The Phenotypic and Molecular Responses of *Listeria monocytogenes* to Stressors

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II Authorship

The accompanying thesis submitted for the degree of PhD entitled:

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is based on work conducted by the author in the School of Health at the University of Northampton mainly during the period between:

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All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. If necessary for the deposit of this thesis in the institutional repository, permission to disseminate third party material has been sought and granted by copyright holders.

None of the work has been submitted for another degree in this or any other University

Signed ..... Date .....

III Abbreviations

µg	Microgram
µg/ml	Micrograms per millilitre
µl	Microlitre
µM	Micromolar
μ_{max}	Maximal growth rate
ATR	Acid tolerance response
BHI	Brain heart infusion
bp	Base pairs
BPW	Buffered peptone water
$CaCl_2$	Calcium chloride
CDC	Centers for disease control and prevention
cDNA	Complementary DNA
cfu/ml	Colony forming units per millilitre
CO_2	Carbon dioxide
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGD-e	Strain designation for *L. monocytogenes* first isolated by E.G.D. Murray (1924) (genome strain)
FSA	Food Standards Agency
g/l	Grams per litre
dH_2O	Sterile distilled water
Fw.	Forward primer
H_2O_2	Hydrogen Peroxide

HCl	Hydrochloric acid
HEPES	*n*-heptadecanoic acid methyl ester
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kV	Kilovolts
Kb	Kilobases
LB	Luria-Bertani
M	Molar
MAP	Modified atmosphere packaging
mg/ml	Milligrams per millilitre
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
mm	Millimeter
ms	Milliseconds
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nM	Nanomolar
NTC	No template control
OD	Optical density
O_2	Oxygen
PCR	Polymerase chain reaction
ppm	Parts per million
qRT-PCR	Quantitative reverse transcription PCR
RNA	Ribonucleic acid
RTE	Ready-to-eat
Rv.	Reverse primer
SD	Standard Deviation

SEOM	Standard error of the Mean
SGJ	Simulated gastric juice
TAE	Tris-acetate-EDTA
TE	Tris-EDTA buffer
T_m	Melting temperature
TSP	Trisodium phosphate
UV	Ultraviolet
V	Volts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

IV Abstract

Listeria monocytogenes is a food-borne pathogen and the causative agent of listeriosis, a severe infection resulting in septicaemia, meningitis and still birth. Infection typically arises through the consumption of contaminated foodstuffs. *L. monocytogenes* is a hardy organism which can survive several food control measures. Psychotrophic and facultatively anaerobic properties permit growth under refrigeration conditions and within modified atmosphere packaging. Through transcriptional and translational changes *L. monocytogenes* is able to mount adaptive responses against stressors. Such responses typically cross protect against subsequent stresses, including effectors of the human immune system. The aim of this study was to assess the ability of *L. monocytogenes* to adaptively respond to stressors, and to assess the phenotypic and molecular responses that such exposures have on resistance and virulence potential.

Using adaptation and repeated exposure assays *L. monocytogenes* cells were assessed for their ability to develop resistance and to adaptively respond to stressors. Using qRT-PCR and insertional mutagenesis the roles of several candidate genes in stress response were assessed. Using a simulated gastro-intestinal transit model the effects of refrigeration and oxygen limitation on virulence potential were investigated, while microarray analysis allowed elucidation of the molecular mechanisms accounting for altered resistance properties and virulence potential.

Stationary phase *L. monocytogenes* cells were not found to adapt to sub lethal exposure to citric acid, TSP, NaClO and H_2O_2. Susceptibility to stressors was increased or unchanged following sub-lethal pre-exposure.

Sub-lethal exposure to NaClO increased expression of *lmo0669* (oxidoreductase) by 4.6-fold, while a 2-fold increase in *gadA* was

observed during TSP exposure. These responses permit survival under NaClO and TSP stress, and may have implications in subsequent stress exposure and/or virulence potential. Inactivation of *ctsR*, *hfq*, *lisR* and *lisK* by site-directed mutagenesis gave rise to mutant cells with increased sensitivity to H_2O_2. Citric acid resistance was impaired by *ctsR* and *hfq* disruption. Pre-conditioning under oxygen limiting conditions significantly increased acid tolerance in *L. monocytogenes* FSL R2-499.

When assessing the effects of pre-conditioning on gastro-intestinal transit *L. monocytogenes* FSL R2-499 displayed growth phase, pre-conditioning and pH dependant resistance profiles. Cells grown under oxygen limiting conditions typically demonstrated increased resistance towards simulated gastric juice (pH 2.5), however, only stationary phase cells were able to survive bile salt exposure. *L. monocytogenes* cells grown under oxygen limiting conditions displayed increased acid tolerance but decreased H_2O_2 resistance. Transcriptional analysis revealed significant up-regulation of the acid response gene, *gadA*, under oxygen limiting conditions, while catalase was significantly down-regulated.

The findings of this study provide important information for food manufacturers who can use this data to "intelligently apply" food control measures, or hurdles, to improve food safety.

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Chapter 1

General introduction

1.1 Overview

Listeriosis is a rare but severe infection caused by the Gram positive bacterium *L. monocytogenes* and is characterised by the presence of *L. monocytogenes* cells in areas considered sterile (Todd and Notermans, 2011. While *L. monocytogenes* is ubiquitous, cases of listeriosis are low compared to other foodborne pathogens (FSA, 2011), however systemic infection often results in hospitalisation and death (Rocourt, 1996). Those with a compromised immune system are at the greatest risk of listeriosis, most notably the elderly, pregnant women or neonates (Swaminathan and Gerner-Smidt, 2007). By having dampened cell mediated immunity, the intracellular nature of *L. monocytogenes* permits cell invasion and subsequent systemic infection (Bakardjiev *et al.*, 2004).

Infection from *L. monocytogenes* generally occurs through consumption of contaminated foods. Compromised foods include those which do not undergo thermal processing/cooking prior to eating (Jamali and Thong, 2014). This is because *L. monocytogenes* is able to overcome several of the food controls commonly employed to maintain the microbiological safety of foods, including low pH, high salinity and low temperatures (Gandhi and Chikindas, 2007), thus microbial load in non-cooked/ready-to-eat foods can reach levels which compromises human health in the susceptible population.

While *L. monocytogenes* can be controlled by some food manufacturing processes; consumer demand for 'natural' and 'fresh' foods which have

undergone only minimal processing increases the risk to the consumer. In minimally processed products the listerial load may be sufficient to cause listeriosis in those who are pre-disposed. Further exacerbating the risks of *L. monocytogenes* in foods is its ability to adaptively respond to unfavourable environments, a response process termed "stress hardening" (Koutsoumanis *et al.*, 2003). During stress hardening cells become increasingly resistant to stressors if they have been previously stressed at a sub-lethal level (Lou and Yousef, 1997). This is problematic for the food industry where, in an attempt to meet consumer demands for natural end products, manufacturers use hurdle technology to maximise food safety while minimising processing intensity (Leistner, 2000). When applying hurdle technology, several processes are incorporated at low intensity. Working in an additive or synergistic manner, hurdle technology can achieve microbial safety while meeting consumer demands (Leistner and Gorris, 1995). However, the application of such technology is paradoxical, whereby application of the first hurdle may in fact increase resistance to the next. Therefore hurdle technology and sub-lethal food processes may give rise to cells with enhanced resistance properties, increasing the potential for listeriosis infection and outbreaks.

1.2 Listeria monocytogenes

1.2.1 Taxonomy, ecology and physiology

The genus *Listeria* contains ten species of Gram positive, catalase positive rods which are non spore-forming, facultative anaerobes (Hellberg *et al.*, 2013). Of these species only *Listeria monocytogenes* is considered a human pathogen although *Listeria ivanovii* is of human interest due to its pathogenic nature towards livestock, making it an economically important species (Vázquez-Boland *et al.*, 2001a; Schmid *et al.*, 2005). The genus *Listeria* is characterised by a low GC content (~38%) and is phylogenetically grouped with the genera *Staphylococcus, Streptococcus, Lactobacillus and Brochothrix* in the *Clostridium* sub-branch of Gram positive eubacteria (Zunabovic *et al.*, 2011). While the exact phylogenetic position of *Listeria* remains elusive, various sequencing methods have identified the genera *Bacillus* and *Brochothrix* as its closest relatives (Rocourt and Buchrieser, 2007).

Physiologically *L. monocytogenes* is a hardy organism. It is capable of growth at temperatures below 0°C, in both acidic and alkaline conditions (pH 4.6 – 9.5) and at water activity levels as low as 0.92 (Carpentier and Cerf, 2011).

L. monocytogenes is ubiquitous in nature owing to its high resilience to a number of environmental stresses and adverse conditions. As such *L. monocytogenes* has been isolated from a number of sources including biological matter (soil and plants surfaces), surface water, sewage and

industrial run-off; as well as from a number of livestock species including cattle and poultry (Farber and Peterkin, 1991).

1.2.2 Evolutionary lineages

Listeria monocytogenes strains derive from one of four distinct evolutionary lineages. These are generally isolated from differing, but overlapping, niches (Orsi *et al.*, 2011). Understanding these evolutionary pathways is important as pathogenicity appears to be lineage dependant, with serotypes 1/2a (lineage II), 1/2b (lineage I) and 4b (lineage I) responsible for both outbreaks and isolated listeriosis cases in humans (Fig. 1-1) (McLauchlin *et al.*, 2004). Lineages III and IV are rare in humans and while prevalence of such strains is highest in animals, it is low compared to isolates belonging to lineages I and II (Orsi *et al.*, 2011).

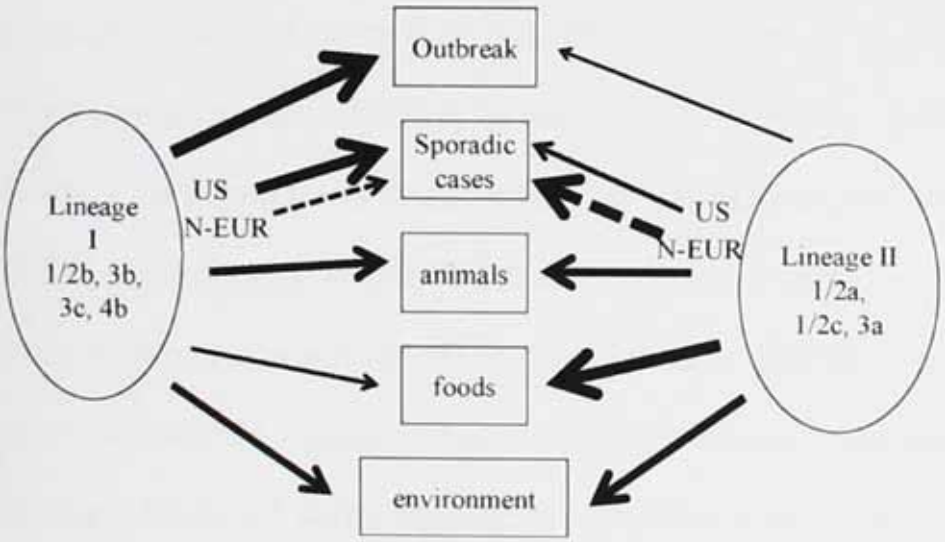

Fig. 1-1: Distribution of lineage I and II *L. monocytogenes* isolates by source (Taken from Osri *et al.*, 2011). Arrows indicate ecology of isolates; thickness of arrows indicate the proportion of each isolate belonging to a particular lineage within an ecological group; dashed lines indicate isolates derived from Northern Europe (N-EUR); solid lines indicate isolates derived from the USA (US).

Lineage III strains are isolated less frequently than those from lineages I and II, with Roberts *et al.* (2006) reporting that just 3% of their 1800 strain culture collection belonged to this lineage. While these strains are seldom of clinical importance, accounting for less than 1% of human listeriosis, they are of potential economic importance as they represent over 10% of isolates obtained from morbid animals (Jeffers *et al.*, 2001). Owing to the low prevalence of lineage III strains in human disease it has been speculated that these strains have diminished pathogenic capacities compared to those deriving from lineages I and II (Wiedmann *et al.*, 1997). However conflicting studies suggest this may be the result of decreased exposure to lineage III strains rather than their decreased virulence (Ward *et al.*, 2004).

Lineage IV strains were first described by Ward *et al.* (2008). This lineage was designated due to distinct phylogenetic differences between lineage III strains and lineage IIIB strains (Liu *et al.*, 2006). Lineage IV (IIIB) strains have received little acknowledgment due to their low prevalence, although research from Liu *et al.* (2006) reported such strains to be virulent in mouse models. These findings, coupled with prevalence studies, have led some to suggest (Jeffers *et al.*, 2001; Ward *et al.*, 2004) that it is low exposure rather than low virulence which accounts for this under-representation of both lineage III and IV strains in human disease.

Ward *et al.* (2008) briefly review the impact of lineage on human *L. monocytogenes* isolation and conclude that lineage I strains are typically

associated with listeriosis outbreaks while lineage II strains are frequently isolated from various foods and are often responsible for sporadic listeriosis cases.

1.2.3 Infection and disease

Infection from *L. monocytogenes* may lead to the onset of listeriosis. This condition is defined by *L. monocytogenes* isolated from 'sterile' sites such as the liver, spleen, cerebral spinal fluid and blood. An infection of this nature will often result in hospitalisation (Todd and Notermans, 2011). Listeriosis typically presents as meningitis or septicaemia and is particularly problematic for pregnant women, where cell mediated immunity is believed to be suppressed (Bakardjiev *et al.*, 2004), in the elderly and in the immuno-compromised (McLauchlin, 1996).

Unlike many food-borne pathogens *L. monocytogenes* can be considered atypical. As stated by Zunabovic *et al.* (2001), infections caused by food-borne micro-organisms are generally defined by low mortality rates with high prevalence rates, however this is not the case for *L. monocytogenes* which generally has a low prevalence rate but high mortality. This statement is supported by the findings of the Food Standards Agency (FSA) (2011) who published a report in which *L. monocytogenes* was found to cause a negligible percentage of cases of food borne illness within the UK while being accountable for the highest number of deaths, approximately 40% of all UK deaths caused by food-related pathogens

(Fig. 1-2). However, Disson *et al.* (2008) reported increasing incidence rates for listeriosis across Europe (Fig. 1-3) (Little *et al.*, 2009).

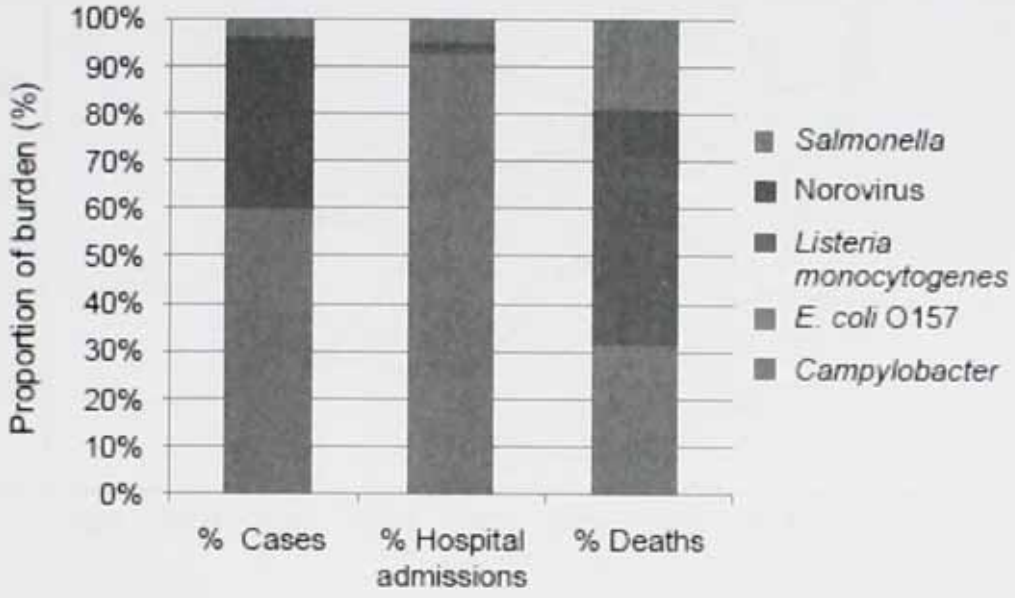

Fig. 1-2: Estimated percentage of cases, hospital admissions and deaths accountable to food borne pathogens in 2009 in England and Wales (FSA, 2011).

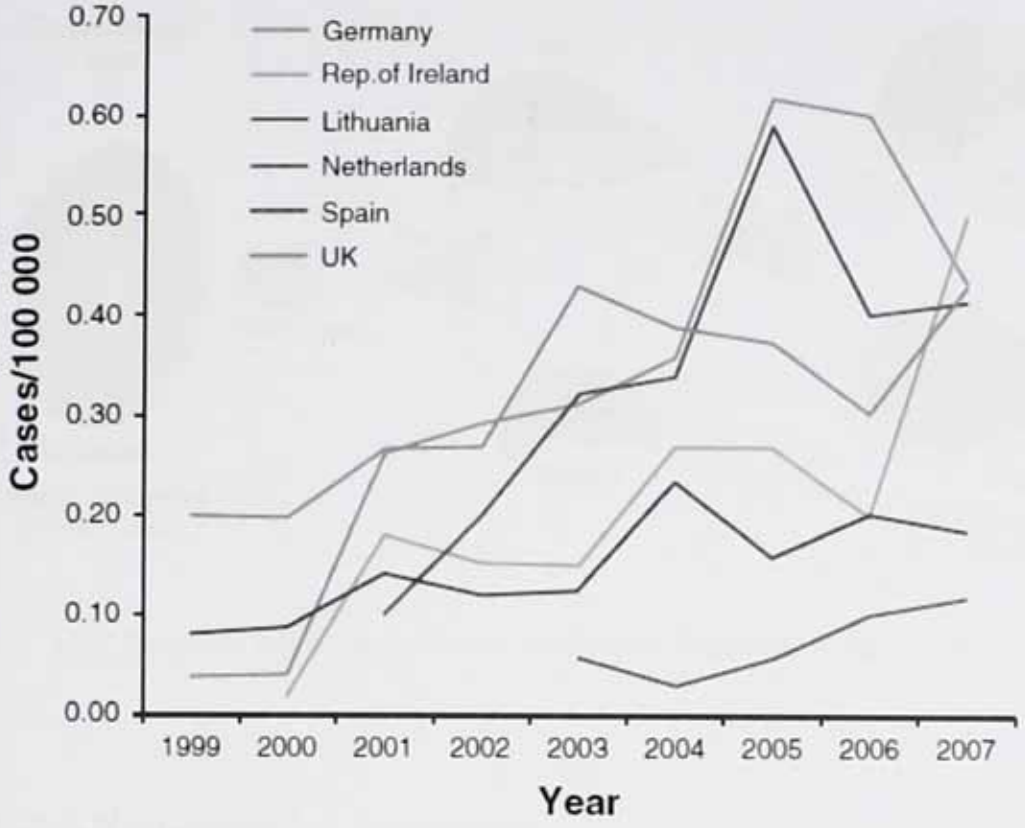

Fig. 1-3: Listeriosis incidence by country; 1999-2007 (Little *et al.*, 2009).

Listeria monocytogenes causes two main infection types, invasive infection, in which organisms spread from the digestive system to the central nervous system and brain via the bloodstream (Fig. 1-4); and non-invasive infection in which microbes remains confined to the digestive system (NHS, 2009).

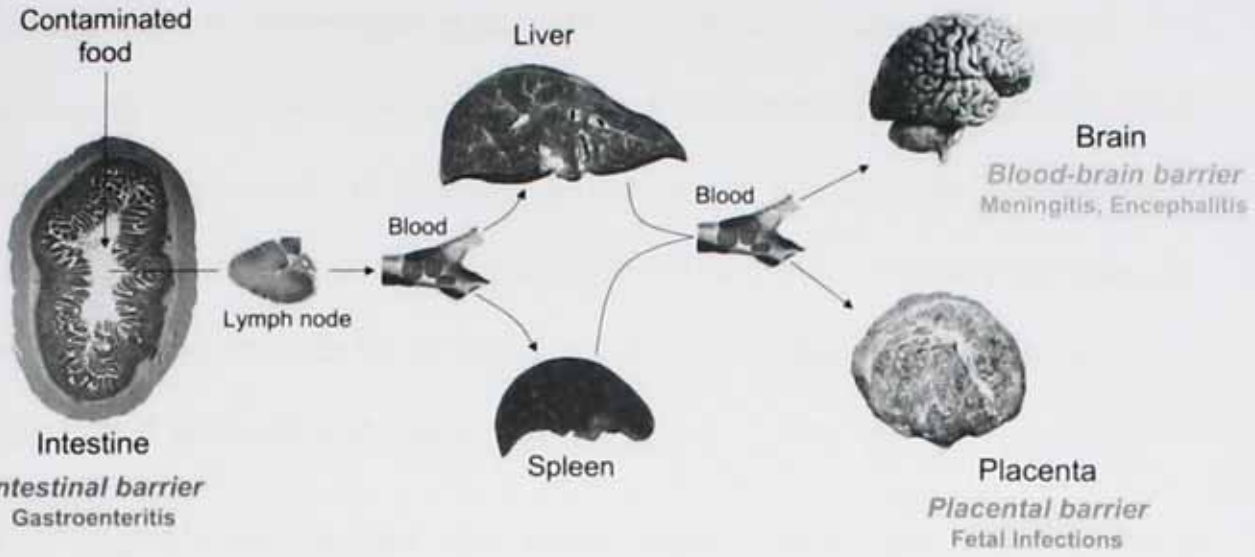

Fig. 1-4: Common infection pathways in human listeriosis (Lecuit, 2007).

1.2.4 Non-invasive listeriosis

Listeriosis was initially believed to be an invasive disease which was largely confined to individuals with a predisposing susceptibility (Franciosa *et al.*, 2001). However, it was later discovered that *L. monocytogenes* can be the causative agent of febrile gastrointestinal disease in otherwise healthy individuals (Sim *et al.*, 2002). Unlike invasive listeriosis, the non-invasive form of the disease is considered less severe and typically presents as fever and headache coupled with typical gastroenteritis symptoms such as diarrhoea, stomach pains and nausea/vomiting (Dalton *et al.*, 1997), although 'flu-like' symptoms and sore throat in the absence of gastrointestinal symptoms have also been reported (Salamina *et al.*, 1996).

The discovery of non-invasive listeriosis has far reaching implications. It is suggested by Franciosa *et al.* (2001) that non-invasive listeriosis increases public health interest in *L. monocytogenes* as epidemiology is likely to be affected. It is plausible that there has been an underestimation of the disease burden caused by *L. monocytogenes* as well as an incomplete understanding of the 'at risk' food products associated with *Listeria* transmission.

A number of food vehicles have been responsible for the transmission of *L. monocytogenes* and the subsequent development of non-invasive listeriosis. These include fish and seafood (Misrachi *et al.*, 1991; Riedo *et al.*, 1994; Miettenen *et al.*, 1999; Farber *et al.*, 2000), salad products (Salamina *et al.*, 1996; Aureli *et al.*, 2000) and milk (Dalton *et al.*, 1997).

While non-invasive listeriosis is not considered a major health risk there have been reports of the development of potentially fatal *L. monocytogenes* associated meningitis and invasive disease following non-invasive listeriosis in both sporadic cases and outbreak situations (Salamina *et al.*, 1996). Furthermore, non-invasive listeriosis cases and outbreaks can put a significant cost on public health services due to hospital admissions, diagnosis and treatment (Aureli *et al.*, 2000).

1.2.5 Invasive listeriosis

Unlike non-invasive listeriosis, invasive listeriosis is generally limited to those with a compromised immune system, of extreme age (old and young) or pregnant (Swaminathan and Gerner-Smidt, 2007). Invasive

listeriosis is considerably more severe than the non-invasive form and can present itself as a number of clinical manifestations including abortion, meningitis, septicaemia and encephalitis (Allerberger and Wagner, 2010). *L. monocytogenes* targets the central nervous system, meningeal and brain parenchymal infections regularly occur in those suffering from listeriosis (Rocourt *et al.*, 2000). The prognosis for those suffering from listeriosis varies with age and pre-disposing factors but case fatality is generally reported to be between 20-30% in both sporadic and outbreak cases, although Nolla-Salas *et al.* (1993) reported fatality rates as high as 51% (Rocourt, 1996). It is suggested by Lecuit (2007) that mortality rates of up to 30% are seen even with the administration of suitable antimicrobial agents. Furthermore, those who survive listeriosis which has invaded the central nervous system frequently develop long term neurological sequelae (Armstrong and Fung, 1993; McLauchlin *et al.*, 2004).

1.3 Listeria and the food industry

1.3.1 Introduction

Listerial infection typically arises through consumption of contaminated food products such as cheese, seafood, vegetables and refrigerated ready-to-eat (RTE) products including deli meats, salads and spreads (Barbosa *et al.*, 2012; Ivy *et al.*, 2012; Zhu *et al.*, 2005). Such consumption represents a significant risk to human health because of the high mortality rate compared to other food-borne pathogens (Zunabovic *et al.*, 2011; CDC, 2013; Lecuit, 2007; FSA, 2011; Garner *et al.*, 2006).

RTE foods represent a particular listeriosis risk as they do not undergo any further treatment post-processing/pre-consumption (Cabedo *et al.*, 2008). As such, RTE foods typically rely on refrigeration to minimise microbial load, however as *L. monocytogenes* is capable of growth at refrigeration temperatures loads may be sufficiently high to put the consumer at risk. In an attempt to ensure a safe end product, the food industry typically adopts the use of modified atmosphere packaging or vacuum packaging to control both spoilage and pathogenic micro-organisms by limiting oxygen availability (Lungu *et al.*, 2009). Its use is multifaceted, predominantly improving food safety and preventing food spoilage but also enhancing the sensory properties of products (Chun and Song, 2013; Mastromatteo *et al.*, 2011; Puligundla *et al.*, 2012). However, refrigeration and oxygen limitation is ineffective at controlling psychotrophs and facultative anaerobes such as *L. monocytogenes* (Oliveira *et al.*, 2010).

The ability of *L. monocytogenes* to survive and adapt to a range of unfavourable conditions, make it problematic for the food industry. Many processed and non-processed foods have been compromised including dairy and meat products, fish and seafood, and fresh produce (Gandhi and Chikindas, 2007). The threat of listeriosis from food is attributable to the organism's ability to overcome many of the systems put in place to ensure food safety, such as low temperature storage, high salt concentration and low pH (Cotter *et al.*, 2000; Duché *et al.*, 2002; Mastronicolis *et al.*, 2006).

1.3.2 The burden of *Listeria* in food

Product recalls are a major problem for the food industry. Recalls can be due to biological, chemical or operational hazards which compromise consumer health and safety. Potter *et al.* (2012) extensively reviewed product recall data from the UK, USA and Ireland and found that recalls not only impacted human health but also consumer perception of a given supplier, with drops in sales of up to 30% following a product recall, indicating a significant economic cost for a company. Biological agents are a major issue, accounting for 36% of recalls, with *Salmonella*, *Listeria* and *E. coli* accounting for 86% of recalls of this nature (Potter *et al.*, 2012). Via Foodnet, the Centers for Disease Control and Prevention (CDC) (2011) reported a total of 19,089 food related infections in 2010, over 4000 of which resulted in hospitalisations and 68 resulting in death. While a majority of these infections were attributable to *Salmonella* and *Campylobacter*, 7,564 and 6,365 cases respectively, 90% of the 125

cases of *Listeria* infection resulted in hospitalisation, highlighting the human health burden of this organism.

1.3.3 *Listeria* biofilms

Biofilms are bacterial communities which exist on biotic or abiotic surfaces. These structures typically consist of micro-organisms and extracellular polymeric substances which protect cells from unfavourable conditions as well as acting as a nutrient trap (Poulsen, 1999). Biofilms are difficult to control as they can form in most places where moisture is present (Djordjevic *et al.*, 2002), and once formed biofilmed cells have been found to possess an elevated resistance to many antimicrobial agents when compared to their planktonic counterparts (Mah and O'Toole, 2001).

Within food processing environments biofilms are able to form on a range of materials including stainless steel, glass, plastic and rubber (Brooks and Flint, 2008). *Listeria* spp. have been isolated from a wide range of processing plants, and in many cases this contamination is the result of biofilm formation (Chmielewski and Frank, 2006).

Environmental screening for *Listeria* spp. in processing plants has been extensively undertaken. Pagadala *et al.* (2012) sampled a range of seafood processing plants and isolated *Listeria* spp. in 69.5% of environmental samples, including samples taken from floor drains, gloves, storage vessels, for both cooked and raw product, and preparation tables. This was considerably greater than the 19.5% and 10.8% prevalence

reported for raw crab and crab meat respectively, highlighting the problem of biofilms in processing plants.

1.3.4 *Listeria* persistence

Persistence refers to the presence of a given bacterial strain at a facility or processing plant, which is of the same molecular type, over a period of time (Carpentier and Cerf, 2011). These persistent strains are over-represented as food contaminants compared to sporadic, or transient, strains. This is largely believed to be attributable to persistent strains possessing enhanced adhesive capabilities compared to their sporadic counterparts (Verghese *et al.*, 2011).

Given that several countries, including the USA, operate a zero-tolerance policy on *L. monocytogenes* in food, persistence represents a potential financial burden through repeated product contamination/recall. However, even in countries where *L. monocytogenes* is tolerated at low levels, such as in the UK where currently <100 cfu/g is acceptable in RTE foods (FSA, 2013), persistence represents a significant financial and health risk as *L. monocytogenes* contaminants may reach excessively high levels within a product even if the cold chain is maintained.

While persistent strains have not been found to possess increased virulence or resistance, their over-representation in food products make them a major risk for food producers as well as a potential risk to human health.

1.4 Stress responses

1.4.1 Introduction

Micro-organisms have evolved to function optimally within their own specific niche. However, most environments are prone to fluctuations and change, the extent of which can result in cell death, growth cessation or a greatly reduced growth rate (Beales, 2004). Even with fluctuations and continuously changing environmental conditions such as temperature, pH, osmolarity, nutrient availability and the presence of antimicrobials, bacteria have managed to colonise every planetary niche (Marles-Wright and Lewis, 2007). The ability of micro-organisms to persist in these changing environmental conditions relies upon cells to both sense and appropriately respond to the altered environment (Boor, 2006).

Upon encountering unfavourable conditions bacteria will rapidly change their gene expression patterns to alleviate the stress being placed upon them (Foster, 2005). This transcriptional change is often followed by a change in protein expression, resulting in an effector response.

The ubiquitous nature of *L. monocytogenes* can be largely attributed to its array of stress response genes, such genes encode various proteins which act to alleviate a given unfavourable condition. *Listeria* stress response genes are extensively reviewed by Chaturongakul *et al.* (2008). The ability of the organism to be a potentially life-threatening, intracellular pathogen to humans and animals, as well as being able to survive and replicate in a range of hostile, non-host environments has led to *L.*

monocytogenes being described as a "Jekyll and Hyde" organism (Gray *et al.*, 2006).

Various regulatory systems and genes are responsible for *L. monocytogenes* being capable of survival and growth in unfavourable conditions. These response mechanisms include intracellular changes at various levels including transcriptional, post-transcriptional and post translational changes (Chaturongakul *et al.*, 2008). At the forefront of these stress responses are a class of protein known collectively as sigma (σ) factors. These proteins make up sub-units of prokaryotic RNA polymerase, the driving force behind the changes in gene expression (Kullik *et al.*, 1998; Kazmierczak *et al.*, 2005). The sigma factor subunits of RNA polymerase are responsible for the recognition of highly conserved DNA sequences found in promoter regions, and during various initiation events, such as environmental stresses, sigma factors will associate with core polymerase, activating the transcriptional machinery (Britton *et al.*, 2002). Promoter recognition allows a number of genes to be regulated by a single sigma factor. This factor may account for the cross protection sometimes seen in adaptive responses to stress (Lou and Yousef, 1997).

1.4.2 Adaptive responses

Adaptive response refers to the ability of a cell or micro-organism to become more stress resistant following exposure to a previous sub-lethal stress (Crawford and Davies, 1994). Food-borne pathogens regularly encounter sub-lethal, or mild, exposures to stressful situations during food processing, which could increase the resistance properties of the

organism, a phenomenon coined "stress-hardening" (Lou and Yousef, 1997).

Owing to consumer demand for minimally processed foods (Leistner and Gorris, 1995), and the sensitive nature of many food products, manufacturers are now required to adopt milder methods of food preservation. These mild decontamination treatments will often inactivate only a portion of a bacterial population present or sub-lethally injure cells, resulting in cells with altered survival properties (Rajkovic *et al.*, 2009). A population which have been previously stressed can develop homologous resistance, increasing resistance to the initial stressor; or heterologous resistance in which a cross protection against a previously un-encountered stress is developed (Rowan, 1999).

A number of studies have confirmed the ability of both food-borne and non food-borne pathogens to adapt in both a homologous and heterologous manner to a wide array of stresses (O'Driscoll *et al.*, 1996; Bokhorst-van de Veen *et al.*, 2011; Vanlint *et al.*, 2013). Lou and Yousef (1997) reported that *L. monocytogenes* was able to adapt to lethal acidic conditions when pre-treated sub-lethally at low pH or with ethanol. Furthermore they found that sub-lethal incubation at low pH, high temperature; or pre-treatment with ethanol, salt (NaCl) or hydrogen peroxide could induce an adaptive response against lethal levels of hydrogen peroxide.

One of the most well studied adaptive responses is that of sub-lethal, or mild, exposure to heat followed by either exposure to lethal temperatures (homologous response) or to various heterologous lethal factors. Sergelidis and Abrahim (2009) extensively reviewed this and concluded that decimal reduction time (*D*-value) was significantly increased following exposure to mild, sub-lethal heating. This assertion was made on the basis of a number of studies in which researchers found increased heat resistance in *L. monocytogenes* cells following pre-incubation at temperatures above optimal in both stationary phase (Pagàn *et al.*, 1997; Novak and Juneja, 2003) and when exponentially growing (Jorgensen *et al.*, 1999; Walsh *et al.*, 2001).

The existence of heterologous adaptive responses to heat has been reported by Lou and Yousef (1997) and Lin and Chou (2004). The former reported that mild heat shocking initiated a cross protection against lethal doses of ethanol, hydrogen peroxide and NaCl in *L. monocytogenes*. These findings were confirmed by those of Lin and Chou (2004) who also reported increased tolerance to ethanol and NaCl following heat shocking, as well as finding an increased tolerance to lethal concentrations of crystal violet following prior exposure to sub-lethally high temperatures. However, Lin and Chou found that heat shocking increased the susceptibility of cells to hydrogen peroxide, contrary to the findings of Lou and Yousef where heat shocking increased hydrogen peroxide tolerance, suggesting that there may be strain differences regarding heat adaptation in *L. monocytogenes*.

1.4.3 Sigma Factors

Listeria monocytogenes contains five sigma factors, the most well defined being alternative sigma factor σ^B (Cossart and Archambaut, 2009). A number of studies have investigated the multi-faceted role of this sigma factor with regards to control of both virulence and stress response genes in *L. monocytogenes*. The use of σ^B knockout experiments has provided unequivocal evidence for the role of σ^B in stress tolerance and virulence. Zhang *et al.* (2011) used this approach to demonstrate the importance of SigB (σ^B) in bile response. The results indicated that *L. monocytogenes* Δ_{sigB} null mutants had a significantly lower minimum inhibitory concentration (MIC) to bile salt when compared to the wild-type highlighting the importance σ^B in bile tolerance. Bile salts are capable of cellular disruption through the disruption of cell membrane phospholipids and proteins, making it an effective host defence against micro-organisms (Begley *et al.*, 2005). This suggests that σ^B is not only a stress response gene regulon, but also a virulence regulon.

The dual importance of σ^B in *Listeria* stress response and virulence was further supported by Wiedmann *et al.* (1998). In this study *L. monocytogenes* $_{\Delta sigB}$ deletion mutants were compared to their wild-type parent strain and assessed for acid tolerance as well as invasiveness in mice. In this study the invasiveness of murine liver and spleen was not affected by deletion of *sigB* however, cells were significantly more susceptible to acid stress. The implications of this are two-fold; *sigB* expression allows cells to tolerate acidic conditions, such as those

commonly adopted by the food industry to decontaminate food surfaces (Russell and Gould, 2003) as well as allowing survival in acidic host environments, such as the stomach, with its acidic secretions and within macrophage vacuoles (Kindt *et al.*, 2007).

1.4.4 Cold Stress Response

One of the defining characteristics of *L. monocytogenes* is its ability to grow at refrigeration temperatures. This is atypical for pathogens and makes *L. monocytogenes* a significant problem for the food industry (Bayles *et al.*, 1996; Wemekamp-Kamphuis *et al.*, 2002). Refrigeration is the primary process by which microbial load in controlled in a number of ready-to-eat (RTE) and heat sensitive foods. The psychotrophic nature of *L. monocytogenes* compromises the safety of a variety of foods including dairy, meat and meat products, and produce (including RTE) (Ramaswamy, *et al.*, 2007; Pesavento *et al.*, 2010; World Health Organisation, 2012).

The psychotrophic nature of *L. monocytogenes* can be largely attributed to a family of proteins known collectively as the cold shock proteins (CSPs). These are small, conserved nucleic acid binding proteins which are believed to have important roles in cellular homeostasis (Schmid *et al.*, 2009). However, survival and growth at low temperature is not a singular process, it utilises the cellular membrane components, transcripts and cryoprotectant solutes to maintain cell viability and division capabilities at low temperatures (Gandi and Chikindas, 2007). Of particular importance is the lmo0501 protein; this is a transcriptional

regulator similar to mannitol transcriptional regulator protein found in some *Bacillus* sp.. The gene is part of a multi-gene operon cluster which is believed to possess genes related to carbon assimilation and metabolic functions (Michel *et al.*, 2011). Michel *et al.* (2011) reported that inactivation of the *lmo0501* gene was found to impact growth at low temperatures, under organic acid stress and in the presence of NaCl.

As transcriptional regulators such as *lmo0501* have been identified as crucial for survival in a range of unfavourable conditions; it would therefore be envisaged that these may provide cross protection against subsequent stresses. The results of a recent study by Bergholz *et al.* (2012) demonstrated low temperature pre-conditioning to increase resistance to hydrogen peroxide. However, Ivy *et al.* (2012) reported that low temperature pre-conditioning increased susceptibility acidic conditions, suggesting a complex relationship between cold stress and heterologous stresses.

1.4.5 Acid Tolerance Response

Of particular interest for the food industry is the ability of pathogens to survive in conditions made unfavourable through adjustment of pH. The interest in bacterial response to low pH/the acid tolerance response is due to the frequent use of organic acids for surface decontamination, as well as the use of low pH as a growth retardant in a number of foods (Gahan *et al.*, 1996; O'Driscoll *et al.*, 1996; Park and Chen, 2011). Figure 1-5 depicts the mechanism by which *L. monocytogenes* is able to withstand decreased environmental pH.

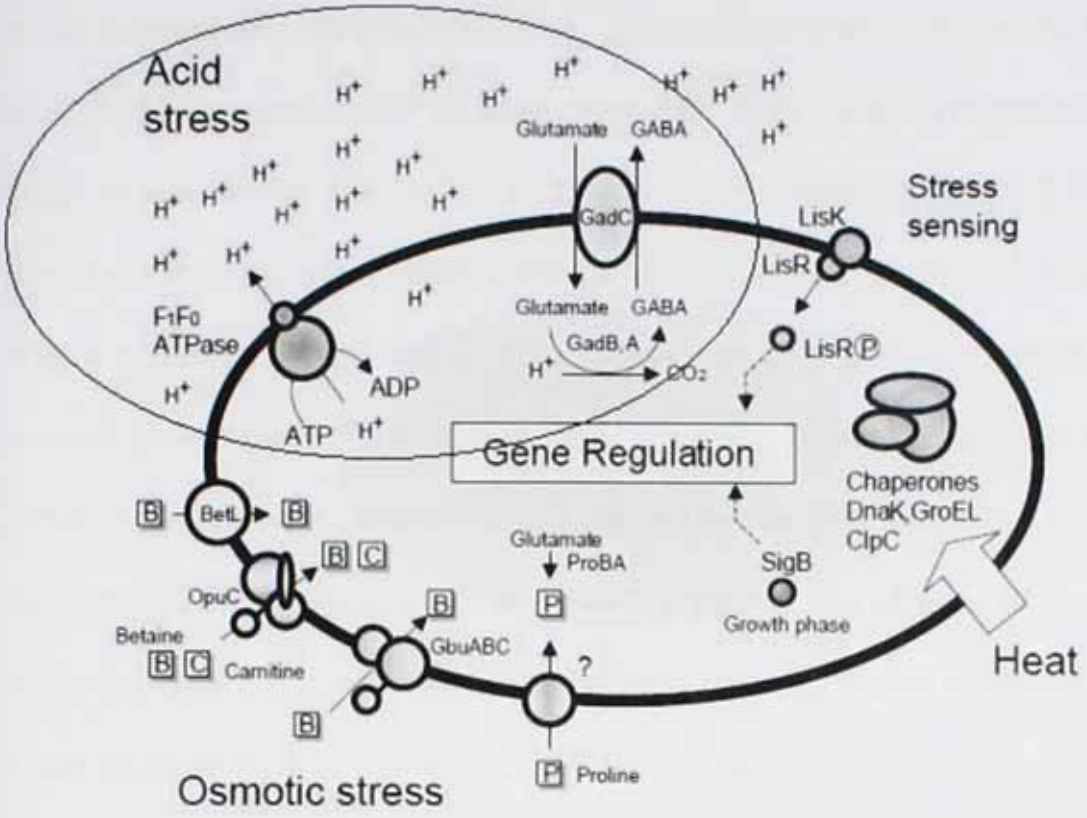

Fig. 1-5: Major stress response mechanisms adopted by *Listeria monocytogenes* to overcome commonly encountered stressors. Highlighted are the mechanisms to alleviate acid stress. H^+ indicates protons. Adapted from Hill *et al.* (2002).

It is suggested by Cotter and Hill (2003) that the mechanism of acid tolerance is highly complex involving a number of regulatory mechanisms including the use of proton pumps, environmental alkalinisation, changes in cell membrane composition and changes in protein and transcript content; in particular those proteins and transcripts associated with general stress responses.

The response of micro-organisms to low pH has received much attention. The acid tolerance response (ATR) is an adaptive response (see section 1.3.2) in which cells demonstrate an elevated tolerance to an otherwise lethal pH following an initial exposure to unfavourable, yet non-lethal, pH stress (Davis *et al.*, 1996). The physiological aspects of the ATR have

been extensively investigated in *L. monocytogenes*. The work of Davis *et al.* (1996) confirmed the existence of ATR in *L. monocytogenes*. In their study it was found that when cells were 'pre-adapted' to pH 5 for 1 hour they had an increased resistance to otherwise lethal pH 3 exposure. The ability to adapt to sub-lethal acid stress has also been reported to be growth phase dependant (Phan-Thanh *et al.*, 2000). Exponentially growing cells demonstrated a greater adaptive capacity than those in both early and late stationary phase. Furthermore, the effects of weak organic acids were found to be more deleterious to cells than strong inorganic acids such as hydrochloric acid (HCl), although the effect of organic acids on the induction of an ATR were not investigated in this study. However, subsequent research by Faleiro *et al.* (2003) confirmed that organic acid could induce an adaptive response to low pH although this was found to be strain dependant.

The importance of the ATR in virulence has been addressed by both O'Driscoll *et al.* (1996) and Marron *et al.* (1997). The former isolated *L. monocytogenes* cells which had a constitutively increased acid tolerance at all growth stages. Following isolation, acid resistant cells were used in an *in vivo* mouse model. Acid tolerant mutants demonstrated a significantly higher virulence compared to wild-type cells. This increased virulence was attributed to increased survival/resistance to the low pH found in the gut and macrophage phagosome. Marron *et al.* (1997) used transposon mutagenesis to develop mutants which were unable to mount a full ATR. Upon isolation, defective mutants were used to infect mice.

Mutants demonstrated a decreased virulence compared to wild-type. These findings support those of O'Driscoll *et al.* (1996) in that the ability of *L. monocytogenes* cells to successfully respond to low pH is vital for both gastro-intestinal survival and pathogenesis.

While the physiological effects of acid adaptation have been thoroughly researched in *L. monocytogenes* the genetic and molecular mechanisms underpinning this process have not received such attention. This gap has been somewhat filled by the findings of Cotter *et al.* (2000). The aim of this study was to elucidate the importance of F_0F_1 –ATPase in the acid response of *L. monocytogenes*. Cotter *et al.* (2000) utilised the ATPase inhibitor, *N, N'* -dicyclohexylcarbodiimide (DCCD), and assessed the effect that such inhibition has on the ATR of *L. monocytogenes*. The findings suggested that, while F_0F_1 –ATPase is an important factor in the ATR, it is not the sole mechanism behind the response. Acid adapted cells became increasingly sensitised to acid stress following exposure to DCCD, however these cells still displayed an elevated tolerance when compared to non-DCCD, non-adapted cells, suggesting other factors influence the ATR.

1.4.6 Response to oxidative stress

During food processing and pathogenesis micro-organisms are likely to encounter oxidative stress. During food processing this may be experienced through surface decontamination using oxidative chemicals such as sodium hypochlorite or hydrogen peroxide (Neal *et al.*, 2012). During host cell colonisation within macrophages; cells are further

subjected to oxidative stress. Reactive Oxygen Species (ROS) such as hydrogen peroxide are employed by cells to destroy engulfed bacteria within the macrophage phagosome (Gomes *et al.*, 2011).

Oxidising agents are generally multi-targeting chemicals which disrupt a wide array of cellular components, subsequently resulting in micro-organism inactivation. It is suggested by Wang *et al.* (2009) that sodium hypochlorite may inactivate cells through disruption to electron transport systems, DNA replication, enzymes and the overall membrane structure. In agreement with this, Finnegan *et al.* (2010) states that the physiological mechanisms of oxidative biocides are largely similar and target cell wall components, cause oxidation of nucleosides, disrupt protein synthesis and inhibit enzyme activity.

1.4.7 Response to low oxygen

One of the most commonly used methods of food preservation adopted by the food industry is that of modified atmosphere (MAP), controlled atmosphere packaging (CAP), equilibrium modified atmosphere packaging (EMA) and vacuum packaging (VP). These are different technologies which aim to reduce oxygen levels within/surrounding a foodstuff with the intention of controlling growth of both spoilage and pathogenic micro-organisms (Phillips, 1996). The facultative nature of *L. monocytogenes* allows it to overcome the application of low oxygen packaging and persist. Furthermore, *L. monocytogenes* is required to survive low oxygen conditions within the gastrointestinal tract if it is to cause disease (Lungu *et al.*, 2009).

For effective survival and proliferation in low oxygen environments *L. monocytogenes* is not only required to survive the unfavourable conditions, but also successfully compete with other organisms found within these environments. The mechanisms of successful survival and competition are not well defined but are not believed to be universal to all species. This was demonstrated by Jydegaard-Axelson *et al.* (2004) in which the acid tolerant LO28 *L. monocytogenes* strain responded to a low oxygen environment through increased expression of glutamate decarboxylase related genes and significant changes in the membrane fatty acid composition while these changes were not found in the acid sensitive EGD strain. It was postulated by the authors that the low oxygen response in EGD resulted from 'more general stress responses including filamentation'.

The low oxygen response of *L. monocytogenes* is not only of interest as a persistence determinant. It has been repeatedly reported that low oxygen tension leads to increased expression of various adhesion factors. One such study, conducted by Burkholder *et al.* (2009), reported that anaerobiosis significantly increased *in vitro* adhesion to both human intestinal Caco-2 cells and human epithelial intestinal HCT-8 cells. This *in vitro* response has been translated to *in vivo* studies in which prior adaptation to low oxygen tension has been found to increase the infective potential of *L. monocytogenes* Scott A (Bo Andersen *et al.*, 2007). In this study a significantly increased number of guinea pigs were found to possess *L. monocytogenes* cells within their organs if bacterial cells had

undergone anaerobic pre-conditioning compared to their aerobically grown counterparts. It should be noted that no significant difference was found in the number of *L. monocytogenes* cells found within these organs. As such it was postulated that anaerobiosis may be linked to initial translocation from gut to internal organs, through increased expression of internalin genes. However, this pre-conditioning was not found to increase the ability of cells to proliferate within host organs.

1.5 Stress response genes

1.5.1 Hfq

Hfq is an RNA binding protein which has been found to be an important regulator of physiological processes such as general stress response and virulence. While its role in stress response is not well understood in *L. monocytogenes*, Hfq has been found to enhance stabilisation and translation of mRNA molecules in *E. coli* (Christiansen *et al.*, 2004). Further studies into the role of Hfq on stress response have been undertaken by Tsui *et al.* (1994) who found that *E. coli* Hfq null mutants demonstrated elevated osmosensitivity, increased susceptibility to UV light and decreased growth rates. Further research by Muffler *et al.* (1997) found that *Hfq* null mutants also exhibited increased sensitivity to oxidative stress from H_2O_2 and had a reduced thermotolerance. The role of Hfq has been extensively reviewed by Chao and Vogel (2010). In which it was reported that *L. monocytogenes* cells with knock-out mutations in *Hfq* had increased sensitivity to starvation, ethanol, osmolarity and detergents as well as elevated virulence potential. However, *Hfq* was not found to influence sensitivity to heat or antimicrobial peptides/antibiotics.

1.5.2 ATPase

F_0F_1 –ATPase is a multi-subunit enzyme with roles in ATP synthesis/hydrolysis and proton translocation (Santana *et al.*, 1994). ATPase primarily acts as an ATP generator in organisms capable of oxidative phosphorylation. In aerobic conditions ATP generation is achieved through the development of a proton motive force (PMF)

resulting from protons passing into cells via ATPase. This process is reversed in anaerobic conditions and ATP is generated through the expulsion of H^+ ions (Cotter *et al.*, 2000). The ability of ATPase to discharge H^+ ions is utilised by cells as a means of maintaining homeostasis in low pH environments (Matsumoto *et al.*, 2004). The mode of action of organic acids and their salts relies upon accumulation of H^+ ions, resulting in intracellular hyper-acidification and disruption of the ATPase enzyme; detrimentally affecting ATP synthesis (Silva *et al.*, 2012) and preventing the expulsion of intracellular protons.

1.5.3 groESL

The groESL operon encodes two major heat shock proteins, groES and groEL. These proteins work in synchrony to maintain protein integrity in unfavourable conditions (Gahan *et al.*, 2001). These molecular chaperones have been extensively studied and have been found to be up-regulated in response to ethanol stress, (Bokhorst-van de Veen *et al.*, 2011), alkaline stress (Giotis *et al.*, 2008), osmotic stress (Bergholz *et al.*, 2012), bile stress (Begley *et al.*, 2002) and acid stress (Gahan *et al.*, 2001). However, the role of the *groESL* operon is not limited to protein folding during conditions of stress; *groEL* has been found to be expressed during growth even under optimal conditions, suggesting a much broader role for the *groESL* operon in survival, growth and stress response (Fayet *et al.*, 1999).

1.5.4 recN

recN is a highly conserved protein among bacteria with similarities to the SMC (structural maintenance of chromosomes) protein family; playing an essential role in the repair of breaks to double stranded DNA. As such, recN is vital to ensure genome integrity is maintained (Grove *et al.*, 2009). recN has not been extensively studied, however, a study by Wang and Maier (2008) found recN to be important for *Helicobacter pylori* growth under a host of conditions, including aerobic atmosphere, low pH and in the presence of mitomycin C, a DNA targeting agent. This is supported by the results of a study by Courcelle *et al.*, (2001) in which *recN* was found to be highly expressed in response to UV exposure, which typically targets DNA. This provides further evidence of recN as a DNA repair protein. In *Listeria* sp. recN remains a predicted protein under the ordered locus name (OLN) *lmo1368*; however, van der Veen *et al.* (2007) found significant up-regulation of this locus in response to heat-shocking, suggesting a functional role for recN, lmo1368, in *Listeria*.

1.6 Control of Listeria in Foodstuffs

1.6.1 Low temperature storage and transport

Refrigeration is an important tool used by the food industry. Maintaining optimal temperatures of foodstuffs during transport and storage maintains organoleptic properties, delays food degradation, helps alleviate food spoilage, and improves food safety (James and James, 2010). Low temperature affects a wide array of cellular structures and functions. These include; negative DNA supercoiling, which influences the ability of cells to perform transcription; changes in the membrane composition, increasing the proportion of short chained unsaturated fatty acids to maintain membrane fluidity; greatly slowed metabolism and altered protein folding (Wouters *et al.*, 2000).

Refrigeration retards the growth of several pathogenic and spoilage organisms and therefore using refrigeration in transport and storage enhances the shelf life of perishables (James *et al.*, 2006). However, the psychotrophic nature of *L. monocytogenes* allows it to grow under conditions of refrigeration. Therefore other processing measures, such as the use of hurdle technology, are employed in when storing and/or transporting *Listeria* compromised foodstuffs

1.6.2 Hurdle technology

Within the food industry a 'hurdle' is the common term used to describe a sub-lethal, or mild, process which must be overcome by a microbe for it to be able to thrive or survive within the food stuff (Hill *et al.*, 2002). The application of hurdle technology aims to improve both the stability and

microbiological safety of products through the additional or synergistic effects of combined preservative measures (Leistner, 2000; Zhu *et al.*, 2005). This is important as there is an increasing expectation by consumers for the delivery of fresh, safe foods which have undergone only minimal processing; allowing the "naturalness" of the product to be maintained (Leistner and Gorris, 1995). As such food manufacturers are continually challenged to develop strategies which deliver contamination free food while maintaining both the food quality and naturalness (Leistner, 2000). Hurdle technology aims to address this consumer demand by allowing decreased intensity of any one preservation technology by combining it with others. Fig. 1-6 (from Leistner and Gorris, 1995) shows a number of hypothetical hurdle regimes resulting in complete pathogen inactivation, providing a safe end product (A-C); or non-successful regimes in which pathogens remain, compromising food safety (D,E).

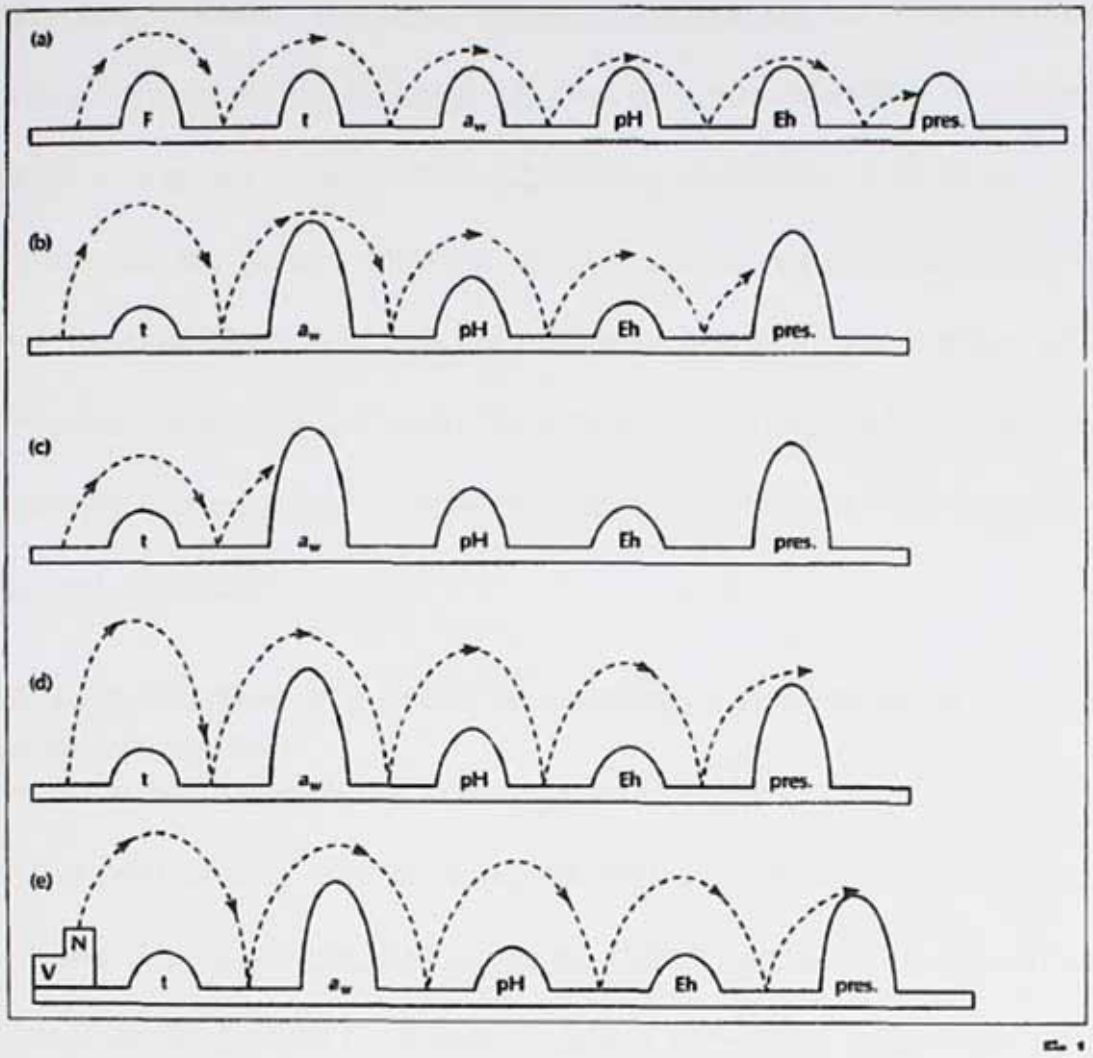

Fig. 1-6: Hypothetical examples of Hurdle technology.

t- chilling; a_w- water activity; Eh- redox potential; F- heating; pH- acidification; pres.- preservatives; V- vitamins, N- nutrients. Height of hurdle indicates intensity; arrows indicate surviving organisms (Leistner and Gorris, 1995).

There are a number of potential hurdles at the disposal of food manufacturers, the most common being heating, adjusted pH, altered water activity, use of preservatives, competitive microflora and low temperature (Leistner, 1999). However it is proposed by Leistner (2000) that as many as 60 hurdles have been described and this list is not

exhaustive. Within the food industry hurdles are 'intelligently applied'. Intelligently applying hurdles requires an understanding of the mode of action of a given preservation process or chemical; it aims to simultaneously target different cellular components increasing the effectiveness of each individual process/chemical in a method of preservation known as 'multi-targeting' (Leistner, 1995). By using this approach it is possible to deliver safe food which has undergone only minimal processing.

1.6.3 Application of hurdle technology for control of *Listeria monocytogenes*

A number of studies have investigated the effects of combined treatments for the control of *L. monocytogenes* both in real-food and in model systems. For example, Raibey *et al.* (2013) adopted the use of hurdle technology to control *L. monocytogenes* growth in model fish system. In their study, the combined effects of NaCl, essential oil and smoke compounds were synergistically greater than any component alone. Likewise Hereu *et al.* (2011) used hurdle technology to increase the food safety of dry-cured ham. In their study the combined effects of nisin and high hydrostatic pressure were greater than either hurdle alone, thus resulting in a safer end product. Hurdle technology also proves a useful tool in the processing of food products which are tarnished by thermal process. For example, Forghani and Oh (2013) combined slightly acidic electrolysed water, ultrasonication and sterile water washes to maximise the safety of several salad vegetables. Again, combined treatment with

several processes was found to enhance antibacterial activity compared to each hurdle individually.

The hurdle concept is well established and the process of targeting several bacterial cell components/processes allows each individual food processing measure to be decreased in intensity (Leistner and Gorris, 1995), thus providing consumers with more natural, and safer, end products. Moving forward it will be important to develop our understanding of new food processing measures and how these measures can interact to maximise food safety and quality.

1.7 Aims and Objectives

1.7.1 Aim

The overall aim of this project was to investigate the phenotypic and molecular responses of *Listeria monocytogenes* when subjected to food-related stressors.

1.7.2 Objectives

- To investigate if *L. monocytogenes* adaptively responds to sub-lethal stressor exposure in a model system.
- To determine if cells and/or sub-populations with increased resistance could be developed in response to stressor exposure.
- To assess the transcriptional responses of *L. monocytogenes* to sub-lethal stressor exposure.
- To investigate the effects of pre-conditioning on the phenotypic properties of *L. monocytogenes* and elucidate the transcriptional changes responsible for altered phenotypes.
- To develop *L. monocytogenes* mutants with disrupted stress response genes and assess the effects that such disruptions have on stress responses.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Growth media

All chemicals used were purchased from Sigma-Aldrich Company Ltd (Dorset, England) or Fisher Scientific UK Ltd (Loughborough, UK). All chemicals used were of the highest available grade. Bacterial culture media was obtained from Fisher Scientific UK Ltd (Loughborough, UK) or Oxoid Ltd (Basingstoke, UK).

2.1.1.1 Brain Heart Infusion (BHI) broth

BHI broth powder (CM1135: Oxoid Ltd, UK) was added at a concentration of 3.7g per 100ml of distilled water, before sterilisation by autoclaving at 121°C for 15 minutes.

2.1.1.2 Luria-Bertani (LB) broth

Two grams of LB broth powder (L3022: Sigma-Aldrich Company Ltd, UK) was suspended in 100ml of distilled water, mixed thoroughly, and sterilised by autoclaving 121°C for 15 minutes. After autoclaving broth was cooled to below 50°C before the addition of antibiotics at required usage concentrations.

2.1.1.4 Brain Heart Infusion (BHI) agar

Twenty six grams of BHI agar powder (70138: Sigma-Aldrich Company Ltd, UK) was suspended in 500ml of distilled water, mixed thorough, and sterilised by autoclaving at 121°C for 15 minutes. Agar was allowed to cool to 50°C before being aseptically transferred to sterile Petri dishes.

2.1.1.5 *Listeria* selective agar base (Oxford formulation)

Listeria selective agar base (Oxford formulation) powder (CM0856: Oxoid Ltd, UK) was added at a concentration of 27.75g per 500ml of distilled water, dissolved by boiling and autoclaved at 121°C for 15 minutes for sterilisation. Following sterilisation agar was cooled to 50°C before the addition of one vial of *Listeria* selective supplement (Oxford formulation) (SR0140: Oxoid Ltd, UK). Agar was then aseptically transferred to sterile Petri dishes.

2.1.1.6 LB Agar

Sixteen grams of LB agar powder (27025: Life Technologies Ltd, UK) was added to 500ml of distilled water, dissolved by boiling and autoclaved at 121°C for 15 minutes for sterilisation. Following sterilisation agar was cooled to 50°C before the addition of antibiotics at required usage concentration. Agar was then aseptically transferred to sterile Petri dishes.

2.1.2 Supplements and Antibiotics

2.1.2.1 *Listeria* selective supplement (Oxford formulation)

Listeria selective supplement (Oxford formulation) (SR0140: Oxoid Ltd, UK) was resuspended by the addition of 5ml of 70% ethanol under sterile conditions.

2.1.2.2 Erythromycin (ERY)

Erythromycin (E5389: Sigma Aldrich Company Ltd, UK) was prepared at a stock concentration of 50mg/ml in absolute ethanol and stored at -20°C. Working concentrations were prepared by direct addition of ERY into sterile media.

2.1.2.3 Chloramphenicol (CHL)

Chloramphenicol (C0378: Sigma Aldrich Company Ltd, UK) was prepared at a stock concentration of 10mg/ml in absolute ethanol and stored at -20°C. Working concentrations of CHL were prepared by direct addition into sterile media.

2.1.3 Diluents and Neutralisers

2.1.3.1 Phosphate Buffered Saline (PBS)

Five PBS tablets (BR0014: Oxoid Ltd, UK) were suspended in 500ml of distilled water and dissolved by heating. Aliquots of 900µl or 9ml were transferred to micro-centrifuge tubes or McCartney bottles respectively and were sterilised by autoclaving at 121°C for 15 minutes. Following sterilisation PBS was stored at room temperature. PBS was used as a general purpose diluent.

2.1.3.2 Buffered Peptone Water (BPW) w/ 4% (w/v) Sodium thiosulphate

Ten grams of BPW powder (CM0509: Oxoid Ltd, UK) was added to 500ml of distilled water and dissolved by heating. Once dissolved 20g sodium thiosulphate (Sigma-Aldrich Company Ltd, UK) was added to the BPW and dissolved by stirring. Nine millilitre aliquots were transferred to McCartney bottles and sterilised by autoclaving at 121°C for 15 minutes. Following sterilisation the solution was stored at room temperature. BPW with 4% (w/v) sodium thiosulphate was used as a neutraliser in H_2O_2 assays.

2.1.3.3 Buffered Peptone Water (BPW) with 0.5% (w/v) Sodium thiosulphate

Ten grams of BPW powder (CM0509: Oxoid Ltd, UK) was added to 500ml of distilled water and dissolved by heating. Once dissolved 2.5g sodium thiosulphate (Sigma-Aldrich Company Ltd, UK) was added to the BPW and dissolved by stirring. Nine millilitre aliquots were transferred to McCartney bottles and sterilised by autoclaving at 121°C for 15 minutes. Following sterilisation the solution was stored at room temperature. BPW

with 0.5% (w/v) sodium thiosulphate was used as a neutraliser in citric acid assays.

2.1.3.4 1.1% (w/v) Sodium thiosulphate solution

Sodium thiosulphate (Sigma-Aldrich Company Ltd, UK) was added at a concentration of 5.5g per 500ml of distilled water and dissolved by stirring. Nine millilitre aliquots were transferred to McCartney bottles and sterilised by autoclaving at 121°C for 15 minutes. Following sterilisation the solution was stored at room temperature. 1.1% (w/v) sodium thiosulphate was used as a neutraliser in NaClO assays.

2.1.4 Antimicrobial chemicals

2.1.4.1 Hydrogen peroxide (H_2O_2)

Thirty percent (w/v) hydrogen peroxide solution was obtained from Sigma Aldrich (216763: Sigma-Aldrich Company Ltd, UK). Usage concentrations were prepared aseptically using sterile distilled water and were used on day of preparation.

2.1.4.2 Sodium hypochlorite (NaClO)

Sodium hypochlorite solution (13% w/v) was purchased from Fisher scientific (10401841: Fisher Scientific UK Ltd, UK). Solutions at usage concentrations were prepared daily, using sterile distilled water, under aseptic conditions.

2.1.4.3 Citric acid

Citric acid was purchased from Sigma Aldrich (251275: Sigma-Aldrich Company Ltd, UK). Stock solutions were prepared daily by addition of Citric acid to distilled water. Citric acid solution was then filter sterilised

and usage concentrations prepared using sterile distilled water in aspetic conditions.

2.1.4.4 Trisodium Phosphate (TSP)

Trisodium phosphate was purchased from Sigma Aldrich (71911: Sigma-Aldrich Ltd, UK). Stock solutions were prepared daily by addition of TSP to distilled water. Trisodium phosphate solution was then filter sterilised and test concentrations prepared with sterile distilled water under aseptic conditions.

2.2 Methods

2.2.1 Culture techniques

2.2.1.1 Sub-culturing

Listeria monocytogenes EGD-e was kindly sent as a live culture on BHI agar by Jana Haase (University College Cork). The culture was aseptically streaked over a pre-dried BHI agar plate and incubated at 37°C for 24 hours under aerobic conditions. Cultures were maintained by repeated re-streaking on BHI agar and incubating as above.

Listeria monocytogenes J0161; FSL R2-499 was kindly sent as a live culture by Professor Martin Wiedmann (Cornell University). The culture was aseptically streaked over a pre-dried BHI agar plate and incubated at 37°C under aerobic conditions. Cultures were maintained by repeated re-streaking on BHI agar and incubating as above.

Escherichia coli DH5α was from University of Northampton archives and was used for routine cloning work. From glycercol stocks single colonies were obtained by streaking onto pre-dried LB agar. *E. coli* was maintained on LB agar and was incubated at 37°C unless stated otherwise.

E. coli DH5α containing the temperature sensitive vector, pAUL-A, was maintained as glycerol stocks by addition of 800µl of growing culture to 200µl glycerol. Single colonies were obtained by streaking onto pre-dried LB agar supplemented 300µg/ml erythromycin (Sigma-Aldrich Limited, UK) and incubated at 28°C overnight.

2.2.1.2 Long term storage of cultures

Stock cultures of non-transformed cells were stored at -80°C on Microbank beads (ProLab Diagnostics, Merseyside, UK). Colonies from BHI agar plates were aseptically picked using a sterile loop and added to a vial of Microbank beads. The vial was then capped, inverted a number of times. The cryopreservation fluid was then aseptically removed by pipetting, vial recapped and stored at -80°C.

Stock cultures of transformed cells were stored at -80°C as glycerol stocks. Eight hundred microliters of growing culture were aseptically added to 200µl of pre-sterilised glycerol in screw-topped cryotubes and stored at -80°C.

2.2.1.3 Recovery of bacterial cultures from Microbank beads

Every three months cultures were regenerated from Microbank beads to limit phenotypic variation. This was performed by aseptic removal of a single Microbank bead which was streaked onto BHI agar and grown in aerobic conditions at 37°C for 24 hours after which plates were checked for culture purity. Single colonies were then re-streaked onto BHI agar, incubated as before, after which plates were stored at 4°C.

2.2.1.4 Recovery of bacterial cultures from streak plates

On a bi-weekly basis a single colony from a BHI agar stock plate would be aseptically streaked onto a fresh pre-dried BHI agar plate using a sterile loop and incubated aerobically for 24 hours at 37°C.

2.2.1.5 Culture purity

On regular occasions the purity of cultures was checked and *Listeria* was identified through Gram staining using light microscopy, KOH string test, the catalase test, plating onto Oxford agar and the use of API *Listeria* test strips (bioMérieux UK Ltd, Hampshire, UK).

2.2.1.6 Test culture preparation

Test cultures were prepared by aseptically transferring a single colony from a sub-cultured BHI agar plate to 100ml of BHI broth. After which broth was incubated statically at 37°C under aerobic conditions for 24 hours.

2.2.2 Plating and enumeration techniques

Various plating methods were used to obtain total viable counts throughout experimentation. Following serial dilutions in PBS cells were plated via the spread plate method, Miles and Misra method (Miles *et al.*, 1938) or by spiral plating (Don Whitley Scientific Ltd, UK). Plates were incubated at 37°C under aerobic conditions for 24 or 48 hours, after which cfu/ml was calculated.

2.2.2.1 Spread plating

Unless stated elsewhere, 100µl of cell suspension was pipetted onto pre-set agar plates under aseptic conditions. Suspension was spread evenly across the plate surface and allowed to air dry before being incubated and subsequently enumerated.

2.2.2.2 Drop plating

Cell counts were enumerated by drop plate method as per Miles *et al.* (1938). In brief, twenty microlitres of cell suspension was aseptically pipetted onto pre-set agar plates in triplicate. Drops were allowed to air dry before being incubated and subsequently enumerated.

2.2.2.3 Spiral plating

200µl of cell suspension was plated onto pre-set agar plates using a Don Whitley spiral plater (Don Whitley Scientific Ltd, UK). Plates were allowed to dry before being incubated and subsequently enumerated

2.2.3 General Molecular techniques

2.2.3.1 DNA Extraction

Genomic DNA was extracted from stationary phase *L. monocytogenes* cultures using a phenol-chloroform extraction method with isopropanol precipitation as described by Liu *et al.* (2004). One colony was aseptically transferred into 10ml BHI broth and incubated statically at 37°C under aerobic conditions overnight. Following incubation cells were pelleted by centrifugation at 8,000x *g* for 10 minutes and supernatant was discarded. The pellet was resuspended in 500µl of a 1X TE (10 mM Tris–HCl pH 8.0 and 1 mM EDTA pH 8.0) buffer containing 20 mg/ml lysozyme (L6876: Sigma-Aldrich Company Ltd, UK) and 50U mutanolysin (M9901: Sigma-Aldrich Company Ltd, UK). Each tube was incubated at 37°C for 30 min, and 50 µl of 10% SDS (L3771: Sigma-Aldrich Company Ltd, UK), 25 µl of 20 mg/ml proteinase K (Qiagen, UK) and 4µl RNase A (Qiagen, UK) were added. After incubation for 2 hours at 55°C one volume of 1X TE followed by 1 volume phenol/chloroform/isoamyl alcohol (25:24:1) (P2069:

Sigma-Aldrich Company Ltd, UK) were added. Tubes were mixed by repeated inversion and centrifuged at 13,000x *g* for 10 minutes. Following centrifugation the upper aqueous phase was transferred to a new tube by pipetting after which one volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the tube. This was again mixed by repeated inversion and centrifuged as before. The upper aqueous phase was again transferred to the new tube. One volume of chloroform (C2432: Sigma-Aldrich Company Ltd, UK) was then added to the tube containing the removed aqueous phase, mixed by repeated inversion and centrifuged as previously described. The upper aqueous phase was removed and this step was repeated.

Following final clean step one volume of pre-chilled isopropyl alcohol and 0.1 volumes of 5M NaCl were added and tubes incubated at -80°C for 1 hour. DNA was pelleted by centrifugation at 13,000x *g* for 30 minutes at 4°C, supernatant was decanted and DNA pellet was washed twice with 1ml of 70% ethanol before air drying and resuspension in 100µl molecular biology grade water.

2.2.3.2 Primer design and preparation

PCR primers were either taken from previously published research or were designed using Clone Manager software. Gene/genome sequences were obtained from the National Center for Biotechnology Information (NCBI). Primers were purchased from MWG Eurofins and were resuspended at a concentration of 100µM using molecular biology grade water. After which a tenfold dilution was performed with molecular grade

water to give a working stock concentration of 10μM. Primers were designed to have a T_m of 50-55°C.

2.2.3.3 Polymerase Chain Reaction (PCR) and Quantitative reverse transcription PCR (qRT-PCR)

PCR was performed using a Bio-Rad MyCycler thermal cycler (Bio-Rad, Hemel Hempsted, UK) or Techne TC-512 thermal cycler (Bibby Scientific Ltd, Staffordshire, UK). Unless otherwise stated cycle conditions consisted of an initial denaturation at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 60 seconds and a final extension at 72°C for 10 minutes.

qRT-PCR was conducted on an Applied Biosystems StepOne Plus device (Life Technologies Ltd, UK) using the pre-set 'fast' cycling conditions. qRT-PCR primers were designed to have a T_m of ~60°C.

2.2.3.4 Plasmid Extraction

Plasmids were extracted from bacterial cells using a method modified from a Qiagen QIAprep spin miniprep kit (27104: Qiagen UK). Isolates were first re-streaked onto pre-prepared LB agar plates supplemented with antibiotics at correct concentration to inhibit growth of wild-type cells. Following overnight incubation at suitable temperature a single colony was aseptically transferred to 10ml sterile LB broth supplemented as previously described. Tubes were next incubated for 12-16 hours at appropriate temperature with shaking before being centrifuged (5,400x *g* for 10 minutes at 4°C) and supernatant discarded. Cell pellets were then

resuspended in 250µl of buffer P1 and mixed by inversion. Once homogenised, 250µl of buffer P2 was added. Suspensions were carefully mixed by repeated tube inversion and 350µl of buffer N3 was added. Again, suspensions were homogenised by repeated inversion before cell debris were pelleted by centrifugation (15,000x *g* for 10 minutes at room temperature). Supernatants were then transferred to QIAprep spin columns by pipetting and were centrifuged at 13,000x *g* for one minute. Flow-throughs were discarded and columns washed by addition of 500µl of buffer PB to spin column before centrifugation at 13,000x *g* for one minute. After initial wash, a second wash was performed by adding 750µl of buffer PE and centrifuging as previously described. Following both wash steps the column underwent a one minute centrifugation (13,000x *g* for one minute) to ensure all residual buffer had been removed; after which plasmid DNA was eluted by addition of 50µl of molecular grade water, a one minute incubation (room temperature) and centrifugation as previously described.

DNA yield was assessed using a nanodrop device (NanoDrop 2000: Fisher Scientific, Loughborough, UK) and quality assessed by gel electrophoresis.

2.2.4 Statistical Analysis

Statistical analyses of data were performed using IBM SPSS Statistics version 20. Data was first assessed for normality, after which appropriate statistical tests were applied to data. Significance was set at $p \leq 0.05$.

Chapter 3

Listeria monocytogenes: adaptation to stressors

3.1 Introduction

Listeria monocytogenes is regularly isolated from foodstuffs even though control measures are in place, such as the use of surface decontaminants (Lunden *et al.*, 2003). This continued persistence is largely attributed to the development of sub-populations which have elevated 'resistance' compared to the general population (Medrala *et al.*, 2003). In this context, 'resistance' can be defined as micro-organisms which are better suited to survive repeated cleaning programmes than those within the general population (Holah *et al.*, 2002).

It is thought that much in the same way as resistance to antibiotics develops, resistance to biocides/antimicrobials may also be attributed to adaptive responses whereby cells encounter sub-lethal concentrations of a given compound, initiating changes at both the transcriptional and translational level to alleviate an unfavourable scenario (Kastbjerg and Gram, 2012). Alternatively, it is proposed that exposing bacterial populations to antimicrobials above the MIC but below the MBC provides a 'mutant selection window'. This mutant selection window can drive the development of antimicrobial resistance and the development of resistance sub-populations (Drlica, 2003); which may account for bacterial persistence within the food processing environment and within the food chain. It is suggested that exposure to antimicrobial concentrations which are between the MIC and the MBC may drive the selection/development of cells with elevated resistance towards the given antimicrobial (Drlica, 2003). Using various stepwise training regimes,

several antimicrobials have been assessed for their capacity to develop and/or select for resistant cells and sub populations. Using repeated cycles of decontamination procedures Garcia-Gonzalez *et al.* (2010) were able to develop sub-populations of both *L. monocytogenes* and *E. coli* which displayed enhanced resistance to high pressure CO_2. Likewise, Rajkovic *et al.* (2009) were able to develop resistant sub-populations of *L. monocytogenes* and *E. coli* to various mild decontaminants using the same process of repeated passaging. Walsh *et al.* (2003) developed cells with altered resistance properties through repeated exposure to antimicrobials. 'Mutants' demonstrated decreased zones of inhibition when compared to parent strains in disc diffusion assays or elevated MICs on gradient plates.

By inactivating only a proportion of the microbial load during minimal processing it is conceivable that cells and/or sub populations with altered resistance properties may be generated. This in turn may impact persistence as well as altering the virulence potential of surviving cells (Rajkovic *et al.*, 2009).

3.2 Aims and Objectives

3.2.1 Aims

The aims of the study described in this chapter were to investigate the ability of *L. monocytogenes* to adapt to sub-lethal stressor exposure and for resistant sub-population to emerge during repeated exposure to stressors.

3.2.2 Objectives

- To determine lethal and sub-lethal treatment intensities (times and concentrations) for antimicrobial agents against *L. monocytogenes*.
- To assess the ability of *L. monocytogenes* cells to adaptively respond to lethal homologous and heterologous stresses following sub-lethal exposures.
- To determine MICs of trisodium phosphate (TSP), citric acid (C.A), sodium hypochlorite (NaClO) and hydrogen peroxide (H_2O_2) against *L. monocytogenes* EGD-e.
- To investigate the effects of sub-MIC growth on the development of resistant/tolerant cells.
- To develop resistant sub populations through repeated exposure to antimicrobials

3.3 Methods

3.3.1 Neutralisation assays

In order to determine the efficacy of various surface decontaminants (citric acid, trisodium phosphate, H_2O_2 and NaClO) effective chemical neutralisation was required. The method of Rutala *et al.* (2000) was used to assess both the toxicity and capacity of the neutralisers used. See section 2.1.3 for neutralisers used in this study.

3.3.1.1 Neutraliser capacity

The capacity of neutralisers to halt bactericidal effect of biocides was verified by addition of 100µl of biocide to 900µl of neutraliser (Section 2.1.3). Neutraliser-biocide mixtures were incubated at room temperature for 10 minutes after which 10µl aliquots of bacterial culture were added, giving final cell densities of approximately 10^3 cfu/ml. At intervals, aliquots were removed, serially diluted in PBS and enumerated by spread plating method. Following aerobic incubation at 37°C for 24 hours counts were obtained and compared to PBS controls.

