The contribution of the σ^E-regulated putative stress response protein STM1250 to the *Salmonella* acid tolerance response.

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Abstract

Salmonella are enteric pathogens which frequently infect both humans and animals, resulting in various clinical manifestations. Fundamental to their pathogenesis is the ability of *Salmonella* to survive in acidic environments experienced throughout the digestive system and inside macrophages. Survival in acidic conditions is credited to several acid tolerance response (ATR) mechanisms, regulated by multiple transcriptional regulators including σ^E, also a major regulator of the envelope stress response.

The σ^E-regulated small heat shock proteins (sHsps) IbpA, IbpB, AgsA and the putative stress response protein STM1250, have recently been implicated in *Salmonella* survival against hydrogen peroxide-associated oxidative stress and the antimicrobial peptide polymyxin B. This same study reported a link between STM1250 and the *Salmonella* ATR induced by hydrochloric acid. We hypothesised in this study that IbpA, IbpB, AgsA and STM1250 may have unidentified roles in protecting *Salmonella* against commonly encountered stresses, which may have implications for food safety.

In this study we have corroborated previous findings that STM1250 functions in the initial stages of the *Salmonella* ATR in the presence of hydrochloric acid. We also established a novel role of STM1250 during the *Salmonella* ATR in the presence of citric acid. These phenotypic results suggest that STM1250 functions in the *Salmonella* ATR regardless of environmental factors, enabling potential future structural and functional investigations and studies utilising STM1250 as a therapeutic target for vaccine development.

Investigation into STM1250s importance in the *Salmonella* ATR during the storage of apple and orange juice identified a trend in which the STM1250 mutant had reduced survival when incubated in these juices, suggesting STM1250s potential implications for *Salmonella* survival in acidic food products. These initial findings in connection with continued research will facilitate the identification of improved food safety regulations, with more specialised implications for the inactivation of *Salmonella* specific to the type of food product.

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List of abbreviations

A	Apple Juice
AA	Acid adapted
ASPs	Acid shock proteins
ATR	Acid tolerance response
AMPs	Antimicrobial peptides
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BAM	β-barrel-assembly machinery
CDC	Centre for Disease Control and Prevention
CFU	Colony forming units
CPS	Capsular polysaccharide
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum β-lactamase
ESRs	Envelope stress responses
Fur	Ferric uptake regulator
HACCP	Hazard Analysis and Critical Control Point
HIV	Human immunodeficiency virus
HCl	Hydrochloric acid
IM	Inner membrane
iNTS	Invasive non-typhoidal Salmonella
Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid
LPS	Lipopolysaccharide
LB	Luria-Bertani
MDR	Multi-drug resistant
NAA	Non-acid adapted
NCE	Non-carbon essential
NTS	Non-typhoidal serovars
O	Orange Juice
OM	Outer membrane
OMP	Outer membrane proteins
O/N	Overnight
ANOVA	One way Analysis of Variance
OD	Optical density
OMPs	Outer membrane proteins
PAMPs	Pathogen-associated molecular pattern molecules
PBS	Phosphate-buffered Saline
POTRA	Polypeptide-transport-associated
rcf	Relative centrifugal force
RNA	ribonucleic acid
rpm	Rotations per minute
SCV	*Salmonella* containing vacuole
SEM	Standard error of the mean
SPSS	Statistical Package for the Social Sciences
TCS	Two component systems
TSB	Tryptic soy broth
UDP	Uridine diphosphate
UVA	Ultraviolet A
UV-Vis	Ultraviolet/ Visible area
Und-P	Undecaprenyl phosphate
uOMPs	Unfolded OMPs
UTR	Untranslated region
ECDC	European Centre for Disease Preventing and Control

Vi CPS	Vi capsular polysaccharide
w/v	Weight per volume
WHO	World Health Organisation
WT	Wild type
XDR	Extensively drug-resistant
µG	Microgram
µL	Microlitre

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Chapter 1: Introduction

1.1 *Salmonella* nomenclature

Salmonella is a member of the Enterobacteriaceae family of Gram-negative bacteria. The genus of *Salmonella* is divided into two species, *Salmonella enterica* and *Salmonella bongori* each of which contain many serovars*. S. bongori* is rarely associated with human disease and is instead predominately found in the environment and cold-blooded animals, and therefore will not be discussed in depth in this review (Brenner *et al*., 2000). *S. enterica* is split into six different subspecies (subsp.) referred to using roman numerals. The subspecies consist of: *enterica* (I), *salamae* (II)*, arizonae* (IIIa), *diarizonae* (IIIb) *houtenae* (IV), and *indica* (VI). The most up to date classification system used by the Centres for Disease Control and Prevention (CDC) uses names for all serotypes in subspecies I, and antigenic formula for unnamed serotypes discovered post 1966 for subspecies II, IV and VI. The antigenic formula is based on the presence of O and H antigens on the bacterium (Brenner *et al*., 2000). I, *S. enterica* subsp. *enterica* is split into different named serovars and are usually grouped into either those which cause typhoidal or non-typhoidal disease, examples are provided in Figure 1.

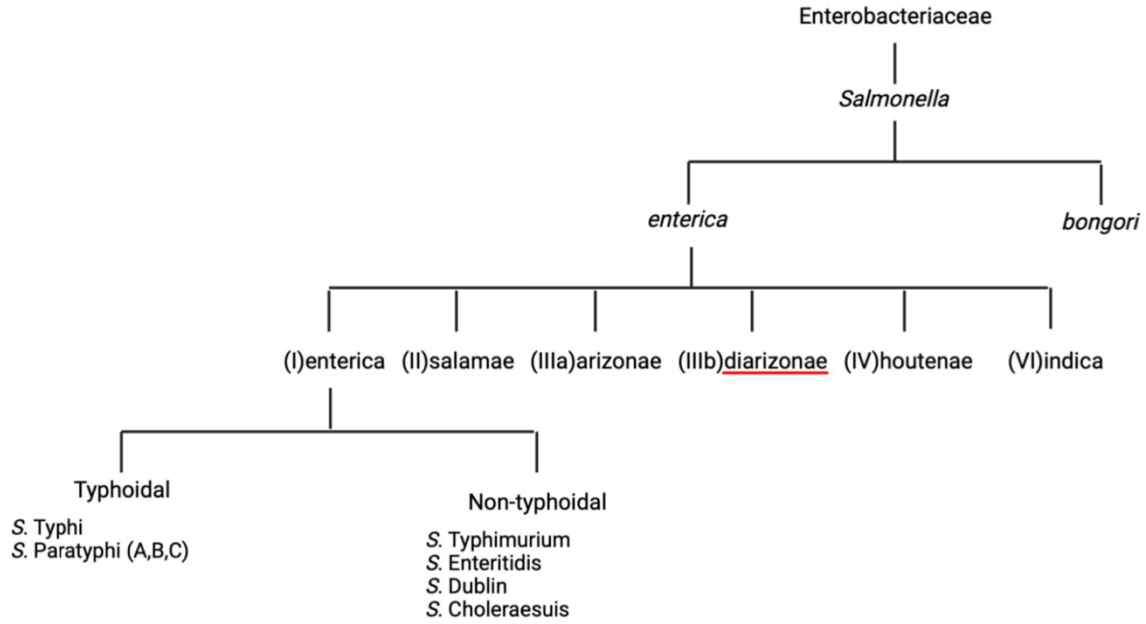

Figure 1. *Salmonella* taxonomy and nomenclature. *Salmonella* belongs to the Enterobacteriaceae family of bacteria, the genus of *Salmonella* is split into two species *S*. bongori and *S*. enterica which is subdivided into six subspecies. Subspecies I (enterica) can then be grouped based on serovars which cause typhoidal disease or not (typhoidal or Non-typhoidal) with examples provided for both.

1.2 *Salmonella* pathogenesis

Salmonella serovars are one of the most frequently isolated foodborne pathogens posing a significant threat to worldwide public health, causing global morbidity and mortality (Eng *et al.,* 2015). *Salmonella* spp. are accountable for approximately 1.3 billion cases of disease each year (Coburn *et al*., 2007). This approximate likely under-represents the true value of disease annually, this is as a result of inadequate surveillance systems, low sensitivity of diagnostic tools and healthcare inequalities causing populations at the highest risk not to seek medical intervention (Amicizia *et al*.,2017). *Salmonella* serovars range in their clinical implications with disease manifestations including, gastroenteritis (also known as salmonellosis), enteric fever (typhoid), bacteraemia, and asymptomatic chronic carrier state. Non-typhoidal serovars (NTS) have a broad host range, with human infection generally resulting in self-limiting gastroenteritis (Balasubramanian *et al*., 2019). NTS serovars cause a greater proportion of disease in comparison to typhoidal serovars, with an estimated 93 million enteric infections and 155,000 associated deaths occurring annually (Majowicz *et al*., 2010). In developed countries, *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most frequently occurring serovars associated with human illness (Duggan *et al*., 2012). *Salmonella* Typhi and *Salmonella* Paratyphi A are the major causative agents for enteric fever. An investigation into the global burden of disease caused by *Salmonella* Typhi and Paratyphi reported an estimated 14.3 million cases of the disease and a death rate of 198 per 100,000 in 2017 (Stanaway *et al*., 2019).

1.2.1 Sources of infection

NTS serovars infect various animals many of which are used as livestock by humans, including cattle and poultry which act as reservoirs for *Salmonella*. NTS serovars can cause zoonotic infection which is the primary cause of enteric and systemic diseases, with infected animal products transmitting the disease to humans (Giannella, 1996; Stevens *et al*., 2009). Different serovars have been associated with contamination of certain food products, with infection by *S.* Enteritidis linked to the consumption of contaminated eggs and poultry meat. When cases of *S.* Typhimurium are more strongly associated with the consumption of contaminated poultry, pork and beef products (Duggan *et al*., 2012). *Salmonella* infection has also been directly linked to contaminated water and non-animal products including raw vegetables and fruits, with a growing body of evidence suggesting *Salmonella* can infect various plants utilising them as hosts (Schikora *et al*., 2012). Typhoidal serovars are host restricted to humans and are unable to colonise animals. Therefore, as humans are the only possible reservoir for

typhoidal serovars the transmission route occurs directly from human to human, with chronic carriers able to shed *Salmonella* contaminating water or food sources (Bhan *et al*., 2005).

1.2.2 Clinical manifestations

Human infection by NTS serovars results in localised gastroenteritis, also known as salmonellosis which is an inflammatory condition of the gastrointestinal tract. Symptoms usually arise 6 to 72 hours post initial infection and consist of diarrhoea, vomiting, nausea, headaches and abdominal cramps (Eng *et al*., 2015). Gastroenteritis is typically restricted by the host immune system, self-limiting the disease within 4 to 7 days post infection. However, many complications can arise from this initial infection causing significant morbidity in patients, especially those who are more susceptible such as children, the elderly and immunocompromised patients (Scallen *et al*.,2011). In cases of NTS infection approximately 5% of patients with gastrointestinal disease develop the more lethal illness, bacteraemia (Acheson and Hohmann, 2001). Bacteraemia refers to the presence of viable bacteria in the blood which can turn into a bloodstream infection, leading to various complications (Smith and Nehring, 2022). Severe complications as a result of bloodstream infection included sepsis, osteomyelitis, lung infections and septic arthritis (Crump *et al*., 2015). Treatment of bacteraemia resulting in *Salmonella* infection typically includes antibiotics such as ampicillin or quinolones. Though, due to the emergence of antibiotic resistant strains infections are harder to treat with third-generation antibiotics such as cephalosporins (Acheson and Hohmann, 2001).

Host-restricted *Salmonella* serovars *S*. Typhi and *S*. Paratyphi (A, B, and C) result in enteric fever disease manifestations. Symptoms associated with infection by these serovars first include mild fever, headaches, and fatigue. However, in more progressed infections vomiting and diarrhoea may arise with a high mortality rate associated (Kumar and Kumar, 2017). Serovars responsible for enteric fever can colonise the gall bladder establishing an asymptomatic chronic infection known as chronic carrier state. *Salmonella* is able to survive and evade the host immune system in this niche acting as host-restricted reservoirs for these serovars (Gunn *et al*., 2014). Enteric fever and chronic carrier state will not be discussed at length due to the NTS serovars being the priority for this investigation.

1.3 Invasive non-typhoidal *Salmonella*

Of emerging importance are NTS serovars which are resulting in frequent cases of invasive disease in many sub-Saharan African countries (Morpeth *et al*., 2009). Invasive non-typhoidal *Salmonella* (iNTS) infection is caused by serovars typically responsible for salmonellosis, including *S*. Typhimurium with the strain ST313 being strongly implicated in iNTS cases in sub-Saharan Africa (Kingsley *et al*., 2009; Feasey *et al*., 2012; Okoro *et al*., 2012). The global burden inferred by iNTS is difficult to accurately implicate, this is as a result of the distinct lack of laboratories capable of microbiological diagnostic reporting in regions where iNTS is epidemic (Balasubramanian *et al*., 2019). An estimation into the global burden however suggests an annual 3.4 million cases of disease and 681,316 deaths annually, with roughly 2/3 of all cases occurring in children younger than five (Ao *et al*., 2015).

The clinical presentation of iNTS infection is nonspecific although distinct from both salmonellosis and typhoid fever, characterisation is instead based on non-specific fever, respiratory symptoms, splenomegaly, hepatomegaly and diarrhoea with microbiological confirmation essential for diagnosis (Kingsley *et al*., 2009: Feasey *et al*., 2012). A problematic clinical overlap exists with many symptoms shared with pneumonia and malaria, in many cases iNTS infection is indistinguishable from malaria. Complicating things further patients typically present with focal infection, predominately of the respiratory tract, this is as a result of co-infection with other pathogens. (Feasey *et al*., 2012; Okoro *et al*., 2012). Due to the fact iNTS cannot be reliable distinguished and the frequent co-infection with other pathogens, World Health Organisation (WHO) guidelines suggest treatment of all children presenting with severe malaria with broad-spectrum antibacterials in order to treat potential iNTS infections (WHO, 2015). Many risk factors are associated with contraction of iNTS, age is a considerable factor with children below the age of three and older patients being particularly at risk. Co-morbidities such as malnutrition, malaria, anaemia and HIV are thought to also be risk factors in both children and adults (Morpeth *et al*., 2009). Due to the niche immunological defects established in HIV patients provide, frequent bacterial colonisation by iNTS occurs. This is thought to be more specifically due to dysregulated cytokine production the impaired killing of bacteria in the serum and dysregulated epithelial barrier maintenance, enabling *Salmonella* to persist and enter the bloodstream more readily (Feasy *et al*., 2012).

1.4 *Salmonella* treatment

Salmonella poses a significant risk to global public safety, the efficient control of disease resulting from *Salmonella* infection through both treatment and prevention is therefore highly

important. Timely diagnosis of disease resulting from *Salmonella* infection as well as public surveillance is paramount, with precise and efficient diagnosis avoiding the need for antibiotic treatment (Vollaard *et al*., 2005).

1.4.1 Antibiotic treatment

For the most part healthy individuals infected with NTS *Salmonella* serovars rarely require clinical intervention as gastroenteric symptoms are typically self-limiting. However, for children the elderly and immunocompromised individuals, treatment with antibiotics may be required. Infection with typhoidal serovars is more serious and therefore it is more common for patients to be treated with antibiotics (Sánchez-Vargas *et al*., 2011). The first antibiotic identified for treatment of typhoid and paratyphoid fever in 1948 was chloramphenicol, revolutionising the prognosis of individuals with the disease. As the trend with many widely used antibiotics, resistant strains of *S*. Typhi soon emerged. Some of which boasted multi-drug resistance to many other common first line antibiotics such as ampicillin, amoxicillin, and trimethoprim-sulfamethoxazole. The first line antibiotics of choice therefore progressed to fluoroquinolones, ofloxacin and ciprofloxacin drugs in the 1990s (Levine and Simon, 2018). The current recommended antibiotics include chloramphenicol, ampicillin and cotrimoxazole, fluoroquinolones, azithromycin and third-generation cephalosporines such as ceftriaxone and cefixime (Browne *et al*., 2020). Global resistance towards many antibiotics is dramatically increasing with resistance being reported to many of the commonly used antibiotics. For example, an outbreak of an extensively drug-resistant (XDR) S. Typhi has been documented in Pakistan, conferring resistance to three first-line antibiotics chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole in addition to fluoroquinolones and third-generation cephalosporins (ceftriaxone/cefixime). Cephalosporin resistance is suggested to be mediated by a harboured $bla_{CTX\text{-}M\text{-}15}$ extended-spectrum β-lactamase (ESBL) gene. Fortunately, these isolates still remain treatable with azithromycin and parenteral meropenem (Klemm *et al*., 2018). The frequency and association with more severe disease outcome of antibiotic resistant NTS infections is becoming a reason of concern. Multiple NTS serovars have been reported as having acquired resistance to many commonly prescribed antibiotics including ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Mukherjee *et al*., 2019). The increasing prevalence of multi-drug resistant (MDR) and XDR strains of *Salmonella* pose a significant threat to public health, highlighting the requirement of new treatment methods and research into therapeutic targets.

1.4.2 Vaccines

Due to the increasing threat to public safety posed by *Salmonella* and the emerging prevalence of MDR and XDR strains, an increase in demand for preventative methods has arisen. The most adept method of preventing serious disease caused by pathogenic bacteria is vaccination. The first *Salmonella* vaccine targeting *S*. Typhi was a parenteral inactivated whole cell vaccine, which was introduced to England and Germany in 1896. Conceptually the vaccine was based on heat inactivation of *S*. Typhi using phenol as a preservative, acetone was then determined as an improved inactivation agent avoiding the destruction of heat-labile antigens which improved the overall efficacy of the vaccine (Guzman *et al*., 2006). Although the efficacy of these vaccines was adequate global use was halted due to issues with reactogenicity, causing local inflammation, pain, systemic fever, malaise and disease like symptoms (Marathe *et al*., 2012).

Currently, two different types of vaccine are available for S. Typhi and therefore typhoid fever these include a subunit vaccine known as Vi capsular polysaccharide (Vi CPS) and a live attenuated vaccine deemed Ty21a (Marathe *et al*., 2012). The Vi CPS vaccine was developed from the wild type (WT) *S*. Typhi Ty2 strain, utilising non-denatured purified Vi polysaccharide. Capsular polysaccharides (CPS) are a major cell-surface component of many bacteria including *Salmonella*, contributing to virulence and proving barrier protection. There are two types of CPS in *Salmonella,* the Vi capsule found in *S*. Typhi and the O-antigen of NTS serovars. The Vi capsule functions as a virulence factor by enabling immune evasion, this is by interfering with complement deposition reducing bacterial killing as a result of neutrophil chemotaxis. The Vi CPS vaccine predominately creates an antibody response towards the Vi capsule, therefore creating an immunity towards *S*. Typhi specifically (Perera *et al*., 2021). Significantly, the vaccine is not licensed for children under the age of two, this is due to the Vi capsule being a T-independent antigen and therefore unlikely to be immunogenic in infants (MacLennon *et al*., 2014).

The Ty21a live attenuated vaccine is sold under the brand name Vivotif®, it is administered orally in capsular form on alternating days (1,3,5) for a total of either 3 or 4 days (Jackson *et al*., 2015). The vaccine was developed through non-specific chemical mutagenesis of *S*. Typhi Ty2 strain, producing a Ty21a mutant lacking the *galE* gene which encodes uridine diphosphate (UDP)-galactose-4-epimerase. This consequently means *S*. Typhi can no longer convert UDP-glucose to UDP-galactose and vice versa, imported exogenous galactose is then able to accumulate intracellularly resulting in lysis and cell death rendering the Ty21a strain avirulent. The additional implication of the *galE* mutation is that when galactose is supplied

exogenously, immunogenically important lipopolysaccharides are synthesised and form on the cell surface eliciting an immune response (Germanier and Fiirer, 1975). Emerging evidence is also suggesting possible cross-protective humoral immune responses of the Ty21a vaccine to *S*. Paratyphi A and B. However, further investigation is needed to assess the efficacy of these responses (Wahid *et al*., 2012).

Although effective in providing protective immunity against typhoid fever the aforementioned vaccines still have major issues associated with them. Crucially, both types of vaccines are not licenced for young children, this poses an issue as young children are one of the most susceptible demographics for typhoid fever. Another consideration is that vaccine boosters are recommended every 2-3 years, making both vaccines a non-suitable method for continuous prevention. This predominantly impacts developing countries which might not have the infrastructure or funds available to provide boosters (Khan *et al*., 2017). Finally, both vaccines are not thermostable over long periods of time and require a cold chain (continuous cold storage) to prolong stability and effectiveness, this again is difficult in developing countries where access to cold storage might not be readily available (Perera *et al*., 2021). A separate issue is also evident with the current Vi CPS and Ty21a vaccines, as they only provide immunological protection against *S*. Typhi and consequential typhoid fever. No confirmed protection is afforded towards other globally important *Salmonella* serovars including *S*. Paratyphi, NTS and iNTS (MacLennon *et al*., 2014). The failure of any new vaccines for typhoid fever and iNTS disease is as a result of a lack of commercial interest, due to the diseases predominately impacting low-income countries which don't have the funds to invest in vaccine development. The emergence of conjugate vaccines is however showing promise in resolving some of the issues faced by current vaccines such as the effective treatment of children. However, to date licensing of these vaccines for use is only approved in India (Sahastrabuddhe and Saluja *et al*., 2019).

