A Novel Approach for Controlling Foodborne Pathogens Using Modified Atmosphere and *Lactobacillus reuteri* DPC16

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Abstract

The current trend of increasing demand for minimally processed food requires more effective preservation technologies than are presently used. In this study, an investigation has been made into a novel strategy to control some common foodborne pathogens, and therefore, to provide an alternative means for enhancing the safety and extending the shelf lives of food products.

Modified atmosphere is able to extend the shelf life of seafood and meat products. In this study, a simulated controlled atmosphere (CA) broth system was used to investigate the potential of a modified atmosphere rich in CO_2 at a concentration of 40%, supplemented with N_2, to control common foodborne pathogens, such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* and *Vibrio parahaemolyticus*. Controlled atmosphere significantly reduced the exponential growth rates of all tested pathogens, while the effects on other growth parameters (eg. lag phase duration and maximum population density) depended on the individual species and the specific growth conditions. The CA significantly extended the lag phase durations of *S. aureus* and *V. parahaemolyticus* at 20^{o}C at both pH 6.3 and 6.8, and that of *L. monocytogenes* at both 7^{o}C and 20^{o}C, and at both pH 6.3 and 6.8. The CA also significantly lowered the maximum population densities of *S. aureus* and *V. parahaemolyticus* at 20^{o}C, at pH 6.3 and 6.8, *S.* Typhimurium at pH 6.8, and *L. monocytogenes* at pH 6.3 and 7^{o}C. *E. coli* O157:H7 and *S.* Typhimurium were more resistant to the inhibitory effect of the CA, while *S. aureus* and *V. parahaemolyticus* were most sensitive. The inhibitory effect of CA was due mainly to the extensions of the lag phase duration and the reduction of the exponential growth rates of the test pathogens. This study confirms other studies that CA as a means for food preservation provides potential to control foodborne pathogens and therefore enhance the safety of a food product.

The use of lactic acid bacteria (LAB) in controlling spoilage microorganisms and pathogens in foods has been a popular research theme worldwide. In this study, the antimicrobial effects of 18 lactic acid bacteria strains were evaluated *in vitro*, with emphasis on the most effective strain, the newly characterised *Lactobacillus reuteri* DPC16. The results demonstrated antagonistic effects of many strains against *L. monocytogenes*, *E. coli* O157:H7, *S.* Typhimurium and *S. aureus*. *L. reuteri* DPC16 showed the strongest antimicrobial activity against the tested pathogens including both Gram-positive and Gram-negative bacteria. Co-cultivation of *L. reuteri* DPC16, and co-incubation of its spent culture supernatant (DPC16-SCS), with the pathogens have demonstrated that the antimicrobial effect is bactericidal and valid at pH 4 - 6.5 and at a temperature as low as 10^{o}C. Further characterisation of the antimicrobial effect of *L. reuteri* DPC16 showed it to be mainly due to the presence of reuterin (β-hydroxypropionaldehyde), although lactic acid may have also played a role. These characteristics of *L. reuteri* DPC16 and its metabolite reuterin make it an unique and potent candidate as a biopreservative to control both Gram-positive and Gram-negative bacteria in foods.

The combination of *L. reuteri* DPC16 and CA was assessed for its inhibitory effect on *L. monocytogenes* using DPC16-SCS and the fermentative supernatant of *L. reuteri* DPC16 from a glycerol-water solution (DPC16-GFS). The results showed that both of these supernatants, at 25 AU/mL, in combination with CA (60% CO_2:40% N_2) had a combined inhibitory effect on *L. monocytogenes* which could not be achieved by any one of the individual factors alone.

Analysis of the levels of expression of some stress response genes of *L. monocytogenes*, after growth in the presence of *L. reuteri* DPC16 supernatant and/or CA, showed that the expression of some genes was affected including genes *betL*, *gbuA* and *opuCA* responsible for osmosis adaptation and genes *gadA*, *gadB* and *gadC* responsible for acid tolerance. Induction of *gbuA*, *gadB* and *gadC* by the culture supernatant suggests activation of osmotic and acid adaptation and that these genes play a major role in the culture supernatant-induced stresses.

An investigation was also carried out to determine if the changes in gene expression conferred a cross-protection to heat. The result showed that the survival of *L. monocytogenes* grown in the presence of the culture supernatant and CA was significantly increased after exposure to heat treatment at 56^{o}C, suggesting that a cross-protection to thermal stress had been induced.

Based on these findings it is proposed that a comprehensive novel strategy incorporating both *L. reuteri* DPC16 or its fermentative products and a modified atmosphere rich in CO_2 could be developed to potentially control foodborne pathogens in food products.

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Abbreviations

A	Adenine
AH	Acid habituation
ANOVA	Analysis of variance
AR	Acid resistance
ASPs	Acid shock proteins
AT	Acid tolerance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATR	Acid tolerance response
bBHI	Buffered BHI
bMMRS	Buffered MMRS
bTSBS	Buffered TSBS
BLAST	The Basic Local Alignment Search Tool
C	Cytosine
C	*Clostridium*
CA	Controlled atmosphere
CDC	Centres for Disease Control and Prevention of USA
CFU/mL	Colony forming units per milliliter
CO_2	Carbon dioxide
CO	Carbon monoxide
cDNA	Complementary deoxyribonucleic acid
Bifido	*Bifidobacterium*
BLIS	Bacteriocin-like inhibitory substance
BHI	Brain Heart Infusion
bp	Base pair
^{o}C	Degree Celsius
cm	centimetre
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides: dATP, dCTP, dGTP and dTTP

DPC16-GFS	*L. reuteri* DPC16 glycerol fermentative solution
DPC16-SCS	*L. reuteri* DPC16 spent cultural supernatant
DTT	Dithiothreitol
E	*Escherichia*
EDTA	Ethylenediamine tetra-acetic acid
e.g.	*exempli gratia*, mean "for example"
EGR	Exponential growth rate
Entero	*Enterococcus*
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration of the US
FSANZ	Food Standard Australia New Zealand
g	Gram or acceleration due to gravity (9.8 m/s^2)
G	Guanine
GABA	γ-aminobutyrate
GAD	Glutamate decarboxylase
GI	Gastrointestinal
GRAS	generally recognised as safe
GroEL	chaperonin, a heat shock protein
GT	Generation time
IU	International unit
h	Hour
HACCP	Hazard analysis and critical control points system
H_2O	Water
HAV	Hepatitis A virus
HCl	Hydrogen chloride
H_2O_2	Hydrogen peroxide
HSPs	Heat shock proteins
KCl	potassium chloride
Kg	kilogram
kGy	kiloGrays
L	*Listeria* or *Lactobacillus*
L	Litre

Lb	*Lactobacillus*
Leuco	*Leuconostoc*
L-lactic acid	Levorotatory isomer of lactic acid
LAB	Lactic acid bacteria
Log	Logarithm
LPD	Lag phase duration
M	Molar
MA	Modified atmosphere
MAP	Modified atmosphere packaging
MDOs	Membrane-derived oligosaccharides
mg	Milligram
MPa	Million pascal
MPD	Maximum population density
MRS	de Man, Rogosa, Sharpe
MMRS	Modified MRS
MRSg	MRS broth supplemented with 250 mM glycerol
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar
mm	Millimetre
mM	Millimolar
min	Minute
mL	Millilitre
MviA	a protein of 38 kDA, the product of the mouse virulence regulatory gene *MviA*
N_2	Nitrogen
Na	Sodium
NAG	non-agglutinable *vibrio*
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information

NCV	non-cholera *vibrio*
NFPA	The National Food Processors Association, USA
NLV	Norwalk-like virus
NoV	Norovirus
OD	Optical density
O_2	Oxygen
OpuC	Carnitine transporter
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P	P value
pH	$-Log[H^+]$
ppm	Part per million
%	Per cent
% v/v	per cent by volume/volume
% w/v	per cent by weight/volume
rmp	Rotation per minute
R	Purines
RNA	Ribonucleic acid
RT	Reverse transcription
rDNA	rRNA gene
rRNA	Ribosomal RNA
S	*Salmonella* or *Staphylococcus*
σ^B	Sigma B factor
σ^H	Sigma H factor
σ^S	RpoS, the alternative sigma factor ς^{38}
SCS	Spent culture supernatant
sec	Second
SO_2	Sulphur dioxide
spp	Species
SRSVs	Small round structured viruses
SSE	lowest sum of squares error
T	thymine or temperature

TAE	Tris-Acetate-EDTA
Tris	Tris (hydroxymethy)amiomethane
TSA	Trypticase soy agar
TSB	Trypticase Soy Broth
TSBS	TSB supplemented with 3% sodium chloride
U	unit
UV	Ultra violet light
V	*Vibrio*
vs	Versus
WHO	The World Health Organisation of the United Nations

Amino Acid Abbreviations

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Iosleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1 General Introduction

1.1 Literature review

1.1.1 The New Zealand seafood industry

The New Zealand seafood industry covers the traditional inshore finfish, shellfish and rock lobster fisheries, the more recent deep-water fisheries, and the rapidly growing aquaculture industry (Anon., 2001).

The seafood industry is important to the New Zealand economy. It is the 4th biggest New Zealand export earner behind dairy, meat and forestry (Anon., 2001). New Zealand seafood is sold worldwide successfully despite stiff competition, with export revenues totalling NZ$1.51 billion for the year of 2002 (Anon., 2003). However, the New Zealand seafood trade accounts for less than 2% of the world seafood trade.

Aquaculture is one of the fastest growing areas of the New Zealand seafood industry, making up around 20% of the total fisheries value. Aquaculture production has risen exponentially over the past decade and further dramatic increases are predicted before 2010 (Anon., 2001).

New Zealand's seafood products have a strong reputation for high quality and good food safety. In recent years, improved seafood storage and handling techniques have brought great leaps in export returns, as have developments in value-added products. Improving storage techniques for live and fresh seafood is particularly important because of the large distances to New Zealand's markets. However, it is understood that seafood remained an important potential source of foodborne disease (Simmons *et al*., 2001; Thornton *et al*., 2002).

1.1.2 Spoilage of seafood

Spoilage is defined as any change in the condition of food in which the latter becomes less palatable, or even toxic; these changes may be accompanied by alterations in taste,

smell, appearance or texture (Cheenivasagam & Vidanapathirana, 1986). A number of factors may contribute to food spoilage, but the most common cause is the deterioration caused by microorganisms (bacteria, yeasts, and moulds).

Seafood is highly perishable due to its autolytic enzymes and post-mortem pH changes that favour bacterial growth (Ward & Baj, 1988). It is stated that: "Fish is an extremely perishable food, and should be handled at all times with great care and in such a way as to inhibit the growth of microorganisms" (Ashie *et al*., 1996). Under normal refrigerated storage conditions, the shelf life of a seafood product is limited by enzymatic and microbiological spoilage.

The spoilage of seafood can result from changes brought about by both biological reactions (such as oxidation of lipids and activities of its own enzymes) and the metabolic activities of microorganisms (Ashie *et al*., 1996). The spoilage mechanisms of seafood can be divided into three main types: microbial, enzymatic, and chemical. Church (1998) postulated that the spoilage of a fish can be a process of four phases, including: phase 1 - Very fresh, sweet, seaweedy and delicate taste; Phase 2 - Loss of characteristic odour and taste, flesh neutral (no off-flavours), texture pleasant; Phase 3 - Texture becomes either soft and watery or dry and tough, production of volatile, unpleasant (smelly odours/flavours starting with slightly sour), fruity, bitter off-flavours; Phase 4 - fish spoiled and putrid. Phases 1 and 2 involve major changes due mainly to autolytic reactions, while phases 3 and 4 include changes resulting mainly from bacterial activity.

The spoilage of a fish begins when it dies as its body defences cease to function. The microorganisms present in the gut, gills, and skin, in conjunction with the activities of endogenous enzymes, begin to metabolise the surrounding low molecular weight compounds, resulting in off-flavours, texture deterioration, discolorations, and other changes characteristic of fish spoilage (Jay, 1986). This is a surface phenomenon under chill storage conditions. However, in the situation when temperature abuse and cuts in the fish skin occur as a result of improper handling, microorganisms invade the muscle tissue, resulting in rapid spoilage.

The microorganisms associated with spoilage of seafood reflect the microbial population in the environment (Ashie *et al*., 1996). Freshly caught fish and shellfish from warm waters generally carry a microbial population composed of mesophilic Gram-positive bacteria such as *Micrococcus*, *coryneforms*, and *Bacillus*. On the other hand, cold-water fish harbour predominantly psychrophilic Gram-negative microbes including *Moraxella/Acinetobacter*, *Pseudomonas*, *Flavobacterium* and *Vibrio* genera.

There are other microorganisms that, although not directly related to seafood spoilage, are of public health concern as they are capable of producing hazardous toxins in seafood products or they cause direct infection in humans after consumption of contaminated foods. These organisms are so-called foodborne pathogens and will be discussed in the following section.

1.1.3 Seafood-borne pathogens

Large numbers of outbreaks of infectious diseases and illnesses have been identified to be associated with the consumption of seafood. However, there is severe underreporting worldwide for the total cases. It has been estimated that as few as 1% of the actual cases of foodborne diseases are reported (Mossel, 1982). Recent study in British Columbia, Canada has found that for every case of infectious gastrointestinal illness reported, a mean of 347 community cases have occurred (Macdougall *et al*., 2007). In the USA, the etiological agent was identified in approximately 50% of the outbreaks caused by shellfish (both molluscan shellfish and crustaceans) whereas the cause of disease was identified in almost 90% of the outbreaks related to fish (Olsen *et al.*, 2000).

The causative agents of seafood-associated illnesses may be bacteria or viruses. These agents may be natural inhabitants of the aquatic environment, introduced into the aquatic environment, or introduced into seafood during harvesting and processing (Fletcher, 1996). At least ten genera of bacterial pathogens have been implicated in seafood-borne diseases (Lipp & Rose, 1997). Seafood-associated outbreaks and illness are associated with the consumption of infected or contaminated shellfish, finfish and crustaceans (Wallace, 1999; Gillespie *et al*., 2001). In the following section, only those causative agents contributed to outbreaks of seafood-borne illness are reviewed.

1) *Salmonella* spp.

Salmonella is a major foodborne agent, which occurs widely in animals, especially in poultry and swine. Raw seafoods, as well as water, soil, insects, factory surfaces, kitchen surfaces, animal faeces, raw meats, and raw poultry are the main environmental sources of the organism (http://www.cfsan.fda.gov/~mow/chap1.html). *Salmonella* spp. reach seafood mainly through faecal contamination of the aquatic environment. Infections caused by *Salmonella* spp. occur throughout the world. In the UK, *Salmonella enterica* serovar Typhi was detected in more than 1.6% of shellfish sampled from open harvesting waters (Wilson & Moore, 1996). In Japan, a survey showed that *Salmonella* spp. were present in 21% of eel culture ponds (Saheki *et al*., 1989). In India, 52 out of 100 samples collected from the southwest coast of India were detected as positive for Salmonella using polymerase chain reaction (PCR) (Shabarinath et al., 2007). The presence of *Salmonella* in fish and fishery products has also been reported from other countries (Rattagool *et al*., 1990). In New Zealand, *Salmonella* spp. were responsible for 10.7% of total outbreaks related to foods in 2002, and accounted for 22.1% of all hospitalisations (Boxall & Ortega, 2003). However, *Salmonella* infections due to seafood consumption are still low compared with salmonellosis associated with other foods.

2) *Clostridium botulinum*

Clostridium botulinum is a spore-forming bacterium and can be classified into types A to G based on the antigenic specificity of the toxin produced by each strain (Rhodehamel *et al*., 1992). The types pathogenic to humans (types A, B, E and F) are generally divided into two groups: (1) the proteolytic types A, B and F, which are heat resistant, mesophilic, NaCl-tolerant and have the general environment as their natural habitat; (2) the non-proteolytic types B, E and F, which are heat sensitive, psychrotolerant, NaCl-sensitive and have the aquatic environment as their natural habitat. Type E is dominant of this species in the marine environment (Hielm *et al*., 1998; Hyytia *et al*., 1998).

C. botulinum exists widely in nature, and is a natural contaminant of fish and shellfish. The vegetative cells of all types are easily killed by heat. However, the spores are heat-resistant and can survive in foods that are incorrectly or minimally processed. *C. botulinum* produces a potent neurotoxin, causing botulism, and seafood products may

serve as vehicles for human botulism. Most of the outbreaks are associated with inadequately processed, home-canned foods, but commercially-produced foods can be also involved in outbreaks. Botulism cases arising from seafood consumption have been associated primarily with home-processed, smoked or fermented fish products (Hobbs, 1976). In the hot-smoking process of fish, the temperatures are usually too low to destroy the spores (Lindstrom *et al.*, 2003). Cell growth and toxin production from spores in vacuum-packed, smoked fish products, with an anaerobic atmosphere and limited preservative factors, are likely during extended storage at temperatures above 3°C. In New Zealand, the last reported case of botulism in 1985 was caused by the consumption of improperly preserved mussels and watercress (Flacks, 1985). This was from *C. botulinum* type A, an organism not usually associated with the marine environment so most likey to have been associated with the watercress.

The most important factors controlling *C. botulinum* growth and toxin production are efficient heat treatment and sterilisation, restricted shelf life and continuous storage below 3°C. Also, the control of botulism lies not in detecting the presence of spores or toxins in products in commerce, but in ensuring that spores cannot germinate to produce toxin while the food is still regarded as edible.

3) *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive bacterium. *L. monocytogenes* and related species are ubiquitous in the environment and can colonise any man-made environments they find favourable (Anon, 1988). Studies have suggested that humans may be intestinal carriers of *L. monocytogenes* (http://www.cfsan.fda.gov/~mow/chap6.html). The organism has been found in mammals, birds, fish and shellfish (Ben Embarek, 1994; Lawrence & Gilmour, 1994; Tapia de Daza & Diaz, 1994; Arvanitidou *et al*., 1997; Heinitz & Johnson, 1998; Monfort *et al*., 1998; Destro, 2000; Dominguez *et al*., 2001). Bernagozzi *et al*. (1994) suggested that higher *L. monocytogenes* concentrations in the environment are probably a result of human or animal activity. A recent survey has found that the prevalence of *L. monocytogenes* increased in line with the degree of human activity: 2% in seawater fish farms, 10% in freshwater fish farms, 16% in fish slaughterhouses, and 68% in a fish smokehouse (Hansen *et al*., 2006). However, a low prevalence of *L. monocytogenes* was found in the natural environment, and the bacteria

did not survive well in natural environments (Hansen *et al*., 2006).

Listeriosis is the name of the general group of disorders caused by *L. monocytogenes* (http://www.cfsan.fda.gov/~mow/chap6.html), with the target population being most often elderly people, people with underlying diseases, and neonates (Rocourt *et al*., 2000). It is also a high risk for pregnant women, resulting in abortion. Although the reported cases of listeriosis are few, the mortality rate is high (Farber & Peterkin, 1991). It is estimated that *L. monocytogenes* accounts for 28% of all deaths caused by foodborne pathogens (Rocourt *et al*., 2000). Refrigerated ready-to-eat products are the main sources of listeriosis, as such products usually have a long shelf life, allowing *L. monocytogenes* to grow to high numbers. *L. monocytogenes* has been associated with raw and smoked fish, and shellfish or raw fish consumption has been suggested as a source of infection (Lennon *et al*., 1984). Although low number of cells carry some risk of infection, the majority of cases (>99%) are caused by food products with high levels of the bacterium (Buchanan *et al.,* 1997; FAO/WHO, 2001a; FDA, 2001). Thus, the real risk is the growth of the organism in the product rather than its mere presence. Despite this knowledge and the understanding that low levels are unlikely to cause disease, several countries, including the United States, have a regulation of so-called “zero tolerance” (not detected).

In New Zealand, two perinatal listeriosis cases were diagnosed during November and December 1992 with histories of consuming smoked mussels (Brett *et al*., 1998). This cases indicated that although listeriosis was rare from consuming seafood, but seafood could be a source of listerial infection.

4) *Vibrio* spp.

Vibrio spp. are members of the *Vibrionaceae*, known as aquatic bacteria. The members in this group include *Vibrio cholerae* serotype O1, *Vibrio cholerae* serotype non-Ol, *Vibrio parahaemolyticus, Vibrio vulnificus* and other marine *Vibrio* spp. These members are halophilic or halotolerant and common organisms in warm marine and estuarine waters. *Vibrio* spp. find reservoirs in the intestinal tract of fishes, within shellfish, in sediments and plankton (Epstein, 1993; DePaola *et al*., 1994; Epstein, 1995; Shukla *et al*., 1995). In general, *Vibrio* spp. (except for *V. cholerae*) are not associated with faecal

contamination and therefore faecal indicators do not correlate with the presence of *Vibrio* spp. (Koh *et al*., 1994). Raw oyster consumption is the most common route for human infection, with 95% of cases in the USA associated with the American oyster (Rippey, 1994). The genus comprises 34 species, of which 13 species can cause human disease (Kaysner, 2000; FAO/WHO, 2001b). Seafood-borne diseases are primarily caused by *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* (Oliver & Kaper, 1997). These three are the dominant and emerging pathogenic species within the *Vibrionaceae*:

Vibrio cholerae

This species includes two groups: serotype O1 and serotype non-O1. Serotype O1 is responsible for Asiatic or epidemic cholera (http://www.cfsan.fda.gov/~mow/chap7. html), an acute diarrhoeal disease endemic in India and Southeast Asia leading to severe dehydration in a matter of hours unless quickly treated. Consumption of raw shellfish harvested from faecally polluted coastal waters often causes sporadic cases (http://www.cfsan.fda.gov/~mow/chap7.html).

V. cholerae serotype non-Ol comprises both pathogenic and nonpathogenic strains of the organism and are normal inhabitants of marine and estuarine environments (http://www.cfsan.fda.gov/~mow/chap8.html). This organism causes a disease less severe than cholera and has previously been referred to as non-cholera *Vibrio* (NCV) and nonagglutinable *Vibrio* (NAG). Non-Ol *V. cholerae* gastroenteritis is the name associated with this illness.

Both types of *V. cholerae* have been reported in New Zealand, with the sufferer having been previously overseas or having consumed imported food (Baker & Wilson, 1993; Frazer *et al*., 1993). However, some cases of non-O1 *V. cholerae* identified in New Zealand have suggested that New Zealand seafood could be a potential source of the infection (Fletcher, 1996). Non-O1 strains are believed to inhabit the New Zealand environment causing concerns for the safety of shellfish consumption.

Vibrio parahaemolyticus

This bacterium is a common inhabitant in the estuarine and marine environment. It can be easily isolated from marine and estuarine environments and from fish and shellfish

dwelling in these environments (http://www.cfsan.fda.gov/~mow/chap9.html). Infections with this organism have been associated with the consumption of raw, improperly cooked, or cooked, recontaminated fish and shellfish. *V. parahaemolyticus* has been a major cause of food poisoning in Japan (Shinoda, 1992). *V. parahaemolyticus* is present in New Zealand oysters at a relatively low level; however, the bacterium can develop high numbers in a short time under conditions caused by mishandling of the contaminated food (Fletcher, 1985).

Vibrio vulnificus

Research conducted in Japan has found that this bacterium is widely distributed in sea water, sea mud, and oysters with isolation rates of 54.8%, 40.8% and 30.3%, respectively from these samples (Oonaka *et al*., 2002). Seafood-borne *V. vulnificus* infections are almost exclusively caused by consumption of raw bivalve molluscs such as oysters. *V. vulnificus* has been isolated from New Zealand shellfish and it has been suggested to be associated with necrotising fasciitis, septicaemia and wound infections (Wright, 1991; Upton & Taylor, 2002). However, it seems that the New Zealand environmental conditions (seawater temperature and high salinity) are not suitable for the organism to reach a number high enough to be epidemic (McCoubrey, 1996).

5) *Aeromonas* spp.

Aeromonas spp. such as *Aeromonas hydrophila* have been recognized as potential or emerging foodborne pathogens for more than 20 years (Isonhood & Drake, 2002). Aeromonads are estuarine bacteria and are ubiquitous in fresh water, fish and shellfish, meats, and fresh vegetables. Most Aeromonads are psychrotrophic and can grow in foods during cold storage. The bacteria are not resistant to food processing regimes and are readily killed by heat treatment. A survey of New Zealand seafood in retail condition found that motile Aeromonads were present in 66% of shellfish and 34% of finfish (Hudson & Delacy, 1991). Epidemiological evidence suggests that the bacterium can cause self-limiting diarrhoea, with children being the most susceptible population.

6) *Escherichia coli* O157:H7

Escherichia coli O157:H7 was first recognized as a human pathogen in 1982 when two outbreaks in the US were associated with consumption of undercooked hamburgers

from a fast-food restaurant chain (Riley *et al*., 1983). The pathogen has since emerged as a major cause of bloody and nonbloody diarrhoea and Occurrences of infection have been reported worldwide (Altekruse *et al*., 1997). The reservoir is thought to be cattle and poultry (Dipineto *et al*., 2006). Most of the infections to date have been associated with the consumption of ground beef, lettuce, raw cider, raw milk, and untreated water (Riley *et al*., 1983; Besser *et al*., 1993; Boyce *et al*., 1995). Shellfish can act as a vehicle for *E. coli* O157:H7 transmission. This was demonstrated by recent survey from which that *E. coli* O157:H7 was isolated from six of 40 *stx*-positive enrichments of samples collected from French coastal environments (Gourmelon *et al*., 2006). The first case of *E. coli* O157:H7 infection in New Zealand was in 1993 (Wright *et al*., 1993). It poses a risk to public consuming seafood as it is difficult to avoid contamination of all fish and shellfish in the wild and in farms affected with sewage contamination. The main means of prevention of this type of food poisoning lies in proper cooking before consumption.

7) *Campylobacter* spp.

Campylobacters are microaerophilic, Gram-negative, small vibrioid or spiral-shaped cells with a rapid, darting, reciprocating motility and are classified as the genus *Campylobacter* (Sebald & Veron, 1963). The organisms primarily include *C. jejuni* subsp. *jejuni* and *C. coli* and were originally identified as human diarrhoeal pathogens. These organisms are recognized as the leading cause of human diarrhoea in many countries including New Zealand (Atabay & Corry, 1998; Altekruse *et al*., 1999). *C. jejuni* has the widest reservoir and is commensal in the intestines of sheep, pigs, cattle, goats, chickens, turkeys and wild birds (Blaser, 1982). The vast reservoir in animals is probably the ultimate source for most infections of humans. Consumption of contaminated food and water has become the most common transmission route of infection (Perkins-Jones *et al*., 1980; Arumugaswamy & Proudford, 1987; Arvanitidou *et al*., 1995). Consumption of raw shellfish has been reportedly associated with illness in many countries (Morris *et al*., 1980; Griffin *et al*., 1983; Arumugaswamy & Proudford, 1987; Abeyta *et al*., 1993; Geldreich, 1996). In New Zealand, the first case of illness from *Campylobacter* was reported in 1979 (Brougham & Meech, 1979). Since then, *Campylobacter* infection has become the most commonly notified enteric disease in this country (Brieseman, 1994; Brieseman *et al*., 2000). Outbreaks have been found to be associated with the consumption of raw milk, fresh chicken, contaminated water and

food (Brieseman, 1984; Stehr-Green *et al*., 1991). Although no outbreak has been directly associated with seafood as a source of infection in this country, the potential of this organism to be a safety concern in seafood cannot be ignored due to the environmental contamination with this microorganism.

8) Norovirus

Norovirus (NoV), also called Norwalk-like virus (NLV), includes a group of unclassified small round structured viruses (SRSVs) (Kapikian *et al*., 1972). These viruses are responsible for a self-limiting viral gastroenteritis, acute nonbacterial gastroenteritis, food poisoning, and food infection (Mead *et al*., 1999). Water is the most common source of outbreaks and may include water from municipal supplies, wells, recreational lakes, swimming pools, and water stored aboard cruise ships. Shellfish and salad ingredients are the foods most often implicated in Norovirus outbreaks. Ingestion of raw or insufficiently steamed clams and oysters poses a high risk for infection with Norovirus (Brieseman *et al*., 2000; Simmons *et al*., 2001). In New Zealand, Norovirus is considered as a leading cause of communicable disease outbreaks (Anon., 2000; Greening *et al*., 2001). The majority of NoV strains found in New Zealand since August 1995 were similar to those occurring overseas and the predominant New Zealand strain is genetically similar to the Bristol/Lordsdale virus group (Greening *et al*., 2001). Several New Zealand outbreaks were attributed to Auckland virus, a Mexico-like NoV strain identified as the most likely cause of gastroenteritis after consumption of contaminated oysters in 1994. A new strain, designated Napier virus, has been identified in six outbreaks since 1996. A number of strains closely resembling internationally recognised strains, including Southampton virus, Saratoga virus, Desert Shield virus and Melksham virus have been associated with gastroenteritis outbreaks across New Zealand (Greening *et al*., 2001).

9) Hepatitis A virus

Hepatitis A virus (HAV) is classified with the Enterovirus group of the *Picornaviridae* family (Minor, 1991). HAV causes a mild illness characterised by sudden onset of fever, malaise, nausea, anorexia, and abdominal discomfort, followed in several days by jaundice. Compared to other enteric viruses hepatitis A virus has an extended incubation period of about 4 weeks (range 2-6 weeks) and is self-limiting and rarely causes death,

although patients may be incapacitated for several months. Hepatitis A is a common endemic infection in developing countries with most children being seropositive by 6 years of age. However improving sanitary conditions in developed countries have led to declining prevalence and has resulted in large sectors of the population being susceptible to infection.

HAV is excreted in faeces of infected people and causes a contaminated environment (http://www.cfsan.fda.gov/~mow/chap31.html). Cold cuts and sandwiches, fruits and fruit juices, milk and milk products, vegetables, salads, shellfish, and iced drinks are commonly implicated in outbreaks. Hepatitis A is the most serious virus infection linked to shellfish consumption (Lees, 2000). Contamination of foods by infected workers in food processing plants and restaurants is common (CDC, 1990, 1993; Hutin *et al*., 1999; Massoudi *et al*., 1999; Dentinger *et al*., 2001) and therefore, personal hygiene is important in preventing it from spreading.

1.1.4 Strategies for shelf life extension and controlling seafood spoilage and pathogens

Because of the perishable nature of seafood, development of satisfactory methods for shelf life extension has been the focus of attention of food technologists, to ensure the maintenance of seafood quality and safety, and a continuous supply of self-stable quality products with minimum losses. Techniques that offer variable degrees of success have evolved over the years. For the preservation of fish and fish products, smoking, drying, salting, and freezing are traditional practice, aimed at inhibiting the growth of spoilage microorganisms and inactivating autolytic enzymes (Gram, 1991). These methods, combined with refrigeration, can extend the shelf life from days to weeks, albeit with drastic changes to the freshness of the fish. Other methods, such as low temperature storage, chemical treatments, low dose irradiation, high pressure treatment, and modified atmosphere storage, are generally aimed at extending the shelf life while still retaining the characteristics of fresh fish. These methods should also be applicable to seafood. In this section, the preservative methods that are able to extend the shelf life while still retaining the characteristic of freshness of seafood are reviewed.

1.1.4.1 Low-temperature storage

Low-temperature storage has been used to retard microbial spoilage of seafood since the mid-nineteenth century and is still a routine practice in the seafood industry. It slows down spoilage or contaminating microorganisms from metabolizing, growing and causing spoilage, without killing them. Refrigeration has the advantage of maintaining freshness and overall organoleptic quality of seafood products, but its inability to kill or completely eliminate microorganisms poses potential health risks, as some pathogens, such as *L. monocytogenes* and some *C. botulinum* strains, are able to grow and in the case of *C. botulinum* produce toxin at refrigeration temperatures. Because of the rapid spoilage of seafood, it has been recommended that fish be cooled to the temperature of melting ice as quickly as possible as the shelf life can be reduced by one day for each hour's delay in icing or exposure to ambient temperatures of 28 to 30^{o}C (FAO, 1973; Barile *et al*., 1985). However, this iced storage also has disadvantages, such as a tendency to injure and bruise the flesh, leaching of flavour components and nutritionally desirable materials and water soluble proteins (Holston & Slavin, 1965).

1.1.4.2 Chemical treatments

Chemical reagents have been studied in the past to extend the shelf life of a variety of foods, and in the food industry, chemical preservation plays a prominent role. However, the use of chemical preservatives is often combined with other methods, such as cooling, drying, smoking, freezing, and heating.

Although the application of chemical preservatives has a long history, they are considered as food additives and are subject to regulations at international and national levels. The preservatives now in use have been thoroughly tested for their toxicological properties and are subject to stringent legal regulations (Luck, 1985). In New Zealand, Food Standards Australia New Zealand (FSANZ) carries out a safety assessment before a food additive can be used in food. FSANZ checks that the food additive is safe at the level proposed to be used, and that there are good technological reasons for the use of the additive. If FSANZ agrees that a food additive should be permitted, approval of government is sought. It is only after government approval that a food additive can be used in foods. This process applies to all chemical preservatives. The Food Standards

Code (a joint food law between Australia and New Zealand) says which additives can be used and in what foods.

Potassium sorbate is a generally recognised as safe (GRAS) food preservative and has been proven effective when used on chilled fish to inhibit spoilage bacteria (Robach, 1979). Its antimicrobial activity is found in the undissociated acid, making the pH a very important factor for its effectiveness. Studies have shown that the combined use of sorbates and polyphosphates prior to storage improves the shelf life even further (Statham *et al*., 1985). Examples of polyphosphates used include sodium triphosphate, disodium orthophosphate, tetrasodium pyrophosphate, and hexametaphosphate.

Sulfites and SO_2 have been the preferred dipping solutions in the fish/crustacean industry as they are very effective as antimicrobial agents and as inhibitors of both enzymatic and nonenzymatic browning (Ashie *et al*., 1996). However, the use of these agents has been restrained or entirely prohibited in the US because of their potential health hazards such as allergic reaction and potential mutagenicity (Sullivan & Smith, 1985; Taylor *et al*., 1986).

Ethylenediamine tetra-acetate (EDTA) is a chelating agent and has been used in a wide variety of food products to prevent oxidation and other deteriorative reactions catalyzed by metal ions (Branen & Davidson, 2004). It has been reported that EDTA enhances the activity of nisin, lysozyme, and monolaurin against Gram-negative microorganisms (Hughey & Johnson, 1987; Stevens *et al*., 1991; Razavi-Rohani & Griffiths, 1994). It also has antimicrobial activity (Reidmiller *et al*., 2006) and is known to potentiate the activity of antimicrobials and antibiotics against meat spoilage and pathogenic bacteria, especially against Gram-negative microorganisms (Gill & Holley, 2003). EDTA is being used in combination with other treatments in fish processing (Miller & Brown, 1992).

Many other chemicals, such as glucose oxidase and chitosan (deacylated chitin) have also shown their potential as food preservatives. Glucose oxidase has been demonstrated as a potentially useful preservative that does not exhibit the risks involved with bisulfites (Field *et al*., 1986; Kantt *et al*., 1993). Chitosan can be an effective preservative because of its antimicrobial activity and the wide distribution in nature,

therefore providing an economically viable tool to the food industry (Chang *et al*., 1989; Jeon *et al*., 2002).

1.1.4.3 Low-dose irradiation

This technique was proven to maintain the quality and the freshness of seafoods in the mid-1950s. Food irradiation involves the exposure of food products to ionizing radiation to improve the shelf life of the product as a result of the antimicrobial effects of ionizing radiations. These improvements were attributed to marked reductions in levels of *Pseudomonas* and other spoilage microorganisms (Bruns & Maxcy, 1979).

Irradiation has been demonstrated effective in controlling the levels of pathogenic microflora, thereby reducing the risk of infection. For instance, *A. hydrophila* has been shown to be sensitive to radiation as low as 1.5 kiloGrays (kGy) on fresh bluefish (Palumbo *et al*., 1986), and spores of *C. botulinum* inoculated on fresh Gulf Coast shrimp and irradiated at a dose of 1.5 kGy failed to produce toxin over a 31-day iced storage period.

The time of exposure and irradiation dose are of critical importance in the application of irradiation to control microorganisms in seafoods, as microorganisms respond differently depending on their specific cellular characteristics. Thus, three levels of control have been developed: radappertization, radurization, and radicidation (Urbain, 1986).

Radappertization involves the use of high doses of about 50 kGy to completely eliminate all microorganisms in the seafood, which can then be kept almost indefinitely. At such high doses, the flavour and texture of the product are altered, and it does not comply with the WHO acceptable upper limit of 10 kGy for food processing and makes the application of such high doses rather unlikely. Radurization, on the other hand, inactivates a proportion of the spoilage microorganisms (about 90 - 95%), so that spoilage will eventually occur if the product is stored over long periods, even in ice. The dose levels used are between 1 - 5 kGy. Radicidation involves treatment with dose ranges between 5 to 8 kGy, which is usually adequate for eliminating nonspore-forming

pathogenic microorganisms. However, doses over 5 kGy often result in undesirable changes in colour, flavour, and odour, especially in the presence of oxygen. Hence, radurization is the preferred application of radiation treatment for shelf life extension. It is technologically effective for extending the shelf life of most fish and fish products with optimum doses ranging from 0.75 - 2.5 kGy (Urbain, 1986).

Despite the obvious advantages that could be derived from low dose irradiation, its application to food products is still a hotly debated issue. In the European community, legislation ranges from a total ban on food irradiation to a substantial list of foods for which irradiation is permitted (Ehlermann, 1991). In the US, legislation allows irradiation of some foodstuffs with doses up to 3 kGy (eg. for chicken), however, for some other products, eg. spices, the allowable dose is up to 30 kGy. Food irradiation also has restricted application in Japan and Australia (Furuta *et al*., 1992). FSANZ stipulates which foods can undergo irradiation. Currently in Australia and New Zealand, only herbs, spices and herbal infusions have been approved by FSANZ for irradiation (http://www.foodstandards.gov.au/mediareleasespublications/factsheets/factsheets2002/foodirradiationjune21581.cfm).

1.1.4.4 High pressure treatment

High pressure treatment in the food industry is becoming more popular and is currently under intense investigation. The effects of high pressure, particularly hydrostatic pressure, on the viability of microorganisms and on protein denaturation have been known for several decades. Pressure influences most biochemical reactions because they often involve changes in volume. This change leads to inhibition of availability of energy to microorganisms by affecting energy-producing enzymatic reactions, thereby reducing the viability of cells. High pressure also causes denaturation of proteins due to the pressure-induced unfolding of the protein chains, and affects cellular morphology in a reversible or irreversible manner depending on the applied pressure.

The required pressure to inactivate microorganisms is dependent on the target microorganism. Generally, bacterial vegetative cells, yeasts, moulds, and some viruses are sensitive to pressures between 200 and 700 MPa. However, bacterial spores are more resistant and can survive pressurization above 1,000 MPa (Sale *et al*., 1970;

Cheftel, 1992; Arroyo *et al*., 1999). The critical site of pressure damage leading to inactivation of microorganisms is the cytoplasmic membrane. The cell permeability is altered and ion exchange is disrupted due to crystallization of membrane phospholipids and protein denaturation (Cheftel, 1995; Yuste *et al*., 2001). Between 200 and 400 MPa, irreversible changes such as cell leakage will occur, leading to cell death.

High hydrostatic pressure equipment has been developed for research and industry. In the food industry, high pressure treatments have so far been used for preservation of fruits and vegetables, reducing the microbial population in milk, and tenderisation of prerigor beef (MacFarlane, 1985). In the fish industry, it has been applied during the gelation of Japanese surimi from Pollack, sardine, skipjack, and tuna (Farr, 1990). The application of high pressure treatment to whole oyster processing appears attractive, as pathogens likely to be associated with raw oysters, notably *Vibrio* spp., and hepatitis A virus, are sensitive to inactivation by high pressure treatment (Styles *et al*., 1991; Kingsley *et al*. 2002; Calci *et al*., 2005; Koo *et al*., 2006). Also, the refrigerated shelf life of harvested oysters is limited so that any extension of the shelf life without altering sensory quality is highly desirable. In addition, it has been reported that pressure-treated oysters were "slightly more voluminous with a very pleasant appearance" (Lopez-Caballero *et al*., 2000).

Although the application of high pressure treatment in the seafood industry is still under investigation, it has been proposed to have strong potential on the basis of its antimicrobial and biochemical influences (Shearer *et al*., 2000). For instance, the freezing point of water decreases with increasing pressure; the freezing point of water is $-5^{o}C$ at 700 kg/cm^2, $-10^{o}C$ at 1250 kg/cm^2, and $-20^{o}C$ at 2000 kg/cm^2. Thus, the application of moderate pressures in combination with subzero temperatures could be exploited as a means of treatment not only for seafoods but for foods in general without the formation of intracellular ice, thus eliminating damage due to freezing while preventing microbial spoilage. Furthermore, pressure-treated foods result in products that retain their natural qualities such as flavour and texture better than those processed by traditional methods (Shearer *et al*., 2000).

1.1.4.5 Modified atmosphere packaging (MAP)

The inhibition of microorganisms on foods, and therefore extension of the shelf life, can be achieved through packaging. Currently, two major forms of packaging system exist, namely vacuum packaging and MAP. In this section, only a brief introduction is given on vacuum packaging, and the focus of the review will be centred on MAP.

1. Vacuum packaging

Vacuum packaging involves placing a product in a film of low oxygen permeability, the removal of air from the package and the application of a hermetic seal (Smith *et al*., 1990). As two of the major modes of spoilage , namely aerobic bacteria and oxidative reactions, require oxygen, its unavailability will therefore inhibit spoilage and thus maximize quality and/or storage life. Some deterioration, however, will occur due to anaerobic/microaerophilic organisms and non-oxidative reactions. This is usually minimized by chilled storage. However, product compression is unavoidable in this packaging, due to its vacuum condition, and makes it unsuitable for many products.

2. Modified atmosphere packaging (MAP)

MAP has been defined as “the enclosure of food products in gas-barrier materials, in which the gaseous environment has been changed or modified” (Young *et al*., 1988), in order to inhibit spoilage agents and thereby either maintain a higher quality of a perishable food during its natural life or actually extend the shelf life. MAP is an extension of the vacuum packaging process and is specially designed to overcome some of the problems associated with (or in fact caused by) vacuum packaging, namely to inhibit a wide range of microbiological spoilage agents and avoid compression damage (Smith *et al*., 1990).

It has been recognised for many years that MAP enables a significant shelf life extension of foods. The first patented application of a CO_2/CO gas mixture to extend the shelf life of meat was granted 100 years ago. However, the first major commercial application of MAP took place only in 1974, when the French company SCOPA started to sell MAP meat (Church, 1994). Since then, the use of MAP has thrived due to the increasing consumer demand for fresh and chilled convenience foods. Today, MAP

foods including raw and cooked meats, poultry, fish, crustaceans, vegetables, fruits, dairy products, bakery products, and crisps, are commonly found in supermarkets.

The microbiological flora on the product has influenced the choice of gases used in MAP. In addition, the sensitivity of the product to O_2 and CO_2, and the colour-stabilising requirements need to be considered. The gases most commonly used in MAP are those found in the atmosphere (O_2, CO_2 and N_2) and each of them has a specific function (Church, 1994):

Oxygen (O_2): Oxygen promotes the growth of aerobic bacteria and inhibits the growth of strictly anaerobic bacteria. Oxygen is important in the storage of fresh meats as it maintains the meat pigment myoglobin in its oxygenated form, oxymyoglobin, which keeps the bright red colour of fresh meat which consumers prefer (Church, 1994). Also, the inclusion of O_2 is essential in the packaging of fresh fruits and vegetables as these continue to respire (consume O_2 and produce CO_2) post-harvest, and in the absence of O_2 anaerobic respiration occurs, the effect of which is to accelerate senescence and spoilage (Church, 1994). However, for fish products, the presence of O_2 may cause oxidative rancidity (Church, 1994), which influences the quality and shelf life of MAP fish products and so is normally excluded from these products.

The inclusion of 5 - 10% O_2 in some certain MAP products can add safety value against the growth of anaerobic pathogens, in particular *Clostridium botulinum* (Hotchkiss, 1989). Also, its inclusion at the beginning of packaging may favour the growth of competitive microflora, helping to inhibit the growth of any anaerobic pathogens present.

Carbon dioxide (CO_2): CO_2 is both water- and lipid- soluble and is a bacterial and fungal growth inhibitor (Wolfe, 1980; Dixon & Kell, 1989). It is mainly responsible for the bacteriostatic effect on microorganisms in modified atmospheres. The overall effect on microorganisms is an extension of the lag phase of growth and a decrease in the growth rate during the logarithmic growth phase. Although the bacteriostatic effect of CO_2 has been known for many years, the precise mechanism of its action is still an area of considerable active research.