3.3.1.2 Neutraliser toxicity

To ensure neutralisers had no toxic effects on cells 100µl of PBS was added to 900µl of neutraliser. This mixture was incubated at room temperature for 10 minutes before 10µl aliquots of approximately 10^3 cfu/ml bacterial culture was added. At various time-points aliquots were removed, serially diluted in PBS and enumerated by spread plating. Plates were incubated aerobically for 24 hours at 37°C after which counts were obtained and compared to PBS controls.

3.3.2 Adaptation to sub-lethal stress

3.3.2.1 Determination of lethal parameters

A single colony was aseptically transferred from stock plates into 100ml BHI broth and grown statically overnight at 37°C. To determine lethal bactericidal concentrations of antimicrobial chemicals time-kill assays were performed. One millilitre of culture was aseptically transferred to 9ml of sterile antimicrobial treatment in centrifuge tubes. Tubes were vortexed and stored at 21°C (mimicking room temperature), with shaking (120rpm), using a thermomixer (Thermomixer comfort, Eppendorf). At hourly intervals, 100µl aliquots were aseptically removed and added immediately to 900µl of suitable neutraliser (H_2O_2= BPW w/4% (w/v) sodium thiosuphate, NaClO= 1.1% (w/v) sodium thiosulphate, citric acid= BPW w/0.5% sodium thiosulphate, trisodium phosphate= PBS). Serial dilutions were performed in pre-sterilised PBS and suspensions plated by Miles and Misra method (Section 2.2.2.2), with the following adjustment, both 20µl and 50µl were spotted onto plates to lower the detection limit from $1.5x10^3$ to $6x10^2$ cfu/ml. After plating, cells were incubated at 37°C under aerobic conditions for 24 and 48 hours before counts were obtained.

Non-treated control cells were prepared by addition of 1ml overnight culture into 9ml sterile PBS.

3.3.2.2 Determination of sub-lethal parameters

To determine sub-lethal concentrations of test antimicrobials time-kill assays were performed (section 3.3.2.1.) However, to ensure that sub-

lethal treatments were not sub-lethally injuring cells the method of Jasson *et al.* (2007) was used. In brief, cells were subjected to various concentrations of antimicrobial after which appropriate dilutions were plated on both non-selective media (BHI) and selective media (Oxford agar) and incubated as in section 3.3.2.1. After incubation counts were compared from non-selective and selective media. Concentrations at which counts were not different were considered sub-lethal.

3.3.2.3 Determination of Adaptive responses

To assess the ability of *L .monocytogenes* to respond to sub-lethal stress exposure the method of Lou and Yousef (1997) was undertaken with modifications. Cells were prepared by overnight incubation of a single colony in 100ml of sterile BHI broth, statically incubated at 37°C under aerobic conditions. Following incubation 10ml aliquots were taken and cells were pelleted by centrifugation at 5000x *g* (5 minutes, 37°C) and the supernatant decanted. Cell pellets were resuspended in 10ml of antimicrobial solution (prepared in dH_2O) at the pre-determined sub-lethal concentrations. Cells were statically incubated at 37°C for 1 hour in the presence of antimicrobial supplemented broth to allow adaptions to occur. Following a one hour 'adaptation' period, cells were again pelleted by centrifugation and washed twice with 10ml sterile PBS, Cell pellets were resuspended in 10ml of antimicrobial solution at the pre-determined lethal concentration. At defined time intervals 100µl aliquots were added to 900µl of neutraliser, serial dilutions were performed and 20µl of each dilution was plated onto pre-dried BHI agar plates. Plates were incubated

at 37°C for 24 hours after which total viable counts were enumerated as per Miles *et al.* (1938). 'Protection areas' were defined as the area generated below each kill curve, and these areas were used for statistical analyses.

Controls for this experiment included cells which were subjected to sterile dH_2O during the adaptation period and cells which were subjected to dH_2O during both the adaptation and inactivation period.

3.3.3 Development of resistant sub-populations

3.3.3.1 Macro-broth dilution method for Minimum Inhibitory Concentration (MIC) determination

The MIC of antimicrobial chemicals was determined as per Wiegand *et al.* (2008). Overnight cultures were prepared by addition of a single colony into 100ml sterile BHI broth with subsequent incubation at 37°C for 14-16 hours under static conditions.

To prepare antimicrobial supplemented BHI, 20ml 'stock' solutions were prepared at 2x the desired starting concentration. Following preparation, stock solutions were membrane filtered (10009230: Fisher Scientific, Loughborough, UK) after which 10ml aliquots were serially diluted 1:1 into sterile BHI broth. From the final dilution 10ml aliquots were discarded giving final volumes of 10ml in each tube.

For test cell suspensions, overnight cultures were diluted to approximately $1x10^6$ cfu/ml. 10ml aliquots were transferred to tubes containing antimicrobial supplemented BHI, giving final cell densities of approx. $5x10^5$ cfu/ml. Tubes were statically incubated at 37°C for 24 hours after

which the MIC was determined as the lowest concentration in which visible growth was inhibited.

Controls for this experiment included non-inoculated BHI broth (sterility control) and non-supplemented BHI broth (growth control).

3.3.3.2 Minimum Bactericidal Concentration (MBC) determination

In parallel to MIC determination, MBCs were determined. After performing MIC assays, tubes which showed no visible growth were screened for the MBC of each antimicrobial. From each tube three 100µl aliquots were spread onto pre-dried BHI agar plates. Plates were incubated at 37°C for 24 hours and the MBC was determined as the lowest concentration in which no growth was seen on the plate.

Controls for this experiment included plating out of the sterility control, the growth control and the tube containing cells subjected to the MIC concentration.

3.3.3.3 Stepwise training for mutant cells/resistant sub-populations

Rajkovic *et al.* (2009) generated resistant sub-populations through repeated exposure of cells to antimicrobial treatments. This method was adopted in an attempt to generate resistant sub-populations with enhanced resistance to citric acid, trisodium phosphate and hydrogen peroxide. Treatment intensities (times and concentrations) were determined by time-kill assay and were optimised to give 1-5 log reductions in cfu/ml.

Overnight cultures were prepared by addition of a single colony into 100ml sterile BHI broth. Cultures were statically incubated at 37°C for 14-18 hours after which 1ml aliquots were transferred into 9ml of antimicrobial solution. Cell suspensions were incubated at room temperature for an appropriate length of time to give a 1-5 log reduction in viable cells. Following exposure, 100µl aliquots were added to 900µl of appropriate neutraliser, serial dilutions were performed, and cells were plated onto pre-dried BHI agar plates. Plates were incubated for 24 hours and total viable counts were obtained by drop plate method. Log reductions were calculated relative to non-treated cells. In parallel to neutralisation and enumeration, 100µl aliquots were transferred into 20ml sterile BHI broth and grown overnight at 37°C. Following incubation the inactivation process was repeated. Log reductions were calculated over 10 days and compared to those obtained on day 1 to assess resistant sub-population development.

3.4 Results

3.4.1 Neutraliser suitability testing

Fig. 3-1a to Fig. 3-1d demonstrate the suitability of selected neutralisers to halt the antimicrobial activity of test chemicals. None of the neutralisers demonstrated toxicity towards to *L. monocytogenes* EGD-e as there were no significant differences in $\log_{10}$ cfu/ml between control cells and cells subjected to neutralisers alone ($p>0.05$).

All neutralisers demonstrated a capacity to halt the antimicrobial activity of test chemicals. There were no significant differences in the number of recoverable cells when comparing control cells with cells exposed to neutralised antimicrobial chemicals over 90 minutes ($p>0.05$). However, after 180 minutes there was significantly lower recovery of cells subjected to neutralised TSP compared to control cells ($p<0.05$). As such, in subsequent assays concerning TSP all plating out was conducted within 90 minutes of neutralisation.

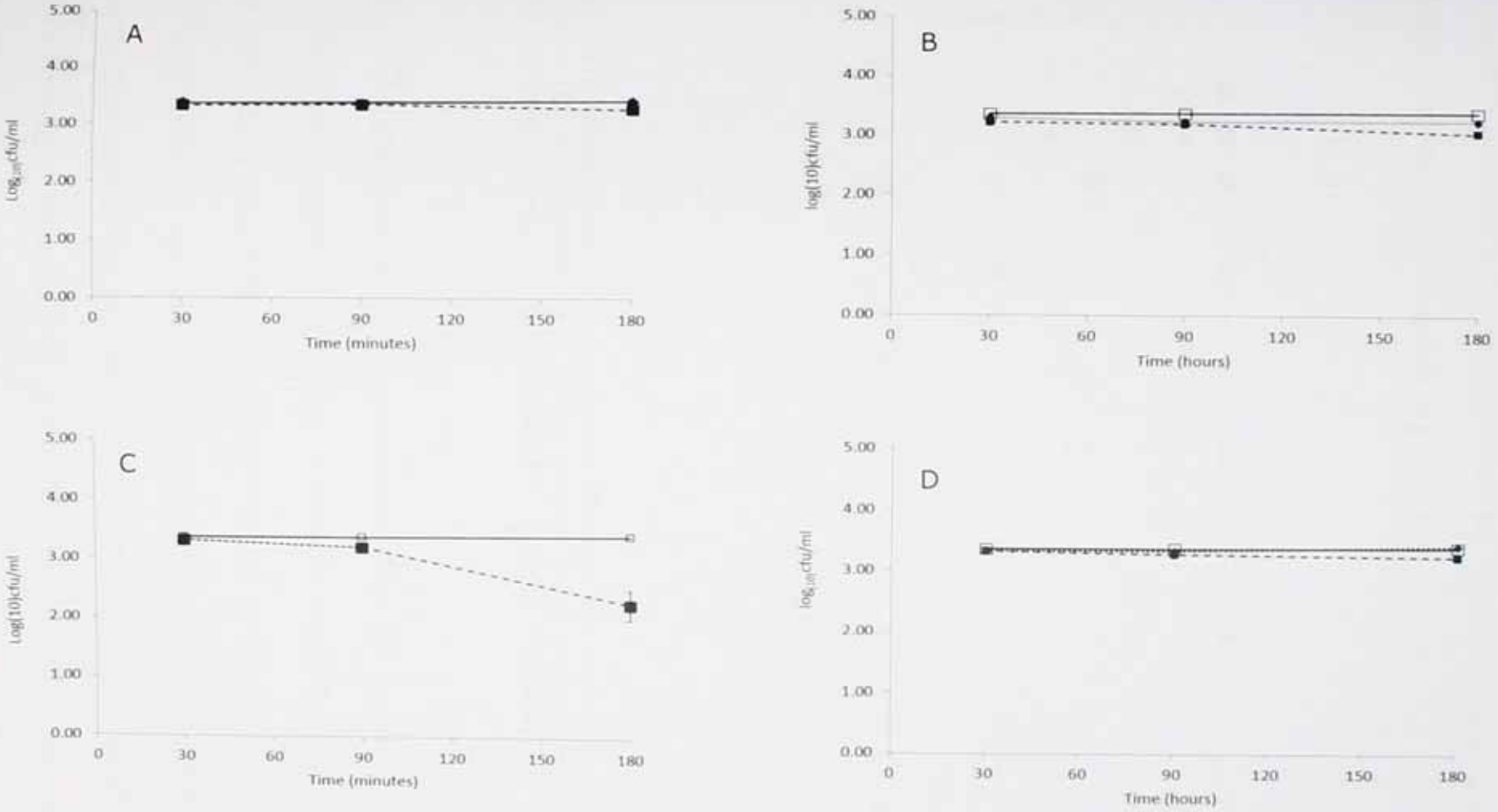

Fig. 3-1: The capacity (··■··) and toxicity (–□–) of selected neutralisers to halt the activity of NaClO (a), citric acid (b), TSP (c) and H_2O_2 (d). PBS controls (··●··) were also included. Error bars indicate the SEOM of three independent experiments (n=3) plated in triplicate.

3.4.2 Determination of 'lethal' treatment intensities

Time kill assays were used to determine 'lethal' antimicrobial intensities (concentrations and times) sufficient to inactivate cells to below the detection limit over a period of up to four hours.

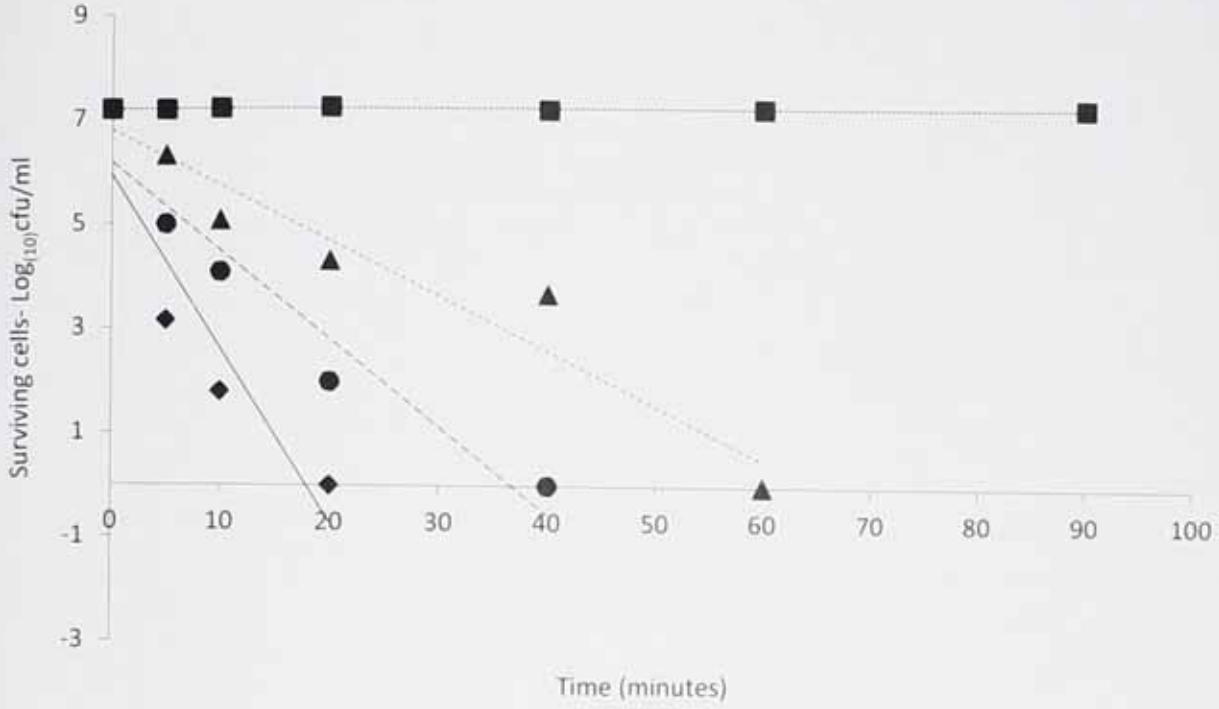

Fig. 3-2:Inactivation of *L. monocytogenes* EGD-e by NaClO at 50ppm (■), 100ppm (▲), 125ppm (●) and 150ppm (◆). Plotted values represent the mean number of surviving cells from four independent experiments (n=4) plated in triplicate.

Fig. 3-2 shows the results of a time-kill assay for *L. monocytogenes* EGD-e cells when subjected to NaClO at various concentrations. Throughout 90 minutes of sampling, cultures subjected to 50ppm NaClO demonstrated no reduction in the number of surviving cells. D-values were calculated using the slope of the death curve for each NaClO concentration. Mean D-values of 9.5±0.2 minutes, 5.9±1.8 minutes and

3.0±1.1 minutes were calculated for NaClO concentrations of 100ppm, 125ppm and 150ppm respectively. These differences were found to be statistically significant ($p<0.05$) demonstrating concentration dependent antimicrobial activity.

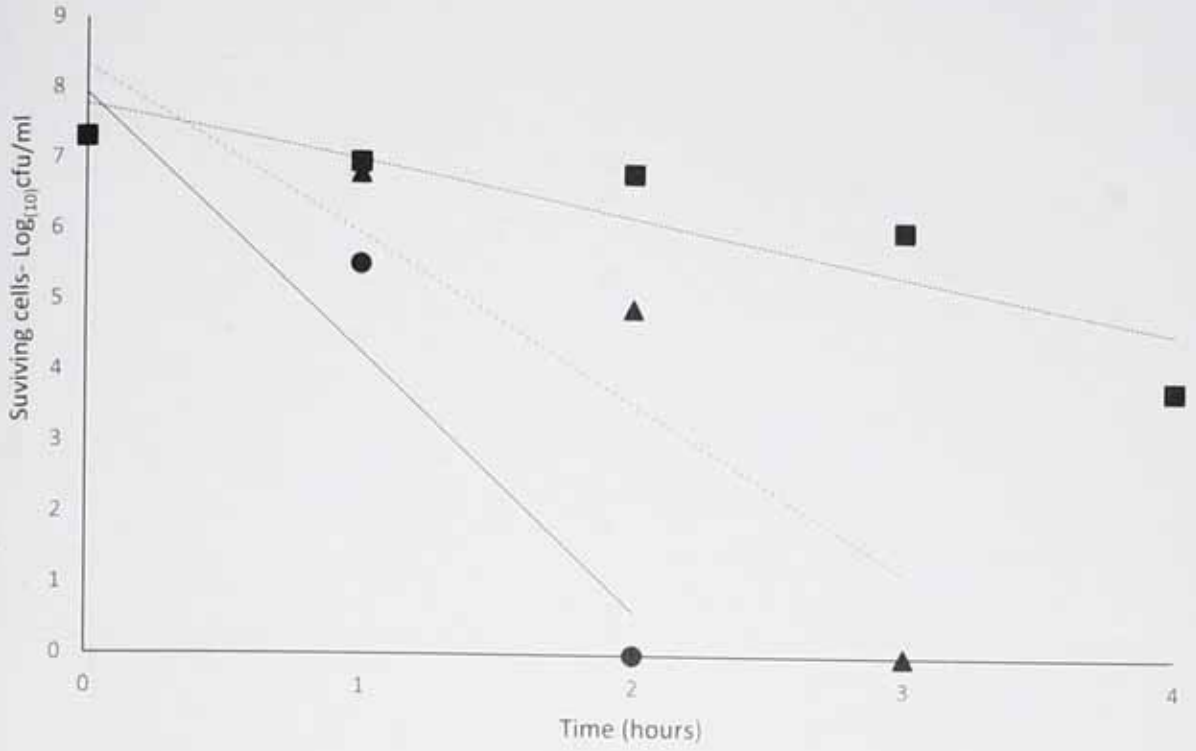

Fig. 3-3: Inactivation of *L. monocytogenes* EGD-e by H_2O_2 at 0.05% (w/v) (■), 0.1% (w/v) (▲), 0.2% (w/v) (●). Plotted values represent the mean number of surviving cells from four independent experiments (n=4) plated in triplicate.

When subjected to H_2O_2 at concentrations of 0.05% and greater *L. monocytogenes* EGD-e cells were inactivated (Fig. 3-3). D-values were determined from the slopes of kill curves. Mean D-values of 2.7±0.70 hours, 0.4±0.02 hours and 0.3±0.00 were calculated for H_2O_2 concentrations of 0.05%, 0.1% and 0.2% respectively. These values were found to be significantly different (p<0.05) from one another, demonstrating concentration dependent antimicrobial activity.

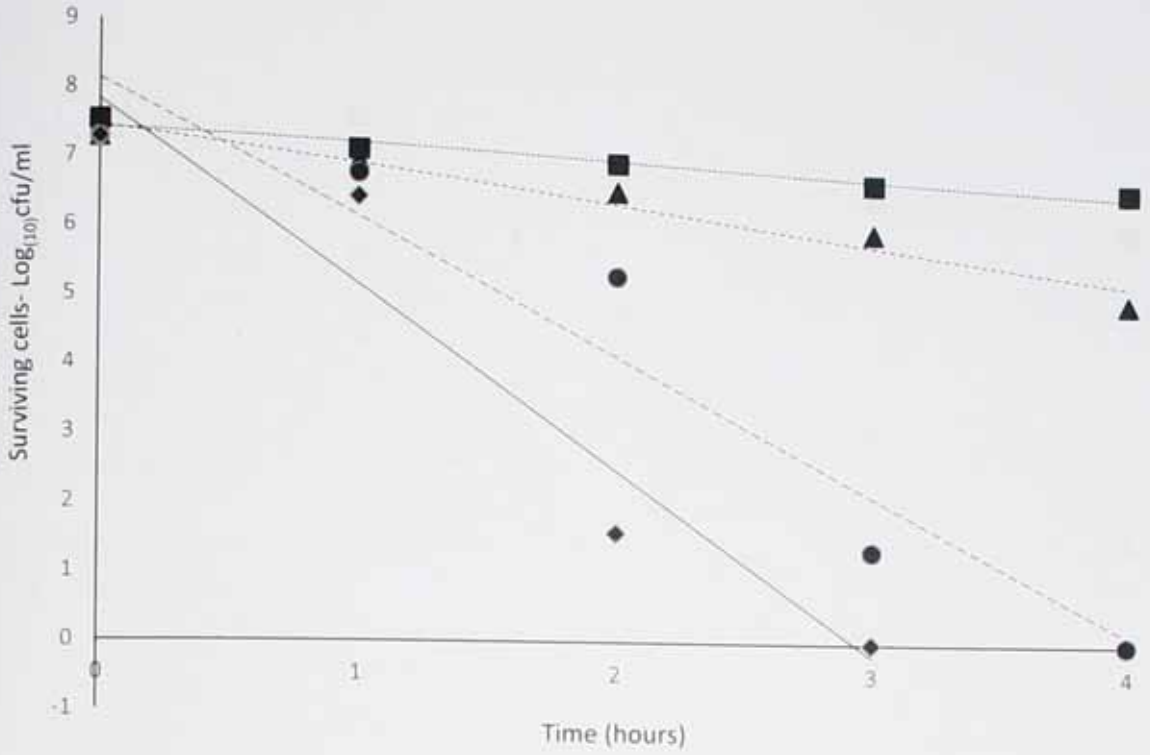

Fig. 3-4: Inactivation of *L. monocytogenes* EGD-e by TSP at 0.5% (w/v) (■), 1% (w/v) (▲), 2% (w/v) (●), 3% (w/v) (◆). Plotted values represent the mean number of surviving cells from four independent experiments (n=4) plated in triplicate.

Fig. 3-4 demonstrates the inactivation of *L. monocytogenes* EGD-e cultures when subjected to TSP at various concentrations over 4 hours. D-values of 4.5±1.2 hours, 1.8±0.9 hours, 0.5±0.06 hours and 0.4±0.02 hours were calculated for TSP concentrations of 0.5%, 1%, 2% and 3% respectively. These D-values were significantly different from one another ($p<0.05$).

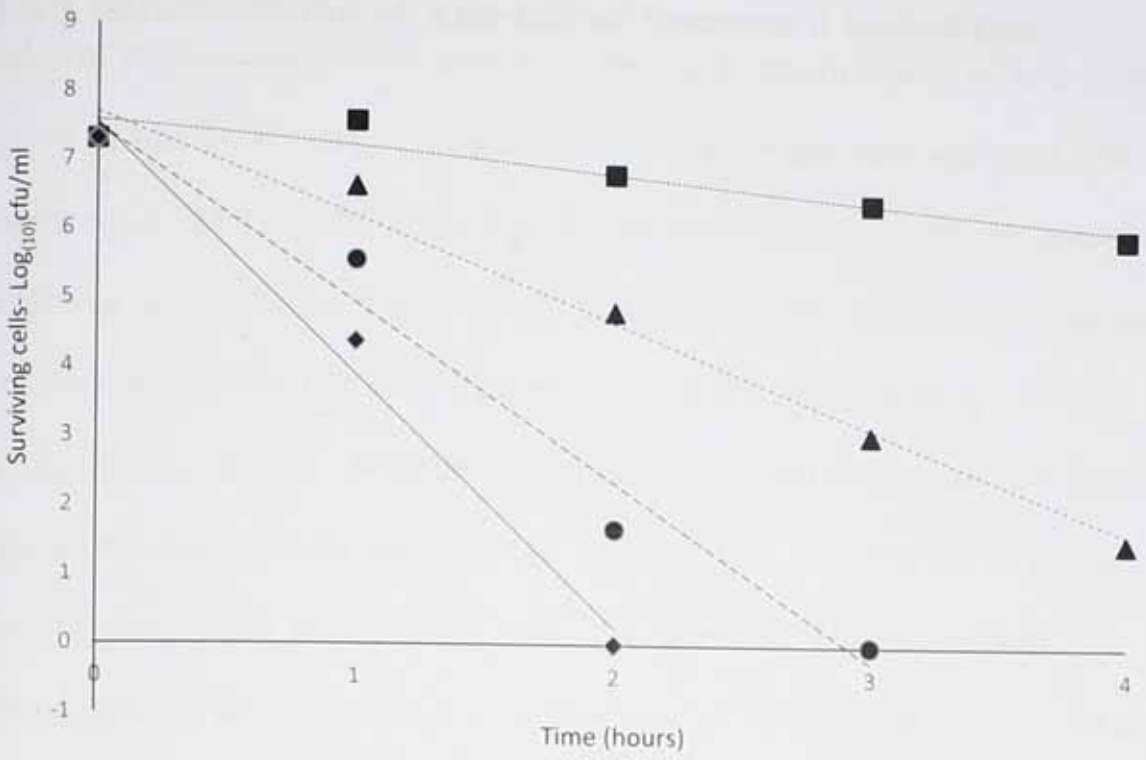

Fig. 3-5: Inactivation of *L. monocytogenes* EGD-e by citric acid at 1% (w/v) (■), 2% (w/v) (▲), 3% (w/v) (●), 4% (w/v) (◆). Plotted values represent the mean number of surviving cells from four independent experiments (n=4) plated in triplicate.

When subjected to citric acid *L. monocytogenes* cells demonstrated dose and time dependent inactivation (Fig. 3-5). D-values were calculated from the slope of kill curves and were found to be significantly impacted by citric acid concentration. Mean D-values of 2.7±1.5 hours, 0.6±0.2 hours, 0.4±0.1 hours and 0.3±0.0 hours were calculated for citric acid treatment at 1%, 2%, 3% and 4% (w/v) respectively. These D-values were found to be significantly different from one another (p<0.05).

3.4.3 Determination of 'sub-lethal' treatment intensities

Several studies have used selective media as an indicator of sub-lethal injury in bacterial cells. Many stressors impact the cell wall and cell membrane, damage to these structures may sensitise cells to some of the selective components found within selective media, thus inhibiting and/or preventing growth (Gnanou Besse *et al.*, 2000; Jasson *et al.*, 2007; Smigic *et al.*, 2009). In order to ensure pre-treatment was not causing sub-lethal injury, cells were plated on both BHI (non-selective) and Oxford (selective) media after 1 hour exposure. The sub-lethal concentration was determined as the concentration in which no difference was observed in recoverable cells plated onto BHI and Oxford following 1 hour stress exposure.

Fig. 3-6 demonstrates the effects of sub-lethal stress exposure on cellular recovery on selective (oxford) and non-selective (BHI) media. When untreated control cells were plated on both media types, no significant difference in counts was observed between those plated on BHI and those plated on Oxford agar ($p>0.05$). This demonstrated that non-injured cells recovered equally well regardless of media.

For H_2O_2 the concentration which did not induce injury, i.e. there was no significance difference ($p>0.05$) between recoverable cells on BHI and Oxford agar was 0.05% (w/v). Upon subjecting cells to 0.075% and 0.1% (w/v) significant differences in recoverable cells was observed ($p<0.05$). As such 0.05% (w/v) was chosen as the sub-lethal

concentration for subsequent assays using H_2O_2 at sub-lethal concentrations.

When subjecting cultures to NaClO for one hour, at concentrations above 50ppm viable cells were not detected, irrespective of recovery media used. When subjected to 50ppm NaClO no significant difference in recoverable cells were observed between those plated on BHI and oxford agar ($p>0.05$). As such this concentration was chosen as the concentration to be used for further studies requiring NaClO exposure at a sub-lethal concentration.

At 0.5% (w/v) citric acid no significant difference was observed in the number of recoverable cells when plated on BHI and Oxford agar ($p>0.05$). At 1% and 2% (w/v) concentrations the number of recoverable cells was significantly greater ($p<0.05$) on BHI compared to Oxford agar. As such 0.5% (w/v) was chosen as the sub-lethal concentration for assays using citric acid at sub-lethal levels.

After one hour exposure to TSP at 0.5% (w/v) no significant difference was observed in the number of recoverable cells which had grown on BHI and Oxford agar ($p> 0.05$). At concentrations greater than 0.5% (w/v) significantly higher recovery occurred on BHI agar compared to Oxford agar ($p<0.05$), indicating that TSP induced sub-lethal injury above 0.5% (w/v). As such, 0.5% (w/v) was chosen as the sub-lethal concentration for sub-lethal TSP treatment.

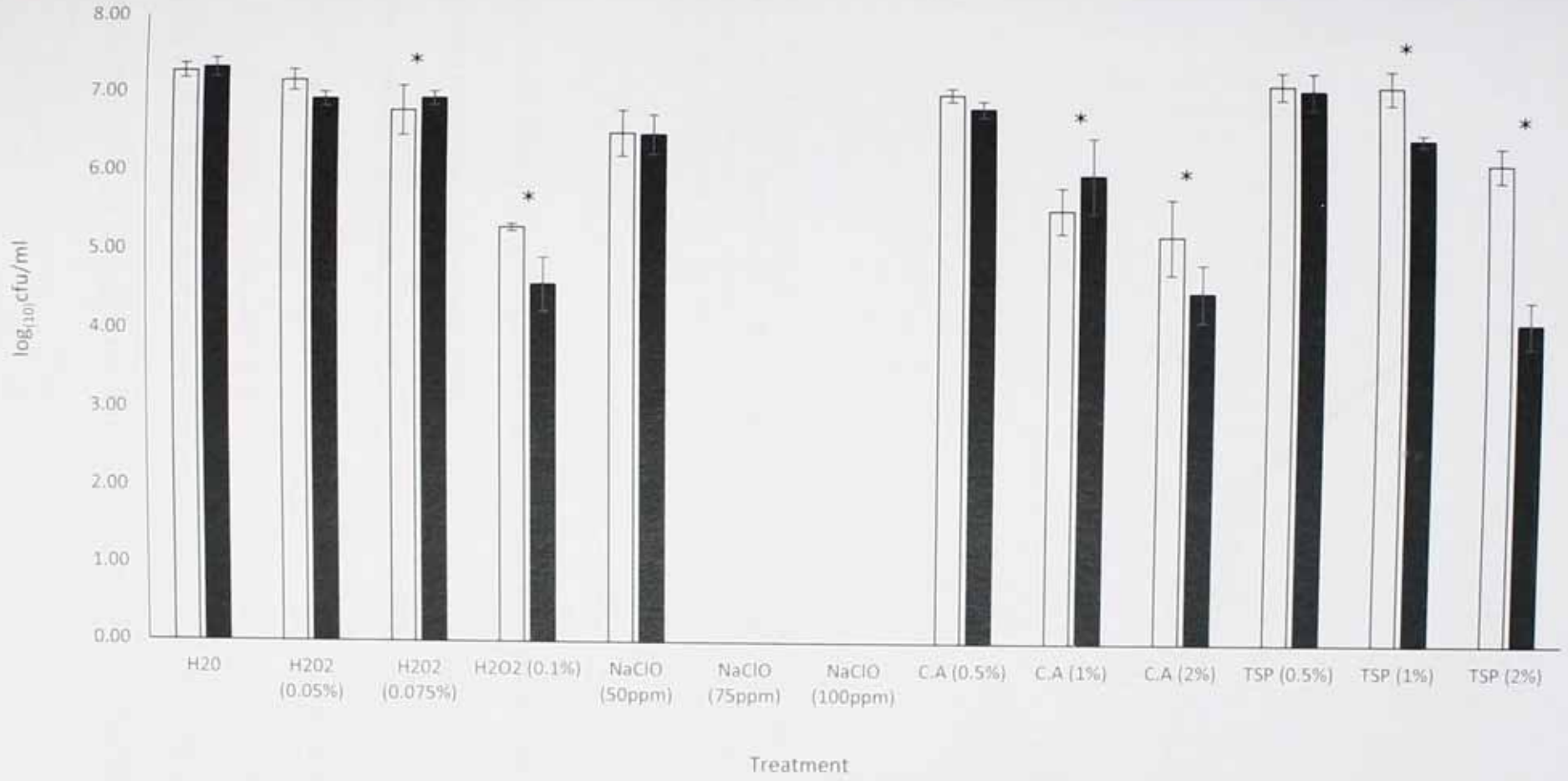

Fig. 3-6: Recoverable *L. monocytogenes* cells following one hour exposure to antimicrobials at various concentrations. Cells were plated on BHI (non-selective) (open) and Oxford agar (selective) (filled). Error bars indicate the SEOM of 3 independent experiments, plated in triplicate (n=3). Asterisks indicate significant difference (p<0.05) between recoverable cells on BHI and Oxford agar.

3.4.4 Adaptive responses of *L. monocytogenes* to sub-lethal stressor exposure

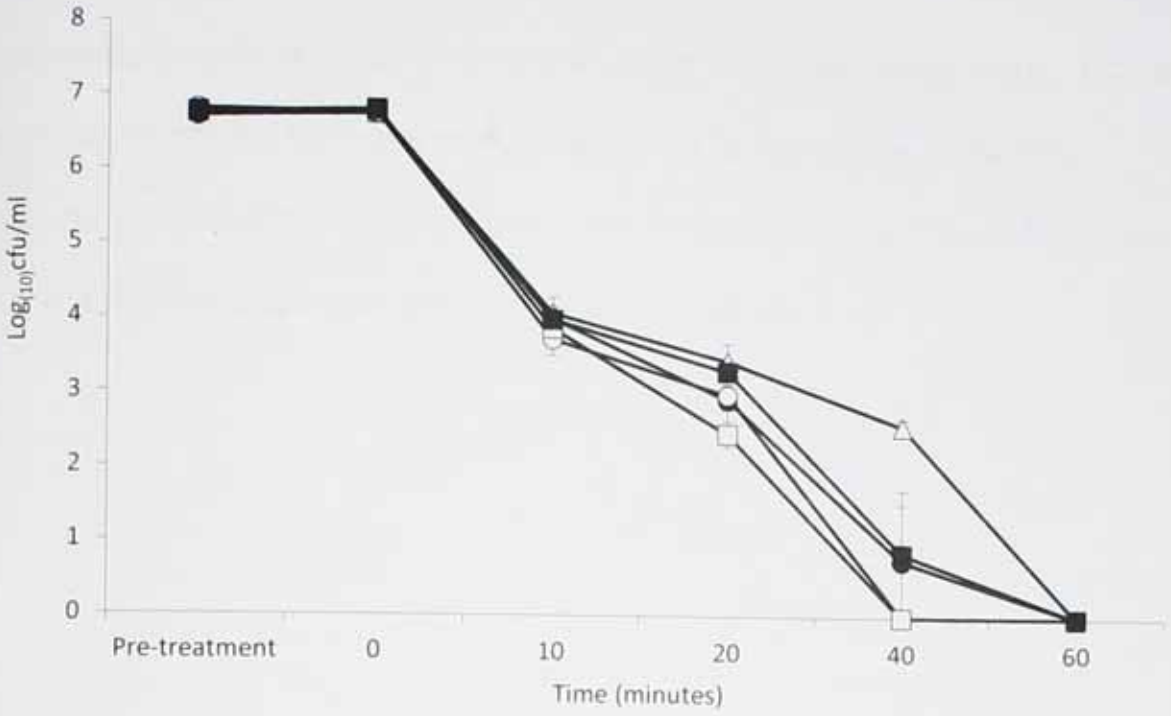

Fig. 3-7: Effects of sub lethal exposure to H_2O_2 (-●-), Citric acid (-○-), Trisodium phosphate (-□-) and NaClO (-■-) followed by lethal concentrations of NaClO on *L. monocytogenes* cells. Controls (-△-) were cells subjected to H_2O during pre-treatment. Error bars indicate the SEOM of three independent experiments (n=3) plated in triplicate.

Adaptive responses to sub-lethal antimicrobial exposure were assessed by comparing the protection area in pre-treated cultures compared to non-pre-treated controls. By conducting an ANOVA it was apparent that significant differences occurred as a result of pre-treatment (F (4, 40) = 12.6, p<0.000). Post hoc analysis revealed that pre-exposure to citric acid, H_2O_2, NaClO and trisodium phosphate significantly decreased the protection area compared to untreated controls (p<0.05). There was no

significant differences in the protection areas of pre-treated cells ($p>0.05$).

These results indicate that pre-conditioning with citric acid, H_2O_2, TSP and NaClO significantly increase susceptibility to lethal levels of NaClO, although no differences were found when assessing the extent to which each pre-treatment impacted NaClO susceptibility.

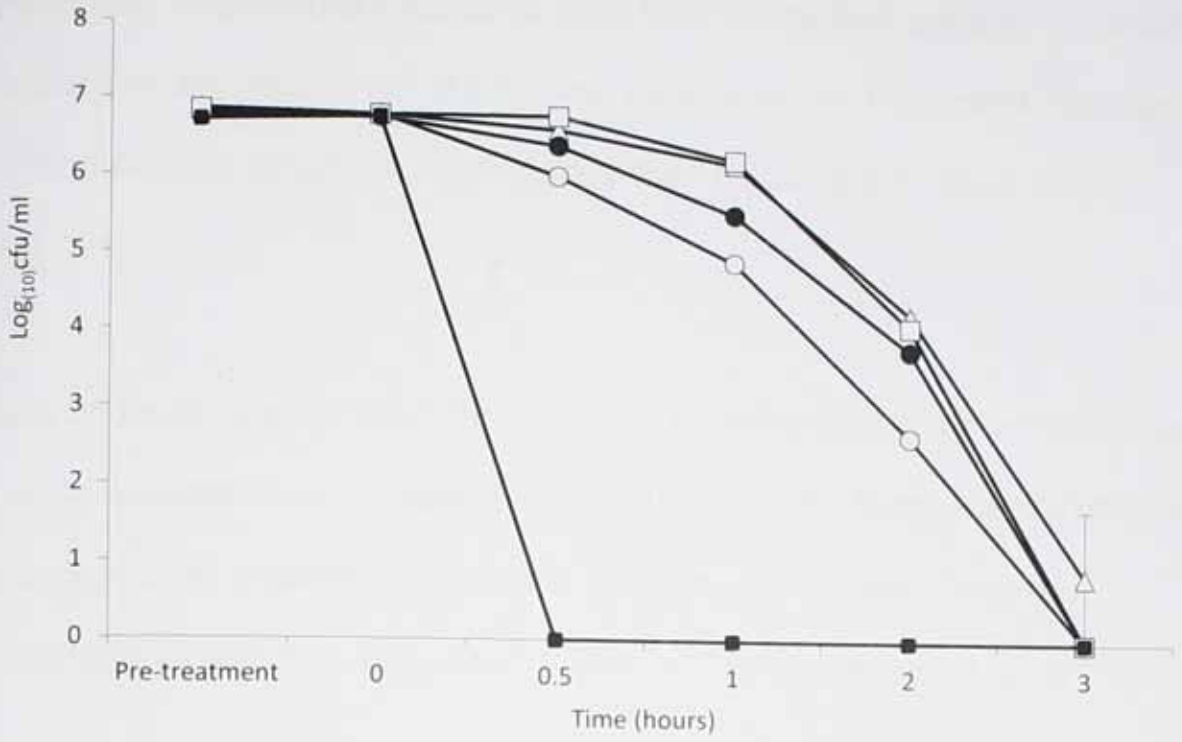

Fig. 3-8: Effects of sub lethal exposure to H_2O_2 (-●-), Citric acid (-○-), Trisodium phosphate (-□-) and NaClO (-■-) on exposure of *L. monocytogenes* cells to lethal concentrations of H_2O_2. Control cells (-△-) were subjected to H_2O during pre-treatment. Error bars indicate the SEOM of three independent experiments (n=3) plated in triplicate.

The results of an ANOVA analysis revealed that pre-conditioning had a significant effect on H_2O_2 resistance (F (4, 40) = 2427.6, p<0.000), as determined by differences in protection area. Post hoc analysis revealed that citric acid, H_2O_2 and NaClO pre-conditioning significantly increased sensitivity to lethal concentrations of H_2O_2 (p< 0.05). However, pre-exposure to sub-lethal levels of trisodium phosphate had no effect on cellular resistance to lethal levels of H_2O_2 (p>0.05). The protection area (Lou and Yousef, 1997) in TSP pre-treated cells was significantly greater (p<0.05) than cells which had undergone pre-treatment with citric acid, H_2O_2 and NaClO. Cells which had undergone H_2O_2 pre-conditioning had a

significantly larger protection area than cells which had been pre-exposed to citric acid and NaClO ($p<0.05$); and citric acid pre-treatment resulted in a significantly larger protection area than NaClO pre-treated cells ($p<0.05$).

These findings suggest that pre-exposure to sub-lethal antimicrobials can increase susceptibility to lethal levels of H_2O_2. The effects were treatment dependent with regards to the effect that pre-conditioning had on susceptibility (NaClO > citric acid > H_2O_2 > TSP > untreated controls).

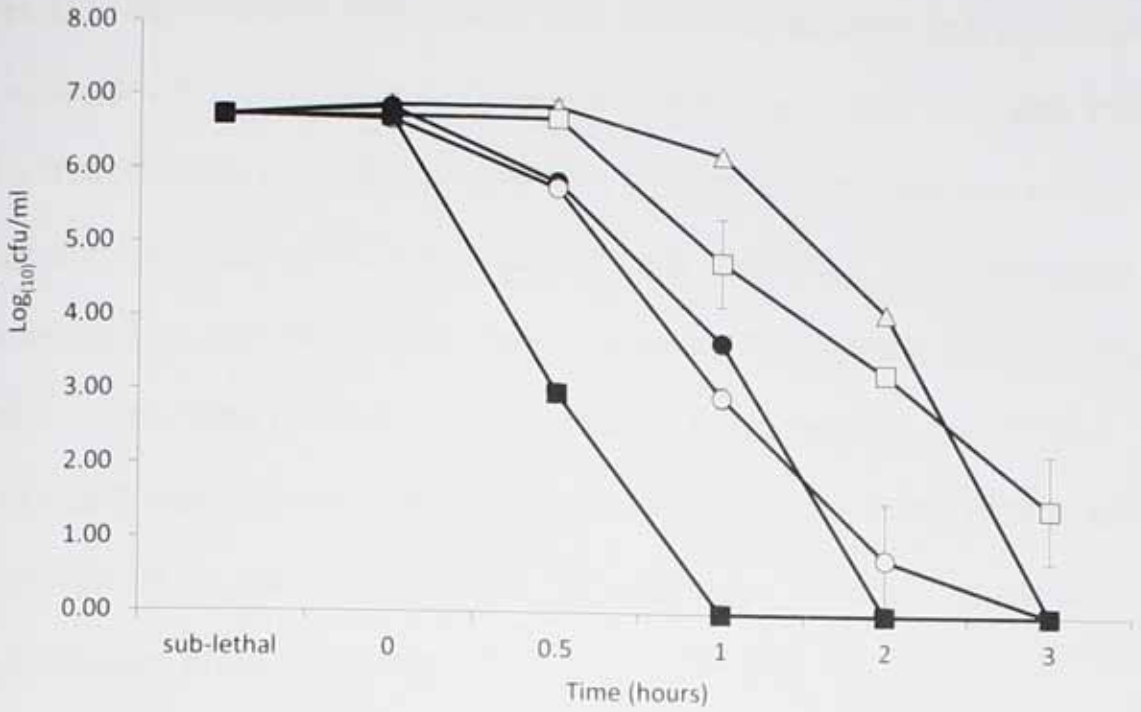

Fig. 3-9: Effects of sub lethal exposure to H_2O_2 (-●-), Citric acid (-○-), Trisodium phosphate (-□-) and NaClO (-■-) on exposure of *L. monocytogenes* cells to lethal concentrations of TSP. Control cells (-△-) were subjected to H_2O during pre-treatment. Error bars indicate the SEOM of three independent experiments (n=3) plated in triplicate.

ANOVA analysis revealed that significant differences occurred in the protection area generated by pre-treated vs. control cells when subjected to lethal levels of trisodium phosphate (F (4, 40) = 641.9, p<0.000). Post hoc analysis revealed that sensitivity to lethal concentrations of TSP was significantly greater (p<0.05) in pre-treated cells compared to cell which had not undergone pre-treatment. This altered sensitivity was pre-treatment dependant. When pre-treated with sub-lethal levels of TSP for one hour, the effects of lethal TSP exposure was significantly greater than control cells which had not undergone pre-treatment (p<0.05). However, the protection area was significantly greater (p<0.05) in TSP pre-treated cells than those which had been pre-treated with citric acid, H_2O_2 and

NaClO. Pre-treatment with citric acid and H_2O_2 resulted in a significantly smaller protection area than those generated by control cells and TSP pre-treated cells ($p<0.05$). However, the protection area was larger than that obtained from NaClO pre-treated cells ($p<0.05$). The protection area generated by cells which had been pre-treated with citric acid and H_2O_2 were comparable ($p>0.05$). Cells which had undergone NaClO pre-treatment were significantly more susceptible to the lethal effects of TSP than TSP, H_2O_2 and citric acid pre-treated cells ($p<0.05$), as indicated by a decreased protection area.

From Fig. 3-9 it can de deduced that pre-treatment with antimicrobials at sub-lethal concentrations influences TSP susceptibility in a treatment dependent manner. The extent in which susceptibility was increased was pre-treatment dependent, whereby NaClO > citric acic/H_2O_2 > TSP > control cells with respects to TSP sensitivity.

that was significantly smaller than control cells and those pre-treated with citric acid ($p<0.05$), however the protection area was significantly larger than those subjected to TSP and NaClO during pre-exposure ($p<0.05$). Both TSP and NaClO pre-treatments resulted in significantly smaller protection areas than control cells, H_2O_2 pre-treated cells and citric acid pre-treated cells ($p<0.05$). However. No differences were found in the protection area of cells which underwent pre-treatment in TSP and NaClO ($p>0.05$).

Citric acid resistance is influenced by pre-conditioning in a treatment dependant manner; the extent to which susceptibility is increased is dependent upon the pre-treatment (TSP/NaClO > H_2O_2 > citric acid > untreated control).

3.4.5 Development of Resistant/Tolerant sub-populations through stepwise 'training'

Table 3-1: MICs and MBCs of antimicrobials against *L. monocytogenes* EGD-e

Treatment	MIC (% w/v)	MBC (% w/v)
NaClO	0.15	0.15
H_2O_2	0.06	0.06
Citric acid	0.25	1
Trisodium phosphate	1	2

Results obtained from three independent experiments (n = 3) conducted in replicates of 6.

MICs and MBCs were determined by broth macro dilution method. The MIC for NaClO was 0.15% (active chlorine), with an MBC of 0.15% (active chlorine), indicative of the bactericidal nature of NaClO. Likewise, H_2O_2 demonstrated typical bactericidal properties, with an MIC and MBC value of 0.06% (w/v). Citric acid and trisodium phosphate demonstrated bacteriostatic properties with MICs of 0.25% and 1%, and MBCs of 1% and 2% (w/v) respectively.

Table 3-2: Effects of sub-MIC incubation on *L .monocytogenes* tolerance/resistance to antimicrobials at the MIC.

Treatment	0.125x MIC	0.25x MIC	0.5x MIC	MIC	1.5x MIC**	MBC
Control*	+	+	+	-	-	-
NaClO	+	+	+	-	nt	-
H_2O_2	+	+	+	-	nt	-
Citric acid	+	+	+	-	-	-
Trisodium phosphate	+	+	+	-	-	-

* Control indicates wild-type culture subjected to each treatment at stated proportion of the MIC.
** 1.5x MIC was only tested when a difference between MIC and MBC was observed; nt-not tested; Results obtained from a triplicate experiment (n = 3)

Upon determining the MIC and MBC for each antimicrobial agent cells underwent stepwise 'training' in an attempt to develop or isolate cells with altered resistance properties. Cells were prepared by overnight incubation in BHI broth and were subjected to progressively increasing proportions of the MIC value. When undergoing this 'training' cells were able to grow at all sub-MIC concentrations, however, cells did not develop increased resistance to any of the antimicrobials tested. At each stage cells were subjected to both the MIC and MBC concentrations but no increases were observed.

3.4.6 Repeated Exposure for resistant sub-population development

Using the method of Rajkovic *et al.* (2009) an attempt was made to develop resistant sub-populations.

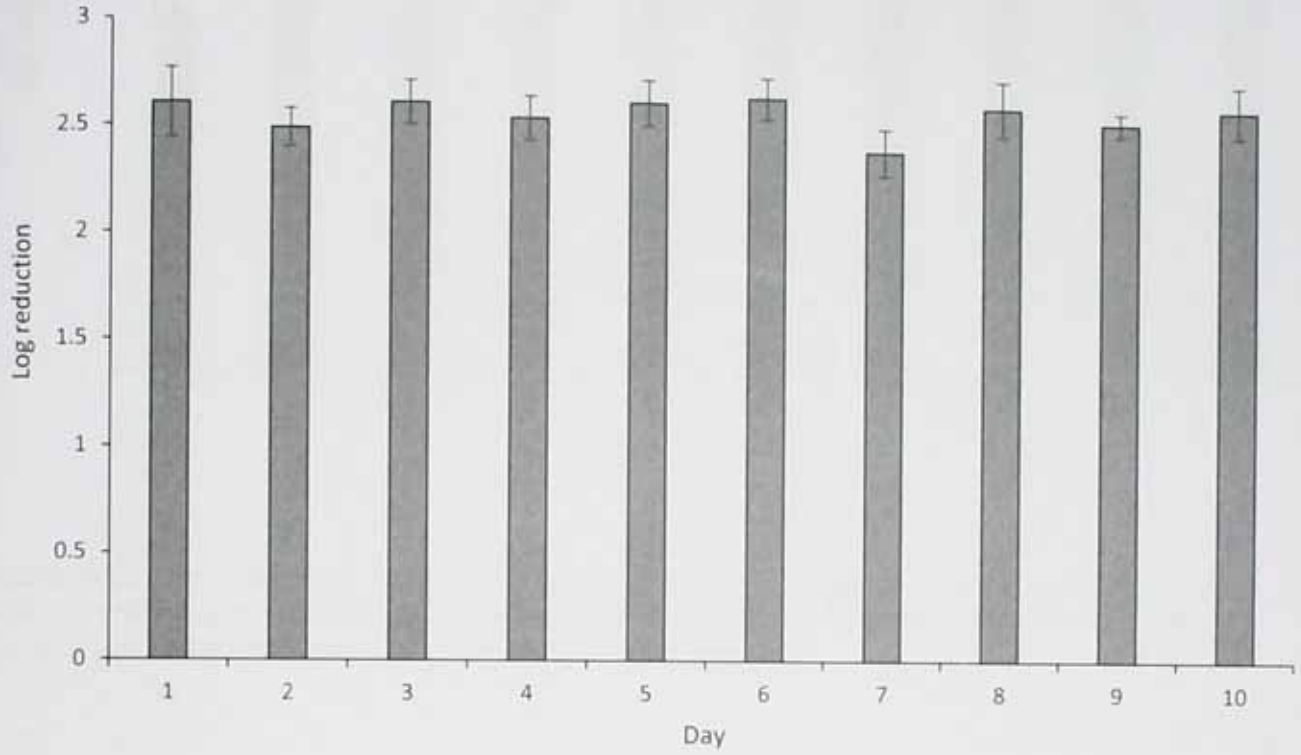

Fig. 3-11: Log reductions of *L. monocytogenes* EGD-e following serial subjection to NaClO. Error bars indicate the SD of three independent experiments plated in triplicate (n=3). Log reductions calculated relative to time-point '0'.

Fig. 3-11 demonstrates the inability of *L. monocytogenes* EGD-e to develop resistance to NaClO through repeated exposure. Cells were grown for 24 hours before undergoing antimicrobial challenge, after challenge an aliquot was taken for enumeration while a second aliquot was transferred to fresh growth media for subsequent exposure. Over 10 days/cycles of exposure and growth no change in population resistance was apparent.

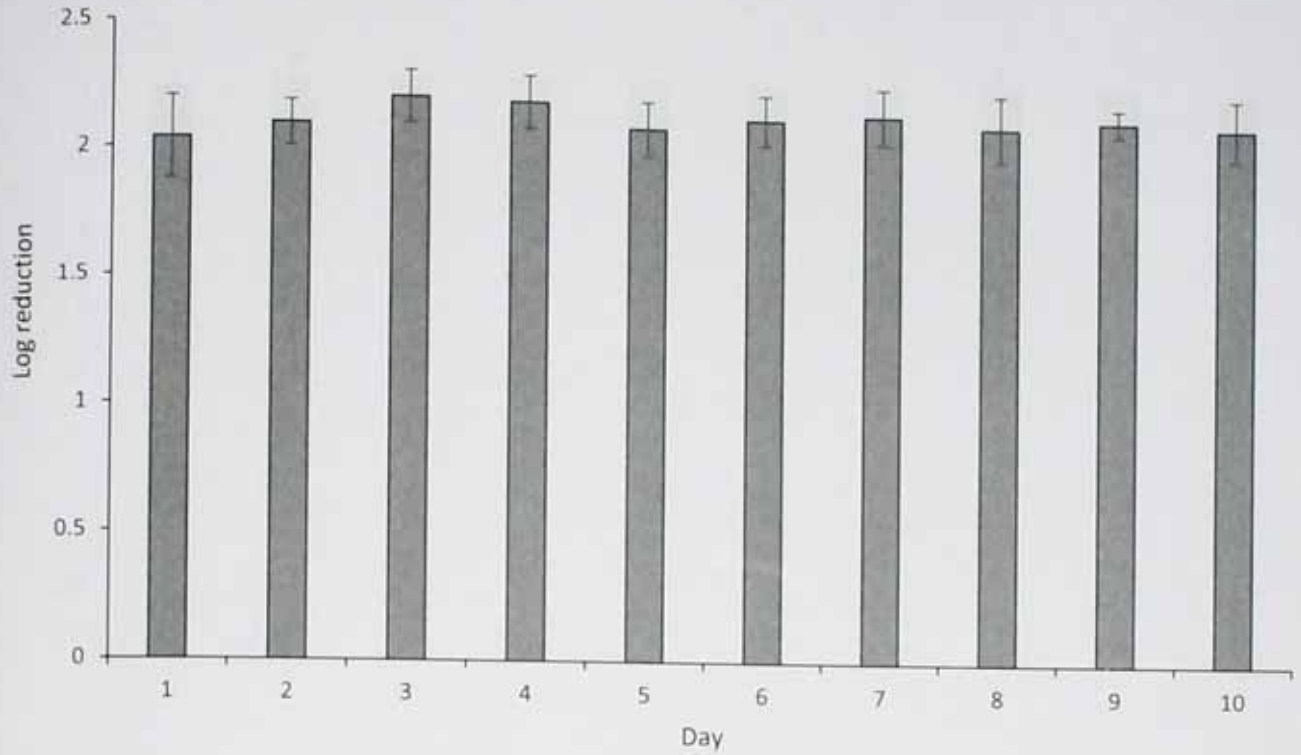

Figure 3-12 Log reductions of *L. monocytogenes* EGD-e following serial subjection to H_2O_2. Error bars indicate the SD of three independent experiments plated in triplicate (n=3). Log reductions calculated relative to time-point '0'.

When undergoing repeated exposure to H_2O_2 *L. monocytogenes* EGD-e population resistance did not increase. A treatment intensity (concentration and time) was pre-determined to give approximately a 2 log reduction in cfu/ml. Over 10 days of passaging no difference in population resistance was apparent.

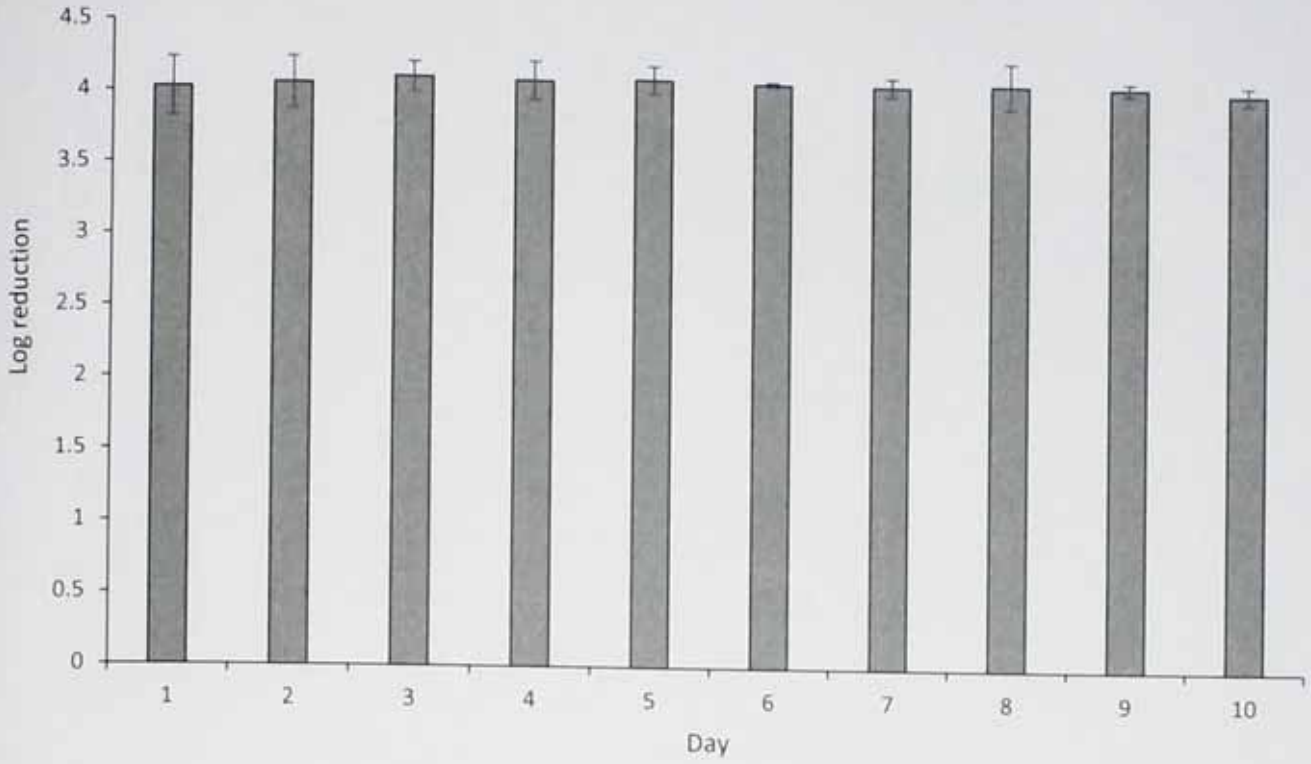

Fig. 3-13: Log reductions of *L. monocytogenes* EGD-e following serial subjection to TSP. Error bars indicate the SD of three independent experiments plated in triplicate (n=3). Log reductions calculated relative to time-point '0'.

When subjected to repeated rounds of exposure to TSP, *L. monocytogenes* EGD-e cells were unable to develop/enhance resistance. A pre-determined treatment intensity was assigned which resulted in approximately a 4 log reduction in cfu/ml following exposure. This reduction did not decrease following passaging of exposed cells.

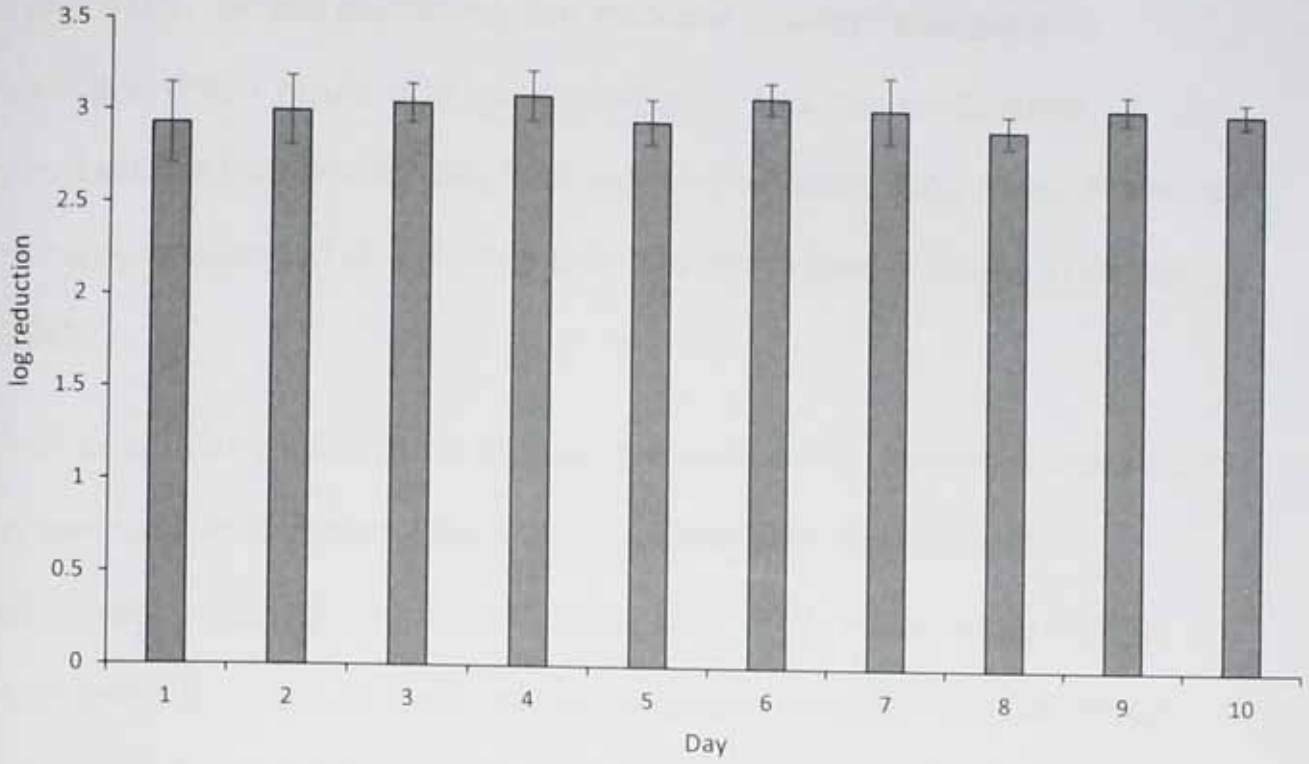

Fig. 3-14: Log reductions of *L. monocytogenes* EGD-e following serial subjection to citric acid. Error bars indicate the SD of three independent experiments plated in triplicate (n=3). Log reductions calculated relative to time-point '0'.

When subjected to repeated rounds of exposure to citric acid at a pre-determined intensity sufficient for approximately a 3 log reduction in cfu/ml, *L. monocytogenes* EGD-e cells were unable to develop resistance over a 10 day 'training' programme.

3.5 Discussion

3.5.1 Adaptive Responses in *L. monocytogenes* EGD-e

It is well documented that under the correct conditions bacterial cells can respond to sub-lethal exposures in such a way that cells become 'stress hardened' (Lou and Yousef, 1997; Sergelidis and Abrahim 2009; Cebrián

et al. 2010). Stress hardening can increase cellular resistance to conditions which would otherwise be lethal. This has particularly important implications for the food industry whereby food manufacturers are being challenged to provide minimally processed products (Ohlsson, 1994).

When conducting adaptation assays, pre-treatment intensities were first determined. Preliminarily each antimicrobial was tested for its antimicrobial activity by time-kill assay (Fig. 2-5). Upon determining lethal and sub-lethal concentrations, each antimicrobial was tested to ensure pre-treated cells were not being sub-lethally injured (Fig. 3-6). By plating cells onto selective and non-selective media an estimation of cellular injury could be determined (Jasson *et al.*, 2007). By ensuring sub-lethal pre-treatments were not inducing injury it was envisaged that cellular adaptions were most likely to occur, as injured cells were likely to be more susceptible to lethal exposure than control cells which were not compromised. However, this data suggests that under these test conditions cells were unable to adapt to sub-lethal pre-treatments (Fig. 3-7 to 3-10). Under these test conditions antimicrobial susceptibility generally increased following pre-conditioning compared to untreated control cells. These findings support the hurdle concept whereby exposure to multiple antimicrobial agents/processes increases the efficiency of the complete hurdle regime.

There are several proposed mechanisms accounting for the effectiveness of the hurdle concept. These are reviewed by Leistner (2000) and

include, disruptions to cellular homeostasis, metabolic exhaustion and multi-component targeting. It is suggested that by adopting several processes organisms cannot maintain cellular homeostasis, exhaust energy supplies by attempting to overcome several stresses and have insufficient energy and/or macronutrients to repair several cellular components if targeted simultaneously. These results support these proposed mechanisms, particularly when considering the results for citric acid and TSP, as susceptibility was impacted least by homologous pre-conditioning. This is probable, as the mechanisms put in place to alleviate the sub-lethal stress are likely to be similar to those adopted to overcome lethal exposure to the same agent.

The observation that, under these test conditions, cells were unable to demonstrate adaptive responses may be explained by several factors. Many studies have investigated the effects of sub-lethal stress exposure on subsequent lethal challenge (O'Driscoll *et al.*, 1996; Lou and Yousef, 1997; Bergholz *et al.*, 2012), however, these studies are often concerned with metabolically active exponential phase cells. As cells are rarely in conditions/environments which permit exponential growth (Navarro Llorens *et al.*, 2010), it was decided that these studies would be performed on stationary phase cells. Davis *et al.* (1996) suggest that low metabolic activity in stationary phase cells makes it improbable that cells could initiate a rapid and effective acid tolerance response. It is therefore conceivable that the growth phase of cells prevented initiation of adaptive responses under the conditions tested here.

Furthermore, in order to keep these studies more representative of 'real-life', both the adaptation and lethal phases of antimicrobial challenge were performed in solution as opposed to adjusted culture media such as BHI. There is a nutritional requirement for protein synthesis to occur (Scott and Hwa, 2011), and protein synthesis is often reported as a requirement for adaptive responses (Davis *et al.*, 1996; O'Driscoll *et al.*, 1996). Furthermore, Samelis *et al.* (2003) reported that stationary phase *L. monocytogenes* cells were better able to mount an acid tolerance response in the presence of glucose, a fundamental energy source, than those 'adapted' in the absence of glucose. As such, the lack of adaptive responses seen in this study may be accountable to the absence of nutrient rich culture media during the adaptation phase.

When considering these findings, it has been demonstrated that under 'real-life' conditions, stationary phase cells are unable to mount adaptive responses against citric acid, TSP, H_2O_2 and NaClO. However, evidence has been presented to support the concept of hurdle technology, whereby bacterial susceptibility to decontaminants was increased following pre-exposure to sub-lethal stress.

3.5.2 Development of mutants and resistant sub populations of *L. monocytogenes* EGD-e

As previously mentioned, minimal processing is sufficient to inactivate a portion of the present microbial load (Rajkovic *et al.*, 2009). However, while the application of minimal processes allows the freshness of a

product to be maintained it represents a food safety risk. It has been documented that cells surviving minimal processing possess altered resistance characteristics, virulence properties and growth dynamics compared to non-exposed cells (Uyttendaele *et al.*, 2008; Van Houteghem *et al.*, 2008; Wesche *et al.*, 2009).

In this study, resistant sub-population were not generated through repeated exposure to test antimicrobials. In a similar study by Rajkovic *et al.* (2009) resistant sub-populations were generated in an organism and treatment dependant manner. In this study intense light pulse and lactic acid resistant populations of *L. monocytogenes* were generated as were intense light pulse resistant *E. coli* populations. This suggests that the development of resistant sup-populations by repeated exposure is complex, and is dependent upon several factors.

In order to generate resistant sub-populations, further passages could have been included. However, as no apparent increase in antimicrobial resistance had occurred after 10 days (Fig. 3-11 to 3-14), it seemed unlikely that such resistance would develop. Use of additional strains and/or antimicrobials may have given rise to resistant sub-populations, however, given the findings of previous studies this would not have guaranteed the development of such populations.

In an attempt to generate *L. monocytogenes* EGD-e cells with enhanced resistance properties compared to parent cells a typical 'stepwise training' approach was adopted. This techniques is commonly employed when

studying antibiotic resistance. By subjecting cells to an increasing concentration of antimicrobial/antibiotic it is proposed that cells can adapt to otherwise lethal, or inhibitory, concentrations of the test agent (Russell, 2003). A second suggestion has been made regarding cell training around the MIC. The mutant selection window suggests that exposure to an agent above the MIC but below the MBC provides the optimal conditions for mutant selection (Drlica, 2003). Both techniques have been reported to have varying levels of success (Firsov *et al.*, 2006; Walsh *et al.*, 2003). However, under these test conditions resistant cells were not developed.

Chapter 4

Effects of sub-lethal stress on gene expression

4.1 Introduction

Several mechanisms are adapted in stressed organisms to allow survival and permit growth in sub-optimal or potentially lethal environments (Neales, 2004). Such mechanisms include 'generic' stress responses; changes in gene expression (Boor, 2006), induction of stress response proteins (Gandhi and Chikindas, 2007), alterations to cellular structures (e.g. cell wall) (Jordan *et al.*, 2007), as well as stress specific responses. For example, both the glutamate decarboxylase system and F_0F_1 ATPase enzyme have been found to be of importance for acid tolerance in several organisms (De Biase *et al.*, 1999; Audia *et al.*, 2001). Compatible solute uptake systems have been implicated in bacterial stress response to high salinity, freeze-thawing and drying (Hill *et al.*, 2002).

L. monocytogenes has been repeatedly found to adaptively respond to unfavourable conditions (Koutsoumanis *et al.*, 2003; Bergholz *et al.*, 2010; Bergholz *et al.*, 2012; Shen *et al.*, 2013) as such, cells which are sub-lethally exposed to stress can adapt and become resistant to subsequent lethal stresses (Bradley *et al.*, 2012); Making the issue of stress adaptation of importance to the food industry.

As well as providing cross protection against subsequent stressors, sub-lethal stress exposure has been implicated in the ability of *L. monocytogenes* to cause human disease. Several studies have highlighted altered resistance of *L. monocytogenes* when subjected to simulated gastric fluid after pre-exposure to sub-optimal environments (Garner *et al.*, 2006; Ilhak *et al.*, 2011; Barbosa *et al.*, 2012; Ivy *et al.*,

2012). Furthermore, the ability of *L. monocytogenes* to invade host cells has been found to be influenced by certain environmental stresses (Garner *et al.*, 2006; Burkholder *et al.*, 2009; Neuhaus *et al.*, 2013). As such, the responses of *L. monocytogenes* to sub-optimal and/or sub-lethal conditions is of interest when considering human health.

4.2 Aims and Objectives

4.2.1 Aim

The aim of this chapter was to elucidate the transcriptional mechanisms permitting growth and survival in the presence of antimicrobial agents at sub-lethal concentrations. Using a bioinformatic approach potential target genes were selected for transcriptional analysis using quantitative real-time PCR.

4.2.2 Objectives

- To determine antimicrobial treatment intensities (concentrations and times) which were sufficient to slow growth to ~60% of maximal growth rate.
- To optimise RNA extraction procedure for *L. monocytogenes*.
- To develop suitable primers to perform qRT-PCR on target genes.
- To assess changes in target gene expression following antimicrobial challenge using qRT-PCR.

4.3 Methods

4.3.1 Determination of treatment intensities sufficient to slow growth

Overnight cultures of *L. monocytogenes* EGD-e were prepared by addition of a single colony into 100ml BHI broth with overnight incubation at 37°C. Cells were diluted 1:100 into sterile, pre-warmed BHI broth (37°C) which had been supplemented with antimicrobial agents at various concentrations. Cultures were incubated statically at 37°C for 8 hours. At hourly intervals 1ml aliquots were taken and the optical density (OD_{595}) was determined relative to a blank (sterile BHI broth) using a cell density meter (WPA biowave, Biochrom Ltd, Cambs, UK).

The control for this experiment was untreated cells which were transferred into pre-warmed BHI broth (37°C) which had not been supplemented with antimicrobial chemicals.

Maximal growth rate (μ_{max}) was calculated using the equation:

$$\mu = \log_{10}(N\text{-}N_0)\ 2.303 / t\text{-}t_0$$

Where μ = maximal growth rate; N_0 = number of cells (OD_{595}) at the beginning of exponential phase; N = number of cells (OD_{595}) during exponential phase; t_0 = time at the beginning of exponential phase; t = time at 'N'

4.3.2 Primer Design

PCR primers were designed to amplify 50-200bp within the central regions of the target genes. Prior to qRT-PCR primer specificity and target amplification were assessed by end-point PCR w/gel electrophoresis.

Table 4-1: Primer sequences for qRT-PCR analysis

Target	Fw. sequence (T_m)	Rv. sequence (T_m)	Amplicon Length (bp)
16S rRNA	TGGTAGTCCACGCCGTAAAC (65°C)	TCAACCTTGCGGTCGTACTC (65°C)	111
groESL	AGAACCAGTTCGCCAAATCG (64°C)	CCGTTTGCTGCATTGAAACC (64°C)	105
Hfq	AAACAAGGTGGACAAGGGTTAC (64°C)	TTACAACGCGTCCTCTTAACTG (64°C)	112
sigB	CAAAGCTCGCCGCAAATTAG (63°C)	TCATCCGTACCACCAACAAC (63°C)	176
recN	TGCGCTTGGTCTTCTTGTTG (64°C)	AGCCCTTGAAGCTCTAAACG (63°C)	81
gadA	AACCGGAAGCTGAGCAAATC (64°C)	AGCCATTCCACCAAGCATAC (63°C)	197
lmo0501	GCCGCTGTTATTGCAACAAG (64°C)	GCGTGTGGAATGGCAATATC (63°C)	114
lmo0669	ATGACGCCTGTTCCAGATAC (63°C)	ACATCAGCACCTTCTCTAGC (62°C)	140
lmo0956	AGCGGACAAAGCAGAAGAAG (64°C)	TTCGTGTGATTGCGTCATCG (64°C)	196

transferred to 1ml of Tri reagent (T9424: Sigma-Aldrich Company Ltd, UK), mixed by inversion, and incubated at room temperature for 5 minutes. Following incubation, 100µl chloroform (Sigma-Aldrich Company Ltd, UK) was added, mixed by gentle inversion, and incubated for a further 3 minutes at room temperature. Tubes were then centrifuged at 13,000x *g* for 10 minutes (4°C) and the upper aqueous phase transferred to a tube containing 200µl chloroform. Tubes were gently mixed and incubated at room temperature for 5 minutes before centrifugation at 13,000x *g* for 10 minutes (4°C). Samples were twice subjected to chloroform phase separation after which the upper aqueous phase was precipitated for 15 minutes at room temperature in 0.1 volumes of 2M NaCl, 0.5µl glycogen and 1 volume (~700µl) isopropanol. RNA was pelleted by centrifugation at 13,000x *g* for 15 minutes (4°C) before being washed twice with ice-cold 75% ethanol. RNA pellets were resuspended in 100µl of molecular biology grade water and a Qiagen RNeasy kit (74104: Qiagen, UK) was used to 'clean' RNA using the protocol designated 'RNA clean-up'. The optional on-column DNase step was incorporated

RNA yield and purity was assessed using a nanodrop device. Only RNA with a 260/230 and 260/280 ratio of at least 1.8 was used for subsequent cDNA synthesis and qRT-PCR. Where necessary, RNA underwent ethanol precipitation to remove salt and protein contamination, thus improving 260/230 and 260/280 ratios.

4.3.4 Reverse transcription

Prior to reverse transcription, RNA was subjected to DNase using amplification grade DNase I (AMPD1: Sigma-Aldrich Company Ltd) to remove contaminating DNA. One microgram of RNA extract was reverse transcribed into cDNA using a high capacity cDNA reverse transcription kit (4368814: Life Technologies UK Ltd) as per manufacturer's recommendations.

No enzyme control (RT-) samples were included during reverse transcription.

cDNA was stored at -20°C until required.

4.3.5 Quantitative Real-Time PCR (qRT-PCR)

4.3.5.1 Defining input cDNA range and PCR efficiency

To determine the input range of cDNA for qRT-PCR, the 'Stock I' method was employed (Gallup and Ackermann, 2008). Equal volumes of cDNA from each sample (including un-treated control) were pooled. From this, serial dilutions (1:10) were performed in molecular biology grade water. Using this dilution series standard curves were performed for each of the gene targets.

PCR efficiency was calculated by standard curve analysis and, where necessary, upper and lower input concentrations were omitted to ensure maximum PCR efficiency. For subsequent quantitative analysis, only input concentrations giving suitable PCR efficiencies were used.

4.3.5.2 Relative quantification of target gene expression

Quantitative RT-PCR was performed in 10µl reactions using Fast SYBR green mastermix (4385612: Life Technologies UK Ltd). PCR reactions were prepared as per manufacturers recommendations using 5ng of cDNA as template material and forward and reverse primers at 200nM.

Relative expression of target genes was assessed in treated cells relative to untreated control cells using the method of Pfaffl (2001). A region of the *16S rRNA* gene was used as a reference (Michel *et al.*, 2011).

Controls for qRT-PCR analysis included the use of RT- samples (RNA samples which had undergone reverse transcription in the absence of RT enzyme) and No Template Controls (NTC), in which molecular biology grade water was added to PCR reactions. Additionally, melt curve analysis was used to confirm specificity of PCR primers following qRT-PCR.

4.4 Results

4.4.1 Effects of antimicrobials on growth of *L. monocytogenes* EGD-e

The effects of various antimicrobials on growth of *L. monocytogenes* EGD-e were assess by comparing optical density (OD_{595}) in treated vs. control cells.

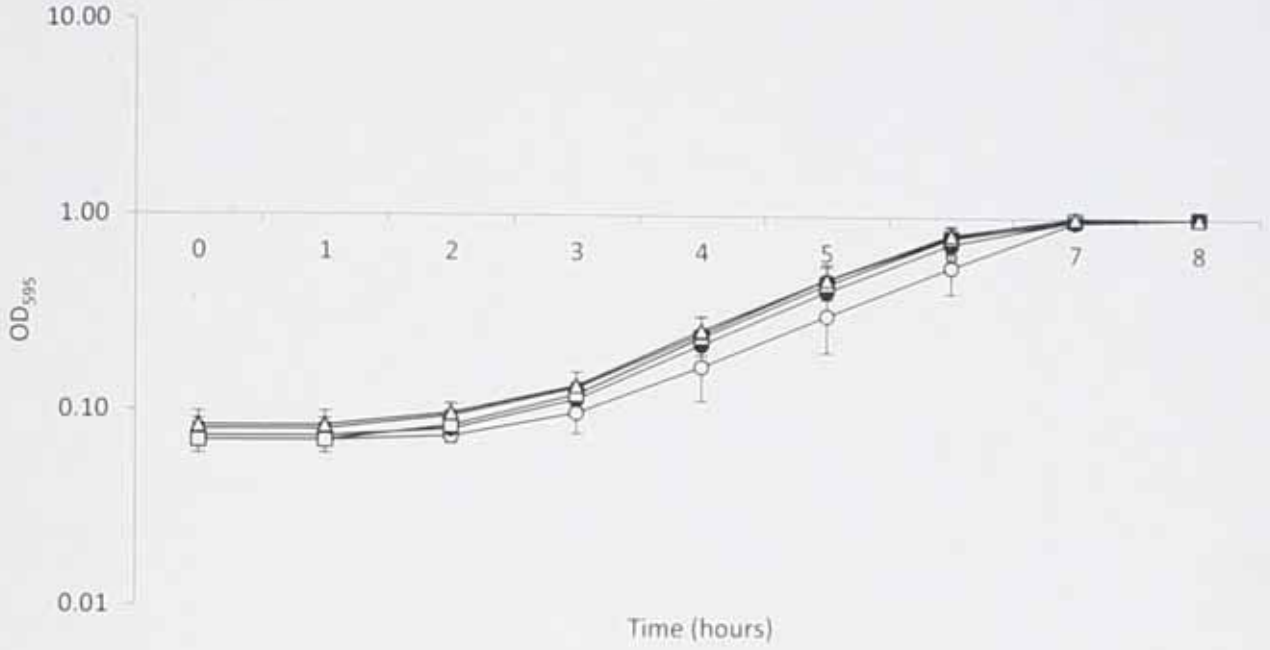

Fig. 4-1: The effects of NaClO at 0.06 % (—○—), 0.05% (—●—), 0.04% (-□-), 0.03% (—■—) on growth of L. monocytogenes EGD-e compared to un-treated controls (-Δ-). Error bars indicate the SD of three independent experiments (n=3).