1.5 The Gram-negative envelope

Bacterial cell envelopes are complex, multilayered, highly functional structures that provide protection to bacteria from a variety of hostile environments and stresses. Dissimilar to cells of other high organisms, bacterial cells come into direct contact with a vast array of unpredictable, interchanging and often potentially lethal environments. Faced with constant environmental pressure bacteria evolved an elaborate and synergistic cell envelope that not only defends against stressful conditions, but also enables the selective interchange of nutrients and chemicals with the extracellular space. The Gram-negative cell envelope is comprised of three principal layers; the outer membrane (OM), the aqueous periplasmic space

containing a layer of peptidoglycan, and the inner membrane (IM) (Figure 2) (Silhavy *et al.*, 2010).

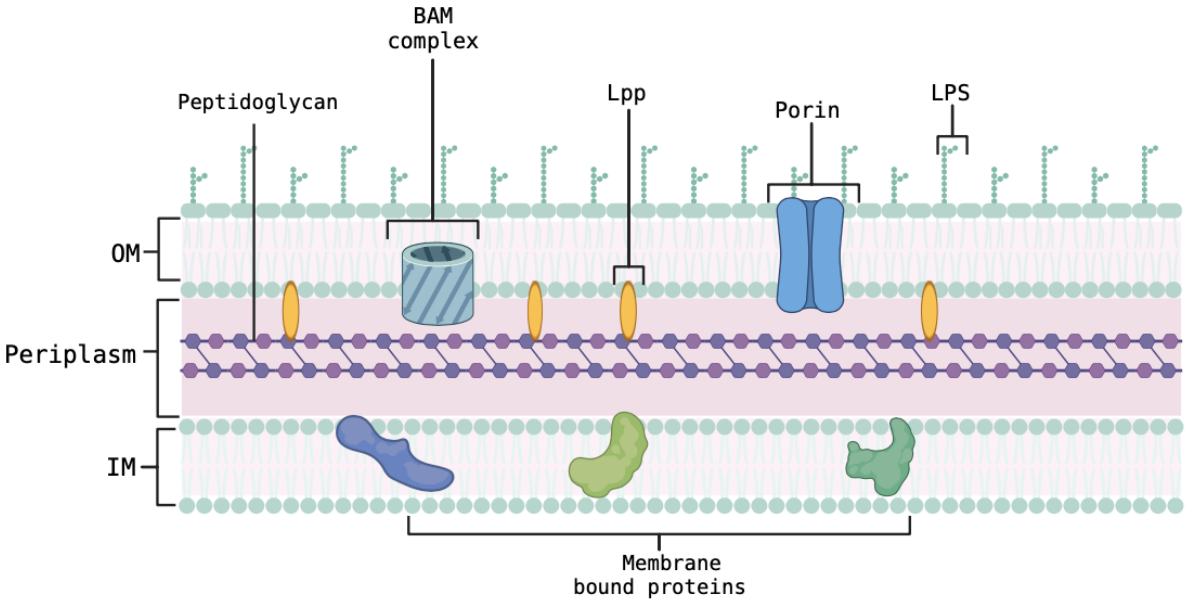

Figure 2. The cell envelope of Gram-negative bacteria. Key structures of the Gram-negative envelope are depicted, the three predominant layers consist of the outer membrane (OM), the peptidoglycan layer emersed in the periplasm, and the inner membrane (IM). The outer leaflet of the OM consists of lipopolysaccharides (LPS), within the OM contains various proteins including transmembrane porins, β-barrel-assembly machinery (BAM) complexes and lipoproteins known as Lpp. The IM contains various membrane bound proteins.

The OM is exclusive to Gram-negative bacteria, similar to other typical biological membranes the OM is lipid bilayered. However, in comparison only the inner leaflet of the membrane is comprised of phospholipids, when the outer leaflet is formed of lipopolysaccharide (LPS) (Figure 2) a highly negatively charged molecule which extends into the bacterial environment (Rollauer *et al.*, 2015). This atypical membrane contains various lipoproteins and outer membrane proteins (OMPs) crucial for the correct functioning of the membrane. Lipoproteins contain lipid moieties which typically result in them being embedded in the inner leaflet of the OM, further translocation can occur onto the cell surface, or they can be assembled into OM protein (OMP) complexes (Konovalova and Silhavy, 2015). Functions of the majority of lipoproteins still remain unresolved, although they are predicted to play important roles in the periplasm (Miyadai *et al.*, 2004). The majority of OMPs functioning in the OM as transmembrane proteins are classified as β-barrel proteins. These proteins are exposed to the extracellular environment and therefore have a plethora of roles ranging from molecular

transport, signalling, adhesion used in virulence, and acting as degradative enzymes (Rollauer *et al*., 2015).

The Peptidoglycan layer is situated directly below the OM, in fact it is anchored onto the OM via lipoproteins called Lpp, murein lipoprotein, or Braun's lipoprotein (Braun, 1975). Peptidoglycan itself functions by providing cell integrity, withstanding turgor pressure. Additionally, it contributes to cell shape and acts as a scaffold for other important cell envelope proteins and teichoic acids (Silhavy *et al*., 2010). Peptidoglycan is specifically made up of many repeating units of the disaccharide N-acetyl glucosamine-N-actyl muramic acid, which are cross-linked by pentapeptide side chains (Vollmer *et al*., 2008). The IM is characterised as a phospholipid bilayer which is the more typical cellular membrane, located within consists of a myriad of proteins. The functioning of these IM proteins is crucial for the survival of the bacterial cell due to their range of implications including energy production, lipid biosynthesis, protein secretion, and transport (Silhavy *et al*., 2010).

1.5.1 Envelope biogenesis

The constituents of the Gram-negative cell envelope including proteins, LPS and phospholipids are synthesised remotely in either the cytoplasm or the inner surface of the IM. Components therefore must be transported across the IM; different processes are utilised depending on the final destination. Periplasmic components must be released from the IM, peptidoglycan components must be released and polymerised and OM components must be transported across the periplasm and assembled (Silhavy *et al*., 2010).

Proteins are synthesised in the cytoplasm via ribosomes, the majority of proteins are transported across the IM in their unfolded state through the general secretion pathway (Sec pathway). This pathway is comprised of SecYEG IM channel and the SecA motor adenosine triphosphatase (ATPase) which provides the energy for the translocation by hydrolysing adenosine triphosphate (ATP) (Tsirigotaki *et al*., 2017). Once translocated across the IM, periplasmic chaperones such as SurA, Skp and DegP protect unfolded OMPs (uOMPs) and aid in their further transport. uOMPs are transported by these chaperones to the inner surface of the OM where they are recognised by the β-barrel-assembly machinery (BAM) complex, which functions by folding and inserting these proteins into the OM. The BAM complex is comprised of an integral OMP called BamA and four lipoproteins (BamB,C,D, and E), BamA contains a set of five Polypeptide-transport-associated (POTRA) domains at the N terminus and a β-barrel domain embedded in the OM at the C terminus. Although the functional roles of the BAM components and the overall mechanism of OMP substrate membrane insertion

remains unresolved based on structural analysis it is thought that OMPs integrate into the OM via a lateral opening of the BamA β-barrel (Han *et al*., 2016). The genes which encode the BAM complex components are each regulated by the extracytoplasmic sigma factor σ^E (Skovierova *et al*., 2006).

LPS is comprised of three major regions; Lipid A, the oligosaccharide core and the O-antigen. Lipid A, is a highly acylated β-1′-6-linked glucosamine disaccharide that is localised to the outer leaflet forming the hydrophobic region of the molecule. The oligosaccharide is nonrepeating in structure and is linked to the glucosamines of lipid A, contained within this structure are typically 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residues, heptoses, and hexoses. Attached to the core oligosaccharide is the O-antigen which is an extended polysaccharide comprised of repeating oligosaccharides made from two to eight sugars (Bertani *et al*., 2018). The overall composition of LPS is not fully conserved across Gram-negative bacterial species. Although the structure of lipid A is highly conserved at the species level, the core oligosaccharides and even more so the O-antigen structurally vary at the species level of bacteria and can even vary between strains of the same species. Interestingly, the O-antigen is completely omitted within some Gram-negative bacteria (Bertani *et al*., 2018). Synthesis of lipid A follows the Raetz pathway occurring at the inner leaflet of the IM (Raetz *et al*., 2009). Biosynthesis of the oligosaccharide core is intertwined with that of lipid A, with the formation of the lipid A complex bound to two Kdo groups which is then translocated across the IM to the periplasm by the ATP-binding cassette (ABC) transporter (MsbA) (Whitfield and Trent, 2014). Distinct from the lipid-core biogenesis the O-antigen is synthesised with the sequential addition of monosaccharides onto the lipid carrier molecule undecaprenyl phosphate (Und-P), this newly created polysaccharide is then transferred to the outer leaflet of the IM and ligated to the lipid A-core acceptor by the O-antigen ligase WaaL (Raetz and Whitfield, 2002).

As previously mentioned LPS is localised to the outer leaflet, through extensive research the functioning and biogenesis of LPS in the OM has been unravelled. It is now understood that LPS molecules interact with each other on the cell surface through divalent cations, forming a permeable barrier. This barrier selectively prevents entry to small hydrophobic compounds including bile salts, detergents and antibiotics overall enabling survival in harsh conditions (Zhang *et al*., 2013). Due to the formation of an effective permeability barrier LPS is considered a key virulence factor of Gram-negative pathogenic bacteria, due to its importance in virulence LPS is recognised by the host immune system and is hence classified as pathogen-associated molecular pattern molecules (PAMPs). Genes involved in the biosynthesis and assembly of LPS have therefore emerged as good therapeutic targets (Srinivas *et al*., 2010). Damage to

the LPS and accumulation of LPS components in the periplasm induces the σ^E envelope stress response, as σ^E takes part in the regulation of genes involved in LPS biogenesis and transport (Rhodius *et al*., 2006).

1.6 Bacterial stress responses

Bacteria inevitably encounter a variety of environments during their lifecycle, many of which confer harsh stressful conditions. Some of which include extreme temperature and pH changes, antimicrobials, osmotic pressure and nutrient limitation. Due to the damage these stresses can impose on bacteria such as the damage of crucial cellular proteins and membranes, key adaptive mechanisms known as bacterial stress responses have been developed providing resilient protection. Bacterial stress responses respond to stress leading to the precise induction of regulatory networks, which consequently control the expression of genes involved in the response to disrupted cellular homeostasis (Dawan and Ahn, 2022). These networks ultimately lead to the regulation of transcription regulons through either the activation of an alternative sigma factor subunit of RNA polymerase or DNA-binding two-component response regulators (Marles-Wright and Lewis, 2007). These stress responses are therefore characterised as being either sigma (σ) factors or two component systems (TCS). Bacterial stress responses are not just crucial for survival, helping withstand harsh stressful conditions in the environment but are also fundamental in pathogenesis. Certain host-associated stresses such as pH, temperature and reactive oxygen species can additionally induce the expression of virulence genes via stress response mechanisms. An overall understanding of stress responses and their precise regulatory mechanisms is therefore crucial in advancing our understanding of bacterial pathogenesis (Fang *et al*., 2016).

1.6.1 Two component systems

TCSs are a fundamental mechanism in all bacteria including pathogenic bacteria such as *Salmonella*, while some TCSs are crucial for cell growth and fitness others have stronger implications to virulence and drug resistance responding to stress-inducing environmental conditions (Hirakawa *et al*., 2020). TCSs are comprised of at least two proteins, typically a sensor kinase which functions to sense external stimuli and a response regulator which can primarily bind to DNA altering transcription but is also involved in other regulatory interactions (Gao *et al*., 2007). More specifically following external stimulus such as changes in but not limited to nutrient levels, pH, osmotic pressure a conserved histidine residue on the sensor kinase becomes phosphorylated via auto-phosphorylation (Gao and Stock, 2009). This phosphate group can then be transferred to a conserved aspartate residue on the response

regulator inducing a conformational change, enabling the binding to a target DNA region therefore resulting in the regulation of gene expression through modulation of promoter sequences (Kenny and Anand, 2020). Although many TCSs exist, of importance in this study are the PhoPQ and OmpR/EnvZ TCSs of *Salmonella* and will therefore be discussed below and further in chapter 3.

The PhoPQ TCS is comprised of the sensor kinase PhoQ and its cognate response regulator PhoP. PhoQ typically forms a dimer and is found in the bacterial IM, it is comprised of two transmembrane regions which span the IM providing periplasmic and cytoplasmic sensor domains. Induction of PhoQ results in its autophosphorylation on a histidine residue on the cytoplasmic domain, this phosphate is then transferred to an aspartate on PhoP enabling the regulation of *phoP*-activated (pag) and -repressed (prg) genes (Monsieurs *et al*., 2005). PhoPQ has been determined to respond to various environmental stresses, including low concentrations of divalent magnesium (Mg^{2+}) and divalent calcium (Ca^{2+}) as well as acidic pH and cationic antimicrobial peptides (AMPs) (Lu *et al*., 2020). The PhoPQ two-component system controls a range of different genes implicated in numerous cellular activities, this includes genes implicated in survival and resistance towards AMPs and pH (Groisman, 2001). The implications of the PhoPQ system towards acid stress will be discussed further in chapter 3.

The EnvZ/OmpR TCS similarly to PhoPQ is comprised of a sensor kinase EnvZ and its cognate response regulator OmpR. EnvZ is located at the IM and consists of two transmembrane domains that flank a periplasmic domain and a cytoplasmic domain, which contains the histidine residue that becomes phosphorylated and a OmpR binding region. The EnvZ/OmpR TCS is best characterised for its involvement in responding to osmotic stress, with EnvZ capable of responding to low osmolarity and activating OmpR which regulates expression of many genes including OM porins including OmpF and OmpC. It is thought that this regulation of porins has implications to virulence in *E. coli* and *Salmonella* enabling the recognition of host environments (high osmolarity) and dilute environmental conditions (low osmolarity) (Kenny and Anand, 2020). Of relevance to this study is that OmpR has also been described as an acid shock protein (ASP) involved in the ATR of *Salmonella,* this will be discussed more in chapter 3 (Bang *et al*., 2003).

1.6.2 Sigma factors

Much of the research into bacterial stress responses including sigma factors have been conducted in the Gram-negative bacteria *E. coli*. Most of the processes determined in this model organism are highly conserved with *Salmonella* due to it also being a Gram-negative bacterium, although crucial differences are evident which will be highlighted later. As previously mentioned, bacterial sigma factors activate RNA polymerase via binding to the core creating a holoenzyme which initiates transcription (Bono *et al*., 2017). The classification of sigma factors primarily falls into two distinct groups either the σ^{70} family, or the structurally distinct σ^{54} family. Both σ^{70} (RpoD) and σ^{54} (RpoN) represent specific sigma factors, the primary sigma factor σ^{70} is responsible for the majority of transcription during growth, when σ^{54} is concerned with directing transcription is response to environmental stress. (Paget, 2015). Categorisation of sigma factors is based on their homology towards either σ^{70} or σ^{54}. Interestingly the σ^{54} family only contains one member being itself, σ^{54} (RpoN) (Bono *et al*., 2017). In *S.* Typhimurium six sigma factors are present controlling gene expression this includes: the primary sigma factor σ^{70} (*rpoD*), and the alternative sigma factors, σ^{24} (*rpoE*), σ^{32} (*rpoH*), σ^{38} (*rpoS*), σ^{28} (*fliA*), and σ^{54} (*rpoN*) (Gruber and Gross, 2003). Of importance in this study are the alternative sigma factors contained within the σ^{70} family specifically σ^{E} and σ^{S}, beyond the scope of this investigation are σ^{H} and FliA therefore they will not be discussed.

The alternative sigma factor, σ^{S} encoded by *rpoS* is regarded as the general stress response. During optimal growth conditions levels of σ^{S} are relatively low, however during the stationary phase of bacterial growth expression is greatly increased (Battesti *et al*., 2011). In addition to its functioning in stationary phase cells σ^{S} also functions in response to environmental stress, with expression induced following but not limited to extreme pH, temperature, osmolarity, and DNA damage (Battesti *et al*., 2011). The σ^{S} regulon has been studied in depth, a large percentage of at least 10% of the total bacterial genome in *E. coli* is positively controlled by σ^{S} (Weber *et al*., 2005). Although regulon members are well conserved between bacterial species such as *Salmonella*, some members are however unique to the species. Therefore, responses towards certain stresses are different, highlighting the importance of species specific research (Meléndez *et al*., 2022). Due to its expansive regulon transcription, translation and post-translational control of σ^{S} is tightly regulated. σ^{S} synthesis and downregulation of its degradation occurs during unfavourable conditions for the cell as mentioned above. Many regulatory cascades are involved in the induction of the σ^{S} system. This includes a response which utilises the small molecule guanosine pentaphosphate (ppGpp) which promotes the increased transcription, translation and inhibition of degradation of σ^{S} following nutrient starvation conditions through maintenance of mRNA stability (Traxler

et al., 2008). Negative regulation of σ^S is also evident during specific stress responses, with cyclic AMP (cAMP) and catabolite response protein (CRP) acting antagonistically towards σ^S (Battesti *et al*., 2011).

The extracytoplasmic sigma factor, σ^E encoded by *rpoE* is fundamental for the maintenance of cell envelope homeostasis and is hence considered an envelope stress response (ESR) (Hews *et al*., 2019). Misfolded OMPs in the OM or periplasm and unlocalised LPS are signs of unbalanced envelope homeostasis, the σ^E ESR can sense these unbalances and initiate transcription of specific genes to resolve the issue. Damage to the cell envelope caused by various environmental stresses can initiate the induction of the σ^E pathway including oxidative stress, heat and cold shock, carbon starvation (Rowley *et al*., 2006), acid stress (Muller *et al*., 2009) ultraviolet A (UVA) radiation, P22 phage, and hypo-osmotic shock (Amar *et al*., 2018). Under typical physiological conditions σ^E is sequestered to the transmembrane protein RseA, inhibiting its ability to interact with RNA polymerase to induce transcription, RseB has also been determined to negatively regulate σ^E by increasing the affinity of RseA towards σ^E (De Las Peñas *et al*., 1997; Collinet *et al*., 2000). Induction of σ^E occurs through regulated intramembrane proteolysis. More specially the site-1 protease DegS can interact with uOMPs in the periplasm causing a conformation change which exposures a protease domain for RseA, a cleavage event occurs removing RseB bound to RseA (Li *et al*., 2009). A site-2 protease RseP is then recruited to cleave the cytoplasmic domain of RseA, producing unsequestered σ^E (Alba *et al*., 2002).

σ^E-regulated genes are fundamental to virulence in many bacterial pathogens and as previously indicated are also crucial in OM biogenesis, regulating genes involved in LPS biogenesis and transport and the five genes which encode the BAM complex components (Rhodius *et al*., 2006; Skovierova *et al*., 2006). The σ^E regulon has been well established in different bacterial species including *Salmonella*, although the regulon has been fully characterised there are still many genes which have not been investigated and therefore have unknown function some of which are unique to *Salmonella* (Skovierova *et al*., 2006). Of primary interest to this study is the σ^E-regulated putative stress response protein STM1250, and the small heat shock proteins (sHsps) IbpA, IbpB and AgsA. In a study by Skovierova *et al*. (2006) assessing the σ^E regulon, IbpA, IbpB, AgsA and STM1250 were all identified as having unknown function in *Salmonella*, both STM1250 and AgsA were identified as being specific to *Salmonella*. Significantly, IbpA, IbpB, AgsA had all been somewhat characterised either in *E. coli* or Salmonella, STM1250 however was still regarded as a hypothetical protein. Due to their location in the σ^E regulon IbpA, IbpB, AgsA and STM1250 have been hypothesised to have a role in *Salmonella* σ^E stress responses, this was shown to be true by

Hews (2020) who described novel roles for each of the proteins including STM1250s apparent role in ATR. STM1250 and the sHsps will be discussed at length in chapter 3.

1.7 Bacterial stress responses and the acid tolerance response

Bacterial stress responses discussed in this chapter, including sigma factors and TCSs all respond to a variety of environmental conditions. Significantly, in common is their involvement against acid stress in *Salmonella* by regulating the expression of ASPs which elicit resistance towards extreme pH levels. The expression of ASPs is a fundamental stage of a stress response deemed the ATR, which is a crucial adaptive mechanism utilised by many bacteria including *Salmonella* where it was first identified and characterised (Foster, 1999). It is defined as the mechanism whereby exposure to mildly acidic conditions (pH 4-6), primes the cell for survival in highly acidic usually toxic acidic levels (pH 3-4) (Foster, 1999). The ATR as well as the precise regulation of ASPs via bacterial stress responses will be more deeply discussed in chapter 3. Of importance to this study are also the implications of this adaptive response in food safety, as the ATR provides resistance to pathogenic bacteria including *Salmonella*. This enables survival in food products during typical food processing and thus posing a public health risk. The ATR and its implications on food safety will be discussed at length in chapter 4.

1.8 Aims and objectives

The characterisation of the σ^E regulon in *Salmonella* by Skovierova *et al*. (2006) identified various protein coding genes with no recorded function. Based on σ^E and its regulon of genes having importance for *Salmonella* resistance to oxidative stress and being highly expressed during acid stress, a previous unpublished thesis conducted by Hews (2020) in the Rowley laboratory set about the characterisation of some of these σ^E-regulated genes with unknown function namely IbpA, IbpB, AgsA and STM1250. Significantly, findings by Hews (2020) implicated one of these uncharacterised genes STM1250 in the ATR of *Salmonella*. Work conducted in this thesis therefore aimed to extend these original findings. Firstly, by confirming the reproducibility of findings that STM1250 is important for *Salmonella* ATR, then by enhancing our phenotypic understanding of STM1250 in the ATR. Further, the project aims to assess the contributions of STM1250 in the long-term survival of *Salmonella* in apple and orange juice, determining whether this has implications for food safety.

Project aims where achieved through:

- Assessing the survival of the Δ*STM1250* mutant in acid shock conditions. Determining whether previous findings by Hews can be replicated, confirming a specific role of STM1250 in the *Salmonella* acid tolerance response.