The effect of CO_2 in MAP is dependent upon the dissolution of the gas into the packaged product, which has a number of consequences:

1) The inhibitory effect is directly related to the amount of CO_2 present. It increases linearly with concentrations up to 50 - 60% (of total atmosphere in terms of volume) above which there is little or no further effect on the majority of microorganisms (Gill & Tan, 1980). An amount above that which will dissolve into the product is required for optimal effect (Gill, 1988). This indicates that the packaging volume and the permeability and surface area of the packaging materials are important considerations;

2) The solubility of CO_2 is inversely proportional to the storage temperature and thus low temperatures have a synergistic effect upon its action (Gill & Tan, 1980);

3) Unpleasant acidic tastes (souring) can be caused by using high concentrations of CO_2, some of which exists as carbonic acid (Daniels *et al*. 1985);

4) Excessive absorption of CO_2 can cause pack collapse with some high moisture foods, such as meat, poultry and seafood. Packaging in high CO_2 concentrations can also cause increased drip in fresh meat, fluid release in ham, product separation in cream, physiological damage to fruit and vegetables, and a sherbet-like taint in fatty fish (Church, 1993).

Nitrogen (N_2): N_2 is normally used to displace the O_2 in the packs and storage vessels to delay oxidative rancidity and inhibit the growth of aerobic microorganisms (Church, 1994). Because of its low solubility in water and lipids and its lack of taste, it also acts as a filler gas in MAP products to prevent the collapse of the pack containing high concentrations of CO_2.

Other gases: Gases such as sulphur dioxide, carbon monoxide, nitrous oxide, nitric oxide, ozone, helium, hydrogen, neon, propylene oxide, ethylene, and chlorine have been investigated experimentally for their potential use in MAP applications. However, the use of these gases has been limited by safety concerns, legislation, adverse consumer response, cost and negative effects on the organoleptic properties of packaged products (Church, 1993).

MAP has been proved beneficial in extending the shelf life of a wide variety of products. Other advantages may also make MAP beneficial, such as easier slice separation and improved product presentation. However, proper refrigeration is required during product storage in order for MAP to be successful and safe. Good manufacturing practices should be followed for all MAP products (Church, 1994).

3. MAP of Seafood

The perishable nature of seafood makes it prone to spoilage. A shelf life range from 2 - 14 days has been reported if it is stored under refrigeration in air, depending upon the species, harvest location and season (Stammen *et al*., 1990). The technique of coupling refrigerated storage with MAP has been in use to extend the shelf life of fresh fish product since the 1930s (Bremer & Fletcher, 1999). The use of MAP, specifically elevated CO_2 levels, has been shown to inhibit normal spoilage bacteria, such as *Pseudomonas*, *Aeromonas*, *Shewanella*, *Moraxella* and *Acinetobacter* in fish from cold and temperate waters, and thus double or triple the shelf life (Stammen *et al*., 1990). Claims have been made for shelf lives of up to 3 - 4 weeks for refrigerated MAP fish, but this is generally considered unrealistic. However, with the use of pretreatment, such as dipping with potassium sorbate or sodium chloride, and irradiation, such a shelf life may become achievable (Stammen *et al*., 1990). More typically, shelf lives are in the range of 3 - 14 days depending upon the product (Church, 1998).

4. Safety concerns of MAP

Storage of fresh or processed fish products using MAP technologies has gained widespread application worldwide. However, the technology is approached with a great deal of caution by regulatory authorities. The major concern about MAP seafood is the relative safety problem of the anaerobic systems. The risk is significantly higher than in meat (Garcia & Genigeorgis, 1987; Stammen *et al.*, 1990; Reddy *et al*., 1992) due to the greater incidence of *C. botulinum* in marine environments (Huss, 1980; Hackney & Dicharry, 1988). The conditions often encountered in MAP fish products are conducive to the growth of, and toxin production by, *C. botulinum* type E even at temperatures as low as 3.3^{o}C (Hobbs, 1976). However this risk only appears to exist in cases of either temperature abuse or extended shelf life (Garcia *et al.*, 1987). One approach that may provide the required safety with respect to *C. botulinum* in MAP fish is the use of a

pretreatment step in combination with MAP. Potassium sorbate, sodium chloride and irradiation have all been shown to be effective, but the treatment with potassium sorbate dips is not legal in the majority of countries using MAP (Stammen *et al*., 1990).

Concerns have also been expressed about the ability of other psychrotrophic pathogens (e.g. *Aeromonas, Listeria* and *Yersinia* spp.) to grow in MAP products (Anderson, 1990). However, studies with MAP fish have demonstrated that the growth/survival of any of the pathogens examined (*L. monocytogenes, Aeromonas* spp. and *Salmonella* spp.) was in no instance greater under MAP than in the aerobically stored control, and frequently the growth was reduced under MAP (Anderson, 1990).

The majority of the studies reported in the literature indicate that the risks from foodborne pathogens in MAP are no greater, and frequently less, than those from aerobically stored foods (Church, 1993). As is the case for almost all aspects of food production, the hazard analysis and critical control points (HACCP) system is likely to play a major role in ensuring the safety of MAP foods (Church, 1994).

1.1.4.6 Biopreservation related to control of foodborne pathogens

Lactic acid bacteria (LAB) have been used for thousand of years to extend the shelf life of foods (Budde *et al*., 2003). The purposes of using LAB include: (1) to improve safety (inactivation of pathogens); (2) to improve stability (extension of shelf life by inhibiting undesirable changes brought about by spoilage microorganisms or abiotic reactions); (3) to provide diversity (modification of the raw material to obtain new sensory properties); and (4) to provide health benefits (through positive effects on the intestinal flora) (Lucke, 2000). The main factor that LAB possess for successful preservation of food products is its atagonistic activity against other microorganisms by production of a low pH (<4.5 to prevent growth of unwanted bacteria) and a substantial amount of non-dissociated organic acid molecules, production of reuterin, production of hydrogen peroxide, production of antibiotics and bacteriocins, competition with other bacteria for nutrients, and the ability to reduce the redox potential (Urlings *et al*., 1993). The term “Biopreservation” has been introduced to refer to the use of natural or controlled microflora and/or their antibacterial products to extend the storage life and enhance the

safety of foods (Stiles, 1996). In this section, the use of LAB and its product - bacteriocin as well as other nonbacteriocin antimicrobial substances - will be discussed.

1. Use of LAB:

During the last decade numerous papers have been published on the use of biopreservation, including examination of the production and characterisation of bacteriocins from different LAB, which have shown that the bacteriocin-producing LAB reduce the number, or inhibit the growth, of *L. monocytogenes* in meat and meat products (Holzapfel *et al*., 1995; Schillinger *et al*., 1996; Stiles, 1996; Hugas, 1998; Ennahar *et al*., 1999; Lucke, 2000; Budde *et al*., 2003). An antilisterial effect, either by bacteriocin-producing LAB or partially purified bacteriocins, has been shown to be achieved to some extent in raw meat (Nielsen *et al*., 1990; Schillinger *et al*., 1991; Hugas *et al*., 1998), fermented sausages (Foegeding *et al*., 1992; Luchansky *et al*., 1992; Schobitz *et al*., 1999), cooked sliced meat products (Hugas *et al*., 1998; Krockel, 1998; Amezquita & Brashears, 2002; Budde *et al*., 2003) and cold-smoked salmon and other fish products (Nilsson *et al*., 1999; Nilsson *et al*., 2004; Alves *et al*., 2005). Non-bacteriocin-producing LAB strains have also been shown to possess antilisterial effects and the ability to extend the shelf life of food products (Andersen, 1995; Kotzekidou & Bloukas, 1996; Bredholt *et al*., 1999; Nilsson *et al*., 2005). Metaxopoulos *et al*. (2002) investigated the antagonistic activity of two LAB strains, *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442, against the spoilage microflora of cooked cured meat products in vacuum or modified atmosphere packaging at 4^{o}C. They demonstrated that the LAB strains had an inhibitory effect on the growth of the spoilage microflora while having no negative effect on the organoleptic properties of the products.

Preservation of fish products from bacterial spoilage, and especially from *L. monocytogenes*, by bacteriocin-producing LAB has also been investigated. Duffes *et al*. (1999b) investigated 23 isolates selected among 160 for their antagonistic activity against *Listeria*. Among these 23 isolates, 22 belonged to the genus *Carnobacterium* and one to *Lactobacillus*. The inhibitory action of *Lactobacillus* was due to acidification and that of *Carnobacterium* due to a bacteriocinic effect. Bacteriocin producer *C. divergens* V41 was the most effective strain in inhibiting *L. monocytogenes*, reducing numbers of *L. monocytogenes* from 2 x 10^3 to less than 10 CFU/mL after 11 days of

culture in a simulated cold fish system at 4°C. Schobitz *et al*. (2003) demonstrated that the antagonism of a bacteriocin-like inhibitory substance (BLIS) produced by *C. piscicola* L103 was effective against all tested *L. monocytogenes* strains isolated from salmon and human samples. Nilsson *et al.* (1999) evaluated *L. sake* strain LKE5 and four strains of *C. piscicola* as biopreservation cultures to control the growth of *L. monocytogenes* on vacuum-packed, cold-smoked salmon stored at 5°C, and found that all five strains were antilisterial as live cultures in an agar diffusion assay. A bacteriocin-producing strain of *C. piscicola* (A9b) initially caused a 7 day lag phase of *L. monocytogenes*, followed by a reduction in numbers from 10^3 CFU/mL to below 10 CFU/mL after 32 days of incubation. The presence of a non-bacteriocin-producing strain of *C. piscicola* A10a also prevented the growth of *L. monocytogenes* during the 32 day incubation. The growth of *L. monocytogenes* was strongly repressed on cold-smoked salmon in the presence of both *C. piscicola* A9b and A10a. The results suggest that specific LAB strains can play an important role in the preservation and safeguarding of fish and fish products and that LAB provide an option for biopreservation of fish products.

2. Use of bacteriocins:

Consumption of food that has been formulated with chemical preservatives has increased consumer concern and created a demand for more "natural" and "minimally processed" food. As a result, there has been a great interest in naturally produced antimicrobial agents. The application of bacteriocins from LAB that target food pathogens without toxicity or other adverse effects has, thus, received much attention.

Bacteriocins are a heterogeneous group of antibacterial proteins that vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties (Abee *et al*., 1995). Bacteriocins have been divided into four classes, I to IV (Klaenhammer, 1993; Nes *et al*., 1996). Class I, termed lantibiotics, typically have 19 to more than 50 amino acids characterised by their unusual amino acids such as lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine. Class II contains small heat-stable, non-modified peptides, and can be further subdivided into the categories IIa, IIb, IIc, and IId (Nes *et al*., 1996; Moll *et al*., 1999). According to conventional classification, Class IIa includes pediocin-like *Listeria* active peptides with

a conserved N-terminal sequence Tyr-Gly-Asn-Gly-Val and two cysteines forming a S-S bridge in the N-terminal half of the peptide. The large and heat labile bacteriocins make up the Class III bacteriocins for which there is much less information available. A fourth class consisting of bacteriocins that form large complexes with other macromolecules has been proposed (Klaenhammer, 1993). Most bacteriocins derived from LAB cause cellular death through a cell membrane pore formation mechanism, which allows the leakage of cellular components and, consequently, a loss of membrane potential (Dykes, 1995; Montville & Winkowski, 1997) which leads to a bacteriocidal effect. The LAB bacteriocins and the producing strains isolated from humans and foods are expected to be promising food preservatives for preventing the growth of harmful bacteria in food.

Nisin attracts most of the interest in research and is currently the only bacteriocin approved for use in the United States (Wessels *et al*., 1998), and is also approved for use in various foods throughout the world, including New Zealnd.

Nisin has been demonstrated to be effective in a range of food products which include processed cheese and cheese spreads, milk products, canned foods, fish and meat products, brewing, wine manufacture, liquid egg and confectionery (Ross *et al*., 2002). Nisin or its combination with lower levels of nitrate can prevent the growth of *Clostridium* and some other Gram-positive pathogens such as *Listeria* and *Staphylococcus* in frankfurters, pork slurries and raw meat (Rayman *et al*., 1981; Rayman *et al.*, 1983; Chung *et al.*, 1989b). However, some researchers concluded that nisin is not effective in meat applications due to the high pH of meat (Rayman *et al*., 1983), the inability to uniformly distribute nisin, and interference by meat components such as phospholipids (de Vuyst & Vandamme, 1994).

Other bacteriocins, such as Leucocin A and B, enterocins, sakacins and the carnobacteriocins A and B have been examined to prolong the shelf life of fresh meat. The most promising results in meats were obtained using pediocin PA-1 (which has an amino acid sequence identical to pediocin AcH) (Nielsen *et al*., 1990). Pediocin PA-1 was proven active against *L. monocytogenes* in both meat and raw chicken (Nielsen *et al*., 1990; Goff *et al*., 1996). Reutericin 6, a bacteriocin produced by a specific strain of *Lactobacillus reuteri* LA6, has been demonstrated to have lytic activity against

Lactobacillus delbrueckii subsp. *bulgaricus* JCM 1002^T and NIAI B6 (Kabuchi *et al*., 1997). However, the molecular weight, primary amino acid sequence, and cyclic structure of reutericin 6 were found to be identical to those of gassericin A, a bacteriocin produced by *Lactobacillus gasseri* LA39 which has a broad spectrum and high activity and is active against *L. monocytogenes*, *B. cereus* and *S. aureus* (Toba *et al*., 1991; Itoh *et al*., 1995).

Bacteriocins have also been assessed as preservatives in fish products. Nykanen *et al*. (2000) demonstrated that nisin and lactate both inhibited the growth of *L. monocytogenes* in smoked fish under vacuum packaging at chilled temperature, but the combination of the two compounds was even more effective. Other experiments showed that crude bacteriocins produced by *Carnobacterium* strains could prevent *L. monocytogenes* growth on sterile and commercial vacuum-packed cold-smoked salmon stored at 4^oC and 8^oC (Duffes *et al*., 1999a). Paludan-Muller *et al*. (1998) demonstrated that the addition of nisin with a CO_2 atmosphere increased the shelf life to five or six weeks, whereas nisin had no effect on the extension of shelf life in vacuum packages. These data suggest that bacteriocins can be a potential source of biological preservatives in fish and fish products combined with appropriate packaging systems. However, there is increasing concern about the use of bacteriocins because of the ubiquitous existence of proteases in the food products and the development of resistance to bacteriocin by foodborne pathogens (Nilsson *et al*., 2005).

3. Use of nonbacteriocin antimicrobial substances

Many anaerobic and facultative anaerobic microorganisms produce weak organic acids such as lactic, succinic, acetic, citric, butyric, or propionic acids (Okino *et al*., 2005). Typically, LAB produces fairly large amounts of lactic, propionic, or acetic acid, as well as other metabolites such as hydrogen peroxide, reuterin, and reutericyclin during fermentation under anaerobic conditions (Slininger *et al*., 1983; Talarico *et al*., 1988; Ray & Sandine, 1992; Ganzle *et al*., 2000; Holtzel *et al*., 2000; Tomas *et al*., 2003a, b). These compounds are nonbacteriocin-like in nature and have long been recognised as having a broad spectrum of antimicrobial activity (Ray & Sandine, 1992; Drago *et al*., 1997; Ganzle *et al*., 2000; Tomas *et al*., 2003a, b; Ganzle, 2004; Fayol-Messaoudi *et al*., 2005; Makras *et al*., 2006).

Many weak organic acids have been found to be antimicrobially effective and some of them are currently being used in food preservation. Their antimicrobial effects are considered to be combined effects produced by both the undissociated and dissociated acid molecules through the acidification of the cytoplasm, destruction of the transmembrane proton motive force, and loss of active transport of nutrients through the membrane (Ray & Sandine, 1992). However, only lactic, propionic, and acetic acid fall in the category of biopreservatives of microbial origin and have been given GRAS status for use in foods, and thus are currently being vigorously investigated for biopreservative application (Ray & Sandine, 1992).

Reuterin (β-hydroxypropionaldehyde) is a nonpeptide low-molecular-weight, heat labile compound produced by some strains of *L. reuteri* during the anaerobic metabolism of glycerol (Slininger *et al*., 1983; Talarico *et al*., 1988; Vollenweider & Lacroix, 2004). It has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria (Talarico *et al*., 1988; El-Ziney & Debevere, 1998; Vollenweider & Lacroix, 2004). It has been postulated that reuterin may be able to compete with ribonucleotides for binding to the ribose recognition site of ribonucleotide reductase, the first enzyme involved in DNA synthesis, and thus inhibit the conversion of ribonucleotides into deoxyribonucleotides and hence exert its antimicrobial effect (Vollenweider & Lacroix, 2004). Due to its broad antimicrobial activity, it is attractive for use as a food preservation agent.

Reutericyclin is a tetramic acid produced by sourdough isolates of *L. reuteri* (Holtzel *et al*., 2000; Ganzle, 2004). It is structurally but not functionally related to naturally occurring tetramic acids with bacteriostatic or bactericidal effects to Gram-positive bacteria. However, reutericyclin does not affect the growth of Gram-negative bacteria because of the barrier properties of their outer membrane (Ganzle *et al*., 2000; Ganzle, 2004). The antimicrobial effect of reutericyclin is mainly due to its activity as a proton ionophore that selectively dissipates the transmembrane proton potential (Ganzle & Vogel, 2003). Based on its antimicrobial property, it and its producing strains may have potential applications in the biopreservation of foods (Ganzle, 2004).

1.1.5 Stress responses and adaptation of foodborne pathogens

Bacterial species are considered the most versatile living organisms, inhabiting almost every environmental niche known to, and including, man (Sleator & Hill, 2002). The successful inhabitation of the often hostile environments can be attributed in part to the development of complex stress management strategies, which allow the bacterial cells to respond and adapt to changes in their external environment. Foodborne pathogens are repeatedly exposed to multiple, sequential or concurrent stressors. However, these pathogens have the potential to adapt to a wide variety of food preservation related stress conditions, e.g. starvation, temperature extremes and (weak) acids, high osmolarity and high hydrostatic pressure (Foster, 1999). The adaptive responses of pathogens to environmental stresses give them an increased ability to survive in the gastrointestinal (GI) tract and during food processing, and to modulate their viability and virulence. Therefore, understanding the responses of the pathogens to stressors is of significant importance in the practice of food preservation and food safety. The most important adaptive responses of microorganisms include acid tolerance, thermotolerance and osmotolerance.

1.1.5.1 Acid tolerance response (ATR)

Exposures to acid stress occurs in a variety of ecological niches occupied by food pathogens. Acid stress can be described as the combined biological effect of low pH and weak (organic) acids, such as acetate, propionate and lactate present in the environment (food) as a result of fermentation, or alternatively, when added as preservatives (Bearson *et al*., 1997). Acid resistance (AR), acid tolerance (AT) and acid habituation (AH) have been used to describe acid stress response systems. The exposure to mild or moderate acid stress results in the synthesis of proteins that protect the bacterium from a more severe acid challenge and can potentially allow the organism to survive a subsequent lethal stress of a different type (cross protection) (Leyer & Johnson, 1993; Abee & Wouters, 1999). This phenomenon is termed the acid tolerance response (ATR) (Goodson & Rowbury, 1989). ATR exists in both Gram-positive and Gram-negative bacteria (Rowbury, 1995). Evidence supports the fact that this stress response is an important component of survival of bacterial pathogens within the host niche and the encountered stressful environmental conditions, in which it has been shown that

exposure to sublethal pH induces the expression of numerous acid shock proteins (ASPs) that promote bacterial survival in subsequent extreme acid environments (Rowbury, 1995).

Acid tolerance response has been well characterised in *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (Hickey & Hirshfield, 1990; Foster, 1991; Lin *et al*., 1995). Protection against extreme acid pH requires prior exposure to a moderately acid pH (adaptive pH). In *S.* Typhimurium, both log phase and stationary phase acid tolerance response systems exist, which protect cells at pH 3 for several hours (Bearson *et al*., 1997). This inducible ATR is a two-stage process involving overlapping acid protection systems triggered at different levels of acidity. Exponential phase cells adapted to pH 5.8 produce a pH homeostasis system (the first stage) that maintains the internal pH at levels compatible with life once cells encounter lethal acid stress (pH 3) (Bearson *et al*., 1997). Lowering the adaptive pH further to pH 4.5 results in the induction of approximately 50 acid shock proteins (ASP) (the second stage) that are believed to prevent or repair macromolecular damage that is induced during this stage (Bearson *et al*., 1997).

Both pathogenic and nonpathogenic *E. coli* have the ability to survive acid stress, though it has been shown that many strains of pathogenic *E. coli* are substantially more resistant to acid and other stresses (Benjamin & Datta, 1995). *E. coli* possesses log phase and stationary phase ATR mechanisms (Bearson *et al*., 1997), but they are different to those of *Salmonella*. In complex medium, *E. coli* cells in stationary phase are more acid resistant than *Salmonella*. The survival of *Salmonella* is very poor below pH 3, while *E. coli* can survive for several hours at pH 2 (Foster, 1999).

Induction of ATR has also been found in other pathogens, including *A. hydrophila* (Karem *et al*., 1994), *L. monocytogenes* (O'Driscoll *et al*., 1996), *V. parahaemolyticus* (Wong *et al*., 1998), *V. cholerae* (Merrell & Camilli, 1999), *B. cereus* (Jobin *et al*., 2002) and *C. jejuni* (Murphy *et al*., 2003).

Most of the ASPs are not synthesized at pH 5.8; rather this pH induces an elevated pH homeostasis system functional at external pH values below 4.0, and that allows second

stage ASP synthesis at external pH 3.3 (Foster, 1993; Foster, 1995). Some of the regulatory proteins which facilitate expression of the ASPs have been identified, e.g. σ^S (RpoS), Fur (Foster, 1995), Ada, PhoPQ (Bearson *et al*., 1998) and OmpR (Bang *et al*., 2000) in *Salmonella*; σ^B in *L. monocytogenes* (Wiedmann *et al*., 1998) and σ^H in *B. subtilis* (Liu *et al*., 1999). Acid tolerance is correlated with the virulence of *S.* Typhimurium (Bearson *et al*., 1998) and *L. monocytogenes* (Gahan & Hill, 1999).

Bearson *et al*. (1997) have summarised the various acid tolerance systems and regulatory circuits in enteric microorganisms as illustrated in Figure 1.1. The figure shows a composite cell containing all the known components of inducible acid stress response mechanisms. Low external pH results in leakage of protons and therefore lowering of the internal pH, which will induce several amino acid decarboxylases, if they are present, in a given bacterial species. These systems act as inducible pH homeostasis systems to elevate the internal pH by consuming a proton during decarboxylation and then exchange the decarboxylation end product for new substrate via a membrane-bound antiporter. Also, low pH will increase the accumulation of at least two important regulators; σ^S (RpoS), through the modulation of σ^S proteolysis by MviA; and PhoP via an unknown mechanism. These two regulators control distinct sets of ASPs defining partially redundant systems of acid tolerance. The Fur protein as an independent sensor of acidic pH controls a third set of ASPs. The function of the ASPs is presumed to include the prevention and/or repair of acid-induced damage to macromolecules.

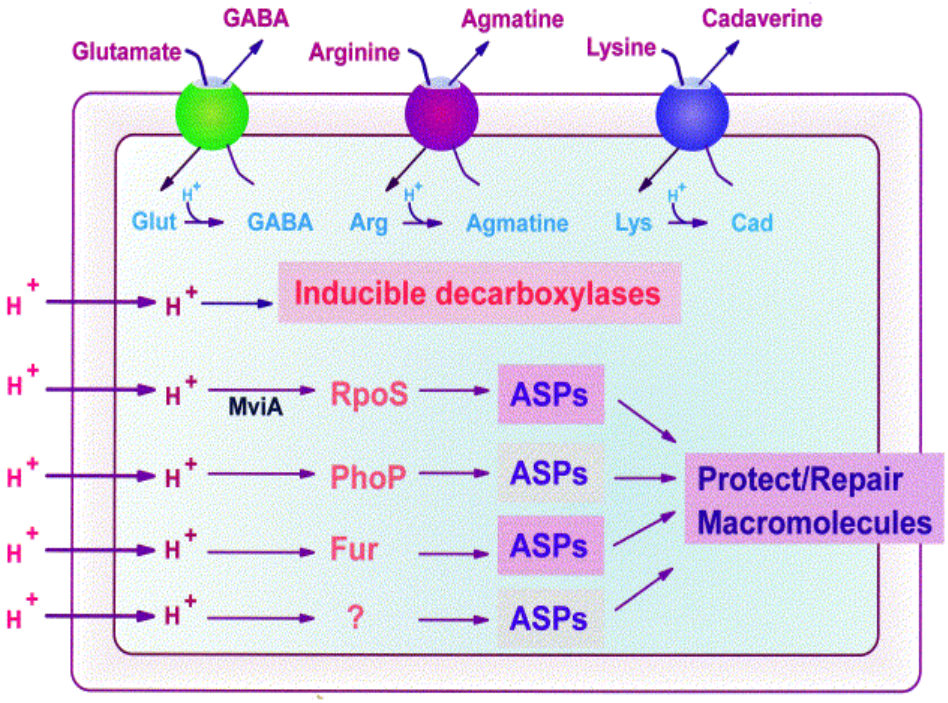

Figure 1.1 Diagram of acid tolerance responses in enteric microorganisms. This figure shows all the known components of inducible acid tolerance and acid resistance in a composite cell. (Adapted from Bearson *et al.*, 1997).

1.1.5.2 Thermotolerance

Bacteria have evolved adaptive networks to face the challenges of a changing environment and to survive under conditions of stress (Abee & Wouters, 1999). An initial non-lethal heat dose can induce transient resistance to subsequent heat treatment, a phenomenon termed thermotolerance (Periago *et al.*, 2002). It is supposed that any temperature above the optimum growth temperature has some lethal effect. However, it has been shown that in most microbial species slow heating or heating for short periods of time at temperatures above the optimum temperature for growth induces higher thermotolerances (Pagan *et al.*, 1997). Such heat-induced thermotolerance has been found in several food pathogens such as *L. monocytogenes* (Pagan *et al.*, 1997), *E. coli* O157:H7 (Byrne *et al.*, 2002), *V. parahaemolyticus* (Wong *et al.*, 2002), *B. cereus* (Mahakarnchanakul & Beuchat, 1999).

Mahakarnchanakul & Beuchat (1999) reported that an increased thermotolerance at 50°C is observed in both psychrotrophic and mesophilic *B. cereus* variants, after

incubation under mild heat conditions (37 or 40°C) for several hours. Periago *et al.* (2002) demonstrated that a pre-exposure of *B. cereus* ATCC 14579 to 42°C could induce thermotolerance to the lethal temperature of 50°C. In *L. monocytogenes*, the temperature at which it is grown has influence on its response to heat shock and can therefore affect its thermotolerance (Pagan *et al.* 1997). Pagan *et al.* (1997) reported that *L. monocytogenes* grown at 37°C responds to heat shock at 42.5^{o}C with a fourfold increase in thermotolerance, whereas it has a sevenfold increase in thermotolerance in response to heat shock at 42.5^{o}C when grown at 4^{o}C.

In a wide variety of bacteria, thermotolerance includes increased synthesis of a set of conserved heat shock proteins (HSPs) (Hecker *et al*., 1996; Yura *et al*., 2000). The molecular genetics of the heat shock response has been most extensively studied in *E. coli* and *B. subtilis*. Using two-dimensional gel electrophoresis, Periago *et al.* (2002) observed 31 heat-induced proteins in *B. cereus* ATCC 14579 during pre-exposure to 42°C. Wong *et al*. (2002) found changes in protein profiles and toxin production in *V. parahaemolyticus* under sublethal heat shock. Logarithmically grown *V. parahaemolyticus* cells heat-shocked at 42^{o}C for 30 minutes were more resistant to thermal inactivation at 47^{o}C than were unshocked cells, and 24 species of proteins were induced, while 13 species were inhibited, as indicated by polyacrylamide gel electrophoresis. Classical HSPs are the molecular chaperones (e.g., DnaK, GroEL, and their cohorts) or ATP-dependent proteases (e.g., ClpP). These proteins play roles in protein folding, assembly, and repair and prevention of aggregation under stress and nonstress conditions. The chaperones and proteases act together to maintain quality control of cellular proteins (Gottesman *et al*., 1997).

It has been observed that stress exposures other than heat, such as exposure to ethanol, acid, or oxidative stress or during macrophage survival, and starvation might result in increased thermotolerance (Bernhardt *et al*., 1997; Abee & Wouters, 1999; Arsene *et al*., 2000; Yura *et al*., 2000; Zhang & Griffiths, 2003).

The increased use of mild heat preservation treatments and hurdle technology makes food products more susceptible to existing bacterial contamination than heavily processed foods. Within the concept of hurdle technology, it is important to keep in mind that microorganisms can be more resistant to adverse conditions after a previous

stress exposure and thus survive normally lethal conditions that occur during food processing. Understanding these heat-adaptive responses of food borne pathogens might be instrumental in optimizing processing conditions and applying hurdle preservation approaches to guarantee the microbial safety of food products.

1.1.5.3 Osmoadaption and osmotolerance

Food pathogens often suffer from changes in their external environment. One such environmental parameter is the osmolarity of the extracellular medium. Bacterial cells are, in principle, required to maintain an intracellular osmotic pressure greater than that of the growth medium in order to generate cell turgor, generally considered to be the driving force for cell extension, growth and division (Csonka, 1989; Taiz, 1984). The ability to adapt to changes in the osmolarity of the external environment is therefore of fundamental importance for growth and survival. In this section, only the aspects of osmoadaption and osmotolerance of food pathogens in an elevated osmolarity environment will be reviewed.

Microorganisms have evolved a number of osmoadaptive strategies to cope with fluctuations in this environmental parameter. The term osmoadaptation describes both the physiological and genetic manifestations of adaptation to low and high osmolarity water environments (Galinski, 1995). In principle, two strategies of osmoadaptation have evolved to cope with elevated osmolarity: (i) the salt in cytoplasm type and (ii) the organic osmolyte type (Galinski & Troper, 1994). The first strategy was discovered in, and is typical of, members of the *Halobacteriaceae* (Galinski & Troper, 1994; Martin *et al*., 1999), which achieve osmotic equilibrium by maintaining a cytoplasmic salt concentration (KCl) similar to that of the bathing solution as a consequence of the cytoplasm exposure to high ionic strength (up to 7 M KCl has been recorded in species of *Halobacterium*) (Lanyi, 1974). This requires extensive structural adaptations and amino acid substitutions, involving enrichment in aspartyl, glutamyl and weakly hydrophobic residues (Lanyi, 1974). The second strategy involves a bi-phasic response in which increased levels of K^+ (and its counter-ion glutamate) have been observed as a primary response phenomenon (Epstein, 1986), followed by a dramatic increase in the cytoplasmic concentration (either by synthesis and/or uptake) of osmoprotective

compounds, representing the secondary response. Such compounds are a restricted range of low molecular mass molecules, given their compatibility with cellular functions at high internal concentrations, and referred to as compatible solutes (Brown, 1976). These compatible solutes are often highly soluble molecules which carry no net charge at physiological pH (Galinski, 1995) and do not interact with proteins or disrupt vital cellular processes such as DNA repair, DNA-protein interactions or the cellular metabolic machinery (Strom & Kaasen, 1993; Record *et al*., 1998a, b). Such compounds, including glycine betaine, carnitine and proline, have emerged as the principal compatible solutes in bacterial osmoadaptation (Beumer *et al*., 1994). These compatible solutes serve as osmotic balancers and function as effective stabilisers of enzyme function, providing protection against salinity, high temperature, freeze-thaw treatment and even drying (Brown, 1976; Lippert & Galinski, 1992; Welsh, 2000).

Sensing and adaptation to chemical and physical fluctuations of the environment are essential processes for the survival of microorganisms. To an elevated osmolarity in the environment, the most rapid response, in both Gram-positive and Gram-negative bacteria, is a stimulation of potassium (K^+) uptake (Epstein, 1986; Whatmore *et al*., 1990). This is the primary response representing the initial phase of osmoadaptation. The function of K^+ accumulation may be to signal induction of the secondary response, especially in Gram-positive bacteria (Booth & Higgins, 1990). A cut-off point for the primary response, at least in Gram-negative bacteria, appears set at around 0.5 M NaCl (Galinski, 1995). Increase in the salt concentration above this level triggers the secondary response; i.e. accumulation of neutral osmoprotectants which, in contrast to the ionic osmolytes of the primary response, can be accumulated to high intracellular concentrations without adversely affecting cellular processes (Brown, 1976; Yancey *et al*., 1982). The accumulation of these osmoprotectants includes a process for the synthesis, conversion and transport of these compounds, e.g. *E. coli* can convert choline to betaine in a two-step enzymatic reaction (Landfald & Strom, 1986). In addition to endogenous synthesis, bacteria have evolved sophisticated mechanisms for the uptake and accumulation of osmolytes released into the external environment either by primary microbial producers upon dilution stress, by decaying plant and animals, or by mammals in the form of excretion fluids (e.g. urine) (Galinski & Troper, 1994; Ventosa *et al*., 1998).

The response of microorganisms to osmotic shock also involves non-compatible solute accumulation. Two structurally related Gram-negative outer membrane channel proteins, OmpC and OmpF, facilitate the non-specific diffusion of small (≤ 500 Dalton) hydrophilic molecules across the outermost permeability barrier of the cell (Nikaido & Vaara, 1987), responding in a reciprocal fashion to the external osmolarity (expression of ompF being depressed while that of ompC is enhanced at elevated osmolarity) (Csonka, 1989). This response also relates to adaptive membrane adjustment by increasing the proportion of anionic to zwitterionic phospholipids (Sutton *et al*., 1991), the accumulation of membrane-derived oligosaccharides (MDOs) (Kennedy, 1982; Miller *et al*., 1986), and catabolism of disaccharides including sucrose, maltose, cellobiose, gentibiose, turanose and palatinose. The latter are a new class of osmoprotectants, produced during early exponential growth, and which contribute indirectly to enhance the levels of two endogenously synthesised osmolytes, glutamate and *N*-acetylglutaminylglutamine amide (Gouffi *et al*., 1999; Gouffi & Blanco, 2000). These responses facilitate an increased ability of stressed microorganisms to grow at elevated osmolarities.

1.2 Objectives of this study

Currently, about 98% of New Zealand export fish fillets are shipped as frozen product despite the fact that fresh fillets earn more than 1.5 times the income generated from frozen fillets. This means that the New Zealand seafood industry could earn more from technologies that will extend the shelf lives of fresh seafood products.

Conditions have been established for extending the shelf life of salmon using modified atmosphere packaging (MAP) (Fletcher *et al*., 2002; Fletcher *et al*., 2003a, b; Fletcher *et al*., 2004; Wierda *et al*., 2006). However, no particular consideration was made of the effect of this technology on foodborne pathogens.

Other work has shown that salmon can occasionally carry *L. monocytogenes*, a foodborne pathogen of considerable concern. This organism is a facultative anaerobe, and it has been hypothesised that it may not grow well or survive under the strict anaerobic conditions of a MAP system. However, there is a need to test this hypothesis

and evaluate the efficacy of this packaging system in controlling the common foodborne pathogens.

LAB and their antimicrobial metabolic products (such as bacteriocin and reuterin) have been proven to be effective against food spoilage microorganisms and foodborne pathogens. The use of LAB and their antimicrobial metabolic products as biopreservatives for control of food spoilage microorganisms and foodborne pathogens has attracted much attention in recent years, although the use of any given LAB and antimicrobial metabolic products for a particular purpose needs specific studies. The seafood industry is continuously looking for new approaches which could lead to a safer seafood product with extended shelf life. The use of LAB for these purposes would provide an alternative choice for seafood preservation. Therefore, the selection and evaluation of specific LAB strains will provide information on whether these strains are effective for use as biopreservatives.

Bacterial species are considered to be the most versatile of all living organisms and able to adapt to a wide variety of food preservation-related stress conditions, e.g. starvation, temperature extremes and (weak) acids, high osmolarity and high hydrostatic pressure. These adaptive responses of pathogens to environmental stresses give them an increased ability to survive in the gastrointestinal tract and during food processing. Therefore, understanding the responses of a pathogen to such stressors would have significant importance in the practice of food preservation and food safety.

It has been shown that the underlying mechanism of a stress response includes increased synthesis of a set of conserved specific proteins. In *S.* Typhimurium, the acid stress response induces the synthesis of 50 acid shock proteins. These proteins are thought to contribute to acid survival at a lethal acid shock. The increased synthesis of responsive stress proteins is also found in other microorganisms under different stresses, providing increased survival under stress.

Given this background, this study will focus on the following objectives:

- Optimising MAP conditions that could potentially provide effective means to control foodborne pathogens.

- Testing the concept of using LAB as food biopreservatives.

- Assessing the efficacy of MAP in combination with LAB on the growth and survival of foodborne pathogens.

- Assessing the combined effect on gene expression and heat survival of *L. monocytogenes* upon exposure to MAP and LAB culture supernatants.

Chapter 2 Effect of controlled atmosphere on growth of foodborne pathogens

2.1 Introduction

Modified atmosphere packaging (MAP), in which the ambient atmosphere around the product is replaced by one that is modified to be more inhibitory to the growth of spoilage bacteria, is one of the most widely accepted technologies for extending the shelf life of chilled food. MAP has been intensively investigated for extending the shelf life of New Zealand king salmon (*Oncorhynchus tshawytscha*) products (Fletcher *et al*., 2002; Fletcher *et al*., 2003a, b; Fletcher *et al*., 2004; Wierda *et al*., 2006). Fletcher *et al*. (2002) have found a delayed spoilage of king salmon packed in 40% CO_2:60% N_2 gas mixture compared to air or nitrogen (N_2), when stored at 0^{o}C. Further study has shown that the growth of spoilage microorganisms in fresh king salmon was progressively more rapid in 100% CO_2, 40% CO_2:60% N_2, N_2 and air, when stored in melting ice (0 ± 0.05^{o}C) (Fletcher *et al*., 2003a). Wierda *et al*. (2006) have monitored the changes of the volatile compounds of fresh king salmon stored in ambient air or in a gas mixture of 40% CO_2:60% N_2 in melting ice (0 ± 0.1^{o}C) over time. It was found that the levels of several of the volatile compounds changed during storage, with some showing a clear difference between storage in air and storage in a CO_2:N_2 mixture and some specific volatile compounds were identified as potential markers for salmon freshness and spoilage. This research has demonstrated that MAP can change and control the number of the spoilage organisms and therefore improve the shelf life of fish. However, in those studies, no particular consideration was given to the effects of this technology on foodborne pathogens.

Food such as seafoods can be a source of some pathogens, such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* spp., *Staphyloccocus aureus* and *Vibrio parahaemolyticus* when contamination with these pathogens occurs, although this is not common. People can be exposed to pathogens through the consumption of contaminated foods and suffer foodborne illness. Seafood such as molluscan shellfish

may become contaminated in the growing waters but most pathogens found on other food are due to contamination with a limited number of pathogenic organisms during the processing stages. Therefore, effective means to control the growth of pathogens during storage and processing are important to reduce the risk of foodborne illness.

In this study, a simulated controlled atmosphere (CA) system was developed to investigate the potential of a modified atmosphere (40% CO_2:60% N_2) to control some common foodborne pathogens in broth. The results obtained would provide useful information for using modified atmosphere packaging in food preservation.

2.2 Materials and Methods

2.2.1 Bacterial strains, growth media and sub-culture

Escherichia coli O157:H7 strain 2988 (Shu & Gill, 2002) and *Salmonella enterica* serovar Typhimurium ATCC1772 (Gill *et al*., 2001) were kindly provided by Bioactives Research New Zealand Ltd. (Auckland, New Zealand). *Listeria monocytogenes* Scott A ATCC49594, *Staphylococcus aureus* ATCC25932 and *Vibrio parahaemolyticus* (local isolate) were held in this laboratory. The stock cultures were stored at -80^{o}C.

The stock bacterial strains were sub-cultured aerobically in Brain Heart Infusion (BHI) broth (CM225, Oxoid, Hampshire, UK) or on BHI agar (BHI broth supplemented with 1.5% agar from Sigma, St. Louis, MO, USA), except *Vibrio parahaemolyticus* which was sub-cultured and assayed aerobically in Trypticase Soy Broth (TSB; CM129, Oxoid, Hampshire, UK) supplemented with 3% sodium chloride (TSBS) (Heinis *et al*., 1977) or TSBS agar (TSBS broth supplemented with 1.5% agar from Sigma, St. Louis, MO, USA).

For subculture, each of the test strains was streaked onto an agar plate and incubated for 24 h at 37^{o}C. A single colony of each strain was re-streaked onto a fresh agar plate and incubated for 24 h at 37^{o}C. An isolated colony was used to inoculate 10 mL of broth medium and then incubated for 16 h at 37^{o}C. The resulting cultures were used as stock for further use.

2.2.2 Enumeration of bacterial samples

The concentrations of bacterial samples were enumerated by plate count on BHI or TSBS agar plates. A drop plate method was employed for enumeration of bacteria throughout this study, following the descriptions of Herigstad *et al*. (2001) and Chen *et al*. (2003) with modifications. Briefly, 25 µL of bacterial suspension was loaded into the first well of each row in a 96-well plate (Nunc, Roskilde, Denmark) pre-loaded with 225 µL of sterile 0.1% peptone (Bacto^{TM}, Becton Dickinson, MD, USA) water per well. Serial dilutions (10 fold) were made using a multi-channel pipette (CAPP, Odense, Denmark) by transferring 25 µL from column *i* into medium in the next column {*i* + 1} after mixing 10 times. This process was repeated until the last dilution. Pipette tips were changed between dilutions. Thereafter, three replicates of 25 µL from each of the selected dilutions were plated onto an agar medium using a single-channel pipette. Plates were allowed to dry, and then placed into an incubator for 24 - 48 hours at 35^{o}C. Colonies were enumerated and colony forming units per mL (CFU/mL) were calculated as:

The number of bacteria
= number of colonies x dilution factor x 1000 x 25^{-1} (CFU/mL).

The count was recorded as the mean of three replicates.

2.2.3 Preparation of the inocula

For preparation of the inocula, each of the test pathogens was sub-cultured on BHI agar plates and BHI broth, except *V. parahaemolyticus* which was grown and enumerated on TSBS agar plates or broth. One hundred microlitres of the resulting culture was then used to inoculate 10 mL of fresh broth medium which was incubated at 37^{o}C for 16 h. After 16 h, the culture was distributed in 1 mL portions in 2 mL sterile tubes and stored at -80^{o}C. A frozen aliquot was thawed in a water bath (37^{o}C) for 10 min and then serially diluted in pre-warmed 0.1% (w/v) peptone water. The diluted bacterial suspension was enumerated by standard plate count using the drop plate method described in Section 2.2.2 on standard plate count agar (CM463, Oxoid, Hampshire, UK) or TSBS agar (TSBS, supplemented with 1.5% agar).

2.2.4 Preparation of growth media

The media used in the experiments were buffered BHI and TSBS in 0.2 M phosphate buffer (bBHI and bTSBS, respectively) at a final pH of 6.8 and 6.3, respectively, after an overnight equilibration under CA (see Section 2.2.5). The bBHI and bTSBS were made by constitution of the required amounts of BHI and TSBS media powder in 0.2 M phosphate buffer at initial pH values of 7.6 and 6.6, respectively, which were predetermined to give pH values of 6.8 and 6.3, respectively, after autoclaving and equilibration under CA.

2.2.5 CA system and growth conditions

The determination of growth curves was performed using 600 mL glass jars specially constructed for this purpose, provided with a central opening which was sealed with a silicone septum. The jars were filled with 100 mL of media and autoclaved (121^{o}C, 15 min). The jars were connected in series with sterile tubing and isolated from each other using sterile 0.2 µm filters (Millex®-FG, Millipore, Billerica, USA) (Figure 2.1).

Figure 2.10 The controlled atmosphere system for the evaluation of its effect on growth of foodborne pathogens. The compressed gases were premixed through a tubing system and introduced into individual jars, which were separated by filters (Millex®-FG). The inoculation and collections of samples were carried out aseptically by syringes through a septum on each of the jars.

