:H7, with survival rate after 180min contact with MRSg being 0.014% and 0.001%, respectively.

Table 3.6 Sensitivity of pathogens to MRSg-8h treatment. Data represent survival rate of pathogen cells, calculated by comparing the viable cells in the treated pathogen cultures with the untreated cultures, and are presented as mean of triplicates.

Contact Time (min)	15	35	50	70	90	120	150	180
S. Typhimurium	75.2%	59.8%	54.3%	42.9%	15.2%	17.9%	0.3%	0.014%
E. coli	74.3%	59.7%	62.6%	53.1%	25.3%	14.2%	0.1%	0.001%
S. aureus	3.3%	0.832%	0.533%	0.121%	0.047%	0.01%	0.004%	0.000%
L. monocytogenes	78.6%	16.2%	15.1%	7.0%	1.6%	4.0%	1.1%	0.221%

3.3.6 Viability of DPC16 resting cells in glycerol-fermenting cultures

The viability of DPC16 cells from different stages of glycerol-fermentation in both MRSg- and SGF-production culture is displayed in Figure 3.14.

The freeze-drying process used in this study (without supporting medium) had a very significant affect ($P<0.001$) on the cell viability, and resulted in a 72.3% reduction in the number of viable cells.

Decreases in cell viability were observed in both cultures producing MRSg or SGF, but cell death pattern were completely different. Both coming from the process, (starving and then freeze-drying), the cells producing MRSg lost their viability in a steady process, while those in SGF-producing cultures started to die almost immediately after inoculation and in at a much faster rate. After 4h of incubation in glycerol-water, the number of viable DPC16 cells had dropped by more than 7 log scale. A statistical analysis showed that the viability of cells after 6h of producing MRSg was still significantly higher than that detected after the first hour incubation in glycerol-water ($P<0.001$), even though the initial viability of cells inoculated pre-incubation was the same for both. These results signify that cell death occurred earlier and faster in SGF than in MRSg. It is also noted that, during the later incubation (10h-20h) in the MRSg-producing culture, the viability of DPC16 cells had significantly decreased, and by the end of production (20h), the number of viable cells had dropped more than 4.5 log scale.

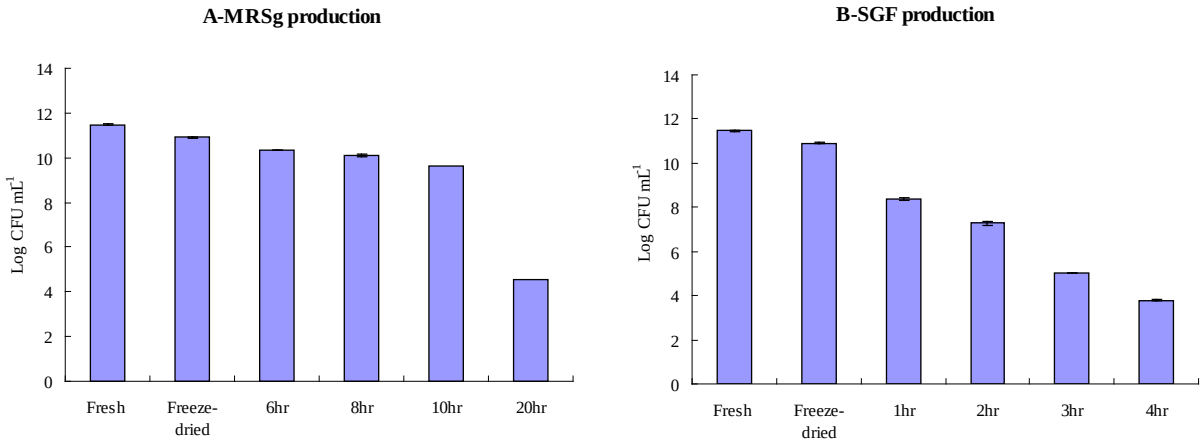

Figure 3.14 A time trend of cell viability of *L. reuteri* DPC16 resting cells in batch culture incubation of glycerol supplemented media. Values are mean log CFU/mL with SEMs represented by vertical bars (n=4).

3.4 Discussion

The objective of this chapter was to evaluate the antimicrobial activities of various cell-free supernatants from DPC16 against selected pathogens, *in vitro*. The tested supernatants are described in Table 3.1.

Prior to antimicrobial assays, the pH measurement of each candidate supernatant showed that the MRSc (pH 4.4) and FZMRSc (pH 4.2) from non-glycerol incubation of DPC16 in MRS were more acidic than those from incubations with glycerol, which indicates that the cells might have taken an alternative metabolic pathway and produced less acidic end-products when glycerol is present, as reported by Talarico *et al.* (1990). The pH change in MRSg was a steadily decreasing process, which took a longer time and resulted with a much lower pH value at the final stabilised state than in SGF. This has suggested that resting cells have undergone more metabolic processes in glycerol-MRS than in glycerol-water. The oscillatory behaviour of pH values in SGF indicates a process in which few acidic products were produced at the initial stage, but such production ceased early and the acids were quickly neutralised leaving the culture with a near-neutral pH with a very low buffering strength (due to the low concentration of salts).

The results of a primary antimicrobial assay have shown that MRSc and FZMRSc significantly inhibited the growth of all selected pathogens, whereas their pH-adjusted counterparts did not show any inhibition. This means the major antimicrobial activity in these two supernatants was most likely due to their acidic components. The role of SCFA (*e.g.* lactic acid, acetic acid, formic acid, succinic acid, *etc.*) produced in probiotic *Lactobacillus* species have been reported to be responsible for strong antimicrobial activity against all the pathogens studied in this chapter (Jin *et al.*, 1996; Lehto and Salminen, 1997; De Keersmacker *et al.*, 2006; Hutt *et al.*, 2006; Millette *et al.*, 2007). The acidic effect of SCFA on the growth of pathogens has been intensively studied and the mechanism is believed to be associated with the dissociation of SCFA across transmembrane pH-gradient. LAB can produce SCFA and secret them extracellularly, hence decrease the pH value of local environment. At relatively low pH, these SCFA remain undissociated; and as being lipophilic, undissociated SCFA are able to penetrate the bacterial membrane and subsequently dissociate when encountering the relative higher intracellular pH, hence resulting in releasing large amounts of free hydrogen ions

and generating a large transmembrane proton gradient which interferes with essential metabolic functions of affected bacteria (Baird-Parker, 1980; Booth, 1985). The pathogens tested in this experiment (*S.* Typhimurium, *E. coli* O157:H7, *S. aureus*, and *L. monocytogenes*) are all neutrophilic bacteria, and they are sensitive to changes of extracellular pH values.

In addition, it is also found that there was no difference between MRSc and FZMRSc in their inhibitory effect against selected pathogens, so the freeze-drying process does not affect the substances in exerting their antimicrobial activity in solution.

In addition to MRSc and FZMRSc, the same antimicrobial assay was carried out with a kinetic profile (a series of supernatant harvested from designated time points during incubation) of MRSg and SGF from glycerol fermentation with two-fold concentrated DPC16 resting cells obtained from the stationary phase of a previously incubated DPC16 culture. The MRSg series produced by glycerol-MRS fermentation (molar concentration ratio of glycerol:glucose = 360:110 mM) has demonstrated a very strong antimicrobial activity against all pathogens both before and after the pH-adjustment. This inhibitory effect of MRSg developed slowly during the first few hours of incubation, but an exponential increase was observed between 6-8h (4-6h for *S. aureus*) of incubation resulting nearly 100% inhibition thereafter. A post-treatment viability count of pathogens treated between the ends of the exponential increase confirmed that the antimicrobial activity of MRSg has turned from moderate inhibition into complete bactericidal. These results suggested that certain antimicrobial-potent substances other than SCFA were synthesised and started to exhibit activity during this period of incubation. Salameh *et al.* (2003) reported the observation of total growth inhibition of *E. coli* O157:H7 when treated with supernatant obtained from a variety of strains of *L. reuteri* incubated in glycerol-supplemented MRS. They also reported that this total inhibition of pathogen could be observed within 4h of treatment. Interestingly, the result of the antimicrobial efficiency test using the MRSg-8h has agreed with this report in that the supernatant was able to, almost-totally, inhibit the pathogen growth within 3h (180 min) of contact in solution.

It is also noticed that this strong antimicrobial activity appeared suddenly after a few hours of initial incubation, where the antimicrobial activity was similar to that of the acidic effect. It is assumed that this is due to either the adaption of DPC16 cells to the

environment prior to its subsequent production of a certain potent substance, or the existence of a certain tolerance threshold where pathogens can only tolerate certain substances in the media to a certain level, and when the amount of such substances exceeds this threshold, the pathogen growth is largely inhibited. Also, antimicrobial activity of this synthesised substance is strain specific.

The exact identification of the potent antimicrobial agent(s) produced in MRSg during the exponential increase of antimicrobial activity is unknown. However, it (or one of them) may be reuterin. Talarico *et al*. (1990) identified reuterin from glycerol-glucose co-fermentation of *L. reuteri* 1063 by means of HPLC and FTIR, and they claimed that *L. reuteri* could utilise exogenous glycerol as a hydrogen acceptor during carbohydrate fermentations, resulting in higher growth rates and cell yields than those obtained during growth on carbohydrates alone. According to Axelsson *et al*. (1989), reuterin, from *L. reuteri*, is a broad spectrum antimicrobial compound. Arques *et al*. (2004) studied the antimicrobial activity of reuterin against different food-borne Gram-positive and Gram-negative pathogens in milk, and found that reuterin (8 arbitrary units /mL) exhibited bacteriostatic activity against *Listeria monocytogenes*, whereas bactericidal activity was detected against *E. coli* O157:H7, *Salmonella choleraesuis* subsp. *choleraesuis*, *Yersinia enterocolitica*, *Aeromonas hydrophila* subsp. *hydrophila* and *Campylobacter jejuni*. Rasch (2002 a) observed an increased inhibitory activity of reuterin against *E. coli* as the temperature was increased within the range of 10-30°C, whereas variations in pH (4.5–6.5) and salt content (0.5–3%) did not influence the inhibitory activity. The results of pH-independent and strong antimicrobial activity of MRSg harvested after 8h of secondary fermentation have been consistent with the above findings.

Although both MRSg and SGF were produced from glycerol fermentation using published methods, SGF showed different antimicrobial activity when compared with the MRSg. Instead, it only transiently inhibited *S.aureus* and *S.* Typhimurium with SGF from 2h incubation. This difference in activity is probably due to the difference in the composition of the media. In the production of MRSg, the DPC16 resting cells were able to continue metabolising while excessive glycerol was supplied. In contrast, the same set of resting cells had no fermentable source of carbohydrate, and this might result in metabolism ceasing shortly after the start of incubation as seen in the transient pH drop, and eventual cell death. This result suggests that this strain of *L. reuteri* may

not be able to utilise glycerol alone. Latest research has reported that the efficacy of reuterin production is highly variable amongst different strains of *L. reuteri* due to variations in gene expression of associated enzymes (Spinler *et al*., 2008).

With the minimum effective dose of each cell-free supernatant determined in a dose response assay, a time course of pathogen growth was carried out to further characterise the supernatants' antimicrobial effect. It was found that treatment with MRSc and FZMRSc did not affect the length of lag phase of pathogen growth, but lowered the growth rate, and as a result, the total pathogen cell numbers were decreased. This indicates that the acidic effect from SCFA could affect the exponential phase of pathogen growth by interfering with cell-division resulting in lower cell numbers in the stationary phase. However, MRSg, which possibly contained reuterin, acted quite differently. By adding the lowest effective dose of MRSg into the pathogen cultures, prolonged lag phase was observed for each pathogen. It is assumed that MRSg, at this very low concentration (320- to 640-fold), has introduced a stress response to the pathogens, so that the cells had to spend more time adapting to the media environment. It is interesting to note that only the growth rate of *L. monocytogenes* was affected by MRSg-8h. This may be due to a low level of glycolysis by the resting cells, as seen with high pH value (pH 5.1) of MRSg-8h. However, the total cell number of each pathogen treated with MRSg-8h was significantly reduced in its stationary phase. Recently, Rasch *et al*. (2007) reported that reuterin elongated the lag phase and induced cell variance to *Listeria innocua*, and at last completely prevented the cell division, irrespective of different pH values and NaCl concentrations in the media environment.

The transient antimicrobial activity of SGF-2h was also tested in the time course against its target pathogens with the original undiluted dose. However, no significant antimicrobial effect was detected with either the lag phase, the growth rate or the final cell numbers of the target pathogens' growth. The completely different antimicrobial activity of SGF from MRSg has pointed to the possibility that there may be no reuterin in SGF, and that there is no SCFA produced from normal cell metabolism either, since the acidic effect was not seen.

An efficiency test for the strong antimicrobial activity of MRSg-8h showed that all selected pathogens were very susceptible to this supernatant at its undiluted dose, and a bactericidal effect was observed with 3h of treatment. This suggests that at relatively

high concentration, the MRSg (putatively containing reuterin presence) has bactericidal or bacteriolytic effect; while at lower concentration, MRSg suppresses pathogen growth by introducing certain stress response to impair and delay pathogen cell replication. The reason for such difference was not investigated during this study, but it could possibly be attributed to the change of molecular structure of the putative reuterin in an aqueous solution. For example, Vollenweider *et al.* (2003) reported that the distribution of the three β-hydroxypropionaldehyde (HPA) compounds forming the reuterin system was concentration-dependent in aqueous solution. At high concentrations, reuterin predominantly exists in the cyclic dimer form; while at lower concentrations, the majority of reuterin is in the hydrated form.

Finally, a viability test on the DPC16 cells producing MRSg and SGF was conducted. The large decrease of cell viability in MRSg-producing cells, observed after the huge increase of extracellular antimicrobial activity, has indicated that MRSg is not only competent to inhibit the pathogen growth, but also it can exert a significant impact on the viability of its own cells. Lethal effect of accumulated reuterin on *L. reuteri* cells in prolonged incubation has been previously reported (Talarico *et al.*, 1988; Chung *et al.*, 1989; Rasch *et al.*, 2002 b). However, it was also believed that *Lactobacillus* species, including *L. reuteri* itself, are about 4-5 times less sensitive to reuterin than the pathogens (Chung *et al.*, 1989), and this decrease in viability occurs only when the *L. reuteri* cells have been encountered with a very high concentration of reuterin for a long duration (Doleyres *et al*., 2005). In addition, the death of *L. reuteri* cell was reported to be independent of reuterin concentration (Rasch *et al*., 2002 b). These published findings have provided more evidence, from another perspective, of the presence of reuterin, since the cell death pattern in MRSg coincided with that described in the above reports.