- Extending our phenotypic understanding of STM1250 by assessing the Δ*STM1250* mutant in acid shock conditions acidified with citric acid.

- Investigating the survival of acid adapted and non-acid adapted Δ*STM1250* mutants in the long-term storage in apple and orange juice over a range of temperatures.

Chapter 2: Materials and methods

2.1 Strains

Table 1. Bacterial strains used in this study.

Strain	Description	References
***Salmonella* strains**		
SL1344	*Salmonella enterica* subsp. *enterica* serovar Typhimurium 4/74, *hisG, rpsL*	(Hoiseth and Stocker, 1981)
***Salmonella* mutant strains**		
Δ*STM1250*	SL1344 Δ*STM1250* :: Kan	(Hews *et al*., 2019)
Δ*STM1250*Δ*agsA*	SL1344 Δ*STM1250*Δ*agsA* :: Cm	(Hews *et al*., 2019)
Δ*STM1250*Δ*ibpA*Δ*ibpB*Δ*agsA*	SL1344 Δ*STM1250*Δ*agsA* :: Cm Δ*ibpA*Δ*ibpB* :: Kan	(Hews *et al*., 2019)

2.2 Media

Luria-Bertani (LB) media was prepared by dissolving 10 g NaCl, 10 g tryptone and 5 g yeast extract in 1 L of distilled water (dH_2O) and sterilised via autoclaving. LB agar plates were prepared the same with the addition of 1.5% (w/v) agar to LB media.

NCE minimal media was prepared by first dissolving 3.94 g KH_2PO_4, 6.46 g K_2HPO_4 and 3.5 g $NaNH_4HPO_4$ in 1 L distilled water (dH_2O) and autoclave to sterilise. After sterilisation 1 mM $MgSO_4$, 0.4% (w/v) glucose and 400 µg/mL when required.

Phosphate-buffered Saline (PBS) was prepared by dissolving 9.93 g of powdered PBS in 1 L of distilled water (dH_2O) and autoclaved to sterilise.

2.3 Culturing and bacterial storage

For short term storage of bacteria, cultures were maintained on LB agar at 4 °C for up to 2 weeks. Stationary phase cultures were prepared using single colonies from short term storage plates, colonies were used to inoculate 10 mL of either non-carbon essential (NCE) media or LB which were then grown overnight at 37 °C and 180 rpm.

For long term storage, bacterial strains were stored at -80 °C in Microbank™ beads (ProLab Diagnostics) according to manufacturer's instructions.

2.4 Growth curves

Growth curves were produced according to optical density (OD) measurements conducted on a Spectronic Helios Gamma ultraviolet- visible area (UV-Vis) Spectrophotometer (Thermo Scientific). An O/N culture of bacteria was used to inoculate 50 mL of either NCE or LB media were stated at a final OD_{600} of 0.1. Flasks containing inoculated media were aerobically incubated at 37 °C and 180 rpm, an OD_{600} reading was recorded every hour over an 8 hour duration and then at 24 hours with 3 seconds of agitation before each reading. Assays were conducted in biological triplicate to allow for statistical analysis.

2.5 Percentage survival

Multiple survival assays were conducted in this study determining the percentage survival of different *Salmonella* strains in varying environmental conditions. For assays requiring the calculation of percentage survival the following equation was used.

Percentage survival = (CFU/mL surviving cells) / (CFU/mL initially challenged cells) * 100

Equation 1. Calculation of percentage survival in survival assays. (CFU; colony forming units)

2.6 pH range survival assay

For pH range survival assays, O/N cultures of WT *S*. Typhimurium were standardised in pH 7.0 NCE medium to an OD_{600} of 0.4 (6 x 10^8 CFU/mL). Cultures were washed twice by centrifugation at 13,500 x *g* and resuspended in NCE media adjusted with hydrochloric acid (HCl) or citric acid to the appropriate pH (Either pH 3.5, 4.0, 4.4, 5.0 and 5.5 for HCl or 4.5,

5.0, 5.5, 6.0 and 6.5 for citric acid). Aliquots of the individual cultures were taken, and 10-fold serially diluted in pH 7.0 NCE and 10 µL spots plated on LB agar to act as a t=0 control. Cultures were aerobically incubated at 37 °C and 180 rpm for 2 hours and plated as for the t=0 control, percentage survival was calculated as per Equation 1. pH range survival assays were performed in technical triplicates.

2.7 Acid tolerance assays

Stationary phase ATR assays were conducted according to Lee *et al*. (2007) and Hews (2020) with alterations. WT SL1344 and respective mutant strains were grown O/N in 10 mL NCE medium (pH 7.0).

For acid adapted assays, O/N cultures were first standardised to an OD_{600} of 0.4 (6 x 10^8 CFU/mL). 3 mL of each the standardised cultures were washed by centrifugation at a relative centrifugal force (rcf) of 13,500 x *g*. Washed cultures were then resuspended in NCE medium adjusted with HCl to pH 4.4 and aerobically incubated at 37 °C and 180 rotations per minute (rpm) for 2 hours. After the incubation, cultures were washed at 13,500 x *g* and resuspended in NCE medium adjusted with HCl to pH 3.0. An aliquot of each culture was taken to act as the t=0 mins control, the aliquot was 10-fold serially diluted in NCE medium (pH 7.0) and 10 µL spots were plated onto LB agar. Cultures were incubated aerobically at 37 °C and 180 rpm, with aliquots taken every 15 minutes for 60 minutes total and serially diluted and plated similarly as the t=0 control.

For non-acid adapted assays, O/N cultures were standardised to an OD_{600} of 0.4 (6 x 10^8 CFU/mL). Standardised cultures were washed at 13,500 x *g* and resuspended in NCE medium adjusted with HCl to pH 3.0. t=0 controls were conducted following the same steps as for the adapted assay, aliquots were instead taken every 30 minutes for a total of 90 minutes and similarly 10-fold serially diluted in NCE medium (pH 7.0) with 10 µL spots plated onto LB agar.

Assays were performed in biological triplicate and technical triplicates at a minimum to allow for statistical analysis. The percentage survival for each of the cultures was calculated following Equation 1.

2.8 Acid tolerance storage assay

Acid tolerance storage assays were conducted based on Álvarez-Ordóñez *et al*. (2013) with modifications. WT SL1344 and respective mutant strains were grown O/N in 10 mL LB

medium, these fresh cultures were later used to produce acid adapted and non-acid adapted cells.

For acid adapted assays O/N cultures were used to inoculate 50 mL of LB medium adjusted to pH 4.4 with HCl to a final concentration of 10^3 cells/mL, producing acid adapted cells. Inoculated cultures were then incubated for a total of 16 hours at 37 °C and 180 rpm, or the time required to reach the late stationary phase of bacterial growth. Aliquots (50 mL) of apple and orange juice were aseptically transferred to sterile flasks and equilibrated to either 4, 25 or 37 °C. Once the required growth was reached in the cultures, 100 µL was used to inoculate 50 mL of either apple or orange juice to a final concentration of 2 x 10^5 cells/mL. Before inoculation cultures were washed via centrifugation and resuspended in the respective juice. Inoculated flasks of apple and orange juice were then statically incubated at the equilibrated temperature. Aliquots of 100 µL were taken from the 4 °C flasks every hour for 3 hours including a sample prior to incubation to act as a t=0 control, aliquots were taken every 12 hours for 36 hours for both the 25 °C and 37 °C flasks. Aliquots were 10-fold serially diluted in PBS and 10 µL of the appropriate dilutions were plated on LB agar.

For non-acid adapted assays O/N cultures were used to inoculate 50 mL of LB medium adjusted to pH 7.0 to a final concentration of 10^3 cells/mL. The remainder of the assay was conducted following the same steps as for the adapted assay.

Assays were performed in biological triplicate and technical triplicates at a minimum to allow for statistical analysis. The percentage survival for each of the cultures was calculated following Equation 1.

2.9 Statistical analysis

Experiments were performed in biological triplicate where appropriate, with three technical replicates to allow for statistical comparison. Data analysis was conducted using IBM SPSS (v28). Analysis of Variance (ANOVA) with Tukey's post hoc test or student's *t-test* was used to determine statistically significant differences between sets of data, specified in figure legends.

Chapter 3: Elucidating the contribution of STM1250 in the *Salmonella* acid tolerance response

3.1 Introduction

As introduced in Chapter 1, Bacteria including *Salmonella* have many different stress responses enabling them to withstand typically lethal environmental conditions such as acidic pH, which can lead to the degradation of crucial cellular components. A variety of stress responses collectively function by regulating the *Salmonella* ATR, an adaptive mechanism which provides resistance to low acidic pH. The response functions through the precise regulation of a plethora of genes, by various stress responses. Many of the functions of these genes remain unknown, with their specific contributions towards the ATR undiscovered.

An introduction to *Salmonella* stress responses and their implications towards survival and pathogenesis was provided in Chapter 1. This chapter more specifically focuses on the survival of *Salmonella* against acidic pH, with a more in depth introduction to the components and mechanisms possessed by *Salmonella* enabling its survival against acidic conditions including a focus on the acid tolerance response. The σ^E-regulated small heat shock proteins IbpA, IbpB, AgsA and the putative stress response protein STM1250 will be discussed at length in this chapter, STM1250 will be specifically explored for whether and to what extent it functions in the *Salmonella* ATR.

3.1.1 The *Salmonella* acid tolerance response

Bacteria have evolved mechanisms to successfully colonise nearly every environmental niche on earth, this largely depended on the ability to appropriately respond to their local environmental conditions (Lund *et al*., 2020). One of the harshest conditions for microbial survival and growth are environments saturated with protons (acidic conditions), which are measured as pH. Low pH results in the protonation of biological molecules hence altering their charge, this can disrupt the structure and function of these molecules. (Lund *et al*., 2020). Severe acidic conditions of pH 3 or lower, are usually toxic to bacteria as protons leak across cell membranes faster than they can be removed, leading to a disruption of biological processes conferring reduced survival for the bacteria (Bearson *et al*., 1998).

Non-typhoidal Salmonella encounters a variety of environmental conditions during its life cycle many of which are detrimental to its survival, one of the most frequently encountered hostile conditions includes acidic environments such as acidic foods and the digestive tracts of animals. Due to Salmonella being a foodborne enteric pathogen it has evolved the ability to survive in different acidic conditions, including naturally acidic food products such as cheeses

and fruit juices and on food products acidified during fermentation processes (Leyer and Johnson, 1992; Álvarez-Ordóñez *et al.,* 2012). During *Salmonella* pathogenesis the bacteria are exposed to the extreme acidic environment of the stomach, pH levels in the human stomach are usually maintained at roughly pH 3, however, they can get as low as pH 1.5-2.5 (Audia and Forster, 2003; Forster, 2004). HCl is the main acidulent within the gastric fluid and creates a crucial barrier for enteric infection. This is apparent as mice with a mutated gastric H^+/K^+ -ATPase proton pump gene are not able to produce gastric HCl, therefore successfully acidifying the stomach (Tennant *et al.,* 2008). Mice with this genetic mutation were shown to be more susceptible to enteric infection from pathogens including *Salmonella*. (Tennant *et al.,* 2008). Despite these harsh conditions *Salmonella* is still able to persist in the stomach until it passes to the small intestine, causing infection (Fàbrega and Vila, 2013). Once in the small intestine *Salmonella* attach to and invade the epithelial lining, proliferating in the epithelium and lymphoid follicles leading to a localised infection (Giannella, 1996). Phagocytic cells including macrophages are then able to engulf the *Salmonella*, compartmentalising them into the *Salmonella* containing vacuole (SCV) which is acidified to a pH of between 4 and 5 (Allam *et al.,* 2012).

Acid stress is a complex phenomenon involving the interconnected biological effects of different acids in an environment. Although the primary acid in the stomach is HCl different forms of acid are present, including organic acids such as: lactic, propionic, butyric, and acetic acids all of which confer antimicrobial effects (Mikkelsen *et al.,* 2004). Primarily, organic acids can diffuse across the cellular membrane when in their undissociated form (Warnecke and Gill, 2005). Weak organic acids such as acetate and lactate are protonated at low pH, therefore in acidic conditions they remain uncharged and more lipophilic, enabling them to permeate the lipid bilayer of *Salmonella* more freely. The higher intracellular pH of Gram-negative bacteria that remains constant at pH 7.6 to 7.8 causes the dissociation of the acid releasing H+ ions, which lowers the pH of the cytoplasm (Hirshfield *et al.,* 2003; Álvarez-Ordóñez *et al.,* 2012). The type of acid is therefore important to consider when investigating *Salmonella* acid tolerance as different acids have different antibacterial mechanisms. Even a mild acid stress of pH 5 can become lethal when weak organic acids such as acetate are present, due to an increased rate of diffusion across the membrane (Bearson *et al.,* 1998). Survival in acidic conditions is credited to developed resistance mechanisms and stress responses which counteract the lethal pH levels, the predominant mechanism is known as the ATR.

The ATR is a crucial adaptive mechanism employed by many bacteria including *Salmonella* where it was first identified and characterised (Foster, 1999). It is defined as the mechanism

whereby exposure to mildly acidic conditions (pH 4-6), primes the cell for survival in highly acidic usually toxic acidic levels (pH 3-4) (Forster *et al.,* 1999). Pioneering work conducted by Foster (1991) in *Salmonella* Typhimurium LT2 detailed two distinct stages of the ATR in exponential phase cells. They deemed the pre-shock stage as occurring following exposure to pH 5.8, which involves the production of pH inducible homeostatic mechanisms, conferring survival benefits. The second stage of the ATR is initiated through exposure to a pH of 4.3 and results in the expression of acid shock proteins (ASPs). In combination, these two stages were shown to confer increased survival of *Salmonella* during acid challenge at pH 3.3. Follow up work by Foster (1993) suggested that the pre-shock stage of the ATR is not vital, with *Salmonella* being able to survive pH 3.3 following incubation at pH 4-5 just as a result of the expression of ASPs. The understanding of the ATR has significantly developed beyond the original descriptions in log phase cells, with the phenomenon also being established in stationary phase cells. Recent studies have provided new details of these originally established mechanisms, an updated outlook of the different regulatory and homeostatic mechanisms of the ATR will be discussed in the following sections.

3.1.2 The lysine and arginine decarboxylase systems

Gram-negative bacteria such as *Salmonella* attempt to maintain intracellular pH at an optimum constant level of pH 7.6-7.8, this remains true even when extracellular pH changes (Álvarez-Ordóñez *et al.,* 2012). The inducible lysine and arginine decarboxylase systems are homeostatic mechanisms which are partly induced via low pH, *Salmonella* utilises them to help retain a constant intracellular pH enabling survival in environments as low as pH 5.8 (Foster, 1991; Park *et al.,* 1996). Both systems have very similar molecular mechanisms each consisting of an antiporter and an associated amino acid decarboxylase. The mechanisms consist of a decarboxylase enzyme which is able to replace the α-carboxyl groups of their respective amino acid substrate with a cytoplasmic proton. The resulting product is then secreted from the bacteria via the corresponding antiporter and exchanged for a new amino acid substrate (Viala *et al.,* 2011). The consequence of this mechanism is a reduced concentration of intracellular protons, hence intracellular pH conferring acid resistance.

The lysine decarboxylase system is comprised of the lysine decarboxylase CadA and the lysine-cadaverine antiporter CadB, together they form the *cadBA* operon. CadC is a transcriptional regulator which is able to respond to the environment inducing the *cadBA* operon in the presence of either high exogenous concentrations of lysine or a low external pH. It is thought that the CadC signal transduction mechanism consists of the proteolytic cleavage of a DNA-binding domain from the CadC protein, which then acts as a transcriptional activator

of the *cadBA* operon inducing expression of CadA and CadB (Lee *et al*., 2008). CadA catalyses the decarboxylation of intracellular lysine and a proton converting it into cadaverine and carbon dioxide. Intracellular cadaverine is then exported out of the cell by the CadB antiporter and replaced by an extracellular lysine as visualised in Figure 3 (Park *et al.,* 1996). The arginine decarboxylase system instead consists of the arginine decarboxylase AdiA, the arginine-agmatine antiporter AdiC and the transcriptional regulator AdiY. As opposed to lysine, the consumption of a proton occurs through intracellular arginine during its decarboxylation to agmatine by AdiA, agmatine is then exported out of the cell by AdiC in return of the import of arginine which again can be seen visualised in Figure 3 (Kieboom and Abee, 2006).

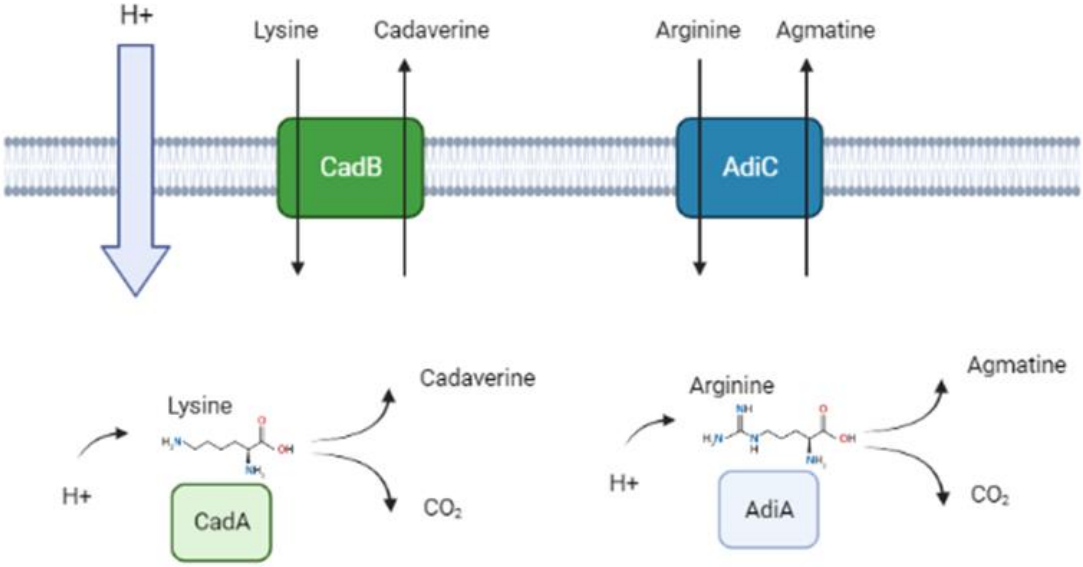

Figure *3. Salmonella* lysine and arginine decarboxylase systems. In response to the increase in intracellular pH due to the influx of protons (H+), CadA and AdiA respectively catalyse the decarboxylation of lysine or arginine in a reaction which removes H+ ions to produce Cadaverine or Agmatine. The resulting product is exported out of the cell via the antiporters CadB or AdiC in exchange for either more lysine or arginine.

The lysine and arginine decarboxylase systems have been experimentally shown to contribute towards the *Salmonella* ATR. Research conducted by Álvarez-Ordóñez *et al.* (2010a) discovered that acid adapted *S.* Typhimurium in adaption media (pH 4-5) had an increased survival in usually lethal acidified media (pH 2.5) when supplemented with either lysine or arginine. These findings were then confirmed to be as a result of the lysine and arginine decarboxylase systems, with genes encoding both decarboxylases being found to be expressed at a significantly higher level in acid adapted cells of which were shown to have enhanced acid resistance. Research conducted by Viala *et al.* (2011) also established that the lysine and arginine decarboxylase systems improved *Salmonella* Typhimurium survival in acid shock media acidified to pH 2.3 and growth in pH 4.5 media. The arginine decarboxylase

system was shown to promote the best survival at pH 2.3, when the lysine decarboxylase system promoted both survival at pH 2.3 and growth at pH 4.5. Interestingly, the ornithine decarboxylase system previously characterised to contribute to acid resistance in E. coli was also shown to improve *Salmonella* survival at pH 2.3, although it was shown to have the least impact on survival compared to the other decarboxylase systems but the most on growth (Viala *et al.,* 2011). Although the importance of these decarboxylase systems has been extensively highlighted for *Salmonella* survival during acid stress, an ATR is still shown in minimal media without the amino acids required for the functioning of the decarboxylase systems (Álvarez-Ordóñez *et al.,* 2010a). This therefore suggests the persistence of additional acid resistance mechanisms enabling *Salmonella* survival, the expression of ASPs have been shown to confer this underlying resistance.

3.1.3 Synthesis and transcriptional regulation of acid shock proteins

The synthesis of ASPs is crucial during the ATR, cumulatively ASPs act by preventing or repairing macromolecular damage to DNA and proteins caused by acid stress (Audia *et al.,* 2001). Although, many ASPs have been associated to the ATR, relatively little is known about their molecular interactions. However, general roles of ASPs consist of transcriptional and translational regulation, molecular chaperoning, energy metabolism, virulence, and regulation of the cellular envelope (Bearson *et al.,* 2006). Four predominant regulatory genes are responsible for regulating the expression of different subsets of ASPs, these master regulator genes include RpoS, PhoPQ, Fur and OmpR/EnvZ (Álvarez-Ordóñez *et al.,*2012). As previously mentioned, the ATR occurs in exponential and stationary phases of cell growth, with acid stress in the form of inorganic and organic acids being capable of inducing the ATR (Bearson *et al.,* 2006). It is now apparent that depending on both the growth stage and the type of acidulent different transcriptional regulators become induced, each of which regulate specific subsets of ASPs. Studies into ASPs expression have discovered that there are 51 ASPs expressed for the log-phase ATR and 15 for the stationary-phase ATR, with only a few thought to be expressed in both (Bearson *et al*., 1996). The type of transcriptional regulator and the corresponding conditions for their induction will be discussed in the next section. The extracytoplasmic sigma factor σ^E has been recently implicated in the regulation of ASPs, and therefore will be discussed alongside the previously mentioned core bacterial stress responses.