The CA was composed of pure compressed nitrogen and CO_2 pre-mixed at a controlled concentration of 40% CO_2 and 60% N_2, with a residual concentration of O_2 less than 40 ppm. The pre-mixed gases were filtered through a 0.2 µm filter (Millex®-FG) and

introduced to the jars at a flow rate of 40 mL/min to produce a CA environment. The gas composition was monitored with a Novatech CO_2/O_2 analyser (Model 1673-5, Novatech Controls Pty Ltd, Cheltenham, Victoria, Australia). The jars in the system were filled with bBHI or bTSBS at initial pH values of 6.8 and 6.3, respectively, after equilibration. The controls consisted of jars with bBHI or bTSBS at pH 6.8 and 6.3, respectively, with free exchange of air without CA. The trials were carried out in triplicate for each condition at 20°C for all 5 pathogens and at 7°C for *L. monocytogenes*, the only one of the five organisms known to grow well at this temperature.

2.2.6 Inoculation

The enumerated stock cultures were thawed and diluted in 0.1% peptone water to a concentration of approximately 1 x 10^5 CFU/mL and used as inocula. Each jar was inoculated with 1 mL of inoculum to give an initial concentration of 1 x 10^3 CFU/mL. The inoculation was carried out aseptically using a spinal needle (Becton Dickinson & Co., Frankin Lakes, NJ, USA) through the silicone septum of the opening on the lid of each jar.

2.2.7 Sampling

Samples were taken aseptically using a spinal needle (Becton Dickinson & Co., Frankin Lakes, NJ, USA) through the silicone septum of the opening on the lid at intervals of 4 to 6 hours after mixing by swirling. A plate count of the sample was carried out immediately as described in Section 2.2.2.

2.2.8 Fitting of growth data to predictive model

The growth data of bacteria in broth were subjected to analysis using Gompertz Solver software (Version 1.0, Agricultural Research Service, USA). A predicted growth curve was then produced and derived growth parameters were obtained for a given data set based at a lowest sum of squares error (SSE). The model is described below:

$$Log(CFU/mL) = A + Ce^{-e[-B(t-M)]}$$

where *e* is the base of the natural logarithm (approximately = 2.71828) and *A*, *B*, *C* and *M* are the parameters that define the shape of the sigmoidal curve. *A* represents the initial (minimum) CFU/mL value for the growth curve data, *B* the slope of the

regression line at the inflection point for the curve, *C* the difference between the minimum and maximum CFU/mL values and *M* the midpoint of the regression line used for the parameter *B* estimate. The *t* represents inoculation time in hours following inoculation.

With bacterial growth data, the parameter values for *A*, *B*, *C* and *M* can be found that make the sigmoidal curve most closely fit the observed data. Therefore, the kinetic parameters, including the exponential growth rate (EGR), lag phase duration (LPD), and the maximum population density (MPD) can be calculated from the parameter estimates (*A*, *B*, *C* and *M*) as described by Buchanan & Phillips (1990):

The exponential growth rate (EGR) = BC/e

The lag phase duration (LPD) = $M-(1/M)$

Maximum population density (MPD) = $A + C$

The observed and predicted growth data were plotted using SigmaPlot software (Version 8.0, SPSS Inc., Chicago, USA).

The predicted growth kinetics parameters under CA and non-CA conditions at different pH values and temperatures were analysed by one-way analysis of variance (ANOVA) using Minitab Software (release 14, PA, USA).

2.3 Results

2.3.1 Growth curve of pathogens under CA

The effect of CA on observed and predicted growth data are shown in Figure 2.2-2.6. It was observed that at the early stage of the growth there were declines in viable counts for *V. parahaemolyticus* at pH 6.3 and 6.8 under CA and *L. monocytogenes* at 7^{o}C, pH 6.3 and 6.8 under CA and non-CA.

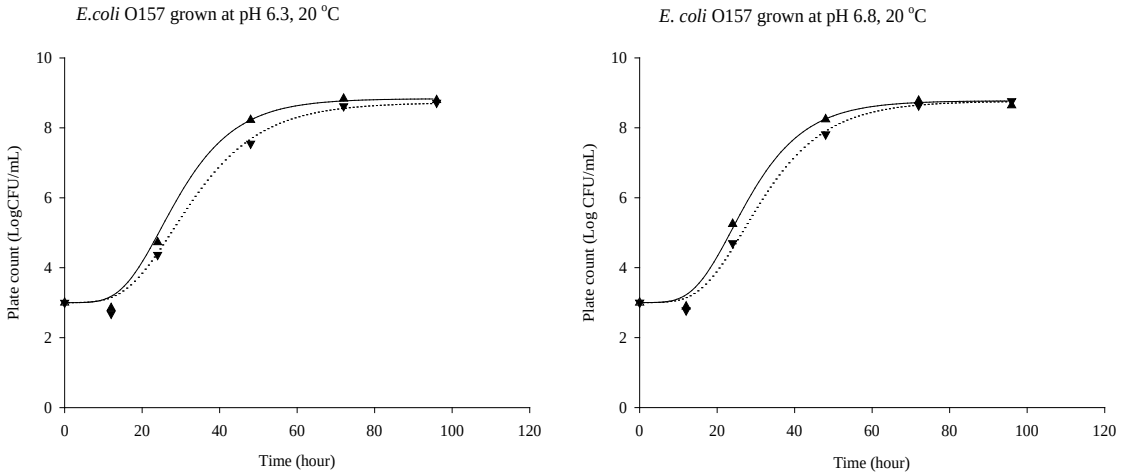

Figure 2.2 The growth curves of *E. coli* O157:H7 grown under controlled atmosphere (CA) at 20°C and different pH values. —: fitted growth curve without CA; …: fitted growth curve under CA; ▲: observed growth data (mean of three replicates) without CA; ▼: observed growth data (mean of three replicates) under CA.

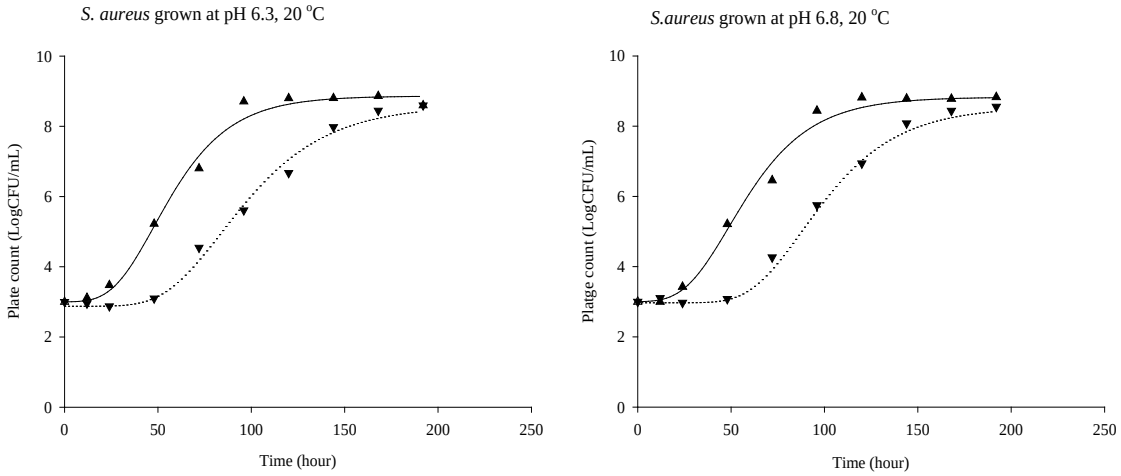

Figure 2.3 The growth curves of *S. aureus* grown under controlled atmosphere (CA) at 20°C and different pH values. —: fitted growth curve without CA; …: fitted growth curve under CA; ▲: observed growth data (mean of three replicates) without CA; ▼: observed growth data (mean of three replicates) under CA.

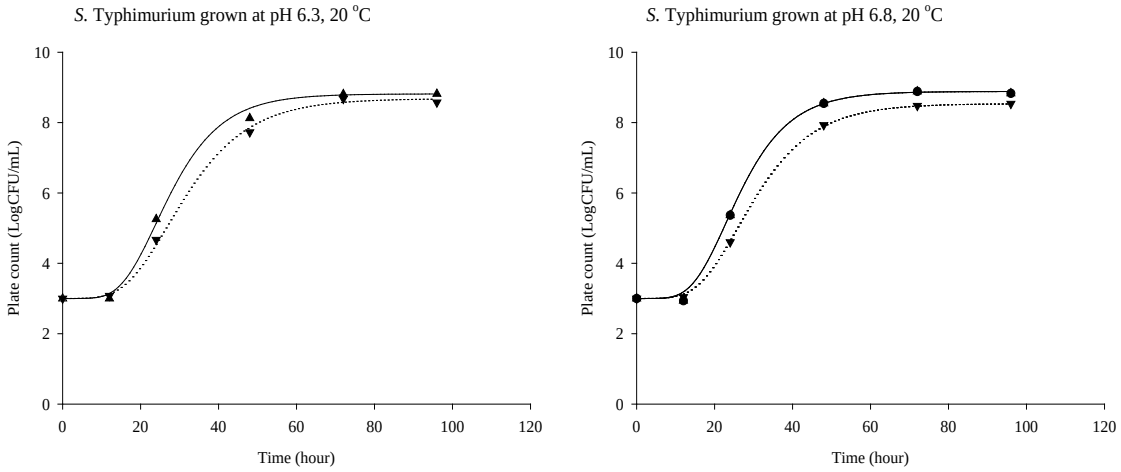

Figure 2.4 The growth curves of *S. enterica* serovar Typhimurium grown under controlled atmosphere (CA) at 20°C and different pH values. —: fitted growth curve without CA; …: fitted growth curve under CA;▲: observed growth data (mean of three replicates) without CA; ▼: observed growth data (mean of three replicates) under CA.

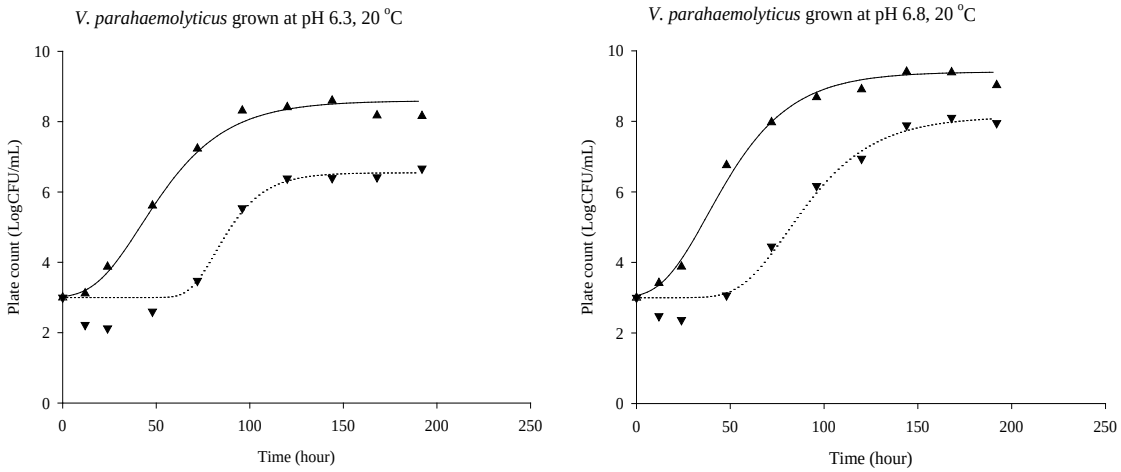

Figure 2.5 The growth curves of *V. parahaemolyticus* grown under controlled atmosphere (CA) at 20°C and different pH values. —: fitted growth curve without CA; …: fitted growth curve under CA; ▲: observed growth data (mean of three replicates) without CA; ▼: observed growth data (mean of three replicates) under CA.

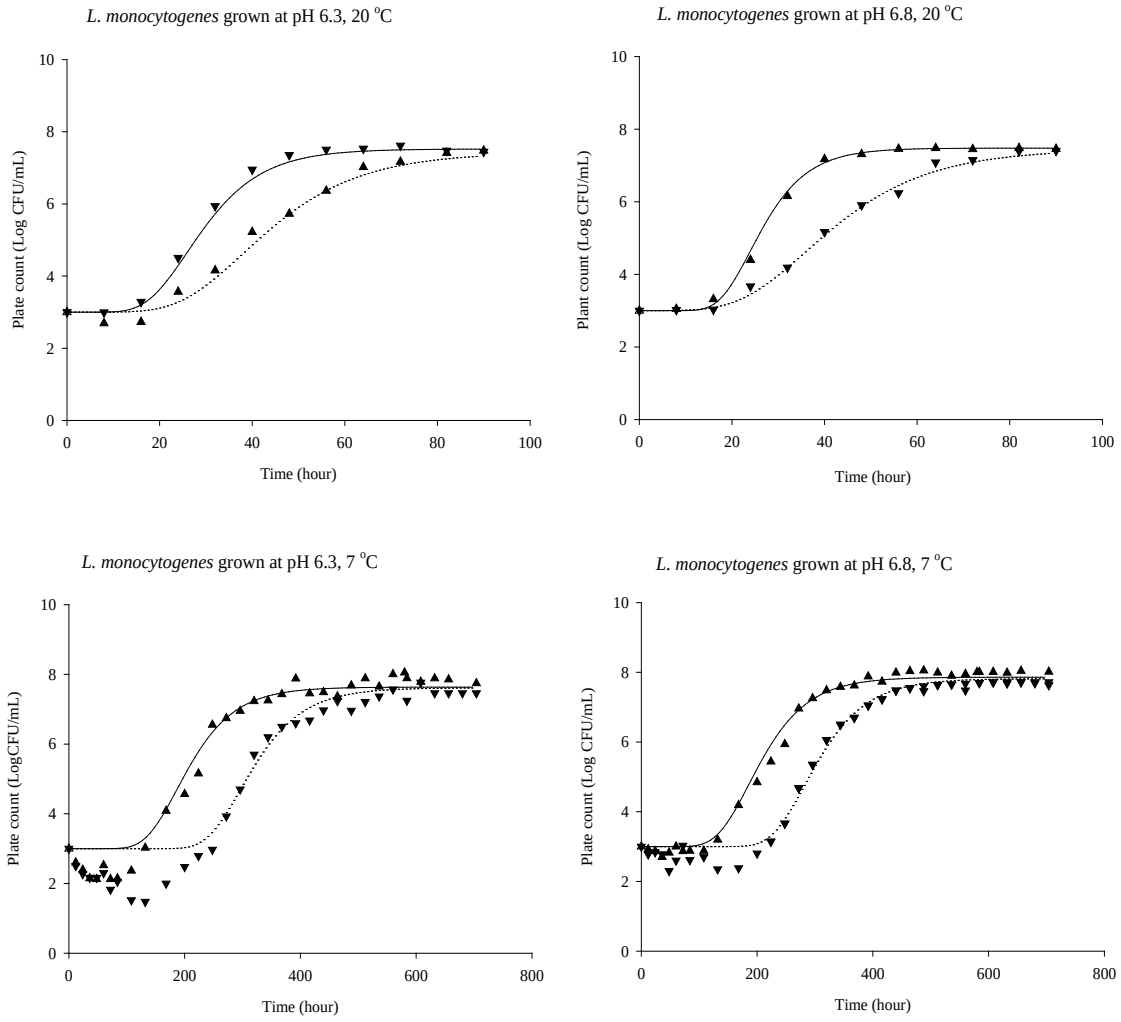

Figure 2.6 The growth curves of *L. monocytogenes* grown under controlled atmosphere (CA) at 20°C or 7°C and different pH values. —: fitted growth curve of pathogens without CA; …: fitted growth curve of pathogens under CA;▲: observed growth data (mean of three replicates) without CA;▼: observed growth data (mean of three replicates) of pathogens under CA.

2.3.2 Effect of CA on the lag phase duration

The lag phase durations (LPD) under CA and non-CA were determined for the five foodborne pathogens grown at 20°C, and for *L. monocytogenes* grown at 7°C, at pH 6.3 and 6.8, using Gompertz solver software (Figure 2.7).

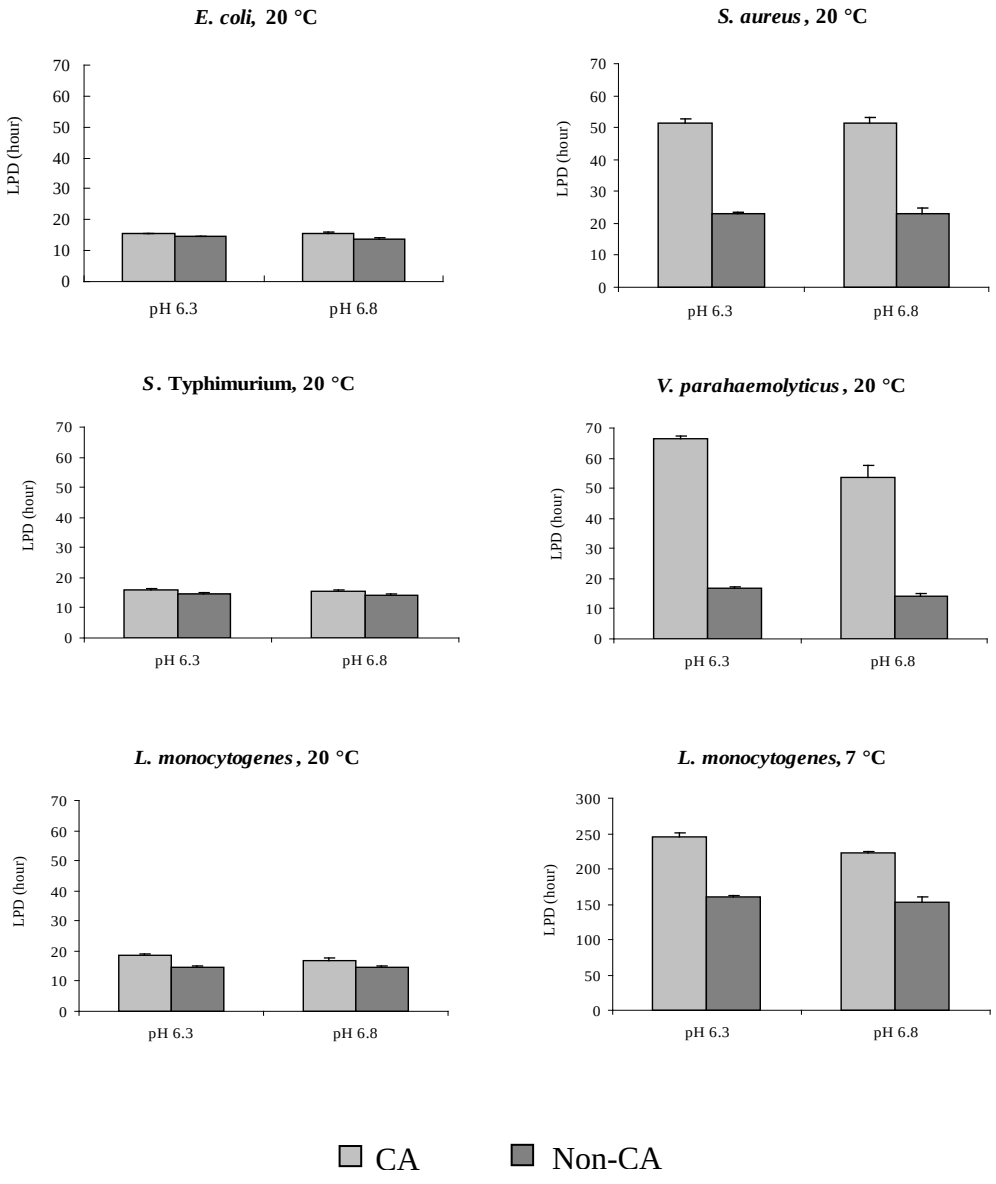

Figure 2.7 Comparison of lag phase duration (LPD) of *E. coli* O157:H7, *L. monocytogenes*, *S. aureus*, *S.* Typhimurium and *V. parahaemolyticus* (20°C), and *L. monocytogenes* (7°C), under CA and non-CA, at pH 6.3 and 6.8. Bars represent standard deviation values.

One-way ANOVA showed that the LPD was significantly longer for three of the pathogens (*S. aureus*, *V. parahaemolyticus* and *L. monocytogenes*) when grown under CA compared to that under non-CA at both pH 6.3 and 6.8 ($P<0.05$), with the exception of *E. coli* O157:H7 and *S.* Typhimurium ($P>0.05$). The effect of CA on LPD for *L. monocytogenes* at 20°C was minor compared to that at 7°C and the effect on *S. aureus* and *V. parahaemolyticus* ($P<0.005$).

The lag phase extensions under CA compared with those under non-CA calculated from the means of the triplicate LPD values are shown in Table 2.1. For *L. monocytogenes*, the extension of lag phase was more prominent at 7°C compared to that at 20°C. However, there were no significant differences in lag phase extension between other tested pathogens at different pH values.

Table 2.1 The lag phase extension (h) of pathogens grown at 20°C or 7°C under CA compared with non-CA

	pH 6.3	pH 6.8
E. coli O157*	1.04[a] (15.61[b] -14.57[c])	1.73 (15.56-13.83)
*S. aureus**	28.63 (51.49-22.86)	28.4 (51.36-22.96)
S. Typhimurium*	0.92 (15.75-14.83)	1.39 (15.49-14.10)
*V. parahaemolyticus**	49.57 (66.39-16.82)	39.56 (53.84-14.28)
*L. monocytogenes**	4.03 (18.64-14.61)	2.53 (17.05-14.52)
*L. monocytogenes***	84.63 (245.49-160.86)	70.19 (223.43-153.24)

[a] lag phase extension; *a=b-c*.

[b] lag phase duration of pathogens grown under CA;

[c] lag phase duration of pathogens grown without CA;

* Lag phase at 20°C;

** Lag phase at 7°C.

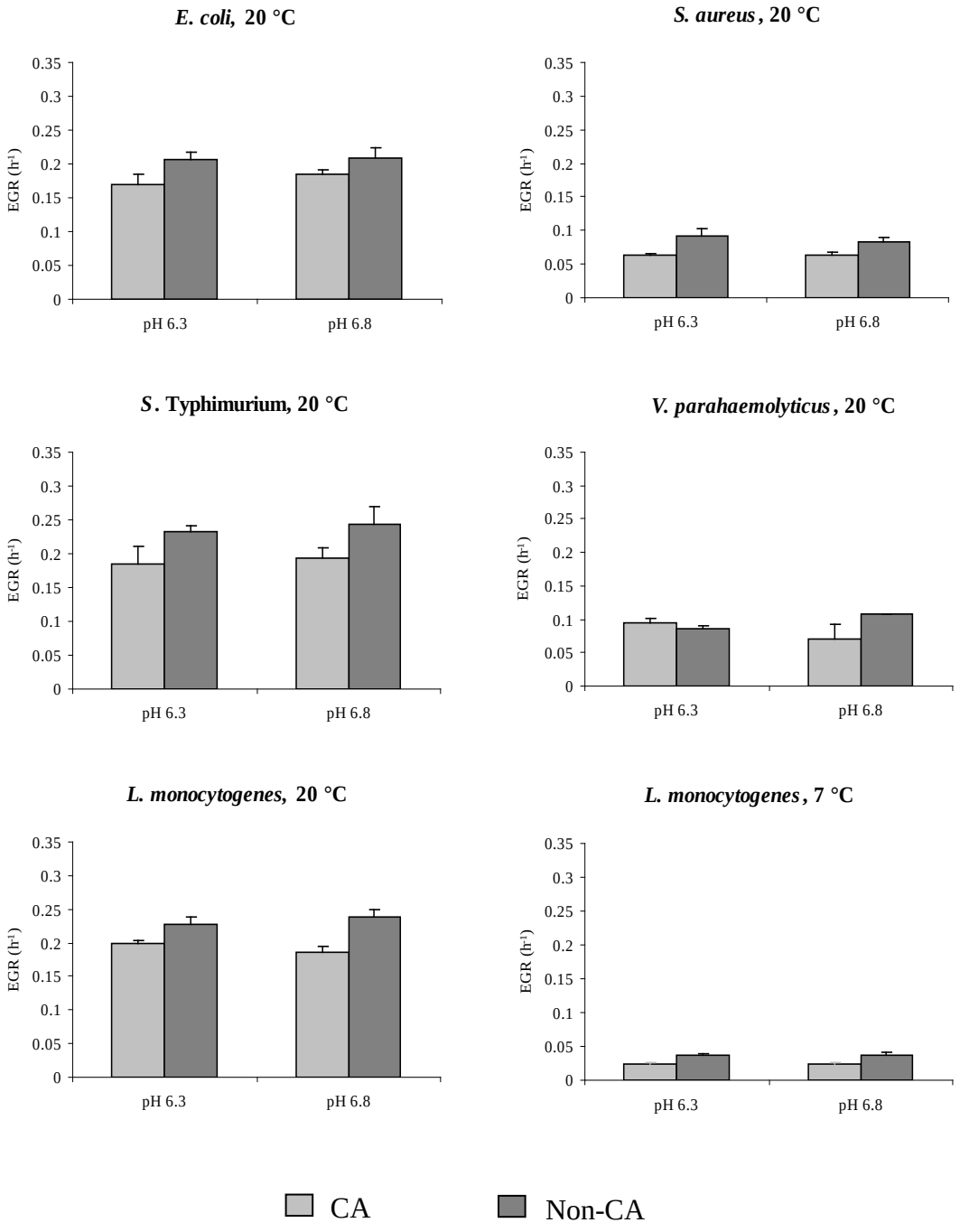

Figure 2.8 Comparison of exponential growth rate (EGR) of *E. coli* O157:H7, *L. monocytogenes*, *S. aureus*, *Salmonella enterica* serovar Typhimurium and *V. parahaemolyticus* (20°C), and *L. monocytogenes* (7°C), under CA and non-CA, at pH 6.3 and 6.8. Bars represent standard deviation values.

2.3.3 Effect of CA on the exponential growth rate

The exponential growth rates (EGR) for the tested foodborne pathogens grown under CA and non-CA at 20^{o}C, as well as for *L. monocytogenes* grown at 7^{o}C, as determined using Gompertz solver software, are shown in Figure 2.8. One-way ANOVA showed that the EGR was significantly lower under CA than that under non-CA for most of the tested foodborne pathogens at 20^{o}C, and *L. monocytogenes* at 7^{o}C, at both pH 6.3 and 6.8 ($P<0.05$). The exception was *V. parahaemolyticus* at pH 6.3, for which CA and non-CA results were not significantly different ($P>0.05$).

2.3.4 Effect of CA on the maximum population density

The maximum population densities (MPD) of the tested foodborne pathogens grown under CA and non-CA as determined by Gompertz solver software are shown in Figure 2.9. One-way ANOVA showed that the MPD was significantly lower for *S. aureus* and *V. parahaemolyticus* grown at 20^{o}C under CA at both pH 6.3 and 6.8 than when grown under non-CA ($P<0.05$). Significantly lower MPDs were also found for *S.* Typhimurium at pH 6.8, and *L. monocytogenes* at pH 6.3 and 7^{o}C, under CA, compared to that under non-CA. No significant differences ($P>0.05$) were observed for the MPDs under CA compared to those under non-CA, for *E. coli* O157:H7 and *L. monocytogenes* grown at 20^{o}C and either pH 6.3 and 6.8, or for *L. monocytogenes* at 7^{o}C, pH 6.8, and *S.* Typhimurium at 20^{o}C and pH 6.3.

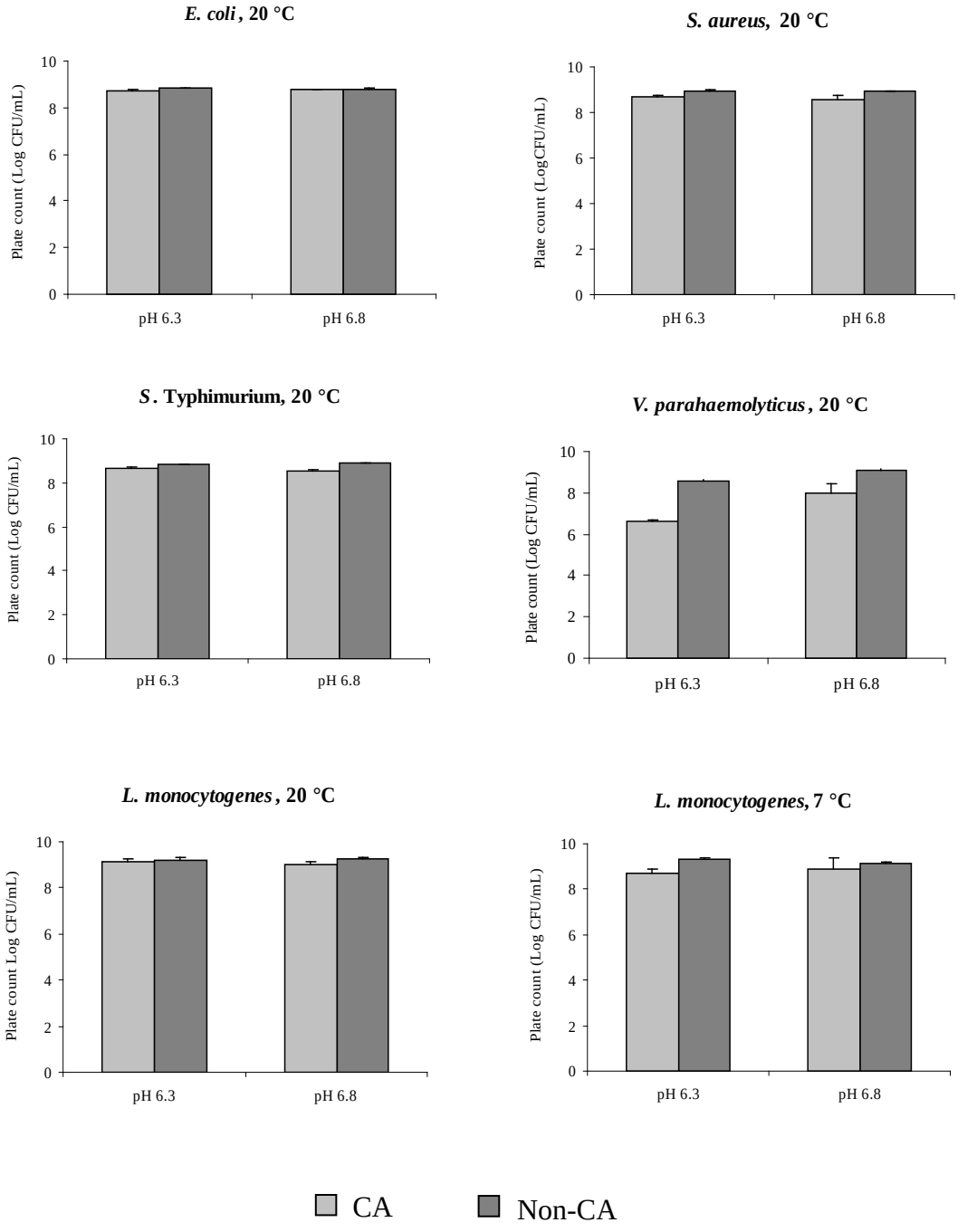

Figure 2.9 Comparison of maximum population density (MPD) of *E. coli* O157:H7, *L. monocytogenes*, *S. aureus*, *S*. Typhimurium and *V. parahaemolyticus* (20°C), and *L. monocytogenes* (7°C), under CA and non-CA. Bars represent standard error values.

2.4 Discussion

Modified atmosphere packaging is considered to be able to extend the shelf life of seafood and meat products (Papa & Passarelli, 1995; Bremer & Fletcher, 1999) and has been widely applied to seafood preservation (Dalgaard, 2000; Sivertsvik *et al*., 2002; Emborg *et al*., 2005). Shelf life extensions of 50 to 300% have been reported for seafood under MAP (Bremer & Fletcher, 1999). The growth of bacteria under modified atmosphere has been extensively investigated in broth in simulated systems using some common foodborne pathogens (Farber *et al*., 1996; Fernadez *et al*., 1997; Devlieghere *et al*., 1998). In those studies, experiments were carried out in jars containing inoculated growth medium under a modified atmosphere with a defined initial proportion of gas mixture of CO_2 and N_2, at controlled pH and temperature. The gas composition was allowed to vary during the study in response to microbial respiration. The effect of such modified atmospheres has mainly been attributed to the antimicrobial activity of dissolved carbon dioxide in the aqueous medium on the bacteria (Devlieghere *et al*., 1998).

In the present study, a similar system was implemented except that the gas mixture was constantly supplied at a concentration of 40% CO_2, supplemented with N_2. The aim was to investigate the effect of the CO_2 gas mix on the growth kinetics of common foodborne pathogens. The study has shown that the growth parameters of the tested pathogens, in terms of lag phase duration (LPD), exponential growth rate (EGR), and maximum population density (MPD) were significantly affected. The effect was more prominent at the lower temperature (7^oC cf 20°C) for *L. monocytogenes*. It was also found that there were declines in viable counts for *V. parahaemolyticus* and *L. monocytogenes* at the early stage of the growth.

The effect of modified atmosphere containing CO_2 on the growth of *L. monocytogenes* has been investigated previously (Farber *et al*., 1996; Fernnadez *et al*., 1997; Martin *et al*., 2003). These researchers generally found that the lag time increased as the CO_2 level increased, compared with that of the tested bacterium grown in air. In the present study, it was also found that the lag phase duration increased, while the exponential growth rate and maximum population density decreased under CA, compared with that

under non-CA. This result is consistent with previous studies (Farber *et al*., 1996; Fernnadez *et al*., 1997; Martin *et al*., 2003). It was found that the effect was more apparent at 7^{o}C indicating that the LPD of *L. monocytogenes* is further extended under CA with decreasing growth temperature. Similar results for the effect of modified atmosphere, incorporating CO_2, on the growth of *L. monocytogenes* have also been reported by others (Nguyen-the & Carlin, 1994; Garcia *et al*., 1995; Kakiomenou *et al*., 1998). It was also found that there was a decline in viable number of *L. monocytogenes* in the early stage of the growth at 7^{o}C, especially under CA. This is probably due to the unfavourable environmental conditions caused by CA and the low temperature to a subpopulation of the total number of bacterium at the early stage of the growth causing death (Yates & Smotzer, 2007). However, the Gompertz model used in this study will not handle this situation, thus, the fitted growth curve will deviate the actual viable counts.

In the present study, CA was found to have limited effect on the growth of *E. coli* O157:H7. The lag phase duration and maximum population density were not significantly affected under the tested CA condition. However, the exponential growth rate was significantly reduced under CA. This result differs somewhat from those reported by others. Sutherland *et al*. (1997) reported that low concentrations of CO_2 (10-20%) had little effect on the lag phase duration and growth rate of *E. coli* O157:H7 at temperatures from 10 to 30^{o}C in TSB, suggesting that this was due to the relatively high tolerance of this organism to CO_2. Similar results were reported by Molin (1983) and Hao & Brackett (1993). However, Kimura *et al*. (1999) reported that a bacteriostatic effect was observed on *E. coli* O157:H7 on Trypticase soy agar (TSA) packed in CO_2 at concentrations above 20%, at pH 8.0 and 15^{o}C. This effect was largely lost at 30^{o}C due to the decreased solubility of CO_2 at higher temperatures (Enfors & Molin, 1981).

The effect of modified atmosphere (MA) on *Salmonella* spp. has been extensively investigated by others (Eklund & Jarmund, 1983; Nychas & Tassou, 1996; Tassou *et al*., 1996). In the present study, the inhibitory effect of 40% CO_2 on the growth of *S.* Typhimurium was investigated in a controlled atmosphere (CA). It was found that the CA affected the exponential growth rate of *S.* Typhimurium grown at 20^{o}C at both pH 6.3 and 6.8, but did not affect the lag phase duration or the maximal population density

(except at pH 6.8). This result is generally consistent with the reported findings of others. Slade & Davies (1997) examined the growth and survival of *S.* Typhimurium on cod and farmed rainbow trout (*Oncorhynchus mykiss*) stored at 0, 5 and 12^{o}C under air, 60% CO_2:40% N_2, 80% CO_2:20% N_2, and 40% CO_2 :30% N_2:30% O_2. They found that the growth of the pathogen was inhibited only slightly in MA, compared with that in the aerobically stored products, and the effectiveness of MA was diminished at higher temperatures. However, the pathogen did not grow better in any MA treatment than in air, and the growth was frequently reduced in MA. Nychas & Tassou (1996) reported that *S.* Enteritidis survived but did not grow significantly in poultry or fish in vacuum-package or packs flushed with 100% N_2, 20% CO_2:80% O_2, and 100% CO_2 at 3^{o}C, but the numbers increased rapidly in samples stored at 10^{o}C. However, Nissen *et al*. (2000) reported that a high CO_2:low CO mixture (0.4% CO:60% CO_2:39.6% N_2) did not inhibit the growth of *Salmonella* spp. in ground beef at 10°C, which is contrary to many other studies (D'Aoust, 1991; Nychas & Tassou, 1996; Slade & Davies, 1997). These differing results could be due to the differences in the composition of MA, the study matrices, and the experimental temperatures. However, overall, these results have demonstrated a somewhat inhibitory effect of MA on the growth of *Salmonella* spp. under certain circumstances.

The inhibitory effect of CA on the growth of *S. aureus* was significant in this study (Figure 2.3), and this effect was more pronounced than for any of the other pathogens except *V. parahaemolyticus*, as shown by greater LPD extension and lower EGR. This result confirmed the findings of others that *S. aureus* is more effectively inhibited by a MA containing CO_2 than the enteric pathogens *E. coli* and *S.* Typhimurium (Molin, 1983; Kimura *et al*., 1997, 1999). Gray *et al*. (1984) also found that *S. aureus* was more sensitive to the inhibitory effect of high levels of CO_2 than was *S.* Enteritidis in chicken at 10^{o}C. Hintlian & Hotchkiss (1987) similarly found a stronger inhibition by 75% CO_2 of *S. aureus* than of *S.* Typhimurium in MAP-stored beef regardless of the presence of O_2 in the atmosphere at 12.8^{o}C. However, Silliker & Wolfe (1980) observed no essential difference in the inhibition of *S. aureus* and *Salmonella* by CO_2 (60% CO_2, 25% O_2, and 15% N_2) in ground beef stored at 10^{o}C or 20^{o}C. This ambiguity and inconsistent results with regard to the different inhibitory effects of MAP with CO_2 to different species of microorganisms has not been fully explained (Dixon & Kell, 1989).

V. parahaemolyticus is an important seafood-borne gastroenteritis-causing bacterium and is frequently associated with the consumption of improperly cooked seafood (Daniels *et al*., 2000; Deepanjali *et al*., 2005). The investigation was only carried out on its responses to inactivation by high hydrostatic pressure and irradiation (Styles *et al*. 1991; Berlin *et al*. 1999). However, no information is available concerning the effect of MA on the growth of *V. parahaemolyticus*. In the present study, the effect of CA with 40% CO_2 on the growth of *V. parahaemolyticus* was investigated. The data obtained showed that the inhibitory effect of CA on *V. parahaemolyticus* was significant (Figure 2.5). The lag phase duration was extended and the maximum population density decreased significantly ($P<0.05$) compared with that in air (Figure 2.7 and 2.9). A decline in viable number was also observed under CA at pH 6.3 and 6.8. It is noteworthy that the lag phase extension of *V. parahaemolyticus* at pH 6.3 was greater than that at pH 6.8 ($P<0.05$), indicating that the growth of *V. parahaemolyticus* was greatly affected at more acidic condition due to the change of physiological status. This is in agreement with the findings of others that *V. parahaemolyticus* most resists inactivation at pH 7.0 and that its sensitivity increases when the pH becomes acidic or alkaline (Beuchat, 1973). Also, Ama *et al*. (1994) reported that pH influences the effectiveness of heat inactivation of *V. vulnificus* in all environmental systems. The current study provides initial information that CA with CO_2 is inhibitory to the growth of *V. parahaemolyticus*.

The inhibitory effect of MA containing CO_2 has also been observed with other microorganisms. Eyles *et al*. (1993) reported that atmospheres containing concentrations of CO_2 as low as 20% inhibited the growth of *Pseudomonas fluorescens* and *Pseudomonas putida* on the surface of buffered Brain Heart Infusion agar plates (pH 6.8), incubated at 5 or 15^{o}C. The modified atmospheres decreased the growth rates and reduced the maximum population densities, but no substantial effect on the lag phase was observed. Devlieghere *et al*. (1998) reported a similar effect of MA on *Lactobacillus sake*.

Spoilage of foods is mainly due to the outgrowth of various contaminating microorganisms in the foods (Gram *et al*., 2002), and contamination with foodborne pathogens hampers the safety of foods. The shelf life of food products is limited mainly

by microbiological spoilage (Ashie *et al*., 1996). Therefore, it is vital to reduce the level of contamination and inhibit the growth of microorganisms to keep the food at higher quality during its natural life or to actually extend its shelf life. MAP is considered as a measure for extending the shelf life of perishable foods such as meat, fish and other food products (Gill & Jeremiah, 1991; Garcia de Fernando *et al*., 1995; Tsigarida *et al*., 2000; Luno *et al*., 2000; Skandamis & Nychas, 2002). The shelf life extension is mainly due to the effect of the modified atmosphere in extending the lag phase of bacteria rather than slowing the rate of growth (Fletcher *et al*., 2002).

In this study, it has been found that a modified atmosphere incorporating 40% CO_2 has significant inhibitory effects on the tested pathogens, in terms of lag phase extension, exponential growth rate reduction, and maximal population density decrease. This result supports the findings of other researchers that the inhibitory effect of MA on foodborne pathogens can be applied to achieve shelf life extended food products.

In summary, this study has demonstrated the inhibitory effect of CA on the foodborne pathogens *E. coli*, *L. monocytogenes*, *S. aureus*, *S.* Typhimurium and *V. parahaemolyticus*. The inhibitory effect of CA is mainly due to both the extension of the lag phase durations and the reduction of the exponential growth rates of the pathogens. The CA significantly reduced the exponential growth rates of all tested pathogens, while the effect of CA on other growth parameters (eg. LPD, MPD) of the pathogens depends on their individual species and the specific growth conditions. The CA significantly extended the lag phase durations of *S. aureus* and *V. parahaemolyticus* at 20^{o}C and both at pH 6.3 and 6.8, and that of *L. monocytogenes* at both 7^{o}C and 20^{o}C, and both at pH 6.3 and 6.8. The CA also significantly minimised the maximum population densities of *S. aureus* and *V. parahaemolyticus* at 20^{o}C, both at pH 6.3 and 6.8, and *S.* Typhimurium at pH 6.8, and *L. monocytogenes* at pH 6.3 and 7^{o}C. However, the inhibitory effect of CA on LPD and MPD of the tested pathogens was less significant. It was found that *E. coli* and *S.* Typhimurium were more resistant to the inhibitory effect of CA, while *S. aureus* and *V. parahaemolyticus* were most sensitive. This study suggests that CA as a means for food preservation provides potential to inhibit the growth of foodborne pathogens and therefore extend the shelf life of a food product.

Chapter 3 Evaluation of lactic acid bacteria strains as biopreservatives for controlling foodborne pathogens

3.1 Introduction

Numerous papers have been published on the use of different lactic acid bacteria (LAB) in controlling the growth of *L. monocytogenes* in meat and meat products (Holzapfel *et al*., 1995; Schillinger *et al*., 1996; Stiles, 1996; Hugas, 1998; Ennahar *et al*., 1999; Lucke, 2000; Budde *et al*., 2003). The antilisterial effect of bacteriocin-producing LAB or partially purified bacteriocins has been demonstrated in raw meat (Nielsen *et al*., 1990; Schillinger *et al*., 1991; Hugas, *et al*., 1998; De Martinis & Franco, 1998), fermented sausages (Foegeding *et al*., 1992; Luchansky *et al*., 1992; Schobitz *et al*., 1999) and cooked sliced meat products (Hugas *et al*., 1998; Krockel, 1998; Amezquita & Brashears, 2002; Budde *et al*., 2003). Non-bacteriocin-producing LAB have also been demonstrated to be antilisterial and able to extend the shelf life of vacuum-packed sliced ham (Kotzekidou & Bloukas, 1996), vacuum-packed bologna-type sausage (Andersen, 1995), saveloys and ham (Bredholt *et al*., 1999). Also, Metaxopoulos *et al*. (2002) demonstrated that *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442 inhibited the growth of spoilage microflora in cooked cured meat products but did not adversely affect the organoleptic properties of the products.