Maximal growth rate (μ_{max}) was not affected by exposure to NaClO at concentrations <0.06% (w/v). The μ_{max} for untreated cells was 0.60±0.03 h^{-1}. When subjected to NaClO at 0.03%, 0.04% and 0.05% (w/v) the μ_{max} was 0.60±0.03, 0.63±0.03 and 0.62±0.02 h^{-1} respectively. These were not significantly different from controls

($p>0.05$). However, when subjected to NaClO at 0.06% (w/v) there was a significant decrease in μ_{max} ($0.37\pm0.08\ h^{-1}$) ($p<0.05$).

As such 0.06% (w/v) NaClO was used for subsequent gene expression analysis.

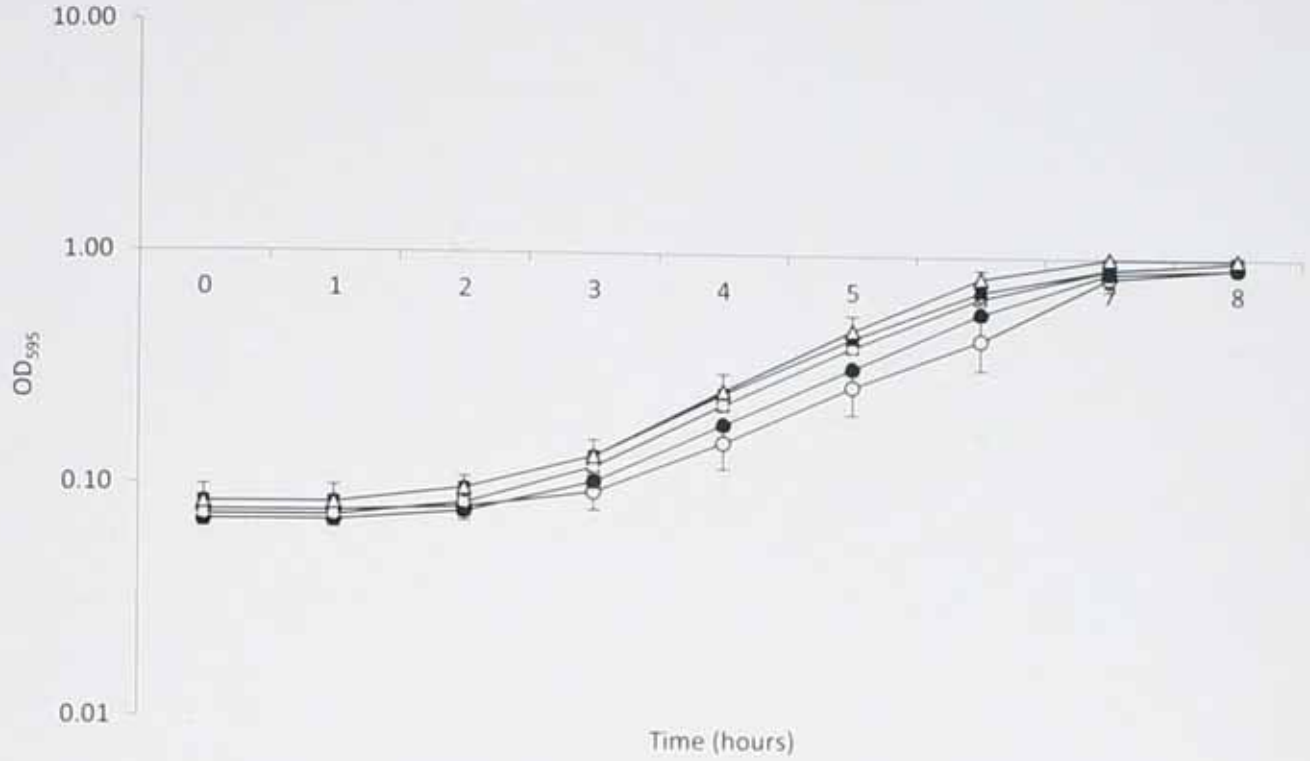

Fig. 4-2: The effects of H_2O_2 at 0.03 % (-○-), 0.02% (-●-), 0.015% (-□-), 0.01% (-■-) on growth of *L. monocytogenes* EGD-e compared to un-treated controls (-△-). Error bars indicate the SD of three independent experiments (n=3).

When subjected to H_2O_2 the maximal growth rate remained unchanged at concentrations <0.03% (w/v). The μ_{max} for cells exposed to H_2O_2 at 0.01%, 0.015% and 0.02% (w/v) were 0.56±0.06, 0.57±0.03 and 0.57±0.01 (h^{-1}) respectively. These were not significantly different from untreated controls (p>0.05).

When subjected to H_2O_2 at 0.03% (w/v) there was a significant decrease in μ_{max} compared to untreated controls (0.39±0.09 vs. 0.60±0.03 h^{-1}) (p<0.05). As such, cells were subjected to H_2O_2 at 0.03% (w/v) for subsequent gene expression analysis.

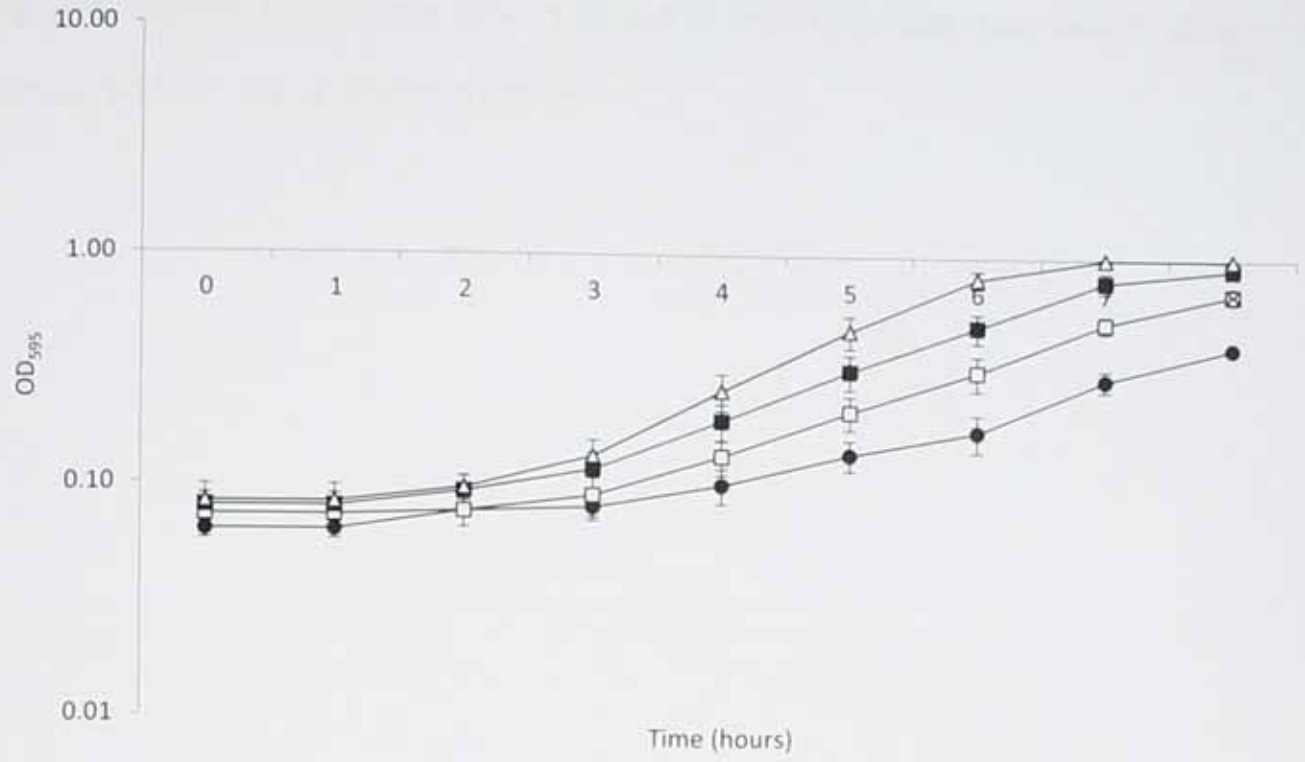

Fig. 4-3: The effects of citric acid at 0.2 % (-●-), 0.15% (-□-), 0.1% (-■-) on growth of *L. monocytogenes* EGD-e compared to un-treated controls (-Δ-). Error bars indicate the SD of three independent experiments (n=3).

Citric acid affected maximal growth rate in a concentration dependent manner. When subjected to citric acid at 0.1% (w/v) there was a significant decrease in μ_{max} (0.47±0.05 vs. 0.60±0.03 h^{-1}) (p<0.05). At 0.15% (w/v) μ_{max} was decreased to 0.40±0.04 (h^{-1}), which was significantly less than untreated controls (p<0.05). When exposed to 0.2% (w/v) citric acid there was a further decrease in growth rate to 0.26±0.03 (h^{-1}), this was again significantly less than untreated controls (p<0.05).

To allow consistency in treatment intensity, cells were exposed to citric acid at 0.15% (w/v) for subsequent gene expression analysis. As seen in

Fig. 4-6, 0.15% (w/v) citric acid resulted in a comparable decrease in μ_{max} compared with other antimicrobials.

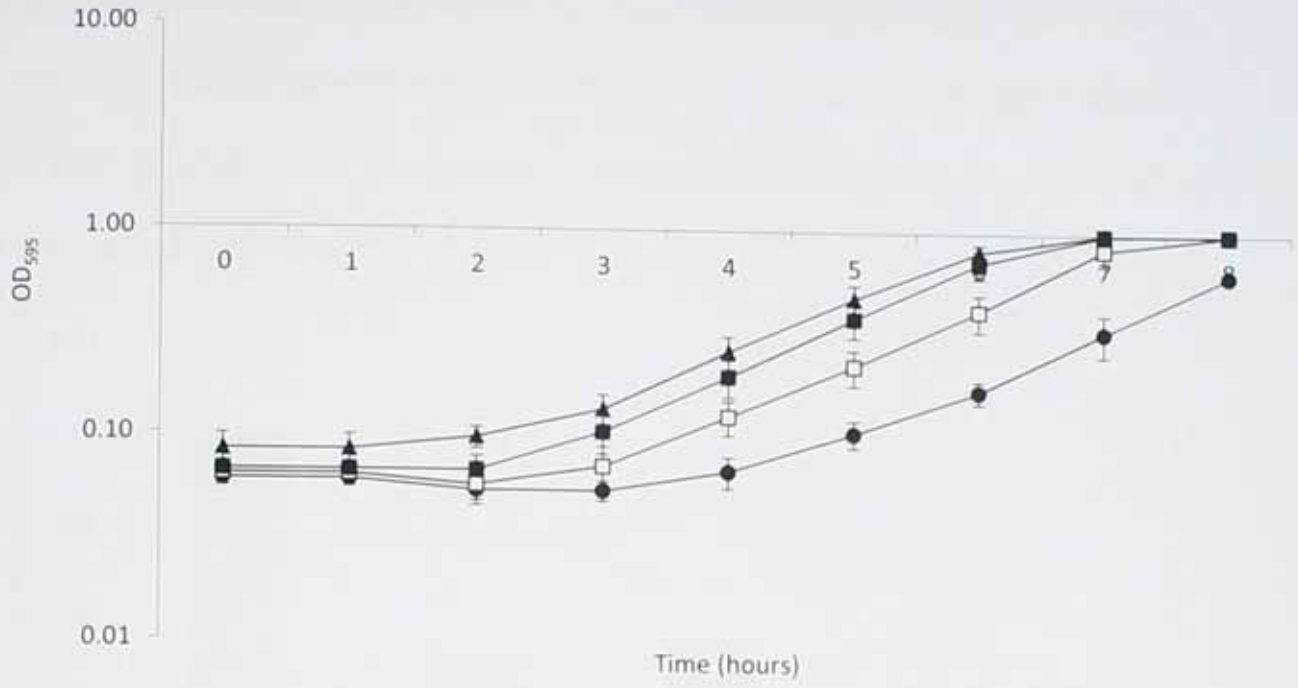

Fig. 4-4: The effects of trisodium phosphate at 1% (-●-), 0.75% (-□-), 0.5% (-■-) on growth of *L. monocytogenes* EGD-e compared to un-treated controls (-Δ-). Error bars indicate the SD of three independent experiments (n=3).

When subjected to trisodium phosphate at 0.5% (w/v), maximal growth rate was not significantly different from untreated controls (0.63±0.06 vs. 0.60±0.03 h^{-1}) (p>0.05). At 0.75% (w/v) μ_{max} was decreased to 0.41±0.03 (h^{-1}), this was significantly different from untreated controls (p<0.05). When subjected to 1% (w/v) trisodium phosphate the μ_{max} decreased further to 0.38±0.01 (h^{-1}), again, this was significantly less than the μ_{max} in untreated controls (p<0.05).

For subsequent gene expression analysis 0.75% (w/v) trisodium phosphate was chosen as this concentration had a comparable effect on μ_{max} compared to other treatments (Fig. 4-5 and 4-6).

To allow comparison of the effects of each antimicrobial agent on gene expression levels, treatment intensities were chosen which gave similar maximal growth rates, thus giving comparable decreases in μ_{max} relative to untreated controls.

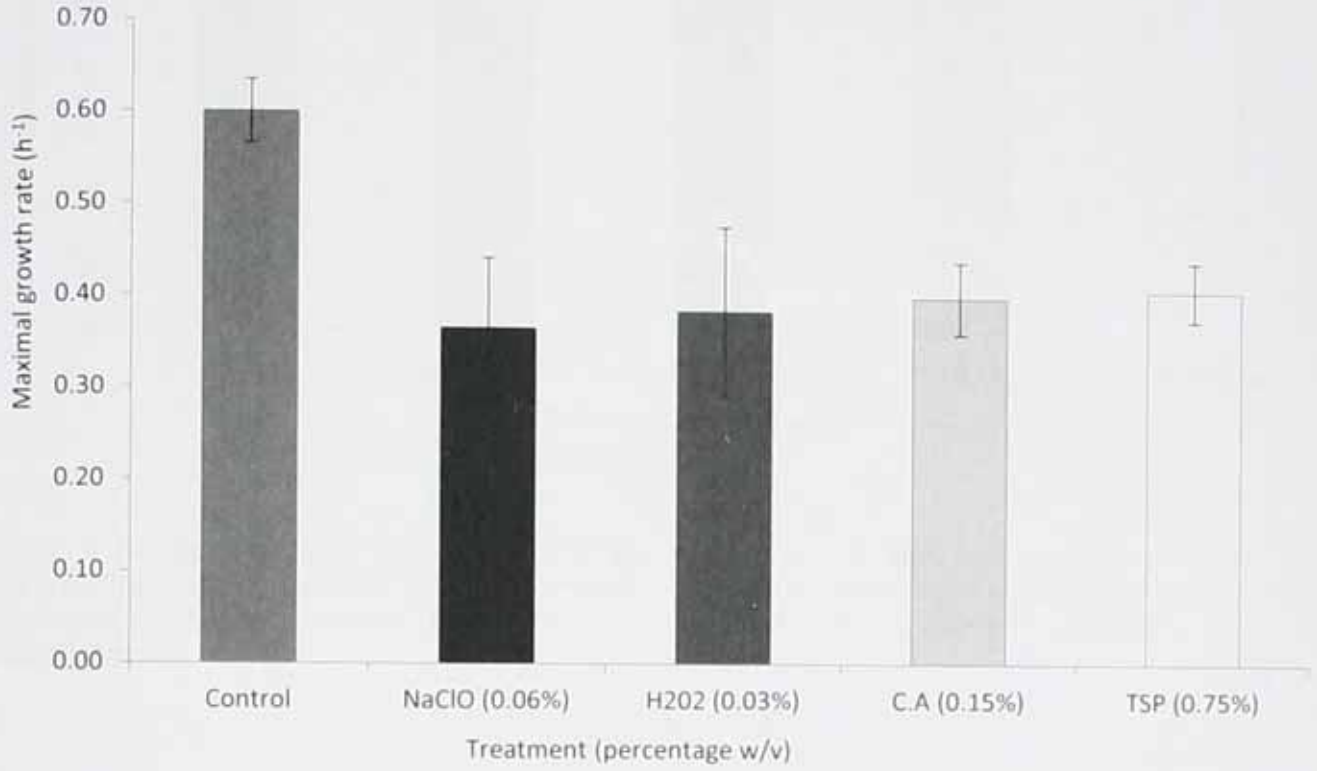

Fig. 4-5: The effects of NaClO (0.06%) (■), H_2O_2 (0.03%) (■), citric acid (0.15%) (□) and trisodium phosphate (0.75%) (□) on maximal growth rate of L. monocytogenes EGD-e compared to un-treated controls (■). Error bars indicate the SD of three independent experiments (n=3).

Treatment intensities were chosen which resulted in comparable decreases in maximal growth rate. There was no significant difference in μ_{max} between treatment intensities (p>0.05).

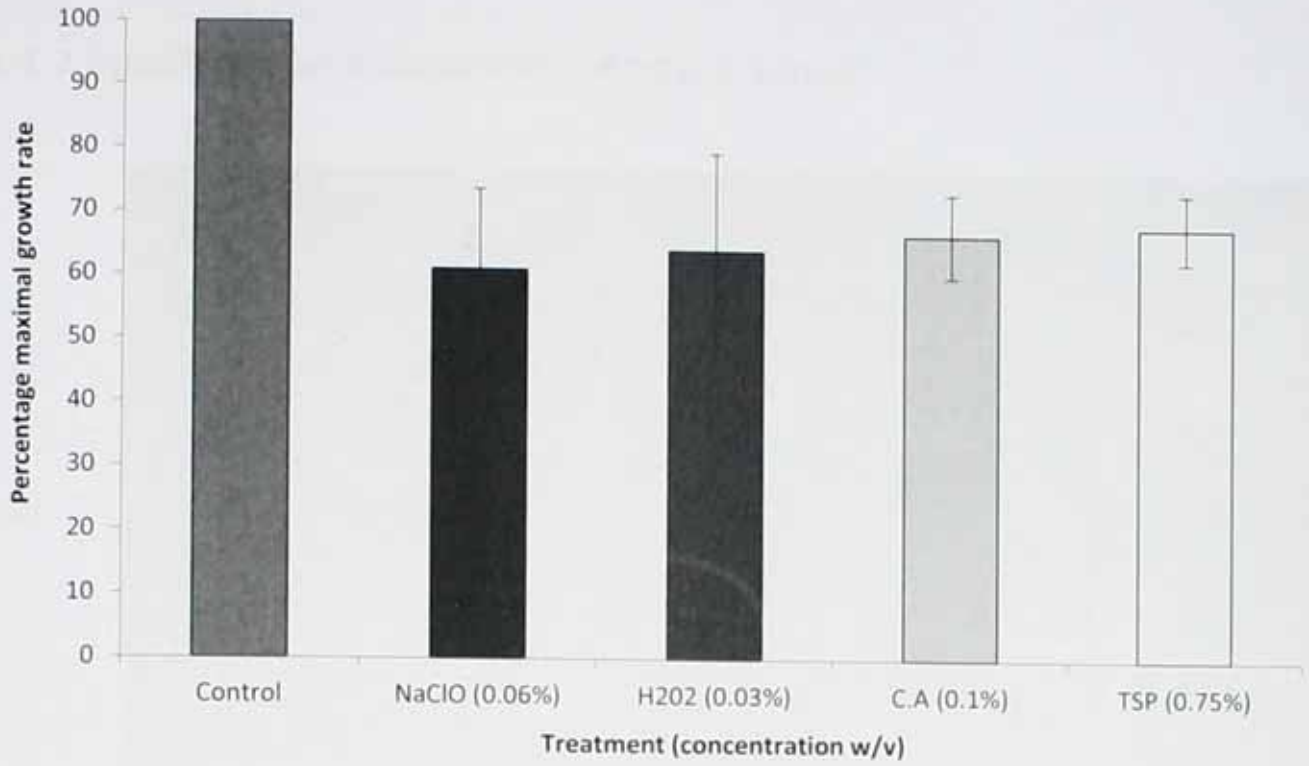

Figure. 4-6: The effects of NaClO (0.06%) (■), H_2O_2 (0.03%) (■), citric acid (0.1%)(□) and trisodium phosphate (0.75%) (□) on the percentage of maximal growth rate relative to un-treated controls (■). Error bars indicate the SD of three independent experiments (n=3).

All treatment intensities gave maximal growth rates which were approximately 60% of the μ_{max} in untreated controls.

4.4.2 Specificity and suitability of PCR primers

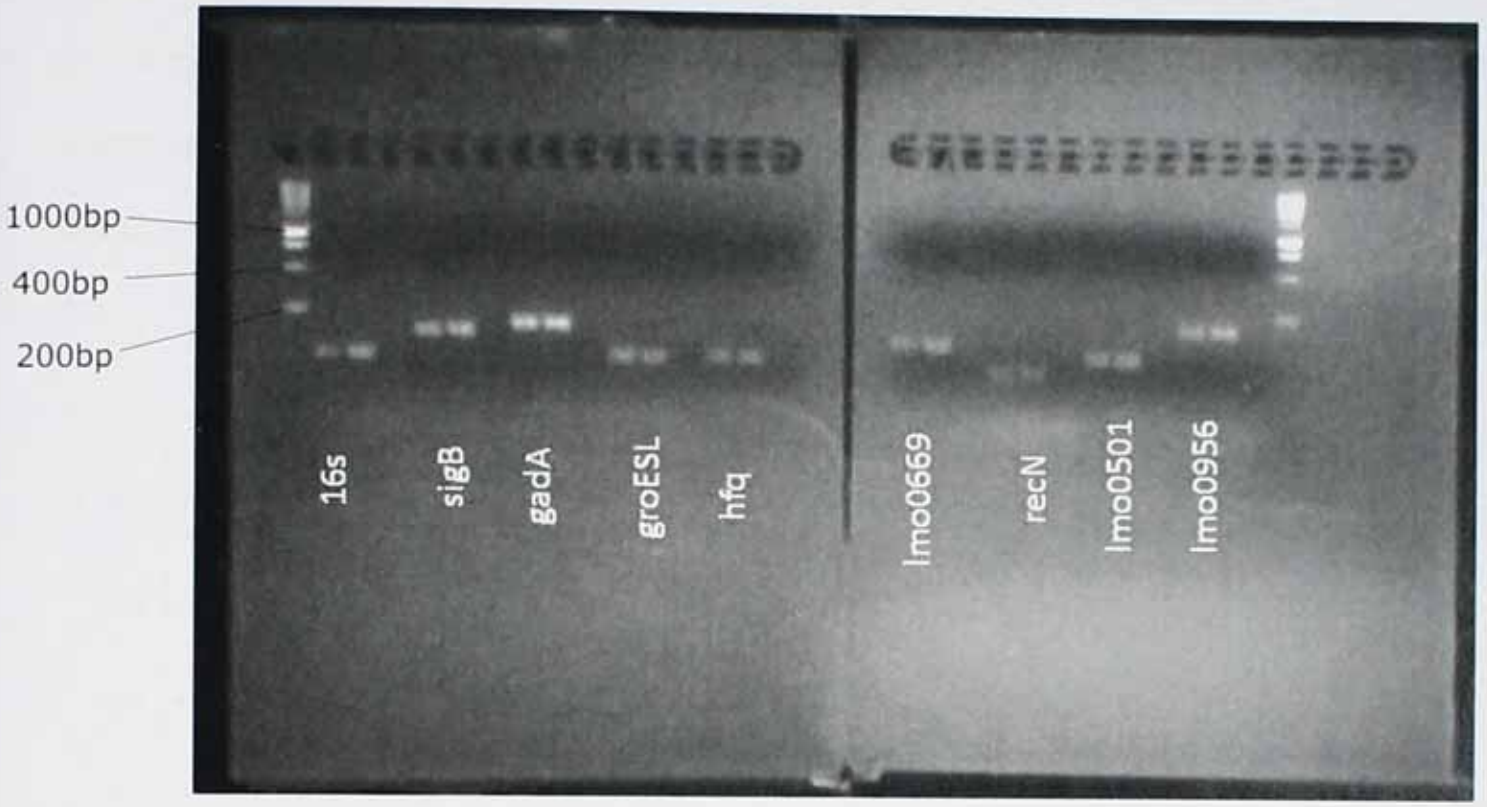

Fig. 4-7: 3% Agarose gel displaying PCR products generated after PCR of genomic DNA using primer pairs for specified targets (see Table 1). 200bp markers (far left/far right) were used as an indicator of product size.

PCR was conducted using genomic DNA as the template. Genomic DNA was extracted as per section 2.2.3.1. Standard PCR cycling conditions and reaction composition were used (section 2.2.3.3.). PCR products were separated by gel electrophoresis using a 3% TAE gel which was supplemented with SYBR safe gel stain. Gels were run at 70V for 1 hour and were visualised using a transluminator.

Each sample was run in duplicate and primer sequences were deemed suitable by the presence of single bands (Fig. 4-7). These primers were taken forward for subsequent gene expression analysis using qRT-PCR.

4.4.3 Determination of target input range and RT PCR efficiency

For qRT-PCR analysis it is advisable to correct for PCR efficiency (Pfaffl, 2004). As such, standard curve analysis was used to determine the PCR efficiency of each primer set. Using the Stock I method (Gallup and Ackermann, 2008) it was possible to determine both the PCR efficiency and the usable input range of cDNA template.

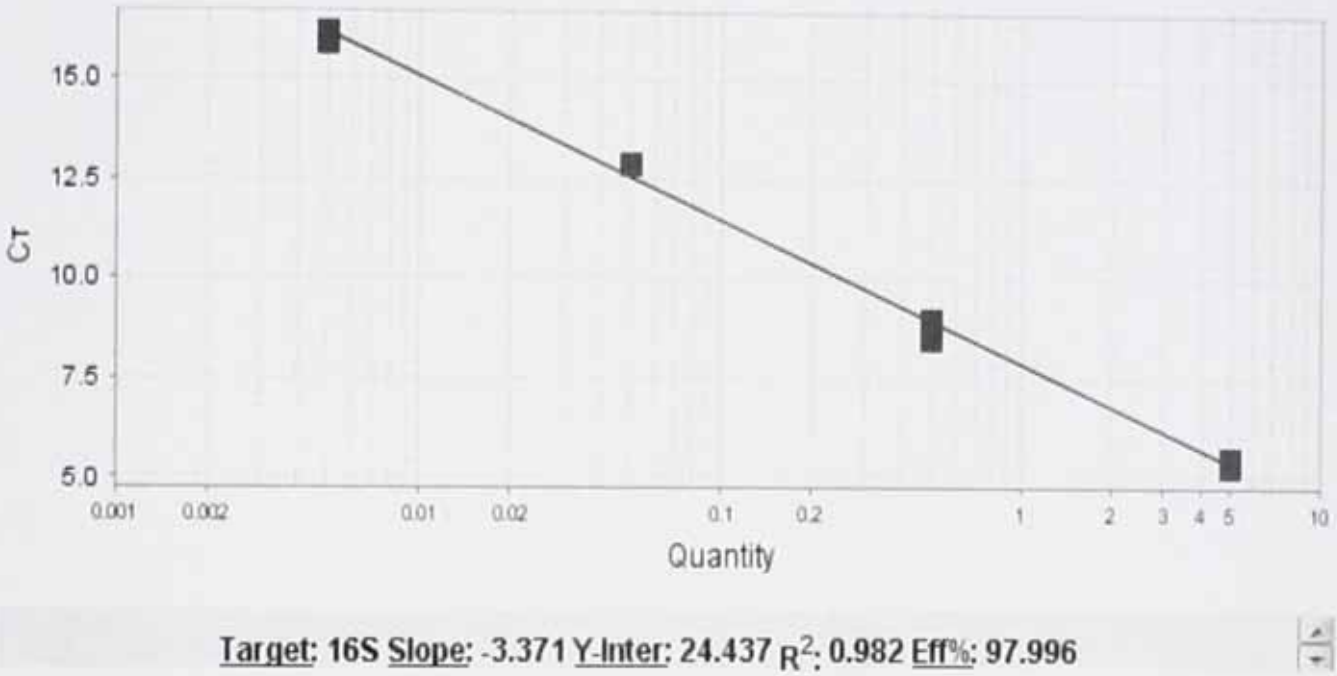

Fig. 4-8: Standard curve for 16s generated from Stock I cDNA dilutions.

Fig. 4-8 shows the standard curve generated by various dilutions of Stock I cDNA when amplified by the *16s rRNA* primer pair (Table 1). In order to achieve a suitable PCR efficiency, the Ct value obtained when inputting 50ng of Stock I cDNA template was omitted as this skewed the curve. As such, subsequent PCR reactions concerning the *16s rRNA* target had an input range of 0.005ng – 5ng. All targets were assessed for slope, R^2

value and percentage efficiency. Using this information relative gene expression could be corrected for PCR efficiency and cDNA input range could be determined.

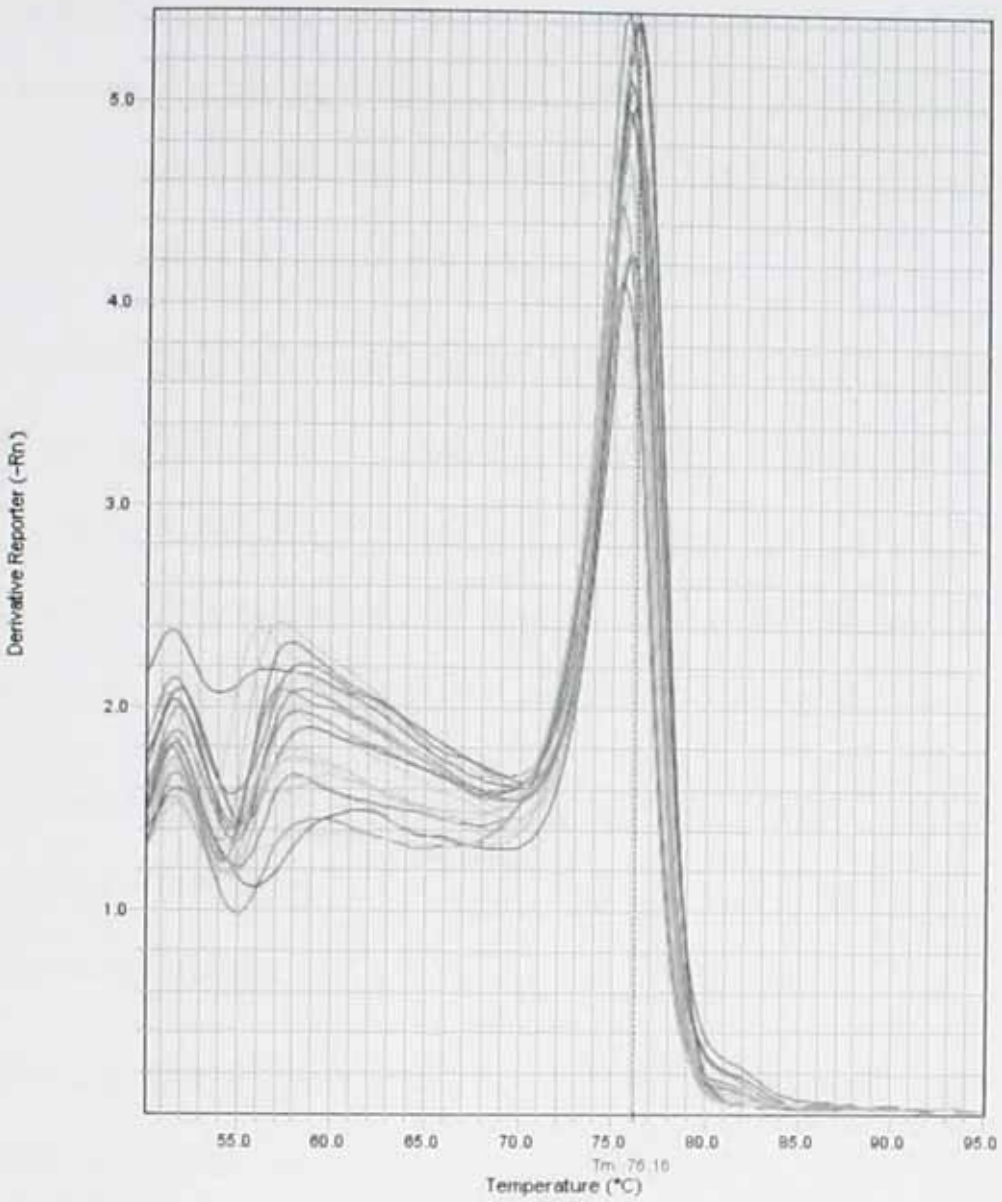

Fig. 4-9: Melt curve for 16s target

Following qRT-PCR reactions melt curve analysis was performed. Using the in-built function of the Applied Biosystems StepOne plus software PCR products were assessed for specificity. A single, prominent peak as shown in Fig. 4-9 was indicative of specific primer binding. All qRT-PCR reactions were assessed by melt curve analysis.

4.4.4 Transcriptional changes arising from sub-lethal stressor exposure

Using the method of Yuan *et al.* (2006) the statistical significance of changes in transcript levels were assessed. By comparing the difference in Ct value (housekeeper Ct – target Ct) between treated and untreated cells statistical analysis was performed.

When subjected to antimicrobials at sub-lethal concentrations, expression of the *GroESL* operon remained unchanged. When exposed to trisodium phosphate and NaClO a small, but non-significant ($p>0.05$), increase in relative fold change was observed. However, exposure to citric acid and H_2O_2 did not result in altered expression of *GroESL*.

Likewise, exposure to trisodium phosphate, NaClO, citric acid and H_2O_2 had no effect ($p>0.05$) on *sigB*, *Hfq*, *recN*, *lmo0501* and *lmo0956* transcript levels.

When subjected to trisodium phosphate there was an approximately 2-fold increase in expression of *gadA* ($p<0.05$). However, *gadA* transcription remained unaltered when exposed to NaClO, citric acid and H_2O_2 ($p>0.05$).

The greatest alteration of gene expression was an up-regulation of *lmo0669* in response to NaClO exposure. Upon exposure to NaClO an approximate 4.5 fold increase in transcript levels was found. This was significantly greater expression compared to untreated controls ($p<0.05$). Expression of *lmo0669* remained unchanged in response to trisodium phosphate, citric acid and H_2O_2.

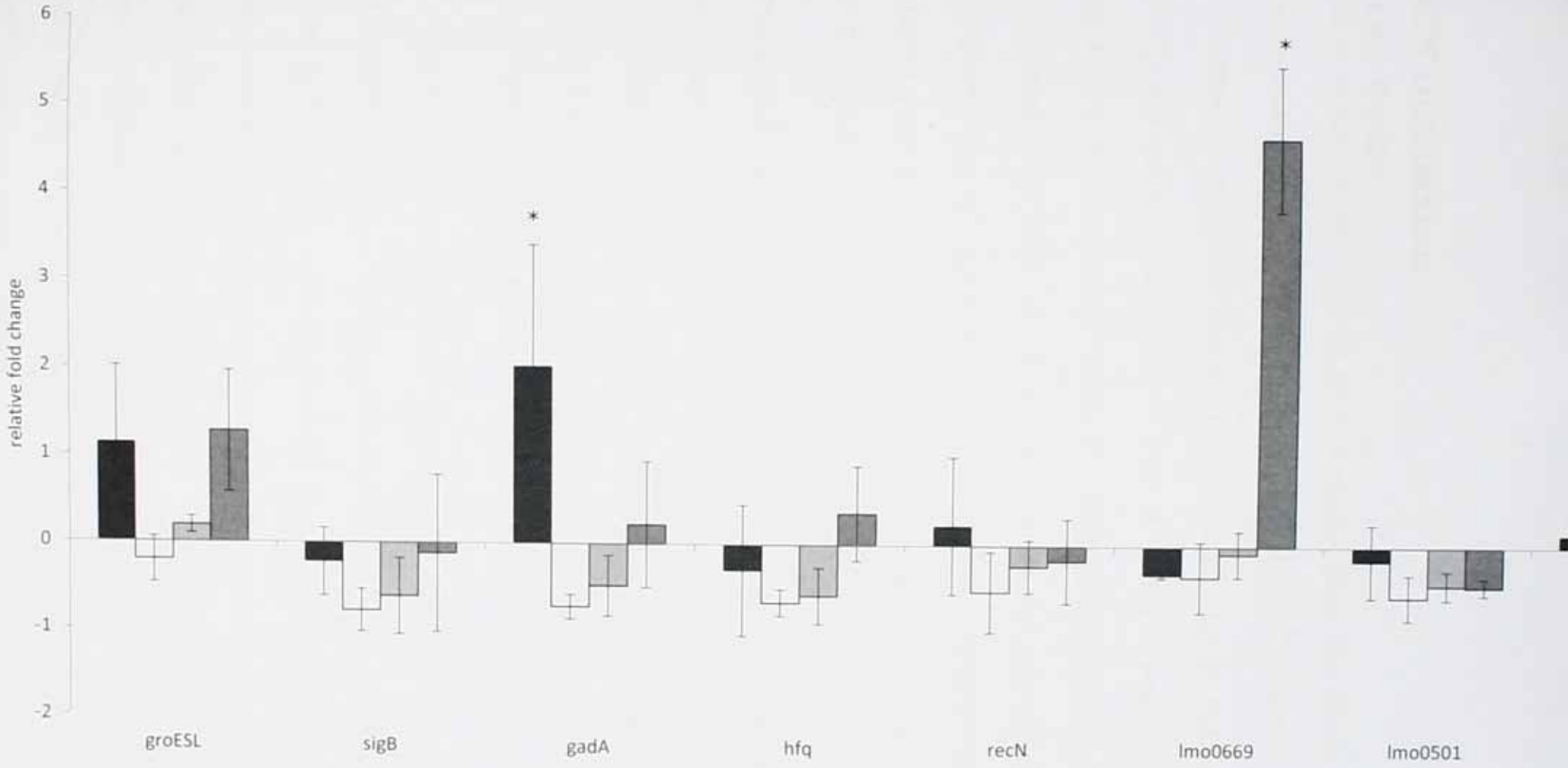

Fig. 4-10: Relative fold change in stress response gene expression following exposure to TSP (■), citric acid (□), H_2O_2 (▨) and NaClO (▩). Error bars indicate the SE of three independent experiments (n=3) conducted in triplicate. Asterisks indicate significant difference in expression level (p<0.05).

4.5 Discussion

4.5.1 *GroESL*

Several biocides act through protein degradation and disruption of the bacterial membrane. These include many surface decontaminants such as trisodium phosphate (Sampathkumar *et al.*, 2003), H_2O_2 (Rea *et al.*, 2005) and NaClO (McDonnell and Russell, 1999) as well as low pH conditions (Beales, 2004). When subjected to such agents, a range of responses occur which aim to alleviate the deleterious effects of biocide exposure. These responses include changes in membrane composition, altered gene expression and induction of stress response proteins (Gandhi and Chikindas, 2006).

The antimicrobial activity of trisodium phosphate attributed to its high pH which causes alteration/damage to cell membranes (Mendonca *et al.*, 1994). Given the role of *GroESL* in membrane stabilisation (Török *et al.*, 1997), it was anticipated that expression would be up-regulated in response to trisodium phosphate exposure.

In this study, the expressional change in *GroESL* was not found to be significant following trisodium phosphate exposure. In a study by Giotis *et al.* (2010), the expression of *GroEL* and *GroES* was up-regulated in response high pH. However, this response was time-dependent and had diminished after 30 minutes exposure. This suggests that the *GroESL* operon acts as an initial alkaline stress responder. The *GroESL* operon does not appear to be responsible for the long term growth and survival of *L. monocytogenes* in high pH environments, however, its role in short

term adaptation to alkaline stress may be of interest to food manufacturers using high pH agents during surface decontamination.

It has been proposed that H_2O_2 targets several cellular components, resulting in its high efficiency as an antimicrobial agent. One such target is the bacterial cell membrane (Finnegan *et al.*, 2010). As previously mentioned, it has been reported that *GroESL* can act by stabilising the membrane of stressed bacterial cells. As such it was hypothesized that its expression would be increased in response to H_2O_2 challenge.

In this study no increase in *GroESL* expression was found. This was unexpected given the mode of action of H_2O_2 and the role of *GroESL* in membrane stabilisation and protein folding. Likewise, Chang *et al.* (2005) did not detect transcriptional changes in *GroESL* when challenging *Pseudomonas aeruginosa* with H_2O_2. Contrary to these findings, in a study concerning the transcriptional response of *E. coli* to H_2O_2, Zheng *et al.* (2001) reported significant up-regulation of both *GroEL* and *GroES* following H_2O_2 exposure.

While the *GroESL* operon seems a logical choice when selecting possible candidate genes responsible for survival and growth in the presence of H_2O_2; the findings here suggest this is not necessarily the case.

As with H_2O_2, NaClO is a potent antimicrobial which targets several cellular structures and processes. These targets include intracellular proteins and cell membranes (McDonnell and Russell, 1999). As such, it was hypothesised that the *GroESL* operon would be initiated during

exposure to NaClO. In this study NaClO increased expression of *GroESL* by ~1.2 fold, which was found to be statistically insignificant. However, in a study by Wang *et al.* (2009) a similar increase in *GroEL* and *GroES* expression was found. In their study *E.coli* cells were subjected to sub-lethal concentrations of NaClO resulting in ~1.4-1.5 fold increases in *GroESL* expression. This suggests that the *GroESL* operon may be of some importance in bacterial response to NaClO challenge.

The mechanism of action of organic acids as antimicrobials is multi-faceted, causing disruption to cellular homeostasis, leading to metabolic exhaustion and disrupting cell membranes (Brul and Coote, 1999). As *GroESL* has roles in membrane stabilisation it was therefore anticipated that an up-regulation would be seen in response to organic acid exposure. During acid stress the *GroESL* operon has been found to be up-regulated in several bacterial genera, including *L. monocytogenes* (Phan-Thanh and Mahouin, 1999; Gahan *et al.*, 2001). However, this was not consistent with these findings in which no change in *GroESL* expression was detected. Likewise, Ivy *et al.* (2012) did not detect changes in *GroEL/GroES* expression during acid exposure of *L. monocytogenes*. This suggests that the role of *GroESL* in acid response may be strain dependent.

4.5.2 *sigB*

Sigma factors are key stress response proteins which have roles in transcription mediation through interaction with RNA polymerase enzymes (Nadon *et al.*, 2002). In *L. monocytogenes* the alternative sigma factor, σ^B, is a key stress response regulator which has roles in several stress responses, including heat, acid, ethanol and oxidative stress (Ferriera *et al.*, 2001).

In *Bacillus subtilis* sub-lethal stress response and adaptation has been found to be largely σ^B dependent. Expression of σ^B was found to permit survival during initial stress exposure, but also induce cross protection against subsequent, otherwise lethal, stress exposure (Völker *et al.*, 1999).

In *L. monocytogenes* several studies have highlighted the importance of σ^B in both stress response and virulence. Using a *sigB* null mutant Sue *et al.* (2004) found that several key stress response and virulence genes under the control of σ^B were significantly down-regulated compared to a wild-type control when subjected to osmotic and acid stress. While the phenotypic effects of this inactivation were not reported, it was hypothesised that the σ^B mutant would be less able to survive environmental stress or cause human disease.

Several studies have highlighted the importance of σ^B in stress response. Moorhead and Dykes (2004) reported the importance of *sigB* during cold stress, whereby a null mutant was less able to survive and recover from

chill stress compared to a wild-type control. Giotis *et al.* (2008) found that *sigB* was important in alkaline tolerance responses in *L. monocytogenes*. In this study *sigB* null mutants were less able to mount an alkaline tolerance response than wild-type parent cells. The induction of σ^B enhanced the ability of cells to adapt to otherwise lethal high pH environments. Furthermore, *sigB* was required for osmotic cross protection induced by alkaline stress (Giotis *et al.*, 2008a).

The role of σ^B in stress response was extensively studied by Wemekamp-Kamphuis *et al.* (2004). In this study *sigB* null mutants were found to be more sensitive to acid stress, high hydrostatic pressure and freezing than wild-type controls, further highlighting the role of σ^B in stress response.

σ^B has also been found to be of importance for cellular survival during exposure to cell membrane targeting agents. Using a Δ*sigB* mutant, Begley *et al.* (2006) concluded that *L. monocytogenes* required σ^B activation for optimal response to cell membrane targeting antimicrobials including nisin, ampicillin and lacticin. While the role of σ^B during such stresses was not elucidated, it was suggested that *sigB* may function by co-ordinating necessary changes in cell membrane composition, thus permitting survival in otherwise lethal conditions.

Given its generic role in stress response, its role in acid and alkaline tolerance and its importance during exposure to cell membrane damaging components, *sigB* was deemed a suitable target for gene expression

analysis in this study. This was particularly relevant given the mode of action of the test antimicrobials used.

σ^B has been also implicated in host cell invasion and disease onset caused by *L. monocytogenes* (Nadon *et al.*, 2002; Kim *et al.*, 2004; Garner *et al.*, 2006). Given its frequent induction during sub-lethal stress exposure (Chaturongakul and Boor, 2006; Bowman *et al.*, 2010; Bergholz *et al.*, 2012), food processing practices which are 'sub-lethal' may inadvertently induce σ^B expression, giving rise to potentially more virulent organisms.

In this study, *sigB* expression was not altered during exposure to citric acid, trisodium phosphate, NaClO nor H_2O_2. Given its universal role in stress response, this was not expected. Several studies have found increased induction of σ^B during exposure to antimicrobials similar to those used in this study. Therefore, it is possible that the treatment intensity was insufficient to induce detectable changes in transcript levels during antimicrobial exposure.

4.5.3 *gadA*

The glutamate decarboxylase (GAD) system is an important response mechanism for low pH survival and growth. During exposure to low pH environments the GAD system converts a molecule of extracellular glutamate to extracellular γ-aminobutyrate while expending an intracellular proton, thus decreasing intracellular pH (Cotter *et al.*, 2001).

The GAD system has been found to play an important role in both growth and survival during mild and extreme acid exposure (Cotter *et al.*, 2005;

Karatzas *et al.*, 2012). This is of interest to the food industry where low pH is commonly used in surface decontamination, or as an intrinsic food preservative.

Furthermore, the GAD system permits survival of *L. monocytogenes* through gastric transit, after which it can invade intestinal epithelial cells which may lead to the onset of potentially fatal listeriosis (Cotter *et al.*, 2001.

The absence of citric acid induced GAD induction in this study was not expected, but may have been attributed to an insufficient exposure intensity. While the treatment was sufficient to slow bacterial growth, this may have been insufficient to induce significant changes in gene expression.

While less well documented the GAD system has also been implicated in alkaline stress response. A study by Giotis *et al.* (2010) found the GAD system to be induced during alkaline shock. In their study, an initial induction of the GAD system occurred during the first 30 minutes of high pH exposure, however, this was found to be short term. In their study, Giotis *et al.* (2010) reported a 14-fold increase in GAD expression after 15 minutes of alkaline exposure decreasing to ~7-fold after 30 minutes. In alkaline adapted cells, those which had been grown in high pH media, no GAD induction was reported. Therefore the 2-fold increase in GAD expression found in this study is feasible.

The GAD system has not been implicated in oxidative stress response, and therefore an altered expression was not anticipated during H_2O_2 challenge.

The antimicrobial activity of NaClO is largely attributed to hypochlorous acid (HOCl), therefore it was hypothesised that GAD expression may be up-regulated in response to NaClO exposure. In a study by Wang *et al.* (2009) *gadA* expression was up-regulated in response to sub-lethal NaClO exposure when challenging *E.coli*. However, the results in this study did not detect a change in *gadA* transcript levels following such exposure.

4.5.4 *Hfq*

Hfq is an RNA binding protein which has a key role in the control of gene expression. By influencing RNA binding, Hfq is able to affect the rates of both transcription and translation, thus controlling gene expression (Vogel and Luisi, 2011).

The role of Hfq is multifaceted, in *E. coli Hfq* has been implicated in both stress response and virulence (Christiansen *et al.*, 2004). In their study, Muffler *et al.* (1997), found that a *Hfq* defective *E. coli* mutant displayed increased sensitivity to several stresses including heat, starvation and H_2O_2. The importance of *Hfq* in *E. coli* stress response was further supported by the findings of Tsui *et al.* (2006) who report increased osmosensitivity and sensitivity to UV exposure in *Hfq* mutants compared to wild-type parents.

In *L. monocytogenes*, *Hfq* has also been found to play an important role in stress response and virulence. When investigating *Hfq* defects in *L. monocytogenes* Christiansen *et al.* (2004) found that disruption to *Hfq* resulted in increased sensitivity to several commonly encountered stresses; including ethanol, NaCl and starvation. Furthermore, cells with defective *Hfq* had a diminished ability to survive in a mouse model.

Given that environmental stresses are capable of inducing *Hfq* expression in *L. monocytogenes*, and that *Hfq* has roles in several stress responses (Raengpradub *et al.*, 2008; http://www.uniprot.org/uniprot/J7NGY2), it is possible that sub-lethal stress exposure could induce cross protection against subsequent stress through *Hfq* expression. Furthermore, this up-regulation could give rise to cells with increased virulence potential compared to cells which had not undergone previous stress exposure.

In this study, *Hfq* expression remained unchanged by exposure to citric acid, H_2O_2, NaClO and trisodium phosphate ($p>0.05$). Given its generic role in stress response it was envisaged that *Hfq* would be up-regulated in response to antimicrobial exposure. The absence of such transcriptional change suggests that *Hfq* is not involved in the response to *L. monocytogenes* EGD-e to the test antimicrobials. However, further work using additional strains and treatment intensities would be needed to confirm this suggestion.

4.5.5 *recN*

Both environmentally and during food processing bacterial cells may encounter conditions of stress which can cause damage to DNA. For example, both oxidative and acid stress are known to cause damage to bacterial DNA (O'Rourke *et al.*, 2003). In order to overcome these deleterious effects bacterial cells have evolved coping mechanisms to permit survival and growth in sub-optimal or stressful conditions.

Double-stranded breaks in bacterial DNA are commonly caused by stressful environments, including exposure to DNA targeting antimicrobials (Ayora *et al.*, 2011; Wang and Maier, 2008). When challenged with antimicrobial agents such as H_2O_2, NaClO, and low pH environments, bacterial cells require mechanisms to repair damaged DNA molecules. While the precise mechanism governing DNA repair by *recN* is not known, it is suggested to be due to DNA fragment alignment (Wang and Maier, 2008). By aligning broken fragments, DNA recombination can occur and DNA molecules can be reassembled/repaired (Meddows *et al.*, 2005).

While research surrounding *recN* in *L. monocytogenes* is limited, several studies have highlighted the importance of *recN* in other species. Wang and Maier (2008) used defective mutants to confirm the importance of *recN* in oxidative stress response, survival in low pH environments and gastric colonisation using *Helicobacter pylori*. Likewise, Stohl *et al.* (2005) found that *recN* defective *Neisseria gonorrhoea* cells displayed increased sensitivity to oxidative stress than its parent strain; while

Kidane *et al.* (2004) similarly found *recN* defective *Bacillus subtilis* cells had increased sensitivity to oxidative stress.

Given its widespread role in oxidative protection, it was hypothesised that *recN* would be up-regulated in response to both H_2O_2 and NaClO exposure. It was also anticipated that citric acid exposure may increase *recN* expression given the findings of Wang and Maier (2008). As displayed in Fig. 4-10, *recN* expression was not changed following exposure to citric acid, H_2O_2, NaClO nor trisodium phosphate. This suggests that either *L. monocytogenes* EGD-e does not require increased transcription/translation of *recN* in response to these antimicrobials, or the treatment intensities were sufficient to slow bacterial growth, but insufficient to cause significant DNA damage. In the absence of such damage there would be no need for increased recN expression.

4.5.6 *lmo0669*

lmo0669 encodes a putative protein with similarities to oxidoreductase enzymes (Milohanic *et al.*, 2003). Such enzymes are typically concerned with the maintenance of the redox potential within cells (Chan *et al.*, 2006). Disruption of this redox potential can ultimately result in cell death.

As this study was concerned with the response of *L. monocytogenes* to H_2O_2 and NaClO, two oxidising agents, it was hypothesised that *lmo0669* expression would be increased in response to exposure to these antimicrobials. However, as seen in Fig. 4-10, *lmo0669* expression

remained unchanged following exposure to citric acid, trisodium phosphate and H_2O_2. While citric acid and trisodium phosphate were not expected to induce *lmo0669* expression, it was anticipated that exposure to H_2O_2 would cause significant up-regulation.

As with these results, Sue *et al.* (2004) found that *lmo0669* was not induced by acid exposure, suggesting the *lmo0669* gene does not contribute to acid resistance and/or tolerance. Oxidoreductase genes were found to be significantly up-regulated in response to H_2O_2 exposure in *S. aureus* (Chang *et al.*, 2006), *N. gonorrhoea* (Stohl *et al.*, 2005) and *B. subtilis* (Mostertz *et al.*, 2004) suggesting an important role for this class of enzyme during oxidative challenge.

Unlike oxidative stress induced by H_2O_2, NaClO exposure did result in a significant up-regulation of *lmo0669*. A 4.6 fold increase in *lmo0669* expression was detected in NaClO exposed cells compared to untreated controls. This indicates that *L. monocytogenes* EGD-e utilises *lmo0669* to overcome NaClO stress. Given its intracellular nature, *L. monocytogenes* must overcome conditions of oxidative stress during pathogenesis (Feld *et al.*, 2012), therefore the application of antimicrobials which induce expression of oxidoreductase enzymes may inadvertently impact virulence. Further work investigating the effects of sub-lethal NaClO exposure on intracellular survival would be of human health interest.

4.5.7 *lmo0501*

The *lmo0501* gene locus encodes a protein similar to the mannitol transcription regulator found in *B. subtilis* (Michel *et al.*, 2011). Given the importance of transcriptional regulators in stress response (Chaturongakul *et al.*, 2008; Gandhi and Chikindas, 2007) it was anticipated that *lmo0501* may have been involved in *L. monocytogenes* survival during antimicrobial challenge. However, there are limited reports of the role *lmo0501* in *L. monocytogenes* stress response.

As seen in Fig. 4-10 there was no change in *lmo0501* transcript levels during exposure to citric acid, trisodium phosphate, NaClO nor H_2O_2. This is contradictory to the findings of Michel *et al.* (2011) who report that an approximately 5-log increase in *lmo0501* expression occurred during lactic acid exposure. Furthermore, in their study disruption to the *lmo0501* locus significantly impaired the growth of *L. monocytogenes* compared to its wild-type parent strain in the presence of organic acids. It should be noted that in their study, Michel *et al.* (2011) did not test citric acid, but tested acetic and lactic acids. Further work using additional organic acids as well as inorganic acids would provide information to ascertain if *lmo0501* is required for all acid responses.

4.5.8 *lmo0956*

lmo0956 encodes *N*-acetylglucosamine-6-phosphate deacetylase (*NagA*), an enzyme which is required for cell wall biosynthesis in *L. monocytogenes* (Popowska *et al.*, 2012). The enzyme acts by catalysing the first step in cell wall synthesis, the conversion of *N*-acetyl-

glucosamine-6-phosphate to glucosamine-6-phosphate and acetate (Park and Uehara, 2008). With glucosamine-6-phosphate being a vital precursor in cell wall synthesis (Komatsuzawa *et al.*, 2004) it was hypothesised that *lmo0956* would be up-regulated in response to cell wall targeting agents.

In this study, no differences in *lmo0956* expression were detected following exposure to citric acid, trisodium phosphate, NaClO nor H_2O_2. In a study by Popowska *et al.* (2012) *lmo0956* defective mutants were found to have increased sensitivity to cell wall hydrolase enzymes as well as cell targeting antibiotics. Given these findings it was expected that cell wall targeting antimicrobials such as NaClO and H_2O_2 would have induced expression of *lmo0956*.

As with the *recN*, it is possible that while the antimicrobial exposure was sufficient to slow the growth of *L. monocytogenes* cells it was insufficient to cause significant enough damage to induce *lmo0956* expression. Further work with additional treatment intensities may confirm the role of *lmo0956* during antimicrobial exposure.

4.6 Conclusion

By subjecting *L. monocytogenes* EGD-e cells to citric acid, NaClO, H_2O_2 and trisodium phosphate it is possible to significantly alter the maximal growth rate (μ_{max}). This may be of interest to the food industry when considering preservation factors during food processing.

However, it is known that several pathogens adapt to unfavourable environments giving rise to cells with elevated resistance and/or tolerance to otherwise lethal levels of the same (homologous) or different (heterologous) stresses.

It was the aim of the study described in this chapter to identify stress response genes which had altered transcript levels following sub-lethal exposure to commonly encountered antimicrobial agents, thus providing evidence for potential cross-protections induced by sub-lethal stressor exposure. However, only *gadA* and *lmo0669* expression were significantly up-regulated in response to trisodium phosphate and NaClO respectively.

Further work might include selection of additional target genes, use of additional *L. monocytogenes* strains or adjustments in treatment intensities.

Chapter 5

Effects of site direct gene inactivation on stress resistance

5.1 Introduction

Since its design by Chakraborty *et al.* (1992) the temperature sensitive vector pAUL-A has been a useful tool for the study of gene inactivation/disruption in *L. monocytogenes*. Several studies have utilised this vector to elucidate the role of target genes in stress response and virulence in *Listeria*. For example, using the pAUL-A vector Bigot *et al.* (2009) were able to identify the putative gene *lmo1273* as an important virulence determinant in *L. monocytogenes*. Similarly, Machata *et al.* (2008) used the pAUL-A vector to identify prolipoprotein diacylglyceryl transferase (*lgt*) as a key virulence determinant of *L. monocytogenes* while Karatzas *et al.* (2003) used the pAUL-A vector to disrupt the *ctsR* locus and elucidate its role in virulence.

As well as virulence studies, the pAUL-A gene disruption system has been used to investigate the roles of specific genes on stress responses Using the pAUL-A vector, Van der Veen *et al.* (2010) were able to identify *recA* as an important stress response gene for *L. monocytogenes*, needed for survival during heat, acid and H_2O_2 challenge. Wemekamp-Kamphuis *et al.* (2004) disrupted the *L. monocytogenes sigB* gene using pAUL-A, in doing so they were able to identify its role in stress resistance against several commonly encountered food processing stresses. Gomes *et al.* (2011) used the pAUL-A vector to inactivate several universal stress protein (USP) encoding genes (*lmo0515*, *lmo1580* and *lmo2673*), these inactivations led to increased susceptibility to oxidative and acid stresses

as well as compromising the ability of *L. monocytogenes* to grow both *in vivo* and *in vitro*.

Studies using inactivation/disruption vectors allow elucidation of the role of a given target gene to a particular stressor. As such, this study was concerned with the roles of *ctsR*, *Hfq*, *LisR*, *LisK*, *GroEL* and *recA* in *L. monocytogenes* resistance to NaClO, H_2O_2, citric acid and trisodium phosphate. These targets were identified as key stress response genes by *in silico* analysis.

5.2 Aim and Objectives

5.2.1 Aim

The aim of this study was to assess the roles of selected target genes in stress resistance in *L. monocytogenes* EGD-e.

5.2.2 Objectives

- To identify potential targets for inactivation using *in silico* analysis.
- To modify a suitable vector for the purpose of site-directed mutagenesis
- To develop potential mutants which had undergone transformation and vector incorporation
- To screen potential mutants for altered phenotypic properties (antimicrobial resistance)
- To identify the disruptions responsible for altered phenotypic properties

5.3 Methods

5.3.1 Strains, plasmids and culturing

For this study *L. monocytogenes* EGD-e was used as the wild type (WT) strain. This is a 1/2a serotype strain which has had its full genome sequenced (Glaser *et al.*, 2001). Routine culturing of this organism was performed as in section 2.2.1. When selecting for transformed *L. monocytogenes* cells BHI media was supplemented with erythromycin (Sigma-Aldrich Company Ltd, UK) at 5µg/ml (BHI w/ERY). Due to the temperature sensitive nature of the pAUL-A vector, culturing was performed at 28°C following transformation of cells.

For vector manipulation *E. coli* strain DH5α was used. Routine culturing of this organism was performed on Lurani Burani (LB) broth and agar. Where necessary LB media was supplemented with erythromycin (LB w/ERY) at 300µg/ml and cells cultured at 28°C

For site directed mutagenesis the temperature sensitive vector, pAUL-A, was used (see appendix 9 for plasmid map). The vector was maintained in *E. coli* DH5α on LB w/ERY, cultured at 28°C.

pAUL-A plasmid was kindly provided by Professor Birgitte Kallipolitis, University of Southern Denmark. pAUL-A was sent as an aliquot of purified DNA which was heat shocked into $CaCl_2$ competent *E. coli* DH5α cells which were subsequently stored as glycerol stocks at -80°C.

5.3.2 Preparation and transformation of $CaCl_2$ competent *E. coli* DH5α cells

A single colony of *E. coli* DH5α was aseptically transferred from a stock plate into 50ml of LB broth and statically incubated overnight at 37°C. Following incubation cells were diluted 1:100 (OD_{595} ~0.1) into 300ml pre-warmed LB broth. Cultures were incubated at 37°C with shaking for approximately 3 hours.

Cells were dispensed into pre-chilled centrifuge tubes, incubated on ice for at least 10 minutes. Following ice incubation cells were collected by centrifugation (3000x *g*, 10 minutes, 4°C), supernatant discarded, and pellets gently resuspended in an equal volume of ice cold 0.1M $CaCl_2$. Cells were again incubated on ice (20 minutes) and pelleted before finally resuspending in 5ml of ice cold 0.1M $CaCl_2$ supplemented with 15% (v/v) glycerol to allow for long term storage. Aliquots of 300µl of competent cells were dispensed for immediate use or -80°C storage.

5.3.3 Heat shock transformation of competent *E.coli* DH5α

For heat shock transformation of $CaCl_2$ cells, cells were kept on ice (or thawed on ice if stored at -80°C). One hundred microliter aliquots were transferred to pre-chilled microtubes and 1µl (plasmid DNA), or an entire ligation mix, were added to cells. This cell/DNA mix was incubated on ice for 30 minutes after which cells were heat shocked in a thermomixer (Eppendorf, Stevenage, UK) at 42°C for 2 minutes. Following heat shock cells were placed back on ice and 900µl of sterile LB added. Cells were incubated for 30 minutes at 37°C prior to plating on LB w/ERY. Transformants were selected after 24 hours incubation at 28°C.

No DNA control cells were included which had undergone heat shock with distilled water in place of vector DNA. All heat shocked cells were plated onto LB w/ERY and LB to ensure cell viability and antibiotic activity.

5.3.4 Extraction of vector DNA from *E.coli* DH5α

Both non-modified and recombinant pAUL-A plasmids were extracted from *E.coli* DH5α using a commercially available mini-prep kit (Qiagen, Manchester, UK). Owing to the large plasmid size (>9kb), the protocol for low copy number/large plasmids was followed. Extracted plasmid DNA was stored at -20°C until required.

5.3.5 PCR amplification of insert DNA

Nucleotide sequences for each gene of interest were obtained from NCBI databases. PCR primers were designed to amplify a central region of each gene (Table 5-1). PCR was performed as per section 2.2.3.3. PCR products were purified using a commercially available kit (MinElute PCR clean-up kit; Qiagen, Manchester, UK) as per manufacturer's recommendations.

Table 5-1: PCR Primer sequences designed to amplify central regions of target genes selected for disruption.

Target	Fw[a,b] (T_m)	Rv[a,b] (T_m)	amplicon (bp)
ctsR	**G**GAATTC**C**ACGAGGGTATATTGT TGAAAG (59°C)	**GG**GGTACC**CC**AAATATCTCTATC CGGTAAAGG (64°C)	257
hfq	**G**GAATTC**C**TGAAACAAGGTGGA CAAG (58°C)	**GG**GGTACC**CC**ATTGCGTGTTTAA ATACAAGC (63°C)	181
GroEL	**G**GAATTC**C**CCGTAGCAACAGCTA TCG (61°C)	**GG**GGTACC**CC**TTCAAGTCCAGC GTTATGTG (66°C)	1010
recA	**G**GAATTC**C**GTCAAGCGGCATTA GACC (61°C)	**GG**GGTACC**CC**TCAGCAGCCATAT CAACAAG (66°C)	832
lisK	**G**GAATTC**C**CAAATGCCGATTGTA GCAG (60°C)	**GG**GGTACC**CC**ATTGTTTAGCAAT CGCAAGTC (64°C)	916
lisR	**G**GAATTC**C**GCGGTTGCTAATGAT GGAC (61°C)	**GG**GGTACC**CC**ACGATACTGTAG CGTTGTTTG (66°C)	318

a- Restriction sites are indicated by underlined text
b- Bold typeface indicates restriction site overhangs as recommended by neb.

5.3.6 Preparation of vector and insert DNA for ligation

Following purification, vector and insert DNA underwent double digestion with EcoRI and KpnI restriction enzymes. (New England Biolabs, Herts, UK) performed in CutSmart™ buffer as per manufacturer's recommendation.

Following digestion, insert DNA was again purified using a MinElute kit (Qiagen, Manchester, UK) as per manufacturer's protocol. Linearized vector was assessed for complete digestion by gel electrophoresis; after which DNA was extracted from agarose using a QIAquick gel extraction kit (Qiagen, Manchester, UK) as per manufacturer's recommendations. Purified, digested DNA was stored at -20°C until required.

5.3.7 Ligation

Insert and vector DNA was thawed on ice and was ligated at a 3:1 (insert:vector) ratio using a commercially available 'quick' ligation kit (New England Biolabs, Herts, UK). Ligations were performed as per manufacturer's recommendations.

The ligation mix was immediately used to transform $CaCl_2$ competent *E. coli* DH5α cells as described previously.

5.3.8 Selection of cells containing recombinant pAUL-A vectors

Using blue-white selection, *E.coli* cells containing recombinant pAUL-A vectors were selected. LB agar supplemented with erythromycin at 300μg/ml were spread with 50μl of 50mg/ml X-gal (Sigma-Aldrich Company Ltd, UK) and 100μl of 0.1M IPTG (Sigma-Aldrich Company Ltd, UK) and allowed to air dry. Following outgrowth of transformed *E.coli* cells, aliquots were plated onto supplemented agar and were incubated at 28°C for 24-48 hours. After incubation, single colonies containing recombinant plasmid (white colonies) were purified on LB w/ERY.

Recombinant plasmids were extracted from *E.coli* as previously described. Such DNA was stored at -20°C until needed for transformation of *L. monocytogenes* EGD-e.

5.3.9 Preparation of electrocompetent *L. monocytogenes* EGD-e

To allow transformation of *L monocytogenes* EGD-e, electrocompetent cells were prepared as described by Park and Stewart (1990). Overnight cultures were prepared by addition of a single colony into 100ml of BHI broth, which were statically incubated at 37°C overnight. Following

overnight incubation cells were diluted 1:100 (v/v) into 500ml of BHI supplemented with 0.5M sucrose. Cells were incubated at 37°C with shaking until grown to an OD_{595} 0.2. Upon reaching the desired OD_{595} penicillin G was added at 10µg/ml and cells were further incubated for 2 hours under shaking conditions at 37°C. After incubation cells were aliquoted into pre-chilled centrifuge tubes and collected by centrifugation (8,000x*g*, 10 minutes, 4°C), supernatants were discarded and cells were gently re-suspended in a 0.5x volume of ice cold 1mM HEPES solution (pH 7) supplemented with 0.5M sucrose. This wash step was repeated on 2 further occasions after which cells were once again collected by centrifugation and re-suspended in 0.0025x volume of ice cold 1mM HEPES solution (pH 7) supplemented with 0.5M sucrose. These cells were kept on ice for no more than 30 minutes prior to electroporation.

5.3.10 Electrotransformation of *L. monocytogenes* EGD-e

For transformation of electrocompetent cells 1µg of plasmid DNA was added to a pre-chilled electroporation cuvette with a 0.2cm path width (Cell Projects Limited, Kent, UK). One hundred microliters of electrocompetent cells were added to the electroporation cuvettes and were gently mixed with plasmid DNA by pipetting. Suspensions were kept on ice until electroporation using a micropulser electroporator (Bio-Rad, Hertfordshire, UK). Cells were electroporated at 2.5kV using the Ec2 device setting, giving time constants of 5-6ms. After pulsing, 900µl of BHI supplemented with 0.5M sucrose was added to electroporated cells

and outgrowth was performed at 37°C for 1 hour under shaking conditions.

Following outgrowth cells were plated on BHI agar and BHI agar supplemented with erythromycin at 5µg/ml. Transformants were selected after 48 hours growth at 28°C.

No DNA control cells electroporated with water instead of vector DNA. All electroporated cells were plated on BHI w/ERY and BHI to ensure viability and antibiotic activity.

5.3.11 Integration of pAUL-A vectors

The temperature sensitive nature of pAUL-A was used to integrate the vectors into the chromosomal DNA of *L. monocytogenes* EGD-e. Serial passages at the non-permissive temperature of 42°C on BHI w/ERY initiated Campbell-like homologous recombination and integration of the vector into the host. After three passages at the non-permissive temperature (42°C) integrants were selected for disintegration and target gene disruption.

Controls during integration included the plating of wild-type cells onto both BHI and BHI w/ERY at each passage and plating of potential integrants onto BHI (viability control) as well as BHI w/ERY at each passage.

5.3.12 Disintegration of pAUL-A

Upon isolating suitable integrants, single colonies were used to inoculate 10ml BHI broths. Cultures were incubated overnight at 28°C in a shaking incubator. Following overnight incubation, 5µl of growing culture was transferred into 10ml of BHI broth and was incubated as before. These passages were performed a total of 10 times in an attempt to disintegrate vectors from host genomes. After the tenth passage cells were plated onto BHI agar to obtain single colonies. Following overnight incubation at 28°C single colonies were spot plated onto BHI and BHI w/ERY. Spot plates were incubated at 28°C for 24-48 hours and cells which grew on BHI but not BHI w/ERY were deemed 'disintegrated'. Such disintegrants were stored at -80°C on microbank beads, were screened by colony PCR using cloning primers and were screened for altered phenotypic properties compared to wild-type controls.

As controls, wild-type cells were plated onto both BHI and BHI w/ERY when spot plating; sterility controls were included throughout passaging, and purity plates were performed alongside each passage.

5.3.13 Colony PCR

Disintegrants were subjected to colony PCR using cloning primers (Table 5-1) to determine if loss of bases had occurred. For colony PCR, single colonies were suspended in 100µl of sterile molecular biology grade water. Cell suspensions were used as template material in subsequent PCR. PCR cycle conditions were used as previously described. PCR

products were separated by gel electrophoresis and viewed by translumination.

PCR products generated from disintegrants were compared to those generated from wild-type controls.

5.3.14 Phenotypic analysis of potential mutants- MIC analysis

Potential mutants were screened for resistance to citric acid, trisodium phosphate, H_2O_2 and NaClO using a standard microtitre plate method to determine the MICs (Wiegand *et al.*, 2008).

MIC values for wild-type cells were also determined. Positive growth controls (BHI without antimicrobial) and sterility controls (BHI without inoculum) were also included in each microplate.

5.3.15 Phenotypic analysis of potential mutants- Disc diffusion assay

Potential mutants were also screened for H_2O_2 and NaClO resistance by disc diffusion assay. Overnight cultures were diluted to ~$1x10^6$ cfu/ml and 100µl spread onto the surface of a pre-dried BHI agar plate. Plates were allowed to air dry for 5 minutes after which 1cm sterile filter paper discs were aseptically placed onto the centre of each plate. Fifty microliters of H_2O_2 solution (30% w/v) (Sigma-Aldrich Company Ltd, UK) and NaClO solution (13% active chlorine) (Fisher Scientific, Loughborough, UK) was pipetted onto filter paper discs before incubation at 37°C for 24 hours. After incubation zones of inhibition were determined by measuring the diameter of 'clear zones' using Vernier callipers.

Wild type cultures were screened alongside potential mutants to act as controls. Further controls included filter paper discs treated with sterile H_2O.

5.4 Results

5.4.1 Extraction of pAUL-A vector from *E. coli* DH5α and linearization by restriction digest

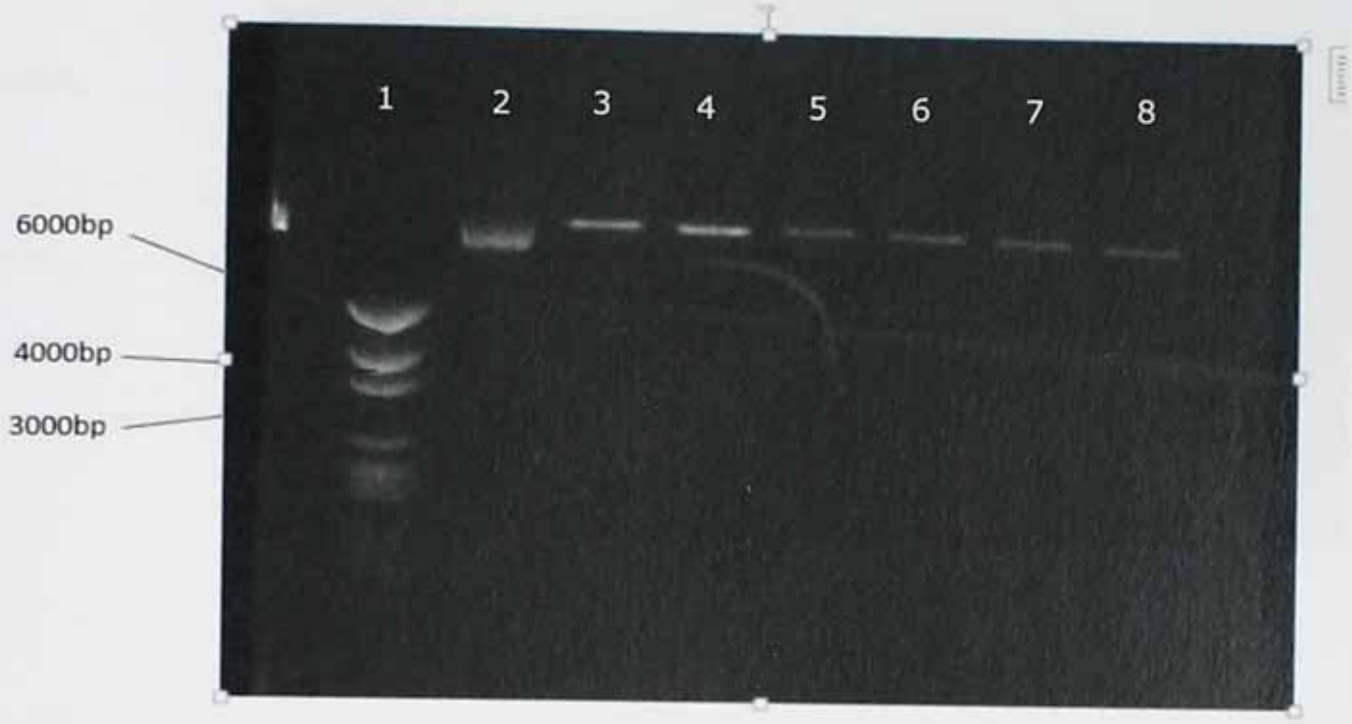

Fig. 5-1: 1% Agarose gel displaying undigested pAUL-A (Lane 2), KpnI digested pAUL-A (Lane 3), EcoRI digested pAUL-A (Lane 4) and KpnI:EcoRI double digested pAUL-A (Lanes 5-8). Lane 1 contained a 2Kb marker.

pAUL-A vector was extracted from *E.coli* DH5α as per section 2.2.3.4. Following extraction plasmid DNA underwent single and double digestion using KpnI and/or EcoRI restriction enzymes. Following digestion DNA was run through a 1% (w/v) agarose gel supplemented with SYBR safe (Invitrogen, Paisley, UK) and was visualised using a transluminator (Syngene, Cambridge, UK). Double digested vector (lanes 5-8) was excised from the gel and was purified for subsequent ligation reactions.

5.4.2 Preparation and purification of insert DNA

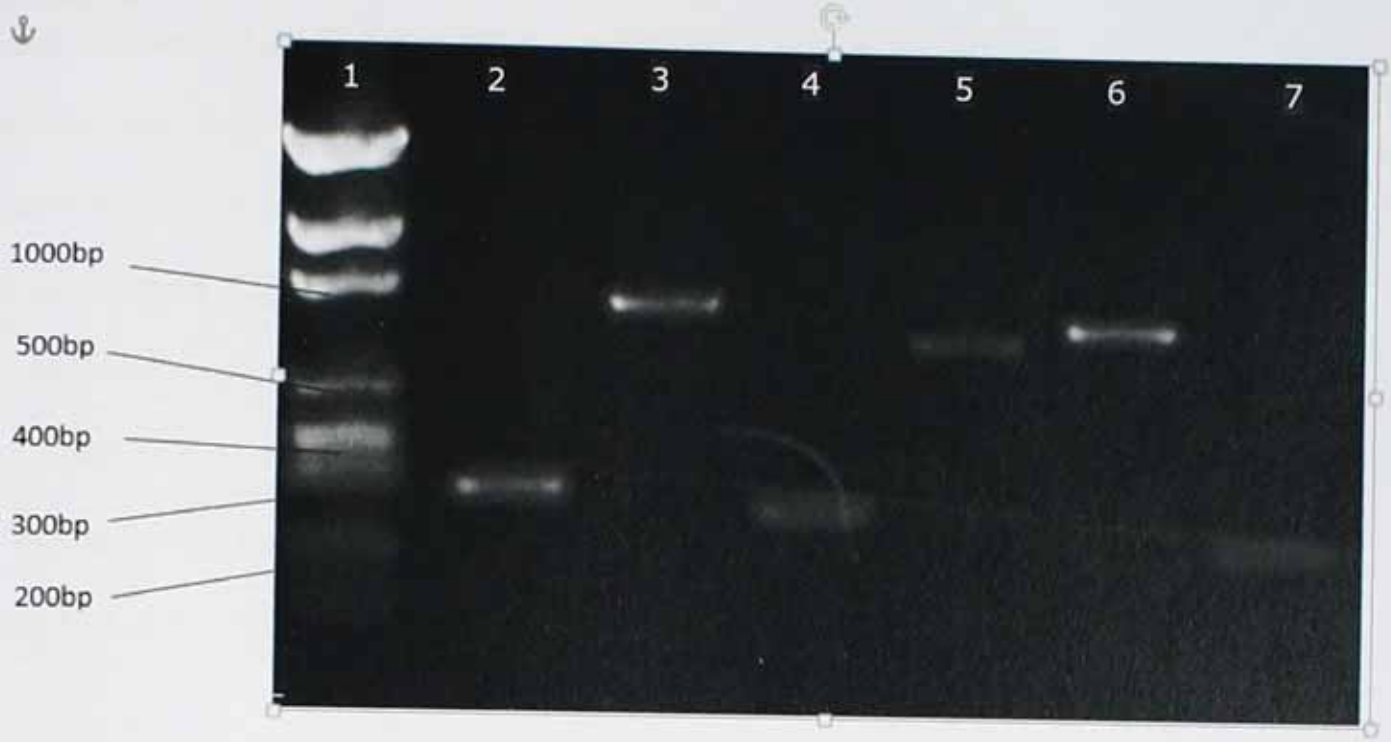

Fig. 5-2: 1% Agarose gel displaying KpnI:EcoRI double digestion of LisR (Lane 2), GroEL (Lane 3), ctsR (Lane 4), LisK (Lane 5), recA (Lane 6) and Hfq (Lane 7) gene fragments. Lane 1 contained a 2Kb marker.

PCR was performed using *L. monocytogenes* EGD-e genomic DNA as the template material. PCR products were first purified as per section 5.3.5. Following purification, PCR products underwent double digestion using KpnI and EcoRI restriction enzymes; after which digested DNA was again purified. An aliquot of digested/purified insert DNA was run through a 1% (w/v) agarose gel supplemented with SYBR safe and visualised using a transluminator. The remainder of the purified insert DNA was used for subsequent ligation reactions with linearised vector DNA.