The cells in SGF-producing culture, however, behaved completely different from those producing MRSg, even though they had the same initial viability. The cell death in glycerol-water fermentation occurred as soon as the incubation started, and the starved cells died very fast in this non-growth medium. This provides evidence to the previous explanation that *L. reuteri* DPC16 cannot metabolise in a solution where only glycerol was supplied, hence the starved resting cells died due to lack of nutrients. However, the transient inhibitory effect of SGF to the most susceptible *S. aureus* was observed, and a transient pH drop was evidenced. Whether, or not, there is reuterin produced in the

glycerol-water fermentation can only be determined with reuterin quantification of the SGF kinetic profile. .

In summary, the *L. reuteri* DPC16 cell free supernatants MRSc and FZMRSc, harvested from MRS broth without glycerol, showed significant inhibitory activity against all selected pathogens, and it is thought to be mainly due to the acidic effect of SCFA as evidenced by the loss of activity in their pH-adjusted counterparts. This acidic effect was shown to reduce the pathogen growth rate and decrease the total pathogen cell numbers in a time course study. The kinetic profile of MRSg, obtained during incubation of DPC16 resting cells in glycerol-supplemented MRS broth has shown very strong antimicrobial activity against all target pathogens. This antimicrobial activity is pH-independent, and impacted on both Gram-negative and Gram-positive pathogens. It is assumed that reuterin may be present in MRSg. By comparing the antimicrobial activity of MRSg in high and low doses, it was found that MRSg, at a high dose, had a potent bactericidal effect resulting in pathogen cell death within 3h of treatment, while, at a low dose, MRSg was bacteriostatic by inducing a stress response to pathogen cells during lag phase and hence delaying cell replication. However, this strong antimicrobial activity can also act on DPC16 itself by significantly reducing its cell viability. But, this self-inhibiting activity occurred moderately and was tolerable until late in the fermentation. SGF, another glycerol fermentation product tested in this study, did not show a clear antimicrobial activity, did not develop a normal acidic cultural pH, and the cells producing SGF died very quickly. These suggest that there was no normal cell metabolism by the DPC16 cells in glycerol-water, and this strain may not be capable of producing reuterin without the presence of fermentable carbohydrate.

The interesting findings of the strong antimicrobial activity of MRSg has led to additional studies in the next chapter, where the growth kinetics of DPC16 (*e.g.* growth rate and biomass) were studied, reuterin productivity was measured, the relationship between reuterin concentration and the antimicrobial activity of MRSg was obtained, and the DPC16 cell viability during reuterin production was examined.

Chapter 4

Kinetics of *Lactobacillus reuteri* DPC16 growth and *in situ* reuterin production in a two-step fermentation process

4.1 Introduction

Lactobacillus reuteri is a Gram-positive lactic acid bacterium belonging to the obligate heterofermentative group, which preferentially uses the phosphoketolase pathway for metabolism. It resides as a major component of enterolactobacilli in the GI tract of various hosts ranging from birds to humans, where it establishes a symbiotic relationship (Casas and Dobrogosz, 2000). Anaerobic resting cells of this species can convert glycerol into a broad-spectrum antimicrobial substance of 3-hydroxy-propionaldehyde, termed reuterin (Chung *et al*., 1989). Reuterin, from *L. reuteri*, is synthesised intracellularly as an intermediate product from glycerol by a cobalamin-dependent enzyme, glycerol dehydratase. Some of the reuterin is further reduced to 1,3-propanediol by an NAD-dependent oxidoreductase (Talarico *et al*., 1990; Luthi-Peng *et al*., 2002 a), while the rest is accumulated and subsequently excreted to extracellular environment under normal physiological conditions of temperature and pH (Talarico *et al*., 1988). A common practical method to measure reuterin is a colorimetric assay introduced by Circle *et al*. (1945) to measure acrolein, which is a dehydration derivative of reuterin produced in equimolar concentrations to reuterin in a heated acid aqueous solution. This method has been widely used for detection and quantitative determination of reuterin; is simple to conduct and delivers accurate results (Slininger *et al*., 1983; Talarico *et al*., 1990; Luthi-Peng *et al*., 2002 a, 2002 b; Vollenweider and Lacroix, 2004).

Several approaches have been made to characterise the growth kinetics of *L. reuteri.* A mathematical model consisting of four coupled differential equations has been developed to predict concentrations of biomass, glucose and glycerol as well as reuterin production with time, in a batch culture fermentation of *L. reuteri* (Rasch *et al.*, 2002 b). Another batch fermentation model was recently established (Tobajas *et al*., 2007) to describe the production kinetics (biomass concentration, utilisation of glycerol substrate, reuterin and 1,3-propanedial production) by using starved *L. reuteri* PRO137 resting cells in a two-step process under controlled conditions.

Experiments in this chapter were conducted with the aim of understanding the growth of *L. reuteri* DPC16 in terms of specific growth rate, biomass production and reuterin productivity under standardised growth conditions. Attempts were made to confirm the existence of reuterin in culture supernatants (MRSg and SGF as prepared in Chapter 3), as well as to establish a correlation between reuterin and its corresponding antimicrobial activity specified in quantitative terms.

4.2 Materials and Methods

4.2.1 Culture preparation

Cultivation of DPC16 followed the method introduced in Section 3.2.2, and a 20h culture was prepared in pre-reduced MRS and subsequently enumerated on MRS agar plate using the drop-plate method. The culture was then binary diluted with peptone water up to 32-fold, and the turbidity of each dilution reflected by optical density (OD_{620}) was measured, in triplicate, using a spectrophotometer (MK3, Genova, Jenway; UK). Thus, a correlation curve of OD_{620} and viable cell counts (CFU/mL) was established. The pH value of the 20h DPC16 culture was also measured using a pH meter (Orion, Thermal Scientific, USA) at room temperature.

4.2.2 Growth curve of *L. reuteri* DPC16 in pre-reduced MRS broth

Based on the number of viable cells in the 20h culture, a dilution containing approximate 10^4 CFU/mL of DPC16 cells was prepared as a subculture inoculum capable of producing a full growth curve with defined length of lag phase. Then, the growth curve was developed over a time course experiment by monitoring the changes of OD_{620} during the subculture incubation. Briefly, 1% (v/v) of the DPC16 subculture inoculum (10^4 CFU/mL) was aseptically transferred into a pre-reduced MRS broth, and incubated anaerobically at 37°C for up to 20h. During the incubation, the OD_{620} value was measured, in triplicate, at 2h time intervals, and the growth curve was plotted with the mean OD_{620} value collected at each time point against incubation time. The growth of DPC16 under the above standardised conditions was characterised by the length of lag phase, slope of the exponential phase (based on optical density), and the total viable cells at the endpoint of incubation (stationary phase).

4.2.3 Measurement of DPC16 dry matter by freeze-drying

At the end of the above incubation, the culture was centrifuged and the concentrations of dry materials were measured by freeze-drying. Following the same process as described in Section 3.2.3, total biomass (dry cells) and lyophilized culture supernatant of this DPC16 culture were measured, in quadruplicate. A correlation curve between OD_{620} and cell dry weight (CDW) was developed using the same culture. Briefly, the culture was binary diluted up to 8-fold, and each dilution was measured for OD_{620} value,

and then centrifuged at 4,000 x g for 10 min at 4°C in pre-weighed centrifuge tubes. The supernatants were discarded; and the cell pellets were washed twice with PBS, centrifuged again and finally freeze-dried for 48h. Hence, the total CDW for each dilution was measured, and the relationship between culture OD_{620} and CDW was obtained.

4.2.4 Colorimetric determination of glycerol and reuterin

The DPC16 cell-free supernatants of MRSg and SGF, in their kinetic profiles, produced from glycerol fermentation as introduced in Chapter 3, were quantitatively analysed for their glycerol residue and reuterin content after the assays of their antimicrobial activity. The determinations of glycerol and reuterin are described as below.

4.2.4.1 Glycerol

The colorimetric method of Spagnolo (1953) was used with some modifications. It was based upon the formation of blue colour developed by the cupric-glycerol complex in basic solution. Briefly, a series of glycerol standards were firstly prepared, in duplicate, with 0-20 mg/mL (0-217.4 mM) of stock glycerol (95% analytical grade, LabServe, Biolab, New Zealand) in 10 mL distilled water. An aliquot of each standard (1 mL) was subsequently added into a mixture containing 1 mL of 20% sodium hydroxide and 6.0 mL of 95% (v/v) ethanol. Then, with vigorous swirling, a volume of 0.6 mL of 10% cupric chloride was added slowly into the above mixture. This mixture was finally diluted to a 10 mL volume with 95% ethanol. These standard solutions were vortex-mixed vigorously for 2 min, and the well-mixed solutions were centrifuged at 420 x g for 5 min. The clear supernatants were collected and transferred into matched spectrophotometer cuvettes to measure the absorbance at 635 nm against a reagent control (similarly prepared using 1 mL of water in place of the glycerol standard). Thus, a standard curve of glycerol in aqueous solution was produced spectrophotometrically. Details on preparation of the glycerol standards are shown in the Appendix 3.

The original MRSg and SGF were firstly neutralised to pH 7 using 0.1 M NaOH. Then, an aliquot of each neutralised supernatant (0.5 mL) was diluted with an equal volume of distilled water to bring the possible glycerol residue concentration within the detectable range of the standard curve prepared above. Subsequently, aliquots of each diluted supernatant (1 mL) were subjected to the above procedure to measure colour formation,

and the glycerol content in each supernatant was determined by interpolation within the glycerol standard curve, in duplicate. A medium control replacing DPC16 supernatants with respective glycerol-supplemented medium (glycerol-MRS or glycerol-water) was also prepared for each supernatant.

4.2.4.2 Reuterin

The colorimetric assay for reuterin followed the acrolein method of Circle *et al.* (1945) with some modifications. Stock acrolein solution (BioChemika, Fluka, Sigma; EU) was provided by Crop and Food Research, New Zealand. Prior to test, stock acrolein was 1000-fold diluted with 95% ethanol, and this dilution was used for preparation of an acrolein standard. Acrolein standards, containing 0-120 µg/mL in 10 mL final volume, were firstly prepared in 95% ethanol (2mL each). A tryptophan solution was made up to 0.01 M by dissolving crystalline *DL*-tryptophan (Sigma, USA) in 0.05 M HCl, and 0.5 mL of the tryptophan solution was added to each acrolein standard. Subsequently, 6.3 mL of 12 M HCl (37%, J. T. Baker Chemical Co.; USA) was added to each acrolein standard. Lastly, an additional 1.2 mL of 95% ethanol was added to each standard mixture to make up to the total volume of 10 mL. All standards were prepared in an ice bath to prevent premature heating. Once prepared, the standards were transferred immediately into a 40°C water bath, and incubated for 50 min to reach the maximum colour development. After incubation, all standards were placed immediately back to the ice bath to stabilize the colours, and subsequently measured for absorbance at 560 nm in matched spectrophotometer cuvettes. A reagent control containing 2 mL of ethanol replacing the acrolein standard was used for adjustment of the spectrophotometer. Details on preparation of the acrolein standard are shown in the Appendix 3.

Original DPC16 supernatants of MRSg and SGF were diluted 2000- and 1000-fold, respectively, with 95% ethanol. An aliquot of each diluted supernatant (1 mL) was then mixed with 95% ethanol (1 mL) and subjected to the same procedure as above. Acrolein concentrations were measured in triplicate, and the molar equivalent of reuterin kinetically produced in each DCP16 glycerol-fermentation culture was then determined.

4.2.5 Relationship between reuterin and the antimicrobial activity in DPC16 supernatants from glycerol fermentation

Having measured the reuterin concentration in both MRSg and SGF profile, the synthesis profile of reuterin was compared with the degree of antimicrobial activity in each supernatant from glycerol fermentation as determined in Section 3.3.2, and hence a relationship between reuterin and cultural antimicrobial activity was established.

4.3 Results

4.3.1 Viable cell enumeration and pH measurement of DPC16 culture

Enumeration showed that the population density of the DPC16 culture, after 20h incubation in reduced MRS broth, reached 9.62 ± 0.01 log CFU/mL (4.23 x 10^9 CFU/mL). The culture pH value was 4.44 ± 0.03. A correlation curve of OD_{620} and concentration of viable cells in the above DPC16 culture was displayed in Figure 4.1, and this was used to predict the concentration of viable DPC16cells in culture.

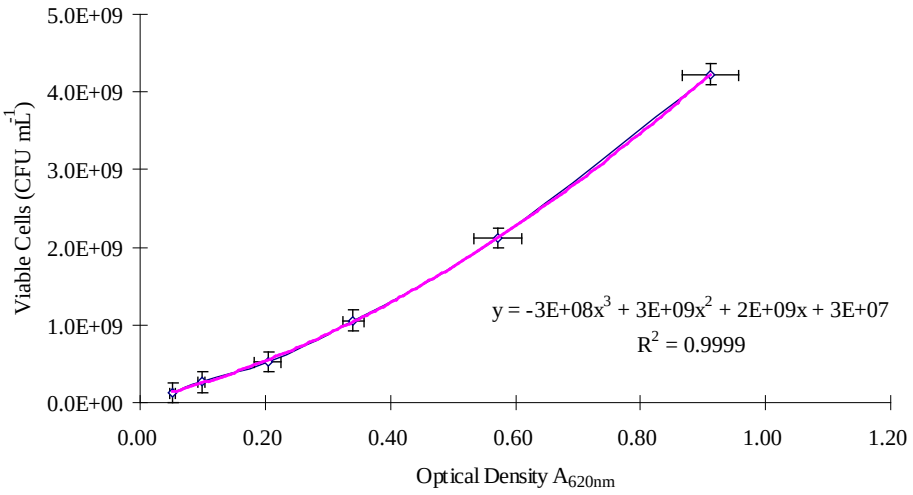

Figure 4.1 Correlation curve of OD_{620} and the numbers of viable cells, expressed in CFU/mL, in a DPC16 culture incubated in MRS for 20h. Data points are means of triplicate measurements in both scales, with SEMs represented as vertical or horizontal bars for CFU/mL or OD_{620}, respectively. The mathematical equation with a fitting coefficient R^2 indicates the correlation.