Protection of fish products from bacterial spoilage and from *L. monocytogenes* by bacteriocin-producing LAB has also been investigated. Duffes *et al*. (1999b) investigated twenty-three isolates for their antagonistic activity against *L. monocytogenes* in a simulated cold fish system at 4^{o}C. The inhibitory action of *Lactobacillus* was due to acidification and that of *Carnobacterium* due to a bacteriocinic effect. Schobitz *et al*. (2003) demonstrated that the antagonism of a bacteriocin-like inhibitory substance (BLIS) produced by *Carnobacterium piscicola* L103 was effective

against all tested *L. monocytogenes* strains isolated from salmon and human samples. This research showed that specific LAB strains can play an important role in the preservation and safeguarding of fish and fish products and that LAB may provide an option for biopreservation of fish products.

Mechanisms of action include the production of acid and other metabolic products. *Lactobacillus reuteri* is a normal inhabitant of human and animal gastrointestinal tracts and is present in a variety of foods such as milk and meat. This organism may inhibit pathogenic organisms by the production of lactic acid, reutericin 6, reutericyclin, or reuterin (Slininger *et al*., 1983; Talarico *et al*., 1988; Kabuchi *et al*., 1997; Ganzle *et al*., 2000; Holtzel *et al*., 2000; Vollenweider & Lacroix, 2004). Reuterin has shown a broad spectrum of activity against both Gram-positive and Gram-negative bacteria including *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes*, while reutericyclin is only effective against Gram-positive bacteria (Talarico *et al*., 1988; El-Ziney & Debevere, 1998; Ganzle *et al*., 2000). *Lactobacillus reuteri*, in the presence of glycerol, has been reported to extend the shelf life of herring and to inhibit *E. coli* O157:H7 in ground beef and milk (El-Ziney & Debevere, 1998; Muthukumarasamy *et al*., 2003).

In this study, the antimicrobial effects of four groups of LAB strains were assessed *in vitro*, with emphasis being placed on a newly characterised strain, *Lactobacillus reuteri* DPC16, for its antimicrobial effects against four common foodborne pathogens: *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, and *S. aureus*.

3.2 Materials and Methods

3.2.1 Bacterial strains, culture medium and growth conditions

The LAB strains were kindly provided by Bioactives Research New Zealand Limited, Auckland, New Zealand. These included *Lactobacillus reuteri* P2, P3, P4, P10, P11, P12, P13, P17, P19, P20, P21, P23 and P26 (subsequently designated as DPC16) strains, *Lactobacillus mucosae* P7, *Enterococcus faecium* P9, and *Leuconostoc garlicum* P18,

P24 and P27 strains. Reference LAB strains *Bifidobacterium lactis* (DR10) and *Lactobacillus rhamnosus* (DR20) were kindly provided by Dr Quan Shu (Bioactives Research New Zealand Ltd., Auckland, New Zealand). The stock cultures were maintained at -80^{o}C in de Man, Rogosa, Sharpe (MRS) broth (Difco^{TM}, Becton Dickinson, MD, USA) containing 15% glycerol. The bacterial strains were sub-cultured on MRS agar (MRS broth supplemented with 1.5% agar) in an anaerobic condition generated by GasPak^{TM} EZ Gas Generating Pouch Systems (BD, Sparks, MD, USA) in an anaerobic jar (BD, Sparks, MD, USA) and MRS broth supplemented with 250 mM glycerol (MRSg).

For sub-culture of LAB strains, each of the strains was streaked onto MRS agar and incubated anaerobically for 24 h at 37^{o}C. A single colony of each strain was re-streaked onto a fresh agar plate and incubated at the same condition. An isolated colony was used to inoculate 10 mL of MRS broth supplemented with 250 mM glycerol (MRSg) and incubated for 16 h at 37^{o}C. The resulting cultures were used as stock for further use.

The pathogenic bacterial strains *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* serovar Typhimurium and *Staphylococcus aureus* were used to evaluate the antimicrobial effects of the above-described LAB strains. The origin of these pathogenic strains and their sub-culture are described in Section 2.2.1.

3.2.2 Preparation of culture inocula of *L. reuteri* DPC16 and pathogenic bacterial strains

Cultures of *L. reuteri* DPC16 and the pathogenic strains were grown for 16 h at 37^{o}C, and were centrifuged at 3,000 x g for 10 min. The pellets were washed in sterile 0.1% peptone water, centrifuged at 3,000 x g for 10 min. The pellets were resuspended in 0.1% peptone water to their original volumes. The bacterial suspensions were stored at 4^{o}C as inocula and subjected to enumeration following the method described in Section 2.2.2 on MRS agar plates for *L. reuteri* DPC16 and on BHI agar plates for the pathogens. When required, the bacterial suspensions were diluted to their required concentrations and used immediately as inocula.

3.2.3 Molecular confirmation of LAB strains

3.2.3.1 Genomic DNA extraction from bacteria

For amplification of 16S rRNA gene, genomic DNA was extracted from each of the LAB strains using a DNeasy Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Briefly, sub-cultured LAB cells (maximum 2 x 10^9 cells) were recovered in a micro-centrifuge tube by centrifugation for 10 min at 5,000 x *g* (7,500 rpm) and resuspended in 180 µL enzymatic lysis buffer (20 mM Tris·Cl, pH 8.0; 2 mM Sodium EDTA; 1.2% Triton X-100; 20 mg/mL lysozyme). The suspension was incubated for 30 min at 37°C followed by the addition of 25 µL proteinase K (supplied) and 200 µL Buffer AL (supplied) and mixed by vortexing. The sample was incubated at 70°C for 30 min and 200 µL of ethanol (96-100%) was added. The sample was mixed thoroughly by vortexing. The mixture was transferred into the DNeasy Mini spin column (supplied) placed in a 2 mL collection tube (supplied). The mixture was centrifuged at 6,000 x *g* (8,000 rpm) for 1 min and the flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 mL collection tube followed by addition of 500 µL Buffer AW1 (supplied). The column was centrifuged for 1 min at 6,000 x *g* (8,000 rpm) and the flow-through and collection tube were discarded. The column was re-inserted in a new 2 mL collection tube followed by the addition of 500 µL of Buffer AW2 (supplied). The column was centrifuged for 3 min at 20,000 x *g* (14,000 rpm) to dry the DNeasy membrane. The DNeasy Mini spin column was carefully removed and placed in a clean 1.5 mL or 2 mL microcentrifuge tube 200 µL of pure water (GIBCO, Invitrogen, Carlsbad, USA) added directly onto the DNeasy membrane. The column was incubated at room temperature for 1 min, and then centrifuged for 1 min at 6,000 x *g* (8,000 rpm) to elute. The DNA sample was stored at -20^{o}C for future use.

3.2.3.2 Amplification of 16S rRNA gene by polymerase chain reaction (PCR)

PCR amplifications of 16S rRNA gene were carried out using an Eppendorf machine (Mastercycler gradient, Hamburg, Germany) with universal primers M27F [5' AGA GTT TGA TCC TGG CTC AG 3'; positions 8-27, (using the *Escherichia coli* numbering system)] and 1522R (5' AAG GAG GTG ATC CAA/G CCG CA 3';

positions 1541-1522) (Mori *et al*., 1997). The PCR mix contained 5 µL of 10 x PCR buffer (Invitrogen, Carlsbad, USA), 2.5 mM $MgCl_2$ (Invitrogen), 0.2 µM each forward and reverse primer, 200 µM each deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase (Invitrogen), and 1 µL of genomic DNA as a template in a total volume of 50 µL.

The following temperature profiles were applied: 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec; and 1 cycle of 72°C for 7 min. PCR products were analysed by gel electrophoresis.

3.2.3.3 Gel electrophoresis of PCR products

The PCR products were analysed on 1% agarose gel by electrophoresis. To prepare 1% agarose gel, 1 g of agarose (Invitrogen, Carlsbad, USA) was placed a glass beaker or flask followed by the addition of 100 mL 0.5 x TAE buffer (see Appendix I). The solution was heated in a microwave oven until the agarose was dissolved and the solution was clear. The solution was allowed to cool to about 50^{o}C and ethidium bromide (Bio-Rad, Hercules, CA, USA) was added to a concentration of 0.5 µg/mL before pouring. The gel tray was assembled on the tray stand with the combs placed appropriately. The cooled gel solution was poured into the tray to a depth of about 5 mm and allowed to solidify at room temperature.

To run the electrophoresis, the combs were gently removed and the tray was placed in the electrophoresis chamber and covered (until the wells were submerged) with electrophoresis buffer (0.5 x TAE buffer).

To prepare samples for electrophoresis, one µL of 6 x gel loading dye (see Appendix I) was mixed with every 5 µL of DNA solution. The mixture of 10 µL was loaded into the well. Electrophoresis was run at 100 volts in a Gel Electrophoresis device (Bio-Rad, Hercules, CA, USA) until the dye markers had migrated an appropriate distance. The gel was visualised under UV light and photographed using a Kodak gel documentation system (Kodak, New Haven, CT, USA).

3.2.3.4 Purification of PCR products and DNA sequencing

PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for the option using a microcentrifuge. Briefly, PCR sample (1 volume) was mixed with 5 volumes of Buffer PB (supplied). The mixed sample was applied to a QIAquick spin column (supplied) in a 2 mL collection tube and centrifuged at 13,000 rpm (~17,900 x *g*) in a conventional tabletop microcentrifuge for 30-60 sec. The flow-through was discarded and the QIAquick column was placed back into the same tube. The QIAquick column was replenished with 0.75 mL Buffer PE (supplied) and centrifuged for 30-60 sec. The flow-through was discarded and the QIAquick column was centrifuged for 60 sec. The QIAquick column was transferred to a clean 1.5 mL micro-centrifuge tube and 50 μL Buffer EB (supplied) or H_2O was added to the centre of the QIAquick membrane. The QIAquick column was centrifuged for 1 min and the resulting purified DNA was stored at -20°C for analysis.

Sequencing of the DNA fragment was done by the Allan Wilson Centre, Massey University, Albany, using primers M27F, 1522R, and two additional primers designed in this study ProbluF1 (5' AAC AGG ATT AGA TAC C 3') and ProbluR1 (5' GGT ATC TAA TCC TGT T 3'), using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kits in Gene Amp PCR system 9700 machines, and analysed on an ABI PRISM 377 DNA Seqencer. The 16S rDNA sequences for each of the selected LAB strains were assembled using program BioEdit Sequence Alignment Editor Version 7.0.4.1 (Hall, 1999).

3.2.3.5 Phylogenetic analysis of LAB strains

The obtained 16S rDNA sequences of the selected LAB strains were subject to similarity searches against the NCBI database using the Basic Local Alignment Search Tool (BLAST) programme (Altschul *et al*., 1997), available on the internet (http://www.ncbi.nlm.nih.gov/BLAST/). The closest relatives identified in the searches were included in further phylogenetic analyses. Alignment of the sequences was carried out using ClustalX version 1.83 (Thompson *et al.*, 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al*., 2004),

and the Neighbour-Joining tree was constructed based on bootstrap analysis of 2000 trees using *Bifidobacterium sp*. h12 16S rRNA gene as the outgroup sequence.

3.2.3.6 Detection of glycerol dehydratase gene in LAB strains using polymerase chain reaction (PCR)

PCR was conducted using genomic DNA extracted from selected isolates to detect the presence of the glycerol dehydratase-encoding gene involved in reuterin production, using degenerate primers GD1 (5' AAA/G GAC/T AAA/T/C CCC/G/A/T GTC/G/A/T CAA/G ATA/T/C GCC/G/A/T GC 3') and GD2 (5' CCA C/G/A/TGG C/G/A/TGT A/GTC A/GTC C/G/A/TCC A/GTC C/G/A/TGT A/GAA C/G/A/TAC 3') (Claisse & Lonvaud-Funel, 2001). The sequences of the primers correspond to the deduced amino acids of a 60-kDa glycerol dehydratase subunit at positions 149 to 157, KDNPVQIAA, and 232 to 241, VFTDGDDTPW, respectively. The PCR produced a 279-bp fragment (Claisse & Lonvaud-Funel, 2001).

The genomic DNA extracted from the selected LAB strains (Section 3.2.3.1) was used as a template for PCR amplification. The PCR reaction and the following detection of the PCR products were conducted following the methods described in Sections 3.2.3.2 and 3.2.3.3. Specifically, the PCR amplification was carried out following the temperature profile consisting of an initial denaturation step at 94^{o}C for 5 min; 30 cycles of 30 sec at 94, 52 and 72^{o}C; a final extension at 72^{o}C for 7 min; and then soaking at 4^{o}C until analysis.

The PCR products were purified and sequenced using the GD1 and GD2 primers following the method described in Section 3.2.3.4.

3.2.4 Antimicrobial effect of LAB strains on pathogens

3.2.4.1 Preparation of LAB spent culture supernatant (SCS) in broth

A 1% inoculum of an overnight broth culture of each of the test and reference LAB strains was grown for 16 h in MMRS supplemented with glycerol at a concentration of

250 mM. The spent culture suspensions were centrifuged at 4,000 x g for 10 min. The supernatant fluids were filtered through an Acrodisc filter (0.45 µm) (Pall, East Hills, NY, USA) to remove any remaining cells. Filtered spent culture supernatants were frozen at -20°C until the time of use. The SCS of *L. reuteri* DPC16 was designated as DPC16-SCS.

3.2.4.2 Gel diffusion assay

Agar diffusion assays were carried out to determine the antimicrobial activities and characterise the antimicrobial substances. The methods of Juarez *et el.* (2002) and Ghrairi *et al*. (2004) were used in this study with modification.

Briefly, Brain Heart Infusion (BHI) broth containing 1% agar was melted and sterilised by autoclaving. BHI agar was cooled to 48^{o}C in a water bath. Each of the indicator pathogens was added to BHI agar at a final concentration of approximately 1 x 10^5 CFU/mL. After mixing thoroughly, the agar (20 mL) was poured into petri dishes. The agar in the plates was allowed to set at room temperature and wells of 4 mm in diameter were made by aseptically cutting into the gel.

Forty microlitres of each of the filter-sterilised spent culture supernatants (SCS) were added into each well aseptically. The plates were incubated at room temperature until appearance of a clear inhibition zone and the diameter of the inhibition zone across the well was measured and recorded.

3.2.4.3 Characterisation of antimicrobial substances produced by *L. reuteri* DPC16

The nature of the antimicrobial substance produced by *L. reuteri* DPC16 in the DPC16-SCS was determined following the methods described by Ghrairi *et al*. (2004). Briefly, the DPC16-SCS was subjected to each of the following treatments: pH adjustment (4.4 and 6.5); heating (80^{o}C, 10 min); incubation with pronase E (Sigma, St. Louis, MO, USA), proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) and trypsin (Sigma, St. Louis, MO, USA) at a concentration of 1 mg/mL for 2 h at 37^{o}C, and with

catalase (Sigma, St. Louis, MO, USA) at a concentration of 500 U/mL for 30 min at 30^{o}C. After each treatment, the remaining antimicrobial activity against *L. monocytogenes*, *E. coli* O157:H7, *S.* Typhimurium, and *S. aureus* was determined using the agar diffusion assay described in Section 3.2.4.2. Untreated DPC16-SCS was used as a control.

3.2.4.4 Determination of reuterin production

The method for acrolein (2-propenal) determination, using the acid catalysed formation of a coloured complex between acrolein/reuterin and tryptophan (Circle *et al*., 1945) was used for the determination of reuterin. This method has been adopted by other authors to accurately determine the concentration of reuterin (Smiley & Sobolov, 1962; Slininger *et al*., 1983; Barbirato *et al*., 1996; Sauvageot *et al*., 2000; Lüthi-Peng *et al*., 2002; Vollenweider *et al*., 2003).

The full method with modification is described in Appendix III using acrolein (Sigma, St. Louis, MO, USA) as a standard. The concentration of reuterin in the samples was based on this (Circle *et al.*, 1945).

The production of reuterin by *L. reuteri* DPC16 in MMRS with 250 mM glycerol was determined when grown alone or in co-culture with *L. monocytogenes*, either at unadjusted pH or at a constant pH of 6.5.

3.2.4.5 Determination of lactic acid production of *L. reuteri* DPC16

The production of lactic acid by *L. reuteri* DPC16 was determined when grown both alone or in co-culture with *E. coli* O157 or *S.* Typhimurium. Lactic acid was quantified using a commercial lactate kit (Cat No: 11822837, Roche Diagnostics GmbH, Basel, Switzerland) following the manufacturer's instructions.

3.2.4.6 Inhibitory effect of DPC16-SCS against foodborne pathogens by co-incubation

The procedure followed was that of Ito *et al*. (2003) with modification. The SCS of *L. reuteri* DPC16 (DPC16-SCS) was tested for its antimicrobial activity by co-incubation with *L. monocytogenes*, *E. coli* O157:H7, *S.* Typhimurium and *S. aureus*. The DPC16-SCS was sterilised using an Acrodisc filter (0.2 µm) (Pall, East Hills, NY, USA) and the pH was adjusted to 4.4, 5.0, or 6.5. Fifty microlitres of pathogen suspension at a concentration of approximately 2.1 x 10^7 CFU/mL was mixed with 1 mL of DPC16-SCS. The mixture was incubated for up to 4 h at 37^{o}C or 48 h at 10^{o}C. Samples were withdrawn at 0.5 - 1 h or 4 h intervals to evaluate the cell viability of the test pathogens by the drop plate count described in Section 2.2.2 using BHI agar plates. The viable cell counts of the pathogens were determined as colony-forming units (CFU) per mL.

Controls were set up to verify the role of pH in the viability of the pathogens, using MMRS supplemented with 250 mM glycerol, with pH adjusted to 4.4, 5.0 and 6.5 with 1 N HCl, followed by sterilisation using Acrodisc filters (0.45 µm) (Pall, East Hills, NY, USA). The sterilised MMRS broth was incubated with pathogens following the same procedures as for DPC16-SCS at 37^{o}C above.

3.2.4.7 Co-cultivation of *L. reuteri* DPC16 and pathogens in broth

To facilitate the co-culture of both *L. reuteri* DPC16 and pathogenic strains, a modified MRS broth (without tri-ammonium-citrate and sodium-acetate, MMRS) was used (Annuk *et al*., 2003). The MMRS contained 10 g peptone, 8 g 'Lab-Lemco' powder, 4 g yeast extract, 20 g glucose, 1 mL 'Tween-80', 2 g di-potassium hydrogen phosphate, 0.2 g magnesium sulphate $7H_2O$, 0.05 g manganese sulphate $4H_2O$, per litre, pH 7.2, supplemented with 250 mM glycerol.

Co-cultivations of *L. reuteri* DPC16 with *L. monocytogenes*, *E. coli* O157:H7, *S.* Typhimurium, and *S. aureus* were carried out following the methods described by Garcia *et al*. (2004) with modification. Briefly, portions of *L. reuteri* DPC16 culture and each of the pathogenic cultures in the exponential phase were mixed in MMRS, pre-

warmed to 37°C, to final concentrations of approximately 1 x 10^4 CFU/mL for pathogens and approximately 1 x 10^6 CFU/mL for *L. reuteri* DPC16. The mixed cultures were incubated at 37°C. At 4 h intervals of incubation, aliquots of co-cultures were removed and the pH was measured and recorded. Aliquots of co-cultures were serially diluted in sterile 0.1% peptone water. Dilutions were plated on MacConkey agar (Tomas *et al*., 2003b), BHI agar (supplemented 1.5% lithium chloride) (Garcia *et al*., 2004), Salmonella Shigella (SS) agar, Mannitol salt agar (Tomas *et al*., 2003a) or MRS agar plates and incubated aerobically at 37°C for viable counts of *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus*, respectively, and incubated anaerobically at 37°C for viable counts of *L. reuteri* DPC16. The drop plate count method described in Section 2.2.2 was used and colony characteristics were used to confirm the identity of the colonies on the plates.

Aliquots of co-cultures were also clarified by centrifugation at 12,000 x g for 10 min, and the supernatants were analysed for reuterin and lactic acid production.

3.2.4.8 Co-cultivation of *L. reuteri* DPC16 and *L. monocytogenes* in broth at constant pH value

Co-cultivation of *L. reuteri* DPC16 with *L. monocytogenes* at a constant pH value was carried out following the method described in Section 3.2.4.7, except that the pH of co-cultivation was controlled manually at 0.5 - 1 h intervals to maintain the pH at approximately 6.5 (6.2 - 6.8) using filter-sterilised 1 M NaOH solution. The growth of *L. reuteri* DPC16 and *L. monocytogenes*, both alone and in co-cultivation, with pH unadjusted, was carried out as controls.

3.3 Results

3.3.1 Molecular confirmation of LAB strains

To confirm the identities of the 18 LAB strains, the partial 16S rDNA sequence (approximately 1,500 bp) for each of the strains was determined (see sequences and alignment in Appendix IV), which allowed the preliminary identification of bacterial

strains to the species level (Table 3.1). Phylogenetic analysis revealed that the 18 strains fell into 4 groups, belonging to three genera of bacteria, *Lactobacillus, Leuconostoc* and *Enterococcus.* A tree illustrating the phylogenetic allocation of the 18 strains was produced (Figure 3.1). The majority of the strains, P2, P3, P4, P10, P11, P12, P13, P17, P19, P20, P21, P23 and P26, were closely related to *Lactobacillus reuteri* with similarity from 99.3 - 99.6%. Three strains, P18, P24 and P27 were grouped closely with *Leuconostoc garlicum* (similarity 99.7 - 99.8%). One strain, P7, closely matched *Lactobacillus mucosae* (99.6%), and one strain, P9 matched *Enterococcus faecium* (99.5%).

Table 3.1 The closest phylogenetic relatives of the selected LAB strains

Strain code	Closest phylogenetic relative	Accession number	Similarity (%)
P2	*Lactobacillus reuteri*	X76328	99.5
P3	*Lactobacillus reuteri*	X76328	99.4
P4	*Lactobacillus reuteri*	X76328	99.3
P10	*Lactobacillus reuteri*	X76328	99.4
P11	*Lactobacillus reuteri*	X76328	99.6
P12	*Lactobacillus reuteri*	X76328	99.6
P13	*Lactobacillus reuteri*	X76328	99.6
P17	*Lactobacillus reuteri*	X76328	99.5
P19	*Lactobacillus reuteri*	X76328	99.6
P20	*Lactobacillus reuteri*	X76328	99.6
P21	*Lactobacillus reuteri*	X76328	99.5
P23	*Lactobacillus reuteri*	X76328	99.5
P26 (DPC16)	*Lactobacillus reuteri*	X76328	99.5
P7	*Lactobacillus mucosae*	AF126738	99.6
P9	*Enterococcus faecium*	AY172570	99.5
P18	*Leuconostoc garlicum*	AY456086	99.8
P24	*Leuconostoc garlicum*	AY456086	99.7
P27	*Leuconostoc garlicum*	AY456086	99.8

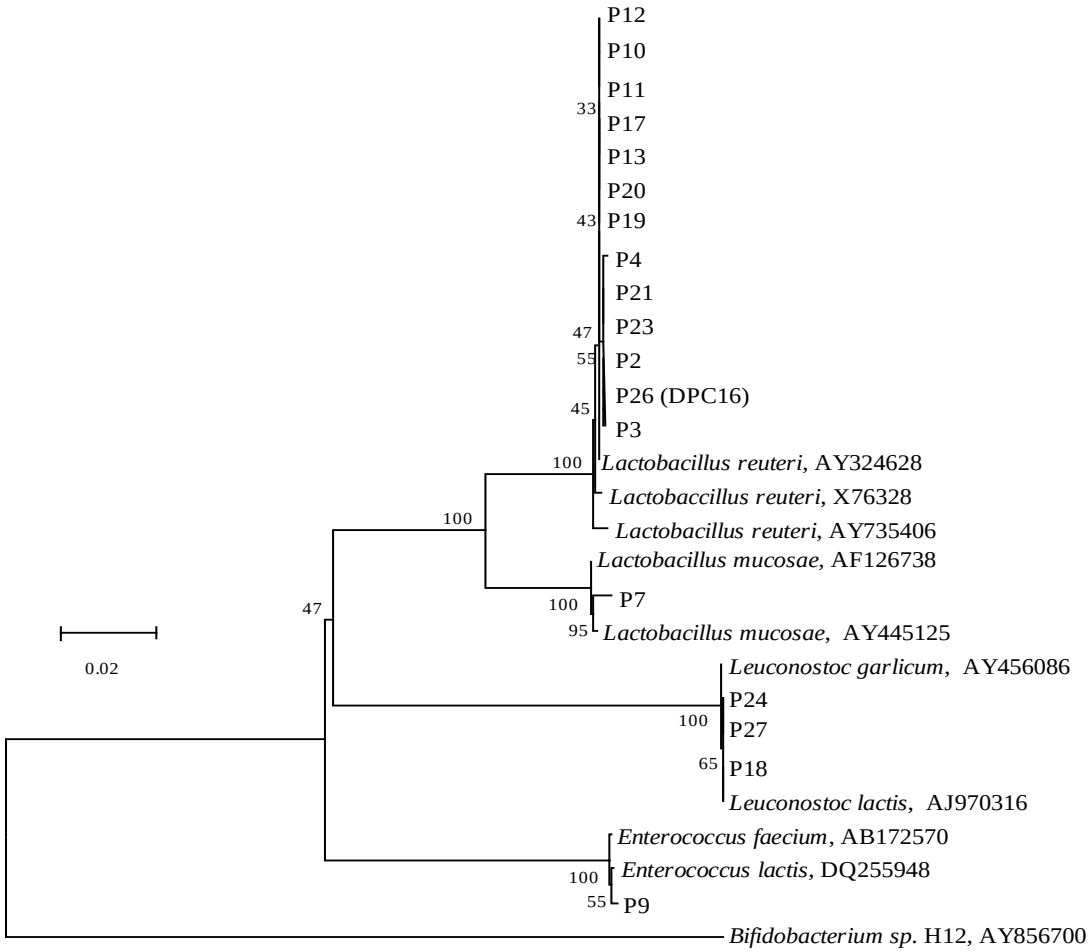

Figure 3.1 Neighbour-joining tree based on 16S rDNA sequences of the 18 LAB strains and selected sequences representing 3 genera of bacteria from GenBank. Values on branches denote bootstrap support (analysis of 2000 trees). The scale bar represents 0.02 inferred substitutions per nucleotide position. The 16S rDNA sequence of *Bifidobacterium sp.* H12 (Accession number: AY856700) was arbitrarily chosen as the outgroup sequence.

3.3.2 Antimicrobial effect of LAB strains against foodborne pathogens using agar diffusion assay

The antimicrobial effects of the LAB strains against some foodborne pathogens are summarised in Table 3.2. The agar diffusion assay revealed that 14 strains had antimicrobial activities of various strengths against the tested pathogens. The inhibition zones ranged from 6 to 12 mm in diameter. *L. reuteri* strains P4, P10, P11, P12, P17,

Table 3.2 Inhibitory effect[a] of spent culture supernatants of LAB strains against some foodborne pathogens

LAB strain		Foodborne pathogen			
		L. monocytogenes	***E.coli* O157:H7**	***S.* Typhimurium**	***S. aureus***
L. reuteri	P2	0	0	0	0
	P3	0	0	0	0
	P4	8	8	8	6
	P10	8	8	9	9
	P11	9	8	8	8
	P12	7	6	6	10
	P13	0	0	6	0
	P17	8	7	6	7
	P19	0	0	0	0
	P20	8	7	8	7
	P21	0	0	0	0
	P23	10	10	8	9
	P26	10	10	10	11
L. garlicum	P18	8	7	9	12
	P24	8	8	8	9
	P27	8	7	8	11
L. mucosae	P7	0	0	8	0
E. faecium	P9	8	0	7	0
B. lactis	DR10	7	8	8	8
L. rhamnosus	DR20	8	9	8	8

[a] Inhibitory effect was measured by the size in diameter (mm) of the inhibition zone across a 4 mm diameter well. A zero value indicates no clear zone observed.

P20, P23 and P26, and *L. garlicum* strains P18, P24 and P27 showed antimicrobial activities against all tested pathogens. *L. mucosae* strain P7 had an antimicrobial effect on *S.* Typhimurium only while *E. faecium* strain P9 showed antimicrobial activity against *L. monocytogenes* and *S.* Typhimurium. *L. reuteri* strains P2, P3, P19 and P21

showed no antimicrobial effect against any of the tested pathogens. Notably, strains P23 and P26 (designated DPC16) showed the highest overall antimicrobial activities against all tested pathogens, with average inhibition zones of 9 and 10.25 mm, respectively, compared to the rest of the *L. reuteri* strains (less than 8.5 mm). The inhibition zones of these two strains were higher than those of two commercial LAB strains *Bifidobacterium lactis* (DR10) and *Lactobacillus rhamnosus* (DR20) which had mean inhibition zones of 7.75 and 8.25 mm, respectively, against all test pathogens.

3.3.3 Characterisation of antimicrobial substances produced by *L. reuteri* DPC16

The effects of different treatments on the antimicrobial substances in the SCS of *L. reuteri* DPC16 are shown in Figure 3.2. Increasing the pH of the SCS from 4.4 to 6.5 (well 2) did not eliminate the antimicrobial effect, suggesting that it is not merely the presence of acids. Only minimum inhibition was observed around well 3 of A and B and no clear zone was observed, indicating that the antimicrobial activity occurring in these wells was small compared with wells 1 and 2 and that heating (80^{o}C, 10 min) had eliminated much of the inhibitory effect. This suggests that the antimicrobial substance in DPC16-SCS is heat-labile.

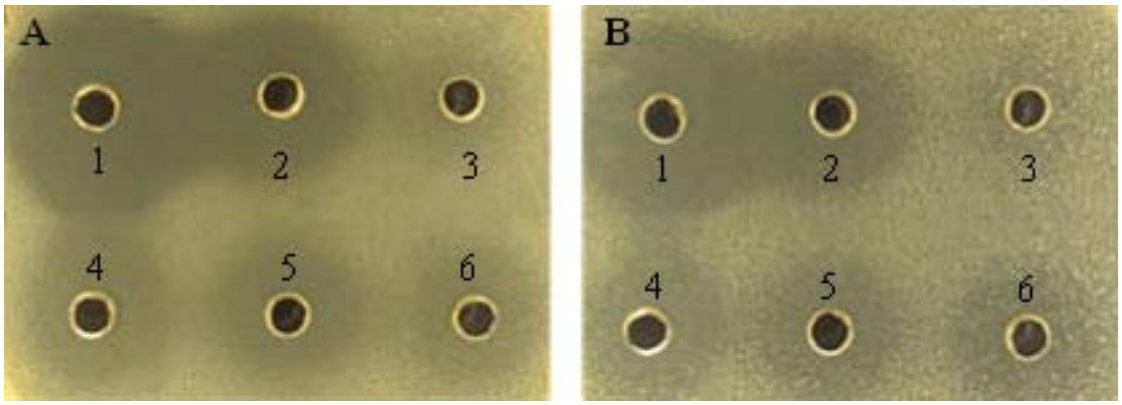

Figure 3.2: Agar diffusion assay for detection of antimicrobial activity of DPC16-SCS subjected to different treatments against *E. coli* O157:H7 (A) and *S.* Typhimurium (B). 1: original SCS at pH 4.4; 2: SCS with pH adjusted to 6.5; 3: SCS with pH adjusted to 6.5 and heat treated at 80^{o}C for 10 min; 4: SCS with pH adjusted to 6.5 and treated with catalase; 5: SCS with pH adjusted to 6.5 and treated with pronase E; 6: SCS with pH adjusted to 6.5 and treated with proteinase K.

A similarly sized clear zone (no pathogen growth) was seen in well 4, suggesting that the antimicrobial substance was not eliminated by catalase, and is therefore not H_2O_2. The zones of inhibition in wells 5 and 6 also showed that the antimicrobial activity was not eliminated in the treatment by pronase E (Well 5) or proteinase K (Well 6). This indicated that the inhibitory effect was due to the production of non-proteinaceous substances and was therefore unlikely to be due to bacteriocin.

These results are consistent with the antimicrobial substance being reuterin, an antimicrobial substance known to be produced by some strains of *L. reuteri*.

3.3.4 Production of reuterin

The concentration of reuterin (β-hydroxypropionaldehyde) in the DPC16-SCS was determined by comparing the spectral extinction values from the assay to those of a standard curve of acrolein (2-Propenal) (Figure 3.3).

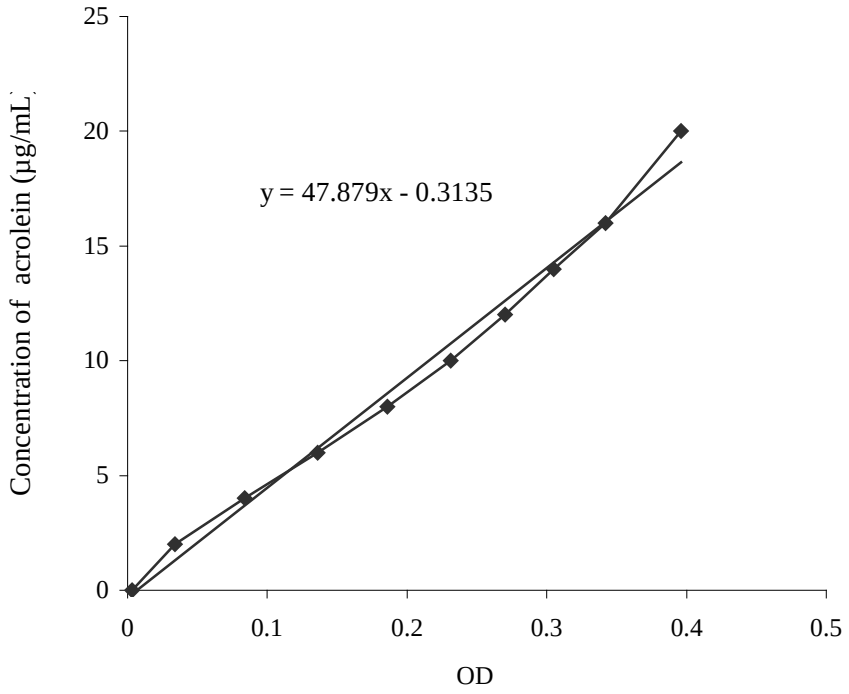

Figure 3.3 Standard curve of acrolein solution determined using the colorimetric method described by Circle *et al.* (1945) with modification.

The production of reuterin by *L. reuteri* DPC16 in MMRS with 250 mM glycerol was determined under different growth conditions and the result was shown in Table 3.3 and Figure 3.4. The maximum concentration of reuterin was observed after 12-16 h of growth, whether *L. reuteri* DPC16 was cultured alone or in co-cultivation with *L. monocytogenes*, with or without pH control at pH 6.5. The maximum concentrations observed ranged from 30.6 to 32.0 µg/mL.

Table 3.3 Production of reuterin (µg/mL) *L. reuteri* DPC16 in MMRS with 250 mM glycerol (mean ± SD)[a]

	Growth Condition		
Time (h)	*L. reuteri* DPC16 grown alone, pH unadjusted	In co-culture with *L. monocytogenes*, pH unadjusted	In co-culture with *L. monocytogenes*, pH constant
4	1.5 ± 0.03	1.70 ± 0.05	1.75 ± 0.03
8	24.63 ± 0.80	28.89 ± 0.38	9.74 ± 0.04
12	28.08 ± 0.16	31.33 ± 0.39	31.33 ± 0.26
16	30.62 ± 0.45	32.00 ± 0.98	31.19 ± 0.26
20	25.40 ± 0.39	25.83 ± 0.51	31.09 ± 0.21
24	10.94 ± 0.56	13.28 ± 0.21	28.70 ± 0.28
36	2.85 ± 0.06	4.28 ± 0.25	14.19 ± 0.21

[a] Mean of two replicate measurements.

SD: Standard deviation.

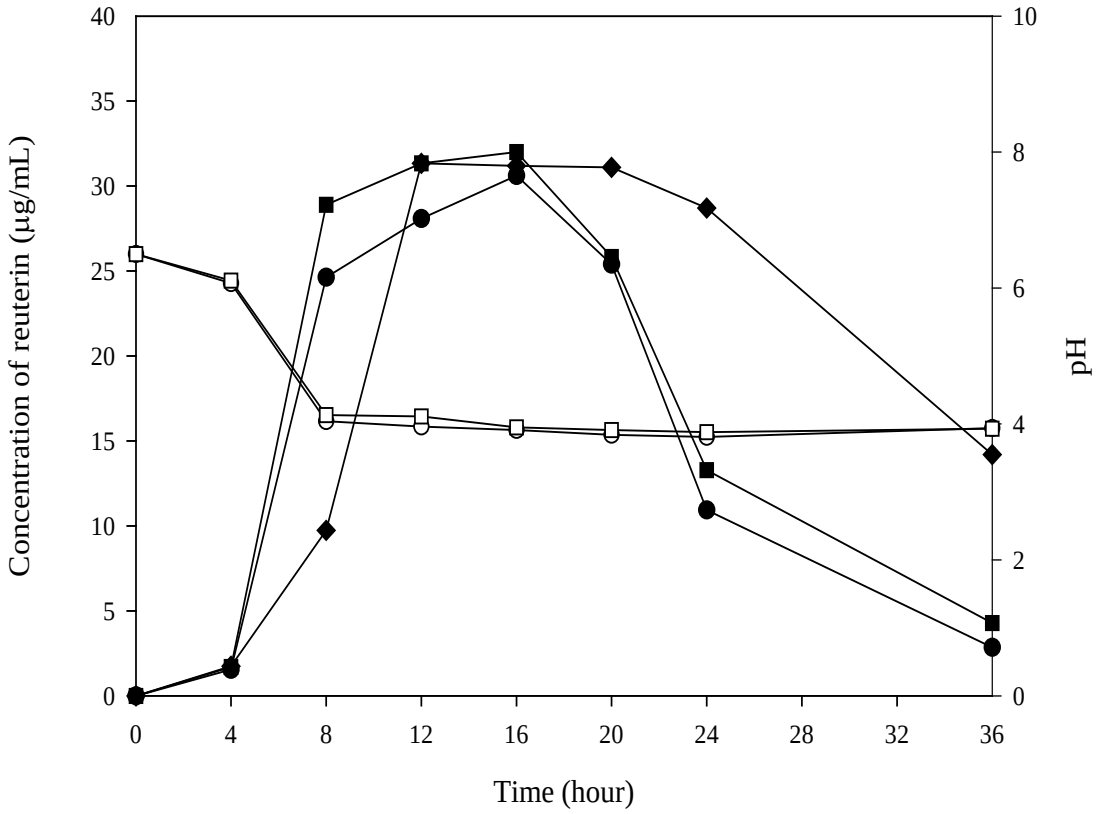

Figure 3.4 Production of reuterin by *L. reuteri* DPC16 in MMRS with 250 mM glycerol and *L. reuteri* DPC16 alone (●), in co-cultivation with *L. monocytogenes* at uncontrolled pH (■), and in co-cultivation with *L. monocytogenes* at a constant pH of 6.5 (♦). The pH changes for *L. reuteri* DPC16 grown alone (○), in co-culture with *L. monocytogenes* at uncontrolled pH (□). Data presented as mean of two replicate measurements.

3.3.5 Determination of lactic acid production by *L. reuteri* DPC16

The production of lactic acid by *L. reuteri* DPC16 grown in MMRS with 250 mM glycerol, both alone and in co-cultivation with *E. coli* O157:H7 or *S.* Typhimurium, at 37^{o}C with an initial pH 6.5 is shown in Table 3.4 and Figure 3.5. The maximum production of lactic acid was observed after 16 h of growth. The maximum concentrations of L-lactic acid produced by *L.reuteri* DPC16 alone, or in co-cultivation with *E. coli* O157:H7 or *S.* Typhimurium, ranged from 2.6 to 2.8 g/L.

Table 3.4 Determination of lactic acid production (g/L) by *L. reuteri* DPC16 (mean ± SD)[a]

Time (h)	Growth Condition		
	L. reuteri DPC16 grow alone	In co-culture with *E. coli* O157:H7	In co-culture with *S.* Typhimurium
4	0.29 ± 0.00	0.27 ± 0.01	0.32 ± 0.02
8	1.27 ± 0.02	1.75 ± 0.01	1.33 ± 0.04
12	1.88 ± 0.02	2.19 ± 0.01	2.27 ± 0.02
16	2.52 ± 0.01	2.64 ± 0.02	2.57 ± 0.02
24	2.60 ± 0.02	2.81 ± 0.04	2.53 ± 0.01
48	2.62 ± 0.01	2.48 ± 0.04	2.70 ± 0.01

[a] Mean of two replicate measurements.

SD: Standard deviation.

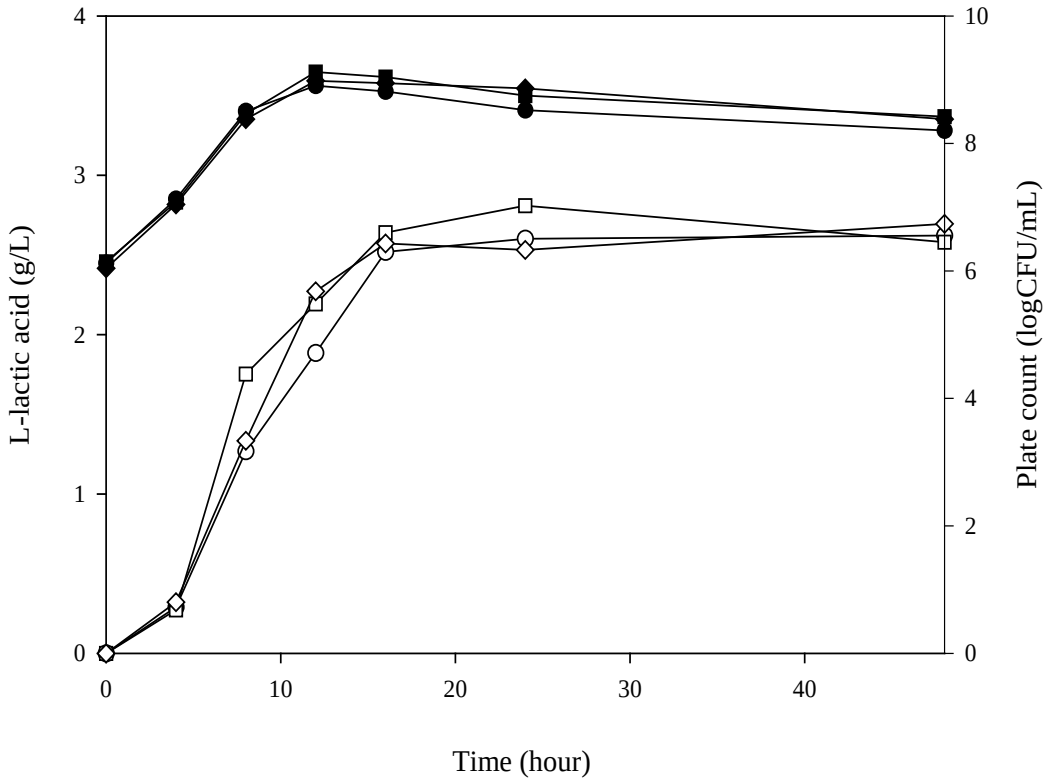

Figure 3.5 L-lactic acid production by *L. reuteri* DPC16 grown at 37°C in MMRS at an initial pH of 6.5, alone (○), in co-cultivation with *E. coli* O157:H7 (□) or *S.* Typhimurium (◊). The corresponding growth curves are recorded as *L. reuteri* DPC16 alone (●), in co-cultivation with *E. coli* O157:H7 (■) or *S.* Typhimurium (♦), by plate count on MRS agar plates for *L. reuteri* DPC16, on BHI agar plates for *E. coli* O157:H7 and *S.* Typhimurium. Data presented as mean of two replicate measurements.

3.3.6 Survival of pathogens in DPC16-SCS

The antimicrobial effects of DPC16-SCS against the pathogens at different pH values and incubation temperatures are presented in Figures 3.6 and 3.7. The counts of viable pathogens incubated at 37°C in MMRS at different pH values without DPC16-SCS are shown in Figure 3.8. At 37°C, antimicrobial activity by DPC16-SCS was observed against all tested foodborne pathogens at all tested pH values, as shown by the significant decline in numbers of the test pathogens. In contrast, in the absence of

DPC16, the counts of the pathogens were little changed after incubation at pH 4.4, 5.0 and 6.5, suggesting that pH alone has little effect on the survival of the pathogens over this pH range.

