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“Mode of action of organic acids against bacterial foodborne pathogens and investigation of improved disinfection methods."

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A DISSERTATION

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DECLARATION

I confirm this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Ruth Barnes

…….

For my family

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Abstract

Mode of action of organic acids against bacterial foodborne pathogens and investigation of improved disinfection methods.

Assuring the microbiological safety of fresh produce can present a huge challenge for food producers. Organic acid washes, are commonly used as antimicrobial treatments. However, our understanding of how foodborne pathogens interact with these acids is limited. Bacteria have evolved a variety of mechanisms to promote survival under acidic conditions presented in various environments (e.g. animal stomach). These mechanisms contribute to maintaining a tolerable intracellular pH when the organism is presented with an acidic challenge.

This work explores the effect of weak organic acids notably fumarate on the inhibition of amino acid decarboxylase systems which are the most potent acid resistance mechanisms. We demonstrate that sodium fumarate reduces survival in planktonic cultures and biofilms of *E. coli*, *L. monocytogenes* and *Salmonella* under acidic conditions. This effect stems from effects on glutamate amino acid decarboxylase system function or transcription. In *E. coli* fumarate inhibited the glutamate decarboxylase (GAD) activity and output resulting in lower survival. In *L. monocytogenes* it also inhibited GAD activity although the organism responded by *gadD2* upregulation resulting in higher GABA export, suggesting that possible effects on intracellular GAD activity or other systems might be responsible. In *Salmonella*, fumarate inhibited the activity of the lysine decarboxylase (LDAR) system under acidic stress resulting in reduced survival.

Subsequently, an examination of the possible usage of fumarate in decontamination procedures on fresh produce. Fumarate significantly improved the efficacy of a commercial acidic disinfectant, which was also significantly higher than that of chlorine.

Furthermore, with the use of the phenotypic microarrays we explored the effect of significant stress genes (e.g. *sigB*) in carbon source utilisation and osmotic tolerance of *L. monocytogenes*. The work demonstrated for first time a self-preservation and nutritional competence (SPANC) balance in a Gram-positive bacterium particularly in the absence of *sigB*, which enhanced carbon source utilisation in expense to lower stress tolerance.

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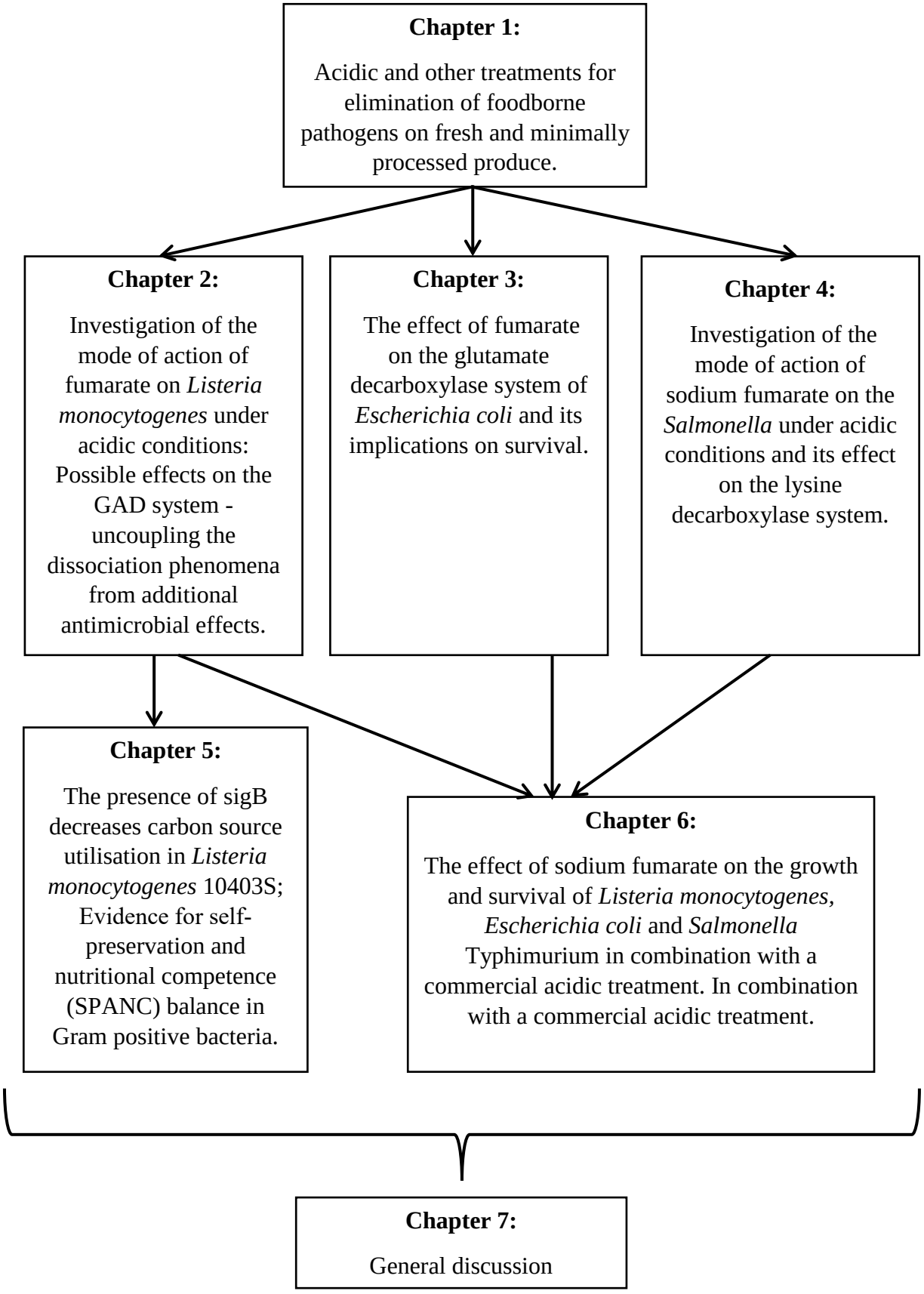

List of Abbreviations

ADAR	**Arginine dependent mechanism of acid resistance**
AM	**Acid mix (an organic acid based commercial disinfectant)**
BHI	**Brain Heart Infusion**
cDNA	**Complementary DNA**
CFU	**Colony forming unit**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
EO's	**Essential oils**
FA	**Fumaric acid**
GABA	**γ-aminobutyric acid**
$GABA_e$	**Extracellular GABA**
$GABA_i$	**Intracelular GABA**
GAD	**Glutamate decarboxylase system**
GC-MS	**Gas chromatography mass spectrometry**
GDAR	**Glutamate dependent mechanism of acid resistance**
HUS	**Haemolytic-uraemic syndrome**
HCl	**Hydrochloric acid**
H_2O_2	**Hydrogen peroxide**
LB	**Lysogeny broth**
LDAR	**Lysine dependent acid resistance**
MA	**Maleic acid**
MIC	**Minimum inhibitory concentration**
MMP	**Minimal processed produce**
MSG	**Monosodium glutamate**
NaCl	**Sodium chloride**
PAA	**Peracetic acid**
ppm	**Parts per million**
pKa	**Acid dissociation constant**

qRT-PCR	**Quantitative Real-Time Reverse Transcription PCR**
rpm	**Revolutions per minute**
SF	**Sodium fumarate**
SPANC	**Self-Preservation and Nutritional Competence**
TSB	**Tryptic Soy Broth**
TSBY	**Tryptic Soy Broth Yeast**
WT	**Wild-type**

CHAPTER 1: Introduction

Acidic and other treatments for elimination of foodborne pathogens on fresh and minimally processed produce.

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Abstract

The consumption of fresh or minimal processed produce (MPP), such as fresh fruits, salads and vegetables, are being promoted by many governments, including the UK and the US, as a strategy for improving the diet and health of their populations The UK is the largest consumer of fresh cut products in Europe However, this type of produce can contain a wide range of pathogenic bacteria such as *E. coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* and *Clostridium botulinum*. By the very nature of fresh and MPP it can be difficult to ensure that these organisms do not enter the food chain.

A wide range of post-harvest treatments and technologies are available to deal with this problem including chlorine washes, atmospheric cold plasma (ACP), electrolysed water, hydrogen peroxide (Mahmoud et al., 2007) and weak acid washes. However it has been noted that many of these treatments only achieve a limited level of reduction, typically 2- 3 logs CFU/ml.

This study focuses on three organisms, *E. coli*, *L. monocytogenes* and *Salmonella* and their ability to survive acidic conditions. Low pH washes are increasingly used on fresh produce as an alternative to chlorine-based products in order to reduce pathogen levels without influencing the nature of the product.

1.1 MPP and fresh produce

MPP and fresh produce receive minimal intervention before human consumption and are becoming more popular with consumers. This has been driven by several factors including improvements in the distribution chain, and hence better availability, greater awareness of the health benefits of fresh produce and changes in social trends (Lynch et al., 2009, Olaimat and Holley, 2012).

The UK is one of the largest consumers of fresh produce in Europe (Wiley, 2017) and whilst, nutritionally, this may be having a positive impact on certain aspects of health, this produce can present a serious risk to other aspects. This comes from pathogens that are commonly found throughout the food chain and that, in other foods they are eliminated through processing, however since fresh produce is consumed with minimal treatment there is a higher chance of these pathogens being present. It is difficult to remove these pathogens from fresh produce without damaging or changing the nature of the product and even minimal processing, such as cutting or peeling, may increase levels of pathogens by increasing the availability of key nutrients (Harris et al., 2003).

The number of outbreaks associated with fresh or MPP has shown a rapid increase over the last two decades (Yaron and Römling, 2014). This is linked with its increased consumption but may also be, in part, due to better detection and reporting methods (Beuchat and Ryu, 1997), alterations in agricultural practices leading to increased contamination and an increase in the number of immunocompromised consumers (Beuchat, 2002).

A wide variety of different organisms have been linked to outbreaks of foodborne illness arising from the consumption of fresh produce or MPP. These include *Bacillus cereus, L. monocytogenes, Campylobacter jejuni, S. aureus,* Salmonella and *E. coli 0l57:H7* (Beuchat,

1996). Of these organisms it has been noted that *Salmonella* and *E. coli* are both frequently associated with larger outbreaks of food borne illness (Buck et al., 2003).

1.2 Treatments for MPP

MPP and fresh produce presents a number of issues in terms of food safety, any selected treatment method must be capable of removing dirt and pathogens without damaging or altering the nature or flavour of the produce. Today a number of effective treatments have been developed providing choice to both produces and consumers. These treatments can be divided in to three main categories, chemical, biological and physical. Each has different advantages and disadvantages. Here we discuss the more commonly used chemical antimicrobial treatments although these may be used in combination with other treatments and systems.

1.2.1 Chlorine Treatments

Chlorine solutions are commonly used within the food industry for disinfecting fresh produce, MPP, food preparation surfaces and washing equipment. The effectiveness of such treatments is dependent upon the quantity of free chlorine available within the solution. To achieve a significant level of bacterial reduction a concentration of free chlorine above 50 ppm is required and no significant advantage is achieved over a concentration of 200 ppm. Such solutions are effective against a range of pathogens including *Salmonella*, *E. coli* and *L. monocytogenes* (Beuchat and Ryu, 1997, Ruiz-Cruz et al., 2007).

The efficacy of chlorine based sanitisers depends upon a number of different factors including, pH, initial bacterial load, and presence of organic matter and physical method of treatment used in combination with the chemical treatments (Sanz et al., 2002). Chlorine washes generally come in three forms, dissolved chlorine gas, calcium hypochlorite and sodium hypochlorite.

Whilst chlorine is known to have highly antimicrobial properties, its mechanism of actions has yet to be fully elucidated (Goodburn and Wallace, 2013, Virto et al., 2005).

Although chlorine a commonly used sanitiser, some sources have raised safety concerns both for the environment and for consumers, although the evidence for the latter is limited (Simons and Sanguansri, 1997, Gil et al., 2009). There has been a drive from some quarters to find alternative effective treatments with potentially fewer negative impacts. As with many treatments the use of chlorine washes may alter the flavour or nature of the produce treated (Goodburn and Wallace, 2013).

1.2.2 Chlorine dioxide

Chloride dioxide ClO_2 commonly used in solution is a powerful oxidising disinfectant currently used as a disinfectant for portable water but has recently been adopted as an effective sanitiser for fresh produce (Gomez-Lopez 2012). It has been suggested that it does not produce by-products that might be harmful if left within the food chain in the same way as other treatments.

The mechanism of ClO_2 is based on its properties as an oxidising agent acting on the proteins in the bacterial cell wall. This causes the cell to lose its ability to maintain its trans-membrane ionic gradient (Berg et al., 1986). One problem with this treatment, however, is that chlorine dioxide is highly explosive and thus impracticable for many food producers.

1.2.3 Weak acid disinfectants

Weak organic acids are known to provide a high degree of antimicrobial action against both bacterial and fungal cells. The generally accepted theory for the mechanism of the action of weak acids derives through their nature of being present in two states dissociated (charged) and undissociated (uncharged) in aqueous solutions. Both states exist in an equilibrium which is

affected by the surrounding environmental pH and the protonation state of the acid which is determined by its acid disassociation constant (pK_a). At low pH, weak acids favour the undissociated state which is able to pass freely across the plasma membrane and enter the cell. Once the molecule enters the cell it normally encounters a high pH of the intracellular environment and it will then dissociate causing the release of protons, which can disrupt cellular processes. As such, the acid will continue to diffuse into the cell until the intracellular pH comes into balance with the extracellular pH. Fig.1.1 offers a generalised representation of the most commonly accepted action of a weak acids' antimicrobial action (Brul and Coote, 1999, Hirshfield et al., 2003).

However, this theory does not appear to solely explain all differences displayed by various weak organic acids. In general, weak acids that dissociate less are more antimicrobial but this is not always the case. Furthermore, it has been shown that different weak organic acids may display different antimicrobial abilities despite the same internal pH being achieved which does not fit with the theory described previously (Young and Foegeding, 1993). It has been shown that *E. coli* grown in various different organic acids is capable of achieving the same level of growth despite different internal pH being recorded which is in contrast to the above theory.

As such, a number of other mechanisms have been proposed to explain deviations from the main theory explaining the antimicrobial activity of weak acids. One of the most popular is that weak organic acids cause a disruption of membrane function. Another theory suggests that the antimicrobial action of weak acids is due to the inhibition of key metabolic functions through the accumulation of weak acid anions with in the cytoplasm creating osmotic stress. It is likely that their mode of action may vary depending upon the exposed organism and the prevailing environmental condition making this a difficult area to fully explore (Brul and Coote, 1999, Hirshfield et al., 2003, Roe et al, 1998, Stratford and Anslow, 1998, Ricke, 2003).

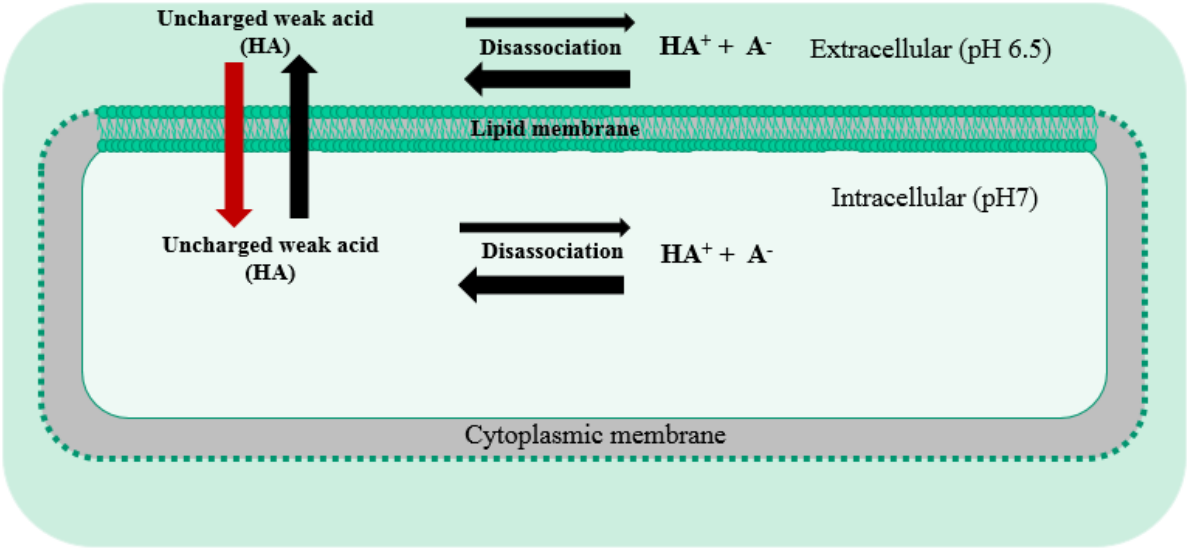

Fig.1.1: Representation of traditional model of the antimicrobial action of weak organic acids. The undisassociated form of the weak organic acid can penetrate the lipid membrane and is theorised to equilibrate across the membrane. The weak acid is then able to disassociate in to protons (HA+) and anions (A^-) Intracellular environment depending on a variety of factors including the properties of the acid such as pK_a and environmental conditions such as pH. This disassociation results in a reduction in intracellular pH causing issues such as the inability to sustain functional macromolecules and increased energy consumption caused by the organism's attempts to maintain a suitable intracellular pH (Hirshfield et al., 2003, Ricke, 2003).

In spite of their debated mode of action, weak organic acids provide an effective method of treating fresh produce and MPPs, there is some concern that an organism constantly treated with a selected weak organic acid may be capable of adapting to it. Thus constant or inappropriate exposure might increase some organism's ability to survive the treatment as well as making it less susceptible to natural defences found in humans in the form of stomach acids (Leyer et al., 1995, Hirshfield et al., 2003).

1.2.4 Electrolysed oxidising water

Electrolysed oxidising water is a comparatively new technique that focuses on improving the effectiveness of water-based washing techniques. It has been applied in a number of different industries including, experimentally, in the food industry (Al-Haq et al., 2002, Bari et al., 2003, Park et al., 2002). It is generated by the electrolysis of an aqueous salt solution, such as sodium chloride, creating a sodium hydroxide solution at the cathode and an acidic solution at the anode. Other salts may be used such as potassium chloride or magnesium chloride. This process disinfects by increasing the concentrations of free chlorine although its full method of disinfection has yet to be elucidated. It has been noted as providing significant reductions in a range of bacteria commonly found on fresh produce (Al haq and Gomez-Lopez, 2012, Ongeng et al., 2006, Guentzel et al., 2008).

1.2.5 Ozone

Ozone (O_3), like ClO_2, is a powerful oxidising agent that acts as a disinfectant in both its gaseous and aqueous forms (Glowacz et al., 2015, Al-Hashimi et al., 2015). Ozone is thought to kill bacteria through a reaction called an oxidative burst (Rao and Davis, 1999) which disrupts the bacterial cell wall. This causes the bacterial structure to break down leading to cell death (Olmez, 2012). Ozone has been shown to be effective on bacteria, spores and vegetative cells (Dosti et al., 2005). One of ozone's major advantages is that it decomposes quickly and does not produce chemical residues (Olmez 2012).

Peroxygens (HP and Peracetic acid)

1.2.6 Hydrogen peroxide

Hydrogen peroxide, H_2O_2, has been utilised as a sanitiser for both MPP, fresh produce and food preparation surfaces (O Ukutu et al., 2012) It has been demonstrated to be effective against a range of organisms including bacteria, yeasts and spores (Back et al., 2014) although it is particularly effective against Gram positive bacteria.

H_2O_2 is a strong oxidising agent and acts through the formation of hydroxyl free radicals (-OH) thus disrupting key cell structures such as lipids and proteins as well as effecting cellular DNA (McDonnell and Russell, 1999, Rios-Castillo et al., 2017). It has been suggested as particularly useful for the food industry that H_2O_2 degrades quickly into water and oxygen resulting in minimal impact upon the environment and creating minimal levels of residues (Daft, 1991). While it can provide an effective treatment for fresh produce it has been shown to have a sygnifcant negative impact on a number of types of fresh produce limiting its uses as an antimicrobial(Ölmez and Kretzschmar, 2009).

1.2.7 Peracetic acid (PAA) PCH_3COOOH

Peracetic acid has strong disinfectant properties and is effective against bacteria, viruses, fungi and spores. It has been found to be effective against *E. coli* and *Salmonella* in concentrations as low as 40 ppm (Park and Beuchat, 1999). The mode of action of PAA has yet to be completely understood, but it has been suggested that it acts upon the lipoproteins found in the cell membranes (Leaper, 1984). It is effective under less than ideal conditions and is capable of operating at a variety of temperatures and pH's. PAA like hydrogen peroxide is considered to have some environmental impact as its decomposition results in acetic acid and oxygen (Gonzalaz- Aquilar et al., 2012).

1.2.8 Essential oils

Essential oils (EO's) are aromatic, volatile oil-based liquids that may be extracted from a wide variety of natural plant materials (Barry-Ryan and Bourke, 2012). They may be utilised by plants to provide protection against pathogens and attract pollinators (Nazzaro et al., 2013). They have been shown to have antimicrobial activity against bacteria, yeasts and moulds (Kwon et al., 2017). Essential oils cover a wide range of compounds with some differences in their mode of action, although generally their antimicrobial activity is based upon their highly hydrophobic nature (Karatzas, 2002). EO's have been shown to degrade or destroy a number of key cellular structures including cell walls, phospholipid bilayers, disrupt enzymatic processes and degrade DNA (Barry-Ryan and Bourke, 2012).

EO's in general are less effective upon gram negative bacteria because of their lipopolysaccharide outer membrane which prevents their diffusion limiting the impact upon this type of bacteria (Nazzaro et al., 2013).

The exploration of EO's as antimicrobials has been driven in part by concerns related to the residues arising from other chemical treatments. Essential oils are viewed by consumers as a natural and safe treatment (Foley and Lassak, 2004). One major issue with them, however, is that by their very nature they often have strong odours and may alter the flavour of treated produce (Goni et al., 2009) which limits significantly their usage to a specific food where the relevant .

1.3 *Escherichia coli*

Organism

E. coli is a rod shaped, gram negative, facultative anaerobe predominantly found in the mammalian digestive tract and is an important part of the mammalian gut microbiota (Evans and Evans, 1996). *E. coli* which is the most studied and characterised organisms in the *Enterobacteriaceae* family (Jang et al., 2017). Whilst the majority of strains are relatively harmless, some are pathogenic and can cause severe symptoms ranging from diarrhoea and vomiting to kidney damage and death (Liaqat, I. 2011).

Whilst *E. coli* is generally found in the digestive tract of warm blooded organisms, it is capable surviving a wide range of environmental conditions persisting within the environment in soil, manure and slurry (Kudva et al., 1998, Jiang et al., 2002). While *E. coli* prefers the mammalian gut temperature in the range of 20 – 37 °C, it is capable of surviving as high as 40 °C and as low as 10°C (Farewell and Neidhardt, 1998, Van Derlinden et al., 2008). Under ideal conditions *E. coli* is capable of replicating its self within ~ 20 minutes (Jang et al., 2017).

One of the greatest environmental challenges that foodborne organisms face is the acidic environment of the mammalian digestive system. This can reach a pH of between 1.5 -3.5 and yet many foodborne organisms including *E. coli* are capable of surviving these extreme conditions (Foster, 2004).

Virulent *E. coli* strains can be sub divided into six different groups: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAggEC), diffusely adherent *E. coli* (DAEC), and Enterohemorrhagic (EHEC) *E. coli* (also known as vero-cytotoxin-producing *E. coli* (VTEC) or sometimes shiga toxin-producing *E. coli* (STEC). Of these the most commonly highlighted as a danger to human health is VTEC which includes *E. coli* O157:H7 this has been recognised as an important cause of

foodborne illness with a number of significant outbreaks. It has been suggested that the incidence of foodborne infections from this group is on the rise in Europe although this may be driven by better identification and more sensitive detection methods (Enternet annual report, 2006).

1.3.1 Incidence

The global incidence of *E. coli* outbreaks are uncertain however, EHEC is one of the most frequently recorded cause of foodborne illness because of the severity of the symptoms associated with it. Strains belonging to this group have been estimated to be globally responsible for 230 deaths and 3,890 cases of haemolytic uraemic syndrome (HUS) per year. A significant number of patients with HUS are left with permanently damaged kidneys and require haemodialysis regime for the rest of their lives which can be resolved with kidney transplantation. This strain (Majowicz et al., 2014), has been identified as a key cause of foodborne disease particularly in low income communities (Havelaar et al., 2015).

One of the clearest examples of the issues caused when fresh and MPP are contaminated with food pathogens occurred in 2011 with an outbreak of *E. coli* O104:H4 that occurred started in North Germany and spread rapidly through Europe. This incident was estimated to have affected 3,816 people, caused over 800 cases of HUS and resulted in 82 deaths across 15 countries (Frank et al., 2011, Mariani-Kurkdjian and Bingen, 2012).

1.3.2 Stress /Acid resistant mechanisms of *E. coli*

There are four amino acid decarboxylase systems present in *E. coli* the glutamate decarboxylase (GAD) system or Glutamate dependent mechanism of acid resistance (GDAR), the lysine decarboxylase system or lysine dependent acid resistance (LDAR), the arginine dependent

mechanism of acid resistance (ADAR) and the ornithine-dependent mechanism of acid resistance (ODAR). These systems are the organism's major mechanisms of acid resistance. All four systems function in similar ways utilizing as a substrate an amino acid which is then decarboxylated and this reaction consumes a proton. The products of this reaction may then be exported via an antiporter as shown in Fig 1.2. This conversion helps to remove protons and assists the survival of *E. coli* in low pH conditions (Kanjee et al., 2011, Feehily et al., 2012, Audia et al., 2001, Cotter et al., 2001).

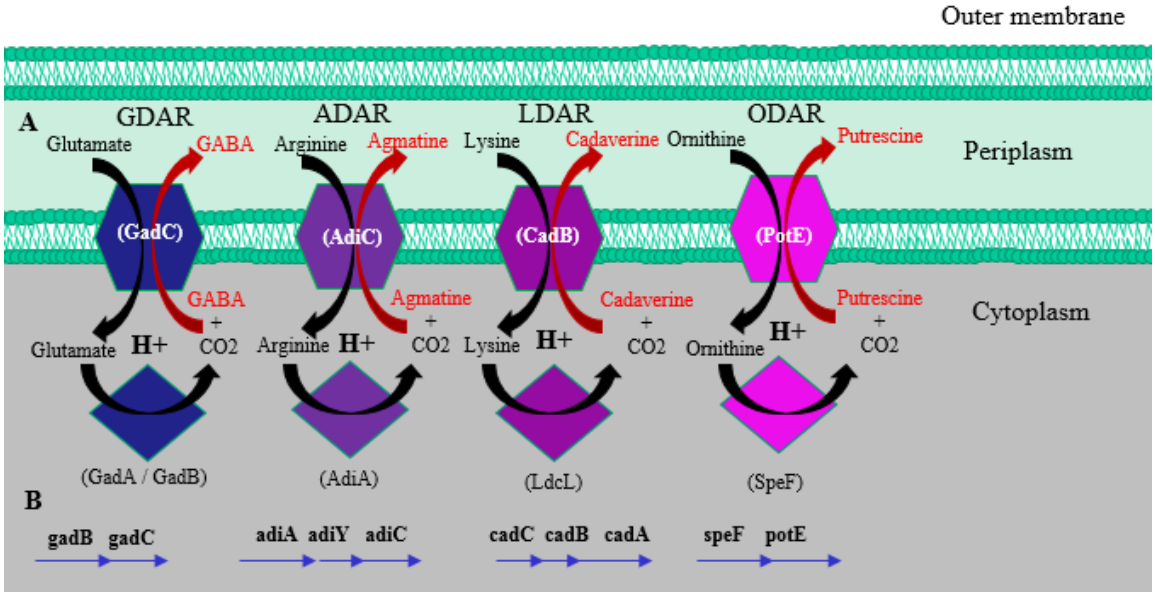

Fig 1.2: A representation of the amino acid decarboxylase systems and their antiporters. **1.** Glutamate-dependent mechanism of acid resistance (GDAR) comprising a glutimate/γ-aminobutyric acid (GABA) antiporter (GadC) and the two cytoplasmic glutamate decarboxylases (GadA and GadB). **2.** Arginine-dependent mechanism of acid resistance (ADAR) the arginine decarboxylase system comprising an arginine/agmatine antiporter (AdiC) and its inducible cytoplasmic arginine decarboxylase (AdiA). **3.** The lysine decarboxylase mechanism of acid resistance (LDAR) comprising a lysine/cadaverine antiporter (CadB) and its cytoplasmic inducible lysine decarboxylase (Ldcl). **4.** The ornithine-dependent mechanism of acid resistance (ODAR) one ornithine/putrescine antiporter (PotE) and its cytoplasmic inducible ornithine decarboxylase (SpeF) (Kanjee and Houry, 2013).

The GAD system which is thought to be the most robust of these four systems, functions through the conversion of glutamate to γ-aminobutyrate which consumes a proton, which is then removed to the extracellular environment (Cotter et al., 2001) and helps to maintain a high intracellular pH in this organism (Smith et al., 1992, Kanjee and Houry, 2013, Castanie-Cornet et al., 1999). The induction of this specific mechanism of acid resistance relies on a number of key proteins which are summarised in Table 1.1 This mechanism is one of the best described representations of the activation of a glutamate dependent acid resistance response in a foodborne pathogen. The large number of regulatory components required by the GAD system may indicate that it is linked to a variety of aspects of an organism's cell physiology making it difficult to fully describe its regulation even in *E. coli* where it is best understood (Sayed et al., 2007). In *E. coli* the GAD system comprises two PLP-dependent, hexameric, enzyme glutamate decarboxylases (GadA and GadB) in combination with one antiporter, GadC, which is used to exchange GABA with glutamate (De Biase et al., 1999). GABA produced via this mechanism may also be utilised via the GABA shunt pathway. This pathway uses two further enzymes a GABA/-ketoglutarate aminotransferase (GABA-AT) (GabT) and succinic semialdehyde dehydrogenase (SSDH) (GabD). The GabT first removes the amino acid group from the GABA molecule converting it to succinic semialdehyde (SSA) and glutamate. The SSA is then oxidised by the GabD to form succinate (Dover and Halpern, 1972, Dover and Halpern, 1972b, Fait et al., 2008)

Whilst Gad A, B and C are the key structures of the glutamate decarboxylase as previously stated, there are a number of key regulatory factors that have been identified. Out of these GadE is considered the most important and acts as an essential transcriptional GAD activator which in turn is controlled by two further activators GadX and GadW which are AraC-family of regulators (Ma et al., 2003a) and part of a complex network summarised in Table 1.1 (Foster

2004). These other systems all operate under a variety of different conditions related to stage of growth, available nutrients and the level of pH stress the organism exposed too.

Table 1.1 Describing the key Genes involved in regulating glutamate-dependent acid resistance in *E.coli* adapted from Foster (2004).

Genes involved in the regulation of the GAD system of *E.coli*			
Protein	**Descriptor**	**Function in acid resistance**	**Reference**
GadE	LuxR- related activator	Required for the acid resistance, binds to *gad* box, activates transcription of *gadA/BC*, auto activates transcription *gadE*, represses *ydeO*	(Ma et al., 2003a, Hommais et al., 2004)
GadX	AraC- like regulator	Activator of the *gadE*, co-activator of *gadA/BC*, represses *gadW*	(Tucker et al., 2002, Shin et al., 2001, De Biase et al., 1999, Hommais et al., 2004)
GadW	AraC-like regulator	Inhibits RpoS production, activator of *gadE*, can co-activate *gadA/BC* at pH 8	(Ma et al., 2002)
GadY		Activator of the acid resistance genes *gadA*, *gadB*, and *gadC* and upregulates GadX	(Negrete and Shiloach, 2015)
Rpos	σ^{38}	Transcription of gadX	(Ma et al., 2002)
EvgAS	Two-component signal transduction	Activates YdeO and gadE transcription	(Masuda and Church, 2003)
YdeO	ArC-like regulator	Activates *gadE* transcription	(Masuda and Church, 2003)
Crp	cAMP- receptor protein	Inhibits Rpos production	(Ma et al., 2002, Ma et al., 2003b)
TrmE	Era- like GTPase	Activates *gadE* mRNA production, stimulates translation of *gadA* and *gadB* mRNA	(Cabedo et al., 1999)
HNS	Histone-like protein	Negative regulator	(De Biase et al., 1999, Ma et al., 2002, Tramonti et al., 2002)
TorR	Response regulator of trimethylamine *N*-oxide (TMAO) reductase	Negative regulator of *gadE*	(Bordi et al., 2003)

The arginine decarboxylase system comprises arginine decarboxylase, AdiA which converts arginine to agmatine which can then be exported using an agmatine antiporter, AdiC, for more arginine. This system assists in the survival of *E. coli* below pH 3 (Iyer et al., 2003).

Under mildly acidic conditions, *E. coli* preferentially uses the lysine decarboxylase rather than the GAD and the arginine systems. This system comprises two lysine decarboxylases, Ldci and CadA which convert lysine to cadaverine and then can be exchanged for more lysine using a lysine/cadaverine, CadB antiporter (Diez - Gonzalez and Karaibrahimoglu, 2004, Kanjee et al., 2011) achieving a similar consumption of protons to other systems maintaining a high intracellular pH.

The ornithine-dependent acid resistance system is the most recently described mechanism of acid resistance in *E. coli* but is one of its weaker defenses (Aquino et al., 2017). This system decarboxylases ornithine using SpeF, an ornithine decarboxylase and the ornithine/putrescine antiporter PotE (Kanjee et al., 2011, Kanjee and Houry, 2013). This is thought to operate under similar conditions to the lysine mechanism although less is known about it. All of these systems and their functions are illustrated in Fig 1.2.

While the amino acid decarboxylase systems described above provide *E. coli* with the majority of its protection under acidic conditions there are a number of other systems linked with the ability of *E. coli* to survive and grow in acidic environments. The first of these are a glucose-dependent oxidative system which is dependent on the alternative sigma factor RpoS. This has been associated with environmental stresses in *E. coli*, such as acidic conditions (Merrell and Camilli, 2002, Richard and Foster, 2003). This factor is a transcriptional regulator operating at a genetic level and can alter the state of the organism thus preparing it for a current or similar future stresses. It activates different genes, depending upon the needs of the organism, in order to help it deal with adverse conditions. This sigma factor is required for oxidative and acid stress resistance and can aid in providing protection down to a pH of 2.5 (Lin et al., 1996). The

RpoS sigma factor has been associated with the expression of the GAD system under specific conditions; however, RpoS is not the sole sigma factor responsible for the σ^S and σ^{70} which also plays a role in the regulation of the GAD system. in *E. coli* have been shown to work in concert with the other systems (Waterman and Small, 2003). This system is induced upon the organism's entry to the stationary phase and is not dependent upon the presence of low pH conditions (Castanie-Cornet et al., 1999).

The formation of cyclopropane fatty acids (CFA), which are a constituent of phospholipids commonly, found in bacteria, have also been linked with acid resistance in *E. coli.* They form when a methylene group is transferred to phospholipids found in the bacterial membrane, causing modification of this membrane. This reaction generally occurs as the bacteria enters into stationary phase and it has been noted that the presence of high levels of CFA appears to confer a strong level of acid resistance on wild-type *E. coli* strains (Brown et al., 1997, Chang and Cronan, 1999). The operation of this mechanism relies on the presence of RpoS and on a functional *cfa* gene (Chang and Cronan, 1999).

1.4 *Listeria monocytogenes*

Organism

L. monocytogenes is Gram positive, facultative anaerobic rod found in a wide variety of different environments, notable in soil and water. It thrives in the presence of decaying vegetation such as animal feeds and plant based fertilisers and can easily infect animals that orally ingest contaminated food (Fenlon, 1999). In the genus *Listeria* there have been, to date, 10 recognised species, (Paudyal and Karatzas, 2016) although other species have been tentatively recognised indicating that there may be more to be discovered (den Bakker et al., 2014, Weller et al., 2015). Of those identified only *L. monocytogenes* presents a risk to human health although other such as *L. ivanovii* present a risk to both pets farm animals including cattle, chickens, horses and in very exceptional cases it can affect humans (Weber et al., 1995).

L. monocytogenes is an organism of special concern to the food industry because whilst it is not frequently identified as the cause of major outbreaks it is one of the most deadly pathogenic organisms, causing listeriosis which has a mortality rate of 20 -30%. Listeriosis in healthy individuals manifests as febrile gastroenteritis which whilst not generally serious may have sygnifcant adverse health impact, in compromised individuals and neonates, where it may result in sepsis, meningitis, or encephalitis. In addition to these issues, *L. monocytogenes* is particularly dangerous to pregnant individuals in whom this organism may cause spontaneous abortions and other health complications (Swaminathan and Gerner-Smidt, 2007, de Noordhout et al., 2014, Abram et al., 2003).

1.4.1 Incidence

While *L. monocytogenes* is less commonly associated with large scale outbreaks from fresh produce, because of its high mortality rate (20-30%) (Swaminathan and Gerner-Smidt, 2007, Zhu et al., 2017) it is one of key concern. It is difficult to estimate the global health burden of

L. monocytogenes as this organism generally only causes issues when it enters the food chain and as such does not have the prevalence of some organisms found more widely in nature such as *Salmonella* Typhi. However, the World Health Organisation (WHO) has suggested that, globally, in 2010 *L. monocytogenes* infected 23,150 people and led to approximately 5463 deaths. It has been stated that it is difficult to estimate the true global burden of this organism due to lack reporting of its incidence (de Noordhout et al., 2014).

This lack of reporting is exemplified by probably the deadliest outbreak in history that occurred in Southern Africa and caused by *L. monocytogenes*. This outbreak was associated with polony sausages produced in S. Africa, consumed there, but also in 15 other African countries where they were exported. This resulted in 978 laboratory-confirmed listeriosis cases with a fatality rate of 27%, which were all reported in S. Africa despite a significant amount of these sausages being consumed in many different African countries. The WHO suggested that a significant number of cases have affected various African countries, but due to the lack of surveillance systems in these countries, only S. Africa was able to trace these listeriosis cases and deaths (WHO, 2018). Since listeriosis normally manifests as meningitis which is caused by a variety of microorganisms' only countries that have a reporting system for the cause of meningitis will be able to report listeriosis cases. If a country does not have this requirement the listeriosis cases will be reported as bacterial meningitis making it impossible for the authorities to trace the source of the outbreak.

L. monocytogenes has been identified in a wide variety of MPP on products including common staples such as carrots, cucumber, parsley and salad leaves (Ruiz-Cruz et al., 2007, Meldrum et al., 2009, Scallan et al., 2011). In the USA alone in the last 20 years there have been 312 reported cases of *L. monocytogenes* associated with fresh produce, resulting in 56 deaths (a mortality rate of 17 %). This is recognised as a significant foodborne issue in the USA and is an indicator of global significance (Zhu et al., 2017).

1.4.2 Stress / Acid resistant mechanisms of *L. monocytogenes*

Similarly to other organisms described above, *L. monocytogenes* possesses a wide variety of acid resistance mechanisms which is a key to its success as a food pathogen. The majority of these systems are based on maintaining a viable intracellular pH (Ryan et al., 2008).

Similarly to *E.coli* one of the most important mechanisms are the decarboxylases systems (Hill et al., 2002). The glutamate decarboxylase system has been noted as the main mechanism of acid resistance in *L. monocytogenes* although the protection varies from strain to strain (Hill et al., 2002). *L. monocytogenes* also has an arginine deiminase (ADI) system using arginine (Ryan et al., 2009) and an agmatine deiminase (AgDI) system using agmatine while both of which help to regulate intracellular pH (Chen et al., 2011).

The GAD system has been identified in a number of organisms including higher animals such as animals (including humans), plants, fungi, yeasts, and archaea (Karatzas et al., 2012). Animals use the system as part of their nervous system, plants use it during hypoxia and by some microorganisms as an acid resistance system (Satyanarayan and Nair, 1985, Feehily and Karatzas, 2013, Erlander and Tobin, 1991). *L. monocytogenes* normally possesses three decarboxylases GadD1, GadD2 and GadD3 and two antiporters GadT1 and GadT2 and its function is demonstrated in Fig 1.2. While this is generally true there variation between strains and it has been noted that some strains belonging to serotype 4 and 1/2 in general do not to possess the gadD1T1 operon which has been suggested to promote growth under milder acidic conditions (Cotter et al., 2005).

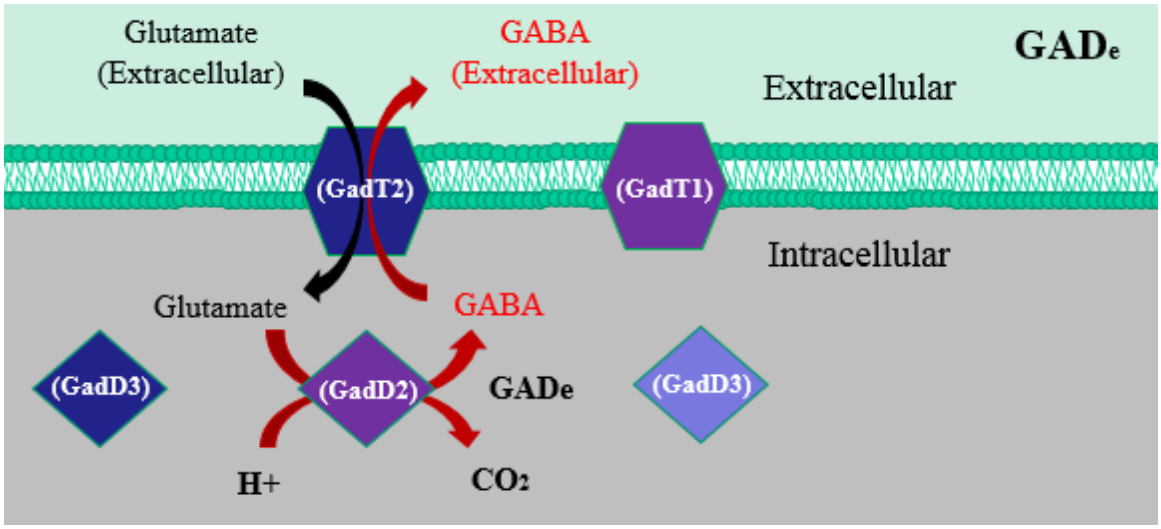

Fig: 1.3 Depiction of the extracellular (GAD_e) glutamate decarboxylase system showing the current model of operation of this system while under severe acidic stress (< pH 4.5). The organism imports extracellular glutamate, in this case via the GadT2 antiporter, which is then decarboxylated by the corresponding GadD2 to GABA. This reaction consumes one proton (H^+). The GABA product is then exported by the same antiporter and replaced with a new molecule of glutamate. (Karatzas et al., 2012).

Until recently, the mechanism in Fig 1.3 was accepted as the model for the function of the glutamate decarboxylase system of *L. monocytogenes*. However, the discovery of an intracellular GAD system (GAD_i) has extended the understanding of this system (Karatzas et al., 2012). This system provides the organism with a greater level of protection allowing this acid resistance mechanism to function even in the absence of a functional antiporter (Fig 1.3). Some organisms such as *Mycobacterium tuberculosis* have been shown to possess a decarboxylase but have no known antiporters working solely on intracellular glutamate (Cotter et al., 2001, Cole et al., 1998). GAD_i functions with the use of glutamate that is already present within the organism and, much like the GAD_e, operates by converting this intracellular glutamate to GABAi and CO_2. In *L. monocytogenes* this function is undertaken by GadD3 which so far has only been identified in *L. monocytogenes* (Feehily and Karatzas, 2013) as illustrated in Fig 1.4. With the absence of an appropriate antiporter the $GABA_i$ remains trapped

within the intracellular environment where it is then metabolised and utilised via the GABA shunt pathway (Feehily et al., 2012). While the GABA shunt pathway of *L. monocytogenes* is less well-described than that of *E. coli* it has been demonstrated that it appears to play a role in the survival of this organism under acidic conditions and may present a method by which *L. monocytogenes* over comes its incomplete citric acid cycle shown in Fig 1.5 (Feehily et al., 2012, Dover and Halpern, 1972a Castanie-Cornet et al., 1999).

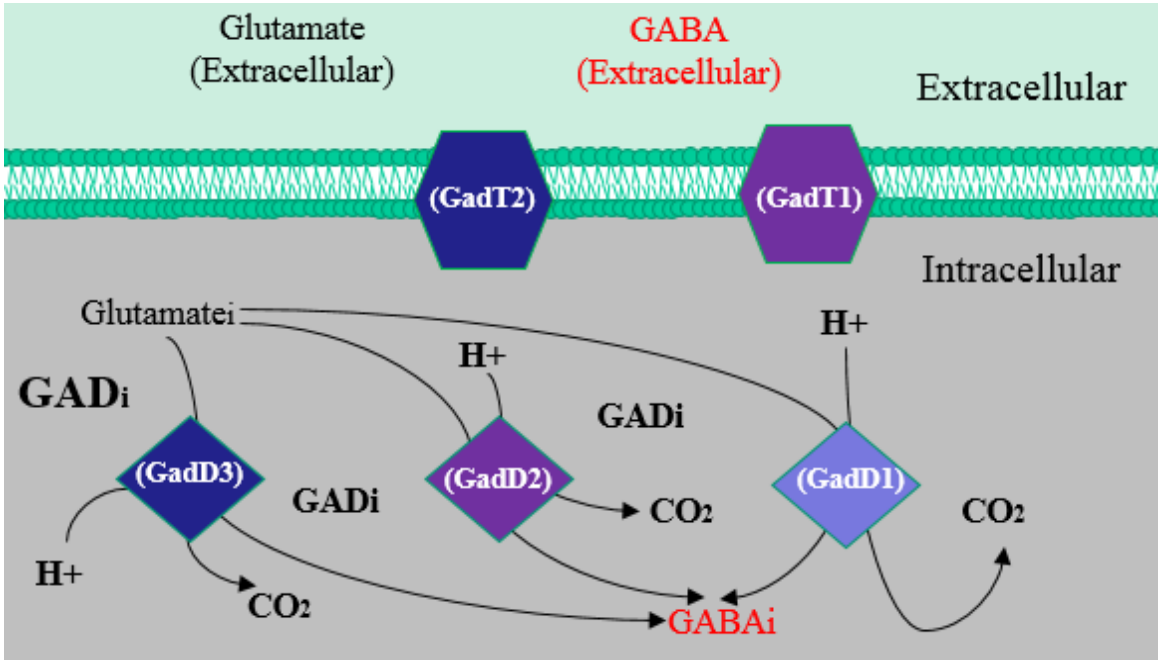

Fig 1.4: Depiction of the function of the intercellular GAD system when functioning under acidic conditions (pH < 4.5). Intracellular glutamate is then decarboxylated by both GadD3 and GadD2, resulting in the accumulation of $GABA_i$. The latter process is carried out by GAD_i, which is depicted by the black arrows. The contribution of GadD1 and GadT1 in both intra and extracellular systems has been demonstrated to be limited (Karatzas et al., 2012).

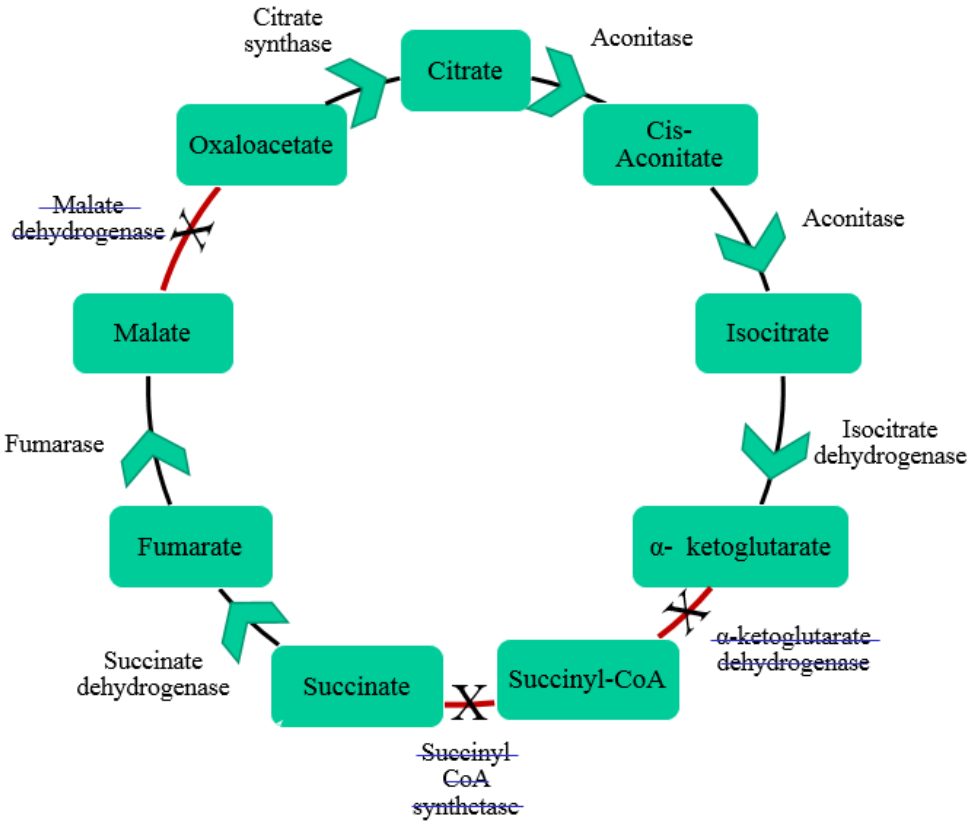

Fig 1.5: A simplified diagram of the citric acid cycle or Krebs cycle of *L. monocytogenes* the missing steps in the cycle are highlighted in red and the missing enzymes indicated with an X.

Although the GAD system has been demonstrated to be present in various organisms, the level of protection that it provides under acidic stress varies from species to species and even within strains. Some strains of *L. monocytogenes* utilise both the intra- and the extra-cellular systems including LO28 and 10403S. While EGD-e is only capable of utilising the GAD_i system this in turn may affect the ability of different organisms, and even different strains of the same organism, to survive in different environmental conditions (Karatzas et al., 2010, Feehily et al., 2013).

The regulation of the GAD system requires the use of a variety of regulatory elements, as demonstrated by Table 1.1, which shows the key regulators in *E. coli.* These elements are not found in *L. monocytogenes.* The regulation and control of the GAD system in *L. monocytogenes* has been less fully explored than that of *E. coli.* The upregulation of *gadT2D2* and *gadD3* appears to be dependent upon SigB (σ^B) expression which occurs during the stationary phase in rich media environments (Wemekamp-Kamphuis et al., 2004, Karatzas et al., 2010). Further research may provide a better explanation of how this system operates in *L. monocytogenes* since its regulation and functionality shows clear differences from the current model based on the GAD system of *E. coli.*

The alternative sigma factor σ^B is present in a number of Gram positive microorganisms such as *Bacillus subtilis*, *S. aureus* and *L. monocytogenes* and functions like the alternative sigma factor Rpos found in *E. coli* and other gram-negative bacteria (Wiedmann et al., 1998). It has been shown that σ^B helps to regulate numerous genes linked with coping with environmentally stressful conditions and has been linked with acid tolerance in *L. monocytogenes* via the regulation of the GAD system of acid resistance (Wemekamp-Kamphuis et al., 2004, Becker et al., 1998).

The arginine deaminase system (ADI System) has also been shown to influence the ability of *L. monocytogenes* to survive acidic stresses. This system relies on three enzymes, arginine deaminase (ADI), catabolic ornithine transcarbamylase (cOTC) and carbamate kinase (CK) which are names as ArcA, ArcB and ArcC. These enzymes act as catalysts for the conversion of arginine to ornithine with the production of NH_3, CO_2 and adenosine triphosphate (ATP). The NH_3 can then combine with intracellular protons to create ammonia ions (NH_4^+). This reaction causes an intracellular increase in the pH thus maintaining a pH closer to neutral under external acid conditions (Liu, 2008, Gurtler et al., 2017). The ATP that is produced from this reaction may be used to export protons via the F0F1-ATPases, which plays an important role

in pH homeostasis (Cotter et al., 2000) .This system promotes growth and survival much like the GAD system although it is considerably less effective. This system in *L. monocytogenes* appears to be the most complex yet identified (Ryan et al., 2009).

The agmatine deiminase system has been identified in *L. monocytogenes*, *Enterococcus faecalis* and *Streptococcus mutans.* Agmatine enters bacterial cells via an agmatine-putrescine antiporter (aguD). AgDI converts agmatine to n-carbamoyl putrescine and NH_3 by one of two putative agmatine deiminases (aguA1 and aguA2) whose transcription is pH dependent (pH < 5). Although two deiminases have been identified it appears that only aguA1 plays a role in the acid resistance of *L. monocytogenes* (Chen et al., 2011).

Another mechanism that plays a role in acid resistance in associated with acetoin biosynthesis. This system is based on a proton consuming-reaction by which α-acetolactate synthase which is encoded by alsS and merges with molecules of pyruvate to create acetolactate which is then converted by α-acetolactate decarboxylase (alsD) to form acetoin in a reaction that also consumes one proton helping to support the organism at low pH. It has also been suggested that the increased production of acetoin prevents acidification by specific organic acids such as acetate or lactate by preventing their interference with the transcription of key enzymes such as pyruvate dehydrogenase (Stasiewicz et al., 2011). It has been noted that both AlsS and AlsD are upregulated under low pH conditions and are dependent upon the presence of thiamine which is commonly found in foods and as such available to food pathogens. (Smith et al., 2012).