3.1.3.1 PhoPQ

The PhoPQ two-component system controls the expression of a large regulon of genes that regulate numerous cellular activities (Groisman, 2001). The PhoQ sensor kinase is activated by AMPs and low pH, it functions directly as a pH sensor for the intracellular environment and upon stimulation phosphorylates the response regulator protein PhoP enabling it to bind to DNA (Bearson *et al.,* 1998; Prost *et al.,* 2007). Expression of PhoP is induced by a moderate acid pH of 4.4, which consequently promotes the transcription of many PhoP regulated genes involved in the exponential phase ATR in *Salmonella* (Bearson *et al.,* 1998). Studies into the precise mechanism of PhoQ induction uncovered that under acidic conditions the periplasmic domain of PhoQ undergoes a conformational change, this is distinct to the conformational change which occurs from AMP activation. However, disruption and loss of AMP activation does not affect the ability of PhoQ to respond to pH activation, with the two mechanisms in fact being additive (Prost *et al.,* 2007).

An early study on the ATR determined that *Salmonella* ΔphoP mutants are highly sensitive to acid stress, therefore suggesting the importance of PhoP in the ATR (Foster and Hall, 1990). A later study conducted by Bearson (1998) confirmed these results and suggested that phoP mutants failed to induce four ASPs which otherwise would be induced in wildtype cells, one of these ASPs was determined as being PhoP itself the other three however had unknown identifies. Interestingly, they detailed that PhoP is crucial for resistance towards inorganic acids but not for organic acids. The PhoPQ system has also been linked to regulating the genes adiA, adiY, cadA and cadB involved in the lysine and arginine decarboxylase systems, with a phoP mutant being shown to significantly reduce the levels of the genes in acid adapted cells (Lang *et al.,* 2021). This is significant as is suggests the PhoPQ system has a role in the ATR by sensing pH and either directly or indirectly regulating the lysine and arginine decarboxylase systems as a mechanism to reduce intracellular acidity.

3.1.3.2 RpoS

The alternative sigma factor RpoS is the master regulator of the general stationary phase stress response in *Salmonella*, conferring resistance to a multitude of different environmental stresses including acidic pH. Significantly, RpoS also plays important roles in the exponential growth phase when under stressful conditions (Melendez *et al.,* 2022). The cellular level of RpoS is regulated by transcription, translation, and posttranslational stability, with the type of regulation directly correlating to the stress condition present (Ibanez-Ruiz *et al.,* 2000). RpoS is induced under different circumstances, with the expression of ASPs dependent on whether

an acid-inducible log-phase or pH independent stationary phase ATR is present (Lee *et al.,* 1995). The low pH log-phase induction of RpoS at pH 4.3 leads to the expression of 8 ASPs and is separated into two distinct mechanisms of induction depending on the type of acidulent, either organic or inorganic (Lee *et al*., 1995). Inorganic stress leads to the inhibition of RpoS proteolysis via the mouse virulence gene *mviA*, resulting in an increased cellular level of RpoS which then drives expression of ASPs (Bearson *et al*., 1996). However, organic acid stress results in the stabilisation of the RpoS protein and increases the translation of *rpoS,* this process is controlled by secondary structures in the untranslated region (UTR) of *rpoS* mRNA (Audia and Foster, 2003). RpoS also functions in the pH-independent stationary-phase ATR, with the RpoS protein being expressed upon entry into stationary-phase as part of the general stress response (Lee *et al.*, 1994). Interestingly, due to the nature of the general stress response expressed RpoS also confers *Salmonella* with cross protection to other stresses including heat, osmolarity and hydrogen peroxide. However, cross resistance to acid stress is not provided during exposure to these stresses (Lee *et al*., 1995).

3.1.3.3 Fur

The ferric uptake regulator (Fur) is a transcription factor which was first characterised by its ability to bind Fe^{2+} utilising it as a corepressor to repress the synthesis of iron-chelating sideophores. Fur was also established to either directly or indirectly control the expression of enzymes which protect the cell from reactive oxygen damage caused by the acquisition of iron (Troxell and Hassan, 2013). Later studies have however since discovered further roles of Fur in cellular physiology, including in the regulation of the ATR. An investigation conducted by Foster (1991) first identified that fur mutations in *Salmonella* Typhimurium confer acid-sensitive phenotypes. Follow-up work by Foster (1993) determined that this phenotype was as a result of aberrant synthesis of some ASPs. The induction of Fur following acid stress is not deeply understood. However, it is clear that Fur is able to sense low intracellular pH, with the presence of iron alone not enough to trigger the ATR. Similarly to RpoS, Fur also plays a role in the ATR of log phase cells and predominantly responds to organic acid stress (Bearson *et al.,* 1998). The role of Fur in acid and iron regulation is genetically separable, this is evident from site directed mutagenesis experiments which determined that the H90R Fur mutant is able to mediate the ATR but not iron regulation (Hall and Foster, 1996).

3.1.3.4 OmpR/EnvZ

OmpR and EnvZ are two proteins which together form the two component system OmpR/EnvZ, EnvZ functions as a membrane bound sensor kinase when OmpR acts as the

corresponding response regulator (Forst and Roberts, 1994). The OmpR/EnvZ signal transduction system has been well characterised in its ability to regulate osmolarity associated stress via the transcriptional regulation of the OmpC and OmpF porins. EnvZ is able to sense changes in osmolarity, with high osmolarity resulting in its autophosphorylation (Taylor *et al.,* 1981). EnvZ then transfers the phosphate group to OmpR which enables DNA binding and therefore the transcriptional activation of target genes (Hsing and Silhavy, 1997). The OmpR/EnvZ TCS has been described as also regulating the stationary phase ATR. ΔompR mutants were shown to elicit a normal exponential phase ATR, cells were however not able to survive during stationary phase growth (Bang *et al.,* 2000). Interestingly, the sensor kinase EnvZ alongside the porin genes OmpC and OmpF were not required for the stationary phase ATR. Instead, the alternate phosphodonor acetyl phosphate seems to instead play a role in the phosphorylation of OmpR, leading to the expression of ASPs (Bang *et al.,* 2000). The mechanism of acid shock induction of ompR is different from basal expression, with a different promoter being used depending on whether a low or neutral pH is present in the cell (Bang *et al.,* 2002). Although OmpR has been determined to play a crucial role in the stationary phase ATR, little research has been conducted on OmpR-regulated ASP genes and how they are involved in this ATR.

3.1.3.5 RpoE

The extracytoplasmic sigma factor σ^E encoded by *rpoE* is important for the maintenance of cell envelope homeostasis in *Salmonella* and other Gram-negative bacteria. RpoE expression is induced via many different stress factors which change or damage the cell envelope. Specifically, the presence of misfolded and/or mis-translocated OMPs or LPS within the periplasm results in the activation of the RpoE pathway (Bang *et al.,* 2005; Rowley *et al.,* 2006). Acid stress has been identified as one of the factors which induces the expression of RpoE, this was identified through investigation of σ^E-deficient *Salmonella* mutants which displayed increased susceptibility to acid pH (Muller *et al.,* 2009). The mechanism of RpoE induction as a result of acid stress occurs in a non-canonical way, first shown in a study which investigated the impact of DegS which is important for the induction of RpoE on virulence. A mutant strain deficient in DegS was demonstrated to not be as attenuated in mice compared to rpoE mutants, this is significant as it suggests that rpoE activation still occurs just via an alternative pathway (Rowley *et al.,* 2005). A follow up study also determined that DegS was dispensable in the acid activation of RpoE, the unfolded OMP signal was also not required. However, the processing of RseA by RseP is required, suggesting that during the absence of DegS an inhibitory reaction between RseA and RseP enable RseA proteolysis leading to the loss of RpoE inhibition (Muller *et al.,* 2009).

3.1.4 σ^E-regulated proteins

The ability to sense different stresses is paramount for bacterial survival both in a host and in the environment. The characterisation of different *Salmonella* stress responses and their involvement in responding to different stresses is well established in the literature. Although the regulons of these stress responses are somewhat studied, the majority of regulon members are poorly described or have unknown roles. The σ^E response previously discussed has been thoroughly investigated in *Salmonella* with the σ^E regulon being identified. Significantly, an investigation into the regulon identified 23 genes not previously shown to be dependent on σ^E, many of which specific to *Salmonella* (Skovierova *et al.,* 2006). Many isolated genes were also determined as having unknown functions including *ibpA*, *ibpB* and *agsA*. One of the genes *STM1250*, specific to *Salmonella* was reported as only encoding a hypothetical protein with its precise intracellular role also unknown (Skovierova *et al.,* 2006).

The *Salmonella* Ibp (Inclusion body protein) A and B (IbpAB) and AgsA (aggregation suppression protein A) proteins are classified as sHsps and are deemed as such due to a conserved α-crystalline domain, a molecular weight of 12 to 42 kDa and have the ability to both function as ATP independent chaperones and to form multimetric structures (Narberhaus, 2002). The role of sHsps is to inhibit the aggregation of unfolded and folded proteins caused by conditions which negatively affect protein conformation such as heat and oxidative stress, the nomenclature of sHsps is misleading as these proteins are utilised during many different stresses including heat stress (Haslbeck *et al*., 2019).

IbpAB are encoded by the genes *ibpA* and *ibpB* separated by 110 nt, they have been shown to be induced as a result of increased levels of insoluble proteins, consequently causing the formation of inclusion bodies which are large aggregates of proteins (Allen *et al*., 1992). In addition to binding to overexpressed heterologous proteins IbpAB can also bind endogenous protein aggregates arising from stress such as heat shock (Laskowska *et al.,* 1996). They function as a mediator of denatured proteins facilitating the initiation step of the refolding pathway (Haselbeck *et al*., 2019). Co-dependency has also been suggested to occur between IbpA and IbpB, with IbpA in-fact forming inactive fibrils in the absence of IbpB, further investigation is needed to establish the implications of protein interactions of IbpAB on their ability as chaperones (Ratajczak *et al*., 2010). The expression of *ibpAB* is regulated by the heat shock sigma factor σ^H and as previously suggested additionally by σ^E (Kuczynska-Wisnik *et al*., 2001).

Unlike IbpAB, AgsA (STM1251) is specific to *Salmonella,* functionally it can reduce thermally-aggregated intracellular proteins hence increasing survival in response to heat stress

(Tomoyasu et al., 2003). Similarly to IbpAB, AgsA can been shown to be implicated in the prevention of aggregation of endogenous proteins, including prevention of protein aggregates arising from heat stress increasing survival (Tomoyasu et al., 2003). Regulation of *agsA* expression like *ibpAB* is controlled by the heat shock sigma factor σ^H and the extracytoplasmic sigma factor σ^E. However, unique to *ibpAB* is the regulation of *agsA* by the housekeeping sigma factor σ^D (Tomoyasu et al., 2003; Skovierova *et al.,* 2006). Through investigation *agsA* has been determined to have high sequence similarity and shared transcriptional regulators to both *ibpA* and *ibpB*, therefore it has been predicted that functional overlap exists between the proteins. Interestingly, *agsA* also forms an operon with *STM1250* so is hypothesised to function in conjunction with AgsA and therefore also interact with IbpAB (Skovierova *et al*., 2006).

Due to their location in the σ^E regulon IbpA, IbpB, AgsA and STM1250 have been hypothesised to have a role in *Salmonella* σ^E stress responses. In a recently published article Hews *et al*. (2019) investigated the roles of STM1250 as well as other sHsps IbpA, IbpB and AgsA in the *Salmonella* envelope stress response, with an attempt to elucidate if and under what stresses these proteins function in. It was discovered that a Δ*STM1250*Δ*ibpA*Δ*ibpB*Δ*agsA* quadruple mutant was significantly more sensitive to hydrogen peroxide, had a lower minimum bactericidal concentration to the cationic antimicrobial peptide polymyxin B and is attenuated in macrophages. Interestingly, it was identified that single gene mutants were not similarly affected, suggesting functional redundancy exists between these proteins.

In an unpublished thesis by Hews (2020) working in the Rowley laboratory STM1250 was shown to be important in the *Salmonella* ATR. An acid adapted STM1250 mutant had a significant reduction in survival in the first 30 minutes of exposure to inorganic acid shock of pH 3.0, this was true for mutant strains both grown to exponential and stationary phases. Similar findings were also established in response to cells adapted and shocked in the organic acid, acetic acid. These findings suggest that STM1250 is important in the *Salmonella* ATR during the initial stages of acid exposure, with the growth phase or acidulent seeming irrelevant. Consequently, this was also the first evidence suggesting a specific role of STM1250 in *S.* Typhimurium. These interesting findings identified by Hews warrant further investigation to both confirm her results and to fully elucidate the contributions of STM1250 on the *Salmonella* ATR.

3.1.5 Chapter Aims

The majority of work conducted on sHsps including IbpAB and their functions has been conducted in non-pathogenic strains of *E*. *coli*, leaving only a very limited understanding of the proteins in *Salmonella.* Hews *et al.* (2019) was the first to conduct preliminary functional work in *Salmonella* on the sHsps IbpAB and the *Salmonella* specific proteins AgsA and STM1250, identifying previously undiscovered roles for the proteins in envelope stress responses. In an unpublished thesis by Hews (2020) STM1250 was discovered to be important for the early stages of the *Salmonella* ATR in response to the inorganic acid HCl and the organic acid, acetic acid. As outlined in this chapter IbpAB, AgsA and STM1250 are each regulated via σ^E, highlighting the feasibility of their involvement in stress responses in some capacity. Therefore, we hypothesise that STM1250 does in-fact contribute to the *Salmonella* ATR by acting in the initial stages of acid exposure in a generalised fashion not being dependent on the environmental condition or growth phase.

The aims of this chapter extend from the previous unpublished findings of Hews (2020) in the Rowley laboratory. Since STM1250 was shown to be associated to the *Salmonella* ATR, initial confirmative experimentation will be conducted determining the reproducibility of findings that STM1250 solely is important, and that functional redundancy is not present between the other sHsps. This chapter will also expand on initial findings attempting to provide a better understanding of the physiological roles of STM1250, and under what stress conditions it functions in. Specifically, STM1250s range of influence will be assessed, determining whether it is also required in the ATR mounted against organic acids commonly found in both naturally and artificially acidic foods.

Theses aims where achieved through:

- Assessing the survival of Δ*STM1250* single, Δ*STM1250*Δ*IbpAB* double and Δ*STM1250*Δ*ibpA*Δ*ibpB*Δ*agsA* mutants in acid shock conditions acidified with HCl. Determining whether previous findings by Hews (2020) can be replicated, confirming a specific role of STM1250 in the *Salmonella* acid tolerance response.

- Expanding previous findings assessing the survival of the Δ*STM1250* mutant in acid shock conditions acidified with citric acid. Dertermining whether STM1250 functions against acidic conditions commonly associated to naturally acidic foods and in food preservation and sterilisation.

3.2 Results

3.2.1 Growth of mutant strains is not significantly reduced in minimal media

Growth curves were first conducted in order to confirm that any significant differences between mutant strains (Single Δ*STM1250* mutant, Double Δ*STM1250*Δ*agsA* mutant and quadruple Δ*STM1250*Δ*ibpA*Δ*ibpB*Δ*agsA* mutant) and the WT in later assays utilising NCE media are due to the loss of the gene, and not as a result of the strains ability to grow in minimal media. Growth curves were performed alongside a WT strain in order to determine their baseline growth in pH 7.0 no-carbon essential (NCE) minimal media supplemented with 0.4% (w/v) glucose and 400µg/mL histidine. The growth of the mutant strains followed that of the WT in pH 7.0 NCE (Figure 4). This suggests that none of the mutants had any growth restrictions in NCE media.

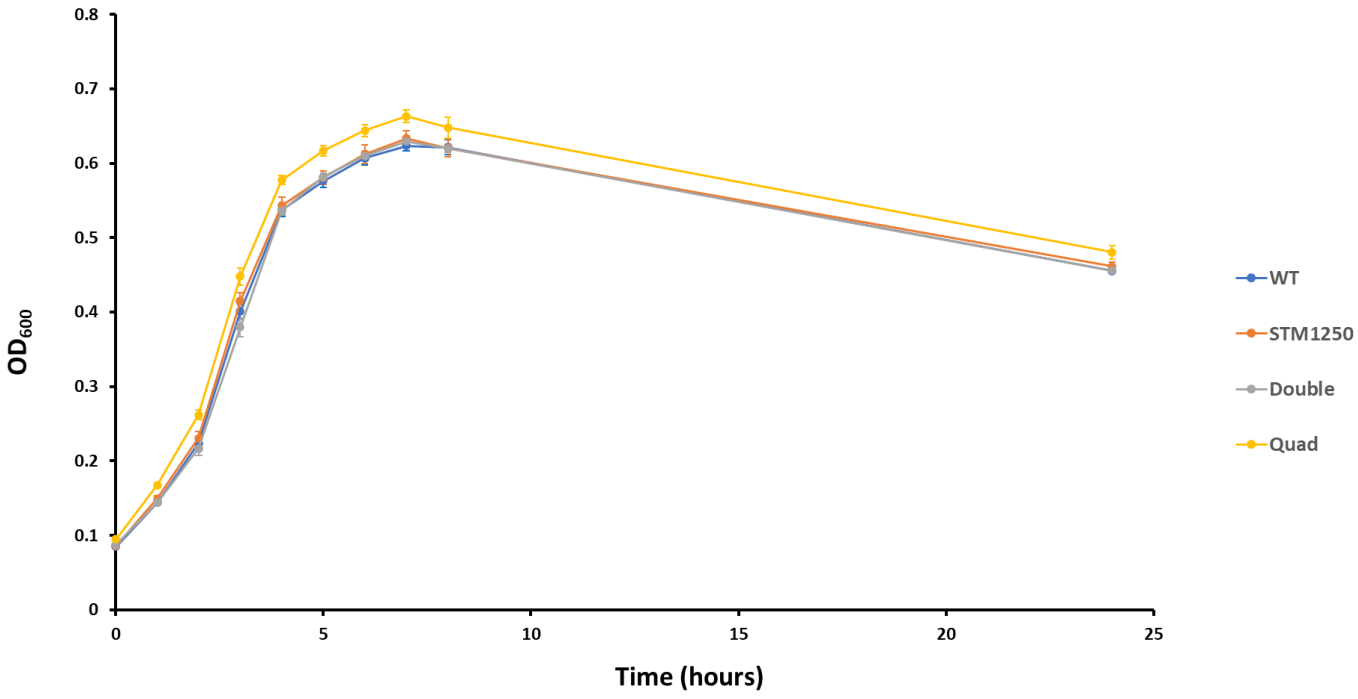

Figure 4. Growth of WT and *Salmonella* mutant strains are not significantly reduced in pH 7.0 NCE medium. Respective strains of *Salmonella* were used to inoculate NCE medium and incubated at 37 °C, 180 rpm for 24 hours. Aliquots (1 mL) were taken every hour for 8 hours with a final sample taken at 24 hours. OD_{600} readings were measured after collection.

Data points are the culmination of three means produced from three individual experiments performed in triplicate. Error bars are SEM.

3.2.2 HCl and citric acid pH range survival assay

During the acid tolerance assay there is a 2-hour adaption period in a mild acidic pH of 4.4 which induces the *Salmonella* ATR. As incubation in different acids results in different levels of bacterial inactivation a pH range survival assay was conducted on both HCl and citric acid, this was to determine an acceptable pH to limit the level of bacterial inactivation during the adaption period. WT *Salmonella* cultures were inoculated into a range of media adjusted to different pHs ranging from 3.5 – 5.5 for HCl, and 4.5 – 6.5 for citric acid and then incubated for 2 hours. Prior to incubation a sample was taken and CFU were determined acting as the base line, post incubation another sample was taken to allow for the calculation of percentage survival.

The HCl assay displayed a correlation between pH and bacterial inactivation, with a higher pH resulting in lower bacterial inactivation (Figure 5). At pH 4.4 the bacterial inactivation was relatively low with the percentage survival being 85.24%. Similarly, the same correlation was displayed in the citric acid assay (Figure 5). However, a much greater bacterial inactivation was evident at the corresponding pHs compared to the HCl assay. pH 5.0 produced the closest percentage survival to that of pH 4.4 in the HCl assay with 88.39%.

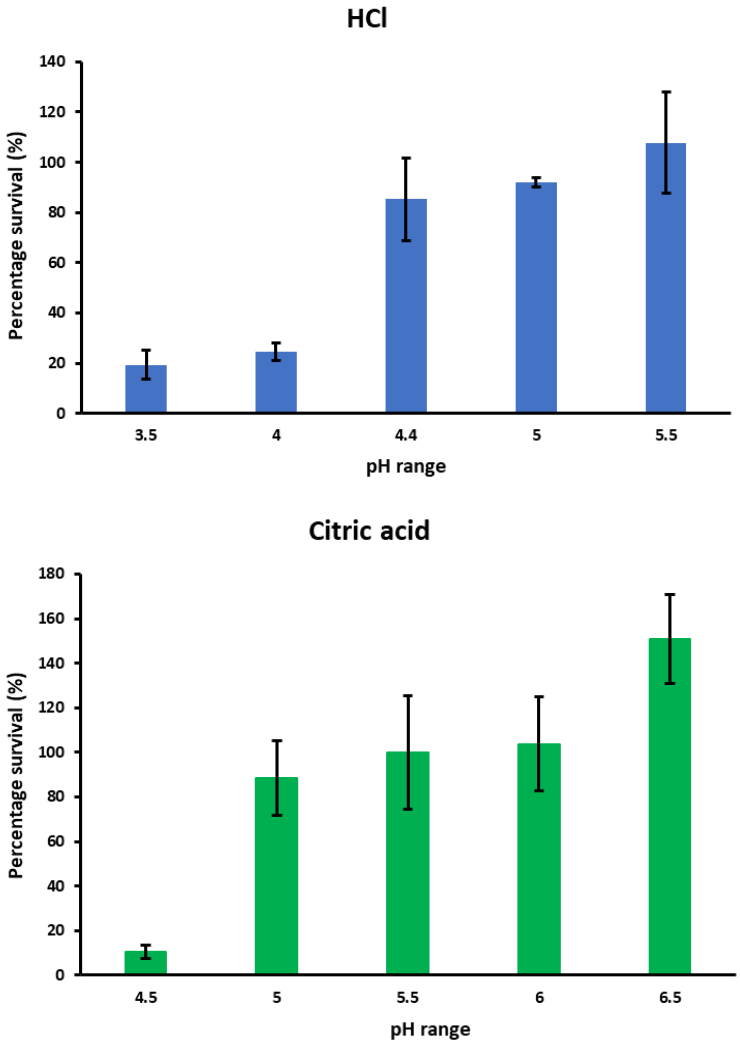

Figure 5. Survival of WT *Salmonella* in NCE medium adjusted to a range of pHs with either HCl or citric acid. NCE medium adjusted to a range of pHs with either HCl or citric acid was inoculated with WT *Salmonella* and incubated at 37 °C, 180 rpm for 2 hours. Samples were taken prior and post incubation in order to calculate percentage survival. Data points are the means of a single experiment performed in triplicate.