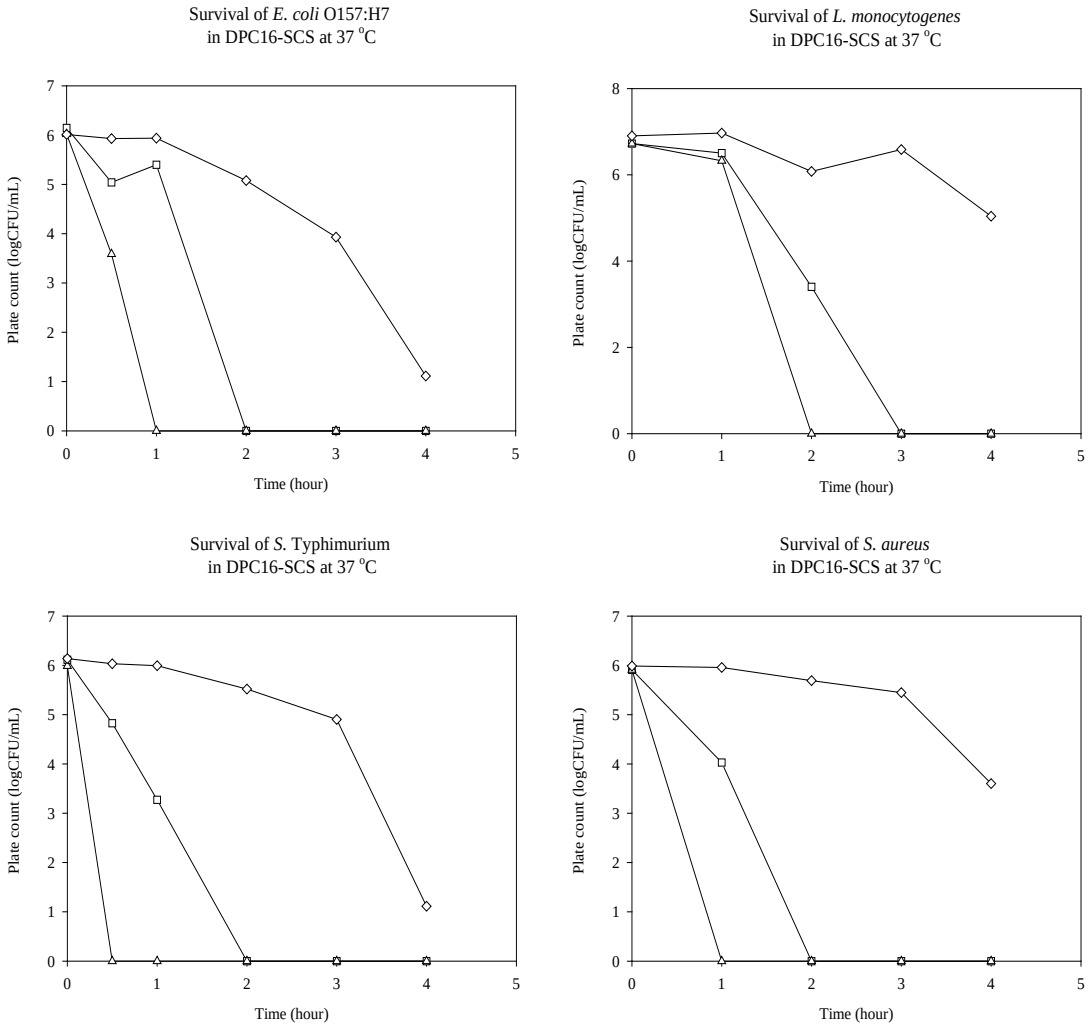

Figure 3.6 Effect of DPC16-SCS on the survival of foodborne pathogens at 37°C. The antimicrobial activity of DPC16 was evaluated at the original culture pH of 4.4 (Δ), and at the adjusted pH values of 5.0 (□) and 6.5 (◊). Data represent the mean of three replicates.

The antimicrobial activity of DPC16-SCS was more pronounced with lower pH. A rapid decrease of viable cells was observed at pH 5.0 and 4.4, while there was a delay before substantial decreases occurred at the pH of 6.5. No detectable viable bacteria were found for *E. coli* O157:H7, *S.* Typhimurium or *S. aureus* after 1 h incubation in DPC16-SCS at

pH 4.4 or after 2 h at pH 5.0. However, *L. monocytogenes* became undetectable only after 2 h incubation at pH 4.4, and 3 h at pH 5.0, suggesting that this pathogen is more resistant to the antimicrobial substances in DPC16-SCS than are the other tested pathogens.

The antimicrobial effect of *L. reuteri* DPC16 was less pronounced at a temperature of 10 ^{o}C than at 37^{o}C. At this temperature, the pathogens were detected for 10 to 24 h of incubation with DPC16-SCS at pH 4.4, and for approximately 50 h at a pH value of 5.5 (Figure 3.7).

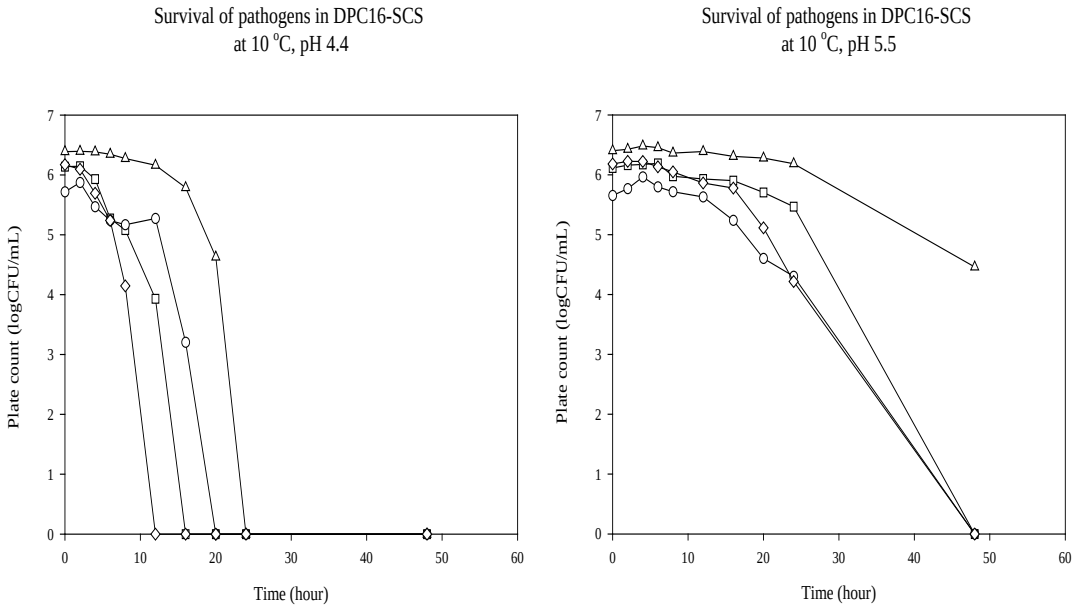

Figure 3.7 *In vitro* effect of *L. reuteri* DPC16-SCS on the survival of foodborne pathogens at 10^{o}C. The antimicrobial activity of DPC16 was evaluated at pH 4.4 and 5.5. *E. coli* O157:H7 (□), *L. monocytogenes* (Δ), *S. aureus* (○) and *S*. Typhimurium (◊). Data represent the mean of three replicates.

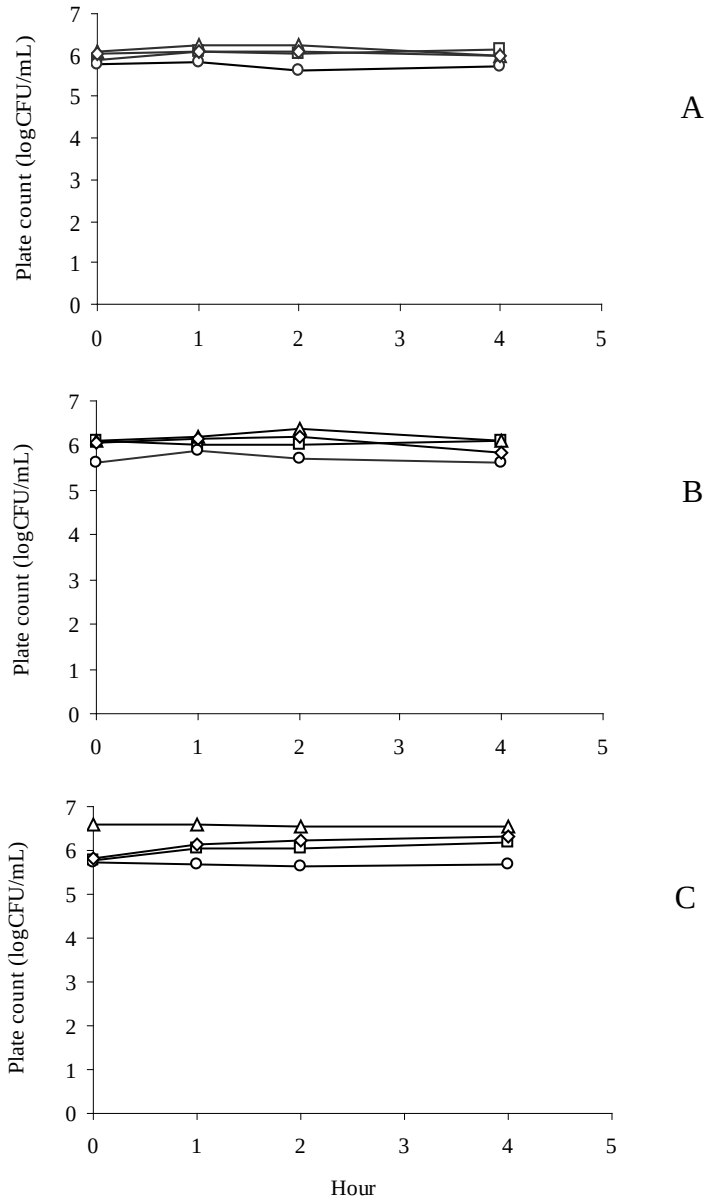

Figure 3.8 The viable counts of foodborne pathogens in MMRS at 37°C, at pH 4.4 (A), 5.0 (B) and 6.5 (C). *E. coli* O157:H7 (□), *L. monocytogenes* (∆), *S. aureus* (○) and *S.* Typhimurium (◊). Data represent the mean of two replicates.

3.3.7 Co-cultivation of *L. reuteri* DPC16 and pathogens in broth

The antimicrobial effects of the *L. reuteri* DPC16 against *L. monocytogenes*, *E. coli* O157:H7, *S.* Typhimurium and *S. aureus* in co-cultures are shown in Figure 3.9.

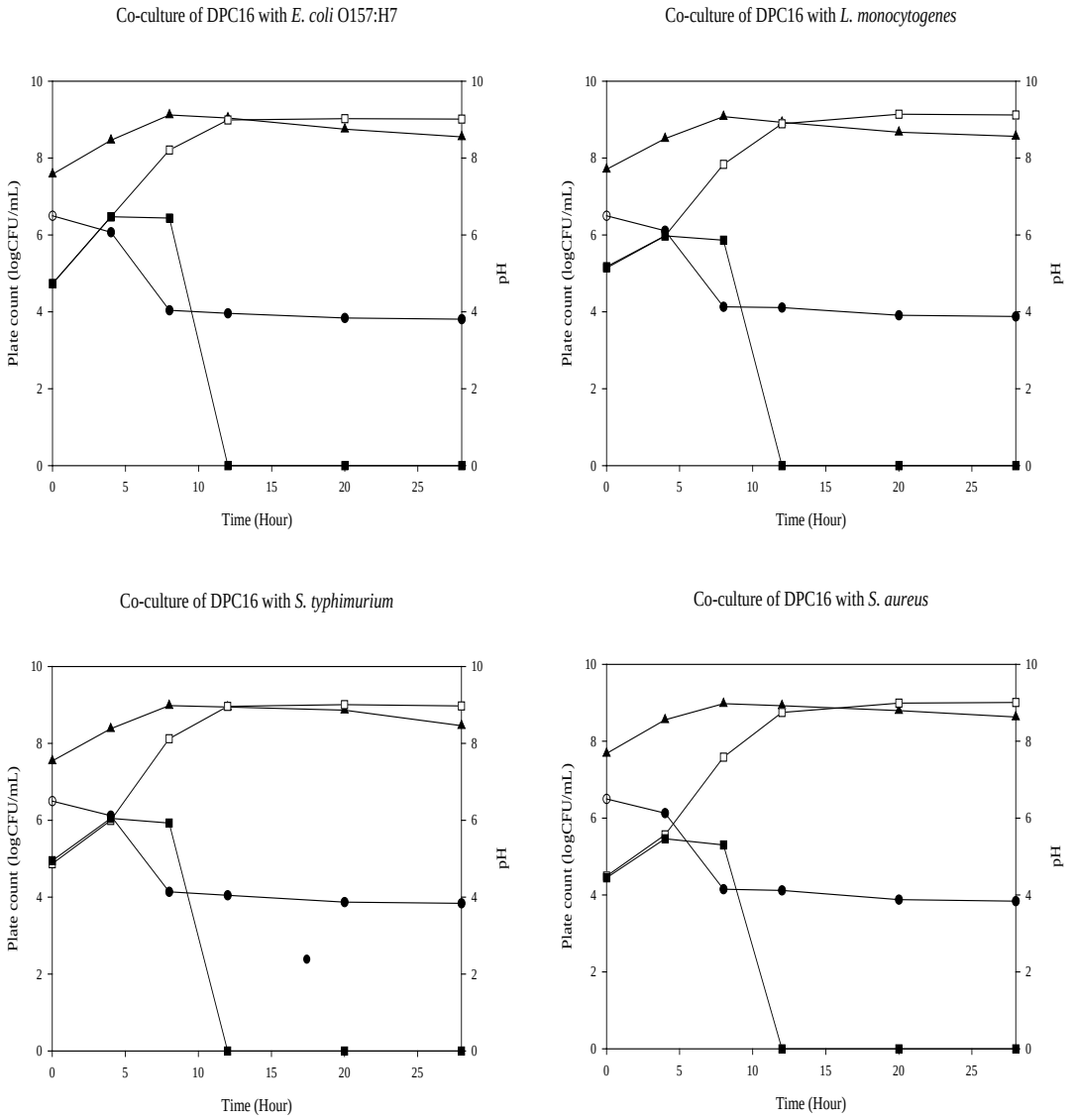

Figure 3.9 Effect of co-cultivation on viable counts of *L. reuteri* DPC16 and the test pathogens in MMRS with an initial pH of 6.5 at 37°C. *L. reuteri* DPC16 grown alone (▲); the test pathogen grown alone (□); the growth of test pathogen in co -culture with *L. reuteri* DPC16 (■); and pH change during co-culture (●). Data represent the mean of three replicates.

The growth of *L. reuteri* DPC16 reached its highest cell density after approximately 8 h, with the pH dropping from the initial pH of 6.5 to a minimum level of approximately 4, when co-cultured with all tested pathogens. After reaching its peak concentration, the number of viable *L. reuteri* DPC16 cells gradually declined (Figure 3.9) but by less than 1 log CFU/mL in the following 16 h. This indicates that *L. reuteri* DPC16 can tolerate pH 4.

The tested pathogens were observed to grow during the early stage (about 4 h after inoculation) in the co-cultivation, but their growth was then inhibited. When the concentration of *L. reuteri* DPC16 reached its highest cell density (about 8 h after inoculation), the pathogens started a rapid decline in their viable numbers and became undetectable by 12 h (Figure 3.9).

3.3.8 Co-cultivation of *L. reuteri* DPC16 and *L. monocytogenes* in broth at a constant pH value (pH 6.5)

The growth patterns of *L. reuteri* DPC16 were similar, whether grown alone, or in co-cultivation with *L. monocytogenes* at 37^{o}C, with or without pH control at 6.5 (Figure 3.10). This observation suggests that the growth of *L. reuteri* DPC16 is independent of pH in this specific co-cultivation condition. However, for *L. monocytogenes*, when grown in co-culture, growth slowed after 4 - 8 h of incubation, and the viable count decreased rapidly after 10 - 20 h, depending on the pH value.

These results demonstrate that *L. reuteri* DPC16 is inhibitory and bactericidal to *L. monocytogenes*, but the effect is less marked when the culture pH value was maintained at pH 6.5.

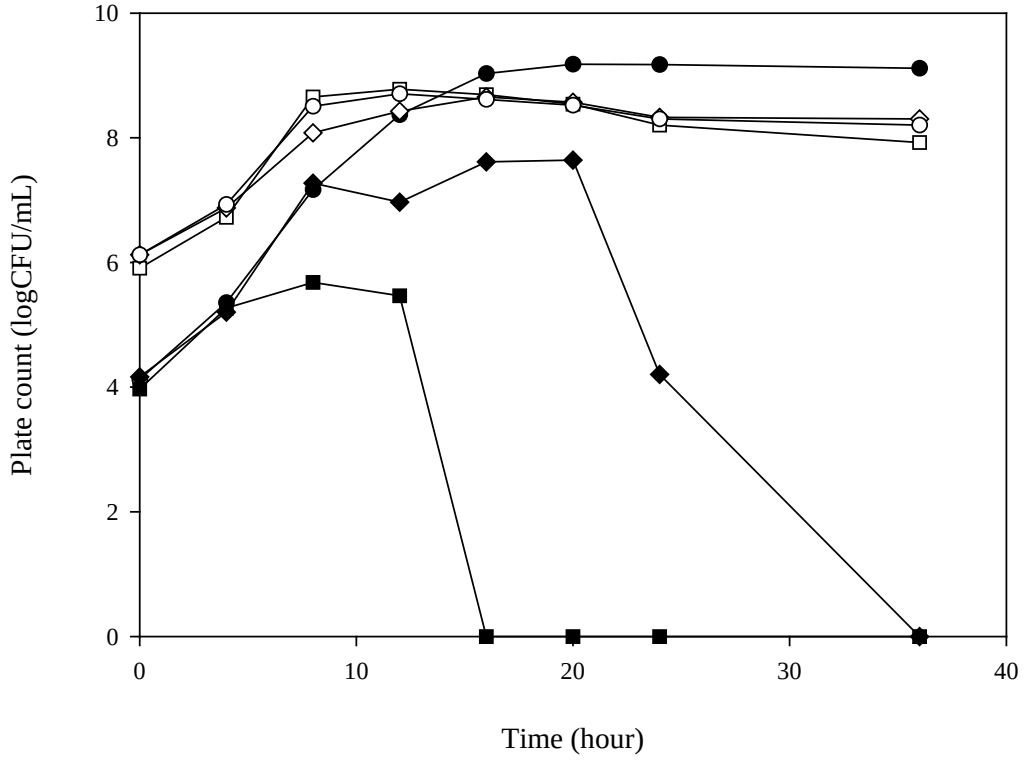

Figure 3.10 Growth of *L. reuteri* DPC16 and *L. monocytogenes* alone, or in co-cultivation, with or without pH control at pH 6.5, 37°C. The growth of *L. reuteri* DPC16 alone (O), in co-cultivation with pH unadjusted (□), or pH control at pH 6.5 (◊). The growth of *L. monocytogenes* alone (●), in co-cultivation with pH unadjusted (■), or pH control at pH 6.5 (♦). Data presented as mean of two replicates.

3.3.9 Detection of glycerol dehydratase (GD) gene in LAB strains

A 279 bp fragment from 2 strains of *L. reuteri* (P23 and DPC16) was amplified by PCR with primers GD1 and GD2 (Figure 3.11). The sequence of the fragment corresponds to a glycerol dehydratase subunit in *L. reuteri*. Thus, these two strains were confirmed to have glycerol dehydratase activity and to be able to produce reuterin from glycerol (Claisse & Lonvaud-Funel, 2001). This result supports the ability of the two *L. reuteri* strains to produce reuterin and explains the high level of antimicrobial activity observed for these two strains.

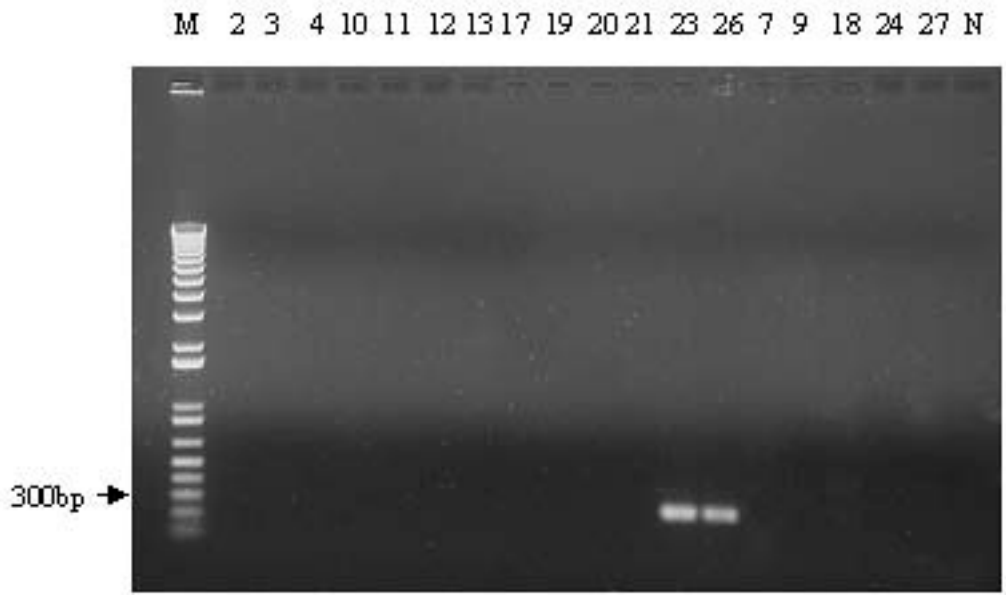

Figure 3.11 PCR detection of glycerol dehydratase gene in selected LAB strains. M: 1kb plus DNA molecular marker from Invitrogen; Lanes 2, 3, 4, 10, 11, 12, 13, 17, 19, 20, 21, 23 and 26 correspond to *L. reuteri* strains P2, P3, P4, P10, P11, P12, P13, P17, P19, P20, P21, P23 and DPC16, respectively; Lane 7: *L. mucosae*; Lane 9: *Enterococcus faecium*; Lanes 18, 24 and 27 correspond to *Leuconostoc garlicum* strains P18, P24 and P27, respectively. N: Negative control.

Nucleotide sequence analysis showed that the amplicon has 79, 74.7, 73, 70, and 70.8% identities with the gene (gldC) of the large subunit (GldC) of glycerol dehydratase of *Lactobacillus collinoides* (GenBank assession #AF166493), *Lactobacillus diolivorans* (GenBank assession #AY061968), *Lactobacillus hilgardii* (GenBank assession #AY061969), *Listeria innocua* Clip11262 (GenBank assession #AL596167), and *Listeria monocytogenes* 4b F2365 (GenBank assession #AE017325), respectively (Figure 3.12). The deduced amino acids encoded by the amplicon have 85.7, 83.1, 88,3, 79.2, and 79.2% identities with the same gene of *L. collinoides, L. diolivorans, L. hilgardii, L. innocua* Clip11262, and *L. monocytogenes* 4b F2365, respectively (Figure 3.13).

```
L. reuteri DPC16              GCGGCTGATG CTGCTGATGC TGCGCTTCGT GGTTTCCCAG AACAAGAAAC
L. collinoides                GCGGCTGATG CTGCCGAAGC TGCATTACGT GGGGTTCCTG AAGAAGAAAC
L. diolivorans                GCCGCTGATG CTGCTGAAGC TTCACTACGT GGATTTCCTG AACAAGAAAC
L. hilgardii                  GCCGCTGATG CTGCAGAAGC TGCTCTTAGA GGATTCCCGG AACAGGAAAC
L. innocua Clip11262          GCAGCAGATG CAGCAGAGGC AGCAATTCGT GGTTTTGACG AGCAAGAAAC
L. monocytogenes 4b F2365     GCTGCAGATG CGGCAGAAGC AGCAATTCGT GGTTTTGACG AGCAAGAAAC

L. reuteri DPC16              TACTACTGCC GTTGCCCGTT ATGCACCATT TAATGCTATT TCAATCTTAA
L. collinoides                CACCACTGCC ATTGCTCGGT ATGCGCCAAT GAACGCTATT TCAATCATGG
L. diolivorans                CACGACTGCG ACTGCTCGGT ACGCACCTTT AAATGCGATT TCAATTATGG
L. hilgardii                  GACGACCGCA GTCGCTCGTT ATGCACCGAT GAATGCTATT TCGATCATGG
L. innocua Clip11262          AACCGTTGCT GTAGTTCGTT ATGCACCTTT CAACGCACTT AGCTTATTGG
L. monocytogenes 4b F2365     AACCGTTGCG GTAGTTCGTT ATGCACCTTT TAACGCGCTT AGTTTATTAG

L. reuteri DPC16              TTGGTGCTCA AACAGGTCGT CCTGGTGTAT TAACACAATG TTCTGTTGAA
L. collinoides                TTGGGGCCCA AGCAGGCCGT CCTGGTGTTA TCACCCAATG TTCAGTTGAA
L. diolivorans                TTGGTTCACA AACTGGTCGT CCAGGTGTTA TCTCCCAGTG TTCAGTTGAG
L. hilgardii                  TTGGTTCGCA AACGGGGCGT CCAGGGGTGA TTACACAATG TTCCGTTGAG
L. innocua Clip11262          TAGGTTCGCA AACAGGCCGT GGTGGCGTAT TAACCCAATG TTCTCTTGAA
L. monocytogenes 4b F2365     TAGGTTCGCA AACTGGCCGT GGTGGCGTAT TAACGCAATG TTCTCTCGAA

L. reuteri DPC16              GAAGCAACCG AATTGCAATT AGGAATGCGT GGCTTTACCG CTTATGCTGA
L. collinoides                GAAGCTGACG AATTGAGTTT GGGGATGCGT GGGTTTACTG CCTATGCTGA
L. diolivorans                GGATCAGAGG AATTATCATT AGGGATGCGT GGCTTTACGG CCTATGCTGA
L. hilgardii                  GAATCGGAAG AACTCAGTTT GGGGATGCGT GGTTTCACTG CATACGCAGA
L. innocua Clip11262          GAAGCAACAG AATTAGAGCT TGGTATGCGC GGTTTAACTT GTTACGCTGA
L. monocytogenes 4b F2365     GAAGCAACAG AATTAGAACT CGGTATGCGT GGTTTAACTT GTTATGCAGA

L. reuteri DPC16              AACTATTTCA GTTTATGGTA CTGACCGGGT CTT
L. collinoides                AACCATTTCA GTTTATGGGA CTGACCGGGT CTT
L. diolivorans                AACCATTTCA GTTTATGGGA CCGATCGAGT ATT
L. hilgardii                  AACCATTTCA GTTTATGGCA CTGATCGTGT ATT
L. innocua Clip11262          AACGATTTCT GTTTATGGTA CAGAACCTGT ATT
L. monocytogenes 4b F2365     AACGATTTCT GTTTATGGTA CAGAACCTGT ATT
```

Figure 3.12 Nucleotide sequence alignment of the 233-bp fragment with a region of the glycerol dehydratase genes of *L. collinoides* (GenBank assession #AF166493), *L. diolivorans* (GenBank assession #AY061968), *L. hilgardii* (GenBank assession #AY061969), *L. innocua* Clip11262 (GenBank assession #AL596167), and *L. monocytogenes* 4b F2365 with identities of 79, 74.7, 73, 70, and 70.8%, respectively. Distinct bases are shaded dark.

```
L. reuteri DPC16           AADAADAALR GFPEQETTTA VARYAPFNAI SILIGAQTGR PGVLTQCSVE
L. collinoides             AADAAEAALR GVPEEETTTA IARYAPMNAI SIMVGAQAGR PGVITQCSVE
L. diolivorans             AADAAEASLR GFPEQETTTA TARYAPLNAI SIMVGSQTGR PGVISQCSVE
L. hilgardii               AADAAEAALR GFPEQETTTA VARYAPMNAI SIMVGSQTGR PGVITQCSVE
L. innocua Clip11262       AADAAEAAIR GFDEQETTVA VVRYAPFNAL SLLVGSQTGR GGVLTQCSLE
L. monocytogenes 4b F2365  AADAAEAAIR GFDEQETTVA VVRYAPFNAL SLLVGSQTGR GGVLTQCSLE

L. reuteri DPC16           EATELQLGMR GFTAYAETIS VYGTDRV
L. collinoides             EADELSLGMR GFTAYAETIS VYGTDRV
L. diolivorans             GSEELSLGMR GFTAYAETIS VYGTDRV
L. hilgardii               ESEELSLGMR GFTAYAETIS VYGTDRV
L. innocua Clip11262       EATELELGMR GLTCYAETIS VYGTEPV
L. monocytogenes 4b F2365  EATELELGMR GLTCYAETIS VYGTEPV
```

Figure 3.13 Sequence alignment of a fragment of 77 amino acids in a region of the glycerol dehydratase of *L. collinoides* (GenBank assession #AF166493), *L. diolivorans* (GenBank assession #AY061968), *L. hilgardii* (GenBank assession #AY061969), *L. innocua* Clip11262 (GenBank assession #AL596167), and *L. monocytogenes* 4b F2365 with identities of 85.7, 83.1, 88,3, 79.2, and 79.2%, respectively. Distinct amino acids are shaded dark.

3.4 Discussion

The objective of this study was to screen selected LAB strains for their antimicrobial capacity towards some foodborne pathogens and to evaluate their potential for use as biopreservatives. Evaluation was achieved using *in vitro* assessment techniques to determine their ability to produce antagonistic substances against four foodborne pathogens: *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*.

Eighteen LAB strains were phylogenetically identified. Among them, 13 strains belong to *L. reuteri*, 3 strains belong to *Leuconostoc garlicum*, one belongs to *L. mucosae*, and one belongs to *Enterococcus faecium*, according to their 16S rRNA gene sequences. Their antimicrobial activity were screened and most of them (11/18) had antimicrobial activity against all 4 test pathogens, while some of them (3/18) were active against only one or two of the tested pathogens in an agar diffusion assay.

L. reuteri DPC16 showed the strongest antimicrobial activity against the tested pathogens in agar diffusion assays and this organism was further characterised. Although it has been reported that *L. reuteri* strains can produce a variety of antimicrobial compounds, including reutericin 6 (Kabuchi *et al*., 1997), reutericyclin (Ganzle *et al*., 2000; Holtzel *et al*., 2000) and reuterin (Talarico *et al*., 1988; Axelsson *et al*., 1989), the antimicrobial activity of *L. reuteri* DPC16 appeared to be due mainly to the production of reuterin as well as the production of lactic acid. In the present study, the antimicrobial substance of *L. reuteri* DPC16 was found to be non-proteinaceous, active at a wide range of pH values, insensitive to hydrogen peroxidase, and heat labile. Therefore, the antimicrobial effect presented by *L. reuteri* DPC16 is unlikely to be due to the proteinaceous bacteriocins (eg. reutericin 6) (Kabuchi *et al*., 1997; Kawai *et al*., 2004), lactic acid, or hydrogen peroxide. On the other hand, the efficient inhibitory activity against Gram-negative bacteria (eg. *S*. Typhimurium and *E. coli* O157:H7) excludes the role of reutericyclin which is ineffective against Gram-negative bacteria (Axelsson *et al*., 1989; Ganzle *et al*., 2000).

Detection of reuterin in the culture supernatant confirmed its major role in the antimicrobial activity against the tested pathogens. Detection of the glycerol dehydratase gene also supports the production of reuterin by *L. reuteri* DPC16 and the role of reuterin in the antimicriobial activity (Rodriguez *et al*., 2003).

The antimicrobial effect of *L. reuteri* DPC16 was demonstrated during co-cultivation with the test pathogens, and also in the spent culture supernatant. It was found in the co-cultivation experiments that *L. reuteri* DPC16 inhibited the growth of all of the pathogens under study. In addition, *L. monocytogenes* was inhibited when grown at a constant value of pH 6.5.

The inhibitory effect of *L. reuteri* DPC16 during co-cultivation with the test pathogens coincided with its production of reuterin, thus supporting the postulated role of reuterin in the inhibitory activity. The maximum concentration of reuterin was reached between 12 and 16 hours of growth when *L. reuteri* DPC16 was grown at 35°C. In the co-cultivation of *L. reuteri* DPC16 and *L. monocytogenes*, an inhibitory effect on *L.*

monocytogenes was observed after 4 to 8 hours when incubated without pH control, and after 8 to 12 hours with pH control at 6.5. This was when the concentration of reuterin in the co-cultivation solution had reached approximately 30 µg/mL. It was found that the concentration of reuterin gradually declined after it reached the maximum concentration. This is probably because of the conversion of reuterin into 1,3-propanediol, the end-product of glycerol fermentation, by enzymatic reaction of an NAD^+-dependant oxidoreductase (Biebl *et al*., 1999; Daniel *et al*., 1999; Zeng & Biebl, 2002), whose expression could possibly be triggered by the accumulation of reuterin.

The antimicrobial effect of *L. reuteri* DPC16 was also demonstrated in this study by incubation of the spent culture supernatant of *L. reuteri* DPC16 with the pathogens. There was a strong bactericidal effect against the tested pathogens at pH values of 4.4 and 6.5 and at both 10 and 37^{o}C. This effect was impaired with increasing pH and decreasing temperature. It has previously been suggested that reuterin might be less reactive at low temperatures (Doleyres *et al*., 2005). The results in the present study are consistent with the findings of others, who also found a higher antimicrobial effect of reuterin at higher temperatures and at lower pH against *Escherichia coli* K12 (MG1655) (Rasch, 2002). A similar result was found by Liang *et al*. (2003) with increasing effectiveness of reuterin against *Pseudomonas aeruginosa* at increasing temperature (25 - 45^{o}C). Talarico *et al*. (1988) reported that reuterin was more stable under acidic conditions than the corresponding neutral sample and degraded immediately when exposed to pH 11. This is consistent with the results of the present study where there is stronger antimicrobial activity at lower pH. Similar results have been reported for the antimicrobial activity of some other LAB which presented pronounced antimicrobial activity at low pH (5.2 - 6.1) and no inhibitory effect at an increased pH (6.6 - 7.1) (Jeppesen & Huss, 1993).

Lactic acid production is a common characteristic of lactobacilli. In this study, the capability of lactic acid production by *L. reuteri* DPC16 was assessed. The concentration of L-lactic acid was 2.6 - 2.8 g/L at optimal growth condition, which is comparable to other lactobacilli (Tomas *et al*., 2003b; Fayol-Messaoudi *et al*., 2005). There have been reports that lactic acid itself might be antimicrobial (Fayol-Messaoudi

et al., 2005; Wilson *et al*., 2005; Makras *et al*., 2006). In the present study, production of lactic acid by *L. reuteri* DPC16 contributed to the decrease of pH to around 4.0, thus creating a detrimental acidic environment. It, therefore, presented a synergistic antimicrobial activity along with reuterin. Lactic acid may have played a secondary rather than a major role in the inhibitory activity against the test pathogens, as this inhibitory effect remains evident at a pH of 6.5.

In recent years, biopreservation using lactobacilli as a potential strategy to promote food safety and shelf life has been studied extensively. It is known that lactobacilli might exhibit their antimicrobial effect through their metabolites, such as bacteriocin, lactic acid, hydrogen peroxide, and short chain fatty acids (Drago *et al*., 1997), and these have been the main targets for such applications. However, concerns have been expressed over the use of bacteriocinogenic LAB (or pure bacteriocin) due to the ubiquitous existence of proteases and the development of bacteriocin resistance (Nilsson *et al.*, 2005). It has been found that reuterin has a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria. In this study, *L. reuteri* DPC16 and/or its metabolite reuterin have been demonstrated to be inhibitory against the tested foodborne pathogens *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*. This characteristic of *L. reuteri* DPC16 and reuterin have made it an unique and potent candidate to control both Gram-positive and Gram-negative bacteria on foods. Thus, the application of *L. reuteri* DPC16 and/or reuterin as a biopreservative could be a feasible option to extend the shelf life and reduce the microbiological risk of foods.

In summary, this study has demonstrated the antagonistic effects of four groups of LAB strains against common foodborne pathogens. The research focused on one of the most antagonistic, *L. reuteri* DPC16, to explore its antimicrobial effects. The result showed that *L. reuteri* DPC16 exerted a strong antimicrobial activity against both Gram-positive and Gram-negative pathogens due to the production of reuterin. This characterisation of *L. reuteri* DPC16 could make it an advantageous alternative for use in biopreservation.

Chapter 4 Combined effects of controlled atmosphere and culture supernatant of *L. reuteri* DPC16 for controlling *L. monocytogenes*

4.1 Introduction

Lactobacillus reuteri is an obligatory heterofermentative microorganism that resides in the gastrointestinal tract of humans and most animals (Axelsson & Lindgren, 1987; Naito *et al*., 1995). Reuterin (β-hydroxypropionaldehyde) is the heterofermentative product of some *L. reuteri* strains (Axelsson *et al*., 1987; Talarico *et al*., 1988; Axelsson *et al*., 1989). Reuterin has antimicrobial activity against a broad range of foodborne pathogens (Axelsson *et al*., 1989; Chung *et al*., 1989a; Talarico & Dobrogosz, 1989). Reuterin is water soluble, effective in a wide range of pH values, resistant to proteolytic and lipolytic enzymes (Axelsson *et al*., 1989). Overall, it is therefore suitable for use as a biopreservation agent.

The antimicrobial activity of reuterin against foodborne pathogens has been investigated in milk and cottage cheese (El-Ziney & Debevere, 1998), meat (El-Ziney *et al*., 1999), and sausage (Kuleasan & Cakmakci, 2002). El-Ziney & Debevere (1998) demonstrated that addition of reuterin at 50 to 250 units per g to milk and cottage cheese decreased numbers of *L. monocytogenes* and *E. coli* O157:H7. The inactivation rate was more pronounced with *E. coli* O157:H7 than with *L. monocytogenes* and it was dependent on reuterin concentration. Decreased numbers of *L. monocytogenes* and *E. coli* O157:H7 due to the addition of reuterin has also been shown in ground pork (El-Ziney *et al*., 1999). Kuleasan & Cakmakci (2002) found that application of reuterin to the surface of sausage considerably inhibited the growth of *L. monocytogenes* but had no effect on the growth of *Salmonella* spp. under the same conditions. Muthukumarasamy *et al*. (2003) investigated the application of *L. reuteri* in vacuum packaged refrigerated (4^{o}C) ground beef for its ability to reduce the number of *E. coli* O157:H7 during storage and found

that *L. reuteri* was highly effective against *E. coli* O157:H7 in the presence of glycerol. More recently, it was found that reuterin increased the lag time of single cells and completely prevented the cell division of *Listeria innocua* grown on the surface of Brain Heart Infusion Agar (Rasch *et al*., 2007). These results indicate that *L. reuteri* and/or its fermentative product, reuterin, could be useful as biopreservation agents.

Research has demonstrated that modified atmospheres (MA) offers multiple advantages to the food industry and to the consumer. MA has been applied to extend the shelf life of a wide range of food products via either controlled atmosphere (CA) or modified atmosphere packaging (MAP) (Church, 1993). MA usually includes carbon dioxide, nitrogen and oxygen (Randell *et al*., 1997; Gimenez *et al*., 2002). Carbon dioxide acts as an antimicrobial agent (Stammen *et al*., 1990) and is able to inhibit the growth of microorganisms during the logarithmic phase and extend the lag phase (Genigeorgis, 1985; Church, 1994). The use of MA with an enhanced carbon dioxide level has been shown to extend the shelf life of fresh fishery products by retarding microbial growth (Farber, 1991; De La Hoz *et al*., 2000; Emborg *et al*., 2002). However, it has been suggested that high concentrations of CO_2 should be avoided in packed fish products with MA as it dissolves into the fish juice and then deforms the package (Stenstrom, 1985). Therefore, it would be desirable to find a condition in which a minimised concentration of CO_2 was used while the maximum food safety and shelf life were achieved.

The combined effect of MA with other reagents on the growth of bacteria has been investigated by several groups. Pothuri *et al*. (1996) found that a combination of modified atmosphere (74.8% CO_2, 10.4% O_2, and 14.8% N_2) and lactic acid (1 - 2%) at 4^oC inhibited the growth of *L. monocytogenes* on packed crayfish tail meats. Skandamis & Nychas (2002) reported that the combination of MAP (40% CO_2:30% N_2:30% O_2, 80% CO_2:20% air, or 100% CO_2) with a piece of filter paper soaked with volatile compounds of oregano essential oil (in the package but not touching the product) extended the shelf life of fresh meat when stored at 5 and 15^oC. There was a synergistic effect between the volatile compounds of oregano essential oil and the modified atmosphere packaging. Szabo & Cahill (1998) found, in a controlled atmosphere broth system, that a combination of MA (40% CO_2:60% N_2, or 100% CO_2) and nisin (400

IU/mL, or 1250 IU/mL) or ALTA^{TM} 2341 (a commercial crude fermentation product of LAB manufactured by Quest International) (0.1%, or 1.0%) at 4 and 12^{o}C affected the growth of *L. monocytogenes* in terms of extending the lag phase and decreasing the exponential growth rate that could not be achieved by MA alone. Paludan-Muller *et al*. (1998) reported that a combination of nisin (1,000 IU/g sample) and CO_2 atmosphere (60% CO_2:40% N_2) at 5^{o}C increased the shelf life of cold-smoked salmon, with nisin inhibiting the growth of Gram-positive bacteria while the CO_2-atmosphere inhibited the growth of Gram-negative bacteria (nisin having no effect on Gram-negative bacteria). Also, Muthukumarasamy *et al*. (2003) found that *L. reuteri* (at input levels of 3 and 6 log CFU/g) in the presence of glycerol (250 mM) killed *E. coli* O157:H7 at medium (3 log CFU/g) or high (6 log CFU/g) inoculated levels before day 20 in vacuum packed ground beef during refrigerated storage (4^{o}C) showing that *L. reuteri* is highly effective against *E. coli* O157:H7. All of this research suggested that there is substantial potential for the effective combination of MA and antimicrobial compounds to prevent the growth of *L. monocytogenes*.

The results of the present study (chapter 3) have shown that the spent culture supernatant of *L. reuteri* DPC16 (DPC16-SCS) inhibited the growth of common foodborne pathogens (*E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus*). The characterisation of the DPC16-SCS showed that the antimicrobial substance was most likely reuterin, as it is resistant to proteolytic enzymes and effective at a wide range of pH values. However, the antimicrobial substance was less effective at low temperatures and weak acidic pH values, conditions normally encountered during the storage of fish and many other food products. No research has been published on any combined inhibitory effect of the antimicrobial substances (including reuterin) produced by *L. reuteri* and MA against foodborne pathogens, whereas the latter is commonly used in the food industry.

In this study, the combined antimicrobial effect of *L. reuteri* DPC16 and a controlled atmosphere (CA) against *L. monocytogenes* was assessed using DPC16-SCS and the fermentative supernatant of *L. reuteri* DPC16 from a glycerol-water solution (DPC16-GFS). DPC16-SCS would be expected to contain reuterin plus other antimicrobial substances, whereas reuterin should be the only antimicrobial substance in DPC16-GFS.

The aim was to explore the relative potential of these combinations to control *L. monocytogenes* and thereby to improve the safety and shelf life of food products.

4.2 Materials and Methods

4.2.1 Bacterial strain, growth medium, and LAB culture supernatant

L. monocytogenes (see Section 2.2.1 for detail) was used as the test pathogen to evaluate the antimicrobial effect of combining CA with the culture supernatant of *L. reuteri* DPC16. *L. monocytogenes* was sub-cultured in MMRS following the procedures described in Section 2.2.1. The resulting subculture was stored at 4^{o}C and used as inoculum. The concentration of the inoculum was determined following the method described in Section 2.2.2.

The spent culture supernatant of *L. reuteri* DPC16 (DPC16-SCS) was prepared following the method described in Section 3.2.4.1.

4.2.2 Preparation of glycerol fermentative solution of *L. reuteri* DPC16 (DPC16-GFS)

The DPC16-GFS was prepared following a two-step fermentation method described by Luthi-Peng *et al*. (2002) with modifications. Briefly, a 1% inoculum of an overnight broth culture of *L. reuteri* DPC16 was grown for 16 h in MRS supplemented with glycerol at a concentration of 250 mM. Bacterial cells were harvested by centrifugation at 4,000 x g for 10 min and washed twice with 50 mM phosphate buffered solution (PBS) (pH 7.5). After being washed, cells from the centrifuge pellet were weighed and suspended to a concentration of 10 mg of cells per mL of deionised water. Glycerol, previously sterilised by autoclaving, was added to a concentration of 250 mM, and this suspension was incubated in a screw-top tube at 37°C for 2 h to produce and accumulate reuterin. Cells were pelleted at 4,000 x g for 10 min and discarded. The supernatant fluid was filtered through an Acrodisc filter (0.45 µm) (Pall, East Hills, NY, USA) to remove any remaining cells. Filtered supernatant fluid (designated as DPC16 glycerol fermentative solution or DPC16-GFS) was frozen at -20°C until the time of use.