5.4.3 Selection of *E. coli* transformants possessing recombinant pAUL-A

Digested insert and vector DNA were ligated at a 3:1 ratio using a quick ligation kit (New England Biolabs, Hertfordshire, UK) as per manufacturer's recommendations. Ligation mixtures were then heat shocked into $CaCl_2$ competent *E.coli* DH5α as per methods. Transformants containing recombinant plasmids were first selected by blue-white selection and were subsequently purified on LB w/ERY. Single transformants containing recombinant pAUL-A were cultured overnight and the modified vectors were extracted as previously described.

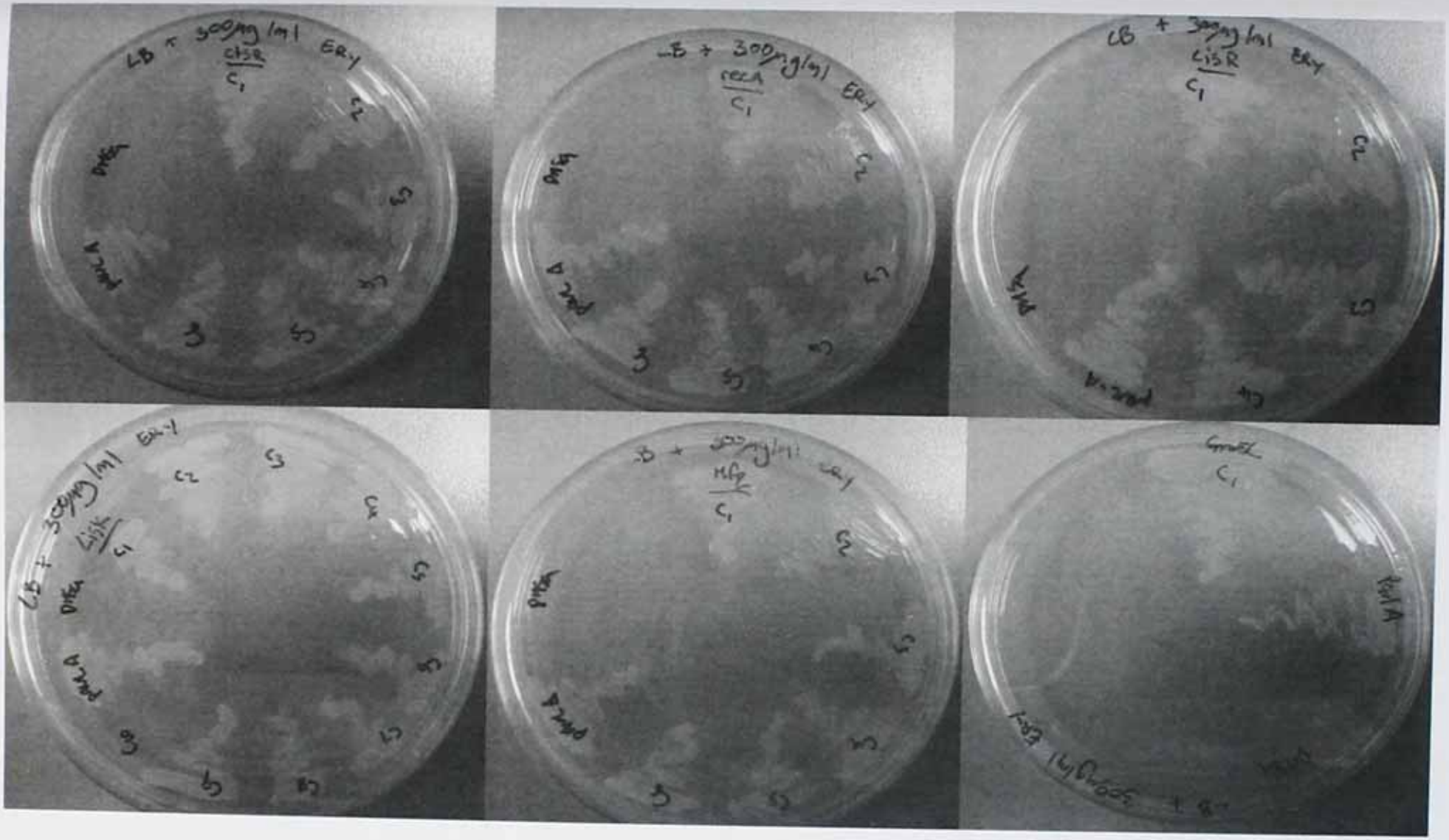

Fig. 5-3: *E. coli* DH5α possessing recombinant pAUL-A vectors containing fragments of *L. monocytogenes* DNA encoding: *ctsR* (top left), *recA* (top middle), *LisR* (top right), *LisK* (bottom left), *Hfq* (bottom middle) and *GroEL* (bottom right). *E.coli* cells containing un-modified pAUL-A (pAUL A) and wild-type *E.coli* DH5α (DH5α) were included as controls.

5.4.4 Electro-transformation of *L. monocytogenes* EGD-e and selection of integrants

Following preparation of electrocompetent *L. monocytogenes* cells, modified pAUL-A was electroporated into *L. monocytogenes* as per section 5.3.10.

Table 5-2: Time constants (msec) obtained during the electroporation of *L. monocytogenes* EGD-e with modified pAUL-A vector.

Vector	Time constant (msec)
DNA negative control (cells only)	5.7
pAUL-A	5.8
pAUL-A:*Hfq*	5.7
pAUL-A:*lisR*	5.8
pAUL-A:*lisK*	5.8
pAUL-A:*GroEL*	5.7
pAUL-A:*ctsR*	5.8
pAUL-A:*recA*	5.9

Time constants between 5 and 6 msec were indicative of successful electroporation (Park and Stewart, 1990).

Following electroporation, *L. monocytogenes* cells were outgrown in BHI broth supplemented with 0.5M sucrose for one hour before plating on BHI w/ERY. Plates were incubated at 28°C for 24-48 hours after which single colonies were streaked onto BHI w/ERY for purification and storage.

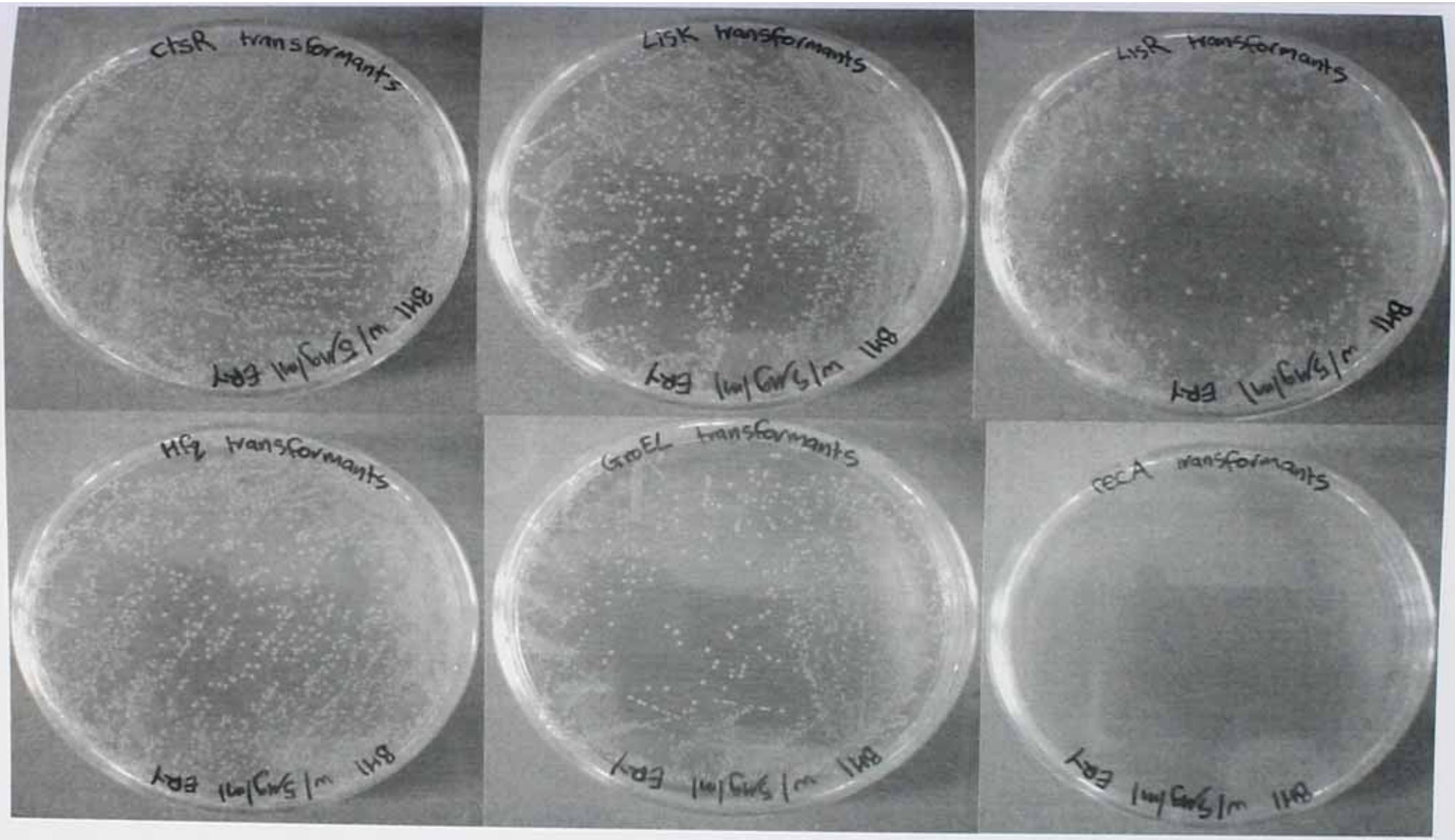

Fig. 5-4: *L. monocytogenes* EGD-e transformants containing recombinant vectors- pAUL-A:*ctsR* (top left), pAUL-A:*LisK* (top middle), pAUL-A:*LisR* (top right), pAUL-A:*Hfq* (bottom left), pAUL-A:*GroEL* (bottom middle) and pAUL-A:*recA* (bottom right). BHI agar plates contained 5µg/ml ERY to select for transformants.

Electrocompetent *L. monocytogenes* EGD-e cells were transformed at an efficiency of approximately $1x10^4$ transformants/µg vector DNA. Transformants were selected on BHI w/ERY following 24-48 hours incubation at 28°C.

On three separate occasions, transformants containing pAUL-A:*recA* were not recovered and so work concerning the disruption of *recA* was discontinued.

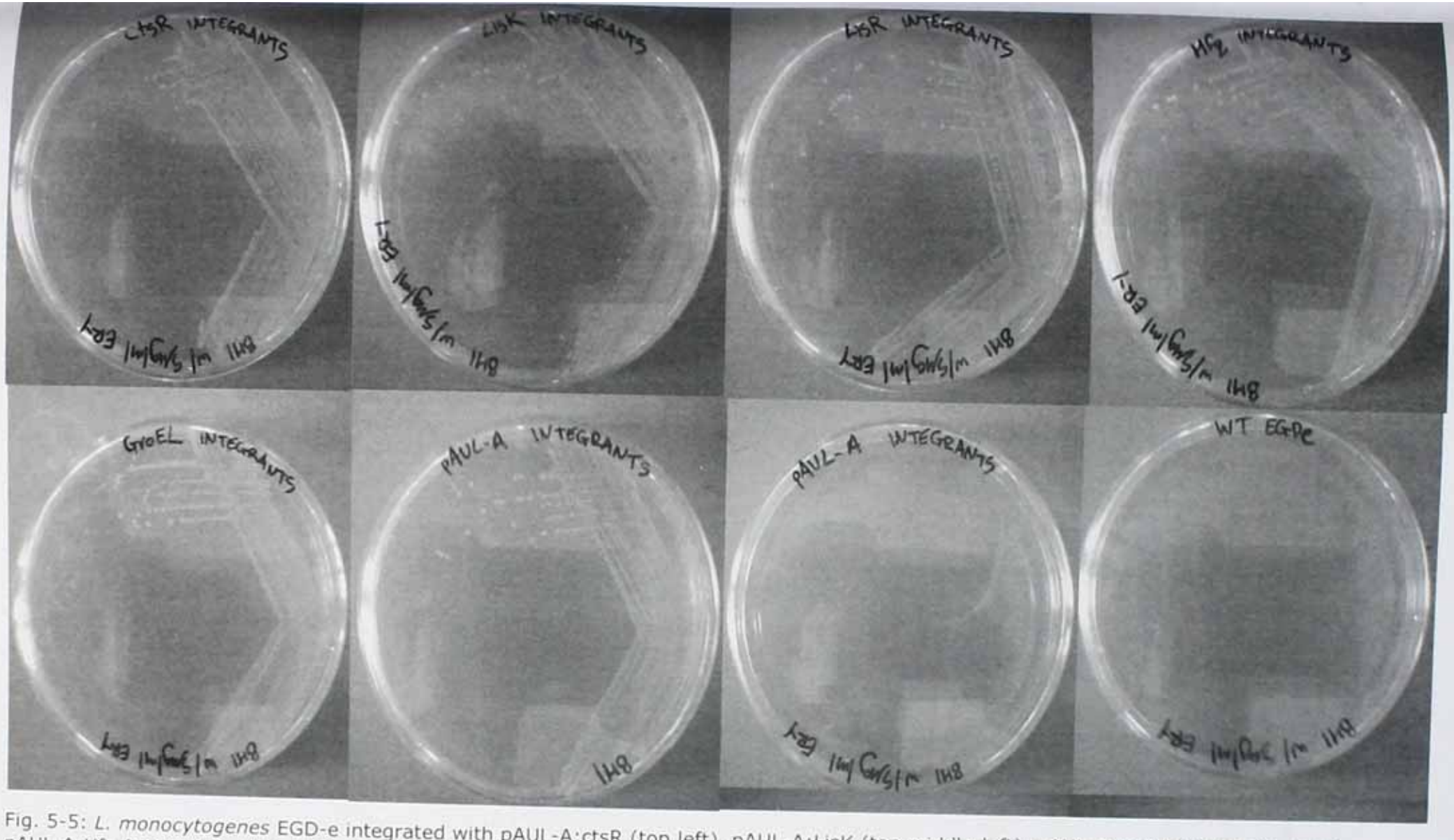

Fig. 5-5: *L. monocytogenes* EGD-e integrated with pAUL-A:ctsR (top left), pAUL-A:LisK (top middle-left), pAUL-A:LisR (top middle-right), pAUL-A:Hfq (top right), pAUL-A:GroEL (bottom left). Controls included; pAUL-A containing cells plated on BHI (bottom middle-left), pAUL-A containing cells plated on BHI w/ERY and wild-type EGD-e cells (bottom right). Cells were plated on 5µg/ml ERY plates and incubated at the non-permissive temperature of 42°C to select only for cells which had integrated the pAUL-A vector chromosomally.

Single transformants were passaged twice onto BHI w/ERY. These plates were incubated at the non-permissive temperature of 42°C for 24 hours. At 42°C pAUL-A is unable to replicate, therefore only cells which incorporate the plasmid by Campbell-like recombination possess the erythromycin resistance cassette (Schäferkordt and Chakraborty, 1995). Resistant cells were subsequently passaged 10 times in BHI as per section 5.3.12 to disintegrate the plasmid and cause target gene disruption.

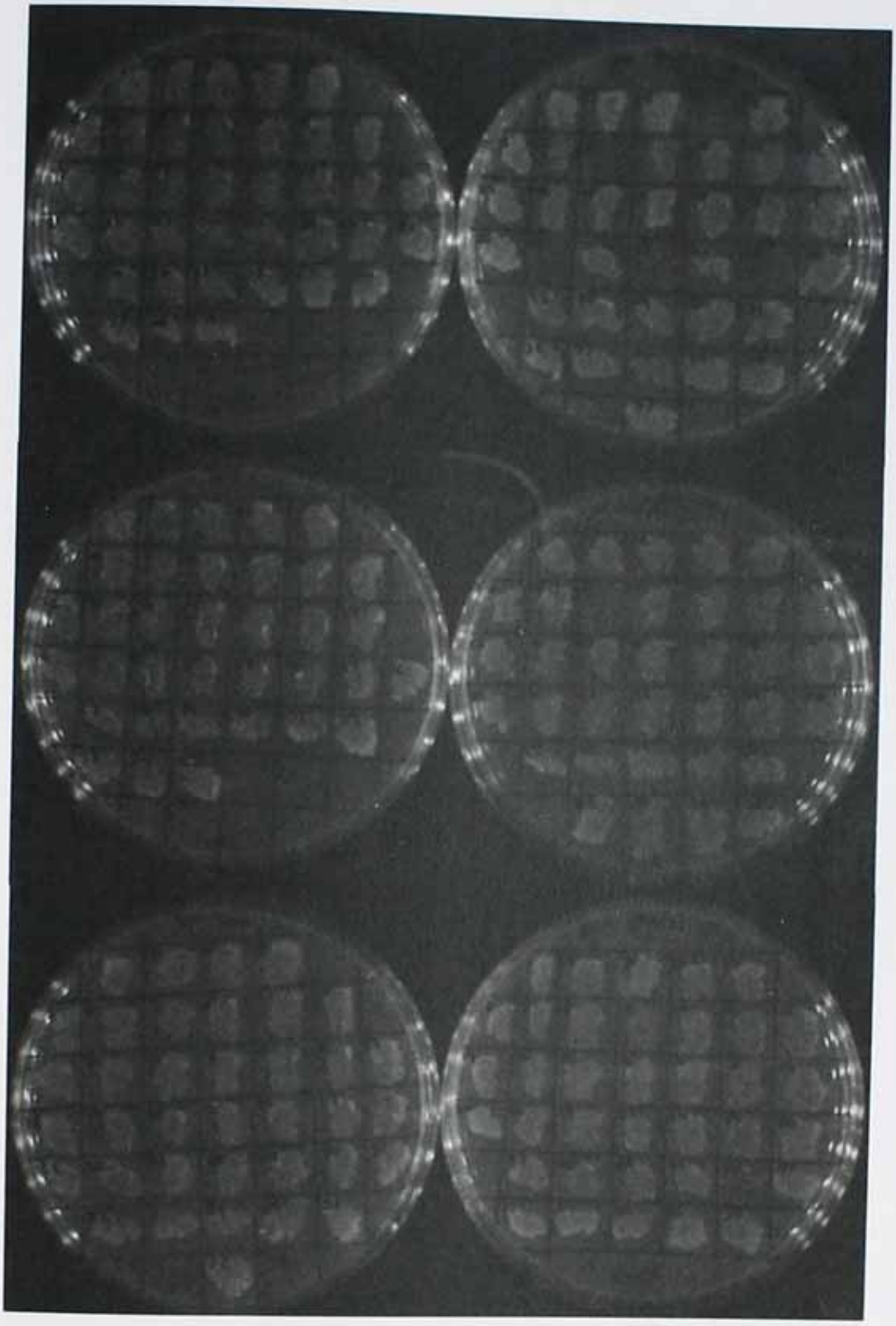

Fig. 5-6: Disintegrants plated on BHI (right) and BHI w/ERY (left). pAUL-A:ctsR (top), pAUL-A:LisK (middle) and pAUL-A:LisR disintegrants were determined as cells which grew on BHI but were unable to grow on BHI w/ERY. Wild-type controls were included (see Fig. 5-7).

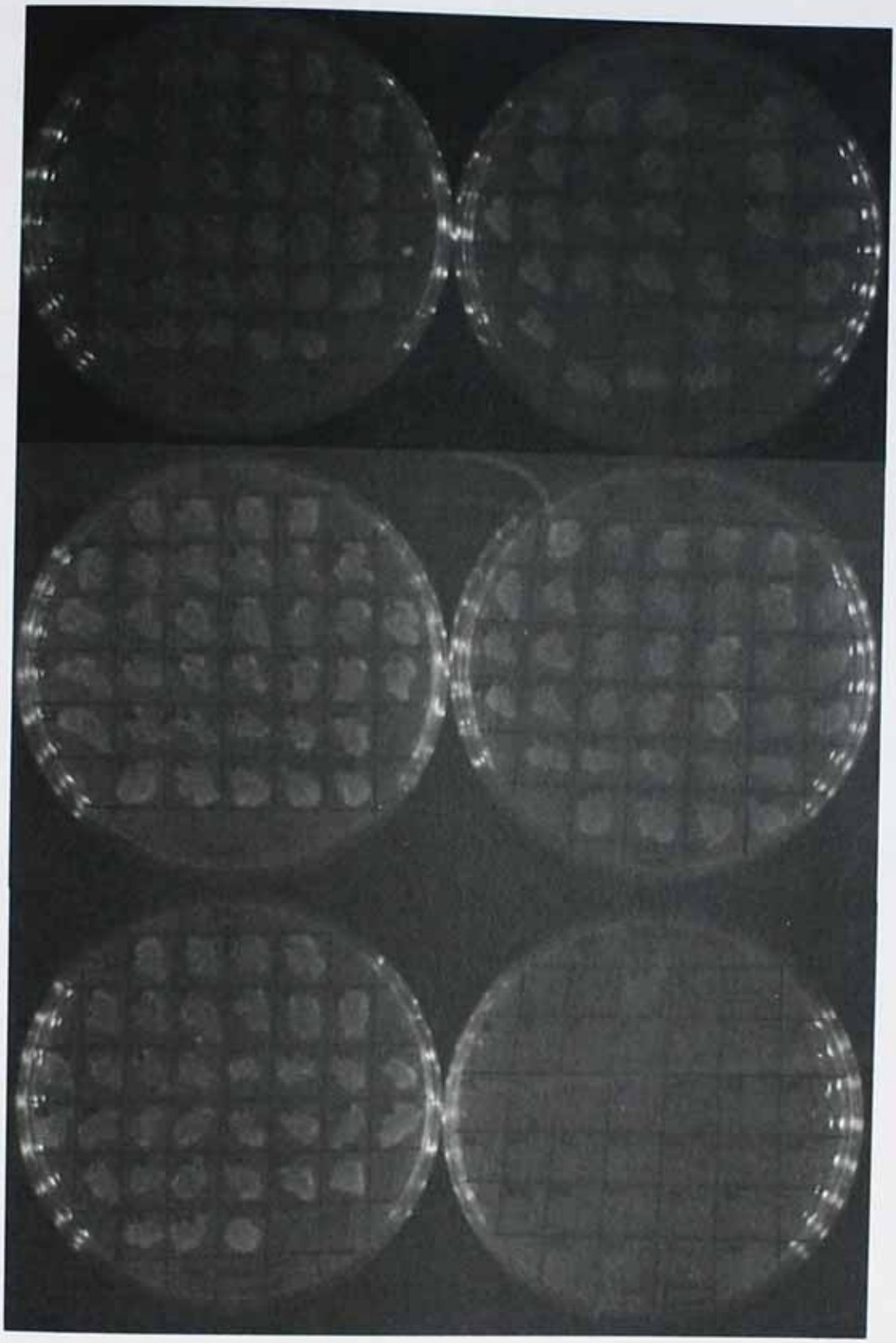

Fig. 5-7: Disintegrants plated on BHI (right) and BHI w/ERY (left). pAUL-A:Hfq (top) and pAUL-A:GroEL (middle) disintegrants were determined as cells which grew on BHI but were unable to grow on BHI w/ERY. Wild-type controls were included (bottom).

Disruptants in *ctsR*, *LisK*, *LisR* and *Hfq* were generated. Up to five further passages were included for *GroEL*; however, no disintegrants were generated. This suggests *GroEL* is vital for *L. monocytogenes* growth and survival, therefore disruption of this gene is not conducive for survival.

Disruptants were screened for altered phenotypic properties compared to wild-type controls and underwent colony PCR for determination of nucleotide deletion/gene disruption.

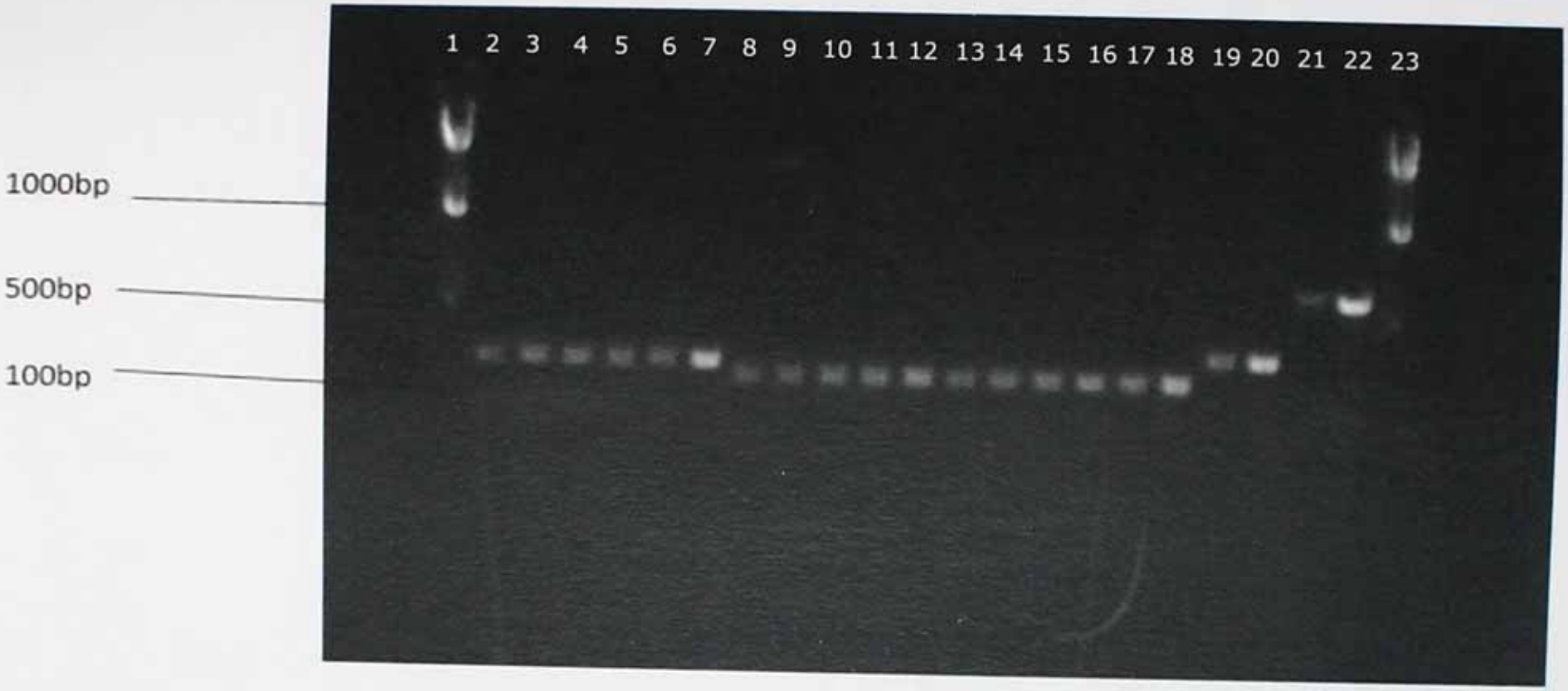

Figure 5-8: Colony PCR of amplifying fragments of ctsR (lanes 2-7), Hfq (lanes 8-18), LisR (lanes 19-20) and LisK (lanes 21-22). Lanes 1 and 23 were loaded with DNA marker. The first well for any given target contained DNA extracted from wild-type cells (lanes 2, 8, 19, 21). DNA was run on a 3% agarose gel stained with SYBR safe.

From Fig. 5-7 it was not apparent if *ctsR* or *Hfq* disintegrants had lost genetic material during disintegration of the pAUL-A vector. This is possibly due to the small amplicon size (<300bp), as such it is likely that only a small number of bases were lost alongside plasmid DNA during the disintegration process. A 3% agarose gel was used in an attempt to discriminate small changes in product size.

LisR and *LisK* amplification in disintegrants appeared to give rise to smaller PCR products relative to wild-type cells. This is indicative of loss of genetic material but was not considered conclusive. Sequence analysis would allow complete characterisation of the effects of integration/disintegration on gene sequence.

Table 5-3: Effects of gene disruption on the MIC (w/v) of citric acid, trisodium phosphate, H_2O_2 and NaClO against *L. monocytogenes* EGD-e.

Isolate	Citric acid	Trisodium phosphate	H_2O_2	NaClO
Wild-type	0.5	2	0.06	0.3
ctsR disruptant '1'	**0.25**	2	0.06	0.3
ctsR disruptant '2'	**0.25**	2	0.06	0.3
ctsR disruptant '3'	**0.25**	2	0.06	0.3
ctsR disruptant '5'	**0.25**	2	0.06	0.3
Hfq disruptant '1'	**0.25**	2	0.06	0.3
Hfq disruptant '2'	**0.25**	2	0.06	0.3
Hfq disruptant '3'	**0.25**	2	0.06	0.3
Hfq disruptant '4'	**0.25**	2	0.06	0.3
Hfq disruptant '5'	**0.25**	2	0.06	0.3
Hfq disruptant '6'	**0.25**	2	0.06	0.3
Hfq disruptant '7'	**0.25**	2	0.06	0.3
Hfq disruptant '8'	**0.25**	2	0.06	0.3
Hfq disruptant '9'	**0.25**	2	0.06	0.3
Hfq disruptant '10'	**0.25**	2	0.06	0.3
LisR disruptant	0.5	2	0.06	0.3
LisK disruptant	0.5	2	0.06	0.3

All experiments were carried out in triplicate, on three separate occasions (n=3).
Bold typeface denotes differences compared to wild-type controls.

Disruption of the *ctsR*, *Hfq*, *LisR* and *LisK* had no effect on the MIC of trisodium phosphate, H_2O_2 and NaClO. The MICs of these agents were 2% (w/v), 0.06% (w/v) and 0.3% (w/v) respectively. These MICs were comparable to the MIC obtained from wild-type controls.

When subjected to citric acid, disruption of the *ctsR* and *Hfq* genes resulted in an increased sensitivity relative to the wild-type control. In wild-type cells, *LisR* disruptants and *LisK* disruptants the MIC of citric acid was 0.5% (w/v); however, all *ctsR* and *Hfq* disruptants had MICs of 0.25% (w/v).

5.4.5 Effects of gene disruption on cellular resistance to H_2O_2, NaClO, citric acid and trisodium phosphate

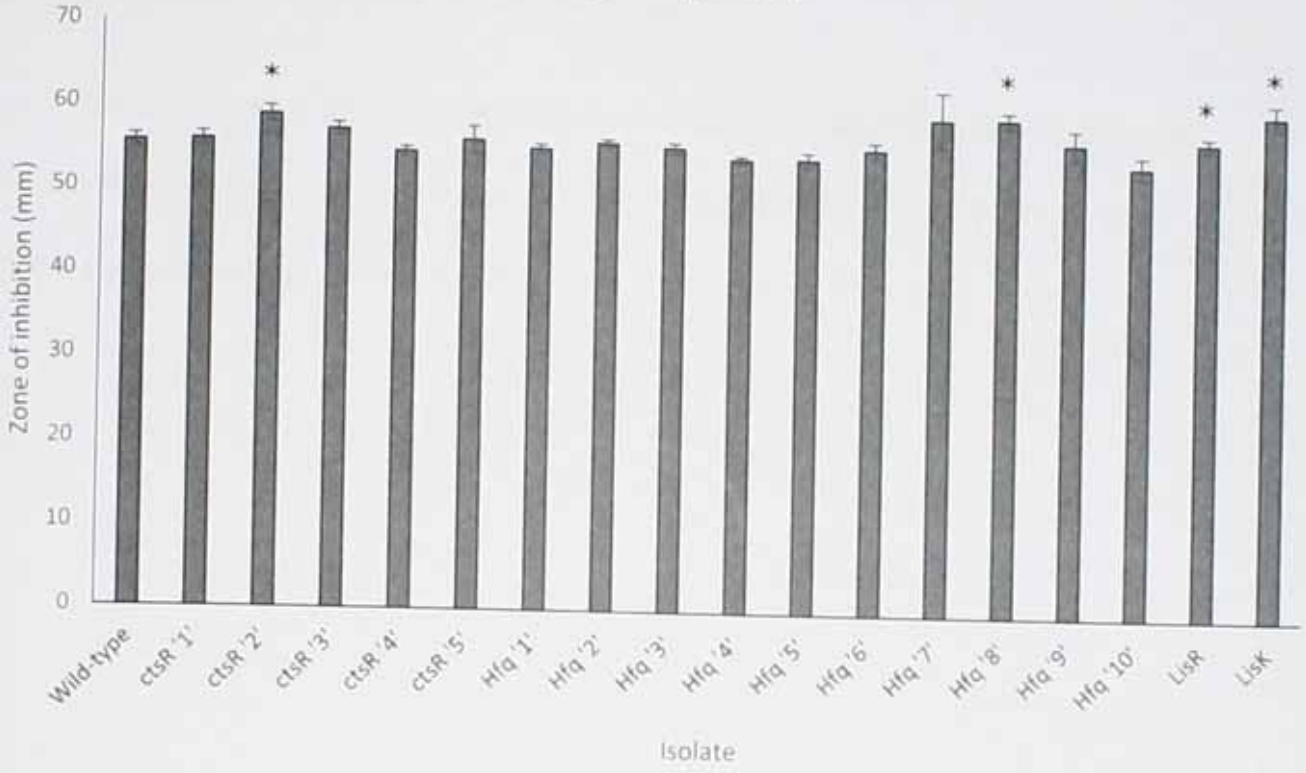

Figure 5-9: Effects of H_2O_2 on wild-type *L. monocytogenes* EGD-e and *ctsR*, *Hfq*, *LisR* and *LisK* disruptants. Data represents mean zone of inhibition (mm) as determined by disc diffusion assay. Error bars indicate the SEOM of 2 independent experiments conducted in triplicate (n=2). Asterisks indicate significant difference from wild-type (p<0.05).

When subjected to H_2O_2 via paper disc diffusion, a zone of inhibition of 55.5±0.7mm occurred in wild-type cells. Of the 5 *ctsR* disruptants which were generated, four were not found to differ in H_2O_2 sensitivity (p>0.05). However, *ctsR* disruptant '2' had a significantly increased sensitivity to H_2O_2 (zone of inhibition =58.7±0.9mm, p<0.05).

Of the 10 *Hfq* disruptants which were obtained, 9 had H_2O_2 sensitivity which was comparable to the wild-type controls (p>0.05). *Hfq* disruptant '8' demonstrated significantly increased sensitivity to H_2O_2 compared to

wild-type controls as indicated by an increased zone of inhibition (60.0±0.9mm vs. 55.5±0.7mm, $p<0.05$).

Both the *LisR* and *LisK* disruptants demonstrated significantly increased zones of inhibition compared to wild-type controls (57.5±0.7mm and 60.8±1.4mm respectively vs. 55.5±0.7mm, $p<0.05$).

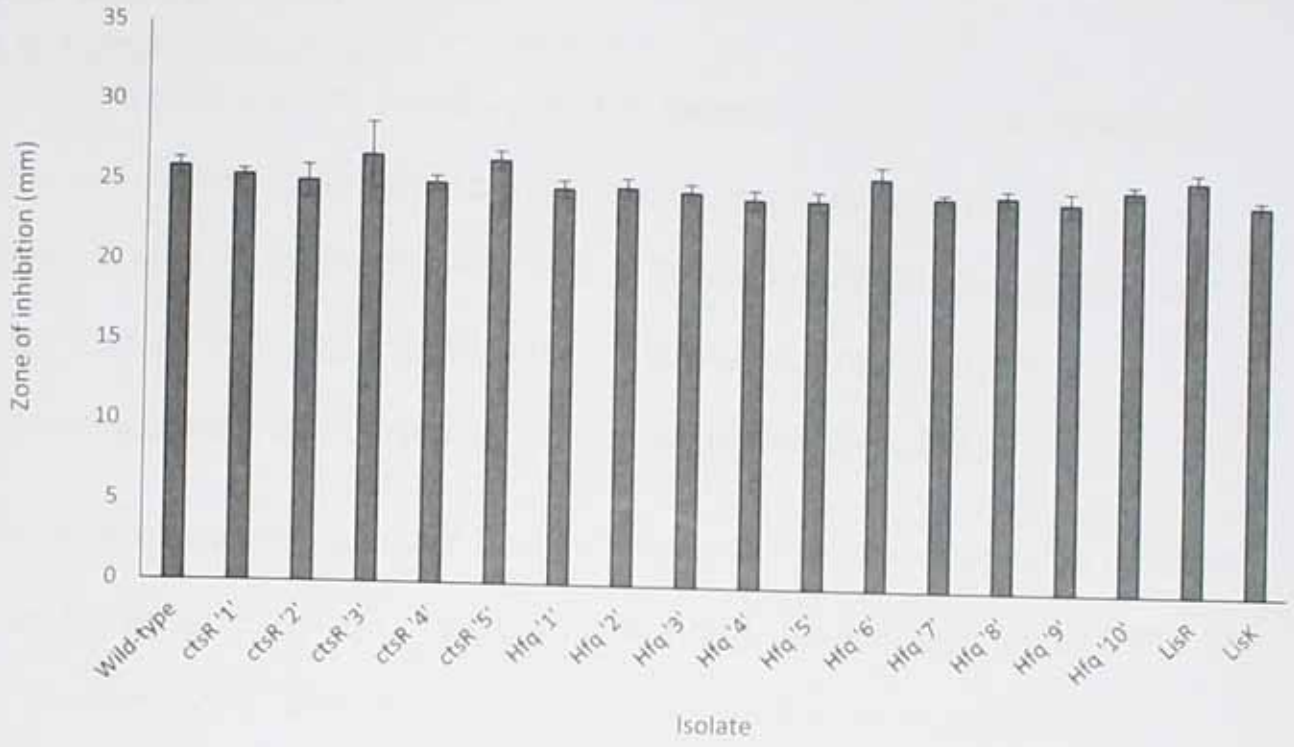

Figure 5-10: Effects of NaClO on wild-type *L. monocytogenes* EGD-e and ctsR, Hfq, LisR and LisK disruptants. Data represents mean zone of inhibition (mm) as determined by disc diffusion assay Error bars indicate the SEOM of 2 independent experiments conducted in triplicate (n=2). Asterisks indicate significant difference from wild-type (p<0.05).

When subjected to NaClO via paper disc diffusion, a zone of inhibition of 25.8±0.8mm was observed in wild-type cells. Disruption in *ctsR*, *Hfq*, *LisR* and *LisK* genes resulted in cells which had zones of inhibition comparable to those obtained from wild-type cells (p>0.05). Therefore NaClO resistance/tolerance did not appear to be influenced by *ctsR*, *Hfq*, *LisR* or *LisK*.

5.5 Discussion

5.5.1 *CtsR* disruption

Using the temperature sensitive shuttle vector, pAUL-A it was possible to generate potential mutants through vector manipulation (Fig. 5-3), insertion (Fig. 5-4), integration (Fig. 5-5) and finally disintegration (Fig. 5-6). Colony PCR was insufficient to indicate how many, if any, nucleotides were lost during vector disintegration (Fig. 5-8).

Phenotypic analysis revealed that any disruption to the *ctsR* locus resulted in an increased sensitivity to citric acid relative to wild-type cells (Table 5-3). Furthermore, *ctsR* disruptant '2' demonstrated an increased sensitivity to H_2O_2 compared to wild-type controls (Fig. 5-9).

Since the *ctsR* gene encodes the class three stress gene regulator, a negative regulator of class III heat shock genes (Karatzas *et al.*, 2003), it was envisaged that a disruption would result in the development of a mutant with increased stress resistance compared to the wild-type.

In several other studies stress resistance has been elevated by *ctsR* disruption. For example, Karatzas *et al.* (2003) first isolated a pleizotolerant mutant and characterised the mutation as being the result of a deletion at the *ctsR* locus. By inserting a functional *ctsR* sequence the pressure sensitivity increased. Likewise, Joerger *et al.* (2006) found disruption to the *ctsR* locus when screening a pleizotolerant *L. monocytogenes* mutant. However, in their study Joerger *et al.* (2006) also report increased resistance to H_2O_2, low pH and nisin. This suggests

a generic role in stress response but is in conflict with the findings of this study in which *ctsR* disruptants had increased sensitivity towards citric acid and, on one occasion, increased sensitivity to H_2O_2.

The effects of *ctsR* disruption on stress response appear to be complex, for example, Hu *et al.* (2007) found that disruption to the *ctsR* locus alone had no effect on stress response (including acid stress); however, when involved in a double disruption alongside *sigB*, sensitivity was greatly increased. Furthermore, when studying the effects of *ctsR* disruption in *Lactobacillus plantarum*, Fiocco *et al.* (2010) reported increased sensitivity to heat and ethanol stress in the Δ*ctsR* mutant relative to the wild-type control.

5.5.2 *recA* disruption

recA was successfully cloned into pAUL-A (Fig. 5-3). However, following pAUL-A:*recA* extraction and subsequent electroporation of *L. monocytogenes* no transformants were recovered (Fig. 5-4). This may be attributed to the low transformation efficiency achieved by electroporation.

The SOS response is an inducible DNA repair system which can be activated in response to excessive DNA damage (Michel, 2005). The system consists of 29 genes with roles in DNA synthesis and repair; and is regulated by RecA and LexA (Van der Veen and Abee, 2011). LexA acts as an autoregulatory SOS repressor, binding to the promoter region of the SOS operon while RecA is a highly conserved recombinase protein

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concerned with DNA repair and SOS initiation (Cox, 2007). Accumulation of intracellular single-stranded DNA results in RecA up-regulation, this cleaves LexA, initiating the expression of the SOS operon (Van der Veen *et al.*, 2010).

Several studies have highlighted the importance of *recA* in *Listeria* stress response. Using deletion mutants, Van der Veen and Abee (2011) found that *recA* was important in both acid and bile tolerance as well as impacting invasiveness and adhesion in *L. monocytogenes*. Likewise, Van der Veen *et al.* (2010) demonstrated that *recA* was important for heat, acid and H_2O_2 tolerance in *L. monocytogenes*, again using a *recA* deletion mutant. The role of *recA* in heat shock was further supported by the findings of Van der Veen *et al.* (2007) in which *recA* was significantly up-regulated in response to heat exposure in *L. monocytogenes*.

Given the multi-faceted role of *recA* in *Listeria* stress response, and the DNA damaging nature of the antimicrobials being tested, *recA* was deemed a suitable candidate gene for these studies.

5.4.3 *Hfq* disruption

Hfq is an RNA binding protein present in many bacterial species. Its primary role is concerned with regulation of gene expression by facilitating the interactions between small RNAs and target mRNAs (Vogel and Luisi, 2011). In many bacterial species Hfq is involved in several cellular processes including stress response and virulence (Chambers and

Bender, 2011; Ding *et al.*, 2004; Guisbert *et al.*, 2007; Sittka *et al.*, 2007; Sonnleitner *et al.*, 2003).

In *L. monocytogenes hfq* has been implicated in stress response and virulence. Christiansen *et al.* (2004) found Δ*hfq* mutants to display significantly greater sensitivity to several commonly encountered stresses compared to wild-type counterparts. These included osmotic stress, ethanol stress and Triton X-100 exposure. Furthermore, Δ*hfq* mutants were more sensitive to starvation and had a diminished capacity to survive *in vivo* using a mouse model.

Given this, and its relationship with the well characterised stress response gene, σ^B (Chaturongakul *et al.*, 2011; Kazmierczak *et al.*, 2006), *hfq* was deemed a suitable candidate gene for inactivation studies and phenotypic analysis.

hfq was successfully cloned into pAUL-A and subsequently used to transform *L. monocytogenes*. Integration was achieved by passaging at a non-permissive temperature and the integrated plasmid was subsequently disintegrated from the host genome. Loss of genetic material could not be determine by colony PCR, however, all Δ*hfq* mutants displayed increased sensitivity to citric acid, as determined by MIC analysis vs. wild-type. Furthermore, *hfq* disruptant '8' demonstrated significantly enhanced sensitivity to H_2O_2 compared to the wild-type control.

5.4.4 *groEL* disruption

A central portion of the *groEL* gene was amplified and cloned into the pAUL-A (Fig. 5-3). pAUL-A:*groEL* vectors were replicated in *E.coli* DH5α after which plasmids were extracted and used to transform *L. monocytogenes* EGD-e (Fig. 5-4). The *groEL* locus was disrupted through plasmid integration by passaging at the non-permissive temperature of 42°C (Fig. 5-5); however, it was not possible to generate disintegrants (Fig. 5-6). This may be due to a necessity for *L. monocytogenes* cells to maintain an intact *groEL* gene for survival. This is supported by the findings of Fayet *et al.* (1989) who found the GroE operon was necessary for *E.coli* growth across a range of temperatures (17-42°C). As such, its inactivation may have prevented/inhibit *L. monocytogenes* growth.

GroEL is a class I heat shock protein which acts in with an important role as a molecular chaperone. During heat shock, and exposure to several environmental stresses, GroEL is up-regulated alongside the co-chaperone GroES to maintain protein integrity by facilitating correct protein folding (Hill *et al.*, 2002; Gahan *et al.*, 2001).

Several studies have highlighted the importance of *groEL* in both stress response and virulence. Using protein analysis Phan-Thanh *et al.* (2000) found that GroEL was up-regulated in response to acid shock. Similarly, using protein analysis, Kilstrup *et al.* (1997) demonstrated that GroEL was up-regulated in *Lactococcus lactis* during salt challenge. When investigating *Leuconostoc esenteroides* stress response, Salotra *et al.* (1995) found several stresses induced the expression of GroEL. This

response was considered a general response as expression was increased by heat, cold and chemical stress. Likewise, Gahan *et al.* (2001) reported increased expression of *groEL* in response to several environmental stress including heat, acid, ethanol and bile when challenging *L. monocytogenes*.

Given the generic role of GroEL in stress response it was deemed an ideal target gene for inactivation studies. However, Δ*groEL* mutants were not obtained during this study.

5.4.5 *LisR/LisK* disruption

lisR and *lisK* make a two-component signal transduction system which is involved in both stress response and virulence (Sleator and Hill, 2005). This system is highly important for listerial survival under a variety of unfavourable conditions.

Using *L. monocytogenes* deletion mutants, Cotter *et al.* (1999) demonstrated that disruption to *lisRK* locus resulted in increased sensitivity to several commonly encountered food processing stresses, including ethanol and acid, while also causing a diminished capacity to infect mice. In a later study Cotter *et al.* (2002) also found *lisRK* to be important in both nisin and cephalosporin resistance. Again using a deletion mutant, Δ*lisK L. monocytogenes* cells had significantly impaired growth capabilities in the presence of nisin, while zones of inhibition were significantly greater for Δ*lisK* cells during cephalosporin disc diffusion assays.

Similarly, Kallipolitis and Ingmer (2001) generated *lisR* mutants using the pAUL-A vector system. Like in other studies, the Δ*lisR* mutants displayed diminished virulence and altered stress response properties compared to wild-type counterparts.

In accordance with previous findings, the *lisR* and *lisK* mutants produced in this study displayed altered phenotypic properties compared to wild-type controls. No differences were found in MICs when challenging cells with citric acid, trisodium phosphate, H_2O_2 nor NaClO (Table 5-3). However, in disc diffusion assays both Δ*lisR* and Δ*lisK* cells displayed increased sensitivity to H_2O_2. From PCR and subsequent gel electrophoresis it appears that genetic material was lost during the process of plasmid disintegration (Fig. 5-8). Further characterisation of these disruptions would provide important information in determining the cause of these altered phenotypes. Furthermore, the response to additional stressors would allow a more complete understanding of the generic role of *lisRK* in stress response and virulence.

5.6 Conclusion

The pAUL-A vector has been, and continues to be an important tool in the study of the molecular biology of *L. monocytogenes*. Using this vector system a variety of candidate genes can be targeted for inactivation and their roles elucidated. Using this approach the roles of *ctsR*, *Hfq*, *lisR* and *lisK* in stress response have been investigated.

From the findings presented in this chapter it can be inferred that disruption to the *ctsR* and *Hfq* loci results in impaired citric acid tolerance. Further work would inform if these genes are required for all acid responses, or the response is citric acid specific. It can also be concluded that disruption to the *lisR* and *lisK* loci result in increased sensitivity to oxidative stress by means of H_2O_2 exposure.

Many of these genes are important for several stress responses. These general stress responders may cause significant problems for the food industry, where sub-lethal exposures may lead to the induction of one, or many, 'general stress response' genes. As such, surviving cells may possess increased resistance properties when subsequently challenged by additional food processing stresses (as per hurdle technology), or may display enhanced virulence characteristics.

Chapter 6

The effects of anaerobiosis on growth, resistance and virulence potential of *L. monocytogenes* FSL R2-499

6.1 Introduction

Listeria monocytogenes possesses high intrinsic resistance to many of the processes employed by the food industry to ensure quality and safety. For example, the organism is tolerant to high salt concentrations and low pH environments, as well as having the capacity to survive and grow under conditions of low temperature (<7°C) and oxygen limitation (Lungu *et al.*, 2009; Swaminathan and Gerner-Smidt, 2007). Furthermore, sub-lethal stress has been found to alter cellular resistance properties, further impacting food safety as well as increasing virulence potential (Bergholz *et al.*, 2012; Gahan *et al.*, 1996; Lou and Yousef, 1997).

Upon ingestion *L. monocytogenes* cells must overcome several barriers before reaching the intestinal epithelial cells for adhesion, invasion and subsequent infection (Begley *et al.*, 2002; Ivy *et al.*, 2012). The first of these is survival within acidic gastric juice. The pH of the human stomach varies from pH 1 to pH 3 in healthy individuals; however a pH of 3.5 to 4.5 is more representative of the gastric pH of a susceptible individual, such as the elderly following food ingestion (Garner *et al.*, 2006; Ivy *et al.*, 2012). This pH is sub-lethal to *L. monocytogenes* and is sufficient to initiate an acid tolerance response (Koutsoumanis *et al.*, 2003). Furthermore, such exposure may inadvertently induce adaptive responses, increasing cellular resistance and virulence. Sub-lethal acid exposure induces expression of several genes which may contribute towards these altered resistance properties (Neuhaus *et al.*, 2013). These include stress specific acid responders such as the glutamate

decarboxylase system and F_0F_1 –ATPase (Hill *et al.*, 2002; Chaturongakul *et al.*, 2008), as well as general stress response genes such as transcriptional activators (Michel *et al.*, 2011), DNA repair proteins (van der Veen and Abee, 2011) and sigma factors (Sue *et al.*, 2004).

Following subjection to low stomach pH, cells pass into the small intestine where they must survive exposure to approximately 1 litre of bile excreted daily by the liver (Hofmann, 1994). Bile is a 'biological detergent' consisting of bile salts, phospholipids, cholesterol, protein and bilirubin with a primary role in lipid solubilisation and digestion (Begley *et al.*, 2002; Zhang *et al.*, 2013). However, bile has a secondary role as an antimicrobial agent owing to its capacity to disrupt bacterial cell membranes and interfere with cellular homeostasis (Tsai *et al.*, 2012). *Listeria monocytogenes* has mechanisms in place to overcome the toxic effects of bile; these include the use of bile salt hydrolase to convert bile salts into less toxic forms and bile exclusion systems which prevent excessive intracellular accumulation of bile (Dussurget *et al.*, 2002; Quillin *et al.*, 2011). Furthermore sub-lethal bile salt exposure has been reported to increase resistance to otherwise lethal levels, while also influencing biofilm forming capacity. Such effects may contribute to the ability of *L. monocytogenes* to colonise human hosts and cause disease. Also, bile tolerance is both strain and growth phase dependant which may account for the differences in virulence observed in *L. monocytogenes* (Begley *et al.*, 2002; Payne *et al.*, 2013).

6.2 Aim and Objectives

6.2.1 Aim

The aim of this chapter was to investigate the effects that anaerobiosis has on growth and survival of *L. monocytogenes*.

6.2.2 Objectives

- To determine the extent to which anaerobiosis effects growth in *L. monocytogenes*.
- To ascertain the effects that anaerobic growth has on acid tolerance.
- To investigate the effects of anaerobic pre-conditioning on gastro-intestinal transit using a model *in vitro* system.
- To consider the effects of growth phase on survival during simulated gastro-intestinal transit.

6.3 Materials and Methods

6.3.1 Preparation of *L. monocytogenes* cultures

To anaerobically pre-condition cells, overnight cultures were diluted in pre-reduced BHI broth to an OD_{595} 0.1. Cultures were then incubated under anaerobic conditions to an OD_{595} 0.3-0.5 (exponential phase cells) or for 24 hours (stationary phase cells). Pre-reduction was used to ensure anaerobic conditions were maintained throughout the conditioning stage, and was performed in an anaerobic cabinet (Don Whitley Scientific Limited, West Yorkshire, UK). Anaerobic indicator strips (Oxoid, Basingstoke, UK) were used to ensure anaerobiosis was achieved during incubation and pre-reduction.

Control cells (aerobically incubated) were prepared as above but diluted into pre-warmed BHI broth which had not undergone reduction.

6.3.2 Effect of anaerobiosis on growth of *L. monocytogenes*

Three independent overnight cultures were diluted into pre-warmed/pre-reduced BHI broths to approximately $5x10^2$ cfu/ml.

At hourly intervals, total viable counts were obtained by drop plate method (Miles *et al.*, 1938) on BHI agar in triplicate. After 24-36 hours incubation at 37°C, under aerobic conditions colonies were counted Length of lag phase, generation time and stationary phase cell density were compared aerobically vs. anaerobically.

6.3.3 Anaerobiosis induced acid tolerance- Time Kill Assays

Aliquots of citric acid (2% w/v), lactic acid (2% v/v), acetic acid (2% v/v) (Sigma Aldrich, UK) or 5M HCl (giving a final pH of 3) were directly added

to exponential phase cultures. Washing steps were omitted as it has been previously reported that centrifugation induces expression of several stress response genes which are known to impact acid resistance (Ivy *et al.*, 2012).

Samples were removed at 15, 30, 60 and 90 minute intervals, diluted in sterile Buffered Peptone Water, BPW (Oxoid, Basingstoke, UK) and spiral plated onto BHI agar (Don Whitley Scientific Limited, West Yorkshire, UK). After 24-48 hours incubation at 37°C, under aerobic conditions, colonies were enumerated. Log reductions were calculated relative to time-point zero. The detection limit was set at $3x10^2$ cfu/ml.

6.3.4 Simulated gastro-intestinal conditions

Gastro-intestinal transit was simulated using a combination of the methods of Barbosa *et al.* (2012) and Ivy *et al.* (2012). In brief, 1.25x simulated gastric juice (SGJ) was prepared (8.3g/L proteose peptone, 3.5g/L D-glucose, 2.05g/L NaCl, 0.6g/L KH_2PO_4, 0.147g/L $CaCl_2 \cdot 2H_2O$, 0.37 g/L KCl) and adjusted to pH 1.7 or 2.5 with HCl giving final pH values of 2.5 and 3.5 upon addition of test cultures. Sixteen ml aliquots of pH adjusted SGJ were transferred to 50ml centrifuge tubes and 4ml of test culture added. Tubes were incubated at 37°C throughout sampling.

Cells were assessed for their capacity to survive 'short' or 'long' gastric transit as described by Barbosa *et al.* (2012). Following addition of pre-conditioned test cultures to pH adjusted SGJ (ca. $3\text{-}4x10^8$ cfu/ml), cells were incubated for 60 minutes (short transit) or 120 minutes (long

transit) in SGJ. Prior to addition of bile salts (Sigma Aldrich, UK), SGJ was neutralised to pH 7-7.4 by addition of 1M NaOH after which 0.1x total volume of a 3% (w/v) bile salt solution was added, giving a final concentration of 0.3% (w/v). Following addition of bile salts cells were incubated for a further 60 minutes (short transit) or 120 minutes (long transit).

6.3.5 Gastro-intestinal survival following pre-conditioning

Four ml of either exponential or stationary phase cultures was added to 16ml SGJ in centrifuge tubes (without a wash step), incubated at 37°C and subjected to either short or long gastric transit as described in section 6.3.4. At 30 minute intervals samples were removed, serially diluted in BPW and spiral plated. Plates were incubated at 37°C under aerobic conditions for 24-48 hours and cfu/ml calculated. Detection limit was $3x10^2$ cfu/ml.

Each experiment included the following controls: PBS control (4ml aliquot of culture added to 16ml sterile PBS), neutralised SGJ control (4ml aliquot of culture added to SGJ neutralised to pH 7), bile control (4ml aliquot of culture added to 16ml PBS; at appropriate time-point subjected to 0.3% bile salts).

6.4 Results

6.4.1 Effects of anaerobiosis on growth parameters of *L. monocytogenes*

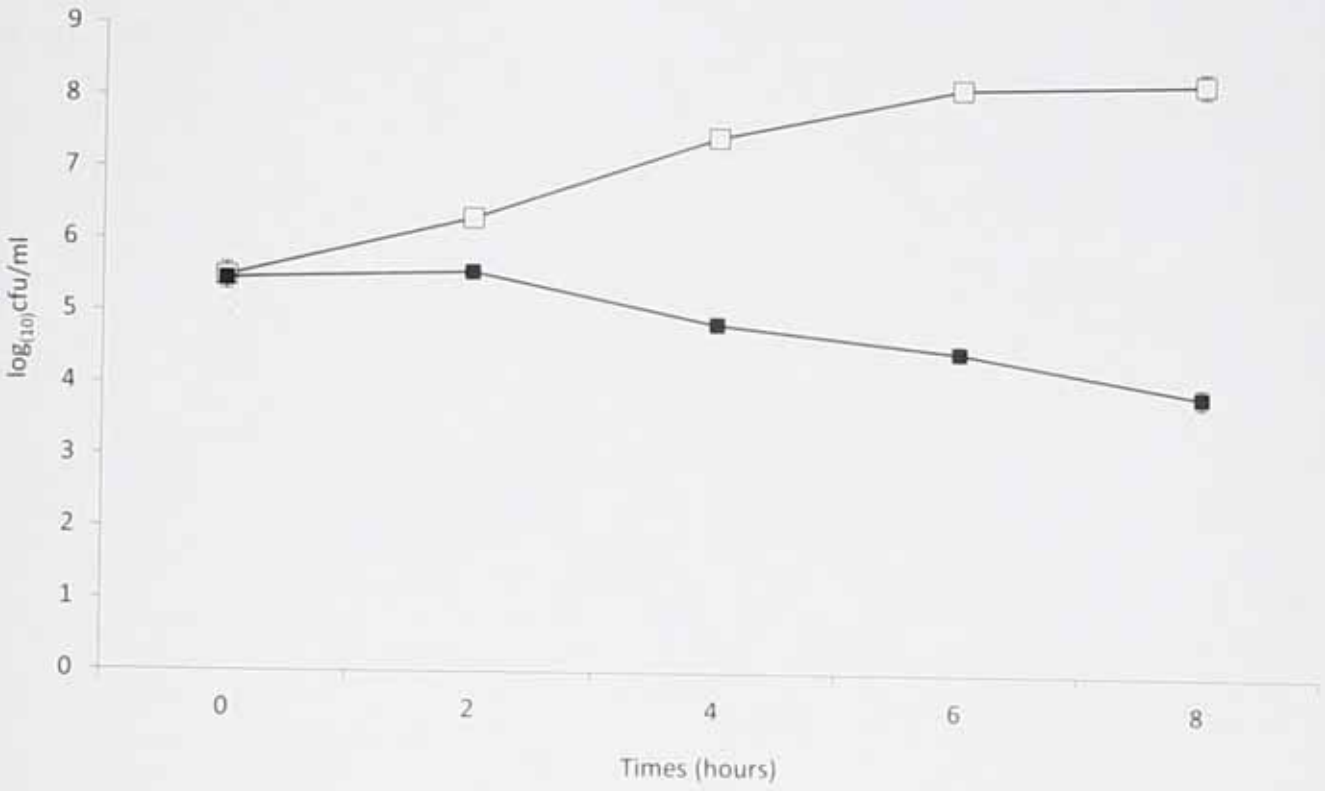

Fig. 6-1: Increase in cell number of *L. monocytogenes* EGD-e grown under aerobic (open) and anaerobic (filled) conditions at 37°C ($log_{(10)}$ cfu/ml ± SEOM, n=3)

When subjected to anaerobic conditions, *L. monocytogenes* EGD-e was unable to grow (Fig. 6-1). This was unexpected due to the facultative nature of *L. monocytogenes*. However, a gradual decline in cells was seen over 8 hours.

The reason for this inability to grow under anaerobic conditions was also reported by Ofer *et al.* (2011), who determined that a deletion in a

ribonucleotide reductase gene (*NrdD*) prevented growth of *L. monocytogenes* EGD-e under anaerobic conditions. As such, future work concerning the responses of *L. monocytogenes* to anaerobic conditions was performed with strain FSL R2-499. This is another 1/2a serotype strain which was associated with a multi-state listeriosis outbreak (Olsen *et al.*, 2005).

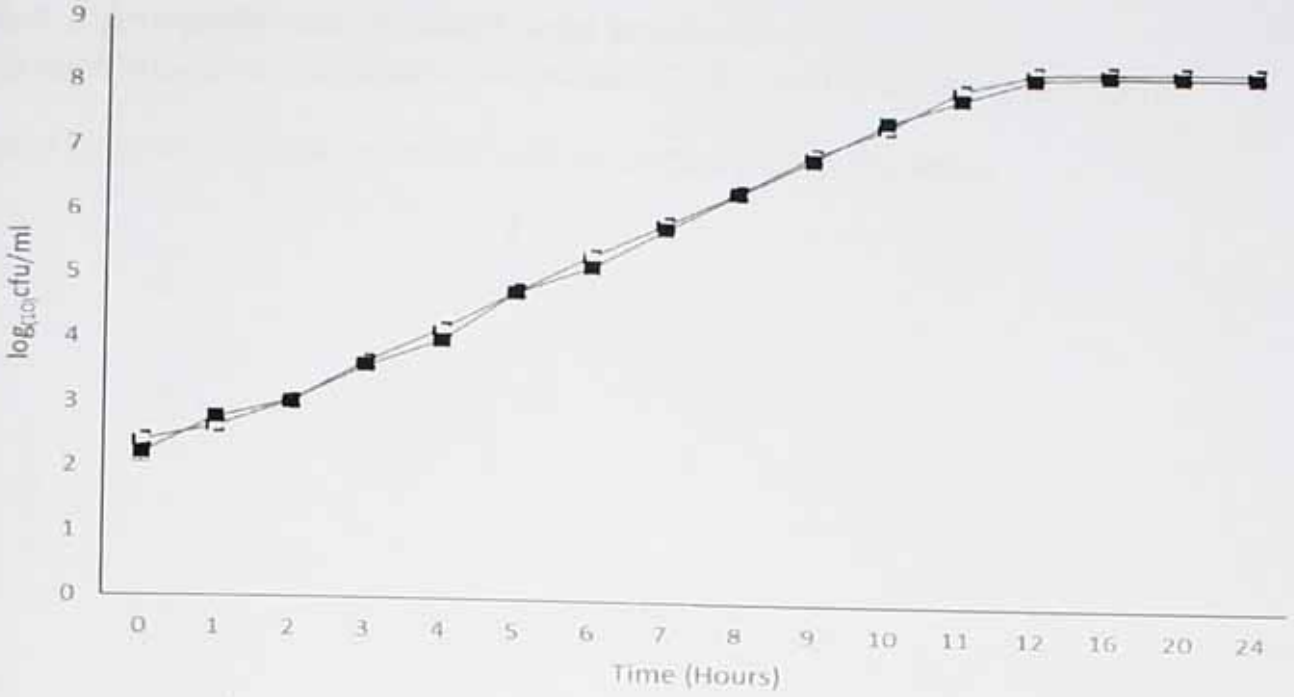

Fig. 6-2: Increase in cell number of *L. monocytogenes* FSL R2-499 grown under aerobic (open) and anaerobic (filled) conditions at 37°C ($log_{(10)}$ cfu/ml ± SEOM, n=3)

Anaerobiosis had no effect on the growth rate, length of lag phase or final cell density in *L. monocytogenes* FSL R2-499 when compared to aerobically grown cultures (Fig. 6-2).

Cultures grown both aerobically and anaerobically showed a non-detectable lag phase. It was envisaged that an extended lag phase would be seen in cells subjected to anaerobic conditions as cellular and metabolic adaptations may be needed to accommodate the switch from aerobic to anaerobic conditions. The generation times for cells grown aerobically and anaerobically were also comparable (36.3±0.5 vs. 35±1.5 mins per generation respectively) as were the maximum cell densities at stationary phase (8.38±0.02 vs. 8.34±0.07 log cfu/ml respectively), $p>0.05$.

6.4.2 Anaerobiosis induced acid tolerance

Exponential phase *L. monocytogenes* FSL R2-499 cells were tested for acid tolerance following anaerobic growth by time kill assay.

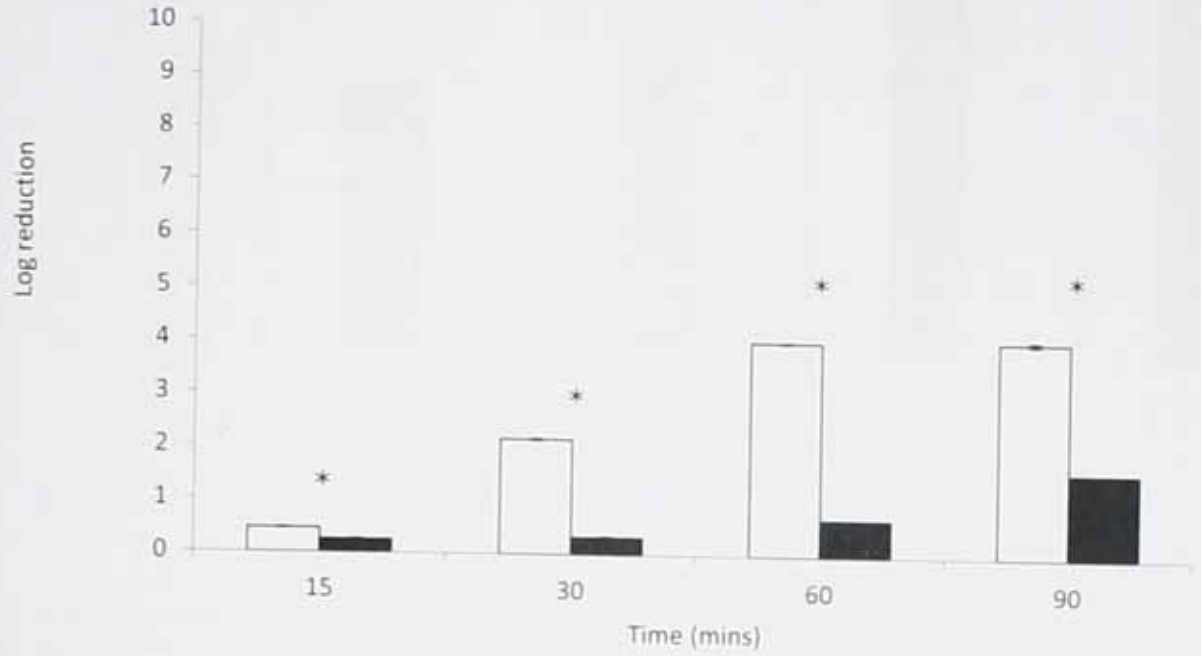

Fig. 6-3: The effect of aerobic (open) and anaerobic (filled) pre-conditioning on the resistance of *L. monocytogenes* FSL R2-499 to 2% (w/v) citric acid. Data represents mean log reduction in cfu/ml relative to time-point '0'. Error bars indicate the SEOM of 3 independent experiments (n=3) plated in duplicate. Asterisks indicate significant difference in log reduction (p<0.05).

When grown under anaerobic conditions, *L. monocytogenes* FSL R2-499 cells demonstrated significantly enhanced resistance to 2% (w/v) citric acid compared with cells grown aerobically (p<0.05). The extent of this difference was time dependent, increasing over the first 60 minutes. After 90 minutes of citric acid exposure the resistance of anaerobically grown cells was still significantly greater than aerobically grown cells (p<0.05).

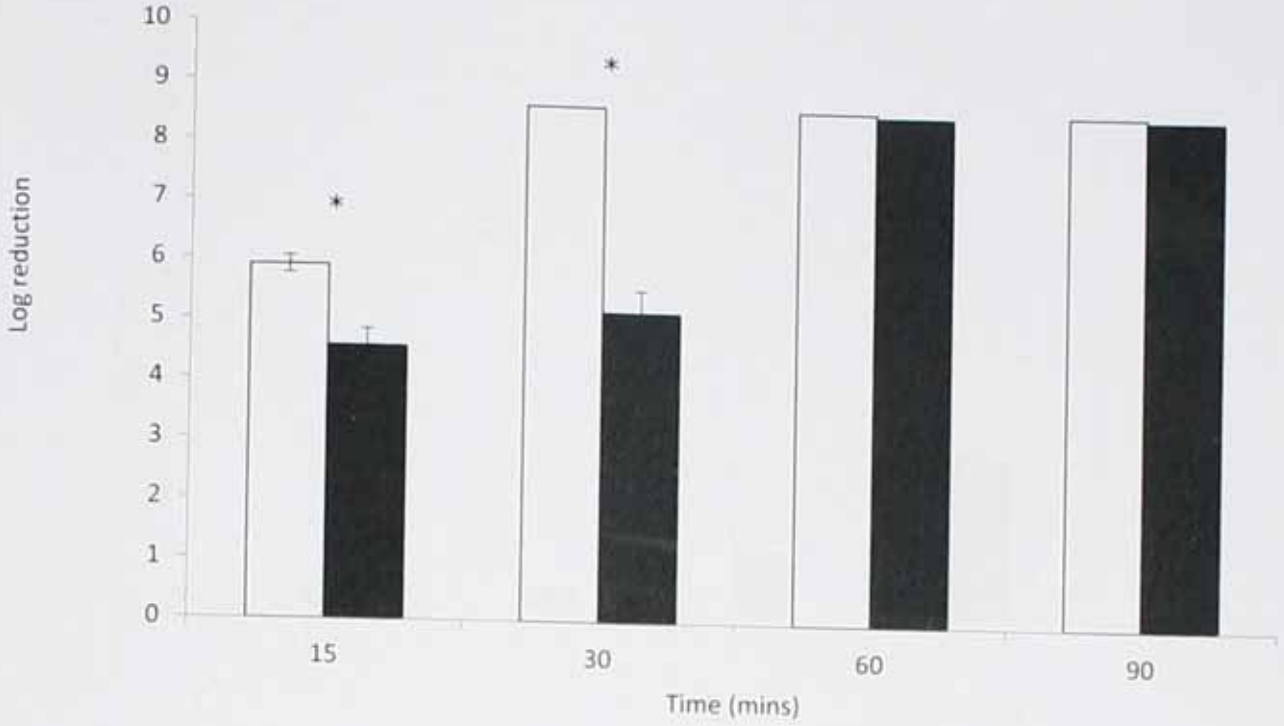

Fig. 6-4: The effect of aerobic (open) and anaerobic (filled) pre-conditioning on the resistance of *L. monocytogenes* FSL R2-499 to 2% (w/v) lactic acid. Data represents mean log reduction in cfu/ml relative to time-point '0' Error bars indicate the SEOM of 3 independent experiments (n=3) plated in duplicate. Asterisks indicate significant difference in log reduction (p<0.05).

When subjected to 2% (w/v) lactic acid for 15 and 30 minutes *L. monocytogenes* FSL R2-499 cells demonstrated significantly enhanced resistance when grown anaerobically compared to aerobically grown cells (p<0.05). However, after 60 minutes no significant difference in log reduction was apparent (p>0.05) as no viable cells were detected, irrespective of pre-conditioning.

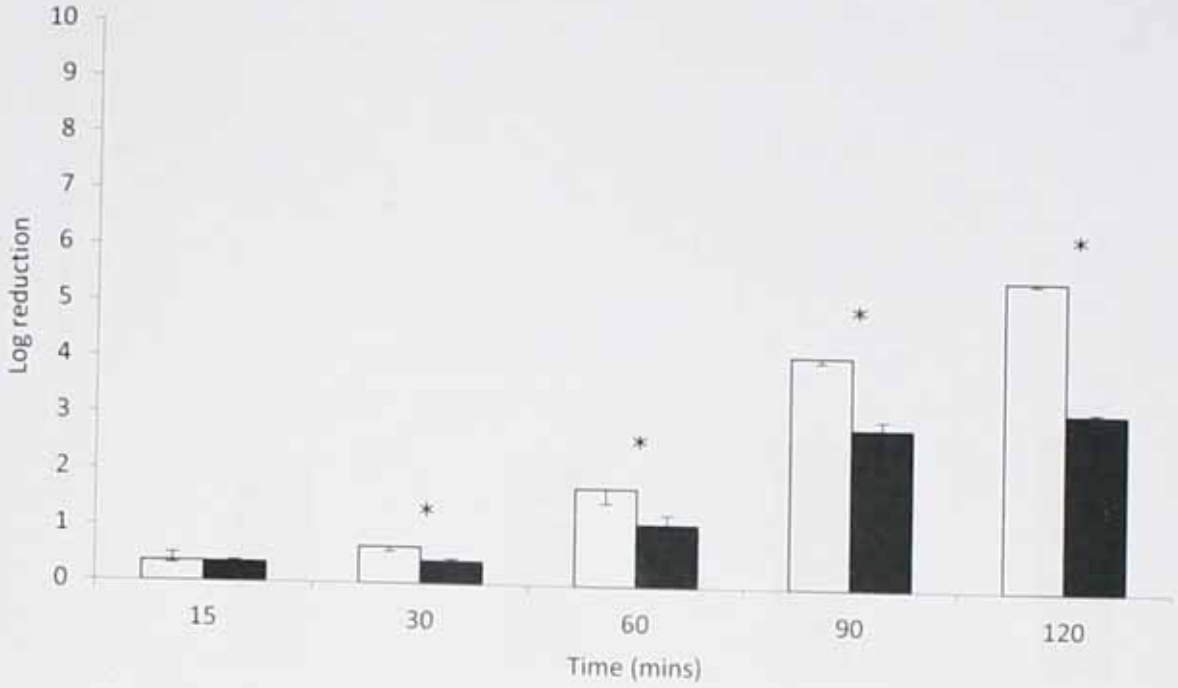

Fig. 6-5: The effect of aerobic (open) and anaerobic (filled) pre-conditioning on the resistance of *L. monocytogenes* FSL R2-499 to 2% (w/v) acetic acid. Data represents mean log reduction in cfu/ml relative to time-point '0' Error bars indicate the SEOM of 3 independent experiments (n=3) plated in duplicate. Asterisks indicate significant difference in log reduction (p<0.05).

When subjected to 2% (v/v) acetic acid differences in viable counts were not statistically significant until 30 minutes (p>0.05); after this point anaerobiosis significantly enhanced acid tolerance compared to cells grown under aerobic conditions. This difference increased with time, with the largest difference seen after 120 minutes exposure. After 30 minutes this difference was ~0.2 log, after 60 minutes this difference increased to ~0.5 log, after 90 minutes the difference further increased to ~1.3 log and finally after 120 minutes there was approximately 2.2 log greater reduction in cfu/ml when cells had been grown in aerobic conditions compared to those grown in an anaerobic environment.

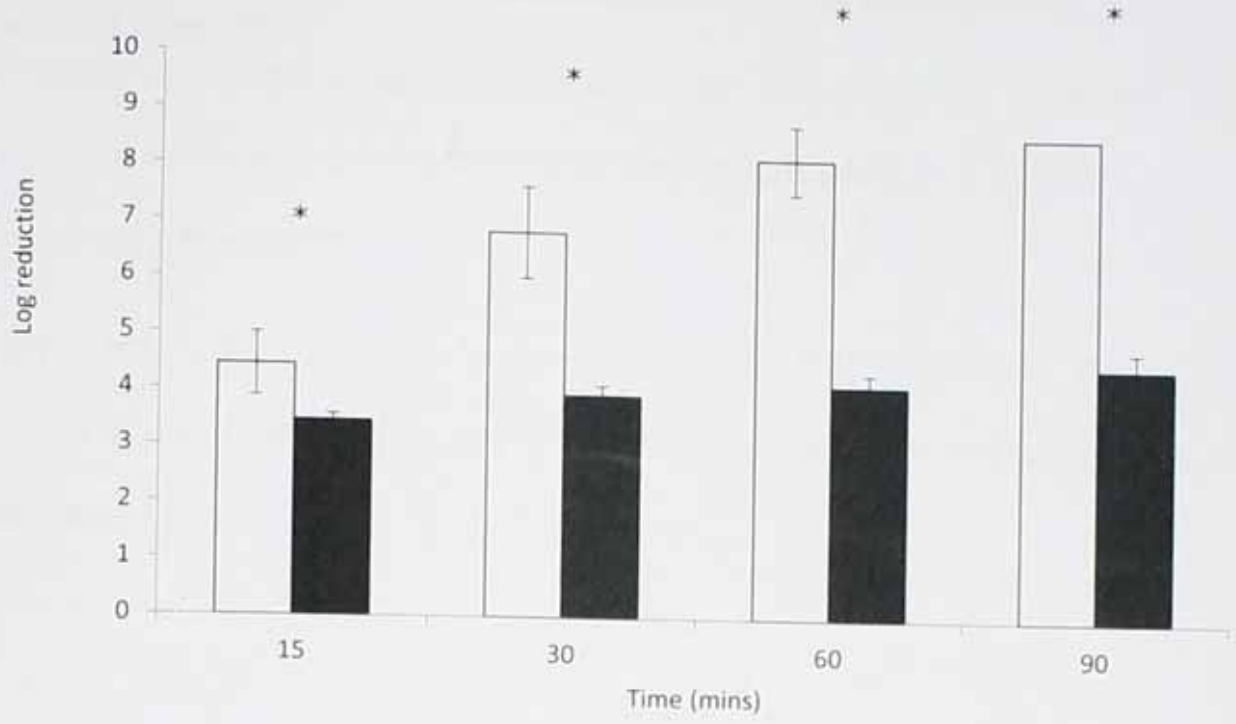

Fig. 6-6: The effect of aerobic (open) and anaerobic (filled) pre-conditioning on the resistance of *L. monocytogenes* FSL R2-499 to HCl (directly added to culture to give a final pH of 3). Data represents mean log reduction in cfu/ml relative to time-point '0' Error bars indicate the SEOM of 3 independent experiments (n=3) plated in duplicate. Asterisks indicate significant difference in log reduction (p<0.05).

To assess whether anaerobiosis induced a generic acid tolerance response, or an organic acid specific response, cells were subjected to low pH (~3) using 5M HCl. As with organic acid exposure (Fig. 6-3 to 6-5) cells displayed a significantly enhanced tolerance to acid stress when grown anaerobically compared to those grown in the presence of oxygen (p<0.05). As with organic acid challenge, the extent of the cross protection induced by anaerobic growth was time dependent, and was most pronounced after 60-90 minutes exposure.

6.4.3 Effects of culture conditions and pH on gastro-intestinal survival of exponential phase *L. monocytogenes* cells during simulated digestion

Upon determining that anaerobiosis initiates an acid tolerance response the effect of anaerobic pre-conditioning was investigated in a model gastro-intestinal system.

The pH of the human stomach is variable, ranging from 1.7 - 4.5. As such pH 2.5 (non-risk individuals) and pH 3.5 (at-risk individuals) were investigated.