4.3.2 Growth of *L. reuteri* DPC16 in MRS under anaerobic condition

Using diluted subculture inoculum (10^4 CFU/mL) as a starter culture, a growth curve of *L. reuteri* DPC16 was developed (Figure 4.2). It shows a lag phase of 8h while slight growth was observed at 10h with exponential growth occurring thereafter, until 14-16h where the growth slowed down and the stationary phase was approached. By plotting a regression line within the clearly linear range of the exponential phase, a slope of 0.137 h^{-1} was determined and subsequently used to calculate the specific growth rate.

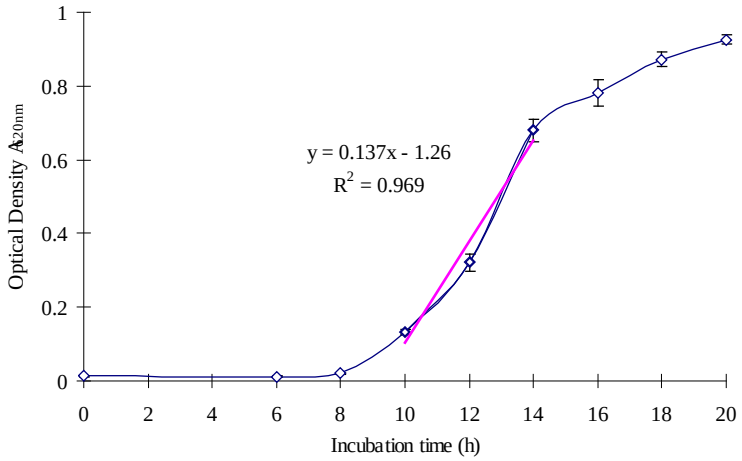

Figure 4.2 Growth curve of DPC16 in pre-reduced MRS during anaerobic incubation at 37°C. Data points are means of triplicate OD_{620} readings, with SEMs represented as vertical bars. A regression line linearly fitting the exponential phase of growth was also plotted, with a mathematical equation indicating the slope.

Dry materials including separated dry cells and lyophilised cell-free supernatant of DPC16 after 20h of incubation were obtained by freeze-drying. The concentration of biomass was 5.5 ± 0.7 g/L and lyophilized cell-free supernatant was 49.6 ± 1.4 g/L, which contributed to the total dry matter of 55.1 ± 1.5 g/L. It was noticed that a slight loss of mass balance occurred during the processes (incubation and subsequent freeze-drying) as the total ingredients input was 55 g/L (comparing the 49.6 g/L of dried supernatant with the 55 g/L of raw material of MRS broth, and excluding weight of bacteria cells). Thus, 5.5 g/L of dry resting cells of DPC16 corresponded to 4.23 x 10^9 CFU/mL, and in the secondary fermentation process for glycerol conversion to reuterin, the two-fold concentrated biomass was about 11 g/L.

A similar freeze-drying process on a dilution series of the same culture has produced a

correlation curve of cell dry weight (CDW) to cultural optical density (OD_{620}). As shown in Figure 4.3, the OD_{620} and CDW of the 20h DPC16 culture had a linear relationship, so it can be used to predict CDW based on culture turbidity.

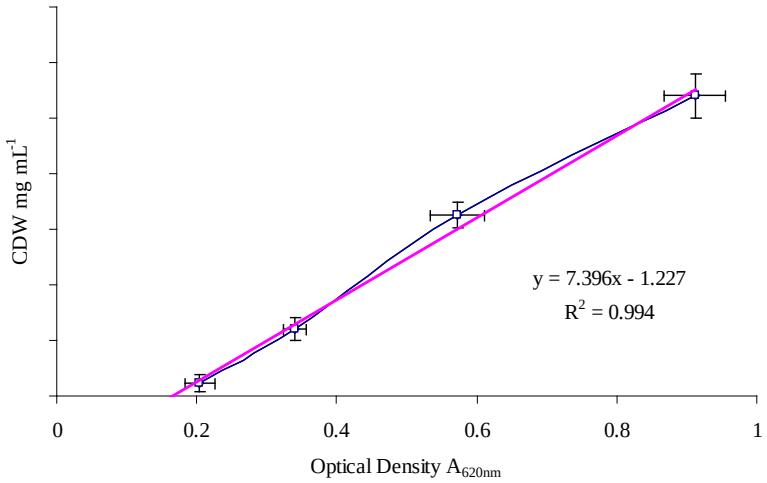

Figure 4.3 Correlation curve of OD_{620} and CDW of DPC16 culture incubated in MRS for 20h. Data points are means of triplicate measurements in both scales, with SEMs represented as vertical or horizontal bars for CDW or OD_{620}, respectively. The mathematical equation with a fitting coefficient R2 indicates the linear correlation.

By incorporating this relationship into the growth curve observed in the 20h DPC16 culture (Figure 4.2), the optical density values in the linear range of the exponential phase were translated into CDW, so that the specific growth rate (h^{-1}) was calculated as follow:

$$\mu = \frac{LnCDWt2 - LnCDWt1}{t2 - t1} = 0.6\ h^{-1}.$$

The population doubling time (Td) was accordingly determined using the following equation:

$$\text{Td} = (t2 - t1) \times \frac{Ln2}{LnCDWt2 - LnCDWt1} = \frac{Ln2}{\mu} = 1.16\text{h}.$$

4.3.3 Bioconversion of glycerol and yield of reuterin from biomass

The production kinetics of bioconversion of glycerol into reuterin in DPC16 glycerol-MRS fermentation is shown in Figure 4.4. A steady increase in glycerol utilisation was accompanied by a sigmoidal increase of reuterin, and the reuterin production increased markedly between 6h to 8h of the secondary fermentation and levelled after 10h. In SGF, however, even though a slight decrease in glycerol concentration was observed, the detectable reuterin was negligible [(no visible colour development and OD_{620} data were not detectable over the reliable range (OD<0.1)] thus it is assumed that glycerol bioconversion did not occur in glycerol-water fermentation and no reuterin was produced. The glycerol utilisation in SGF was considered as being absorbed into DPC16 resting cells and saturation was reached after 2h of incubation with average intracellular glycerol concentration of 10.4 mM.

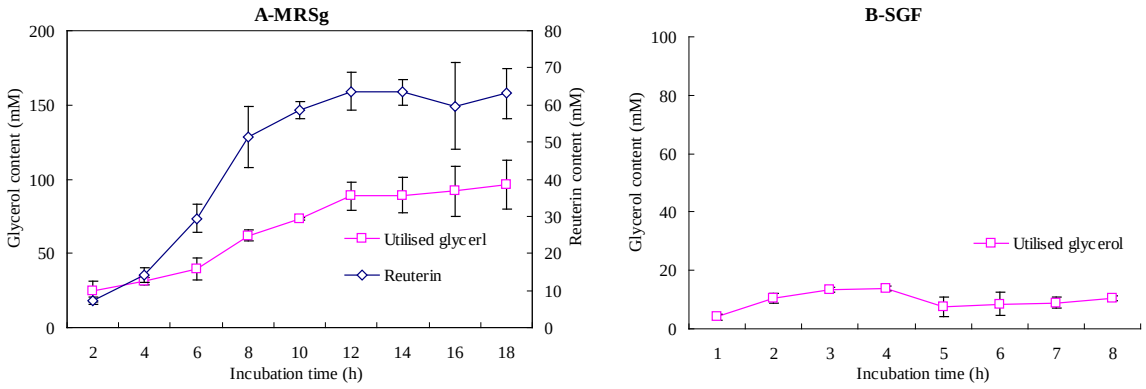

Figure 4.4 Time course of L. reuteri DPC16 resting cells bioconversion of glycerol to reuterin in MRSg (A) and SGF (B). A: concentrations of utilized glycerol relative to medium control (□) and reuterin production (◊) detected in MRSg kinetic profile; B, glycerol utilisation relative to medium control detected in SGF kinetic profile. The data points represent means of duplicate OD_{635} measurements for glycerol in A and B, and triplicate OD_{560} measurements for reuterin in A. SEMs are represented as vertical bars.

Based on the above assumption, an actual glycerol utilisation of 85.8 mM was calculated in glycerol-MRS fermentation while producing MRSg, resulting in 23.8% utilisation of the total glycerol supplementation (360mM). At the end of the 18h fermentation (MRSg profile was tested for reuterin up to 18h), there was 62.5 mM (calculated as an average concentration from 12h to 18h) of reuterin detected in the culture supernatant, and hence the yield of reuterin from utilised glycerol was 72.8%.

From the reuterin concentration detected in MRSg profile, another kinetic parameter of reuterin production rate was obtained, and it was calculated as follow:

$$\text{Production rate (mM/h)} = \frac{reuterin\,2\,(mM) - reuterin1\,(mM)}{T2 - T1}.$$

As shown in Figure 4.5, the reuterin production rate reached a maximum of 10.9 mM/h (811 mg/L.h^{-1}) at 6h of incubation and sharply decreased thereafter.

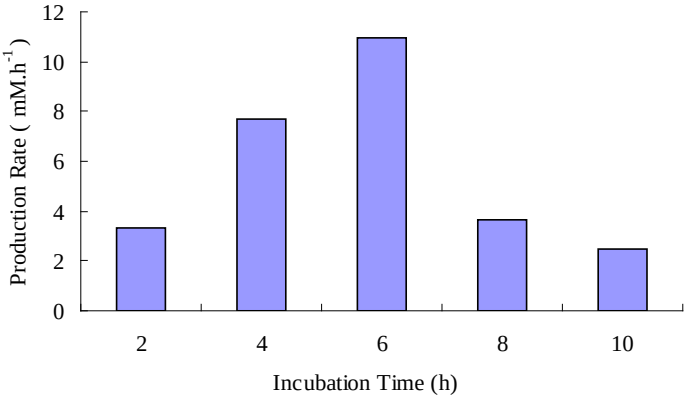

Figure 4.5 Production rate of reuterin during incubation of DPC16 in glycerol-MSR broth under standardised conditions.

Having known from previous biomass determination (Section 4.3.3) that the MRSg was produced from 11 g/L of DPC16 resting cells, then the production rates of reuterin (monomer MW = 74 g/mol) were translated into specific production rate as follow:

$$\text{Specific production rate} = \frac{reuterin\,synthesized\,(mM)}{DPC16\,cell\,dry\,weight\,(g/L)} \text{ x } h^{-1}.$$

A maximal specific rate of 0.99 mmol/g CDW.h^{-1} (73.7 mg/g CDW.h^{-1}) was obtained at 6h of incubation and the total reuterin produced from biomass was 420.4 mg/g CDW after 18h incubation in glycerol supplemented MRS broth.

4.3.4 Reuterin and antimicrobial activity

Figure 4.6 integrates the reuterin produced in MRSg with its antimicrobial activity through the kinetic profile. It shows that the presence of low level of reuterin (<30 mM for most pathogens and <14 mM for *S. aureus*) produced in the early hours of incubation, inhibits pathogens at low levels, however, as reuterin accumulated beyond a certain threshold (specific tolerable level of pathogen), the inhibition of pathogens

largely increased. This result complies with the primary antimicrobial test of MRSg as discussed in Section 3.3.2. Thus, a relationship can be proposed between accumulated reuterin and the antimicrobial activity of MRSg. This result also confirms that the strong antimicrobial activity exhibited by MRSg at 8h incubation and thereafter (6h in case of *S. aureus*) was largely due to production of reuterin, as seen in the development of antimicrobial activity. Additionally, differential growth inhibition has revealed that the reuterin activity on pathogens is strain specific, where *S. aureus* is more susceptible to reuterin than the other pathogens tested.

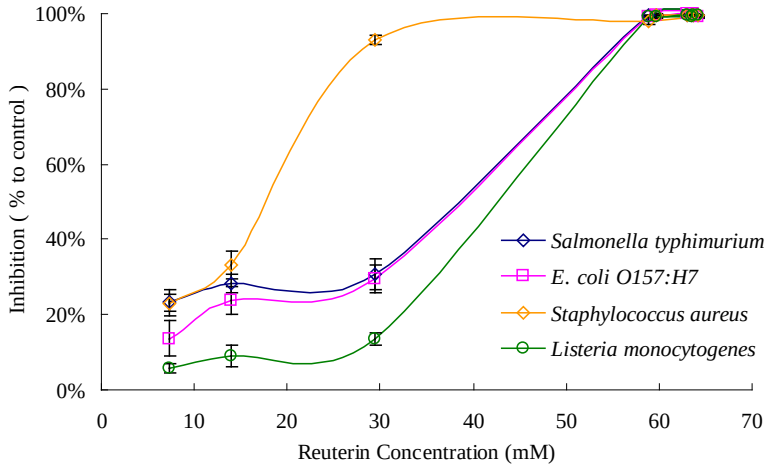

Figure 4.6 Antimicrobial activity of MRSg, expressed as concentration of reuterin, exhibited during the secondary fermentation of glycerol in MRS broth.

4.4 Discussion

The investigations in this chapter covered three parts. The first part reported the growth kinetics of *L. reuteri* DPC16 in batch culture under standardised *in vitro* conditions of anaerobic incubation in pre-reduced MRS broth at 37°C. The second part measured rate of reuterin production from glycerol, in both MRSg and SGF. The last part of this chapter investigated the relationship between reuterin in MRSg and its antimicrobial activity, and provided evidence that reuterin production is the major factor responsible for the strong antimicrobial activity of MRSg.

A 20h incubated DPC16 culture has shown a well-grown population with viable cells reaching a total number of 4.23 x 10^9 CFU/mL in a total biomass of 5.5 g/L. The biomass result indicated that the two-fold concentrated DPC16 resting cells, used for glycerol fermentation, contained about 11 g/L of biomass. This is consistent with the data reported by Talarico *et al.* (1988), where 10 mg/mL of CDW was used to produce reuterin.