3.2.3 The survival of the acid adapted quadruple mutant is reduced compared to WT during stationary phase growth in HCl acidified NCE media

The percentage survival of non-acid adapted and acid adapted (inducing the ATR) Δ*STM1250*Δ*ibpA*Δ*ibpB*Δ*agsA* quadruple mutants were analysed compared to a WT in acid shock media (pH 3.0). This was to determine whether the genes of interest were associated

with unadapted survival in acidic conditions and if they are associated with the stationary ATR in adapted cultures. The quadruple mutant was grown to stationary phase and then assessed for its ability to survive an acidic challenge of pH 3.0. Strains were either pre-incubated at a pH of 4.4 in order to induce the ATR, or challenged in acid shock without pre-incubation. Strains were first grown overnight in NCE medium (pH 7.0) and then standardised to an OD_{600} of 0.4, the respective strains were then transferred to either adaption media inducing an ATR or to acid shock media. For the adapted ATR induced cultures, the bacteria were incubated for 2 hours at 37 ℃ in NCE media adapted to pH 4.4 with the inorganic acid HCl. Cultures were then transferred to acid shock media of pH 3.0 and further incubated at 37 ℃, aliquots were taken every 15 minutes over an hour period. For acid shock cultures the bacteria were added straight to acid shock media, aliquots were taken every 30 minutes over a 90 minute time period. Percentage survival was calculated relative to samples taken at t=0 control time points.

Both the WT and quadruple mutant unadapted strains exhibited a reduction in percentage survival over the 90-minute time period, with both strains approximately falling by 85%. Crucially, there was no significant difference between WT and quadruple mutants ability to survive acid challenge (Figure 6). Acid adapted strains contrastingly only had a mean overall reduction in percentage survival of approximately 20%. Interestingly, a significant difference between WT and mutant strain percentage survival was displayed (Figure 6). The WT strain experienced an increase in survival over the first 15 minutes, followed by a steady reduction and then another increase at 60 minutes. In comparison the quadruple mutant did not show this initial increase in percentage survival, with a significant difference in survival being observed over the first 15 minutes.

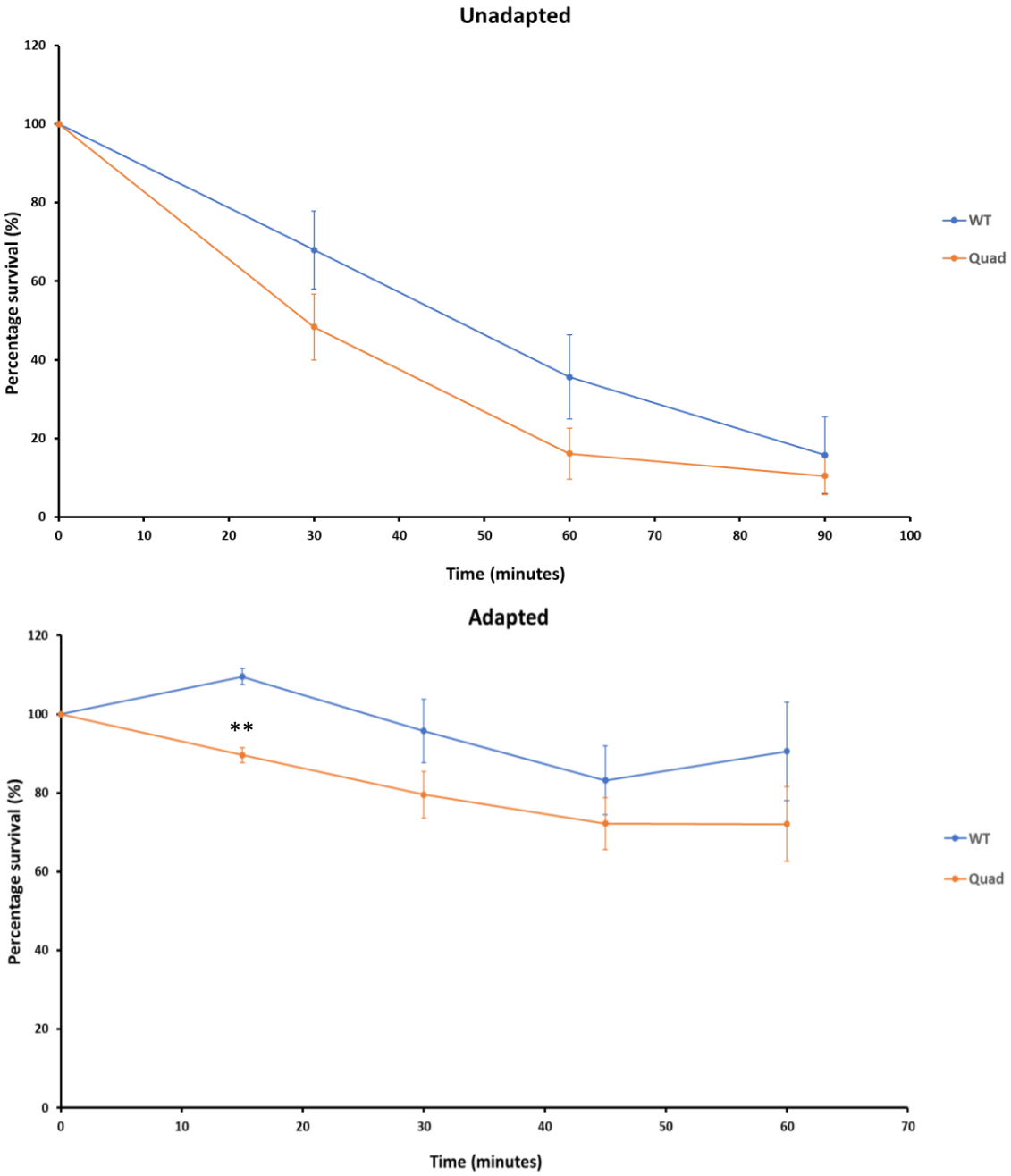

Figure *6*. Survival of the acid adapted ΔSTM1250ΔibpAΔibpBΔagsA mutant was significantly reduced after 15 minutes during acid shock adjusted with HCl, compared to the WT. Un-adapted cultures were added to pH 3.0 acid shock media acidified with HCl and incubated for 90 minutes. Aliquots of 1 mL were taken every 30 minutes and a percentage survival calculated relative to the control t=0 sample. Adapted cultures were instead added to pH 4.4 adaption media following standardisation and incubated for 2 hours. Strains were then transferred to acid shock media and incubated for 60 minutes, with aliquots taken every 15 minutes. Data points are the means of 5 experiments performed in triplicate, error bars are SEM. Data points were analysed for significant difference using Student's t-test, p values below 0.05 were deemed significant. ** $p < 0.005$.

3.2.4 STM1250 is solely required for the stationary phase ATR response in S. Typhimurium in the presence of HCl

The previous identification of the acid adapted quadruple mutant having reduced survival warranted further investigation of the Δ*STM1250* single and Δ*STM1250*Δ*agsA* double mutants, this was in order to determine individual importance of the genes in the stationary phase ATR. The same procedure was used in the investigation of the single and double mutants as with the quadruple mutant. Similar to the previous experiment unadapted strains did not show a significant difference in percentage survival compared to the WT (Figure 7). Interestingly, the overall reduction in percentage survival of the mutant strains was approximately 70% when the WT was approximately 50%, although this was deemed as not being significantly different. In contrast a significant difference in percentage survival was deemed between the single mutant and the WT at both 15- and 30-minute time points. However, the double mutant was determined to not have any significant difference from either the WT or the single mutant. Interestingly, each strain showed a distinct increase in percentage survival at the 60-minute time interval, with the double mutant in-fact returning to 100%.

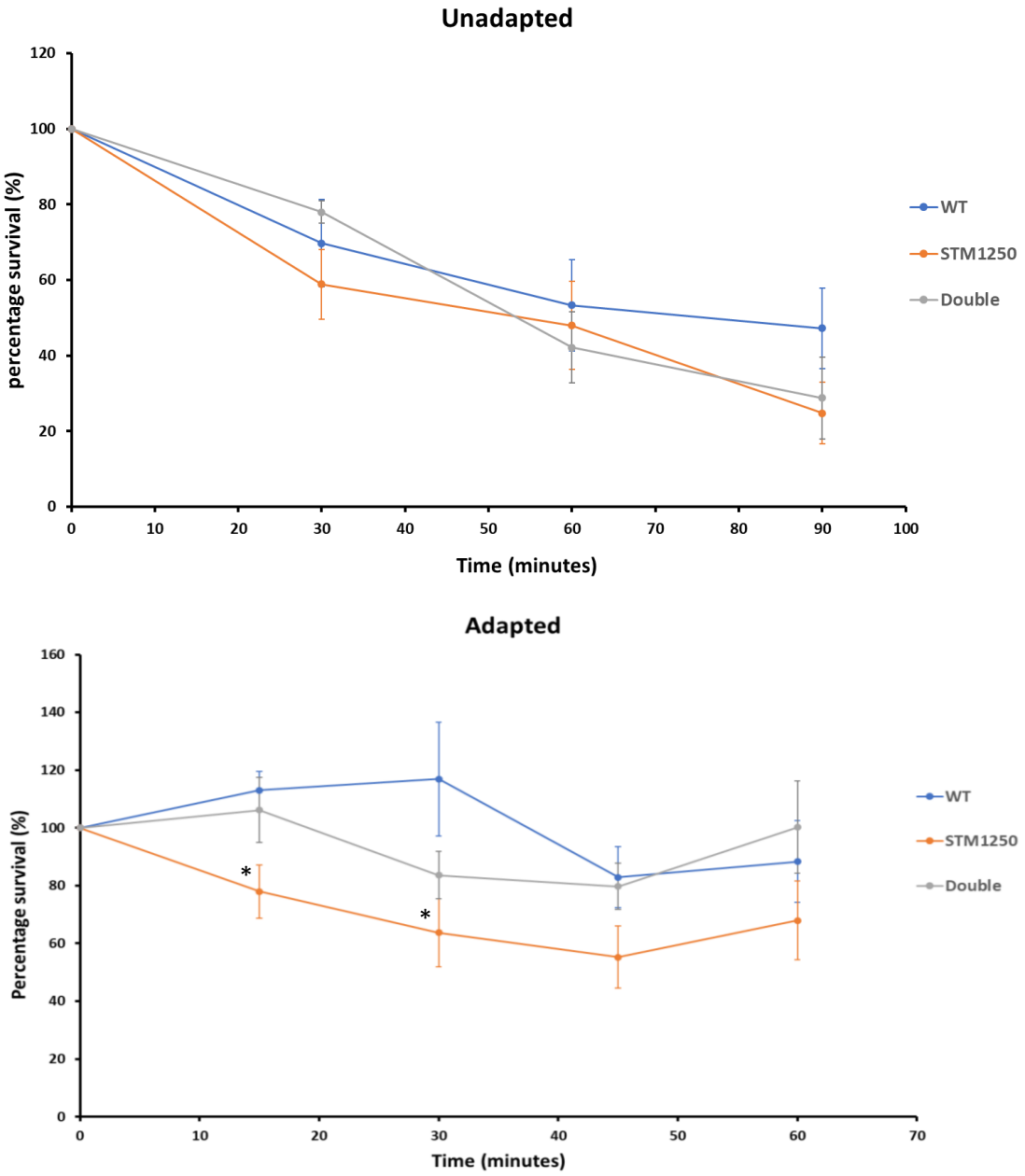

Figure 7. Survival of the acid adapted Δ*STM1250* mutant but not the Δ*STM1250*Δ*agsA* double mutant was significantly reduced after 15 and 30 minutes in its ability to survive acid shock of pH 3.0 adjusted with HCl, compared to WT. Unadapted cultures were added to pH 3.0 acid shock media acidified with HCl and incubated for 90 minutes. Aliquots of 1 mL were taken every 30 minutes and a percentage survival calculated relative to the control t=0 sample. Adapted cultures were instead added to pH 4.4 adaption and incubated for 2 hours. Strains were then transferred to acid shock media and incubated for 60 minutes, with aliquots taken every 15 minutes. Data points are the means of 7 experiments performed in triplicate, error bars are SEM. Data points were analysed for significant difference using one-way ANOVA with Tukey's post-test, p values below 0.05 were deemed significant. * $p < 0.05$.

3.2.5 STM1250 is involved in the stationary phase ATR response in the presence of citric acid

The previous ATR assays used the inorganic acid HCl to acidify the adaption and shock media. To get a deeper understanding of the influence of STM1250 in the *Salmonella* ATR the assay was repeated using the organic acid, citric acid. Due to the findings that STM1250 was solely responsible for the *Salmonella* ATR, the survival of only WT and Δ*STM1250* was investigated in the presence of citric acid. Bacterial cultures were adapted in NCE media adjusted to pH 5.0 with citric acid, this is due to the previous results collected from the pH range assay which determined that adaption in pH 4.4 as used with HCl would lead to an unacceptable amount of bacterial inactivation. The acid shock media was additionally altered to a pH of 3.5 as to not result in excessive bacterial inactivation.

In accordance with previous ATR assays there was no significant difference in survival between unadapted WT and the Δ*STM1250* mutant cultures. Interestingly, despite the alteration of the adaption and shock media pH, the overall findings were replicated. The percentage survival of the Δ*STM1250* mutant was significantly lower in comparison to the WT after 15 minutes of incubation at pH 3.5 (Figure 8). Similar to previous assays the reduction of percentage survival of both *Salmonella* strains in the adapted assay were smaller to that of the unadapted assay. With percentage survival only reducing by a mean 58.7% in the adapted assay compared to 82.43% in the unadapted assay.

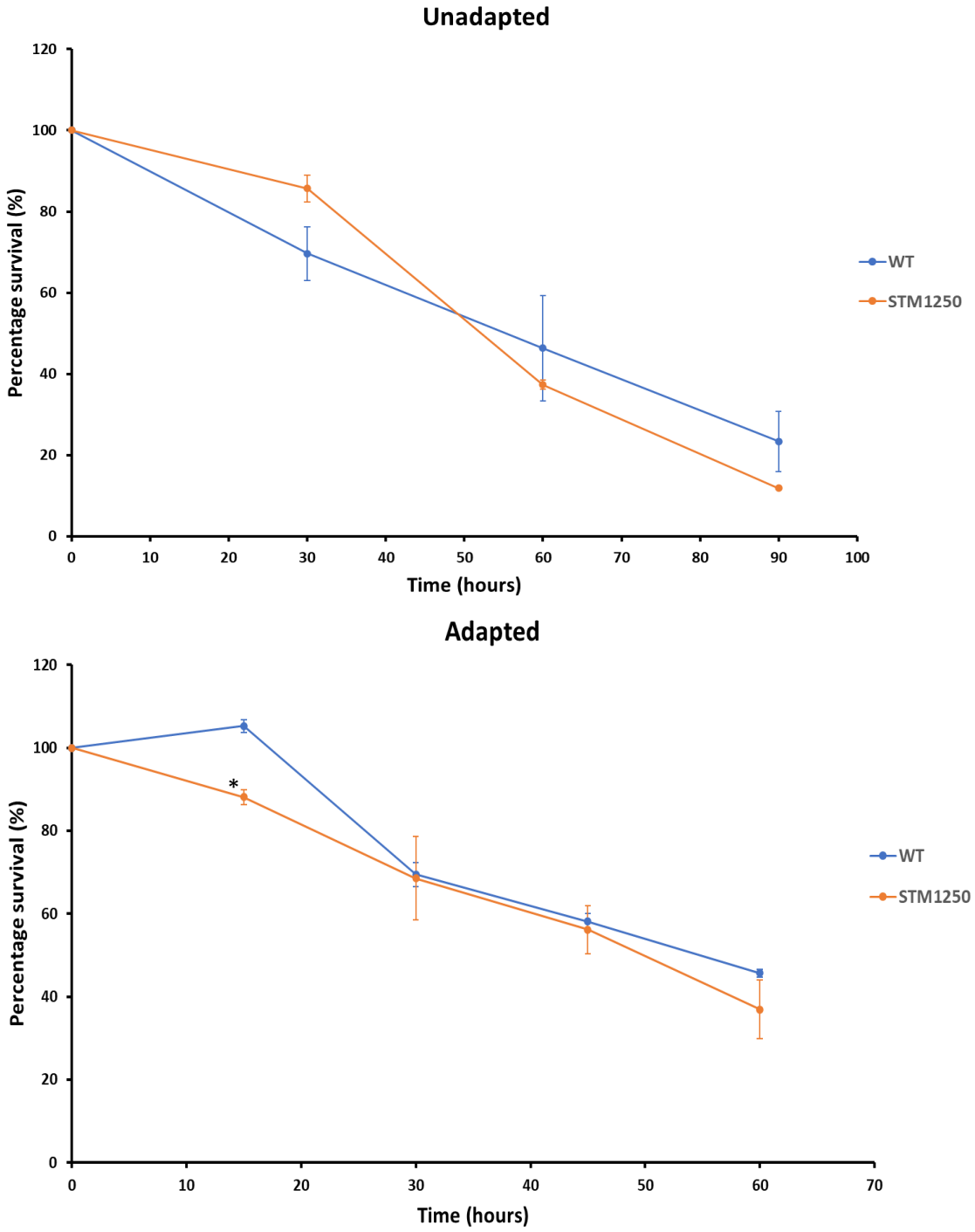

Figure 8. Survival of the acid adapted Δ*STM1250* mutant was significantly reduced after 15 minutes in its ability to survive acid shock of pH 3.0 adjusted with citric acid, compared to WT. Unadapted cultures were transferred to pH 3.5 acid shock media (adjusted with citric acid) and incubated for 90 minutes. Aliquots of 1 mL were taken every 30 minutes and a percentage survival calculated relative to the control t=0 sample. Adapted cultures were instead added to pH 5.0 adaption media and incubated for 2 hours. Strains were then transferred to acid shock media and incubated for 60 minutes, with aliquots taken every 15 minutes. Data points are the means of 3 experiments performed in triplicate, error bars are SEM. Data points were analysed for significant difference using Student's t-test, p values below 0.05 were deemed significant. * $p < 0.05$.

3.3 Discussion

Overall, this chapter aimed to confirm previous unpublished findings by Hews (2020). Predominately, confirming that STM1250 solely is important for the *Salmonella* ATR in the presence of either inorganic acid (HCl) which is host associated due to its abundance in host digestive systems, or organic acid (citric acid) which is associated with certain food products and food preservatives. The possibility that functional redundancy exists between the sHsps and STM1250 in the *Salmonella* ATR was additionally assessed. RpoE has been shown to be induced by acid shock in *Salmonella,* therefore suggesting an involvement of RpoE in the ATR (Muller *et al*., 2009). However, further information in the literature surrounding RpoE-regulated proteins is very limited, with the extent of their contribution or roles in the acid shock response undefined. In this chapter, we confirmed the contribution of STM1250 in the *Salmonella* ATR determining it functions in the ATR in the presence of citric acid.

3.3.1 STM1250 is important for the *Salmonella* ATR in the presence of HCl

The previous association of STM1250 to the *Salmonella* ATR was highlighted by Hews (2020), previous to this no role specific to STM1250 had been determined. To assess the significance of these findings confirmatory studies were conducted, determining whether the findings are reproducible. The survival of WT and Δ*STM1250*Δ*ibpA*Δ*ibpB*Δ*agsA* quadruple mutants following acid shock in pH 3.0 NCE acidified with HCl, with or without a 2-hour adaption period in pH 4.4 NCE acidified with HCl were first assessed. Acid adapted quadruple mutants were first confirmed to indeed have a reduced percentage survival following incubation in acid shock media (Figure 6), with a marked reduction in survival occurring within the 15 minutes of incubation. These results suggest a role for the genes in the mutant to mount the ATR. In addition, the unadapted assay confirmed there was no significant difference between the WT and quadruple mutant, suggesting that none of the genes *ibpAB*, *STM1250* or *agsA* are important for non-acid adapted survival in acid stress conditions acidified with HCl. Further ATR experiments performed in the same manner identified that the Δ*STM1250* mutant was independently responsible for the reduction in survival within the first 30 minutes of acid shock. This was in accordance with the findings in Hews (2020), therefore we conclude that STM1250 is important for the early stages of acid exposure in the ATR.