One system that is not based on the enzymatic consumption of protons is the SOS regulon which is an inducible pathway involved in DNA repair as well as in helping to restart stalled DNA replication. The SOS regulon is regulated by LexA (repressor) and RecA (activator), and is active under only certain stress conditions including low pH and heat shock (Castanie-Cornet et al., 1999, van der Veen et al., 2010).

1.5 *Salmonella*

Organism

Salmonella are a gram-negative, rod shaped, bacterium which is member of the *Enterobacteriaceae* family and is a key food pathogen globally with over 2600 known serovars. Approximately 60 % of these serovars can be classified as *Salmonella enterica subsp. enterica* (Sánchez-Vargas et al., 2011, Batista et al., 2015, Gal-Mor et al., 2014, Humphrey et al., 2012). Infection with *Salmonella* can cause salmonellosis, which may result in diarrhoea, fever, and abdominal cramps. This organism has an incubation time of approximately 12--72 hours (Control and Prevention, 2007) and are generally capable of growth at a range of temperatures with a lower limit of 5–10 °C and an upper limit of 42–50 °C (Juneja et al., 2007). *Salmonella* can effect a wide range of foods and is most commonly associated with poultry and poultry products (Keerthirathne et al., 2016) but has also be shown to affect fresh produce and MPP (Abadias et al., 2008) .

Salmonella typically follow the faecal/oral route of infection and once is ingested it utilises a complex selection of virulence factors that allows it to invade the epithelial lining of the intestine. Some organisms e.g. *L. monocytogenes,* are able to express molecules that interact with the host cells receptors leading to the pathogen being absorbed through phagocytosis, commonly described as ‘zipper’ entry. Some *Salmonella*, such as *S.* Typhimurium are capable of skipping this process through the use of bacterial effector molecules which the pathogen injects in to the host cell through a kind of biological syringe called Type III secretion system, allowing it to manipulate the target cytoplasm. This causes significant alterations to the actin cytoskeleton and allows the creation of macropinosomes which are then internalised by the target. The host’s structures quickly recover and the invading salmonella is safe inside a membrane bound vacuole where it can replicate (Ly and Casanova, 2007).

1.5.1 Incidence

Salmonella has been identified as the leading cause of gastroenteritis worldwide but it is difficult to establish the full extent of the incidence of non-typhoidal *Salmonella*. It has been estimated that annually 93.8 million cases of gastroenteritis are caused by various non-typhoidal *Salmonella* species and approximately 155,000 deaths. From these estimates it has been proposed that approximately 75 % of the cases were due to foodborne *Salmonella (Majowicz et al., 2010).* This suggests that this organism presents a major health burden globally and as such this pathogen is of high interest to food producers and processors.

A clear example of the issues *Salmonella* can cause when present in fresh produce occurred in the United States in 2015 involving cucumbers imported from Mexico and distributed widely throughout America. In total a reported 838 people became ill, 165 people were hospitalised and 4 died. The causative agent was subsequently identified as *Salmonella* enterica serovar *Poona* (Zuraw, 2015).

1.5.2 Stress / Acid resistant mechanisms of *Salmonella*

Like many successful foodborne pathogens *Salmonella* are capable of growth and survival in a wide range of environmental conditions and have developed numerous different mechanisms to assist in this. The majority of studies focus on *Salmonella* cells grown under optimal conditions which die rapidly when exposed to acid shocks below pH 4. In contrast cells grown in mildly acid conditions have a significantly increased ability to survive under acidic conditions (Bearson et al., 1997).

Salmonella are capable of surviving a wide range of pH values although the optimal pH range is between 6.5 and 7.5 (Chung and Goepfert, 1970, Foster, 1991). They have a number of mechanisms that assist in survival under acidic conditions. The first line of defence under mildly acidic conditions is the modulation of Na^+/H^+ and K^+/H^+ antiporters that allow the

organism to maintain a satisfactory intracellular pH (Foster and Hall, 1991). This system is of limited capability, though, and is less effective under more extreme acid conditions (Bearson et al., 1998).

Salmonella, like *E. coli* and *L. monocytogenes*, possesses amino acid decarboxylase systems that help it to survive acidic stresses. Although it lacks the GAD system, *Salmonella* typically have three amino acid decarboxylase systems, the arginine system the lysine decarboxylase system and the ornithine decarboxylase system.

The arginine decarboxylase system consists of and arginine decarboxylase (AdiA), an arginine/agmatine antiporter (AdiC) for the export and import of arginine and the export of agmatine and a transcriptional activator (AdiY) this system is typically active under aerobic conditions and plays a role in maintaining intracellular pH. The lysine decarboxylase system is also responsible for helping *Salmonella* to maintain a suitable intracellular pH and is composed of lysine decarboxylase enzyme (CadA), a lysine–cadaverine antiporter (CadB) which is used for the import of lysine and the export of cadaverine and a transcriptional regulator of the *cadBA* operon (CadC) (Álvarez-Ordóñez et al., 2012) the operation of these systems can be seen in Fig 1.2. The ornithine decarboxylase system (SpeF) is present although the literature surrounding this system in *Salmonella* is limited (Viala et al., 2011). These systems are activated by exposure to low pH and as such are often described as inducible acid resistance mechanisms (Viala et al., 2011).

In *Salmonella*, like most organisms does not rely solely on amino acid decarboxylase systems to provided protection from challenging environmental conditions. In *Salmonella* a number of responses to acidic conditions have been noted and the response may vary depending upon the organism's stage of growth. *Salmonella* have been shown to have three key regulatory proteins RpoS, and Fur and PhoP that help the organism adapt to low pH environments (Bearson et al., 1997).

The alternative sigma factor σ^S, encoded by *rpoS* is thought to be key to the regulation of the acid tolerance response of *Salmonella* in both stationary and exponential growth phases. The σ^S, is thought to be regulated by a 38-kDa protein which in turn is encoded by the virulence gene MviA which is thought to control the accumulation of σ^S. The σ^S is suspected of controlling between 10 and 50 acid shock proteins (ASP) making it key to this organisms response to low pH. Interestingly mutations in the *rpoS* or *mviA* reduce the virulence of *Salmonella* (Bearson et al., 1997, Spector and Kenyon, 2012, Benjamin et al., 1996, Becker et al., 1998, Bang et al., 2005, Hengge, 2009). *Salmonella* also have a number of other sigma factors which are responsible for dealing with other environmental stresses including nitrogen metabolism, flagella synthesis, heat shock and cytoplasmic stress (Shen and Fang, 2012).

One regulatory protein that is involved in the response of *Salmonella* to acidic conditions is the PhoP which is independent of the σ^S. The PhoP system consists of two components the PhoP and the response regulator sensor-kinase PhoQ (ASP 29) working in concert with a further regulator PhoPQ and helps to regulate a further 4 ASP's. This system is active when concentrations of low Mg^{2+} are low or under acidic conditions and is thought to be able to detect low pH conditions via H^+ ions on the conformation of the Mg^{2+} ion-binding site resulting in the phosphorylation of PhoP via histidine kinase (Bearson et al., 1998, Spector and Kenyon, 2012).

The Fur is a ferric uptake regulator which helps to repress the expression of iron–regulated genes in the presence of high levels of intra cellular levels of Fe^{2+} found in *Salmonella* and helps to prevent the oxidative damage to cytoplasmic macromolecules (Park et al., 1996, Spector and Kenyon, 2012).

These systems together provide a wide range of tools that *Salmonella* may utilise under acidic conditions making their removal using acids a challenge.

1.6 Legislative controls for safe food production and fresh produce

The food industry has a legal, and moral, duty to eliminate or to reduce pathogens to safe levels in their products although this can be difficult with fresh and minimally processed produce. To achieve this, a number of different tools, including the treatments described above, can be used but should be supported by microbiological testing, good manufacturing practices and food management systems such as Hazard Analysis and Critical Control Point (HACCP) systems.

The microbiological criteria for fresh produce in the UK is currently in line with EU standards and falls under the European Commission (EC) Regulation on microbiological criteria for foodstuffs ([EC] No. 2073/2005 ((EC), 2005). In annex one specific criteria are detailed for fresh and MPP. Under this legislation standards are described for *Salmonella* levels in pre-cut fruit, vegetables, unpasteurised fruit and vegetable juices *Salmonella* should be absent in a 25g sample. In similar products that are able to support *L. monocytogenes* bacteria present should not exceed 100 cfu/g. In products that are intended for special medical purposes or intended for consumption by infants no *L. monocytogenes* should be found. For *E. coli* there is less stringency and it would be acceptable to find < 100 cfu/g in 5 samples, 100 cfu/g - 1 000 cfu/g found in no more than 2 samples and an unsatisfactory would be higher than 1 000 cfu/g more than 2 samples ((EC), 2005).

1.7 Biofilms

Biofilms are formed when unicellular organism come together to form a complex community that then attaches itself to a suitable surface (O'Toole et al., 2000) such as those on fresh produce or food preparation areas (Mah and O'Toole, 2001). These structures may comprise one or multiple organisms and are capable of forming single or multi layered structures (Chmielewski and Frank, 2003). This protective mechanism allows pathogens to resist stressful environments

including conventional antimicrobial treatments and, allowing them to persist within the food chain thus contributing to the incidence of foodborne illness (Yaron and Römling, 2014, Costerton et al., 1999).

A wide variety of foodborne pathogens have demonstrated the ability to form biofilms on fresh produce, including *E. coli, L. monocytogenes* and *Salmonella.* (Niemira and Cooke, 2010, Aruscavage et al., 2006). Biofilm formation may also lead to the internalisation by plants of key food pathogens providing them with further protection from many conventional antimicrobial treatments (Lapidot et al., 2006). It has been estimated that between 10 to 40 % of bacteria that are found on some types of fresh produce, such as broad-leaf endive and parsley, may be in a form of biofilm (Morris et al., 1998).

Because of the nature of fresh produce and MPP, thermal treatments are inappropriate. The complex nature of the surface presented by fresh produce to which biofilms may attach presents a real challenge to the food industry. Investigations show that listerial biofilms are able to resist common treatments such as free chlorine solutions and sodium hypochlorite (Norwood and Gilmour, 2000, Morris et al., 1998).

1.8 Key Compounds used in study/ Potential Inhibitors

1.8.1 Fumaric acid ($HO_2CCH=CHCO_2H$) and sodium fumarate ($Na_2C_4H_2O_4$)

Fumaric acid (shown in Fig 1.6, A) is considered one of the strongest weak organic acids with pK_a of 3.03 and 4.54 and as such it might be expected not to be as effective a bactericide as some other organic acid but has demonstrated a higher degree of bactericidal ability than other organic acids including acetic and lactic acids (Podolak et al., 1996).

Fumaric acid is produced by fermentation or the isomerisation of its cis isomer maleic acid (Saltmarsh et al., 2013). It's utilised within the food industry both in the EU and the US sometimes for its own merits and sometimes as a substitute for other acids such as acetic and citric both as an acidulant, flavour enhancer and for its low hygroscopic properties (Saltmarsh et al., 2013, Lee, 2014). Fumaric acid has been used in a number of food products including baked goods, confectionery, juices and dried powdered products (Lee, 2014). It is also used as supplement in some animal feeds and within the polymer industry (Lee, 2014).

One major issue with Fumaric acid which prevents it being more widely utilised within the food industry is its low level of solubility which significantly limits its potential applications (Lee, 2014). However fumaric acids salts such as sodium fumarate in water demonstrates a considerably higher level of solubility (Roa Engel et al., 2013) and while sodium fumarate is not an acceptable food additive within the EU it is applied by the food industry in other regions in a similar way to fumaric acid (Ishiwata et al., 2002, Smith and Hong-Shum, 2011).

Finding new inhibitory agents, is key in attempting to control levels of foodborne pathogens on both produce and food preparation surfaces. One such compound is fumaric acid, a dicarboxylic organic acid, used within the food industry as an acidity regulator (Hemat, 2003). It has also been noted for its antimicrobial activity in low pH conditions and is effective against a number of different organisms including *E.coli* O157:H7, *L. monocytogenes,* and *Salmonella.* This has

been demonstrated on a number of food products including salads, apple ciders and meat products (Kondo et al., 2006, Miller and Kaspar, 1994, Podolak et al., 1996). While a number of papers have described its antimicrobial effects under a wide variety of conditions and on a range of different food products, its mechanism of action has yet to be elucidated.

It has been suggested that carboxylic acids might be capable of inhibiting the action of the glutamate decarboxylase enzyme this has been demonstrated in *E. coli* (Fonda, 1972). Low concentrations of some of these acids have been suggested to be capable of inhibiting GAD extracted from bacterial sources and it has previously been demonstrated that the cis-isomer of fumaric acid, maliec acid can inhibit the GAD system (Paudyal et al., 2018, Martin, 1987, Wu and Roberts, 1974, Fonda, 1972, Satyanarayan and Nair, 1985).

1.8.2 Maleic acid ($HO_2CCH=CHCO_2H$)

Maleic acid, (shown in Fig 1.6, B) the cis-isomer of fumaric acid, (Kawamura and Ikushima, 1993) has been suggested as having unique antimicrobial properties that may be useful for the food and medical industries. Recent studies have suggested that maleic acid may have equal disinfectant properties to ethylenediaminetetraacetic acid (EDTA) an anti-microbial used to remove organic materials during dental treatments. Thus, it may also be useful in removing bacteria from surfaces (Ballal et al., 2009) from other items such as fresh produce. It has previously been demonstrated that under acidic conditions maleic acid has the ability to interfere with the activity of the glutamate decarboxylase system in *L. monocytogenes* and has been indicated as influencing the GAD enzyme of *E. coli* (Paudyal et al., 2018, Fonda, 1972)**.**

1.8.3 Calcium hypochlorite Ca $(ClO)_2$

Chlorine sources, used for the disinfection of produce and wash water, can come in a number of forms including a chlorine gas, sodium hypochlorite solution or as a dry solid in the form of calcium hypochlorite. Chlorine gas is generally not used as it requires specialised equipment, training of operatives and can be dangerous to handle (Garcia-Villanova et al., 2010). While sodium hypochlorite is generally favoured because it is easy to use, it does not always provide a high level of free available chlorine which is required for disinfection. Calcium hypochlorite (shown in Fig 1.6, C) has been suggested since it provides a higher level of free chlorine and is more stable and as such was the subject of one study (Connell, 2006). Chlorine washes are generally used to sanitise fresh produce at concentrations of between 50-200 ppm (Beuchat et al., 1998). It is important to note that increasing the amount of chlorine available does not always increase its efficiency as a disinfectant and it is important to monitor and maintain the pH of a solution to ensure this (Monsalve-Gonsalez et al., 1995).

1.8.4 Sodium chloride (NaCl)

NaCl has been used for millennia for the preservation of food. Its mechanism of action is to reduce the water activity in the food product to the point where it becomes an inhospitable environment for the bacteria resulting in their dehydration preventing both spoilage and reducing the levels of pathogens (Albarracín et al., 2011). In recent years because of its effects on human health there has been a drive towards reducing levels within many food products. Some organisms have a greater ability than others to survive high concentrations of salt. *L. monocytogenes* and *E. coli* have been shown to be able to survive up to 10% (Liu et al., 2005, Hajmeer et al., 2006) and *Salmonella* a tolerance of between 6-8% (Matches and Liston, 1972).

It has also been noted that the presence of NaCl under low pH conditions may influence the growth of organisms promoting some and inhibiting others (Shabala et al., 2008).

1.8.5 Glutamic ($C_5H_9NO_4$) acid and monosodium glutamate ($C_5H_8NO_4Na$)

Glutamic acid (shown in Fig 1.6, D), normally usually found as monosodium glutamate, is an abundant non-essential amino acid (Ault, 2004). It can be synthesised from a wide number of different sources and is widely utilised as a flavour enhancer in foods (Sano, 2009). Glutamate or monosodium glutamate is key to the functionality of the glutamate decarboxylases system and is important for some pathogens, such as *E. coli* and *l. monocytogenes*, when exposed to severe acidic conditions.

1.8.6 Lysine ($C_6H_{14}N_2O_2$)

Lysine (shown in Fig 1.6, E) is an essential amino acid, key to good nutrition, produced by the hydrolysis of many common proteins (Encyclopædia Britannic, 2016). In animals it must be consumed but plants and bacteria are capable of producing lysine from aspartic acid (Mahmood, 2010, Yang and Ludewig, 2014). Lysine is key for the functionality of the lysine decarboxylase system (LDS) which confers a moderate level of acid resistance on a number of food pathogens including *Salmonella.*

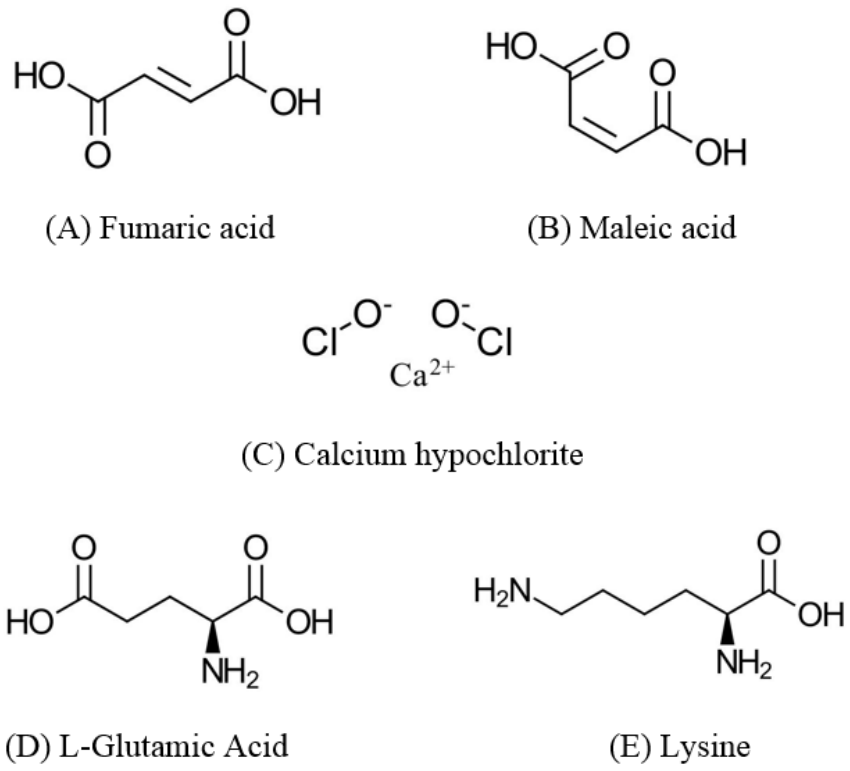

Fig 1.6 Structures of key compounds used in this study

1.8.7 Organic acid mix (AM)

The organic acid mix (AM) used in this study is a commercially available produce wash for whole fruit, vegetables, and salad leaves and is referred to hereafter as AM. This product is made up of three weak organic acids commonly used within the food industry. In appearance AM is a colourless and odourless liquid that has pH of < 1.1.

AM is suitable for use with organic produce and it meets the criteria of a number of consumer groups being classified as kosher and halal. When used it is added to water to produce a solution with a pH of 2.4 to 2.8 which, depending upon local water chemistry, is a solution consisting of around 1 to 1.5 % AM.

This product is used in some experiments as a method of altering the pH of the environment of the selected organism as an alternative to using hydrochloric acid (Sigma Aldrich). AM provides a more realistic low pH testing condition than HCl which is not used industrially as a disinfectant for food products.

1.9 Conclusion

The presence of microorganisms on food and food preparations surfaces is a major concern to the food industry. Alterations in how food is produced and the types of food demanded by consumers has led to an increased risk associated with fresh produce. Dealing with this issue has driven the need for new antimicrobial agents and techniques on produce intended for human consumption.

By examining the mechanisms by which the most common and the deadliest food pathogens survive, current anti-microbial processing techniques may be made more effective. This review covers the most common chemical anti-microbial techniques and focuses on the stress mechanisms that help three major pathogens to survive acidic environmental stresses. In particular this study focuses on amino acid decarboxylase systems which may be the most important of these mechanisms. Fumaric acid has previously demonstrated a unique ability to reduce the survival of key food pathogens under acidic conditions. It has also been shown that fumaric acid and other weak organic acids are capable of inhibiting the activity of the decarboxylase enzymes upon which the previously discussed amino acid decarboxylase systems rely. It is possible that the interaction of fumaric acid with amino acid decarboxylase may offer some form of explanation for the previously unexplained antimicrobial action of fumaric acid.

Through a greater understanding of the amino acid decarboxylase systems and their interaction with acidic environments it may be possible to influence their effectiveness by increasing the sensitivity of selected pathogens to acidic treatments. This in turn, could lead to the development of more effective decontamination regimes for fresh and minimally processed produce.

1.10 Hypotheses of the study

- Organic acids may be used to target key mechanisms of acid resistance increasing the efficacy of antimicrobial treatments
- Sodium fumarate can inhibit the function of the glutamate decarboxylase system in *L. monocytogenes* and *E. coli.*
- Sodium fumarate can inhibit the function of the lysine decarboxylase system of *Salmonella.*
- Key stress genes may influence the ability of *L. monocytogenes* to utilise key nutrients.

1.11 Aims of this study:

- To investigate which organic acids provide higher antimicrobial activity towards foodborne pathogens in planktonic and biofilm state.
- To investigate the mode of action of organic acids with higher antimicrobial activity (fumarate) and if this could be linked with effects on amino acid decarboxylase systems since they are the most potent acid resistance mechanisms.
- To assess the efficacy of fumarate as an anti-microbial treatment for minimally processed produce and static surfaces.
- Investigate for first time the self-preservation and nutritional competence (SPANC) hypothesis in a Gram positive bacterium such as *L. monocytogenes*.

1.12 Justification of study:

This study focuses on the use of weak organic acids such as fumaric acid to disrupt the function of key acid resistance mechanisms based on various organisms. The increased incidence of foodborne illness associated with fresh produce has led to an increased interest and need for effective antimicrobial treatments by gaining a greater understanding of the stress mechanisms and their regulation on key food pathogens. Previous studies have demonstrated that weak organic acids are effective antimicrobial however there full mechanistic properties have yet to be fully elucidate. Through this knowledge it might be possible to develop more effective or efficient decontamination regimes reducing incidence of foodborne illness.

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CHAPTER 2:

Investigation of the mode of action of fumarate on *Listeria monocytogenes* under acidic conditions: Possible effects on the GAD system - uncoupling the dissociation phenomena from additional antimicrobial effects.

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Abstract

Organic acids such as fumarate are commonly used as antimicrobials in foods. Here we demonstrate in *L. monocytogenes* a great discrepancy between the experimentally observed (higher) and the expected (lower) antimicrobial activity of fumarate based on its chemical properties. We observed that fumarate increases GABA levels in *L. monocytogenes* cultures under acidic conditions, suggesting an increased extracellular GAD activity and therefore increased acid resistance. This is in contrast to the observed acid sensitive phenotype, while fumarate did not affect intracellular GABA pools. Further work in cell lysates showed that fumarate is indeed a *L. monocytogenes* GAD inhibitor, but the microorganism counteracts the inhibition by upregulating *gadD2* expression which, clearly contributes to the increased GABA export.

Ultimately, the significant bactericidal effect of fumarate might be linked to effects on the intracellular GAD system which is difficult to assess since intracellular GABA pools are affected by other pathways (e.g. GABA shunt) or, possibly effects on other acid resistance systems or metabolic pathways. Furthermore, we show that maleate, a highly bactericidal fumarate isomer and GAD inhibitor which however reduces GABA export, also results in upregulation of *gadD2*. In addition, similarly to maleate, fumarate is able to eliminate *L. monocytogenes* in biofilms under acidic conditions.

2.1 Introduction

Food borne illness is a significant public health problem both in the UK and globally. The World Health Organisation (WHO) estimates that foodborne illness is responsible for 2.2 million deaths annually (Food standards agency, 2011). The majority of this illness in the UK is caused by *Campylobacter* and *Norovirus* whilst most deaths are due to *Listeria monocytogenes* and *Escherichia coli* infections. (Food standards agency, 2011)

Various strategies are employed to eliminate these pathogens in foods aiming to reduce the incidence of foodborne illness. One such strategy is the addition of organic acids which have been used for millennia to prevent the growth of pathogenic bacteria. Today many of these acids are used or being examined for their ability to control microbial contamination within the food industry (Ricke, 2003). Organic acids are believed to affect microorganisms through rapid diffusion of undissociated molecules across the cell membrane followed by intracellular dissociation and release of protons causing death or growth inhibition (Comes and Beelman, 2002; Lambert and Stratford, 1999; Podolak et al., 1996).

One of the well-known organic acids with antimicrobial activity is fumaric acid, which is a food grade, dicarboxylic acid found widely in nature and active against a number of foodborne pathogens including *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* sp. (Comes and Beelman, 2002; Kim et al., 2009; Kondo et al., 2006; Miller and Kaspar, 1994; Pérez‐Díaz and McFeeters, 2010; Podolak et al., 1996). In the EU and the US besides as an antimicrobial is also used as an acidulant, and a flavour enhancer (Lee, 2014; Saltmarsh et al., 2013). Fumaric acid is regularly used in various products including baked goods, confectionery, juices and dried powdered foods as well as in animal feeds (Lee, 2014). Fumaric acid is considered as one of the relatively strongest weak organic acids which however has low solubility in aqueous solutions (Arnold et al., 2001; Roa Engel et al., 2013) while its salts are highly soluble (Zhou

et al., 2002). Based on its low dissociation constants (pK_{a1} = 3.02 and pK_{a2} = 4.38; Lohbeck et al., 2000; Okuyama and Maskill, 2013; Szalka et al., 2013) it should be expected that fumarate has low antimicrobial activity which is not the case, if compared to other organic acids including acetic and lactic acid (Podolak et al., 1996). This additional antimicrobial activity of fumarate, beyond what could be explained by the intracellular dissociation theory of weak acids, is normally attributed to unknown factors such as interference with metabolic activities or other cellular functions. Understanding these additional effects could increase our knowledge and allow us to enhance the antimicrobial activity of these compounds and consequently achieve higher levels of hygiene or develop novel and improved antimicrobials regimes. Furthermore, it is important to understand in detail in which organisms this additional effect occurs and what the mode of action is.

The current study focuses on the foodborne pathogen *L. monocytogenes*, a Gram positive, facultative anaerobic bacterium that is ubiquitous in the environment (Posfay-Barbe and Wald, 2009) causing listeriosis, that mainly affects pregnant women, neonates and immunocompromised individuals (Posfay-Barbe and Wald, 2009). The organism is capable of surviving a wide range of environmental conditions and can grow under refrigeration temperatures affecting ready-to-eat products (Liu et al., 2002; O'Driscoll et al., 1996). It is also able to survive extreme acidic environments such as the stomach or acidic foods through the use of key mechanisms of acid resistance of which the main one is the GAD system (Davis et al., 1996; Foster, 2004).

The GAD system converts glutamate to γ-amino butyric acid (GABA) with the removal of a proton resulting in an increase in the intracellular pH (Cotter et al., 2001; Karatzas et al., 2012). The architecture of the GAD system is highly variable and in *L. monocytogenes* it typically comprises two antiporters, GadT1 and GadT2 and three decarboxylases GadD1, GadD2 and GadD3. The GadD1T1 operon is typically associated with growth under mild acidic conditions,

the GadT2D2 promoting survival under extreme acidic conditions (Cotter et al., 2005) while the GadD3 is the main part of the intracellular GAD system (GAD_i) utilising solely intracellular glutamate to produce intracellular GABA which is catabolised to glutamate by the GABA shunt (Cotter and Hill, 2003; Feehily et al., 2014; Feehily and Karatzas, 2013)

Given that fumarate has previously been described as an inhibitor of the *E. coli* GAD system (Fonda, 1972) we investigate here the antimicrobial activity of fumarate on *L. monocytogenes* under acidic conditions and the possibility that this stems from effects on the GAD system and possibly other amino acid decarboxylase systems (Grobelny, 1995). Furthermore, we look at the ability of fumarate to remove biofilms of *L. monocytogenes* and investigate further the effects of the cis-isomer of fumarate, maleic acid on the GAD system which has also been previously shown to affect it in *L. monocytogenes* (Paudyal et al., 2018).

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

All strains used (Table 2.1) were stored in 2 ml cryovials with a 7% dimethyl sulfoxide (DMSO) at -80^{o}C. *L. monocytogenes* 10403S and EGD-e were cultured onto Brain Heart Infusion (BHI) agar (LABM, Lancashire UK) and *L. monocytogenes* LO28 onto Tryptic Soy Broth (Oxoid, UK) supplemented with 5% yeast extract (TSBY; Oxoid, UK) and incubated at 37^{o}C overnight. Three colonies from each plate were transferred, with an inoculation loop, into BHI and TSBY broth respectively in 10 ml bijous and incubated overnight at 37^{o}C with shaking (150 rpm). These overnight cultures were used to inoculate 20 ml cultures of the corresponding media (1% inoculum) in 250 ml conical flasks which then were subsequently incubated overnight at 37^{o}C with shaking at (150 rpm) for 18 h.

Table 2.1: list of strains used in these experiments

List of strains	Relevant properties	Source
L. monocytogenes 10403S	Serotype 1⁄2a, WT	Wiedmann et al., 1998
L. monocytogenes 10403S Δ*gadD1*	10403S with *gadD1* deleted	Wiedmann et al., 1998
L. monocytogenes 10403S Δ*gadD2*	10403S with *gadD2* deleted	Wiedmann et al., 1998
L. monocytogenes 10403S Δ*gadD3*	10403S with *gadD3* deleted	Wiedmann et al., 1998
L. monocytogenes EGD-e	Serotype 1⁄2a, WT	Feehily et al., 2013
L. monocytogenes EGD-e Δ*gadD1*	EGD-e with *gadD1* deleted	Feehily et al., 2013
L. monocytogenes EGD-e Δ*gadD2*	EGD-e with *gadD2* deleted	Feehily et al., 2013
L. monocytogenes EGD-e Δ*gadD3*	EGD-e with *gadD3* deleted	Feehily et al., 2013
L. monocytogenes LO28	Serotype 1/2c, WT	Cotter et al., 2001
L. monocytogenes LO28 Δ*gadD1*	LO28 with *gadD1* deleted	Cotter et al., 2001
L. monocytogenes LO28 Δ*gadD2*	LO28 with *gadD2* deleted	Cotter et al., 2001
L. monocytogenes LO28 Δ*gadD1/2*	LO28 with *gadD1/2* deleted	Cotter et al., 2001
E. coli K-12	Wild type	KEIO collection

2.2.2 Calculation of undissociated acids using Henderson-Hasselbalch equation

The percentage of undissociated acid present at pH 3 was determined using the Henderson-Hasselbalch equation as adapted by Wemmenhove et al., (2016) and presented at Table 2.2

$$[\text{Undissociated acid}] = [\text{Total acid}] / 1 + 10^{(\text{pH-pKa})}$$

2.2.3 Determination of minimum inhibitory concentration

A range of concentrations (0, 1, 2, 4, 8, 16 and 32 mg/ml) of selected organic acids (Table 2.3) were used in BHI inoculated at 1% with overnight culture of *L. monocytogenes* 10403S WT. Cultures were then placed into 96 well plates and had their OD_{620nm} analysed over a 24 h period using a Sunrise plate reader machine (Tecan, Mannedorf, Switzerland) operated by Magellan software (Tecan, Mannedorf, Switzerland) with 20 min intervals between measurements. The plates were kept at 37^{o}C with shaking to assess the MIC.

2.2.4 Survival under acidic conditions

Survival experiments were undertaken with *L. monocytogenes* 10403S WT. Twenty ml cultures were prepared in BHI, using stock cultures prepared as described previously, and grown in 250 ml Erlenmeyer flasks at 37^{o}C with agitation at 150 rpm. Acid challenge took place with the addition of 8.6 mM fumaric acid, and a variety of organic acids (Table 2.3). Control experiments were performed in the absence of any chemicals in overnight cultures. The pH of the cultures was then adjusted to pH 3.0 using 1 M HCL.

One hundred µl samples were obtained prior to the acid challenge and every 20 min for 1 h and placed in 900 µl MRD (Oxoid Limited, Hampshire UK). Ten-fold serial dilutions were prepared and 10 µl of each dilution were placed onto BHI agar plates using the spot plate method and

incubated at 37ºC overnight. Subsequently, colonies were counted to assess the cell concentration in the culture at each time point. All experiments were performed in triplicate.

2.2.5 Survival in the presence of sodium fumarate

Following initial survival experiments, further survival experiments were performed focusing on the effect of fumaric acid and its salt sodium fumarate on *L. monocytogenes* 10403S WT, and its isogenic mutants Δ*gadD1*, Δ*gadD2*, Δ*gadD3*, on EGD-e WT and its isogenic mutants Δ*gadD1*, Δ*gadD2*, Δ*gadD3* and on LO28 WT with its isogenic mutants Δ*gadD1*, Δ*gadD2*, Δ*gadD1/2*.

Cultures were prepared in BHI or TSBY for LO28, using stock cultures, prepared as described previously and grown in 250 ml Erlenmeyer flasks at 37ºC with agitation at 150 rpm. Due to the significantly differences in strain sensitivity, different concentrations of sodium fumarate were used for 10403S (8.6 mM) and for EGD-e and LO28 (4.3 mM). Control cultures were also prepared containing no additional antimicrobials. Subsequently, all *L. monocytogenes* 10403S cultures had their pH adjusted to 3.0 and *L. monocytogenes* EGD-e and LO28 to pH 3.3.

One hundred μl samples were taken immediately prior to the acid challenge and every 20 or 5 min thereafter for 10403S or EGD-e and LO28 respectively. Samples were subsequently added in 900 μl Maximum Recovery Diluent (MRD; Oxoid Limited, Hampshire UK) to prepare decimal serial dilutions and 10 μl of each dilution was plated onto BHI agar or TSBY agar respectively and incubated at 37ºC overnight. Following incubation, colonies were counted to assess the cell concentration at every time point.

2.2.6 GABAse assay

GABase assay was used to determine the concentrations of intracellular ($GABA_i$) and extracellular ($GABA_e$) GABA in 10403S and LO28 WT as described by O'Byrne et al., (2011). *E. coli* K-12 samples were assayed following the same methodology with the modification of the initial culture being grown in Lysogeny broth (LB LAB M, Lancashire, UK) supplemented with 10 mM monosodium glutamate (MSG; Steinheim, Germany), $GABA_e$ was quantified according to Tsukatani et al. (2005) as modified by Karatzas (2010).

2.2.7 GAD activity in protein lysates

WT 10403S cultures were prepared in 20 ml BHI in 250 ml Erlenmeyer as described previously by Paudyal et al (2018), while for LO28 and EGD-e 40 ml cultures were used. All cultures were then transferred to 50 ml falcon tubes (VWR, Leighton Buzzard UK) with 10 µg/ml chloramphenicol (Sigma-Aldrich, Steinheim, Germany). The samples were then centrifuged at 12,000 xg for 15 min and washed with a buffer solution, as described previously (Abrams et al., 2008; Boura et al., 2016). Suspensions were then incubated at 37°C in an orbital shaker at 150 rpm (Gallenkamp, Germany).

A 2 ml cryovial (Sarstedt, Germany) was filled with 0.07g acid washed glass beads (< 106 µm diameter Sigma-Aldrich, Steinheim, Germany) together with 1 ml cell suspension. The sample was then agitated using a Mini-Beadbeater (Biospec, Bartesville, USA), thrice for 1 min, followed by 1 min on ice. DNAse I (Thermo Fisher Scientific, California, USA) was then added up to 0.1% in the cell lysates and were then incubated at 37°C in an orbital shaker at 150 rpm for 30 min. One ml sample was then transferred to an Eppendorff tube and centrifuged at 5,000 xg for 15 min. The supernatant was then transferred to an Eppendorf tube and the pellet discarded. Subsequently, 100 µl of the supernatant was added to a pyridine hydrochloride buffer

(Fonda, 1972) supplemented with 30 mM MSG and with or without 20 mM sodium fumarate. All samples were then adjusted to a pH of 4.5. The GABase assay was then used to assess GABA levels. It had previously been established using standard concentrations of GABA, that the presence of sodium fumarate does not affect the accuracy of this assay.

2.2.8 Amino acid analysis by GC-MS

As the activity of the GABase enzyme could be affected by the presence of other molecules, GABA concentrations were also assessed in the supernatant or the bacterial lysates with the use of gas chromatography mass spectrometry (Elmore et al., 2005). The method also assessed the concentration of a wide range of amino-acids. Intra- and extra-cellular samples taken from *L. monocytogenes* 10403S were assessed in the presence and absence of sodium fumarate. Previous work by Paudyal et al. 2018 has shown that in similar conditions to those described here, GABA levels quantified by GC-MS and GABase were always within ± 5%.

2.2.9 Real-time PCR determination of GAD gene expression

The transcription of the *gad* genes in the presence and absence of sodium fumarate was assessed in *L. monocytogenes* 10403S WT (*gadD1*, *gadD2* and *gadD3*) using real time reverse transcription-PCR (RT-PCR) as previously described by Karatzas et al., (2010). The transcription of the antiporter-encoding genes (*gadT1* and *gadT2*) was not examined as it has previously been demonstrated that it is similar to the corresponding glutamate decarboxylases (*gadD1* and *gadD2*) belonging to the same corresponding operon (Karatzas et al., 2012). Overnight cultures of *L. monocytogenes* 10403S WT grown for 24 h until stationary phase in BHI were treated with 10 mM of either sodium fumarate or maleic acid for 40 min. Samples were taken and prepared as previously described by Karatzas et al (2010). Relative expression

of the data was calculated as a ratio between expression of each of the target genes and the expression of the 16S rRNA which was used as the reference gene for each cDNA sample. Calculations were carried out following the advanced relative quantification settings of the Light Cycler 480 SW 1.5.1 software programme, with PCR efficiencies of the primer pairs gadD1F-gadD1R, gadD2F-gadD2R, gadD3F-gadD3R and 16SF-16SR being 2.12, 2.09, 2.03 and 2.27 respectively (Karatzas et al., 2010).

2.2.10 Biofilm removal by sodium fumarate

Biofilm formation was assessed using *L. monocytogenes* 10403S WT, EGD-e WT and LO28 WT overnight cultures grown in their corresponding BHI or TSBY agar as described previously and then inoculated to 1% in a 2 ml of BHI broth or TSBY broth. The broth was mixed thoroughly and placed in a 24-flat-bottom-well Corning Costar cell culture plate and sealed using petrifilm. Following incubation at 37°C for 48h, the culture was removed and the wells washed thrice with sterile water. Subsequently a fourth treatment was applied using 2.5 ml of either water, 100 ppm free chlorine from calcium hypochlorite, HCl (pH 2.4), HCl (pH 2.4) with 25 mM sodium fumarate, AM (an organic acid disinfectant) at pH 2.4 and AM at pH 2.4 with 25 mM of sodium fumarate.

The biofilm was exposed to these solutions in the well for 5 min and then the supernatants were discarded and wells were rinsed with 2.5 ml deionized water. Subsequently, 500 µl MRD was placed in the well and the bottom of the well was scraped using a 200 µl pipette tip for 30 s in a pattern covering the whole well bottom. This was repeated 4 times to provide a total volume of 2 ml which was serially diluted 10-fold and then 10 µl was plated onto BHI or TSBY agar and incubated at 37°C for 24 h and then growth was assessed (Ramírez et al., 2015). The impact

of the treatments on the biofilm was assessed using the following calculation (Hamilton, 2003; Ramírez et al., 2015).

Density = (Average count/Volume plated) * Dilution * Volume of MRD scraped into *(1/well surface area)

2.2.11 Statistical analysis

In all cases all experiments were run in triplicate unless stated otherwise. Subsequently results were assessed using paired Student t-tests. A P value below 0.05 indicated a statistically significant result accompanied by an asterisk.

2.3 Results

2.3.1 Calculation of the percentage of undissociated acid

The pKa of sodium fumarate is low compared to the other acids tested (maleic acid tartaric acid and oxaloacetic acid). This suggest that fumaric acid has a lower level of undissociated acid (51.7 %) and therefore, lower antimicrobial activity. This is in contrast to the results and suggests additional antimicrobial activity beyond that explained by the dissociation phenomena (Table 2.2).

Table 2.2: Percentage of undissociated acids at a concentration of 8.6 mM and pH 3.

Compound	pKa_1	pKa_2	pKa_3	%undissociated /total acid (pKa_1)	%undissociated /total acid (pKa_2)	%undissociated/ total acid (pKa_3)
Maleic acid	1.9	6.07		7.35	99.91	
Fumaric acid	3.03	4.44		51.72	96.49	
Sodium fumerate	3.55			78.01		
Pimlic acid	4.71	5.58		98.08	99.73	
Valeric acid	4.82			98.50		
Adipic acid	4.43	5.41		96.41	99.61	
Glutaric acid	4.34	5.22		95.62	99.40	
Malic acid	3.4	5.44		71.52	99.63	
Citric acid	3.13	4.76	6.39	57.42	98.29	99.95
Tartaric acid	2.98	4.34		48.84	95.62	
Oxaloacetic acid	2.22	3.89		14.23	88.58	
Alpha ketoglutaric acid	3.08			54.59		
Valeric acid	4.82			98.50		
Levulinic acid	4.59			97.49		

2.3.2 Growth in the presence of selected acids

The MICs of a variety of organic acids on *L. monocytogenes* 10403S WT were assessed (Table 2.3). However, tartaric acid seemed to be the most bacteriostatic as it had the lowest MIC (14.9 mM). Sodium fumarate as a salt did not inhibit *L. monocytogenes* under the conditions of the current experiment.

Table 2.3: MICs of compounds tested

Potential inhibitor	MIC
Maleic acid	34.4 mM
Fumaric acid	34.4 mM
Sodium fumarate	Above solubility threshold 0.22 mg/ml
Glutaric acid	30mM
Pimelic acid	24.9 mM
Adipic acid	27.3 mM
Malic acid	14.9 mM
Citric acid	20.8 mM
Tartaric acid	26.6 mM
Oxaloacetic acid	60.5 mM
α-Ketoglutaric acid	27.3 mM
Valeric acid	13 mM
Levulinic acid	60 mM

2.3.3 Acid survival of *L. monocytogenes* 10403S, LO28 and EGD-e in the presence of different organic acids.

Of all tested organic acids, under acidic conditions (pH 3) fumaric acid, its sodium salt and maleic acid showed the most significant bactericidal effect on survival of the 10403S at 8.6 mM (Fig. 2.1 A). Similar results were obtained with both EGD-e and LO28 (Fig. 2.1 B and 2.1C) assessed at pH 3.3 as more acid-sensitive than 10403S (Karatzas et al., 2012). Based on previous work, it was expected that EGD-e might be the most sensitive strain, however it displayed a similar response with LO28 to sodium fumarate (Fig. 2.1 B and 2. 1 C; Karatzas et al., 2012).

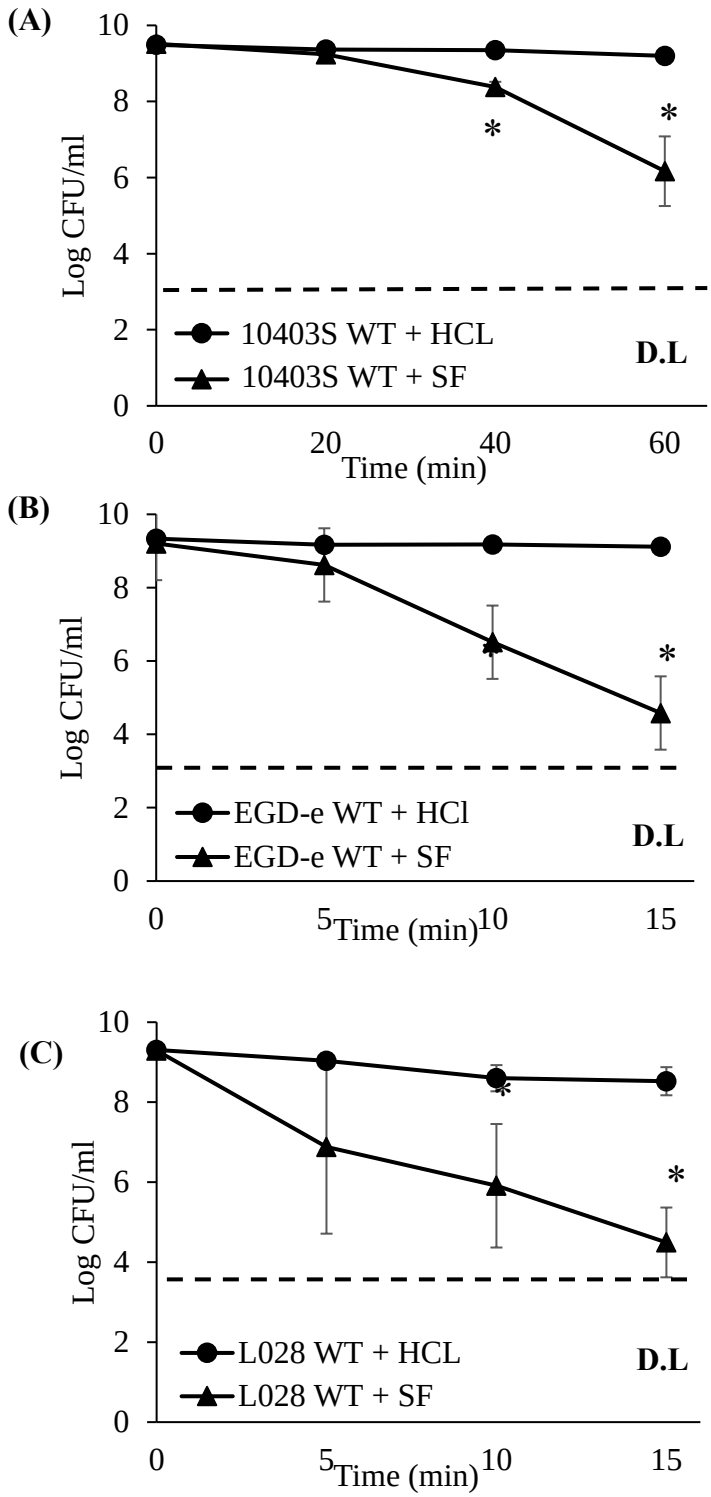


Fig. 2.1 Survival of *L. monocytogenes* (A) 10403S WT in the presence (black triangles) and absence (black circles) of 8.6 mM sodium fumarate adjusted to pH 3 using 1 M HCl (B) EGD-e WT and (C) LO28 WT in the presence (black triangles) and absence (black circles) of 4.3 mM sodium fumarate at pH 3.3 using 1 M HCl. Asterisks represent statistically significant result ($P < 0.05$ paired student T-test) while D.L denotes detection limit of the experimental setup.

2.3.4 Survival of *L. monocytogenes* 10403S LO28 and EGD-e and their isogenic mutants under acidic conditions in the presence and absence of sodium fumarate.

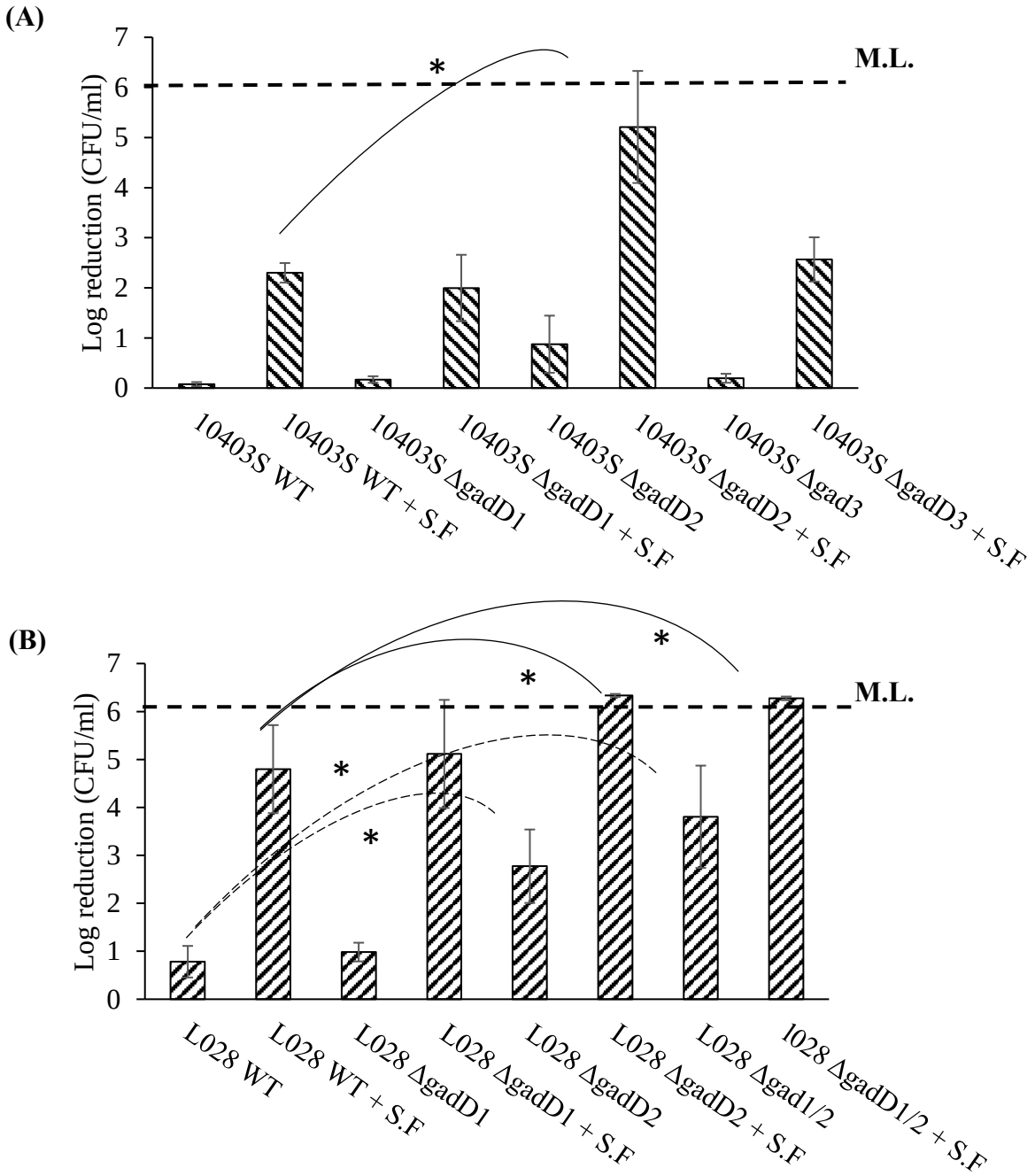
Once it was determined that sodium fumarate conferred the highest bactericidal activity on three strains of *L. monocytogenes*, the role of the GAD genes in the presence of sodium fumarate was assessed. In all cases, the presence of sodium fumarate resulted in significant increase in the log reduction in all strains and mutants (Fig. 2.2).

In 10403S the absence of sodium fumarate at pH 3 with HCL, minor log reductions in survival occurred with Δ*gadD2* being the most sensitive. In the presence of 8.6 mM sodium fumarate (pH 3) a significantly higher log reduction occurred for all strains while a similar trend occurred, with all mutants except Δ*gadD2*, behaving similarly to the WT (10403S WT, Δ*gadD1* and Δ*gadD3* showed 2.29, 1.99, and 2.56 log reduction of CFU/ml respectively; Fig. 2.2 A). Δ*gadD2* was the most sensitive strain and impacted more by the presence of sodium fumarate (5.21 log reduction of CFU/ml).

In LO28 the effect of sodium fumarate showed a similar trend to 10403S, although this strain was more sensitive and the effect was significantly more pronounced. Also, in this case the addition of 4.3 mM sodium fumarate significantly affected survival at pH 3.3 and *gadD2* was also in this case the main determinant either with HCL alone or with sodium fumarate (Fig, 2.2. B). In the presence of 4.3 mM sodium fumarate (pH 3.3), LO28 WT and Δ*gadD1*, showed a 4.80 and 5.11 log reduction CFU/ml respectively while that of Δ*gadD2* and Δ*gadD1/2* was higher than the maximum of 6 logs that could be determined with this protocol (Fig. 2.2 B).

In EGD-e the major difference compared to the other two strains was that removal of *gadD2* did not result in increased sensitivity. In the presence of 4.3 mM sodium fumarate (pH 3.3), EGD-e WT, Δ*gadD1*, Δ*gadD2* and Δ*gadD3* showed 4.62, 2.65, 2.60 and 5.07 log reduction

CFU/ml respectively (Fig. 2.2 C). In the presence of sodium fumarate, Δ*gadD3* was the most sensitive strain, while Δ*gadD1* and Δ*gadD2* appeared significantly more resistant than the WT probably due to the activation of another acid resistance mechanism. This trend had also been observed in the presence of maleic acid the GAD_i system may play a survival role in the presence of sodium fumarate (Paudyal et al., 2018).