The culture pH was observed to drop from 6.50 to 4.44 during incubation, which indicated the growth of DPC16 as evidenced by production of metabolic by-product (SCFA). According to Lu (2007), lactic acid produced by *L. reuteri* DPC16 in modified MRS broth grown under optimal anaerobic conditions reached a maximum of 2.6 to 2.8 g/L after 16h of growth. Based on the viable cell count and weight of dried cells, two correlation curves were developed, namely optical density (OD_{620}) with viable cells and optical density with biomass (CDW), to characterise the culture growth. There was a good linear relationship between the cultural optical density and the biomass. Such linear correlation of *L. reuteri* culture has been reported previously by El-Ziney *et al*. (1998) and Rasch *et al*. (2002 b).

A time course produced a sigmoidal growth curve for DPC16 in MRS broth. It showed that the length of the lag phase was 8-10h; the exponential phase was initiated from 10h and continued over a 4-6h period of linear growth; then the culture entered the stationary phase where the growth curve levelled. The slope of the linear range in the exponential phase was determined as 0.137 h^{-1}. By converting the optical density values into cell dry weight in the linear range of the exponential phase, the specific growth rate of DPC16 culture was mathematically described as 0.6 h^{-1}, with a population doubling

time of 1.16h. Talarico *et al*. (1990) reported a generation time of approximately 66 min when growing a strain of *L. reuteri* on glucose in a modified *Lactobacillus* carrying medium. El-Ziney *et al.* (1998) reported a specific growth rate of 0.43 h^{-1} in a batch culture fermentation of *L. reuteri* 12002 in a medium containing 60 mM of glucose.

In this study, production of reuterin from glycerol was measured to characterise the production kinetics during a secondary fermentation. Glycerol utilisation in both MRSg and SGF were observed from the beginning of the secondary fermentation; however, the amount of reuterin produced varied greatly between the two supernatants. Reuterin was detected in MRSg but not in SGF, even though some decrease of glycerol concentration was observed in SGF. As mentioned in Section 2.4.4, reuterin is produced as an intermediate during glycerol dissimilation, and it can be further reduced to 1,3-propanediol. However, according to Talarico *et al.* (1988, 1990), *L. reuteri* is unique in its ability to accumulate reuterin in cultural supernatant, and no reuterin was detected over the entire incubation period in producing SGF. Thus it can be considered that reuterin was not produced in glycerol-water fermentation, even though other by-products (*i.e.* 1,3- propanediol) were not measured in this study. This small amount of glycerol was assumed to have auto-diffused (*i.e.* been absorbed) into the DPC16 cell until a saturation concentration was reached. Based on this assumption, glycerol utilisation in MRSg was calculated, and together with the concentration of reuterin, a series of kinetic parameters were obtained.

From the kinetic profile of MRSg supernatants, it was found that 85.8 mM (23.8% of the total supplementation) of glycerol was utilised, other than auto-diffusion, during the production MRSg, where a total amount of 62.5 mM of reuterin was produced at the end of the incubation, accounting for the 72.8% of the utilised glycerol. Again, it was observed that the amount of glycerol utilised was not equal to the reuterin synthesised. According to Talarico *et al.* (1988), 1 mol of glycerol is transformed into 1 mol of reuterin. The difference observed here may be due to further reduction in some of the synthesised reuterin, as mentioned by Talarico *et al.* (1990). However, the exact mechanism of this was not investigated further.

Varied magnitudes of glycerol bioconversion to reuterin in glucose-containing media have been previously reported on different strains of *L. reuteri*. El-Ziney *et al.* (1998) obtained 38 arbitrary units/mL of reuterin in batch culture containing 60 mM of glucose

and 150 mM of glycerol. Luthi-Peng *et al.* (2002 b) obtained a total of 24.32 mM of reuterin in a *L. reuteri* ATCC53608 culture accounting for 40.5% of the supplemented glycerol (60 mM). The differences in reuterin production within *L. reuteri* species is reported to be strain specific (Spinler *et al.*, 2008).

In the present study, it was also found that reuterin was produced as early as 2h in the secondary fermentation and the production rate continued to increase until a maximum of 10.9 mM/h (811 mg/L.h^{-1}) was reached at 6h of fermentation, Then the production rate decreased sharply with a constant reuterin concentration for the rest of the fermentation process. A similar result was reported by El-Ziney *et al*. (1998) who detected reuterin after 2h of incubation in a co-culture fermentation of glycerol and glucose, and an exponential increase occurred between 2-4h, after which glycerol continued to decrease while the reuterin remained constant. Rasch *et al*. (2002 b) studied growth kinetics of a batch culture of *L. reuteri* with glycerol-supplementation, and reported that there is no decease in reuterin concentration from maximum level with time.

Based on the biomass quantification (11 g/L), the reuterin production was further translated into a maximum specific production rate of 73.7 mg/g CDW.h^{-1} obtained at 6h of incubation, and the total reuterin produced from biomass was 420.4 mg/g CDW during incubation in glycerol supplemented MRS broth.

Thus, the results of this study have shown that the bioconversion of glycerol to reuterin only occurred in glycerol-MRS fermentation whereas no reuterin was produced in the glycerol-water fermentation, based on the reuterin production in DPC16 supernatants of MRSg and SGF. Even though some *L. reuteri* strains may be able to produce reuterin in glycerol-water medium, this study has provided evidence that this DPC16 strain of *L. reuteri* does not have this capability. For this strain, as mentioned by Talarico *et al.* (1990), fermentable carbohydrate (glucose) is essential for metabolism and subsequent reuterin production, if glycerol is provided. Consequently, SGF, the supernatant produced from glycerol-water fermentation, did not show any antimicrobial activity, which may be attributed to the lack of reuterin as well as SCFA from cell metabolism.

On the contrary, MRSg, the supernatant containing reuterin has shown strong antimicrobial activity. Based on the observation of a similar sigmoidal pattern of development in both reuterin synthesis and antimicrobial activity, reuterin concentration

in MRSg profile was integrated with its antimicrobial activity, and a relationship between reuterin and the antimicrobial activity was established. Additionally, it was observed that the most susceptible pathogen, *S. aureus*, responded to the increase in reuterin production in the same way it responded to the increase in antimicrobial activity observed in MRSg. These results have provided further evidence that the major antimicrobial component in MRSg that accounted for its potent antimicrobial activity is very much likely to be reuterin. However, because reuterin was not purified in this thesis, the true antimicrobial activity of pure reuterin remains to be explored.

Finally, in response to the assumption of pathogen threshold as discussed in Chapter 3 (Section 3.4), if reuterin was the major antimicrobial factor, the sudden increase of pathogen growth inhibition may be due to a specific reuterin-tolerance threshold of each pathogen, where accumulated reuterin has to exceed a certain level to reach total inhibition. Again, this assumption remained unexplored because there was no purified reuterin available in this study.

In summary, the growth kinetics and reuterin production kinetics of *L. reuteri* DPC16 in batch culture under standardised *in vitro* conditions were studied. The growth of this strain had a 8-10h lag phase, followed by a 4-6h exponential phase with a specific growth rate of 0.6 h^{-1} (or a doubling time of 1.16h), and resulted in a total number of viable cells of 4.23 x 10^9 CFU/mL weighing 5.5 g/L and a pH drop from 6.50 to 4.44, after 20h of anaerobic incubation at 37°C in pre-reduced MRS broth. The reuterin production from glycerol bioconversion was analysed with supernatants harvested from a secondary fermentation of DPC16 resting cells in either growth medium of glycerol-MRS or non-growth medium of glycerol-water, namely MRSg and SGF respectively. The results showed that, in MRSg, 72.8% of used glycerol was converted to reuterin with a maximum production rate of 10.9 mM/h (or a maximum specific production rate of 73.7 mg/g CDW h^{-1}) at 6h of secondary fermentation and a total amount of 62.5 mM of reuterin accumulated in the supernatant. However, there was no reuterin detected in SGF, even though glycerol has been absorbed by the concentrated resting cells.

The kinetics of growth and reuterin production studied in this chapter were obtained under standard *in vitro* conditions where sufficient nutrients were provided and no natural competition was present. The actual bacterial growth in the host GI tract may be

totally different in terms of specific growth rate and reuterin production. However, these *in vitro* findings may provide evidence for industrial application of products from this DCP16 strain, based on the examined safety status.

Chapter 5

Evaluating the impacts of *Lactobacillus reuteri* DPC16 on mucin degradation and on the growth of other probiotics, *in vitro*

5.1 Introduction

The human GI tract harbours a variety of abundant microorganisms, namely the gastrointestinal microflora. There is a growing consensus that the composition of the microflora can affect human health and cause disease due to their close association during co-evolution (Hooper and Gordon, 2001; Magalhaes *et al*., 2007). The bacterial inhabitants of the human gastrointestinal tract constitute an enormously complex ecosystem with an approximate total number of 10^{14} CFU/mL, representing more than 500 species of bacteria (Magalhaes *et al.*, 2007). These microorganisms perform extremely diverse metabolism, growing on food components ingested or secreted by the host, converting substances into compounds beneficial or detrimental to the host, and live together in symbiotic or antagonistic relationships (Mitsuoka, 1992 a, 1992b). A predominance of harmful bacteria leads to aging and various severe bowel diseases, while "good" bacteria subsequently act as scavengers against the induced damages. Hence the microbial interaction is a major factor in regulating the indigenous intestinal microflora (Simon and Gorbach, 1986). A healthy gastrointestinal microbial regulation can be achieved by stimulating beneficial bacteria to locally synthesise enzymes, organic molecules, and short chain fatty acids, which inhibit the proliferation of detrimental bacteria hence maintaining a satisfactory balance of the local microflora towards host health and longevity. To qualify to be a probiotic bacterium, a novel indigenous bacterial strain must demonstrate efficacy of being non-pathogenic to other members of the local microflora and to the host, and preferably possess functional characteristics to suppress pathogenic bacteria as well as conferring other benefits to the host (Donohue and Salminen, 1996; Saarela *et al*., 2000; Puupponen-Pimia *et al*., 2002).

The intestinal mucosa facing the intestinal lumen possesses a layer of mucus, which is hydrophilic and has a complex structure with a viscoelastic property (Hoskins *et al*., 1985). This mucus immobilises nutrients and provides a microhabitat for colonization by a majority of the local microflora. The mucus is synthesised by the underlying

epithelia, and contains a high molecular weight (~10^3 kDa) glycoprotein, termed mucin (Bengmark and Jeppsson, 1995; Smith *et al*., 1995; Macfarlane *et al.*, 2005). Mucin is composed of a peptide core rich in serine and threonine residues, which are decorated by tandem oligosaccharide repeats *via O*- or *N*-glycosidic bonds. The exterior oligosaccharides that tightly wrap around the peptide core are terminated by sialic acids or sulphate groups, which together with the flanking oligosaccharide can exhibit antigenic specificity for mucoadhesivity (Hoskins *et al.*, 1985; Rosendale, 1997; Derrien *et al*., 2004; Bansil and Turner, 2006). The mucus structure acts as an intestinal protective barrier that tightly controls the trans-cellular and para-cellular transports by membrane pumps, ion channels and tight junctions. It also regulates the host defence mechanisms at the mucosal interface with "the outside world" (Baumgart and Dignass, 2002). By covering the underlying epithelial enterocytes, mucus provides protection against potential damages caused by corrosive acids, bile salts and enzymes of digestive juice (Allen *et al*., 1984; Zhou *et al.*, 2001; Macfarlane *et al.*, 2005), luminal flow shearing forces generated by the digestive process from the stomach duodenum and the gut lumen (Smith and Podolsky, 1986; Smith *et al*., 1995), and invasion by pathogenic microorganisms (Cohen and Laux, 1995; Gibson *et al.*, 1997).

However, the mucus can be damaged by colonised pathogenic bacteria through degrading the mucin side chains. Studies have reported that a viable subpopulation of faecal flora collected from healthy human subjects is capable of degrading mucin based on their capacity to grow on mucin-containing basal media (Hoskins and Boulding, 1981; Hoskins *et al.*, 1985; Macfarlane and Gibson, 1991; Gibson *et al*., 1997; Ruas-Mediedo *et al*., 2008). Some of these subpopulations, belonging to the genera of *Ruminococcus, Bacteroides, Clostridium* and *Bifidobacterium*, have been shown to secret extracellular sialidase and a series of glycosidases, which recognise the terminal receptors of mucin and exfoliate the oligosaccharides. Impaired mucin structure, from enzymatic degradation, may provide opportunity for invading microorganisms to traverse to the underlying enterocytes, damage the cells, and secret toxic and inflammatory compounds into the blood vessels which circulate throughout the body. In addition, mucin degradation releases monosaccharides and amino acids, and these nutrients can be utilised directly by those invading pathogens to worsen the damage. Ruseler-van Embden *et al.* (1995) claimed that any change in mucus content and structure may compromise the mucosal defence barrier and increase the numbers of bacteria adhering to the enteric cell surface, and hence is regarded as pathogenic and

locally toxic. Therefore, mucin degradation has been considered as a useful indicator for distinguishing between commensal and pathogenic bacteria, and thus can be used as a safety assessment for potential probiotics (Magalhaes *et al.*, 2007). Several probiotic strains including *Lactobacillus acidophilus*, *Lactobacillus casei, Lactobacillus rhamnosus* and *Bifidobacterium lactis* have been cultured on hog gastric mucin, *in vitro*, and none has been reported to degrade gastric mucin glycoprotein, thus far (Ruseler-van Embden *et al*., 1995; Zhou *et al*., 2001).