4.2.3 Determination of arbitrary units of DPC16-SCS and -GFS

The determination of arbitrary units (AU) of DPC16-SCS and -GFS against the test pathogens was carried out following the method described by Juarez *at el*. (2002). Briefly, DPC16-SCS and -GFS were neutralised to pH 6.5 with 2 M HCl and subjected to sterilisation using Acrodisc filters (0.2 µm) (Pall, East Hills, NY, USA). A series of 2-fold dilutions was made in 0.1% sterile peptone water from 1:1 to 1:128.

The diluted solutions were subjected to an agar diffusion assay as described in Section 3.2.4.2. The arbitrary units were calculated as the reciprocal of the highest dilution that produced a distinct inhibition zone per mL (AU/mL).

4.2.4 Preparation of buffered MMRS

The medium used in the experiments was buffered MMRS (bMMRS) at a final pH value of 6.5. It was made by constitution of the required amount of reagents for MMRS in 0.2 M phosphate buffer at an initial pH value of 7.0, which was predetermined to give a pH of 6.5 after autoclaving and equilibration under CA. When the bMMRS (pH 6.5) was used as the control under non-CA, it was prepared in 0.2 M phosphate buffer, pH 6.5 (For detail, see Appendix I).

4.2.5 Inhibitory activity of DPC16-SCS and DPC16-GFS in combination with CA against *L. monocytogenes* in buffered MMRS broth

The inhibitory activities of DPC-SCS and DPC-GFS, and their combination with controlled atmosphere (CA), against *L. monocytogenes* were investigated in bMMRS in triplicate experiments following the same procedures as described in Section 2.2. DPC-SCS and DPC-GFS were diluted in bMMRS to a final concentration equivalent to 25 AU/mL against *L. monocytogenes* and filter-sterilised through an Acrodisc filter (0.2 µm) (Pall, East Hills, NY, USA). The diluted DPC-SCS or DPC-GFS (100 mL) was added into flasks and then equilibrated overnight under CA (60% CO_2:40% N_2) at a flow rate of 40 mL/min. *L. monocytogenes* was inoculated to give a final concentration of approximately 1 x 10^4 CFU/mL. Flasks containing bMMRS broth inoculated with *L. monocytogenes* without DPC-SCS or DPC-GFS were also prepared. The bMMRS broth,

with or without addition of DPC-SCS or DPC-GFS, but inoculated with *L. monocytogenes* and held in air, served as a control.

Microbiological counts were determined on BHI agar plates periodically at intervals based on the growth conditions (4 to 8 h intervals) following the method described in Section 2.2.2.

4.2.6 Experimental design

The experiments were carried out with different levels of the independent variables (Table 4.1). The controls included bMMRS under CA only, bMMRS under non-CA only, DPC16-SCS under non-CA (air), and DPC16-GFS under non-CA (air). The variables at different levels produced 12 combinations of experiment. Each of the combinations was carried out in triplicate, except bMMRS under CA only and bMMRS under non-CA only, which have two replicates respectively.

Table 4.1 Variables and their usage levels

Variable	**Values**
Temperature	4^oC, 20^oC
Concentration of LAB culture supernatant[a]	None
	DPC16-SCS 25% (25 AU/mL)[b]
	DPC16-GFS 12.5% (25 AU/mL)[b]
Initial pH	6.5[b]
Gas mix	Air, 60% CO_2

[a] The concentrations of the LAB culture supernatant are equivalent to their minimum inhibitory concentrations determined by agar gel diffusion assay.

[b] The values are all the initial values in the broth.

4.2.7 Microbiological analysis, curve fitting and analysis of growth parameters

A standard plate count (drop plate method) was carried out on BHI agar plates to enumerate the growth of *L. monocytogenes*, following the method described in Section 2.2.2.

The growth data of *L. monocytogenes* were fitted using the Gompertz model (Gompertz solver 1.0, Agricultural Research Service, USA) and the predicted kinetic growth parameters such as lag phase duration (LPD) and exponential growth rate (EGR) were generated for comparison.

4.3 Results

4.3.1 Effect of controlled atmosphere on the growth of *L. monocytogenes* in the broth system

The controlled atmosphere alone, at 60% CO_2, decreased the exponential growth rate (EGR) of *L. monocytogenes* in bMMRS broth at 4 and 20^{o}C and increased the lag phase duration (LPD) (Figure 4.1 and Table 4.2). These effects of CA on EGR and LPD were more pronounced at 4^{o}C than at 20^{o}C.

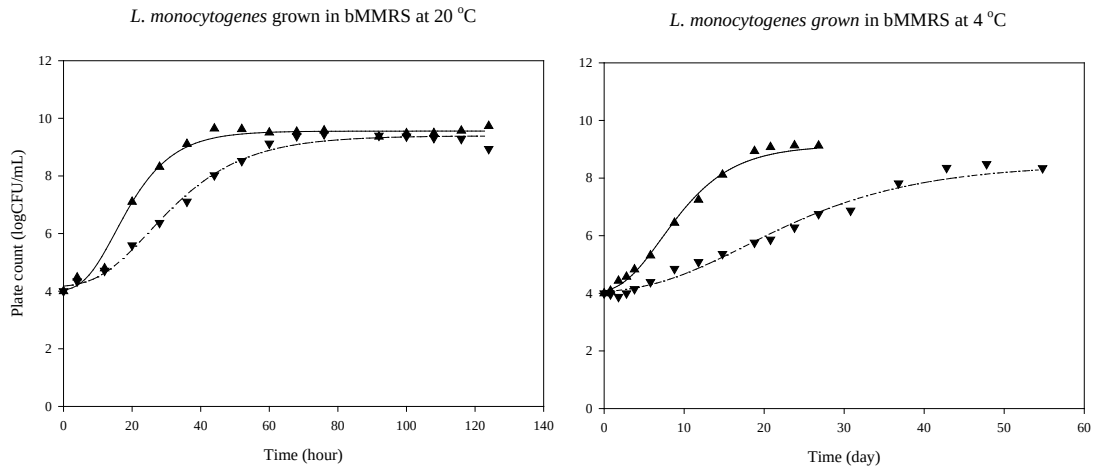

Figure 4.1 Fitted curves of the growth of *L. monocytogenes* in bMMRS under CA of 60% CO_2 (▼) or non-CA (▲) at 4 or 20^{o}C. The data points are the mean of two replicates.

Table 4.2 Parameters generated by Gompertz model for the growth of *L. monocytogenes* (mean ± SD)

Kinetic Parameters, 60% CO_2, 20°C						
	bMMRS, DPC16-SCS, CA[a]	bMMRS, DPC16-GFS, CA[a]	bMMRS, CA[b]	bMMRS, DPC16-SCS, non-CA[a]	bMMRS, DPC16-GFS, non-CA[a]	bMMRS. Non-CA[b]
EGR (h^{-1})	0.052 ± 0.004	0.066 ± 0.0119	0.13 ± 0.017	0.12 ± 0.017	0.097 ± 0.011	0.222 ± 0.026
LPD (h)	16.94 ± 0.959	53.69 ± 4.556	9.98 ± 0.865	14.80 ± 1.153	29.43 ± 0.810	5.94 ± 0.422
Kinetic Parameters, 60% CO_2, 4°C						
	bMMRS, DPC16-SCS, CA[a]	bMMRS, DPC16-GFS, CA[a]	bMMRS, CA[b]	bMMRS, DPC16-SCS, non-CA[a]	bMMRS, DPC16-GFS, non-CA[a]	bMMRS , Non-CA[b]
EGR (h^{-1})	0.0037 ± 0.001	0.0047 ± 0.0006	0.0058 ± 0.0012	0.011 ± 0.001	0.0083 ± 0.0011	0.0159 ± 0.002
LPD (h)	202.22 ± 7.60	192.09 ± 7.99	137.88 ± 13.02	63.23 ± 4.623	94.19 ± 6.091	52.93 ± 5.035

[a] Mean of three replicate values;

[b] Mean of two replicate values.

SD: Standard deviation.

4.3.2 Combined effect of DPC16-SCS and controlled atmosphere on the growth of *L. monocytogenes* in the broth system

The combined effect of DPC16-SCS with CA on the growth of *L. monocytogenes* at 4 and 20°C is shown in Figure 4.2 and summarised in Table 4.2. At both 4 and 20°C, the combination of DPC16-SCS (25 AU/mL) and CA (60% CO_2) increased the LPD and decreased the EGR of *L. monocytogenes* when compared to that with either CA or DPC16-SCS alone, or non-CA without DPC16-SCS. The effect of the combination of DPC16-SCS and CA on the growth of *L. monocytogenes* was more pronounced at 4°C than at 20°C. The impact of the growth condition combinations on both EGR and LPD at 20°C were: DPC16-SCS plus CA > DPC16-SCS alone > CA alone > non-CA without DPC16-SCS. At 4°C, the order was: DPC16-SCS plus CA > CA alone > DPC16-SCS alone > non-CA without DPC16-SCS.

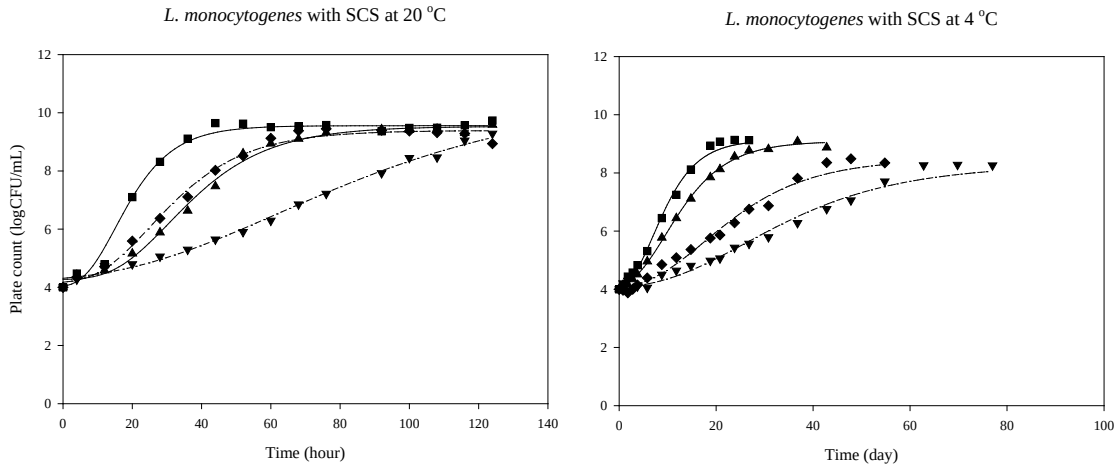

Figure 4.2 Fitted curves of the growth of *L. monocytogenes* with DPC16-SCS (25 AU/mL) under CA of 60% CO_2 (▼) or non-CA (▲), without DPC16-SCS under CA (♦) or non-CA (control) (■) at 4 or 20°C. The data points are the mean of three replicates, except the controls (♦) and (■) which are the mean of two replicates.

4.3.3 Combined effect of controlled atmosphere and DPC16-GFS on the growth of *L. monocytogenes* in the broth system

The combined effect of DPC16-GFS with CA on the growth of *L. monocytogenes* at 4 and 20°C is shown in Figure 4.3 and summarised in Table 4.2. At both 4 and 20°C, the combination of DPC16-GFS (25 AU/mL) and CA of 60% CO_2 increased the LPD and decreased the EGR of *L. monocytogenes* when compared to that with either CA or DPC16-GFS alone, or non-CA without DPC16-GFS. The effect of the combination of DPC16-GFS and CA on the growth of *L. monocytogenes* was more pronounced at 4°C than at 20°C. The impact of the growth condition combinations on the EGR and LPD at 20°C are: DPC16-GFS with CA > DPC16-GFS alone > CA alone > non-CA without DPC16-GFS. At 4°C, the order is: DPC16-GFS with CA > CA alone > DPC16-GFS alone > non-CA without DPC16-GFS.

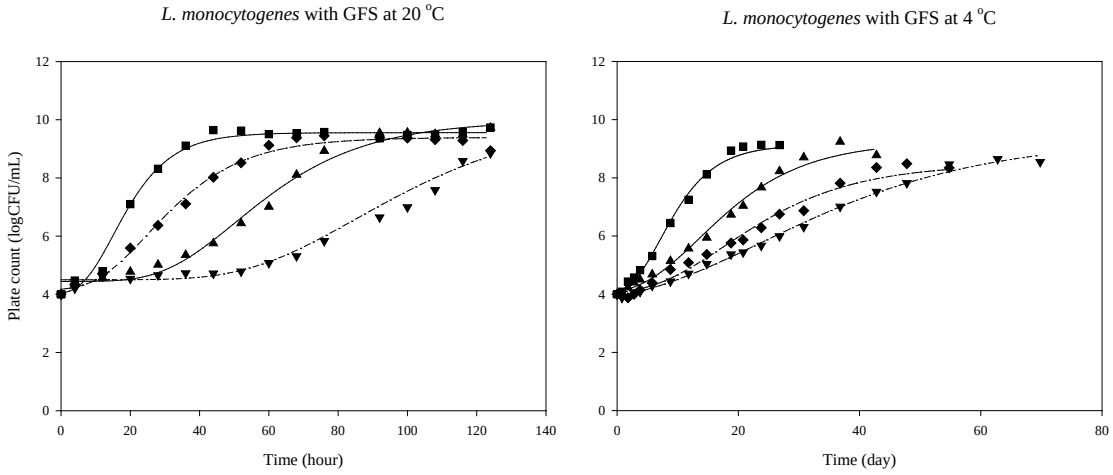

Figure 4.3 Fitted curves of the growth of *L. monocytogenes* with DPC16-GFS under CA of 60% CO_2 (▼) or non-CA (▲), without DPC16-GFS under CA (♦) or non-CA (control) (■) at 4 or 20°C. The data points are the mean of three replicates, except the data points for (♦) and (■) which are the mean of two replicates.

4.4 Discussion

In this study, the effect of DPC16-SCS and -GFS of *L. reuteri* DPC16 combined with controlled atmosphere (CA, 60% CO_2:40% N_2) on the growth of *L. monocytogenes* was evaluated in a system similar to that described in Chapter 2. MMRS broth was chosen as the basal growth medium to support the growth of *L. monocytogenes* so that the results would be comparable with those found in Chapter 3, but the MMRS was further buffered to minimise the pH effect of the acidic DPC16-SCS or -GFS on the growth of *L. monocytogenes*. The DPC16-SCS and -GFS were used at their minimal inhibitory concentrations (25% and 12.5%, respectively) equivalent to 25 AU/mL. These concentrations of DPC16-SCS and DPC16-GFS were chosen due to the fact that both DPC16-SCS and DPC16-GFS were bactericidal at a higher concentration and this might have overshadowed the effect of CA on the growth of bacteria.

In Chapter 2, CA (40% CO_2:60% N_2) was found to be effective in extending the LPD as well as reducing the EGR of selected foodborne pathogens (including *L. monocytogenes*) grown in buffered BHI broth (bBHI). In the present study, the controlled atmosphere (60% CO_2:40% N_2) alone was found to extend the LPD and reduce the EGR of *L. monocytogenes* in bMMRS broth, in the absence of DPC16-SCS or DPC16-GFS, at both 4 and 20^{o}C. These results were similar to the findings in the previous study in bBHI broth (Chapter 2), except that the lag phases of *L. monocytogenes* grown in bMMRS at both 4 and 20^{o}C in this study were shorter than in the previous study when the organism was grown in buffered BHI broth in a similar atmosphere (40% CO_2:60% N_2) and at similar temperatures (7 and 20^{o}C). The likely reasons for this difference are that the medium used (bMMRS) in this study different from that (bBHI) of the previous study, and the freshly made inoculum used in this study compared with the frozen (-20^{o}C) inocula used in the previous studies.

An inhibitory effect on growth was found when the experiment was carried out with DPC16-SCS or -GFS alone in air. This confirms the results from the previous experiments which had demonstrated the inhibitory effect of the reuterin-containing DPC16-SCS (Chapter 3). However, in contrast to the earlier finding, this effect was neither bactericidal nor completely inhibitory to *L. monocytogenes* which is probably

due to the lower concentration of DPC16-SCS or -GFS that was used in the current study.

It was found that the combination of DPC16-SCS or DPC16-GFS and CA had an additive antimicrobial effect on the growth of *L. monocytogenes* by extending the LPD and reducing the EGR. The combination had a greater inhibitory effect than that of DPC16-SCS, DPC16-GFS or CA alone. This result is consistent with the results from other studies on combining MA with other antimicrobial substances, such as nisin and ALTA^{TM} 2341 in buffered medium (Szabo & Cahill, 1998), nisin on cold-smoked salmon (Paludan-Muller *et al*., 1998) or lactic acid/sodium lactate on fresh poultry and crayfish meat (Zeitoun & Debevere, 1992; Pothuri *et al*., 1996). Altieri et al. (2005) reported a similar result using a *Bifidobacterium bifidum* strain and thymol as preservatives for the preservation of fresh plaice (*Pleuronectes platessa*) packed in modified atmosphere, where the data revealed that *B. bifidum* had a strong synergistic effect with thymol in controlling the biological markers (total viable count, total coliforms count, and sensory evaluation) of the fresh plaice under MA. The present study suggests that the combination of DPC16-SCS or DPC16-GFS and CA have an additive inhibitory effect against the growth of *L. monocytogenes*.

It was noted that the inhibitory effect of DPC16-SCS or DPC16-GFS alone was greater (in terms of longer LPD and lower EGR) than that of CA alone against the growth of *L. monocytogenes* at 20^{o}C. However, this effect was reversed at 4^{o}C (Figure 4.2 and 4.3). This was not unexpected, given that the reuterin-containing DPC16-SCS or DPC16-GFS was less active at lower temperatures, as shown in earlier experiments (Chapter 3). It was also noticeable that the DPC16-GFS had a greater inhibitory effect against *L. monocytogenes* (in terms of extending the lag phase duration and reducing the exponential growth rate) than DPC16-SCS had in this experiment. This could be because DPC16-GFS would have only contained antimicrobial, glycerol-fermented, reuterin, whereas the DPC16-SCS would contain both reuterin and lactic acid and the latter was less effective in the buffered medium at near neutral pH (6.5).

There is great interest in using *L. reuteri* and/or the fermentative products of *L. reuteri* as biopreservative agents. This is generally due to its wide spectrum of antimicrobial

activity against both Gram-positive and Gram-negative foodborne pathogens and its resistance to proteinase. Also, a great deal of effort has been put into the study of modified atmosphere in combination with other antimicrobial substances to control the growth of spoilage and pathogenic microorganisms in foods. However, this is the first *in vitro* study on the additive inhibitory effect of the culture supernatant of *L. reuteri* and modified atmosphere on foodborne pathogens. The result strongly suggests that the combination of the culture supernatant of *L. reuteri* DPC16 and modified atmosphere can be used with an additive inhibitory effect to control foodborne pathogens in foods.

In summary, this study has investigated the combined effect of DPC16-SCS or DPC16-GFS and CO_2-enriched CA against the growth of *L. monocytogenes* at 4^oC and 20^oC. The results showed an additive inhibitory effect (in terms of LPD extension and EGR reduction) by combining DPC16-SCS or DPC16-GFS with modified atmosphere, an inhibitory effect beyond that which could be achieved by any one of the individual factors alone. With this finding, a comprehensive effective strategy could be developed to improve the safety and extend the shelf life of food products.

Chapter 5 Stress responsive gene expression and heat survival of *L. monocytogenes* during growth under a controlled atmosphere in a medium containing culture supernatant of *L. reuteri* DPC16

5.1 Introduction

Listeria monocytogenes is a Gram-positive, rod-shaped, facultatively anaerobic bacterium that causes listeriosis, a serious illness from which pregnant women, infants, and elderly and immunocompromised individuals are at greatest risk (Vazquez-Boland *et al*., 2001). *L. monocytogenes* is of particular concern for the food industry due to its wide distribution in the environment and its ability to survive and proliferate under adverse environments, including acidic conditions, refrigeration temperatures and high osmolarity (Wemekamp-Kamphuis *et al*., 2004). These characteristics of *L. monocytogenes* make it a problematic foodborne pathogen in the food production industry (Wemekamp-Kamphuis *et al*., 2004).

The ability of bacteria to survive in harsh environmental conditions requires their ability to respond rapidly to environmental changes. These responses are often coordinated at the transcription level. In bacteria, the global changes in transcription are usually coordinated by specific sigma factors, with fluctuations in levels and activities in response to the environmental changes. In some Gram-positive genera (e.g. *Staphylococcus* and *Bacillus*), the stress-inducible sigma factor σ^B plays a central role in regulating the transcription of genes required for protection against environmental stresses, such as high osmolarity, low pH and low temperatures (Hecker *et al*., 1996; Gertz *et al*., 2000). The gene encoding σ^B in *L. monocytogenes* (*sigB*), which is homologous to the *sigB* gene from *B. subtilis*, plays the same role as those in other Gram-positive genera in resistance to low-pH stress (Wiedmann *et al*., 1998), cryotolerance (Becker *et al*., 2000), oxidative stress resistance and survival of carbon starvation (Ferreira *et al*., 2001) and osmotolerance (Becker *et al*., 1998). Studies have

also found that other regulatory systems, such as *LisRK* two-component regulatory system (Sleator & Hill, 2005) and alternative sigma factor RpoN (Okada *et al*., 2006) have played roles in osmotolerance in *Listeria monocytogenes*.

The ability of the organism to survive and grow at elevated osmolarities and reduced temperatures have been found to be due to the accumulation of osmo- and cryoprotective compounds, osmolytes or compatible solutes (Ko *et al*., 1994; Sleator & Hill, 2002). Glycine, betaine and carnitine have been considered as the preferred osmolytes in *L. monocytogenes*, and have been shown to stimulate the growth of *L. monocytogenes* in high salinity and low temperature (Ko *et al*., 1994). After an osmotic upshift, these osmolytes accumulate to high intracellular concentrations via active transport rather than by synthesis, and relieve turgor pressure without affecting the activity and function of the cellular components (Yancey *et al*., 1982; Verheul *et al*., 1995; Verheul *et al*., 1997; Ko & Smith, 1999).

The compatible solute accumulation is controlled both by transporter gene expression and transporter activity (Wood, 1999; Sleator *et al*., 2003). An ATP-dependant carnitine transport system (Verheul *et al*., 1995) and an ATP-dependant betaine transporter system have been found in *L. monocytogenes* (Ko & Smith, 1999). An additional sodium ion-driven betaine transport system has also been identified (Gerhardt *et al*., 1996). These transporter systems have been confirmed by the identification of three distinct transporter genes, *betL*, *gbu*, and *opuC* (Ko & Smith, 1999; Sleator *et al*., 1999; Fraser *et al*., 2000; Angelidis *et al*., 2002). The translational product of *betL* is a betaine transporter, and the deletion of *betL* may slow the growth of *L. monocytogenes* in media with elevated osmolarity (Sleator *et al*., 1999; Sleator *et al*., 2000). The gene *gbu*, encoded by the *gbuABC* operon, is an ATP-dependent transporter, and it is a homolog of OpuA in *Bacillus subtilis* and ProU in *E. coli* (Ko & Smith, 1999). The *gbuABC* operon encodes three protein subunits including GbuA, which is an ATPase. Disruption of *gbuA* results in reduced growth and a low rate of accumulation of betaine when cells are grown in high salinity media or at low temperatures (Ko & Smith, 1999). OpuC is the primary carnitine transporter in *L. monocytogenes* and is activated at low temperatures (Angelidis *et al*., 2002; Angelidis & Smith, 2003).

L. monocytogenes displays an active acid tolerance response upon exposure to low, non-lethal pH values and subsequent exposure to a lethal pH value. This response is considered to be the major role of the glutamate decarboxylase (GAD), which constitutes a special acid resistance mechanism and acid adaptation tool in *L. monocytogenes* (Cotter *et al*., 2001; Wemekamp-Kamphuis *et al*., 2004). It involves an antiporter at the cell membrane (GadC) and a cytoplasmic GAD (GadA or GadB). An additional GAD encoding gene (*gadD*) and antiporter encoding gene (*gadE*) are also involved in this acid tolerance response (Conte *et al*., 2002; Wemekamp-Kamphuis *et al*., 2004). The antiporter transports glutamate into the cell and then the GAD converts it into γ-aminobutyrate (GABA) upon consumption of a proton (Cotter *et al*., 2001). The antiporter subsequently excludes GABA from the cell. The effect of this process is to reduce the proton concentration in the cell and thus alleviate the acidification of the cytoplasm when the bacteria are exposed to a low pH environmental niche. It has been found that the survival of the σ^B null mutant of *L. monocytogenes* was 10,000-fold lower than the wild-type cells at pH 2.5 and the mutant even failed to show an acid tolerance response, which suggested that this mechanism was highly σ^B dependant (Wemekamp-Kamphuis *et al*., 2004).

Earlier in this study, the combined effects of DPC16-SCS or DPC16-GFS and CO_2-enriched CA against *L. monocytogenes* were investigated and an additive inhibitory effect on the growth of *L. monocytogenes* was observed. In the present work, the levels of expression of some stress responsive genes of *L. monocytogenes*, and the ability of *L. monocytogenes* to survive a heat treatment, were investigated after exposure to a growth condition with combined DPC16-SCS or DPC16-GFS and CO_2-enriched CA.

5.2 Materials and Methods

5.2.1 Samples

For gene expression profiling, samples were collected during the late stage of exponential growth, at a cell concentration of approximately 5 x 10^7 CFU/mL, in the experiment on the combined effect of DPC16-SCS or DPC16-GFS and CO_2-enriched controlled atmosphere on the growth of *L. monocytogenes* at 4^oC (Chapter 4). At the

Table 5.1 *L. monocytogenes* concentrations at the last count before sampling of the cells grown at 4°C

	Treatment					
	bMMRS, DPC16-SCS, CA[a]	bMMRS, DPC16-GFS CA[a]	bMMRS, CA[b]	bMMRS, DPC16-SCS, non-CA[a]	bMMRS, DPC16-GFS, non-CA[a]	bMMRS. Non-CA[b]
Bacterial count (logCFU/mL)	8.28	8.55	8.35	8.87	8.78	8.93

bMMRS: Buffered modified MRS;

DPC16-SCS: Spent culture supernatant of *L. reuteri* DPC16;

DPC16-GFS: Fermentative supernatant of glycerol water solution of *L. reuteri* DPC16;

CA and non-CA: Controlled atmosphere and non-controlled atmosphere, respectively.

[a] The mean of three replicates;

[b] The mean of three replicates.

time of sampling, 10 mL of culture was withdrawn and quickly placed on ice. The bacterial cells were harvested by centrifugation (12,000 x g, 3 min, 4^{o}C). The supernatant was discarded and the cell pellet was immediately stored at -80^{o}C until required for total RNA extraction.

For cell survival under heat treatment, samples were collected during the stationary phase, which was on the day following a plate count for cell concentration that had not increased from the previous count. The cell concentrations of the last counts before sampling are listed in Table 5.1.

5.2.2 RNA extraction from *L. monocytogenes*

RNA was extracted with an RNeasy Protect Bacteria Mini Kit (Cat. No. 74524, Qiagen, Hilden, Germany) following Protocol 4 of the manufacturer's instruction. Briefly, the sample previously stored at -80^{o}C was thawed and resuspended in ice-cold phosphate buffered saline (PBS, pH 7.2) to a final concentration of approximately 5 x 10^{8} cells/mL. Cell suspension (0.5 mL) was taken and added to a tube containing 1 mL of RNAprotect Bacteria Reagent. The cell suspension was mixed immediately by vortexing for 5 sec and then incubated for 5 min at room temperature (15-25^{o}C). The mixture was centrifuged for 10 min at 5,000 x *g*. The supernatant was decanted and the residual supernatant was removed by gently dabbing the inverted tube once onto a paper towel and leaving the tube inverted on a paper towel for 10 sec. Supplied proteinase K (20 µL) was mixed with 200 µL of TE buffer (30 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 15 mg/mL lysozyme. The mixed solution was added to the pellet, which was then carefully resuspended by pipetting up and down several times followed by vortexing for 10 sec. The mixture was incubated at room temperature for 10 min on a shaker-incubator. Supplied buffer RLT (700 µL) was added to the sample and vortexed vigorously. Ethanol (500 µL of 95%) was added to the lysate and mixed by pipetting. The resulting cell lysate was used for total RNA purification.

The lysate, including any precipitate that may have formed, was applied to an RNeasy Mini Column placed in a 2 mL collection tube (supplied) with maximum loading volume of 700 µL. The tube was centrifuged for 15 sec at 8000 x *g*. The flow-through

was discarded. The remaining cell lysate was loaded to the same column followed by centrifugation. Supplied buffer RW1 (700 µL) was added to the RNeasy column and the column was centrifuged for 15 sec at 8,000 x *g*. The flow-through was discarded. The column was transferred into a new 2 mL collection tube. Supplied buffer RPE (500 µL) was added to the RNeasy column and the column was centrifuged for 15 sec at 8,000 x *g* to wash the column. The flow-through was discarded and the column was washed once more with another 500 µL of buffer RPE as above. The column was transferred to a new 1.5 mL collection tube and 30 µL of RNase-free water was added directly onto the RNeasy silica-gel membrane. The tube was centrifuged for 1 min at 8,000 x *g* to elute the RNA. The resulting RNA solution was stored at -20^{o}C for future testing.

5.2.3 Reverse transcription-polymerase chain reaction

5.2.3.1 Reverse transcription

First-strand cDNA was synthesized using SuperScript™ II RT (Cat. No. 18064-022, Invitrogen, Carlsbad, USA) by random primers (Cat. No. 48190-011, Invitrogen, Carlsbad, USA) following the manufacturer's instructions. A 20 µL reaction volume was used for approximately 5 µg of total RNA. Briefly, the reaction components, including 250 ng random primers, 5 µg total RNA, 1 µL of dNTP Mix containing 10 mM each of the four deoxynucleotides, and ultra pure DNAse and RNAse free water (Invitrogen, Auckland, New Zealand) to top up to total volume of 12 µL, were added to a nuclease-free microcentrifuge tube. The mixture was heated to 65°C for 5 min and then quickly chilled on ice. Four microlitres of 5x First-strand buffer and 2 µL of 0.1 M DTT were added to the mixture. The mixture was gently mixed and incubated at 25°C for 2 min. One microlitre (200 units) of SuperScript™ II RT was added and mixed by pipetting gently up and down, and the mixture was incubated at 25°C for 10 min followed by incubation at 42°C for 50 min and heating at 70°C for 15 min. The resulting mixture was used as a template for amplification by PCR.

5.2.3.2 Quantification of stress responsive gene expression by PCR

PCR was carried out using the primers listed in Table 5.2. The concentration of cDNA was normalised against the amount of product generated by primers against the 16S rRNA gene.

PCR of 25, 30, and 35 cycles was performed to allow optimal quantification of PCR products by running on ethidium bromide-stained gel and photographed under UV illumination. Template cDNA was used in the reaction mixtures at levels that gave similar band intensities for 16S rRNA reactions. The experiment was performed in duplicate, and representative results are described.

Table 5.2 Primers used for gene expression study

Gene	Primer	Sequence (*5'→3'*)
*betL**	Forward	AAG TCC GAT TGG CTC GAT TC
	Reverse	ATC AAG TCC GGA CAT AGC CG
*gbuA**	Forward	TGG GCC GAA TTT TTG ACC TAG
	Reverse	CGC TCT TCT TTG TCC ATT CC
*opuC**	Forward	AAT GGA GGT GTG TAG GCG TG
	Reverse	GTA ATT GGA TCT AGC GCG CC
gadA (GAD, *lmo0447*)**	Forward	CGG TGT TTG GCT CTT TT GA
	Reverse	CTC CGA TTC ATC CAC ATT CC
gadB (GAD, *lmo2363*)**	Forward	GGC ATG CAC CTA AGG ACC AAA AAT
	Reverse	GAT ACC GAG GAT GCC GAC CAC AC
gadC (antiporter, *lmo2362*)**	Forward	AAA TGG CGA CGG TGG ATG GT
	Reverse	TTT TGC GAT TTT AGC CGT GTT TT
*16S rRNA***	Forward	TTA GCT AGT TGG TAG GGT
	Reverse	AAT CCG GAC AAC GCT TGC

* Cetin *et al*. (2004). ** Wemekamp-Kamphuis *et al*. (2004).

5.2.4 Survival during heat treatments

The collected samples were immediately placed on a heat block preheated to 56^{o}C. Heat-treated cells were removed at 0, 2, 3, 4, 5, 6 min during the heat treatment and diluted in MMRS broth. The cell viability was measured on MMRS agar plates following the method described in Section 2.2.2. The survival of *L. monocytogenes* was described as percentage of the cells that survived after heat treatments. The heat treatment was performed in triplicate and the survival of *L. monocytogenes* was analysed by ANOVA using Minitab Software (release 14, PA, USA).

5.3 Results

5.3.1 Expression levels of genes involved in osmotic and cold stresses

The transcriptional levels of genes (*betL*, *gbuA*, and *opuCA*) that encode the compatible solute transporters are shown in Figure 5.1. The expression of *gbuA* was increased (increased expression is indicated by higher intensity of band) when grown with either DPC16-SCS (Lane S) or DPC16-GFS (Lane G) under CA and non-CA, compared to that grown in bMMRS without DPC16-SCS or DPC16-GFS (control, Lane C).

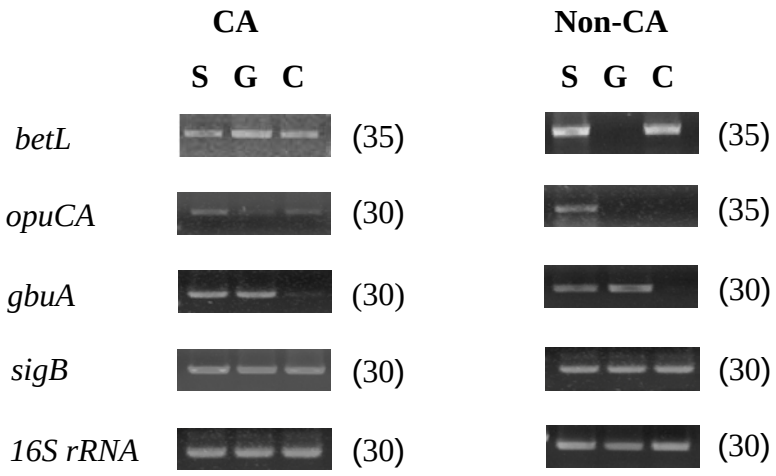

Figure 5. 1 RT-PCR transcriptional analysis of compatible solute transporter genes (*betL*, *gbuA*, and *opuCA*) and *sigB* encoding alternative σ^B factor in *L. monocytogenes* grown in bMMRS supplemented with DPC16-SCS (Lane S) or DPC16-GFS (Lane G) under CA or non-CA, compared to the control (Lane C). The cDNA concentration was normalised against the product generated by primers for 16S rRNA gene. The numbers shown in parentheses indicate the number of PCR cycles required to differentiate the levels of gene expression. An increased expression of a gene is indicated by a higher intensity of the corresponding band resulted from PCR amplification of the gene with the same or less number of cycles, and vice versa. No difference of gene expression is indicated by the same band intensity of a gene after PCR amplification at the same number of cycles.

No difference was found for the transcription of *betL* of *L. monocytogenes* when grown with DPC16-SCS (Lane S) or DPC16-GFS (Lane G) under CA. However, under non-

CA, although there was no difference when grown in the presence of DPC16-SCS (Lane S), the *betL* was repressed when grown with DPC16-GFS (Lane G). The expression of *opuCA* was induced under CA, compared to that under non-CA. However, under non-CA, the expression of *opuCA* was slightly induced in the presence of DPC16-SCS (Lane S), but not with DPC16-GFS (Lane G).

The transcription of *sigB* was induced under different growth environments, indicating that the regulator σ^B factor is necessary for directing the transcription of various stress responsive genes under different growth environments.

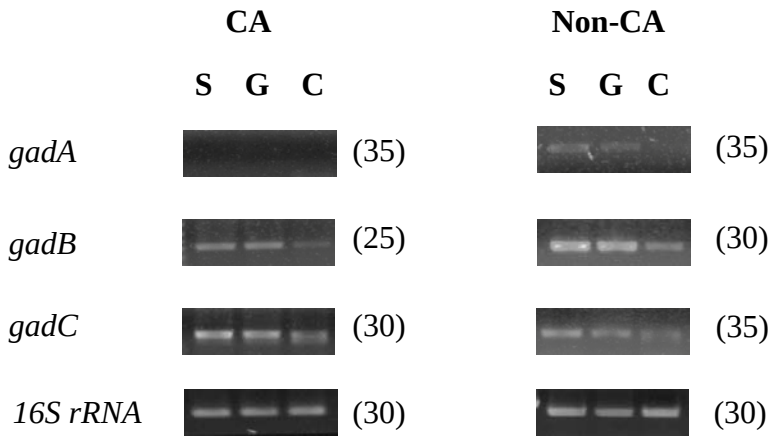

Figure 5.2 RT-PCR transcriptional analysis of gad genes (*gadA, gadB,* and *gadC*) in *L. monocytogenes* grown in bMMRS supplemented with DPC16-SCS (Lane S) or DPC16-GFS (Lane G) under CA or non-CA, compared to the control (Lane C) with neither DPC16-SCS or DPC16-GFS. The cDNA concentration was normalised against the PCR product generated by primers for 16S rRNA gene. The numbers shown in parentheses indicate the number of PCR cycles required to differentiate the levels of gene expression.

5.3.2 Expression levels of genes involved in acid stress

The expression profiles of the genes responsible for acid adaptation are shown in Figure 5.2. The expression levels of *gadB* and *gadC* genes were increased when grown with

DPC16-SCS (Lane S) or DPC16-GFS (Lane G) under both CA and non-CA, compared to bacteria grown in bMMRS without DPC16-SCS or DPC16-GFS (control, Lane C). The expression of *gadA* gene was not induced under CA, but was slightly induced when grown with either DPC16-SCS (Lane S) or DPC16-GFS (Lane G) under non-CA, compared to the control (Lane C). Overall, the expression of *gadB* and *gadC* appeared to be increased under CA, compared to that under non-CA, as shown by the PCR detection at fewer cycles (Figure 5.2).

5.3.3 Survival of *L. monocytogenes* during heat treatment

The survival of *L. monocytogenes* grown with DPC16 antimicrobial supplements, after heat treatment at 56^{o}C is shown in Figure 5.3 and Table 5.3. The survival of *L. monocytogenes* grown with either DPC16-SCS or DPC16-GFS under CA was significantly increased after exposure to heat treatment for up to 4 min, compared to the control ($P<0.05$). However, this difference in survival may have lessened after longer heat exposure (after 4 min).

The survival of *L. monocytogenes* grown with either DPC16-SCS or DPC16-GFS under CA was significantly higher, compared to that of *L. monocytogenes* grown with either DPC16-SCS or DPC16-GFS under non-CA, after exposure to heat treatment for up to 4 min ($P<0.05$).

The survival of *L. monocytogenes* grown with either DPC16-SCS or DPC16-GFS under non-CA was not significantly different after exposure to heat treatment, compared to the control ($P>0.05$).

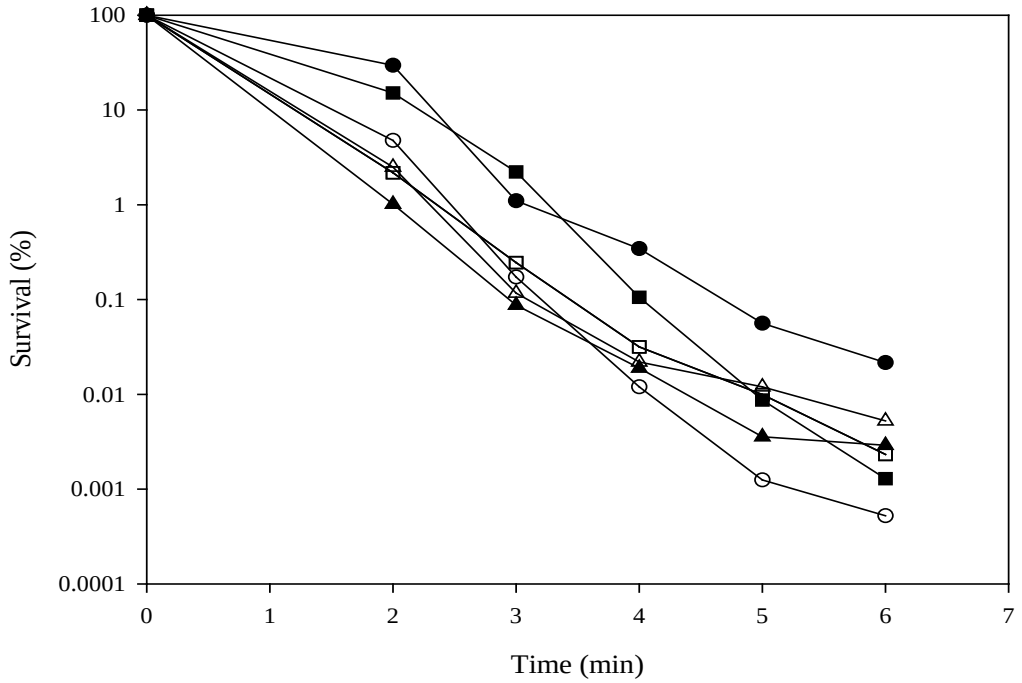

Figure 5.3 Survival of *L. monocytogenes* at 56°C after growth in bMMRS supplemented with DPC16-SCS under CA (■) or non-CA (□), or with DPC16-GFS under CA (●) or non-CA (○). The controls were cells grown in bMMRS without DPC16-SCS and -GFS under CA (▲) or non-CA (∆). The data points are the mean of three replicates, except the data points for (▲) and (∆) which are the mean of two replicates.

Table 5.3 Survival (%) of *L. monocytogenes* during heat treatment at 56°C after growth in bMMRS supplemented with DPC16-SCS or DPC16-GFS under CA or non-CA (mean ± SD)

Treatment time (min)	Growth condition					
	bMMRS, DPC16-SCS, CA[a]	bMMRS, DPC16-GFS, CA[a]	bMMRS, CA[b]	bMMRS, DPC16-SCS, non-CA[a]	bMMRS, DPC16-GFS, non-CA[a]	bMMRS. Non-CA[b]
0	100	100	100	100	100	100
2	15.1303 ± 1.05	29.3780 ± 2.80	1.0417 ± 0.27	2.1759 ±1.00	4.7778 ± 0.69	2.5000 ± 0.00
3	2.2273 ± 0.06	1.1041 ± 0.13	0.0908 ± 0.04	0.2676 ± 0.07	0.1733 ± 0.03	0.1250 ± 0.04
4	0.1074 ± 0.02	0.3470 ± 0.07	0.0199 ± 0.01	0.0343 ± 0.00	0.0121 ± 0.00	0.0266 ± 0.02
5	0.0087 ± 0.00	0.0564 ± 0.07	0.0037 ± 0.00	0.0118 ± 0.01	0.0012 ± 0.00	0.0137 ± 0.01
6	0.0013 ± 0.00	0.0217 ± 0.01	0.0030 ± 0.00	0.0026 ± 0.00	0.0005 ± 0.00	0.0060 ± 0.00

[a] Mean of three replicates;

[b] Mean of two replicates.

SD: Standard deviation.

5.4 Discussion

It is known that *L. monocytogenes* is able to adapt and grow in adverse environmental conditions, such as acidic conditions, refrigeration temperatures, and high osmolarity. These conditions are often encountered during food processing and storage. The ability of micro-organisms to adapt to the adverse growth conditions depends on a rapid response to the environmental changes through activation of a variety of enzymes and enhanced and/or repressed rates of transcription of some genes, resulting in physiological changes and elevated levels of protective proteins (Jydegaard-Axelsen *et al*., 2004; Wemekamp-Kamphuis *et al*., 2004).

The combined inhibitory effect of the antimicrobial supernatants DPC16-SCS and -GFS was demonstrated on the growth of *L. monocytogenes* in buffered MMRS in a controlled atmosphere rich in CO_2 (Chapter 4). In the current study, the expression levels of some genes, namely those responsible for a compatible solute transportation system for osmosis adaptation (eg. *betL*, *gbuA*, and *opuCA*) and those encoding for glutamate decarboxylase, essential for acid adaptation (eg. *gadA*, *gadB*, and *gadC*), were investigated for their responses to growth in the presence of DPC16-SCS and -GFS combined with CO_2.