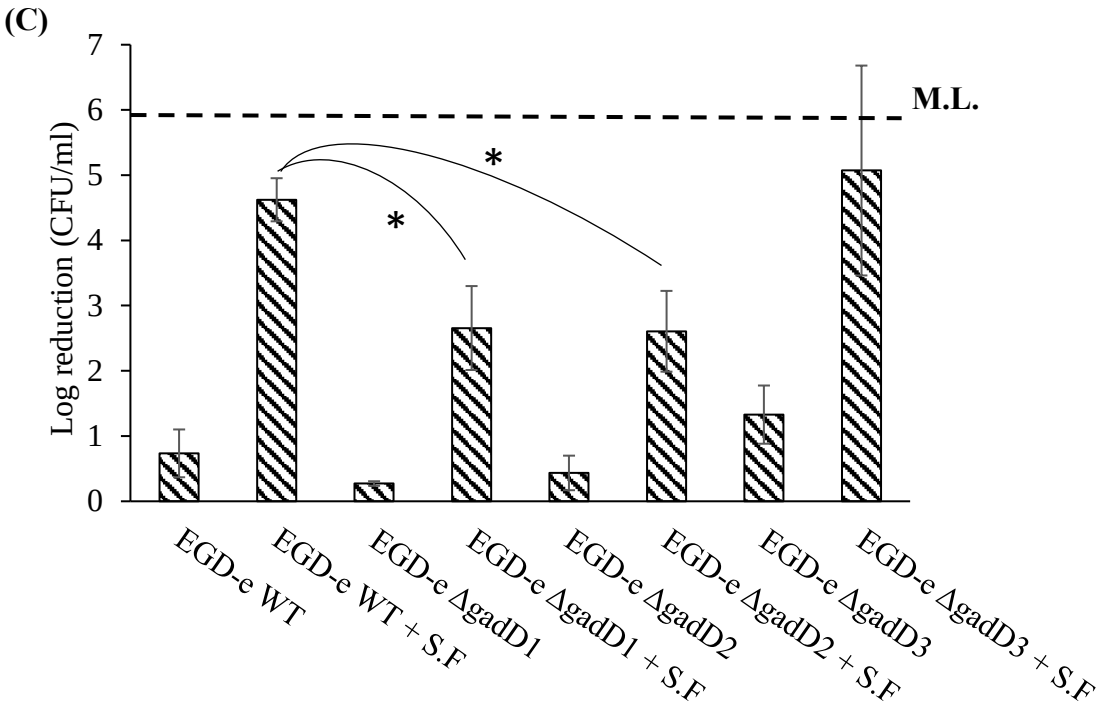


Fig. 2.2 Survival of *L. monocytogene*s and its GAD mutants in the **(A)** 10403S background in the presence and absence of 8.6 mM of sodium fumarate adjusted to pH 3 for 60 min, in the **(B)** LO28 and **(C)** EGD-e background in the presence and absence of 4.3 mM of sodium fumarate adjusted to pH 3.3 for 15 min. Adjustment of pH was done using 1 M HCl. Asterisks represent statistically significant result as assessed with paired student T-test ($P < 0.05$) and M.L. denotes the maximum log reduction could be recorded with the current protocol.

2.3.5 Extracellular GABA of *L. monocytogenes* 10403S, LO28 and *E. coli* K-12.

To assess the possible influence of sodium fumarate on the activity of the GAD system an examination of the effect of this compound on the levels of $GABA_e$ was undertaken**.** In *L. monocytogenes* 10403S WT the presence of sodium fumarate caused significant increases in $GABA_e$ levels from 2.01 mM to 4.11 mM for the WT (2.04-fold increase $P < 0.05$; paired T-test, Fig. 2.3 A). Similar increases were achieved with all isogenic mutants except Δ*gadD2* (data not shown). Similarly, LO28 WT also showed an increase in $GABA_e$ levels in the presence of sodium fumarate, from 1.24 mM to 2.89 mM (2.33-fold increase Fig. 2.3B) although this result was not statistically significant. Also, its isogenic mutants followed the same pattern (data not shown).

Finally, when this experiment was undertaken using *E. coli* K-12 WT in contrast to the above, $GABA_e$ levels showed a significant decrease from 9.2mM to 4.5 mM in the presence of sodium fumarate (2.01-fold reduction, $P < 0.05$; paired T-test; Fig. 2.3 C). $GABA_i$ was also examined however no significant difference in any of the strains tested was observed in the presence of sodium fumarate.

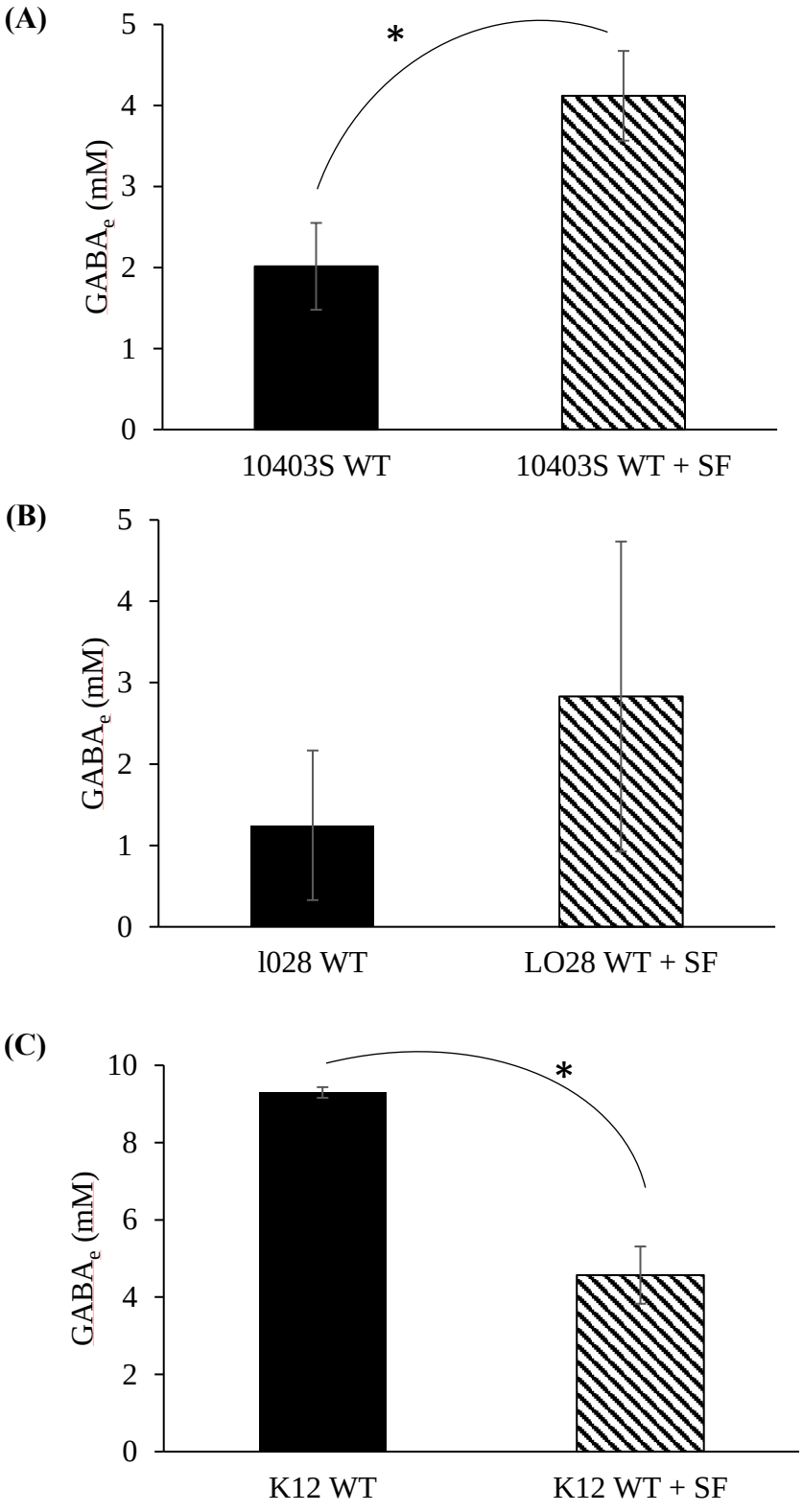


Fig. 2.3 GABA$_e$ levels of overnight cultures grown to stationary phase (~18 h at 37°C) with shaking in the presence or absence of 10 mM sodium fumarate (SF) for **(A)** *L. monocytogenes* 10403 WT at pH 4.2, **(B)** *L. monocytogenes* LO28 WT at pH 4.2 and **(C)** *E. coli* K-12 WT at pH 4. pH was adjusted with the addition of 1 M HCl. Asterisk represents statistically significant result. P <0.05 paired student T-test.

2.3.6 10403S WT GAD activity in protein lysates

The GAD activity of protein lysates was assessed by monitoring GABA production in the presence of MSG (Sigma-Aldrich, Steinheim, Germany). The results indicate that, sodium fumarate inhibited the GAD system activity in 10403S WT, resulting in reduced levels of GABA from 3.4 mM to 2.7 mM (0.79-fold reduction; P <0.05; Fig. 2.4). A similar protocol was attempted with EGD-e and LO28 however, GABA levels were below the detection limit of the GABase assay and despite protocol alterations in the pH, the buffer used, higher glutamate supplementation or increasing the volume of culture utilised no improvement occurred (Fig. 2.5).

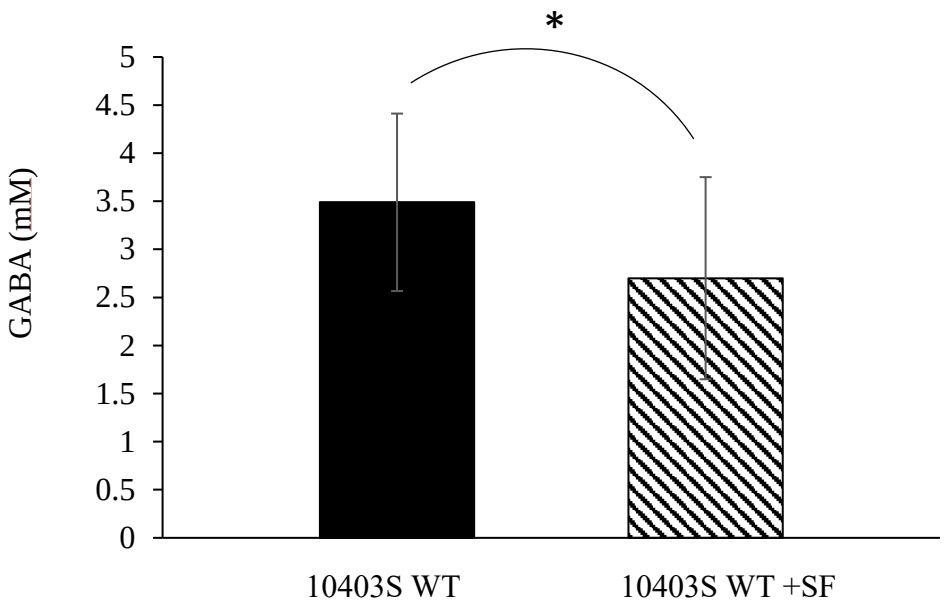


Fig. 2.4 GAD activity in cell lysates of *L. monocytogenes* 10403S WT cells grown overnight until stationary phase (~18h) at 37°C with agitation (150 rpm) in the presence or absence of 20 mM sodium fumarate at pH 4.2. Lysates were prepared and then levels of GAD activity were assessed using GC-MS. Asterisk represents statistically significant result. P <0.05 paired student T-test.

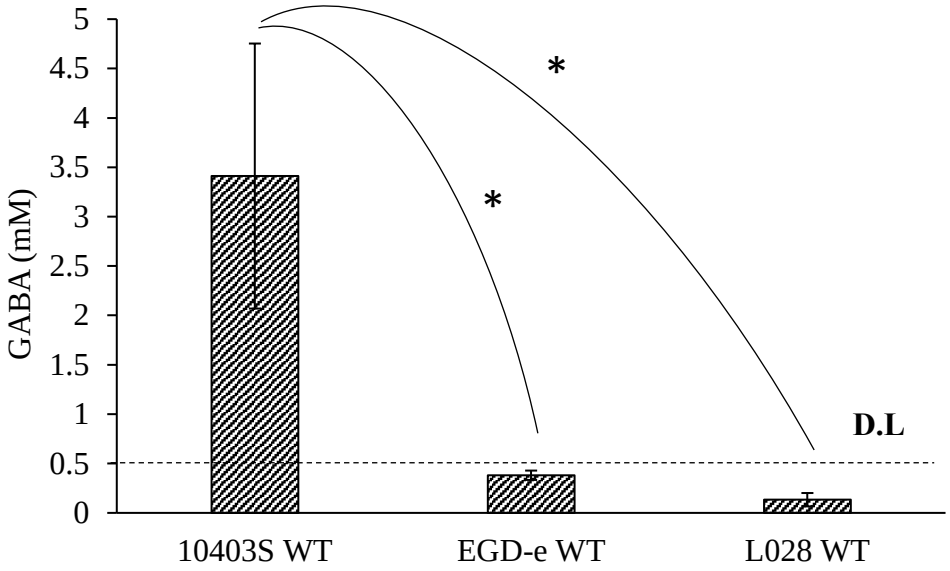


Fig. 2.5 GAD activity in cell lysates of *L. monocytogenes* 10403S WT, EGD-e WT and LO28 WT cells grown overnight until stationary phase (~18h) at 37°C with agitation (150 rpm). Lysates were produced and then levels of GAD activity were assessed using the GABase enzymatic assay. Asterisks represents statistically significant result. P <0.05 paired student T-test.

2.3.7 Real-time PCR determination of GAD gene expression.

Real time quantitative Polymerase Chain Reaction (RT-qPCR) was used to quantify the transcription of the *L. monocytogenes* 10403S WT GAD system genes in the presence of sodium fumarate and its cis-isomer maleic which has previously been shown to inhibit the listerial GAD system (Paudyal et al., 2018). Transcription of *gadD1* was very low and not affected by the presence of sodium fumarate or maleic acid (Fig. 2.6 A). In contrast, both sodium fumarate and maleic acid resulted in a significant upregulation (P <0.05) of the main component of the GAD_e system, *gadD2* by 9.44- and 48.51-fold respectively (Fig. 2.6 B). The latter gene also showed the highest expression compared to the other two decarboxylases. Regarding *gadD3,* expression was not affected by the presence of sodium fumarate although that of maleic acid showed to result in an increase of 22.33-fold which however, was not statistically significant (Fig. 2.6 C).

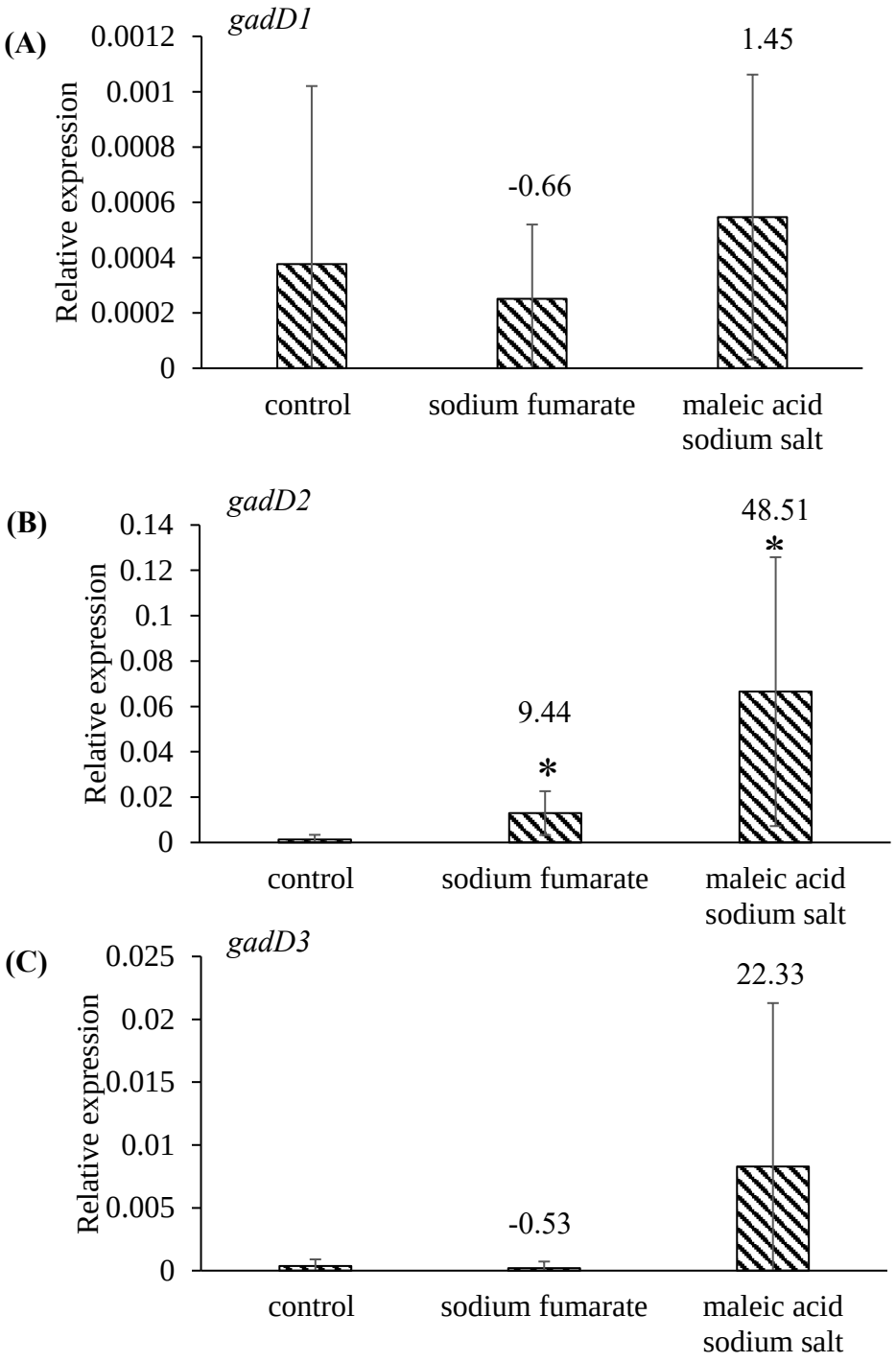


Fig. 2.6 Expression of **(A)** *gadD1* **(B)** *gadD2* and **(C)** *gadD3* gene in *L. monocytogenes* 10403S WT in the absence or presence of 10 mM sodium fumarate or 10 mM maleic acid. Relative expression of each gene was calculated by comparing expression relative to 16S rRNA gene in each strain. Numbers above the bars represent fold difference in relative expression compared to control. Markers represent an average of triplicate measurements and error bars represent standard deviations.Asterisks * denote statistical significant difference compared to the control (P <0.05 paired student T-test).

2.3.8 Biofilm formation

The survival of *L. monocytogenes* biofilms was assessed after the application of various antimicrobial treatments including 100 ppm chlorine and an acidic disinfectant (AM).

When *L. monocytogenes* 10403S was assessed, all treatments did not affect the survival in the biofilm with the exception of those with 25 mM sodium fumarate, either alone at pH 2.4 (1M HCl; 1.49 log reduction of CFU/cm^2) or in combination with AM at the same pH (1.98 log reduction of CFU/cm^2; Fig. 2.7 A).

In *L. monocytogenes* EGD-e three treatments achieved a statistically significant reduction of CFU/ml. Those were the same ones that affected 10403S and the AM disinfectant alone (2.35 log reduction of CFU/cm^2; Fig. 2.7 B). Sodium fumarate alone at pH 2.4 resulted in a significant 3.72 log reduction of CFU/cm^2 while in combination with AM resulted in a 4.7 log reduction of CFU/cm^2.

In *L. monocytogenes* LO28 all treatments resulted in a significant reduction in biofilm survival. However, this was due to a lower variability between the replicates and overall the results were similar to EGD-e with the exception of the AM treatment which seemed to be highly effective against this strain. In this case also the two treatments with sodium fumarate were the most effective along with 100 ppm chlorine (2.96 log reduction of CFU/cm^2). Sodium fumarate alone at pH 2.4 resulted in a significant 2.67 log reduction of CFU/cm^2 while in combination with AM resulted in a 3.40 log reduction of CFU/cm^2. The AM disinfectant treatment at pH 2.4 resulted in a 2.23 log reduction of CFU/cm^2 while pH 2.4 alone resulted in 1.13 log reduction of CFU/cm^2 (Fig. 2.7C).

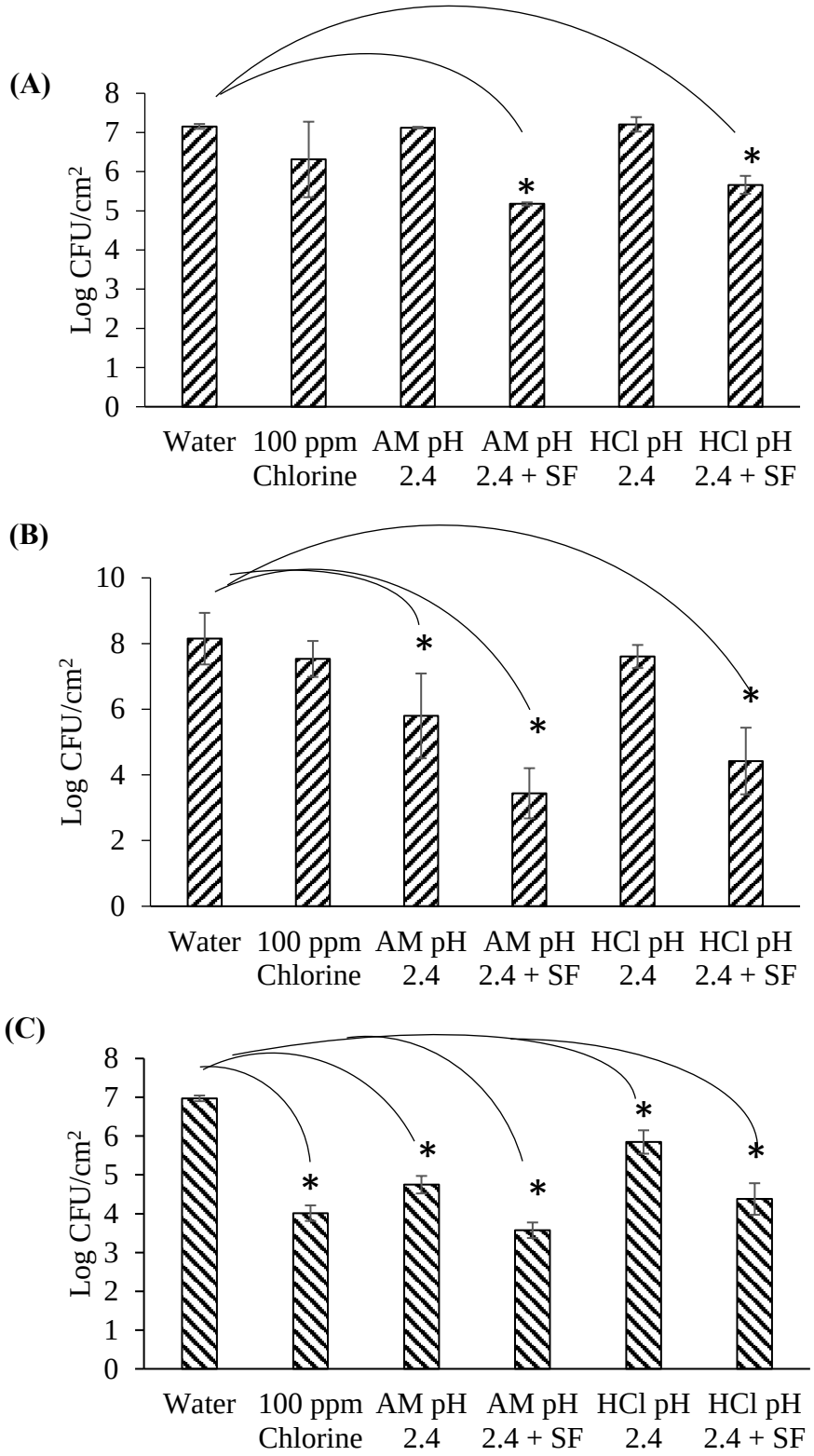


Fig. 2.7 Survival of cells in biofilms of *L. monocytogenes* **(A)** 10403S WT **(B)** *L.* EGD-e WT and **(C)** LO28 WT following no treatment (water) or treatment with an acidic disinfectant (AM), AM together with 25 mM sodium fumarate (SF), HCl and HCl together with 25 mM SF. All treatments were at pH 2.4. Asterisks represent statistically significant difference between no treatment and a treatment ($P < 0.05$; paired student T-test).

2.4 Discussion

Organic acids exert antimicrobial effects through passive diffusion of their undissociated molecules through the membrane, followed by increased dissociation intracellularly leading to a drop of intracellular pH resulting in death, or growth inhibition (Foster, 2004). However, not all antimicrobial effects of organic acids can be explained by this theory (Ricke, 2003), since some organic acids that dissociate more (higher Ka) would be expected to be less antimicrobial while this is not the case and vice versa. Therefore, these additional effects are highly important for our understanding of the mode of action of various organic acids and their more efficient use in foods.

The present work focuses on fumaric acid which is widely used in foods. It is one of the relatively strongest among the weak organic acids and based on its low pKa of 3.02 it should have low antimicrobial activity as it dissociates more (Table 2.2). To assess this, we looked at the MIC of fumarate in comparison to a variety of organic acids against *L. monocytogenes* 10403S WT, a highly acid resistant strain. As expected, the MIC of fumaric acid was among the highest (34 mM; Table 2.3) suggesting a low antimicrobial activity with only oxaloacetic acid (60.5 mM) and sodium fumarate having higher MICs. The latter suggests that the antimicrobial effects of fumarate only occur at low pH and most probably its additional antimicrobial activity beyond that stemming from intracellular dissociation occurs only at acidic conditions.

Subsequently we looked at the bactericidal activity of these compounds against three strains of *L. monocytogenes* namely 10403S, a highly acid resistant strain (challenged at pH 3), LO28 a median acid resistance strain (challenged at pH 3.3) and EGD-e, a highly acid sensitive strain (challenged at pH 3.3; Feehily and Karatzas, 2013). Interestingly, despite its low inhibitory effects and the low pKa, fumarate (both acid and its sodium salt) showed high bactericidal activity against all three strains of *L. monocytogenes* (Fig. 2.1 A, B & C). This level of

antimicrobial activity could not be predicted by the pKa and this suggests an additional mode of action beyond that stemming from the intracellular dissociation as in similar pH and concentration, fumarate would have less undissociated molecules than other acids and therefore it would be less antimicrobial. This high antimicrobial activity of fumarate has been noted previously (Chikthimmah et al., 2003; Comes and Beelman, 2002; Podolak et al., 1996; Kondo et al., 2006) and our aim is to identify why this is the case and the mode of action in terms of this additional antimicrobial activity apart from this that stems from dissociation phenomena.

The most obvious hypothesis to explain this is possible inhibitory effects of fumarate on the GAD system, the major mechanism of acid resistance in of *L. monocytogenes*, since fumarate is a known inhibitor of the *E. coli* GAD system (Fonda, 1972) and the fumarate cis-isomer maleic acid is an inhibitor of *L. monocytogenes* GAD system (Paudyal et al., 2018).

To investigate this hypothesis, we used deletion mutants in GAD genes in all three strains of *L. monocytogenes* used in this work. In 10403S the removal of *gadD1*, and *gadD3* resulted in similar levels of reduction when compared to the WT in the presence of sodium fumarate. The removal of *gadD2* caused the greatest level of reduction both in the presence and in the absence of sodium fumarate as expected since most strains rely on the GadD2 for the operation of the dominant GAD_e system (Fig. 2.2 A; Karatzas, Brennan et al. 2010). Similarly, to 10403S, in LO28 the removal of the key *gadD2* significantly reduced survival under acidic conditions (Fig. 2.2 B). EGD-e does not possess a GAD_e system associated with GadD2, and only utilises the GAD_i which is mediated by GadD3 (Feehily et al., 2014; Karatzas et al., 2012). Therefore, Δ*gadD3* was the most sensitive either in the presence or absence of sodium fumarate, without statistical significance though, whereas the removal of the *gadD1* and *gadD2* significantly increased the organism's ability to deal with the additional stress provided by sodium fumarate resulting smaller log reductions CFU/ml when compared to the WT (Fig. 2.2 C). It should be noted that the removal of the *gadD1* in all tested organisms did not appear to significantly

influence their ability to survive acidic conditions. This gene has been linked with growth at mild acidic conditions (Cotter et al., 2005) and is a designated glutamate decarboxylase based on genetic similarity, while further evidence is required to support this. GadD2 and GadD3, depending on the strain, where significant for survival against sodium fumarate under acid conditions, and they did not show a different function in the presence of fumarate compared to its absence under acidic conditions (Fig. 2.2 A, B & C).

The presence of 10 mM sodium fumarate resulted in a significant increase in the levels of $GABA_e$ exported by *L. monocytogenes* 10403S (2.04-fold increase) under pH 4.2 ($P < 0.05$; Fig. 2.3 A), underpinning an increased GAD_e activity which however, does not confer increased survival under acidic conditions (Fig. 2.2 A). This high GAD_e output by *L. monocytogenes* in the presence of fumarate clearly contributes to an increased acid resistance since each GABA molecule exported removes one intracellular proton, suggesting that fumarate increases acid resistance in *L. monocytogenes* (Karatzas et al., 2012) which is not the case (Fig. 2.3 A, B & C). A similar trend was observed with LO28 although it was not statistically significant ($P < 0.05$; Fig. 3C). However, in contrast to *L. monocytogenes*, when *E. coli* K12 was challenged, sodium fumarate resulted in a significant decrease in levels of $GABA_e$ (-2.01-fold decrease; $P < 0.05$; Fig. 3B). The latter is expected as fumarate has been previously identified as an inhibitor of the *E. coli* glutamate decarboxylase enzyme (Fonda, 1972).

The explanation for the antilisterial effects of fumarate might lie in the effects on the GAD_i system (Feehily and Karatzas, 2013), or other possible effects on other acid resistance systems or on cell metabolism that in its turn could affect acid resistance. To assess this, we first looked at the amino-acid profile in the presence or absence of fumarate and the only difference found was the increased levels of $GABA_i$ in the presence of fumarate confirming the GABase results, suggesting that the other amino-acid decarboxylase systems are possibly not affected. We also measured $GABA_i$ levels, and we observed no significant difference in the presence or absence

of sodium fumarate. At first glance, this might suggest no effects however, $GABA_i$ levels apart from GAD_i activity, are also affected by metabolic flux through the GABA shunt pathway and therefore the above results are not conclusive.

To investigate further the effects of fumarate on the GAD system we looked at its effects on the enzymatic GAD decarboxylases activity in cell lysates. Surprisingly, we observed that sodium fumarate significantly inhibited GAD activity as measured through GABA levels in the lysates ($P < 0.05$; Fig. 2.4). This coincides with its role as GAD inhibitor in *E. coli* (Fonda, 1972) and in plants (Ohno and Okunuki, 1962). We further investigated these inhibitory effects of sodium fumarate in lysates of LO28 and EGD-e but unfortunately, we were not able to get measurable GABA levels (Fig. 2.5) and GAD activity even in the absence of sodium fumarate, despite various protocol modifications (usage of higher cell numbers, higher levels of glutamate, different buffer pH values). This might be related to lower GAD activity or a different optimal pH of the GAD enzymes in these strains.

Subsequently, we looked at possible effects of fumarate and its cis-isomer maleic acid on the transcription of GAD genes *gadD1*, *gadD2* and *gadD3*. RT-qPCR showed no effect of fumarate or maleate on *gadD1* and *gadD3* (Fig. 2.6 A and C) however, *gadD2*, the key component of GAD_e system in *L. monocytogenes* 10403S WT (Cotter et al., 2001; Cotter et al., 2005) was upregulated by sodium fumarate and even more by sodium maleate ($P<0.05$, paired t-test, Fig. 2.6 B). This can clearly increase acid resistance and maybe a way for the organism to counteract the inhibitory effects of both above-mentioned compounds. All the above might suggest that the key might be linked to effects on the GAD_i system or another system.

Furthermore, the ability of sodium fumarate to act on cells in a biofilm was examined. It has previously been shown that maleic acid can act on biofilms of *L. monocytogenes* and *E. faecalis* (Ferrer-Luque et al., 2010; Paudyal et al., 2018). Due to these properties it has been suggested that maleic acid could be an effective alternative to the more toxic EDTA which is commonly

used to remove biofilms from the oral cavity and dental equipment (Ballal et al., 2009; Ferrer-Luque et al., 2010). However, fumarate has no toxicity and therefore further work could investigate other potential applications. Our results showed the striking ability of sodium fumarate (25 mM) to eliminate cells of three different strains of *L. monocytogenes* in a biofilm, which was significantly higher than that of hypochlorite and a commonly used organic acid disinfectant AM at pH 2.4 (Fig. 2.7). Furthermore, the addition of fumarate together with the AM disinfectant increased significantly the ability of the disinfectant to eliminate cells in biofilm. Our results also show that the more acid resistant strain 10403S survived the treatments better than the other two acid sensitive strains (EGD-e and LO28), underpinning the important role of acid resistance and GAD system in survival in a biofilm. Furthermore, we also show that LO28 was highly sensitive to chlorine. This coincides with previous reports suggesting a high variation in resistance to chlorine-based sanitisers among different strains (Brackett, 1987; Jacquet and Reynaud, 1994) and that mixed culture strains of *L. monocytogenes* are better able to resist chlorine treatments (Vaid et al., 2010). Our results suggest that fumarate has a great potential for removal of biofilms of *L. monocytogenes* while it is also nontoxic.

2.5 Conclusions

Up to now a number of studies have demonstrated the antimicrobial effect of fumaric acid under acidic conditions. Fumaric acid is an *E. coli* GAD enzyme inhibitor (Fonda, 1972) and as such, affects the ability of this organism to survive acidic stress. However, a similar effect might be taking place in other organisms such as *L. monocytogenes* and this is investigated here. We demonstrate that sodium fumarate is not so inhibitory but highly bactericidal against *L. monocytogenes* under acidic conditions. We also show in cell lysates that it is a GAD enzyme inhibitor on the one hand, while it increases *gadD2* (key GAD_e component) transcription on the other. The final result is an increase in the GAD_e output which however, cannot explain the

high bactericidal activity of this compound. We also show that similarly to fumarate, maleate which is also a *L. monocytogenes* GAD inhibitor, upregulates *gadD2* transcription, although the final result is opposite as it impacts negatively on the GAD_e output contributing to impaired acid resistance. It is possible that the bactericidal effect of fumarate might be associated with effects on the GAD_i system which is not as potent as GAD_e but it plays an important role. It is also possible that fumarate affects other acid resistance or even functional metabolic systems impairing acid resistance in this microorganism. For example, fumarate is antimicrobial against organisms such as *Salmonella* (Kondo et al., 2006) that lack GAD system suggesting these additional effects (Park et al., 1996). Furthermore, we demonstrate that fumarate has a major impact on *L. monocytogenes* biofilms which is superior to chlorine and an organic acid-based commercial disinfectant. Further work is required to elucidate the full extent of the antimicrobial activity, the mode of action of fumarate that stems beyond its intracellular dissociation and new possible applications against biofilms or planktonic cells of *L. monocytogenes* and possibly other organisms.

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CHAPTER 3:

The effect of fumarate on the glutamate decarboxylase system of *Escherichia coli* and its implications on survival

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Abstract

Organic acids are commonly used as antimicrobials while some of them are able to affect the glutamate decarboxylase enzyme which is key mechanism of acid resistance in *E. coli*. Fumaric acid and its sodium salt, sodium fumarate have been identified as having antimicrobial properties in excess of those that could be predicted from their physical properties stemming from the general theory explaining their antimicrobial action. Here we look at the effect of fumarate on the acid resistance of *E. coli* and how this could be associated with the possible inhibition of its GAD system.

We were able to identify that sodium fumarate equally affected the GABA output of both GAD decarboxylases resulting in decreased survival of *E. coli,* while it had no influence on levels of intracellular GABA. Interestingly we demonstrate for first time an effect of the antiporter GadC on the levels of intracellular GABA despite the latter being involved only on the extracellular GABA production and not the intracellular. Furthermore, we demonstrated that sodium fumarate under acidic conditions is able to partly remove biofilms of *E. coli*.

Despite the clear effects of sodium fumarate, throughout the rest of the study it was not possible to establish if the presence of sodium fumarate influenced the transcription of *GAD* system in *E. coli.*

3.1 Introduction

Food borne illness is estimated to affect 1,000,000 people in the UK every year and as such is considered a major element of the national public health. In the UK the Food Standards Agency has identified a number of key food pathogens including *Campylobacter, L. monocytogenes, E. coli* O157, *Salmonella* and *Norovirus* (Food Standards Agency, 2011). *E. coli* in recent years has been linked with a number of high profile food safety incidences making it a key concern when managing food safety (Buchholz et al., 2011).

Organic acids have been used for thousands of years as food preservatives to prevent the growth of pathogenic bacteria. Today, many of these acids are being examined for their ability to help control microbial contamination within the food industry (Ricke, 2003). Today organic acids are used in medical settings to decontaminated surfaces and are being investigated for their potential as novel antibacterial treatments (Zhitnitsky et al., 2017, Hughes and Webber, 2017). Organic acids are thought to function as antimicrobials through the rapid diffusion of undissociated molecules across the bacterial cell membrane swiftly followed by their disassociation within the cell liberating protons and causing a rapid increase in the intracellular pH which, in turn, results in restriction of growth of the organism or its death (Podolak et al., 1996, Comes and Beelman, 2002, Lambert and Stratford, 1999, Cotter and Hill, 2003).

Several organic acids have been noted to have good antimicrobial properties and are widely used for controlling foodborne pathogens. One of these is fumaric acid which is a food-grade dicarboxylic acid that participates in in a number of biological processes including the citric acid cycle (Krebs et al., 1938). Fumaric acid (E297) is used in the food industry as an acidulant, flavour enhancer and for its low hygroscopic properties (Saltmarsh et al., 2013, Lee, 2014) and is used in a wide range of products including baked goods, confectionery, juices and dried powdered products (Lee, 2014). It is also used as supplement in some animal feeds and within the polymer industry (Lee, 2014). While fumaric acid is legally allowable food additive in

numerous regions including EU where it has an acceptable daily intake of 0–6 mg/kg body weight. It is also used in other regions including America, Australasia and New Zealand although allowable concentrations and applications vary (Saltmarsh et al., 2013, Lee, 2014).

Fumaric acid has been shown to have significant bactericidal effect on a number of food pathogens including *E. coli, Listeria monocytogenes and Salmonella* (Miller and Kaspar, 1994, Pérez‐Díaz and McFeeters, 2010, Podolak et al., 1996, Kondo et al., 2006, Comes and Beelman, 2002, Kim et al., 2009). It is considered one of the strongest among weak organic acids with pK_as of 3.03 and 4.54. Based on the latter properties it is not expected to be as antimicrobial as some other organic acids however some studies suggest that fumaric acid may be an effective antimicrobial (Podolak et al., 1996, Comes and Beelman, 2002).

One problem with fumaric acid, which prevents it being more widely used within the food industry, is its low level of solubility (Lee, 2014). However salts of fumaric acid, such as sodium fumarate, are highly soluble in water (Roa Engel et al., 2013).

One organism against which fumaric acid has shown to be effective against is *E. coli,* a well-studied rod shaped, gram negative, facultative anaerobe predominantly found in the mammalian digestive tract. While a large proportion of *E. coli* strains are harmless, some of them, such as *E. coli* O157, are pathogenic and can cause severe symptoms ranging from diarrhoea and vomiting to kidney damage and death (Peterson and Rogers, 2011)

One of the most severe environmental challenges that foodborne organisms face is the acidic environment of the mammalian digestive system (pH of 1.5-3.5). Despite the adverse conditions many foodborne organisms are capable of surviving passage through the stomach (Foster, 2004) and *E. coli* is one of these. It is capable of growth under mildly acidic conditions and survival under extreme acidic conditions. A number of separate mechanisms of acid

resistance have been identified and one of these is the glutamate decarboxylase (GAD) system (Biase et al., 1999, Kanjee and Houry, 2013).

The GAD system, which is found in a number of bacteria, typically functions by converting glutamate to γ-aminobutyric acid (GABA) and carbon dioxide. This reaction results in the consumption of protons that are present intracellularly due to the acid environment. These can be removed from the intracellular environment through an antiporter, allowing the organism to maintain a more neutral intracellular environment (Karatzas et al., 2012).

The structure of the GAD system is variable between different organisms and sometimes even within the same species (Feehily and Karatzas, 2013). In *E. coli* the GAD system consists of two PLP-dependent, hexameric enzyme glutamate decarboxylases (*gadA*, and *gadB*,) that function together with one antiporter, *gadC*, which exchanges GABA for glutamate (De Biase et al., 1999)

In *E. coli* the GABA shunt pathway catabolises GABA that is produced by the GAD system. This system utilises two further enzymes in addition to those found in the GAD system (Dover and Halpern, 1972a, Dover and Halpern, 1972b, Prell et al., 2002). The first of these is GABA/α-ketoglutarate aminotransferase (GABA-AT) GabT which acts by converting the GABA amino group to glutamate and succinic semialdehyde (SSA). Subsequently, the SSA is then oxidised through the action of succinic semialdehyde dehydrogenase (SSDH) encoded by *gabD* to succinate (Fait et al., 2008). This system has also been identified in *L. monocytogenes* to contribute in acid resistance (Feehily et al., 2012).

It has been demonstrated that the GAD system in *E. coli* is regulated by a network requiring various genes including the *gadX* and *gadW*, which are located downstream of *gadA*. Both of these encode an AraC-type transcription factor which are thought to work in tandem as regulators of the GAD system of acid resistance (Tucker et al., 2002) It has been demonstrated

that previously that maleic acid fumaric acid the cis-isomer is capable of disrupting the GAD system in *L. monocytogenes* (Paudyal et al., 2018).

It is possible that the bactericidal action of fumaric acid may not be limited to its ability to disassociate as it has been previously demonstrated by Fonda (1972) that fumaric acid is capable of directly inhibiting the glutamate decarboxylase enzyme. Such an effect would render this organism sensitive to acidic environmental conditions. This current paper attempts to elucidate the antimicrobial action of fumaric acid and its cis isomer, maleic acid, and their effects on the GAD system of *E. coli* as well as the possible role of fumarate in the removal of biofilms.

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

All strains used (See Table 3.1) were stored in 2 ml cryovials with a 7% dimethyl sulfoxide (DMSO) at -80^{o}C. *E. coli* K-12 and all mutants were cultured onto Lysogeny broth (LB) agar (LABM, Lancashire UK). All KEIO collection mutants were cultured in the presence of 30 μg/ml kanamycin (Baba et al., 2006) and samples were then incubated at 37^{o}C overnight. Subsequently three colonies from each plate were transferred, with an inoculation loop, into 3 ml of Lysogeny broth (LB) broth in 10 ml Bijous and incubated overnight at 37^{o}C with shaking (150 rpm). These overnight cultures were used as inocula for 20 ml cultures of the corresponding media (1% inoculum) in 250 ml conical flasks which then were subsequently incubated overnight at 37^{o}C with shaking (150 rpm) for 15 h.

Table 3.1: Strains used in this study.

List of strains	Relevant properties	Source
E. coli K-12 WT	Wild type strain (Baba et al., 2006)	KEIO collection
E. coli K-12 Δ*gadA*	Deletion on glutamate decarboxylase enzyme A (De Biase et al., 1996)	KEIO collection: JW3485
E. coli K-12 Δ*gadB*	Deletion on glutamate decarboxylase enzyme B (De Biase et al., 1996)	KEIO collection: JW1488
E. coli K-12 Δ*gadC*	Deletion on putative GABA/glutamate antiporter (Capitani et al., 2003)	KEIO collection: JW1487
E. coli K-12 Δ*gadW*	Deletion of AraC-like regulator, activator of *gadE*, can co-activate *gadA/BC* (Ma et al., 2003, Foster, 2004)	KEIO collection: JW3483
E. coli K-12 Δ*gadX*	Deletion on AraC- like regulator, activator of the *gadE*, co-activator of *gadA/BC*, represses *gadW* (Foster, 2004, Ma et al., 2003)	KEIO collection: JW3484
E. coli K-12 Δ*gabD*	Deletion on succinate semialdehyde dehydrogenase which oxidizes succinate semialdehyde (Kurihara et al., 2010)	KEIO collection: JW2636
E. coli K-12 Δ*gabT*	Deletion on GABA aminotransferase converting GABA to succinate semialdehyde (Kurihara et al., 2010)	KEIO collection: JW2637
E. coli O157:H7	Non-verocytotoxic strain not possessing either *stx1* or *stx2* shiga toxin genes (Woodward et al., 2003).	Central Public Health Laboratory, London. National Culture Type Collection (NCTC)12900

3.2.2 Calculation of undissociated acids using Henderson-Hasselbalch equation

The percentage of undissociated acid present a pH 3.8 was determined using the Henderson-Hasselbalch equation as adapted by Wemmenhove et al (2016) and presented in Table 3.2

$$[\text{Undissociated acid}] = [\text{Total acid}] /1 + 10\ (\text{pH-pKa}) \quad (1)$$

3.2.3 Determination of minimum inhibitory concentration

A range of concentrations (0, 1, 2, 4, 8, 16 and 32 mg/ml) of selected organic acids (Table 3.3) were used in BHI inoculated at 1% with overnight culture of *E. coli* K-12 WT. Cultures were then placed into 96 well plates and had their OD_{620nm} analysed over a 24 h period using a Sunrise plate reader machine (Tecan, Mannedorf, Switzerland) operated by Magellan software (Tecan, Mannedorf, Switzerland) with 20 min intervals between measurements. The plates were kept at 37^{o}C with shaking to assess the MIC.

3.2.4 Survival under acidic conditions

Survival experiments were undertaken with only *E. coli* K12 WT. Twenty ml cultures were prepared in LB, using stock cultures as described previously and grown in 250 ml Erlenmeyer flasks at 37^{o}C and 150 rpm agitation. Acid challenge took place with the addition of 8.6 mM fumaric acid and a variety of organic acids. Control experiments were performed in the absence of any of these chemicals in overnight cultures. Cultures then acid challenged through the adjustment of their pH 3.8 with the addition 1 M HCl.

One hundred μl of the samples were obtained prior to the acid challenge and every 20 min for 1h. Samples were placed in 900 μl of maximum recovery diluent (Oxoid Limited, Hampshire UK). Ten-fold serial dilutions were prepared and 10 μl of each dilution were plated onto LB agar plates and incubated at 37^{o}C overnight. Subsequently, colonies were counted to assess the concentration of cells in the culture at each time point.

3.2.5 Survival in the presence of sodium fumarate

Following initial survival experiments, further survival experiments were performed focusing on the effect of fumaric acid and its salt sodium fumarate on *E. coli* K - 12 WT, and its isogenic mutants Δ*gadA*, Δ*gadB*, Δ*gadC*. Cultures were prepared in LB supplemented with 10 mM monosodium glutamate (MSG;Steinheim, Germany) using stock culture, prepared as previously described and grown in 250 ml Erlenmeyer flasks at 37^{o}C with agitation at 150 rpm. Ten mM of sodium fumarate was used for all strains. Control cultures were also prepared containing no potential inhibitors. Subsequently, all *E. coli* K12 cultures had their pH adjusted to 3.8.

One hundred µl samples were taken immediately prior to the acid challenge and every 20 minutes thereafter. Samples were subsequently added in to 900 µl MRD. Decimal serial dilutions were prepared and 10 µl of each dilution was plated onto LB agar and incubated at 37^{o}C overnight. Following incubation, colonies were counted to assess the concentration of cells at every time point.

3.2.6 GABAse assay

A GABase assay was used to determine the concentrations of intracellular GABA ($GABA_i$) and extracellular GABA ($GABA_e$) in *E. coli* K12 and the mutants described in Table 3.1 and cultured as described previously in Lysogeny broth (LB) (LAB M, Lancashire UK) supplemented with 10 mM monosodium glutamate (Steinheim, Germany) . Levels of $GABA_i$ were quantified as described by O'Byrne et al, (2011) while GABAe was quantified according to the method of Tsukatani et al, (2005) as modified by Karatzas et al (2010).

The GABase reaction was monitored using a Sunrise plate reader (Tecan, Männedorf, Switzerland) operated by Magellan software (Tecan, Männedorf, Switzerland). Absorbance

was measured at 340 nm every 2 min at 37°C for a 3-hour period. All reagents used for the GABase assay were obtained from Sigma-Aldrich (Steinheim, Germany).

3.2.7 Biofilm removal by sodium fumarate

Biofilm formation was assessed using *E. coli* O157:H7 in LB, prepared as previously in bacterial strains and growth conditions and then inoculated to 1% in 2 ml of LB. This was mixed thoroughly and placed in one cell of a Corning Costar cell culture plate with 24 wells with flat bottoms and sealed using petrifilm. This process was repeated for all cells. The plate was then incubated at 37°C for 48 hours. Following incubation, the culture was removed and the wells were washed three times with sterile water. Subsequently the selected treatment was applied to the biofilm 2.5ml of either water, hypochlorite 100 ppm, HCl (pH 2.4), HCl (pH 2.4) + 25 mM SF, organic acid mix (AM) at pH 2.4 or AM + 25 mM SF was placed into a well of the 24 well plate. The biofilm was exposed to each treatment for 5 minutes. The treatment was then removed and discarded. Each well was then washed again using 2.5ml of deionised water to remove any residue of the treatment.

Subsequently, 500 µl MRD was then placed in each well and the bottom of the well was scraped using a 200 µl pipette tip for 30 seconds in a pattern covering the whole of the bottom of the well. This was repeated 4 times to provide a total volume of 2 ml which was used for decimal serial dilutions with 10 µl placed onto LB agar plates which were then incubated at 37°C for 24 hours. Subsequently, the colonies that grew on the plates were counted and survival was assessed (Ramírez et al., 2015) with the use of the following calculation showing the impact of the individual treatments on the biofilm (Ramírez et al., 2015, Heersink J., 2003).

Density = (Average count/Volume plated) * Dilution * Volume of MRD scraped into *(1/well surface area)

3.2.8 Real-time PCR determination of GAD gene expression

The transcription of the GAD genes in the presence and absence of 10 mM of sodium fumarate dibasic, maleic acid sodium salt and sodium chloride was assessed in *E. coli* K12 WT (*gadA*) using real time reverse transcription-PCR (RT-PCR) following the procedure as previously described by Karatzas et al ,. (2010) using Primer described by Krin et al (2010).

Overnight cultures of *E. coli* K12 WT were grown for 15 h until stationary phase in LB supplemented with 10 mM monosodium glutamate (Steinheim, Germany). After 15 h cultures were prepared by the addition of 10 mM of either sodium fumarate, maleic acid, or sodium chloride, with an exposure time of 40 minutes. At this point samples either had their pH adjusted to 4 which was the pH used to analyse the GABA production or the culture was left unaltered (pH 8.5). Samples were then taken and prepared following the protocol previously described by Karatzas et al (2010).

Relative expression of the data was calculated as a ratio between expression of each of the target genes and the expression of the 16S rRNA, which was used as the reference gene for each cDNA sample. Calculations were carried out following the advanced relative quantification settings of the Light Cycler 480 SW 1.5.1 software program, with PCR efficiencies of the primer pairs 16Srt3- 16srt5 and Gadabrt3- Gadabrt5 (Krin et al., 2010) 1.88 and 2.15 respectively.

In this study only the *gadA* was investigated as it has been shown that *gadA* and *gadB* are highly homologous showing a 98% similar at the nucleotide level making them difficult to differentiate. Furthermore, *gadB* and *gadC* are co-transcribed as part of the same *gadBC* operon (Krin et al., 2010, Kanjee and Houry, 2013, Smith et al., 1992).

3.2.9 Statistical analysis

In all cases all experiments were run in triplicate unless otherwise stated. Subsequently the results were assessed using paired Student's T-test. A P-value of < 0.05 denotes statistically significant results which are also indicated by asterisk in the relevant figures.

3.3 Results

3.3.1 Calculation of the percentage of undissociated acid

The pKa of fumaric acid is low, compared to the other acids tested, and it is considered one of the relatively stronger weak organic acids, however its pKa is similar to some of the other acids initially examined (e.g. tartaric acid). This would suggest that fumaric acid would contain low levels of undissociated fumarate (51.7 %) and therefore be less effective as an antimicrobial. Despite that, this compound, and its salt sodium fumarate, possessed a much higher level of antimicrobial activity than might be predicted solely based upon their pKas (Table, 3.2)

Table 3.2: Percentage of undissociated acids at a concentration of 10 mM and pH 3.

Compound	**pKa_1**	**pKa_2**	**pKa_3**	**%undissociated /total acid (pKa_1)**	**%undissociated /total acid (pKa_2)**	**%undissociated/ total acid (pKa_3)**
Maleic acid	1.9	6.07		7.35	99.91	
Fumaric acid	3.03	4.44		51.72	96.49	
Sodium fumerate	3.55			78.01		
Pimlic acid	4.71	5.58		98.08	99.73	
Valeric acid	4.82			98.50		
Adipic acid	4.43	5.41		96.41	99.61	
Glutaric acid	4.34	5.22		95.62	99.40	
Malic acid	3.4	5.44		71.52	99.63	
Citric acid	3.13	4.76	6.39	57.42	98.29	99.95
Tartaric acid	2.98	4.34		48.84	95.62	
Oxaloacetic acid	2.22	3.89		14.23	88.58	
Alpha ketoglutaric acid	3.08			54.59		
Valeric acid	4.82			98.50		
Levulinic acid	4.59			97.49		

3.3.2 *Growth in the presence of selected acids*

The MICs of a variety of acids against *E. coli* K12 WT were assessed (Table 3.3). Sodium fumarate did not show any antimicrobial activity against *E. coli* (no MIC was found in the concentration range tested). However, α-ketoglutaric acid seemed to be the most bacteriostatic as it had the lowest MIC (7.5 mM).