Lactobacillus reuteri is believed to be one of the few true autochthonous *Lactobacillus* species in humans (Reuter, 2001), and resides frequently as a heterofermentative lactic acid producing-bacterium in the GI tract of many species of animals. Strains of *L. reuteri* have been shown to possess high affinity for mucin binding *via* membrane-associated protein (Satoh *et al*., 1999; Jonsson *et al.*, 2001), and no observable mucin degradation was reported in *L. reuteri*-primed mice after oral administration (Norin and Casas, 1999). *Bifidobacterium* is another genus of common inhabitants of the gastrointestinal tract. This genus has received special attention because of its health-promoting effects and long history of safe use in the functional food industry (Ruseler-van Embden *et al*., 1995; Salminen *et al*., 1998 a), even though several strains (*i.e. B. bifidum*, and *B. longum*) have been reported for mucinolytic activity due to the possession of the genes accountable for extracellular glycosidase (Ruas-Mediedo *et al*., 2008). *Bifidobacterium lactis* HN019 is a patented probiotic strain trade-marked as *B. lactis* DR10, which has been intensively studied for function, safety and production characteristics important to its ability to function as a probiotic (Gopal *et al.*, 2005). This strain is now commercially available in selected dairy products (Activate.co.nz, 2008). In particular, this DR10 strain has been studied for mucus degradation and found to be safe for the gastrointestinal mucosal environment (Gopal *et al*., 2001; Zhou *et al*., 2001). Hence, this strain was used as a reference culture indicating non-mucin-degradation in the mucus degradation study for DPC16 cultures in this study.

The objectives of this chapter were to provide preliminary safety assessment by evaluating the effects of *L. reuteri* DPC16 cultural supernatants, especially MRSg, on the growth of some common commensal probiotic bacteria; and determining if this strain is capable of degrading mucin.

5.2 Materials and Methods

5.2.1 The effect of DPC16 supernatants on growth of the selected probiotic strains

The probiotic bacteria *Lactobacillus acidophilus* (*L. acidophilus*), *Lactobacillus plantarum* (*L. plantarum*), *Pediococcus acidilactici* (*P. acidilactici*) and *Bifidobacterium lactis* DR10 (*B. lactis* DR10) were kindly provided by BRNZ, as 30% glycerol stocks stored at -80°C. The stock cultures were activated, sub-cultured and enumerated following the method of described in Section 3.2.2 and 4.2.1, except that DR10 was incubated for 48h. The subcultures were then used as master inocula for subsequent assays (these master inocula were always freshly made from stock prior to assay).

Based on the enumeration, an inoculum containing *ca*. 10^4 CFU/mL of each probiotic strain was prepared from the master inocula, and subsequently inoculated into pre-reduced MRS broth in 96-well microtitre plates, in quadruplicate. By incubating the probiotic cultures under anaerobic conditions with shaking (250 RPM) at 37°C, and measuring the OD_{620} at designated time intervals, a growth curve of each probiotic culture was developed spectrophotometrically.

DPC16 cultural supernatants, which had been previously tested for antimicrobial activity against selected pathogens (Chapter 3), were used to evaluate their effects on the growth of selected probiotic strains following the method described in Section 3.2.4 (with exception of longer incubations: *B. lactis* DR10 was incubated for 48h; the other probiotic strains were incubated for 24h) in the rich growth medium of pre-reduced MRS broth. The DPC16 cultural supernatants as listed in Table 3.1 and their pH-adjusted counterparts (except SGF) were also investigated.

While a preliminary antagonistic assay was processed with undiluted supernatants, MRSg-8h (containing reuterin as proven in Chapter 4) and its pH adjusted counterpart were further studied in a highly diluted form [4000-fold, the probiotic growth-stimulatory concentration of methylglyoxal as introduced by Rosendale (2007)] to assess their stimulatory effects on the growth of target probiotics in a time course investigation, as in Section 3.2.5.

5.2.2 Mucin degradation

L. reuteri DPC16 cultures from incubation in pre-reduced MRS broth, and from secondary fermentation using concentrated resting cells in glycerol-supplemented MRS broth, were used to test their mucinolytic activity on porcine gastric mucin (PGM), *in vitro*. This assay followed the method introduced by Zhou *et al*. (2001) with some modifications.

5.2.2.1 Mucin purification

Partially purified porcine gastric mucin (Type III, Sigma; USA) with 0.5-1.5 % (w/v) bound sialic acid was further purified by stirring 10 g in 500 mL of PBS for 1h. The pH value of this solution was adjusted to 7.2 using 0.1 M NaOH. With addition of a few drops of toluene for preservation, this solution was continuously stirred for another 24h at room temperature, and was subsequently centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was removed and immediately put in an ice bath. The pellet was washed with pre-chilled (4°C) ethanol (99%, molecular biology grade, J.T. Baker Chemical Co.; USA) and re-centrifuged to collect the resulting supernatant. This resulting supernatant was added to the previously collected supernatant to obtain the final supernatant, which contained dissolved mucin. Pre-chilled ethanol was again added to the cooled final supernatant to a final concentration of 60% (v/v). The precipitated mucin was collected and washed two more times with ethanol to remove trace sialic acid and other salts. The final precipitate was then rotary-evaporated to remove ethanol and dissolved in distilled water. This mucin solution was frozen and then freeze-dried as described in Section 3.2.2, and the lyophilized mucin was used as a source of purified mucin for the mucinolytic assay in this chapter.

5.2.2.2 Basal medium for anaerobic cultures

A basal medium was prepared according to the methods of Zhou *et al*. (2001), as shown in Table 5.1. *L*-cysteine-HCl was filter-sterilized and added into the medium after autoclaving at 120°C for 15 min, followed by pH adjustment using 0.1 M NaOH. The final pH value of this medium was 7.2 ± 0.2. Purified PGM and/or glucose were supplemented as required to a concentration of 10 g/L.

Table 5.1 Ingredients of basal medium for mucin degradation assay.

Ingredients	Amount (g per litre of distilled water)
Tryptone (Bacto, BD; USA)	7.5
Trypticase Peptone (Bacto, BD; USA)	7.5
Yeast Extract (Fluka, EU)	3.0
Beef extract (Acumedia, Michigan, USA)	5.0
NaCl (Sigma, USA)	5.0
K_2HPO_4 (BDH, USA)	2.3
KH_2PO_4 (UniVar, USA)	0.5
$MgSO_4 \cdot 7H_2O$ (Sigma, USA)	0.5
L-cysteine-HCl	0.5
Resazurin (anaerobic indicator)	0.002
Medium pH value: 7.2 ± 0.2	

5.2.2.3 Bacterial cultures

The cultures of the potential probiotic strain *L. reuteri* DPC16 and the reference strain *B. lactis* DR10 were prepared anaerobically. Briefly, *L. reuteri* DPC16 was prepared as a 20h culture in pre-reduced MRS broth and as an 8h culture from secondary fermentation with 11 g/L of resting cells in glycerol-supplemented MRS broth. These DPC16 cultures were designated as LR and LRg, respectively. The 48h culture of the reference strain *B. lactis* DR10 was prepared in pre-reduced MRS broth, and designated as BbL.

A mucinolytic faecal culture was prepared from a fresh faecal sample of a healthy young adult female who had not received antibiotics for the previous 3 months. Preparation of the faecal sample followed the method described by Darrien *et al*. (2004) with some modifications. Briefly, the fresh faecal samples were collected in a sterile disposable stomacher blender bag (Bagpage+ 400, Interscience, France), weighed and suspended to 10% (w/v) in anaerobic Ringer solution (containing 8.6 g/L NaCl, 0.3 g/L KCl, 0.33 g/L $CaCl_2$, and 0.5 g/L of *L*-cysteine in distilled water, pH 7.2; diluted with water to quarter strength prior to use). The faecal suspension was homogenized twice, using a Stomacher Blender (Masticator 400 lab blender, IUL instrument; Spain) for 2min each time, in a sealed stomacher blender bag. After 5 min standing, the faecal liquid was centrifuged at 600 x g for 5 min at 4°C. The supernatant was collected and stored at -20°C as a faecal flora suspension until the time of use (maximum storage period of one month).

Subsequently, 1% (v/v) of the faecal culture was inoculated into BHI broth and incubated at 37°C for 24h. This subculture, enumerated by haemocytometer to contain approximately 2 x 10^{10} CFU/mL of total bacteria, was used as a positive control for the mucin degradation assay, and designated as FF. An aliquot of this subculture was heat-killed at 120°C for 15 min, and used as a negative control, designated as HFF.

5.2.2.4 Mucin degradation assays

The quantitative mucin degradation assay in basal media and the qualitative assay on agar plates were both based on the methods of Zhou *et al*. (2001). Cultures of the LR, the LRg, the BbL, and the controls of fresh faecal flora (FF) and the heat-killed faecal flora (HFF) were all included in this assay. Briefly, 1% (v/v) of each culture was incubated at 37°C for 48h in basal media containing either PGM (10 g/L) with or without glucose (10 g/L), or glucose (10 g/L) only. Basal medium containing no inoculum but PGM or glucose supplementations were used as blank or medium controls. At the end of incubation, the cell concentrations in the different media were measured by OD at 620 nm, and the pH changes were measured. Each culture, in every designated growth medium, was assayed in triplicate.

To confirm the results, a mucin degradation assay on agar plates was conducted to demonstrate the mucinolytic activity more objectively, using the method described by Colina *et al*. (1996) with some modifications. Briefly, purified PGM (10 g/L) was incorporated into a basal medium containing agarose (15 g/L) (DNA-grade, BDH, Electran, England) with or without glucose (10 g/L). After allowing this medium to harden, aliquots of each test culture were inoculated onto the surface of the agarose medium using the drop plate technique. The plates were allowed to dry, and then were incubated anaerobically at 37°C for 72h. After incubation, all plates were stained with Coomassie Blue R-250 staining solution[*] [containing per L, 2.5 g of Coomassie Blue R-250; 455 mL of analytical grade ethanol; 455 mL of distilled water; and 100 mL of glacial acetic acid (100%, BDH; UK)] for 45 min, and subsequently washed with 1.2 M acetic acid to de-stain until the discoloured zone appeared. Mucin degradation was indicated by the appearance of the mucin lysis zone around the colonies, and the diameters of zones were measured from triplicate angles.

[*] Like the use of amido black as described elsewhere (Colina *et al*., 1996; Zhou *et al*., 2001), Coomassie blue was employed in this study for detecting the appearance of core protein, exposed from polysaccharide wrapping, of degraded mucin.

5.3 Results

5.3.1 The effects of DPC16 supernatants on growth of the selected probiotic strains

A growth curve of each probiotic strain was initially developed in pre-reduced MRS broth, as shown in Figure 5.1. Most probiotic cultures showed a similar growth pattern to *L. reuteri* DPC16 (Figure 4.2), except for *B. lactis* DR10 which had as longer period of lag phase to 18h. The cell concentration of each strain, reflected by the OD_{620} at the endpoint of incubation, was also in a similarly high level to that of the *L. reuteri* DPC16 in its stationary phase.

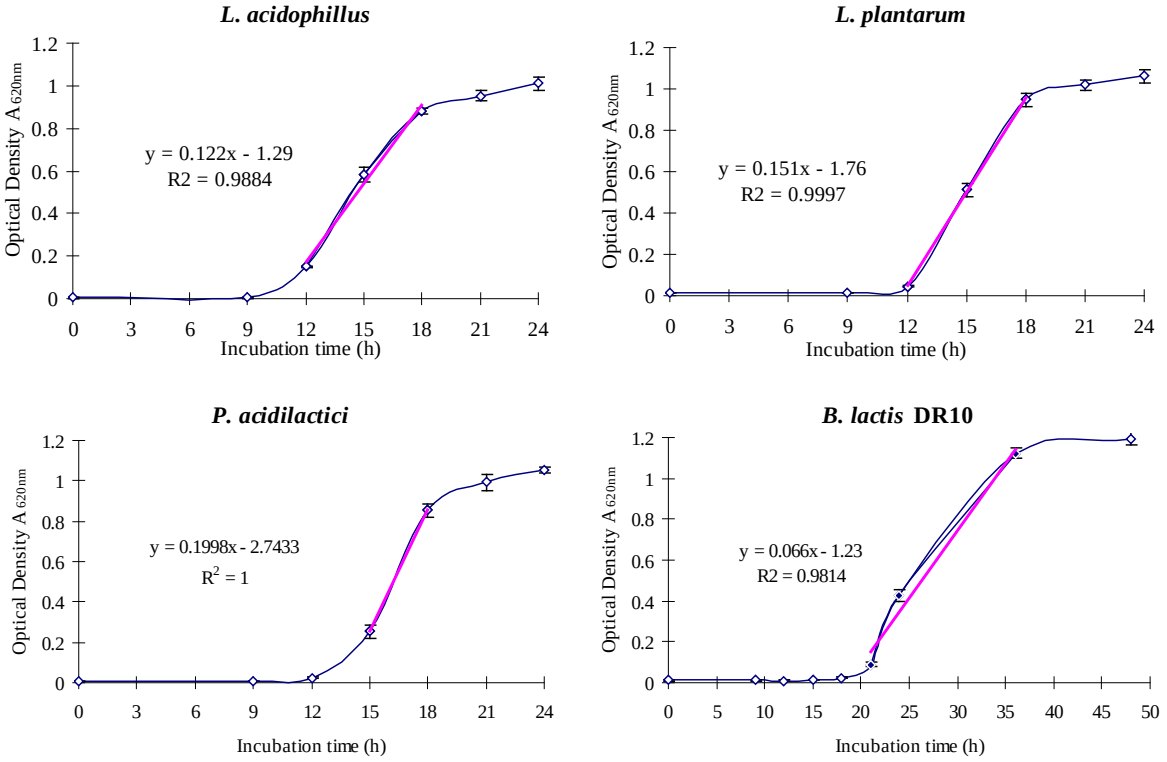

Figure 5.1 Growth curves of probiotic strains in MRS during anaerobic incubation at 37°C. Data points are mean of OD_{620} with SEMs represented as vertical bars (n=4). A regression line linearly fitting the exponential phase is plotted for each strain, with a mathematic equation indicating the slope.

The preliminary test on effects of selected DPC16 supernatants on the growth of target probiotic strains is shown in Figures 5.2 and 5.3. No significant growth inhibition was observed from any of the tested DPC16 supernatants (P>0.05), regardless of pH adjustment. Additionally, as seen in Figure 5.4, the time course of probiotic strains showed that the 4000-fold-diluted MRSg-8h had no significant stimulatory effect on the growth of any of the tested probiotics, which suggests that the reuterin, at this concentration, does not stimulate the growth of the tested probiotic strains.