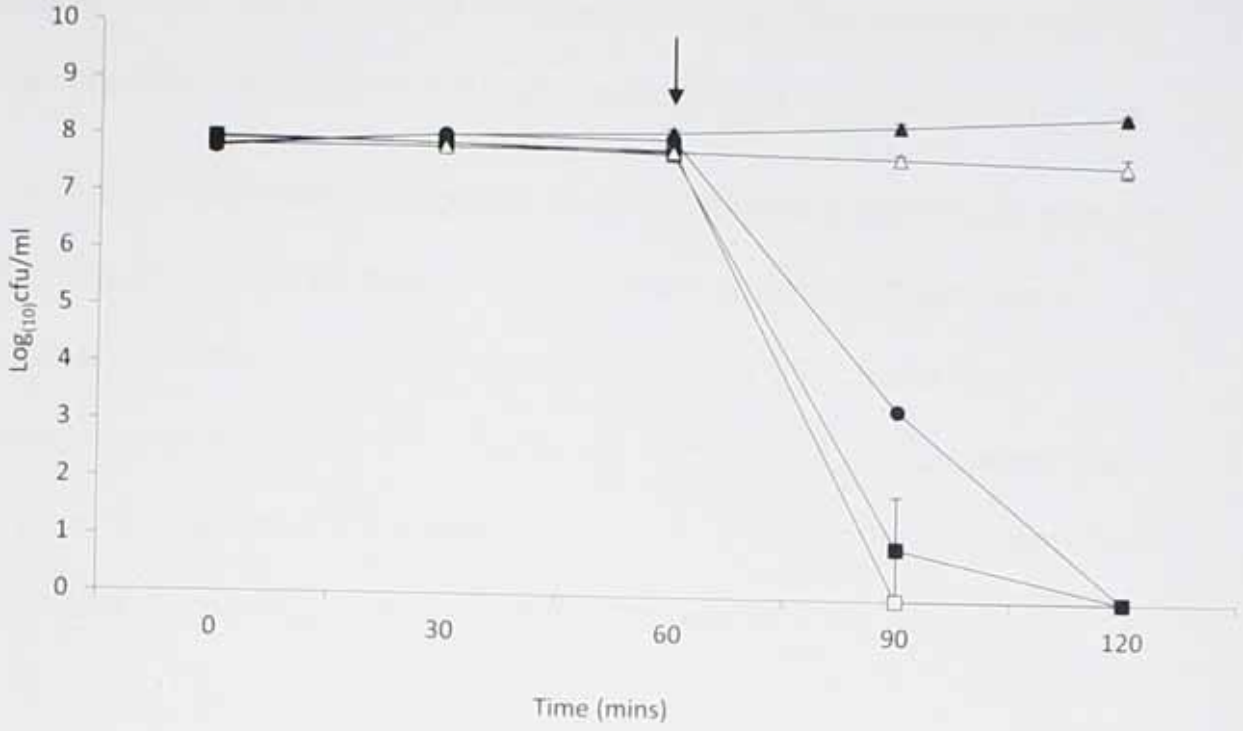

Fig. 6-7: Effect of aerobic (-□-) and anaerobic (-■-) pre-conditioning on the ability of exponential phase *L. monocytogenes* FSL R2-499 to survive short gastric transit at pH 3.5 (n=3 ± SEOM). PBS (-▲-), SGJ (-△-) and bile (-●-) controls were also included. Limit of detection= $3x10^2$cfu/ml. Arrow indicates neutralisation to pH 7 and addition of bile salts.

Exposure to pH 3.5 artificial gastric juice had no significant effect on exponential phase *L. monocytogenes* cells ($p>0.05$). Cells remained viable throughout gastric juice exposure at pH 3.5 and no differences ($p>0.05$) were observed between cells grown aerobically or anaerobically prior to exposure. Following subjection to the acidic pH of the stomach, surviving cells are next exposed to bile salts in the small intestine. Exponential phase cells demonstrated extreme sensitivity to bile salts at 0.3% (w/v) in that exposure to *in vivo* concentrations resulted in a significant decrease in viable cells ($p<0.05$), with complete cellular

inactivation occurring within 30 minutes; an effect that was observed in both aerobically and anaerobically pre-conditioned cells ($p<0.05$).

Cells displayed increased sensitivity to bile salts when previously exposed to pH 3.5 gastric juice compared to untreated controls. There were significantly greater number of recoverable ($p<0.05$) cells after 30 minutes bile salt exposure in control cells compared to those which had undergone acid exposure previously, indicating that mild acid exposure pre-disposes *L. monocytogenes* cells to the toxic effects of bile salts.

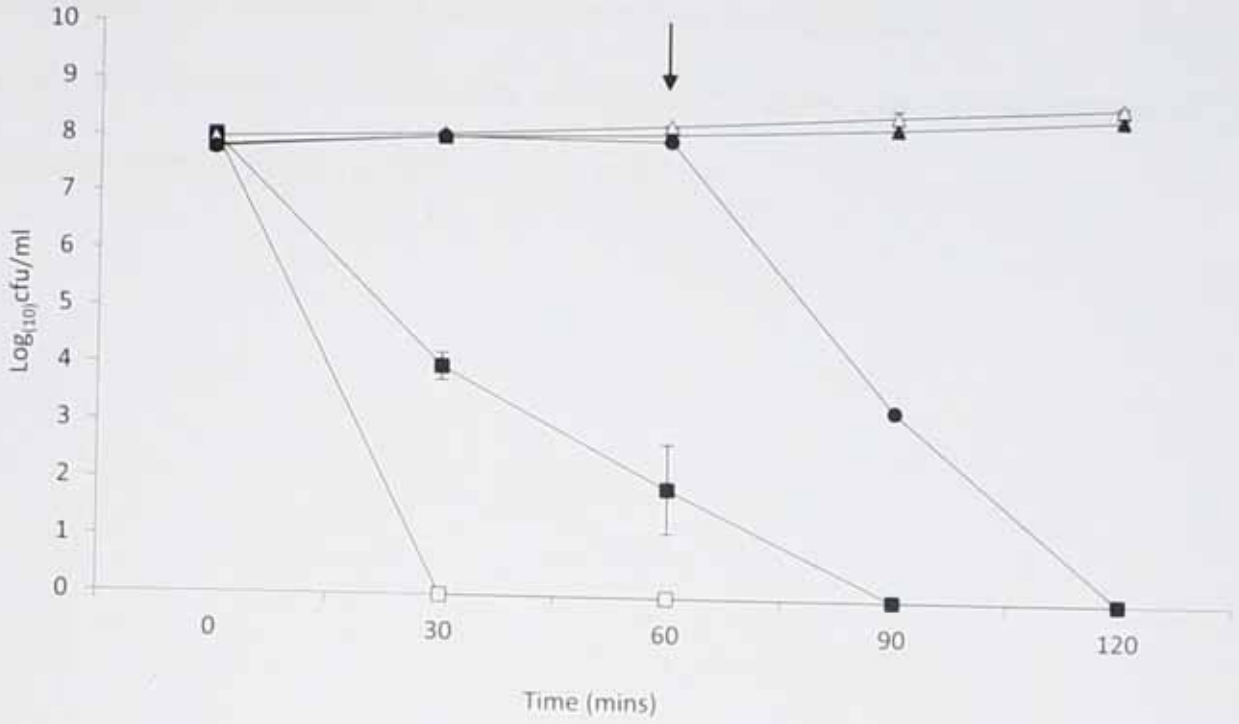

Fig. 6-8: Effect of aerobic (-□-) and anaerobic (-■-) pre-conditioning on the ability of exponential phase *L. monocytogenes* FSL R2-499 to survive short gastric transit at pH 2.5 (n=3 ± SEOM). PBS (-▲-), SGJ (-△-) and bile (●) controls were also included. Limit of detection= $3x10^2$cfu/ml. Arrow indicates neutralisation to pH 7 and addition of bile salts.

Exponential phase cells were unable to tolerate short digestion with exposure to pH 2.5 artificial gastric juice. However, the previously observed anaerobiosis induced acid tolerance response was evident After 30 minutes exposure to pH 2.5 artificial gastric juice 4.0±0.2 log cfu/ml remained viable when cells were grown under anaerobic conditions. This was significantly greater (p<0.05) than aerobically pre-conditioned cells, which could not be detected after 30 minutes. Inactivation was also time-dependant with 1.9±0.8 log cfu/ml remaining viable after 60 minutes. This suggests that gastric juice at pH values found in 'healthy' individuals (pH 2.5) is effective at controlling *L.*

monocytogenes. However, it should be noted that anaerobic pre-conditioning significantly enhances resistance to otherwise lethal exposure to artificial gastric juice ($p<0.05$) (Fig. 6-8).

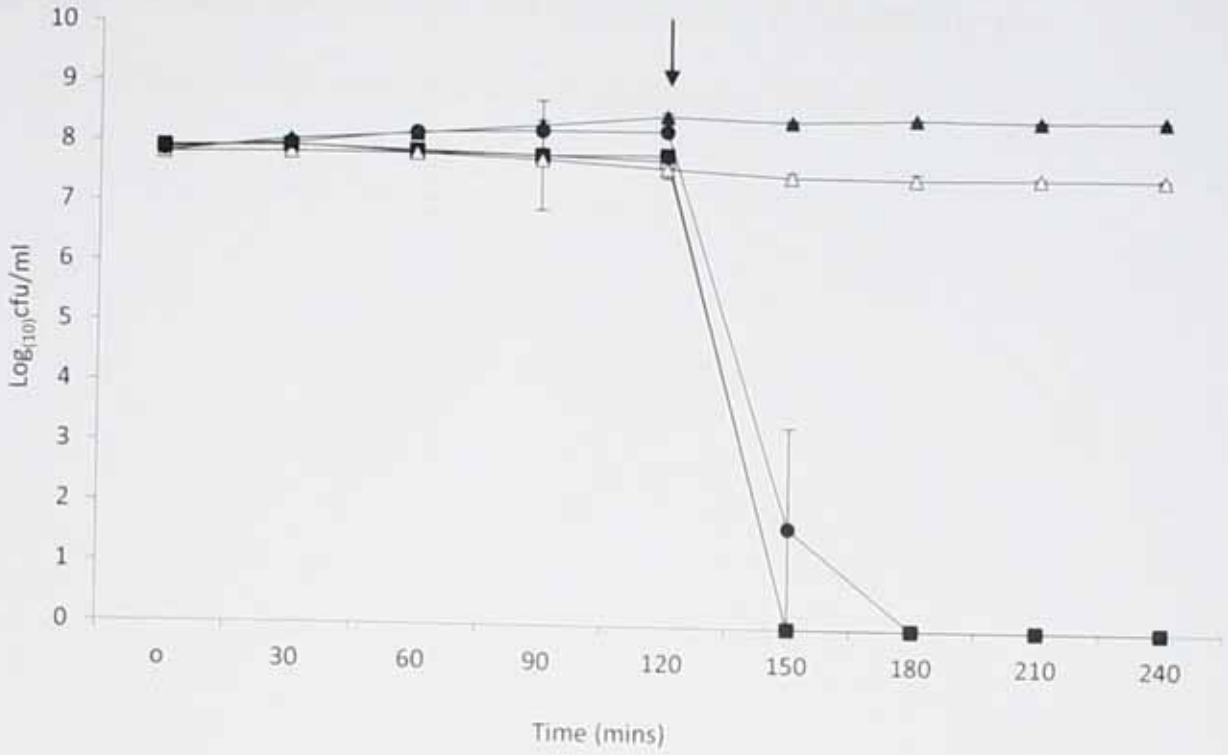

Fig. 6-9: Effect of aerobic (-□-) and anaerobic (-■-) pre-conditioning on the ability of exponential phase *L. monocytogenes* FSL R2-499 to survive long gastric transit at pH 3.5 (n=3 ± SEOM). PBS (-▲-), SGJ (-△-) and bile (-●-) controls were also included. Limit of detection= $3x10^2$cfu/ml. Arrow indicates neutralisation to pH 7 and addition of bile salts.

As with short gastric transit, pH 3.5 artificial gastric juice had no significant effect on cellular inactivation ($p>0.05$) during long exposure. No decrease in viable cells was seen throughout 120 minutes and no differences occurred between cells grown aerobically vs. anaerobically ($p>0.05$). Cells showed significantly enhanced sensitivity to bile salts, with complete inactivation occurring within 30 minutes of exposure to 0.3% (w/v) bile salts ($p<0.05$). Anaerobic pre-conditioning had no effect on subsequent bile exposure compared to aerobically grown cultures. As with short gastric transit, cells displayed increased sensitivity to bile salts following simulated gastric juice exposure than bile control cells ($p<0.05$).

These findings suggest that bile salt sensitivity is enhanced by pre-exposure to the low pH environment found in the stomach.

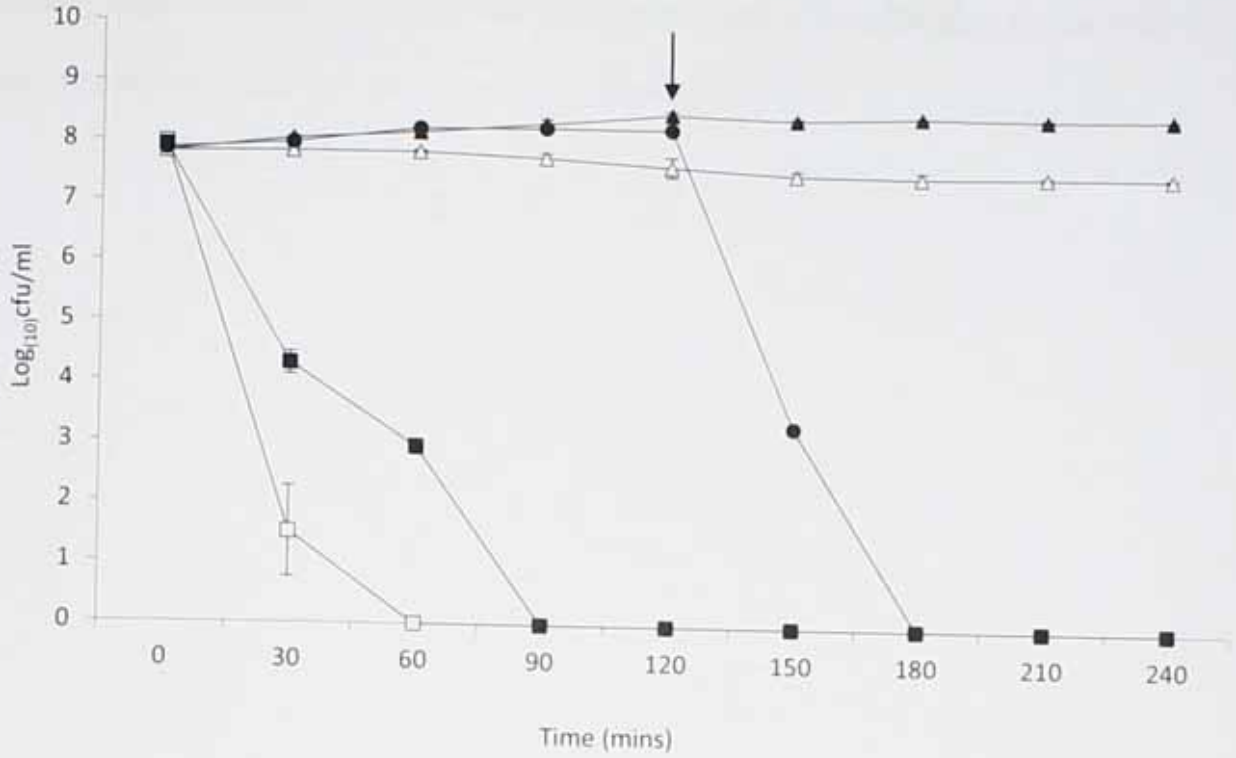

Fig. 6-10: Effect of aerobic (-□-) and anaerobic (-■-) pre-conditioning on the ability of exponential phase *L. monocytogenes* FSL R2-499 to survive long gastric transit at pH 2.5 (n=3 ± SEOM). PBS (-▲-), SGJ (-△-) and bile (-●-) controls were also included. Limit of detection= $3x10^2$cfu/ml. Arrow indicates neutralisation to pH 7 and addition of bile salts.

As with short gastric transit at pH 2.5, exponential phase cells were unable to survive long exposure to simulated gastric juice. Anaerobiosis significantly enhanced cellular resistance ($p<0.05$) compared to aerobic growth, but this protection was insufficient to allow survival beyond 60 minutes exposure. After 30 minutes simulated gastric juice exposure 1.5±0.8 log cfu/ml remained viable in aerobic cultures while 4.3±0.2 log cfu/ml survived exposure following anaerobic growth. Within 60 minutes aerobically grown cultures were completely inactivated while 2.9±0.1 log cfu/ml remained viable in anaerobically pre-conditioned cultures. This suggests that an anaerobiosis induced acid tolerance response occurs in

L. monocytogenes FSL R2-499, but this response is insufficient to permit gastro-intestinal passage in a 'healthy' individual.

6.4.4 Effects of culture conditions and pH on gastro-intestinal survival of stationary phase *L. monocytogenes* cells during simulated digestion

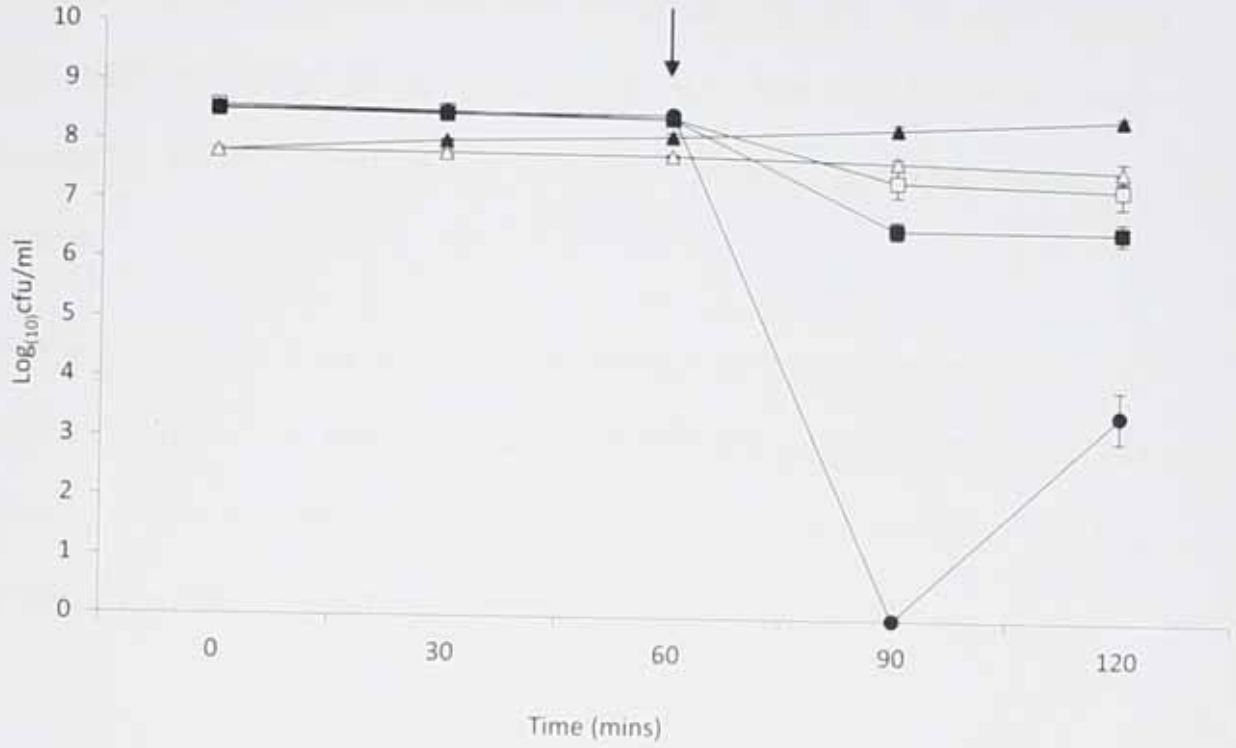

Fig. 6-11: Effect of aerobic (-□-) and anaerobic (-■-) pre-conditioning on the ability of stationary phase *L. monocytogenes* FSL R2-499 to survive short gastric transit at pH 3.5 (n=3 ± SEOM). PBS (-▲-), SGJ (-△-) and bile (-●-) controls were also included. Limit of detection= $3x10^2$cfu/ml. Arrow indicates neutralisation to pH 7 and addition of bile salts.

Exposure of stationary phase cells to simulated gastric juice at pH 3.5 had no significant effect on cell number ($p<0.05$) throughout 60 minutes exposure. Pre-culture condition also had no effect on susceptibility of cells to simulated gastric juice at pH 3.5 with no significant differences ($p<0.05$) found between cells which had been grown to stationary phase under aerobic or anaerobic conditions.

However, unlike exponentially grown cells, those which were grown to stationary phase were readily able to survive exposure to 0.3% (w/v) bile salts. When stationary phase bile control cells were challenged with bile salt a rapid decline in viable cells was observed, cell numbers were below the detection limit after 30 minutes exposure to bile salt, however, when sampled after 60 minutes bile salt exposure 3.5 log cfu/ml cells were detectable.

When previously exposed to pH 3.5 simulated gastric juice, cells displayed enhanced resistance to bile salts compared to bile control cells ($p<0.05$). Aerobically grown cells displayed greater resistance towards bile salt than anaerobically grown cultures ($p<0.05$).

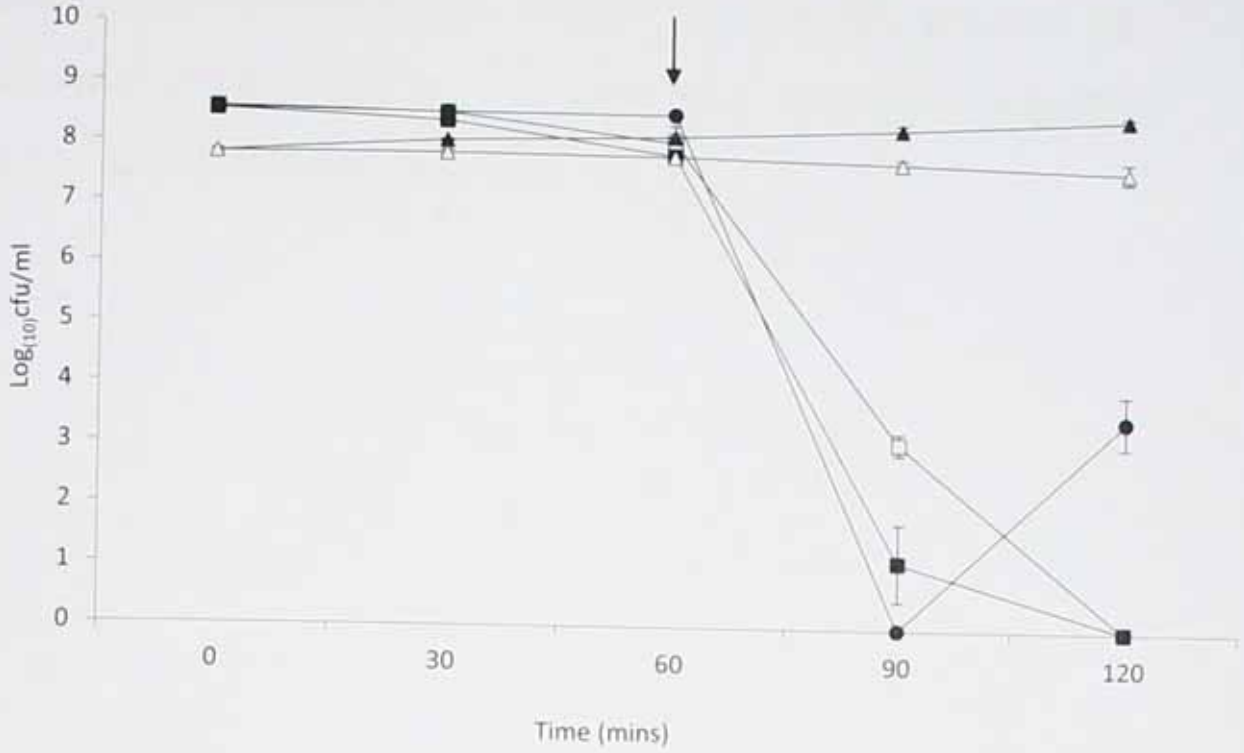

Fig. 6-12: Effect of aerobic (-□-) and anaerobic (-■-) pre-conditioning on the ability of stationary phase *L. monocytogenes* FSL R2-499 to survive short gastric transit at pH 2.5 (n=3 ± SEOM). PBS (-▲-), SGJ (-△-) and bile (-●-) controls were also included. Limit of detection= $3x10^2$cfu/ml. Arrow indicates neutralisation to pH 7 and addition of bile salts.

Unlike their exponential counterparts, cells grown to stationary phase were able to survive exposure to simulated gastric juice at pH 2.5 during short digestion. Pre-culture condition had no effect on cellular tolerance to pH 2.5 simulated gastric juice (p>0.05). While exposure to pH 3.5 simulated gastric juice enhanced resistance to bile salts (Fig. 6-12), pH 2.5 simulated gastric juice exposure did not confer resistance to bile salts. Following addition of bile salts a rapid decline in viable cells was seen in both aerobically and anaerobically pre-conditioned cells.

Thirty minutes post bile salt addition, aerobically pre-conditioned cells displayed enhanced resistance to bile salt exposure compared to

anaerobically grown cells and bile control cells ($p<0.05$). However, 60 minutes post bile salt exposure, 'test' cells were completely inactivated, while bile control cells had recovered.

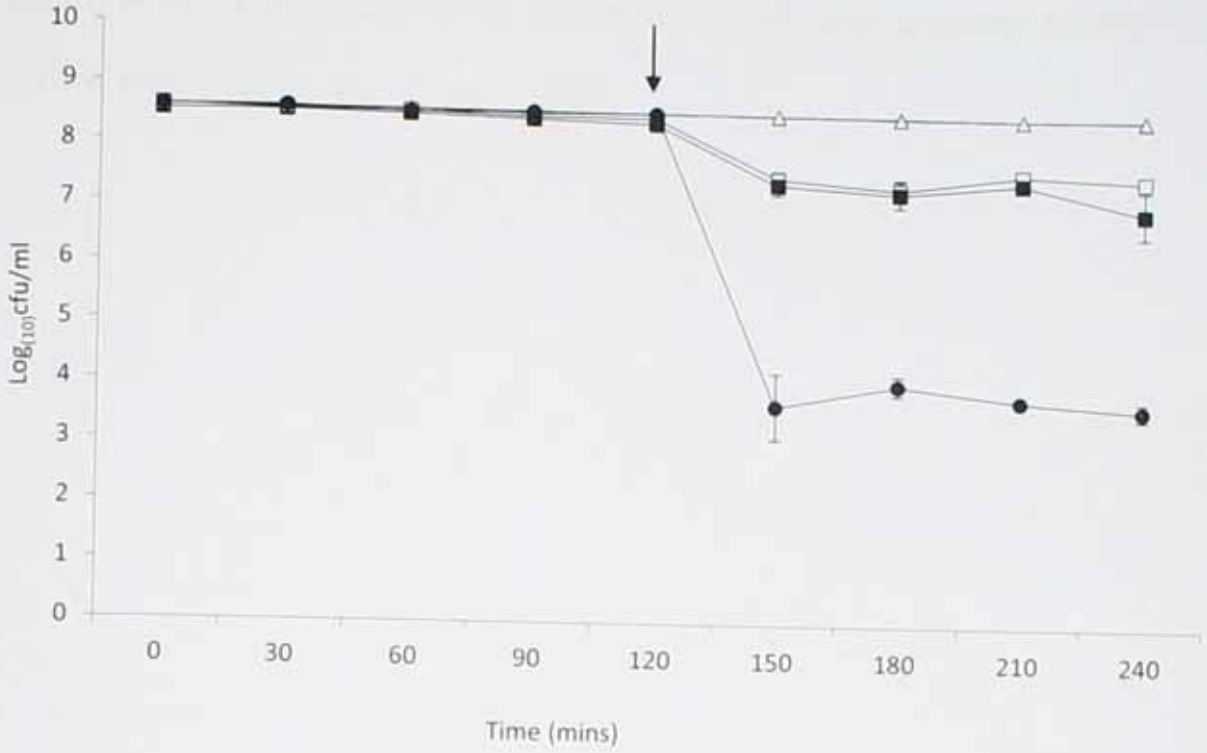

Fig. 6-13: Effect of aerobic (-□-) and anaerobic (-■-) pre-conditioning on the ability of stationary phase *L. monocytogenes* FSL R2-499 to survive long gastric transit at pH 3.5 (n=3 ± SEOM). PBS (-▲-), SGJ (-△-) and bile (-●-) controls were also included. Limit of detection= $3x10^2$cfu/ml. Arrow indicates neutralisation to pH 7 and addition of bile salts.

Stationary phase cells were able to survive prolonged exposure to pH 3.5 Simulated gastric juice with no significant reductions in cfu/ml occurring during the 120 minutes exposure prior to addition of bile salts ($p>0.05$). Pre-culture condition had no effect on pH 3.5 survival ($p>0.05$).

As with short digestion, cells exposed to pH 3.5 simulated gastric juice demonstrated significantly enhanced resistance to bile salts compared to bile control cells ($p<0.05$). Following a small decline in viable cells (~1 log cfu/ml) cells counts were maintained at approximately 7.5 and 7.4 log cfu/ml for aerobic and anaerobically grown cells respectively. These were significantly higher counts than those obtained from bile control cells

($p<0.05$) in which a decline of 5 log cfu/ml occurred upon addition of bile salts before stabilisation at ~4 log cfu/ml.

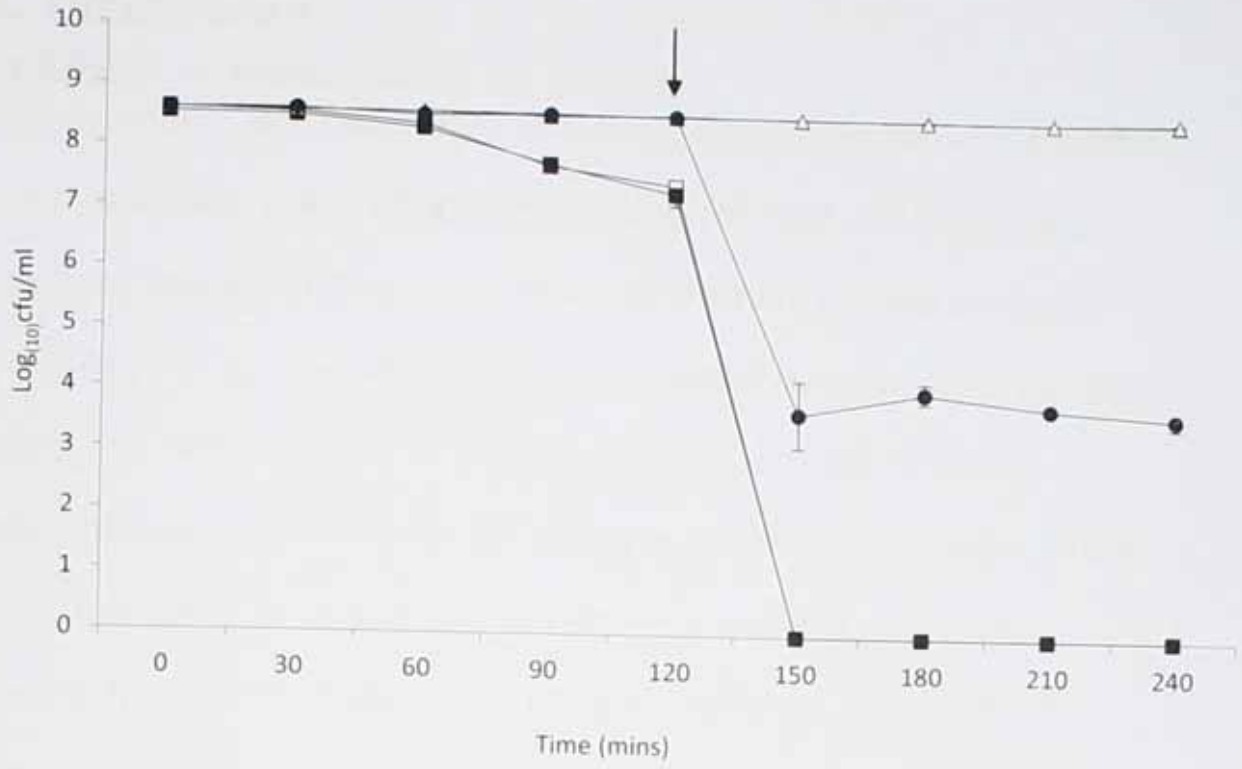

Fig. 6-14: Effect of aerobic (-□-) and anaerobic (-■-) pre-conditioning on the ability of stationary phase *L. monocytogenes* FSL R2-499 to survive long gastric transit at pH 2.5 (n=3 ± SEOM). PBS (-▲-), SGJ (-△-) and bile (-●-) controls were also included. Limit of detection= $3x10^2$cfu/ml. Arrow indicates neutralisation to pH 7 and addition of bile salts.

When subjected to simulated gastric juice at pH 2.5 for 120 minutes cell viability gradually decreased by approximately 1 log cfu/ml for both aerobically and anaerobically grown cells. After exposure to pH 2.5 simulated gastric juice, cells were unable to tolerate bile salts at 0.3% (w/v). Within 30 minutes of addition of bile salts cells were completely inactivated. Exposure to pH 2.5 SGJ significantly enhanced sensitivity of cells to bile salts (p<0.05) compared to bile control cells. No differences (p>0.05) were found between cells grown aerobically and anaerobically.

6.5. Discussion

6.5.1 Effects of Anaerobiosis on growth

L. monocytogenes is a facultative anaerobe, which may provide a problem for the food industry who often adopt the use of MAP and Controlled Atmosphere Storage (CAS) to increase the shelf life of food products (Macé *et al.*, 2013). By generating oxygen limiting conditions through the application of MAP/CAS, the growth of obligate aerobes, including potential pathogens and spoilage organisms is inhibited (McKillin, 2008). As such, the use of MAP and CAS is a growing trend across Europe, with a number of food commodities being transported under oxygen limiting conditions including meats, seafood and produce (Jacxsens *et al.*, 2010; Karabagias *et al.*, 2011; Macé *et al.*, 2012).

While the benefits of using MAP are clear (Galić *et al.*, 2011) its application does little to inhibit the growth of facultative organisms such as *L. monocytogenes*. Of interest to the food industry, the results presented in this chapter suggest that the use of oxygen limitation alone cannot be used to increase shelf life as growth parameters in *L. monocytogenes* FSL R2-499 were unaffected by such application (Fig. 6-2). No differences in length of lag phase, generation time or maximum cell density were observed. *L. monocytogenes* EGD-e growth was inhibited by anaerobic conditions (Fig. 6-1), which can be explained by a deletion in *NrdD*, a gene required for anaerobic respiration in *L. monocytogenes* (Ofer *et al.*, 2011). This inability to grow anaerobically is atypical for *Listeria* sp.

6.5.2 Induction of anaerobiosis-induced acid tolerance

Facultative anaerobes such as *L. monocytogenes* can adopt complex mechanisms to overcome oxygen limiting environments. These include metabolic adjustments (Lungu *et al.*, 2009), structural changes (Jydegaard-Axelson *et al.*, 2005) and altered transcription of several genes, including an increase in the expression of glutamate decarboxylase (GAD) (Cotter *et al.*, 2001).

The GAD system is crucial for acid survival in *L. monocytogenes*. The system operates by expending intracellular protons during the conversion of glutamate to γ-aminobutyrate (GABA). This decreases cytosolic pH (Cotter *et al.*, 2005). By means of an antiporter, GABA is then exchanged for extracellular glutamate, allowing more intracellular protons to be consumed and the toxic effects of acidification to be further alleviated (Cotter *et al.*, 2005).

The fact that the GAD system is important in anaerobic growth and acid tolerance suggests a cross protection between anaerobiosis and acid tolerance may exist. This is supported by the findings presented here whereby cells grown under anaerobic conditions displayed increased acid resistance when challenged by several acid types (Fig. 6-3 to 6-6). When challenged by both organic and inorganic acids, *L. monocytogenes* FSL R2-499 was considerably more resistant following anaerobic growth than when grown aerobically.

Given their potential to be used in combination during a hurdle regime, this anaerobiosis induced acid tolerance response is of importance to food manufacturers and should be considered during the development and application of food preservation measures.

6.5.3 The effects of an anaerobiosis-induced acid tolerance response on virulence potential

Acidity plays an important role in host immunity and offers protection against gastro-intestinal infection. The role of pH is highlighted by Cotter and Hill (2003) in which they describe stomach neutralisation by food and/or bicarbonate as causing increased *Listeria* survival throughout gastric transit. Furthermore elevated gastric pH is associated with listeriosis, with both antacid consuming populations and the elderly at greater risk (Garner *et al.*, 2006; Glupczynski, 1996). Gastric pH is largely age dependant, with elderly individuals possessing a resting pH of ~4.5 compared to ~2.5 in younger individuals. This difference is more pronounced after food consumption, where the gastric pH of the elderly remains unchanged while that of younger individuals reduces to around pH 1.7 (Dressman *et al.*, 1990; Russell *et al.*, 1993; Garner *et al.*, 2006). Failure of stomach pH to inactivate cells may account for subsequent infection from *L. monocytogenes* in pre-disposed individuals.

In this study *L. monocytogenes* cells were able to overcome pH 3.5 SGJ in both exponential and stationary phases of growth which may account for the listeriosis predisposition found in the elderly. However when subjected to pH 2.5 SGJ, exponential phase cells were unable to survive

even short gastric transit (120 minutes). Significant reductions in viable cells occurred during gastric juice exposure and any remaining cells were rapidly inactivated by exposure to bile salts. While anaerobiosis enhanced cellular tolerance to pH 2.5, this was not sufficient to allow survival throughout simulated gastric transit.

Exponential phase cells are commonly used to demonstrate adaptive responses arising from pre-conditioning, stress exposure or fluctuating environmental conditions. However, this is not necessarily representative of 'real-life' events, where most cells exist in a stationary phase state (Kolter *et al.*, 1993). This is important as numerous studies have reported acid stress to be influenced by growth phase (Arnold and Kaspar, 1995; Benjamin and Datta, 1995; King *et al.*, 2010). Similarly, this study found that when grown to stationary phase, *L. monocytogenes* cells demonstrated enhanced acid tolerance when challenged by both organic and inorganic acids. Furthermore, stationary phase cells were able to tolerate exposure to SGJ at pH 2.5 whereas exponential phase cells were readily inactivated. Upon entry into stationary phase several genetic and physiological changes occurs which generally deem cells more resistant to environmental stresses, including acid stress (Metselaar *et al.*, 2013; Sewell *et al.*, 2011). This phenomenon may arise from the induction of the GAD system upon entering stationary phase, and is beneficial to cells surviving gastric transit (Karatzas *et al.*, 2012; Metselaar *et al.*, 2013). Furthermore, pH 3.5 exposure enhanced the tolerance of stationary phase cells to bile salts.

Bile salts are vital for mammalian digestion, emulsifying fats and lipids. It is this capacity to disrupt lipids which make bile salts potent antimicrobials (Quillin *et al.*, 2011). The findings demonstrate that when grown to exponential phase *L. monocytogenes* cells were highly sensitive to *in vivo* concentrations of bile salts. Exponential phase cells were unable to survive gastric transit, regardless of pre-conditioning. However, when grown to stationary phase, cells readily overcame bile salt exposure. These findings are consistent with those of Begley *et al.* (2002) who also reported high sensitivity of exponential phase cells to bile with increased tolerance exhibited by stationary phase cells. In their study Begley *et al.* report that bile adaptation is seen in the presence of protein synthesis inhibitors, and as such cell wall structural changes are likely to be responsible for bile tolerance.

Many factors influence bacterial cell wall structure including biocide exposure, entry into stationary phase and low pH environments (Lou and Yousef, 1997; Martínez-Rodriguez and Mackey, 2005; Van Schaik *et al.*, 1999). These structural changes may account for the elevated bile resistance displayed by stationary phase cells which had been previously subjected to pH 3.5 SGJ. However, excessive pH exposure was found to be detrimental to bile salt tolerance and subsequent gastric transit survival. The findings of this study suggest that the ability of *L. monocytogenes* to survive gastric transit is a complex mechanism, involving several adaptations which alter the resistance properties of bacterial cells to host immune defences.

Further research, using additional strains is needed to determine if this response is strain specific or a generic response to growth under oxygen limitation. Furthermore, the molecular mechanisms underlying these responses is of interest to improve the understanding of the resistance and virulence profile of *L. monocytogenes*.

Chapter 7

The phenotypic and transcriptomic responses of *L. monocytogenes* FSL R2-499 to low temperature and anaerobiosis alone and in combination

7.1 Introduction

The psychotrophic properties of *L. monocytogenes* makes it particularly problematic for the food industry. The organism not only survives, but can grow at temperatures typically adopted by both the food industry and consumers to prevent food spoilage and minimise microbial load (Burall *et al.*, 2012). At temperatures below 0°C *L. monocytogenes* is able to multiply (Carpentier and Cerf, 2011), which compromises the safety of chilled RTE foods. A majority of cases of listeriosis arise from the consumption of chilled RTE foods (Ramaswamy *et al.*, 2007), where initial levels of *L. monocytogenes* are low. However, prolonged storage at refrigeration temperatures allows the microbial load to become sufficiently high to cause human disease (Chan and Wiedmann, 2009).

In an attempt to minimise the risk of food-related disease, food processors typically adopt the use of MAP alongside refrigerated storage to control microbial load (Sandhya, 2010). By reducing available oxygen through the use of vacuum packaging, oxygen absorbers, selectively permeable films or gas replacement it is possible to restrict the growth of both food spoilage and pathogens alike (McMillin, 2008). However, the deprivation of oxygen is only effective at inhibiting the growth of strictly aerobic organisms, for those which are anaerobes or facultative anaerobes the removal of oxygen is not an effective food control measure. Given that *L. monocytogenes* is both psychotrophic and a facultative anaerobe, the application of MAP in combination with refrigeration does not prevent growth in food products.

For food manufacturers, the ability of *L. monocytogenes* to overcome food processing hurdles, such as refrigeration and MAP, is of importance as they want to ensure they comply with legislation dictating tolerable microbial load, i.e. <100 cfu/g within the EU (EFSA, 2013). However, from a human health perspective the altered phenotypic properties arising from 'sub-optimal' environments is equally important.

Exposure to antimicrobials/food processing stresses (Lou and Yousef, 1997; Hill *et al.*, 2002; Vanlint *et al.*, 2013), harsh environments (Hahne *et al.*, 2010; Durack *et al.*, 2013) or antibiotic challenge (Raivio *et al.*, 2013; Andersson and Hughes, 2014) can lead to transcriptional adaptations. These may cause organisms to become stress hardened, making cells more resistant to antibiotic treatments or host defences, thus making them a greater risk to human health.

While the response of *L. monocytogenes* to low temperature is fairly well characterised and understood (Neales, 2004; Tasara and Stephan, 2006) our understanding of the adaptations and responses of *L. monocytogenes* to anaerobiosis/oxygen limitation are extremely limited (Lungu *et al.*, 2009). Therefore it was the aim of this chapter to investigate the effects of low temperature and anaerobiosis, alone and in combination, on the phenotypic response of *L. monocytogenes* to food-related antimicrobials. Furthermore, the transcriptional changes arising from adaptation to such environments was investigated in the hope of enhancing our understanding of the anaerobic response of *L. monocytogenes*.

7.2 Aims and Objectives

7.2.1 Aim

The aim of this chapter was to determine the effects of low temperature and anaerobic growth, both alone and in combination, on the resistance properties of *L. monocytogenes* FSL R2-499.

7.2.3 Objectives

- To determine the effects of low temperature and anaerobiosis, alone and in combination, on the growth of *L. monocytogenes*.
- To determine if phenotypic changes occur as a result of sub-optimal growth, with emphasis on resistance to antimicrobial agents.
- To elucidate the transcriptional mechanisms which permit growth under 'sub-optimal' conditions.
- To assess which transcriptional changes account for altered phenotypic properties using *in silico* analysis.

7.3 Methods

7.3.1 Generation and maintenance of 'test' environments

Anaerobic conditions were generated through the use of an anaerobic cabinet (Don Whitley Scientific Limited, West Yorkshire, UK). Anaerobic indicator strips were used to ensure anaerobic conditions were generated and maintained throughout testing (Oxoid, Basingstoke, UK). Test broths were pre-reduced overnight before use.

Low temperature environments (7°C) were achieved through the use of a refrigerated incubator.

For low temperature and anaerobic conditions in combination media were first pre-reduced overnight in an anaerobic cabinet. Upon inoculation with test cultures broths were transferred to anaerojars (Oxoid, Basingstoke, UK) containing anaerogen sachets (Oxoid, Basingstoke, UK). Cultures were then transferred to a refrigerated incubator (7±2°C). Anaerobic indicator strips were added to each jar to ensure anaerobic conditions were maintained.

7.3.2 Growth curves

L. monocytogenes FSL R2-499 was routinely cultured (section 2.2.1.6). Overnight cultures were prepared by addition of a single colony into 100ml of BHI broth followed by overnight incubation at 37°C under static, aerobic conditions.

Overnight cultures were diluted 1:100 (~5 x 10^5 cfu/ml) into pre-tempered and/or pre-reduced BHI broth and at regular intervals, 100µl aliquots were taken, serially diluted in PBS and plated by drop plate

method. Plates were air dried and total viable counts obtained after aerobic incubation at 37°C for 24 hours.

For low temperature and low temperature/anaerobic conditions samples were taken for the first 12 hours at two hourly intervals. Samples were then taken at 24 hours intervals.

7.3.3 Phenotypic analysis of pre-conditioned cells

Overnight cultures were diluted 1:100 into pre-tempered and/or pre-reduced BHI broth to give a final concentration of approximately 5×10^5 cfu/ml. Cells were grown to mid-exponential phase (as determined by growth curves) under pre-conditioning conditions. Upon reaching exponential phase 10ml aliquots were taken and subjected directly to citric acid, trisodium phosphate, H_2O_2 and NaClO at previously determined lethal concentrations. At regular intervals appropriate dilutions were made in BPW and plated using a spiral plater. Plates were air dried before 24 hours incubation at 37°C under aerobic conditions. Following incubation total viable counts were obtained and log reductions were calculated relative to time point '0'. Log reductions in pre-treated cells were compared to control cells grown to exponential phase under optimal conditions prior to antimicrobial challenge.

7.3.4 RNA extraction and purification

To elucidate the transcriptomic changes responsible for the altered phenotypic properties observed in cells following pre-conditioning, microarray analysis was conducted.

Upon reaching exponential phase under test conditions (anaerobiosis, refrigeration, anaerobiosis/refrigeration combined) 3ml aliquots of cultures were transferred into centrifuge tubes containing 2 volumes (6 ml) RNA Protect solution (Qiagen, Manchester, UK) for RNA stabilisation. Cells were pelleted by centrifugation (5000x *g*, 10 minutes, 4°C), supernatants discarded, and pellets resuspended in 700µl of TE buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA pH 8.0). Suspensions were transferred to matrix B lysis tubes (MP Biomedicals, UK) and underwent three rounds of mechanical disruption using a Fastprep device at speed setting 6 for 45 seconds. Tubes were briefly centrifuged to pellet the matrix and supernatant was transferred to a new microtube.

Extracted RNA was purified by use of RNeasy kits as per manufacturer's instructions. The optional on-column DNase step was omitted as contaminated DNA was eliminated using an amplification grade DNase kit (Sigma-Aldrich Company Ltd, UK)

7.3.5 Microarray analysis

Transcriptional changes occurring during pre-conditioning were assessed using a pan *Listeria* species microarray design kindly shared by the Bacterial Microarray Group at St George's (BµG@S). DNA microarrays were purchased from Agilent technologies (Agilent Technologies UK Ltd, Berkshire, UK) and, along with RNA samples, were sent to the Functional Genomics, Proteomics and Metabolomics Facility (University of Birmingham) for processing.

Prior to labelling and hybridisation RNA integrity was assessed using an Agilent Bioanalyser RNA 6000 Nano Kit (Agilent Technologies UK Ltd, Berkshire, UK). Samples yielding an RNA Integrity Number (RIN) of 7 or above were deemed un-degraded and were used for subsequent processing.

Fifty nanograms of good quality RNA (260/230 and 260/280 ratio ≥ 1.8) was then labelled using an Agilent Low Input Quick Amp WT kit as per manufacturer's instructions (5991-0879EN, 2013). Labelling efficiency and yield were assessed using a nandrop device, and samples yielding 600ng cRNA with a labelling efficiency of 6pmol/ugRNA were taken forward for hybridisation.

Labelled RNA was hybridised to the microarray slide using an Agilent Hybridisation Kit as per manufacturer's recommendations. Hybridisations were performed in an Agilent oven by baking for 18 hours at 65°C. Following baking, slides were washed and subsequently scanned on an Agilent scanner using the Agilent 1 colour HD setting for 15K arrays, using a scan resolution of 5µm.

The generated scan image was imported into Agilent Feature Extraction software and data analysis was performed.

Transcriptome data obtained from pre-conditioned cells was compared to control cells which had been grown under optimal conditions. Differences were assessed as relative fold change in transcript level.

7.3.6 Microarray validation by qRT-PCR

In order to validate the findings obtained by microarray, qRT-PCR was performed on cDNA generated from the same batch of RNA sent for microarray analysis.

PCR primers were designed to amplify fragments of several genes with altered transcript levels in pre-conditioned cells relative to untreated controls (Table 7-1). These targets were subjected to qRT-PCR and the correlation in fold change as determined by microarray and fold change as determined by qRT-PCR was calculated.

cDNA was reverse transcribed from RNA using a commercially available kit (as per section 4.3.4). Following reverse transcription, PCR efficiency and cDNA input range was determined as per section 4.3.5.1. qRT-PCR was conducted as in section 2.2.3.3 and changes in transcript level in pre-conditioned cells was calculated relative to untreated control cells.

Table 7-1: Primer sequences for qRT-PCR for validation of microarray data

Target	Fw. sequence (T_m)	Rv. sequence (T_m)	Amplicon Length (bp)
16s rRNA	TGGTAGTCCACGCCGTAAAC (65°C)	TCAACCTTGCGGTCGTACTC (65°C)	111
lmo2785	TCGCTAACAACCAACGTGAC (59°C)	TGCTCCGGTTCGATAAATGC (59°C)	121
lmo2016	AATTCAACGCTTTGACCTTCG (58°C)	AAGGCGGAGATGATGTATTCG (58°C)	85
lmo0690	GCTGCTGAAATGTCCGAAATG (58°C)	TTTGCGGTGTTTGGTTTGC (58°C)	88
lmo2363	ATCCAACATTCGCCACTTCC (59°C)	AATACCGCCTTCTTGCCAAC (59°C)	131
lmo2101	TGATGCCGCACTAATGATGG (58°C)	TGGCTTGAACAATCGCACTG (59°C)	169
lmo1007	ACCTGGTAAATACGAAGCTCTC (58°C)	CCACAGGGATTAAAGCCATAAG (57°C)	81
lmo1880	TCATTCGTCGGTGCGATTAG (58°C)	CTGCTGGATTTGGCTTTGTC (58°C)	151
lmo1055	TCCGATGCTTACCCAGAAAC (58°C)	GGCAGCCACTTCTTTGAAAC (58°C)	165
lmo2105	CAGGACTCGACGTACCAATG (58°C)	ACCTAGCGGAACGAGTAGTG (59°C)	80

No enzyme controls (RT-) and no template controls (NTC) were included in all assays. Furthermore, melt curve analysis was included to ensure specific amplification by PCR primers.

7.4 Results

7.4.1 Effects of refrigeration and anaerobiosis, alone and in combination, on growth of *L. monocytogenes* FSL R2-499

Table 7-2: Maximal growth rates of *L. monocytogenes* FSL R2-499 grown under various conditions

Condition	Maximal growth rate (μ_{max})*
37°C, aerobic	1.27 ± 0.05^{a}
37°C, anaerobic	1.01 ± 0.12^{a}
7°C, aerobic	0.07 ± 0.00^{b}
7°C, anaerobic	0.07 ± 0.00^{b}

*-μ_{max} values with the same superscripted letter are not significantly different ($p>0.05$).

Anaerobic conditions did not affect the maximal growth rate of *L. monocytogenes* FSL R2-499 ($p>0.05$). However, refrigeration significantly slowed maximal growth rate when compared to growth at 37°C ($p<0.05$).

Cells grown aerobically at 37°C had a μ_{max} of 1.27±0.05 h^{-1} which was comparable to the μmax in cells grown anaerobically at 37°C (1.01±0.12 h^{-1}) as determined by t.test ($p>0.05$). Cells grown aerobically at 7°C had a μ_{max} of 0.07± 0.00 h^{-1} compared to 0.07± 0.00 h^{-1} in cells grown anaerobically at 7°C ($p>0.05$).

7.4.2 Effects of anaerobic pre-conditioning on subsequent chemical treatment

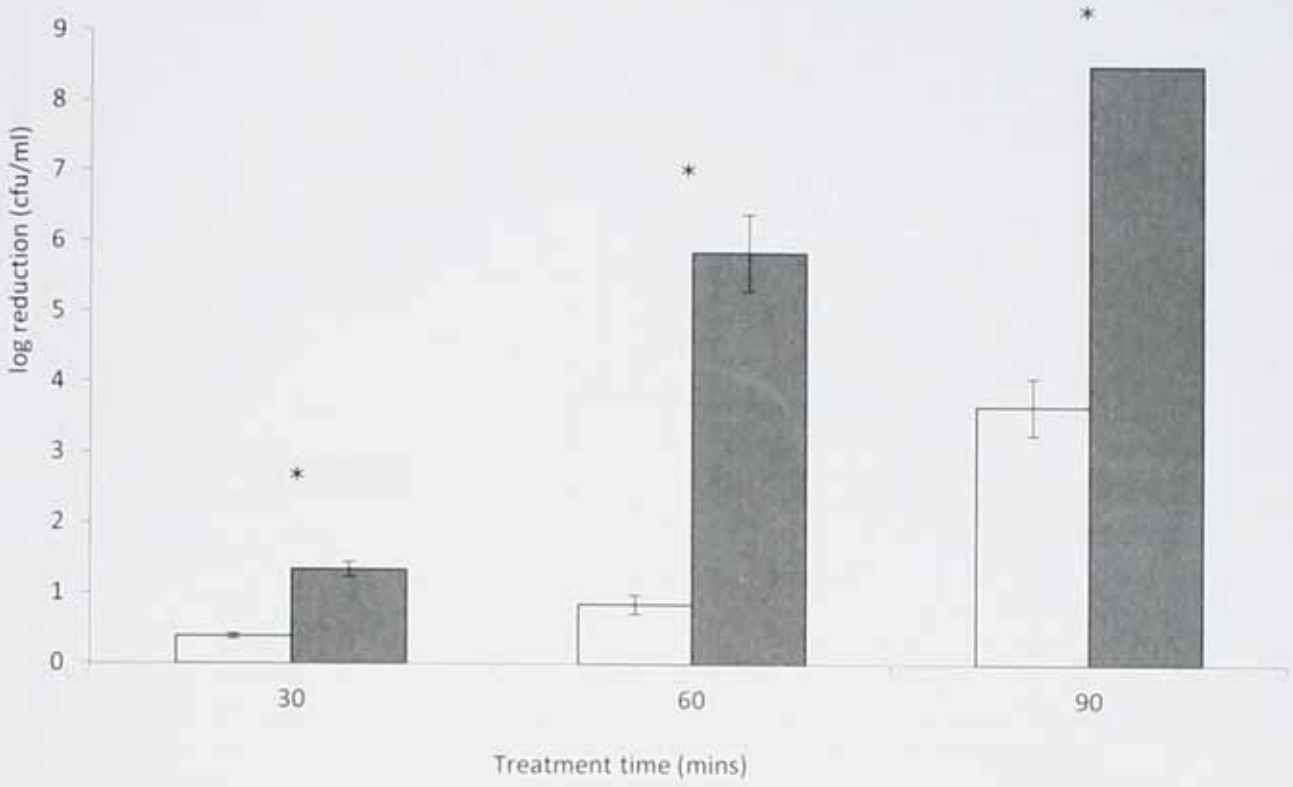

Fig. 7-1: Effects of anaerobic pre-conditioning on H_2O_2 sensitivity of *L. monocytogenes* FSL R2-499. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth under aerobic (open) or anaerobic (filled) conditions. Log reductions were calculated relative to time-point '0'. Asterisks indicate significant difference in log reduction (p<0.05).

Resistance properties of *L. monocytogenes* FSL R2-499 were altered following pre-conditioning in the absence of oxygen. When subjected to H_2O_2 cells which were subjected to anaerobic conditions displayed significantly enhanced sensitivity compared to those which were pre-conditioned under aerobic conditions (p<0.05) (Fig. 7-1). After 30 minutes there was ~1 log greater reduction in anaerobically pre-conditioned cells compared to aerobically pre-conditioned cells. This difference increased to ~5 log greater reduction after 60 and 90 minutes.

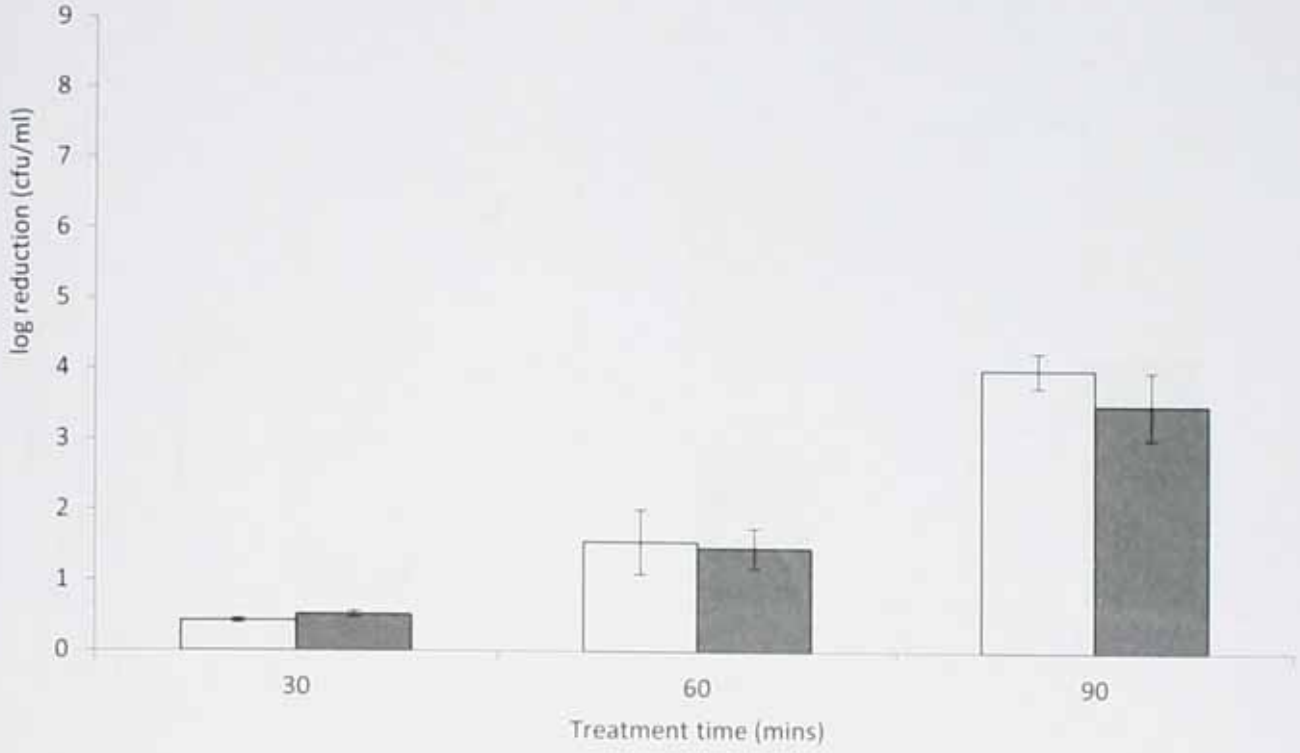

Fig. 7-2: Effects of anaerobic pre-conditioning on trisodium phosphate sensitivity of *L. monocytogenes* FSL R2-499. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth under aerobic (open) or anaerobic (filled) conditions. Log reductions were calculated relative to time-point '0'.

When challenged with trisodium phosphate anaerobic pre-conditioning had no effect on sensitivity relative to control cells which were grown in the presence of oxygen (p>0.05) (Fig. 7-2). Trisodium phosphate inactivation was time dependent, with approximately 0.5 log reduction after 30 minutes and 1.5 log reduction after 60 minutes. After 90 minutes there was ~3.5 log reduction in anaerobically pre-conditioned cells compared to ~4 log reduction in aerobically pre-conditioned cells, although this difference was not significant (p>0.05).

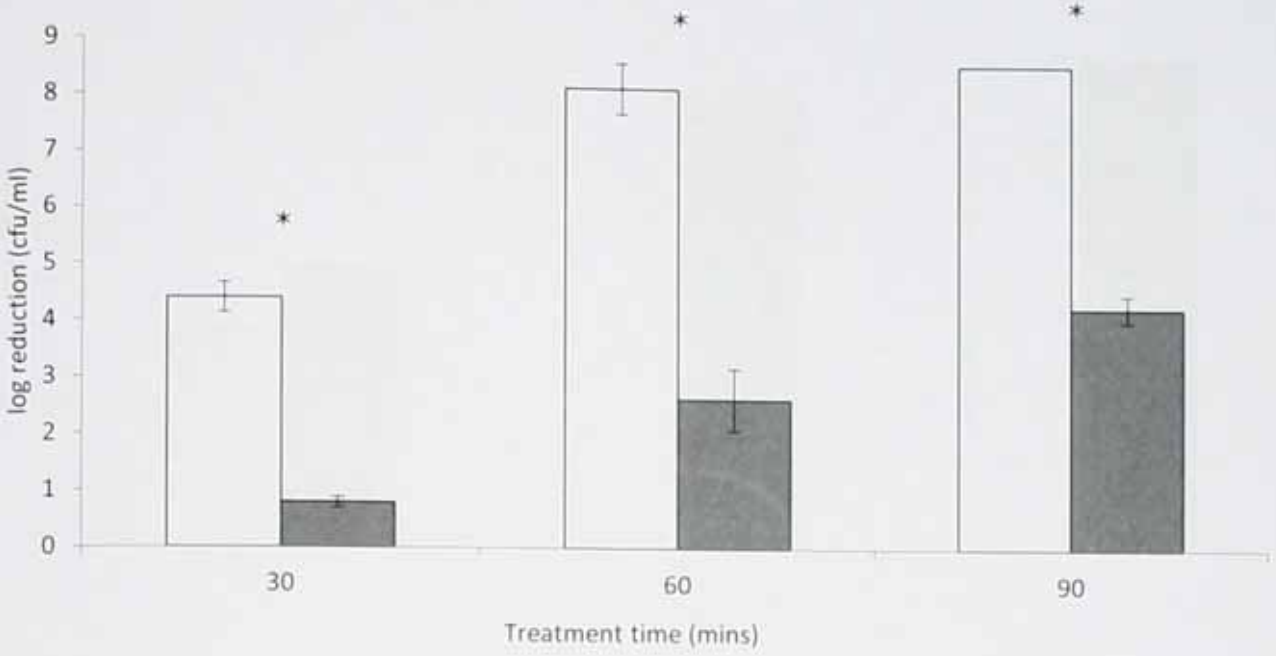

Fig. 7-3: Effects of anaerobic pre-conditioning on citric acid sensitivity of *L. monocytogenes* FSL R2-499. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth under aerobic (open) or anaerobic (filled) conditions. Log reductions were calculated relative to time-point '0'. Asterisks indicate significant difference in log reduction (p<0.05).

Following anaerobic pre-conditioning, cells displayed significantly enhanced resistance to citric acid exposure compared to aerobically pre-conditioned cells (p<0.05) (Fig. 7-3). After 30 minutes citric acid exposure, approximately 4 log greater reduction occurred in cells grown in the presence of oxygen compared to anaerobically grown cells. This increased to approximately 5.5 log greater reduction after 60 minutes. By 90 minutes citric acid exposure, cells which were pre-conditioned aerobically were no longer detectable while approximately 4 log cfu/ml cells remained viable following anaerobic pre-conditioning.

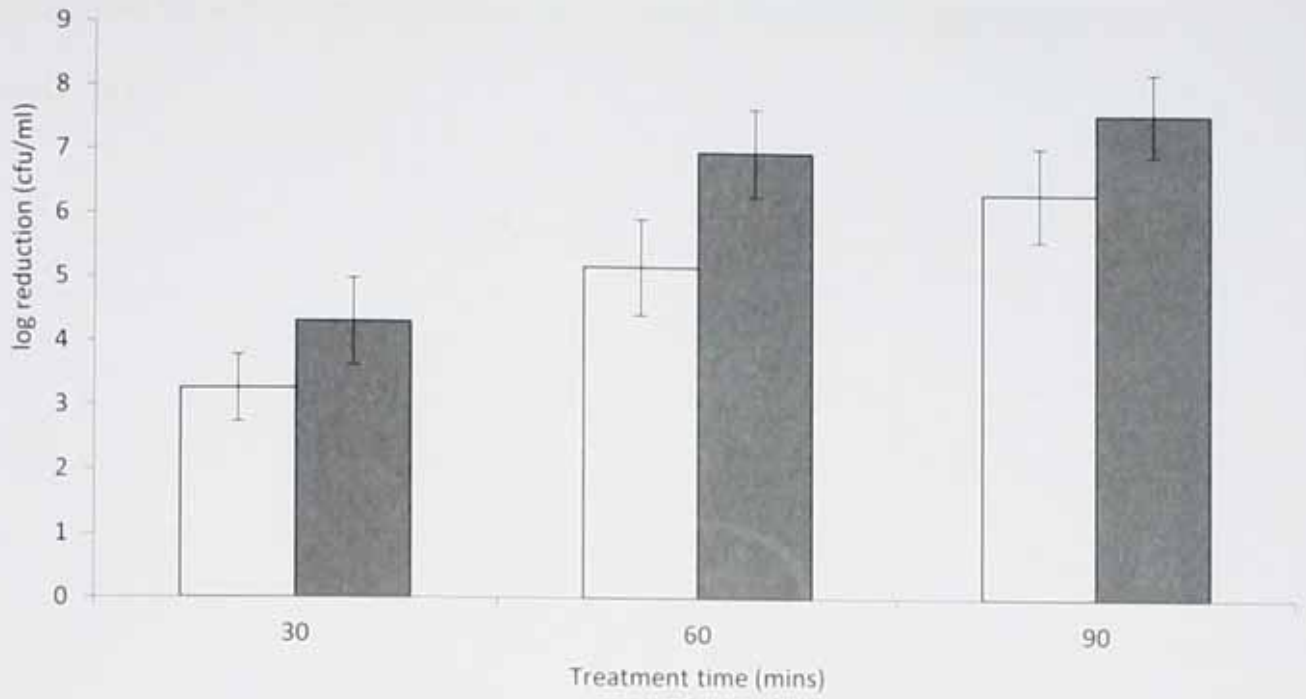

Fig. 7-4: Effects of anaerobic pre-conditioning on NaClO sensitivity of *L. monocytogenes* FSL R2-499. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth under aerobic (open) or anaerobic (filled) conditions. Log reductions were calculated relative to time-point '0'.

Following anaerobic pre-conditioning sensitivity to NaClO remained unchanged compared to aerobically pre-conditioned control cells (p>0.05) (Fig. 7-4). Inactivation by NaClO was time dependent, after 30 minutes there was ~3 log reduction in aerobically pre-conditioned cells compared to ~4 log reduction in anaerobically pre-conditioned cells; this difference was not significant (p>0.05). Following 60 minutes exposure ~5 log reduction was seen in aerobically pre-conditioned cells compared to ~7 log reduction in anaerobically pre-conditioned cells, again this was not found to be significant (p>0.05). After 90 minutes NaClO exposure, there was approximately a 6.5 log reduction in aerobically pre-conditioned cells

compared to ~7.5 log reduction in anaerobically pre-conditioned cells (p>0.05).

7.4.3 Effects of refrigeration on subsequent chemical exposure

When exposed to low temperatures *L. monocytogenes* FSL R2-499 displayed altered resistance properties when challenged with citric acid, trisodium phosphate, H_2O_2 and NaClO compared to cells grown at the 'optimal' temperature of 37°C.

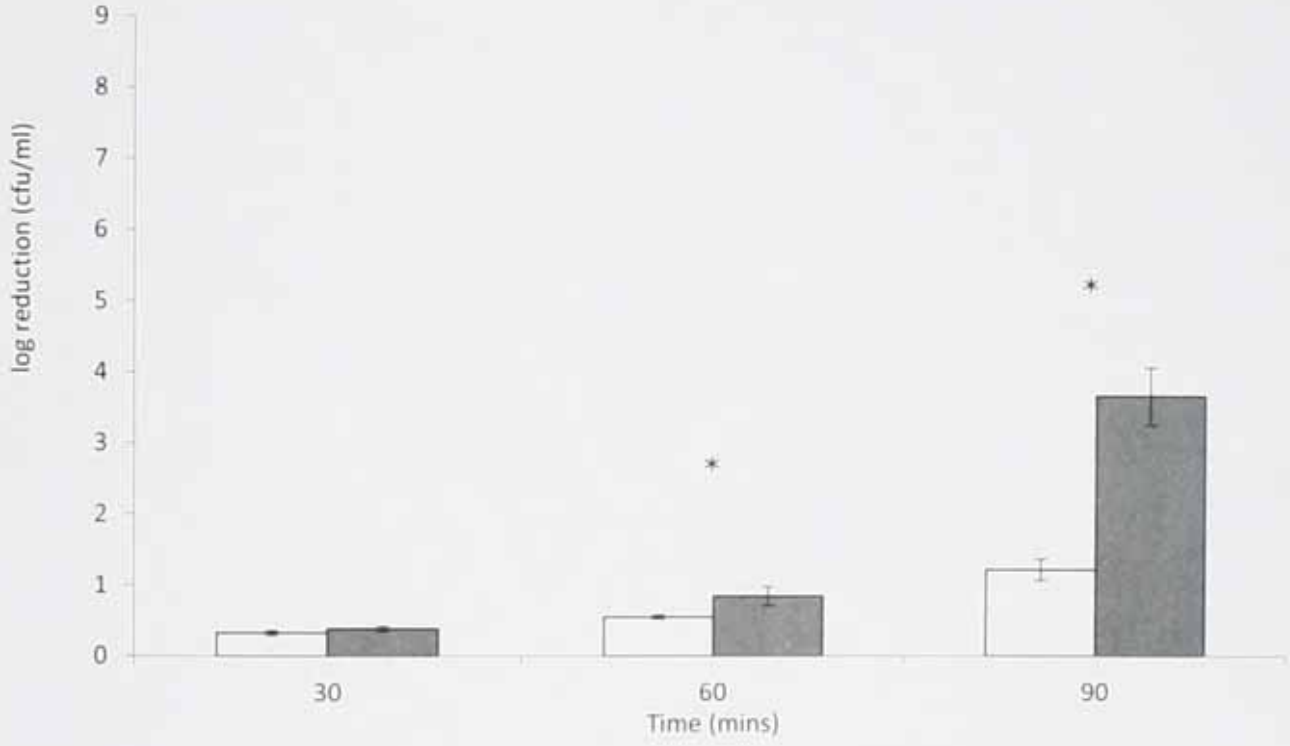

Fig. 7-5: Effects of refrigeration on H_2O_2 sensitivity of *L. monocytogenes* FSL R2-499. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth at 7°C (open) or 37°C (filled). Log reductions were calculated relative to time-point '0'. Asterisks indicate significant difference in log reduction (p<0.05).

When subjected to H_2O_2 cells pre-conditioned at 7°C displayed enhanced resistance compared with cells pre-conditioned at 37°C (Fig. 7-5). After 30 minutes exposure ~0.3 log reduction occurred in cells exposed to refrigeration temperature, while ~0.4 log reduction occurred in control

cells; this difference was not found to be significant ($p>0.05$). After 60 minutes of H_2O_2 exposure ~0.5 log reduction occurred in cells pre-conditioned at 7°C while ~1 log reduction was seen in cells pre-conditioned at 37°C ($p<0.05$). Finally, after 90 minutes of H_2O_2 challenge a 1.2 log reduction occurred in cells pre-conditioned at 7°C while a 3.7 log reduction occurred in control cells ($p<0.05$).

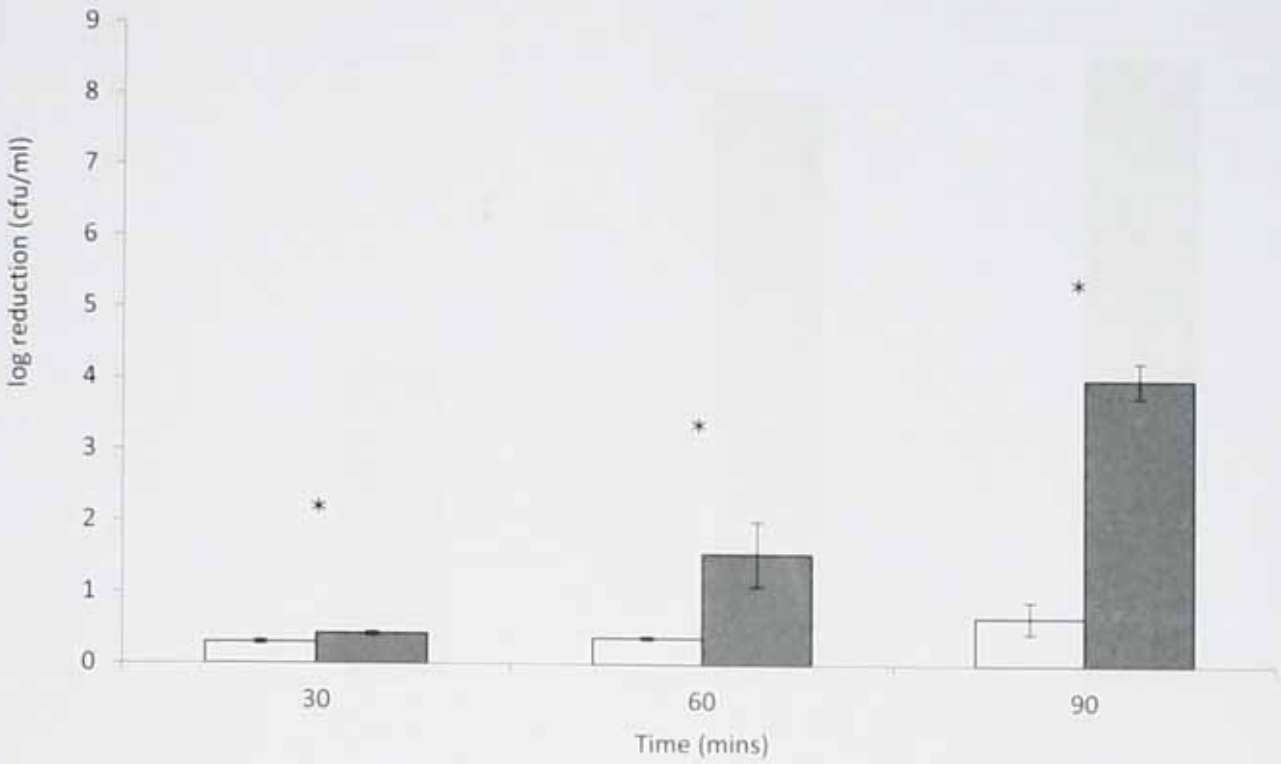

Fig. 7-6: Effects of refrigeration on trisodium phosphate sensitivity of *L. monocytogenes* FSL R2-499. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth at 7°C (open) or 37°C (filled). Log reductions were calculated relative to time-point '0'. Asterisks indicate significant difference in log reduction (p<0.05).

When subjected to trisodium phosphate, cells exposed to 7°C displayed increased resistance compared to those grown at 37°C (Fig. 7-6). After 30 minutes exposure ~0.3 log reduction occurred in cells pre-conditioned with low temperature compared to ~0.4 log reduction in 'control' cells (p<0.05). After 60 minutes exposure no further reduction occurred in cells pre-conditioned at 7°C (~0.3 log reduction), while approximately 1.5 log reduction occurred in control cells (p<0.05). When subjected to trisodium phosphate for 90 minutes, cells which were pre-conditioned at 7°C displayed log reductions of 0.6 while control cells had log reductions of ~4 (p<0.05).

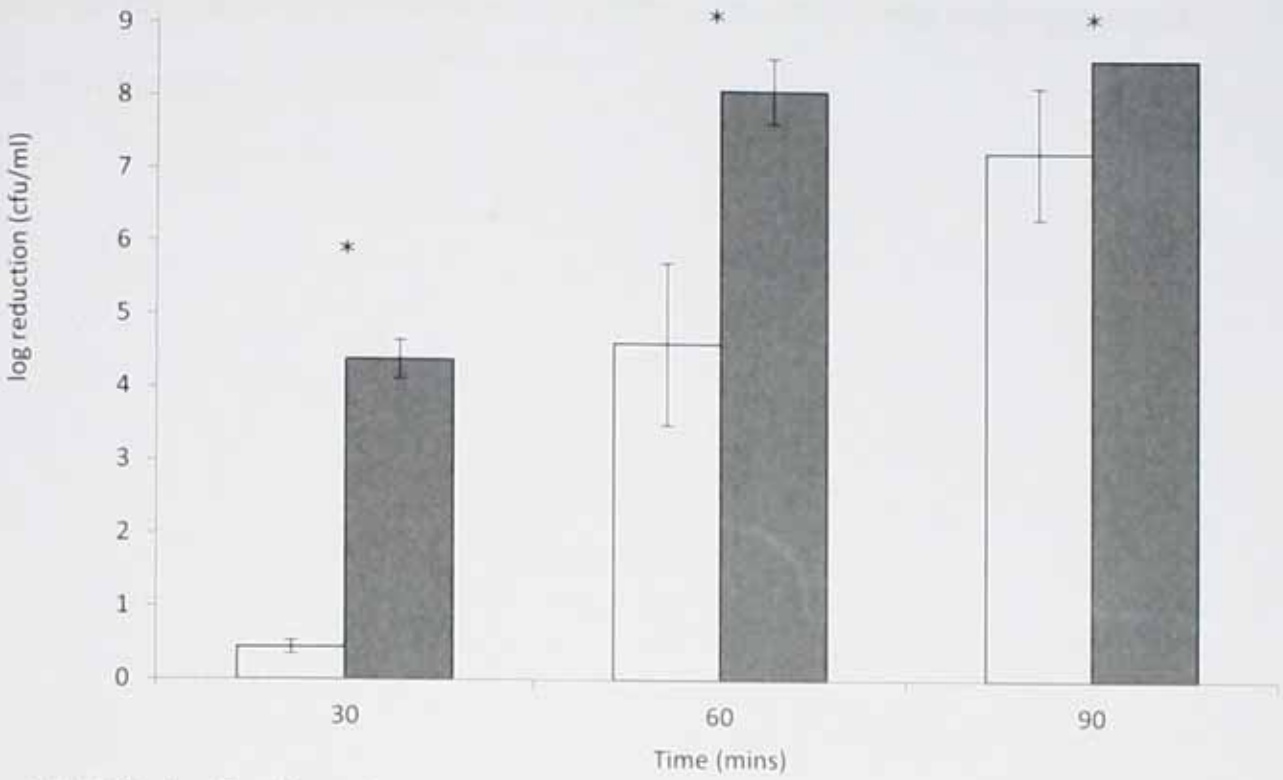

Fig. 7-7: Effects of refrigeration on citric acid sensitivity of *L. monocytogenes* FSL R2-499. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth at 7°C (open) or 37°C (filled). Log reductions were calculated relative to time-point '0'. Asterisks indicate significant difference in log reduction (p<0.05).

Citric acid resistance was significantly enhanced by low-temperature pre-conditioning (Fig. 7-7) (p<0.05). After 30 minutes citric acid exposure a 0.4 log reduction occurred in test cells compared to a >4 log reduction in control cells, this difference was determined as significant by t-test (p<0.05). After 60 minutes citric acid exposure ~4.6 log reduction occurred in cells pre-conditioned at 7°C, this was significantly less than the ~8.1 log reduction seen in control (p<0.05). Following subjection to citric acid for 90 minutes, a 7.3 log reduction occurred in test cells, while control cells were no longer detectable (log reduction ≥ 8.6), indicating a

significantly enhanced resistance to citric acid when cells were grown at low temperature ($p<0.05$).