Table 3.3: List of acids tested with assessed MIC and pKas

Potential inhibitor	MIC of potential inhibitor
Maleic acid	34.4 mM
Fumaric acid	34.4 mM
Sodium fumarate	Above solubility limit MIC not identified
Glutaric acid	15.1 mM
Pimelic acid	12.4 mM
Adipic acid	13.6 mM
Malic acid	14.9 mM
Citric acid	20.8 mM
Tartaric acid	13.32 mM
Oxaloacetic acid	30.28 mM
α-Ketoglutaric acid	7.5 mM
Valeric acid	13 mM
Levulinic acid	30 mM

3.3.3 Acid survival of *E. coli* K12 WT in the presence of different organic acids.

The effect of selected organic acids on the survival on *E. coli* K12 WT was assessed. Of all organic acids tested, maleic acid, and sodium fumarate showed a significant effect on the survival of *E. coli* K12 WT. This was under an extreme acid stress of pH 3.8 adjusted with 1 M HCl and 10 mM of the assessed compound (Fig. 3.1) with maleic acid resulting in a reduced survival with statistical significance after 60 min of treatment while sodium fumarate resulted in this after 40 min.

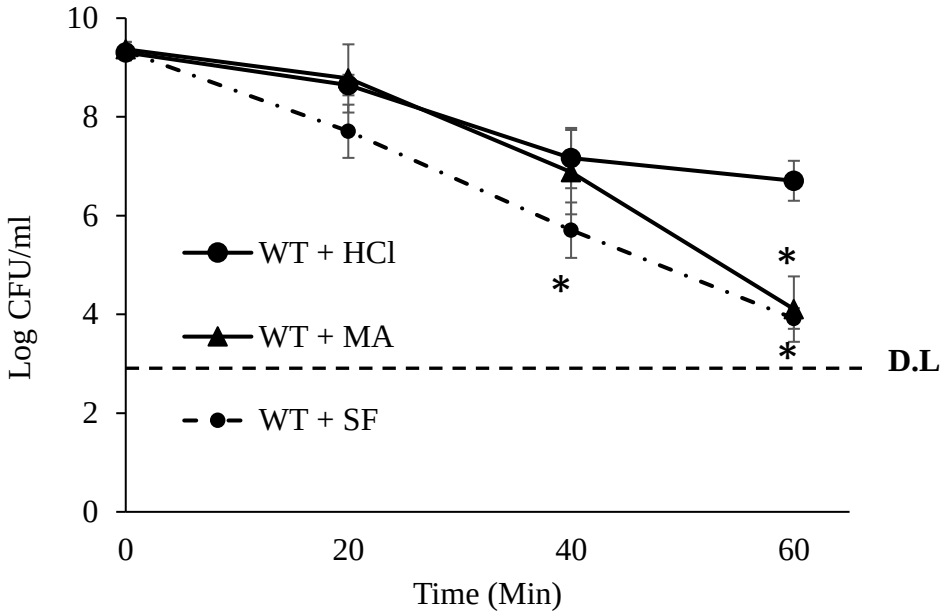


Fig. 3.1. Survival of *E. coli* K12 WT in the presence and absence of 10 mM of sodium fumarate (SF) or maleic acid (MA) adjusted to a pH 3.8 using 1 M HCl. Asterisk (*) denotes statistical significance using a paired student T-test ($P < 0.05$) while D.L denotes detection limit of experimental set up.

3.3.4 Survival of *E. coli* K12 WT and its isogenic mutants under acidic conditions in the presence and absence of sodium fumarate.

Once it had been determined that sodium fumarate had a significant bactericidal effect on *E. coli* K12 WT in low concentrations (10 mM) under acidic conditions (pH 3.8). The effect of sodium fumarate on key genes of the GAD system was assessed. The removal of *gadA*, *gadB*, *gadC* significantly reduced the survival of *E. coli* with differences of 2.1, 1 and 2.1 log reductions (CFU/ml) (Fig 3.2). The presence of 10 mM sodium fumarate significantly increased the observed Log reductions (CFU/ml) observed for *E. coli* K12 WT, Δ*gadA*, Δ*gadB*, Δ*gadC* all showed increased log reductions of 1.8, 0.7, 1.4 and 1.8 CFU/ml respectively. This indicates that sodium fumarate increases the sensitivity of *E. coli* K12 to acidic conditions in all tested mutants.

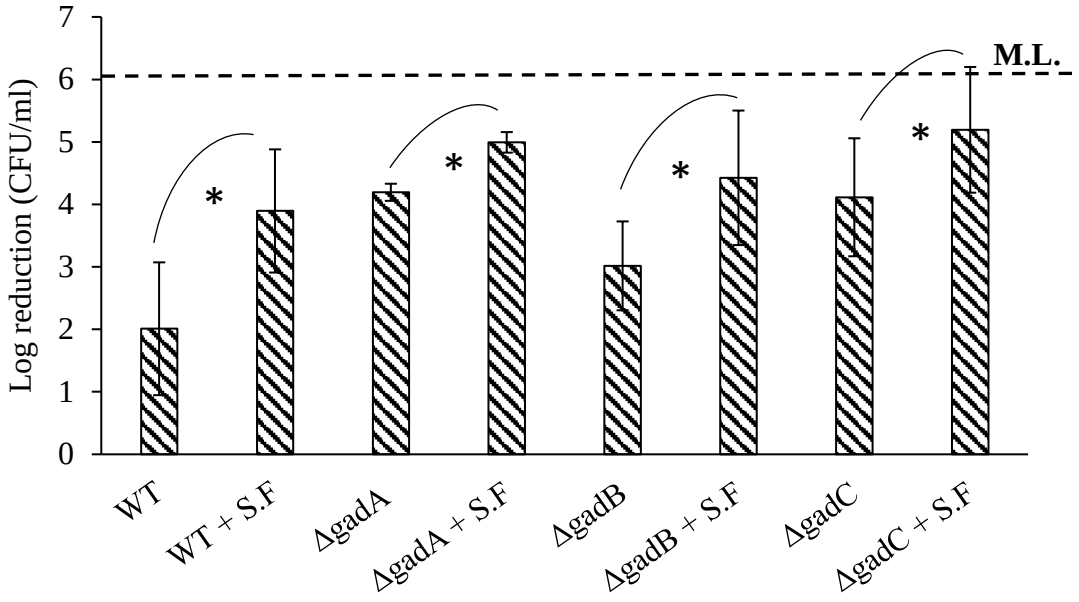


Fig. 3.2. Survival of *E. coli* K12 and its GAD mutants in the presence and absence of 10 mM of sodium fumarate (S.F.) adjusted to pH 3.8 after 40 min Adjustment of pH was done using 1 M HCl. Asterisks represent statistically significant result as assessed with paired student T-test ($P < 0.05$) and M.L. denotes the maximum log reduction could be recorded with the current protocol.

3.3.5 Examination of $GABA_i$ and $GABA_e$ in *E. coli* K12 and its isogenic mutants.

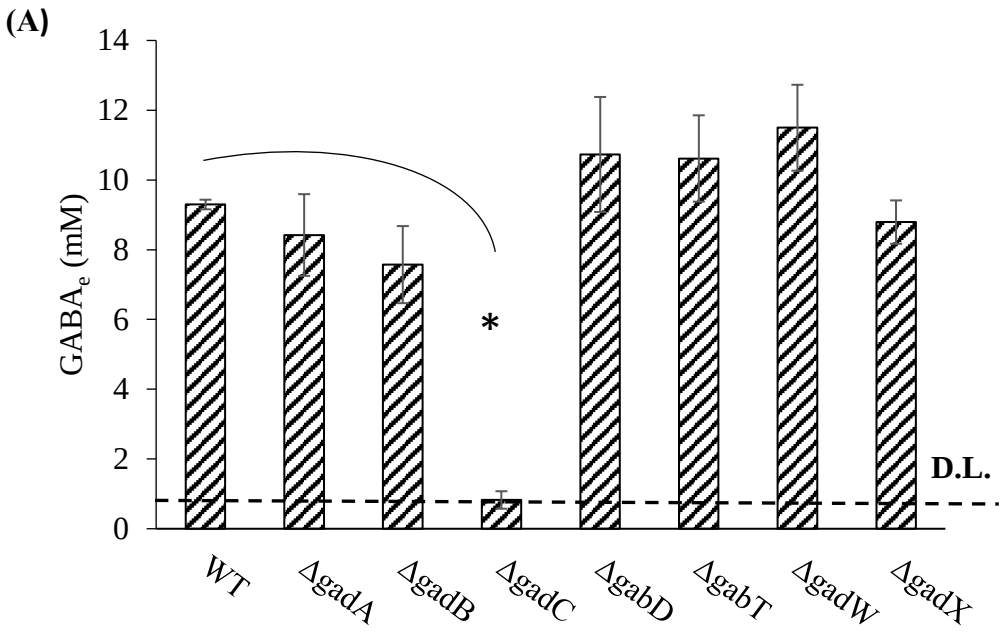
To assess the influence of the removal of key components for the GAD system of *E. coli,* an examination of the levels of both intracellular GABA ($GABA_i$) and extracellular GABA ($GABA_e$) was undertaken using isogenic mutants of *E. coli* K12 (Table 3.1).

The removal of the majority of the GAD genes had no effect on the levels of $GABA_e$ except for *gadC* gene (glutamate-GABA antiporter) whose removal caused a significant reduction in $GABA_e$ levels from 9.2 to 0.82 mM, representing a reduction of 91.09 % (Fig 3.3 A; paired student T -test $P < 0.05$).

The removal of the *gadC, gadD* and *gadW* all had an effect on levels of $GABA_i$. Levels of $GABA_i$ were significantly decreased in Δ*gadC* and Δ*gadD* (paired student T -Test $P < 0.05$).

The removal of *gadC* caused a significant reduction from 10.05 mM to 4.63 mM in $GABA_i$ levels representing a 54% reduction. The removal of *gadD* resulted in a 1.96 mM reduction in $GABA_i$ representing 19.50 % reduction (Fig. 3.3 B).

The removal of *gadW* caused a slightly significant increase in the levels of $GABA_i$ from 10.05 mM to 10.77mM a small increase of 7.10% (paired student T-test < 0.05). The *gadW,* gene is an AraC-like regulator that inhibits RpoS production, activator of *gadE*, can co-activate *gadA/BC* (Fig. 3.3 B).



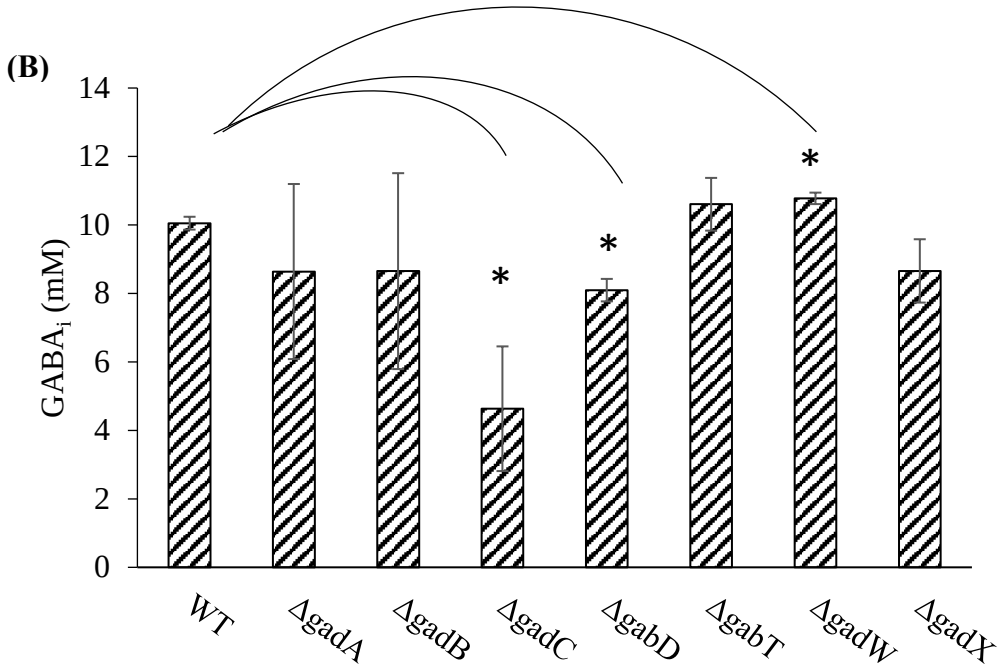


Fig. 3.3 (A) Extracellular GABA ($GABA_e$) **(B)** Intracellular GABA ($GABA_i$) production in *E. coli* K-12 WT and its isogenic mutants at pH 4. Asterisk (*) denotes statistical significance using a paired student T-test ($P< 0.05$) and D.L denotes the detection limit recorded for $GABA_e$ quantification of 0.8 mM and the D.L for $GABA_i$ is 0.1 mM and is not displayed

3.3.6 Examination of $GABA_i$ and $GABA_e$ in *E. coli* K12 and its isogenic mutants in the presence and absence fumaric acid.

To better understand the role of GAD genes in the GAD system output which is linked with acid resistance in *E. coli* and its role in possible effects by the presence of fumaric acid, the GABA output of *E. coli* K12 WT and its isogenic GAD mutants was assessed. Fumaric acid appeared to have a significant effect on the levels of $GABA_e$ produced by both the WT and all of the isogenic mutants tested with the exception of Δ*gadC* where $GABA_e$ production is abolished and no effect was observed (Fig. 3. 4 A, B & C).

While sodium fumarate had a significant impact on $GABA_e$ levels in all but the Δ*gadC,* its presence had no effect upon the levels of $GABA_i$ (Fig 3.5 A, B & C).

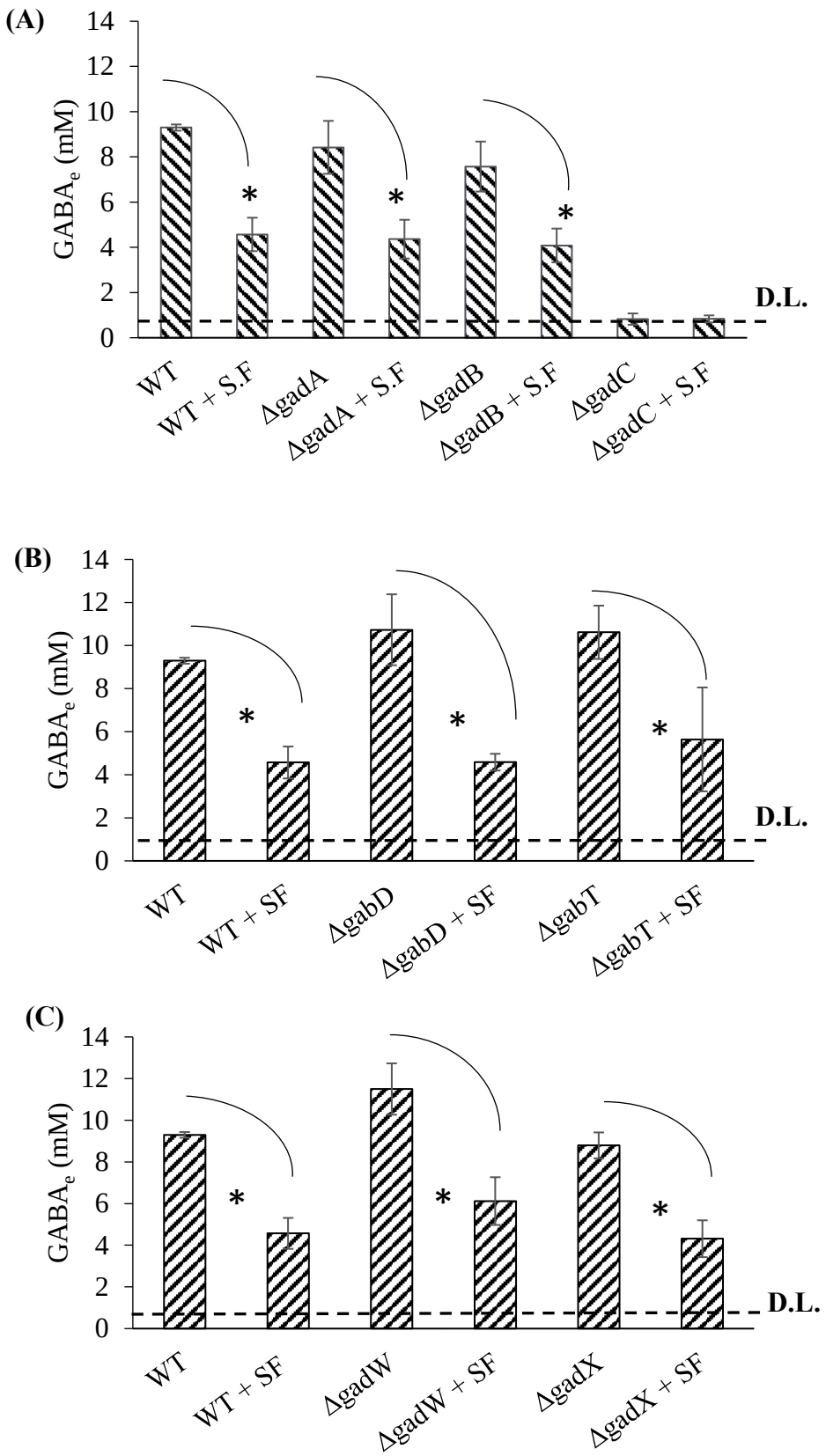


Fig 3.4 A, B & C: Extracellular ($GABA_e$) production in *E. coli* K-12 WT and its isogenic mutants with and without 10 mM sodium fumarate at pH 4. Asterisk (*) denotes statistical significance using a paired student T-test ($P< 0.05$) and D.L denotes the detection limit recorded for $GABA_e$ quantification of 0.8 mM.

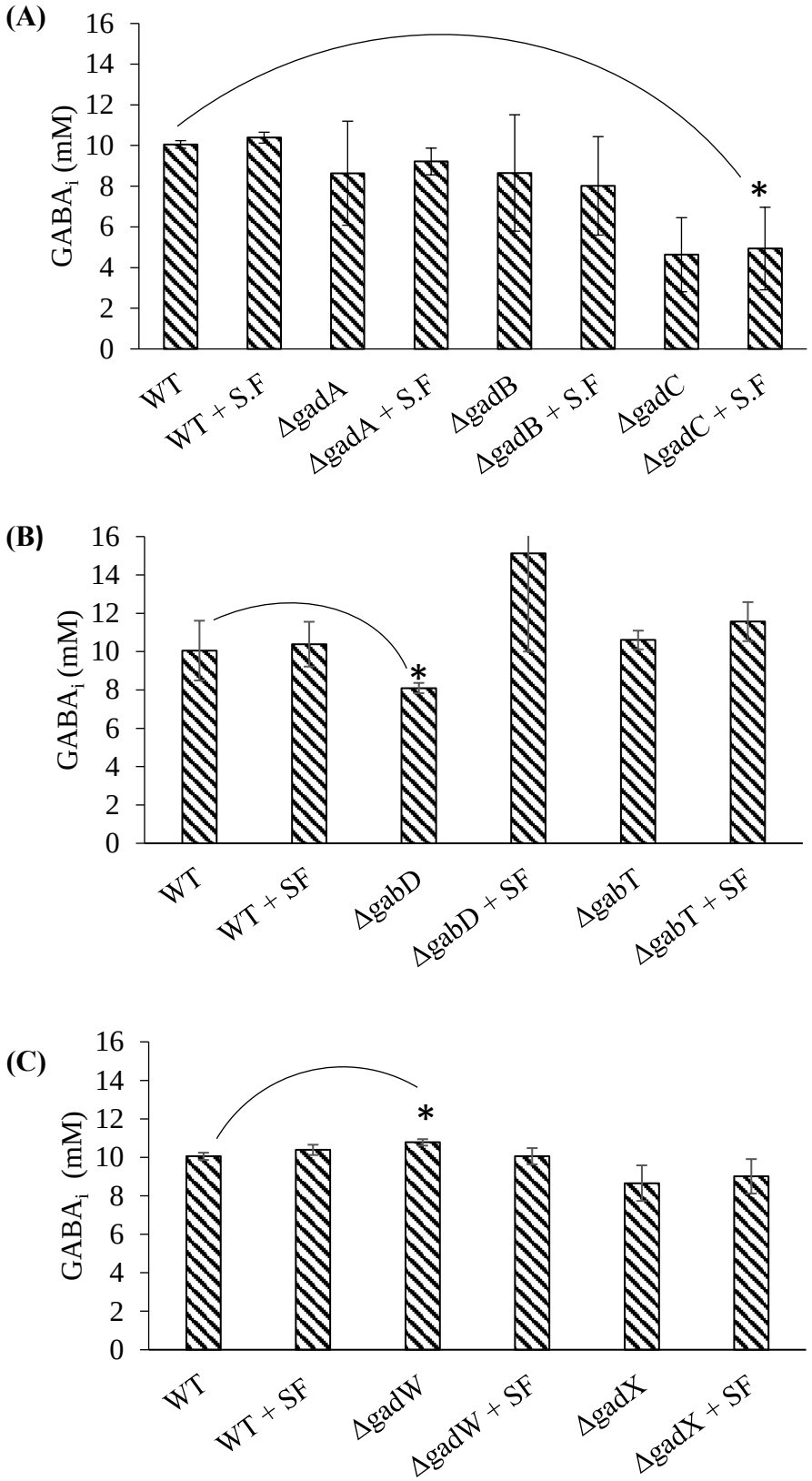


Fig 3.5 A, B & C: $GABA_i$ levels of *E. coli* K-12 WT and its isogenic mutants with and without 10 mM sodium fumarate (SF) at pH 4. Asterisk (*) denotes statistical significance using a paired student's T -test ($P< 0.05$). The detection limit recorded for $GABA_i$ quantification is 0.1 mM.

3.3.7 Examination of $GABA_i$ and $GABA_e$ in *E. coli* K-12 and its isogenic mutants in the presence and absence maleic acid.

To better understand the role of different genes associated with the GAD system in *E. coli* in acid resistance and possible effects elicited by the presence of maleic acid (M.A) an assessment of levels of $GABA_i$ and $GABA_e$ in *E.coli* K12 WT and its isogenic GAD mutants was performed.

Maleic acid appeared to cause slight but significant increases in the levels of $GABA_e$ in the *E. coli* K-12 WT strain with an increase from 8.67 mM to 10.15 mM representing an increase of 17% (student's T -test $P < 0.05$). This trend was mirrored in the isogenic mutants but was not shown to be significant **(**Fig. 3.6 A).

A similar and significant increase in levels of $GABA_i$ from 8.21 mM to 9.65 representing an increase of 14% being observed with *E. coli* K12 WT when in the presence of maleic acid. However this increase was not observed in the intracellular levels of $GABA_i$ in Δ*gadA* and *ΔgadB*. Furthermore, a small decrease of from 5.1 mM to 4.51 representing an decrease of 11.5 % (student's T -test, $P < 0.05$) was observed in Δ*gadC* in the presence of maleic acid (Fig. 3.6 B).

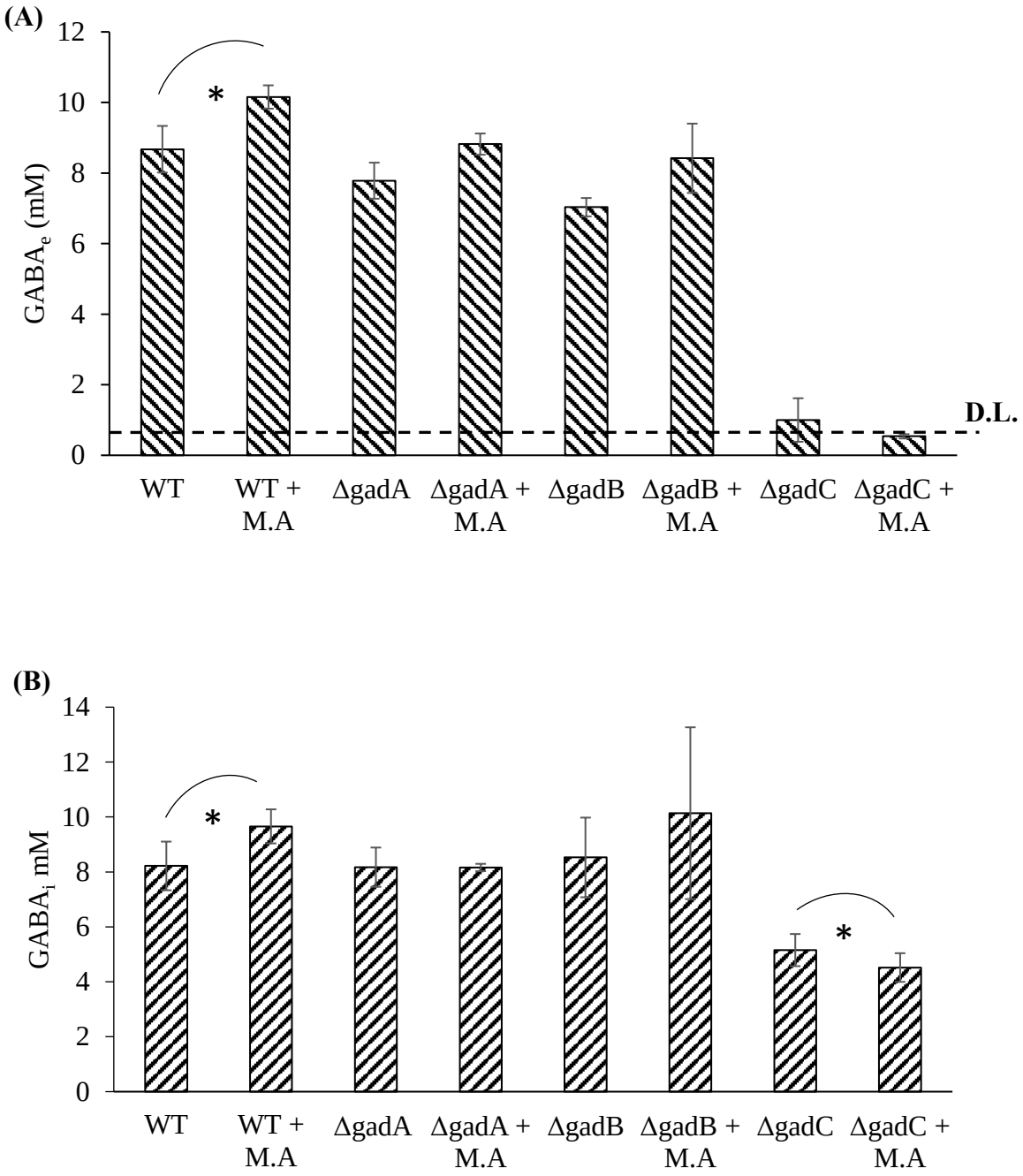


Fig 3.6 A. $GABA_e$ levels in *E. coli* K12 WT with and without 10 mM maleic acid at pH 4. Asterisk (*) indicates statistical significance using a paired student's T-test ($P< 0.05$) **B.** $GABA_i$ levels in *E. coli* K12 with and without 10 mM sodium fumarate at pH 4. Asterisks (*) show statistical significance using a paired student T-test ($P < 0.05$).

3.3.8 Treatment of biofilms

The survival of *E. coli* O157:H7 biofilms formed on 24 well plates was assessed in the presence of a variety of treatments. Following each treatment, the biofilms were scraped in to MRD. The solution was then plated and following incubation allowed enumeration of the viable counts.

In these experiments a commercially produced disinfectant (AM) and chlorine were used. Although 100 ppm chlorine did not have any significant effect, the commercially produced disinfectant (AM) showed a 3.21 log reduction of CFU/cm^2 at pH 2.4 ($P<0.05$), while upon supplementation with 25mM sodium fumarate at the same pH, a 3.44 log reduction of CFU/ml^2 ($P<0.05$; Fig. 3.7). A treatment of pH 2.4 alone (adjusted with 1M HCl) resulted in a 1.02 log reduction of CFU/cm^2 ($P<0.05$), while 25 mM sodium fumarate at the same pH a 3.56 log reduction of CFU/cm^2 ($P<0.05$; Fig. 3.7).

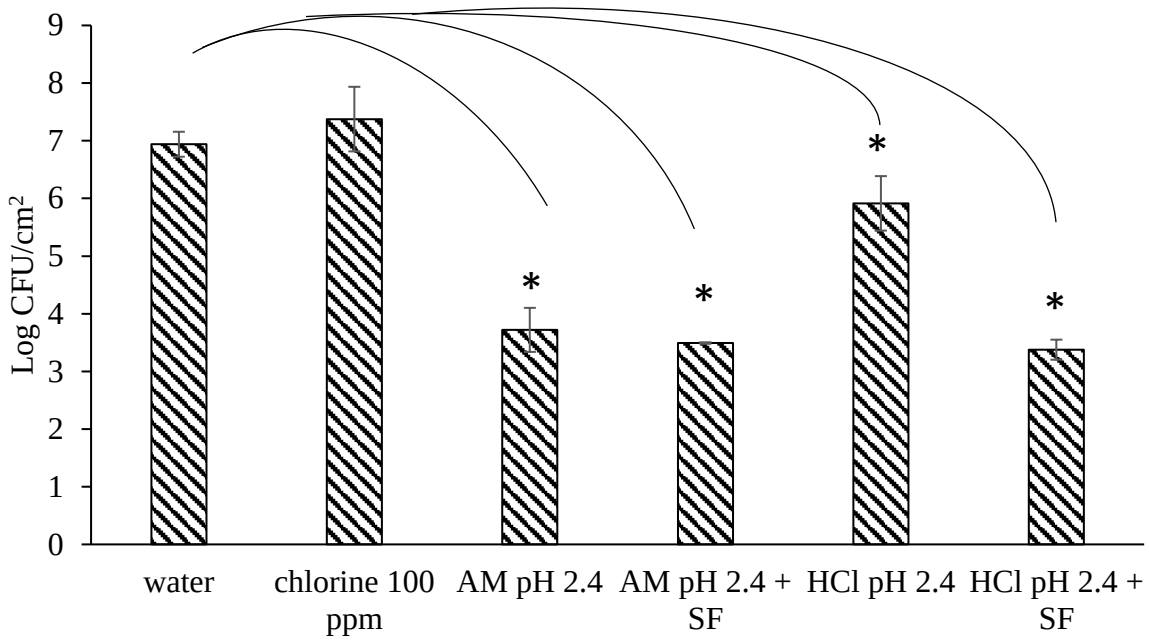


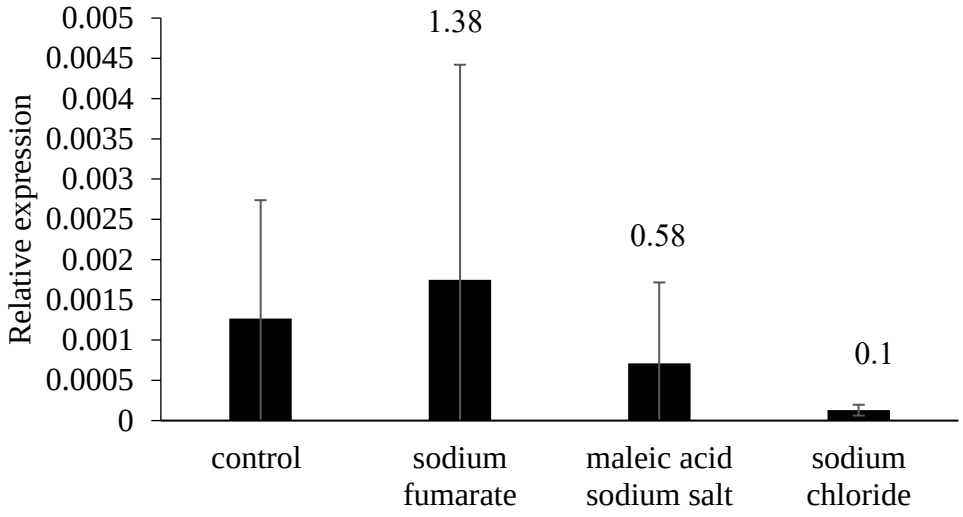
Fig 3.7. *E. coli* O157:H7 WT biofilm scraping samples a comparison of treatments and their impact on biofilms. Treatments tested water, free chlorine 100 PPM, organic acid mix pH 2.4 and organic acid mix NS pH 2.4 supplemented 25mM of Sodium fumarate (SF), HCL pH 2.4, HCL pH 2.4 + 25 mM of Sodium fumarate (SF) shows statistical significance using a paired student T -Test * < 0.05.

3.3.9 Real-time PCR determination of GAD gene expression

Real Time quantitative Polymerase Chain Reaction (RT-qPCR) was used to quantify the transcription of *gadA* gene in *E. coli* K12 WT. There were no significant alterations caused by the presence of 10 mM of sodium fumarate, maleic acid or sodium chloride when the cultures were tested at their natural pH of 8.2 (Fig. 3.8 A).

The presence of 10 mM of sodium fumarate, maleic acid sodium salt and sodium chloride also did not have any significant influence on the expression of *gadA* at pH 4.2 although small decreases were observed (Fig. 3.7 B).

(A)



(B)

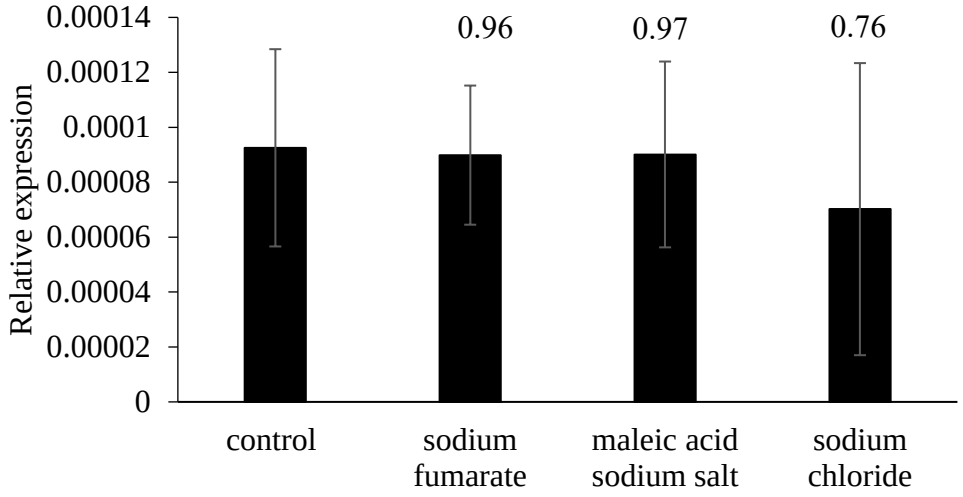


Fig. 3.8. Expression of *gadA*, gene in *E. coli* K12 WT in the absence or presence of 10 mM of sodium fumarate or maleic acid sodium salt or 10 mM sodium chloride either at pH 8.2 (A) or pH of **4.2** (B). Relative expression of each gene was calculated by comparing expression relative to that of 16S rRNA gene in each strain. Numbers above the bars represent the fold difference in relative expression. Transcription was measured and calculations were carried out from the Light Cycler 480 SW 1.5.1 software programme, with PCR efficiency as described in Materials and Methods. Markers represent an average of measurements performed in triplicate, and error bars represent standard deviations.

3.4. Discussion

This study examines the antimicrobial action of fumaric acid and its cis-isomer maleic acid (Keeler and Wothers, 2013) on *E. coli.* Maleic acid has been shown by Paudyal et al 2018 to affect the GAD system, which is the major mechanism of acid resistance in *L. monocytogenes* and in *E. coli* (Paudyal et al., 2018). While a number of studies have identified the ability of fumaric acid to provide a high level of antimicrobial activity particularly when used in combination with other antimicrobial treatments, its precise mechanism of action has yet to be fully elucidated (Kim et al., 2009, Comes and Beelman, 2002, Kondo et al., 2006, Podolak et al., 1996). This study seeks to explore the ability of maleic and fumaric acid to affect this system in *E. coli*. Previous studies such as Fonda, (1972) demonstrated that the glutamate decarboxylase of *E. coli* may be inhibited by carboxylic acids including maleic and fumaric acid as such it was possible that by applying these acids to an *E. coli* culture it might be possible to disrupt the function of the GAD system and limiting *E. coli's* ability to survive acidic conditions.

This present work focused on maleic and fumaric acids both used within the food industry. These acids which are respectively trans and cis isomers of each other, both have low pKas fumaric (3.03) and maleic (1.9) (Table 3.2). Based solely on these values these acids should have a low level of antimicrobial activity as they would more freely dissociate than other acids (Table 3.2). To access the antimicrobial activity of malic and fumaric acids against *E.coli* K - 12 WT a comparison with to a variety of other common organic acids was made acids were selected based on having the potential of effecting glutamate decarboxylase (Table 3.3) (Fonda, 1972).

Of the acids tested, maleic, fumaric both had high MICs (34 mM) when compared to the other acids suggesting a low antimicrobial activity. The low antimicrobial activity of maleic and fumaric acid were anticipated based on their pKas (Table 3.2). Under mildly acidic conditions

maleic acid and fumaric acids would be expected to remain mostly in its un-disassociated form which under the standard theory of the antimicrobial action of these acids would make it one of the least antimicrobial. An attempt was made to identify the MIC of sodium fumarate because in later survival experiments it was used as an substitute for fumaric acid because of its greater degree for solubility (220 g/l), however an MIC was not establish as its solubility limit was reached before the MIC could be identified Sodium fumarate also possesses a relatively low pKa of 3.55 (Roa Engel et al., 2013).

Based on the MICs all of the acids in Table 3.2 were assessed for their antimicrobial properties using *E. coli* K-12 under acidic conditions. This provided interesting results, maleic acid and sodium fumarate at pH 3.8 both demonstrated significant antimicrobial activity. None of the other acids assessed provided any significant reductions and as such were not investigated further. It has been shown in some previously that fumaric acid under acidic conditions could provide a higher degree of antimicrobial activity than might be predicted although no substantial explanation for this has been proposed (Kim et al., 2009, Kondo et al., 2006).

To investigate the hypothesis that this antimicrobial activity might be linked to an effect on the GAD system, an assessment was made of the effect of sodium fumarate on the survival of mutants in GAD genes in *E. coli* K12 Δ*gadA*, Δ*gadB* and Δ*gadC*. In all strains tested, the presence of sodium fumarate caused significant log reductions in the survival of the mutants at a pH of 3.8 in the presence of 10 mM of sodium fumarate (Fig 3.2). These results suggested that the presence of these selected genes was not affecting the ability of sodium fumarate as an antimicrobial. Fig 3.2 seemed to suggest that the Δ*gadA* had low levels of survival when compared to the WT however due to variation in the WT this could not be established although previous studies suggest that GAD activity strongly increases at the start of the stationary phase and this may have caused some variation in results (De Biase et al., 1999). It has been shown that although these two enzymes are functionally identical, GadA is less abundant and may

have a lower level of expression this fits with results shown here where the removal of GadA caused a smaller reduction in survival than other components (De Biase et al., 1996). It has been shown by De Biase 1999 that under acidic conditions the removal of *gadA* did not influence the survival of *E. coli*, but that the insertional inactivation of *gadB* could strongly decrease the survival of *E. coli* in acidic conditions. This effect was not observed here as shown in Fig 3.2 where no significant difference was observed with either strain this could be due to the use of insertional inactivation mutants as opposed to the deletion mutants used here or possibly strain variation as in the previous study *E. coli* ATCC11246 was used as opposed the *E. coli* K-12 used in this study (De Biase et al., 1999).

Due to these variations it was necessary to assess the levels of GABA produced under acidic conditions. In Fig 3.3 A & B we show the effects of the removal of a variety of genes including the key components of the GAD system and a number of regulatory genes associated with its function. Here we show that the removal of the genes encoding for the glutamate decarboxylase, *gadA* and *gadB* caused small but not significant alterations in levels of $GABA_e$ and $GABA_i$. However these results further demonstrated that the removal of the *gadC* had a catastrophic effect on $GABA_e$ reducing levels by 91% which confirms the established view that this antiporter is key to the export of GABA (Richard and Foster, 2004). We explored this further adding to the evidence supporting the function of this gene by examining the $GABA_i$ levels. In the absence of *gadC* we found that levels of $GABA_i$ decreased significantly (54%). This result suggests for first time that GadC antiporter might play a key role to the function of the GAD enzymes in *E. coli*. It has been demonstrated previously that GadB enzyme is found in the cytoplasm at neutral pH, but recruited to the membrane when the pH drops where it anchors the GadC antiporter and works in association with it (Capitani et al. 2003). However, it would be expected that the absence of the antiporter would not affect the function of the GAD enzyme on intracellular pools of glutamate however, this is not the case. It is possible

that the interaction with the antiporter affects the function of the enzyme or its transcription through a feedback mechanism. It should be stated that the deletion of the *gadC* could not have resulted in downstream effects to *gadA* and *gadB* since as *gadA* is in another locus from *gadC* while *gadB* is before *gadC* in the operon and the *gadC* deletion would not affect it.

None of the other genes assessed showed any significant variation in levels of $GABA_e$ from the *E. coli* K-12 (Fig 3.3 A). However two genes showed some variation in levels of $GABA_i$ (Fig 3.3 B) Firstly the *gabD* showed a significant reduction in levels of $GABA_i$ (19%) The *gabD* encodes for succinic semialdehyde dehydrogenase which is one of two enzymes used to form the GABA shunt pathway in *E. coli* which catabolises $GABA_i$ (Somasundaram et al., 2016). Although it should be expected that this would result in an increased accumulation of $GABA_i$ it has the opposite effect which might be caused by a feedback mechanism reducing the levels of $GABA_i$. The removal of *gadW* was also noted to have a small but statistically significant increase on the levels of $GABA_i$ (6.6 %). This gene is an AraC-like regulatory protein and is thought to act as a regulator for *gadA* and *gadX*. It has been shown to act as a repressor for *gadX* which in turn activates expression of *gadA*, *gadB* and *gadC* and as such its removal might increase the activity of these gene possibly resulting in this small increase (Ma et al., 2002, Ma et al., 2003).

A key point about these regulatory genes is that, despite their presence in the GAD system of *E. coli* which is the best described and most frequently studied of any bacterial GAD system genes including the *gadW* and *gadX*, assessed here are not found in other organisms, with the exception of *Shigella*. This can be demonstrated by undertaking a search of National Center for Biotechnology Information (NCBI) databases for homologues of these regulators. For example, if this search is undertaken for *GadX*, one of the main regulatory gene examined here, this results in only two matches for *E. coli* and *Shigella* (Appendix 1).

To further investigate the effects of sodium fumarate and maleic acid on the GAD system of *E. coli* K-12, an examination of is effects on the levels of GABA produced by the mutants previously described was undertaken (Fig 3.4 A, B & C). All mutants except *gadC* followed the previously observed pattern with sodium fumarate causing significant reductions in levels of $GABA_e$. It is probable that levels of $GABA_e$ were very low and as such it may not have been possible to observe a significant alteration. These results suggest that sodium fumarate affected equally both GadA and GadB driven export. As *E. coli* is able to use both GadA and GadB interchangeably it might suggest that levels of inhibition should be similar. In the presence of sodium fumarate, levels of $GABA_i$ were completely unaffected suggesting that sodium fumarate may not be affecting the intracellular mechanisms of the GAD system (Fig 3.5 A, B & C).

Interestingly, when the effect of maleic acid on the GAD system of *E.coli* was examined it achieved the opposite result to sodium fumarate causing small increases in both $GABA_e$ and $GABA_i$. It had been anticipated based on the results observed in the presence of sodium fumarate and the effect demonstrated in Paudyal et al (2018) where maleic acid caused significant reductions in levels of $GABA_e$ in *L. monocytogenes*. With *E. coli* K-12 WT, 10 mM of maleic acid resulted in statistically significant increases in $GABA_e$ levels of 17 % (Fig. 3.6 A). Similar results were noted with Δ*gadA* and Δ*gadB* but it was not found to be significant, however with Δ*gadC* a small decrease was observed (Fig 3.6 A). This difference could also possibly be related to the previously discussed differences in the regulation of the GAD systems of *E. coli* and *L. monocytogenes*.

The pattern seen in levels of $GABA_e$ with maleic acid was repeated in levels of $GABA_i$ with the *E. coli* K-12 WT showing an increase of $GABA_i$ of 17% (Fig 3.6 B). This potentially suggests that the mode of action of sodium fumarate and maleic acid on the GAD system may differ possibly interacting with different components of this organism's mechanism of acid

resistance although further investigation is required. Furthermore, a small but significant reduction in levels of $GABA_i$ of 11.7% was observed with Δ*gadC* in the presence of maleic acid. This suggests that the absence of the antiporter may increase the effects on maleic acid on the GAD system of *E. coli* (Fig 3.6 B).

An examination was made of the effect of sodium fumarate on biofilms. It has been shown that maleic acid, the cis-isomer of fumaric acid, is an inhibitor of the GAD system in *L. monocytogenes* (Paudyal et al., 2018) and that it can act on biofilms of the latter organism and those of *E. faecalis* (Paudyal et al., 2018, Ferrer-Luque et al., 2010). It has also been identified as effective against biofilm formation in the oral cavity and on dental equipment. Fumarate offers advantages when compared to other treatments that are used to combat biofilms such as EDTA as it has no toxicity and therefor may be more widely utilised than some current treatments (Ferrer-Luque et al., 2010, Ballal et al., 2009).

Our results show the marked ability of a mix of organic acids at a pH of 2.4 (AM) and fumarate at (25 mM) to eliminate cells of *E. coli* O157:H7 (Fig 3.7). This strain was used as an alternative to *E. coli* K-12 in an attempt to provide a more robust and realistic challenge for the selected treatments. The results obtained with hypochlorite (100 PPM free chlorine) demonstrated that this commonly used treatment was not effective at removing the prepared biofilms. While low pH alone in the form of HCl (pH 2.4) did prove to be an effective treatment the presence of sodium fumarate (25 mM) in addition to HCl (pH 2.4) caused a reduction of 2.53 log $CFU/cm^{2.}$ (Fig 3.7). These results suggest that acid treatments can provide an effective treatment for biofilms and that the presence of sodium fumarate may heighten the antimicrobial effect of acid-based disinfectants.

Finally, in an attempt to establish nature of the effects of both sodium fumarate and maleic acid on the GAD system an examination on the transcription of *gadA* a key component of the GAD system was made. This was done using RT-qPCR this was done at two different pH's as

previous research suggests that the expression of *gadA* may be repressed at low pH (pH 4) (Parry-Hanson et al., 2010).

No significant effect on *gadA* was observed with either maleic acid sodium salt, sodium fumarate or sodium chloride under either set of environmental conditions tested. Sodium chloride was included to see if its combination with the acids might have an effect on the regulation of the tested gene (Fig. 3.8 A & B). It had been thought that if the tested compounds were directly influencing the regulation of the GAD system then an alteration in expression may have been observable but no such effect was seen. Interestingly although no significant effect under both sets of tested conditions small reductions were observed in *gadA* in the presence of sodium chloride. It is possible that in higher concentrations than those tested here that sodium chloride might reduce the expression of *gadA.*

3.5. Conclusions

There have been a number of studies that have demonstrated the antimicrobial effects of fumaric acid under acidic conditions. It has been demonstrated that fumaric acid could inhibit *E.coli* (Fonda, 1972) GAD enzyme and therefore, affect the ability of this organism to survive acidic stress. However, a similar effect as that previously seen on the GAD enzyme might be taking place in within the organisms thus influencing the GAD systems ability to deal with acidic environments. These experiments show that sodium fumarate is highly bactericidal against *E. coli* and also inhibitory. However, we found no evidence that it increases the transcription of the *gadA* and by extension the other key components of the GAD. These two conflicting pieces of evidence cannot explain the high bactericidal activity of sodium fumarate against this microorganism. The antimicrobial effect of fumaric acid has also been noted in organisms such as *Salmonella* (Kondo et al., 2006), which does not have a GAD system, and as such may be operating in a similar fashion on other acid resistance systems, such as the

lysine decarboxylase system or possibly the arginine decarboxylase system which are found in both *E. coli* and *Salmonella* (Park et al., 1996, Richard and Foster, 2003). Further work is required to elucidate the full extent of the antimicrobial activity and mode of action of sodium fumarate which seems to go beyond the effect of intracellular dissociation after passive diffusion across the cell wall.

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CHAPTER 4:

Investigation of the mode of action of sodium fumarate on the *Salmonella* under acidic conditions and its effect on the lysine decarboxylase system.

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Key words: *Salmonella*, fumaric acid, lysine decarboxylase, inhibition, biofilm.

Abstract

Organic acids are frequently used as antimicrobials, particularly within the food industry. This paper describes the antimicrobial properties of fumaric acid which has demonstrated a higher degree of antimicrobial action against *Salmonella* than what might be expected, based solely on its physical properties.

Experiments examining the antimicrobial activity of weak organic acids such as the cis-isomer of fumaric acid, maleic acid have demonstrated that weak organic acids are capable of inhibiting amino acid decarboxylase of some food pathogens. These systems represent a key acid tolerance mechanism for many organisms including *Salmonella*. This paper demonstrates the antimicrobial action of sodium fumarate against *Salmonella* under acidic conditions.

An examination of the function of the lysine decarboxylase system of four *Salmonella* to in the presence and absence of sodium fumarate indicated that the presence of sodium fumarate significantly reduced the ability to increase its environmental pH suggesting an inhibition of the lysine decarboxylase system (LDAR). However, an examination of the expression of key components of the LDAR system, *cadA* and *cadB*, when exposed to sodium fumarate did not show a significant effect on their expression.

In addition, sodium fumarate demonstrated the ability to reduce the survival of *Salmonella* biofilms under acidic conditions.

4.1. Introduction

Bacterial food borne illness is a key factor for public health and is a consistent and preventable threat to human health. The World Health Organisation (WHO) estimates that foodborne illness is responsible for 2.2 million deaths annually and the UK Food Standards Agency estimates that 500 people in the UK die of foodborne illness every year (Food Standards Agency, 2011). In the UK, the largest number of food related illnesses are associated with *Campylobacter* and *Norovirus* (Food Standards Agency, 2011). However, it is *Salmonella*. that cause the largest number of hospitalisations with an estimated 2490 admissions per year (O'Brien et al., 2016).

A wide range of treatments for raw food is available to help prevent foodborne illness. One process that has been used for thousands of years is the use of organic acids to prevent the growth of pathogens thus preventing foodborne illness. Currently there is a wide range of organic acids used by the food industry as preservatives, stabilisers and flavour enhancers (Ricke, 2003).

Organic acids are thought to act as effective antimicrobials because in their undissociated form they are capable of passing freely through the cell membrane of foodborne organisms. The pH of the internal environment of the target organism such as *E. coli* and *Salmonella* is generally slightly alkaline or closer to neutral then external environment. As such, once the organic acid, has entered the cell it then will dissociate, releasing a proton, resulting in acidification of the cell's internal environment (Cotter and Hill, 2003).

Some organisms such as acid-tolerant fermentative bacteria may allow the intracellular pH to decrease in parallel with the external pH allowing the organism to maintain a pH gradient rather than a constant internal pH. This strategy can offers an advantage to the organism as it

requires less energy. The action of weak organic acids against such organism can be limited and has not yet been fully elucidate (Siegumfeldt et al., 2000).

Studies have identified fumaric acid as having a high antimicrobial activity against a range of food pathogens including *E. coli*, *L. monocytogenes* and *Salmonella*. (Kim et al., 2009, Kondo et al., 2006, Podolak et al., 1996). Fumaric acid has been used in a number of food products including baked goods, confectionery, juices and dried powdered products (Lee, 2014). It is also used as a supplement in some animal feeds and in the polymer industry (Lee, 2014). This study focuses on the food pathogen *Salmonella*, a gram-negative, rod shaped, bacterium, a member of the *Enterobacteriaceae* family. It is a key foodborne pathogen globally with over 2600 known serovars and can cause salmonellosis, which may result in diarrhoea, fever, and abdominal cramps (Sánchez-Vargas et al., 2011, Batista et al., 2015, Gal-Mor et al., 2014).

Foodborne pathogens, including many *Salmonella*, are capable of tolerating a wide range of environmental conditions and *Salmonella* have a number of mechanisms to help them to resist acidic environments (Bearson et al., 1997, Neely et al., 1994, Meng and Bennett, 1992). These include inducible stress proteins including pH-response regulators such as the two component regulators PhoP and OmpR, the sigma factor RpoS, and the iron regulator Fur (Park et al., 1996). *Salmonella*. Typically possess three inducible amino acid decarboxylase systems that may help support survival and growth under acidic conditions. These systems function through the use of pyridoxal phosphate-containing enzymes. In the case of *Salmonella* the arginine (*AdiA*), lysine (*cadA*) and ornithine (*SpeF*) decarboxylases replace the α-carboxyl groups of their cognate amino acid substrates (arginine, lysine or ornithine). These reactions consume a proton helping to reduce and maintain intracellular pH and produce agmatine, cadaverine, and putrescine, respectively. These products may then be exported from the cell via an antiporter.