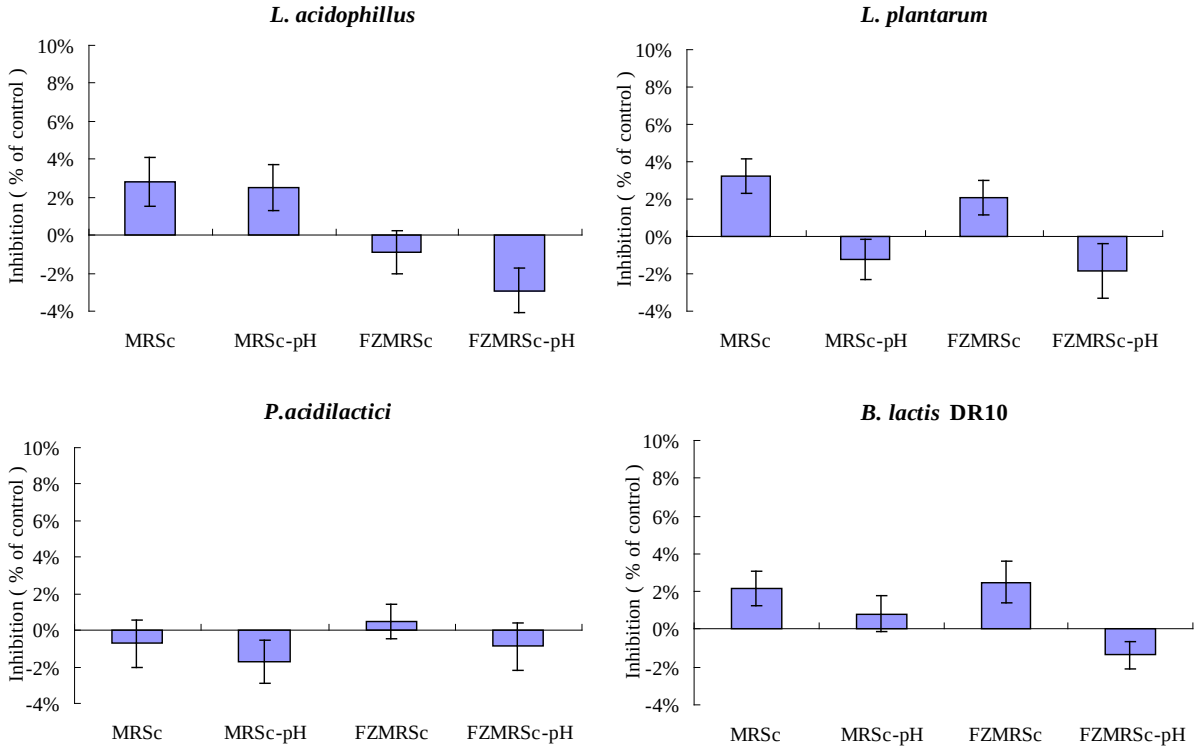

Figure 5.2 Effects of MRSc, FZMRSc and their pH-adjusted counterparts on the growth of selected probiotic cultures. Values are the mean percentage of inhibition relative to untreated controls, calculated from OD_{620} of probiotic strains with or without treatments, with SEMs represented by vertical bars (n=9).

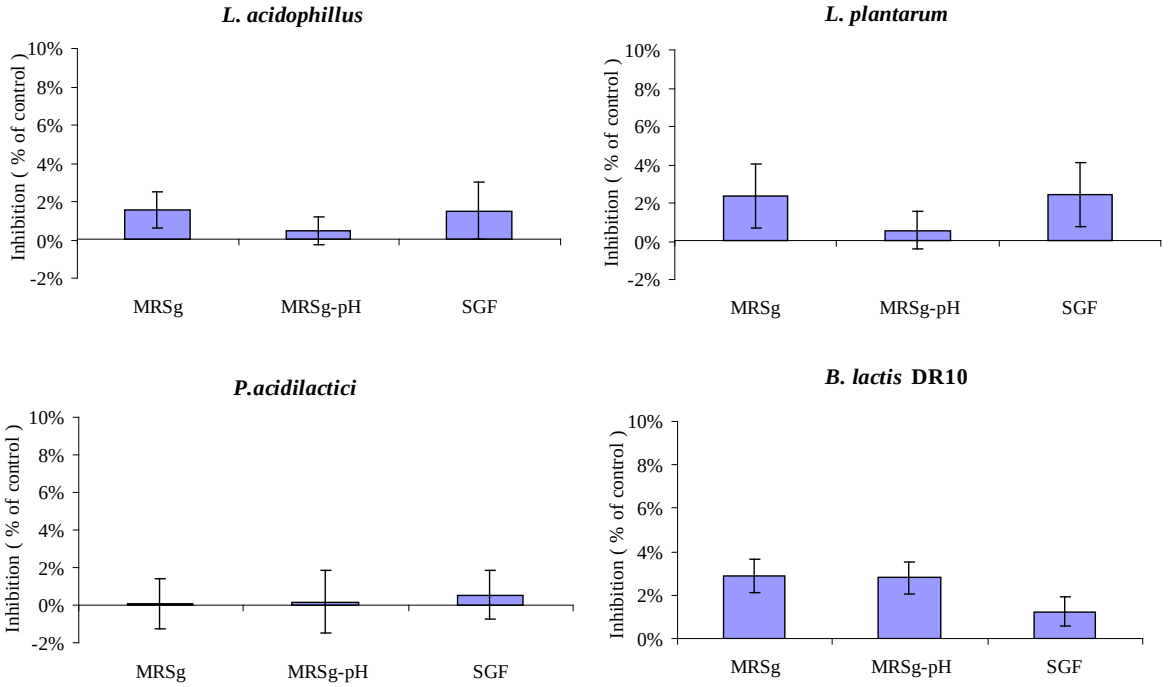

Figure 5.3 Effects of MRSg, SGF and pH-adjusted MRSg on the growth of selected probiotic cultures. Values are the mean percentage of inhibition relative to untreated controls, calculated from OD_{620} of probiotic strains with or without treatments, with SEMs represented by vertical bars (n=9).

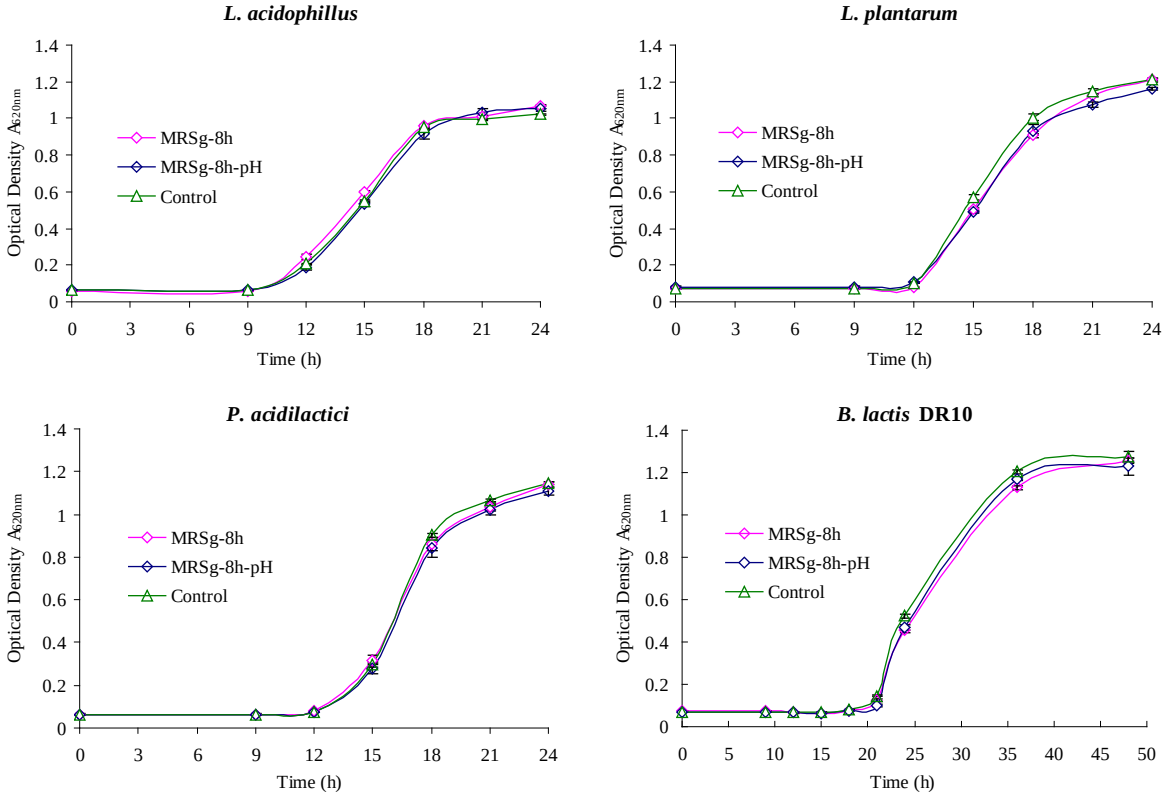

Figure 5.4 A time course of probiotic strains treated with and without MRSg-8h or its pH-adjusted counterpart. Data points are means OD_{620} of quadruplicate measured at time intervals during incubation, with SEMs represented by vertical bars.

5.3.2 Degradation of mucin in liquid media

The growth of LR, LRg, BbL and the faecal flora controls in different media were indicated by the changes of OD_{620} (Figure 5.5) and the pH values (Table 5.2) after an anaerobic incubation for 48h. No growth occurred in the heat-killed faecal flora control. A statistical analysis showed that there was no significant difference in the growth of BbL (P= 0.194) in the medium containing purified PGM as the sole carbon source, when comparing with the heat-killed faecal flora control. Negligible growth (OD_{620}<0.1) in LR and LRg was observed, whereas an apparent growth of the faecal flora in the same medium was detected (P=0.004). All cultures showed good growth in glucose-containing media, regardless of the presence of PGM. There was no pH change detected in any culture incubated in the basal medium containing no carbohydrate, nor in the heat-killed faecal flora control. The pH decreases of *L. reuteri* cultures (LR or LRg) were detected only in media containing glucose, and were similar to the reference culture (BbL). The faecal flora culture, however, showed lowered pH values in growth media with or without glucose.

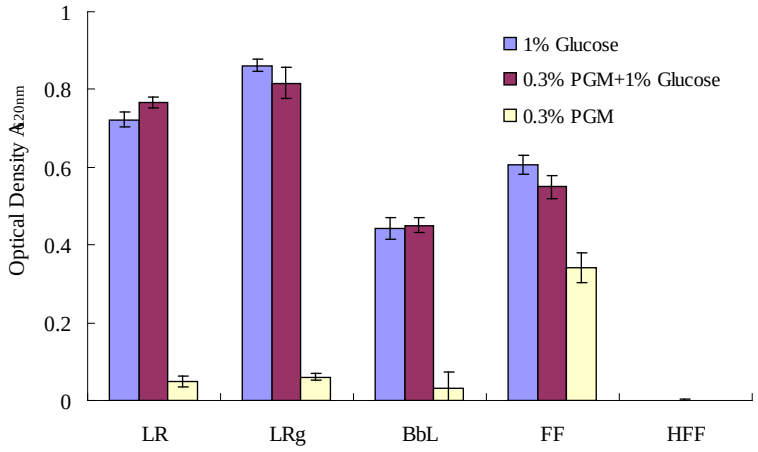

Figure 5.5 Growth of potential probiotic cultures and faecal flora cultures in basal medium supplemented with designated carbohydrate sources. Bar values are means of triplicate OD_{620} readings measured at the end of the 48h incubation, with SEMs represented by vertical bars. Abbreviations of culture inocula: LR, 20h culture of DPC16 in MRS broth; LRg, 8h culture of DPC16 in glycerol-supplemented MRS using concentrated resting cells; BbL, 48h culture of B. lactis DR10; FF, 20h incubated faecal flora culture; HFF, heat-killed faecal flora culture; PGM, purified porcine gastric mucin.

Table 5.2 The final pH values of cultures incubated in various media comparing to their original cultures which indicate the starting pH values.

	Original Cultural pH	PGM + Glucose	PGM	Glucose	Basal Medium
LR	4.4	4.4	7.2	4.3	7.2
LRg	4.3	4.3	7.2	4.3	7.2
BbL	3.9	3.8	7.3	3.9	7.3
FF	6.7	4.1	5.1	4.1	7.2
HFF	6.9	7.1	7.4	7.2	7.1

5.3.3 Degradation of mucin on agarose plate

Figure 5.6 shows the results of the mucin degradation assay on agarose plates containing PGM only or PGM with glucose. Only the faecal flora culture grew on the plate containing PGM only; whereas colonies of the other cultures appeared on the medium where glucose was available. No growth of the HFF control was observed on either of the agar media. More objectively and with better resolution, the Coomassie blue-staining showed significant mucinolytic activity of faecal flora on both media. Again, no mucin degradation was observed for any other cultures. Measurements show that the faecal flora developed a lysis zone with diameter of 1.68 cm on PGM-only medium, and a smaller lysis zone with a diameter of l.16 cm on medium containing both PGM and glucose. It is worth noting that the mucin degradation of faecal flora on agarose medium containing both PGM and glucose was less intensive than that on medium containing PGM only.

Figure 5.6 Degradation of mucin on agarose plate supplemented with PGM only (left) and PGM with glucose (right). The plates were stained with Coomassie-blue and de-stained with 1.2 M acetic acid. The discoloured zones indicate mucinolytic activity of the faecal flora culture. Abbreviation: PGM, purified porcine gastric mucin; NG, medium containing no glucose but PGM; G, medium was supplemented with both PGM and glucose.

5.4 Discussion

Lactobacillus reuteri belongs to the genus *Lactobacillus*, which has a long history of safe usage in various food industries. There is no report for *L. reuteri* or the previously classified *L. fermentum* of any clinically adverse effect (Wolf *et al*., 1995, 1998). *L. reuteri* has also been demonstrated *in vitro*, to be able to resist pH 1.5 and 0.3% oxgall bile (Kim *et al.*, 2006; Casas and Dobrogosz, 2000), to adhere strongly to epithelial mucus (Satoh *et al*., 1999; Jonsson *et al.*, 2001) and to colonise the human GI tract for up to 2 months (Wolf *et al*., 1995). Additionally, *L. reuteri* has been found, in a probiotics screening test, to have no adverse effect on normal GI bacteria (Jacobsen *et al.*, 1999). Hence, *L. reuteri* species can be generally regarded as safe for the host.