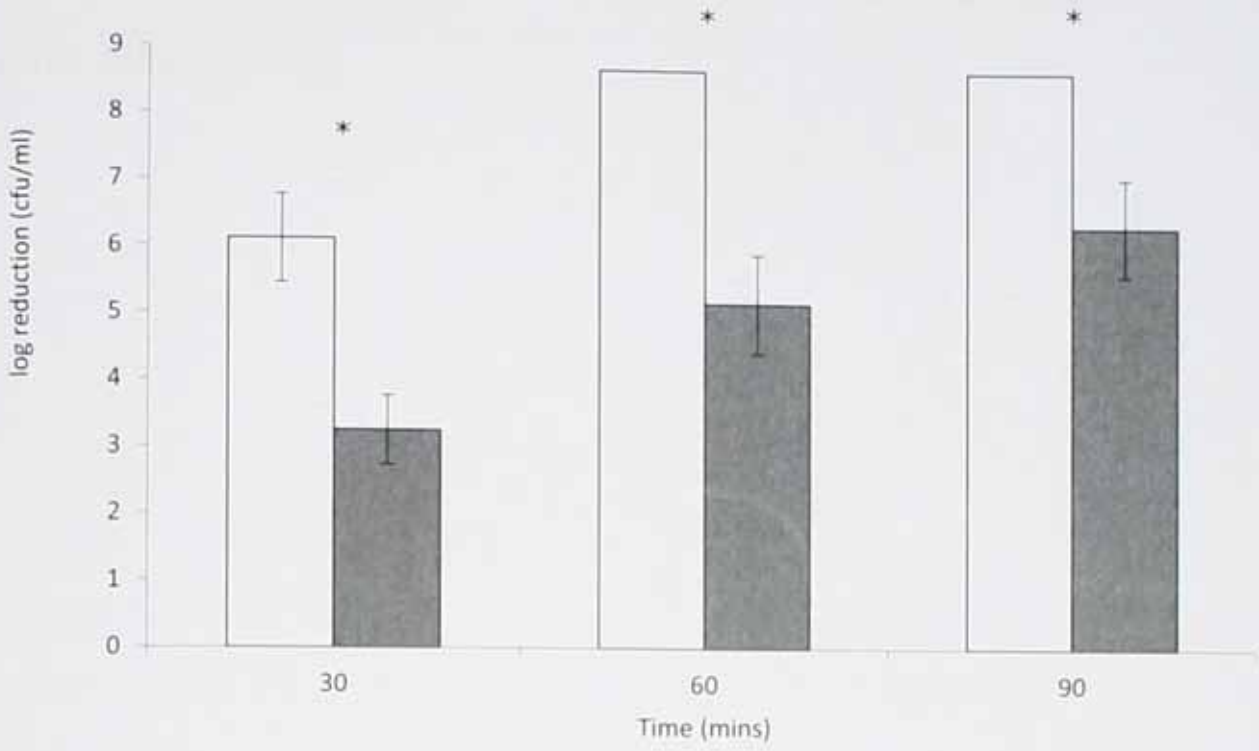

Fig. 7-8: Effects of refrigeration on NaClO sensitivity of *L. monocytogenes*. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth at 7°C (open) or 37°C (filled). Log reductions were calculated relative to time-point '0'. Asterisks indicate significant difference in log reduction (p<0.05).

When subjected to NaClO, low temperature pre-conditioning significantly decreased resistance compared to growth under optimal conditions (Fig. 7-8) (p<0.05). After 30 minutes exposure approximately 6 log reduction occurred in cells pre-conditioned at 7°C compared to ~3 log reduction in control cells (p<0.05). Following NaClO exposure for 60 minutes cells which had been pre-conditioned were no longer detectable (log reduction ≥ 8.6) compared with a 5.1 log reduction seen in control cells, indicating significantly reduced resistance following low temperature pre-conditioning (p<0.05). After 90 minutes pre-conditioned cells remained

undetectable while a log reduction of approximately 6.3 had occurred in control cells ($p<0.05$).

7.4.4 Combined effects of refrigeration and anaerobiosis on subsequent chemical exposure

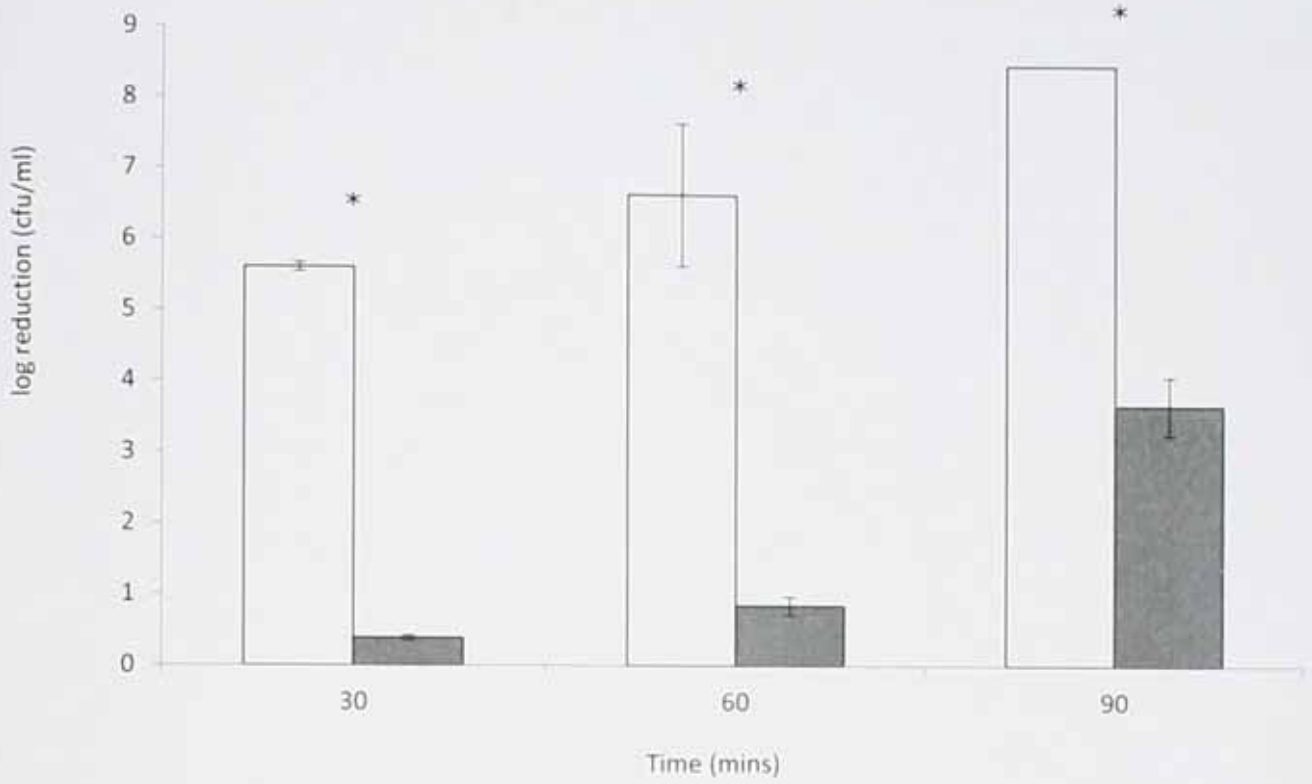

Fig. 7-9: Combined effect of refrigeration and anaerobiosis on H_2O_2 sensitivity of *L. monocytogenes*. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth in anaerobic conditions at 7°C (open) or in aerobic conditions at 37°C (filled). Log reductions were calculated relative to time-point '0'. Asterisks indicate significant difference in log reduction (p<0.05).

When cells were pre-conditioned in anaerobic conditions and at low temperature in combination cells displayed significantly enhanced sensitivity to H_2O_2 exposure compared to control cells (Fig. 7-9) (p<0.05). After 30 minutes exposure a >5 log reduction was seen in test cells while the log reduction in control cells was ~0.4 (p<0.05). After 60 minutes H_2O_2 exposure cells grown under test conditions had a log reduction of

6.6 while control cells had a log reduction of 0.8 ($p<0.05$). Finally, after 90 minutes exposure test cells were no longer detectable (log reduction ≥ 8.5) while control cells had a log reduction of 3.7 ($p<0.05$).

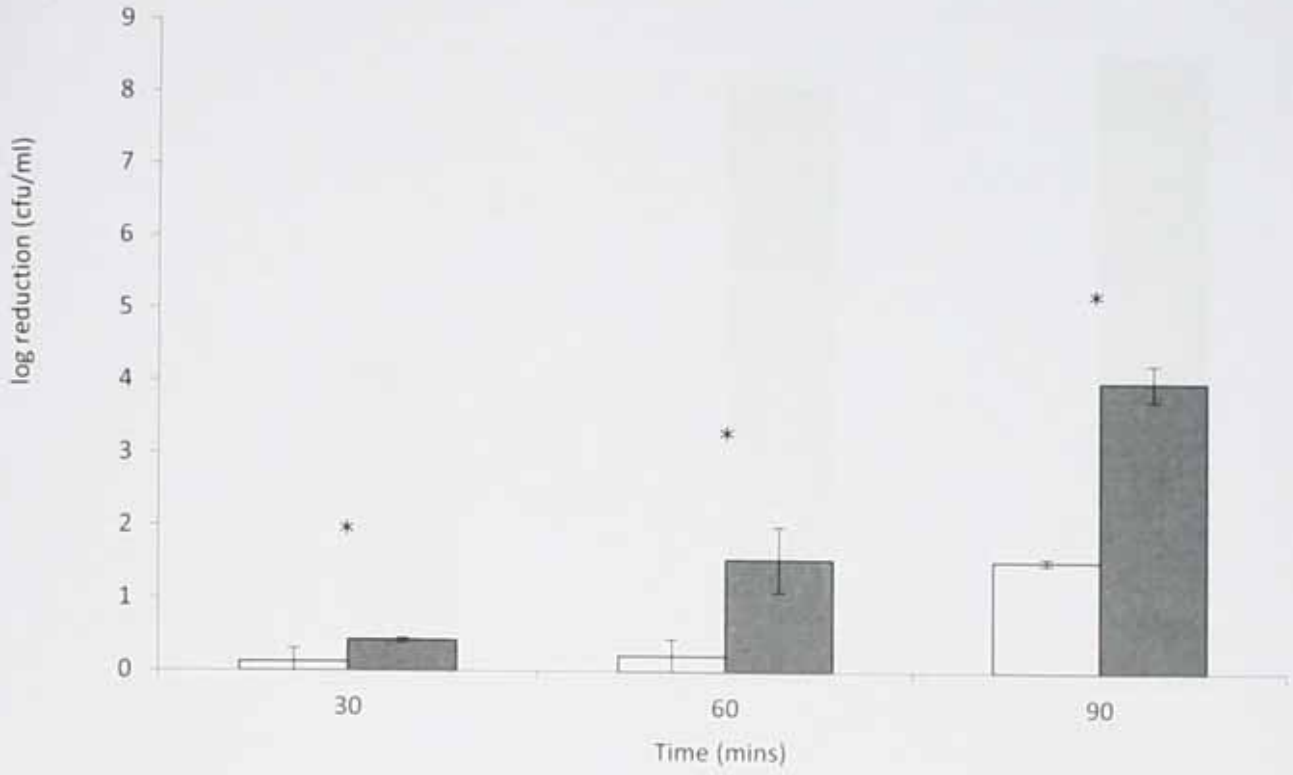

Fig. 7-10: Combined effect of refrigeration and anaerobiosis on trisodium phosphate sensitivity of *L. monocytogenes*. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth in anaerobic conditions at 7°C (open) or in aerobic conditions at 37°C (filled). Log reductions were calculated relative to time-point '0'. Asterisks indicate significant difference in log reduction (p<0.05).

Anaerobic, low temperature pre-conditioning significantly enhanced trisodium phosphate resistance compared to pre-conditioning under 'optimal' conditions (Fig. 7-10) (p<0.05). After 30 minutes exposure to trisodium phosphate there was a 0.1 log reduction in test cells compared to 0.4 log reduction in control cells. After 60 minutes this reduction increased to a 0.2 log reduction in test cells compared to a 1.5 log reduction in control cells. When subjected to trisodium phosphate for 90 minutes, a 1.5 log reduction occurred in test cells compared to a 4 log reduction in controls. These differences were all deemed significant as determine by t.test (p<0.05).

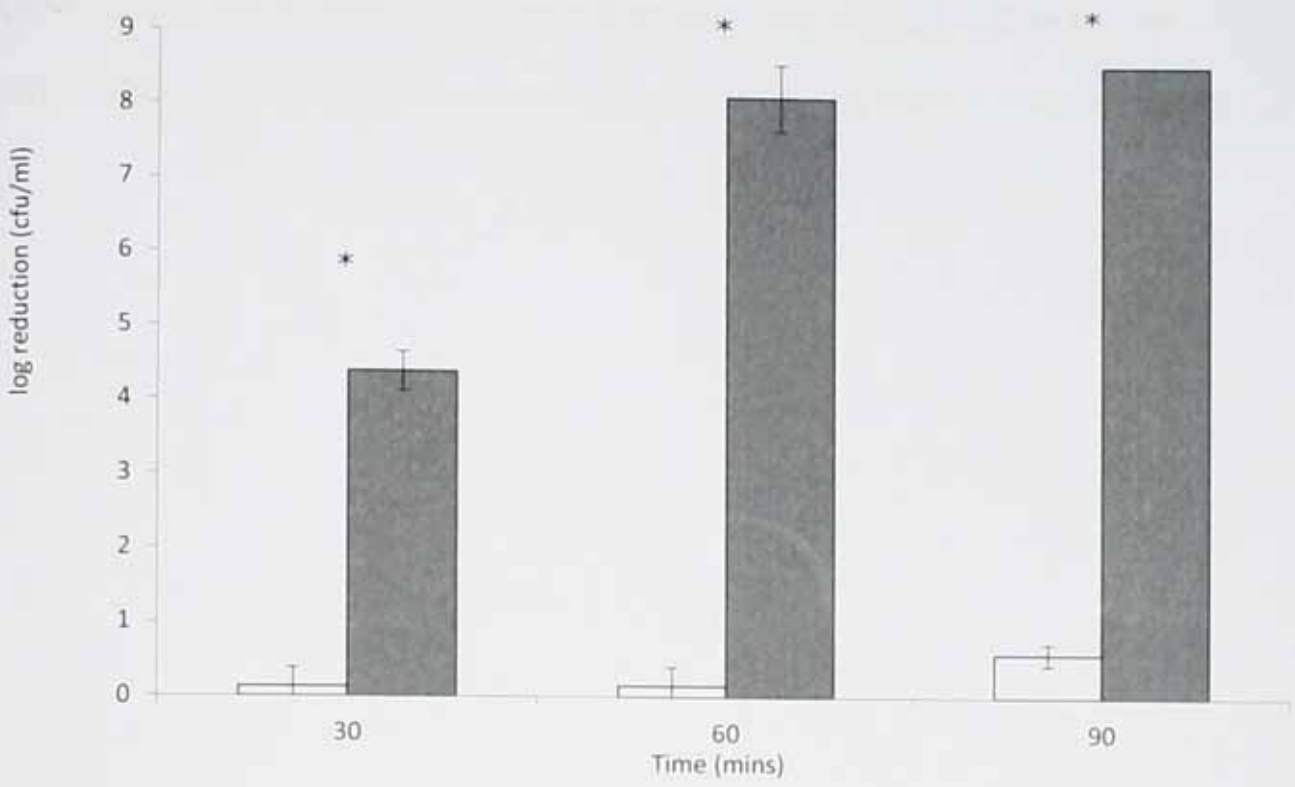

Fig. 7-11: Combined effect of refrigeration and anaerobiosis on citric acid sensitivity of *L. monocytogenes*. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth in anaerobic conditions at 7°C (open) or in aerobic conditions at 37°C (filled). Log reductions were calculated relative to time-point '0'. Asterisks indicate significant difference in log reduction (p<0.05).

Cellular resistance to citric acid resistance was enhanced by pre-conditioning under anaerobic, low temperature conditions compared to control cells (Fig. 7-11). After 30 minutes exposure, cells grown under test conditions reduced by 0.1 log reduction compared to ~4.4 log reduction in controls. Following subjection to citric acid for 60 minutes, cells pre-conditioned at low temperature under anaerobic conditions had a log reduction of 0.15 while those grown under 'optimal' conditions had a log reduction of approximately 8. Finally, after 90 minutes exposure to citric acid cells grown under test conditions showed a 0.6 log reduction

while control cells were no longer detectable (log reduction ≥ 8.6). All differences were statistically significant ($p<0.05$) as determined by t.test.

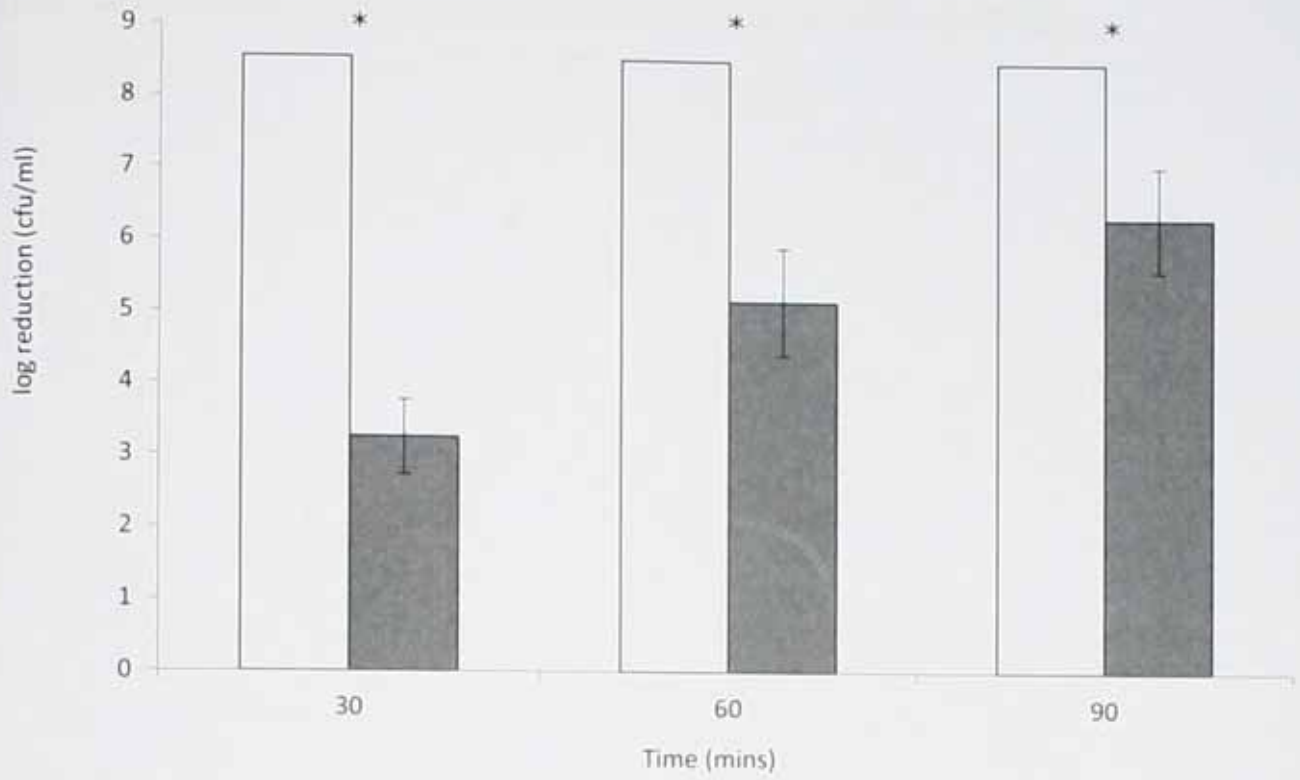

Fig. 7-12: Combined effect of refrigeration and anaerobiosis on NaClO sensitivity of *L. monocytogenes*. Error bars indicate the SEOM of three independent experiments plated in duplicate (n=3). Cells were pre-conditioned by growth in anaerobic conditions at 7°C (open) or in aerobic conditions at 37°C (filled). Log reductions were calculated relative to time-point '0'. Asterisks indicate significant difference in log reduction (p<0.05).

Cells which were pre-conditioned by growth at low temperature in the absence of oxygen where significantly more sensitive to NaClO exposure compared to control cells (p<0.05). Within 30 minutes of NaClO exposure, cells grown under oxygen limiting conditions at refrigeration temperature were inactivated to a level in which no cells could be detected (log reduction ≥ 8.5) while cells grown under optimal conditions were reduced by 3.2 log. After 60 minutes pre-conditioned cells remained undetected while reduction in control cells increased to 5.2 log. Finally, after 90 minutes exposure log reduction in control cells increased to ~6.4 while cells which had been pre-conditioned remained undetectable. T.test

analysis revealed that all differences were statistically significant ($p<0.05$).

7.4.5 Transcriptomic responses to anaerobic conditions

Several genes had significantly altered transcript levels in response to anaerobic growth compared to those grown aerobically ($p<0.05$). Fifteen genes were found to be positively regulated by greater than two-fold under anaerobiosis when compared to aerobic controls. These included genes concerned with amino acid transport (*lmo2362*), glutamate metabolism (*lmo2361* and *lmo2363*), transcription regulation (*lmo1251*), protein folding (*lmo0903*) and cell envelope biogenesis (*lmo1999*). As well as these, hydrolytic enzymes (*lmo2567*), virulence determinants (*lmo1388* and *lmo0725*) and several genes with unknown functions were up-regulated.

Over 100 genes were significantly down-regulated by at least two-fold in response to anaerobic growth ($p<0.05$). These included several genes associated with stress response (*lmo2673*, *lmo1422*, *lmo2067*, *lmo2436*, *lmo2067*, *lmo2152*, *lmo1427* and *lmo1428*), virulence (*lmo0171*, *lmo2085*, *lmo2463*, *lmo0434*, *and lmo0943*), energy production/maintenance (*lmo1293*, *lmo2717*, *lmo0014*) and metabolic processes (*lmo0784*, *lmo0782*, *lmo0781*, *lmo1421*, *lmo0783*, *lmo1421 and lmo1539*).

Table 7-3: Selected stress response and virulence genes with altered transcript levels during anaerobic growth relative to 'control' conditions.

Locus Id	Fold change	Adjusted *p* value	Function
lmo2363	+10.48	<0.000	Glutamate decarboxylase- response to low pH, virulence
lmo2362	+12.74	<0.000	Glutamate decarboxylase- response to low pH, virulence
lmo2361	+2.70	<0.000	Glutamate decarboxylase- response to low pH, virulence
lmo0903	+2.75	0.05	Post translational regulator/chaperone-stress response
lmo1251	+2.39	<0.000	Fnr/Crp family transcriptional regulator- stress response
lmo1388	+2.17	<0.000	Antigenic lipoprotein- virulence
lmo0725	+2.05	0.003	Peptidoglycan binding protein- virulence determinant
lmo2067	-2.03	<0.000	Bile acid hydrolase- stress response, virulence
lmo1422	-2.03	<0.000	Osmoprotection, cell envelope biosynthesis
lmo0171	-2.06	0.007	Internalin- virulence
lmo1439	-2.14	<0.000	Superoxide dismutase- Antioxidant, virulence
lmo2463	-2.24	<0.000	Multidrug transporter- Virulence and resistance
lmo0610	-2.78	<0.000	Internalin like protein- virulence
lmo0434	-3.03	0.004	Internalin B- invasion and virulence
lmo1425	-3.84	<0.000	Glycine-betaine transporter- osmotolerance/stress response
lmo1428	-4.01	<0.000	opuCA- stress response
lmo0263	-4.12	<0.000	Internalin H- invasion and virulence

lmo0669	-4.26	<0.000	Oxidoreductase- stress response and virulence
lmo2673	-5.91	<0.000	Universal stress protein (USP) like protein- stress response
lmo2785	-27.30	<0.000	Catalase- oxidoreductase, stress response and virulence

7.4.6 Transcriptomic responses to low temperature environments

Extensive changes in the transcriptome of *L. monocytogenes* FSL R2-499 occurred in response to low temperature growth. A total of 439 genes had altered two fold or greater changes in transcript level, which were also statistically significant ($p<0.05$), 118 of these were down-regulations while the remaining 321 were up-regulations in response to low temperatures.

Genes with significant ($p<0.05$) up-regulations in response to low temperatures included those concerned with flagellation/motility (*lmo0690*, *lmo0699*, *lmo0696*, *lmo0693*, *lmo0697*, *lmo0698*, *lmo0700*, *lmo0686*, *lmo0685*, *lmo0705*), transcriptional regulation (*lmo1251*, *lmo0570*, *lmo2766*, *lmo2436*, *lmo0599*, *lmo2243*) and chemotaxis (*lmo0699*, *lmo0691*, *lmo1699*, *lmo0689*, *lmo0723*).

As predicted, genes concerned with heat shock were significantly down-regulated in response to growth at low temperatures. These included the heat shock proteins GrpE (*lmo1474*), GroES (*lmo2069*) and hrcA (*lmo1475*). As well as these, genes regulating DNA structure and topology were also down-regulated (*lmo1412*, *lmo1473*), as were stress response genes concerned with camphor resistance (*lmo2082* and *lmo2081*). Low temperature also caused down-regulation in the key virulence genes clpE (*lmo0997*) and Listeriolysin O precursor (*lmo0202*).

Table 7-4: Selected stress response and virulence genes with altered transcript levels during low temperature growth relative to 'control' conditions.

Locus Id	Fold Change	Adjusted *p* value	Function
lmo0690	+249.6985	<0.000	(*flaA*) flagella component- motility, virulence
lmo1250	+107.9432	<0.000	Major facilitator protein family- membrane transport, antibiotic resistance, virulence
lmo0699	+95.96173	<0.000	Flagella component- chemotaxis, cell motility, secretion, virulence
lmo0697	+71.21956	<0.000	*flgE* – Flagella hook protein- chemotaxis, cell motility, secretion, virulence
lmo1251	+11.166	<0.000	Fnr/Crp family transcriptional regulator
lmo1364	+10.97631	<0.000	cspL- cold-shock protein
lmo1864	+3.84943	<0.000	Similar to hemolysin III proteins- virulence
lmo0835	+4.303034	<0.000	Peptidoglycan binding protein- virulence determinant
lmo2219	+3.079187	<0.000	Similar to foldase protein (PrsA)- pathogenesis
lmo1460	+3.18619	<0.000	Similar to *B. subtilis* RecO protein- DNA repair and recombination
lmo0211	+3.41143	<0.000	ctc- similar to *B. subtilis* general stress protein
lmo0599	+2.620084	<0.000	PadR family transcriptional regulator
lmo1388	+2.45082	0.048	tcsA- T cell-stimulating antigen- virulence
lmo1428	+2.308824	<0.000	opuCA- stress response
lmo0640	+2.184319	<0.000	Similar to oxidoreductase
lmo2081	-2.18127	<0.000	Camphor resistance protein CrcB

lmo1475	-2.43049	<0.000	hrcA- heat-inducible transcription repressor
lmo1474	-2.44271	<0.000	heat shock protein GrpE (Molecular chaperone)- general stress response
lmo0790	-2.66855	<0.000	Similar to putative transcription regulator
lmo0202	-2.56287	<0.000	Hly- Listeriolysin O precursor- virulence
lmo2069	-4.0716	<0.000	Co-chaperonin GroES- general stress response
lmo0997	-6.457	<0.000	clpE- ATP-dependent protease- virulence
lmo1473	-2.78544	<0.000	Molecular chaperone DnaK

7.4.7 Transcriptomic responses to anaerobiosis and low temperature in combination

Growth under anaerobiosis at low temperature caused altered expression in many genes. Of these 461 were altered by two-fold or greater and were statistically significant compared to controls which had been grown under 'optimal' conditions ($p<0.05$).

Growth under oxygen limiting conditions at refrigeration temperatures resulted in up-regulations in 357 genes. Of these up-regulations, several genes were associated with flagella production and cellular motility (*lmo0690*, *lmo0693*, *lmo0696*, *lmo0697*, *lmo0679*, *lmo0680*, *lmo0686*, *lmo0685*), chemotaxis (*lmo0699*, *lmo0683*, *lmo0691*, *lmo1699*, *lmo0689*) and transcriptional regulation (*lmo1251*, *lmo2436*, *lmo2766*, *lmo0597*, *lmo0599*, *lmo2243*). In addition to these functional groups, genes associated with membrane transport (*lmo1250*), stress response (*lmo0211*, *lmo1172*, *lmo2243*, *lmo1428*, *lmo1427*, *lmo1426*), DNA repair/topology (*lmo1275*, *lmo1460*, *lmo1302*) and virulence (*lmo0117*, *lmo0433*, *lmo1864*).

The remaining 104 genes which showed significantly altered transcript levels were down-regulations. These included heat shock induced chaperones GrpE and GroES (*lmo1474*, *lmo2069*), the heat induced transcriptional repressor hrcA (*lmo1475*) and several genes with roles in membrane transport (*lmo1210*, *lmo1671*, *lmo2254*, *lmo0153*, *lmo1849*, *lmo2569*, *lmo1848*). Unexpectedly, cold shock protein B, CspB

(*lmo2016*), showed the greatest decrease in fold change in response to growth at low temperature and anaerobiosis.

Table 7-5: Selected stress response and virulence genes with altered transcript levels during anaerobic/low temperature growth relative to 'control' conditions.

Locus Id	Fold change	Adjusted *p* value	Function
lmo1250	+190.8599	<0.000	Major facilitator protein family- membrane transport, antibiotic resistance, virulence
lmo0690	+101.2387	<0.000	(*flaA*)- flagella component- motility, virulence
lmo0699	+49.11214	<0.000	Flagella component- chemotaxis, cell motility, secretion, virulence
lmo0117	+45.03744	<0.000	*lmaB* – *Listeria* antigen - virulence
lmo0691	+31.8334	<0.000	*cheY* – chemotaxis response regulator – motility, virulence, chemotaxis
lmo2363	+18.64863	<0.000	Glutamate decarboxylase- response to low pH, virulence
lmo1251	+9.701428	<0.000	Fnr/Crp family transcriptional regulator
lmo1364	+8.760029	<0.000	cspL- cold-shock protein
lmo0433	+6.113946	<0.000	(inlA) Internalin A- virulence
lmo1864	+5.290748	<0.000	Similar to hemolysin III protein- virulence
lmo0211	+4.836132	<0.000	ctc- similar to *B. subtilis* general stress protein
lmo0540	+5.689453	<0.000	Similar to penicillin-binding protein- virulence
lmo0640	+3.08885	<0.000	Similar to oxidoreductase
lmo2818	+2.942437	<0.000	Multidrug resistance protein

lmo2766	+2.706593	<0.000	Hypothetical transcriptional regulator
lmo1460	+2.858685	<0.000	Similar to *B. subtilis* RecO protein-DNA repair and recombination
lmo1428	+2.837483	<0.000	opuCA- stress response
lmo2766	+2.706593	<0.000	Similar transcriptional regulator
lmo1427	+2.184902	0.001	opuCB- osmotic stress response
lmo1302	+2.161337	0.044	LexA repressor- SOS response
lmo1413	-2.22985	<0.000	Peptidoglycan binding protein-virulence
lmo1475	-2.25596	0.001	hrcA- heat-inducible transcription repressor
lmo0790	-2.29827	0.001	Putative transcription regulator
lmo1474	-2.60951	<0.000	Heat shock protein GrpE (molecular chaperone)
lmo1974	-2.96823	0.001	GntR family transcriptional regulator
lmo2069	-5.507	<0.000	Co-chaperonin GroES- General stress response
lmo0997	-6.74303	<0.000	clpE- ATP-dependent protease-Virulence
lmo2785	-27.7332	<0.000	Catalase- Oxidoreductase, stress response and virulence

7.4.8 Validation of Microarray data by qRT-PCR

To validate the transcriptional changes as determined by microarray analysis qRT-PCR was conducted on cDNA generated from the RNA used for microarrays.

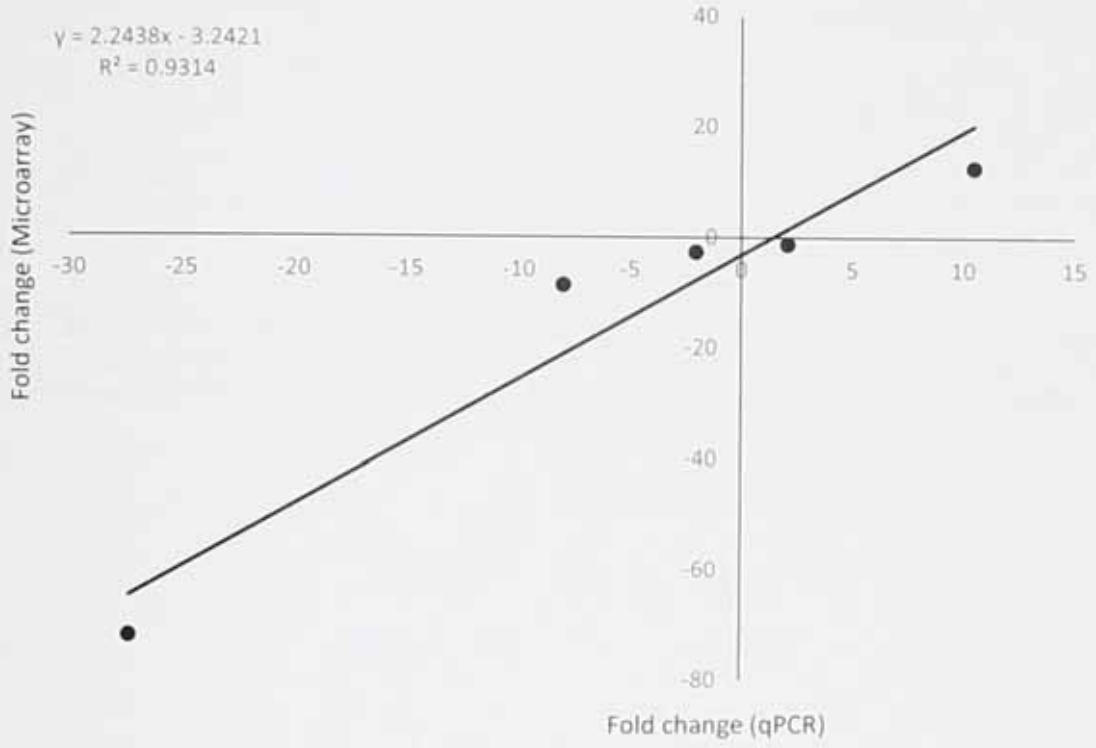

Fig. 7-13: Validation of microarray data by qRT-PCR (37°C, anaerobic conditions vs. 37°C, aerobic conditions). Data points represent the mean fold change of an individual target determined by qRT-PCR and microarray (n=4). qRT-PCR was performed in triplicate.

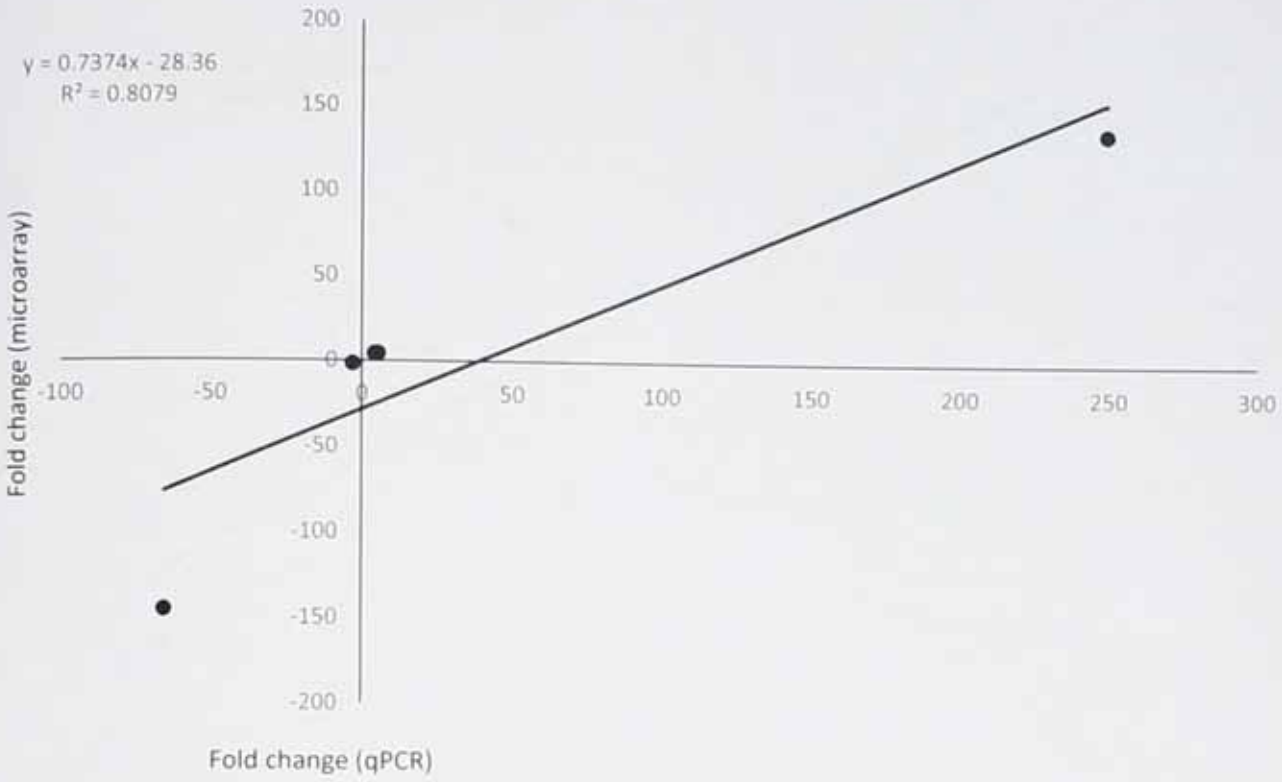

Fig. 7-14: Validation of microarray data by qRT-PCR (8°C, aerobic conditions vs. 37°C, aerobic conditions). Data points represent the mean fold change of an individual target determined by qRT-PCR and microarray (n=4). qRT-PCR was performed in triplicate.

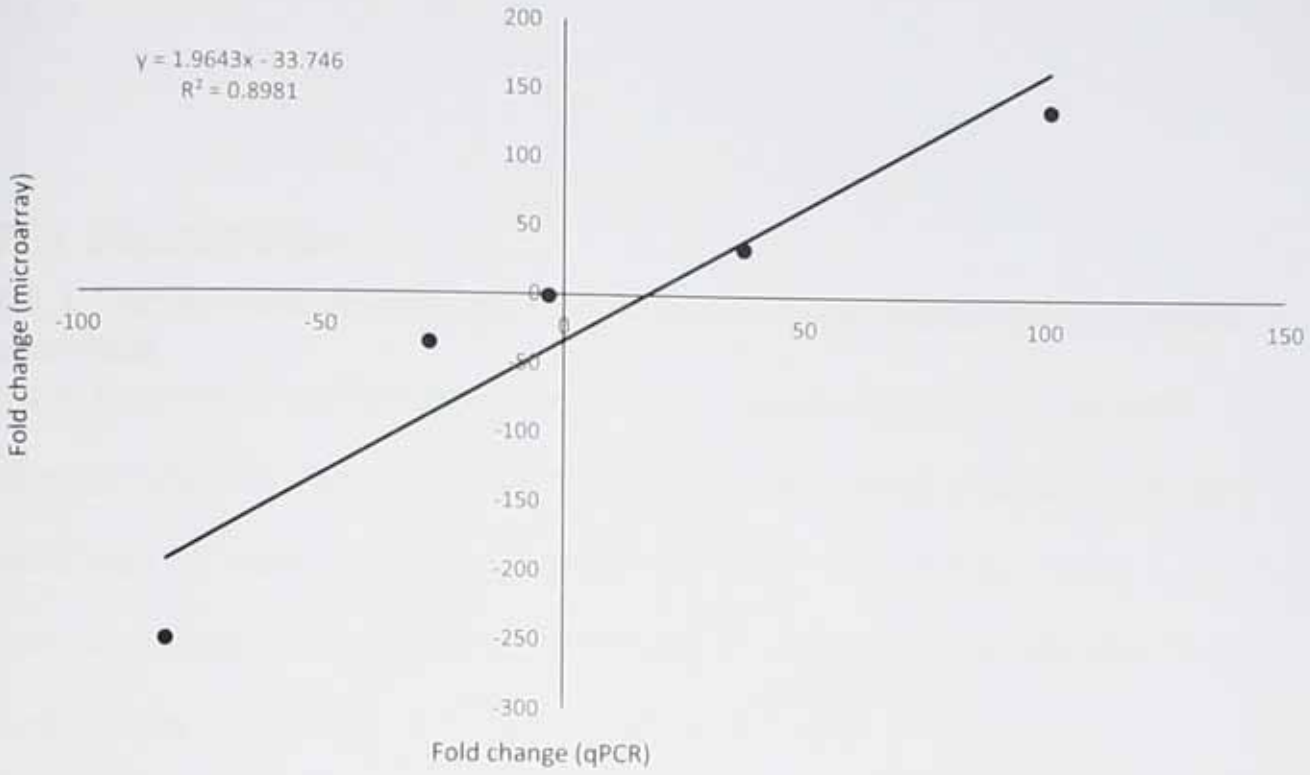

Fig. 7-15: Validation of microarray data by qRT-PCR (8°C, anaerobic conditions vs. 37°C, aerobic conditions). Data points represent the mean fold change of an individual target determined by qRT-PCR and microarray (n=4). qRT-PCR was performed in triplicate.

qRT-PCR confirmed the data generated by microarray. By assessing the correlation between transcript fold change obtained by qRT-PCR and microarray it was possible to validate the data generated by microarray. An R^2 value of >0.8 is generally considered the threshold for a strong correlation between data obtained by qRT-PCR vs. microarray (van der Veen *et al.*, 2007). All correlations (Fig. 7-13 to 7-15) showed R^2 values of at least 0.8, therefore fold changes as determined by microarray could be accepted.

7.5 Discussion

7.5.1 The effects of anaerobiosis on stress response and virulence potential

When subjected to anaerobic conditions *L. monocytogenes* FSL R2-499 demonstrated an altered resistance profile to cells which had been grown under aerobic conditions (Fig. 7-1 to 7-4). While the sensitivity to trisodium phosphate and NaClO remained unchanged, anaerobic pre-conditioning significantly enhanced H_2O_2 sensitivity.

During aerobic respiration organisms face potential damage from oxygen or one of its partially reduced forms such as superoxide anions (O_2^-) (Mobley *et al.*, 2001). The process of aerobic respiration generates H_2O_2, a highly reactive compound which interacts with several intracellular components, potentially leading to cell death (Finnegan *et al.*, 2010). Many aerobic organisms use catalase to overcome the detrimental effects of H_2O_2 exposure (Vergauwen *et al.*, 2003). In the absence of oxygen, *L. monocytogenes* can generate energy by substrate level phosphorylation (Nilsson *et al.*, 2013), respiration of this nature does not generate H_2O_2, thus catalase is redundant. This was supported by the findings of this study where a 27-fold decrease in catalase gene expression was found following anaerobic pre-conditioning.

While catalase is not required for respiration in the absence of oxygen, its down-regulation leaves *L. monocytogenes* cells highly susceptible to exposure to H_2O_2 which it may encounter as an antimicrobial surface decontaminant, or during host immune response during infection. Given the widespread use of oxygen limitation through the use of MAP, the use of H_2O_2 surface decontaminants may serve as a useful tool in the control of *L. monocytogenes* both at the manufacturing level and in the home.

While H_2O_2 sensitivity increased following anaerobic growth, citric acid sensitivity was significantly decreased when cells were grown in the absence of oxygen. Transcriptional analysis revealed significant increases in several loci making up the glutamate decarboxylase (GAD) system (*lmo*2061, *lmo*2062 and *lmo*2063). The GAD system is an important component of many bacterial species which must transit the stomach prior to colonisation of the gut lining (Cotter *et al.*, 2001). By using an intracellular H^+ ion, the GAD system converts extracellular glutamate to γ-aminobutyrate (GABA), this results in a net decrease in intracellular H^+ ions, thus decreasing the pH of the intracellular environment (Small and Waterman, 1998). Given this role, the GAD system has been repeatedly found to influence the acid tolerance of bacterial cells, including *L. monocytogenes*.

While predominantly reported as an acid stress response mechanism, the GAD system has also been found to be activated in response to CO_2 rich environments (Francis *et al.*, 2007; Jydegaard-Axelson *et al.*, 2004). The

mechanism behind GAD expression during CO_2 exposure is believed to be due to acidification of the surrounding environment, therefore making cross-resistance between anaerobiosis and acid exposure feasible (Jydegaard-Axelson *et al.*, 2004). The findings presented here support this as acid resistance was enhanced by anaerobic growth, and several GAD system loci were up-regulated during anaerobic pre-conditioning. This has implications for the food industry where food packaged under low oxygen/high CO_2 may cause induction of the GAD system, this in turn may lead to cells with increased resistance to acid challenge, or may possess elevated virulence potential due to an enhanced ability to survive exposure to the low pH of the stomach (Gahan and Hill, 2014).

7.5.2 The effects of low temperature on stress response and virulence potential

When grown at low temperatures, *L. monocytogenes* FSL R2-499 demonstrated altered resistance characteristics compared to when grown at 37°C (Fig. 7-5 to 7-8). Sensitivity to citric acid, trisodium phosphate and H_2O_2 was significantly decreased following growth at low temperatures. Contrary to this, sensitivity to NaClO was greater in cells grown at low temperatures compared to those grown at 37°C.

Transcriptional analysis revealed that several loci associated with flagella assembly and motility were up-regulated. This is in agreement with a number of studies which have identified temperature dependant motility and flagella expression in *L. monocytogenes* (Peel *et al.*, 1988; Way *et al.*, 2004; O'Neil and Marquis, 2006). While this is a potential risk in

terms of epithelial adhesion (virulence) and biofilm formation (persistence) (O'Neil and Marquis, 2006; Lemon *et al.*, 2007), it is unlikely that flagella expression would influence antimicrobial resistance in this system. It is more feasible that the increased expression of transcriptional regulators (*lmo1251*, *lmo0599*), general stress responders (*lmo1460*, *lmo0211*) and the >100-fold increased expression of a major facilitator protein contributed to the increased resistance to H_2O_2, citric acid and trisodium phosphate.

Transcriptional regulators are vitally important for stress response in a range of bacteria, including *L. monocytogenes*. By altering the transcription of effector proteins bacterial cells can adapt in order to overcome unfavourable environments. The role of transcriptional regulators in *L. monocytogenes* stress response has been extensively reviewed by Chaturongakul *et al.* (2008) and are implicated in both stress response and virulence.

General stress response proteins (GSPs), such as *lmo0211* (similar to *B. subtilis* ctc) are up-regulated in response to a range of stressful environments including heat shock, osmotic stress, ethanol exposure and low pH, as well as during starvation conditions (Petersohn *et al.*, 2001), therefore it is likely that increased GSP expression could contribute towards citric acid, H_2O_2 and trisodium phosphate resistance. In *L. monocytogenes* expression of GSPs have been found to be involved in virulence as well as stress response (Rouquette *et al.*, 1998), therefore

low temperature storage may inadvertently elevate resistance properties and virulence potential in *L .monocytogenes*.

Major Facilitator Superfamily (MFS) proteins are highly conserved molecular transporters (Pao *et al.*, 1998). A diverse range of MFS groups exist, each group transporting a range of similar compounds. MFS proteins are responsible for the transport of anions and cations (Reddy *et al.*, 2012), as well as contributing towards H_2O_2 resistance/tolerance (Frawley *et al.*, 2013). Given that accumulation of antimicrobial chemicals such as citric acid, trisodium phosphate and H_2O_2 is believed to cause cellular inactivation, it is likely that the up-regulation of MFS protein (*lmo1250*) contributed to the elevated resistance when challenged by these antimicrobials.

While several stress response genes were up-regulated in response to low temperature growth, others were found to be down-regulated. These included the heat induced molecular chaperones, GrpE, GroES and dnaK. These genes are known to contribute to oxidative stress resistance (Mols and Abee, 2011), therefore their down-regulation may have contributed toward the decreased resistance towards NaClO found in this study. However, the resistance to H_2O_2, another oxidising agent, was increased following low temperature growth.

While not assessed phenotypically, the expression of two key virulence determinants, Listeriolysin O precursor (*lmo0202*) and *clpE* (*lmo0997*) were significantly down-regulated. This is relevant as it indicates that

refrigerated growth may lead to diminished virulence, thus lessening the risk to human health.

7.5.3 The combined effects of low temperature and anaerobiosis on stress response and virulence potential

When pre-conditioned under anaerobic conditions at refrigeration temperatures *L. monocytogenes* FSL R2-499 cells had altered resistance properties compared to their 'control' counterparts (Fig. 7-9 to 7-12). Resistance to H_2O_2, citric acid and trisodium phosphate were all enhanced by anaerobiosis/low temperature while NaClO resistance was diminished by pre-conditioning.

Transcriptional analysis revealed altered expression levels in several stress response genes which may account for the altered resistance properties seen after pre-conditioning (Table 7-5) as well as indicating transcriptional changes in several virulence associated genes.

When grown at low temperature under anaerobic conditions the transcriptional changes showed similarity to the changes which occurred as a result of growth at low temperature alone. These similarities included up-regulations in Major facilitator protein (*lmo1250*), Fnr/Crp family transcriptional regulator (*lmo1251*), general stress proteins (*lmo0211*, *lmo1460*). In addition to these changes cells grown anaerobically at low temperature also had up-regulation in loci associated with anaerobic pre-conditioning. Most notably *lmo2363*, encoding a GAD

system component was found to be up-regulated during low temperature/anaerobic growth. This probably explains the greater citric acid resistance displayed when cells were grown in conditions of low temperature and anaerobiosis combined, compared to either condition alone. This is relevant as the GAD system has been implicated not only stress resistance, but also in virulence by enhancing the ability of cells to survive gastric transit (Cotter *et al.* 2001; Biase and Pennacchietti, 2013).

In addition to stress response genes, growth under anaerobic conditions at low temperature increased expression of several virulence genes. These included genes associated with flagellation and motility (*lmo0690*, *lmo0691*) which have been found to contribute towards cellular adhesion (O'Neil and Marquis, 2006); haemolysin genes (*lmo1864*) and genes associated with antibiotic protection (*lmo2818*, *lmo0540*). As such, it is conceivable that cells grown at low temperature under anaerobic conditions may have enhanced virulence potential compared to those grown under 'optimal' conditions. This is relevant for the food industry where MAP uses both oxygen limitation and refrigeration in an attempt to increase the shelf life of perishable foods. While this may prevent the growth of several spoilage and pathogenic organisms, it may give rise to *L. monocytogenes* cells with increased virulence potential, thus increasing the risk of food-borne disease arising from consumption of such products.

While resistance to citric acid, H_2O_2 and trisodium phosphate was increased by low temperature/anaerobic growth, NaClO resistance was

diminished. This was also the case when cells were grown under low temperature alone. As with low temperature alone, growth at low temperature under anaerobic conditions caused down-regulation in several heat shock protein loci. These HSP loci included *hrcA*, *groES* and *grpE*, which all act as general stress response genes (Hecker *et al.*, 1996). Both *groES* and *grpE* encode molecular chaperones, aiding correct folding and confirmation of proteins (Hartl, 1996), given that the mode of action of NaClO is believed to be related to protein denaturation and degradation (McDonnell and Russell, 1999), it is likely that down-regulation in these chaperones contributes to the decreased resistance to NaClO seen in cells grown at low temperatures in the absence of oxygen. This is supported by the findings of Wang *et al.* (2010) who reported up-regulation of the *groESL* operon in response to NaClO exposure; thus conditions which decrease the expression of this operon, such as growth at low temperature under anaerobic conditions, is likely to lead to increased sensitivity to this chemical.

Microarray analysis revealed that pre-conditioning at refrigeration temperatures and under oxygen limiting conditions, alone and in combination, induces transcriptional changes which alter the phenotypic properties of *L. monocytogenes* FSL R2-499 cells. Altered phenotypes described in this study include altered H_2O_2 tolerance and acid resistance. These are important components in food manufacturing, as well as acting as key components of the human immune system. Therefore, improving our understanding of the molecular responses to food processing stresses

will allow manufacturers to improve food safety measures, increasing processing efficiencies and minimising the likelihood of inducing responses which give rise to hypervirulent cells.

7.6 Conclusions

From this study it has been determined that anaerobiosis has no effect on the growth capacity of *L. monocytogenes* FSL R2-499 at physiological temperatures (37°C) and at temperatures commonly adopted by the food industry to prevent food spoilage and ensure food safety (7°C). The use of low temperatures was capable of slowing the growth of *L. monocytogenes*, but growth still occurred.

'Pre-conditioning' by refrigeration and oxygen limitation, alone and in combination, significantly altered the resistance properties of *L. monocytogenes* FSL R2-499 cells. By adopting a transcriptomic approach key stress response loci were identified which were likely to contribute to the altered phenotypes discovered. Furthermore, several virulence determinants were found to be differentially regulated in response to pre-conditioning.

The data generated in this study provides useful information regarding the effects of pre-conditioning on resistance in *L. monocytogenes* FSL R2-499. Using this information food manufacturers can make better informed decisions when developing hurdle regimes during manufacturing. This data suggests that while certain conditions, such as refrigeration, slow the growth of *L. monocytogenes*; they may give rise to

cells with elevated resistance and disease causing capabilities representing a human health risk.

FSL R2-499 was a *L. monocytogenes* strain associated with a large scale listeriosis outbreak. Further work investigating the effects of pre-conditioning on additional outbreak and sporadic *L. monocytogenes* strains would provide information as to whether these responses are strain specific of generic to all *L. monocytogenes* strains.

Additionally, phenotypic analysis investigating the effects of pre-conditioning on virulence would provide strong supporting evidence that pre-conditioning influences virulence potential in *L. monocytogenes*.

Chapter 8

Final conclusions and further work

8.1 Final conclusions

8.1.1 Responses of *L. monocytogenes* to antimicrobial chemicals

Studies described in chapters 3, 4 and 5 set out to address the important issue of stress hardening / adaptive responses in *L. monocytogenes* following sub-lethal subjection to antimicrobial chemicals; Investigating both the phenotypic responses and molecular mechanisms which permit survival during stressor exposure.

It is well documented that sub-lethal stressor exposure can induce adaptive responses which enhance the resistance properties of pre-exposed cells (Beales, 2004; Bokhorst-van de Veen *et al.*, 2011; Lou and Yousef, 1997). In *L. monocytogenes* this ability to respond to stressors represents a human health risk.

A majority of *L. monocytogenes* infections occur from the consumption of contaminated food stuffs (Carpentier and Cerf, 2011). Such infections arise when the listerial load in a food product is sufficiently high to overwhelm the immune system, thus allowing a systemic infection to occur (Vázquez-Boland *et al.*, 2001). For hardy organisms, such as *L. monocytogenes*, this represents a problem. Many of the common practises adopted for food manufacturers are unable to prevent the growth of *L. monocytogenes*, including refrigerated storage, modified atmosphere packaging and surface decontaminants.

In an attempt to meet consumer demands for minimally processed foods, manufacturers often adopt the use of hurdle technology. By using

multiple preservation factors, the intensity of any given process can be decreased, providing a more 'natural' end product. However, given that organisms can become stress hardened following mild or sub-lethal stressor exposure may deem hurdle technology unsuitable.

It was the primary aim of studies described in chapter 3 to address the issue of sub-lethal stressor exposure in *L. monocytogenes* when subjected to commonly used antimicrobial chemicals.

When conducting adaption assays to assess adaptive responses in bacteria, it is common practise to use exponentially growing cells in a broth culture as well as using supplemented broth during the adaptation phase. Under these conditions several authors have reported strong adaptive responses, whereby the resistance and/or virulence of an organism is enhanced by pre-exposure (Beales, 2004; Bokhorst-van de Veen *et al.*, 2011; Lou and Yousef, 1997). While this answers some important questions, the relevance of these findings in a non-experimental environment are up for debate. It is reported that environmentally bacterial cells are rarely found in exponential phase (Navarro Llorens *et al.*, 2010). Furthermore, it is improbable that bacterial cells would be 'challenged' within a nutrient rich environment such as supplemented culture media. In an attempt to assess if *L. monocytogenes* is capable of mounting an adaptive response in a more relevant scenario, stationary phase cells were used and the 'adaptation phase' was performed in antimicrobial solutions.

Contradictory to the several reports of adaptive responses in *L. monocytogenes*, this study found that sub-lethal pre-exposure did not increase resistance properties of *L. monocytogenes* EGD-e. In fact, cells pre-exposed to sub-lethal levels of antimicrobial chemicals typically displayed decreased resistance when challenged with lethal levels of homologous and heterologous antimicrobial chemicals (Fig. 3-7 to 3-10), although these effects were combination dependant. While these findings are limited to *L. monocytogenes* strain EGD-e they are contradictory to those studies which suggest that hurdle technology enhances the resistance properties of *L. monocytogenes*. These findings generate some important questions regarding the likelihood of adaptive responses occurring in 'real-world' situations, where cells are in stationary and nutrients are not plentiful; under these conditions the application of a primary stressor, or hurdle, appears to have no effect on secondary exposures or enhances sensitivity.

As well as adaptive responses, it is believed that the presence of resistant sub-populations often exist within the wild-type population (Rajkovic *et al.*, 2009; Walsh *et al.*, 2003). By subjecting populations to sub-lethal levels of various stressors it is possible to select for these resistant cell lines, much in the same way that antibiotic resistance can arise (Kohanski *et al.*, 2010). While the emergence of stressor induced sub-populations is extremely rare (Granier *et al.*, 2011), the movement towards minimally processing of foods may make this an emerging risk.

In this study resistance was not acquire / mutants were not selected through repeated exposure (Fig. 3-11 to 3-14), nor through stepwise 'training' (Table 3-2). However, mutant selection is a chance occurrence, therefore the ability of these antimicrobials and/or methods to select for mutant sub populations cannot be discounted. Previous studies have used similar methods with varying levels of success in generating/isolating resistant cells and sub-populations (Condell *et al.*, 2012; Rajkovic *et al.*, 2009; Walsh *et al.*, 2003), therefore careful consideration must be taken by food manufacturers when choosing to adopt minimal processing of food products as there is a small possibility that such practises may inadvertently drive mutant selection.

In summary, the findings of the studies described in chapter 3 suggest that in nutrient limited conditions, in stationary phase, *L. monocytogenes* cells are unable to mount adaptive responses which enhance the resistance to subsequent stress. Contrarily, cells generally displayed increased sensitivity to secondary stress following sub-lethal pre-exposure, supporting the effectiveness of the hurdle concept when used under controlled conditions. Furthermore, while some reports suggest that repeated sub-lethal exposure can give rise to mutant cells with increased resistance towards antimicrobial agents, this was not found in this study. Using several techniques it was not possible to induce mutations or select for sub-populations which possessed enhanced resistance characteristics. While this may occur in *in vitro* systems these

results suggest there is a low possibility of such occurances outside of a laboratory environment.

Listeria monocytogenes is considered a hardy organism which can survive subjection to many food processing practises. This is largely attributed to its extensive repertoire of stress response genes (Chaturongakul *et al.*, 2008). Exposure to sub-lethal stressors has been frequently found to alter the transcription levels of several of these stress response genes (Bergholz *et al.*, 2012; Giotis *et al.*, 2010; Ivy *et al.*, 2012; Michel *et al.*, 2011).

When developing hurdle regimes for a given food product, hurdles are "intelligently applied"; by applying hurdles which target several cellular structures, or processes, it is less likely that bacterial cells will survive the entire regime, thus delivering a microbiologically safe end product (Leistner, 2000).

When considering hurdle regimes it is important to consider the possibility of transcriptional changes which may alter the resistance properties of the present microbial cells. Several stress response genes are considered 'general', which are up-regulated in response to challenge from a range of stressors. Up-regulation in such a gene may have implications for downstream hurdles. Therefore it was the aim of studies in chapter 4 to ascertain the roles of several stress response genes during exposure to antimicrobial chemicals, thus allowing more informed decisions to be made when developing hurdle regimes. Only by understanding the

molecular mechanisms which permit survival during sub-lethal stress exposure can manufacturers truly apply hurdles in an 'intelligent' manner, targeting several cellular components and metabolic systems to allow maximal disruption to the microbial load.

In this study, of the selected candidate genes only *gadA* and *lmo0669* were found to significantly alter in expression following antimicrobial challenge from TSP and NaClO respectively (Fig 4-10). The GAD system is responsible for acid tolerance in *L. monocytogenes* (Cotter *et al.*, 2005) while *lmo0669* encodes a putative oxidoreductase enzyme, with probable roles in oxidative stress response and maintenance of redox potentials within cells (Chan *et al.*, 2006). To put these findings into a broader context, if devising a hurdle regime which utilised TSP it would be pertinent to adopt additional hurdles which do not induce *gadA*, thereby targeting multiple 'systems' within microbial cells. Likewise, if including NaClO in a regime, it would be ill advised to use an additional oxidising agent, as cross protection from *lmo0669* may have been induced.

These findings may be of relevance to food manufacturers as transcriptional responses to any individual hurdle must be realised before regimes can be "intelligently applied".

While transcriptional analysis provides a good indication of the role of stress response genes in stress exposure, it is the translated effector proteins which often permit survival. In addition to transcriptional analysis, the role of a given candidate gene in stress response can be

assessed through gene inactivation/disruption. Several studies have adopted this approach to elucidate the role of a given gene in stress response or virulence (Bigot *et al.*, 2009; Machata *et al.*, 2008; Kallipolitis and Ingmer, 2001).

It was the aim of studies in chapter 4 to use gene disruption to investigate the role of specific stress response genes in antimicrobial chemical exposure. In this study several stress response genes were inactivated using the pAUL-A vector. By inactivating stress response genes it is possible to associate a gene with a response. This provides supporting data to transcriptional analysis in the elucidation of a given genes role in stress response, allowing better informed decisions when devising hurdle regimes.

Potential mutants were generated with disruptions to the *ctsR*, *hfq*, *LisR* and *LisK* genes. Subsequent phenotypic analysis revealed that *ctsR* and *hfq* impacted citric acid resistance (Table 5-3), while disruption in all targets could give rise to mutants with enhanced sensitivity to H_2O_2 (Fig. 5-9). Of the *ctsR* (5) and *hfq* (10) disruptants, only 1 mutant of each displayed increased sensitivity to H_2O_2, this suggests that the nature of the disruption is important when considering the effects of a given gene in stress response, ie. a disruption is only indicative of the effects that a disruption could have. Further characterisation of the nature of each disruption would allow a more complete association between gene and stress response.

The data generated in this study provides evidence elucidating the roles of *ctsR*, *hfq*, *lisR* and *lisK* in stress response. This information could be used alongside previous transcriptomic and gene disruption data to devise a hurdle regimes which are truly multi-targeting, allowing food manufacturers to improve food safety.

While there is a commercial interest in the survival mechanisms of food-borne pathogens, their ability to survive food processing, and persist within food manufacturing plants and food stuffs; there is an equally relevant interest in the effects of food processing practise on microbial virulence.

If microbial cells adaptively respond to sub-lethal stress, or mild exposure selects for mutant sub populations these cells may have an enhanced virulence potential (Bo Andersen *et al.*, 2007; Christiansen *et al.*, 2004; Cotter *et al.*, 1999; Karatzas *et al.*, 2003). This is due to similarities between food manufacturing processes and host immune defences. For example, gastro-intestinal pathogens must overcome the low pH of the stomach if they are to establish an infection (Garner *et al.*, 2006; Ivy *et al.*, 2012), therefore sub-lethal stress exposure which induces acid response mechanisms may inadvertently cross protect against the low pH of the stomach, thus permitting survival and subsequent infection. Likewise, for intracellular organism such an *L. monocytogenes* the ability to survive exposure to H_2O_2 within macrophages is essential for prolonged infection to be sustained (Gomes *et al.*, 2011). Therefore food processing

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stress which induces oxidoreductase enzymes, such as the induction of *lmo0669* by NaClO (Fig 4-10), may enhance the ability of *L. monocytogenes* cells to survive host immune responses. Therefore it was deemed relevant to investigate the potential of sub-lethal stressor exposure to impact virulence potential in this study.

8.1.2 Responses of *L. monocytogenes* to oxygen limitation and refrigeration

Two of the most defining features of *L. monocytogenes* are its ability to grow at refrigeration temperatures and grow under oxygen limiting conditions (Oliveira *et al.*, 2010); these conditions typically inhibit or prevent the growth of food-borne pathogens. As such, this study aimed to investigate the phenotypic and molecular effects of growth under these conditions.

Upon determining that that oxygen limitation enhanced the resistance of *L. monocytogenes* to both organic and inorganic acids (Fig 6-3 to 6-6) it was considered relevant to investigate the effects of oxygen limitation on the ability of *L. monocytogenes* to survive simulated gastric transit.

When grown under oxygen limiting conditions *L. monocytogenes* FSL R2-499 displayed significantly enhanced acid tolerance compared to cells grown aerobically (chapter 6). However, these effects were found to be both pH and growth phase dependant (section 6.4.3 and 6.4.4).

As previously mentioned, the pH of the stomach varies from ~pH 2.5 in 'healthy' individuals up to pH 4.5 in the elderly (Garner *et al.*, 2006). It is

this high stomach pH which is thought to contribute to the pre-disposal of some individuals to *L. monocytogenes* infection. Therefore, this study aimed to assess if the previously observed acid tolerance response, induced by oxygen limiting conditions, was relevant in a model gastro-intestinal transit system.

Following growth under oxygen limiting conditions, exponential phase cells were not affected by pH 3.5 simulated gastric transit, cells were not inactivated (Fig. 6-7 and Fig. 6-9) however cells displayed enhanced resistance to pH 2.5 simulated gastric transit (Fig. 6-8 and Fig 6-10). While simulated gastric juice exposure was effected by growth under oxygen limitation exponential phase cells were unable to survive 'gastric transit' as they were readily inactivated by subjection to bile salts at physiological concentrations (0.3% w/v). However, when cells were grown to stationary phase (Fig. 6-11 to 6-14) they readily overcame artificial gastric juice exposure at both pH 2.5 and 3.5 irrespective of previous growth conditions. When grown to stationary phase, cells which were subjected to pH 3.5 artificial gastric juice were readily able to overcome bile salt exposure, suggesting that mild acid shock in stationary phase cells may inadvertently induce bile tolerance in stationary phase *L. monocytogenes* cells. This increased tolerance to bile salts in stationary phase cells has been reported elsewhere (Begley *et al.*, 2002), and is considered relevant as bacterial cells are often existing in stationary phase.

To put these findings into context, *L. monocytogenes* is a facultative anaerobe which can readily survive and proliferate in oxygen limiting conditions, such as those found in modified atmosphere packaging (Lungu *et al.*, 2009), this represents a consumer health risk. Furthermore, growth under these conditions enhances the ability of *L. monocytogenes* cells to survive the low pH found in the stomach. Should an individual possess a condition which affects bile production/secretion, such as gall bladder removal, gall stones or liver disorders, surviving *L. monocytogenes* cells may be able to cross the intestinal epithelium and establish an infection (Burkholder and Bhunia, 2010). Therefore the use of modified atmosphere packaging should be carefully considered before use in *Listeria* compromised foods.

Chilled RTE foods are common vehicles for the transmission of *L. monocytogenes*. This is largely attributed to its psychotrophic and facultatively anaerobic characteristics which permit its survival and growth during refrigerated transport and storage in MAP products. As previously discussed, growth under 'unfavourable' conditions is able to induce adaptive responses which phenotypically alter the survival properties of several micro-organisms. As such, it was one of the aims of this study to investigate the effects of low temperature and oxygen limitation, alone and in combination, on phenotypic and molecular changes in *L. monocytogenes*.

Phenotypically, oxygen limitation and low temperature 'pre-conditioning' had significant effects on resistant characteristics (section 7.4.2 – 7.4.4). To briefly summarise the key findings, oxygen limitation at any temperature increased susceptibility to oxidative stress (H_2O_2) but reduced susceptibility to low pH (citric acid). These findings represent particular relevance as oxidative stress and low pH are vital components in the innate immune response of potential hosts, where H_2O_2 is employed by macrophages during an innate immune response while the low pH is the stomach acts as an important barrier to gastro-intestinal pathogens. While the effects of anaerobiosis/oxygen limitation on *L. monocytogenes* are not well studied (Lungu *et al.*, 2009), previous studies have also reported that oxygen limitation enhances acid tolerance in *L. monocytogenes* (Francis *et al.*, 2007), suggesting a link between the acid response and the response to low oxygen.

In addition to the effects on low pH and oxidative stress; low temperature, alone and combined with oxygen limiting condition, influenced trisodium phosphate and NaClO susceptibility (Fig. 7-6, 7-8, 7-10 and 7-12). These findings are of relevance to food manufacturers when devising hurdle regimes, as they may wish to 'intelligently' include refrigeration and/or oxygen limitation within a regime to improve food safety.

In order to understand the molecular mechanisms responsible for the altered phenotypic properties observed following low temperature growth

and oxygen limitation transcriptome analysis by microarray was conducted. By understanding these responses on a molecular level, food manufacturing processes can be better applied to improve food safety.

Many genes/loci displayed altered transcript levels in response to pre-conditioning (Table 7-3 to 7-5). In summary of the key findings, oxygen limitation at both temperatures resulted in up-regulation of genes associated with the glutamate decarboxylase (GAD) system. As previously mentioned, the GAD system is important for survival at low pH (section 4.5.3). Therefore the up-regulation of GAD associated genes provides a strong link between oxygen limitation and the increased acid tolerance reported in this study. These findings are supported by Francis *et al.* (2007) who also reported the link between oxygen limitation and acid tolerance using several Δ*gad* mutants. Additionally, growth under oxygen limitation at both temperatures resulted in significant down-regulation of catalase. Catalase acts by converting H_2O_2 to $H_2O + O_2$, thus neutralising H_2O_2 to non-toxic components (Vergauwen *et al.*, 2003). H_2O_2 generation typical arises during aerobic respiration, and thus organisms which utilise molecular oxygen for respiration have evolved mechanisms to protect against these toxic by-products, such as the use of the catalase enzyme. During anaerobic respiration it was hypothesised that catalase would be redundant, thus its expression would be down-regulated. This was confirmed by transcriptome analysis and supports the findings of this study whereby H_2O_2 sensitivity was increased following growth under oxygen limitation. This again provides important

information for food manufacturers, who can apply food control measures, or hurdles, more intelligently.

In conclusion, studies in chapters 6 and 7 highlighted some important phenotypic and molecular responses of *L. monocytogenes* to food related stresses. Most notably, growth under what are considered 'sub-optimal' conditions can have a significant effect on the resistance profiles of *L. monocytogenes* cells. For example, growth under oxygen limitation has been found to increase acid tolerance, thus impacting not only food safety but compromising the health of consumers. However, in a broader context, these findings may aid food manufacturers in making decisions regarding food control measures and hurdle regimes. By understanding both the phenotypic and molecular responses of micro-organisms during food manufacturing, transport and storage, suitable control measures can be applied which decrease the likelihood of infection arising from food consumption. Additionally, food controls can be applied in such a way that virulence potential in contaminating organisms is minimised, thus decreasing the likelihood of severe food-borne outbreaks.

Overall this study has provided novel information and answered important questions surrounding the survival mechanisms adopted by *L. monocytogenes* during stressor exposure. Furthermore the effects on resistance, as well as virulence potential have been investigated. Using this information food manufacturers can improve their practises to

enhance food safety and minimise the financial and public health burden caused by *L. monocytogenes*.

8.2 Future work

- The gastro-intestinal transit model presented here provides novel findings highlighting the effects of anaerobiosis/oxygen limitation on gastro-intestinal transit, with an implication of the effects of pre-conditioning on virulence potential. However, before a definitive association between oxygen limitation and virulence/virulence potential can be drawn, further studies utilising model *in vivo* systems would be necessary. Only by undertaking such studies can the true extent of oxygen limitation on virulence be assessed.
- The findings of this study highlight several stress responses at both the phenotypic and molecular level. However, the work has been conducted on well characterised genome strains. To generalise these findings to all *L. monocytogenes* strains is difficult, further work with additional strains from various sources, including human isolates and persistent strains isolated from food processing plants, would add to the data presented here.