While all three amino acid decarboxylase systems aid in supporting *Salmonella*. These systems can only operate when the appropriate amino acids are present, and under specific environmental conditions. The ornithine decarboxylase system (ODAR) appears to offer the most limited level of protection. Whilst information on this system is limited, it has been shown to function under mildly acidic conditions (4.5 pH) in combination with anaerobiosis (Viala et al., 2011). The arginine decarboxylase system (ADAR) appears to operate under extreme acidic condition (< pH 2.4) and under anoxic conditions (Kieboom and Abee, 2006, Viala et al., 2011).

Out of the three systems acid resistance systems, the lysine decarboxylase system (LDAR) appears to give the broadest level of protection and protects against a wide range of pH values. This system comprises the lysine decarboxylase enzyme (*cadA*), a lysine–cadaverine antiporter (c*adB*) used for the import of lysine and the export of cadaverine and a transcriptional regulator of the *cadBA* operon (*cadC*) (Álvarez-Ordóñez et al., 2012).

It is possible that the bactericidal action of fumaric acid may not be due solely to its ability to disassociate. Fonda (1974b) demonstrated that fumaric acid and related compounds are capable of inhibiting the glutamate decarboxylase enzyme, which functions in a similar fashion to the LDAR system. It is thus possible that this acid is capable of disrupting the function of the LDAR system in *Salmonella*, making this organism more sensitive to low pH environments. This paper examines the action of fumaric acid, and its cis-isomer maleic acid, on the LDAR system of *Salmonella*, as well as assessing its ability to remove biofilms. Biofilm removal is of key concern when dealing with food pathogens as their presence can be a huge challenge to achieve safe food. Maleic acid, the isomer of fumaric acid, is already used for this purpose (Baca et al., 2011, Ferrer-Luque et al., 2010).

4.2. Materials and methods

4.2.1 Bacterial strains and growth conditions

All strains used (Table 4.1) were stored in 2 ml cryovials with a 7% dimethyl sulfoxide solution (DMSO) at -80°C. All strains were cultured onto Lysogeny Broth (LB) agar (LABM, Lancashire UK) and incubated at 37°C overnight. Three colonies from each plate were transferred, using an inoculation loop, into Lysogeny Broth (LB; LAB M, Lancashire UK) in 10 ml bijous and incubated overnight at 37°C with shaking (150 rpm). These overnight cultures were used to inoculate 20 ml cultures of the corresponding media (1% inoculum) in 250 ml conical flasks which then were subsequently incubated overnight at 37°C with shaking at (150 rpm) for 18 h.

Table 4.1: Bacterial strains used

Strains	Isolated from	Source
Salmonella Typhimurium 37938	Pork chop	Payne et al., (1992)
Salmonella Typhimurium DT 104 strain 10	Human	Jørgensen et al., (2000)
Salmonella Typhimurium DT 104 strain 30	Bovine	Jørgensen et al., (2000)
Salmonella Enteritidis P518496	Human	Laboratory of Enteric Pathogens, Public Health Laboratory Service. Colindale, London NW9 5EQ
Salmonella Heidelberg S172457	Chicken	Laboratory of Enteric Pathogens, Public Health Laboratory Service. Colindale, London NW9 5EQ

4.2.2 Survival of various *Salmonella* in the presence of sodium fumarate and maleic acid.

Initial survival experiments were performed to examine the effect of sodium fumarate and maleic acid on three strains namely, *Salmonella* Typhimurium DT 104, strain 10 (*S.* Typhimurium *10*), *Salmonella* Typhimurium DT 104 strain 30 (*S.* Typhimurium 30) and *Salmonella* Heidelberg S172457.

Cultures were prepared in LB using stock culture, prepared as previously described and grown in 250 ml Erlenmeyer flasks at 37°C with agitation at 150 rpm for 18 h. Subsequently 10 mM sodium fumarate or 10 mM maleic acid were added. Control experiments were performed in the absence of sodium fumarate. All cultures were then adjusted to a pH of 3.5 using 1 M HCL.

Samples of 100 µl were taken immediately prior to the acid challenge and every 5 min thereafter for 15 min. Samples were subsequently added into 900 µl maximum recovery diluent (MRD; Oxoid Limited, Hampshire UK). Decimal serial dilutions were prepared and 10 µl of each dilution was plated onto LB agar and incubated at 37°C overnight. Following incubation, colonies were counted to assess the concentration of cells at every time point.

4.2.3 Survival under acidic conditions with varying concentrations of sodium fumarate.

Further survival experiments were undertaken using a further strain *Salmonella* Enteritidis 37938 selected because been isolated from the food chain to provide a realistic challenge for the acid treatment. Twenty ml cultures were prepared in LB, using stock cultures, as previously described in section 2.1. Acid challenges took place with the addition of various concentrations of sodium fumarate (0 mM, 5 mM, 10 mM, 15 mM, 20 mM and 25 mM).

Control experiments were performed in the absence of sodium fumarate. All cultures were then adjusted to a pH of 3.5 using 1 M HCL.

Samples of 100 µl were taken immediately prior to the acid challenge and every 5 min thereafter and were subsequently added into 900 µl maximum recovery diluent (MRD; Oxoid Limited, Hampshire UK). Decimal serial dilutions were prepared and 10 µl of each dilution was plated onto LB agar and incubated at 37°C overnight. Following incubation, colonies were counted to assess the concentration of cells for each time point and each concentration of sodium fumarate.

4.2.4 Lysine decarboxylase activity in the presence of sodium fumarate.

Initial strains of *S.* Typhimurium 10, *S.* Typhimurium 30 and *S.* Heidelberg and *S.* Enteritidis P518496 were grown as described in section 2.2 and used as 1% inocula for 20 mL of lysine decarboxylase medium (LDM) containing (L^{-1}) peptone 5g, yeast extract 3g, lysine monohydrochloride 10g, adjusted to pH 4.5 using 1 mol HCL (Brooker et al., 1973). All cultures were grown under agitation 150 RPM for 20 hours to achieve stationary phase cultures at 37°C.

Once cultures reached stationary phase, they were placed in 50 ml falcon tubes (VWR USA), centrifuged (12,000 xg, 10 min) and then re-suspended in 20 ml LDM with or without 25 mM of sodium fumarate. Cultures were then adjusted to pH 4.8 using 1 M HCl. Subsequently pH readings were taken every 20 min for 100 min to assess the pH change caused by the lysine decarboxylase activity.

4.2.5 Real-time PCR analysis of CAD gene expression.

The transcription of the *gad* genes in the presence and absence of sodium fumarate was assessed in *S.* Typhimurium 30 (*cadA* and *cadB*) using quantitative reverse transcription PCR (RT-qPCR), as described by Karatzas et al., (2010) using primers described by Álvarez-Ordóñez et al, (2010).

Overnight cultures of *S.* Typhimurium 30 were prepared in LB until their stationary phase was achieved. They were treated with 10 mM of either sodium fumarate or maleic acid for 40 min. Samples were taken and prepared as previously described by Karatzas et al (2010). Relative expression of the data was calculated as a ratio between expression of each of the target genes and the expression of 16S rRNA (which was used as the reference gene for each cDNA sample). Calculations were carried out following the advanced relative quantification settings of the Light Cycler 480 SW 1.5.1 software programme, with PCR efficiencies of the primer pairs *cadA* F-*cadA* R and *cadB* F- *cadB* R, and *rrsA* F- *rrsA* R being 2.04, 1.93 and 2.06 respectively. The *rrsA* gene, encoding the 16S ribosomal RNA, was used as a housekeeping gene for normalization (Álvarez-Ordóñez et al., 2012).

4.2.6 Biofilm removal by sodium fumarate under acidic conditions.

Biofilm formation was assessed using *S.* Typhimurium 10 and *S.* Typhimurium 30 in LB, prepared as previously described, and then inoculated to 1% in 2 ml of LB. The broth was mixed thoroughly and placed in a 24-flat-bottom-well Corning Costar cell culture plate and sealed using petrifilm. Following incubation at 37°C for 48h, the culture was then removed, and the wells washed three times with sterile water. A fourth treatment was applied using 2.5 ml of either water, water with 100 ppm free chlorine from calcium hyperchlorite, HCl (pH 2.4), HCl (pH 2.4) with 25 mM sodium fumarate, AM (an organic acid disinfectant) at pH 2.4

or AM at pH 2.4 with 25 mM of sodium fumarate. The biofilm was exposed to these solutions in the well for 5 min and then the supernatant was discarded, and the wells rinsed with 2.5 ml deionized water. Five hundred µl MRD was then placed in the well and the bottom of the well was scraped using a 200 µl pipette tip for 30s in a pattern covering the entire well bottom. This was repeated 4 times to provide a total volume of 2 ml which was serially diluted 10-fold. Ten µl was plated onto LB agar and incubated at 37°C for 24 h and growth was assessed (Ramírez et al., 2015). The impact of the treatments on the biofilm was assessed using the following calculation (Ramírez et al., 2015, Heersink J., 2003).

Density = (Average count/Volume plated) * Dilution * Volume of MRD scraped into *(1/well surface area)

4.2.7 Statistical analysis

In all cases, experiments were run at least in triplicate (unless stated), and the results were assessed with paired Student *t*-test. *P*-values lower than 0.05 indicated results that were statistically significant.

4.3 Results

4.3.1 Acid survival of *S.* Typhimurium 10, *S.* Typhimurium 30 and *S.* Heidelberg in the presence of maleic acid and sodium fumarate.

The effect of selected organic acids on the survival of *S.* Typhimurium 30, *S.* Typhimurium 10 and *S.* Heidelberg was assessed at pH 3.5 in the presence of 10 mM of either sodium fumarate or maleic acid. Only sodium fumarate showed a significant effect on survival of the selected strains over a period of 15 min with log reductions of 2.12, 1.3 and 4.5 of CFU/ml respectively. The presence of maleic acid did not seem to have any significant effect (Fig 4.1. A, B & C). Similar levels of acid sensitivity were observed in the *S.* Typhimurium 30 and *S.* Typhimurium 10. The third strain *S.* Heidelberg appeared to be significantly more sensitive to the presence of sodium fumarate while in the absence of sodium fumarate it showed similar levels of acid tolerance to the other strains.

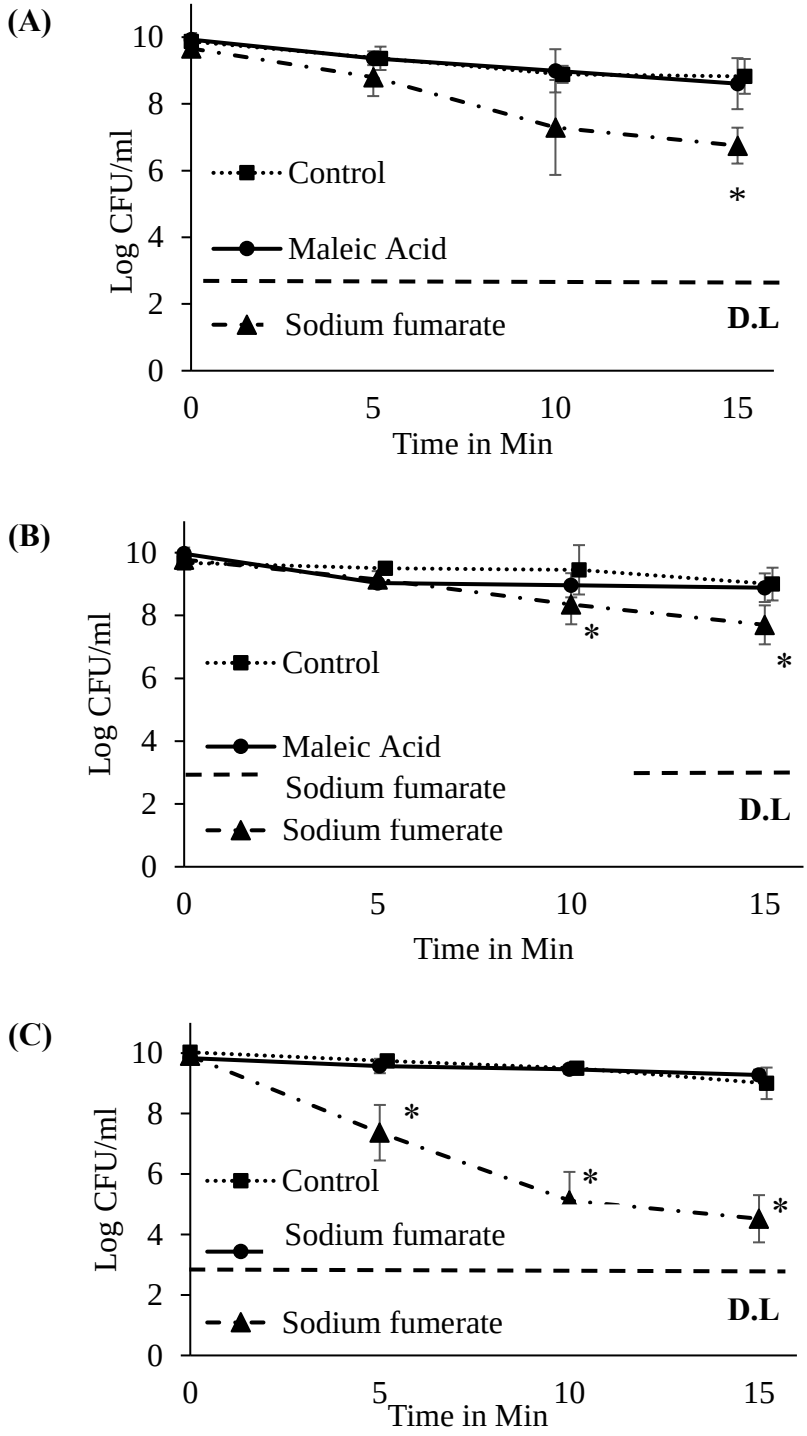


Fig. 4.1. Survival of *S.* Typhimurium 30 **(A),** *S.* Typhimurium 10 **(B)** and *S.* Heidelberg **(C)** in the presence and absence of 10 mM of maleic acid or sodium fumarate adjusted to a pH 3.5 Using 1 M HCl. Asterisks represent statistically significant result ($P < 0.05$ paired student T-test) while D.L denotes detection limit of the experimental setup.

4.3.2 Survival under acidic conditions with varying contractions of sodium fumarate.

The presence of sodium fumarate provided a significant reduction in the survival of *Salmonella* Enteritidis 37938. Increasing the concentration of sodium fumarate rapidly increased the bactericidal effect (Fig 4.2) and this can be clearly seen after the elapse of 5 min. Even with concentrations as low as 5 mM there was a statistically significant reduction of 1.4 log CFU/ml. After 10 min, the highest concentration (25 mM of sodium fumarate) reduced the level of cells to the detection limit of the plating method resulting in a significant 6.33 log reduction of CFU/ml. At 25 min, the sample exposed to 25 mM of sodium fumarate had also reached the detection limit of this method. After 25 min, final reductions compared to initial levels were seen ranging from 0.4, 4.59, 5.6, 6.25, 6.04 and 6.44 log reductions of CFU/ml (Fig 4.2) at concentrations from 0 to 25 mM respectively.

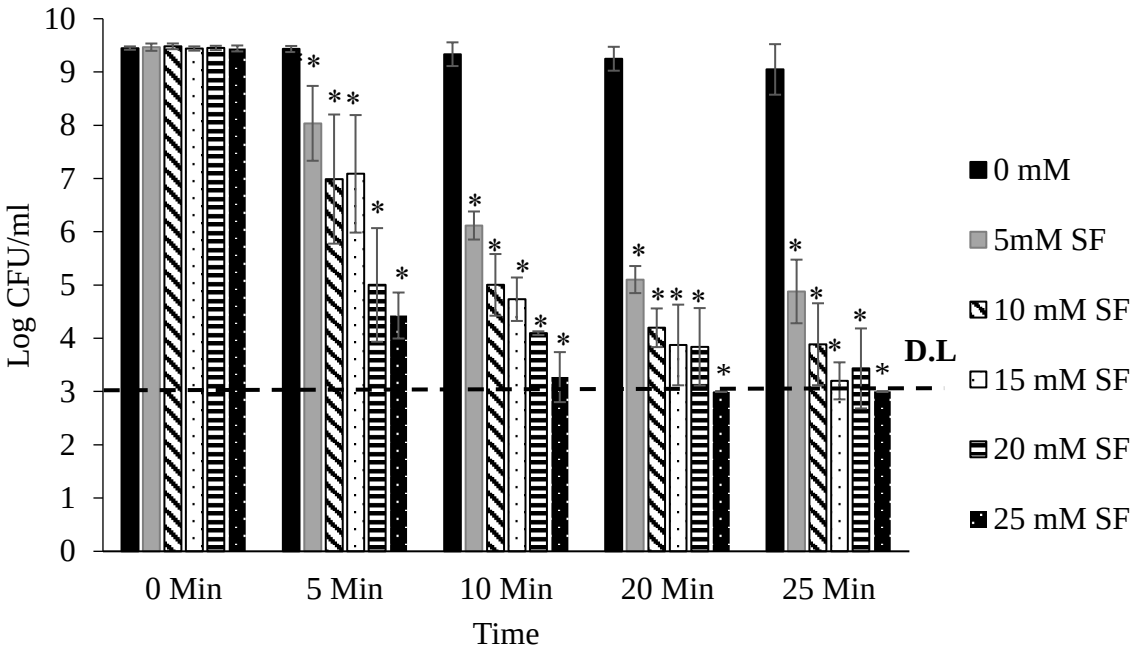


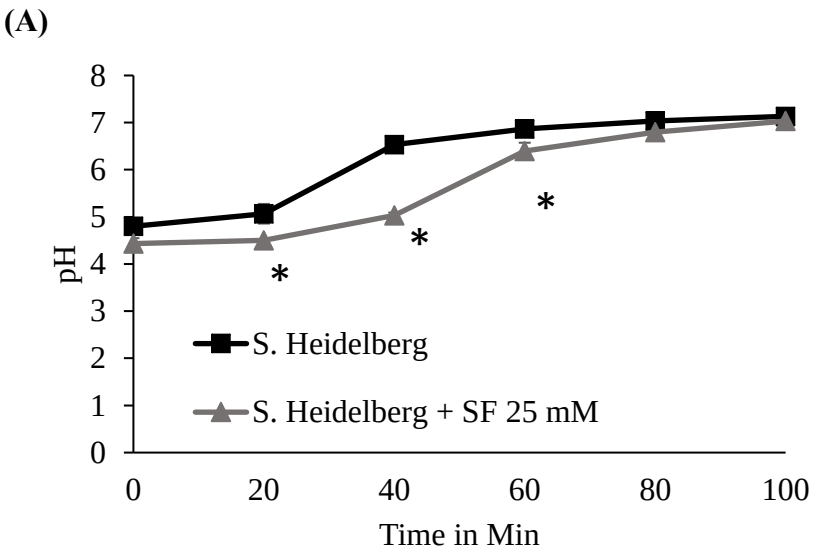
Fig.4.2. Survival of *Salmonella* Enteritidis 37938 in the presence or absence of various concentrations of sodium fumarate (adjusted to a pH 3). Samples were obtained at 5-minute intervals. Asterisks represent statistically significant result ($P < 0.05$ paired student T-test) while D.L denotes detection limit of the experimental setup.

4.3.3 Lysine decarboxylase mediated pH recovery of *Salmonella* in the presence or absence of sodium fumarate.

The acid tolerance response of *S.* Heidelberg, *S.* Typhimurium 10, *S.* Typhimurium 30 and *S.* Enteritidis P518496 was assessed by observing their pH increase as indication of the lysine decarboxylase activity in lysine decarboxylase medium in the presence and absence of sodium fumarate.

Sodium fumarate demonstrated a major impact on the function of the lysine decarboxylase system on three of the tested strains *S.* Heidelberg, *S.* Typhimurium 10 and *S.* Typhimurium 30. In the presence of 25 mM of sodium fumarate, these three strains all demonstrated a significantly slower pH recovery. *S.* Heidelberg (Fig 4.3 A) demonstrated a slower pH recovery at 20, 40 and 60 min ($P < 0.05$) with the greatest difference being observed at 40 min, *S.* Typhimurium 10 (Fig 4.3 B) also showed a slower pH recovery extracellular pH 40 and 60 min ($P < 0.05$). *S.* Typhimurium 30 (Fig 4.3 C) showed lower increase of the extracellular pH 40, 60 and 80 min ($P < 0.05$). Around 100 minute all tested strains had achieved a neutral pH and the rapid increase in pH had halted.

The other strain that was tested *S.* Enteritidis P518496 achieved a similar final pH recovery to the other three strains however no significant differences were seen in the presence or absence of sodium fumarate (Fig 4. 3 D).



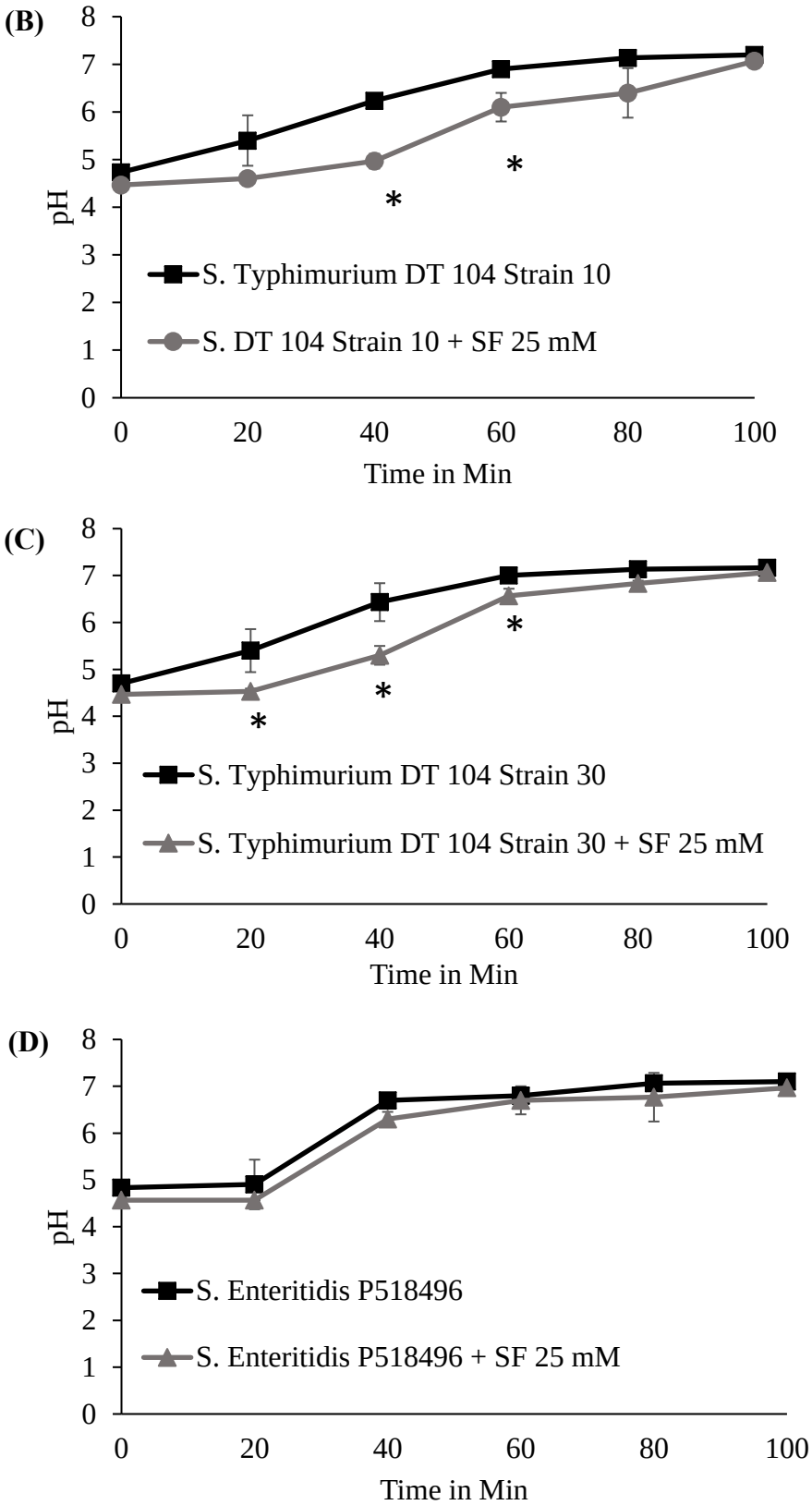


Fig.4.3. pH increase during the lysine decarboxylase test indicating lysine decarboxylase activity in the presence and absence of 25 mM of sodium fumarate for **(A)** *S.* Heidelberg **(B)** *S.* Typhimurium 10 **(C)** *S.* Typhimurium 30 **(D)** and *S.* Enteritidis P518496 Asterisks represent statistically significant result ($P < 0.05$ paired student T-test).

4.3.5 Real-time PCR determination of CAD gene expression.

Real time quantitative Polymerase Chain Reaction (RT-qPCR) was used to assess the transcription of the LDAR system of *S.* Typhimurium 30. The key components of the LDAR system were examined *cadA* and *cadB* genes in the presence of sodium fumarate, and its cis-isomer maleic acid sodium salt, both of which have been shown to previously inhibit the listerial GAD system of *L. monocytogenes* 10403S (Paudyal et al., 2018) (Barnes and Karatzas., unpublished) . Transcription of both *cadA* and *cadB* was very low and not affected by the presence of sodium fumarate, maleic acid or sodium chloride (Fig. 4.4 A & B). While no significant results were observed under assessed conditions, a slight upregulation was observed in the presence of sodium fumarate, with a 4.3-fold increase being seen with *cadA* and 2.8-fold increase with *cadB.* A slight decrease -0.2 -fold in *cadA* and -0.45-fold *cadB* was observed with 10 mM maleic acid. This was not, however, statistically significant (Fig. 4.4 A & B). Only very small changes were observed with sodium chloride.

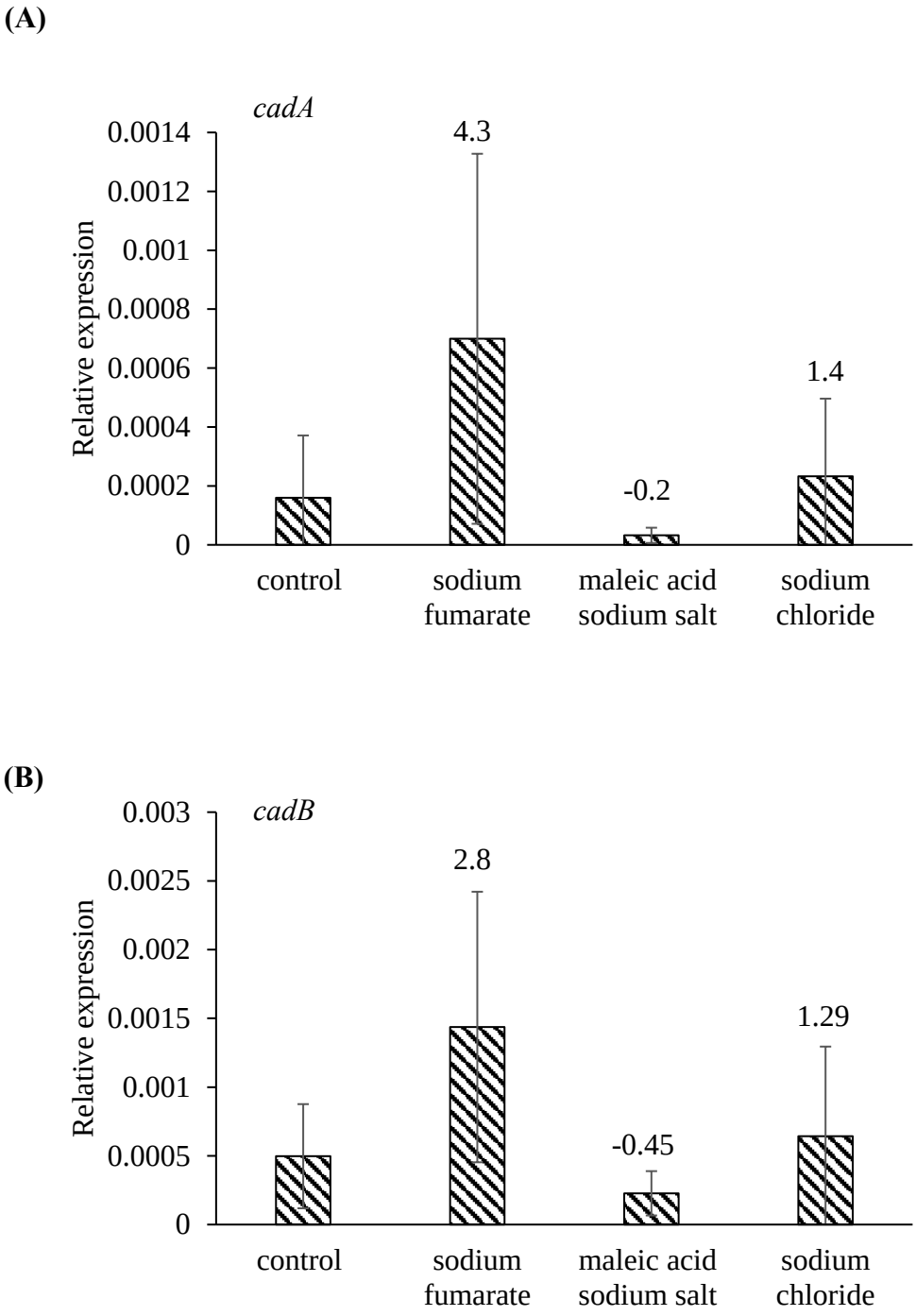


Fig. 4.4. Expression of **(A)** *cadA and* **(B)** *cadB* gene in *S.* Typhimurium *30* in the absence or presence of 10 mM sodium fumarate or 10 mM maleic acid. Relative expression of each gene was calculated by comparing expression relative to 16S rRNA gene in each strain. Numbers above the bars represent fold difference in relative expression compared to control. Markers represent an average of triplicate measurements and error bars represent standard deviations.

4.3.6 Biofilm removal

Experiments with *S.* Typhimurium 10 showed that four of the treatments provided significant reductions in the survival of biofilm ($P < 0.05$). These treatments were100 ppm chlorine, AM pH 2.4 + 25mM of sodium fumarate, HCl pH 2.4 + 25 mM sodium fumarate, and AM pH 2.4. The greatest log reduction of CFU/cm^2 was seen with the use of chlorine which was above 4.2 log CFU/cm^2 as counts reached the detection limit of the plating technique. Altering the pH of 2.4 (adjusted with HCl) did not show a significant reduction, however, with the addition of 25 mM of sodium fumarate a reduction of 2.4 log CFU/cm^2 was achieved when compared to water. While the AM treatment was effective at a pH of 2.4, creating a reduction of 1.9 log CFU/cm^2, the presence of sodium fumarate resulted in an increased reduction of 4.1 log CFU/cm^2 (Fig 4.5 A).

Experiments with *S.* Typhimurium 30 resulted in significant reductions in the survival of biofilm ($P< 0.05$) with four treatments. These treatments were, AM pH 2.4 + 25mM sodium fumarate, AM pH 2.4, HCl pH 2.4 + 25 mM sodium fumarate and 100 ppm chlorine. In this case the greatest reduction was seen with AM pH 2.4 + 25mM of sodium fumarate where a reduction was seen of 4.8 log CFU/cm^2. When compared to water, this treatment provided a significant improvement over the AM alone with an increased reduction of 1.73 log CFU/cm^2 (Fig 4.5 B).

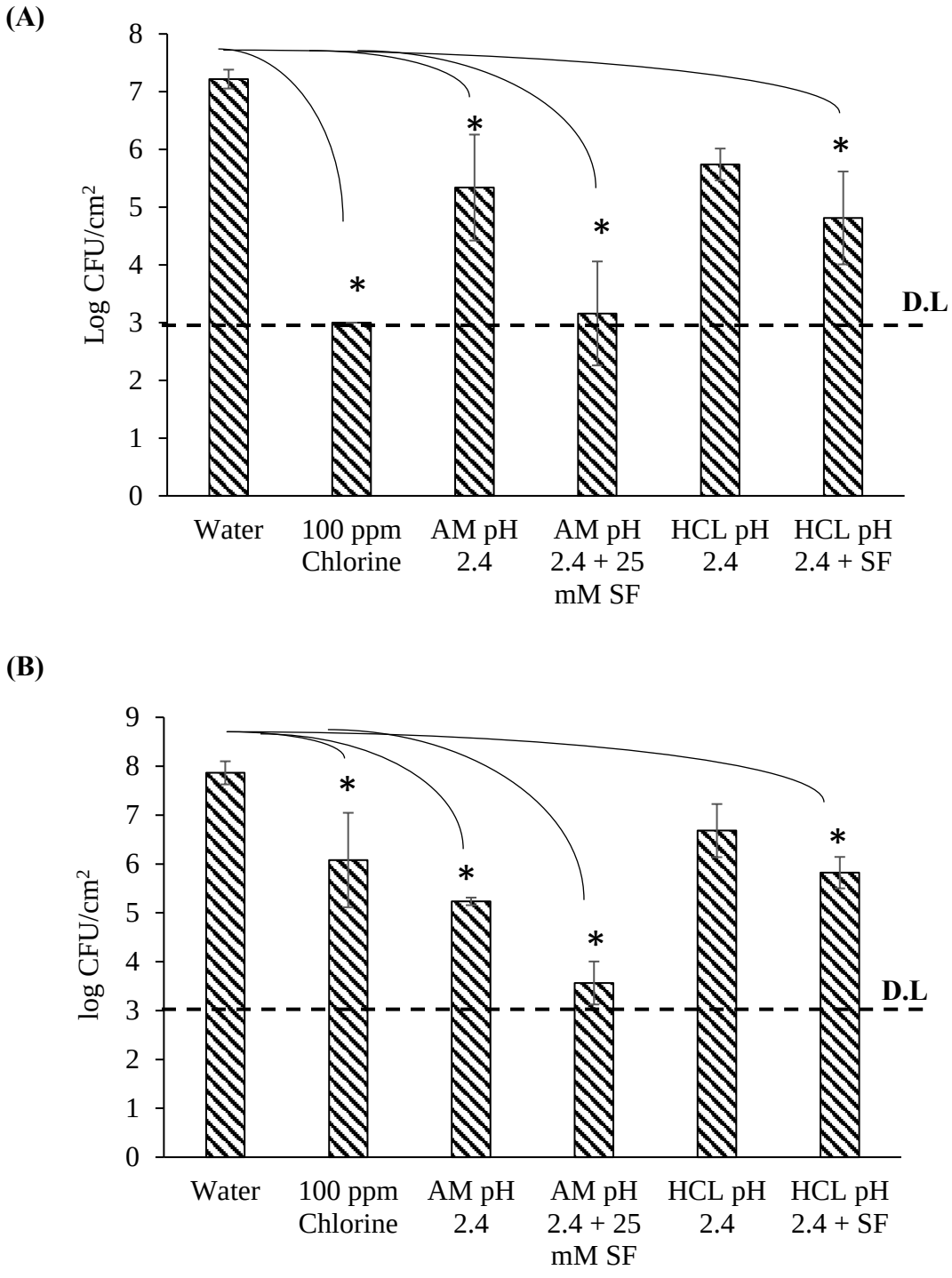


Fig 4.5. (**A)** Survival of cells in biofilms of *S.* Typhimurium 10 (**B)** *S.* Typhimurium 30, following no treatment (water) or treatment with an acidic disinfectant (AM), AM together with 25 mM sodium fumarate (SF), HCl and HCl together with 25 mM SF. All treatments were at pH 2.4. Asterisks represent statistically significant difference between no treatment and a treatment ($P < 0.05$; paired student T-test). While D.L denotes detection limit of the experimental setup.

4.4 Discussion

Organic acids have for many years been widely used within the food industry as additives preservatives and antimicrobials. However, the mechanism of action of weak organic acids as antimicrobials is poorly understood and may be dependent upon many factors including the nature of the organism and the prevailing environmental conditions (Ricke, 2003).

The current theory of how organic acids operate against bacteria is through the passive diffusion of their undissociated molecules through the membrane, followed by intracellular dissociation that leads to a drop of intracellular pH resulting in death, or growth inhibition. It has however been demonstrated that the bactericidal effect of some organic acids is different from what might be predicted by this theory. Some studies have identified acids, such as fumaric, as achieving significant bacterial reductions compared to other acids, such as citric and malic, under acidic conditions (Foster, 2004, Ricke, 2003, Brul and Coote, 1999, Hirshfield et al., 2003).

This additional antimicrobial activity of some of the organic acids such as fumarate might be related to additional effects on metabolic pathways or cellular mechanisms. It is important to identify the additional antimicrobial effects such acids have on bacteria as it may further increase our understanding of the function and operation of bacterial stress responses. Maleic acid has been found to be capable of disrupting the glutamate decarboxylase (GAD) system, which is the major mechanism of acid resistance in *L. monocytogenes* (Paudyal et al., 2018). While the GAD system is not found in *Salmonella*, the latter has a similar amino acid decarboxylase systems such as the lysine, ornithine and arginine decarboxylase systems. These systems may operate as previously described, depending upon which amino acids are available and environmental conditions when the organism is exposed to acid stress, working in concert with a number of acid shock proteins to protect the organism (Bearson et al., 1997, Álvarez-Ordóñez et al., 2012).

Our work focuses on the possible effects of, fumaric acid and maleic acid on the lysine decarboxylase system in *Salmonella*. Fumaric acid was examined because in previous studies it had demonstrated high antimicrobial activity against *S.* Typhimurium although it has generally been examined in combination with non-acidic treatment (Kim et al., 2009, Kondo et al., 2006). Maleic acid was chosen as it has a known inhibitory effect on the GAD system. We decided to investigate if both compounds have inhibitory effects against other amino acid decarboxylase systems. If this is the case, these compounds might have major antimicrobial effects under acidic conditions against a wide variety of microorganisms since most of them rely on a variety of amino acid decarboxylase systems for their acid resistance.

The initial experiments examined the effect of small quantities (10 mM) of sodium fumarate and maleic acid on survival under acidic conditions of three strains of *Salmonella* (Fig. 4.1 A, B & C). This work demonstrated that sodium fumarate, in small quantities was highly antimicrobial against *Salmonella* under acidic conditions ($P < 0.05$; Fig 4.1 A, B & C) over a 15 minute period. Maleic acid, however, achieved only small reductions in survival when compared to control experiments suggesting that it is not influencing the organisms in the same fashion.

Next the bactericidal effect of varying concentrations of sodium fumarate on *S.* Enteritidis 37938 was assessed over the course of 25 min (Fig 4.2). All concentrations of sodium fumarate tested (5-25 mM) demonstrated a significant bactericidal effect after only 5 min when compared to acidic stress alone with the reductions in survival increasing over time. The highest concentration tested (25 mM) showed a reduction of 6.44 Log/CFUml at 20 min. This demonstrated that in small quantities and over short time scales, under acidic conditions, sodium fumarate has a strong bactericidal effect. This bactericidal effect increase at higher concentrations possibly through the inhibition of specific bacterial mechanisms of acid resistance.

Subsequently, we examined the effect of 25 mM sodium fumarate in the activity of the lysine decarboxylase system in *Salmonella* by looking at the increase in the pH due to the production of cadaverine by the decarboxylation of lysine. This reaction is a commonly used test to detect *Salmonella* in food or water (Park et al., 1996). In three of the strains examined, *S.* Typhimurium 10, *S.* Typhimurium 30 and *S.* Heidelberg, the pH increase was delayed by the presence of sodium fumarate suggesting an inhibition of the lysine decarboxylase system ($P < 0.05$; Fig 4.3 A, B & C). A further strain, *S.* Enteritidis P518496, was tested but no significant effect on pH increase (Fig 4.3 D). This indicates that sodium fumarate is capable of inhibiting the lysine decarboxylase system, however this effect on recovery may vary between strains as did the effects of sodium fumarate upon the survival of the different strains tested under acidic conditions (Fig 4.1 A, B, & C). It should also be noted that this delay was significant as a close to neutral pH was achieved within 60 min in the absence of sodium fumarate, while this occurred only after 100 min in the presence of the sodium fumarate. This delay could make the difference between life and death for the cell which could clearly explain the significant antimicrobial effect of sodium fumarate against *Salmonella* under acidic conditions. This may help to explain the unexpectedly high degree of antimicrobial action that has been observed on some foodborne pathogens including *Salmonella* in the presence of fumaric acid.

This result was consistent with previous work on organic acids which has demonstrated the ability of weak organic acids to effect specific amino acid decarboxylases (Fonda ,1972a) where a range of weak organic acid including fumaric were demonstrated as having varying ability to inhibit the glutamate decarboxylase of *E.coli* (Fonda, 1972a). This work suggests that weak organic acids could be having unique effects on organisms based on their metabolism and function and this provided scope for helping to design decontamination regimes aimed at specific organisms.

The possible effects of fumarate and its cis-isomer maleic acid on the transcription of CAD genes *cadA* and *cadB* was also examined. However, the RT-qPCR performed demonstrated no significant effect of fumarate, maleate or sodium chloride on *cadA* or *cadB* (Fig. 4.4 A & C). However, although not significant due to a high statistical variation, a major 4.3-fold increase occurred in *cadA* and a 2.8- fold increase in *cadB* in the presence of fumarate. Although small changes were observed with sodium chloride, they were not as pronounced as those shown which sodium fumarate suggesting a possible effect that should be further explored.

The action of sodium fumarate on the bacterial cells in a biofilm was examined. Biofilms are of key interest in terms of antimicrobials and disinfection as in this form many organisms become extremely resistant to current decontamination techniques. In recent years some organic acids, such as maleic, the cis-isomer of fumaric acid, have been shown to have potential as an effective treatment for biofilms of *E. faecalis* and *L. monocytogenes* (Ferrer-Luque et al., 2010, Paudyal et al., 2018). It has been suggested that maleic acid could make an effective alternative to EDTA, which is used by the dental industry to target biofilms both on equipment and within the oral cavity (Ballal et al., 2009, Ferrer-Luque et al., 2010). If sodium fumarate were effective, it would offer advantages over some current treatments, as it is not toxic. These results demonstrate the striking bactericidal effect of sodium fumarate under acidic conditions when compared to a commonly used organic acid disinfectant AM at pH 2.4. Its effectiveness was comparable to the more commonly used chlorine-based treatments (Fig 4.5 A &. B). *S.* Typhimurium 10 demonstrated a marked sensitivity in this series of experiments to the chlorine-based treatment. This is possibly due to this strain's lack of the RpoS, which is key to the regulation of a large group of genes responsible for variety of stress responses (Abdullah et al., 2017, Nickerson and Curtiss, 1997). In previous studies on *Salmonella*, it has been noted that this organism's reaction to a chlorine stress created an

upregulation in this specific protein. It is possible, therefore, that its absence increased this strain's sensitivity to chlorine under these specific conditions (Wang et al., 2010).

4.5 Conclusions

The antimicrobial effect of fumaric acid, and its action as an inhibitor of the GAD enzyme of *E.coli,* has been noted in a number of studies (Fonda, 1972a). Fumaric acid is capable of affecting this organism's ability to deal with low pH environments. However, a similar effect might be taking place in other organisms that use decarboxylase systems to protect against acid stresses. These experiments demonstrate that sodium fumarate is bactericidal against various *Salmonella* under acidic conditions. It was also demonstrated that sodium fumarate inhibits the ability of the ability of *Salmonella* to increase its environmental pH, an ability associated with the lysine decarboxylase system. While no significant alteration in the regulation of the genes of the lysine decarboxylase system was observed, small increases in regulation were observed which might be supported by further investigation. It was also observed that sodium fumarate under acidic condition has a major impact on *Salmonella* species biofilms. This was found to be comparable or superior to chlorine and an organic acid-based commercial disinfectant. Further work is needed to elucidate the complete mode of action of fumarate however; this work may in part explain its effectiveness as an antimicrobial which may lead to new treatments for the eradication of biofilms of *Salmonella*.

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CHAPTER 5:

The presence of *sigB* decreases carbon source utilisation in *L. monocytogenes* 10403S; Evidence for self-preservation and nutritional competence (SPANC) balance in Gram-positive bacteria.

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Abstract

L. monocytogenes, the causative agent of listeriosis, is a virulent food pathogen and is known for its ability to survive extreme environmental stresses. This study investigates how the absence of key stress genes of the glutamate decarboxylase system (GAD), the alternative sigma factor B (*sigB*) and lmo0913, might affect the ability of *L. monocytogenes* to utilise different carbon sources and grow under high osmotic pressure. This investigation used 96-well phenotypic microarrays (Biolog TM) to provide a wide variety of environmental conditions. Variation in carbon source utilisation was observed when comparing *L. monocytogenes* 10403S WT with Δ*gadD2*, Δ*gadD3* and Δ*sigB*. Interestingly, Δ*sigB* demonstrating an increased ability to utilise 14 of the tested carbon sources in comparison to the WT. This mutant also demonstrated an increased tolerance to specific osmolytes specifically sodium lactate and sodium nitrite. The GAD mutants Δ*gadD1*, Δ*gadD2* and Δ*gadD3* also showed a sensitivity to sodium chloride (NaCl) which had not been observed previously. Throughout these tests, the Δ*Lmo0913* saw no alteration form the profile presented by the WT.

5.1 Introduction

Listeria monocytogenes is a Gram positive, facultative anaerobic bacterium, and is one of the most virulent foodborne pathogens, causing listeriosis, which is associated with a mortality rate of 20-30 % and is of particular danger to the immunocompromised and to pregnant women (Rocourt, 1996).

L. monocytogenes is found widely in nature including in soil and water, making it difficult to remove from the food chain. Within the food chain, it has been identified in a number of different products but is most commonly associated with ready-to-eat products. It is known to be remarkably robust capable of surviving and growing at temperatures below 4°C, while it is also halotolerant and resistant to acidic conditions (Rocourt et al., 2003).

To achieve this high degree of environmental stress tolerance, *L. monocytogenes* relies on a number of key systems and mechanisms. A major part of the overall stress response in *L. monocytogenes* is governed by the alternative sigma factor B (SigB). SigB is a key factor in the transcriptional response of *L. monocytogenes*, to environmental stresses. Including low pH, high pressure, the presence of bile salts and low temperatures (Becker et al., 1998, Becker et al., 2000, Begley et al., 2005, Chaturongakul and Boor, 2004, Ferreira et al., 2001, Herbert and Foster, 2001, Sue et al., 2004, Wemekamp-Kamphuis et al., 2004, Wiedmann et al., 1998).

The *sigB* influences *L. monocytogenes* ability to deal with acidic stress through the glutamate decarboxylase system (GAD system). The GAD system of *L. monocytogenes* generally comprises three decarboxylases GadD1, GadD2 and GadD3 and two antiporters GadT1 and GadT2 (Feehily and Karatzas, 2013). The GAD system provides protection through the conversion of glutamate to gamma amino butyric acid (GABA), a reaction that consumes one intracellular proton thus helping to reduce or maintain the organism's intracellular pH (Cotter et al., 2001). In the absence of key components of the GAD, system including *gadD2 gadD3*

and *gadT2* fail to be transcribed under acidic conditions, limiting the organism's ability to tolerate low pH conditions (Wemekamp-Kamphuis et al., 2004). Previous studies have suggested that acid tolerance may be linked with response to osmotic stresses (Álvarez-Ordóñez et al., 2012).

It was initially suggested that Lmo0913 might play a role in the utilisation of key carbon sources but was later identified as being a succinate-semialdehyde dehydrogenase. This is part of the GABA shunt, which used to metabolise GABA, produced in the GAD system, helping to support the organism under acidic stress, as well as helping to compensate for the interrupted citric acid cycle found in *L. monocytogenes.* The presence of *sigB* has been demonstrated as playing a role in the function of this protein (Abram et al., 2008, Feehily et al., 2014).

This paper examines the effect of the absence of *sigB, gadD1, gadD2 gadD3,* and *lmo0913* in *L. monocytogenes* 10403S on its ability to utilise key carbon sources and to withstand a wide range of osmolytes. The work helps us to better understand the role and function of these genes during environmentally stressful conditions and hence leading to a greater understanding expanding our knowledge of gene function expanding our knowledge of *L. monocytogenes*. It has previously *Escherichia coli* that there is a trade-off between stress response and nutritional ability a concept described as *s*tress *p*rotection *a*nd *n*utritional *c*apability (SPANC) balance (Ferenci, 2005) which has never been observed in a Gram positive organism such as *L. monocytogenese*

This phenotypic analysis was undertaken using Phenotype Microarrays (PMs). This is a technique that allows for a high throughput of samples using 96 well plates with individualised environmental conditions providing the ability to test multiple genes quickly for phenotypic variation. Mutants may display alterations in phenotypes if the deleted gene

played a key role under the tested conditions in this case isolated carbon sources and osmotic pressure.

5.2 Materials and methods

5.2.1 Bacterial strains and growth conditions

All strains used (Table 5.1) were stored in 2 ml cryovials with 7% dimethyl sulfoxide (DMSO) at -80^{o}C. Strains were cultured on to Tryptic soy agar (TS Agar) LAB M, Lancashire UK) and incubated at 37^{o}C overnight. Individual colonies were then selected and sub cultured twice before use on TSA. All strains used are described in Table 1. Mutants were all constructed during previous experiments (Cotter et al., 2001, Feehily and Karatzas, 2013) and have been used extensively in previous work.

Table 5.1: Strains used in this study.

Strain	Relevant properties	Source
L. monocytogenes 10403S	WT	(Wiedmann et al., 1998)
L. monocytogenes 10403S Δ*gadD1*	Glutamate decarboxylase promoting growth under mild acidic conditions (Cotter et al., 2005)	(Wiedmann et al., 1998)
L. monocytogenes 10403S Δ*gadD2*	Glutamate decarboxylase promoting survival under extreme acidic conditions (Cotter et al., 2005)	(Wiedmann et al., 1998)
L. monocytogenes 10403S Δ*gadD3*	Glutamate decarboxylase part of the intercellular GAD system (Karatzas et al., 2012)	(Wiedmann et al., 1998)
L. monocytogenes 10403S Δ*sigB*	*sigB* encodes for the alternative sigma factor ς^{B} which promotes survival mechanism for key environmental stresses including acidic stress and carbon starvation (Moorhead and Dykes, 2003).	(Wiedmann et al., 1998)
L. monocytogenes 10403S Δ*lmo0913*	Putative succinate semialdehyde dehydrogenase (Abram et al., 2008)	(Abram et al., 2008)

5.2.2 Phenotype micro array analysis.

Phenotype micro array (PM) analysis was undertaken using Biolog PM™ technology (Biolog Inc., USA). This technology was used to undertake a comparison between wild type (WT) *L. monocytogenes* 10403S and a selection of mutants in stress-related genes as listed in Table 1.

Experiments were performed using Biolog™ 96 well microtiter plates, PM1 and PM2A carbon source utilization assays, and PM9 Osmolytes. Fig 5.1- 5.3 show the lay out of the plates.

The PM system functions using a tetrazolium based dye, which is used to monitor the active metabolism of the selected organisms. This assesses the extent of the reduction of tetrazolium violet to formazan following metabolic activity of cells. This corresponds to the intracellular reducing rate by NADH, which causes the inoculum to become purple in colour (Chai et al., 2012). The extent of this colour change is monitored and recorded through optical density readings and a charge coupled-device camera every 15 min for the selected incubation time, which in this case was 45 h.

5.2.2.1 Preparation of cell suspension.

All strains used (Table 1) were stored in 2 ml cryovials with a 7% dimethyl sulfoxide (DMSO) at -80°C, cultured onto tryptone soya (TS) agar (LABM, Lancashire UK) and incubated at 37°C overnight. Subsequently they were sub cultured a second time for 24 hours under the same conditions and then, following growth, colonies were harvested using a Longswabz™ (Technopath, Tipperary, Ireland) cotton tipped swabs and suspended in IF-0a inoculating fluid for PM1 and PM2A (Biolog Inc., USA) or IF-0b inoculating fluid for PM9. Suspensions were adjusted to achieve to an optical density of 0.032 at an absorbance of 590 nM equivalent of 85% transmittance using a Biolog turbidometer (Technopath, Tipperary, Ireland).

5.2.2.2 Preparation of additives

An additive solution to support the growth of the organism on the PM plates was prepared depending upon, the type of plate selected, according to manufacturer's instructions as shown in Table 5.2. All solutions were initially prepared at 120x stock solutions and later diluted as specified by manufactures instructions all stock solutions were filter sterilised and stored at 4ºC before being combined with water to 100 ml.

Table 5.2: Composition and Preparation of 12x PM Additive Solutions.