The transcription of *sigB* was induced under different growth conditions, with or without DPC16-SCS and -GFS and under CA or not, as the alternative sigma factor σ^B is necessary for directing the transcription of various stress responsive genes in various growth environments, although other regulatory systems may also contribute to regulate the transcription of various stress responsive genes in the same growth conditions (Chaturongakul & Boor, 2006). This result supports the finding of others that σ^B activity is induced by a number of different stress conditions including non-osmotic stresses, leading to elevated transcriptional levels of σ^B-dependent *gbuA* and *opuC* osmolyte transporters, unless additional mechanisms were present to prevent induction (Becker *et al*., 1998; Cetin *et al*., 2004).

It has been reported that the expression of *gbuA* operon is inducible osmotically and at low temperature (Ko & Smith, 1999; Cetin *et al*., 2004). In the present study,

gbuA, encoding GbuA, an ATPase in the glycine betaine transport system GbuABC responsible for osmosis stress response, was induced when *L. monocytogenes* was grown with either DPC16-SCS or DPC16-GFS regardless of CA or non-CA. The induction of *gbuA* by DPC16-SCS or DPC16-GFS suggests that an elevated activity of the glycine betaine transport system provides an elevated ability of *L. monocytogenes* to grow in a harsh environment. The gene *betL* encodes a Na^+-dependent secondary betaine transporter and is σ^B-independent (Sleator *et al*., 1999; Fraser *et al*., 2003). However, no difference was found for the transcription level of *betL* of *L. monocytogenes* when grown with DPC16-SCS or DPC16-GFS under CA, compared to the controls. However, *betL* was repressed when grown with DPC16-GFS under non-CA. This result suggests that DPC16-SCS and DPC16-GFS have no effect on the induction of *betL* expression. This is plausible because of the Na^+-dependent nature and the σ^B-independence of the *betL* gene (Cetin *et al*., 2004). In other words, the σ^B-dependent *gbuA* may play a major role in the betaine transporter system in response to the stress derived from DPC16-SCS or DPC16-GFS.

The gene *opuCA*, encoding a carnitine transporter, was induced only under CA, indicating that CO_2 may play a role in activating the expression of *opuCA*, although CO_2 does not cause an osmotic change to the growth medium. In the presence of DPC16-SCS but not DPC16-GFS, the expression of *opuCA* was only slightly induced under non-CA and slightly increased under CA, suggesting that some other components, *eg*. lactic acid and/or some other unspecified bacterial metabolite, apart from reuterin (present in DPC16-SCS) may also contribute to the expression of *opuCA*.

The glutamate decarboxylase (GAD) acid resistance system is essential for survival and adaptation of *L. monocytogenes* to acidic conditions, and involves at least a cytoplasmic glutamate decarboxylase (GadA or GadB) and a glutamate/GABA antiporter at the membrane (GadC).

In this study, it was found that the expression of *gadB* and *gadC* were increased when *L. monocytogenes* was grown in the presence of DPC16-SCS or DPC16-GFS under either CA or non-CA with buffered pH at 6.5, compared to their expression without

DPC16-SCS or DPC16-GFS under the same atmosphere conditions. This indicates that both DPC16-SCS and DPC16-GFS might have a direct effect on the expression levels of *gadB* and *gadC*, regardless of whether under CA or non-CA. In other words, reuterin in DPC16-SCS and -GFS is able to trigger the expression of *gadB* and *gadC*, which might play a role in the stress that results from the exposure to reuterin, irrespective of the presence of CO_2. However, *gadA* was not induced in the presence of DPC16-SCS or DPC16-GFS under CA, suggesting that *gadA* plays only a minor role in the stress imposed by the DPC16 system.

It was also found that the expression of *gadB* and *gadC* was increased under CA, with or without DPC16-SCS or DPC16-GFS, compared to that under non-CA, as indicated by the PCR detection at fewer cycles. This is in accordance with the recent report that the expression of the genes *gadB* and *gadC* was increased in *L. monocytogenes* LO28 grown in CO_2 compared to growth in air (Jydegaard-Axelsen *et al*., 2004). This result suggests that some *L. monocytogenes* strains (eg. LO28 and Scott A) respond similarly to CO_2 as they do to acid using the GAD system (Cotter *et al*., 2001; Jydegaard-Axelsen *et al*., 2004).

It has been reported that heat tolerance and cross-protection to heat can be induced by other environmental stresses such as mild acid conditions in *S.* Typhimurium (Foster & Hall, 1990; Leyer & Johnson, 1993), lower concentration of salt in *Bacillus subtilis* (Volker *et al*., 1992) and *Lactococcus lactis* (Kilstrup *et al*., 1997), and nutrient starvation in *V. parahaemolyticus* (Koga & Takumi, 1995). In *L. monocytogenes*, heat tolerance has also been demonstrated to be induced by acid adaptation and other harsh environmental conditions such as starvation conditions, ethanol, acid, alkali, chlorine and H_2O_2 (Farber & Pagotto, 1992; Gahan, *et al*., 1996; Lou & Yousef, 1996; Taormina & Beuchat, 2001). In this study, DPC16-SCS and DPC16-GFS have been shown to induce changes in the expression of stress responsive genes to acid and osmosis in *L. monocytogenes*. Therefore, the investigation was carried out to find out whether these changes in gene expression also provide cross-protection to heat.

The survival of *L. monocytogenes* grown in the presence of either DPC16-SCS or DPC16-GFS under CA was significantly increased after exposure to heat treatment at 56^{o}C, compared to the control (with neither DPC16-SCS nor DPC16-GFS). In addition, the survival of *L. monocytogenes* grown with either DPC16-SCS or DPC16-GFS under CA was significantly increased compared to that of *L. monocytogenes* grown with either DPC16-SCS or DPC16-GFS under non-CA. However, the survival of *L. monocytogenes* grown with either DPC16-SCS or DPC16-GFS under non-CA was not significantly different after exposure to heat treatment, compared to the control. This result suggests that cross-protection to thermal stress was induced in *L. monocytogenes*, but this protection might be associated with the presence of the CO_2-enriched atmosphere.

The induction of this cross-protection to heat may have profound implications in the food industry if DPC16-SCS or DPC16-GFS is applied as a preservative. It is possible that the inclusion of DPC16-SCS or DPC16-GFS in food preservation could increase the ability of bacteria to survive subsequent heat treatments.

In summary, this study has demonstrated the effect of DPC16-SCS or DPC16-GFS, in combination with CA, on the expression of genes of *L. monocytogenes* responsible for osmosis adaptation, such as *betL, gbuA*, and *opuCA*, and for acid tolerance, such as *gadA, gadB,* and *gadC*. The induction of *gbuA, gadB,* and *gadC* by DPC16-SCS or DPC16-GFS suggests the activation of processes of osmosis and acid adaptation and that these genes play a major role in DPC16-SCS or DPC16-GFS induced stresses. This study also demonstrates the elevated thermal tolerance in *L. monocytogenes* induced by DPC16-SCS and DPC16-GFS under a CO_2-enriched atmosphere, which implies an increased ability of bacteria to survive subsequent heat treatments.

Chapter 6 General Discussion

The seafood industry is important to New Zealand as it constitutes the fourth largest export earner to the New Zealand economy (Anon., 2001) and is always looking for more efficient ways of preserving food and promoting food safety. More than 90% of its export fillets are shipped as frozen product due to the long distance from its major markets but fresh fillets earn a lot more than the income generated from frozen fillets. Therefore technologies that allow extended shelf life of chilled product and enhance their food safety could provide a major advantage for the industry. Also, the current trend of increased demand for minimally processed seafood requires more efficient preservation technologies to be able to provide extended shelf life and ensure an enhanced safety for chilled products.

Seafood has always been considered as potential sources or carriers of some foodborne pathogens (Simmons *et al*., 2001; Thornton *et al*., 2002), such as *L. monocytogenes*, *E. coli* O157:H7, *S.* Typhimurium, *S. aureus* and *V. parahaemolyticus*, although food poisoning outbreaks are relatively rare. Most of the pathogens found on seafood occur due to contamination with a limited number of organisms during the processing stages. Therefore, it is important that effective means are developed and implemented to control the growth of pathogens during the storage and processing stages to reduce the risk from seafood-borne illness.

Modified atmosphere packaging (MAP) is now considered a widely accepted technology for extending the shelf life of chilled seafood (Dalgaard, 2000; Emborg *et al*., 2005; Mejlholm *et al*., 2005). Research conducted in New Zealand has found that MA can significantly improve the shelf life of chilled New Zealand king salmon. However, this research has not investigated the effect of MA conditions on seafood-borne pathogens (Fletcher *et al*., 2002; Fletcher *et al*., 2003a, b; Fletcher *et al*., 2004). Other research has found that MA can inhibit the normal spoilage bacteria such as *Pseudomonas*, *Aeromonas*, *Shewanella*, *Moraxella* and *Acinetobacter* in fish (Stammen *et al*., 1990) and hence extend the shelf life of these products. However, the products with extended shelf life may allow a prolonged time for growth of the

contaminating foodborne pathogens (if there are any) and an increase in the concentration of toxins. For example, *L. monocytogenes* can grow close to 0°C and *C. botulinum* type E can grow and produce toxin at 3°C (Mejlholm *et al*., 2005). Therefore, particular consideration should be given to the effects of this technology on seafood-borne pathogens.

In the current study, experiments were conducted in a simulated controlled atmosphere system, in order to investigate the effect of modified atmosphere on the growth of some common foodborne pathogens. This system used buffered broth with a defined pH value and a constant controlled atmosphere to provide defined conditions for the growth of the pathogens. The focus was centred on the common foodborne pathogens *L. monocytogenes, S. aureus, S.* Typhimurium and *V. parahaemolyticus.* The results demonstrated that the controlled atmosphere (40% CO_2:60% N_2) was inhibitory to the growth of the tested pathogens, in terms of extending the lag phases, reducing the growth rates and decreasing the maximum population densities, although the magnitude of this inhibitory effect varied for the individual pathogens. Therefore, MAP as a means for food as well as seafood preservation has potential to control the foodborne pathogens and thereby enhance the safety of food products.

Unfortunately, a review of the literature suggests that high concentrations of CO_2 in MAP should be avoided for fishery products, as it dissolves into the fish juice and therefore deforms the package (Stenstrom, 1985). It can also have negative effects on the sensory properties of the fish (Fletcher *et al*., 2004). However, as a low concentration of CO_2 will compromise its inhibitory effect, conditions in which a minimised concentration of CO_2 are used while the maximum inhibition to seafood-borne pathogens is achieved would be beneficial. Also, MA is less effective in inhibiting Gram-positive bacteria compared with Gram-negative bacteria (Paludan-Muller *et al*., 1998). This characteristic of MA could limit its efficacy in extending the shelf life of seafoods. Therefore, one of the objectives of this study was to explore the possibility of an inhibitory combination that combines MA with other antimicrobial agents, especially with those antimicrobial compounds of microbial

origin (biopreservatives). Research on such combinations is becoming a popular theme in the seafood research.

LAB, the major constituent of biopreservatives, have been used alone for thousand of years to extend the shelf life of foods (Budde *et al*., 2003). LAB can help improve safety by inactivating pathogens, and extend shelf life by inhibiting undesirable changes brought about by spoilage microorganisms (Lucke, 2000). The main factors that LAB contribute to successful preservation of food products include a low pH value (<4.5 to prevent growth of unwanted bacteria), a substantial amount of non-dissociated organic acid molecules, competition with other bacteria for nutrients, and production of antimicrobial substances including antibiotics, bacteriocins, reuterin and other unknown compounds (Urlings *et al*., 1993).

The basis for the selection of antimicrobial preservatives to be used in foods, as recommended by the National Food Processors Association (NFPA), should not only be their effectiveness against both Gram-positive and Gram-negative pathogenic and spoilage microorganisms, but also their proven safety records and their acceptance by health-conscious consumers and regulatory agencies (Anon., 1988). The latter aspects have not been addressed in the current study.

Initially in this study, 18 LAB strains were screened for their antimicrobial activity. Molecular characterisation of their identities confirmed that they belong to 4 groups of LAB, including 13 strains of *L. reuteri*, 3 strains of *L. garlicum*, one strain of *L. mucosae*, and one strain of *E. faecium*. *In vitro* assays found most of these strains had antimicrobial activity against the four common foodborne pathogens *E. coli* O157:H7, *S.* Typhimurium, *S. aureus* and *L. monocytogenes*. *L. reuteri* DPC16 showed the strongest antimicrobial activity against both Gram-positive and Gram-negative bacteria. Further characterisation of *L. reuteri* DPC16 found that it produced reuterin, a compound that is able to inhibit both Gram-positive and Gram-negative bacteria (Axelsson *et al*., 1989). It also produced lactic acid which effectively reduces the pH to less than 4 in the growth environment. These are the important features of *L. reuteri* DPC16 compared to other bacteriocin-producing LAB strains which inhibit only closely-related Gram-positive bacteria through production of bacteriocin.

In vitro assays demonstrated that *L. reuteri* DPC16 can effectively inhibit both Gram-positive and Gram-negative common foodborne pathogens in co-culture, and that the cultural supernatant of *L. reuteri* DPC16 inhibited the tested pathogens in co-incubation and had a strong bactericidal effect. This inhibitory effect of *L. reuteri* DPC16 is valid over a wide spectrum of pH and temperature values. These results provide evidence that *L. reuteri* DPC16 could be a good candidate for use as a biopreservative able to aid in safety enhancement and shelf life extension of food products.

Given that the inhibitory effect of MA on normal spoilage microorganisms selectively allows the growth of Gram-positive microorganisms, and that the inhibitory effect of *L. reuteri* DPC16 is effective on both Gram-positive and Gram-negative microorganisms, a combination of the two could be particularly useful in seafood preservation, allowing the concentration of CO_2 to be restricted without compromising its inhibitory effect on Gram-negative bacteria (Stenstrom, 1985).

To test the hypothesis that the application of *L. reuteri* DPC16 in combination with MA would provide a combined inhibitory effect against foodborne pathogens, an *in vitro* study was conducted on the growth of *L. monocytogenes*, an important foodborne pathogen for the seafood industry. This is the first such study employing a combination of culture supernatant of *L. reuteri* with CO_2-enriched modified atmosphere to investigate its inhibitory effect on a foodborne pathogen.

The results showed that this combination has an additive antimicrobial effect on the growth of *L. monocytogenes* at temperatures of 4 and 20^{o}C, which cannot be achieved by any one of the individual factors alone. Therefore, this result demonstrated that applying the combination of *L. reuteri* DPC16 and MA on food products could offer enhanced safety and stability (in terms of extended shelf life) to the food products. Based on this finding, a comprehensive effective strategy could be developed to improve the safety and extend the shelf life of seafood as well as other food products, which could be very beneficial to the industry.

To analyse the underlying molecular biological mechanism for this combined effect on *L. monocytogenes*, the gene expressions of *L. monocytogenes* after exposure to the combined cultural supernatants of *L. reuteri* DPC16 and CA were investigated. As acid stress, osmosis stress, cold stress and heat stress are the major stresses encountered by foodborne pathogens during food processing and storage, the expressions of the genes that are responsible for acid, osmosis, and cold stresses were analysed. The results showed that this combination has an effect on the expression of the genes of *L. monocytogenes* that are responsible for osmosis adaptation and acid tolerance. This result suggests that the processes of osmosis and acid adaptation have been activated during the growth under induced stresses.

It has been reported that acid adaptation and other harsh environmental conditions induce heat tolerance and cross-protection to heat in L. monocytogenes and other microorganisms, such as S. Typhimurium and *B. subtilis* (Foster & Hall, 1990; Farber & Pagotto, 1992; Volker *et al*., 1992; Leyer & Johnson, 1993; Taormina & Beuchat, 2001). Therefore, this study investigated whether the stresses of the combination of cultural supernatants of *L. reuteri* DPC16 and CA provided cross-protection to *L. monocytogenes* against heat. The results demonstrated an increased thermal tolerance in *L. monocytogenes*, which implies an increased ability of the bacteria to survive subsequent heat treatments. This phenomenon might have important implications in the food industry if *L. reuteri* DPC16 and CA were applied as preservatives, i.e. when the shelf life is extended the subdued potentially pathogenic contaminants may become more tolerant to any subsequent heat treatment and therefore, are required to be inactivated by prolonged or temperature-elevated heat treatment before consumption.

In this study, the work has focused on the *in vitro* evaluation of the antimicrobial property of *L. reuteri* DPC16 and its potential biopreservative application either alone or in combination with CA, especially on the growth of *L. monocytogenes*. It has been found that *L. reuteri* DPC16 alone is inhibitory to the tested pathogens and an additive inhibitory effect against *L. monocytogenes* can be achieved when *L. reuteri* DPC16 is combined with CA. Future work should be carried out to find out whether the combined inhibitory effect can be achieved on other foodborne

pathogens, such as *E. coli* O157:H7, *S.* Typhimurium and *S. aureus.* Also, and more importantly, this combined inhibitory effect should be tested *in vivo*, such as on fish fillets and other seafood products. It is anticipated that, based on these findings, a comprehensive sophisticated strategy incorporating both *L. reuteri* DPC16, or its fermentative products, and MA, for the control the foodborne pathogens can be developed that results in safer food products with extended shelf life.

In summary, this study has investigated a new strategy that can be applied to assist the development of a new generation of seafood products incorporating new barriers to ensure enhanced safety and extended shelf life. The inhibitory effects of modified atmosphere and the selected LAB strains were evaluated against common foodborne pathogens. Further assessment focused on a candidate strain, *L. reuteri* DPC16, for its inhibitory effect on foodborne pathogens and potential use as a biopreservative. The combination of the cultural supernatant of *L. reuteri* DPC16 and MA was evaluated for its inhibitory effect against *L. monocytogenes*. It was found that the cultural supernatant of *L. reuteri* DPC16 or MA alone have an inhibitory effect against some common foodborne pathogens, while the combination of both provides an additive inhibitory effect on the tested pathogen. Based on these findings a comprehensive novel strategy incorporating both *L. reuteri* DPC16 or its fermentative products and MA could be developed to control potential foodborne pathogens in seafood products.

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Appendix I

Media, buffers, solutions

(1) 1% agarose gel

Agarose (Invitrogen, Carlsbad, USA)	1 g
0.5x TAE buffer added to make	100 mL

Agarose is completely dissolved in a flask by a microwave oven, and then cooled to 50^{o}C, following by pouring into the electrophoresis tray and use after solidified.

(2) Agar gel plate

BHI powder (CM225, Oxoid, Hampshire, UK)	37 g
Agar (Sigma, St. Louis, MO, USA)	1 g
Distilled water added to make	100 mL

The BHI powder is dissolved in 80 mL water, followed by addition of the prescribed amount of agar and mix up. The suspension is sterilised by autoclaving at 121^{o}C for 15 min. The sterile BHI agar was cooled to 48^{o}C in a water bath. The sterile BHI agar is shifted to Biosafety hood. Each of the indicator pathogens is added in an aseptic manner to the BHI agar, individually, at a final concentration of 1 x 10^5 CFU/mL. After mixing thoroughly, the agar is poured into petri dishes at 20 mL/each. The agar plates are allowed to set at room temperature and used immediately.

(3) Brain Heart Infusion (BHI) broth

BHI powder (CM225, Oxoid, Hampshire, UK)	37 g
Distilled water added to make	1 L

Mix well and sterilised by autoclave at 121^{o}C for 15 min.

(4) Brain Heart Infusion (BHI) agar plates

BHI powder (CM225, Oxoid, Hampshire, UK)	37 g
Agar (Sigma, St. Louis, MO, USA)	15 g
Distilled water added to make	1 L

Mix well and sterilised by autoclave at 121^{o}C for 15 min. After cooled to 50^{o}C, the agar is poured into petri dishes 18 mL/each.

(5) Brain Heart Infusion (BHI) agar with 1.5% Lithium chloride

BHI powder (CM225, Oxoid, Hampshire, UK)	37 g
Agar (Sigma, St. Louis, MO, USA)	15 g
Lithium chloride	15 g
Distilled water added to make	1 L

Mix well and sterilised by autoclave at 121^{o}C for 15 min. After cooled to 50^{o}C, the agar is poured into petri dishes 18 mL/each.

(6) Buffered BHI broth (bBHI) (pH 6.8)

BHI powder (CM225, Oxoid, Hampshire, UK)	37 g
0.2 M phosphate buffer (pH 7.6)	1 L

The medium is dispensed into jars (100 mL/each) and sterilised by autoclave at 121^{o}C for 15 min, followed by an overnight equilibration under CA. The pH of the equilibrated medium is checked aseptically to be 6.8, otherwise adjusted using sterile 1 N HCl if necessary. When the bBHI (pH 6.8) was used for control set up, it was prepared in 0.2 M phosphate buffer at pH 6.8.

(7) Buffered BHI broth (bBHI) (pH 6.3)

BHI powder (CM225, Oxoid, Hampshire, UK)	37 g
0.2 M phosphate buffer (pH 6.6)	1 L

The medium is dispensed into jars (100 mL/each) and sterilised by autoclave at 121^{o}C for 15 min, followed by an overnight equilibration under CA. The pH of the equilibrated medium is checked aseptically to be 6.3, otherwise adjusted using sterile 1 N HCl if necessary. When the bBHI (pH 6.3) was used for control set up, it was prepared in 0.2 M phosphate buffer at pH 6.3.

(8) Buffered MMRS (bMMRS) (pH 6.5)

Peptone (Bacto^{TM}, Becton Dickinson, MD, USA)	10 g
'Lab-Lemco' powder (LP0029, Oxoid, Hampshire, UK)	8 g
Yeast extracts (Bacto^{TM}, Becton Dickinson, MD, USA)	4 g
Glucose	20 g
Tween-80	1 mL
Magnesium sulphate $7H_2O$	0.2 g
Manganese sulphate 4 H_2O	0.05 g
0.2 M phosphate buffer (pH 7.0) added to make	1 L

The medium is dispensed into jars (100 mL/each) and sterilised by autoclave at 121^{o}C for 15 min, followed by an overnight equilibration under CA. The pH of the equilibrated medium is checked aseptically to be 6.5, otherwise adjusted using sterile 1 N HCl if necessary. When the bMMRS (pH 6.5) was used for control set up, it was prepared in 0.2 M phosphate buffer at pH 6.5.

(9) Buffered TSBS broth (bTSBS) (pH 6.8)

TSB powder (CM129, Oxoid, Hampshire, UK)	30 g
NaCl	30 g
0.2 M phosphate buffer (pH 7.6)	1 L

The medium is dispensed into jars (100 mL/each) and sterilised by autoclave at 121^{o}C for 15 min, followed by an overnight equilibration under CA. The pH of the equilibrated medium is checked aseptically to be 6.8, otherwise adjusted using sterile 1 N HCl if necessary. When the bTSBS (pH 6.8) was used for control set up, it was prepared in 0.2 M phosphate buffer at pH 6.8.

(10) Buffered TSBS broth (bTSBS) (pH 6.3)

TSB powder (CM129, Oxoid, Hampshire, UK)	30 g
NaCl	30 g
0.2 M phosphate buffer (pH 6.6)	1 L

The medium is dispensed into jars (100 mL/each) and sterilised by autoclave at 121^{o}C for 15 min, followed by an overnight equilibration under CA. The pH of the equilibrated medium is checked aseptically to be 6.3, otherwise adjusted using sterile 1 N HCl if necessary. When the bTSBS (pH 6.3) was used for control set up, it was prepared in 0.2 M phosphate buffer at pH 6.3.

(11) Catalase (500 U/mL)

Catalase (1870 U/mg, Sigma, St. Louis, MO, USA)	1.34 mg
Distilled water	5 mL

Sterilised by filtration (0.2 µm)

(12) 0.5 M EDTA

Disodium-EDTA	93 g
Distilled water	350 mL

Adjust to pH 8.0 with sodium hydroxide (about 25 mL of 10M). The EDTA will not all dissolve until pH 8.0 is reached. Bring the volume to 500 ml with distilled water. The solution can be stored indefinitely at room temperature if sterilised by filtration or autoclave.

(13) 6 x gel loading dye

Bromophenol blue	0.25 g
Xylene cyanol FF	0.25 g
Ficoll Type 4000	15 g
0.5 M EDTA	24 mL
Distilled water added to make	100 mL

(14) 1 N HCl

Concentrated HCl (equivalent to 12 N)	8.33 mL
Distilled water	91.27 mL

To 80 mL of H_2O in a beaker, slowly add the prescribed amount of concentrated HCl, stirring continuously, and then adjust the volume to 100 mL with H_2O.

(15) Lysozyme (50 mg/mL)

Lysozyme (Sigma, St. Louis, MO, USA)	0.5 g
Distilled water	10 mL
Sterilised by filtration	

(16) MacConkey agar

MacConkey (Difco™, Becton Dickinson, MD, USA)	50 g
Distilled water added to make	1 L

Suspend the required amount of the prescribed reagents in 1 L of distilled water. Adjust the pH to 7.1. Bring to the boil with frequent agitation to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to about 50°C, and pour into sterile petri dishes.

(17) Mannitol salt Agar

`Lab-Lemco' powder (LP0029, Oxoid, Hampshire, UK)	1 g
Peptone (Bacto™, Becton Dickinson, MD, USA)	10 g
Mannitol	10 g
Sodium chloride	75 g
Phenol red	0.025 g
Agar (Sigma, St. Louis, MO, USA)	15 g
Distilled water added to make	1 L

Suspend the required amount of the prescribed reagents in 1 litre of distilled water. Adjust the pH to 7.5. Bring to the boil with frequent agitation to dissolve

completely. Sterilise by autoclaving at 121°C for 15 minutes. Cool to about 50°C, and pour into sterile petri dishes.

(18) Modified MRS (MMRS) broth (Annuk *et al*., 2003)

Peptone (Bacto™, Becton Dickinson, MD, USA)	10 g
'Lab-Lemco' powder (LP0029, Oxoid, Hampshire, UK)	8 g
Yeast extracts (Bacto™, Becton Dickinson, MD, USA)	4 g
Glucose	20 g
Tween-80	1 mL
Di-potassium hydrogen phosphate	2 g
Magnesium sulphate $7H_2O$	0.2 g
Manganese sulphate 4 H_2O	0.05 g
Distilled water added to make 1 L	

The pH is adjusted to 6.5 using 1 N HCl, and then the medium is sterilised by autoclave at 121^{o}C for 15 min.

(19) MMRS agar plates

MMRS broth	1 L
Agar (Sigma, St. Louis, MO, USA)	15 g

Mix well and sterilised by autoclave at 121^{o}C for 15 min. After cooled to 50^{o}C, the agar is poured into petri dishes 18 mL/each.

(20) MRS broth

de Man, Rogosa, Sharpe (MRS) powder (Difco™, Becton Dickinson, MD, USA)	55 g
Distilled water added to make	1 L

Mix well and sterilised by autoclave at 121^{o}C for 15 min.

(21) MRS agar plates

MRS broth	1 L
Agar (Sigma, St. Louis, MO, USA)	15 g

Mix well and sterilised by autoclave at 121^{o}C for 15 min. After cooled to 50^{o}C, the agar is poured into petri dishes 18 mL/each.

(22) MRSg broth

de Man, Rogosa, Sharpe (MRS) powder (Difco^{TM}, Becton Dickinson, MD, USA)	55 g
Glycerol	18.25 mL
Distilled water added to make	1 L

Mix well and sterilised by autoclave at 121^{o}C for 15 min.

(23) 1 M NaOH

10 M NaOH	10 mL
Distilled water	90 mL

(24) 10 M NaOH

NaOH pellets	40 g
Distilled water	100 mL

To 80 mL of H_2O in a beaker, slowly add 40 g of NaOH pellets, stirring continuously. When the pellets have dissolved completely, adjust the volume to 100 mL with H_2O.

(25) 0.1% (w/v) Peptone water

Peptone (Bacto^{TM}, Becton Dickinson, MD, USA)	1 g
Distilled water added to make	1 L

Mix well and sterilised by autoclave at 121°C for 15 min.

(26) Phosphate buffer

Solution A (1 M):

Na_2HPO_4, $2H_2O$	177.9 g
Distilled water added to make	1 L

Solution B (1 M):

NaH_2PO_4, H_2O	137.99 g
Distilled water added to make	1 L

To make phosphate buffer of desired concentration at desired pH, follow the following table:

For example, to prepare 1 L of 0.1 M sodium phosphate buffer at 25ºC

Desired pH	Volume of 1 M Na_2HPO_4 (mL)	Volume of 1M NaH_2PO_4 (mL)
5.8	7.9	92.1
6.0	12.0	88.0
6.2	17.8	82.2
6.4	25.5	74.5
6.6	35.2	64.8
6.8	46.3	53.7
7.0	57.7	42.3
7.2	68.4	31.6
7.4	77.4	22.6
7.6	84.5	15.5
7.8	89.6	10.4
8.0	93.2	6.8

0.2 M Phosphate buffer, pH 6.3:

Solution A	43.3 mL
Solution B	156.7 mL
Distilled H_2O	800 mL

0.2 M Phosphate buffer, pH 6.5:

Solution A	60.7 mL
Solution B	139.3 mL
Distilled H_2O	800 mL

0.2 M Phosphate buffer, pH 6.8:

Solution A	92.6 mL
Solution B	107.4 mL
Distilled H_2O	800 mL

0.2 M Phosphate buffer, pH 7.0:

Solution A	115.4 mL
Solution B	84.6 mL
Distilled H_2O	800 mL

0.2 M Phosphate buffer, pH 7.6:

Solution A	169 mL
Solution B	31 mL
Distilled H_2O	800 mL

0.1 M Phosphate buffered saline (PBS, pH 7.2):

Solution A	68.4 mL
Solution B	31.6 mL
NaCl	9 g
Distilled H_2O	900 mL

0.05 M PBS (pH 7.5):

Solution A	40.48 mL
Solution B	9.52 mL
NaCl	9 g
Distilled H_2O	950 mL

(27) Pronase E (1 mg/mL)

Pronase E (Sigma, St. Louis, MO, USA)	5 mg
Distilled water	5 mL

Sterilised by filtration (0.2 µm)

(28) Proteinase K (1 mg/mL)

Proteinase K (GibcoBRL, Gaithersburg, MD, USA)	5 mg
Distilled water	5 mL

Sterilised by filtration (0.2 µm)

(29) Salmonella Shigella (SS) agar

`Lab-Lemco' powder (LP0029, Oxoid, Hampshire, UK)	5 g
Peptone (Bacto™, Becton Dickinson, MD, USA)	5 g
Lactose	10 g
Bile salts	8.5 g
Sodium citrate	10 g
Sodium thiosulphate	8.5 g
Ferric citrate	1 g
Brilliant green	0.00033 g
Neutral red	0.025 g
Agar	15 g
Distilled water added to make	1 L

Dissolve the required amount of the prescribed reagents in 1 litre of distilled water. Adjust the pH to 7.0. Bring to the boil with frequent agitation and allow to simmer gently to dissolve the agar. Cool to about 50°C, mix and pour into sterile petri dishes.

(30) Standard plate count agar plates:

Standard plate count agar	
(CM463, Oxoid, Hampshire, UK)	23.5 g
Distilled water added to make	1 L

Mix well and sterilised by autoclave at 121^{o}C for 15 min. After cooled to 50^{o}C, the agar is poured into petri dishes 18 mL/each.

(31) 50x TAE buffer

Tris base (Sigma, St. Louis, MO, USA)	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA (pH 8.0)	100 mL
Distilled water added to make	1 L

(32) 0.5x TAE buffer

50x TAE	10 mL
Distilled water added to make	1 L

(33) Trypsin (1 mg/mL)

Trypsin (Sigma, St. Louis, MO, USA)	5 mg
Distilled water	5 mL

Sterilised by filtration (0.2 µm)

(34) Trypticase Soy Broth (TSB), supplemented with 3% NaCl (TSBS)

TSB powder (CM129, Oxoid, Hampshire, UK)	30 g

NaCl	30 g
Distilled water added to make	1 L

Mix well and sterilised by autoclave at 121^{o}C for 15 min.

(35) TSBS agar plates

TSB powder (CM129, Oxoid, Hampshire, UK)	30 g
NaCl	30 g
Agar (Sigma, St. Louis, MO, USA)	15 g
Distilled water added to make	1 L

Mix well and sterilised by autoclave at 121^{o}C for 15 min. After cooled to 50^{o}C, the agar is poured into petri dishes 18 mL/each.

Appendix II

Data analysis

Chapter 2

2.3.2 One way ANOVA of effect of CA on the lag phase duration

***E. coli* O157:H7, pH 6.3, 20°C**

```
Source      DF       SS       MS       F      P
CA           1  14.0399  14.0399  567.57  0.000
Error        4   0.0989   0.0247
Total        5  14.1388

S = 0.1573   R-Sq = 99.30%   R-Sq(adj) = 99.13%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  ------+---------+---------+---------+---
CA      3  17.738  0.118                              (-*--)
Non-CA  3  14.678  0.188  (--*-)
                          ------+---------+---------+---------+---
                             15.0      16.0      17.0      18.0

Pooled StDev = 0.157
```

***E. coli* O157:H7, pH 6.8, 20°C**

```
Source      DF      SS      MS      F      P
CA           1  11.906  11.906  67.77  0.001
Error        4   0.703   0.176
Total        5  12.609

S = 0.4191   R-Sq = 94.43%   R-Sq(adj) = 93.03%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev    -+---------+---------+---------+--------
CA      3  16.571  0.467                          (-----*-----)
Non-CA  3  13.754  0.365    (-----*----)
                            -+---------+---------+---------+--------
                          13.2      14.4      15.6      16.8

Pooled StDev = 0.419
```

S. aureus, pH 6.3, 20°C

```
Source      DF        SS        MS        F      P
CA           1  1229.587  1229.587  1826.65  0.000
Error        4     2.693     0.673
Total        5  1232.279

S = 0.8204   R-Sq = 99.78%   R-Sq(adj) = 99.73%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  ---+---------+---------+---------+------
CA      3  51.486  1.072                                      (*-)
Non-CA  3  22.855  0.445  (-*)
                          ---+---------+---------+---------+------
                          24.0      32.0      40.0      48.0

Pooled StDev = 0.820
```

S. aureus, pH 6.8, 20°C

```
Source      DF       SS       MS       F      P
CA           1  1210.05  1210.05  328.94  0.000
Error        4    14.71     3.68
Total        5  1224.76

S = 1.918   R-Sq = 98.80%   R-Sq(adj) = 98.50%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev   +---------+---------+---------+---------
CA      3  51.362  1.979                              (--*--)
Non-CA  3  22.959  1.855   (--*--)
                           +---------+---------+---------+---------
                          20        30        40        50

Pooled StDev = 1.918
```

_S_. Typhimurium, pH 6.3, 20°C

```
Source      DF      SS      MS       F      P
CA           1  57.134  57.134  234.16  0.000
Error        4   0.976   0.244
Total        5  58.110

S = 0.4940   R-Sq = 98.32%   R-Sq(adj) = 97.90%


                          Individual 95% CIs For Mean Based on Pooled
                          StDev
Level   N    Mean  StDev    +---------+---------+---------+---------
CA      3  18.924  0.646                                  (---*---)
Non-CA  3  12.752  0.266    (---*---)
                            +---------+---------+---------+---------
                            12.0      14.0      16.0      18.0

Pooled StDev = 0.494
```

_S_. Typhimurium, pH 6.8, 20°C

```
Source      DF      SS      MS       F      P
CA           1  45.910  45.910  112.41  0.000
Error        4   1.634   0.408
Total        5  47.544

S = 0.6391   R-Sq = 96.56%   R-Sq(adj) = 95.70%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  ----+---------+---------+---------+-----
CA      3  17.796  0.522                            (----*----)
Non-CA  3  12.264  0.738  (----*----)
                          ----+---------+---------+---------+-----
                           12.0      14.0      16.0      18.0

Pooled StDev = 0.639
```

***V. parahaemolyticus*, pH 6.3, 20°C**

```
Source      DF        SS        MS        F      P
CA           1  3685.272  3685.272  8216.05  0.000
Error        4     1.794     0.449
Total        5  3687.066

S = 0.6697   R-Sq = 99.95%   R-Sq(adj) = 99.94%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev   +---------+---------+---------+---------
CA      3  66.388  0.882                                      *)
Non-CA  3  16.822  0.346   (*)
                           +---------+---------+---------+---------
                          15        30        45        60

Pooled StDev = 0.670
```

***V. parahaemolyticus*, pH 6.8, 20°C**

```
Source      DF       SS       MS       F      P
CA           1  2335.84  2335.84  305.00  0.000
Error        4    30.63     7.66
Total        5  2366.47

S = 2.767   R-Sq = 98.71%   R-Sq(adj) = 98.38%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  ---+---------+---------+---------+------
CA      3  53.740  3.799                          (--*--)
Non-CA  3  14.278  0.941  (--*-)
                          ---+---------+---------+---------+------
                            15        30        45        60
Pooled StDev = 2.767
```

***L. monocytogenes*, pH 6.3, 20°C**

```
Source      DF      SS      MS       F      P
CA           1  24.374  24.374  106.63  0.000
Error        4   0.914   0.229
Total        5  25.289

S = 0.4781   R-Sq = 96.38%   R-Sq(adj) = 95.48%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  --------+---------+---------+---------+-
CA      3  18.645  0.449                            (----*----)
Non-CA  3  14.613  0.505  (----*-----)
                          --------+---------+---------+---------+-
                               15.0      16.5      18.0      19.5

Pooled StDev = 0.478
```

***L. monocytogenes*, pH 6.8, 20°C**

```
Source      DF      SS     MS      F      P
CA           1   9.660  9.660  31.39  0.005
Error        4   1.231  0.308
Total        5  10.892

S = 0.5548   R-Sq = 88.70%   R-Sq(adj) = 85.87%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  ------+---------+---------+---------+---
CA      3  17.054  0.680                       (------*-------)
Non-CA  3  14.516  0.392  (------*------)
                          ------+---------+---------+---------+---
                             14.4      15.6      16.8      18.0

Pooled StDev = 0.555
```

***L. monocytogenes*, pH 6.8, 7°C**

```
Source      DF       SS       MS       F      P
Atmosphere   1  10744.6  10744.6  753.76  0.000
Error        4     57.0     14.3
Total        5  10801.6

S = 3.776   R-Sq = 99.47%   R-Sq(adj) = 99.34%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  --------+---------+---------+---------+-
CA      3  245.49   4.98                                  (-*--)
Non-CA  3  160.86   1.93  (-*--)
                          --------+---------+---------+---------+-
                                175       200       225       250

Pooled StDev = 3.78
```

***L. monocytogenes*, pH 6.8, 7°C**

```
Source      DF      SS      MS       F      P
CA           1  7388.9  7388.9  236.40  0.000
Error        4   125.0    31.3
Total        5  7513.9

S = 5.591   R-Sq = 98.34%   R-Sq(adj) = 97.92%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  --+---------+---------+---------+-------
CA      3  223.43   1.80                              (--*---)
Non-CA  3  153.24   7.70  (--*---)
                          --+---------+---------+---------+-------
                          150       175       200       225

Pooled StDev = 5.59
```

2.3.3 One way ANOVA of effect of CA on the exponential growth rate:

***E. coli* O157:H7, pH 6.3, 20°C**

```
Source      DF        SS        MS     F      P
CA           1  0.001524  0.001524  3.24  0.146
Error        4  0.001880  0.000470
Total        5  0.003404

S = 0.02168   R-Sq = 44.78%   R-Sq(adj) = 30.97%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N     Mean    StDev    +---------+---------+---------+---------
CA      3  0.21800  0.02651              (-----------*----------)
Non-CA  3  0.18612  0.01539    (-----------*-----------)
                               +---------+---------+---------+---------
                             0.150     0.180     0.210     0.240

Pooled StDev = 0.02168
```

***E. coli* O157:H7, pH 6.8, 20°C**

```
Source      DF        SS        MS     F      P
CA           1  0.000228  0.000228  1.93  0.237
Error        4  0.000473  0.000118
Total        5  0.000701

S = 0.01087   R-Sq = 32.56%   R-Sq(adj) = 15.70%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N     Mean    StDev   -+---------+---------+---------+--------
CA      3  0.20867  0.00611   (--------------*-------------)
Non-CA  3  0.22100  0.01411             (-------------*--------------)
                               -+---------+---------+---------+--------
                             0.192     0.204     0.216     0.228
Pooled StDev = 0.01087
```

S. aureus, pH 6.3, 20°C

```
Source      DF         SS         MS      F      P
CA           1  0.0012669  0.0012669  17.08  0.014
Error        4  0.0002967  0.0000742
Total        5  0.0015636

S = 0.008613   R-Sq = 81.02%   R-Sq(adj) = 76.28%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N     Mean    StDev  --------+---------+---------+---------+-
CA      3  0.06245  0.00317  (---------*--------)
Non-CA  3  0.09151  0.01176                    (--------*--------)
                             --------+---------+---------+---------+-
                                   0.060     0.075     0.090     0.105

Pooled StDev = 0.00861
```

S. aureus, pH 6.8, 20°C

```
Source      DF         SS         MS      F      P
CA           1  0.0005883  0.0005883  33.60  0.004
Error        4  0.0000700  0.0000175
Total        5  0.0006583

S = 0.004184   R-Sq = 89.36%   R-Sq(adj) = 86.70%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level   N      Mean     StDev  ---+---------+---------+---------+------
CA      3  0.064018  0.002760  (------*------)
Non-CA  3  0.083822  0.005234                     (------*------)
                               ---+---------+---------+---------+------
                                0.060     0.070     0.080     0.090

Pooled StDev = 0.004184
```

_S_. Typhimurium, pH 6.3, 20°C

```
Source      DF       SS       MS     F      P
CA           1  0.00859  0.00859  8.19  0.046
Error        4  0.00419  0.00105
Total        5  0.01278

S = 0.03238   R-Sq = 67.20%   R-Sq(adj) = 58.99%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N     Mean    StDev  ------+---------+---------+---------+---
CA      3  0.24700  0.04508                (---------*----------)
Non-CA  3  0.17133  0.00802  (---------*----------)
                             ------+---------+---------+---------+---
                                0.150     0.200     0.250     0.300
Pooled StDev = 0.03238
```

_S_. Typhimurium, pH 6.8, 20°C

```
Source      DF        SS        MS      F      P
CA           1  0.019041  0.019041  41.69  0.003
Error        4  0.001827  0.000457
Total        5  0.020867

S = 0.02137   R-Sq = 91.25%   R-Sq(adj) = 89.06%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level   N     Mean    StDev  -------+---------+---------+---------+--
CA      3  0.30967  0.01504                       (------*------)
Non-CA  3  0.19700  0.02621  (-----*------)
                            -------+---------+---------+---------+--
                                 0.200     0.250     0.300     0.350

Pooled StDev = 0.02137
```

***V. parahaemolyticus*, pH 6.3, 20°C**

```
Source      DF         SS         MS     F      P
CA           1  0.0000927  0.0000927  2.92  0.163
Error        4  0.0001270  0.0000317
Total        5  0.0002196

S = 0.005634   R-Sq = 42.20%   R-Sq(adj) = 27.75%


                            Individual 95% CIs For Mean Based on Pooled
                            StDev
Level   N     Mean    StDev  ---------+---------+---------+---------+
CA      3  0.09430  0.00698            (------------*------------)
Non-CA  3  0.08644  0.00385  (-----------*------------)
                            ---------+---------+---------+---------+
                                   0.0840    0.0910    0.0980    0.1050

Pooled StDev = 0.00563
```

***V. parahaemolyticus*, pH 6.8, 20°C**

```
Source      DF        SS        MS     F      P
CA           1  0.001915  0.001915  7.86  0.049
Error        4  0.000974  0.000244
Total        5  0.002889

S = 0.01560   R-Sq = 66.28%   R-Sq(adj) = 57.85%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level   N     Mean    StDev   -+---------+---------+---------+--------
CA      3  0.07128  0.02203   (---------*---------)
Non-CA  3  0.10701  0.00127                (---------*---------)
                             -+---------+---------+---------+--------
                            0.050     0.075     0.100     0.125
Pooled StDev = 0.01560
```