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Appendices

Appendix 1: Transcriptional changes in cells grown at 37°C under anaerobic conditions relative to 'control cells' (37°C, aerobic conditions)

Locus ID	Fold change	Adjusted *p*-value	Gene/protein function
lmo2362	+12.74	<0.000	Amino acid transporter
lmo2363	+10.48	<0.000	Glutamate decarboxylase- stress response and virulence,
lmo2439	+3.19	0.006	Unknown
lmo0903	+2.75	0.05	Post-translational regulator/chaperone
lmo2361	+2.70	<0.000	Glutamate decarboxylase
lmo1251	+2.39	<0.000	Fnr/Crp family transcriptional regulator
lmo2568	+2.36	0.05	*yxeA* homologue- Unknown function
lmo2567	+2.28	<0.000	5-bromo-4-Chloroindolyl phosphate hydrolysis
lmo2360	+2.22	<0.000	Membrane protein- Unknown function
lmo1999	+2.19	0.03	Cell envelope biogenesis
lmo1388	+2.17	<0.000	Antigenic lipoprotein
lmo2150	+2.10	0.05	Unknown function
lmo2156	+2.08	0.009	Unknown function
lmo1056	+2.08	<0.000	Unknown
lmo0725	+2.05	0.003	Peptidoglycan binding protein- possible virulence determinant
lmo0760	-2.01	<0.000	Esterase homologue
lmo2231	-2.01	<0.000	Unknown function

lmo0279	-2.02	0.001	anaerobic ribonucleoside triphosphate reductase
lmo2213	-2.02	<0.000	Biosynthesis of extracellular polysaccharides
lmo2413	-2.02	<0.000	Aminotransferase
lmo0321	-2.02	<0.000	Similar to unknown proteins
lmo2572	-2.02	<0.002	dihydrofolate reductase
lmo0169	-2.03	<0.000	Glucose transport
lmo2569	-2.03	<0.000	Substrate binding protein- Amino acid transport
lmo2067	-2.03	<0.000	Bile acid hydrolase
lmo1422	-2.03	<0.000	Osmoprotection, cell envelope biosynthesis
lmo1293	-2.03	<0.000	Energy production and conservation
lm02717	-2.03	<0.000	Energy production and conversion
lmo2269	-2.04	0.006	Unknown function
lmo0794	-2.05	0.03	Similar to flavin reductase- macromolecule metabolism/redox sensing
lmo0784	-2.05	<0.000	Regulation of metabolic and transcriptional processes
lmo1375	-2.05	0.008	Aminotripeptidase
lmo0171	-2.06	0.007	Internalin- virulence
lmo0782	-2.06	<0.000	Mannose transport- metabolic and transcriptional processes
lmo0014	-2.07	<0.000	Energy production and conversion
lmo0781	-2.07	<0.000	Mannose transport- metabolic and transcriptional processes
lmo0015	-2.07	0.004	Proton pump, reduction of O_2

lmo1421	-2.08	<0.000	Amino acid transport and metabolism
lmo2684	-2.08	<0.000	Carbohydrate transport and metabolism
lmo0723	-2.08	0.01	Hypothetical protein- similar to methyl-accepting chemotaxis protein
lmo0043	-2.09	<0.000	Similar to arginine deiminase
lmo2573	-2.09	0.006	Zinc binding dehydrogenase
lmo0783	-2.09	<0.000	Mannose transport- metabolic and transcriptional processes
lmo1421	-2.09	<0.000	Glycine-betaine transporter- amino acid transport and metabolism
lmo2414	-2.09	0.01	Post-translational modification, protein turnover, chaperones
lmo0781	-2.10	<0.000	Mannose transport
lmo2415	-2.11	0.003	ABC transporter- Energy production
lmo1539	-2.11	<0.000	Glycerol transporter- Carbohydrate transport and metabolism
lmo2708	-2.12	<0.000	Carbohydrate transport and metabolism
lmo0539	-2.13	<0.000	Galactose metabolism
lmo0596	-2.13	<0.000	Unknown function
lmo1439	-2.14	<0.000	Superoxide dismutase- Antioxidant, virulence
lmo1830	-2.15	0.01	Short chain dehydrogenase
lmo1422	-2.16	<0.000	Glycine-betaine transporter- Cell envelope biogenesis
lmo0782	-2.16	<0.000	Mannose transport-
lmo0724	-2.17	0.005	Hypothetical peptidase (homologue)
lmo0169	-2.17	0.002	Carbohydrate transport and metabolism- glucose transporter

lmo0016	-2.18	0.001	Oxidoreductase activity
lmo0994	-2.18	0.001	Unknown function
lmo0784	-2.18	<0.000	Mannose transporter- metabolic and transcriptional processes
lmo2570	-2.19	<0.000	Predicted membrane protein
lmo0013	-2.22	<0.000	Quinol oxidase- oxidoreductase activity
lmo2733	-2.22	<0.000	Fructose transporter- metabolic and transcriptional processes
lmo2602	-2.23	0.006	Predicted membrane protein
lmo2463	-2.24	<0.000	Multidrug transporter- Virulence and resistance
lmo2153	-2.27	0.03	Flavodoxin- Electron transport
lmo2152	-2.27	0.04	Thioredoxin- oxidative stress response
lmo0943	-2.30	0.01	Ferritin- stress response and virulence
lmo2154	-2.30	0.009	Ribonucleotide reductase- DNA synthesis and regulation
lmo2391	-2.32	<0.000	Amino acid metabolism
lmo2085	-2.32	0.001	Peptidoglycan binding protein- possible virulence determinant
lmo0655	-2.36	0.009	Similar to phosphoprotein phosphatases
lmo1883	-2.40	<0.000	Chitinase- carbon and nitrogen utilisation, virulence
lmo0265	-2.42	0.008	Metallopeptidase activity
lmo2571	-2.50	<0.000	Similar to Nicotinamidase
lmo1539	-2.51	<0.000	Glycerol transporter- Carbohydrate transport and metabolism
lmo2695	-2.51	<0.000	Dihydroxyacetone kinase
lmo2411	-2.52	<0.000	Unknown function

lmo0589	-2.54	<0.000	Predicted membrane protein
lmo0591	-2.54	0.002	Predicted function- Steroid 5-alpha reductase
lmo2155	-2.57	0.009	ribonucleotide-diphosphate reductase-control of dNTP synthesis
lmo2415	-2.62	<0.000	ATPase component- Energy production
lmo0610	-2.78	<0.000	Internalin like protein- virulence
lmo0670	-2.95	<0.000	Conserved, uncharacterised bacterial protein
lmo0434	-3.03	0.004	Internalin B- invasion and virulence
lmo0628	-3.15	<0.000	Hypothetical protein- unknown function
lmo2412	-3.20	<0.000	Iron-sulfur cluster scaffold-like proteins
lmo0019	-3.57	<0.000	Unknown
lmo0590	-3.60	<0.000	Predicted kinase related to dihydroxyacetone kinase
lmo0602	-3.60	<0.000	Acetyltransferase- transcription regulator
lmo1425	-3.84	<0.000	Glycine-betaine transporter- Amino acid transport and metabolism, stress response
lmo2158	-4.00	<0.000	Uncharacterized protein conserved in bacteria
lmo1428	-4.01	<0.000	opuCA- similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)
lmo0263	-4.12	<0.000	Internalin H- invasion and virulence
lmo0133	-4.23	<0.000	Uncharacterized conserved protein
lmo0669	-4.26	<0.000	Oxidoreductase- stress response and virulence

lmo2157	-4.27	<0.000	*sepA*- Secondary metabolites biosynthesis, transport and catabolism
lmo1427	-4.38	<0.000	Glycine-betaine permease- Amino acid transport and metabolism, stress response
lmo0134	-4.42	<0.000	Acetyl transferase
lmo1992	-4.44	<0.000	acetolactate decarboxylase- butanoate metabolism
lmo0433	-4.44	<0.000	inlA- Internalin A
lmo2748	-4.46	<0.000	Uncharacterised stress protein- stress response
lmo1426	-4.47	<0.000	Glycine-betaine transporter- Amino acid transport and metabolism, stress response
lmo2697	-4.54	<0.000	Mannose transporter- metabolic and transcriptional processing
lmo0880	-4.89	<0.000	Cell envelope biogenesis and peptidoglycan binding
lmo0722	-5.04	<0.000	pyruvate oxidase- control of acetate production
lmo0913	-5.20	<0.000	succinate semialdehyde dehydrogenase- Succinate metabolism
lmo1694	-5.57	<0.000	Predicted nucleoside-diphosphate sugar epimerase
lmo2673	-5.91	<0.000	Universal stress protein (USP) like protein- stress response
lmo2434	-6.00	<0.000	Alanine, aspartate and glutamate metabolism
lmo2006	-7.16	<0.000	acetolactate synthase- Amino acid biosynthesis

lmo1052	-7.61	<0.000	pyruvate dehydrogenase subunit- Carbon metabolism, metabolism of secondary metabolites
lmo1054	-7.75	<0.000	Dihydrolipoamide acetyltransferase- Carbon metabolism, biosynthesis of secondary metabolites
lmo1055	-8.00	<0.000	Dihydrolipoamide dehydrogenase- Carbon metabolism, biosynthesis of secondary metabolites
lmo1053	-8.14	<0.000	Similar to pyruvate dehydrogenase subunit E1 beta
lmo2230	-12.11	0.01	Similar to arsenate reductase
lmo2785	-27.30	<0.000	Catalase- Inorganic ion transport and metabolism, oxidoreductase, stress response and virulence

Appendix 2: Transcriptional changes in cells grown at 7°C relative to 'control cells' (37°C, aerobic conditions)

Locus ID	Fold change	Adjusted *p*-value	Gene/protein function
lmo0690	249.6985	<0.000	*flaA*- flagella production, motility, virulence
lmo1250	107.9432	<0.000	Major facilitator protein family- membrane transport, antibiotic resistance, virulence
lmo0699	95.96173	<0.000	Flagella motor switch protein- chemotaxis, cell motility, secretion, virulence
lmo0694	89.53144	<0.000	Hypothetical protein- Unknown function
lmo0696	87.76654	<0.000	*flgD* – Flagella component- motility, virulence
lmo0693	87.5494	<0.000	FliY – Flagella motor switch protein - chemotaxis, cell motility, secretion, virulence
lmo0697	71.21956	<0.000	*flgE* – Flagella hook protein- chemotaxis, cell motility, secretion, virulence
lmo0695	71.07127	<0.000	Hypothetical protein
lmo0698	68.13814	<0.000	Flagella motor switch protein- Flagella rotation control- chemotaxis, virulence, motility
lmo0118	65.28152	<0.000	Antigen A – Secreted protein (unknown function)
lmo0691	64.51798	<0.000	*cheY* – chemotaxis response regulator – motility, virulence, chemotaxis
lmo1699	64.48275	<0.000	ethyl-accepting chemotaxis protein (MCP), signaling domain – ligand binding, light/oxygen sensing
lmo0692	64.27554	<0.000	*cheA* - two-component sensor histidine kinase

lmo0702	63.7775	<0.000	Hypothetical protein (Unknown function)
lmo0683	60.97107	<0.000	Chemotaxis protein methyltransferase
lmo0700	56.44609	<0.000	Similar to flagellar switch protein FliY
lmo0686	54.8634	<0.000	*motB*- similar to motility protein (flagella motor rotation)
lmo0689	54.15873	<0.000	Similar to CheA activity modulating chemotaxis protein CheV
lmo0701	53.09056	<0.000	Hypothetical protein (unknown function)
lmo0685	50.21984	<0.000	Flagella motor protein MotA
lmo0703	49.37793	<0.000	Hypothetical protein (Unknown function)
lmo0688	48.75951	<0.000	Glycosyl transferase
lmo0117	48.25358	<0.000	imaB – Antigen B
lmo0679	46.75628	<0.000	*FlhB*- flagellar biosynthesis protein
lmo0687	46.17648	0.003	Hypothetical protein (Unknown function)
lmo0680	46.04454	<0.000	Similar to flagellar associated protein FlhA
lmo1700	44.2389	<0.000	Conserved hypothetical protein
lmo2091	42.42985	<0.000	argH- argininosuccinate lyase
lmo0712	41.94507	<0.000	Similar to flagellar hook-basal body complex protein FliE
lmo0684	41.9335	<0.000	Hypothetical protein (Unknown function)
lmo0705	41.39669	<0.000	Similar to flagellar hook associated protein FlgK
lmo0723	41.175	<0.000	Hypothetical protein- similar to methyl-accepting chemotaxis protein
lmo0724	41.02503	<0.000	Hypothetical protein- similar to *B. subtilis* YvpB protein
lmo0704	39.49521	<0.000	Hypothetical protein (Unknown function)

lmo0682	38.28963	<0.000	Flagellar basal body rod protein FlgG
lmo0681	37.61094	<0.000	flagellar biosynthesis regulator FlhF
lmo0678	36.86485	<0.000	similar to flagellar biosynthetic protein FliR
lmo0120	36.8196	<0.000	Hypothetic protein (unknown function)
lmo0675	36.69703	<0.000	Hypothetical protein (Unknown function)
lmo0707	35.9627	<0.000	Flagella capping protein FliD
lmo0711	34.63697	<0.000	Flagella basal-body rod protein FlgC
lmo0706	32.6777	<0.000	Flagella hook-associated protein 3 FlgL
lmo0710	31.94117	<0.000	Flagella basal-body rod protein FlgB
lmo2090	31.19518	<0.000	Argininosuccinate synthase
lmo0677	30.96867	<0.000	Flagella biosynthetic protein FliQ
lmo0676	30.92087	<0.000	Flagella biosynthetic protein FliP
lmo0708	30.52353	<0.000	Flagella protein FliS
lmo1179	30.01127	<0.000	Hypothetical protein
lmo0717	28.31375	<0.000	Hypothetical protein
lmo0713	28.10118	<0.000	Flagella M-ring protein FliF
lmo0709	26.18104	<0.000	Hypothetical protein
lmo0718	25.93175	<0.000	Hypothetical protein
lmo1178	25.67654	<0.000	Hypothetical protein
lmo1986	25.63439	<0.000	Ketol-acid reductoisomerase
lmo1983	25.00269	<0.000	Dihydroxy-acid dehydratase- Valine and isoleucine biosynthesis
lmo0716	24.44531	<0.000	Flagellum-specific ATP synthase
lmo1249	23.94163	<0.000	Hypothetical protein
lmo1180	22.88427	<0.000	Ethanolamine utilization protein EutM

lmo0715	22.15028	<0.000	Flagella assembly protein FliH
lmo0122	21.82654	<0.000	Hypothetical protein
lmo0714	21.61173	<0.000	Flagella motor switch protein FliG
lmo0123	20.80283	<0.000	Hypothetical protein
lmo1182	19.93906	<0.000	Hypothetical protein
lmo1185	19.32973	<0.000	Hypothetical protein
lmo1984	18.4686	<0.000	ivlB- Similar to acetolactate synthase
lmo0125	17.58158	<0.000	Hypothetical protein
lmo0119	17.41351	<0.000	Hypothetical protein (Unknown function)
lmo1985	17.20817	<0.000	ilvH- similar to acetolactate synthase, small subunit
lmo1184	16.96572	<0.000	Carbon dioxide concentrating mechanism protein
lmo1183	16.74557	<0.000	Conserved hypothetical protein
lmo0128	16.58997	<0.000	Hypothetical protein
lmo0124	16.05146	<0.000	Hypothetical protein
lmo0126	15.80294	<0.000	Hypothetical protein
lmo1181	15.613	<0.000	Ethanolamine utilization cobalamin adenosyltransferase
lmo0129	15.60994	<0.000	Similar to N-acetylmuramoyl-L-alanine amidase
lmo1175	15.3929	<0.000	Similar to ethanolamine ammonia-lyase large subunit (eutB)
lmo1186	14.78198	<0.000	Similar to *E. coli* ethanolamine utilization protein EutH
lmo0121	14.66113	<0.000	Hypothetical protein
lmo1991	12.91826	<0.000	ilvA- threonine dehydratase

lmo1177	12.68942	<0.000	Similar to ethanolamine utilization protein EutL
lmo2210	12.59074	<0.000	Hypothetical protein
lmo1176	12.43607	<0.000	eutC- ethanolamine ammonia-lyase small
lmo1987	12.25648	<0.000	leuA- similar to 2-isopropylmalate synthase
lmo1990	11.84521	<0.000	leuD- isopropylmalate isomerase small subunit
lmo1187	11.73588	<0.000	EutQ- ethanolamine utilization protein
lmo2125	11.46818	<0.000	Similar to sugar ABC transporter substrate-binding protein
lmo1251	11.166	<0.000	Fnr/Crp family transcriptional regulator
lmo1364	10.97631	<0.000	cspL- cold-shock protein
lmo1989	10.07473	<0.000	leuC- isopropylmalate isomerase large subunit
lmo1174	9.56731	<0.000	eutA- ethanolamine utilization protein
lmo1988	9.219541	<0.000	leuB- 3-isopropylmalate dehydrogenase
lmo2269	7.944972	<0.000	Hypothetical protein
lmo0661	6.88403	<0.000	Hypothetical protein
lmo0322	6.143867	<0.000	Unknown protein
lmo1190	6.11791	<0.000	Hypothetical protein
lmo0189	6.063614	<0.000	Highly similar to *B subtilis* Veg protein
lmo0283	5.978592	<0.000	Similar to D-methionine transport system permease protein
lmo1000	5.823258	<0.000	Hypothetical protein
lmo2375	5.820458	<0.000	Unknown
lmo0321	5.678311	<0.000	Similar to unknown proteins

lmo2852	5.542428	<0.000	Hypothetical protein
lmo1001	5.509939	<0.000	Similar to *B. subtilis* protein YkvS
lmo1007	5.346892	<0.000	Hypothetical protein
lmo2374	5.331363	<0.000	Aspartate kinase
lmo0115	5.26332	<0.000	imaD- Hypothetical protein
lmo2720	5.162165	<0.000	Similar to acetyl-CoA synthetase
lmo0937	5.149137	<0.000	Hypothetical protein
lmo2126	5.021793	<0.000	Hypothetical protein
lmo0269	4.995484	<0.000	Similar to peptide/nickel transport system permease protein
lmo0019	4.949752	<0.000	Unknown
lmo0823	4.832022	<0.000	Hypothetical protein
lmo2256	4.81396	<0.000	Similar to unknown proteins
lmo1429	4.719311	<0.000	Similar to thiamine transporter
lmo1145	4.694376	<0.000	Similar to ethanolamine utilization protein EutP
lmo1917	4.689944	<0.000	pflA- Similar to formate C-acetyltransferase
lmo0284	4.625296	0.002	Similar to D-methionine transport system ATP-binding protein
lmo2832	4.555078	<0.000	Similar to glycerate kinase
lmo0285	4.515244	<0.000	Lipoprotein- D-methionine transport system substrate-binding protein
lmo0835	4.303034	<0.000	Peptidoglycan binding protein
lmo1717	4.293451	<0.000	Hypothetical protein

lmo0116	4.166781	<0.000	Hypothetical protein- ImaC
lmo2101	4.153585	0.009	Pyridoxal 5'-phosphate (PLP) synthase- PLP production
lmo1056	4.112061	<0.000	Unknown
lmo0654	4.021752	<0.000	Hypothetical protein
lmo2102	4.018595	<0.000	Vitamin B6 metabolism
lmo0561	3.901826	<0.000	Similar to phosphorybosil-AMP-cyclohydrolase (HisI2 protein)
lmo0672	3.894708	<0.000	Similar to unknown protein
lmo1618	3.873443	<0.000	Hypothetical protein
lmo1864	3.84943	<0.000	Similar to hemolysinIII proteins, putative integral membrane protein
lmo0525	3.81055	<0.000	Unknown
lmo1617	3.785095	<0.000	Hypothetical protein
lmo0540	3.75157	<0.000	Similar to penicillin-binding protein
lmo1146	3.657618	<0.000	Hypothetical protein
lmo1590	3.620143	<0.000	argJ- glutamate N-acetyltransferase / amino-acid N-acetyltransferase
lmo1171	3.60364	<0.000	Hypothetical protein- pduQ
lmo0642	3.587107	<0.000	Hypothetical protein
lmo1713	3.563207	<0.000	Similar to cell-shape determining proteins
lmo1144	3.561364	<0.000	Similar to ethanolamine utilization protein EutS
lmo0392	3.556415	<0.000	Highly similar to *B. subtilis* YqfA protein
lmo0604	3.496012	<0.000	Similar to *B. subtilis* YvlA protein
lmo1067	3.439317	<0.000	Similar to GTP-binding elongation factor
lmo1588	3.429574	0.001	argD- acetylornithine aminotransferase

lmo0211	3.41143	<0.000	ctc - similar to *B. subtilis* general stress protein
lmo2197	3.386435	<0.000	Hypothetical protein
lmo0562	3.374816	<0.000	Similar to phosphoribosyl-AMP cyclohydrolase (HisI1 protein)
lmo1038	3.353566	0.001	Similar to molybdopterin-guanine dinucleotide biosynthesis protein A
lmo1040	3.347303	<0.000	Similar to molybdenum ABC transporters (permease)
lmo1690	3.335989	<0.000	Similar to hypothetical proteins
lmo2124	3.325154	0.005	Similar to maltodextrin ABC-transport system (permease)
lmo1172	3.315704	<0.000	Similar to response regulator NasT
lmo1039	3.258998	<0.000	Similar to ABC transporter, ATP-binding protein
lmo1173	3.247352	<0.000	Hypothetical protein
lmo2761	3.234252	0.004	Similar to 6-phospho-beta-glucosidase
lmo2123	3.23241	<0.000	Similar to arabinogalactan oligomer / maltooligosaccharide transport system permease protein
lmo0393	3.23097	<0.000	Unknown
lmo1460	3.18619	<0.000	Similar to *B. subtilis* RecO protein involved in DNA repair and homologous recombination
lmo2065	3.160166	<0.000	Hypothetical protein
lmo2460	3.149034	<0.000	Similar to central glycolytic genes regulator
lmo1733	3.148081	<0.000	Similar to glutamate synthase (small subunit)

lmo1589	3.133224	<0.000	argB- acetylglutamate kinase
lmo2825	3.132722	<0.000	serC- phosphoserine aminotransferase
lmo1587	3.12178	<0.000	argF- ornithine carbamoyltransferase
lmo1537	3.119249	<0.000	obgE- GTP-binding protein
lmo0673	3.117902	<0.000	Hypothetical protein
lmo2576	3.096263	<0.000	pepdidoglycan bound protein
lmo2219	3.079187	<0.000	Similar to foldase protein (PrsA)
lmo0282	3.073175	<0.000	Similar to carbon-nitrogen hydrolase family protein
lmo0597	3.046816	<0.000	Crp/Fnr family transcriptional regulator
lmo0834	3.040552	<0.000	Hypothetical protein
lmo1147	3.024252	<0.000	Similar to adenosylcobinamide kinase/adenosylcobinamide-phosphate guanylyltransferase
lmo0563	2.96762	<0.000	imidazole glycerol phosphate synthase subunit (HisF)
lmo2831	2.944163	<0.000	Similar to beta-phosphoglucomutase
lmo2830	2.929907	<0.000	Hypothetical protein
lmo2376	2.921588	<0.000	Similar to peptidyl-prolyl cis-trans isomerase B (cyclophilin B)
lmo0391	2.919879	<0.000	Hypothetical protein
lmo2373	2.917667	<0.000	Similar to PTS system- cellobiose-specific IIB component
lmo2766	2.914166	<0.000	Similar to hypothetical transcriptional regulator
lmo0622	2.913549	<0.000	Hypothetical protein
lmo0564	2.896443	<0.000	hisA- phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase

lmo2250	2.86183	<0.000	arpJ- Similar to polar amino acid transport system substrate-binding protein
lmo0844	2.86076	<0.000	Similar to reactive intermediate imine deaminase
lmo2824	2.858117	<0.000	Similar to D-3-phosphoglycerate dehydrogenase
lmo0160	2.844425	<0.000	Peptidoglycan binding protein
lmo1937	2.814344	<0.000	Similar to unknown protein
lmo2588	2.799352	0.012	Hypothetical protein
lmo2753	2.775959	<0.000	Hypothetical protein
lmo2744	2.744972	<0.000	Hypothetical protein
lmo1148	2.727225	<0.000	Similar to adenosylcobinamide-GDP ribazoletransferase
lmo0565	2.714365	<0.000	hisH- glutamine amidotransferase
lmo1734	2.708658	0.033	Similar to glutamate synthase (large subunit)
lmo1845	2.7058	<0.000	Similar to conserved hypothetical proteins
lmo1449	2.694897	0.042	Similar to endonuclease IV
lmo0869	2.690774	<0.000	Unknown
lmo2845	2.689172	<0.000	Hypothetical protein
lmo0666	2.683488	<0.000	Hypothetical protein
lmo0623	2.678609	<0.000	Hypothetical protein
lmo1526	2.673668	<0.000	Hypothetical protein
lmo2778	2.669836	<0.000	Hypothetical protein
lmo0665	2.653581	<0.000	Hypothetical protein
lmo0533	2.643926	<0.000	Similar to ACT domain-containing protein
lmo0532	2.633857	<0.000	Hypothetical protein

lmo0802	2.630528	<0.000	Hypothetical protein
lmo0664	2.624439	<0.000	Similar to maltose O-acetyltransferase
lmo2436	2.620582	<0.000	Similar to beta-glucoside operon transcriptional antiterminator
lmo0599	2.620084	<0.000	PadR family transcriptional regulator, regulatory protein PadR
lmo0845	2.610795	<0.000	Hypothetical protein
lmo0569	2.607699	<0.000	hisZ- ATP phosphoribosyltransferase
lmo2122	2.597958	<0.000	Hypothetical protein
lmo0433	2.582812	<0.000	inlA- Internalin A
lmo1450	2.580802	<0.000	Hypothetical protein
lmo2243	2.577836	<0.000	Similar to AraC family transcriptional regulator, regulatory protein of adaptative response/methylphosphotriester-DNA alkyltransferase methyltransferase
lmo0405	2.551634	<0.000	Similar to inorganic phosphate transporter, PiT family
lmo0617	2.542971	<0.000	Hypothetical protein
lmo1137	2.540802	0.004	Hypothetical protein
lmo0757	2.521715	<0.000	Similar to ABC-2 type transport system permease protein
lmo2121	2.51775	0.001	Maltose phosphorylase
lmo2085	2.513669	<0.000	Peptidoglycan binding protein
lmo0194	2.50654	<0.000	ABC transporter, ATP-binding protein
lmo2173	2.498106	<0.000	Hypothetical protein
lmo2352	2.488809	<0.000	Hypothetical protein
lmo1872	2.486668	<0.000	Similar to 23S rRNA (guanine745-N1)-methyltransferase

lmo2244	2.478331	<0.000	Similar to 23S rRNA pseudouridine1911/1915/1917 synthase
lmo0600	2.47201	0.001	Hypothetical protein
lmo0867	2.461018	<0.000	Hypothetical protein
lmo1388	2.45082	0.048	tcsA- CD4+ T cell-stimulating antigen, lipoprotein
lmo0624	2.442013	<0.000	Hypothetical protein
lmo1832	2.441899	<0.000	pyrF- orotidine 5'-phosphate decarboxylase
lmo0784	2.439789	<0.000	Similat to PTS system, mannose-specific IIA component
lmo1844	2.438992	<0.000	lspA- lipoprotein signal peptidase
lmo0610	2.434724	<0.000	Hypothetical protein
lmo0149	2.433626	0.010	Hypothetical protein
lmo0567	2.424148	<0.000	hisD- histidinol dehydrogenase
lmo1838	2.423831	<0.000	pyrB- aspartate carbamoyltransferase
lmo1722	2.418835	<0.000	Hypothetical protein
lmo1558	2.418066	<0.000	engB- ribosome biogenesis GTP-binding protein
lmo2786	2.413561	<0.000	bvrC- Similar to ADP-ribosylglycohydrolase
lmo1665	2.389012	<0.000	Hypothetical protein
lmo0077	2.387568	<0.000	Hypothetical protein
lmo2388	2.367947	<0.000	Hypothetical protein
lmo0903	2.365036	0.001	Hypothetical protein
lmo0949	2.352106	<0.000	Hypothetical protein
lmo0193	2.342503	<0.000	Similar to HlyD family secretion protein

lmo2251	2.339287	<0.000	Similar to polar amino acid transport system ATP-binding protein
lmo1843	2.31757	<0.000	Similar to 23S rRNA pseudouridine1911/1915/1917 synthase
lmo1428	2.308824	<0.000	opuCA- similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)
lmo2395	2.306472	<0.000	Hypothetical protein
lmo1491	2.30381	<0.000	Similar to unknown proteins
lmo0843	2.293582	<0.000	Hypothetical protein
lmo0566	2.293434	<0.000	hisB- imidazoleglycerol-phosphate dehydratase
lmo0042	2.290149	<0.000	Similar to membrane-associated protein
lmo1014	2.288493	<0.000	Similar to glycine betaine/proline transport system ATP-binding protein (gbuA)
lmo1955	2.274103	0.002	Similar to integrase/recombinase
lmo1936	2.267831	0.011	gpsA- similar to NAD(P)H-dependent glycerol-3-phosphate dehydrogenase
lmo2021	2.263118	0.001	Hypothetical protein
lmo0913	2.262837	<0.000	Similar to succinate semialdehyde dehydrogenase
lmo0603	2.262007	<0.000	Hypothetical protein
lmo2779	2.255656	<0.000	Similar to phosphotransferase system mannitol-specific enzyme IIBC
lmo1246	2.248765	0.029	Hypothetical protein
lmo0265	2.247628	<0.000	succinyl-diaminopimelate desuccinylase
lmo0625	2.243262	<0.000	Putative lipase/acylhydrolase
lmo0773	2.241966	<0.000	Hypothetical protein
lmo2350	2.237259	0.001	Hypothetical protein

lmo0195	2.231925	0.001	Similar to membrane protein (putative ABC transporter component)
lmo2204	2.228177	<0.000	Hypothetical protein
lmo2252	2.226525	0.007	aspartate aminotransferase
lmo2270	2.217592	<0.000	Similar to competence protein ComK
lmo1149	2.214065	<0.000	Similar to alpha-ribazole phosphatase
lmo0404	2.209728	<0.000	Hypothetical protein
lmo1247	2.208013	<0.000	Hypothetical protein
lmo0601	2.205942	<0.000	Hypothetical protein
lmo1492	2.205497	<0.000	Hypothetical protein
lmo0272	2.194753	<0.000	Hypothetical protein
lmo1015	2.193409	<0.000	Similar to glycine betaine/proline transport system permease protein (gbuB)
lmo0568	2.192273	<0.000	HisG- ATP phosphoribosyltransferase
lmo0640	2.184319	<0.000	Similar to oxidoreductase
lmo0560	2.176134	<0.000	Glutamate dehydrogenase
lmo2238	2.170601	<0.000	Hypothetical protein
lmo1315	2.167675	<0.000	Undecaprenyl pyrophosphate synthase
lmo2762	2.167399	0.017	Similar to PTS system, cellobiose-specific IIB component
lmo0783	2.15977	0.001	Similar to PTS system, mannose-specific IIB component
lmo2585	2.157814	<0.000	Similar to *B. subtilis* YrhD protein
lmo0027	2.156467	<0.000	Similar to PTS system, beta-glucosides-specific IIA component
lmo1193	2.15539	0.002	cbiC- cobalt-precorrin-8X methylmutase
lmo1010	2.152684	<0.000	Hypothetical protein

lmo1619	2.145704	<0.000	daaA- D-amino acid aminotransferase
lmo1831	2.144809	0.013	pyre- orotate phosphoribosyltransferase
lmo2819	2.144176	<0.000	Hypothetical protein
lmo1837	2.131893	<0.000	pyrC- Highly similar to dihydroorotase
lmo1009	2.123423	0.029	Hypothetical protein
lmo0995	2.123166	<0.000	Hypothetical protein
lmo0836	2.11774	<0.000	phosphate-starvation-inducible protein PsiE
lmo1016	2.112066	<0.000	gbuC- Highly similar to glycine betaine ABC transporters (glycine betaine-binding protein)
lmo1270	2.108211	<0.000	Similar to signal peptidase I
lmo0043	2.107584	0.002	Similar to arginine deiminase
lmo0994	2.106731	0.005	Hypothetical protein
lmo0870	2.106039	0.010	Hypothetical protein
lmo1257	2.105861	<0.000	Unknown
lmo0595	2.101085	<0.000	Similar to O-acetylhomoserine (thiol)-lyase
lmo2521	2.101078	<0.000	Similar to N-acetylglucosaminyldiphosphoundecaprenol N-acetyl-beta-D-mannosaminyltransferase
lmo0798	2.09387	<0.000	Similar to lysine-specific permease
lmo1624	2.089294	<0.000	Hypothetical protein
lmo0850	2.0668	<0.000	Hypothetical protein
lmo2673	2.066421	<0.000	Hypothetical protein
lmo0352	2.054393	0.028	Hypothetical protein
lmo0534	2.053371	<0.000	Hypothetical protein

lmo0866	2.05161	0.009	Similar to ATP-dependent RNA helicase (DeaD)
lmo1909	2.044013	<0.000	Hypothetical protein
lmo2157	2.041023	0.002	sepA- hypothetical protein
lmo1490	2.038605	<0.000	Similar to shikimate dehydrogenase
lmo1887	2.0365	<0.000	Similar to ethanolamine utilization protein (EutQ)
lmo2586	2.032389	0.003	Similar to formate dehydrogenase alpha chain
lmo0170	2.03134	0.003	Hypothetical protein
lmo1839	2.03062	0.001	Similar to uracil permease (pyrP)
lmo2480	2.028146	<0.000	Hypothetical protein
lmo0055	2.027999	0.003	purA- adenylosuccinate synthetase
lmo2232	2.026296	0.001	Hypothetical protein
lmo1697	2.026231	<0.000	Hypothetical protein
lmo2103	2.01877	0.044	eutD- phosphotransacetylase
lmo1487	2.017836	<0.000	Hypothetical protein
lmo0161	2.009932	<0.000	Similar to rsbT co-antagonist protein RsbR
lmo0214	-2.00379	<0.000	Mfd- transcription-repair coupling factor
lmo1498	-2.00404	<0.000	Hypothetical protein
lmo1061	-2.01023	<0.000	Similar to histidine kinase
lmo0792	-2.01762	<0.000	Hypothetical protein
lmo2218	-2.02365	0.023	Hypothetical protein
lmo2579	-2.03822	0.024	Hypothetical protein
lmo1074	-2.03874	<0.000	Similar to teichoic acid transport system permease protein
lmo2084	-2.04812	<0.000	Hypothetical protein

lmo1402	-2.05773	<0.000	Hypothetical protein
lmo0464	-2.06321	<0.000	Hypothetical protein
lmo1770	-2.07341	<0.000	Similar to L-threonine kinase
lmo0645	-2.07516	0.002	Similar to basic amino acid/polyamine antiporter, APA family
lmo2731	-2.08573	<0.000	Hypothetical protein
lmo0076	-2.08848	<0.000	Similar to AraC family transcriptional regulator, regulatory protein of adaptative response / methylated-DNA-[protein]-cysteine methyltransferase
lmo2796	-2.09129	<0.000	Hypothetical protein
lmo0075	-2.10344	<0.000	Hypothetical protein
lmo0051	-2.10633	<0.000	Similar to two-component system, AgrA family, response regulator AgrA
lmo2361	-2.12063	0.005	Hypothetical protein
lmo1584	-2.1309	<0.000	Hypothetical protein
lmo2723	-2.13644	<0.000	Hypothetical protein
lmo2082	-2.13984	0.003	Camphor resistance protein CrcB
lmo1764	-2.16253	0.003	purD- phosphoribosylamine--glycine ligase
lmo0735	-2.16416	<0.000	Similar to ribulose-phosphate 3-epimerase
lmo0165	-2.17707	<0.000	Hypothetical protein
lmo0426	-2.17968	<0.000	Similar to PTS system, fructose-specific IIA component
lmo2081	-2.18127	<0.000	Camphor resistance protein CrcB
lmo2578	-2.18755	<0.000	Hypothetical protein
lmo1307	-2.19045	<0.000	Hypothetical protein
lmo0106	-2.20153	<0.000	Hypothetical protein

lmo0441	-2.20634	<0.000	Similar to penicillin-binding protein
lmo2063	-2.20764	<0.000	Hypothetical protein
lmo0489	-2.21558	<0.000	Hypothetical protein
lmo0434	-2.22502	<0.000	inlB- internalin B
lmo1551	-2.22516	<0.000	Similar to dihydrofolate synthase / folylpolyglutamate synthase (folC)
lmo1585	-2.22646	<0.000	Similar to protease IV
lmo2423	-2.23799	0.006	Hypothetical protein
lmo0420	-2.23988	<0.000	Hypothetical protein
lmo2644	-2.24668	<0.000	Hypothetical protein
lmo0483	-2.25786	<0.000	Hypothetical protein
lmo0516	-2.26235	<0.000	Similar to poly-gamma-glutamate synthesis protein (capsule biosynthesis protein)
lmo1078	-2.27949	<0.000	Similar to TP-glucose-1-phosphate uridylyltransferase
lmo2461	-2.29796	<0.000	sigL- RNA polymerase factor sigma-54
lmo0294	-2.31197	<0.000	Hypothetical protein
lmo2828	-2.31233	0.019	Hypothetical protein
lmo0824	-2.3138	<0.000	Hypothetical protein
lmo2798	-2.34214	<0.000	Hypothetical protein
lmo0021	-2.36733	<0.000	Similar to PTS system, mannose-specific IIA component
lmo1457	-2.37391	<0.000	Hypothetical protein
lmo0573	-2.38367	<0.000	Similar to putative MFS transporter, AGZA family, xanthine/uracil permease
lmo1300	-2.39162	<0.000	Similar to arsenical pump membrane protein

lmo2562	-2.40416	<0.000	Hypothetical protein
lmo1534	-2.40681	<0.000	Similar to L-lactate dehydrogenase
lmo0842	-2.4162	<0.000	Peptidoglycan binding protein
lmo0100	-2.43041	<0.000	Hypothetical protein
lmo1475	-2.43049	<0.000	hrcA- heat-inducible transcription repressor
lmo2808	-2.43178	0.001	Hypothetical protein
lmo0153	-2.43247	<0.000	Similar to zinc transport system substrate-binding protein
lmo1474	-2.44271	<0.000	heat shock protein GrpE (Molecular chaperone)
lmo1629	-2.46271	<0.000	trpF- phosphoribosylanthranilate isomerase
lmo2815	-2.48741	<0.000	fabG- 3-ketoacyl-ACP reductase
lmo0737	-2.49677	<0.000	Hypothetical protein
lmo0804	-2.49711	<0.000	Hypothetical protein
lmo1765	-2.50113	0.028	purH- bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
lmo2795	-2.55206	<0.000	Hypothetical protein
lmo2229	-2.558	<0.000	Similar to penicillin-binding protein 2A
lmo0202	-2.56287	<0.000	Hly- Listeriolysin O precursor
lmo0421	-2.56637	<0.000	Hypothetical protein
lmo1412	-2.56708	<0.000	Modulator of DNA topology
lmo0736	-2.57461	<0.000	ribose-5-phosphate isomerase B
lmo2276	-2.5848	<0.000	Hypothetical protein
lmo2687	-2.61281	<0.000	Hypothetical protein

lmo0790	-2.66855	<0.000	Similar to putative transcription regulator
lmo0934	-2.70263	0.001	Hypothetical protein
lmo1413	-2.7086	<0.000	peptidoglycan binding protein
lmo0427	-2.72579	<0.000	Similar to PTS system, fructose-specific IIB component
lmo1473	-2.78544	<0.000	Molecular chaperone DnaK
lmo2112	-2.81454	<0.000	hemE- uroporphyrinogen decarboxylase
lmo1974	-2.94926	<0.000	Similar to GntR family transcriptional regulator
lmo2156	-2.97331	0.001	Hypothetical protein
lmo0822	-2.98862	<0.000	Hypothetical protein
lmo0731	-3.05655	<0.000	Hypothetical protein
lmo1880	-3.09451	0.01	ribonuclease HI
lmo0848	-3.12084	<0.000	Similar to polar amino acid transport system ATP-binding protein
lmo0935	-3.24155	<0.000	Similar to tRNA (cytidine/uridine-2'-O-)-methyltransferase
lmo2275	-3.39277	<0.000	protein gp28
lmo1841	-3.61851	0.001	Hypothetical protein
lmo1298	-3.96286	<0.000	Similar to MerR family transcriptional regulator, glutamine synthetase repressor (glnR)
lmo2803	-3.97145	<0.000	Hypothetical protein
lmo2069	-4.0716	<0.000	Co-chaperonin GroES
lmo0314	-4.18026	<0.000	Hypothetical protein
lmo0847	-5.37379	<0.000	Similar to polar amino acid transport system permease protein
lmo2759	-5.67283	<0.000	Hypothetical protein

lmo0997	-6.457	<0.000	clpE- ATP-dependent protease
lmo1885	-6.94557	<0.000	xanthine phosphoribosyltransferase
lmo2591	-7.29315	<0.000	GW repeat-containing surface protein
lmo2016	-65.156	<0.000	cspB- cold shock protein (beta-ribbon, CspA family)

Appendix 3- anaerobic/low temp combined

Locus ID	Fold change	Adjusted *p*-value	Gene/protein function
*lmo*1250	190.8599	<0.000	Major facilitator protein family- membrane transport, antibiotic resistance, virulence
*lmo*0690	101.2387	<0.000	*flaA*- flagella production, motility, virulence
*lmo*0118	55.72899	<0.000	Antigen A – Secreted protein (unknown function)
*lmo*0693	50.02654	<0.000	FliY – Flagella motor switch protein - chemotaxis, cell motility, secretion, virulence
lmo0694	49.3852	<0.000	Hypothetical protein- Unknown function
*lmo*0699	49.11214	<0.000	Flagella motor switch protein, chemotaxis, cell motility, secretion, virulence
*lmo*0117	45.03744	<0.000	*lmaB – Listeria* antigen - virulence
*lmo*1986	45.02603	<0.000	ketol-acid reductoisomerase
*lmo*1249	44.65633	<0.000	Hypothetical protein
*lmo*0696	44.59835	<0.000	*flgD* – Flagella component- motility, virulence
*lmo*0695	42.17935	<0.000	Hypothetical protein
*lmo*0697	38.57689	<0.000	*flgE* – Flagella hook protein- chemotaxis, cell motility, secretion, virulence
*lmo*2101	37.39495	<0.000	Pyridoxal 5'-phosphate (PLP) synthase- PLP production

*lmo*0698	37.14999	<0.000	Flagella motor switch protein- Flagella rotation control- chemotaxis, virulence, motility
*lmo*2102	36.25897	<0.000	Vitamin B6 metabolism
*lmo*1983	35.63289	<0.000	Dihydroxy-acid dehydratase- Valine and isoleucine biosynthesis
*lmo*0120	35.16858	<0.000	Hypothetic protein (unknown function)
*lmo*0683	34.40201	<0.000	Chemotaxis protein methyltransferase
*lmo*0678	34.19878	<0.000	similar to flagellar biosynthetic protein FliR
*lmo*0692	33.52338	<0.000	*cheA* - two-component sensor histidine kinase
*lmo*0691	31.8334	<0.000	*cheY* – chemotaxis response regulator – motility, virulence, chemotaxis
*lmo*0679	30.59859	<0.000	*FlhB*- flagellar biosynthesis protein
*lmo*0701	29.99263	<0.000	Hypothetical protein (unknown function)
*lmo*1699	29.07322	0.003	ethyl-accepting chemotaxis protein (MCP), signaling domain – ligand binding, light/oxygen sensing
*lmo*0680	28.80633	<0.000	Similar to flagellar associated protein FlhA
*lmo*0700	28.78955	<0.000	Similar to flagellar switch protein FliY
*lmo*0702	28.6406	<0.000	Hypothetical protein (Unknown function)
*lmo*0686	28.24426	<0.000	*motB*- similar to motility protein (flagella motor rotation)

*lmo*0685	27.71481	<0.000	Flagella motor protein MotA
*lmo*1984	27.62546	<0.000	ivlB- Similar to acetolactate synthase
*lmo*0689	26.99055	<0.000	Similar to CheA activity modulating chemotaxis protein CheV
*lmo*0688	25.39076	<0.000	Glycosyl transferase
*lmo*0687	25.31482	<0.000	Hypothetical protein (Unknown function)
*lmo*1985	24.66357	<0.000	ilvH- similar to acetolactate synthase, small subunit
*lmo*1987	24.53125	<0.000	leuA- similar to 2-isopropylmalate synthase
*lmo*0703	23.30961	<0.000	Hypothetical protein (Unknown function)
*lmo*0681	22.44093	<0.000	Fagellar biosynthesis regulator FlhF
*lmo*0682	22.39263	<0.000	Flagellar basal body rod protein FlgG
*lmo*1990	21.69433	<0.000	leuD- isopropylmalate isomerase small subunit
*lmo*2091	21.1281	<0.000	argH- argininosuccinate lyase
*lmo*2362	21.10581	<0.000	Hypothetical protein
*lmo*1991	20.89806	<0.000	ilvA- threonine dehydratase
*lmo*0677	20.74591	<0.000	fliQ- flagella biosynthesis protein
*lmo*0684	20.5186	<0.000	Hypothetical protein (Unknown function)
*lmo*0122	20.29478	<0.000	Hypothetical protein
*lmo*0675	19.61841	<0.000	Hypothetical protein (Unknown function)
*lmo*0705	19.53554	<0.000	Similar to flagellar hook associated protein FlgK

*lmo*1179	18.986	<0.000	Hypothetical protein
*lmo*2363	18.64863	<0.000	Similar to glutamate decarboxylase
*lmo*0704	18.20314	<0.000	Hypothetical protein (Unknown function)
*lmo*0676	18.17424	<0.000	flip- flagella biosynthesis protein
*lmo*0712	18.12392	<0.000	Similar to flagellar hook-basal body complex protein FliE
*lmo*1700	18.10441	<0.000	Conserved hypothetical protein
*lmo*1989	17.85489	<0.000	leuC- isopropylmalate isomerase large subunit
*lmo*0123	17.78181	<0.000	Hypothetical protein
*lmo*1988	17.50702	<0.000	leuB- 3-isopropylmalate dehydrogenase
*lmo*1178	17.38015	<0.000	Hypothetical protein
*lmo*0724	17.1393	<0.000	Hypothetical protein- similar to *B. subtilis* YvpB protein
*lmo*0661	16.37865	<0.000	Hypothetical protein
*lmo*0723	16.00742	<0.000	Hypothetical protein- similar to methyl-accepting chemotaxis protein
*lmo*0707	15.91783	<0.000	flagella capping protein FliD
*lmo*0119	15.84576	<0.000	Hypothetical protein (Unknown function)
*lmo*0711	15.81011	<0.000	flgC- flagella basal body rod protein
*lmo*0125	15.6522	<0.000	Hypothetical protein
*lmo*0710	15.57185	<0.000	flgB- flagella basal-body rod protein
*lmo*0716	15.375	<0.000	flagellum-specific ATP synthase
*lmo*0706	15.3176	<0.000	flgL- flagellar hook-associated protein

*lmo*0128	15.06932	<0.000	Hypothetical protein
*lmo*0708	14.8131	<0.000	Similar to flagella protein FliS
*lmo*2090	14.72795	<0.000	argG- argininosuccinate synthase
*lmo*0713	14.32659	<0.000	fliF- flagella MS-ring protein
*lmo*1180	14.18672	<0.000	Similar to ethanolamine utilization protein EutM
*lmo*0126	13.98685	<0.000	Hypothetical protein
*lmo*0717	13.6853	<0.000	Hypothetical protein
*lmo*2720	13.64725	<0.000	Similar to acetyl-CoA synthetase
*lmo*0129	13.63781	<0.000	Similar to N-acetylmuramoyl-L-alanine amidase
*lmo*0124	13.40416	<0.000	Hypothetical protein
*lmo*0715	13.20428	<0.000	fliH- flagella assembly protein H
*lmo*0121	12.9791	<0.000	Hypothetical protein
*lmo*0718	12.46127	<0.000	Hypothetical protein
*lmo*2269	12.27723	<0.000	Hypothetical protein
*lmo*2210	12.22814	<0.000	Hypothetical protein
*lmo*0714	11.83985	<0.000	fliG- flagella motor switch protein G
*lmo*1182	11.2835	<0.000	Hypothetical protein
*lmo*0709	11.05713	<0.000	Hypothetical protein
*lmo*1185	10.46214	<0.000	Hypothetical protein
*lmo*1175	9.90352	<0.000	Ethanolamine ammonia-lyase large subunit (eutB)
*lmo*1183	9.881764	<0.000	Conserved hypothetical protein
*lmo*1251	9.701428	<0.000	Fnr/Crp family transcriptional regulator

*lmo*1184	9.290833	<0.000	Chapter 9 Carbon dioxide concentrating mechanism protein
*lmo*1181	8.95426	<0.000	Similar to ethanolamine utilization cobalamin adenosyltransferase
*lmo*1176	8.882883	<0.000	ethanolamine ammonia-lyase small subunit (eutC)
*lmo*1364	8.760029	<0.000	cspL- cold-shock protein
*lmo*2125	8.732708	<0.000	Similar to sugar ABC transporter substrate-binding protein
*lmo*1917	8.425621	<0.000	pflA- Similar to formate C-acetyltransferase
*lmo*1177	8.302939	<0.000	Similar to ethanolamine utilization protein EutL
*lmo*0322	7.893457	<0.000	Unknown protein
*lmo*1186	7.70185	<0.000	Similar to *E. coli* ethanolamine utilization protein EutH
*lmo*0019	7.255966	<0.000	Unknown
*lmo*2374	7.206012	<0.000	Aspartate kinase
*lmo*1174	7.187881	<0.000	Ethanolamine utilization protein EutA
*lmo*2256	7.120557	<0.000	Similar to unknown proteins
*lmo*0321	7.106004	<0.000	Similar to unknown proteins
*lmo*1733	6.691899	<0.000	Similar to glutamate synthase (small subunit)
*lmo*2375	6.365469	<0.000	Unknown
*lmo*1187	6.202916	<0.000	EutQ- ethanolamine utilization protein
*lmo*1000	6.147074	<0.000	Hypothetical protein
*lmo*0433	6.113946	<0.000	inlA- Internalin A

*lmo*1056	5.977439	<0.000	Unknown
*lmo*0189	5.976781	<0.000	Highly similar to *B subtilis* Veg protein
*lmo*0540	5.689453	<0.000	Similar to penicillin-binding protein
*lmo*1734	5.614218	<0.000	Similar to glutamate synthase (large subunit)
*lmo*2832	5.551275	<0.000	Similar to glycerate kinase
*lmo*1001	5.443279	<0.000	Similar to *B. subtilis* protein YkvS
*lmo*0835	5.291354	0.002	Peptidoglycan binding protein
*lmo*1864	5.290748	<0.000	Similar to hemolysinIII proteins, putative integral membrane protein
*lmo*0654	5.052038	<0.000	Hypothetical protein
*lmo*0562	5.008585	<0.000	Similar to phosphoribosyl-AMP cyclohydrolase (HisI1 protein)
*lmo*1190	4.922596	<0.000	Hypothetical protein
*lmo*0561	4.908838	<0.000	Similar to phosphorybosil-AMP-cyclohydrolase (HisI2 protein)
*lmo*0115	4.855664	0.009	lmaD- hypothetical protein
*lmo*0604	4.838538	<0.000	Similar to *B. subtilis* YvlA protein
*lmo*0211	4.836132	<0.000	ctc - similar to *B. subtilis* general stress protein
*lmo*0937	4.825045	<0.000	Hypothetical protein
*lmo*2819	4.819486	<0.000	Hypothetical protein
*lmo*1040	4.7934	<0.000	Similar to molybdenum ABC transporters (permease)
*lmo*1038	4.736907	<0.000	Hypothetical protein- molybdopterin-guanine dinucleotide biosynthesis protein A
*lmo*2673	4.689369	<0.000	Hypothetical protein

*lmo*0913	4.654007	<0.000	Similar to succinate semialdehyde dehydrogenase
*lmo*1137	4.650307	<0.000	Hypothetical protein
*lmo*0564	4.614605	<0.000	hisA- phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase
*lmo*1690	4.580904	<0.000	Similar to hypothetical proteins
*lmo*2352	4.509178	<0.000	Hypothetical protein
*lmo*1039	4.45448	<0.000	Similar to ABC transporter, ATP-binding protein
*lmo*2126	4.450846	<0.000	Hypothetical protein
*lmo*2085	4.338683	<0.000	peptidoglycan binding protein
*lmo*1717	4.315693	<0.000	Hypothetical protein
*lmo*2845	4.27518	<0.000	Hypothetical protein
*lmo*1429	4.251639	<0.000	Similar to thiamine transporter
*lmo*2157	4.230401	<0.000	sepA; hypothetical protein
*lmo*2576	4.153014	0.001	pepdidoglycan bound protein
*lmo*0269	4.086272	<0.000	Hypothetical protein- peptide/nickel transport system permease protein
*lmo*0565	4.065241	<0.000	hisH- imidazole glycerol phosphate synthase subunit
*lmo*0563	4.06332	<0.000	HisF- imidazole glycerol phosphate synthase subunit
*lmo*0610	4.036549	0.001	Hypothetical protein
*lmo*0823	4.00148	<0.000	Hypothetical protein
*lmo*0265	3.913432	<0.000	succinyl-diaminopimelate desuccinylase
*lmo*1145	3.887254	0.005	Similar to ethanolamine utilization protein (EutP)

*lmo*0869	3.860078	<0.000	Unknown
*lmo*2825	3.819342	<0.000	serC- phosphoserine aminotransferase
*lmo*1713	3.803681	<0.000	Similar to cell-shape determining proteins
*lmo*0802	3.779321	0.004	Hypothetical protein
*lmo*2852	3.765043	<0.000	Hypothetical protein
*lmo*0569	3.761846	<0.000	hisZ- ATP phosphoribosyltransferase
*lmo*2460	3.729667	<0.000	Similar to central glycolytic genes regulator
*lmo*0568	3.719294	<0.000	hisG- ATP phosphoribosyltransferase
*lmo*2373	3.709326	<0.000	Similar to cellobiose-specific IIB component
*lmo*1007	3.696959	<0.000	Hypothetical protein
*lmo*2350	3.644989	<0.000	Hypothetical protein
*lmo*2824	3.626133	<0.000	Similar to D-3-phosphoglycerate dehydrogenase
*lmo*0525	3.608876	<0.000	Unknown
*lmo*0283	3.574633	<0.000	Similar to D-methionine transport system permease protein
*lmo*0834	3.53015	<0.000	Hypothetical protein
*lmo*0567	3.507627	<0.000	hisD- histidinol dehydrogenase
*lmo*0405	3.504425	<0.000	Similar to inorganic phosphate transporter, PiT family
*lmo*0532	3.479206	<0.000	Hypothetical protein
*lmo*0392	3.440108	<0.000	Highly similar to B. subtilis YqfA protein

*lmo*2779	3.422954	<0.000	Similar to phosphotransferase system mannitol-specific enzyme IIBC
*lmo*0642	3.401809	<0.000	Hypothetical protein
*lmo*0664	3.385576	<0.000	Similar to maltose O-acetyltransferase
*lmo*1067	3.371746	<0.000	Similar to GTP-binding elongation factor
*lmo*0870	3.360949	<0.000	Hypothetical protein
*lmo*1172	3.357794	<0.000	Similar to response regulator NasT
*lmo*2173	3.353267	<0.000	Hypothetical protein
*lmo*0533	3.310312	<0.000	Similar to ACT domain-containing protein
*lmo*0672	3.293964	<0.000	Similar to unknown protein
*lmo*0903	3.269328	<0.000	Hypothetical protein
*lmo*1173	3.246548	<0.000	Hypothetical protein
*lmo*0784	3.220654	<0.000	Similar to mannose-specific IIA component
*lmo*0043	3.210203	<0.000	Similar to arginine deiminase
*lmo*0566	3.200438	<0.000	hisB- imidazoleglycerol-phosphate dehydratase
*lmo*2817	3.182528	<0.000	Hypothetical protein
*lmo*2219	3.176266	<0.000	Similar to foldase protein PrsA
*lmo*1872	3.166382	0.012	Similar to rRNA (guanine745-N1)-methyltransferase
*lmo*0665	3.15948	<0.000	Hypothetical protein
*lmo*0666	3.149171	<0.000	Hypothetical protein
*lmo*1694	3.141291	<0.000	Hypothetical protein

*lmo*0640	3.08885	<0.000	Similar to oxidoreductase
*lmo*1369	3.077761	0.033	Similar to phosphate butyryltransferase
*lmo*1526	3.077287	<0.000	Hypothetical protein
*lmo*0393	3.058069	0.042	Unknown
*lmo*0285	3.034138	<0.000	Lipoprotein- D-methionine transport system substrate-binding protein
*lmo*2585	3.004798	<0.000	Similar to *B. subtilis* YrhD protein
*lmo*1618	2.979403	<0.000	Hypothetical protein
*lmo*0783	2.963854	<0.000	Similar to mannose-specific IIB component
*lmo*0116	2.952264	<0.000	ImaC
*lmo*0284	2.949538	<0.000	Similar to D-methionine transport system ATP-binding protein
*lmo*2818	2.942437	<0.000	Similar to MFS transporter, multidrug resistance protein
*lmo*1634	2.939595	<0.000	acetaldehyde dehydrogenase / alcohol dehydrogenase
lmo2602	2.932922	<0.000	putative Mg^{2+} transporter-C (MgtC) family protein
lmo0932	2.930661	<0.000	Hypothetical protein
lmo1617	2.917227	<0.000	Hypothetical protein
lmo1171	2.916689	<0.000	pduQ like protein
lmo0223	2.896457	<0.000	Similar to cysteine synthase A (cysK)
lmo1146	2.896244	<0.000	Hypothetical protein
lmo0844	2.885981	<0.000	Similar to reactive intermediate imine deaminase

lmo0554	2.862235	<0.000	Similar to NADP-dependent alcohol dehydrogenase
lmo1460	2.858685	<0.000	Similar to *B. subtilis* RecO protein involved in DNA repair and homologous recombination
lmo2376	2.858523	<0.000	Similar to peptidyl-prolyl cis-trans isomerase B (cyclophilin B)
lmo0845	2.850841	<0.000	Hypothetical protein
lmo0603	2.842609	<0.000	Hypothetical protein
lmo1428	2.837483	<0.000	opuCA- similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)
lmo0994	2.830429	0.004	Hypothetical protein
lmo0391	2.809317	<0.000	Hypothetical protein
lmo2204	2.802348	0.001	Hypothetical protein
lmo1257	2.79588	<0.000	Unknown
lmo2230	2.772668	<0.000	Similar to arsenate reductase
lmo0757	2.772068	<0.000	Similar to ABC-2 type transport system permease protein
lmo2436	2.771473	<0.000	Similar to beta-glucoside operon transcriptional antiterminator
lmo2816	2.769586	<0.000	Hypothetical protein
lmo2231	2.765269	<0.000	Hypothetical protein
lmo1740	2.761779	0.001	Similar to polar amino acid transport system permease protein
lmo2586	2.756667	<0.000	Similar to formate dehydrogenase alpha chain
lmo0782	2.7499	0.048	Similar to mannose-specific IIC component (pts system)

lmo0560	2.737253	<0.000	glutamate dehydrogenase
lmo2123	2.732546	<0.000	Similar to arabinogalactan oligomer / maltooligosaccharide transport system permease protein
lmo0995	2.715594	<0.000	Hypothetical protein
lmo2766	2.706593	<0.000	Similar to hypothetical transcriptional regulator
lmo2103	2.703132	<0.000	phosphate acetyltransferase (eutD)
lmo1955	2.700524	0.010	Similar to integrase/recombinase
lmo0170	2.683651	<0.000	Hypothetical protein
lmo2830	2.677172	<0.000	Hypothetical protein
lmo1450	2.666813	<0.000	Hypothetical protein
lmo2238	2.666538	<0.000	Hypothetical protein
lmo2338	2.663344	<0.000	Aminopeptidase (pepC)
lmo1144	2.65888	<0.000	Hypothetical protein: ethanolamine utilization protein (EutS)
lmo0867	2.652362	<0.000	Hypothetical protein
lmo2467	2.645009	<0.000	Hypothetical protein: chitin-binding protein
lmo0161	2.627437	0.001	rsbT co-antagonist protein RsbR
lmo2778	2.623386	<0.000	Hypothetical protein
lmo2021	2.620505	<0.000	Hypothetical protein
lmo2722	2.594359	<0.000	Hypothetical protein
lmo1570	2.580879	<0.000	pyruvate kinase (pykA)
lmo2202	2.57242	<0.000	3-oxoacyl-ACP synthase
lmo0597	2.554475	<0.000	Crp/Fnr family transcriptional regulator

lmo2343	2.549453	<0.000	Hypothetical protein
lmo2197	2.549084	<0.000	Hypothetical protein
lmo2584	2.545996	<0.000	FdhD- formate dehydrogenase accessory protein
lmo1537	2.533617	<0.000	GTPase ObgE
lmo2124	2.533159	<0.000	Similar to maltodextrin ABC-transport system (permease)
lmo0673	2.528687	0.002	Hypothetical protein
lmo2755	2.504347	0.011	Hypothetical protein
lmo1909	2.49714	0.001	Hypothetical protein
lmo1845	2.496106	<0.000	Similar to conserved hypothetical proteins
lmo0773	2.488597	<0.000	Hypothetical protein
lmo0499	2.478646	<0.000	Similar to ribulose-phosphate 3-epimerase
lmo0103	2.476714	0.029	Hypothetical protein
lmo0169	2.470591	<0.000	Similar to glucose uptake protein
lmo2348	2.444835	<0.000	Similar to L-cystine transport system permease protein
lmo2347	2.435395	<0.000	Similar to L-cystine transport system permease protein
lmo2158	2.433083	0.001	Hypothetical protein
lmo0515	2.432558	0.001	Hypothetical protein
lmo1937	2.429744	<0.000	Similar to unknown protein
lmo1147	2.424378	0.007	Similar to adenosylcobinamide kinase/adenosylcobinamide-phosphate guanylyltransferase
lmo2270	2.412775	<0.000	Similar to competence protein ComK

lmo0911	2.40762	<0.000	Hypothetical protein
lmo1722	2.404489	<0.000	Hypothetical protein
lmo0539	2.403114	<0.000	Tagatose 1,6-diphosphate aldolase
lmo2545	2.398647	<0.000	thrB- homoserine kinase
lmo0048	2.397423	<0.000	Accessory gene regulator protein
lmo0292	2.397062	<0.000	Hypothetical protein
lmo1619	2.393864	<0.000	daaA- D-amino acid aminotransferase
lmo2395	2.38472	<0.000	Hypothetical protein
lmo2122	2.378851	<0.000	Hypothetical protein
lmo2065	2.377646	<0.000	Hypothetical protein
lmo1433	2.368743	<0.000	Similar to glutathione reductase (NADPH)
lmo2005	2.363768	<0.000	Hypothetical protein
lmo2171	2.347547	0.017	Hypothetical protein
lmo1879	2.342399	0.001	cspD- cold shock protein (beta-ribbon, CspA family)
lmo0534	2.33815	<0.000	Hypothetical protein
lmo2344	2.33582	<0.000	Hypothetical protein
lmo1009	2.320033	0.002	Hypothetical protein
lmo1624	2.319636	<0.000	Hypothetical protein
lmo0624	2.297116	<0.000	Hypothetical protein
lmo0194	2.296794	0.013	Putative ABC transport system ATP-binding protein
lmo1623	2.296654	<0.000	Hypothetical protein
lmo2743	2.296209	<0.000	Translaldolase
lmo0350	2.296054	0.029	Hypothetical protein

lmo1315	2.29217	<0.000	Undecaprenyl pyrophosphate synthase
lmo2387	2.289952	<0.000	Hypothetical protein
lmo0212	2.288209	<0.000	Hypothetical protein
lmo0949	2.287786	<0.000	Hypothetical protein
lmo0160	2.281761	0.002	Peptidoglycan binding protein
lmo2172	2.28036	0.005	Similar to propionate CoA-transferase
lmo2400	2.268239	0.010	Similar to diamine N-acetyltransferase
lmo1432	2.268179	<0.000	Hypothetical protein
lmo0271	2.26424	<0.000	Similar to 6-phospho-beta-glucosidase
lmo0193	2.260046	<0.000	Hypothetical protein- HlyD family secretion protein
lmo0617	2.259895	<0.000	Hypothetical protein
lmo2346	2.252982	<0.000	Similar to L-cystine transport system ATP-binding protein
lmo0149	2.245368	<0.000	Hypothetical protein
lmo0866	2.238085	<0.000	Similar to ATP-dependent RNA helicase (DeaD)
lmo0622	2.233373	0.028	Hypothetical protein
lmo1571	2.22056	<0.000	pfkA- 6-phosphofructokinase
lmo2388	2.204874	0.009	Hypothetical protein
lmo1270	2.204179	<0.000	Similar to signal peptidase I
lmo2351	2.196421	0.002	Similar to FMN reductase
lmo1730	2.189793	<0.000	Similar to multiple sugar transport system substrate-binding protein

lmo0625	2.18753	<0.000	Putative lipase/acylhydrolase
lmo0794	2.187354	0.003	Hypothetical protein
lmo0595	2.186075	0.003	Similar to O-acetylhomoserine (thiol)-lyase
lmo1427	2.184902	0.001	opuCB- osmoprotectant transport system permease protein
lmo1844	2.183311	<0.000	lspA- lipoprotein signal peptidase
lmo1558	2.176263	0.003	GTP-binding protein
lmo1426	2.166459	0.001	opuCC- osmoprotectant transport system substrate-binding protein
lmo1133	2.163807	<0.000	Hypothetical protein
lmo1302	2.161337	0.044	LexA repressor
lmo0655	2.15003	<0.000	Similar to serine/threonine protein phosphatase 1
lmo0599	2.146349	<0.000	Similar to PadR family transcriptional regulator, regulatory protein PadR
lmo0454	2.140592	<0.000	Similar to MoxR-like ATPase
lmo1590	2.137194	<0.000	argJ- bifunctional ornithine acetyltransferase/N-acetylglutamate synthase
lmo1587	2.136675	<0.000	argF- ornithine carbamoyltransferase
lmo1340	2.133438	<0.000	Hypothetical protein
lmo1738	2.129806	0.023	Similar to polar amino acid transport system substrate-binding protein
lmo1936	2.120785	0.024	gpsA- similar to NAD(P)H-dependent glycerol-3-phosphate dehydrogenase
lmo2547	2.117799	<0.000	Homoserine dehydrogenase
lmo0449	2.116533	<0.000	Hypothetical protein

lmo2674	2.098415	<0.000	Ribose-5-phosphate isomerase B
lmo2345	2.096843	<0.000	Hypothetical protein
lmo0137	2.094369	<0.000	Similar to peptide/nickel transport system permease protein
lmo2697	2.089858	0.002	Phosphotransferase mannnose-specific family component IIA
lmo2399	2.08775	<0.000	Hypothetical protein
lmo0349	2.083616	<0.000	Hypothetical protein
lmo2033	2.083522	<0.000	Cell division protein FtsA
lmo1148	2.072739	<0.000	Similar to adenosylcobinamide-GDP ribazoletransferase
lmo1829	2.06969	<0.000	Hypothetical protein
lmo0335	2.061861	0.005	Hypothetical protein
lmo2191	2.056229	<0.000	Regulatory protein spx
lmo0337	2.048751	<0.000	Hypothetical protein
lmo2244	2.048561	0.003	Similar to 23S rRNA pseudouridine1911/1915/1917 synthase
lmo0102	2.046455	0.003	Hypothetical protein
lmo0336	2.046244	<0.000	Hypothetical protein
lmo0406	2.032453	<0.000	Similar to lactoylglutathione lyase
lmo2243	2.032385	<0.000	Similar to AraC family transcriptional regulator, regulatory protein of adaptative response / methylphosphotriester-DNA alkyltransferase methyltransferase
lmo1149	2.027246	<0.000	Similar to alpha-ribazole phosphatase

lmo1935	2.025935	<0.000	Similar to protein-tyrosine phosphatase
lmo1867	2.025929	<0.000	Pyruvate phosphate dikinase
lmo2022	2.022974	<0.000	Similar to cysteine desulfurase
lmo2234	2.016888	<0.000	Hypothetical protein
lmo0756	2.015348	<0.000	Similar to ABC-2 type transport system ATP-binding protein
lmo0341	2.013386	<0.000	Hypothetical protein
lmo1269	2.005517	<0.000	Trigger factor
lmo2780	2.005274	<0.000	Similar to PTS system, cellobiose-specific IIA component
lmo1275	2.004022	<0.000	topA- DNA topoisomerase I
lmo2473	2.003937	0.006	Hypothetical protein
lmo2742	2.002758	<0.000	Hypothetical protein
lmo1635	2.000634	<0.000	Hypothetical protein
lmo1060	-2.00719	<0.000	Hypothetical protein
lmo1074	-2.00805	<0.000	Similar to teichoic acid transport system permease protein
lmo1410	-2.00893	<0.000	Hypothetical protein
lmo0590	-2.01203	<0.000	Hypothetical protein
lmo0478	-2.01269	<0.000	Secreted protein
lmo2233	-2.02021	0.019	Hypothetical protein
lmo2302	-2.02029	<0.000	Hypothetical protein
lmo1928	-2.02317	<0.000	aroF- chorismate synthase
lmo2798	-2.02561	<0.000	Hypothetical protein
lmo1222	-2.03406	<0.000	pheT- phenylalanyl-tRNA synthetase subunit beta

lmo0106	-2.04057	<0.000	Hypothetical protein
lmo2225	-2.04673	<0.000	fumC- fumarate hydratase
lmo0076	-2.04738	<0.000	Similar to AraC family transcriptional regulator, regulatory protein of adaptative response / methylated-DNA-[protein]-cysteine methyltransferase
lmo0427	-2.05352	<0.000	Similar to PTS system, fructose-specific IIB component
lmo2461	-2.06109	<0.000	sigL- RNA polymerase factor sigma-54
lmo1078	-2.06422	<0.000	Similar to UTP-glucose-1-phosphate uridylyltransferase
lmo1299	-2.07483	<0.000	Similar to glutamine synthetase
lmo2365	-2.08488	0.001	Hypothetical protein
lmo0735	-2.08847	<0.000	Similar to ribulose-phosphate 3-epimerase
lmo1534	-2.08893	<0.000	Similar to L-lactate dehydrogenase
lmo0426	-2.11447	<0.000	Similar to PTS system, fructose-specific IIA component
lmo1210	-2.12834	<0.000	Similar to bacterial/archaeal transporter family-2 protein
lmo1671	-2.13042	<0.000	Similar to zinc transport system substrate-binding protein
lmo0366	-2.13305	<0.000	Similar to iron uptake system component EfeO
lmo0464	-2.14163	0.028	Hypothetical protein
lmo1051	-2.15618	<0.000	Peptide deformylase
lmo1981	-2.16885	<0.000	Hypothetical protein
lmo2808	-2.17308	<0.000	Hypothetical protein

lmo1645	-2.17574	<0.000	Cell surface protein
lmo1453	-2.17594	<0.000	Similar to tRNA (adenine22-N1)-methyltransferase
lmo2731	-2.19864	<0.000	Hypothetical protein
lmo0294	-2.2198	<0.000	Hypothetical protein
lmo0369	-2.22601	<0.000	Probable transcriptional regulatory protein
lmo1292	-2.22831	<0.000	Similar to glycerophosphoryl diester phosphodiesterase
lmo0165	-2.22934	0.001	Hypothetical protein
lmo1413	-2.22985	<0.000	Peptidoglycan binding protein
lmo1745	-2.23417	<0.000	Hypothetical protein
lmo0737	-2.23728	<0.000	Hypothetical protein
lmo2796	-2.24414	<0.000	Hypothetical protein
lmo1714	-2.2466	<0.000	Hypothetical protein
lmo1475	-2.25596	0.001	hrcA- heat-inducible transcription repressor
lmo1632	-2.27145	<0.000	trpG- anthranilate synthase component II
lmo0736	-2.27191	<0.000	Ribose-5-phosphate isomerase B
lmo1381	-2.27201	<0.000	Acylphosphatase
lmo0645	-2.2937	<0.000	Similar to basic amino acid/polyamine antiporter, APA family
lmo0559	-2.2947	<0.000	Hypothetical protein
lmo0790	-2.29827	0.001	Putative transcription regulator
lmo1457	-2.30493	<0.000	Hypothetical protein
lmo0842	-2.32224	<0.000	Peptidoglycan binding protein

lmo2147	-2.32517	<0.000	Hypothetical protein
lmo2063	-2.32891	<0.000	Hypothetical protein
lmo2689	-2.33392	<0.000	Similar to Mg^{2+}-importing ATPase
lmo2254	-2.33962	<0.000	Putative MFS transporter, AGZA family, xanthine/uracil permease
lmo0609	-2.35271	<0.000	Hypothetical protein
lmo0934	-2.35912	<0.000	Hypothetical protein
lmo2390	-2.36676	<0.000	Similar to thioredoxin reductase (NADPH)
lmo2060	-2.38135	<0.000	Hypothetical protein
lmo0516	-2.38881	<0.000	Similar to poly-gamma-glutamate synthesis protein (capsule biosynthesis protein)
lmo0420	-2.42327	<0.000	Hypothetical protein
lmo1633	-2.44246	<0.000	trpE- anthranilate synthase component I
lmo2229	-2.48979	<0.000	Similar to penicillin-binding protein 2A
lmo1412	-2.49009	<0.000	Modulator of DNA topology
lmo2064	-2.5442	<0.000	mscL- large-conductance mechanosensitive channel
lmo1584	-2.56833	<0.000	Hypothetical protein
lmo1939	-2.57098	<0.000	Cmk- cytidylate kinase
lmo1585	-2.57608	<0.000	Similar to protease IV
lmo2562	-2.5842	<0.000	Hypothetical protein
lmo2156	-2.59242	<0.000	Hypothetical protein
lmo2688	-2.59303	<0.000	Hypothetical protein

lmo1551	-2.59491	<0.000	folC- dihydrofolate synthase / folylpolyglutamate synthase
lmo2393	-2.60235	<0.000	Hypothetical protein
lmo1474	-2.60951	<0.000	Heat shock protein GrpE (molecular chaperone)
lmo0100	-2.66013	<0.000	Hypothetical protein
lmo0153	-2.66061	<0.000	Similar to zinc transport system substrate-binding protein
lmo1849	-2.67418	<0.000	Similar to iron/zinc/copper transport system ATP-binding protein
lmo0104	-2.6877	<0.000	Hypothetical protein
lmo2828	-2.73403	<0.000	Hypothetical protein
lmo0573	-2.74131	<0.000	Putative MFS transporter, AGZA family, xanthine/uracil permease
lmo1303	-2.77179	<0.000	Cell division suppressor protein YneA
lmo2276	-2.77552	<0.000	Hypothetical protein
lmo2185	-2.87226	<0.000	Hypothetical protein
lmo0935	-2.8745	<0.000	Similar to tRNA (cytidine/uridine-2'-O-)-methyltransferase
lmo1884	-2.91327	<0.000	Similar to xanthine permease
lmo1629	-2.92705	<0.000	trpF- phosphoribosylanthranilate isomerase
lmo1974	-2.96823	0.001	GntR family transcriptional regulator
lmo0848	-3.00788	<0.000	Similar to polar amino acid transport system ATP-binding protein
lmo0421	-3.02597	<0.000	Hypothetical protein
lmo2569	-3.06601	<0.000	Similar to peptide/nickel transport system substrate-binding protein
lmo1880	-3.13973	0.01	ribonuclease HI

lmo0822	-3.23851	<0.000	Hypothetical protein
lmo1841	-3.24221	<0.000	Hypothetical protein
lmo2685	-3.55332	<0.000	Similar to PTS system, cellobiose-specific IIA component
lmo2275	-3.99152	<0.000	Protein gp28
lmo2006	-4.01849	<0.000	alsS- acetolactate synthase
lmo0314	-4.07082	<0.000	Hypothetical protein
lmo0847	-4.14511	<0.000	Similar to polar amino acid transport system permease protein
lmo1298	-4.58594	<0.000	glnR- MerR family transcriptional regulator, glutamine synthetase repressor
lmo2687	-4.71615	<0.000	Hypothetical protein
lmo1848	-5.23594	<0.000	Similar to iron/zinc/copper transport system permease protein
lmo2069	-5.507	<0.000	Co-chaperonin GroES
lmo0731	-5.54247	<0.000	Hypothetical protein
lmo0997	-6.74303	<0.000	clpE- ATP-dependent protease
lmo2591	-8.70864	<0.000	GW repeat-containing surface protein
lmo1885	-10.9747	<0.000	Xanthine phosphoribosyltransferase
lmo2785	-27.7332	<0.000	Catalase- Inorganic ion transport and metabolism, oxidoreductase, stress response and virulence
lmo2016	-81.4636	<0.000	cspB- cold shock protein (beta-ribbon, CspA family)

Appendix 4- Raw data for figure 4-1

0.06 (A)

OD	
0	0.08
1	0.08
2	0.08
3	0.12
4	0.23
5	0.43
6	0.72
7	0.98
8	1

NaClO 0.06 (B)

time	od
0	0.06
1	0.06
2	0.07
3	0.08
4	0.14
5	0.26
6	0.48
7	0.9
8	0.99

NaClO 0.06 (c)

time	od
0	0.07
1	0.07
2	0.07
3	0.09
4	0.13
5	0.23
6	0.45
7	0.97
8	0.99

0.05 (A)

od	
0	0.08
1	0.08
2	0.09
3	0.14
4	0.28
5	0.52
6	0.85
7	0.99
8	0.99

NaClO 0.05 (b)

time	od
0	0.06
1	0.06
2	0.07
3	0.1
4	0.2
5	0.39
6	0.68
7	0.99
8	1

NaClO 0.05 (C)

time	od
0	0.08
1	0.08
2	0.08
3	0.1
4	0.17
5	0.32
6	0.65
7	0.92
8	0.99

0.04 (A)

od	
0	0.08
1	0.08
2	0.1
3	0.15
4	0.3
5	0.56
6	0.91
7	0.99
8	0.99

NaClO 0.04 (b)

time	od
0	0.06
1	0.06
2	0.07
3	0.1
4	0.21
5	0.41
6	0.72
7	0.97
8	0.99

NaClO 0.04 (C)

time	od
0	0.07
1	0.07
2	0.08
3	0.11
4	0.2
5	0.37
6	0.71
7	0.97
8	1

0.03 (A)

od	
0	0.09
1	0.09
2	0.11
3	0.16

NaClO 0.03 (b)

time	od
0	0.07
1	0.07
2	0.08
3	0.11

NaClO 0.03 (C)

time	od
0	0.08
1	0.08
2	0.09
3	0.12

4	0.31	4	0.22	4	0.21
5	0.58	5	0.43	5	0.4
6	0.92	6	0.74	6	0.71
7	0.99	7	0.97	7	0.92
8	1	8	1	8	0.99

Appendix 5- Raw data for figure 4-2

H2O2 0.03 (A)

time	od
0	0.09
1	0.09
2	0.09
3	0.11
4	0.19
5	0.34
6	0.56
7	0.91
8	0.9

H2O2 0.03 (b)

time	od
0	0.07
1	0.07
2	0.07
3	0.08
4	0.12
5	0.21
6	0.36
7	0.72
8	0.9

H2O2 0.03 (C)

time	od
0	0.07
1	0.07
2	0.08
3	0.09
4	0.15
5	0.26
6	0.38
7	0.83
8	0.92

H2O2 0.02 (A)

time	od
0	0.08
1	0.08
2	0.09
3	0.13
4	0.24
5	0.42
6	0.69
7	0.89
8	0.9

H2O2 0.02 (b)

time	od
0	0.06
1	0.06
2	0.07
3	0.09
4	0.16
5	0.29
6	0.51
7	0.89
8	0.91

H2O2 0.02 (C)

time	od
0	0.07
1	0.07
2	0.07
3	0.09
4	0.15
5	0.26
6	0.5
7	0.8
8	0.9

H2O2 0.015 (A)

time	od
0	0.08
1	0.08
2	0.1
3	0.15
4	0.27
5	0.48
6	0.77
7	0.89
8	0.99

H2O2 0.015 (b)

time	od
0	0.07
1	0.07
2	0.07
3	0.1
4	0.2
5	0.36
6	0.61
7	0.92
8	0.99

H2O2 0.015 (C)

time	od
0	0.07
1	0.07
2	0.08
3	0.11
4	0.2
5	0.36
6	0.61
7	0.87
8	0.92

H2O2 0.01 (A)

time	od
0	0.09
1	0.09
2	0.11
3	0.16
4	0.31
5	0.53

H2O2 0.01 (b)

time	od
0	0.08
1	0.08
2	0.09
3	0.12
4	0.22
5	0.39

H2O2 0.01 (C)

time	od
0	0.08
1	0.08
2	0.09
3	0.12
4	0.22
5	0.39

6	0.84
7	0.92
8	0.99

6	0.65
7	0.9
8	0.96

6	0.63
7	0.87
8	0.95

Appendix 6- Raw data for figure 4-3

C.A 0.2 (A)

time	od
0	0.07
1	0.07
2	0.09
3	0.09
4	0.12
5	0.16
6	0.21
7	0.32
8	0.41

C.A 0.2 (b)

time	od
0	0.06
1	0.06
2	0.07
3	0.08
4	0.09
5	0.12
6	0.16
7	0.26
8	0.4

C.A 0.2 (C)

time	od
0	0.06
1	0.06
2	0.07
3	0.07
4	0.09
5	0.13
6	0.15
7	0.3
8	0.41

C.A 0.15 (A)

time	od
0	0.08
1	0.08
2	0.09
3	0.11
4	0.16
5	0.25
6	0.38
7	0.57
8	0.7

C.A 0.15 (b)

time	od
0	0.07
1	0.07
2	0.07
3	0.08
4	0.12
5	0.19
6	0.29
7	0.49
8	0.71

C.A 0.15 (C)

time	od
0	0.07
1	0.07
2	0.07
3	0.08
4	0.12
5	0.19
6	0.28
7	0.5
8	0.69

C.A 0.1 (A)

time	od
0	0.09
1	0.09
2	0.11
3	0.14
4	0.23
5	0.38
6	0.58
7	0.88
8	0.9

C.A 0.1 (b)

time	od
0	0.08
1	0.08
2	0.09
3	0.11
4	0.17
5	0.29
6	0.45
7	0.73
8	0.9

C.A 0.1 (C)

time	od
0	0.07
1	0.07
2	0.08
3	0.1
4	0.17
5	0.28
6	0.46
7	0.76
8	0.9

Appendix 7- Raw data for figure 4-4

TSP 1 (A)

time	od
0	0.06
1	0.06
2	0.06
3	0.06
4	0.08
5	0.12
6	0.19
7	0.4
8	0.65

TSP 1 (b)

time	od
0	0.06
1	0.06
2	0.05
3	0.05
4	0.06
5	0.1
6	0.16
7	0.33
8	0.6

TSP 1 (C)

time	od
0	0.06
1	0.06
2	0.05
3	0.05
4	0.06
5	0.09
6	0.15
7	0.25
8	0.62

TSP 0.75 (A)

time	od
0	0.07
1	0.07
2	0.07
3	0.09
4	0.15
5	0.27
6	0.51
7	0.99
8	1

TSP 0.75 (b)

time	od
0	0.06
1	0.06
2	0.05
3	0.06
4	0.11
5	0.2
6	0.37
7	0.8
8	0.99

TSP 0.75 (C)

time	od
0	0.06
1	0.06
2	0.05
3	0.06
4	0.11
5	0.19
6	0.36
7	0.73
8	0.99

TSP 0.5 (A)

time	od
0	0.08
1	0.08
2	0.08
3	0.13
4	0.24
5	0.46
6	0.84
7	0.98
8	0.99

TSP 0.5 (b)

time	od
0	0.06
1	0.06
2	0.06
3	0.09
4	0.17
5	0.34
6	0.65
7	1
8	1

TSP 0.5 (C)

time	od
0	0.06
1	0.06
2	0.06
3	0.09
4	0.17
5	0.33
6	0.65
7	1
8	1

Appendix 8- Raw data for untreated controls, Fig 4-1 to Fig 4-4

control (A)

time	od
0	0.1
1	0.1
2	0.11
3	0.16
4	0.31
5	0.56
6	0.89
7	0.99
8	0.99

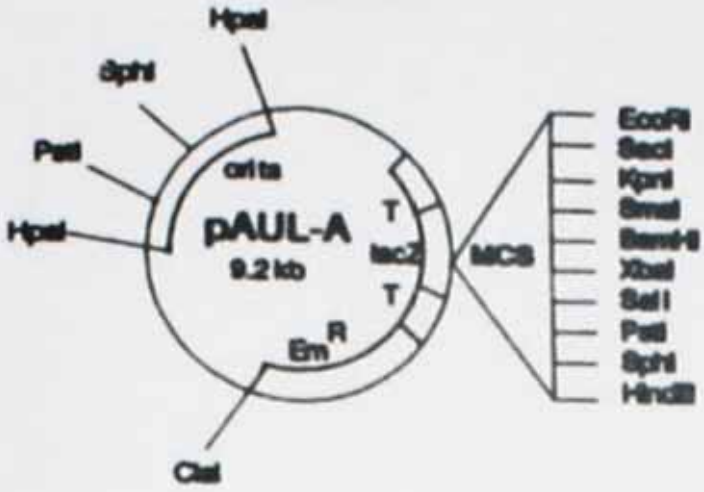
control (b)

time	od
0	0.07
1	0.07
2	0.09
3	0.12
4	0.24
5	0.44
6	0.76
7	1
8	1

control (C)

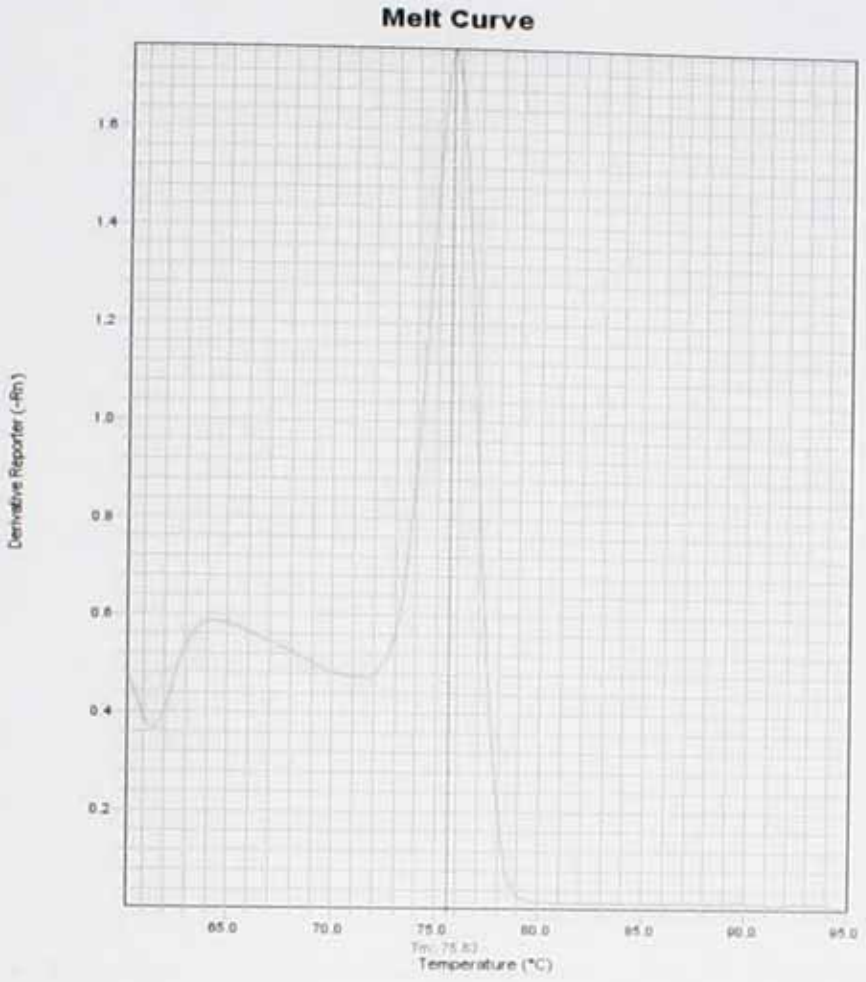
time	od
0	0.08
1	0.08
2	0.09
3	0.12
4	0.22
5	0.42
6	0.78
7	1
8	0.99