In this chapter, experiments were conducted to assess the safety of this novel DPC16 strain of *L. reuteri*, in aspects of effect (inhibitory of stimulatory) of its cell-free supernatants on the growth of selected commensal probiotic bacteria. In addition, the DPC16 cultures from both reuterin-producing and non-reuterin-producing media were tested for mucinolytic activity.

A preliminary antagonistic assay was carried out using selected DPC16 supernatants, which had shown antimicrobial activities (except SGF-2h) against selected pathogens. The results showed that, although some of these supernatants were able to inhibit the growth of pathogenic bacteria (as seen in Chapter 3), no significant inhibitory effect on common probiotics was detected. The results may also suggest that there was no bacteriocin synthesised by this strain, which targets closely related LAB species as seen in reutericin 6 produced by *L. reuteri* LA6. Thus, they can be, so far, regarded as safe to the tested normal commensal intestinal microflora. This result is consistent with the published finding of Jacobsen *et al*. (1999) on another strain of *L reuteri* (DSM 12246). A recent study on compatibility of four strains of *L. reuteri* (ATCC 55730, ATCC PTA 6475, ATCC PTA 4659 and ATCC PTA 5289) with other common *Lactobacillus* strains showed that the *L. reuteri* strains did not potently inhibit the growth of most of the tested *Lactobacillus* strains, except for *L. casei* ATCC 334; and also there was no inter-strain variation among the four *L. reuteri* strains, in terms of inhibition of other *Lactobacillus* species (Spinler *et al.*, 2008).

In addition, the result from this study also showed that the reuterin-containing supernatant of MRSg did not have a stimulatory effect on the growth of the tested probiotic bacteria, even in a highly diluted form. This suggests that reuterin, at the same dilution, is unlike the structurally similar compound of methylglyoxal and may not have a similar stimulatory effect on probiotics' growth

The gastrointestinal mucosa plays an important role as a physical barrier in preventing potential pathogens from translocation and subsequent infection to extra-intestinal epithelia and tissues (Carlsson, 1993). The ability to degrade the mucus basic structure framework, mucin, is considered as one of the valuable indicators of potential pathogenicity and local toxicity of lumen bacteria (Ruseler-van Embden *et al*., 1995; Donohue and Salminen, 1996; Salminen *et al*., 1998 a). A series of extracellular enzymes including sialase, sulfatases, glycosidases, galactosidase, and *N*-acetyl-glucosaminidase, synthesised by a variety of enteropathogens, have been reported to be responsible for extensive mucin degradation (Hoskins *et al.*, 1985; Colina *et al.*, 1996; Darrien *et al.*, 2004), and bacteria capable of producing such enzymes are also recognized to be pathogenic.

In this study, an enzymatic assay was not performed; instead, commercially available porcine gastric mucin was used to assess the mucinolytic activity of two *L. reuteri* DPC16 cultures (reuterin-producing and non-reuterin-producing) in a basal medium, using a previously-assessed mucinolytic-negative strain of *B. lactis* DR10 as a reference and human faecal flora as a positive mucin-degrading control.

DPC16 cultures inoculated in the basal medium, where purified mucin was the only carbohydrate source, exhibited a negligible growth accompanied by stable pH. These results were similar to the reference strain of *B. lactis* DR10. However, the positive control of the faecal flora grew reasonably well in the same medium. Addition of glucose into this mucin-containing medium triggered the normal growth of all cultures containing viable inocula, and large pH drops. This result shows that the DPC16 cultures, regardless of reuterin presence, behaved similarly to the mucinolytic-negative reference strain in that they cannot utilise mucin as a sole energy source due to lack of enzymes required to metabolise the components of complex mucin glycoprotein. For the positive control, the growth of faecal flora occurred in media with or without glucose, which suggests that the faecal flora culture is capable of utilising both mucin and/or glucose by its respective subpopulations.

To confirm the results of the mucin degradation obtained in liquid media, the mucinolytic activity was tested on agarose plates. The results were consistent with the findings from the liquid media in that the DPC16 cultures were unable to grow on medium supplemented with PGM only. Similar result was observed with the reference culture. In contrast, faecal flora colonization was apparent on both the PGM-containing plates with or without glucose. By using the staining and de-staining process, a large discoloured zone around the colony of faecal flora was observed. This indicated the presence of proteins within the discoloured zone. As previously introduced (Section 5.1), mucin structure is composed of a protein core attached to, and tightly wrapped by, repeated links of polysaccharides. The presence of protein suggests that the polysaccharide framework of mucin has been demolished, and the core protein composition was exposed. Hence, according to Zhou *et al.* (2001), it indicates mucinolytic activity. No DPC16 cultures or reference culture showed such activity on either medium containing glucose or not, which corresponds to the result of the mucin degradation assay in liquid media. Hence, this DPC16 strain of *L. reuteri* does not possess mucinolytic activity. This result is consistent with the previous finding of Norin and Casas (1999), who studied the development of microflora-associated characteristics of *L. reuteri* mono-colonised rats, and reported that no observable mucin degradation occurred.

Levels of mucin degradation by the faecal flora were different on agarose media with and without glucose. It was noted that the mucinolytic zone on glucose-supplemented medium (containing PGM) was smaller and less intense than the zone that appeared on PGM-only medium. This may indicate nutrient-dependence of subpopulations in the faecal flora. When mucin is the only carbohydrate source, faecal flora exercised extensive mucinolytic activity to utilise this sole energy source for metabolism and growth purpose. But, when more ready-to-use carbohydrate (glucose) was present, an alternative and preferred metabolic pathway was used by some of the subpopulations, so that the production of mucinolytic enzymes were reduced to a lower level, and thus less mucinolytic activity was exhibited.

In conclusion, the preliminary *in vitro* assays in this chapter have demonstrated that the potential probiotic strain *L. reuteri* DPC16 has no antagonistic effect on selected commensal probiotic bacteria, and does not degrade gastric mucin.

Chapter 6

General discussions and conclusion

This research investigated the antimicrobial activity of four different kinds of DPC16 cell-free supernatants, namely MRSc FZMRSc MRSg and SGF. Paired with their pH-adjusted counterparts, these supernatants were tested in a series of *in vitro* antimicrobial assays against selected Gram-positive and Gram-negative pathogens. By determining their minimum effective dose, these supernatants were further studied for their effects on pathogen growth kinetics.

A primary antimicrobial test showed that supernatants harvested from non-glycerol fermentation, MRSc and its freeze-dried derivative FZMRSc, had significant inhibitory effects against target pathogens, and these effects were mainly due to production of SCFA during incubation as evidenced by the negation of such activity from their pH-neutralized counterparts. In addition, this acidic effect was observed to reduce the pathogen growth rate and decrease the total pathogen cell numbers in a time course study.

By using a two-step fermentation process with two-fold concentrated DPC16 resting cells, kinetic profiles of supernatants MRSg and SGF were obtained from glycerol-supplemented MRS broth and distilled water, respectively. The MRSg series has shown very strong antimicrobial activity against all target pathogens, nearly analogous to commercial antibiotics at its original dose. Moreover, this antimicrobial activity is pH-independent, and impacted on both Gram-negative and Gram-positive pathogens. It is assumed that reuterin may be present in MRSg. The kinetic profile of MRSg also showed that such antimicrobial activity was developed as a function of incubation time in a sigmoidal fashion reaching maximum inhibition by 6-8h of incubation and maintaining the same level thereafter. Using the determined minimum effective dose, a time course investigation provided further evidence of MRSg effectiveness on pathogen growth kinetic in elongating the lag phase, decreasing growth rate and lowering the total number of viable cells against all tested pathogens. Lastly, by comparing the antimicrobial activity of MRSg in high and low doses, it was found that MRSg, at a high dose, had a potent bactericidal effect resulting in pathogen cell death within 3h of treatment, while, at a low dose, MRSg was bacteriostatic by inducing a

stress response to pathogen cells during lag phase and hence delaying cell replication. However, this strong antimicrobial activity was also found to act on DPC16 itself by significantly reducing its cell viability. Nevertheless, this self-inhibiting activity occurred moderately and was tolerable until late in the fermentation.

SGF, another glycerol fermentation product tested in this study, did not show a clear antimicrobial activity, did not develop a normal acidic cultural pH, and the cells producing SGF died very quickly. These suggest that there was no normal cell metabolism by the DPC16 cells in glycerol-water, and this strain may not be capable of producing reuterin without the presence of fermentable carbohydrate.

The antimicrobial activity found in MRSg has matched with previously published characteristics of reuterin. Hence, a kinetic study on the production of reuterin by DPC16 resting cells was carried out. Initially, the growth kinetics of DPC16 was studied and it is found that the growth of this strain had a 8-10h lag phase, followed by a 4-6h exponential phase with a specific growth rate of 0.6 h^{-1} (or a doubling time of 1.16h), and resulted in a total number of viable cells of 4.23 x 10^9 CFU/mL (weighing 5.5 g/L)and a pH drop from 6.50 to 4.44, after 20h of anaerobic incubation at 37°C in pre-reduced MRS broth.

The reuterin production from glycerol bioconversion was analysed with both MRSg and SGF series. The results show that, in MRSg, 72.8% of used glycerol was converted to reuterin with a maximum production rate of 10.9 mM/h (or a maximum specific production rate of 73.7 mg/g CDW h^{-1}) at 6h of secondary fermentation and a total amount of 62.5 mM of reuterin accumulated in the supernatant. However, there was no reuterin detected in SGF, even though glycerol has been absorbed by the concentrated resting cells. The huge difference in reuterin production between MRSg and SGF has suggested that this specific strain does not possess the capability to utilise glycerol in the absence of fermentable carbohydrate.

Formation of reuterin in MRSg series followed a sigmoidal pattern of increase, which was similar to that of its antimicrobial activity; thus, a certain relationship was proposed between reuterin formation and the increased antimicrobial activity. Therefore, the major antimicrobial component in MRSg that accounted for its potent antimicrobial activity may be reuterin.

To assess the safety of this novel strain, an antagonistic assay against selected common probiotic bacteria was used to evaluate the same set of DPC16 supernatants in their effects on the growth of the target bacteria. This assay has shown that no DPC16 supernatant had any significant inhibition effect on the growth of the tested probiotics. This has, one the other hand, proved that there was no bacteriocin synthesis in any DPC16 culture. However, there was no growth stimulatory effect detected in reuterin-containing MRSg supernatant, either. Next, as a second part of safety assessment, a mucin degradation assay was carried out by growing DPC16 together with its resting cells, controls and reference strains in a basal medium where purified porcine gastric mucin was incorporated as the only carbohydrate source. This classic mucin-degradation assay was conducted both in liquid and agarose media, and the results from both tests have shown that, as with the reference strain of *B. lactis* DR10, *L. reuteri* DPC16 does not have mucinolytic activity on gastric mucin, whereas the positive control of faecal flora cultures significantly degraded mucin for energy source due to their possession of responsible enzymes.

Hence, *L. reuteri* DPC16 does not inhibit the growth of normal commensal microflora, and does not degrade epithelial gastric mucus, therefore it is safe to the GI environment of the host.

Several significant findings have been reported in this thesis, especially the potent antimicrobial activity of reuterin-containing MRSg. This pH-independent bactericidal activity, exerted against both Gram-positive and Gram-negative pathogens, has great potential to be applied in food industry as preservative agent. Also, the host-safe character of MRSg has provided it with great advantage in pharmaceutical industry as a potential nature antibiotic reagent.

However, there are still a few issues left unsolved. For example, reuterin in this research was detected as an equivalent of acrolein in supernatant solutions, but the reuterin was not purified. In future tests, a purification method introduced by Vollenweider *et al.* (2003) and the HPLC quantification by Chen *et al*. (2004) are recommended for reuterin analysis with higher accuracy and integrity. Another important kinetic parameter, utilisation of glucose in the reuterin production process, was not measured. This parameter can demonstrate critical information of cell metabolism during growth and the initiation of glycerol utilisation. Also, fermentation by-products, other than reuterin,

during the glycerol fermentation were not analysed in this thesis. To obtain a better understanding of the kinetics of glycerol fermentation by this DPC16 strain in MRS broth, a study on the above factors (*e.g.* HPLC quantification of pure reuterin, glucose and other end-products from glycerol fermentation in MRS broth) is essential and highly recommended for further investigations on this novel strain.

This study has provided some evidence for the safety status of this novel strain of *L. reuteri*. However, to qualify for probiotic concept, there are a lot more safety assessments that need to be done. For example, it is worthwhile to investigate the full sequence the genomic DNA (especially on plasmids) of this strain for any existence of transmissible antibiotic-resistance gene, so that a further step can be made toward the GRAS category of the probiotics concept.

The studies in this thesis only serve as a partial contribution towards the validation of the probiotic status claimed for this novel strain. The ultimate purpose was to gain knowledge and values from scientific discoveries, and then contribute to benefit human life.

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Appendices

Appendix 1 Ingredients of cultural growth media

Table A.1 Composition of BHI (Brain Heart Infusion broth, BBL, BD; USA)

Ingredients	Amount (g per litre of distilled water)
BHI solids	6.0
Peptic digest of animal tissue	6.0
Sodium chloride	5.0
Glucose	3.0
Pancreatic digest of gelatine	14.5
Disodium phosphate	2.5
Medium pH value: 7.2 ± 0.2	

Table A.2 Composition of MRS broth (Difco, Michigan; USA)

Ingredients	Amount (g per litre of distilled water)
Protease peptone	10.0
Beef extract	10.0
Yeast extract	5.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Dipotassium phosphate	2.0
Magnesium sulphate	0.1
Manganese sulphate	0.05
Glucose	20.0 (equivalent to 110mM)
Medium pH value: 6.5± 0.2	

Appendix 2 Raw data and statistics

Table A.3 A statistical analysis of antimicrobial activity of MRSc and FZMRSC against selected pathogens, compared to untreated controls. Percentage of inhibition on pathogen growth was calculated by comparing OD_{620} of treated pathogens with untreated controls, from triplicate readings with SEMs. Pathogen growth was significantly inhibited when symbol * P <0.05 or ** P <0.01 is presented. The P values are calculated from a paired sample T-test (n=3).