Ingredient	1x Conc.	40-120x Conc.	Formula Weight	Grams/ 100 ml	PM 1,2	PM 9+
MgCl2, 6H2O	2mM	240mM	203.3	4.88	10ml	10ml
CaCl2, 2H2O	1mM	120mM	147.0	1.76		
L-arginine, HCl L-	25uM	3mM	210.7	0.063	10ml	-
glutamate, Na	50uM	6mM	169.1	0.101		
L-cystine,(pH8.5[a])	12.5uM	0.5mM	240.3	0.012	30ml	-
5'-UMP, 2Na	25uM	1mM	368.1	0.037		
yeast extract	0.005%	0.6%	-	0.6	10ml	10ml
tween 80	0.005%	0.6%	-	0.6	10ml	10ml
D-glucose	2.5mM	300mM	180.2	5.40	-	10ml
pyruvate, Na	5mM	600mM	110.0	6.6		
Sterile water					30ml	60ml
Total					100ml	100ml

[a] The L-cystine, was adjusted to a pH of 8.5 with using 1 M NaOH.

5.2.2.3 Preparation of final PM inoculation.

Final inoculations were made following the manufacturer's instructions (Table 5.3). In this series of experiments Dye mix F was used as this is recommended for fast growing Gram positive organisms including *L. monocytogenes*.

Following the preparation of each plate final inoculation solution 100 µl of this mix was inoculated on to each well of the plates. The plates were then monitored over a 45 h period in

the OmniLog PM system (Biolog Inc.,USA). Kinetic data was analysed using the OmniLog PM software.

Table 5.3: Recipe for PM Inoculating Fluids from Stock Solution.

PM Stock Solution	PM 1 and 2 (ml)	PM9 (ml)
IF-0a GN/GP (1.2x)	20.0	-
IF-10b GN/GP (1.2x)	-	110.0
Dye mix F, G, or H c (100x)	0.24	1.32
PM additive (12x)	2.0	11.0
Cell suspension (13.64x)	1.76	9.68
Total	24.0	132.0

2.4 Data analysis

The data for all plates and strains was analysed using Omnilog PM Software. The software generated a time course kinetic curve for tetrazolium colour development, using optical density units called Omnilog units (arbitrary colour units due to dye reduction. For PM 1 and PM 2 the A1 zero option was selected during data processing to subtract any background signal found in well A1 (negative control). This option was not possible for PM9 as no negative control was present. Each strain was analysed in triplicate, and the results were examined for consistency by looking for high levels of deviation in the data max of the kinetic curves. In this experiment an average height of below 10 Omnilog units was considered a negative result whilst results between 10 and 100 Omnilog units was ascribed as weak utilisation and over 100 Omnilog units was ascribed as strong utilisation (Fig, 5.1 and 5.2).

For each well of all three types of plate, a number of kinetic properties were calculated, including average height, maximum height, slope and area. These were then used to compare the statistical difference, using a paired T-test, between the *L. monocytogenes* WT strain and each one of the mutants. A $P \leq 0.05$ using a paired student T-test was considered to be statistically significant.

3. Results

3.1 Carbon source utilisation

Using the carbon source plates PM 1 and 2, 190 separate carbon sources were examined. *L. monocytogenes* 10403S WT was able to utilise 57 separate carbon sources and of these, 15 were only able to be used to a limited degree (between 10 and 100 Omnilog units) as indicated in Fig 5.1 and 5.2 as indicated with a cross (+). The remaining 43 sources appeared to be used to a high degree (100 Omnilog units and above) which is indicated with a double cross (++) in Fig 5.1 and 5.2 [Appendix 2].

It was found that of the 57 carbon sources *L. monocytogenes* 10403S was capable of using 22 were monosaccharides, 8 disaccharides, 4 deoxysugars, 3 oligosaccharides, 3 polysaccharides, 2 sugar alcohols and 1 tri-saccharide. The rest of the carbon sources were identified as 7 carboxylic and keto acids, 4 nucleotides, 2 glycosides and 1 monoglyceride (Tables 5.4 and 5.5).

Table 5.4: Average height of growth curves recorded using Omnilog units for *L. monocytogenes* 10403S WT on PM1 this table also identifies the group to which each carbon source belongs.

Plate	WT PM1	Main group	Sub group	Average Height (Omnilog units)	St. dev.
PM1	L-arabinose	Carbohydrate	Monosaccharide	108	4.5
PM1	N-acetyl-D-glucosamine	Carbohydrate	Monosaccharide	174.6	11.9
PM1	D-galactose	Carbohydrate	Monosaccharide	20.6	6.6
PM1	D-trehalose	Carbohydrate	Disaccharide	164.3	6.1
PM1	D-mannose	Carbohydrate	Monosaccharide	175.3	12.5
PM1	glycerol	Carbohydrate	Monoglyceride.	163.3	0.5
PM1	L-fucose	Carbohydrate	Deoxy sugar	30.3	9.8
PM1	D-glucuronic acid	Carbohydrate	Uronic acid	11.6	6.5
PM1	D-xylose	Carbohydrate	Monosaccharide	167.6	25.6
PM1	D-ribose	Carbohydrate	Monosaccharide	177.3	2.5
PM1	L-rhamnose	Carbohydrate	Deoxy sugar	179	1
PM1	D-fructose	Carbohydrate	Monosaccharide	171.3	12.3
PM1	a-D-glucose	Carbohydrate	Monosaccharide	167.3	10.0
PM1	maltose	Carbohydrate	Disaccharide	156	9.5
PM1	thymidine	Nucleic acid	Nucleotide	35.3	6.6
PM1	a-ketobutyric acid	Keto acid	Keto acid	14	7
PM1	a-D-lactose	Carbohydrate	Disaccharide	51	17.0
PM1	uridine	Nucleic acid	Nucleotide	164.3	2.3
PM1	D-fructose-6-phosphate	Carbohydrate	Monosaccharide	63	5.2
PM1	b-methyl-D-glucoside	Carbohydrate	Monosaccharide	161.3	0.5
PM1	maltotriose	Carbohydrate	Trisaccharide	161	5.5
PM1	adenosine	Nucleic acid	Nucleotide	61	4.3
PM1	D-cellobiose	Carbohydrate	Disaccharide	156.3	6.0
PM1	Inosine	Nucleic acid	Nucleotide	95.6	18.7
PM1	acetoacetic acid	Keto acid	Keto acid	42	4.3
PM1	N-acetyl-D-mannosamine	Carbohydrate	Monosaccharide	164.6	3.2
PM1	D-psicose	Carbohydrate	Monosaccharide	184.3	5.1
PM1	L-lyxose	Carbohydrate	Monosaccharide	177.6	5.7
PM1	glucuronamide	Carbohydrate	Monosaccharide	40.6	4.1

Table 5.5: Average height of growth curves recorded using Omnilog units for *L. monocytogenes* 10403S WT on PM2 this table also identifies the group to which each carbon source belongs.

Plate	WT PM2	Main group	Sub group	Average Height (Omnilog units)	St. dev.
PM2	a-cyclodextrin	Carbohydrate	Oligosaccharide	157.3	11.8
PM2	b-cyclodextrin	Carbohydrate	Oligosaccharide	155	8.5
PM2	g-cyclodextrin	Carbohydrate	Oligosaccharide	155.6	10
PM2	dextrin	Carbohydrate	Polysaccharide	172	2.6
PM2	laminarin	Carbohydrate	Polysaccharide	59.3	93.2
PM2	pectin	Carbohydrate	Polysaccharide	38	23.6
PM2	b-D-allose	Carbohydrate	Monosaccharide	156	14
PM2	amygdalin	Carbohydrate	disaccharides (O-glycosides)	163.6	3.5
PM2	D-arabinose	Carbohydrate	Monosaccharide	143.6	26
PM2	D-arabitol	Carbohydrate	Sugar alcohol	147	29.1
PM2	arbutin	Carbohydrate	Glycoside	158.6	3.5
PM2	2-deoxy-D-ribose	Carbohydrate	Deoxy sugar	155.3	2.5
PM2	D-fucose	Carbohydrate	Deoxy sugar	34.3	16.7
PM2	3-0-b-D-galactopyranosyl-D-arabinose	Carbohydrate	Disaccharides (O-glycosides)	105.6	10.6
PM2	gentiobiose	Carbohydrate	Disaccharide	153.6	11.5
PM2	a-methyl-D-glucoside	Carbohydrate	Monosaccharide	74.3	4.6
PM2	3-methylglucose	Carbohydrate	Monosaccharide	24.6	22.4
PM2	a-methyl-D-mannoside	Carbohydrate	Monosaccharide	112.3	5.7
PM2	palatinose	Carbohydrate	Disaccharide	133	26.2
PM2	salicin	Carbohydrate	β-glucoside	143	8.1
PM2	D-tagatose	Carbohydrate	Monosaccharide	85	46.6
PM2	xylitol	Carbohydrate	sugar alcohol	160	1.7
PM2	dihydroxyfumaric acid	Carboxylic acid	Carboxylic acid	172.3	13.6
PM2	4-hydroxybenzoic acid	Mono hydroxybenzoic acid,	Mono hydroxybenzoic acid,	31.3	54.2
PM2	5-keto-D-gluconic acid	Carbohydrate	Monosaccharide	104.6	20.9
PM2	sorbic acid	Carboxylic acid	Carboxylic acid	64.3	44.1
PM2	dihydroxyacetone	Carbohydrate	Monosaccharide	121.6	9.0
PM2	2,3-butanone	Ketone	Ketone	81.6	70.9

5.3.2 Carbon source utilisation difference observed between WT and mutants.

The carbon source utilisation profile demonstrated by *L. monocytogenes* 10403S Δ*gadD1* and Δ*lmo0913* did not show any significant observable differences compared to the WT.

The carbon source utilisation profiles demonstrated by *L. monocytogenes* 10403S Δ*gadD2* and Δ*gadD3* did show any differences from the profile of the WT. The Δ*gadD2* demonstrated four significant differences with a decreased ability to utilise two carboxylic acids (dihydroxyfumaric acid and sorbic) acid and two carbohydrates (3-0-b-D-galactopyranosyl-D-arabinose and D-fucose) (Table 5.6) [Appendix 2, Fig. 5.3 A-D]. The Δ*gadD3* mutant demonstrated a reduced ability to, a sugar derivative laminarin (Table 5.6) [Appendix 2, Fig 5.4]. All differences were identified using a paired student T-test where $P \leq 0.05$ was considered significant.

The carbon source utilisation of Δ*sigB* differed significantly from that demonstrated by the WT with 16 significant differences being observed. In 14 of these cases the Δ*sigB* mutant was observed to have an increased ability to grow compared to the WT strain (Table 5.6) [Appendix 2, Fig 5.5, A-H, J- K and M-P]. With only 2 carbon sources did the WT perform better than the Δ*sigB,* N-Acetyl-D-mannosamine and 2-Deoxy-D-Ribose (Table 4) [Appendix 2, Fig 5.5, I and L- $P \leq 0.05$ using a paired student T-test].

Carbon source	Mode of action	Knockout	Type of difference observed	Fig (see Appendix 2)
3-0-b-D-galactopyranosyl-D-arabinose	Carbohydrate	Δ*gadD2*	WT > Δ*gadD2* (Average height and area)	3.A.
dihydroxyfumaric acid	Carboxylic acid	Δ*gadD2*	WT > Δ*gadD2* (Average height and area)	3.B.
D-fucose	Carbohydrate	Δ*gadD2*	WT > Δ*gadD2* (Average height, maximum height and area)	3.C.
sorbic acid	Carboxylic acid	Δ*gadD2*	WT > Δ*gadD2* (Slope)	3.D.
laminarin	Carbohydrate	Δ*gadD3*	WT > Δ*gadD3*(Max height)	4.A.
thymidine	Nucleic acid	Δ*sigB*	WT < Δ*sigB (*Average height, Maximum height, slope and Area)	5.A.
a-ketobutyric acid	Keto acid	Δ*sigB*	WT < Δ*sigB (*Average height, Maximum height, slope and Area)	5.B.
a-D-lactose	Carbohydrate	Δ*sigB*	WT < Δ*sigB (*Average height, and Area)	5.C.
2`-deoxyadenosine	Nucleic acid	Δ*sigB*	WT < Δ*sigB (*Average height, Maximum height, slope and Area)	5.D.
adenosine	Nucleic acid	Δ*sigB*	WT < Δ*sigB (*Average height, Maximum height, slope and Area)	5. E.
inosine	Nucleic acid	Δ*sigB*	WT < Δ*sigB (*Average height, Maximum height, slope and Area)	5.F.
L-alanine	Amino acid	Δ*sigB*	WT < Δ*sigB (*Average height, Maximum height, and Area)	5.G.
alanine and glycine (ala-gly)	Amino acid	Δ*sigB*	WT < ΔsigB (Maximum height)	5.H.
N-acetyl-D-mannosamine	Carbohydrate	Δ*sigB*	WT > Δ*sigB (*Average height, maximum height, and Area)	5.I.
glucuronamide	Carbohydrate	Δ*sigB*	WT < Δ*sigB (*Average height and maximum)	5.J.
pyruvic acid	Keto acid	Δ*sigB*	WT <Δ*sigB (*Average height and maximum)	5.K.
2-deoxy-D-ribose	Carbohydrate	Δ*sigB*	WT > Δ*sigB (*Slope)	5.L.
a-methyl-D-glucoside	Carbohydrate	Δ*sigB*	WT < Δ*sigB* (Maximum height, and Area)	5.M.
L-sorbose	Carbohydrate	Δ*sigB*	WT < Δ*sigB (*Slope)	5.N.
turanose	Carbohydrate	Δ*sigB*	WT < Δ*sigB (*Average height, Maximum height, and Area)	5.O.
b-hydroxypyruvic acid	Keto acid	Δ*sigB*	WT < Δ*sigB (*Average height, Maximum height, and Area)	5. P.

Table 5.6 : Differences observed in carbon source utilisation of *L. monocytogenes* 10403S WT vs selected knockout mutants areas examined were average height, max height slope and area < indicates increased activity than WT, > indicates decreased activity compared to WT for increases compared to wild type, (All difference $P < 0.05$; paired T-test).

5.3.3 PM9 singular osmolyte challenge

The WT and the mutants all showed an ability to grow on all osmolytes (Fig 5.6) [Appendix 2] with the exception of two, 100 mM and 200 mM of sodium benzoate. The final Omnilog unit readings did not significantly vary from the initial readings and no increase was observed. An issue with the stability of the tetrazolium-based dye was encountered with the Omnilog readings in this series of experiments reducing significantly over time demonstrated, [Appendix 2, Fig 5.7 A-D]

In high concentrations, NaCl inhibited the growth of all three GAD mutants assessed the Δ*gadD1* and Δ*gadD2* showed a similar level of inhibition at a higher concentration of 10% NaCl. The Δ*gadD3* mutant was more inhibited compared to the WT at concentrations of 9% and 10% NaCl (Table 5.7) [Appendix 2, Fig 5.8. A-C] $P \leq 0.05$ using a paired student T-test.

The Δ*sigB* mutant also demonstrated significant differences form the WT in the presence of NaCl with concentrations of 5.5%, 6% [Appendix 2, Fig 5.9. A] and 6.5% showing a lower level of activity in terms of average height, maximum height, and area demonstrated by providing a $P \leq 0.05$ using a paired student T-test. (Table 5.5). This mutant also demonstrated significantly increase growth when compared to the WT when challenged with sodium lactate at concentrations ranging between 4 and 11% (Table 5.7) [Appendix 2, Fig 5.9. B] and sodium nitrite at a concentration of 100 mM (Table 5.7) [Appendix 2, Fig 5.9. C].

The Δ*lmo0913* mutant did not demonstrate any significant or observable differences from the profile of the WT strain under all conditions.

Osmolytes	Mutant	Type of difference observed compared to the WT	Fig (see Appendix 2)
9% NaCl	Δ*gadD1*	WT > Δ*gadD1* (Average height, maximum height and area)	8.A.
10% NaCl	Δ*gadD2*	WT > Δ*gadD2* (Maximum height)	8.B.
9% NaCl	Δ*gadD3*	WT > Δ*gadD3* (Average height, maximum height and area)	Not shown
10% NaCl	Δ*gadD3*	WT > Δ*gadD3* (Average height, maximum height and area)	8.C.
5.5% NaCl	Δ*sigB*	WT > Δ*sigB (*Average height, Maximum height, and Area)	Not shown
6% NaCl	Δ*sigB*	WT > Δ*sigB (*Average height, Maximum height, and Area)	9.A.
6.5% NaCl	Δ*sigB*	WT > Δ*sigB (*Average height, Maximum height, and Area)	Not shown
4% sodium lactate	Δ*sigB*	WT < Δ*sigB* (Maximum height)	Not shown
5% sodium lactate	Δ*sigB*	WT < Δ*sigB (*Average height, Maximum height, and Area)	9.B.
6% sodium lactate	Δ*sigB*	WT < ΔsigB (Average height, Maximum height, slope and Area)	Not shown
7% sodium lactate	Δ*sigB*	WT < Δ*sigB* (Maximum height)	Not shown
8% sodium lactate	Δ*sigB*	WT < Δ*sigB (*Average height, Maximum height, and Area)	Not shown
9% sodium lactate	Δ*sigB*	WT < ΔsigB (Average height, Maximum height, slope and Area)	Not shown
10% sodium lactate	Δ*sigB*	WT < ΔsigB (Maximum height and slope)	Not shown
11% sodium lactate	Δ*sigB*	WT < ΔsigB (Average height, Maximum height, slope and Area)	Not shown
100mM sodium nitrite	Δ*sigB*	WT < ΔsigB (Average height, Maximum height, and Area)	9.C.

Table 5.7 : Differences observed in osmolytes of *L. monocytogenes* 10403S WT vs selected knockout mutants areas examined were Average height, Max height slope and area < indicates increased activity than WT, > indicates decreased activity compared to WT for increases compared to wild type, (All difference $P < 0.05$; paired T-test).

5.4. Discussion.

In this study, we identified that *L. monocytogenes* 10403S WT has a low to moderate ability to utilise a variety of the 190 carbon sources that were contained in PM1 and PM2 plates with only 30% of all tested carbon sources being used to some degree. Other organisms such as *Pseudomonas aeruginosa* have been shown to be capable of using up to 71% of these carbon sources (Johnson et al., 2008). *L. monocytogenes* demonstrated a similar ability as that of *Salmonella enterica* serovar Typhi (*S.* Typhi), which is reported as being capable of using 27% carbon sources suggesting that *L. monocytogenes* may be limited in terms of carbon sources choice (Chai et al., 2012) .

L. monocytogenes was able to metabolise most of the monosaccharides tested. Carbon sources such as D-psicose D-ribose and L-rhamnose were shown to promote a high degree of growth, as shown in Table 5.4 and 5.5. Some pathogens, such as *S.* Typhi, have a carbon source profile comprising almost exclusively substrates available in the gut whilst *L. monocytogenes* was able to utilise a number of plant based carbon sources such as pectin, palatinose and xylitol (Chai et al., 2012). This may reflect this organism's lifestyle as it is commonly found in decaying vegetation in the environment and previous reports suggesting that it is predominantly an environmental organism that when conditions are right it can utilise its pathogenesis mechanisms. On the other hand, *S.* Typhi is adapted to live in the guts of humans, which is also reflected in the carbon source utilisation profile.

It is known that when *L. monocytogenes* operates in the intracellular environment it utilises the pentose phosphate and not glycolysis for its primary source of carbohydrate metabolism and alters the carbon source that it relies on as it starts using phosphorylated glucose and glycerol as key carbon sources (Joseph et al., 2006). Here we show that *L. monocytogenes* may also utilise these carbohydrates as sole carbon sources. This ability to utilise different

sole carbon sources may aid the organism by helping it, to not compete with host cells or environmental competitors (Hain et al., 2007).

This information helps to provide a detailed profile of the carbon source utilisation of *L. monocytogenes* 10403S WT as well as helping to help explore the possible roles and functions of the important stress genes in nutrient utilisation.

Previously, it has been shown that various carbon sources affect key functions such those related to virulence in *L. monocytogenes*. In the presence of freely utilisable sugars such as cellobiose key virulence genes in *L monocytogenes* are suppressed. Some workers have suggested that some sugars may form part of a global catabolite control system. Studying this can help us develop a greater understanding of carbon source utilisation and aid in a greater understanding of the biology of such organisms (Behari and Youngman, 1998).

The ability to use specific carbon sources, such as glutamate, has been linked to key stress responses in a number of bacteria including *L. monocytogenes* (Feehily and Karatzas, 2013). Therefore, we investigated if removal of key stress genes from *L. monocytogenes* might have a significant impact upon the organism's ability use individual carbon sources. The removal of the *gadD1* and *lmo0913* genes did not provide any significant change in their ability to utilise individual carbon sources compared to the WT. The *gadD1* gene, which encodes for one of three decarboxylases, has been identified as a supporting mechanism for organism's ability to grow under mild acidic conditions (Cotter et al., 2005). The *gadD1* has not been previously associated with any metabolic mechanisms and our results are pointing to this direction, as its removal did not affect the utilisation of any carbon sources in comparison to the WT.

lmo0913 who's expression is regulated by SigB, is known to play a role in catabolism of GABA as it encodes for a succinic semialdehyde dehydrogenase (SSDH) which forms a part of the γ-aminobutyrate shunt. The *lmo0913* has previously shown to play a role in acid resistance of *L. monocytogenes* (Feehily et al., 2013). The GABA shunt, might be a key to the recovery of carbon lost through the citric acid cycle (Abram et al., 2008). In *E. coli* it has been shown that key components of the GABA shunt are upregulated during carbon source starvation (Metzner et al., 2004) and as such it was thought that possibly the removal of the *lmo0913* might influence carbon source utilisation in *L. monocytogenes.* However, this experiment suggests that the removal of *lmo0913* does not adversely affect carbon source utilisation in *L. monocytogenes.*

The removal of the *gadD2* did have an impact on carbon source utilisation as Δ*gadD*2 performed less well on four carbon sources, 3-0-b-D-galactopyranosyl-arabinose, dihydroxyfumaric acid, sorbic acid and fucose (Fig 5.3 A-D Appendix 2) two carbohydrates, and two carboxylic acids. The *gadD*2 gene encodes for a glutamate decarboxylase in *L. monocytogenes*. This gene is key to the function of both the intracellular and extracellular GAD system, which is the most important mechanism of acid resistance in *L. monocytogenes*. As it is key when dealing with acidic environments, it is unsurprising that some of the acidic carbon sources such as dihydroxyfumaric acid and sorbic acid would be less suitable metabolites for the Δ*gadD2* mutant.

Interestingly it has been previously observed that *L. monocytogene*s uses a large set of genes for intermediate utilization of carbohydrates such fucose (Schauer et al., 2010), which is commonly found in the mammalian gut it is possible that *gadD*2 may also fall in to this category which has not been observed previously.

The removal of *gadD3*, which encodes for a glutamate decarboxylase dedicated to the intracellular GAD system (Feehily et al., 2014), was noted to be slightly worse at utilising laminarin than the WT. Laminarin is a carbohydrate and is one of the most abundant carbon sources for marine prokaryotes being found in sources such as brown seaweed. Extracts of laminarin have been shown to have and antimicrobial effect upon *L. monocytogenes* and its possible that removing the *gadD3* may have increased the sensitivity of this strain to this stressor (Alderkamp et al., 2007, Kadam et al., 2015).

SigB plays a key role in both virulence and dealing with environmental stress (Chaturongakul et al., 2008). Its removal may cause increased protein expression and has been suspected as being integral to the function of genes involved in carbon metabolism. In addition, it has been shown that SigB plays an important role in dealing with carbon source starvation upon glucose depletion, with a rapid decrease in cell viability being observed in the Δ*sigB* mutant in glucose-depleted media (Ferreira et al., 2001).

Here we demonstrate a broader investigation of the role of SigB upon the nutritional competence of *L. monocytogenes* 10403S with the use of a wide variety of carbon sources. In the Δ*sigB* mutant carbon source utilisation profile was seen to differ greatly from the WT with the mutant being found to grow significantly better than the WT on 14 different carbon sources and significantly worse on 2 carbon sources.

The 14 carbon sources where the Δ*sigB* outperformed the WT in terms of growth [Appendix 2, Fig 5.5, A-H, J- K and M-P] contain a number of interesting groups. The largest of these being a range of carbohydrates including a-D-lactose (a naturally occurring sugar*)*, glucuronamide, a-methyl-D-glucoside (a synthetic analogue of maltose), L-sorbose (a naturally occurring sugar) and turanose (a naturally occurring reducing disaccharide communally utilised by bacteria) (Table 5.6). a-D-lactose is found in milk, which is a

common vehicle for *L. monocytogenes*, and as such it seem counter intuitive for the organism's key stress regulator to limit its ability to utilise such sources.

Furthermore, several nucleosides (thymidine, adenosine, 2`-deoxyadenosine and inosine; table 5.6) when utilised as sole carbon sources seemed to favour the growth of Δ*sigB* compared to the WT. Thymidine and adenosine are both formed from pyrimidine bases found in the nucleic acid of DNA. Thymidine is pyrimidine base formed of thymine and deoxyribose and adenosine is formed of adenine linked to ribose. It is also interesting to note that inosine is involved in purine metabolism, as a precursor and a metabolite of adenosine. It is possible that the removal of *sigB* has a detrimental impact on the systems required for metabolising these nucleotides.

Δ*sigB* also grew better than the WT on keto acids and amino acids. With Δ*sigB* showed increased growth compared to the WT on a-ketobutyric acid (produced by the degradation threonine), pyruvic acid (a key intermediate in several metabolic pathways) and b-hydroxypyruvic acid (a derivative of pyruvic acid; Table 5.7). In addition, the removal of *sigB* increased the organism's ability to grow using the amino acid L-alanine, the L-alanyl and glycine dipeptide (ala-gly). Alanine is required for the synthesis of the mucopeptide found in cell walls of many bacteria including *L. monocytogenes* (Thompson et al., 1998) (Table 5.7)

The two compounds that the Δ*sigB* was less well able to utilise were, N-acetyl-D-mannosamine and 2-deoxy-d-ribose (Table 5.6 ($P \leq 0.05$ using a paired student T-test) [Appendix 2, I and L- $P \leq 0.05$ using a paired student T-test]. Interestingly N-acetyl-D-mannosamine acts as precursor to N-acetylneuraminic acid, which is key to the function of a number of transport systems in pathogenic bacteria and has been linked with the ability of *L. monocytogenes* to adhere to macrophages, a key step in the infectious process of many

intracellular pathogens. As such a lower ability to utilise such sources might have a serious impact for such organisms limiting their life cycle (Maganti et al., 1998) (Fig 5 I, Appendix 2).

The ability of *L. monocytogenes* to utilise 2-deoxy-d-ribose is interesting observation aside from the fact that removing *sigB* reduces this ability since a limited number of organisms are capable of utilising this carbon source (Bordi et al., 2003). As such, it is possible that the removal of *sigB* might affect the regulation of the genes involved in mediating this mechanism (Fig. 5 L Appendix 2).

It is logical, therefore, that the removal of *sigB* could enhance the metabolic activity as resources are directed to this activity in expense of numerous stress mechanisms, which are limited in the absence of *sigB*. Previously it has been observed that the removal of *sigB* resulted in faster growth under sub lethal levels of increased salt concentration (0.5 M NaCl) and hyper-resistance to hydrogen peroxide (Boura et al., 2016). However, in these cases there was a stress present while in our experiments there were no stressful conditions.

Previously this effect of negative correlation between the removal of a key stress gene regulator and nutrient utilisation has been observed in a number of organisms including plants, insects and bacteria (Gudelj et al., 2010). In bacteria has been most widely demonstrated in *Escherichia coli* as a trade-off between stress response and nutritional ability a concept described as *s*tress *p*rotection *a*nd *n*utritional *c*apability (SPANC) balance (Ferenci, 2005). The typical bacterial stress response may involve the expression of a large number of genes depending upon the stress such as acidic or osmotic stress. These genes are regulated by a transcriptional regulator protein such as RpoS which in *E. coli*, plays an equivalent role to that of *sigB* in *L. monocytogenes* (Gomes Neto et al., 2015, Ferreira et al., 2001).

In *E. coli* an increase in RpoS levels leads to a decreased expression of sigma factor RpoD which is involved in the expression of generalised housekeeping genes. The RNA polymerase available in the organism is limited and when the expression of one increases the expression of the other decreases. Some of the genes RpoD regulates are responsible for the organism's ability to metabolise key nutrients and grow on a wide range of substrates, which is sacrificed to provide protection against environmental stresses (Gomes Neto et al., 2015, King et al., 2004, Ferreira et al., 2001). In the absence of a *sigB* other alternative sigma factors (some such as σ^C, σ^H and σ^L are also found in *L. monocytogenes*) (Chaturongakul et al., 2008) normally responsible for housekeeping direct RNA polymerase towards the increased transcription of their own regulons resulting in increased carbon utilisation. To our knowledge, this is the first time that this trade-off between stress and housekeeping functions (SPANC balance) is demonstrated in a Gram positive organism.

The osmotic stress profile of *L. monocytogenes*, as assessed through phenotypic microarrays, showed that the organism was capable of growth under the majority of conditions provided on the PM 9 plate (Fig 5.6), with the exception of high concentrations >100mM of sodium benzoate where all tested strains of *L. monocytogenes* failed to grow (data not shown). It has been shown previously that *L. monocytogenes* can be sensitive to benzoic acid (Heavin et al., 2009). In this study *L. monocytogenes* 10403S displayed this sensitivity but with concentrations above 50 mM presenting a significant challenge to growth with no growth being displayed above this contraction.

The osmotic profiles of the three GAD mutants (Δ*gadD1* Δ*gadD2* and Δ*gadD3)* did not show a significant difference from that of the WT strain with the notable exception of sodium chloride. When exposed to high concentrations, 9% or 10 %, all three mutants did not grow as well as the WT (*P*.> 0.05 using a paired student test) (Table 5.7) [Appendix 2, Fig 5.8 A-

C]. A similar profile was observed in the Δ*sigB* mutant which also performed less well than the WT in the presence of high concentrations of NaCl (Table 5.7) [Appendix 2, Fig 5.9 A]. *L. monocytogenes* is typically capable of growing up to 10% NaCl and while *sigB* has in the past been shown to be key to *L. monocytogenes* growth under osmotically stressful conditions. The above it had been suggested by previous studies and that acid tolerance responses might be linked with osmotic stress response (Hill et al., 2002). However, Δ*gadD1* Δ*gadD2* and Δ*gadD3* have not previously been shown to play a role in osmotic stress tolerance (Gardan et al., 2003, Becker et al., 1998). This provides an important indication that the GAD system may be linked to more than in acid resistance to organisms such as *L. monocytogenes.*

It might have been expected that the removal of *sigB* could have had a deleterious effect on the ability of *L. monocytogenes* to deal with osmotic pressure as its presence has been linked with the regulation of genes associated with osmotic stress response such as *opuC* and *Hfq* (Sue et al., 2003, Christiansen et al., 2004). As such, it might be anticipated that its removal could reduce the organism's ability to cope with a high degree of osmotic stress. However, in this series of experiments the Δ*sigB* mutant demonstrated an ability to deal with some specific osmotic stresses better than the WT strain with increased growth on sodium lactate 4%-11% and sodium nitrite [Appendix 2, Fig 5.9 B and C]. Both of these are commonly utilised as food preservatives. Sodium lactate at concentrations of 4% and above has been shown to be highly effective anti-listerial (Chen and Shelef, 1992). Sodium nitrite has also been used against *L. monocytogenes* (Pelroy et al., 1994). In this study the WT strain did not perform well but was capable of growth in the presence of both osmolytes while Δ*sigB* demonstrated significantly higher levels of growth ($P.> 0.05$; paired student test).

This effect could be due to the previously reported growth advantage of Δ*sigB* in the presence of sub lethal levels salt. Both compounds are salts and could have a similar effect to NaCl while the anions would exert the antimicrobial effects only at acidic conditions, which were not present in the current experimental setup.

5.5 Conclusions.

*L. monocytogene*s carbon source profile indicates that it is capable of surviving in a variety of different environments which enhances its ability to act as an effective pathogen. These experiments demonstrate that *L. monocytogenes* 10403S like *E. coli* sacrifices a degree of nutritional competence to provide its self with a greater degree of self-preservation demonstrated by the increased carbon source utilisation shown by the Δ*sigB* this SPANC balance has not previously been observed in gram positive bacteria. Furthermore, this study also suggests that the GAD genes may also be involved in coping with osmotic stresses as well as acid stresses, which has not previously been explored as shown by the decrease in growth demonstrated by the GAD knockout mutants in the presence of NaCl. The understanding of *L. monocytogene*s methods of dealing with stress conditions provides useful information as to how this organism may be neutralised within the food chain.

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5.7 Referances

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CHAPTER 6:

The effect of sodium fumarate on the growth and survival of *Listeria monocytogenes, Escherichia coli* and *Salmonella* Typhimurium in combination with a commercial acidic treatment.

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Abstract

The efficiency of aqueous-based sanitisers is key to the safety of fresh or minimally processed produce. However, it has been demonstrated that, while existing treatments may inhibit the growth of foodborne pathogens, their effects can be limited resulting in significant number of foodborne illness cases and outbreaks. In the present study, the increased efficacy of low pH treatments when supplemented with fumaric acid or sodium fumarate, compared to current organic acid treatments and chlorine-based sanitisers, is demonstrated. According to the results obtained, the presence of fumaric acid led to significant antimicrobial effects against a wide range of foodborne pathogens including *Listeria monocytogenes, Escherichia coli,* and *Salmonella* Typhimurium on a range of fresh produce. Moreover, under acidic conditions, supplementation with sodium fumarate had a significant bactericidal effect against the food spoilage bacterium *Lactobacillus plantarum.*

6.1. Introduction

Fresh fruits and vegetables, which are classified as minimally processed produce (MPP), have in the past been considered relatively low risk, in terms of food safety. However, incidences of foodborne illness, caused by organisms such as *Escherichia coli*, *Salmonella* and *Listeria monocytogenes*, associated with fresh and MPP appear to have increased rapidly (Murray et al., 2017). Several studies now suggest that up to 46% of all foodborne illness is associated with fruits, nuts and vegetables (Beuchat, 2002, Painter et al., 2013).

The reasons behind this phenomenon are believed to include: a) an increase in the global consumption of MPP, b) wider distribution of produce, thus allowing for more widespread incidents to occur, c) increasingly complex food chains leading to traceability and control issues, combined with poor production and handling techniques (Murray et al., 2017, Berger et al., 2010, Carmichael et al., 1998, Gil et al., 2009, Olaimat and Holley, 2012, Ramos et al., 2013).

There is currently, a wide range of treatments and technologies designed to help reduce levels of food borne pathogens. The most common treatments include washes or dips to solutions designed to reduce levels of pathogens. These solutions may contain chlorine, organic acids (such as citric or acetic), hydrogen peroxide, peroxyacetic acid, calcium, ozone and electrolysed water (Gomez-Lopez,. 2012, Al haq and Gomez-Lopez, 2012 Al-Hashimi et al., 2015, Simons and Sanguansri, 1997, Olaimat and Holley, 2012, Ramos et al., 2013, Ölmez and Kretzschmar, 2009). While these treatments are widely used, it has been suggested that they have limited effectiveness and are therefore often used in combination with other physical treatments (Murray et al., 2017). Examples of such physical treatments may include irradiation, cold plasma pulsed light, ultrasound and high pressure (Niemira, 2012, Lynch et al., 2009, Sagong et al., 2011, Lou et al., 2011).

Chlorine based disinfectants have been popular with producers as an effective and inexpensive sanitiser. However, there has been concern amongst consumers that these may have adverse health impacts associated with some carcinogenic chemical by-products of this type of treatment; this has driven many producers to look for more acceptable alternatives (Rico et al., 2007). Alternatives include organic acids, which are already used by the food industry as preservatives, and flavour enhancers, i.e. citric acid and acetic acid (Murray et al., 2017, Rico et al., 2007). These acids are "Generally Recognised as Safe" (GRAS), making them easily accepted by consumers and producers (Rico et al., 2007) and therefore, they may be used individually or in combination as treatments for MPP (Ramos et al., 2013).

Organic acid treatments such as citric acid, acetic acid, tartaric acid and malic acid have been found to have a strong antimicrobial action (Ölmez and Kretzschmar, 2009, Rico et al., 2007). The mode of action of these acids is based upon the fact that weak organic acids can exist as either charged or uncharged. The uncharged form of the weak acid is lipid permeable and can therefore diffuse easily into the cytoplasm of a bacterium (Hirshfield et al., 2003). As intracellular pH of many organisms is normally close to neutral, further dissociation of the undissociated molecules that entered the cell takes place releasing more protons intracellularly leading to further cellular damage. The acid may also interfere with membrane transport and permeability which can results in the death of the bacteria (Rico et al., 2007, Hirshfield et al., 2003).

An example of such a commercial organic acid treatment referred to here as AM, which is a combination of common organic acids, some of which are assessed here, resulting in a low pH solution. This is used to wash fresh produce at a pH range between 2.4 and 2.8. It has been shown that, some organic acids may have different levels of antimicrobial activity that may not be explained by the classical model proposed for weak acids. For example, it has been previously reported that fumaric acid is capable of interacting with key mechanisms of

acid resistance of certain foodborne pathogens, possibly resulting in an increased antimicrobial action under acidic conditions (Barnes and Karatzas, unpublished)**.** This is considered advantageous compared to other acids such as acetic and lactic acids (Podolak et al., 1996, Comes and Beelman, 2002). Through further exploitation of these additional antimicrobial abilities, obtained with specific organic acids, it might be possible to improve the effectiveness of AM as a treatment for fresh produce.

Fumaric acid is a dicarboxylic organic acid, used within the food industry as an acidity regulator (Hemat, 2003). It is effective against a number of different organisms including *Escherichia coli* O157:H7, *L. monocytogenes,* and *Salmonella.* Its effectiveness has been demonstrated for a number of food products including on fresh produce (Kondo et al., 2006, Miller and Kaspar, 1994, Podolak et al., 1996).

One major issue regarding the use of fumaric acid, preventing it from being widely used within the food industry, is its low level of solubility that significantly limits its range of potential application. However, its salts, including sodium fumarate, may be employed as alternatives (Lee, 2014). The aim of this study was to examine the antimicrobial effect of its more soluble salt, sodium fumarate, when acting in combination with the AM treatment, and to investigate its potential application as an antimicrobial for fresh produce.

6.2 Materials and Methods

6.2.1 Bacterial strains and growth conditions

All strains used (Table 6.1), were revived from 2 ml cryovials stored at -80 °C cryopreserved with the aid of 7% dimethyl sulfoxide (DMSO). *L. monocytogenes* 10403S was subsequently grown on Brain Heart Infusion (BHI) agar (LABM, Lancashire UK), *L. monocytogenes* LO28 was grown on Tryptic Soy Broth Agar (Oxoid, UK) supplemented with 5% yeast extract (TSBY agar; Oxoid, UK). *E. coli* O157:H7, *E. coli* K-12 and *S.* Typhimurium 30 were grown on Lysogeny Broth (LB) (LABM, Lancashire UK) whereas *Lactobacillus plantarum* WCFS1was grown on De Man, Rogosa and Sharpe (MRS) agar (LABM, Lancashire UK). All cultures were incubated at 37°C overnight with *Lb. plantarum* being incubated under anaerobic conditions. Three colonies from each plate were taken with an inoculation loop and transferred into Brain Heart Infusion broth (BHI; LABM, Lancashire UK), TSBY broth (Oxoid, UK), LB broth (LABM, Lancashire UK) or MRS broth (LABM, Lancashire UK) in 10 ml Bijoux bottles as mentioned before. These overnight cultures were used to inoculate 250 ml Erlenmeyer flasks (1% inoculum), containing the appropriate media, which were subsequently incubated overnight at 37°C with shaking at 150 rpm for 18 h for all bacteria, apart from *Lb. plantarum* which was incubated under anaerobic conditions without any shaking.

Table 6.1: List of strains examined in this study.

Strains	Relevant properties	Source
L. monocytogenes 10403S	Serotype 1⁄2a, wild type (WT).	Wiedmann et al., (1998)
L. monocytogenes LO28	Serotype 1/2c, wild type.	Cotter et. al. (2001)
E. coli K-12	Wild type strain	KEIO collection Baba et al. (2006)
E. coli O157:H7	Non verocytotoxic strain not posing either *stx1* or *stx2* shiga toxin genes (Woodward et al., 2003).	Central Public Health Laboratory, London. National Culture Type Collection (NCTC)12900
S. Typhimurium 30	Designation DT104 serotype 30 from bovine source (Payne et al., 1992).	Jorgensen et al. (2000)
Lactobacillus plantarum WCFS1	Originally isolated from human saliva.	Kleerebezem et al. (2003)

6.2.2 Determination of minimum inhibitory concentration (MIC).

Concentrations ranging from 0 – 200 mM of tartaric acid, citric acid, malic acid and sodium fumarate were mixed with a 1% solution of an overnight culture of either *L. monocytogenes* LO28, *E. coli* O157:H7 or *S.* Typhimurium 30. Due to limitations regarding the solubility of fumaric acid, lower concentrations ranging from 0 to 34 mM were prepared and inoculated in the same way. Cultures were inoculated into 96 well plates and incubated at 37°C with shaking. The optical density of the cultures was assessed at 620 nm (OD_{620nm}) after 24 h using a Tecan's Sunrise absorbance microplate reader (Tecan Group AG, Switzerland) and a Magellan™ data analysis software in order to detect the MIC for each case.

6.2.3 Survival under extreme acidic conditions in the presence of sodium fumarate.

Starting cultures of 20 ml volume were prepared in BHI for *L. monocytogenes* 10403S and LB for *E. coli* K –12 and *S.* Typhimurium 30. Stock cultures were prepared as previously

described. Subsequently, cultures were further grown in 250 ml Erlenmeyer flasks at 37°C under agitation at 150 rpm. For all three organisms were an acid challenge was performed using an organic acid mix (AM) at pH 2.8 in the presence of 10 mM of sodium fumarate. For *L. monocytogenes* 10403S an additional acidic challenge was performed using AM at pH 2.4 in the presence of 10 mM of sodium fumarate. Control experiments were performed using unaltered AM at either pH 2.8 or 2.4 in the absence of sodium fumarate.

One hundred microliter samples were taken for *L. monocytogenes* 10403S at 0, 30, 60, and 120 sec for samples adjusted to pH 2.8 and 0, 60, 120, 240 and 360 sec for samples at pH 2.4. Samples for *E. coli* K–12 and *S.* Typhimurium 30 were taken at 0, 30, 60 and 120 sec. All samples were then placed in 900 µl of maximum recovery diluent (MRD; Oxoid Limited, Hampshire UK). Ten-fold serial dilutions were prepared and 10 µl of each dilution was plated onto BHI agar plates and incubated at 37°C for 24 h. Colonies were then counted to assess the concentration of cells in the culture at each time point. All experiments were performed in triplicate.

6.2.4 Comparison of treatment's on fresh produce.

In order to examine the effect of different treatments on fresh produce three types of fruit were selected: a) apples (Granny Smith variety), b) pears (Conference variety) and c) strawberries (Sweet Eve variety). All fruits were washed with de-ionised water and were left to dry. Following this, 10 g samples of each fruit was weighed out and washed with 70% ethanol to inactivate any residual bacteria. Samples were allowed to air dry for 1 h in a laminar flow hood. Each sample was then inoculated with 100 µl of overnight culture of either *E. coli* O157:H7, *L. monocytogenes* LO28 or *S.* Typhimurium 30 prepared as previously described. Samples were then placed in petri dishes and stored at 4°C for 24 hours

to allow for bacterial attachment and to mimic conditions under which such produce might be stored or transported.

The following four treatment solutions were prepared: a) deionised water, b) deionised water with 100 ppm free chlorine, prepared using calcium hypochlorite, c) AM at pH of 2.4 and d) AM at pH of 2.4 supplemented with 25 mM of sodium fumarate. Each sample of inoculated fruit was submerged in 50 ml of each of these treatments for 5 min. Samples were then transferred to a stomacher bag containing 90 ml of MRD. The sample was left for 1 min in a Colworth stomacher 400 (Seaward, UK) in order to homogenise. Samples of 100 µl were then placed in 900 µl of MRD. Ten-fold serial dilutions were prepared and 10 µl of each dilution was plated onto LB agar for *E. coli* O157:H7 and *S.* Typhimurium 30 and TSBY agar for *L. monocytogenes* LO28 (LABM, Lancashire UK). All plates were incubated at 37°C 24 hours. Colonies were then counted to assess the concentration of cells for each sample. All experiments were performed in triplicate.

6.2.5 Survival of *E. coli* O157:H7, *L. monocytogenes* LO28 and *S.* typhimurium 30 against reformulated AM organic acid treatments.

Cultures of *E. coli* O157:H7, *L. monocytogenes* LO28 and *S.* Typhimurium 30 were prepared using the protocol described above (section 6.2.1). All samples were transferred into 50 ml Falcon® tubes (VWR, Leicestershire UK). Samples were then centrifuged (Eppendorf, Hamburg Germany) at 12,000 xg for 10 min. The supernatant was then discarded and the pellet homogenised using an inoculation loop. Next, 20 ml of either AM (pH 2.8), HCL (pH 2.8) or one of the organic acid treatments described in Appendix 3 (pH 2.8) was added to the sample and then vortexed for 10 sec. Subsequently100 µl samples of *E. coli* O157:H7 and *L. monocytogenes* LO28 were taken at 0, 5, 10 and 20 minutes. Samples of *S.* Typhimurium 30

were taken at 0, 30, 60 and 120 sec. The 100 µl samples were then placed in 900 µl of MRD. Ten-fold serial dilutions were prepared and 10 µl of each dilution were plated onto LB agar (LABM, Lancashire UK) for *E. coli* O157:H7 and *S.* Typhimurium 30 or TSBY agar *L. monocytogenes* LO28 and all plates were incubated at 37°C for 24 h.

6.2.6 Survival of *Lb. plantarum* under acidic conditions in the presence of sodium fumarate

Survival experiments were also performed for *Lb. plantarum.* Twenty ml cultures were prepared in MRS, using stock cultures prepared as described previously, and grown in 50 ml Falcon tubes at 37°C under anaerobic conditions. Acid challenge took place following the addition of 0, 5, 10, 15, 20, and 25 mM of sodium fumarate with the pH of the cultures adjusted to pH 3.0 using a 1 M HCl solution.

One hundred µl samples were obtained prior to the acid challenge and every 5 min for a period of 20 min. Samples were placed in 900 µl of MRD (Oxoid Limited, Hampshire UK). Ten-fold serial dilutions were prepared and 10 µl of each dilution were plated onto MRS agar plates and incubated anaerobically at 37°C overnight. Colony counts were carried out to assess the concentration of cells in the culture at each time point. All experiments were performed in triplicate.

6.2.7 Statistical analysis

In all cases, experiments were run in triplicate unless otherwise stated. Subsequently the results were assessed using paired Student's T-test. A P-value of < 0.05 denoted statistically significant results which have been indicated by an asterisk in the relevant figures.

6.3 Results.

6.3.1 Growth in the presence of selected acids

The MIC of a variety of acids against a variety of microorganisms was assessed (Table 6.2). Sodium fumarate did not show an MIC for the range of the concentrations tested, and no inhibition was observed as expected since it is a salt and does not affect the pH. It has to be noted here that the concentrations of fumaric acid solutions tested were low due to limitations regarding its solubility (up to 0.7% w/v) (Gangl et al., 1990). Different concentrations of fumaric acid were tested, compared to the rest of the acids, due to its poor solubility.

Table 6.2: MICs observed for the organic acids tested against *L. monocytogenes*, *E. coli* and *S.* Typhimurium.

Organic acid	***L. monocytogenes* LO28**	***E. coli* O157:H7**	***S.* Typhimurium 30**
Tartaric acid	50 mM	25 mM	25 mM
Citric acid	50 mM	12.5 mM	25 mM
Malic acid	50 mM	25 mM	50 mM
Fumaric acid	34 mM	34 mM	34 mM
Sodium fumarate	> 200 mM	> 200 mM	> 200 mM

6.3.2 Acid survival of *E. coli* K-12, *L. monocytogenes* 10403S *and S.* Typhimurium 30 when exposed to low pH acid treatments.

The addition of 10 mM of sodium fumarate to AM at pH 2.8 significantly increased the effectiveness of the original treatment against *L. monocytogenes* 10403S resulting in

reductions of 1.5 log CFU/ml after 240 sec and 4.9 log CFU/ml after 360 sec when compared to AM alone at a pH of 2.8 alone ($P < 0.05$; paired T-test, Fig. 6.1 A).

The presence of 10 mM sodium fumarate also increased the effectiveness of AM against *L. monocytogenes* 10403S at pH 2.4 with a significant difference ($P < 0.05$; paired T-test) being noted after 30 seconds. In more detail, the additional log reduction of CFU/ml conferred by the supplementation of AM with 10 mM sodium fumarate at pH 2.8 was 1.3, 2.48 and 4.95 after 30, 60 and 120 sec respectively ($P < 0.05$; paired T-test)t, Fig. 6.1 B).

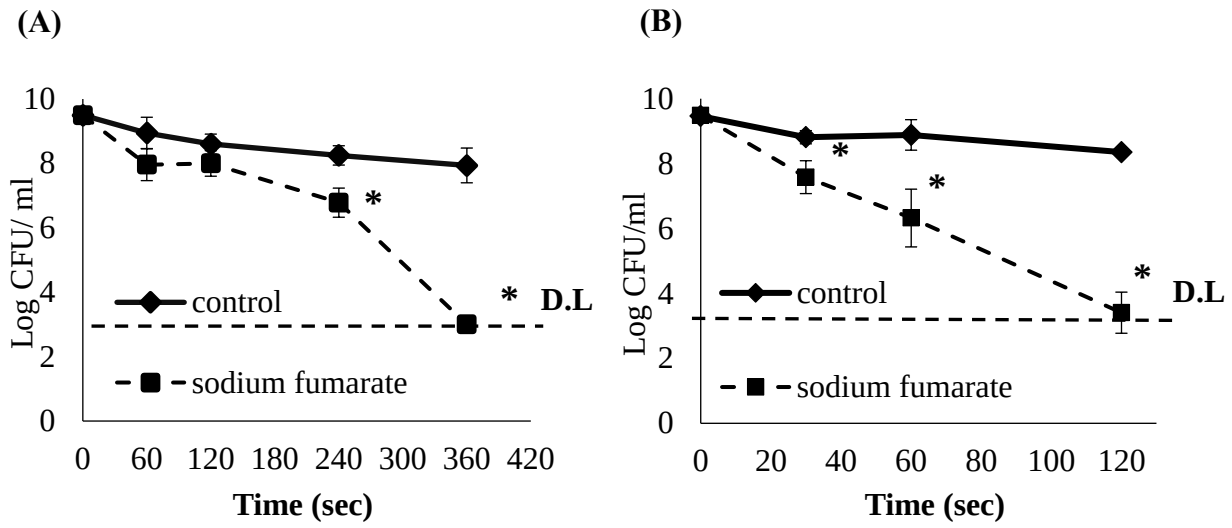

Fig. 6.1 (A) Survival of *L. monocytogenes* 10403S at a pH value of 2.4 using AM in the presence (■) and absence (◆) of 10 mM of sodium fumarate, and **(B)** survival of *L. monocytogenes* 10403S adjusted to a pH of 2.4 using AM in the presence (■) and absence (◆) of 10 mM of sodium fumarate **(***: statistical significance using a paired student T -Test < 0.05, while D.L denotes detection limit of the experimental setup).

The addition of 10 mM sodium fumarate showed to have a dramatic effect on the survival of *E. coli* K-12 when challenged to a pH value of 2.8 using AM. Sodium fumarate caused a significant decrease in the survival of this organism with a difference in reduction of 3.5 log CFU/ml **(**$P < 0.05$; paired T-test) T-test) after 30 sec, by 60 sec the supplemented treatment had reached the detection threshold after only 60 sec (Fig. 6.2 A).

The presence of 10 mM of sodium fumarate decreased the level of survival of *S.* Typhimurium 30 as in the case of *L. monocytogenes* 10403S. Again, significant differences in reduction were observed of 1.3, 1.3 and 2.2 log CFU/ml after 30, 60 and 120 seconds respectively when compared to AM alone at a pH of 2.8 **(**P < 0.05; paired T-test), Fig. 6.2 B).

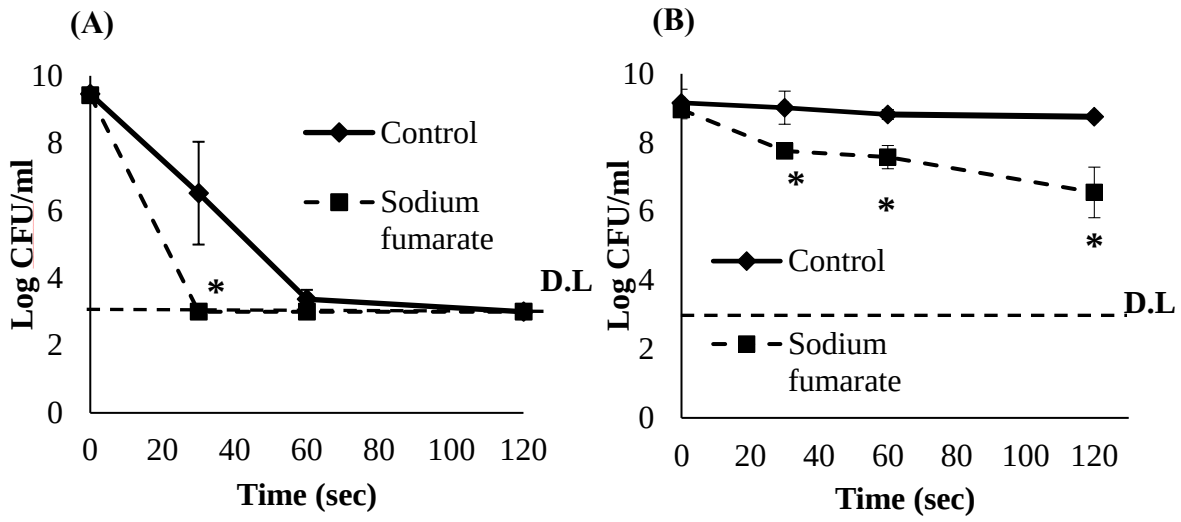

Fig. 6.2. (A) Survival of *E.coli* K-12 at a pH value of 2.4 using AM in the presence (■) and absence (◆) of 10 mM of sodium fumarate, and **(B)** survival of *S.* typhimurium 30 when adjusted to a pH of 2.4 using AM in the presence (■) and absence (◆) of 10 mM of sodium fumarate (*: Statistical significance using a paired student T -Test < 0.05, while D.L denotes detection limit of the experimental setup.