Of importance was the lack of a reduction in survival of the Δ*STM1250*Δ*agsA* double mutant, which instead failed so show a significant difference from either the WT or the single Δ*STM1250* mutant. This is unexpected as the expectation is that the double mutant would share a similar reduction in percentage survival to the single mutant as the same genes are

omitted. However, it is possible that the absence of *agsA* in the double mutant confers a survival benefit to *Salmonella*. This may be as a result of functional redundancy between AgsA and IbpAB, which has been previously shown Hews (2020). Therefore, the double mutant IbpAB may be overexpressed conferring a survival benefit. However, in the same thesis by Hews (2020) no survival disadvantage was identified in acid adapted Δ*ibpAB* mutants following acid challenge, suggesting the genes have no function in the *Salmonella* ATR. Further experimentation would be required to prove whether functional redundancy between AgsA and IbpAB infers a survival advantage towards *Salmonella* and its ability to survive acid challenge.

Transcriptomic analysis has been previously studied in *Salmonella* Typhimurium under the ATR. *STM1250* was discovered as being upregulated after an acid adaption phase which consisted of incubation in minimal E glucose media adjusted to pH 4.4 with HCl (Ryan *et al*., 2015). These findings support data presented in this chapter as STM1250 was determined as having a role in the ATR, this is consistent with it becoming upregulated during the acid adaption period. The use of minimal media and the same pH and acidulent as the acid tolerance assays in this chapter, bolsters the relevance of these supporting claims. Of additional importance was the upregulation of *STM1250* was no longer apparent following acid shock for 1 hour in media adjusted to pH 3.1 by HCl (Ryan *et al*., 2015). This also corroborates findings in this chapter, as STM1250 was only determined as being important in the initial stages of acid shock in the first 30 minutes of acid shock exposure. A more recent transcriptional analysis on *Salmonella* acid tolerance had somewhat contrasting results compared to the previous Ryan *et al*. (2015) study. The investigation conducted by Cao *et al*. (2019) only assessed gene regulation post acid adaption and acid shock. STM1250 was determined as being upregulated after 1 hour of acid shock, contrasting findings in this chapter as STM1250 did not appear to be important in the ATR after an hour post acid shock. Although the adaption and acid shock pHs were the same as the Ryan *et al*. (2015) study, Cao *et al*. (2019) did not mention the type of acid used to adjust the media. Additionally, they utilised tryptic soy broth (TSB) a type of complex media. It may be beneficial to conduct acid tolerance assays as seen in this chapter with complex media, determining whether in fact STM1250 may have a yet unknown function in the ATR only apparent in complex media.

3.3.2 STM1250 is important for the *Salmonella* ATR in the presence of citric acid

Various methods are utilised during food processing to make products safe for consumers. A common technique is the use of antimicrobial agents which preserve food by preventing the growth of pathogenic and deteriorative microorganisms. This includes organic acids which act

by lowering the pH to levels where microorganisms stop proliferating (García-García and Searle, 2016). The organic acid, citric acid is widely used in the food industry due to its antimicrobial properties. This is a result of its acidulation, enabling the preservation of foods such as tomato juice, ice cream, beverages, salad dressing and more (Søltoft-Jensen and Hansen, 2005). However, research has been conducted warning the use of certain antimicrobial agents in food processing including organic acids. This is due to the ability of bacteria especially *Salmonella* in developing greater tolerance to stresses including acid stress, such as the onset of the ATR. Acid adapted cells can also confer cross-protection with other environmental stresses, thus together increasing their persistence throughout the food chain (Dubois-Brissonnet, 2011). Although this is significant for artificially acidified food products, the same applies for naturally acidified food products such as fruit juices. Should *Salmonella* contamination be completely avoided during food processing, a more advanced comprehension of the innate mechanisms of stress responses and especially the ATR are fundamental. Determining the influence of STM1250 in the ATR in response to organic acids may inform on new ways to target *Salmonella* survival during food processing.

After confirmation of STM1250s importance in the *Salmonella* ATR in the presence of the inorganic acid HCl the survival of the Δ*STM1250* mutant to the organic acid, citric acid was assessed. ATR assays were performed with a less acidic pH in the citric acid assays in comparison to the HCl assays, with the citric acid adaption media having a pH of 5.0 and the shock media 3.5. This was in accordance with the findings in the pH range survival assay. Which determined that the bacterial inactivation seen at pH 4.4 in the HCl assay most closely matched that of pH 5.0 in the citric acid assay, a relatively small difference of pH 0.6. In support of the findings in the pH range survival assay, previous studies have highlighted the survival ability of *Salmonella* in citric acid and HCl to be similar at low pH. This is however relative to other organic acids such as acetic acid, which *Salmonella* is highly sensitive to (Álvarez-Ordóñez *et al.,* 2010b). Interestingly an equal response was observed in citric acid adapted *Salmonella* cells regardless of the slight change in pH from the HCl assay, with a significant reduction in percentage survival of the Δ*STM1250* mutant observed after 15 minutes in acid shock. STM1250 is therefore seemingly important in the initial stages of the ATR in the presence of citric acid.

Citric acid is a weak acid defined as triprotic due to its three dissociable protons that undergo sequential ionisation to form three distinct negatively charged species as seen in Figure 9 (Burel *et al.,* 2021). Weak acids are able to diffuse freely across bacterial membranes when it their undissociated form due to their lipophilic nature (Hirshfield *et al.,* 2003). The precise antimicrobial mode of action is still not fully understood for citric acid. However, it is thought

that in its un-charged, undissociated state (CAH_3) citric acid can freely cross microbial membranes. Once within the cytoplasm, citric acid dissociates into citrate anions and protons which causes the acidification of intracellular compartments. This acidification can confer functional and structural damages or modifies enzyme function, structural proteins, DNA, and can destabilise the outer membrane via chelation or intercalation (Mani-López *et al*., 2012). Therefore, the reduction in survival of the Δ*STM1250* mutant in the presence of both inorganic and organic acids suggests STM1250 does not act in a specialist manner restricted to a type of acidulent, but instead may function in a generalised fashion by increasing or maintaining intracellular pH or responding to structural damage caused by low pH.

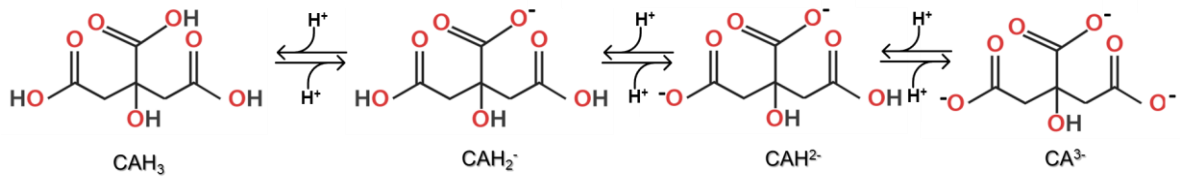

Figure 9. The ionisation states of citric acid. Citric acid exists in its undissociated form CAH_3 at low pH, as pH increases citric acid dissociates releasing a proton at each ionisation state. Adapted from (Burel *et al*., 2021).

3.3.3 Summary

Overall, in this chapter STM1250 has been confirmed to have an independent and significant role in the initial stages of the *Salmonella* ATR in the presence of HCl. Novel roles for STM1250 have also been reported in the ATR in the presence of citric acid. Together these findings are highly suggestive of a generalised role of STM1250 early stages of acid exposure in the ATR, with the type of acidulent not dependent on its functioning. Potential functional redundancy has also been reported between AgsA and IbpAB for the first time in the *Salmonella* ATR. This would be interesting to further investigate determining whether there is in fact functional redundancy between the genes during the *Salmonella* ATR, and if the overexpression of IbpAB confers a survival benefit in *Salmonella's* ability to tolerate acid stress.

Chapter 4: Investigating the importance of the acid tolerance response and STM1250 in food safety

4.1 Introduction

In chapter 3, a novel function of STM1250 was established in the *Salmonella* ATR in response to the organic acid, citric acid. As previously mentioned, citric acid is a common preservative used in the food industry to limit the levels of pathogenic and deteriorative microorganisms. There are also many naturally acidified food products such as fruit juices which contain varying amounts of organic acids including citric acid. Naturally acidified food products such as fruit juices have been associated with outbreaks of *Salmonella.* Therefore, in this chapter we sought out to investigate the importance of the *Salmonella* ATR during exposure to acid stress from naturally acidified orange and apple fruit juices, specifically assessing the importance of STM1250 in the ATR in fruit juice over a range of storage temperatures.

4.1.1 Implications of the ATR on Food safety

Although extensive research has been conducted on the molecular mechanisms governing the *Salmonella* ATR, it is important to note that very few studies have focused on the implications of this response in *Salmonella* survival in foodstuffs and therefore what impacts it might have on food safety measures. The majority of investigations on the *Salmonella* ATR have been conducted using complex or minimal media, this has been successful in identifying specific conditions required in the induction of the ATR. Some studies have started to associate the existence of similar adaptive responses in acidic foods and foods acidified during processing. Many of the studies conducted in the literature however focus on the induction of the ATR during the processing of meat products, with many investigations being conducted on meat extract. Although relevant to this investigation as it informs on the ability of *Salmonella* to mount an ATR in response to artificially acidified products, only a select few organic acids are typically used artificially so limited information can be inferred to naturally acidified food products.

Leyer and Johnson. (1992) first showed that acid adapted cells survived better compared to non-adapted cells in different dairy products such as cheeses or milk during fermentation by lactic acid. Adapted cells displayed increased resistance to organic acids including lactic, propionic and acetic acids commonly found in cheese. Similar observations have also been reported in fruit juices, with acid adapted cells displaying increased survival in orange juice compared to non-acid adapted cells (Álvarez-Ordóñez *et al.,* 2013). Not only have acid adapted cells been shown to have increased survival in dairy products and fruit juices but also in beef products. An investigation by Lang *et al.* (2021) similarly determined that acid-adapted strains had increased survival in long-term storage of meat extract, suggesting the persistence

of the ATR during production and storage. Significantly, low temperatures were again linked to the inhibition of the ATR highlighting the importance of low temperature during slaughter and storage of meat products (Lang *et al.,* 2021).

The evidence that acid adapted cells have increased survival in different acidic food environments is noteworthy, this is because in the food industry bacteria including *Salmonella* can become acid adapted in various ways. The potential induction of the ATR may occur following incubation in low pH environments commonly found during food processing, subsequently leading to prolonged bacterial survival in food products posing a significant risk to food safety. This may additionally enhance *Salmonellas* ability to survive in the digestive system or inside the SCV resulting in disease (Álvarez-Ordóñez et al., 2012). The presence of low pH frequently occurs in food processing. Industrial interventions such as the use of rinsing solutions containing organic acids and acid solutions as antimicrobial sprays on produce and animal carcasses. These processes can create environments with pH values ranging from 4.5 to 5.5, enough to induce an ATR (Samelis *et al.,* 2002).

As previously mentioned, *Salmonella* is a zoonotic pathogen therefore the majority of food borne outbreaks of *Salmonella* are attributed to contaminated meat products such as poultry, beef and pork. *Salmonella* Typhimurium outbreaks have also been reported in naturally acidified food products such as fruit juices, however little attention has been assigned to the reasoning behind these outbreaks. The Centers for Disease Control and Prevention (CDC) receives reports of food-associated outbreaks of illness. A report conducted by Vojdani *et al*. (2008) determined that 5 reported outbreaks in fruit juice were caused by *Salmonella* between 1995 and 2005, leading to 710 cases and 94 hospitalisations. Another reported nationwide outbreak occurred in the United States in 2005, with a reported 152 cases in 23 states. The illness was associated with the consumption of unpasteurised orange juice (Jain *et al*., 2009). Apple juice is another apparent vector for *Salmonella* Typhimurium, with an outbreak in 1974 leading to 296 cases of disease being traced back to commercial apple cider (CDC, 1975). Evidently orange and apple juices provide a vector for *Salmonella,* enabling the bacteria to persist in the products long enough to result in human consumption and infection.

Fruit juices contain natural levels of a variety of organic acids, typically making up 1% of the total weight of the juice. However, certain fruit juices contain a specific organic acid which is predominantly more abundant than the others. For orange juice citric acid accounts for approximately 90% of the total organic acids, for apple juice malic acid is the most abundant (Huang *et al*., 2009). The acidity of fruit juices varies depending on the fruit itself and whether they contain additives, pH generally ranges from 3.0 to 4.0. An investigation into the pH levels

of beverages in the United States determined that Tropicana 100% apple juice had a pH of 3.50 whereas orange juice had a pH of 3.80 (Reddy *et al*., 2015). Exposure to the mild acidic conditions apparent in fruit juices corresponds to the hypothetical induction of the ATR, and therefore an enhanced resistance to extreme pH conditions in host digestive systems.

Storage temperature is also an important component in the induction of the ATR in the presence of organic acids. This is due to the temperature having a direct role in the ability of undissociated organic acids to pass through bacterial membranes, with a higher temperature increasing the rate of passage (Ulgas and Ingham, 1998). The effect of temperature on the *S*. Typhimurium ATR in food products is underreported in the literature, considering the implications it may have in the way we store acidic foods. An investigation by Álvarez-Ordóñez *et al*. (2013) initially assessed the survival of acid adapted and non-acid adapted *S*. Typhimurium cells in orange juice under different storage temperatures. They determined that acid adapted cells with an induced ATR had enhanced survival abilities compared to non-acid adapted cells at higher temperatures of 25 and 37 °C. This initial study highlights how correct storage of fruit juice at appropriate temperatures is vital, stopping the induction of the ATR therefore reducing the level of *Salmonella*.

4.1.2 Chapter aims

Only very limited studies have been conducted assessing the importance of the ATR in *Salmonella* survival in fruit juices during storage. A prominent study conducted by Álvarez-Ordóñez *et al*. (2013) investigated acid adapted and non-acid adapted cells during storage in orange juice, as previously mentioned they determined that acid adapted cells had enhanced survival at higher temperatures. As discussed in chapter 3, STM1250 is important in the ATR in the presence of citric acid. In combination to citric acid being the predominant organic acid in orange juice, we sought out to investigate whether STM1250 may be important for the survival of *Salmonella* in naturally acidified fruit juices. Therefore, we hypothesised that STM1250 would be important for the survival of *Salmonella* during storage of fruit juices.

In this chapter, we aim to investigate the role of the ATR in the survival of *Salmonella* in apple and orange juice over a range of storage temperatures. Specifically assessing for the first time whether STM1250 does in fact also extend its contribution to the ATR from minimal media acidified with citric acid, into more complex naturally acidified media being apple and orange juices during storage.

These aims were achieved through:

- Assessing the percentage survival of each of the strains across the storage temperatures; 37 ℃ representing average body temperature, 25 ℃ representing room temperature and 4 ℃ representing refrigeration temperature.

- Assessing the percentage survival of acid adapted and non-acid adapted Δ*STM1250* single mutants in long term storage in either apple or orange juice stored at either 4, 25 or 37 °C.

4.2 Results

4.2.1 Growth of mutant strains is not significantly reduced in complex media

Growth curves were conducted using Luria-Bertani (LB) medium. Similarly, to the minimal media growth curve this will confirm whether any significant differences are present between mutant strains and the WT. This will inform on later assays utilising complex media such as fruit juice in the apple and orange juice survival assays, confirming that any differences in survival displayed are as a result of the loss of the gene and not as a result of the strains ability to grow in complex media. Growth curves were conducted on each of the mutant strains being investigated (Δ*STM1250* single, Δ*STM1250*Δ*agsA* double and Δ*STM1250*Δ*ibpA*Δ*ibpB*Δ*agsA* quadruple mutants). Growth curves were performed alongside a WT strain in order to determine their baseline growth. The growth of the mutant strains followed that of the WT in LB medium, with no significant difference being displayed between the strains (Figure 10). This suggests that none of the mutants had any growth restrictions in complex LB media.

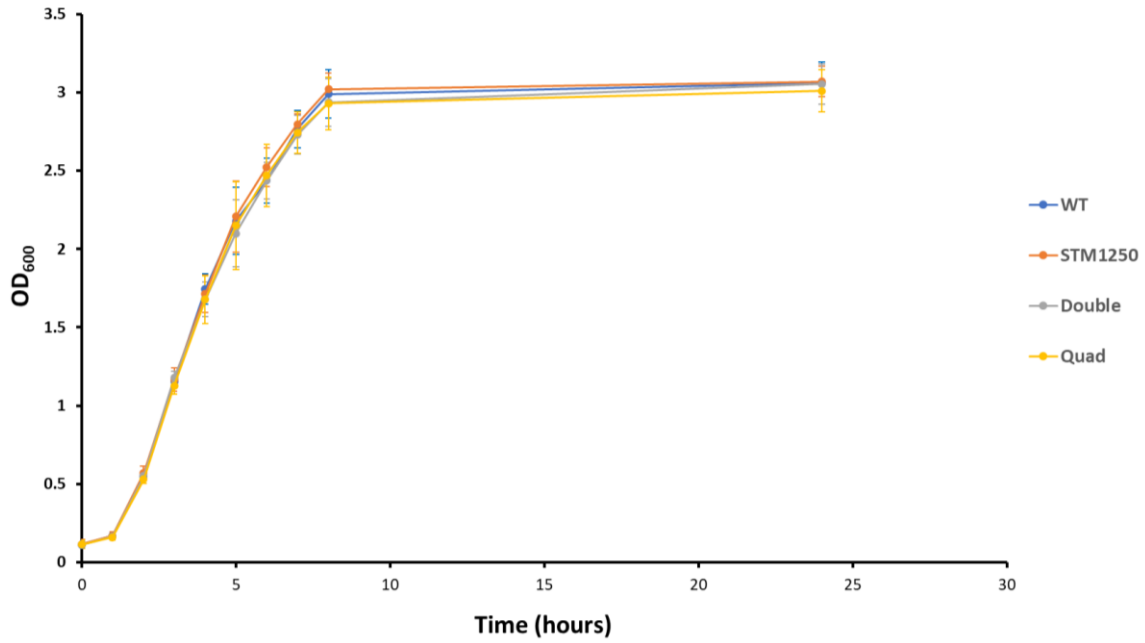

Figure 10. Growth of WT and *Salmonella* mutant strains are not significantly reduced in pH 7.0 LB medium. Respective strains of *Salmonella* were used to inoculate LB medium and incubated at 37 °C, 180 rpm for 24 hours. Aliquots (1 ml) were taken every hour for 8 hours with a final sample taken at 24 hours. OD_{600} readings were measured after collection. Data points are the culmination of three means produced from three individual experiments performed in triplicate. Error bars are SEM.

4.2.2 Survival of acid adapted and non-acid adapted Δ*STM1250* mutants in pasteurised apple and orange juice

The involvement of STM1250 in the *Salmonella* ATR was investigated further, with non-acid adapted and acid adapted Δ*STM1250* mutants being assessed for their percentage survival in apple and orange juice stored over a range of temperatures. This would determine whether STM1250 is important for the ATR in acidic food products stored at specific temperatures. Percentage survival of acid adapted and non-acid adapted Δ*STM1250* mutants were assessed in apple and orange juice at temperatures of 37 °C, 25 °C and 4 °C. Strains of *Salmonella* were first grown overnight in Luria-Bertani (LB) broth (pH 7.0). Strains were then standardised before being transferred into either pH 7.0 LB (non-acid adapted) or pH 4.4 LB broth acidified with HCl (acid adapted) and grown to late stationary phase. Cultures were then added to apple and orange juice and stored at either 37 °C, 25 °C or 4 °C. Aliquots were taken at regular intervals and the CFU/mL determined.

4.2.2.1 Apple and orange juice storage survival assay at 37 °C

Overall, the survival of both the WT and Δ*STM1250* mutants dramatically decreased over the 3-hour time period at 37 ℃, with the percentage survival of all cultures falling a combined 90.3% (Figure 11). Interestingly, acid adapted cultures irrelevant of strain had a reduced survival compared to non-acid adapted cultures, with acid adapted cultures having a 91.6% reduction in percentage survival compared to an 88.96% reduction for unadapted cultures after 3 hours. The type of fruit juice appeared to have no significant impact on survival when stored at 37 °C. However, it can be noted that a trend is visible in the acid adapted assay as cultures stored in orange juice displayed reduced survival compared to apple juice, with a 9.6% difference between the two after 3 hours. The opposite is true in the unadapted assay with a trend suggesting cultures stored in apple juice had greater survival, with a 12.1% difference after 3 hours. Crucially, no statistically significant difference in percentage survival occurred between WT and the Δ*STM1250* mutant in unadapted cultures in either the apple or orange juice. The same can be said of acid adapted cultures with no significant difference determined in either juice between the strains.