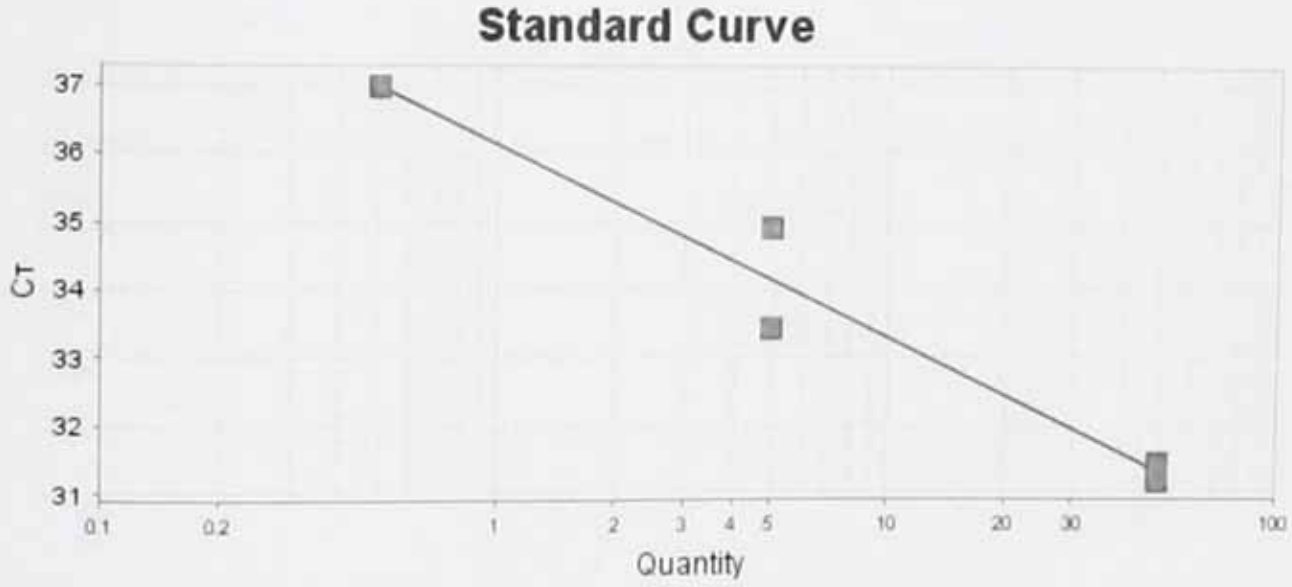
Appendix 9: Plasmid map for pAUL-A vector



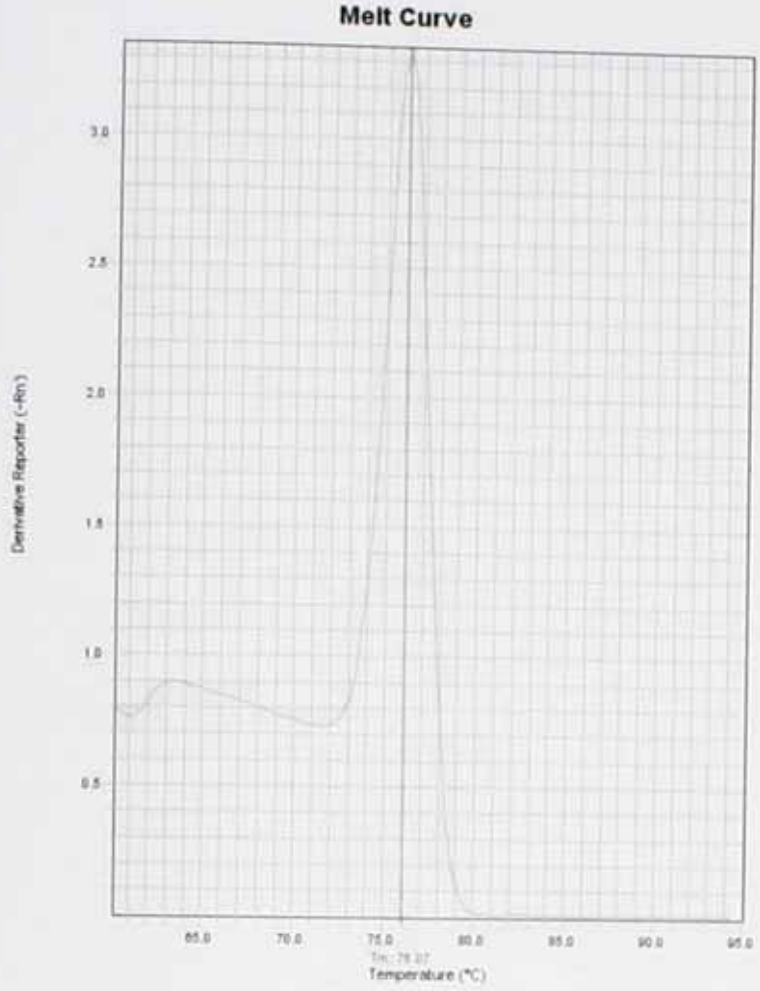
Taken from: Chakraborty *et al.* (1992)

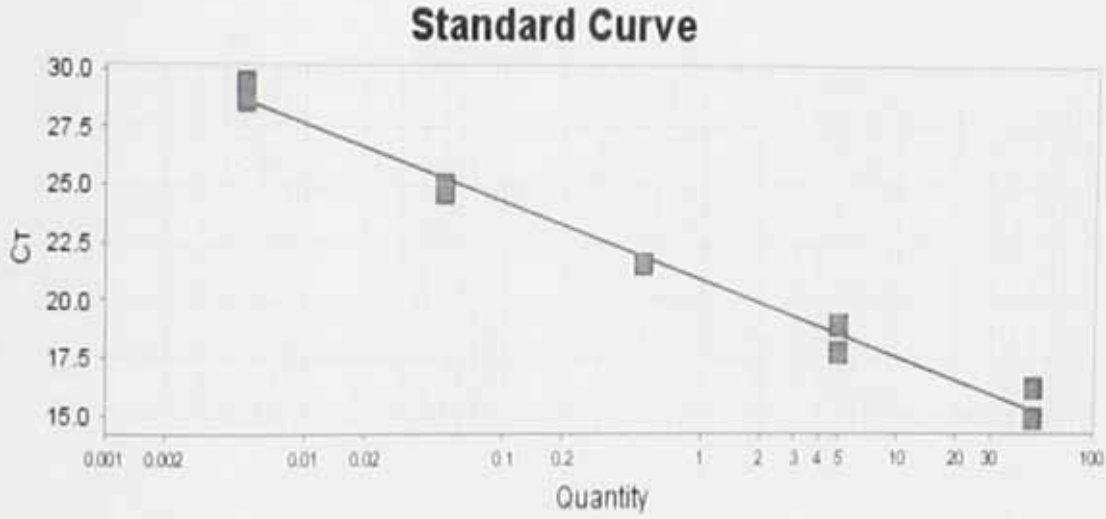
Appendix 10 – qRT-PCR Melt curve and standard curve for gadA



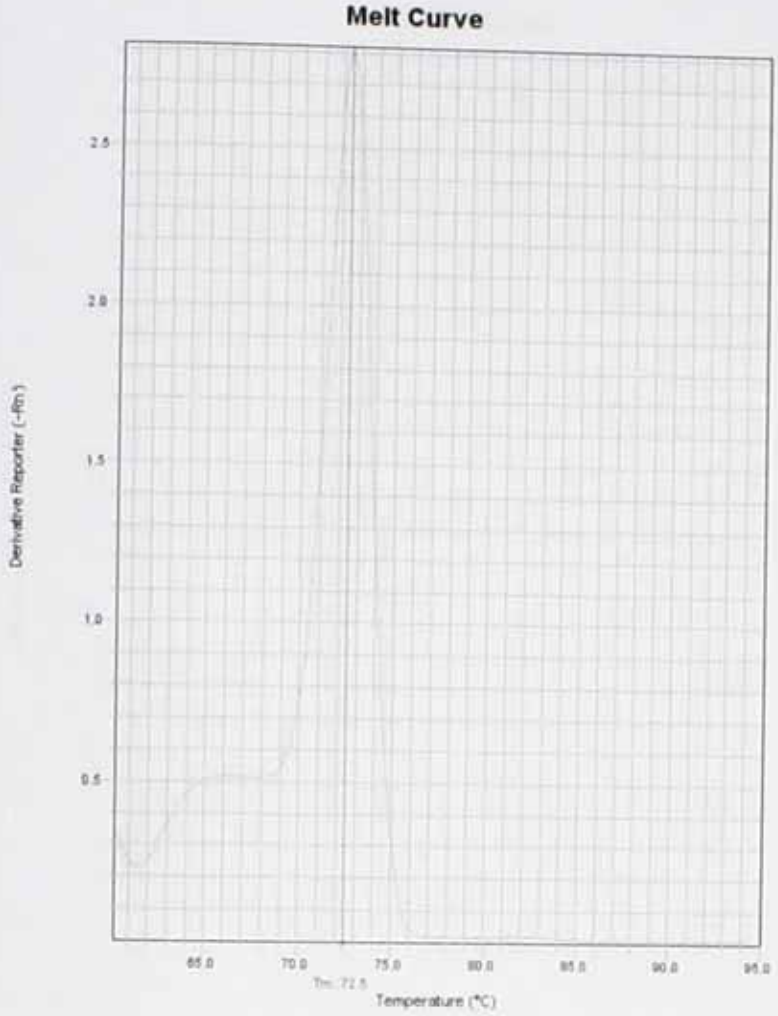


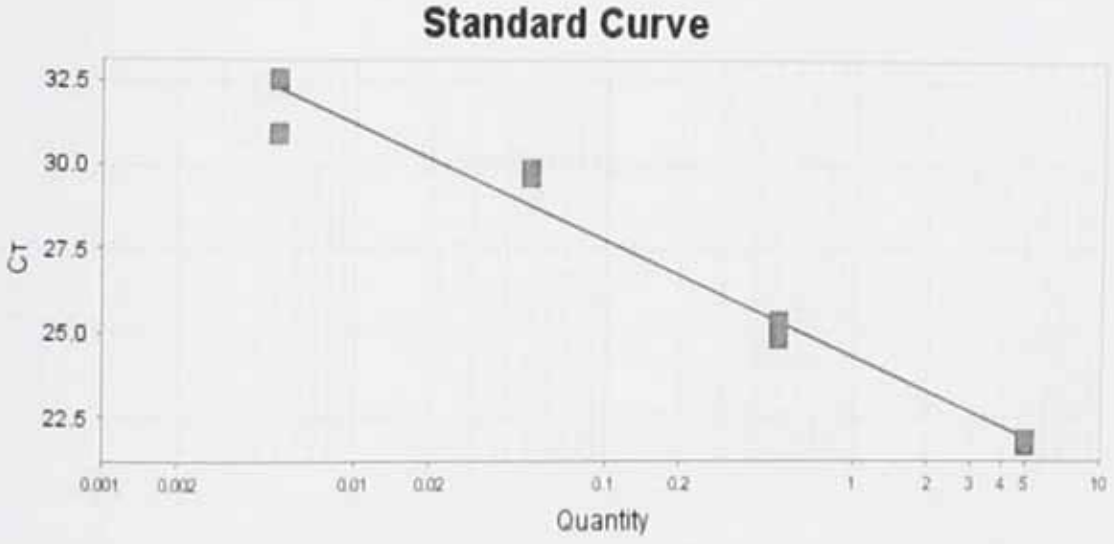
Appendix 11 - qRT-PCR Melt curve and standard curve for groESL



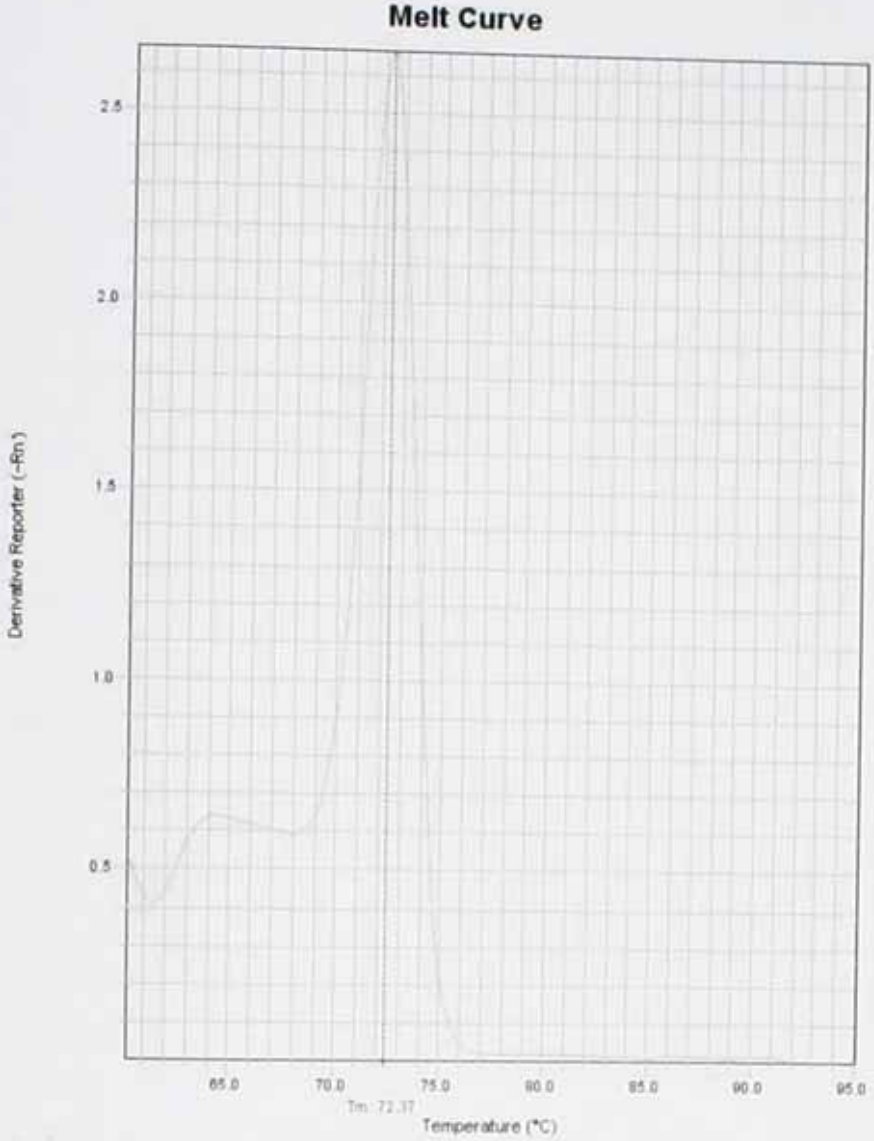


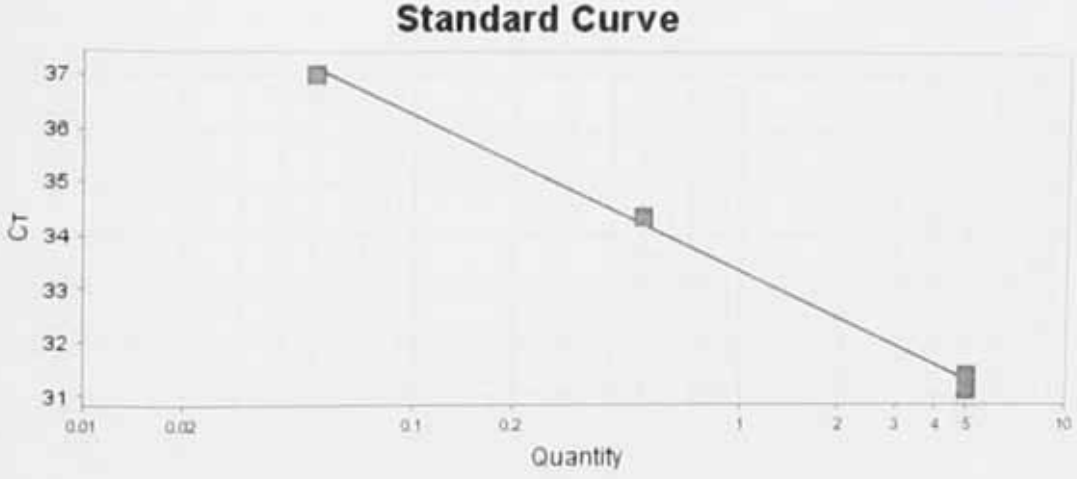
Appendix 12 - qRT-PCR Melt curve and standard curve for Hfq



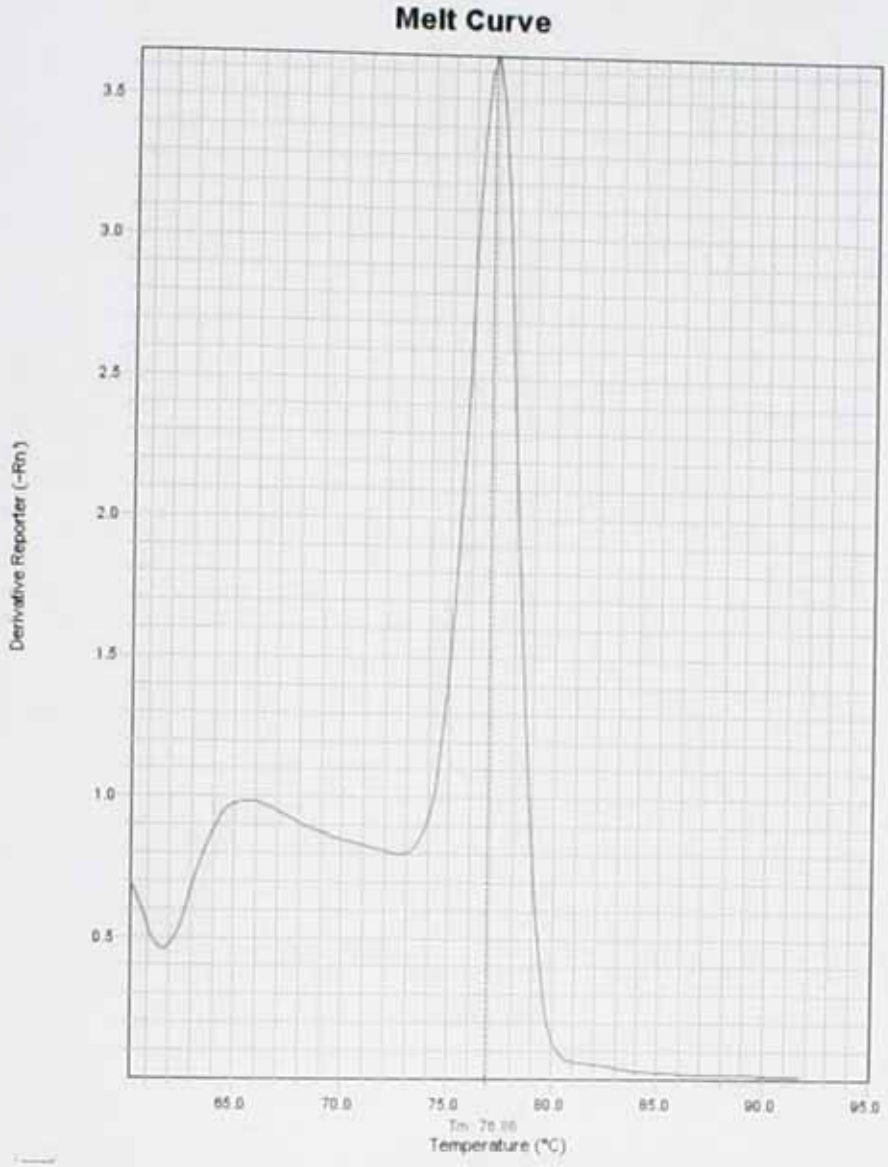


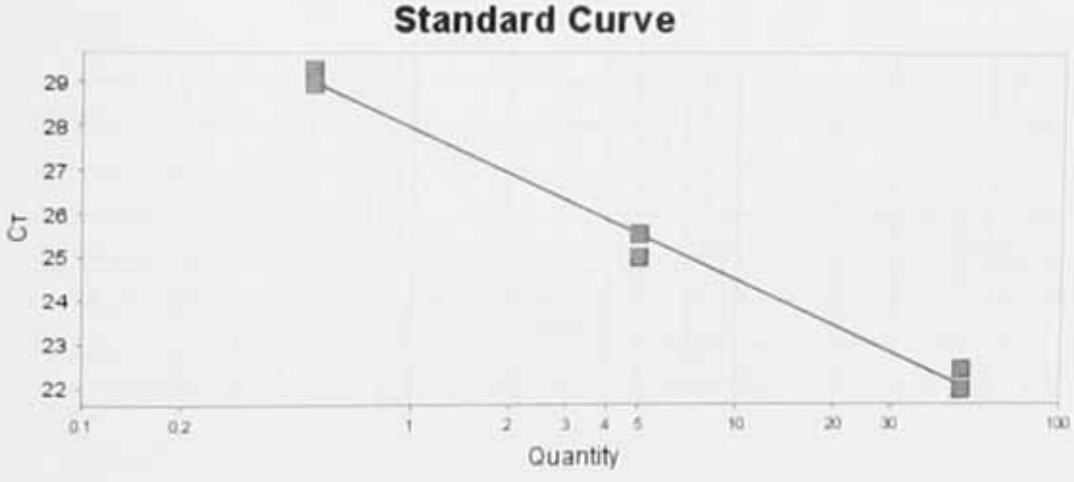
Appendix 13 - qRT-PCR Melt curve and standard curve for lmo0501



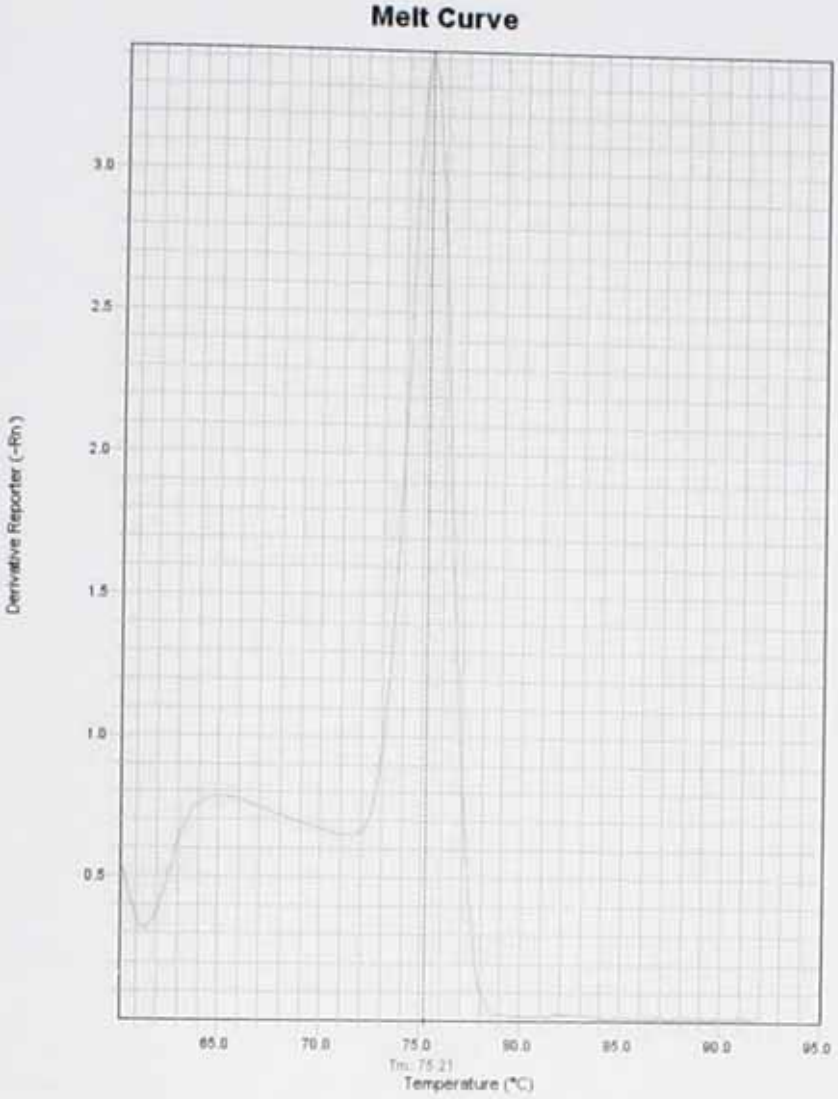


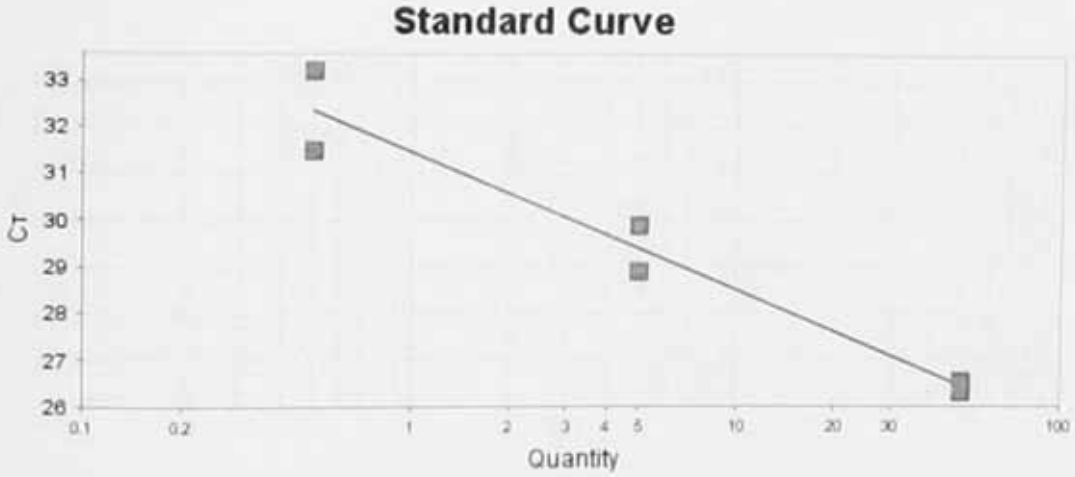
Appendix 14 - qRT-PCR Melt curve and standard curve for lmo0669



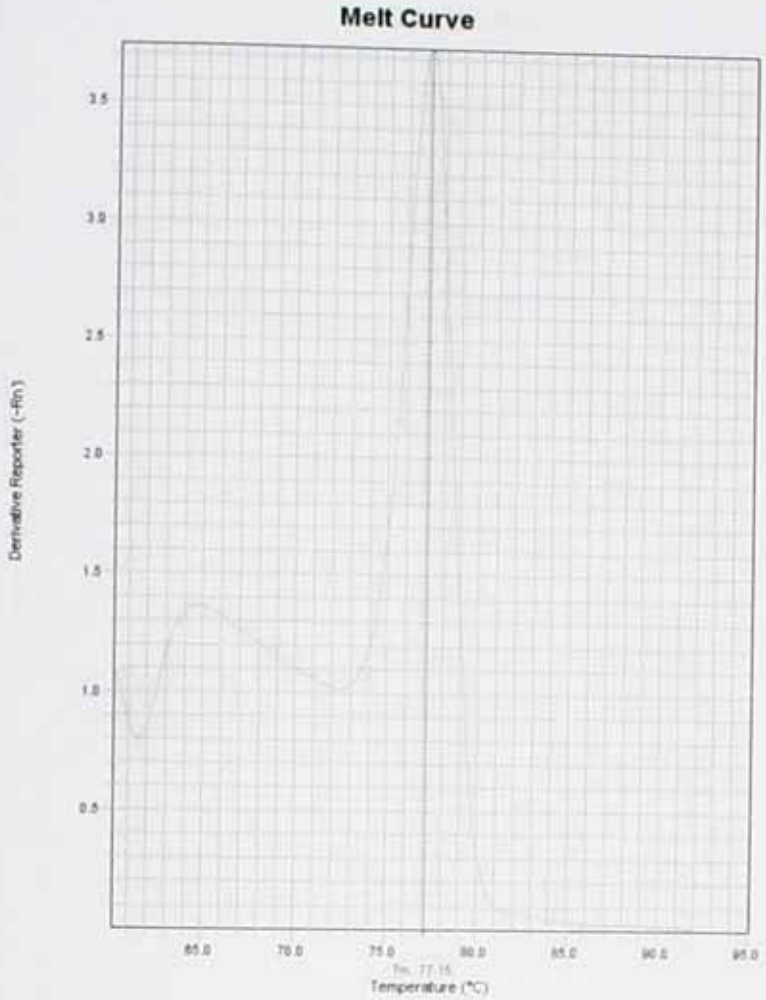


Appendix 15 - qRT-PCR Melt curve and standard curve for lmo0956

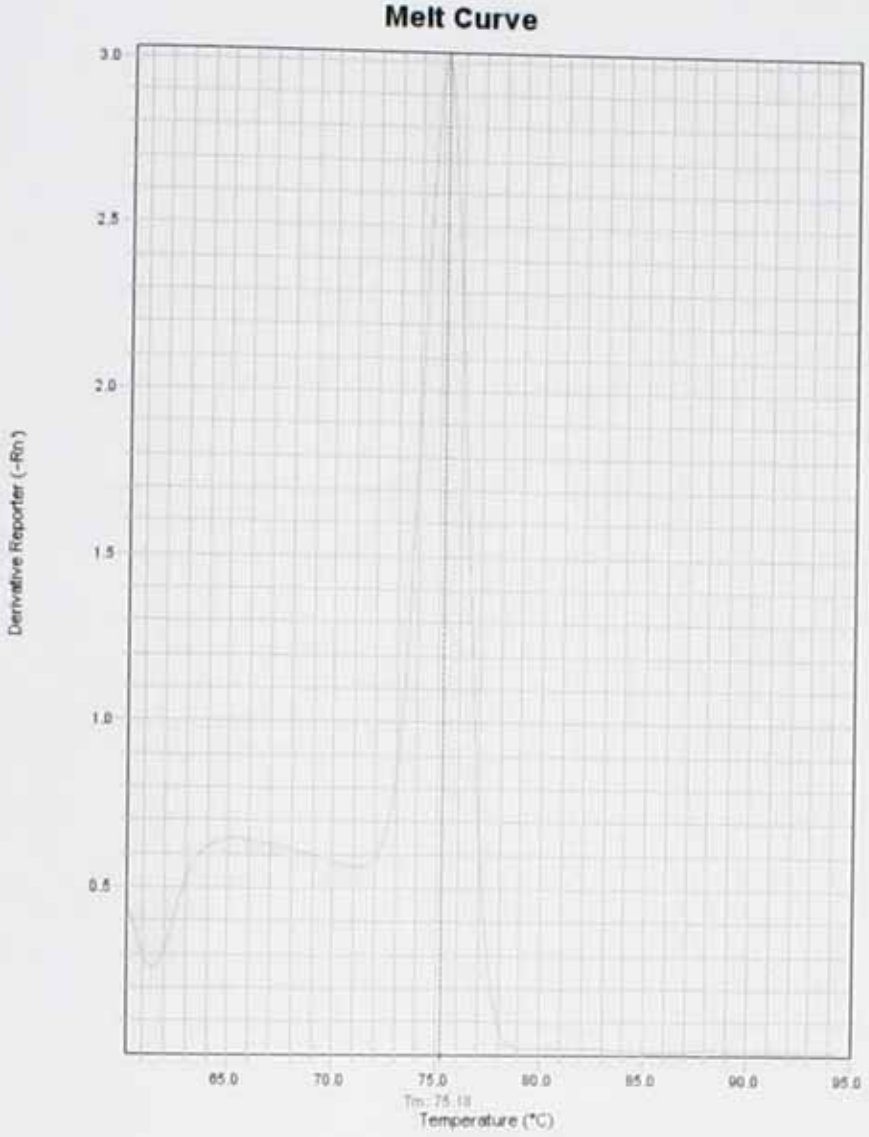


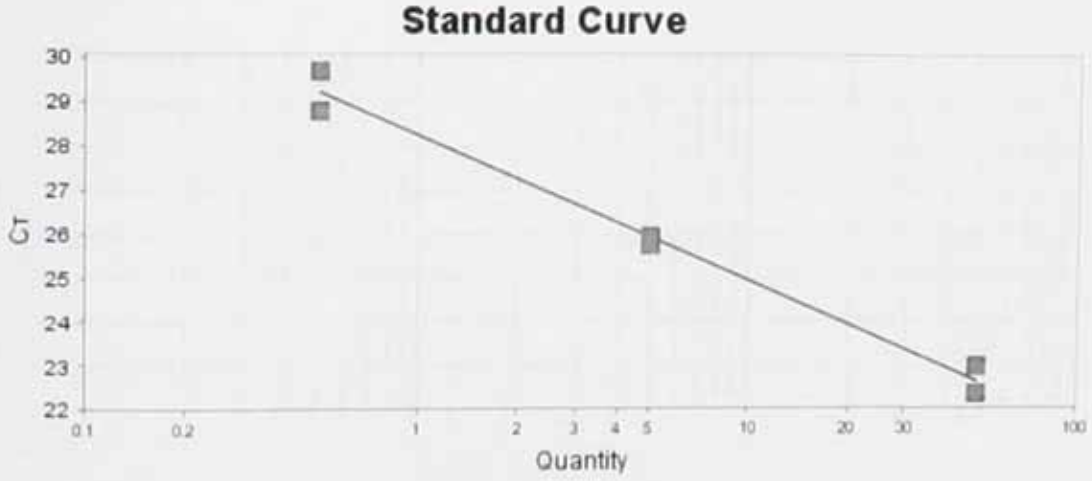


Appendix 16 - qRT-PCR Melt curve and standard curve for recN

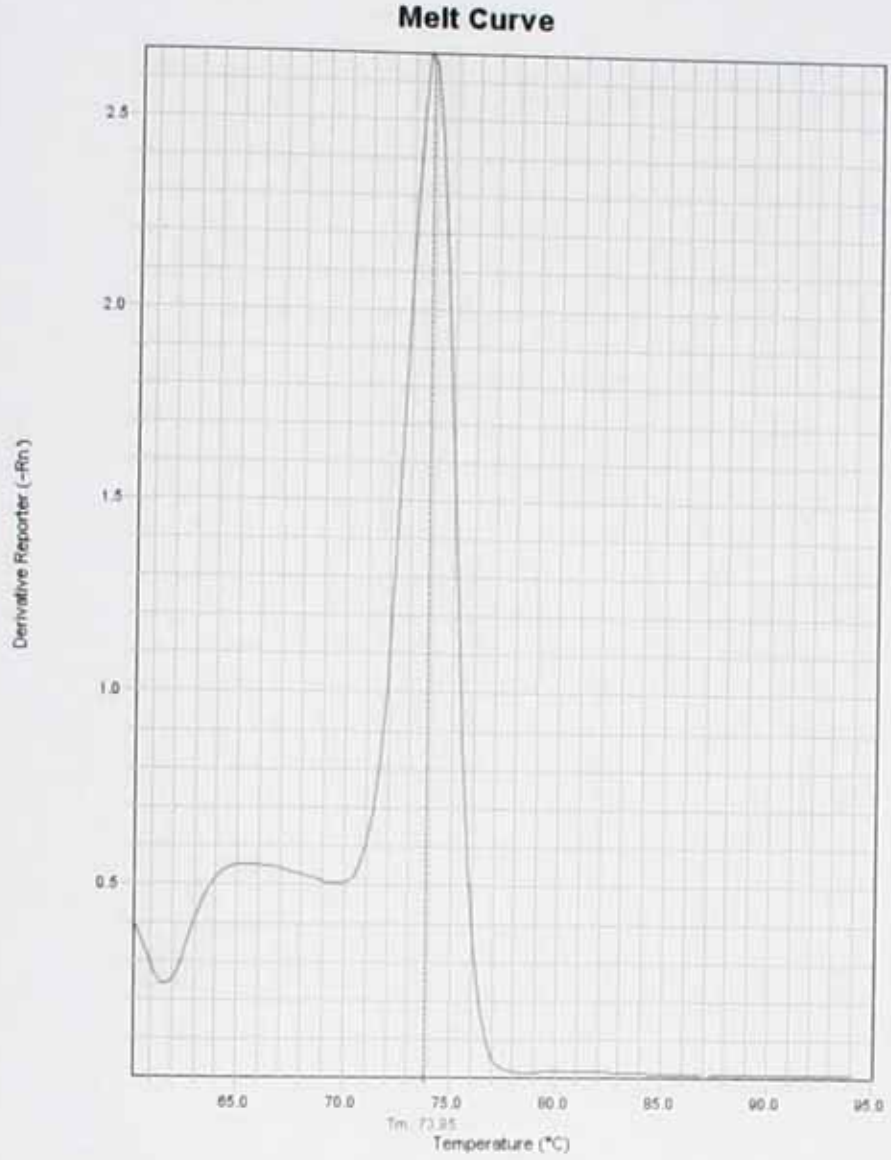


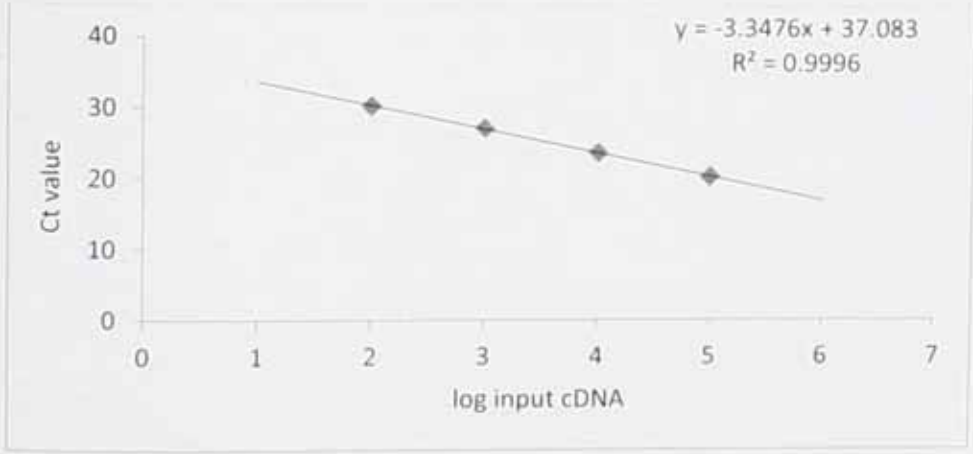
Appendix 17 - qRT-PCR Melt curve and standard curve for sigB



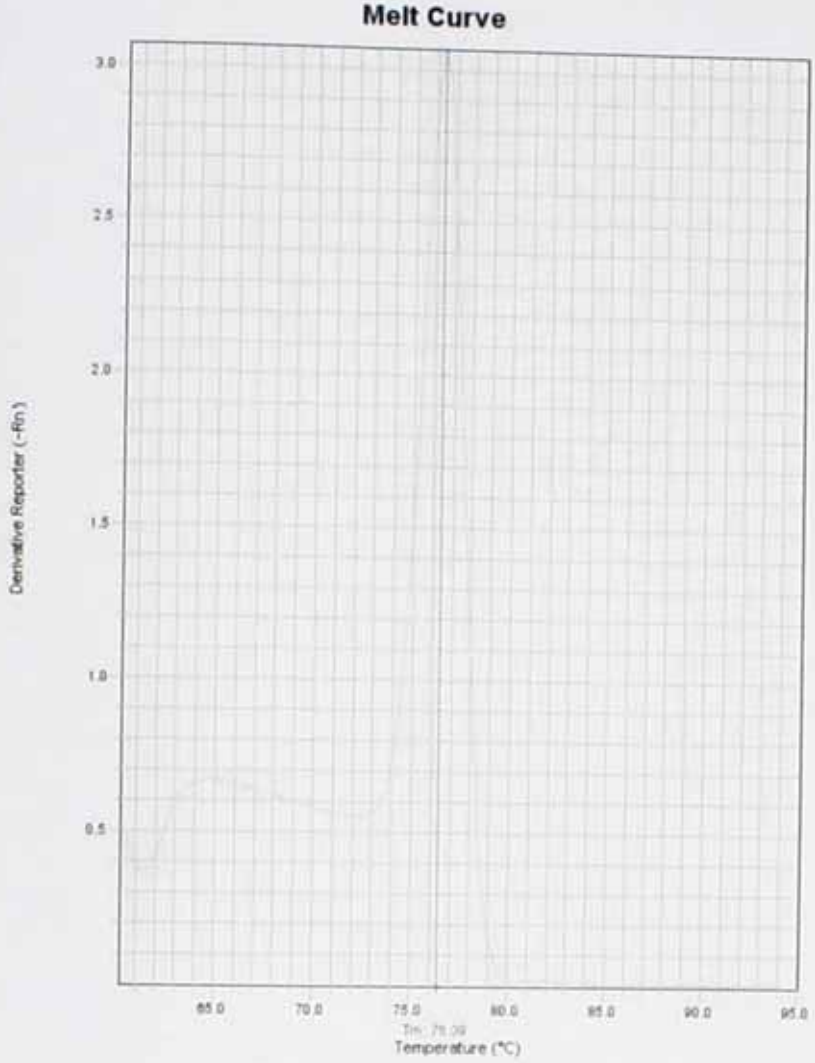


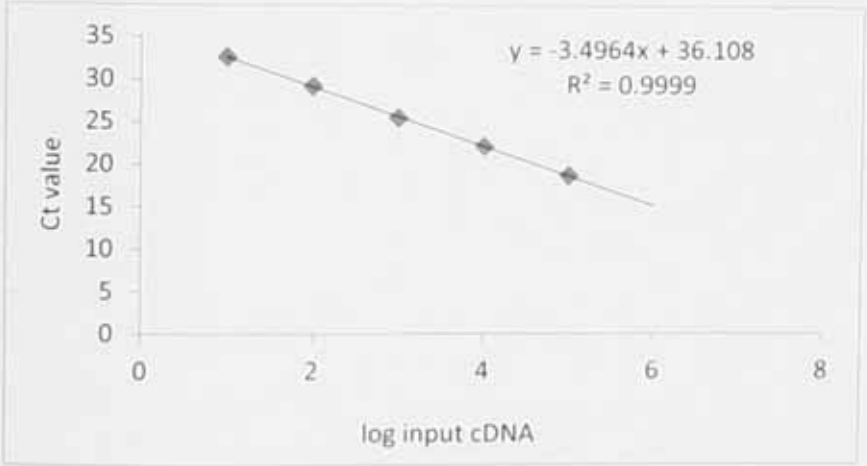
Appendix 18 - qRT-PCR Melt curve and standard curve for lmo2785



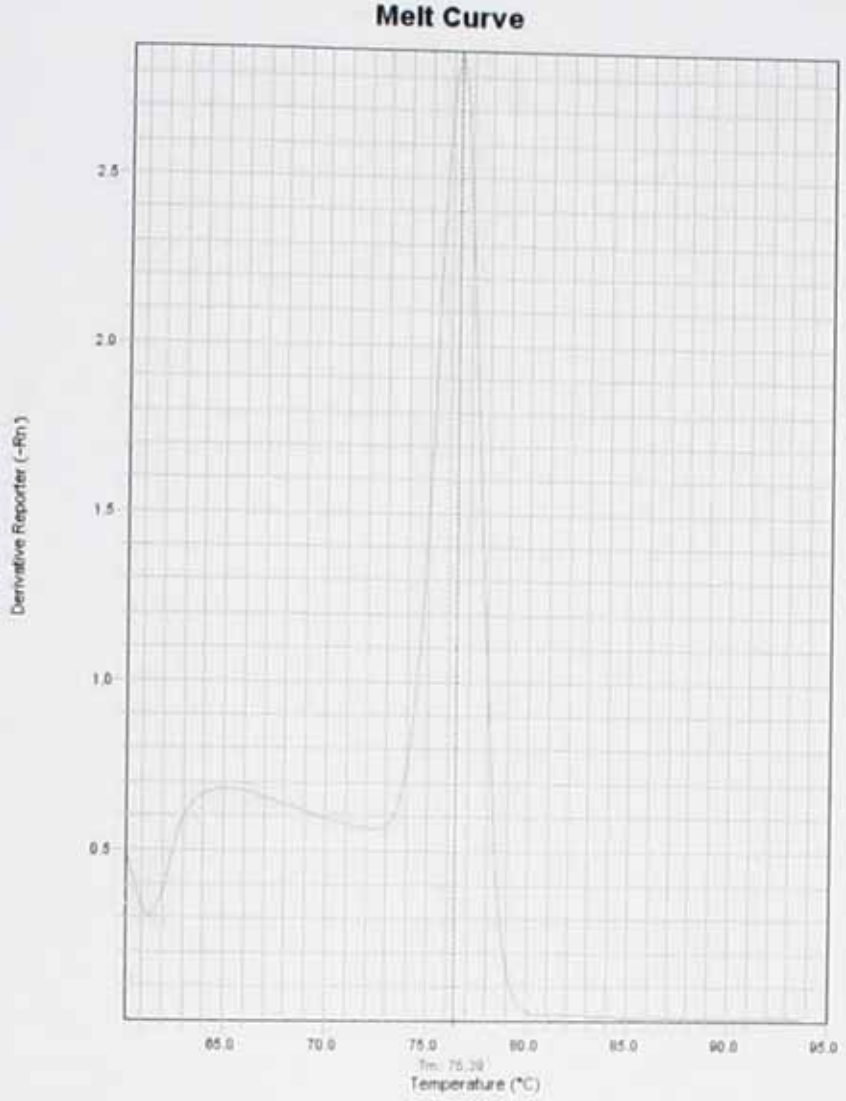


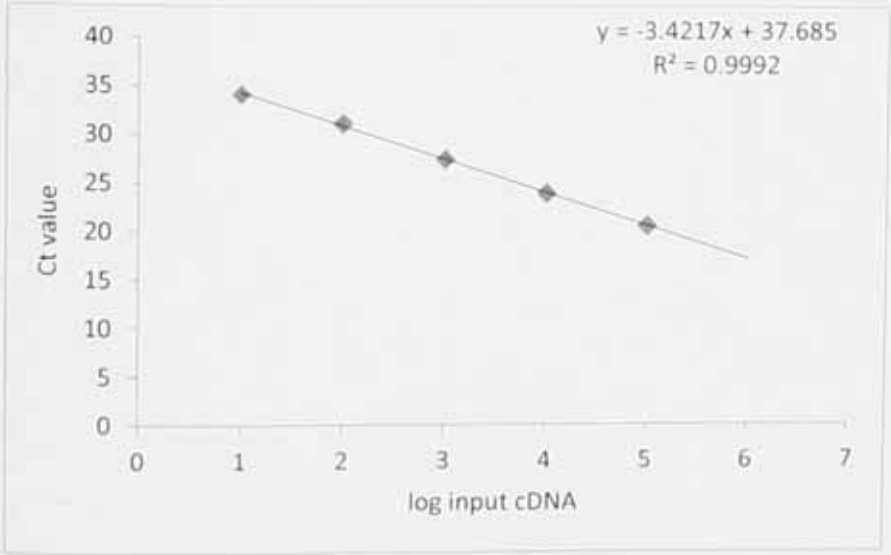
Appendix 19 - qRT-PCR Melt curve and standard curve for lmo1055



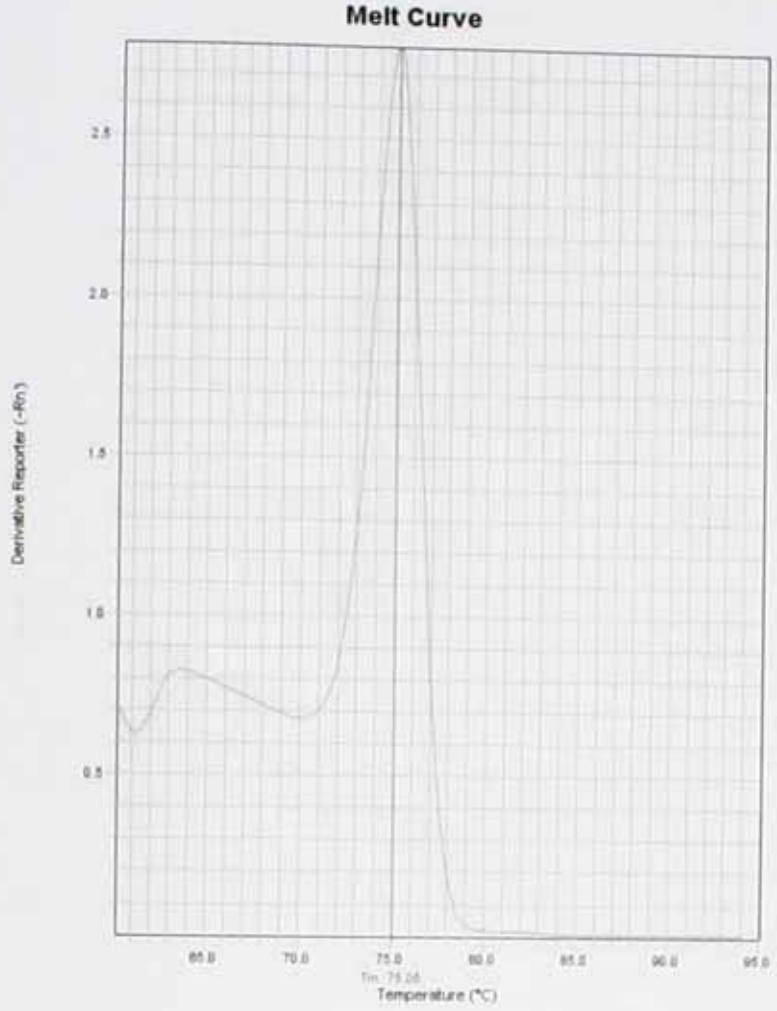


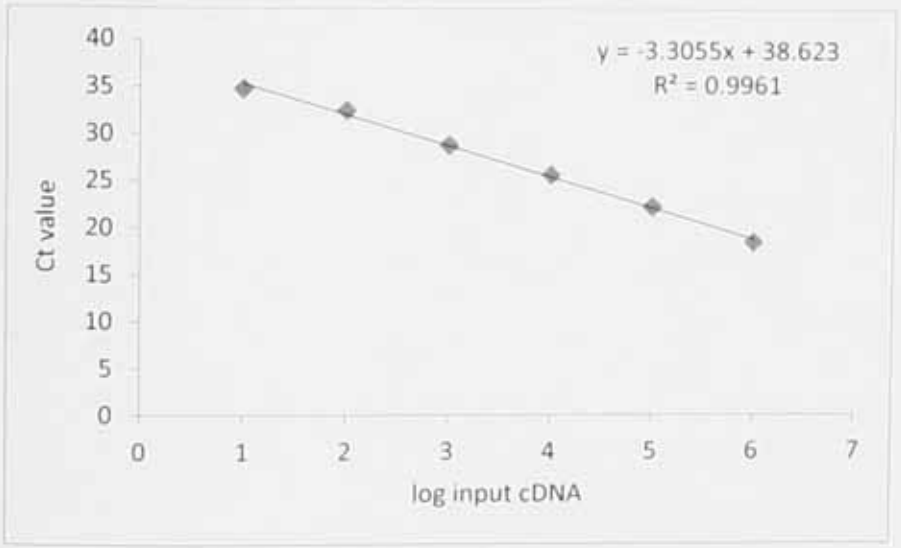
Appendix 20 - qRT-PCR Melt curve and standard curve for lmo2569



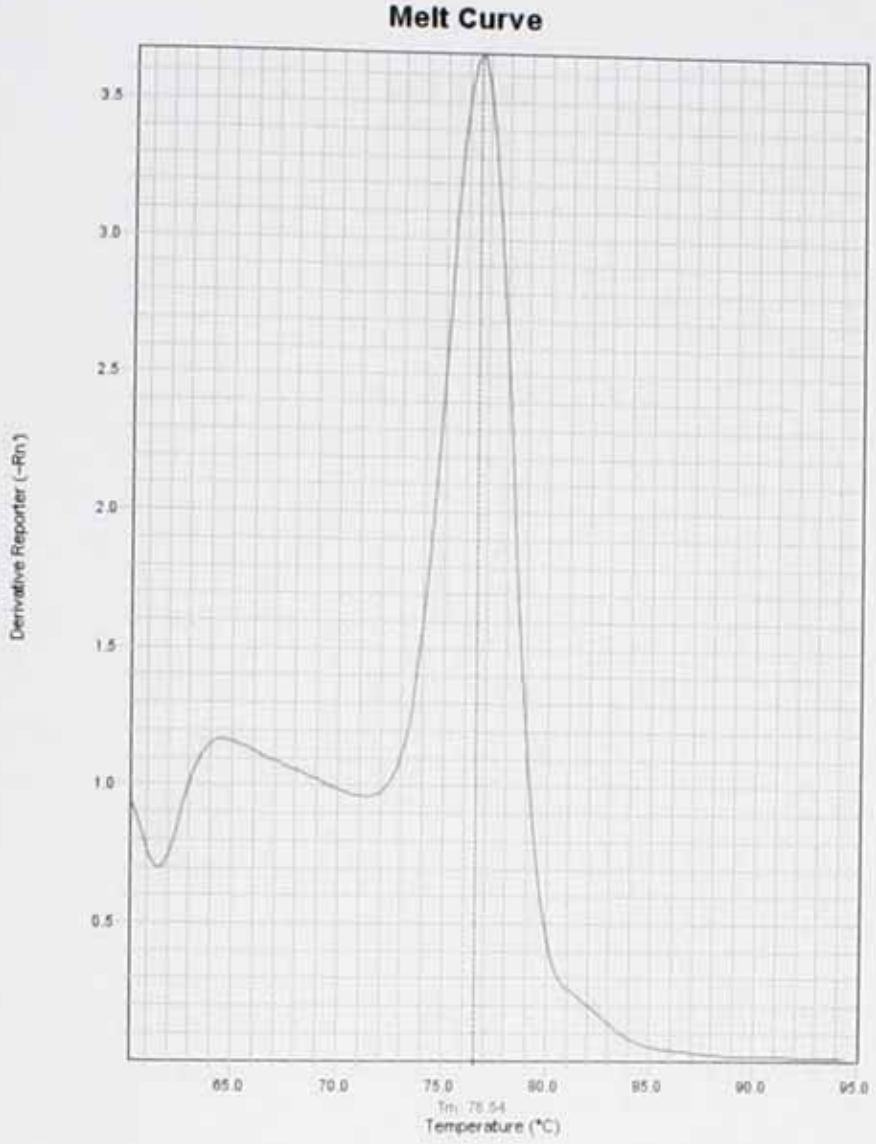


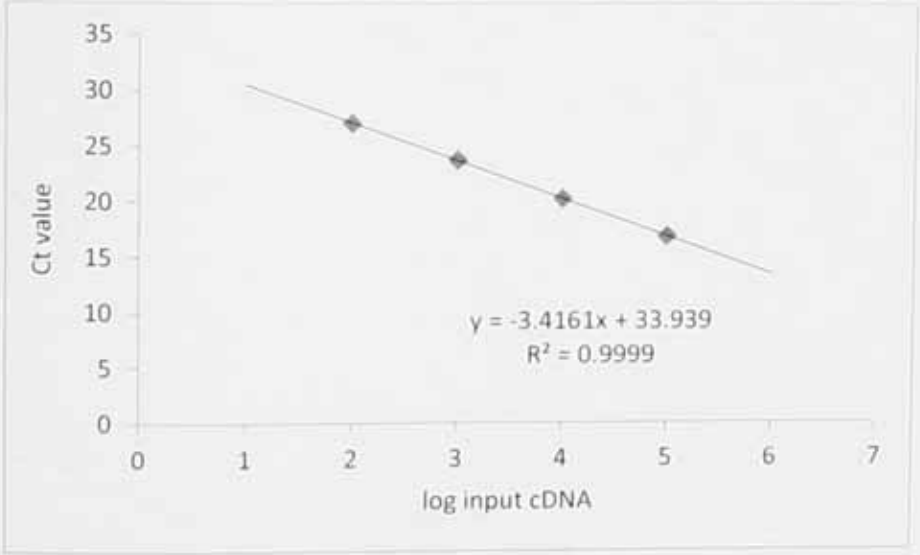
Appendix 21 - qRT-PCR Melt curve and standard curve for lmo2150



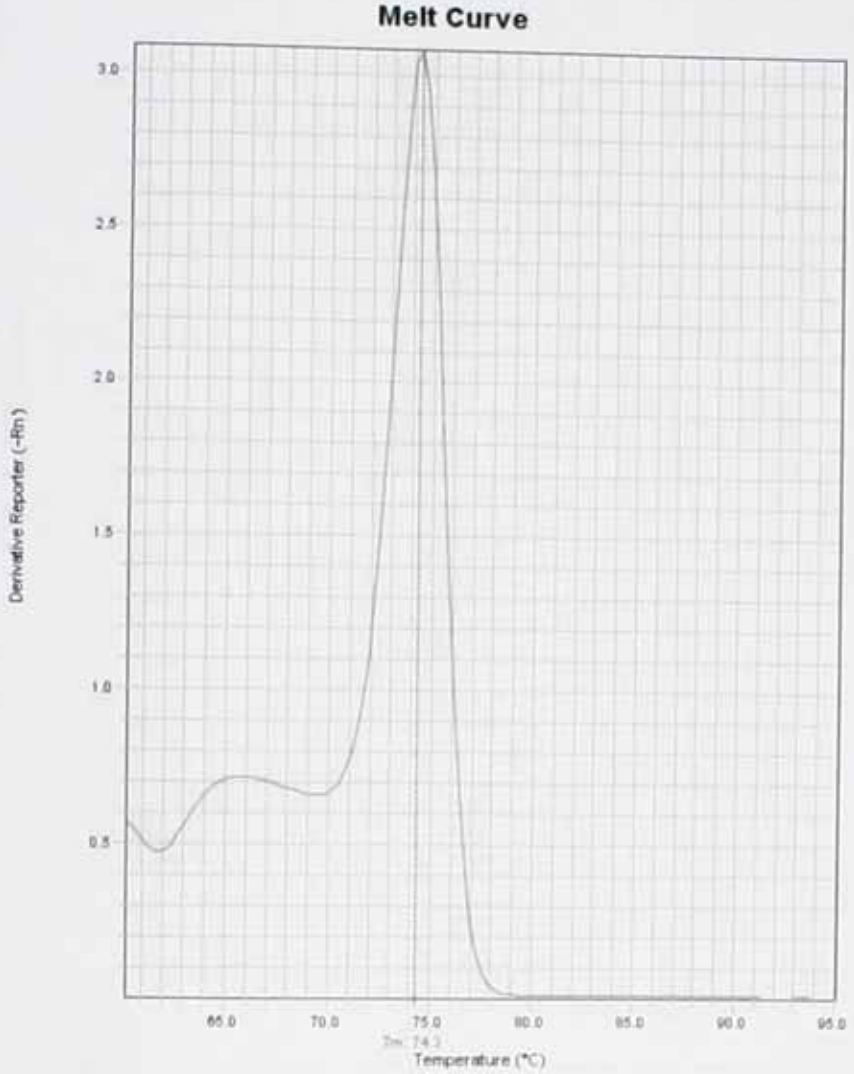


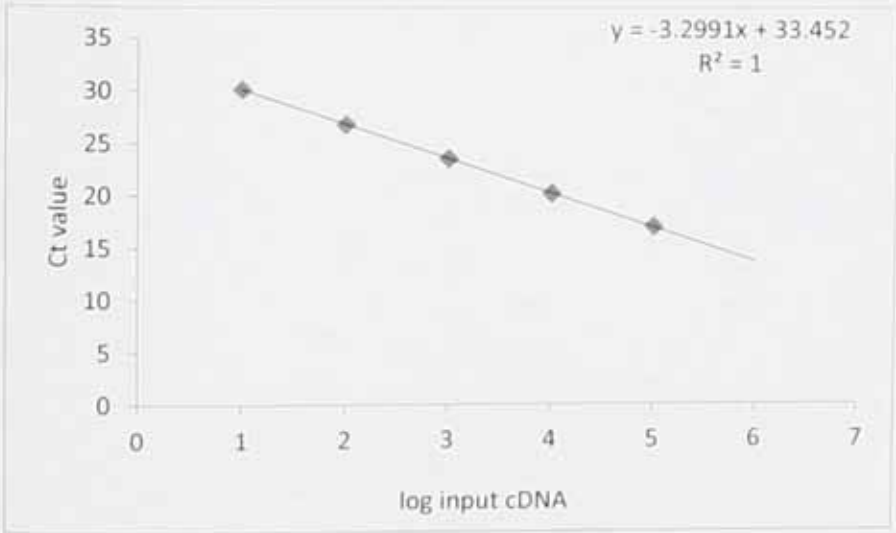
Appendix 22 - qRT-PCR Melt curve and standard curve for lmo2363



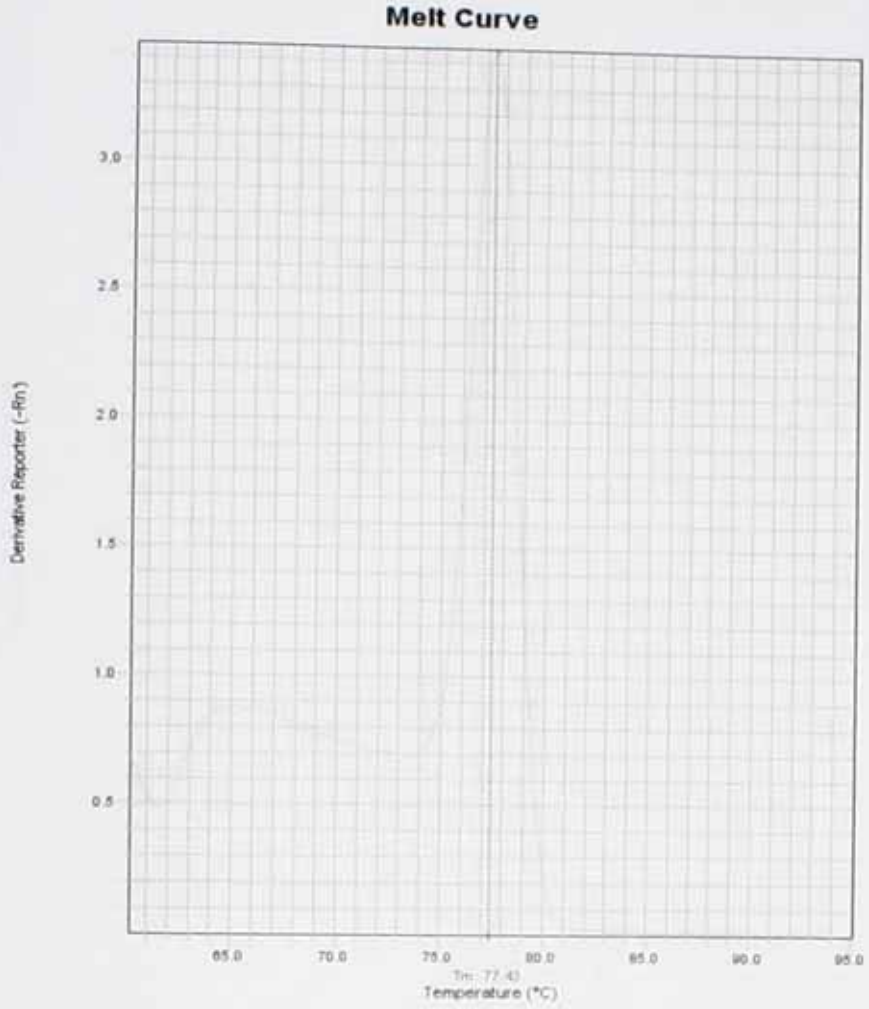


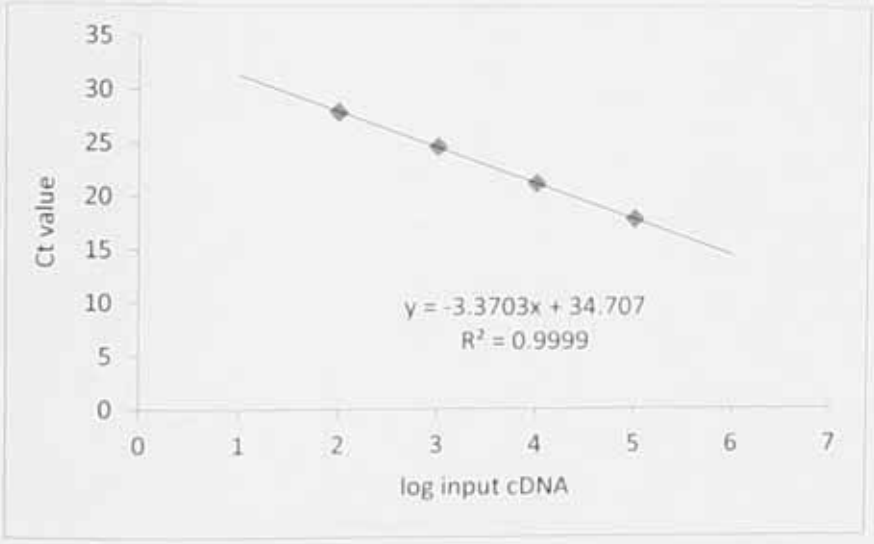
Appendix 23 - qRT-PCR Melt curve and standard curve for lmo2016



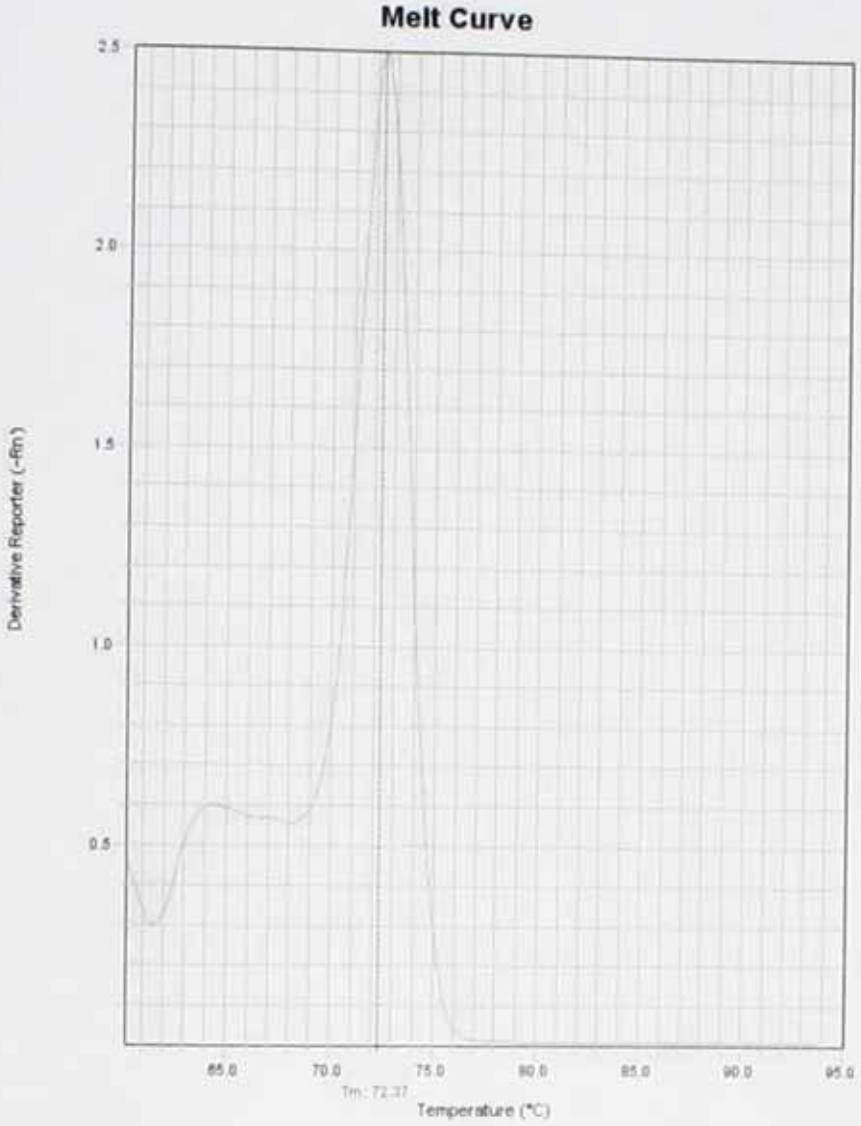


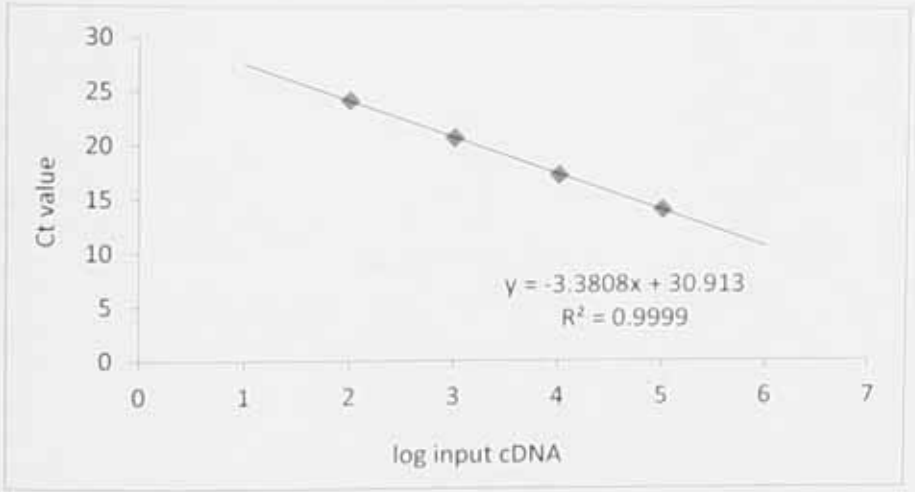
Appendix 24 - qRT-PCR Melt curve and standard curve for lmo1880



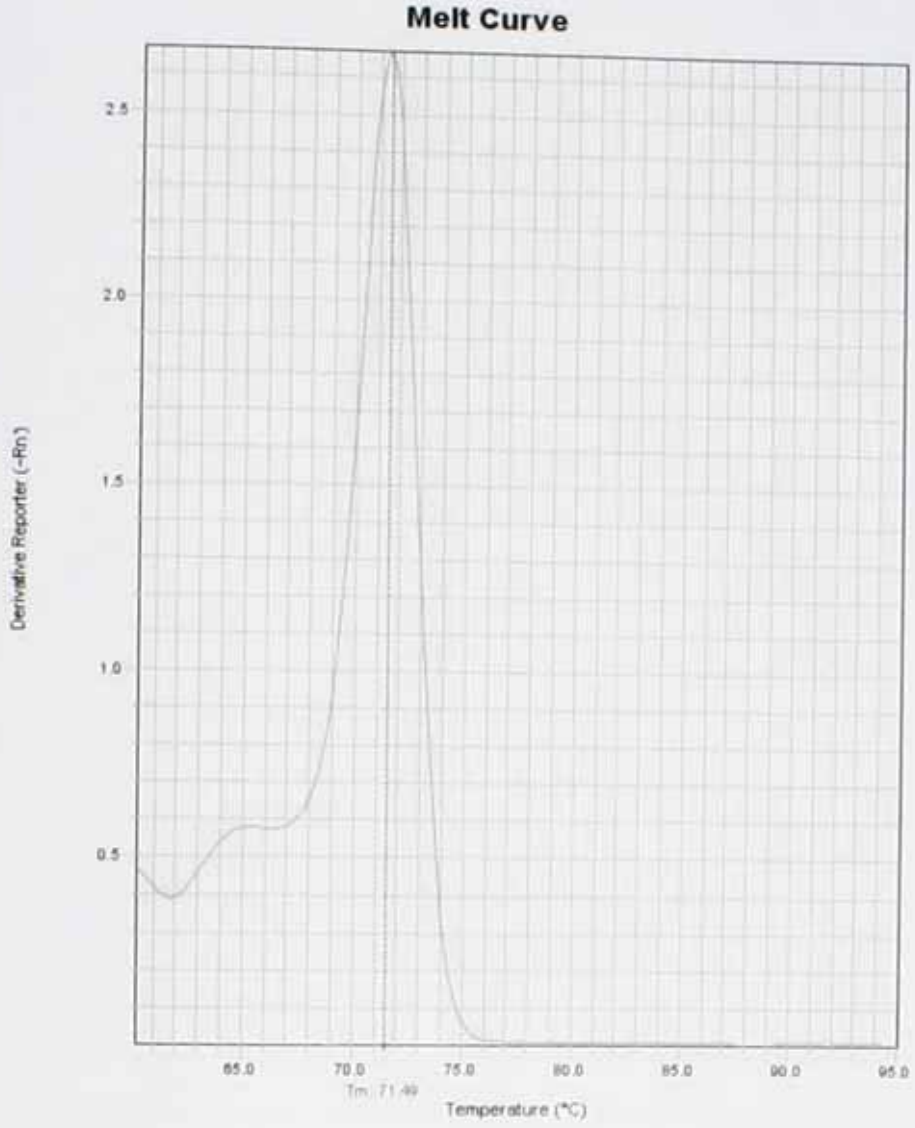


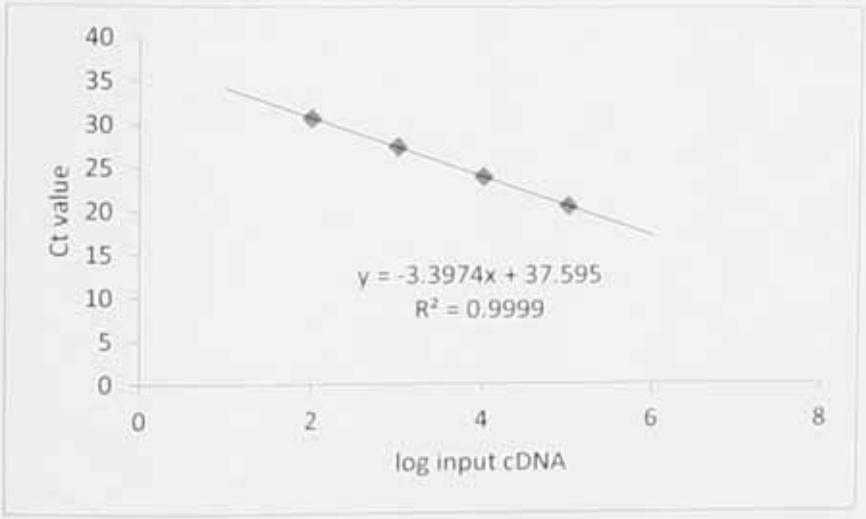
Appendix 25 - qRT-PCR Melt curve and standard curve for lmo0690



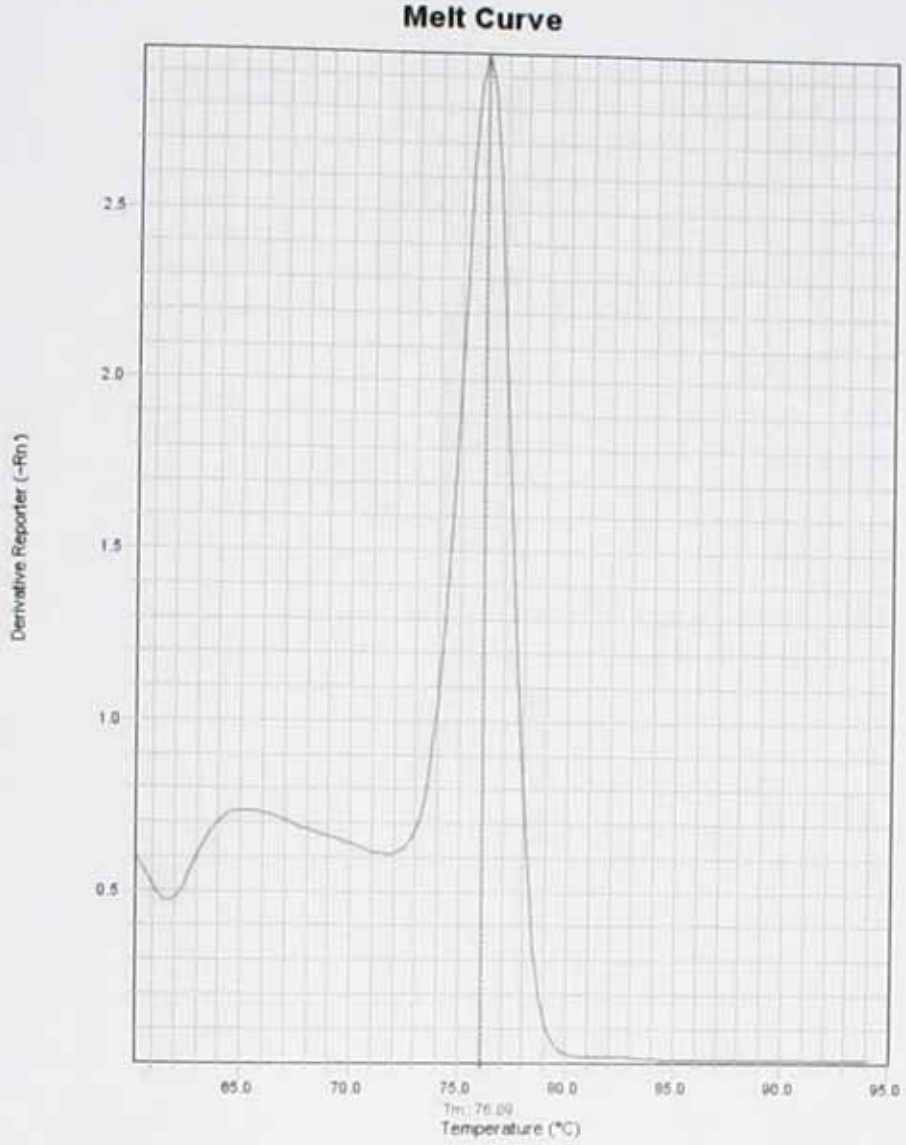


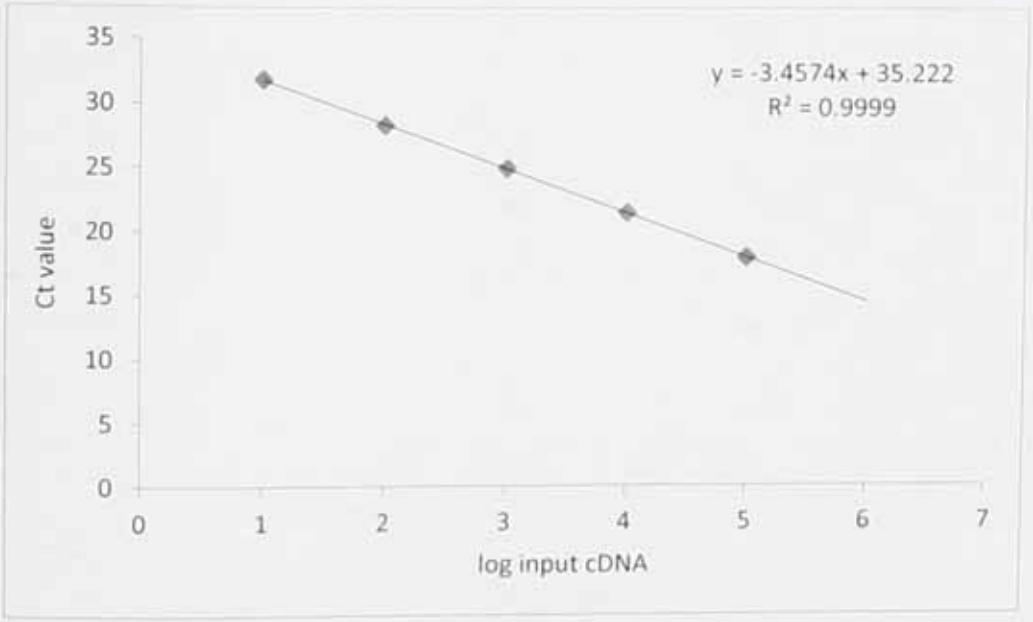
Appendix 26 - qRT-PCR Melt curve and standard curve for lmo1007





Appendix 27 - qRT-PCR Melt curve and standard curve for lmo2101





Appendix 28 - qRT-PCR Melt curve and standard curve for 16s