6.3.3 Survival of *E. coli* O157:H7, *L. monocytogenes* LO28 and *S.* Typhimurium 30 on fresh produce when exposed to various aqueous treatments.

6.3.3.1 Strawberries

In these experiments we tried to assess different treatments on strawberries that were inoculated with the three different foodborne pathogens used in this study. Regarding *L. monocytogenes* LO28, 5 min treatments with water, 100 ppm chlorine and AM at pH 2.4, resulted in minor log reductions of CFU/ml of 0.55, 0.67 and 0.83 respectively. Interestingly,

AM supplemented with 25 mM sodium fumarate (pH 2.4) resulted in a statistically significant 2.4 log reduction of CFU/ml **(**$P < 0.05$; paired T-test). The latter treatment was the most successful and it was significantly more effective than chlorine and AM by 1.7 and 1.6 Log CFU/ml respectively (Fig. 6.3 A).

For *E. coli* O157:H7 a similar pattern was observed as in the case of *L. monocytogenes* LO28 with the 5 min treatments of water, 100 ppm chlorine and AM at pH 2.4, resulting in minor log reductions of CFU/ml of 0.78, 0.89 and 1.03 respectively (Fig. 6.3 B). The only difference was that the latter treatments resulted in statistically significant reductions which however, were not substantial. Similarly, to *L. monocytogenes* AM (pH 2.4) supplemented with 25 mM sodium fumarate resulted in a substantial and statistically significant reduction of 2.28 Log CFU/ml **(**$P < 0.05$; paired T-test). Again, the usage of fumarate together with AM resulted in a more effective treatment than chlorine and AM by 1.4 and 1.25 Log CFU/ml respectively (Fig. 6.3 B).

Also, with *S.* Typhimurium 30 the 5 min treatments of water, 100 ppm chlorine and AM at pH 2.4, resulted in minor log reductions of CFU/ml of 0.17, 0.51 and 0.97 respectively without any statistical significance (Fig. 6.3 C). Once more the 5 min treatment of AM supplemented with 25 mM sodium fumarate (pH 2.4) resulted in a statistically significant reduction of 2.14 Log CFU/ml. The combined treatment had an improved effectiveness of 1.63 and 1.16 Log CFU/ml compared to chlorine and AM respectively (Fig. 6.3 C).

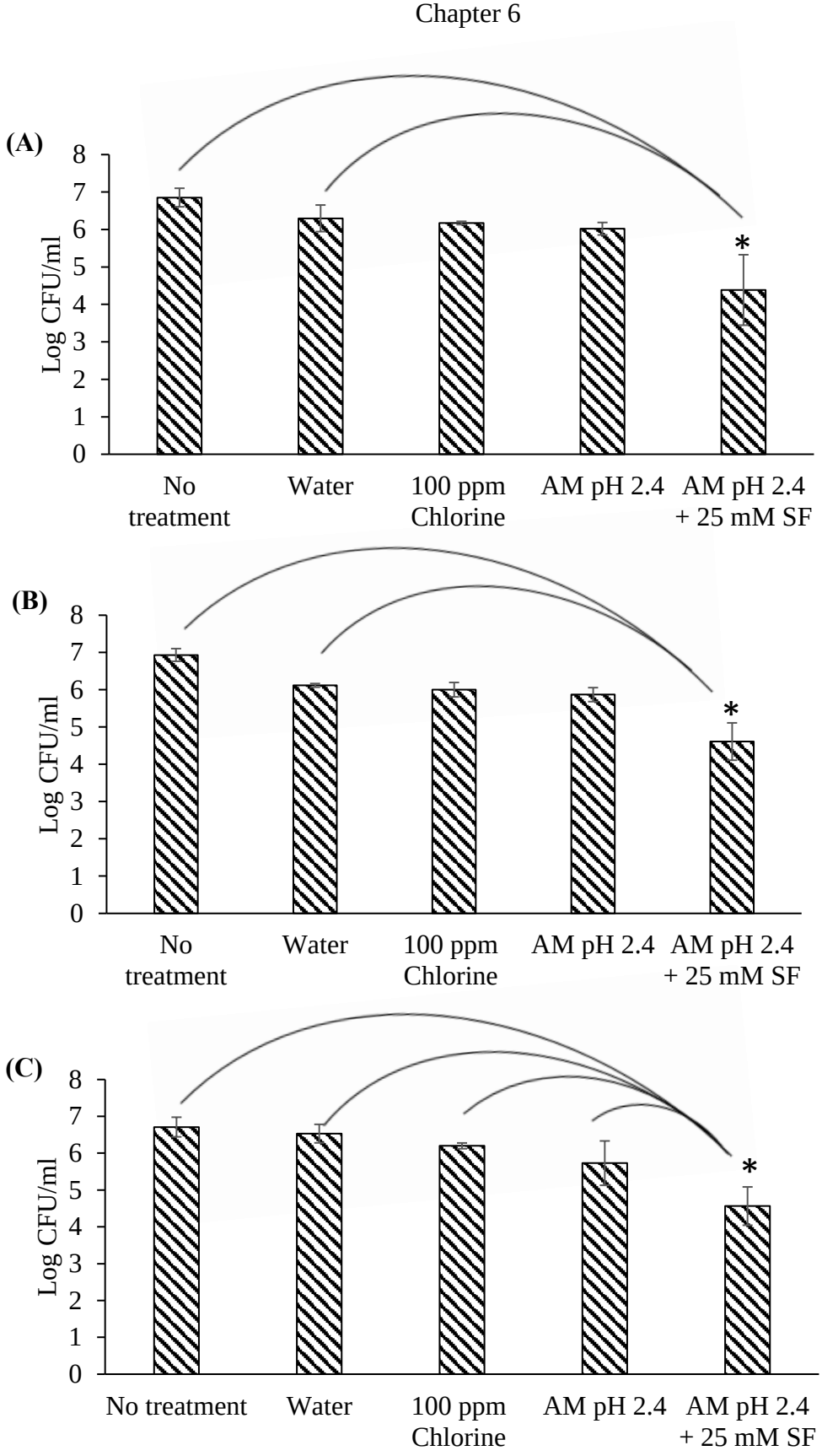

Fig. 6.3. Survival of **(A)** *L. monocytogenes* LO28, **(B)** *E. coli* O157:H7 and **(C)** *S.* typhimurium 30 inoculated on to the surface of strawberries using treatments with water, 100 ppm chlorine, AM (pH 2.4) and AM supplemented with 25 mM sodium fumarate (pH 2.4) (*: statistical significance using a paired student T -Test < 0.05).

6.3.3.2 Pears

In the experiments with pears similarly the combined treatment of AM and sodium fumarate was the most effective. In the case of *L. monocytogenes* LO28, 5 min treatments with water, 100 ppm chlorine and AM at pH 2.4, resulted in minor log reductions of CFU/ml of 0.65, 0.87 and 1.10 respectively, with the latter two being statistically significant **(**$P < 0.05$; paired T-test). The combined treatment of AM and 25 mM sodium fumarate (pH 2.4; 5 min) resulted in a statistically significant 3.21 log reduction of CFU/ml **(**$P < 0.05$; paired T-test). The latter treatment was the most successful and it was significantly more effective than chlorine and AM by 2.30 and 2.11 Log CFU/ml respectively (Fig. 6.4 A).

With *E. coli* O157:H7 the 5 min treatments of water, 100 ppm chlorine and AM at pH 2.4, resulted in minor log reductions of CFU/ml of 0.89, 0.94 and 1.01 respectively (Fig. 6.4 B). All these reductions were statistically significant with the exception of chlorine **(**$P < 0.05$; paired T-test). Once more, the combined treatment of AM and 25 mM sodium fumarate resulted in a substantial and statistically significant reduction of 2.61 Log CFU/ml **(**$P < 0.05$; paired T-test). This treatment was more effective than chlorine and AM by 1.67 and 1.60 Log CFU/ml respectively (Fig. 6.4 B).

In the case of *S.* Typhimurium 30 the 5 min treatments of water, 100 ppm chlorine and AM at pH 2.4, resulted in minor but statistically significant log reductions of CFU/ml of 0.56, 0.68 and 0.79 respectively (Fig. 6.4 C). Again, the 5 min treatment of AM supplemented with 25 mM sodium fumarate (pH 2.4) resulted in a statistically significant reduction of 2.45 Log CFU/ml. The combined treatment was more improved by 1.77 and 1.66 Log CFU/ml compared to that of chlorine and AM respectively (Fig. 6.4 C).

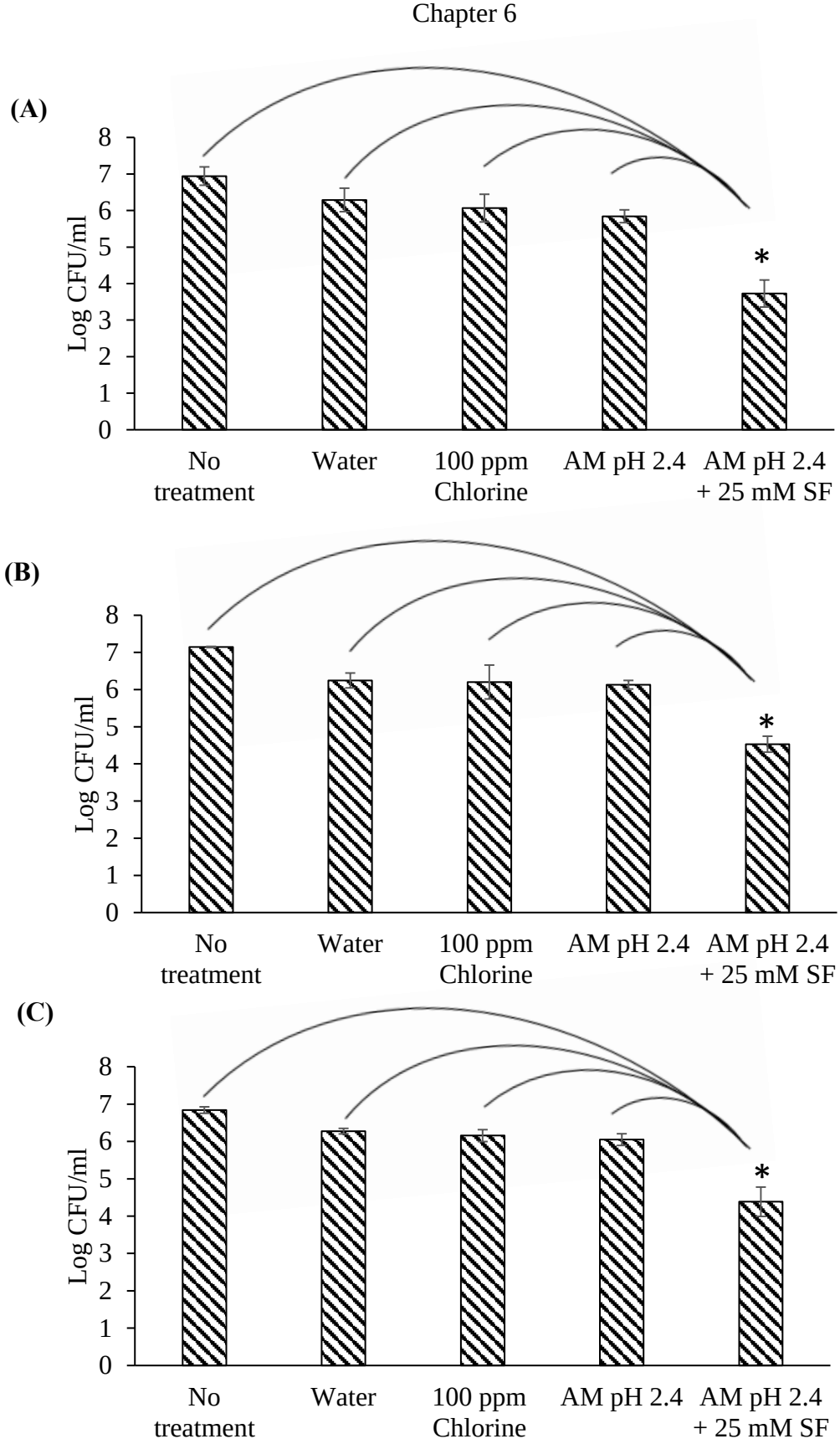

Fig 6.4. Survival of **(A)** *L. monocytogenes* LO28, **(B)** *E. coli* O157:H7 and **(C)** *S.* typhimurium 30 inoculated on to the surface of pears using treatments with water, 100 ppm chlorine, AM organic acid treatment (pH 2.4) and AM supplemented with 25 mM sodium fumarate (pH 2.4) (*: statistical significance using a paired student T -Test < 0.05).

6.3.3.3 Apples

With apples once more, the combined treatment of AM and sodium fumarate was the most effective. When *L. monocytogenes* LO28 was used, 5 min treatments with water, 100 ppm chlorine and AM at pH 2.4, resulted in minor log reductions of CFU/ml of 0.45, 0.68 and 0.67 respectively, with the latter two being statistically significant **(**$P < 0.05$; paired T-test). The combined treatment of AM and 25 mM sodium fumarate (pH 2.4; 5 min) resulted in a statistically significant 2.79 log reduction of CFU/ml **(**$P < 0.05$; paired T-test). The latter treatment was the most successful and it was significantly more effective than chlorine and AM by 2.11 and 2.12 Log CFU/ml respectively (Fig. 6.5 A).

With *E. coli* O157:H7 the 5 min treatments of water, 100 ppm chlorine and AM at pH 2.4, resulted in minor and not statistically significant log reductions of CFU/ml of 0.28, 0.28 and 0.84 respectively (Fig. 6.5 B). Once more, the combined treatment of AM and 25 mM sodium fumarate resulted in a substantial and statistically significant reduction of 2.64 Log CFU/ml **(**$P < 0.05$; paired T-test). This treatment was more effective than chlorine and AM by 2.36 and 1.80 Log CFU/ml respectively (Fig. 6.5 B).

In the case of *S.* Typhimurium 30 the 5 min treatments of water, 100 ppm chlorine and AM at pH 2.4, resulted in minor log reductions of CFU/ml of 0.67, 0.45 and 0.94 respectively (Fig. 6.5 C). From the above only the AM treatment was statistically significant **(**$P < 0.05$; paired T-test). The 5 min treatment of AM supplemented with 25 mM sodium fumarate (pH 2.4) resulted in a statistically significant reduction of 2.93 Log CFU/ml **(**$P < 0.05$; paired T-test). The combined treatment was more improved by 2.47 and 1.98 Log CFU/ml compared to that of chlorine and AM respectively (Fig. 6.5 C).

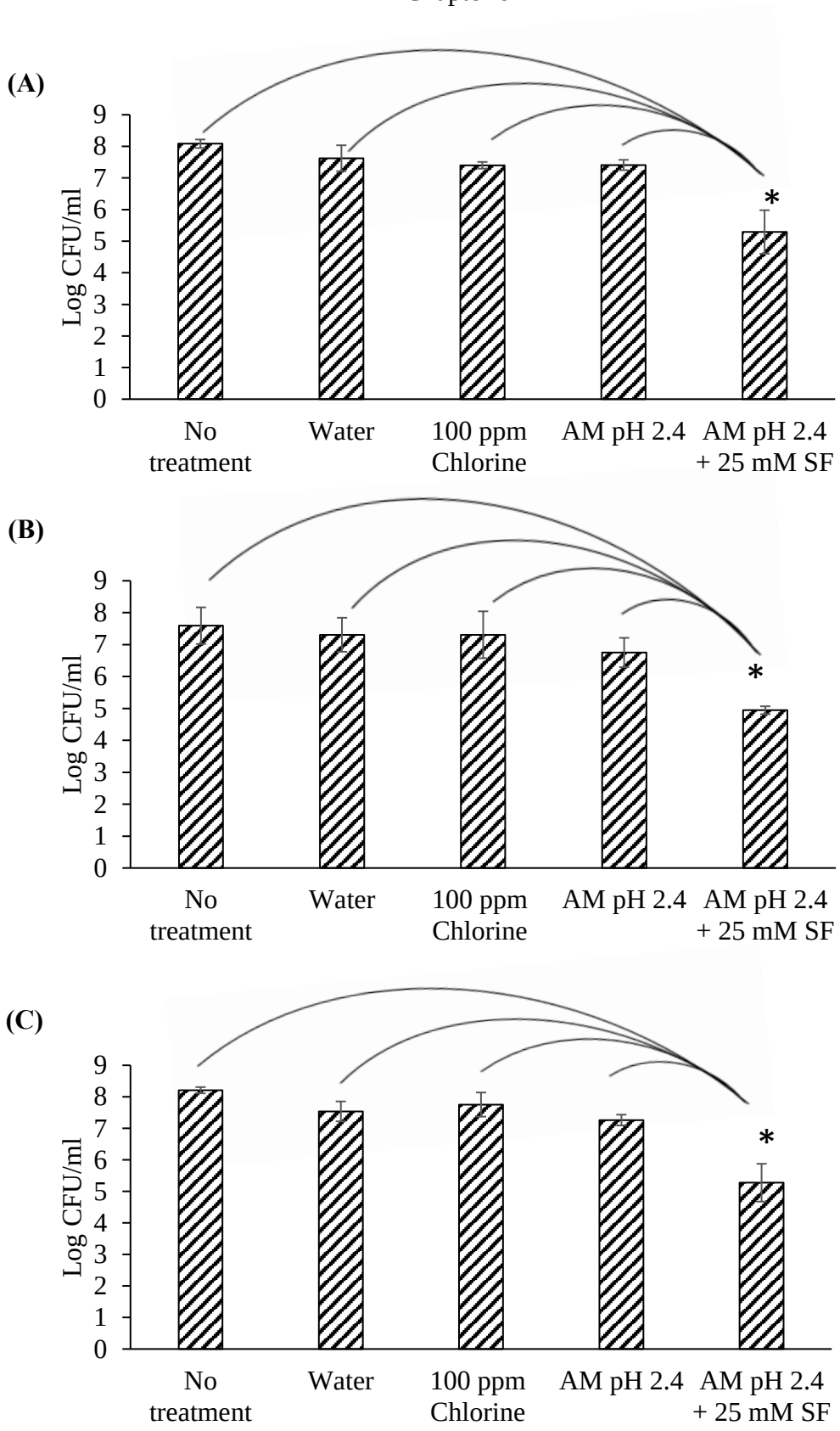

Fig 6.5. Survival of **(A)** *L. monocytogenes* LO28, **(B)** *E. coli* O157:H7 and **(C)** *S.* typhimurium 30 inoculated on to the surface of apples using treatments with water, 100 ppm chlorine, AM (pH 2.4) and AM supplemented with 25 mM sodium fumarate (pH 2.4) (*: statistical significance using a paired student T -Test < 0.05).

6.3.4 Survival of *L. monocytogenes* LO28, *E. coli* O157:H7 and *S.* Typhimurium 30 against various reformulated AM organic acid treatments.

In total, six alternative treatments were examined and tested for their bactericidal effect against *L. monocytogenes* LO28, *E. coli* O157:H7 and *S.* Typhimurium 30. These combinations were based on variations of the composition of the AM (pH 2.8) treatment, with alterations in the proportions of key constituent acids (malic, tartaric, fumaric, citric and trisodium citrate) or with the addition of sodium fumarate. The details regarding the composition of the treatments are given in Appendix 3.

In the case of *L. monocytogenes* LO28, AM treatment (pH 2.8) and HCL (pH 2.8) resulted in minor but significant log reductions of CFU/ml of 1.38 and 0.57 after 20 min. The six reformulated treatments 1, 2, 3, 4, 5 and 6 that were based on the original AM (pH 2.8) brought larger log reductions of CFU/ml 6.10, 3.23, 2.68, 6.10, 3.80 and 2.48 respectively. Of these, four treatments (1, 3, 4 and 5) were found to confer a significant improvement over the original AM treatment after 20 min treatments **(**$P < 0.05$; paired T-test; Fig. 6.6 A).

Against *L. monocytogenes* LO28, treatments 1 and 4 resulted in the most rapid reductions in survival, with log reductions of 6.1 CFU/ml being observed for both treatments within 5 and 10 min CFU/ml **(**$P < 0.05$; paired T-test; Fig. 6.6 A).

In the case of *E. coli* O157:H7, AM treatment (pH 2.8) and HCL (pH 2.8) resulted in significant log reductions CFU/ml of 2.52 and 2.05 after 20 min. The six reformulated treatments 1, 2, 3, 4, 5 and 6 that were based on the original AM (pH 2.8) resulted in 6.07, 2.31, 3.14, 6.07, 2.00 and 3.48 log reduction of CFU/ml respectively. Three of the reformulated treatments (1, 3 and 4) were found to offer a significant improvement over the original AM treatment (pH 2.8) after 20 min **(**$P < 0.05$; paired T-test) (Fig. 6.6 B). Similarly to *L. monocytogenes* LO28, treatments 1 and 4 resulted in the most rapid reductions in

survival, with a log reduction above 6.07 CFU/ml (detection limit) being observed for both treatments at 5 and 10 min CFU/ml **(**$P < 0.05$; paired T-test) (Fig. 6.6 B).

When *S.* Typhimurium 30 was challenged with AM (pH 2.8) and HCL (pH 2.8) after 120 sec log reductions of CFU/ml of 2.25 and 0.48 occurred but these treatments were not found to offer significant reduction. With the six reformulated treatments 1, 2, 3, 4, 5 and 6 that were based on the original AM (pH 2.8) a 6.05, 3.78, 3.42, 6.22, 3.24 and 1.48 log reduction of CFU/ml occurred respectively. In the case of *S.* Typhimurium 30 treatments 1, 2, 4 and 5 were found to offer significant reductions after 120 seconds. Treatments 1,2,4 and 5 were also found to offer significant improvements over the original AM treatment (pH 2.8). Again, as with *L. monocytogenes* LO28 and *E. coli* O157:H7, treatments 1 and 4 resulted in the most rapid reductions in survival, with significant log reductions of above 6.07 CFU/ml being observed for both treatments at 5 and 10 min CFU/ml **(**$P < 0.05$; paired T-test) (Fig. 6.6 B).

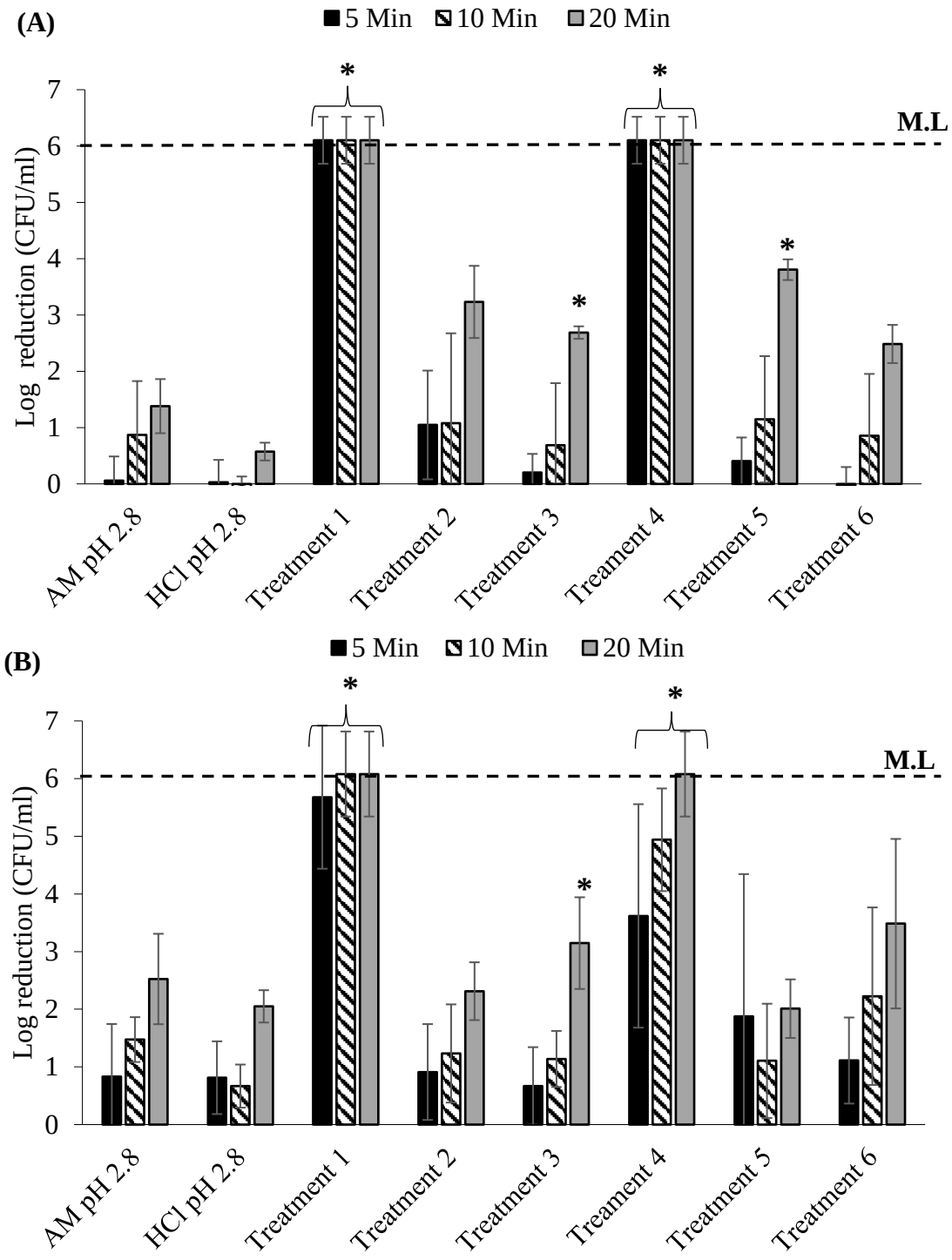

Fig 6.6. Cultures challenged with AM treatment, HCL and six reformulated treatments based on the original AM l. **(A)** *L. monocytogenes* LO28 sampled at 0,5,10 and 20 min **(B)** *E. coli* O157:H7 sampled at 0,5,10 and 20 min. Asterisks denote statistical significance compared to AM pH 2.8, using a paired student T -Test < 0.05; M.L denotes detection limit of the experimental setup.

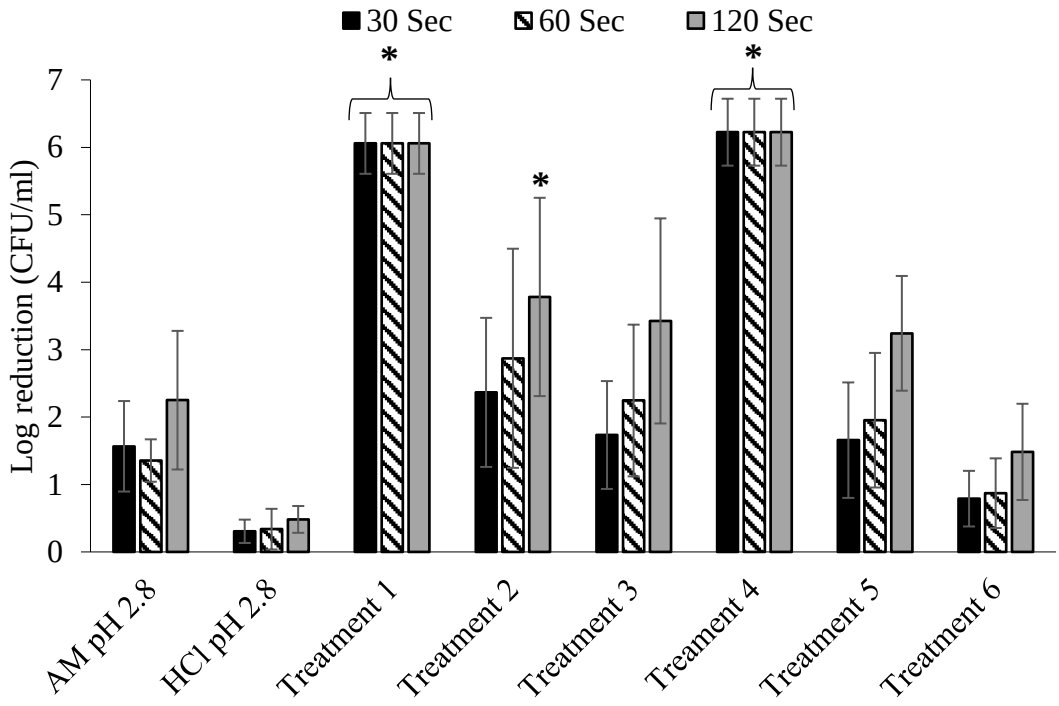

Fig 6.7. Survival of *S.* Typhimurium 30 when challenged with AM treatment, HCL and six reformulated treatments based on the original AM, sampled at 0,30,60 120 sec. Asterisks denote statistical significance compared to AM pH 2.8, using a paired student T -Test < 0.05; M.L denotes detection limit of the experimental setup.

6.3.5 Survival of *Lb. plantarum* WCFS1 when challenged with various concentrations of sodium fumarate under acidic conditions.

The effect of various concentrations of sodium fumarate (0 mM, 5 mM, 10 mM, 15 mM, 20 mM and 25 mM) under low pH conditions (< pH 3) on the survival of *Lb. plantarum* WCFS1 was assessed. The addition of sodium fumarate provided a significant reduction in the survival of *Lb. plantarum* WCFS. Increased concentrations of sodium fumarate reduced survival, as shown in Fig. 6.7. The lowest concentration of sodium fumarate, where a statistically significant level of reduction was achieved, was found to be at 15 mM. This concentration resulted to a reduction of 1.3 Log CFU/ml after 20 min (P, 0.05; paired T-test; Fig. 6.8). When this concentration was increased to 20 mM of sodium fumarate, reductions

observed at 15 and 20 min were 1.94 and 3.3 CFU/ml respectively ($P < 0.05$; paired T-test; Fig. 6.8). The greatest and most rapid effect was observed in the presence of 25 mM sodium fumarate resulting in 1.4, 3.1 and 4.7 log reduction of CFU/ml after 15, 20 and 25 minutes respectively ($P < 0.05$; paired T-test, Fig. 6.8).

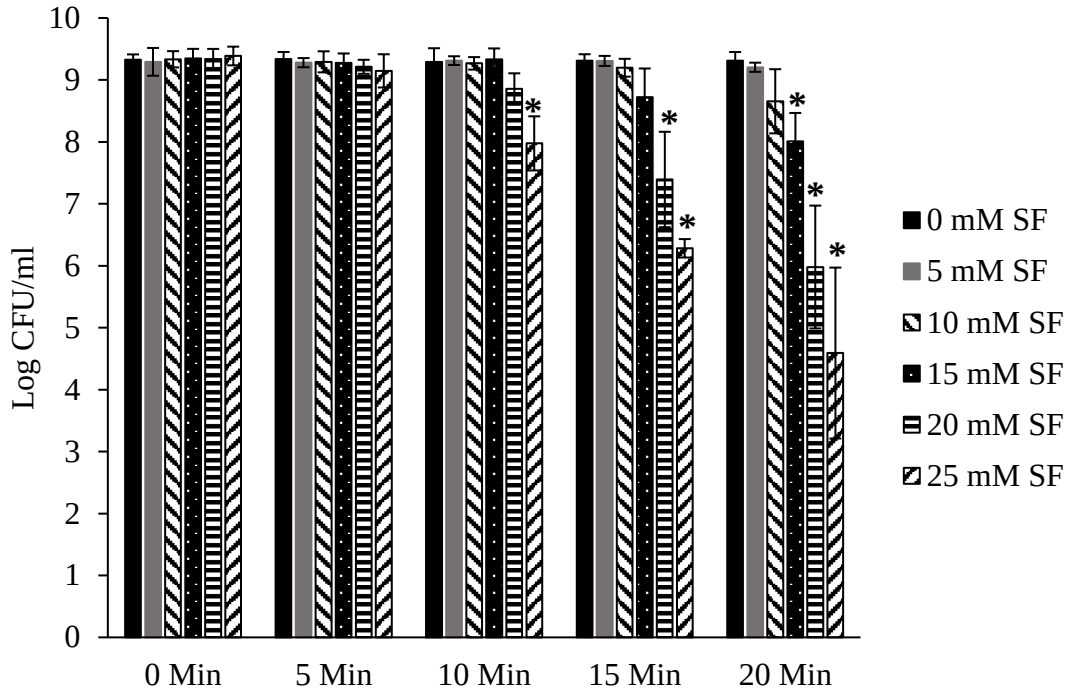

Fig 6.8. Survival of *Lb. plantarum* WCFS1 in the presence of various concentrations of sodium fumarate (SF) at a pH value of 3 (Asterisks statistical significance, using a paired student T -Test < 0.05)

6.4 Discussion.

This series of experiments was aimed at improving the antimicrobial (AM) effect of a commercially available organic acid treatment for fresh produce. Initial experiments were undertaken to establish the MIC's of the key components of this treatment (tartaric, citric and malic) and to establish the MIC's of fumaric acid and sodium fumarate. These have been selected as potential supplements for this treatment based on previous observations that fumaric acid when examined at low pH may have a higher degree of AM action than other commonly used weak acids including citric and malic (Comes and Beelman, 2002).

The MIC's indicate that fumaric acid was not the most antimicrobial of the acids tested, the most was citric acid with a MIC of 12.5 against *E. coli* O157:H7. Sodium fumarate had higher solubility than fumaric acid allowing tests to be performed at higher concentrations, up to 22% w/v compared to 0.7% w/v (Gangl et al., 1990). Sodium fumarate as a salt does not possess a MIC at low concentration (> 200 mM) as it requires acidic conditions to inhibit the growth of pathogens (Skřivanová et al., 2006) and it does not reduce the pH of the environment (Ma et al., 2018).

These results demonstrate that while fumaric acid can be inhibitory to growth its profile does not differ dramatically from other weak organic acids and the impact of and sodium fumarate is extremely limited even in high concentrations. As such they would not offer any advantage over the other acids currently in the treatment of preventing growth on fresh produce.

It has previously been observed that high concentrations of fumaric acid alone may have a significant bactericidal effect on a range of foodborne pathogens, in comparison with other treatments, e.g. sodium hypochlorite or mild heat. However, some issues have been identified in using high concentrations, e.g. 50 mM, where fumaric acid has been found to damage fresh produce and has a limited solubility which makes it difficult to combine with other treatments (Kondo et al., 2006, Comes and Beelman, 2002, Kim et al., 2009).

So, our experiments focused on examining low concentrations of sodium fumarate with its high degree of solubility in combination with AM to assess if low concentrations of this compound could be used to increase the AM ability of this commercial treatment.

These experiments clearly demonstrate that the presence of just 10 mM of sodium fumarate under acidic condition does significantly increase the bactericidal effect of AM against *L. monocytogenes* 10403S, *E. coli* K-12 and *S.* Typhimurium 30 when under acidic conditions (Fig. 6.1 A & B and Fig. 6.2 A & B), and within the short time scales that are required by processors of MMP (Beuchat et al., 1998, Roa Engel et al., 2013).

The series of experiments focused upon the ability of the AM treatment supplemented with sodium fumarate to remove foodborne pathogens (*L. monocytogenes* LO28, *E. coli* O157:H7 and *S.* Typhimurium 30) from the surface of fresh produce (strawberries, pears and apples). The strains of the organisms selected for these experiments were changed to more representative ones of what would normally be encountered on fresh produce. In this experiment a comparison between four treatments consisting of a) water, b) chlorine 100 ppm, c) AM (pH 2.4) and d) AM (pH 2.4) supplemented with 25 mM sodium fumarate.

The quantity of sodium fumarate used to supplement the AM treatment was increased to 25 mM, because preliminary tests on fresh produce using 10 mM of sodium fumarate showed a limited bactericidal effect on fresh produce (data not shown). This more limited impact on the presence of food pathogens may be explained by the complex nature presented by fresh produce. It has been shown that bacteria may embed themselves on the irregular surface of fresh produce or bind to specific sites such as the stomata (Gil et al., 2009, Li et al., 2001).

The AM treatment supplemented with 25 mM sodium fumarate at a pH of 2.4, was found to provide a greater effect than the original treatment (AM alone) at the same pH. On apples and pears, the combined treatment was found to be significantly more effective than 100 ppm free

chlorine and the original AM treatment (Fig. 6.4 and 6.5) ($P < 0.05$; paired T-test). The decontamination of strawberries presented a more complex picture while the AM treatment supplemented with 25 mM sodium fumarate at a pH of 2.4 was still found to provide the highest log reduction CFU/ml. It was not found to be significantly more effective at removing *L. monocytogenes* LO28 and *E. coli* O157:H7 than 100 ppm free chlorine and the original AM treatment (Fig. 6.3 A & C) ($P < 0.05$; paired T-test). This could be partially attributed to the nature of the surface of a strawberry which has been shown to be problematic to decontaminate as observed in other studies (Han et al., 2004)

It was also noted that the use of low pH treatments resulted in some depigmentation of the strawberries as evidenced by an alteration in the colour of the wash water. Ensuring microbiological safety as well as maintaining the quality of the product is a key issue for both producers and consumers (Ramos et al, 2013)

The results presented here demonstrate the potentially limited effects of washing treatments methods. Here a treatment of 100 ppm chlorine which is typically used in industry at concentrations ranging for 50 -200 ppm (Olaimat and Holley, 2012) and a commercial organic acid treatment at a pH of 2.4, both demonstrated small reductions pathogen levels (Fig. 6.3, 6.4 and 6.5). These results highlight the need for further developments in to effective methods for removing foodborne pathogens from fresh produce.

In an attempt to assess if further improvements to the AM treatment might be achieved six reformulations of the original AM treatment were produced, the details of which are described in Appendix 3.

It was demonstrated that treatments 1, 3, 4 and 5 offered significant improvements over the original AM treatment (pH 2.8) at 20 min against *L. monocytogenes* LO28. Only two treatments achieved significant results at 5 and 10 min these were treatments 1 and 4 ($P <$

0.05; paired T-test) (Fig 6.6 A). This suggests that these treatments might be more effective at targeting *L. monocytogenes* LO28. Both Treatments 1 and 4 contain fumarate suggesting that, fumarate may offer significant reductions compared to other acid treatments.

When *E. coli* O157:H7 was challenged with the reformulated treatments 1, 3 and 4 offered significant improvements over the original AM treatment at 20 min. As with *L. monocytogenes* treatments 1 and 4 achieved significant results at 5 and 10 min demonstrating the most rapid antimicrobial effect ($P < 0.05$; paired T-test) (Fig 6.6 B). Treatment 3 was also found to provide an increased antimicrobial action against *L. monocytogenes*.

In the experiments with *S.* Typhimurium 30, a shorter time scale was utilised in initial experiments. Initial tests resulted in rapid reduction in survival across all treatments, making a comparison between unachievable. Over this shorter time scale treatments 1, 2 and 4 proved to be more effective than the original AM treatment against *S.* Typhimurium ($P < 0.05$; paired T-test) (Fig 6.7). One treatment that performed very poorly was treatment 6, which against *S.* Typhimurium 30 performed worse than the original AM. This suggests that the alterations for this reformulation might not be suitable for commercial use.

Against all three tested pathogens treatments 1 and 4 had the citric acid used in the original AM treatment replaced with trisodium citrate while both treatments also had 50 mM and 25 mM fumaric acid fumaric acid added respectively. Fumaric acid has been shown to have a high degree of antimicrobial effect under acidic conditions and might be expected to improve the effectiveness of an acidic treatment. Although in high concentrations such as 50 mM it has been shown to cause a browning effect on some types of produce (Kondo et al, 2006).

During this examination of the effects of the reformulations of AM saw similar levels Log reductions CFU/ml against *L. monocytogenes* LO28 and *E. coli* O157:H7. The *S.* Typhimurium 30 appeared to be much more sensitive to acidic stresses with rapid reductions

necessitating reduced time intervals between samples to differentiate between the antimicrobial abilities of the tested treatments.

The final experiments assessed the effect of various concentrations of sodium fumarate on *Lb. plantarum* WCFS1 under acidic conditions (pH 3). Whilst this is not a foodborne pathogen it is commonly responsible for food spoilage and is thus of interest to producers. Fumaric acid has been shown to affect the growth of lactobacilli and its use as an inhibitor appears to be where the majority of research is focused. (Perez-Diaz and McFeeters, 2010)

The survival of *Lb. plantarum* was affected by the presence of sodium fumarate at a pH of 3, with increasing concentrations of sodium fumarate providing greater and more rapid reductions in survival than compared to the control at the same pH. Under the conditions tested, 15 mM of sodium fumarate or above showed significant reduction after 20 min (Fig. 6.8). This demonstrated that sodium fumarate has a bactericidal effect on *Lb. plantarum* under acidic conditions and that increases of the concentration of fumarate enhance this result.

The relationship between Lactobacilli and organic acid salts is an interesting one and it has been reported for *Lactobacillus casei* that the presence of 30 mM of malate (the salt of malic acid) can actively increase the survival of *L. casei* under acidic conditions through a process called malolactic fermentation. The malonate decarboxylase system, decarboxylases malate through the use of malolactic enzyme to produce L-lactate and CO_2, leading to an alkalization of the cytoplasm promoting survival (Broadbent et al., 2010). This shows that while the formulation of organic acid treatments may be tailored for each organism as it is important to consider many factors including the physical attributes of the organism, the produce and the effect the treatment may have upon them. It is possible in the case of organic acid which has

a complex relationship with some bacteria that some specific organisms and advantage either in terms of growth or survival may be offered.

6.5 Conclusions

In this study we examined the effect of fumaric acid and sodium fumarate as antimicrobial compounds used in combination with other organic acids. It was demonstrated that the presence of low concentrations of sodium fumarate, could increase the bactericidal effects of a current commercial treatment on a range of fresh produce such as strawberries, apples and pears. It is possible that such a treatment might find its way to these products in the future.

Our study also demonstrated that organic acid washes especially when optimised as shown here, can provide a rapid and effective disinfection compared to more conventional treatments, such as chlorine washes.

It is key to note that while an acid can be used to target a mechanism of acid resistance, previously reported results suggest that some organic acids such as malate may potentially offer advantages to some organisms in terms of survival under acidic stress. As such, the selection of weak organic acids that are used to treat fresh produce must be carefully considered to ensure safe and effective disinfection protocols.

The selection of the most effective acids may result in the formulation of treatments targeted at specific types of produce or specific organisms. Through formulating more affective treatments such as those here that contained fumarate it may be possible to achieve effective decontamination at higher pH, reducing the impact of such treatments upon the environment and upon the produce.

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CHAPTER 7:

General Discussion

7.1 Mode of action of organic acids against bacterial foodborne pathogens and investigation of improved disinfection methods

This final summary chapter will examine the outputs of this research and highlight some of the key findings. The results presented in the preceding chapters highlight the issues surrounding the consumption of fresh and minimally processed produce and the techniques used to ensure the microbiological safety of this type of produce. This issue has become an increasing challenge for both producers and consumers in recent years due to the international nature of the food chain providing greater opportunities for contamination, a global increase in the consumption of fresh or minimally processed produce (MMP) and changes in agricultural practices (Tauxe et al., 1997, Olaimat and Holley, 2012).

Although a range of treatments and technologies are available to help reduce the presence of food pathogens on fresh and MMP, they are not always effective or appropriate for this type of produce. This thesis has focused upon the effect of organic acids notably fumaric acid and its salt, sodium fumarate and how they interact with key mechanisms of acid resistance commonly found in food pathogens; notably the glutamate decarboxylase (GAD) system of *Listeria monocytogenes* and *Escherichia coli* and the lysine decarboxylase system (LDAR) of *Salmonella*.

This thesis also explored how weak organic acid treatments could be tailored by using specific acids such as fumaric that have been shown to have specific antimicrobial affects against key pathogens and biofilms.

Finally, it examined the influence that key stress genes in *L. monocytogenes* might have on the ability of this organism to grow in nutritionally limited and osmotically stressful environments.

7.2 Fumaric acid and the GAD system of *L. monocytogenes*.

Organic acids such as fumarate are commonly used as antimicrobials in foods. Here we demonstrate in *L. monocytogenes and E. coli* a great discrepancy between the experimentally observed (higher) and the expected (lower) antimicrobial activity of fumarate based on its chemical properties.

It has previously been demonstrated by Fonda (1972) that fumaric acid is capable of inhibiting the activity of *E. coli* GAD enzyme and as such it could reduce the survival of this pathogen under acidic conditions.

When examined the acid tolerance of both *E. coli* K-12 and *L. monocytogenes* 10403S significantly decreased in the presence of sodium fumarate resulting in reduced survival during acidic stress. However, an examination of the output of the GAD system extracellular GABA ($GABA_e$) demonstrated that the GAD systems of the two organisms were behaving quite differently to fumarate. The presence of fumarate resulted in *E. coli* producing lower levels of $GABA_e$ as would be expected if the system was inhibited while in *L. monocytogenes* it resulted in increased levels of $GABA_e$. The result observed with *L. monocytogenes* was in direct conflict with the increased acid sensitivity as increased $GABA_e$ output should increase acid resistance.

In an attempt to elucidate the effect of fumarate on the GAD system by examining its effect on GAD activity in cellular lysate, surprisingly, we observed that sodium fumarate significantly inhibited GAD activity of *L. monocytogenes* this result tallied with previous studies showing fumarate having a similar ability in *E. coli* (Fonda, 1972). Based upon this

result the fumarate should have reduced the function of the GAD system of *L. monocytogenes* not increased it, as shown by increased levels of $GABA_e$.

Through an examination of the regulation of the GAD system of *L. monocytogenes* it was possible to establish identify that *L. monocytogenes* was upregulating *gadD2* the key decarboxylase of this system, which must be contributing to the increased GABA export. Ultimately, the bactericidal effect of fumarate might be linked to effects on the intracellular GAD ($GAD_{i)}$ system, which is difficult to assess since intracellular GABA pools are affected by other pathways (e.g. GABA shunt).

While this work attempted to establish a similar effect of fumarate, upon the regulation of the GAD system of *E. coli* as that demonstrated in *L. monocytogenes* it was not possible to establish a direct effect of fumarate upon the transcription of the GAD system of *E. coli.* This difference was possibly due to *E. coli* possessing a number of regulatory genes associated with the operation of the GAD system that *L. monocytogenes* and many other organisms lack which might in part explain some of the differences highlighted in this study.

During the assessment of the impact of fumarate on the GAD system of *E. coli* an interesting insight into the function of the antiporter GadC was made. Here we demonstrated for first time an effect of the antiporter GadC on the levels of intracellular GABA ($GABA_i$) despite the latter being involved only on the extracellular GABA production. This suggests that GadC antiporter might play a key role to the function of the GAD enzymes in *E. coli*.

7.3 Fumarate and the lysine decarboxylase acid resistance system.

Although initially this project focused on *E. coli* and *L. monocytogenes* we expanded the work to include a Gram negative bacterium such as *Salmonella* and the effects of fumarate on this organism. *Salmonella* does not possess a GAD system but other amino acid

decarboxylase systems such as the LDAR system which was investigated for effects on its activity in the presence of fumaric acid (Kondo et al., 2006, Kim et al., 2009). This thesis demonstrates that sodium fumarate has a significant affect upon *S*. Typhimurium and *S*. Heidelberg. As this affect was not observed in all serovars tested (e.g. *Salmonella* Enteritidis P518496), this suggests that there may be some variation in the function of the LDAR system of different serovar of *Salmonella*. Although it was not possible to confirm an effect on the transcription of a specific component of the LDAR system (*cadA* and *cadB*) it seems likely that further investigation would identify the full extent of this specific acid on *Salmonella*.

As this work demonstrates that sodium fumarate appears to have broad effect on amino acid decarboxylase systems such as the GAD system and the LDAR system it is possible that it might also be capable of disrupting other mechanisms such as the arginine or the ornithine decarboxylase systems. These systems although less potent than the GAD system have also been shown to support organisms such as *E. coli* during acidic stress a greater understanding of their mechanism and function may offer advantage in helping to control food pathogens (Foster, 2004).

7.4 Fumarate and fresh produce

It is clear both from this work and from previous studies that fumaric acid can be used to provide a high degree of antimicrobial effect against key foodborne pathogens (Kim et al., 2009, Kondo et al., 2006, Comes and Beelman, 2002). In this study we demonstrate that sodium fumarate can provide a high degree of antimicrobial action against foodborne pathogens on fresh produce when combined with a commercially available organic acid disinfectant. This work attempted to provide conditions more closely associated with the

processing of fresh produce than has been demonstrated previously. To this end this study utilised the short time scales required by food possessors, while assessing sodium fumarate in combination with a commercially available organic acid mix against selected foodborne pathogens. It was found that sodium fumarate significantly improved the bactericidal effects of the organic acid mix on all forms of produce and offered improvements in comparison to a chlorine-based wash. In addition, it was possible to demonstrate that using similar concentrations of other acids with in the AM treatment did not offer such a high degree of antimicrobial effect once again demonstrating the unique abilities of sodium fumarate in this area.

While chlorine continues to be the most commonly used sanitiser for fresh produce, our work demonstrated that significant improvement is possible that can ensure the safety of fresh produce. Furthermore, additional studies using combined methods or using competitive microflora to extend and enhance the safety of this kind of products are crucial.

7.5 Fumarate and the inactivation of biofilms

Bacteria in the form of biofilms present a significant challenge to the fresh and minimally processed produce market. Biofilms have been suggested as representing a high proportion of the microbiota found on fresh produce and are thought to be the key factor limiting the efficacy of liquid sanitisers (Olaimat and Holley, 2012, Gil et al., 2009).

This work examined the ability of sodium fumarate to aid in the removal of biofilms of *L. monocytogenes*, *E. coli* and *Salmonella* under acidic conditions. It has been previously shown that maleic acid which is the cis isomer of fumaric acid, can have a significant effect on both listerial biofilms and those found within the oral cavity making it a good alternative to Ethylenediaminetetraacetic (EDTA) which is currently used by the dental industry to remove

biofilms (Paudyal et al., 2018, Ballal et al., 2009). However, maleic acid (Wu et al., 2017) has a mildly toxic which could limit its use. This is not an issue associated with fumaric acid which is commonly used within the food industry (Lee, 2014).

The presence of sodium fumarate in a currently available organic acid (AM) treatment consistently improved its bactericidal effect. The effect of a chlorine-based treatment varied considerably from organism to organisms and even between strains. This work was found in some cases to provide a significant effect on some bacterial biofilms notably that of *S.* typhimurium 10. The use of a weak organic acids supplemented with fumarate offered a consistent and effective antimicrobial treatment for biofilms.

7.6 *L. monocytogenes* and self-preservation and nutritional competence (SPANC).

This work provides important insight into the SigB-controlled general stress response of *L. monocytogenes* 10403S. In *L. monocytogenes* the key mediator of the bacterium's stress responses is the alternative sigma factor SigB. This protein helps to regulate the response to a number of environmental stresses including pH, high osmolality and carbon starvation (Begley et al., 2006, Sue et al., 2003). However, in our work we demonstrate that in the absence of SigB, *L. monocytogenes* demonstrated a small but significant increase in its ability to utilise 14 different carbon sources.

This effect has previously been observed in *E. coli* which when its general stress response protein RpoS is absent demonstrates increased utilisation of various carbon sources. The RpoS of *E. coli* functions in a similar fashion to SigB in *L. monocytogenes* by controlling the organism's general stress response. This effect has been theorised as a trade-off between self-preservation and nutritional competence, called A SPANC balance (Ferenci, 2005, Stoebel et

al., 2009). This work represents the first time that this effect has been observed in a Gram positive organism.

It is possible that different strains of *L. monocytogenes* might present with variation in SPANC balances as has been observed in *E. coli*; where some strains are skewed towards a strong stress response and limited nutritional capacity and others to a broader nutritional capability but lower levels of stress resistance. Further investigation would be required to confirm this in *L. monocytogenes*. This effect could offer some survival advantage to the organism by broadening its ability to occupy a variety of environmental niches (King et al., 2004). More generally this work could provide some insight into the stress regulation and nutritional competence of this organism.

7.7 The GAD system and NaCl

An interesting and unexpected outcome of assessing the effect of various osmotic stresses on *L. monocytogenes* 10403S was that the organism appears to rely upon the presence of various components of the GAD system notably *gadD1, gadD2* and *gadD3* to support the organism when exposed to high concentrations of NaCl. It has previously been observed that *L. monocytogenes* is capable of tolerating high levels of NaCl up to 10% (Miller, 1992). However, the absence of the GAD genes significantly reduced this capacity in *L. monocytogenes* 10403S. While the absence of *sigB* had previously been linked with the osmotic stress (Becker et al., 1998) response this is the first time that any of the GAD genes has been linked with this function.