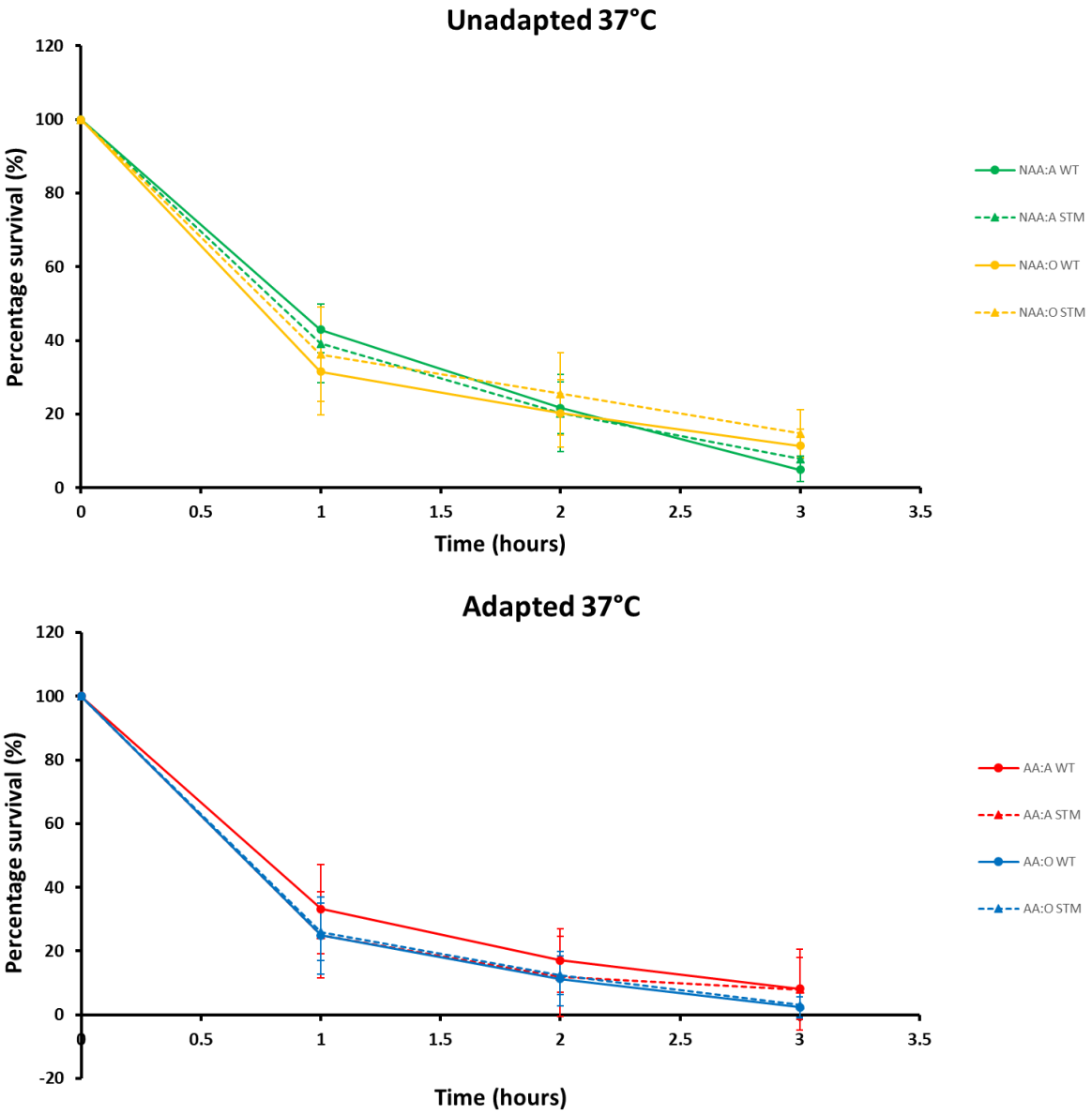

Figure 11. Survival of acid adapted and non-acid adapted Δ*STM1250* mutant and WT *Salmonella* in apple and orange juice at 37 °C. Unadapted cultures were incubated in pH 7.0 LB until late stationary phase then transferred to either apple or orange juice. Acid adapted cultures were incubated in pH 4.4 LB adjusted with HCl. Aliquots were taken every 2 hours and CFU/mL was calculated. Data points are the means of 3 experiments performed in triplicate, error bars are SEM. (AA refers to acid adapted, and NAA refers to non-acid adapted, A/O refers to apple and orange juice respectively).

4.2.2.2 Apple and orange juice storage survival assay at 25 °C

The survival of each culture dramatically decreased over the 24-hour time period at 25 ℃ (Figure 12). Dissimilar to 37 ℃, the percentage survival of cultures reduced at a slower rate, with cultures falling a mean 79.3% over 36 hours. In agreement with the previous assay, acid adapted cultures irrelevant of strain appeared to have a decreased survival advantage compared to unadapted cultures. With the survival of acid adapted cultures falling a mean 80.8% compared to 78.5% of unadapted cultures. The type of juice did however appear to have an impact on survival irrelevant of acid adaption, with cultures stored in apple juice overall having reduced survival. This is evident with a 10.9% difference in adapted cultures and 14.7% in unadapted cultures. Of importance, no statistically significant difference was determined between the mutant strain and WT in adapted and unadapted cultures in either apple or orange juice. However, a clear trend can be visualised in the adapted assay, with the mutant strain having reduced survival compared to WT in both apple and orange juice.

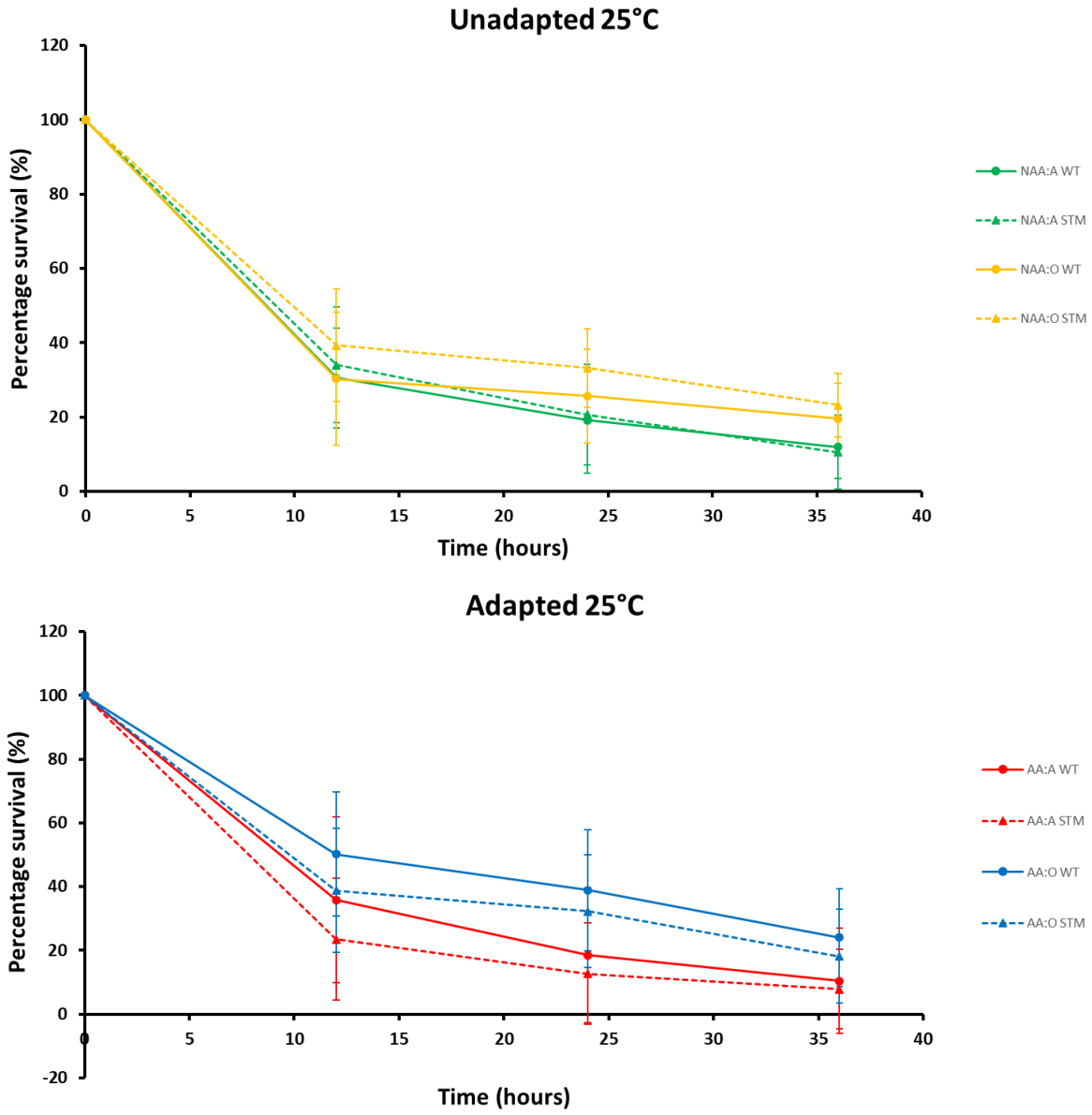

Figure 12. Survival of acid adapted and non-acid adapted ∆*STM1250* mutant and WT *Salmonella* in apple and orange juice at 25 °C. Unadapted cultures were incubated in pH 7.0 LB until late stationary phase then transferred to either apple or orange juice. Acid adapted cultures were incubated in pH 4.4 LB adjusted with HCl. Aliquots were taken every 24 hours and CFU/mL was calculated. Data points are the means of 3 experiments performed in triplicate, error bars are SEM. (AA refers to acid adapted, and NAA refers to non-acid adapted, A/O refers to apple and orange juice respectively).

4.2.2.3 Apple and orange juice storage survival assay at 4 °C

The survival of cultures incubated at 4 ℃ also significantly reduced over the 36-hour period, although on average cultures only decreased by 65.4% (Figure 13). Like the previous assays adapted cells had a greater reduction in survival falling 70.5% compared to only 60.3% in

unadapted cells. Similar to the 4 °C assay cultures incubated in apple juice had a greater reduction in percentage survival, with a 7.0% difference in adapted and 12.7% in unadapted cultures. Again, no statistically significant difference was determined between the mutant strain and WT in adapted and unadapted cultures in either apple or orange juice. However, a clear trend can be similarly visualised in the adapted assay, with the mutant strain having reduced survival.

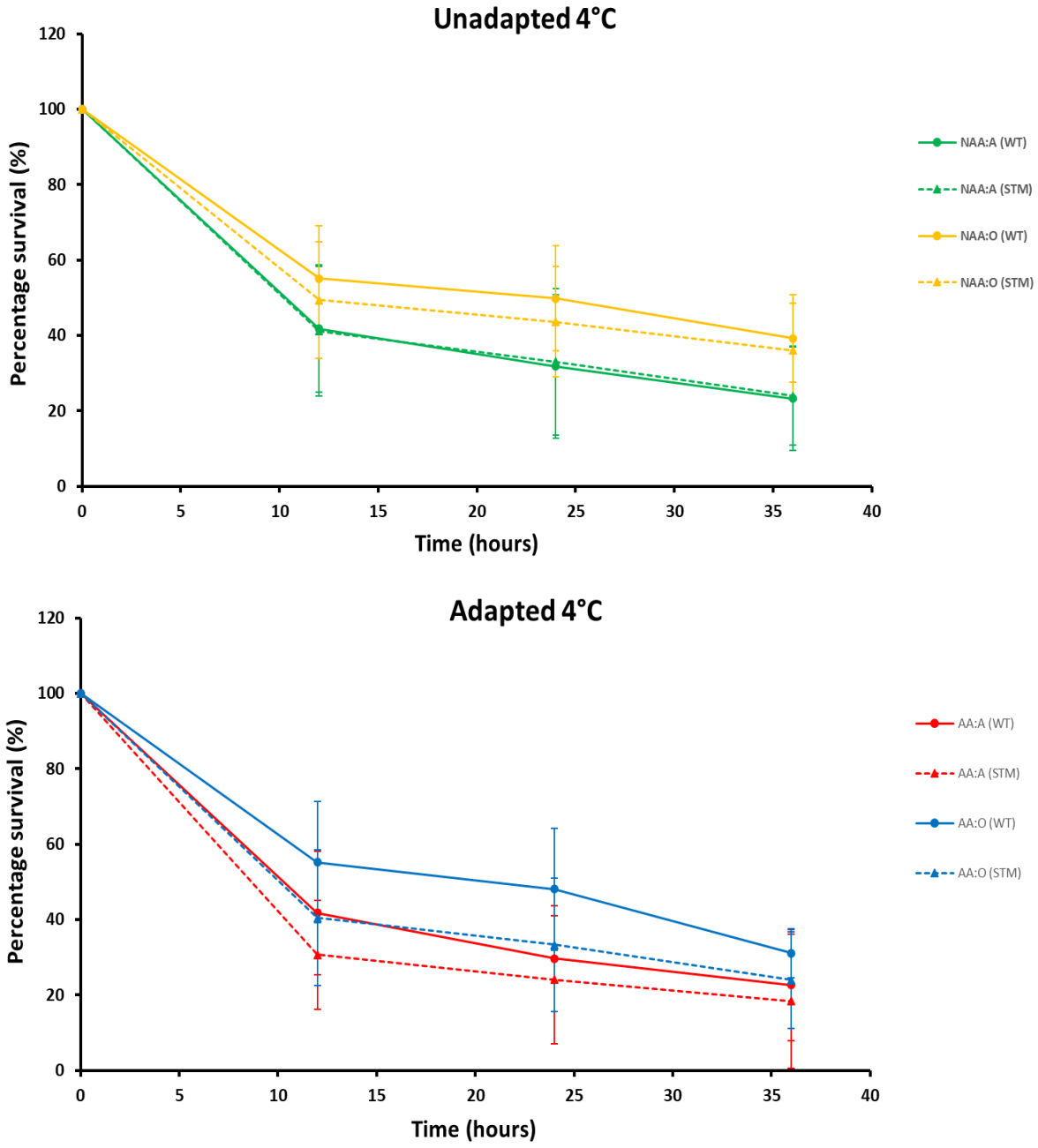

Figure 13. Survival of acid adapted and non-acid adapted Δ*STM1250* mutant and WT *Salmonella* in apple and orange juice at 4 °C. Unadapted cultures were incubated in pH 7.0 LB until late stationary phase then transferred to either apple or orange juice. Acid adapted cultures were incubated in pH 4.4 LB adjusted with HCl. Aliquots were taken every 24 hours and CFU/mL was calculated. Data points are the means of 3 experiments performed in triplicate, error bars are SEM. (AA refers to acid adapted, and NAA refers to non-acid adapted, A/O refers to apple and orange juice respectively).

4.3 Discussion

In this chapter acid adapted and non-acid adapted cultures of ∆*STM1250* and WT *Salmonella* Typhimurium were assessed for their survival capabilities in apple and orange juice, over a range of storage temperatures. In the previous chapter STM1250 was determined as being important in the ATR in the presence of the organic acid, citric acid. As citric acid is the primary acidulent found in orange juice we investigated whether the function of STM1250 was important for *Salmonella* survival in naturally acidified orange juice during storage. We also extended the investigation into apple juice as similarly to orange juice it is also naturally acidified with organic acids, primarily malic acid. In this chapter we determined that temperature significantly impacts the survival of *Salmonella* when incubated in apple and orange juice. We also established that unadapted cultures overall appeared to have a survival advantage compared to adapted cultures, which was unexpected. Importantly, STM1250 was not significantly involved in the survival of *Salmonella* in the storage of apple and orange juice. However, a clear trend was apparent in both 4 and 25 °C assays, with the mutant strain having reduced survival. Furter investigation is required to fully elucidate whether the trend shown in this investigation is significant, and that therefore STM1250 does in fact contribute to *Salmonella* survival in fruit juice storage at 4 and 25 °C.

4.2.1 Survival of the *STM1250* mutant in apple and orange juice

As discussed in the introduction of this chapter several outbreaks of salmonellosis have been attributed to contaminated acidic foods, including fruit juices (CDC, 1975; Jain *et al*., 2009; Vojdani *et al*., 2008). This has raised some concerns over the safety of naturally acid food products, with their inherent acidity potentially not being a satisfactory protective mechanism against pathogenic microorganisms. A more complete understanding of the involvement of ASPs in the ATR is required, informing how bacteria including *Salmonella* which possess this inherit response behave under naturally acidified food products. Therefore, enabling the assessment of microbiological risk in acidic food products.

The apple and orange juice storage survival experiments were designed based on Álvarez-Ordóñez *et al* (2013), and were conducted in order to compare the survival capacity of WT and ∆*STM1250* mutant acid adapted and non-acid adapted *S*. typhimurium cells in orange and apple juice stored under a selection of temperatures. Results gathered suggest that each of the four variables: acid adaption, fruit juice, storage temperature, and strain each had some impact on *Salmonella* survival. The storage temperatures used in this investigation were specifically selected: 4 ℃ was chosen as it mimics normal refrigeration temperature of fruit

juices, 25 ℃ was selected as it mimics room temperature when fruit juice is not stored correctly, and 37 ℃ was selected as it is the optimum growth temperature of *Salmonella*.

Overall, the survival of cultures irrespective of incubation temperature dramatically decreased over the respective experimentation period. This was expected as apple and orange juice contain high amounts of organic acids which have deleterious effects on the growth and viability of bacteria (Baik *et al*., 1996). As previously stated, citric acid is the primary organic acid present in orange juice when malic acid is the main organic acid for apple juice (Huang *et al*., 2009). Citric acid has been previously shown to be the more effective than malic acid in inducing the ATR (Álvarez-Ordóñez *et al*., 2009). Cultures incubated in orange juice appeared to survive better in comparison to those incubated in each of the assays, apart from acid adapted cultures incubated at 37 °C, this may have been as a result of the more effective induction of the ATR by citric acid present in the orange juice compared to the malic acid of apple juice. However, it is also feasible malic acid caused higher bacterial inactivation, this is due to unadapted cultures also appearing to have greater survival in orange juice compared to apple juice. A previous study investigating the antimicrobial activity of organic acids indicated that the smaller molecular size of undissociated malic acid (134.09 Da) may enable easier entry into bacterial cells and therefore more readily reducing internal pH, compared to the larger size of citric acid (192.12 Da) which might not gain entry to the cell as effective (Eswaranandam *et al*., 2004).

Storage temperature also had an evident impact on the survival of cultures, with higher storage temperatures resulting in higher bacterial inactivation. This is evident from the combined reduction in percentage survival of each of the strains, at 37 °C survival fell the most by 90.3% over 3 hours, at 25 °C survival reduced by 79.3% over 36 hours. Finally, survival reduced the least at 4 °C by only 65.4%. A previous investigation into acid adapted and non-acid adapted *Salmonella* survival in orange juice produced similar results, reporting that for challenged cells the higher the storage temperature used, the greater the bacterial lethality (Álvarez-Ordóñez *et al*., 2013). This was the expected result as it is known that high temperatures increase microbial death in acidic environments (Gounadaki *et al*., 2007). The same study by Álvarez-Ordóñez *et al*. (2013) stated that this phenomenon was likely due to the increased diffusion of undissociated organic acids through the bacterial membrane at higher temperatures, this is also reasonable explanation for results produced in this experiment. This finding highlights the importance of correct processing of fruit juices and reinforces how crucial pasteurisation is in limiting the level of pathogenic microorganisms in food products.

An interesting finding in the study by Álvarez-Ordóñez *et al*. (2013) was that non-acid adapted cells survived better at 4 ℃, whereas acid adapted cells survived better at 25 ℃ and 37 ℃. Findings in this chapter somewhat contradict these results. Similarly, at 4 °C unadapted cells had a greater survival reducing by 60.3% compared to 70.5% in adapted cells, however at 25 and 37 °C unadapted cells also had increased survival. Overall suggesting that unadapted cells survived better than adapted cells regardless of storage temperature. These results were highly unexpected as it suggests that the ATR may be deleterious to the survival of *Salmonella* in storage of apple and orange juice, it is also feasible that some other unknown variable in apple and orange juice is resulting in the repression of the ATR. Further investigation is required in order to fully determine the impact of acid adaption for the survival of *Salmonella* in apple and orange juice at different temperatures.

Of importance in this chapter was the assessment of percentage survival for acid adapted and unadapted Δ*STM1250* single mutants. No statistically significant difference was determined between any of the mutant strains compared to WT during storage at either 4, 25 or 37 °C in apple or orange juice. However, a clear trend was apparent in apple and orange juice at both 4 and 25 °C. Acid adapted Δ*STM1250* mutants incubated in apple and orange juice portrayed a clear reduction in survival in comparison to the WT, with the trend establishing itself during the early stages of incubation from 12 hours onwards. Interestingly, this trend is seemingly no longer apparent, or apparent to a much lesser degree in unadapted cells. This may be suggestive of STM1250 having a role in the ATR in the presence of naturally acidified fruit juices, but only at 4 and 25 °C. As previously established STM1250 is important in the initial stages of acid exposure in the ATR in the presence of the organic acid, citric acid. Potential reasoning for the trend of reduced survival for acid adapted Δ*STM1250* mutants compared to the WT at 4 and 25 °C could be that STM1250 functions in the initial stages of the ATR during acid exposure in fruit juices. This fits in with findings as the trend is evident from the first time period at 12 hours, with the difference in percentage survival compared to the WT upheld across the other time periods at 24 and 36 hours.

4.2.2 Summary

In conclusion, we have shown for the first time that STM1250 is implicated in the *Salmonella* ATR during storage of fruit juices. The effect of temperature on the ATR and its impact on *Salmonella* survival was investigated, in agreement with previous studies a higher temperature was aligned with greater bacterial inactivation. However, in contradiction was that irrelevant of temperature unadapted cells had greater survival. The type of fruit juice was also

significant in the survival of *Salmonella*, with cells incubated in orange juice having increased survival over those incubated in apple juice. These findings bear potential implications for food safety, especially the way in which we treat and store naturally acidified fruit juices. With the consideration of the type of food product, the composition of acids and storage temperature highly important during food processing in order to accurately inactive pathogenic microorganisms which have innate ATR systems. It is crucial we gain a better understanding of the proteins and mechanisms involved in the *Salmonella* ATR, in order to be better prepared in finding solutions to overcome the response and reduce the levels of *Salmonella* and other pathogenic microorganisms to safe levels in acidic food products.

Chapter 5: General Discussion

Results produced from each chapter have already been individually discussed at length. Therefore, this final chapter will outline the predominate findings of each chapter and explain how they take place within the wider context of *Salmonella* research and food safety. Furthermore, potential future avenues of research will be discussed to build on the results displayed.