***L. monocytogenes*, pH 6.3, 20°C**

```
Source      DF         SS         MS      F      P
CA           1  0.0012714  0.0012714  24.02  0.008
Error        4  0.0002117  0.0000529
Total        5  0.0014831

S = 0.007275   R-Sq = 85.73%   R-Sq(adj) = 82.16%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level   N     Mean    StDev  -----+---------+---------+---------+----
CA      3  0.19920  0.00387  (-------*-------)
Non-CA  3  0.22832  0.00953                   (-------*-------)
                            -----+---------+---------+---------+----
                               0.195     0.210     0.225     0.240

Pooled StDev = 0.00727
```

***L. monocytogenes*, pH 6.8, 20°C**

```
Source      DF         SS         MS      F      P
CA           1  0.0041443  0.0041443  49.16  0.002
Error        4  0.0003372  0.0000843
Total        5  0.0044815

S = 0.009181   R-Sq = 92.48%   R-Sq(adj) = 90.59%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level   N     Mean    StDev  --+---------+---------+---------+-------
CA      3  0.18567  0.00808  (-----*-----)
Non-CA  3  0.23823  0.01016                     (-----*-----)
                            --+---------+---------+---------+-------
                            0.175     0.200     0.225     0.250

Pooled StDev = 0.00918
```

***L. monocytogenes*, pH 6.3, 7°C**

```
Source      DF         SS         MS       F      P
CA           1  0.0002381  0.0002381  129.48  0.000
Error        4  0.0000074  0.0000018
Total        5  0.0002454

S = 0.001356   R-Sq = 97.00%   R-Sq(adj) = 96.25%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level   N      Mean     StDev  ------+---------+---------+---------+---
CA      3  0.024232  0.001447  (---*----)
Non-CA  3  0.036830  0.001258                        (----*---)
                              ------+---------+---------+---------+---
                                  0.0250    0.0300    0.0350    0.0400

Pooled StDev = 0.001356
```

***L. monocytogenes*, pH 6.8, 7°C**

```
Source      DF         SS         MS      F      P
CA           1  0.0002764  0.0002764  36.06  0.004
Error        4  0.0000307  0.0000077
Total        5  0.0003071

S = 0.002769   R-Sq = 90.01%   R-Sq(adj) = 87.52%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level   N      Mean     StDev  -------+---------+---------+---------+--
CA      3  0.023946  0.002429  (------*------)
Non-CA  3  0.037521  0.003071                    (-------*------)
                              -------+---------+---------+---------+--
                                   0.0240    0.0300    0.0360    0.0420

Pooled StDev = 0.002769
```

2.3.4 One way ANOVA analysis of effect of CA on maximum population density:

***E. coli* O157:H7, pH 6.3, 20°C**

```
Source      DF       SS       MS     F      P
CA           1  0.02765  0.02765  7.11  0.056
Error        4  0.01556  0.00389
Total        5  0.04320

S = 0.06236   R-Sq = 63.99%   R-Sq(adj) = 54.99%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev  -------+---------+---------+---------+--
CA      3  8.7290  0.0852  (---------*---------)
Non-CA  3  8.8648  0.0226             (---------*---------)
                          -------+---------+---------+---------+--
                               8.70      8.80      8.90      9.00

Pooled StDev = 0.0624
```

***E. coli* O157:H7, pH 6.8, 20°C**

```
Source      DF       SS       MS     F      P
CA           1  0.01170  0.01170  4.97  0.090
Error        4  0.00942  0.00235
Total        5  0.02112

S = 0.04852   R-Sq = 55.42%   R-Sq(adj) = 44.27%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev  --------+---------+---------+---------+-
CA      3  8.8570  0.0446             (----------*----------)
```

```
Non-CA  3  8.7687  0.0522  (----------*----------)
                           --------+---------+---------+---------+-
                                 8.750     8.820     8.890     8.960

Pooled StDev = 0.0485
```

***S. aureus*, pH 6.3, 20°C**

```
Source      DF       SS       MS      F      P
CA           1  0.09786  0.09786  22.37  0.009
Error        4  0.01750  0.00437
Total        5  0.11536

S = 0.06614   R-Sq = 84.83%   R-Sq(adj) = 81.04%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  ------+---------+---------+---------+---
CA      3  8.6770  0.0785  (--------*--------)
Non-CA  3  8.9325  0.0509                    (-------*--------)
                           ------+---------+---------+---------+---
                               8.64      8.76      8.88      9.00

Pooled StDev = 0.0661
```

***S. aureus*, pH 6.8, 20°C**

```
Source      DF      SS      MS     F      P
CA           1  0.1980  0.1980  9.06  0.040
Error        4  0.0874  0.0218
Total        5  0.2853

S = 0.1478   R-Sq = 69.37%   R-Sq(adj) = 61.71%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  -------+---------+---------+---------+--
CA      3  8.5544  0.2044  (--------*---------)
Non-CA  3  8.9176  0.0437               (---------*--------)
                           -------+---------+---------+---------+--
                                8.50      8.75      9.00      9.25

Pooled StDev = 0.1478
```

***S*. Typhimurium, pH 6.3, 20°C**

```
Source      DF       SS       MS      F      P
CA           1  0.09400  0.09400  49.16  0.002
Error        4  0.00765  0.00191
Total        5  0.10165

S = 0.04373   R-Sq = 92.48%   R-Sq(adj) = 90.59%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev    +---------+---------+---------+---------
CA      3  8.5700  0.0476    (------*------)
Non-CA  3  8.8203  0.0395                           (------*------)
                             +---------+---------+---------+---------
                           8.50      8.60      8.70      8.80
```

```
Pooled StDev = 0.0437
```

_S_. Typhimurium, pH 6.8, 20°C

```
Source      DF       SS       MS      F      P
CA           1  0.15811  0.15811  54.70  0.002
Error        4  0.01156  0.00289
Total        5  0.16968

S = 0.05376   R-Sq = 93.19%   R-Sq(adj) = 91.48%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev  -----+---------+---------+---------+----
CA      3  8.5640  0.0755  (-----*-----)
Non-CA  3  8.8887  0.0086                      (-----*----)
                          -----+---------+---------+---------+----
                             8.55      8.70      8.85      9.00

Pooled StDev = 0.0538
```

***V. parahaemolyticus*, pH 6.3, 20°C**

```
Source      DF       SS       MS        F      P
CA           1  5.76395  5.76395  3499.04  0.000
Error        4  0.00659  0.00165
Total        5  5.77054

S = 0.04059   R-Sq = 99.89%   R-Sq(adj) = 99.86%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev   -+---------+---------+---------+--------
CA      3  6.6344  0.0559   (-*)
Non-CA  3  8.5946  0.0129                                    (*)
                           -+---------+---------+---------+--------
                          6.60      7.20      7.80      8.40

Pooled StDev = 0.0406
```

***V. parahaemolyticus*, pH 6.8, 20°C**

```
Source      DF     SS     MS      F      P
CA           1  2.015  2.015  15.38  0.017
Error        4  0.524  0.131
Total        5  2.539

S = 0.3620   R-Sq = 79.36%   R-Sq(adj) = 74.20%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev  -------+---------+---------+---------+--
CA      3  7.9594  0.5118  (---------*--------)
Non-CA  3  9.1185  0.0091                    (---------*---------)
                          -------+---------+---------+---------+--
```

```
                                7.80      8.40      9.00      9.60

Pooled StDev = 0.3620
```

***L. monocytogenes*, pH 6.3, 20°C**

```
Source      DF      SS      MS     F      P
CA           1  0.0135  0.0135  0.86  0.407
Error        4  0.0631  0.0158
Total        5  0.0766

S = 0.1256   R-Sq = 17.65%   R-Sq(adj) = 0.00%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  -----+---------+---------+---------+----
CA      3  9.1198  0.1479  (------------*------------)
Non-CA  3  9.2147  0.0983        (------------*-------------)
                           -----+---------+---------+---------+----
                              9.00      9.15      9.30      9.45

Pooled StDev = 0.1256
```

***L. monocytogenes*, pH 6.8, 20°C**

```
Source      DF       SS       MS     F      P
CA           1  0.06147  0.06147  7.39  0.053
Error        4  0.03325  0.00831
Total        5  0.09472

S = 0.09118   R-Sq = 64.89%   R-Sq(adj) = 56.12%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  --------+---------+---------+---------+-
CA      3  9.0326  0.1076  (---------*---------)
Non-CA  3  9.2350  0.0710               (---------*--------)
                           --------+---------+---------+---------+-
                                 9.00      9.15      9.30      9.45

Pooled StDev = 0.0912
```

***L. monocytogenes*, pH 6.3, 7°C**

```
Source      DF      SS      MS      F      P
CA           1  0.4913  0.4913  25.51  0.007
Error        4  0.0770  0.0193
Total        5  0.5683

S = 0.1388   R-Sq = 86.44%   R-Sq(adj) = 83.05%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
```

```
Level   N    Mean   StDev  -------+---------+---------+---------+--
CA      3  8.7273  0.1635  (-------*------)
Non-CA  3  9.2995  0.1086                    (------*------)
                           -------+---------+---------+---------+--
                                8.70      9.00      9.30      9.60

Pooled StDev = 0.1388
```

***L. monocytogenes*, pH 6.8, 7°C**

```
Source      DF     SS     MS     F      P
CA           1  0.094  0.094  0.72  0.443
Error        4  0.519  0.130
Total        5  0.613

S = 0.3603   R-Sq = 15.34%   R-Sq(adj) = 0.00%

                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev  ---+---------+---------+---------+------
CA      3  8.8666  0.4996  (--------------*-------------)
Non-CA  3  9.1170  0.1000        (--------------*-------------)
                           ---+---------+---------+---------+------
                            8.40      8.80      9.20      9.60

Pooled StDev = 0.3603
```

Chapter 5

5.3.3 Survival of *L. monocytogenes* during heat treatment

1. Comparison of survival of *L. monocytogenes* grown with either DPC16-SCS or -GFS with the control (neither DPC16-SCS nor -GFS) under CA by One way ANOVA:

Treated for 2 min:

```
Source      DF      SS      MS       F      P
supplement   2  979.45  489.73  136.04  0.000
Error        5   18.00    3.60
Total        7  997.45

S = 1.897   R-Sq = 98.20%   R-Sq(adj) = 97.47%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean  StDev  --+---------+---------+---------+-------
GFS    3  29.378  2.803                              (-*--)
PB     2   1.042  0.271  (--*--)
SCS    3  15.130  1.051             (--*--)
                          --+---------+---------+---------+-------
                            0        10        20        30
```

```
Pooled StDev = 1.897
```

Treated for 3 min:

```
Source      DF       SS       MS       F      P
supplement   2  5.61237  2.80619  319.55  0.000
Error        5  0.04391  0.00878
Total        7  5.65628

S = 0.09371   R-Sq = 99.22%   R-Sq(adj) = 98.91%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev   -+---------+---------+---------+--------
GFS    3  1.1041  0.1313                (-*-)
PB     2  0.0909  0.0366   (-*--)
SCS    3  2.2273  0.0636                                  (-*-)
                           -+---------+---------+---------+--------
                          0.00      0.70      1.40      2.10


Pooled StDev = 0.0937
```

Treated for 4 min:

```
Source      DF       SS       MS      F      P
supplement   2  0.15059  0.07530  39.74  0.001
Error        5  0.00947  0.00189
Total        7  0.16007

S = 0.04353   R-Sq = 94.08%   R-Sq(adj) = 91.71%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level  N     Mean    StDev  -----+---------+---------+---------+----
GFS    3  0.34703  0.06629                               (----*----)
PB     2  0.01990  0.00962  (------*-----)
SCS    3  0.10743  0.01721           (----*----)
                            -----+---------+---------+---------+----
                               0.00      0.12      0.24      0.36

Pooled StDev = 0.04353
```

Treated for 5 min:

```
Source      DF         SS         MS       F      P
supplement   2  0.0046509  0.0023254  128.63  0.000
Error        5  0.0000904  0.0000181
Total        7  0.0047413

S = 0.004252   R-Sq = 98.09%   R-Sq(adj) = 97.33%


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

```
GFS    3  0.056367  0.006612                              (--*--)
PB     2  0.003750  0.001485  (---*---)
SCS    3  0.008700  0.000608     (--*---)
                               --+---------+---------+---------+-------
                               0.000     0.020     0.040     0.060

Pooled StDev = 0.004252
```

Treated for 6 min:

```
Source      DF         SS         MS      F      P
supplement   2  0.0007306  0.0003653  18.55  0.005
Error        5  0.0000985  0.0000197
Total        7  0.0008291

S = 0.004438   R-Sq = 88.12%   R-Sq(adj) = 83.37%


                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level  N      Mean     StDev  -----+---------+---------+---------+----
GFS    3  0.021633  0.006987                       (------*-----)
PB     2  0.002950  0.000636  (-------*-------)
SCS    3  0.001267  0.000462  (-----*------)
                               -----+---------+---------+---------+----
                                  0.000     0.010     0.020     0.030

Pooled StDev = 0.004438
```

2. Comparison of survival of *L. monocytogenes* grown with either DPC16-SCS or -GFS with the control (neither DPC16-SCS nor -GFS) under Non-CA by One way ANOVA:

Treated for 2 min:

```
Source      DF      SS     MS     F      P
supplement   2  11.586  5.793  9.70  0.019
Error        5   2.986  0.597
Total        7  14.572

S = 0.7728   R-Sq = 79.51%   R-Sq(adj) = 71.31%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level  N    Mean   StDev  ---+---------+---------+---------+------
GFS    3  4.7778  0.6939                    (-------*------)
PB     2  2.5000  0.0000  (---------*--------)
SCS    3  2.1759  1.0058  (-------*------)
                           ---+---------+---------+---------+------
                            1.5       3.0       4.5       6.0

Pooled StDev = 0.7728
```

Treated for 3 min:

```
Source      DF       SS       MS     F      P
supplement   2  0.02701  0.01351  5.21  0.060
Error        5  0.01297  0.00259
Total        7  0.03998
```

```
S = 0.05093   R-Sq = 67.57%   R-Sq(adj) = 54.59%

                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level  N     Mean    StDev  ------+---------+---------+---------+---
GFS    3  0.17333  0.02665         (---------*--------)
PB     2  0.12500  0.03536  (-----------*----------)
SCS    3  0.26763  0.07176                    (--------*---------)
                           ------+---------+---------+---------+---
                               0.080     0.160     0.240     0.320

Pooled StDev = 0.05093
```

Treated for 4 min:

```
Source      DF         SS         MS     F      P
supplement   2  0.0007619  0.0003810  4.31  0.082
Error        5  0.0004417  0.0000883
Total        7  0.0012036

S = 0.009398   R-Sq = 63.30%   R-Sq(adj) = 48.63%

                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level  N      Mean     StDev   -+---------+---------+---------+--------
GFS    3  0.012033  0.003101   (--------*--------)
PB     2  0.026650  0.020011          (-----------*----------)
SCS    3  0.034300  0.003315                   (--------*--------)
                             -+---------+---------+---------+--------
                            0.000     0.015     0.030     0.045

Pooled StDev = 0.009398
```

Treated for 5 min:

```
Source      DF         SS         MS     F      P
supplement   2  0.0002442  0.0001221  4.15  0.087
Error        5  0.0001472  0.0000294
Total        7  0.0003914

S = 0.005426   R-Sq = 62.39%   R-Sq(adj) = 47.35%

                             Individual 95% CIs For Mean Based on Pooled
                             StDev
Level  N      Mean     StDev  ---------+---------+---------+---------+
GFS    3  0.001233  0.000153  (----------*---------)
PB     2  0.013700  0.007212               (-----------*-----------)
SCS    3  0.011767  0.006897               (---------*---------)
                             ---------+---------+---------+---------+
                                    0.0000    0.0080    0.0160    0.0240

Pooled StDev = 0.005426
```

Treated for 6 min:

```
Source      DF         SS         MS     F      P
supplement   2  0.0000359  0.0000179  7.56  0.031
Error        5  0.0000119  0.0000024
Total        7  0.0000478

S = 0.001541   R-Sq = 75.15%   R-Sq(adj) = 65.21%


                              Individual 95% CIs For Mean Based on
                              Pooled StDev
Level  N      Mean     StDev  ------+---------+---------+---------+---
GFS    3  0.000533  0.000058  (-------*------)
PB     2  0.006000  0.003253                  (--------*--------)
SCS    3  0.002600  0.000800         (-------*------)
                              ------+---------+---------+---------+---
                                  0.0000    0.0030    0.0060    0.0090

Pooled StDev = 0.001541
```

3. Comparison of survival of *L. monocytogenes* grown with either DPC16-SCS or -GFS under CA with non-CA by One way ANOVA:

Treated for 2 min:

```
Source      DF      SS      MS      F      P
Atmosphere   1  1057.8  1057.8  31.52  0.000
Error       10   335.6    33.6
Total       11  1393.3

S = 5.793   R-Sq = 75.92%   R-Sq(adj) = 73.51%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level   N    Mean  StDev  --+---------+---------+---------+-------
CA      6  22.254  8.030                        (------*-----)
Non-CA  6   3.477  1.621  (-----*------)
                            --+---------+---------+---------+-------
                            0.0       8.0      16.0      24.0

Pooled StDev = 5.793
```

Treated for 3 min:

```
Source      DF     SS     MS      F      P
Atmosphere   1  6.266  6.266  31.97  0.000
Error       10  1.960  0.196
Total       11  8.226

S = 0.4427   R-Sq = 76.18%   R-Sq(adj) = 73.79%


                             Individual 95% CIs For Mean Based on
                             Pooled StDev
Level   N    Mean   StDev  ---+---------+---------+---------+------
CA      6  1.6657  0.6220                          (------*-----)
Non-CA  6  0.2205  0.0708  (------*-----)
```

```
                          ---+---------+---------+---------+------
                           0.00      0.60      1.20      1.80

Pooled StDev = 0.4427
```

Treated for 4 min:

```
Source      DF       SS       MS      F      P
Atmosphere   1  0.12493  0.12493  12.98  0.005
Error       10  0.09628  0.00963
Total       11  0.22121

S = 0.09812   R-Sq = 56.48%   R-Sq(adj) = 52.12%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level   N     Mean    StDev  -------+---------+---------+---------+--
CA      6  0.22723  0.13820                       (--------*--------)
Non-CA  6  0.02317  0.01253  (--------*--------)
                            -------+---------+---------+---------+--
                                 0.00      0.10      0.20      0.30

Pooled StDev = 0.09812
```

Treated for 5 min:

```
Source      DF        SS        MS     F      P
Atmosphere   1  0.002033  0.002033  5.41  0.042
Error       10  0.003758  0.000376
Total       11  0.005791

S = 0.01939   R-Sq = 35.11%   R-Sq(adj) = 28.62%


                            Individual 95% CIs For Mean Based on
                            Pooled StDev
Level   N     Mean    StDev  -------+---------+---------+---------+--
CA      6  0.03253  0.02644                  (----------*----------)
Non-CA  6  0.00650  0.00723  (----------*----------)
                            -------+---------+---------+---------+--
                                 0.000     0.016     0.032     0.048

Pooled StDev = 0.01939
```

Treated for 6 min:

```
Source      DF         SS         MS     F      P
Atmosphere   1  0.0002930  0.0002930  4.03  0.073
Error       10  0.0007280  0.0000728
Total       11  0.0010210

S = 0.008532   R-Sq = 28.70%   R-Sq(adj) = 21.57%


                              Individual 95% CIs For Mean Based on Pooled
                              StDev
```

```
Level   N      Mean     StDev  ---------+---------+---------+---------+
CA      6  0.011450  0.012002               (----------*----------)
Non-CA  6  0.001567  0.001240  (----------*----------)
                               ---------+---------+---------+---------+
                                      0.0000    0.0070    0.0140    0.0210

Pooled StDev = 0.008532
```

Appendix III

Determination of reuterin concentration

The production of reuterin (β-hydroxypropionaldehyde) was determined following the method described by Circle *et al*. (1945) with modification. The concentration of reuterin can be determined based on a standard curve for the correlation of the acrolein concentrations vs their optical densities obtained by this procedure.

1. Apparatus and materials:

Spectral extinction values were determined on a spectrophotometer (Model: Genova, Jenway Ltd, Essex, UK) at a wavelength of 490 nm, using 10 x 10 mm square cuvettes (Sarstedt, Nümbrecht, Germany) with a path of 10 mm, and water as the comparison standard.

2. Standard solutions:

1) Water
2) 0.01 M Tryptophane solution
3) Ethanol: 95%, aldehyde-free
4) HCl: 37% or 12.0 N
5) Acrolein (2-Propenal) solution (100 µg/mL)

3. Procedure and method of analysis:

To produce a standard curve, a series of concentrations of acrolein (2-propenal, Sigma, St. Louis, MO, USA) solution were made in 10 mL volumetric flasks following the composition of reagents listed in the following table by pipetting into each flask 0.5 mL of 0.01 M tryptophane (Sigma, St. Louis, MO, USA), amounts of acrolein in 95%

ethanol (100 µg/mL) varying from 0 to 2 mL, enough 95% ethanol to make its total quantity in each flask of 2 mL, and 1.2 mL of water.

Dilution (µg/mL)	0	2.0	4.0	6.0	8.0	10	12	14	16	20
0.01 M tryptophane	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Acrolein solution	0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	2.0
95% ethanol	2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0
H_2O	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
HCl	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3
Total volume (mL)	10	10	10	10	10	10	10	10	10	10

The flasks were then cooled in an ice bath and made up to the volume with ice-cold 12 N HCl to avoid premature heating on mixing. If desired, the order of addition of water and HCl may be reversed to give the same results, provided all ingredients are ice cold, that is, 6.3 mL of 12 N HCl may be added to each flask followed by the addition of the required amount of water to make the final volume of 10 mL in each flask.

The flasks were incubated and the development of colour was allowed in an oven with subdued light at 40^{o}C for 50 minutes. The optical density (OD) was read in the spectrophotometer. A standard curve of acrolein vs OD was then produced.

In the final method of analysis adopted, the procedure is identical to that described above, except that in the case of unknown acrolein samples the acrolein must either be concentrated by distillation or other means, or diluted, as indicated by preliminary test, in order for its concentration to fall within the limits of the standard curve.

Appendix IV

Alignment of 16S rDNA sequences of the identified LAB strains

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60         70
P2              ---------- -CTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P3              ----TTTGAT CCTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P4              ---------T CCTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P10             ---GTTTGAT CCTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P11             ---------- -CTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P12             ---------- -CTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P13             ---------T CCTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P17             ----TTTGAT CCTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P19             --------AT CCTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P20             ---------- --TGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P21             ---------- --TGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P23             ---------- -CTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
DPC16           ---------- --TGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGTAC GCACTGGCCC
P7              AGAGTTTGAT CCTGGCTCAG GATGAACGCC GGCGGTGTGC CTAATACATG CAAGTCGAAC GCGTTGGCCC
P9              ----TTTGAT CCTGGCTCAG GACGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGTAC GCTTTGGCTT
P18             --------AT CCTGGCTCAG GATGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAAC GCGCAG----
P24             ---------- CCTGGCTCAG GATGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAAC GCGCAG----
P27             --------AT CCTGGCTCAG GATGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAAC GCGCAG----

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         80         90        100        110        120        130        140
P2              AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P3              AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P4              AACTGATTGA TGGTGCTTGC ACCTGATTGA AGTTGGTTTA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P10             AACTGATTGA TGGTGCTTGC ACCGGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P11             AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P12             AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P13             AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P17             AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P19             AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P20             AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P21             AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P23             AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
DPC16           AACTGATTGA TGGTGCTTGC ACCTGATTGA CGATGGATCA CCAGTGAGTG GCGGACGGGT GAGTAACACG
P7              AACTGATTGA ACGTGCTTGC ACGGACTTGA CGTTGGTTTA CCAACGAGTG GCGGACGGGT GAGTAACACG
P9              TTTCCACCGG A---GCTTGC TC-------- CACCGGAAAA AGAG-GAGTG GCGAACGGGT GAGTAACACG
P18             ---CGAAAGG T---GCTTGC AC-------- ------CTTT CAAGCGAGTG GCGAACGGGT GAGTAACACG
P24             ---CGAAAGG T---GCTTGC AC-------- ------CTTT CAAGCGAGTG GCGAACGGGT GAGTAACACG
P27             ---CGAAAGG T---GCTTGC AC-------- ------CTTT CAAGCGAGTG GCGAACGGGT GAGTAACACG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        150        160        170        180        190        200        210
P2              TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P3              TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P4              TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P10             TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P11             TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P12             TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P13             TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P17             TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P19             TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P20             TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P21             TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P23             TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
DPC16           TAGGTAACCT GCCCCGGAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AACAAAAGCC
P7              TAGGTAACCT GCCCCAAAGC GGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGCATAAC AGTTTGAATC
P9              TGGGTAACCT GCCCATCAGA AGGGGATAAC ACTTGGAAAC AGGTGCTAAT ACCGTATAAC AATCGAAACC
P18             TGGATAACCT GCCTCAAGGC TGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGAATAAA ACTTAGTATC
P24             TGGATAACCT GCCTCAAGGC TGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGAATAAA ACTTAGTATC
P27             TGGATAACCT GCCTCAAGGC TGGGGATAAC ATTTGGAAAC AGATGCTAAT ACCGAATAAA ACTTAGTATC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        220        230        240        250        260        270        280
P2              ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P3              ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P4              ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P10             ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P11             ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P12             ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P13             ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P17             ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
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P19          ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P20          ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P21          ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P23          ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
DPC16        ACATGGCTTT TGTTTGAAAG ATGGCTTTGG CTATCACTCT GGGATGGACC TGCGGTGCAT TAGCTAGTTG
P7           GCATGATTCA AACTTAAAAG ATGGTTTCGG CTATCACTTT GGGATGGACC TGCGGCGCAT TAGCTTGTTG
P9           GCATGGTTTT GATTTGAAAG GCGCTTTCGG GTGTCGCTGA TGGATGGACC CGCGGTGCAT TAGCTAGTTG
P18          GCATGATACA AAGTTGAAAG GCGCTAC--G GCGTCACCTA GAGATGGGTC CGCGGTGCAT TAGTTAGTTG
P24          GCATGATACA AAGTTGAAAG GCGCTAC--G GCGTCACCTA GAGATGGGTC CGCGGTGCAT TAGTTAGTTG
P27          GCATGATACA AAGTTGAAAG GCGCTAC--G GCGTCACCTA GAGATGGGTC CGCGGTGCAT TAGTTAGTTG

                      ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                               290        300        310        320        330        340        350
P2           GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P3           GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P4           GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P10          GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P11          GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P12          GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P13          GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P17          GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P19          GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P20          GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P21          GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P23          GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
DPC16        GTAAGGTAAC GGCTTACCAA GGCGATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P7           GTAGGGTAAC GGCCTACCAA GGCTGTGATG CGTAGCCGAG TTGAGAGACT GATCGGCCAC AATGGAACTG
P9           GTGAGGTAAC GGCTCACCAA GGCCACGATG CATAGCCGAC CTGAGAGGGT GATCGGCCAC ATTGGGACTG
P18          GTGGGGTAAA GGCCTACCAA GACAATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC ATTGGGACTG
P24          GTGGGGTAAA GGCCTACCAA GACAATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC ATTGGGACTG
P27          GTGGGGTAAA GGCCTACCAA GACAATGATG CATAGCCGAG TTGAGAGACT GATCGGCCAC ATTGGGACTG

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                               360        370        380        390        400        410        420
P2           AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P3           AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P4           AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P10          AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P11          AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P12          AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P13          AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P17          AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P19          AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P20          AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P21          AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P23          AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
DPC16        AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P7           AGACACGGTC CATACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCCACAA TGGGCGCAAG CCTGATGGAG
P9           AGACACGGCC CAAACTCCTA CGGGAGGCAG CAGTAGGGAA TCTTCGGCAA TGGACGAAAG TCTGACCGAG
P18          AGACACGGCC CAAACTCCTA CGGGAGGCTG CAGTAGGGAA TCTTCCACAA TGGGCGAAAG CCTGATGGAG
P24          AGACACGGCC CAAACTCCTA CGGGAGGCTG CAGTAGGGAA TCTTCCACAA TGGGCGAAAG CCTGATGGAG
P27          AGACACGGCC CAAACTCCTA CGGGAGGCTG CAGTAGGGAA TCTTCCACAA TGGGCGAAAG CCTGATGGAG

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                               430        440        450        460        470        480        490
P2           CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P3           CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P4           CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P10          CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P11          CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P12          CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P13          CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P17          CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P19          CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P20          CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P21          CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P23          CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
DPC16        CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTG GAGAAGAACG TGCGTGAGAG
P7           CAACACCGCG TGAGTGAAGA AGGGTTTCGG CTCGTAAAGC TCTGTTGTTA GAGAAGAACG TGCGTGAGAG
P9           CAACGCCGCG TGAGTGAAGA AGGTTTTCGG ATCGTAAAAC TCTGTTGTTA GAGAAGAACA AGGATGAGAG
P18          CAACGCCGCG TGTGTGATGA AGGCTTTAGG GTCGTAAAGC ACTGTTGTAT GGGAAGAAAT GCTAGAATAG
P24          CAACGCCGCG TGTGTGATGA AGGCTTTAGG GTCGTAAAGC ACTGTTGTAT GGGAAGAAAT GCTAGAATAG
P27          CAACGCCGCG TGTGTGATGA AGGCTTTAGG GTCGTAAAGC ACTGTTGTAT GGGAAGAAAT GCTAGAATAG

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                               500        510        520        530        540        550        560
P2           TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P3           TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P4           TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P10          TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P11          TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P12          TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P13          TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P17          TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P19          TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P20          TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P21          TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
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P23         TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
DPC16       TAACTGTTCA CGCAGTGACG GTATCCAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P7          CAACTGTTCA CGCAGTGACG GTATCTAACC AGAAAGTCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P9          TAACTGTTCA TCCCTTGACG GTATCTAACC AGAAAGCCAC GGCTAACTAC GTGCCAGCAG CCGCGGTAAT
P18         GGAATGATTC TAGTTCGACG GTACCATACC AGAAAGGGAC GGCTAAATAC GTGCCAGCAG CCGCGGTAAT
P24         GGAATGATTC TAGTTCGACG GTACCATACC AGAAAGGGAC GGCTAAATAC GTGCCAGCAG CCGCGGTAAT
P27         GGAATGATTC TAGTTCGACG GTACCATACC AGAAAGGGAC GGCTAAATAC GTGCCAGCAG CCGCGGTAAT

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                            570        580        590        600        610        620        630
P2          ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P3          ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P4          ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P10         ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P11         ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P12         ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P13         ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P17         ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P19         ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P20         ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P21         ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P23         ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
DPC16       ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTGCTT AGGTCTGATG
P7          ACGTAGGTGG CAAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTTGAT AAGTCTGATG
P9          ACGTAGGTGG CAAGCGTTGT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG GCGGTTTCTT AAGTCTGATG
P18         ACGTATGTCC CGAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG ACGGTTGATT AAGTCTGATG
P24         ACGTATGTCC CGAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG ACGGTTGATT AAGTCTGATG
P27         ACGTATGTCC CGAGCGTTAT CCGGATTTAT TGGGCGTAAA GCGAGCGCAG ACGGTTGATT AAGTCTGATG

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                            640        650        660        670        680        690        700
P2          TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P3          TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P4          TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P10         TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P11         TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P12         TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P13         TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P17         TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P19         TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P20         TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P21         TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P23         TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
DPC16       TGAAAGCCTT CGGCTTAACC GAAGAAGTGC ATCGGAAACC GGGCGACTTG AGTGCAGAAG AGGACAGTGG
P7          TGAAAGCCTT TGGCTTAACC AAAGAAGTGC ATCGGAAACT GTCAGACTTG AGTGCAGAAG AGGGCAGTGG
P9          TGAAAGCCCC CGGCTCAACC GGGGAGGGTC ATTGGAAACT GGGAGACTTG AGTGCAGAAG AGGAGAGTGG
P18         TGAAAGCCCG GAGCTCAACT CCGGAATGGC ATTGGAAACT GGTTAACTTG AGTGTTGTAG AGGTAAGTGG
P24         TGAAAGCCCG GAGCTCAACT CCGGAATGGC ATTGGAAACT GGTTAACTTG AGTGTTGTAG AGGTAAGTGG
P27         TGAAAGCCCG GAGCTCAACT CCGGAATGGC ATTGGAAACT GGTTAACTTG AGTGTTGTAG AGGTAAGTGG

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                            710        720        730        740        750        760        770
P2          AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P3          AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P4          AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P10         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P11         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P12         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P13         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P17         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P19         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P20         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P21         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P23         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
DPC16       AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGTCTGGTC
P7          AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTGCCTGGTC
P9          AATTCCATGT GTAGCGGTGA AATGCGTAGA TATATGGAGG AACACCAGTG GCGAAGGCGG CTCTCTGGTC
P18         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTTACTGGAC
P24         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTTACTGGAC
P27         AACTCCATGT GTAGCGGTGG AATGCGTAGA TATATGGAAG AACACCAGTG GCGAAGGCGG CTTACTGGAC

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                            780        790        800        810        820        830        840
P2          TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P3          TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P4          TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P10         TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P11         TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P12         TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P13         TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P17         TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P19         TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P20         TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P21         TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P23         TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
DPC16       TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
P7          TGCAACTGAC GCTGAGGCTC GAAAGCATGG GTAGCGAACA GGATTAGATA CCCTGGTAGT CCATGCCGTA
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P9          TGTAACTGAC GCTGAGGCTC GAAAGCGTGG GGAGCAAACA GGATTAGATA CCCTGGTAGT CCACGCCGTA
P18         AACAACTGAC GTTGAGGCTC GAAAGTGTGG GTAGCAAACA GGATTAGATA CCCTGGTAGT CCACACCGTA
P24         AACAACTGAC GTTGAGGCTC GAAAGTGTGG GTAGCAAACA GGATTAGATA CCCTGGTAGT CCACACCGTA
P27         AACAACTGAC GTTGAGGCTC GAAAGTGTGG GTAGCAAACA GGATTAGATA CCCTGGTAGT CCACACCGTA

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                       850        860        870        880        890        900        910
P2          AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P3          AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P4          AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P10         AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P11         AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P12         AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P13         AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P17         AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P19         AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P20         AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P21         AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P23         AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
DPC16       AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGGAGCT AACGCATTAA GCACTCCGCC
P7          AACGATGAGT GCTAGGTGTT GGAGGGTTTC CGCCCTTCAG TGCCGCAGCT AACGCATTAA GCACTCCGCC
P9          AACGATGAGT GCTAAGTGTT GGAGGGTTTC CGCCCTTCAG TGCTGCAGCT AACGCATTAA GCACTCCGCC
P18         AACGATGAAT ACTAGGTGTT AGGAGGTTTC CGCCTCTTAG TGCCGAAGCT AACGCATTAA GTATTCCGCC
P24         AACGATGAAT ACTAGGTGTT AGGAGGTTTC CGCCTCTTAG TGCCGAAGCT AACGCATTAA GTATTCCGCC
P27         AACGATGAAT ACTAGGTGTT AGGAGGTTTC CGCCTCTTAG TGCCGAAGCT AACGCATTAA GTATTCCGCC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       920        930        940        950        960        970        980
P2          TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P3          TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P4          TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P10         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P11         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P12         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P13         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P17         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P19         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P20         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P21         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P23         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
DPC16       TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P7          TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P9          TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG
P18         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGACCCG CACAAGCGGT GGAGCATGTG
P24         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGACCCG CACAAGCGGT GGAGCATGTG
P27         TGGGGAGTAC GACCGCAAGG TTGAAACTCA AAGGAATTGA CGGGGACCCG CACAAGCGGT GGAGCATGTG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       990       1000       1010       1020       1030       1040       1050
P2          GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P3          GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P4          GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P10         GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P11         GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P12         GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P13         GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P17         GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P19         GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P20         GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P21         GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P23         GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
DPC16       GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CTAACCTTAG AGATAAG-GC
P7          GTTTAATTCG AAGCTACGCG AAGAACCTTA CCAGGTCTTG ACATCTTGCG CCAACCCTAG AGATAGG-GC
P9          GTTTAATTCG AAGCAACGCG AAGAACCTTA CCAGGTCTTG ACATCCTTTG ACCACTCTAG AGATAGA-GC
P18         GTTTAATTCG AAGCAACGCG AAGAACCTTA CCAGGTCTTG ACATCCTTTG AAGCTTCTAG AGATAGAAGT
P24         GTTTAATTCG AAGCAACGCG AAGAACCTTA CCAGGTCTTG ACATCCTTTG AAGCTTCTAG AGATAGAAGT
P27         GTTTAATTCG AAGCAACGCG AAGAACCTTA CCAGGTCTTG ACATCCTTTG AAGCTTCTAG AGATAGAAGT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1060       1070       1080       1090       1100       1110       1120
P2          GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P3          GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P4          GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P10         GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P11         GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P12         GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P13         GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P17         GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P19         GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P20         GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P21         GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P23         GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
DPC16       GTTCCCTTCG GGGACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P7          GTTTCCTTCG GGAACGCAAT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P9          TTCCCCTTCG GGGGCAAAGT GACAGGTGGT GCATGGTTGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P18         GTTCTCTTCG GAGACAAAGT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
P24         GTTCTCTTCG GAGACAAAGT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
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P27          GTTCTCTTCG GAGACAAAGT GACAGGTGGT GCATGGTCGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA


                    1130       1140       1150       1160       1170       1180       1190
P2           AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P3           AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P4           AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P10          AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P11          AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P12          AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P13          AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P17          AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P19          AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P20          AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P21          AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P23          AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
DPC16        AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTAAGTTGG GCACTCTAGT GAGACTGCCG
P7           AGTCCCGCAA CGAGCGCAAC CCTTGTTACT AGTTGCCAGC ATTCAGTTGG GCACTCTAGT GAGACTGCCG
P9           AGTCCCGCAA CGAGCGCAAC CCTTATTGTT AGTTGCCATC ATTCAGTTGG GCACTCTAGC AAGACTGCCG
P18          AGTCCCGCAA CGAGCGCAAC CCTTATTGTT AGTTGCCAGC ATTCAGTTGG GCACTCTAGC GAGACTGCCG
P24          AGTCCCGCAA CGAGCGCAAC CCTTATTGTT AGTTGCCAGC ATTCAGTTGG GCACTCTAGC GAGACTGCCG
P27          AGTCCCGCAA CGAGCGCAAC CCTTATTGTT AGTTGCCAGC ATTCAGTTGG GCACTCTAGC GAGACTGCCG


                    1200       1210       1220       1230       1240       1250       1260
P2           GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P3           GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P4           GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P10          GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P11          GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P12          GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P13          GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P17          GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P19          GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P20          GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P21          GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P23          GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
DPC16        GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P7           GTGACAAACC GGAGGAAGGT GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P9           GTGACAAACC GGAGGAAGGT GGGGATGACG TCAAATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P18          GTGACAAACC GGAGGAAGGC GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P24          GTGACAAACC GGAGGAAGGC GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG
P27          GTGACAAACC GGAGGAAGGC GGGGACGACG TCAGATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG


                    1270       1280       1290       1300       1310       1320       1330
P2           CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P3           CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P4           CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P10          CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P11          CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P12          CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P13          CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P17          CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P19          CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P20          CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P21          CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P23          CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
DPC16        CTACAATGGA CGGTACAACG AGTCGCAAGC TCGCGAGAGT AAGCTAATCT CTTAAAGCCG TTCTCAGTTC
P7           CTACAATGGA CGGTACAACG AGTCGCGAAC TCGCGAGGGC AAGCTAATCT CTTAAAACCG TTCTCAGTTC
P9           CTACAATGGG AAGTACAACG AGTTGCGAAG TCGCGAGGCT AAGCTAATCT CTTAAAGCTT CTCTCAGTTC
P18          CTACAATGGC GTATACAACG AGTTGCCAAC CCGCGAGGGT GAGCTAATCT CTTAAAGTAC GTCTCAGTTC
P24          CTACAATGGC GTATACAACG AGTTGCCAAC CCGCGAGGGT GAGCTAATCT CTTAAAGTAC GTCTCAGTTC
P27          CTACAATGGC GTATACAACG AGTTGCCAAC CCGCGAGGGT GAGCTAATCT CTTAAAGTAC GTCTCAGTTC


                    1340       1350       1360       1370       1380       1390       1400
P2           GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P3           GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P4           GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P10          GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P11          GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P12          GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P13          GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P17          GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P19          GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P20          GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P21          GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P23          GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
DPC16        GGACTGTAGG CTGCAACTCG CCTACACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P7           GGACTGCAGG CTGCAACTCG CCTGCACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG
P9           GGATTGCAGG CTGCAACTCG CCTGCATGAA GCCGGAATCG CTAGTAATCG CGGATCAGCA CGCCGCGGTG
P18          GGACTGCAGT CTGCAACTCG ACTGCACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA CGCCGCGGTG
P24          GGACTGCAGT CTGCAACTCG ACTGCACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA CGCCGCGGTG
P27          GGACTGCAGT CTGCAACTCG ACTGCACGAA GTCGGAATCG CTAGTAATCG CGGATCAGCA CGCCGCGGTG
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1410       1420       1430       1440       1450       1460       1470
P2              AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P3              AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P4              AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P10             AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P11             AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P12             AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P13             AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P17             AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P19             AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P20             AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P21             AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P23             AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
DPC16           AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAACGCCCAA AGTCGGTGGC
P7              AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA TGAGAGTTTG CAACACCCAA AGTCGGTGGG
P9              AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA CGAGAGTTTG TAACACCCGA AGTCGGTGAG
P18             AATACGTTCC CGGGTCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAATGCCCAA AGCCGGTGGC
P24             AATACGTTCC CGGGTCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAATGCCCAA AGCCRGTGGC
P27             AATACGTTCC CGGGTCTTGT ACACACCGCC CGTCACACCA TGGGAGTTTG TAATGCCCAA AGCCGGTGGC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1480       1490       1500       1510       1520       1530       1540
P2              CTAACCATT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P3              CTAACCATT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P4              CTAACCATT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P10             CTAACCTTT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P11             CTAACCTTT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P12             CTAACCTTT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P13             CTAACCTTT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P17             CTAACCTTT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P19             CTAACCTTT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P20             CTAACCTTT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P21             CTAACCATT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P23             CTAACCATT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
DPC16           CTAACCATT- ATGGAGGGAG CCGCCTAAGG CGGGACAGAT GACTGGGGTG AAGTCGTA-A CAAGGTAGCC
P7              GTAACCCTTC GGGGAGCTAG CCGCCTAAGG TGGGGCAGAT GATTAGGGTG AAGTCGTA-A CAAGGTAGCC
P9              GTAACCTTTT GGGGAGCCAG CCGCCTAAGG TGGGGTAGAT GATTGGGGTG AAGTCGTA-A CAAGGTAGCC
P18             CTAACCTTA- -TGGAGGGAG CCGTCTAAGG CAGGACAGAT GACTAGGGTG AAGTCGTA-A CAAGGTAGCC
P24             CTAACCTTA- -TGGAGGGAG CCGTCTAAGG CAGGACAGAT GACTAGGGTG AAGTCGTA-A CAAGGTAGCC
P27             CTAACCTTA- -TGGAGGGAG CCGTCTAAGG CAGGACAGAT GACTAGGGTG AAGTCGTA-A CAAGGTAGCC

                ....|....| ....|....| ....|....|
                       1550       1560       1570
P2              GTAGGAGAAC CTGCGGCTGG ATCACCTCCT
P3              GTAGGAGAAC CTGCGGCTGG ATCACCTCCT
P4              GTAGGAGAAC CTGCGGCTGG ----------
P10             GTAGGAGAAC CTGCGGCTGG ATCACCTCC-
P11             GTAGGAGAAC CTGCGGCTGG ATCACCTCC-
P12             GTAGGAGAAC CTGCGGCTGG ----------
P13             GTAGGAGAAC CTGCGGCTGG ----------
P17             GTAGGAGAAC CTGCGGCTGG ----------
P19             GTAGGAGAAC CTGCGG---- ----------
P20             GTAGGAGAAC CTGCGGCTGG ATC-------
P21             GTAGGAGAAC CTGCGGCTGG ATCACC----
P23             GTAGGAGAAC CTGCGGCTGG ATCACCTC--
DPC16           GTAGGAGAAC CTGCGGCTGG ATC-------
P7              GTAGGAGAAC CT-------- ----------
P9              GTAGGAGAAC CTGCGG---- ----------
P18             GTAGGAGAAC CTGCGGCTG- ----------
P24             GTAGGAGAAC CTGCGGCTGG ATCAC-----
P27             GTAGGAGAAC CTGCGGCTGG ----------
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