	MRSc		**FZMRSc**		**MRSc-pH**		**FZMRSc-pH**	
	%Inhibition	P value	%Inhibition	P value	%Inhibition	P value	%Inhibition	P value
S. Typhimurium	35.9% ± 2.9%	0.009	42.7 % ± 2.0%	0.002	3.9% ± 2.6%	0.275	6.8 %± 1.9%	0.061
E. coli	35.9% ± 0.7%	0.002	43.3 % ± 3.5%	0.012	3.2% ± 2.8%	0.361	6.3 %± 3.9%	0.243
S. aureus	18.0% ± 0.6%	0.001	24.1 % ± 3.1%	0.021	6.2% ± 2.4%	0.111	4.4 %± 4.8%	0.457
L. monocytogenes	19.1% ± 1.8%	0.018	23.9 % ± 2.4%	0.013	5.5% ± 3.3%	0.227	5.3 %± 6.3%	0.484

Table A.4 A statistical analysis comparing the antimicrobial activities between MRSc and FZMRSc (panel A), and between 8h-MRSg and its pH-adjusted counterpart (panel B). The P values were calculated from paired sample T-test (n=3), with P<0.05 indicating significance.

A.

Freeze-drying effect				
P-value	*S.* Typhimurium	*E. coli O157:H7*	*S. aureus*	*L. monocytogenes*
MRSc versus FZMRSc	0.12	0.15	0.23	0.23

B.

pH adjustment effect				
P-value	*S.* Typhimurium	*E. coli O157:H7*	*S. aureus*	*L. monocytogenes*
MRSg :pH-adjusted MRSg	0.31	0.27	0.18	0.53

Table A.5 A statistical analysis of antimicrobial activity of original SGF series (non-pH-adjusted) against selected pathogens, comparing to non-treatment negative controls. The P values are calculated from a paired sample T-test (n=3), with P<0.05 indicating significance.

	***S.* Typhimurium**		***E. coli*** O157:H7		***S. aureus***		***L. monocytogenes***	
SGF	% Inhibition	P-value	% Inhibition	P-value	% Inhibition	P-value	% Inhibition	P-value
h1	6.9% ± 0.7%	0.089	4.1% ± 0.8%	0.135	9.5% ± 0.6%	0.064	3.9% ± 0.8%	0.174
h2	13.2% ± 0.5%	0.007	6.4% ± 0.4%	0.089	17.3% ± 0.5%	0.002	6.4% ± 0.7%	0.076
h3	9.8% ± 0.6%	0.031	5.6% ± 0.4%	0.099	12.6% ± 0.8%	0.019	5.3% ± 0.6%	0.088
h4	10.3% ± 0.6%	0.045	5.6% ± 0.7%	0.176	12.5% ± 0.8%	0.030	5.3% ± 1.05%	0.229
h5	8.8% ± 0.4%	0.056	5.7% ± 0.9%	0.086	10.1% ± 1.1%	0.059	5.9% ± 0.8%	0.124
h6	8.5% ± 0.7%	0.067	4.9% ± 0.7%	0.082	9.6% ± 0.9%	0.062	3.5% ± 0.9%	0.322

h7	3.8% ± 0.4%	0.261	2.7% ± 0.5%	0.108	8.3% ± 1.0%	0.067	3.4% ± 0.9%	0.348
h8	2.5% ± 0.6%	0.236	1.3% ± 0.4%	0.121	4.1% ± 0.6%	0.156	1.0 % ± 0.8%	0.763

Table A.6 A statistical analysis on the dose response of DPC16 supernatants antimicrobial activity against selected pathogens. Data are P-values calculated from a paired sample T-test (n=3), by comparing the growth of the treated pathogens and that of the untreated controls, where P<0.05 indicates significance effect (inhibition or stimulation).

A. MRSc

Dilution fold	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**1280**	**2560**	**5120**
S. Typhimurium	0.003	0.004	0.015	0.026	0.049	0.239	0.496	0.402	0.774	0.797
E. coli	0.013	0.012	0.012	0.011	0.096	0.047	0.113	0.110	0.144	0.025
S. aureus	0.013	0.067	0.078	0.286	0.854	0.965	0.564	0.447	1.000	0.755
L. monocytogenes	0.010	0.011	0.085	0.037	0.100	0.519	0.826	0.900	0.792	0.980

B. FZMRSc

Dilution fold	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**1280**	**2560**	**5120**
S. Typhimurium	0.007	0.004	0.013	0.043	0.050	0.067	0.092	0.048	0.440	0.515
E. coli	0.000	0.000	0.008	0.007	0.009	0.053	0.080	0.007	0.011	0.034
S. aureus	0.000	0.009	0.058	0.113	0.057	0.553	0.408	0.913	0.883	0.486
L. monocytogenes	0.001	0.006	0.022	0.067	0.371	0.501	0.887	0.595	0.370	0.220

C. MRSg

Dilution fold	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**1280**	**2560**	**5120**
S. Typhimurium	0.000	0.000	0.000	0.013	0.011	0.010	0.075	0.017	0.136	0.128
E. coli	0.002	0.002	0.002	0.009	0.018	0.038	0.071	0.080	0.112	0.049
S. aureus	0.000	0.000	0.000	0.000	0.001	0.003	0.029	0.101	0.453	0.729
L. monocytogenes	0.001	0.001	0.004	0.024	0.012	0.000	0.015	0.053	0.014	0.028

D. SGF

Dilution fold	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**1280**	**2560**	**5120**
S. Typhimurium	0.057	0.793	0.789	0.377	0.649	0.521	0.255	0.145	0.116	0.034
S. aureus	0.217	0.118	0.131	0.254	0.385	0.064	0.283	0.310	0.649	0.819

Table A.7 A statistical analysis on the viability of DPC16 resting cells enumerated at designated time of the secondary fermentation producing either MRSg or SGF. Panel A, cell concentration measured at each time point of the secondary fermentation is expressed as mean log CFU/mL with SEMs, calculated from plate counts of quadruplicate comparing with that in the original inoculum; panel B, a comparison of cell viability between freshly harvested DPC16 cells and the starved and freeze-dried DPC16 cells. P values are calculated from a paired sample T-test (n=3), and indicate significance in reduced cell viability if P <0.01 is presented.

A.

MRSg	**Log CFU/mL**	**P-value**	**SGF**	**Log CFU/mL**	**P-value**
6hr	10.35 ± 0.01	0.0006	1hr	8.36 ± 0.03	0.0001
8hr	10.11 ± 0.03	0.003	2hr	7.27 ± 0.05	1.92×10^{-5}
10hr	9.64 ± 0.01	0.0002	3hr	5.03 ± 0.01	6.23×10^{-7}
20hr	4.56 ± 0.03	2.59×10^{-6}	4hr	3.79 ± 0.02	1.16×10^{-6}

B.

Freeze-drying effect				
Cells viability	Freshly harvested	Starved and freeze-dried	Reduction %	P-value
Log CFU/mL	11.47 ± 0.02	10.91 ± 0.02	72.5	0.0005

Table A.8 A statistic analysis of the effects of DPC16 cell-free supernatants on the growth of selected probiotic cultures. Data are P-values calculated from a paired sample T-test (n=3), by comparing the growth of the treated probiotics to that of the untreated controls, where P<0.05 indicates significance effect (inhibition or stimulation).

P values	MRSc	MRSc pH adjust	FZMRSc	FZMRSc pH adjust	MRSg	MRSg pH adjust	SGF
L. acidophilus	0.058	0.091	0.073	0.059	0.13	0.51	0.34
L. plantarum	0.055	0.28	0.21	0.27	0.20	0.58	0.19
P. acidilactic	0.66	0.19	0.13	0.56	0.59	0.66	0.42
B. lactis DR10	0.069	0.39	0.11	0.101	0.056	0.053	0.098

Table A.9 Growth of LAB cultures, faecal flora and the heat-killed faecal flora in a variety of media with and without PGM supplementation. Values are means of OD_{620} calculated from triplicate readings with SEMs. A statistic analysis compared the growth of LAB cultures and faecal flora with that in the heat killed faecal flora, and P values calculated from a paired sample T-test with P<0.05 indicating significance are also presented.

Culture	1% Glucose	0.3% PGM+1% Glucose	0.3% PGM	
	OD_{620}	OD_{620}	OD_{620}	P-value
LR	0.72±0.02	0.77±0.01	0.05±0.01	0.064
LRg	0.86±0.02	0.82±0.04	0.06±0.01	0.051
BbL	0.44±0.03	0.45±0.02	0.03±0.04	0.194
FF	0.61±0.03	0.55±0.03	0.34±0.04	0.004
HFF	-0.02	-0.01	-0.03	

Appendix 3 Standard curves for colorimetric assays of glycerol and acrolein-equivalent reuterin

Table A.10 Preparation of glycerol standard for colorimetric determination of glycerol in L. reuteri DPC16 supernatants, with glycerol stock (1.25 g/mL, MW 92 g/mol).

Glycerol standard (mg/mL)	0	2	4	6	8	10	12	14	16	18	20
Glycerol standard (mM)	0.0	21.7	43.5	65.2	87.0	108.7	130.4	152.2	173.9	195.7	217.4
Standard Solution (mL)	1	1	1	1	1	1	1	1	1	1	1
20% NaOH (mL)	1	1	1	1	1	1	1	1	1	1	1
95% Ethanol (mL)	6	6	6	6	6	6	6	6	6	6	6
10% CuCl2 (mL)	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
additional Ethanol (mL)	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Final Volume (mL)	10	10	10	10	10	10	10	10	10	10	10

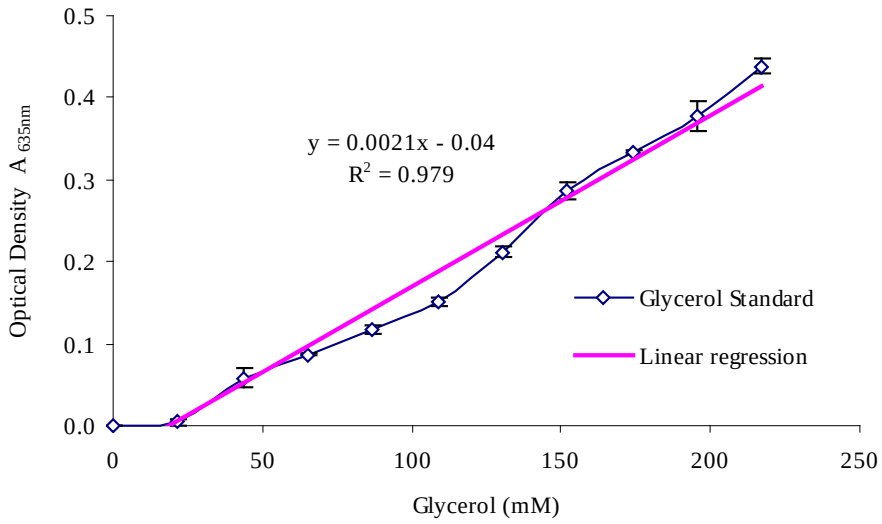

Figure A.1 Standard curve of glycerol in molar concentration. Data points are mean OD_{635} from duplicate readings with SEMs represented as vertical bars. A linear regression line and a mathematics equation with a fitting coefficient R2 are also presented.

Table A.11 Preparation of acrolein standard for colorimetric determination of reuterin in L. reuteri DPC16 supernatants, with acrolein stock (0.839 g/mL, MW 56 g/mol). Acrolein stock was 1:1000 diluted prior to the rest of preparation.

Acrolein standard (µg/10mL)	0	20	40	60	80	100	120
Acrolein (mmol/L)	0.0	0.04	0.07	0.11	0.14	0.18	0.21
Acrolein standard (mL)	0.00	0.24	0.48	0.72	0.95	1.19	1.43
95% Ethanol(mL)	2.00	1.76	1.52	1.28	1.05	0.81	0.57
10mM Tryptophan(mL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
additional 95% Ethanol (mL)	1.2	1.2	1.2	1.2	1.2	1.2	1.2
37%, 12M HCl (mL)	6.3	6.3	6.3	6.3	6.3	6.3	6.3
Final Volume (mL)	10	10	10	10	10	10	10

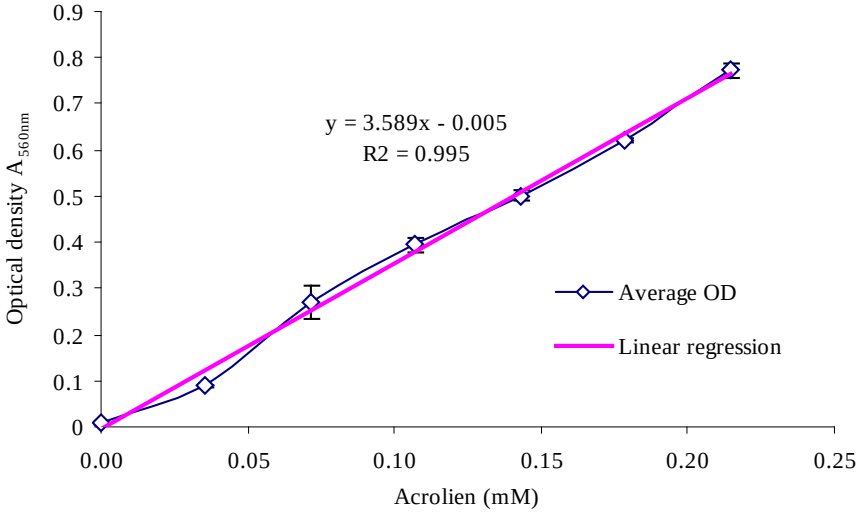

Figure A.2 Standard curve of acrolein in molar concentration. Data points are mean OD_{560} from duplicate readings with SEMs represented as vertical bars. A linear regression line and an mathematics equation with a fitting coefficient R2 are also presented.