5.1 Context

Foodborne diseases continue to pose a significant threat to global public health resulting in extensive morbidity and mortality irrelevant of age, socioeconomic development is also notably impacted (He *et al*., 2023). Most foodborne diseases are caused by specific pathogens such as bacteria, viruses, parasites, fungi and mycotoxins which are able to contaminate food products during processing, storage or transportation (Gallo *et al*., 2020). *Salmonella* is a prevalent microorganism responsible for contamination of a wide array of food products and consequently causes many cases of foodborne disease. Annually, *Salmonella* causes an estimated 1.3 billion cases of disease, with 93 million cases of gastroenteritis and 155,000 deaths (Coburn *et al*., 2007; Majowicz *et al*., 2010). *Salmonella* infection from contaminated food products is a major concern for developing countries, this is due to it being one of the leading causes of morbidity and mortality. The pathogen is able to spread without control as a result of poor hygiene and lack of access to clean and safe water (Ngogo *et al*., 2020). Not only does *Salmonella* pose a significant threat in developing but also to developed countries, in Europe *Salmonella* is one of the most important causes of foodborne illness (Callejón *et al*., 2015). The European Centre for Disease Preventing and Control (ECDC) suggested that in 2018 Salmonellosis was the second most reported gastrointestinal infection in humans in the EU with 91 857 cases, of these 48,365 were as a result of 5,146 foodborne outbreaks of *Salmonella* (ECDC, 2020). As suggested previously, *Salmonella* doesn't just pose a threat to public health but also to the economy. A notable reported example was a major outbreak of *Salmonella* Thompson in the Netherlands in 2012, which cost an approximate €7.5 million due to reduced productivity, outbreak control measures and healthcare costs (Suijkerbuijk *et al*., 2016).

NTS serovars of *Salmonella* including Typhimurium typically cause self-limiting gastroenteritis, although this can develop into more serious complications such as bacteraemia (Balasubramanian *et al*., 2019). The emergence of iNTS serovars which cause invasive disease is significant, resulting in more serious bloodstream infections. These serovars are becoming more prevalent in developing countries especially sub-Saharan Africa, especially in young children and adults suffering with comorbidities such as HIV, malaria and malnutrition

(Gordon, 2011). Crucially, no licensed vaccine is available for NTS or iNTS serovars, highlighting the requirement for further *Salmonella* research identifying novel vaccine strains and therapeutic targets (Baliban *et al*., 2020). Of additional concern to global public health are MDR isolates of *Salmonella*. Antibiotics are the primary treatment method for progressed salmonellosis infections and bacteraemia resulting from iNTS, coupled with the frequent use of antibiotics for livestock is enabling MDR isolates of *Salmonella* to emerge. These isolates may also enter the environment and contaminate fruits, vegetables, and water sources (Punchihewage-Don *et al*., 2022). A notable outbreak of MDR *Salmonella* Heidelberg occurred in the United States in 2013 resulting in a reported 634 cases and 241 hospitalizations. The strain was resistant to ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, sulfisoxazole, and tetracycline (CDC, 2013). This again highlights the importance of continued *Salmonella* research for identification for new therapeutic targets.

Due to the zoonotic nature of *Salmonella* the predominate source of infection occurs from contaminated meat products such as poultry and pork, although other animal related products such as eggs also significantly contribute to salmonellosis (Ehuwa *et al*., 2021). These products make good environments for the growth of *Salmonella* due to the high-water content and abundance of nutrients (Wessels *et al*., 2021). The widespread nature of *Salmonella* enables the pathogen to be present in or on any raw food materials including non-meat products such as fruit and vegetables (Podolak *et al*., 2010). Many reported mechanisms have been suggested for the contamination of fruit and vegetable products. For example, contamination has been suggested to occur through produce contact to soil, this is a result of the ability of *Salmonella* to survive in adverse conditions including the soil for long periods of time (Jacobsen and Bech, 2012). Frequent cross-contamination into fruit and vegetables is also thought to occur from contaminated water used to irrigate plantations, staff and animal manure (Kowalska, 2023).

As established *Salmonella* can survive in a plethora of different conditions, as discussed in chapter 3 *Salmonella* can also contaminate and persist in the low pH environments found in fruit juices. This persistence in fruit juices namely apple and orange juices has led to significant outbreaks of the disease as reported by Vojdani *et al*. (2008) who detailed 5 outbreaks in fruit juice between 1995 and 2005, leading to 710 cases and 94 hospitalisations. Other notable outbreaks have also been reported by Jain *et al*. (2009) and the CDC. (1975) leading to an additional 152 and 296 cases of disease respectively. These outbreaks can be attributed to inadequate food safety regulations and poor food handling, with apple and orange juices clearly providing a stable vector for *Salmonella* contamination. The implication of different regulatory legislations in the early 2000s such as the Hazard Analysis and Critical Control

Point (HACCP) regulation reduced juice-associated *Salmonella* outbreaks, although some juice operations are still exempt from regulations and therefore remain a risk to public health (Vojdani *et al*., 2008; FDA, 2001). Of significance was that many of these outbreaks were associated with unpasteurised apple or orange juices with correct food processing guidelines not followed. Societal changes are resulting in the more frequent consumption of fresh unpasteurised fruit juices because of the nutritional benefits such as antioxidants, fibre, phytonutrients, vitamins, and minerals (Kaddumukasa *et al*., 2017). Therefore, foodborne outbreaks of *Salmonella* in fruit juices still pose a significant threat to public health.

The ability of *Salmonella* to survive within harsh conditions is due to innate stress responses, providing resistance towards antibiotics and environmental stresses such as low pH, temperature changes and osmotic pressure. Stress response mechanisms are highly complex involving the precise functioning of a network of proteins, many of which remain unknown with their specific impact not fully elucidated. Stress responses are crucial during the lifecycle of *Salmonella* enabling survival outside of a host in suboptimal conditions for example the soil, in water or acidic food products. They are also important during host pathogenesis, helping to resist lethal stresses such as the low pH of stomach acid and even host innate and adaptive immune responses.

5.2 The importance of the bacterial stress response

As previously highlighted *Salmonella* frequently encounter various environmental stresses during their lifecycle, requiring the contribution of innate stress responses in order to tolerate or even enable survival in certain conditions. Stress responses including ESRs have been the focus of many recent investigations, implicating them more precisely in *Salmonella* survival against various stresses and even pathogenesis. This is also true for the extracytoplasmic sigma factor σ^E which has been shown to become induced in *Salmonella* following: oxidative stress, heat and cold shock and carbon starvation (Rowley *et al*., 2006), acid stress (Muller *et al*., 2009) ultraviolet A (UVA) radiation, P22 phage and hypo-osmotic shock which have more recently been implicated in the induction of the σ^E ESR (Amar *et al*., 2018).

Investigations have also established the importance of σ^E in *S*. Typhimurium virulence, finding that it is critical for murine infection and survival within macrophages (Humphreys *et al*., 1999). Due to the importance of σ^E it seemed feasible that *Salmonella rpoE* mutants would make good attenuated candidates strains for vaccines. However, the degree of attenuation exceeds that of a functional level with a protective immunity not raised against subsequent challenge with WT *Salmonella* (Rychlik and Barrow, 2005). Attention has therefore been diverted to the

potential use of *rpoE* regulon members as more appropriate vaccine strains, with the reasoning being that they may not result in over attenuation. This is due to many σ^E-regulated proteins being determined to have roles in *Salmonella* pathogenesis. Examples include the σ^E-regulated periplasmic chaperone *skp* which was identified as being required for *S*. Typhimurium murine infection (Rowley *et al*., 2011). Deletion of another σ^E-regulated chaperone *surA* has been shown to lead to a loss of adhesion and invasion of epithelial cells. Mice infected with WT *S*. Typhimurium after exposure to a *surA* mutant were found to be unable to colonise the host (Sydenham *et al*., 2000). More recently the σ^E-regulated proteins STM1250, AgsA, IbpA and IbpB were implicated in *Salmonella* survival in macrophages and in the presence of oxidative stress (Hews *et al*., 2019). The association of the σ^E-regulated proteins to *Salmonella* pathogenesis makes them good targets for vaccine strains, continued elucidation of σ^E-regulated and their precise functioning is beneficial for the identification of novel therapeutic targets.

5.3 The putative stress response protein STM1250 and the *Salmonella* ATR

One of the key stress responses *Salmonella* possesses is the ATR which is responsible for survival under extreme acid conditions. The majority of studies have focused on the ATR of *S*. Typhimurium where much of its characterisation has been conducted. However, similar ATR have also been established in additional serovars of *Salmonella* including but not limited to *S.* Enteritidis *S.* Heidelberg *S.* Senftenberg, and *S.* Typhi and other Gram-negative bacteria such as *E*. Coli (Álvarez-Ordóñez *et al*., 2012). Vast amounts have been deciphered about the *Salmonella* ATR since its first description by Foster and Hall. (1990) including many investigations over the conditions required for the induction of the ATR and the various factors which influence the response such as the phase of growth, temperature, type of acidulant and growth medium. Not only has progress been made in our understanding of the ATR and how it functions in *Salmonella* but also in the molecular mechanisms which regulate the response, with more understood about the intricacies of the response including gene expression and pH homeostatic mechanisms.

As mentioned in chapter 3 the pH homeostatic mechanisms involved in the ATR have been well characterised, with the functioning and implications of the lysine and arginine decarboxylase systems in protecting *Salmonella* from acid stress well known. Extensive research has been conducted over the last couple of decades determining the involvement of ASPs in the *Salmonella* ATR. The expression of ASPs was first descripted as a potential mechanism for *Salmonella's* ability to tolerate extreme acid stress by Foster. (1991). A follow-up study by Foster. (1993) highlighted the crucial impact of ASPs stating that they play an

integral role in the protection against acid stress, also noting that they become induced at a pH of 4.3. Stemming from this research many subsets of ASPs have been characterised in addition to the regulatory genes controlling their expression. Functions of most ASPs include: cellular regulation, molecular chaperoning, energy metabolism, transcription, translation, synthesis of fimbriae, regulation of the cellular envelopes, colonization and virulence (Bearson *et al*., 2006). Although vast improvements in our understanding of ASPs and their contributions to the ATR have been made, further studies are required in order to fully understand the contributions of ASPs in *Salmonella* survival following acid shock.

The core regulatory genes known to control the expression of ASP subsets include the alternative sigma factor RpoS, the iron regulator Fur, the two-component signal transduction system PhoP/PhoQ and the OmpR response regulator (Foster, 2000). Although not widely accepted to be a core regulator of ASPs in the *Salmonella* ATR, research has pointed at a role of RpoE during acid stress. An investigation by Muller *et al*. (2009) determined that σ^E-deficient S. Typhimurium exhibited increased susceptibility to acid pH. In the same study σ^E was shown to become activated by acidic conditions in a non-canonical mechanism independent of DegS, the full mechanism of acid induced activation of σ^E as well as its role in *Salmonella* survival during acid stress remains uncharacterised.

Of significance is that although the σ^E-regulon has been established many of the σ^E-regulated proteins have not been investigated and therefore have unknown functions in *Salmonella* (Skovierova *et al*., 2006). Hews (2020) started to characterise some of these σ^E-regulated proteins including STM1250, where she identified novel phenotypes of a Δ*STM1250* mutant. This was the first time STM1250 had been shown to be important in the *Salmonella* ATR. Continued efforts in this study confirmed STM1250s importance in the initial stages of the ATR during acid exposure in *S*. Typhimurium. Novel phenotypes of the Δ*STM1250* mutant were also identified in the presence of the organic acid, citric acid therefore extending the known implications of STM1250 in the ATR. Put together with previous findings it can be assumed STM1250 is functional in the ATR regardless of environmental factors such as growth phase or the acidulent. However, apart from these initial phenotypic implications of STM1250 little is known about the protein, with it still being regarded as a hypothetical protein (Skovierova *et al*., 2006). To further increase our understanding of this protein biochemical and protein interaction studies should be conducted, enabling a better idea of how STM1250 might interact and function. With no known conserved domains or motifs and lack of any structural data it is difficult to suggest an intracellular mechanism of STM1250. Although, thanks to promising phenotypic results it remains possible that STM1250 could still become a new therapeutic

target with its inhibition potentially leading to *Salmonella* attenuation, further investigation is however required before this can be confirmed.

5.4 The importance of the *Salmonella* ATR and STM1250 in food safety

Societal shifts including consumer preferences for healthy foods is leading to the popularisation of fruit juices. This increase in consumption is partly due to the abundance of vitamins, minerals, phenolic compounds, organic acids, and carotenoids in many fresh fruits and their juices (Dhalaria *et al*., 2020). Due to the high occurrence of pathogenic microorganisms in fruit juices, processing methods such as thermal treatment including pasteurisation are commonly implemented. This ensures microbial safety and also inactivates deleterious enzymes such as polyphenol oxidase and peroxidase which lead to undesirable sensory and nutritive changes (Petruzzi *et al*., 2017). Different legislations have been implemented to control food processing for fruit juices including the HACCP, which states fruit juices must a mandatory 5-$\log_{10}$ reduction of the most resistant microorganism of public health significance (FDA, 2001). This has been successful in drastically reducing the number of food-borne outbreaks of disease in fruit juices. However, retail establishments or businesses that sell directly to consumers and do not sell or distribute juice to other businesses are exempt from the juice HACCP regulations, therefore, not requiring them to thermally treat their juice products. Interestingly, pasteurisation may negatively impact the quality of fruit juices, this is due to the inactivation of some thermos-sensitivity bioactive compounds and alteration of levels of antioxidants, polyphenols and vitamin C (Mandha *et al*., 2023; Petruzzi *et al*., 2017). Therefore, the trend of consuming unpasteurised fruit juices is gaining traction, which poses a significant risk to public health due to the frequent contamination with pathogenic microorganisms.

There has been gaining attention paid over the last decades to the implications of the *Salmonella* ATR towards food safety including that of fruit juices, this is due to the popularisation of unpasteurised fruit juices and the occurrence of food-borne outbreaks of *Salmonella* in different fruit juices (CDC, 1975; Jain *et al*., 2009; Vojdani *et al*., 2008). Doubts have however been cast over the effectiveness of naturally acidified food products in reducing the viability of pathogenic microorganisms, with suggestions that certain bacteria including *Salmonella* can tolerate the low pH environment with innate stress responses such as the ATR. Due to the risk posed on public health a more comprehensive understanding of pathogenic survival in fruit juice products is required, informing on improved methods of food processing specific to the type of juice. Of importance is a more complete understanding of

the involvement of ASPs in the ATR under different food relevant conditions, specifically informing on how bacteria including *Salmonella* which possess this inherit response behave under naturally acidified food products including fruit juice. Therefore, enabling the assessment of microbiological risk in acidic food products. This study aimed to address this issue and build on our understanding of the putative stress response protein STM1250 in the ATR in the presence of fruit juices.

Survival assays in orange and apple juice storage reported in chapter 4 identified various key findings. As expected, a higher storage temperature of apple and orange juice had a deleterious impact on the mean survival of *Salmonella* strains. This is likely due to the increased diffusion of undissociated organic acids through the bacterial membrane at higher temperatures, resulting in more extreme acidification of the *Salmonella* cytoplasm and therefore microbial death (Álvarez-Ordóñez *et al*., 2013). This finding confirms the functionality of high temperature treatment of fruit juices as a viable method of *Salmonella* inactivation. Although, as previously stated thermal treatment of fruit juices negatively impacts their quality and therefore attractiveness to consumers (Mandha *et al*., 2023; Petruzzi et al., 2017). A study by Mandha *et al*. (2023) characterising the implications of pasteurisation on watermelon, pineapple, and mango juices determined considerable variations in physicochemical, chemical composition, and quality parameters of each juice during pasteurisation at different time periods. To overcome the issue conventional techniques such as pasteurisation have on the nutritional benefit of fruit juices, further investigation into the optimal pasteurisation temperature and time period should be conducted specific to the type of juice.

Cultures incubated in orange juice appeared to survive better in comparison to those incubated in each of the survival assays, apart from acid adapted cultures incubated at 37 °C. As suggested earlier this may have been as a result of the more effective induction of the ATR by citric acid in orange juice, or potentially higher bacterial inactivation caused by malic acid in apple juice. Is it evident throughout the literature that acid resistance is enhanced in *S.* Typhimurium when the medium is supplemented with amino acids, including arginine and lysine. This is thought to be due to the contribution they have on the pH homeostatic decarboxylation mechanisms (Kieboom and Abee, 2006). Interestingly, amino acid profiles differ between fruit juices with orange juice typically made up of 0.3%-0.4% amino acids, when in apple juice the level can be as low as 10 parts per million (ppm) (Huang *et al*., 2009). Therefore, another potential reasoning behind these results may be that the higher levels or amino acids in orange juice may more effectively induce the ATR of *Salmonella.* Regardless, the type of fruit juice appears to have an impact on the survival of *Salmonella,* highlighting the importance of food processing regulations specific to the type of fruit juice in order to precisely

inactive *Salmonella* to adequate levels. Further investigation would however be required to assess the reasoning behind the differential survival of *Salmonella* in different fruit juices, the data of which will be crucial in the production of food safety regulations for specific fruit juices.

Significantly, a clear trend was apparent in the fruit juice storage survival assays conducted at 4 and 25 °C. Acid adapted ∆*STM1250* mutants incubated in apple and orange juice portrayed a clear reduction in survival in comparison to the WT, this trend was no longer apparent or apparent to a much lesser degree in unadapted cells. Interestingly, the trend was established from the first time period at 12 hours, with the difference in percentage survival compared to the WT upheld across the other time periods at 24 and 36 hours. These findings overall suggest a possible role of STM1250 initially in the ATR after exposure to organic acids. To confirm whether this apparent trend is a result of an initial function of STM1250 in the ATR after exposure to organic acids present in fruit juices further investigation is required, with smaller time intervals taken over a reduced total storage time. For example, as STM1250 has been shown to be important in the first 15 minutes of citric acid exposure, future investigations should focus on the initial impacts of STM1250 during short term storage of fruit juices. Research towards the identification of new genes and proteins and their involvement in the *Salmonella* ATR is just as important as understanding the implications of environmental factors on the development of the ATR in food items, such as those investigated in this study storage temperature and medium composition. A greater overall understanding of the ATR including the functioning of proteins will facilitate the identification of improved food safety regulations, with more specialised implications for the inactivation of *Salmonella* specific to the type of food product. This study utilised the model NTS serovar *S*. Typhimurium, overall findings in this strain provide the basis and evidence for future extended investigation into alternative serovars. Of importance are serovars which are prevalent in foodborne outbreaks such as *S*. Enteritidis.

Of importance to food safety is the intricate relationship between stress resistance and virulence in many food-borne pathogenic bacteria, especially *Salmonella.* There is accumulating evidence suggesting that induction of the ATR enhances survival under the extreme acidic challenge presented by the gastric fluid in the stomach (Gahan and Hill, 1999; Wilmes-Riesenberg *et al*., 1996). A study by Yuk and Schneider. (2006) determined that the introduction of *Salmonella* to acidic juice environment during processing can consequently enhance its ability to survive in the human stomach, stating that this could potentially increase the risk of *Salmonella* outbreak by juice. Not only has it been suggested that microbial pathogenesis involving the tolerance of the stomachs low pH heavily relies on stress responses, especially the ATR of *Salmonella*. But also other aspects of pathogenesis beyond

the stomach including resistance to oxidative stress and intestinal invasion capacity (Felipe-López and Hensel, 2010; Perez *et al*., 2010). These findings directly suggest an intimate relationship between the development of an ATR in *Salmonella* and its virulence capacity. This has crucial relevance towards food safety as the ability of bacteria such as *Salmonella* to grow, compete for nutrients and tolerate stresses such as low pH would determine the number of viable cells that would reach the gut, and therefore cause disease (Guillén *et al*., 2021). Overall, highlighting the importance of the significant inactivation of *Salmonella* during food processing of fruit juices.

5.5 Study limitations

There are limitations present within this study, this includes the omission of phenotypic complementation strains. Low-copy non-inducible vectors, under the control of a native promoter and overexpression vectors of STM1250 would have been beneficial throughout the study. The low-copy non-inducible vector would be able to confirm whether native expression of STM1250 restores survival of the Δ*STM1250* mutant to WT levels. The use of overexpression vectors of STM1250 would primarily validate apparent phenotypes are due to the specific loss of STM1250. Use in the ATR survival assays would also provide insight into STM1250s functioning in the ATR, assessing whether the protein provides an additive survival advantage when overexpressed. Limitations to this study also include the lack of *Salmonella* serovars utilised with only *S.* Typhimurium investigated. Further characterisation of STM1250 in the ATR of other serovars would be beneficial, providing information on whether STM1250 functions equally irrelevant of serovar. Use of other common food-borne serovars of *Salmonella* such as *S*. Enteritidis would also extend our understanding of the ATR and STM1250 and its association with food safety.

5.6 Conclusions

The results in this thesis have confirmed previous findings that STM1250 is important in the *Salmonella* ATR, showing that it functions as a stress response protein. Our understanding of STM1250 has additionally been advanced through efforts in this thesis, showing further importance on STM1250 in the ATR in the presence of citric acid. Although the intracellular role of STM1250 still evades us, greater evidence has been built suggesting STM1250 functions in the initial stages of the ATR during acid exposure. This thesis has also taken strides in implicating the importance of the *Salmonella* ATR in naturally acidified food products specially fruit juices, with suggestions being made about how this may impact food safety.

STM1250 has also been suggested to be important for the *Salmonella* ATR in the presence of apple and orange juice, providing real implications of the protein outside of the laboratory conditions.

In conclusion, this study contributes to our knowledge of the *Salmonella* ATR and outlines how it impacts upon the food safety of fruit juices. Multiple suggestions for further investigations extending on findings of this study have been included throughout. Research conducted here and suggestions made for future investigations have the potential to identify novel therapeutic targets, and to more precisely tailor food regulations surrounding naturally acidified food products.

Chapter 6: References

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