7.8 Conclusion.

In this thesis, it is shown that fumaric acid and sodium fumarate possess antimicrobial effects in excess of their physical properties. While this has been previously observed, no solid explanations for this effect has been described. Here we demonstrate that one possible explanation is the possible interaction with key mechanisms of acid resistance in a wide range of foodborne organisms, notably via disrupting the function of amino acid decarboxylase systems. This effect could be exploited when considering the formulation of antimicrobial treatments and sanitisers to improve their efficacy. With an improved level of efficacy organic acid sanitisers would make a more attractive choice for food processors and producers as it would be possible to use less chemical to achieve greater affect. This would mean that the food production industry could theoretically improve food safety while minimising the impact of washes on the produce as well as reducing the environmental impacts associated with some washes or sanitisers commonly employed.

It is possible that other organic acids that are commonly used within the food industry may also have additional effects upon bacteria that have yet to be fully explored. How these compounds interact with food pathogens may have some intriguing implications for food safety in the future and provide a fascinating avenue of research.

7.9 Future work:

- Examination of antimicrobial properties of sodium fumarate against a wider range of organisms to include spoilage organisms (ie *Lactobacillus, Campylobacter Staphylococcus* and fungi).
- Examination and exploration of the effect of fumaric and other organic acids as inhibitors on the arginine decarboxylase system.
- Assessment of the effect of sodium fumarate and fumaric acid on fresh produce including shelf life studies and sensory properties.
- Further examine the use of organic acids to remove biofilm formations notably on mixed biofilm matrices.
- Further investigate the influence of the SigB stress response on the nutritional competence of *L. monocytogenes* helping to elucidate the SPANC balance of this organism.
- Examine the response of the GAD system to osmolytes notably sodium chloride.

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Appendix 1. Results of alignment of the *E. coli* K12 GadX protein against the full database of NCBI excluding all *E. coli* results. (NCBI Resource Coordinators (2017) "Database resources of the National centre for Biotechnology Information") Paudyal, R, (2017).

AraC family transcriptional regulator [Shigella sp. FC2175]	490	490	100%	2.00E-175	98%
HTH-type transcriptional regulator gadW [Escherichia sp. KTE172]	493	493	100%	1.00E-176	98%
AraC/XylS family transcriptional regulator [Klebsiella oxytoca]	491	491	100%	1.00E-175	98%
AraC family transcriptional regulator [Shigella sp. FC2045]	487	487	100%	3.00E-174	97%
transcriptional regulator GadW [Shigella sonnei]	344	344	71%	1.00E-118	97%
HTH-type transcriptional regulator gadW [Escherichia sp. 3_2_53FAA]	473	473	100%	2.00E-168	95%
AraC family transcriptional regulator [Shigella sp. FC569]	471	471	100%	6.00E-168	94%
HTH-type transcriptional regulator gadW [Escherichia sp. KTE114]	454	454	100%	3.00E-161	90%
AraC family transcriptional regulator [Escherichia sp. TW14182]	453	453	100%	1.00E-160	90%
AraC family transcriptional regulator [Escherichia sp. B1147]	387	387	100%	2.00E-134	79%
MULTISPECIES: AraC family transcriptional regulator [Escherichia]	385	385	100%	1.00E-133	76%
HTH-type transcriptional regulator gadW [Escherichia albertii TW07627]	374	374	100%	1.00E-129	75%
AraC family transcriptional regulator [Escherichia albertii]	374	374	100%	2.00E-129	75%
AraC family transcriptional regulator [Escherichia fergusonii]	314	314	99%	1.00E-105	63%
AraC family transcriptional regulator [Escherichia fergusonii]	312	312	99%	5.00E-105	63%
AraC family transcriptional regulator [Escherichia fergusonii]	307	307	99%	6.00E-103	62%
hypothetical protein ABI57_19205 [Salmonella enterica subsp. enterica]	142	142	78%	1.00E-38	42%
AraC family transcriptional regulator [Shigella flexneri 2a str. 301]	500	500	100%	3.00E-179	100%
HTH-type transcriptional regulator gadW [Shigella flexneri K-671]	350	350	70%	3.00E-120	100%
AraC family transcriptional regulator [Shigella boydii]	271	271	54%	1.00E-90	100%
AraC family transcriptional regulator [Shigella flexneri]	498	498	100%	2.00E-178	99%
MULTISPECIES: AraC family transcriptional regulator [Shigella]	498	498	100%	2.00E-178	99%
AraC family transcriptional regulator [Shigella flexneri]	498	498	100%	2.00E-178	99%
bacterial regulatory helix-turn-helix s, AraC family protein [Shigella flexneri SFJ17B]	496	496	100%	8.00E-178	99%
HTH-type transcriptional regulator gadW [uncultured bacterium Contig1670]	456	456	91%	2.00E-162	99%
putative ARAC-type regulatory protein [Shigella sonnei Ss046]	496	496	100%	6.00E-178	99%
AraC family transcriptional regulator [Shigella flexneri]	495	495	100%	2.00E-177	99%
transcriptional regulator GadW [Shigella sonnei]	347	347	70%	1.00E-119	99%
putative ARAC-type regulatory protein [Shigella sonnei 4822-66]	347	347	70%	6.00E-119	99%
AraC family transcriptional regulator [Shigella boydii]	494	494	100%	6.00E-177	99%
putative ARAC-type regulatory protein [Shigella boydii Sb227]	493	493	100%	2.00E-176	99%
HTH-type transcriptional regulator gadW [Shigella flexneri K-315]	481	481	97%	4.00E-172	99%
HTH-type transcriptional regulator gadW [Shigella dysenteriae 1617]	317	317	64%	3.00E-108	99%
AraC family transcriptional regulator [Shigella boydii]	445	445	90%	6.00E-158	99%
AraC family transcriptional regulator [Shigella boydii]	261	261	52%	2.00E-86	98%

HTH-type transcriptional regulator gadW [Klebsiella pneumoniae IS22]	136	136	71%	1.00E-36	39%
transcriptional regulator [Salmonella enterica subsp. enterica ser Typhimurium]	134	134	97%	3.00E-35	36%
AraC family transcriptional regulator [Shigella sp. FC2531]	149	149	96%	1.00E-40	36%
AraC family transcriptional regulator [Escherichia sp. KTE52]	167	167	99%	8.00E-48	36%
AraC family transcriptional regulator [Escherichia albertii]	167	167	99%	9.00E-48	36%
HTH-type transcriptional regulator AppY [Escherichia sp. KTE172]	167	167	99%	1.00E-47	36%
MULTISPECIES: AraC family transcriptional regulator [Escherichia]	166	166	99%	1.00E-47	36%
HTH-type transcriptional regulator AppY [Escherichia sp. KTE159]	166	166	99%	2.00E-47	36%
AraC family transcriptional regulator [Shigella sonnei]	132	132	97%	2.00E-34	35%
hypothetical protein G434_04953 [Escherichia sp. KTE172]	150	150	97%	2.00E-41	35%
AraC family transcriptional regulator [Shigella dysenteriae]	134	134	97%	7.00E-35	35%
AraC family transcriptional regulator [Escherichia coli]	142	142	96%	2.00E-38	34%
AraC family transcriptional regulator [Escherichia albertii]	144	144	96%	8.00E-39	34%
AraC family transcriptional regulator [Escherichia coli]	134	134	97%	3.00E-35	34%
transcriptional regulator, AraC family [Shigella dysenteriae 1012]	134	134	97%	6.00E-35	34%
AraC family transcriptional regulator [Shigella sp. SF-2015]	134	134	97%	7.00E-35	34%
HTH-type transcriptional regulator ydeO [Escherichia sp. 3_2_53FAA]	134	134	97%	8.00E-35	34%
AraC family transcriptional regulator [Shigella dysenteriae]	134	134	97%	8.00E-35	34%
AraC family transcriptional regulator [Shigella boydii]	134	134	97%	8.00E-35	34%
transcriptional regulator [Escherichia coli]	134	134	97%	8.00E-35	34%
AraC family transcriptional regulator [Shigella sp. SF-2015]	134	134	97%	9.00E-35	34%
transcriptional regulator YdeO [Shigella flexneri 2a str. 301]	134	134	97%	9.00E-35	34%
AraC family transcriptional regulator [Shigella flexneri]	134	134	97%	9.00E-35	34%
AraC family transcriptional regulator [Escherichia coli]	134	134	97%	9.00E-35	34%
AraC family transcriptional regulator [Escherichia coli]	134	134	97%	9.00E-35	34%
AraC family transcriptional regulator [Escherichia coli]	133	133	97%	9.00E-35	34%
HTH-type transcriptional regulator ydeO [Escherichia sp. KTE172]	133	133	97%	1.00E-34	34%
AraC family transcription regulator [Klebsiella oxytoca]	133	133	97%	1.00E-34	34%
transcriptional regulator YdeO [Shigella sp. FC569]	133	133	97%	1.00E-34	34%
transcriptional regulator YdeO [Shigella sp. FC2045]	133	133	97%	1.00E-34	34%
transcriptional regulator YdeO [Escherichia fergusonii]	133	133	97%	2.00E-34	34%
AraC family transcriptional regulator [Shigella flexneri]	132	132	97%	2.00E-34	34%
AraC family transcriptional regulator [Shigella boydii]	132	132	97%	2.00E-34	34%
HTH-type transcriptional regulator ydeO [Escherichia sp. 4_1_40B]	132	132	97%	2.00E-34	34%
putative ARAC-type regulatory protein [Shigella boydii Sb227]	132	132	97%	2.00E-34	34%
AraC family transcriptional regulator [Shigella boydii]	132	132	97%	2.00E-34	34%
putative ARAC-type regulatory protein [Shigella sonnei Ss046]	132	132	97%	2.00E-34	34%
AraC-family transcriptional regulator [uncultured bacterium]	131	131	97%	9.00E-34	34%

transcriptional regulator YdeO [Shigella sonnei]	145	145	97%	3.00E-39	34%
AraC family transcriptional regulator [Escherichia albertii]	144	144	96%	9.00E-39	34%
AraC family transcriptional regulator [Shigella flexneri]	143	143	96%	1.00E-38	34%
MULTISPECIES: AraC family transcriptional regulator [Escherichia]	143	143	96%	1.00E-38	34%
hypothetical protein WCO_00911 [Escherichia sp. KTE11]	143	143	96%	1.00E-38	34%
AraC family transcriptional regulator [Escherichia fergusonii]	142	142	96%	3.00E-38	34%
AraC family transcriptional regulator [Escherichia fergusonii]	142	142	96%	3.00E-38	34%
AraC family transcriptional regulator [Escherichia fergusonii]	142	142	96%	4.00E-38	34%
Put. araC-type reg. prot. from phage origin [Escherichia fergusonii ATCC 35469]	142	142	96%	5.00E-38	34%
araC-type reg. prot. from phage origin [Escherichia fergusonii ECD227]	142	142	96%	5.00E-38	34%
AraC family transcriptional regulator [Escherichia fergusonii]	141	141	96%	6.00E-38	34%
transcriptional regulator YdeO [Shigella sonnei]	132	132	97%	4.00E-34	34%
AraC family transcriptional regulator [Shigella sonnei]	137	137	97%	3.00E-36	34%
transcriptional regulator YdeO [Shigella sonnei]	126	126	95%	5.00E-32	34%
AraC family transcriptional regulator [Shigella sonnei]	142	142	97%	3.00E-38	33%
putative transcription regulator [Escherichia albertii KF1]	142	142	97%	4.00E-38	33%
hypothetical protein [Salmonella enterica]	151	151	97%	2.00E-41	33%
AraC family transcriptional regulator [Salmonella enterica]	150	150	97%	2.00E-41	33%
transcriptional regulator GadW [Shigella sonnei]	150	150	97%	3.00E-41	33%
AraC/XylS family transcriptional regulator [Klebsiella oxytoca]	140	140	96%	1.00E-37	33%
AraC family transcriptional regulator [Escherichia fergusonii]	140	140	96%	2.00E-37	33%
AraC family transcriptional regulator [Escherichia fergusonii]	140	140	96%	3.00E-37	33%
HTH-type transcriptional regulator gadW [Achromobacter sp. ATCC35328]	141	141	97%	7.00E-38	33%
transcriptional regulator YdeO [Shigella sonnei]	140	140	97%	2.00E-37	33%
AraC family transcriptional regulator [Escherichia albertii]	140	140	96%	2.00E-37	33%
AraC family transcriptional regulator [Escherichia albertii]	139	139	96%	3.00E-37	33%
AraC family transcriptional regulator [Escherichia albertii]	139	139	97%	6.00E-37	33%

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PM1 MicroPlate™ Carbon Sources

A1 Negative Control	A2 L-Arabinose ++	A3 N-Acetyl-D-Glucosamine ++	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose +	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose ++	A11 D-Mannose ++	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol ++	B4 L-Fucose +	B5 D-Glucuronic Acid +	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose ++	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose ++	C5 Tween 20	C6 L-Rhamnose ++	C7 D-Fructose ++	C8 Acetic Acid	C9 α-D-Glucose ++	C10 Maltose ++	C11 D-Melibiose	C12 Thymidine +
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid +	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose ++	D10 Lactulose	D11 Sucrose	D12 Uridine ++
E1 L-Glutamine	E2 m-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate ++	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside ++	E9 Adonitol	E10 Maltotriose ++	E11 2-Deoxy Adenosine	E12 Adenosine ++
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 m-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose ++	F12 Inosine ++
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid +	G8 N-Acetyl-β-D-Mannosamine ++	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose ++	H6 L-Lyxose ++	H7 Glucuronamide +	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

Fig 5.1: Carbon source utilisation of L. monocytogenes 10403S WT ++ indicates strong utilisation above (< 100 Omnilog units) + Weak utilisation (>100 and <10 Omnilog units) and Blank indicates no or very weak utilisation (>10 Omnilog units) based upon the average height growth curve performed in triplicate.

PM2A MicroPlate™ Carbon Sources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin ++	A4 β-Cyclodextrin ++	A5 γ-Cyclodextrin ++	A6 Dextrin ++	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin +	A11 Mannan	A12 Pectin +
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose ++	B4 Amygdalin ++	B5 D-Arabinose ++	B6 D-Arabitol ++	B7 L-Arabitol	B8 Arbutin ++	B9 2-Deoxy-D-Ribose ++	B10 i-Erythritol	B11 D-Fucose +	B12 3-0-β-D-Galacto-pyranosyl-D-Arabinose ++
C1 Gentiobiose ++	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 a-Methyl-D-Glucoside +	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose +	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside ++	C11 β-Methyl-D-Xyloside	C12 Palatinose ++
D1 D-Raffinose	D2 Salicin ++	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose +	D7 Turanose	D8 Xylitol ++	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine ++	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 a-Keto-Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid ++
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid +	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone ++	H10 2,3-Butanediol	H11 2,3-Butanedione +	H12 3-Hydroxy 2-Butanone

Fig 5.2: Carbon source utilisation of L. monocytogenes 10403S WT ++ indicates strong utilisation above (< 100 Omnilog units) + Weak utilisation (>100 and <10 Omnilog units) and Blank indicates no or very weak utilisation (>10 Omnilog units) based upon the average height of growth curve performed in triplicate.

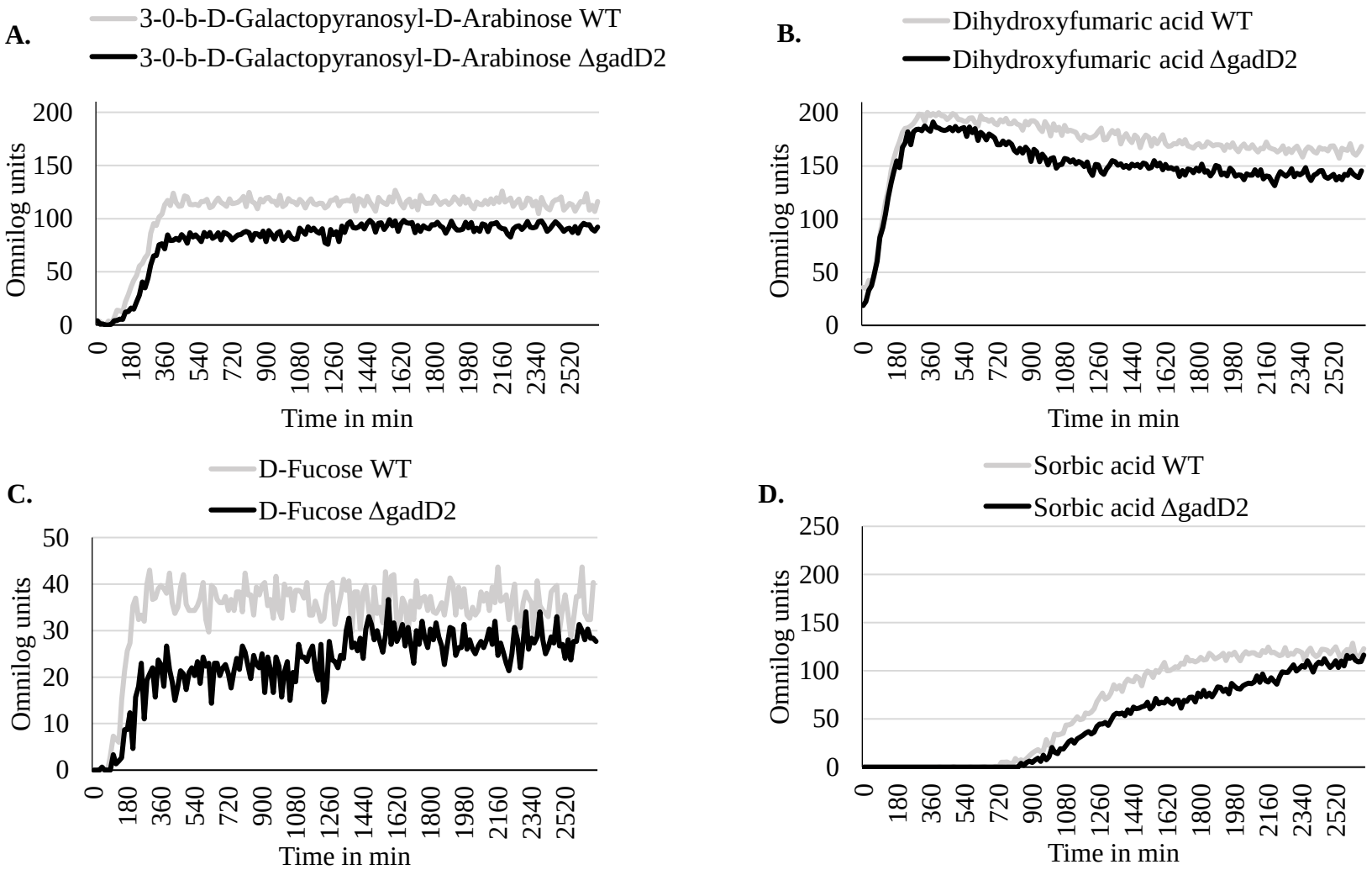


Fig 5.3: Differences observed in carbon source utilisation between *L. monocytogenes* 10403S WT and Δ*gadD2* **A**. 3-0-b-D-Galactopyranosyl-D-Arabinose. **B.** Dihydroxyfumaric acid **C.** Sorbic acid and **D.** Fucose

Fig 5.4: Differences observed in carbon source utilisation between *L. monocytogenes* 10403S WT and Δ*gadD3* laminarin

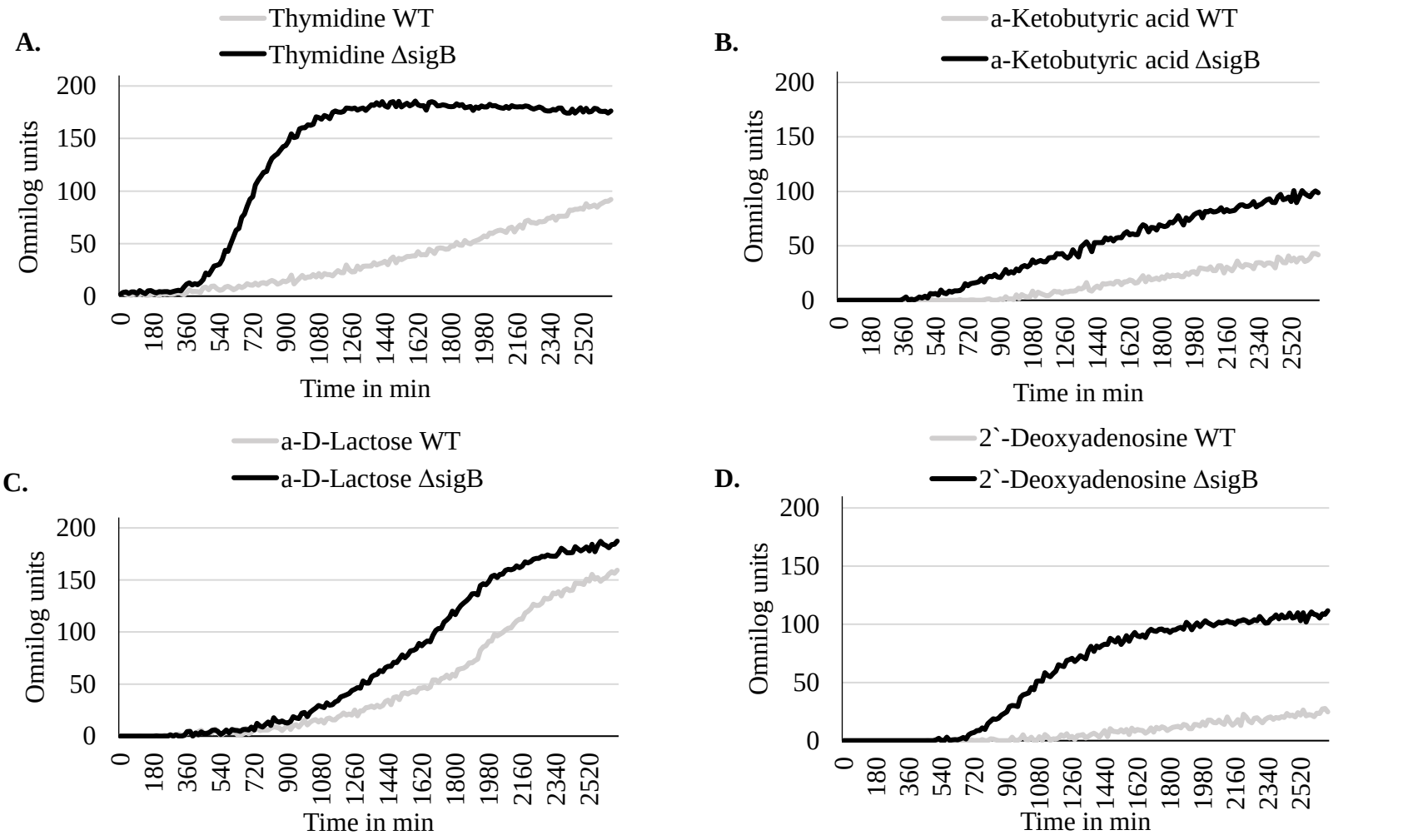


Fig 5.5: Differences observed in carbon source utilisation between *L. monocytogenes* 10403S WT and Δ*sigB* **A**. Thymidine. **B.** a-Ketobutyric acid **C.** a-D-Lactose and **D.** 2`-Deoxyadenosine

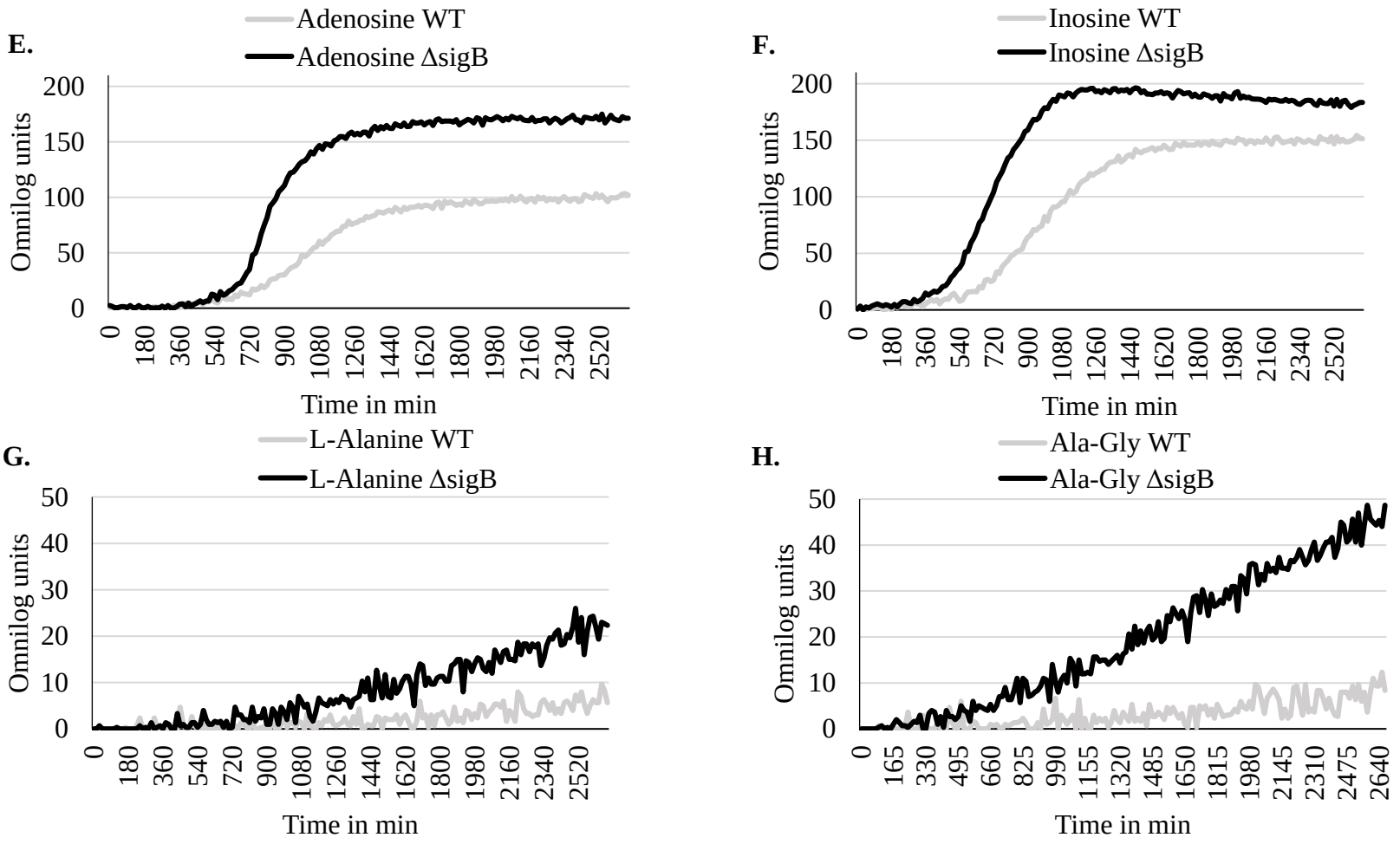


Fig 5.5: Differences observed in carbon source utilisation between *L. monocytogenes* 10403S WT and Δ*sigB* **E**. Adenosine **F.** Inosine **G.** L-Alanine and **H.** Ala-Gly

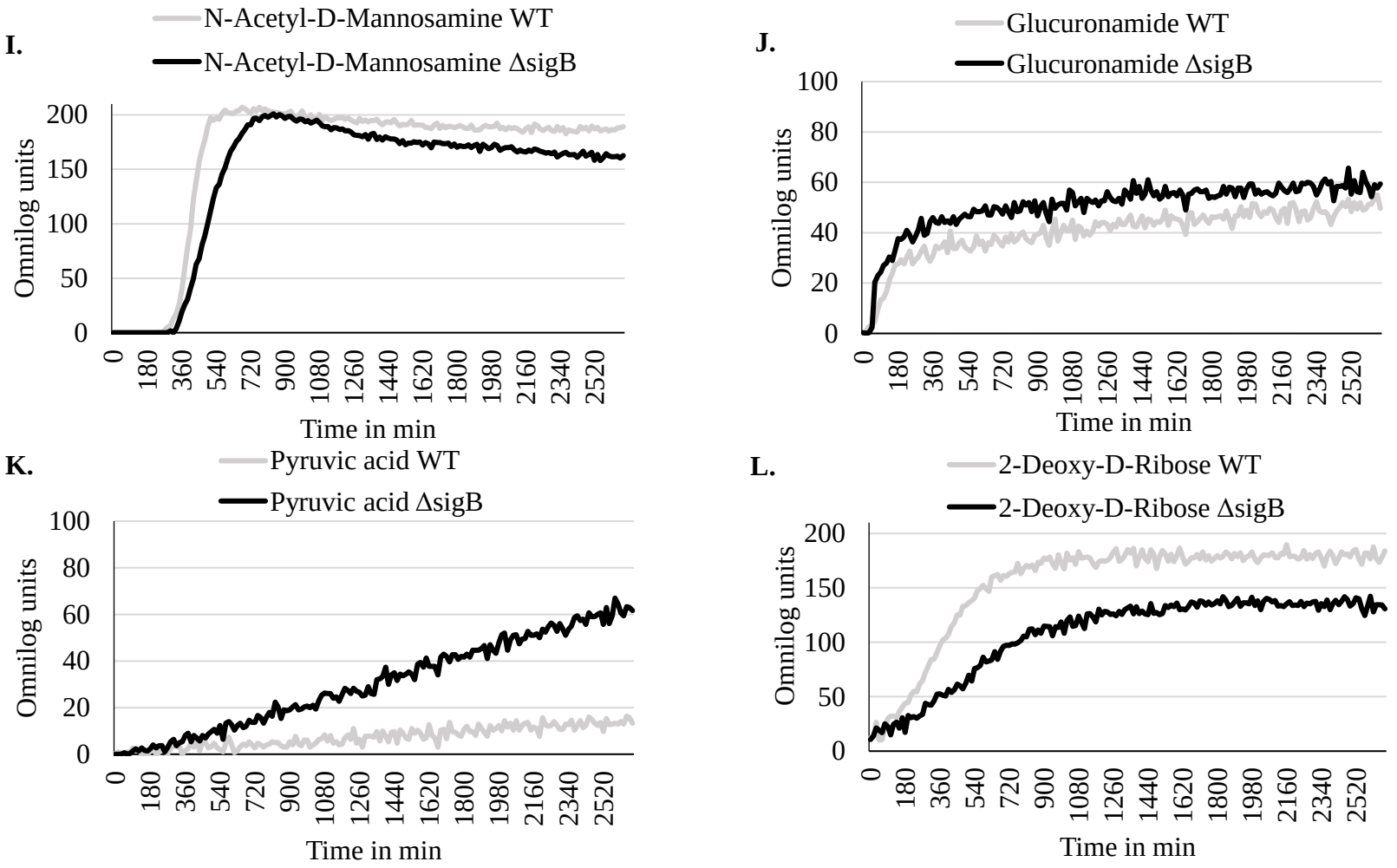


Fig 5.5: Differences observed in carbon source utilisation between *L. monocytogenes* 10403S WT and Δ*sigB* **I**. N-Acetyl-D-Mannosamine **J.** Glucuronamide **K.** Pyruvic acid and **L.** 2-Deoxy-D-Ribose

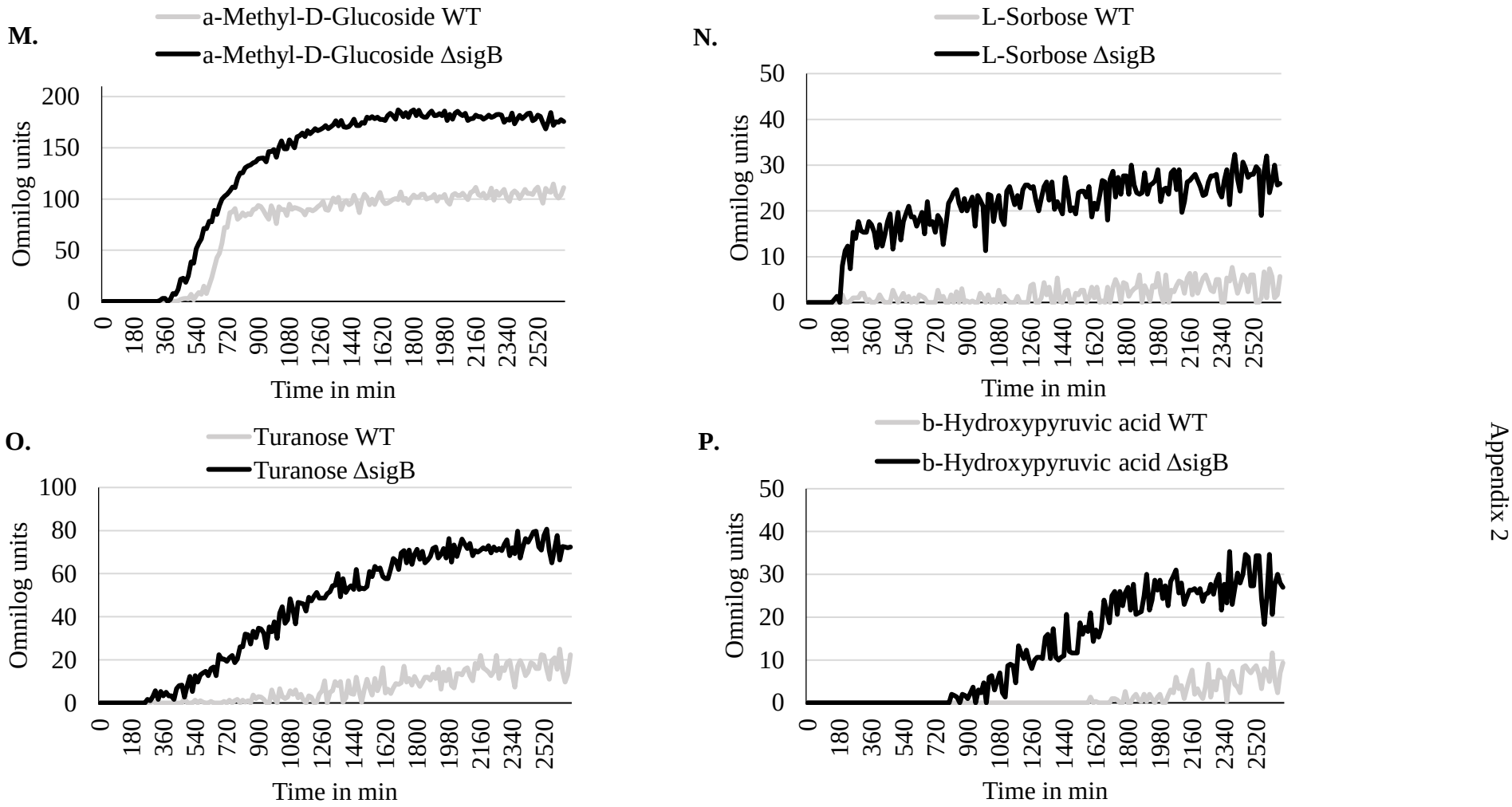


Fig 5.5: Differences observed in carbon source utilisation between *L. monocytogenes* 10403S WT and Δ*sigB* **M**. a-Methyl-D-Glucoside **N.** L-Sorbose **O.** Turanose and **P.** b-Hydroxypyruvic acid

PM9 MicroPlate™ Osmolytes

A1 NaCl 1%	A2 NaCl 2%	A3 NaCl 3%	A4 NaCl 4%	A5 NaCl 5%	A6 NaCl 5.5%	A7 NaCl 6%	A8 NaCl 6.5%	A9 NaCl 7%	A10 NaCl 8%	A11 NaCl 9%	A12 NaCl 10%
B1 NaCl 6%	B2 NaCl 6% + Betaine	B3 NaCl 6% + N-N Dimethyl Glycine	B4 NaCl 6% + Sarcosine	B5 NaCl 6% + Dimethyl sulphonyl propionate	B6 NaCl 6% + MOPS	B7 NaCl 6% + Ectoine	B8 NaCl 6% + Choline	B9 NaCl 6% + Phosphoryl Choline	B10 NaCl 6% + Creatine	B11 NaCl 6% + Creatinine	B12 NaCl 6% + L- Carnitine
C1 NaCl 6% + KCl	C2 NaCl 6% + L-Proline	C3 NaCl 6% + N-Acetyl L-Glutamine	C4 NaC1 6% + β-Glutamic Acid	C5 NaC1 6% + γ–Amino -N- Butyric Acid	C6 NaC1 6% + Glutathione	C7 NaCl 6% + Glycerol	C8 NaC1 6% + Trehalose	C9 NaC1 6% + Trimethylamine- N-oxide	C10 NaC1 6% + Trimethylamine	C11 NaCl 6% + Octopine	C12 NaC1 6% + Trigonelline
D1 Potassium chloride 3%	D2 Potassium chloride 4%	D3 Potassium chloride 5%	D4 Potassium chloride 6%	D5 Sodium sulfate 2%	D6 Sodium sulfate 3%	D7 Sodium sulfate 4%	D8 Sodium sulfate 5%	D9 Ethylene glycol 5%	D10 Ethylene glycol 10%	D11 Ethylene glycol 15%	D12 Ethylene glycol 20%
E1 Sodium formate 1%	E2 Sodium formate 2%	E3 Sodium formate 3%	E4 Sodium formate 4%	E5 Sodium formate 5%	E6 Sodium formate 6%	E7 Urea 2%	E8 Urea 3%	E9 Urea 4%	E10 Urea 5%	E11 Urea 6%	E12 Urea 7%
F1 Sodium Lactate 1%	F2 Sodium Lactate 2%	F3 Sodium Lactate 3%	F4 Sodium Lactate 4%	F5 Sodium Lactate 5%	F6 Sodium Lactate 6%	F7 Sodium Lactate 7%	F8 Sodium Lactate 8%	F9 Sodium Lactate 9%	F10 Sodium Lactate 10%	F11 Sodium Lactate 11%	F12 Sodium Lactate 12%
G1 Sodium Phosphate pH 7 20mM	G2 Sodium Phosphate pH 7 50mM	G3 Sodium Phosphate pH 7 100mM	G4 Sodium Phosphate pH 7 200mM	G5 Sodium Benzoate pH 5.2 20mM	G6 Sodium Benzoate pH 5.2 50mM	G7 Sodium Benzoate pH5.2 100mM	G8 Sodium Benzoate pH 5.2 200mM	G9 Ammonium sulfate pH8 10mM	G10 Ammonium sulfate pH 8 20mM	G11 Ammonium sulfate pH 8 50mM	G12 Ammonium sulfate pH8 100mM
H1 Sodium Nitrate 10mM	H2 Sodium Nitrate 20mM	H3 Sodium Nitrate 40mM	H4 Sodium Nitrate 60mM	H5 Sodium Nitrate 80mM	H6 Sodium Nitrate 100mM	H7 Sodium Nitrite 10mM	H8 Sodium Nitrite 20mM	H9 Sodium Nitrite 40mM	H10 Sodium Nitrite 60mM	H11 Sodium Nitrite 80mM	H12 Sodium Nitrite 100mM

Fig 5.6: Shows all tested osmolytes.

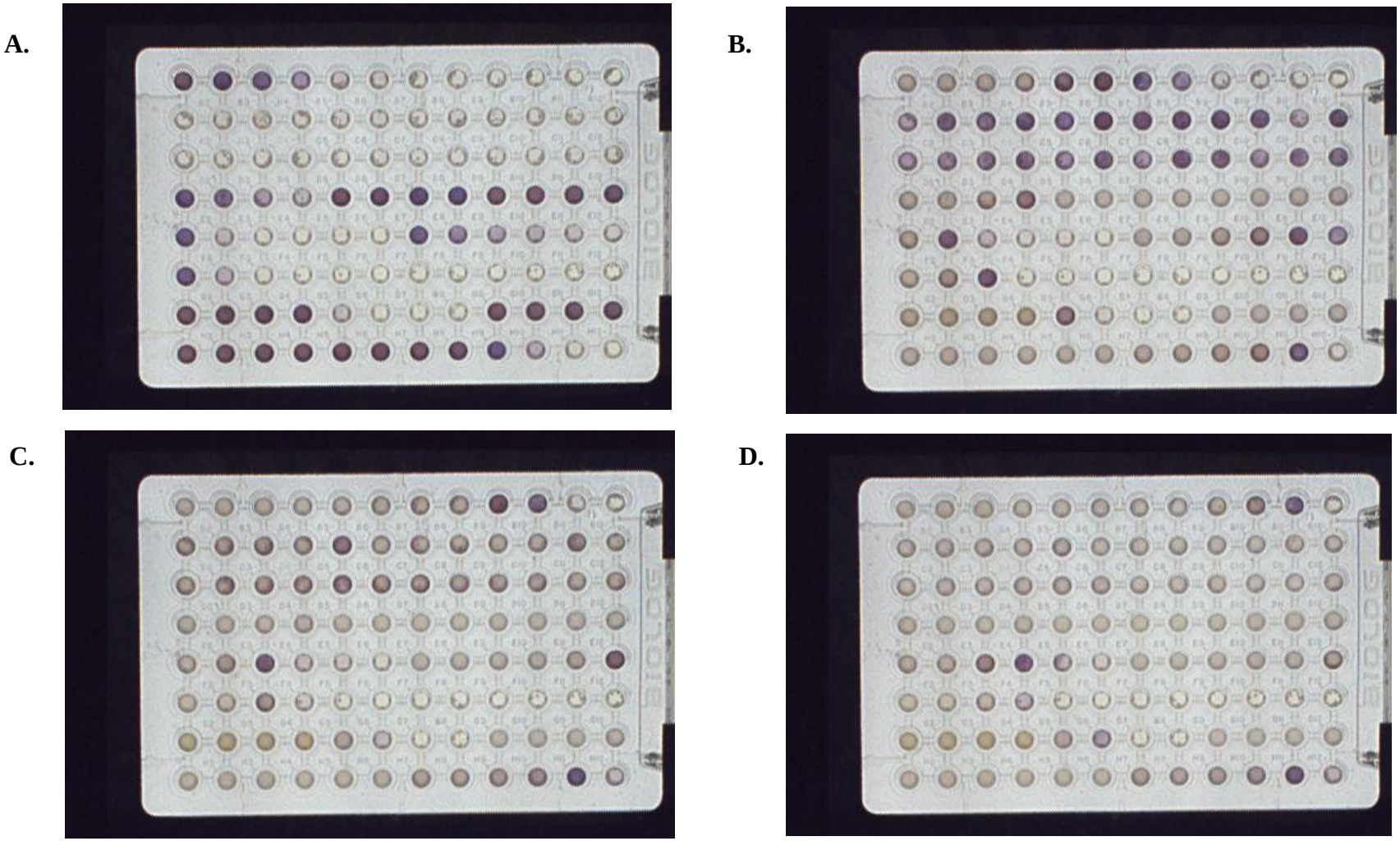


Fig 5.7: L. monocytogenes WT PM9 photographs demonstrating the degradation of the colour of samples resulting in rapid decrease in Omnilog units shown at **A**. 3 hours, **B.** 6 hours, **C.** 9 hours and **D.**12 hours.

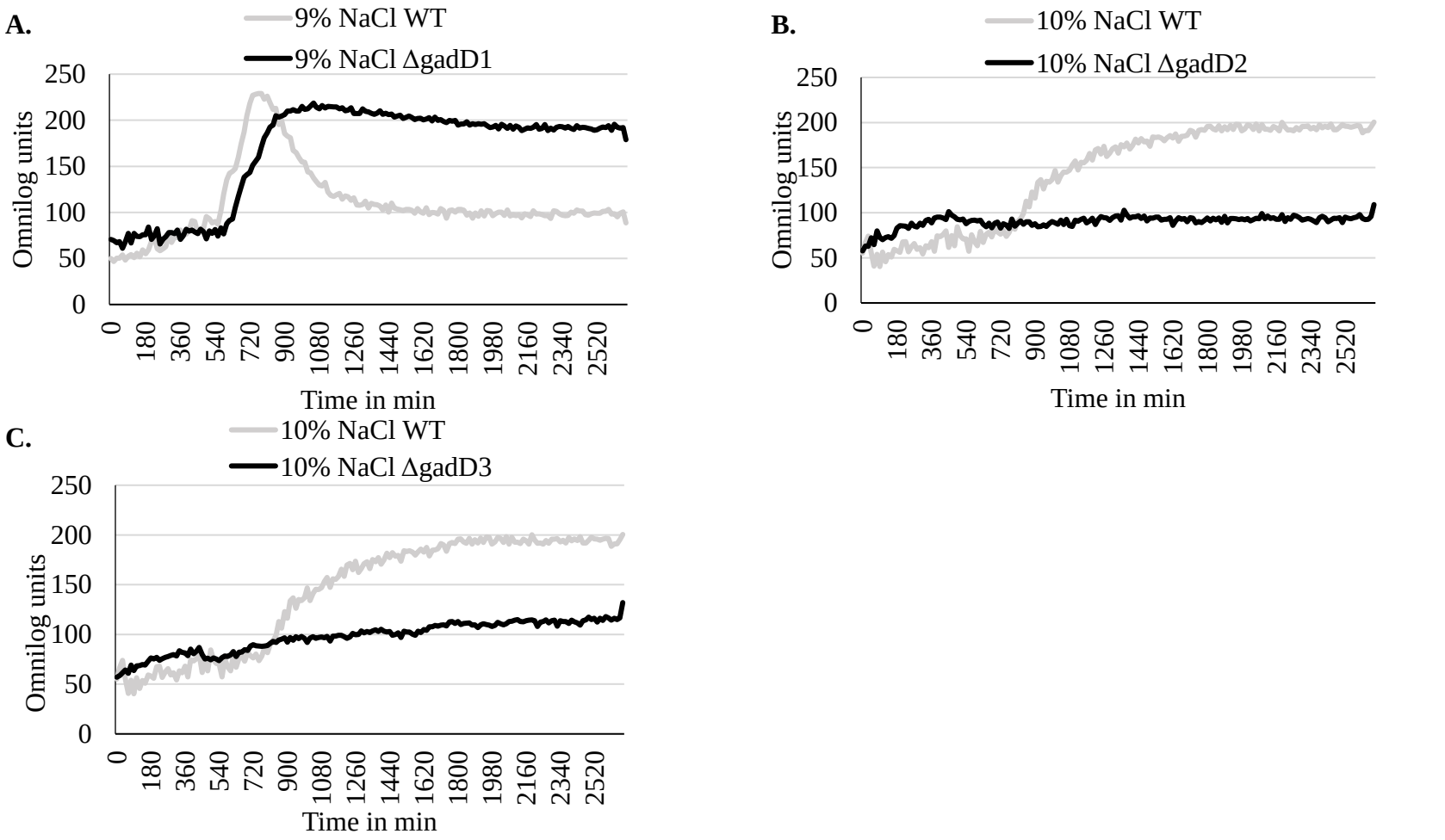
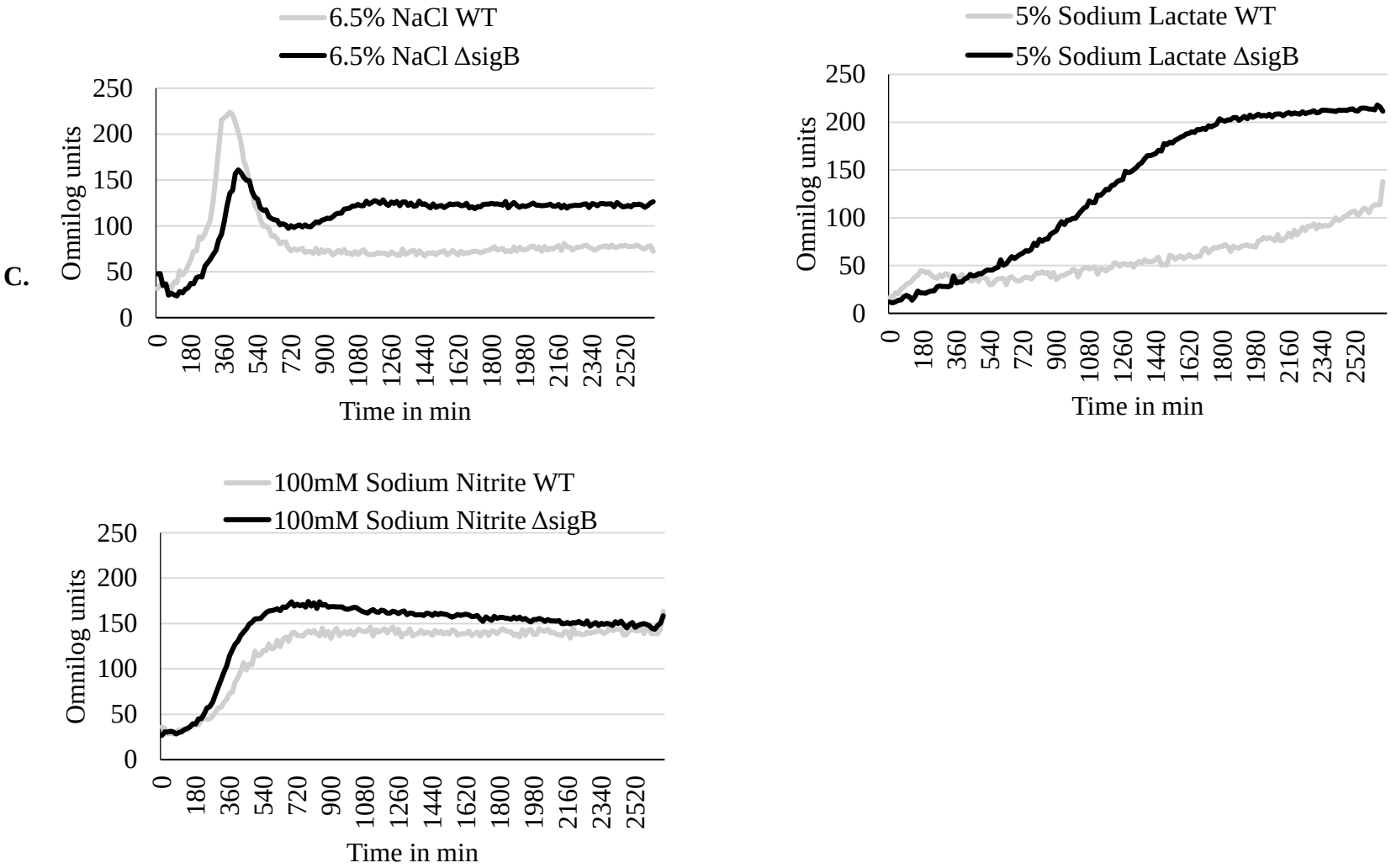


Fig 5.8: Differences observed in the effect of osmolytes betw **B.** *monocytogenes* 10403S WT and Gad knockout mutants with 10 % NaCl **A**. Δ*gadA* **B.** Δ*gadB* **C.** Δ*gadC*



C.

Fig 5.9: Differences observed in the effect of osmolytes between *L. monocytogenes* 10403S and Δ*sigB* **A**. 6.5% NaCl **B.** 5% Sodium Lactate **C.** 100 mM sodium nitrite

Appendix 3

List of commercially reformulated treatments reformulated based upon the original AM formula. All solutions are made up with in solution made up with 1 litre of tap water (pH 2.8).

Treatment 1: AM with citric acid replaced with trisodium citrate with added fumaric acid at 50 mM trisodium citrate 4.26g 14.5 mM (Equivalent to original 3.04g monosodium citrate (14.5 mM))

- Malic acid 1.96g or 14.6 mM.
- Tartaric acid 0.65g or 4.4 mM.
- Fumaric acid 5.80g or 50 mM.

Treatment 2: AM (50mM more malic than standard AM ®)

- Citric acid anhydrous 2.78g or 14.5 mM.
- Malic acid 8.66g or 64.6 mM.
- Tartaric acid 0.65g or 4.4 mM.

Treatment 3: AM (25mM more malic than standard AM ®)

- Citric acid anhydrous 2.78g or 14.5 mM.
- Malic acid 0.65g or 39.6 mM.
- Tartaric acid 4.4g or 4.4 mM.

Treatment 4: AM with citric acid replaced with trisodium citrate with added fumaric acid at 25 mM.

- Trisodium citrate 4,26g or 14.5 mM (equivalent to original 3.04g or 14.5 mM monosodium citrate)
- Malic acid 1.96g or 14.6 mM
- Tartaric acid 0.65g or 4.4 mM
- Fumaric acid 2.9g or 25 mM

Treatment 5: AM with citric acid replace with trisodium citrate with extra malic (50mM more malic than standard AM)

- Trisodium citrate 4,26g or 14.5 mM (equivalent to original 3.04g or 14.5 mM monosodium citrate).
- Malic acid 8.66g or 64.6 mM
- Tartaric acid 0.65g or 4.4 mM

Treatment 6: AM with citric acid replaced with trisodium citrate with extra malic (25 mM more malic than standard AM)

- Trisodium citrate 4,26g or 14.5 mM (equivalent to original 3.04g or 14.5 mM monosodium citrate).
- Malic acid 5.31g or 3.96 mM.

Tartaric acid 0.65 or 4.4 mM